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Attenuated poxviruses expressing a synthetic HIV protein stimulate HLA-A2-restricted cytotoxic T-cell responses

Arnaud Didierlaurent[a,1], Juan-Carlos Ramirez[b,1], Magdalena Gherardi[b], Simone C. Zimmerli[c], Marcus Graf[d], Hans-Acha Orbea[a], Giuseppe Pantaleo[c], Ralf Wagner[d], Mariano Esteban[b], Jean-Pierre Kraehenbuhl[a,*], Jean-Claude Sirard[a,e]

[a] *Swiss Institute for Experimental Cancer Research (ISREC), Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland*
[b] *Centro Nacional Biotecnología, CSIC; Campus Universidad Autonoma de Madrid, 28049 Madrid, Spain*
[c] *Division of Immunology and Allergy, Laboratory of AIDS Immunopathogenesis, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland*
[d] *GENEART GmbH, 93053 Regensburg, Germany*
[e] *Equipe Avenir, Institut de Biologie de Lille, 59021 Lille, France*

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Abstract

Efficient HIV vaccines have to trigger cell-mediated immunity directed against various viral antigens. However little is known about the breadth of the response induced by vaccines carrying multiple proteins. Here, we report on the immunogenicity of a construct harbouring a fusion of the HIV-1 IIIB *gag*, *pol* and *nef* genes (*gpn*) designed for optimal safety and equimolar expression of the HIV proteins. The attenuated poxviruses, MVA and NYVAC, harbouring the *gpn* construct, induced potent immune responses in conventional mice characterised by stimulation of Gpn-specific IFN-γ-producing cells and cytotoxic T cells. In HLA-A2 transgenic mice, recombinant MVA elicited cytotoxic responses against epitopes recognised in most HLA-A2$^+$ HIV-1-infected individuals. We also found that the MVA vaccine triggered the in vitro expansion of peripheral blood cells isolated from a HIV-1-seropositive patient and with similar specificity as found in immunised HLA-A2 transgenic mice. In conclusion, the synthetic HIV polyantigen Gpn delivered by MVA is immunogenic, efficiently processed and presented by human MHC class I molecules.

Keywords: HIV vaccine; HLA-A2; Recombinant poxviruses

1. Introduction

Many HIV candidate vaccines that target both cellular and humoral immunity have been tested for a decade in mice and non-human primates [1,2]. Successful HIV vaccination, however, has been hampered by the complexity of HIV infection and the absence of a defined correlate of protection. More recently, protective immunity against simian immunodeficiency (SIV) challenges in macaques has been reported [3–5]. These data indicate that induction of cytotoxic T cells (CTLs) prevents the spread of the viral infection [6]. Although induction of neutralising antibodies at mucosal surfaces is probably a prerequisite to induce sterile protection, a robust CTL response may lower viral load and, therefore, limit transmission [7].

Among the vectors used to carry HIV antigens, Modified Vaccinia Ankara (MVA), an attenuated vaccinia virus, was shown to induce HIV-specific CTL responses [3,8,9]. Upon MVA infection of mammalian cells, early and late protein synthesis is restricted to the first 24–48 h post-infection [10,11]. MVA is safe for humans [12] and is currently used in several HIV vaccine trials [13,14]. Within the family of attenuated poxvirus, NYVAC carrying multiple SIV/HIV antigens has also been used in monkeys [15,16]. NYVAC is a highly attenuated strain of vaccinia virus generated by precise deletion of 18 open reading frames from the viral genome, which affects non essential genes for virus growth in some cell lines but are important for virulence in animal models [17]. MVA has been generated by passage on

* Corresponding author. Tel.: +41-21-692-58-56; fax: +41-21-652-69-33.
E-mail address: jean-pierre.kraehenbuhl@isrec.unil.ch (J.-P. Kraehenbuhl).
[1] Contributed equally to this work. Present address: Laboratorio de Reumatología, Unidad de Investigación, Hospital Universitario 12 de Octubre, 28041 Madrid, Spain.

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chicken embryo fibroblast cells more than 500 times, loosing about 15% of genetic information and ability to grow in human and most other mammalian cells [18]. Both NYVAC and MVA share common deleted genes, like the serpins (B13R and B14R) and host range (K1L), but most other of the deleted genes are distinct between the two virus strains. A careful evaluation of the differences in MVA and NYVAC biology remains to be done [19].

Peptides corresponding to CTL epitopes administered with adjuvant or via recombinant vectors are immunogenic but their use is limited due to HLA variability and high mutation rate of HIV. A strategy that targets as many viral proteins as possible and that is not restricted to a few HLA molecules is more likely to be successful. So far, few candidate vaccines have used fusion proteins that contain multiple HIV determinants and little is known about the breadth and the magnitude of responses against such vaccines. This information is critical as immunodominance may favour the induction of restricted sets of specificities in vaccinated individuals. In order to develop strategies overcoming immunodominance, it is important to validate mouse model, such as transgenic mice carrying human MHC class I to predict human $CD8^+$ T cell responses [20]. In particular, the HHD mouse, that is H2-$Db^{-/-}$ β2-$microglobulin^{-/-}$ and transgenic for a chimeric HLA-A2 molecule, was shown to be a good model for the prediction of HLA-A2-restricted responses [21].

Within the frame of EuroVacc (http://www.eurovac.net), a European effort to bring HIV vaccines into clinical trials, candidate vaccines harbouring a fusion of the HIV-1 IIIB *gag*, *pol* and *nef* genes (*gpn*) have been constructed. Before entering clinical trials in humans, we sought to verify that immunisation with Gpn induced CTLs with similar specificities than found in natural infections. We show here that MVA and NYVAC expressing Gpn induce strong CTL and IFN-γ-producing T cell responses in conventional mice. HLA-A2 transgenic mice immunised with MVA expressing Gpn developed HLA-A2-restricted responses against two CTL epitopes known to be immunogenic in HIV infected individuals. In addition, the candidate vaccine specifically triggers the in vitro expansion of human HIV-1-specific $CD8^+$ T cells. Therefore, we provide evidence that these vaccines tested for safety in mice [22], are immunogenic and elicit CTL responses against HLA-A2-restricted epitopes recognised by HIV-infected patients.

2. Material and methods

2.1. Construction of the chimeric gpn

The synthetic read-through *gagpolnef* polygene was synthesized and provided by GENEART GmbH (Regensburg, Germany). *Gagpolnef* was generated by back-translating the amino-acid sequence of the HIV-1_{IIIB} (BH10, GenBank Accession no. M15654) using a matrix for the most frequently occurring codons in mammalian cells. By optimising the *gagpolnef* sequence, cryptic splice-donor/acceptor sites and RNA destabilising sequence motifs were eliminated. The synthetic *gagpolnef* DNA was designed in fragments of 300–800 nucleotides, assembled via unique restriction sites and cloned into pCRscript giving rise to pCRscript/*gpn*. The *gagpolnef* fusion protein comprises the group-specific antigen Gag including p7 (aa 1–432; GI: 326388) fused and in frame to Pol (aa 1–793; GI: 326385) lacking the integrase domain. Furthermore, the active site of the reverse transcriptase (RT) was replaced by a scrambled *nef* gene (aa 1–74 fused to aa 75–123; GI: 326393) resulting in an artificial budding defective 1326 aa read-through *gagpolnef* fusion protein. The RT sequence overlapping the active site was translocated in frame to the 3′ end (aa 296–396; GI: 326385) of the *gagpolnef* polygene. Accordingly to prevent myristoylation, the N-terminal glycine was substituted by alanine (G to A). A point mutation was also introduced into the active site of the protease domain (aa 93; GI: 326385) to impair its enzymatic activity.

2.2. Construction of recombinant poxvirus vectors

After digestion with *Eco*RI and *Sac*I, the DNA fragment containing *gagpolnef* sequence was isolated from pCRscript/*gpn* and cloned into the Vaccinia virus (VV) insertion vector pJR101. In the resulting plasmid, the expression of Gpn and the selection marker β-glucuronidase is regulated by the VV synthetic early/late promoter e/l [23] and promoter p7.5, respectively. The synthetic e/l promoter contains 40 bp which largely overlap early and late regulatory elements [23]. This construct was further checked by sequencing and inserted into the VV haemagglutinin gene. The selection of recombinant MVA viruses (MVAgpn) was performed as in [11]. The purity of the recombinant virus was confirmed by PCR analysis. The construction of MVA delivering the luciferase protein is described elsewhere (MVAluc [11]). MVA recombinants were grown in CEF, purified by sucrose-cushion and titrated by immunostaining in CEF [11]. Recombinant NYVAC expressing Gpn (NYVACgpn) was provided by Dr. Frachette (Aventis Pasteur, Lyon). NYVACgpn was grown in CEF and titrated by immunostaining in CEF or by plaque assay in BSC-40 cells. Both MVAgpn and NYVACgpn recombinants represent a homogeneous virus population, as established after PCR analysis. In NYVACgpn, Gpn expression is regulated by the same synthetic e/l promoter as in MVAgpn except that the expression cassette is inserted into the thymine kinase gene. To generate WRgp, the sequences encoding natural HIV Gag and Pol proteins as well as the selection marker β-galactosidase were inserted in the thymine kinase gene. The expression of HIV antigens was assessed in BHK-21 cells infected at a multiplicity of infection (m.o.i.) of five with either sucrose-purified MVAgpn, NYVACgpn or WRgp. Cells were collected at various times after infection and extracts (12 μg) were run on 10% SDS-PAGE.

Gpn was visualized after Western blotting using rabbit polyclonal anti-gag p24 serum (ARP 432, NIBSC, Centralised Facility for AIDS reagents, UK). Alternatively, the analysis of Gpn intracellular location was performed at 24 hpi by immunofluorescence and confocal microscopy on permeabilized BHK-21 cells that were incubated with the antibody ARP 432 and the nuclei staining reagent To-Pro (Molecular Probes).

2.3. Mice immunisation

C57BL/6 mice were purchased from Harlan. Transgenic HHD mice kindly provided by Dr. Lemonnier (Pasteur Institute, France) are double-knockout for H2-D^b and β2-microglobulin and transgenic for a chimeric HLA-A2 molecule, the only MHC class I, therefore, expressed [24]. For peptide immunisation, 25 μmol of each peptide were mixed with 50 μg of tetanus toxin universal helper peptide P30 in 200 μl IFA/PBS 1:1 emulsion and injected at days 0 and 15 at the base of the tail. Recombinant MVA and NYVAC were injected intraperitoneally (i.p.) or subcutaneously (s.c.) at 5×10^7 p.f.u. in 200 μl PBS.

2.4. IFN-γ ELISPOT and CTL assays

Six peptides chosen from the Los Alamos Database (http://hiv-web.lanl.gov/immunology/index.html) were synthesised at the Biochemistry Institute facility: Gag (SLYNTVATL), Pol1 (VLDVGDAYFSV), Pol2 (ILKEPVHGV), Pol3 (ALQDSGLEV), Pol4 (VIYQYMDDL) and Prot1 (VLVGPTPVNI). EL4 cells were a gift of Dr. Pedro Romero (Ludwig Institute, Lausanne branch). RMAS-HHD cells were obtained from Dr. Lemonnier. EL4gpn are stable EL4 clones transfected with the synthetic *gagpolnef* coding region inserted into the in pCi/neo expression vector (Promega) under the transcriptional control of the immediate-early promoter-enhancer of the human cytomegalovirus. EL4gpn cells thus constitutive express Gagpolnef. Cell cultures were performed in RPMI 1640 containing 2 mM L-glutamine, 20 mM HEPES, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 2-β-mercaptoethanol (Gibco BRL), and 10% foetal calf serum (FCS, Myoclone Superplus). Ten days after the last immunisation, spleens from immunised animals were removed and pooled. Fresh IFN-γ ELISPOT analysis was performed as described [25]. Briefly, 10^5–10^6 splenocytes were incubated with either medium containing 10 μM specific peptide (for HHD animals), 10^5 MVA-infected splenocytes, EL4 or EL4gpn (for C57BL/6) depending on the experiments. MVA-infected splenocytes were prepared from naïve splenocytes infected at a m.o.i. of five in FCS-free medium for 1 h. The cells were further incubated for 5 h in medium containing 2% FCS to allow expression of the viral antigens and used in the ELISPOT assay after extensive washing.

The cells were incubated 18 h in 96 well plates coated with IFN-γ antibodies (clone R4-6A2, Caltag). The spots were revealed using biotinylated secondary anti-IFN-γ antibodies (clone XMG1.2, Pharmingen) and extravidin conjugated to the Alkaline Phosphatase (Sigma). The spots were counted using a ProgRes microscope (Zeiss). For CTL assay or expanded IFN-γ ELISPOT assay, effector cells (5×10^6 splenocytes) were stimulated in vitro in 10 ml complete medium for 6 days once or twice. For C57BL/6 effectors, Gpn-specific stimulation was achieved by adding 5×10^5 EL4gpn and 5×10^6 irradiated naïve C57BL/6 splenocytes. For HHD effectors, incubation was performed with 5×10^5 LPS blasts derived from HHD mice and independently loaded with each peptide [26]. Target cells for CTL assays were EL4gpn effector cells and RMA-S/HHD loaded with individual HLA-A2-restricted peptide, respectively for C57BL/6- and HHD-stimulated effector cells [24].

2.5. Stimulation of HIV-1-specific human CD8$^+$ T cells with MVAgpn

The expansion of Pol2- and Gag-specific CD8$^+$ T cells induced by MVAgpn was studied as described in [27] using cryo-preserved peripheral blood mononuclear cells (PBMC) from an HIV-1-infected patient. Patient CNA 2099 is part of a cohort of HIV-1 infected patients with progressive disease enrolled in therapeutic clinical trials with anti-retroviral regimens [28,29]. These studies were approved by the local Institutional Review Board and the subject gave written informed consent. PBMCs from an HIV-seronegative subject (LDH 197) were included in the study as negative control.

PBMCs were cultured at 1×10^6 cells per ml with a sonicated preparation of MVAgpn or MVAluc at the indicated m.o.i. Fresh culture medium supplemented with recombinant human IL-2 (0.02 U/ml, Roche Diagnostics) was added every 2–3 days to the PBMC cultures. On day 10, the expansion of Pol2- and Gag-specific CD8$^+$ T cells was monitored by staining with APC-conjugated anti-CD8 antibodies (clone SK1) and PE-labelled tetramers. Class I-peptide tetramers were produced as previously described [30]. Data were acquired on a FACSCalibur™ system and analysed using CellQuest™ software (Becton Dickinson).

3. Results

3.1. Construction of the attenuated poxviruses candidate vaccines

3.1.1. Characteristics of the chimeric Gagpolnef

Gagpolnef is a fusion protein of 1326 amino acids composed of Gag, Pol and Nef from the HIV-1 clade B clone IIIB [31] that has been modified to enhance its immunogenicity and for safety reasons (Fig. 1). The *gag* sequence was fused in frame to the *polnef* part by creating a 1 frame shift in the natural slippery sequence. A glycine to alanine

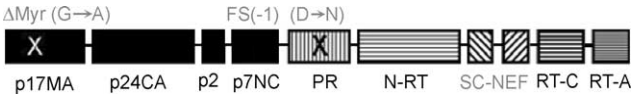

Fig. 1. Schematic drawing of the synthetic HIV IIIB fusion protein Gagpolnef. The Gag derived-domains are constituted of the matrix (p17MA), the capsid (p24CA), p2 and the nucleocapsid (p7Nc). The Pol and Nef derived domains, i.e. Protease (PR), N-terminal (N-RT) and C-terminal (RT-C) portion of the reverse transcriptase (RT), the active site of the RT (RT-A) and a scrambled Nef (SC-NEF) are also depicted. Modifications include the G to A amino acid substitution abolishing N-terminal myristilation (ΔMyr) of Gag, the in frame fusion (FS) of the Gag and Polnef domains and the D to N amino acid substitution inactivating the PR.

substitution in Gag was introduced to prevent formation of virus-like particles [32]. The active sites of protease (PR) and integrase were inactivated by mutagenesis and deletion, respectively. The active site of the RT was translocated at the C-terminus of the fusion protein. The *nef* gene was partially deleted, mixed up to generate a fusion between the C-terminal and N-terminal parts of the protein, and inserted into the fragment coding the RT. In addition, the codon usage was adapted to highly expressed human genes (GENEART GmbH, Germany) based on a previous study showing that such optimisation of an HIV Gag DNA vaccine increases its expression and its immunogenicity [33].

3.1.2. Expression of Gagpolnef

The synthetic *gagpolnef* gene was introduced into the MVA or NYVAC genome, referred to as MVAgpn and NYVACgpn, respectively. After infection of permissive BHK21 cells with MVAgpn, a band corresponding to the full length Gagpolnef protein (~150 kDa) was revealed on immunoblot with anti-Gag polyclonal antibodies (Fig. 2A). Pol- and Nef-specific antibodies recognised the same band (data not shown). BHK-21 infection by WR harbouring wild type HIV IIIB *gag* and *pol* genes (WRgp) resulted in PR-mediated Gag processing as shown by the presence of p55 and p24. In contrast, native HIV proteins resulting from PR activity were not observed in cells infected with MVAgpn. Therefore, according to the construct design, MVAgpn-infected cells produce a Gagpolnef fusion protein that cannot be processed by PR into native and potentially harmful HIV proteins. As compared to MVAgpn, the full length Gpn fusion protein was produced in NYVACgpn infected cells but at different levels depending on the time point after infection (Fig. 2B). In these cells, expression of Gpn was higher at 6 h post infection (hpi) in NYVAC-gpn when compared to MVAgpn-infected cells. We have consistently found that in permissive (CEF, BHK-21) and non-permissive (TK-143, HeLa) cells infected with NYVACgpn, the levels of Gpn at 6 hpi are somewhat higher or similar to those found in cells infected with MVAgpn depending on the cell lines (Fig. 2B and data not shown). This observation rules out the possibility that putative mutations in the early/late promoter of NYVACgpn influences expression. With time (18 and 24 hpi), the Gpn expression level decreases in NYVACgpn versus MVAgpn-infected cells. This results from differences of cytopathic effect and inhibition of virus-induced protein synthesis between both poxviruses (Najera et al, manuscript in

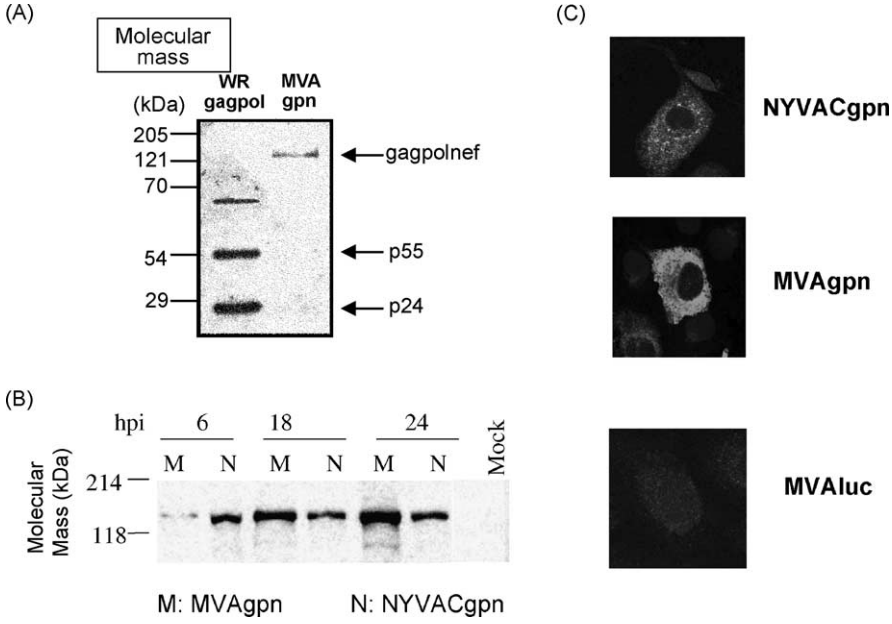

Fig. 2. Gagpolnef expression in MVAgpn and NYVACgpn infected cells. BHK-21 cells were infected either with MVAgpn, MVAluc, NYVACgpn or with recombinant Vaccinia virus WR expressing the HIV IIIB *gag* and *pol* (WRgp) as indicated. Cell extracts were analysed after 24 h (A) or various hpi (B) by SDS-PAGE followed by immunoblotting using anti-Gag serum (A,B) or immunofluorescence (C). Fluorescence analysis was performed at 24 hpi on BHK-21 permeabilized cells for detection of Gagpolnef fusion protein (anti-Gag serum, white) and cell nuclei (grey).

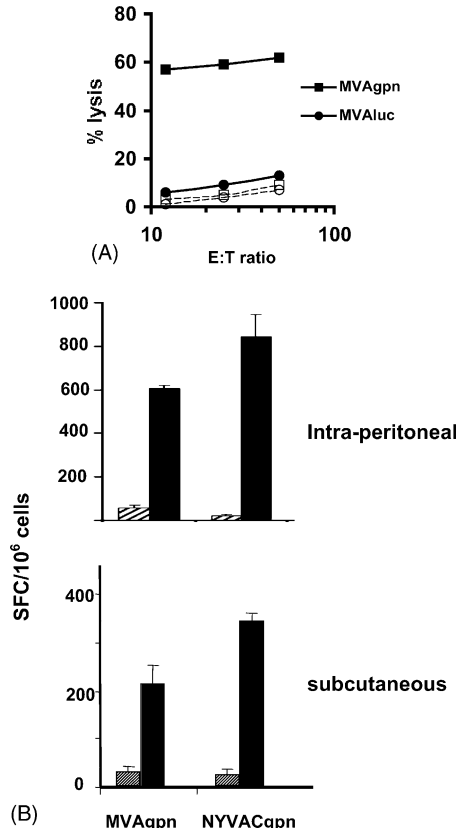

Fig. 3. Immunogenicity of Gagpolnef carried by MVAgpn and NYVACgpn. (A) C57BL/6 mice ($n = 3$) were injected i.p. with MVAgpn or MVAluc as control. Ten days later, splenocytes from immunised animals were stimulated in vitro for 6 days and tested for their ability to kill target cells in a chromium release assay. Target cells were EL4 for non-specific response (dashed line and open symbols) and EL4gpn for Gagpolnef-specific response (filed symbols). E:T stands for effector/target. Data are representative of three experiments. (B) C57BL/6 mice ($n = 3$) were injected i.p. or s.c. with MVAgpn or NYVACgpn. Ten days later, splenocytes from immunised animals were tested in fresh IFN-γ ELISPOT assay. Target cells were EL4 for non-specific response (hatched bars) and EL4gpn for Gagpolnef-specific response (black bars). SFC stands for spot forming cells. Data are representative of two experiments.

preparation). Immunofluorescence analysis by confocal microscopy in infected BHK-21 cells at 24 hpi, revealed that Gpn is localised in the cytoplasm of cells infected by both recombinant viruses but not by a control virus delivering luciferase (MVAluc, Fig. 2C) [11].

3.2. Recombinant MVA and NYVAC induce Gagpolnef-specific CTL response

To assess the immunogenicity of Gpn, C57BL/6 mice (H-2^b) were immunised with MVAgpn and NYVACgpn and the response against the whole fusion protein was assessed using EL4 cells (H-2^b) expressing Gpn (EL4gpn). As shown in Fig. 3A, a significant CTL activity against EL4gpn was detected in mice immunised with MVAgpn but not in animals treated with MVAluc. These results indicate that the fusion protein delivered by MVAgpn is efficiently processed and presented in a MHC class I-restricted pathway to $CD8^+$ T cells.

To allow a direct comparison between immunisation protocols, Gpn-specific T-cell response was analysed by measuring the frequency of IFN-γ-secreting cells using ELISPOT [25]. Gagpolnef-specific IFN-γ-producing splenocytes were found directly ex-vivo after immunisation with MVAgpn (Fig. 3B). This corresponds to a frequency of 0.4% Gagpolnef-specific $CD8^+$ T cells. This response correlates with the CTL activity described in Fig. 3A, confirming that Gagpolnef is an immunogenic protein when expressed in cells infected with MVA. NYVACgpn also induced the development of Gpn-specific IFN-γ-producing cells similar than the one observed with MVA. Specific responses were also monitored after subcutaneous immunisation with both vectors. Altogether, these results indicate that Gpn delivered by recombinant poxvirus vectors induce a robust HIV-specific CTL response.

3.3. MVAgpn induces HIV-specific HLA-A2-restricted CTL in HHD mice

3.3.1. Immunogenicity of selected HLA-A2-restricted peptides

To test whether Gagpolnef expressed by MVAgpn was recognised by human MHC class I molecules, we immunised transgenic HHD mice that exclusively display a chimeric human HLA-A2.1 as MHC class I molecule [24]. Six HLA-A2-restricted peptides recognised by CTLs from HIV-infected individuals were selected throughout the fusion protein (Fig. 4A) and tested for their intrinsic immunogenicity in HHD mice. Seven days after immunisation with a mixture of the peptides, splenocytes were stimulated in vitro with LPS blasts loaded with each peptide. All peptides except Pol4 induced an HLA-A2-restricted IFN-γ-producing (Fig. 4B) and CTL (Fig. 4C) responses against RMAS-HHD cells loaded with each peptide. The magnitude of the response varied among the peptides, Gag, Pol2 and Pol3 being more immunogenic than Prot and Pol1. These results show that the selected HIV-specific CD8 epitopes found to be immunodominant in humans are immunogenic in HHD mice.

3.3.2. MVAgpn triggers CTLs specific for peptides immunogenic in HLA-$A2^+$ HIV-infected individuals

To assess whether HHD mice could respond to MVAgpn immunisation, an IFN-γ ELISPOT assay specific for MVA antigens was first performed using naïve splenocytes infected with MVAluc as MVA-specific antigen presenting cells (Fig. 5A). The frequency of IFN-γ-producing cells was lower in HHD mice than in C57BL/6 animals, which correlates with the lower proportion of total splenic $CD8^+$ T cells in HHD mice (on average 3% of total splenocytes). The response against the different HLA-A2 restricted epitopes spanning Gpn was next investigated after i.p. injection of MVAgpn. After 6 days in vitro expansion with each

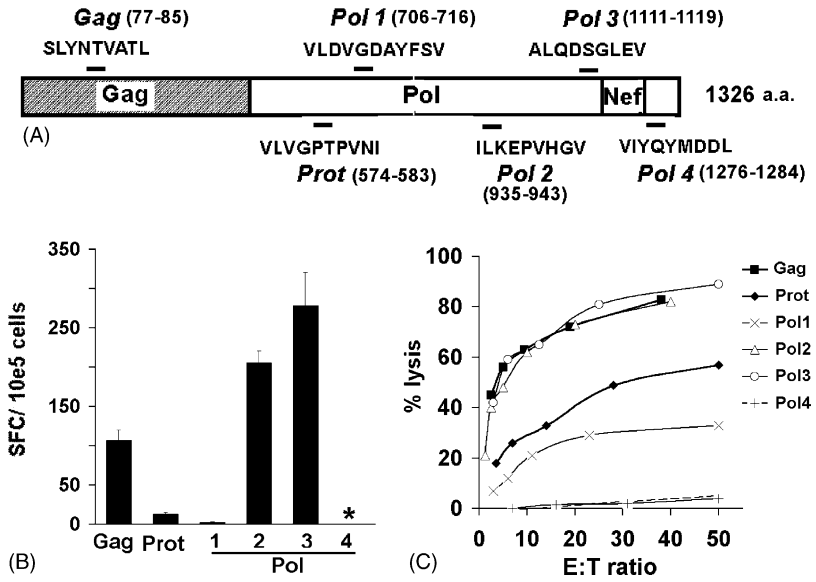

Fig. 4. HLA-A2-restricted peptides from Gagpolnef stimulate CTL responses in HHD mice. (A) The position on Gagpolnef of six HLA-A2-restricted peptides (Gag, Prot, Pol1, Pol2, Pol3 and Pol4) recognised by HIV infected individuals is depicted. (B, C) HHD mice ($n = 5$) were immunised with a mixture of the HLA-A2 peptides with helper peptide P30 in IFA. One week later, splenocytes of immunised animals were collected and stimulated in vitro for 1 (B) or 2 (C) weeks with LPS blasts loaded independently with each peptide. An IFN-γ ELISPOT assay (B) and a chromium release assay (C) were then performed using RMAS-HHD loaded with each peptide as target cells. Values obtained with non-loaded RMAS-HHD as target cells were subtracted from values obtained with peptide-loaded RMAS-HHD. This experiment was reproduced twice. SFC, asterisk and E:T mean spot forming cells, no detectable response, and effector/target, respectively.

peptide, IFN-γ-producing cells specific for Prot (VLVGPTPVNI) and Pol2 (ILKEPVHGV) peptides were only detected (Fig. 5B). These cells were shown to be CTLs since they were able to kill RMAS-HHD cells loaded with Prot and Pol2, respectively (Fig. 5C). No CTL nor IFN-γ-producing cell responses were observed under similar conditions following immunisation with MVAluc. Moreover, two successive injections of MVAgpn did not alter the broadness of Gpn-specific T cell responses (data not shown). Altogether, these data show that MVAgpn induces HLA-A2-restricted CTL responses directed against peptides generated during natural HIV infection.

3.4. MVAgpn stimulates human Pol2-specific CD8$^+$ T cells

To define the relevance of our observations in a human model, we investigated whether MVAgpn could stimulate Pol2-specific human CD8$^+$ T cells. To this aim, we used the in vitro assay developed by Dorrell and co-workers to show that recombinant MVA efficiently stimulates human CTLs [27]. PBMCs from an HLA-A2$^+$ HIV-1-infected patient (CNA 2099) were found to contain Pol2-specific CD8$^+$ T cells as shown by staining with Pol2 HLA-A2 tetramer (Fig. 6). The cells were then stimulated with MVAgpn at increasing m.o.i. or with MVAluc as control. Pol2-specific CD8$^+$ T cells were specifically expanded in the presence of MVAgpn but not in the presence of MVAluc. Moreover, the expansion of Pol2-specific CD8$^+$ cells, ranging from a 5.2- to 16.7-fold increase in cell number, was proportional to the m.o.i. No Pol2-specific T cells were detected in PBMCs from seronegative donors (LHD 197) stimulated with MVAgpn. Thus, these data show that the Pol2 epitope is generated in MVAgpn-infected human cells, loaded onto HLA-A2 and presented to human CD8$^+$ T cells, confirming the results obtained in the HHD mice. Gag-specific CD8$^+$ T cells were also specifically expanded upon stimulation with MVAgpn (13.5-fold increase compared to baseline, at a m.o.i. of 1). This observation suggests that some epitopes found to be cryptic upon immunisation of the HHD mice are however generated in human cells.

4. Discussion

Here we report the construction of a novel antigen containing multiple HIV proteins and its immunogenicity when expressed in poxvirus vectors. The antigen reported in this study has several advantages. First, it is constituted of the major proteins targeted by CTLs in a natural infection and gathered in a single expression cassette. In particular, the Pol protein is often seen by the immune system but its expression level is low during a natural infection. Here, the removal of the frameshift between *gag* and *pol* results in similar expression level of Gag and Pol proteins, i.e. Gag- and Pol-specific epitopes. Second, it has been designed for optimal safety as it does not produce functional proteins and, therefore, no potential virus-like particles (Fig. 1). Third, it has been engineered for human codon usage, which is known to increase both antigen expression and immunogenicity [34–36]. Gpn has been inserted into several vac-

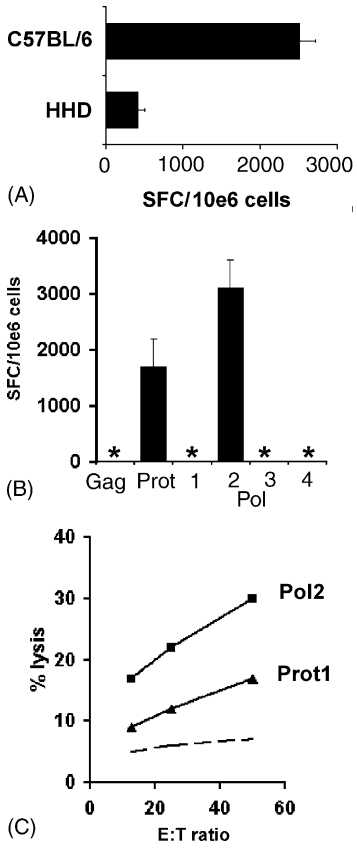

Fig. 5. MVAgpn induces Gagpolnef-specific HLA-A2-restricted CTLs in HHD mice. Mice were immunised i.p. with a single injection of MVAgpn. Ten days later, spleens of treated animals were sampled and assayed for $CD8^+$ T cell responses. (A) An IFN-γ ELISPOT assay specific for MVA antigens (with MVAluc-infected irradiated splenocytes as antigen presenting cells) was performed on pooled splenocytes from three mice. Number of spots per 10^6 cells against non-infected APC is <20. (B, C) Splenocytes were also stimulated *in vitro* for 6 days with LPS blasts loaded independently with each peptide. An IFN-γ ELISPOT assay (B) and a chromium release assay (C) were then performed using RMAS-HHD as target cells. In panel C, squares and triangles represent specific lysis of Pol2- and Prot1-loaded RMAS-HHD cells by Pol2- and Prot1-stimulated effectors, respectively. Dashed line is background lysis of non loaded RMAS-HHD. The difference in the magnitude of the response against the Pol2 and Prot1 epitopes was not significant. SFC, asterisks and E:T mean spot forming cells, no detectable response, and effector/target, respectively. Data are representative of three experiments.

cine candidates which will be tested in a near future in phase I clinical trials within the EuroVacc program. We show that Gpn induces T cell response with peptide specificity similar to that found in $HLA\text{-}A2^+$ HIV-infected individuals. Importantly, the dominance towards the Pol2 epitope was further extended to humans since MVAgpn but not a control MVA stimulated pre-existing Pol2-specific human $CD8^+$ T cells in vitro. Moreover, Gpn was immunogenic when expressed either from NYVAC or MVA. Altogether, these results indicate that Gpn delivered by recombinant poxviruses is a suitable antigen to use in human vaccination.

Recently, Belyakov and colleagues have reported that MVA and NYVAC induce similar levels of protection against virulent Vaccinia virus challenge in mice [37]. However, no studies have thoroughly compared the immunogenicity of both vectors when carrying an heterologous antigen. Our study indicates that Gpn-specific immune responses are similar when induced by NYVACgpn and MVAgpn using two different routes of inoculation. This also indicates that the different insertion sites for the heterologous antigen (TK or HA) have no apparent effect on the strength of the immune response.

Mice transgenic for human HLA molecules are important models to study the immunogenicity of vaccines dedicated to human trials. Our results support the use of HHD mice to study HIV-specific HLA-A2-restricted responses since selected HIV-specific CD8 epitopes found to be immunodominant in humans are immunogenic in HHD mice. Moreover, we provide strong evidence that an immunodominant epitope found after immunisation in the HHD mice is similarly processed in humans cells, thereby reinforcing the relevance of this mouse model in the prediction of vaccine trials.

When the peptides were tested individually for their intrinsic immunogenicity, the magnitude of the response was found to be different between peptides: Gag, Pol2 and Pol3 were more immunogenic than Prot and Pol1. This observation could reflect a variation in HLA-A2 binding affinity within these epitopes. Pol4 for instance is not immunogenic in HHD mice and has indeed a poor binding and stabilising capacity for HLA-A2 [38]. Gagpolnef fusion protein delivered upon infection of mammalian cells with MVAgpn is processed into HLA-A2-restricted epitopes. Among the selected epitopes, Prot and Pol2 are immunodominant. The response against Pol2, also called I9V, is particularly interesting since this peptide has been reported in most HLA-A2 infected patients and is well conserved among HIV isolates, in particular B and C clades (http://hiv-web.lanl.gov/immunology/index.html). This indicates that the CTL response induced by MVAgpn could be cross-reactive in humans. The magnitude of the CTL response observed against Pol2 and Prot was weaker than the level of the Gagpolnef-specific response in conventional mice. The difference could be due to the immunological limitation of the HHD mice, i.e. low $CD8^+$ T cell number and limited $CD8^+$ T-cell repertoire [39]. In addition, the response in conventional mice is assumed to be directed against the three antigenic determinants including Gag, Pol and Nef and to be polyclonal, i.e. specific for various epitopes on each determinant. The strong Gagpolnef-specific response in C57BL/6 supports this hypothesis. Therefore, many HLA-A2-restricted epitopes different from those selected in this study are likely to stimulate CTL responses in the HHD mice.

The Gag peptide (known also as S9L) combined to an adjuvant elicit strong CD8 responses in the HHD mice whereas it is cryptic in the context of the fusion protein delivered

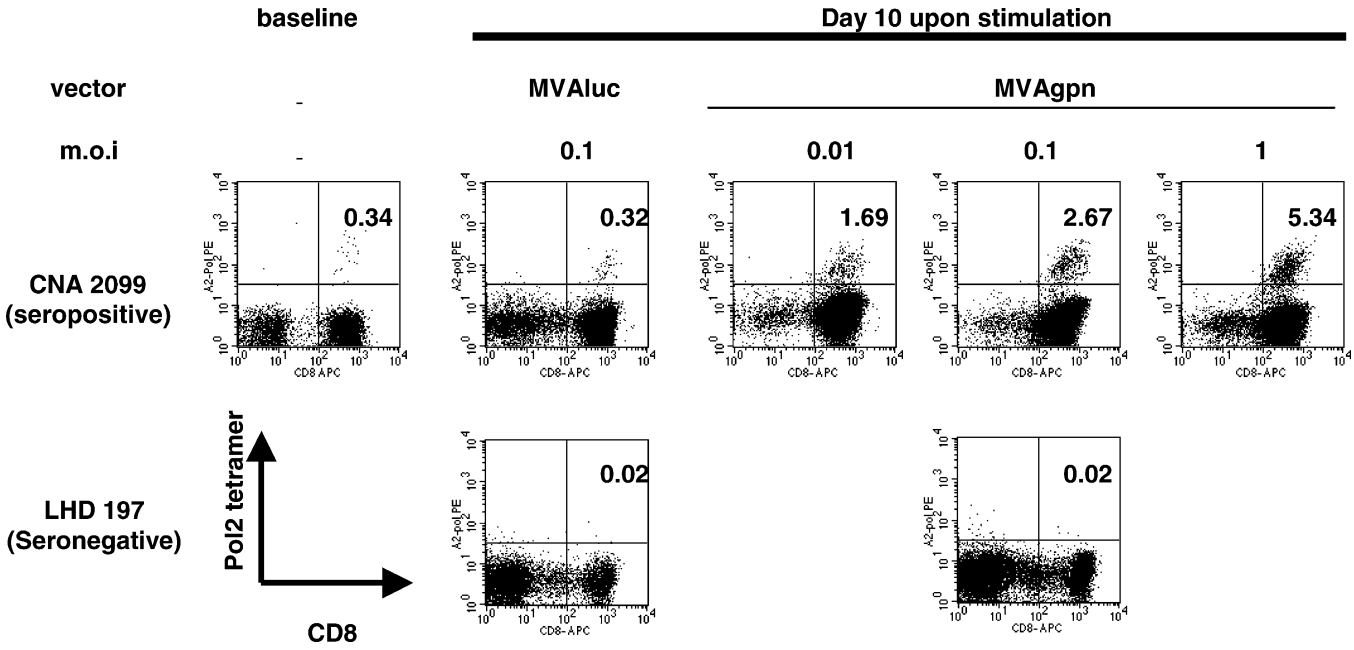

Fig. 6. MVAgpn specifically stimulates Pol2-specific human CD8$^+$ T cells. Diagrams represent flow cytometry analysis of PBMCs from HIV-seropositive (CNA 2099) and seronegative (LHD 197) patients stimulated or not with recombinant MVA for 10 days at indicated m.o.i. The number of Pol2-specific CD8$^+$ T cells was evaluated by staining with anti-human CD8 antibodies and Pol2-specific tetramer before (baseline) and after stimulation. Diagrams represent cells gated on lymphocytes and numbers indicate the percentage of Pol2-specific cells among total lymphocytes.

by MVA. Therefore, the lack of Gag-specific response upon immunisation with MVAgpn can not be explained by the absence of specific naïve cells in the HHD mice. In humans, the Gag epitope emerges only after chronic HIV infection and has been, therefore, referred as subdominant peptide [40]. However, we found that Gag-specific human CD8$^+$ T cells are stimulated by MVAgpn in vitro. The difference between human and mouse could mirror some differences in the respective processing machineries. Overlapping peptide pools spanning the entire Gpn molecule should be useful to address this point. This observation also means that human vaccination with MVAgpn could induce CTL specificities even broader than anticipated using the HHD model.

The induction of a restricted set of CTLs might favour the emergence of viral escape mutants, accelerating disease progression [41]. It is therefore crucial for a successful vaccine to induce the greatest breadth of CTL responses. As it was also shown for hepatitis B DNA vaccination [42], we demonstrate here that a novel complex multi-determinant HIV antigen is efficiently processed and is likely to induce multiepitopic T-cell response in humans. As antibodies may also play an important role in neutralising free viruses, the Gp120 membrane protein will be included in the vaccine to be used in clinical trials. The use of mouse model could be valuable in comparing various vaccines and vaccination protocols. The clinical trials that will be conducted by the EuroVacc consortium are going to establish whether CTL responses in HHD mice are predictive of those that the vaccines will elicit in humans.

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References

[1] Nabel GJ. Challenges and opportunities for development of an AIDS vaccine. Nature 2001;410:1002–7.
[2] Letvin NL, Barouch DH, Montefiori DC. Prospects for vaccine protection against HIV-1 infection and AIDS. Ann Rev Immunol 2002;20:73–99.
[3] Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 2001;292:69–74.
[4] Belyakov IM, Hel Z, Kelsall B, Kuznetsov VA, Ahlers JD, Nacsa J, et al. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. Nat Med 2001;7:1320–6.
[5] Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 2002;415:331–5.
[6] Robinson HL, Montefiori DC, Johnson RP, Manson KH, Kalish ML, Lifson JD, et al. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and

recombinant pox virus booster immunizations. Nat Med 1999;5:526–34.
[7] McMichael A, Hanke T. The quest for an AIDS vaccine: is the CD8+ T-cell approach feasible? Nat Rev Immunol 2002;2:283–91.
[8] Amara RR, Villinger F, Staprans SI, Altman JD, Montefiori DC, Kozyr NL, et al. Different patterns of immune responses but similar control of a simian-human immunodeficiency virus 89.6P mucosal challenge by modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines. J Virol 2002;76:7625–31.
[9] Ourmanov I, Brown CR, Moss B, Carroll M, Wyatt L, Pletneva L, et al. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. J Virol 2000;74:2740–51.
[10] Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc Natl Acad Sci USA 1992;89:10847–51.
[11] Ramirez JC, Gherardi MM, Esteban M. Biology of attenuated modified vaccinia virus Ankara recombinant vector in mice: virus fate and activation of B- and T-cell immune responses in comparison with the Western Reserve strain and advantages as a vaccine. J Virol 2000;74:923–33.
[12] Stickl H, Hochstein-Mintzel V, Mayr A, Huber H, Schäfer CH, Holzner A. MVA vaccination against smallpox:clinical trials of an attenuated live vaccinia virus strain (MVA). Dtsch Med Wochenschr 1974;99:2386–92.
[13] Hanke T, McMichael AJ. Design and construction of an experimental HIV-1 vaccine for a year-2000 clinical trial in Kenya. Nat Med 2000;6:951–5.
[14] Robinson HL. Vaccines: new hope for an aids vaccine. Nat Rev Immunol 2002;2:239–50.
[15] Hel Z, Venzon D, Poudyal M, Tsai WP, Giuliani L, Woodward R, et al. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. Nat Med 2000;6:1140–6.
[16] Hel Z, Nacsa J, Tryniszewska E, Tsai WP, Parks RW, Montefiori DC, et al. Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge CD4+ and CD8+ T-cell responses. J Immunol 2002;169:4778–87.
[17] Tartaglia J, Perkus ME, Taylor J, Norton EK, Audonnet JC, Cox WI, et al. NYVAC: a highly attenuated strain of vaccinia virus. Virology 1992;188:217–32.
[18] Sutter G, Staib C. Vaccinia vectors as candidate vaccines: the development of Modified vaccinia virus Ankara for antigen delivery. Curr Drug Target-Infect Disord 2003;3:263–71.
[19] Gallego-Gomez JC, Risco C, Rodriguez D, Cabezas P, Guerra S, Carrascosa JL, et al. Differences in virus-induced cell morphology and in virus maturation between MVA and other strains (WR, Ankara, and NYCBH) of vaccinia virus in infected human cells. J Virol 2003;77:10606–22.
[20] Faulkner L, Borysiewicz LK, Man S. The use of human leucocyte antigen class I transgenic mice to investigate human immune function. J Immunol Methods 1998;221:1–16.
[21] Firat H, Garcia-Pons F, Tourdot S. HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur J Immunol 1999;29:3112–21.
[22] Ramirez JC, Finke D, Esteban M, Kraehenbuhl JP, Acha-Orbea H. Nasal inoculation of Modified Vaccinia Virus Ankara (MVA) is safe in mice. Arch Virol 2003;148:827–39.
[23] Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. Biotechniques 1997;23:1094–7.
[24] Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 1997;185:2043–51.
[25] Miyahira Y, Murata K, Rodriguez D, Rodriguez JR, Esteban M, Rodrigues MM, et al. Quantification of antigen-specific CD8(+) T-cells using an Elispot assay. J Immunol Methods 1995;181:45–54.
[26] Vitiello A, Sette A, Yuan L, Farness P, Southwood S, Sidney J, et al. Comparison of cytotoxic T lymphocyte responses induced by peptide or DNA immunization: Implications on immunogenicity and immunodominance. Eur J Immunol 1997;27:671–8.
[27] Dorrell L, O'Callaghan CA, Britton W, Hambleton S, McMichael A, Smith GL, et al. Recombinant modified vaccinia virus Ankara efficiently restimulates human cytotoxic T lymphocytes in vitro. Vaccine 2000;19:327–36.
[28] Rizzardi GP, Tambussi G, Bart PA, Chapuis AG, Lazzarin A, Pantaleo G. Virological and immunological responses to HAART in asymptomatic therapy-naive HIV-1-infected subjects according to CD4 cell count. AIDS 2000;14:2257–63.
[29] Bart PA, Rizzardi GP, Tambussi G, Chave JP, Chapuis AG, Graziosi C, et al. Immunological and virological responses in HIV-1-infected adults at early stage of established infection treated with highly active antiretroviral therapy. AIDS 2000;14:1887–97.
[30] Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature 2001;410:106–11.
[31] Ratner L. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 1985;313:277–84.
[32] Göttlinger HG, Sodroski JG, Haseltine WA. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc Natl Acad Sci USA 1989;86:5781–5.
[33] Deml L, Schirmbeck R, Reimann J, Wolf H, Wagner R. Recombinant human immunodeficiency Pr55(Gag) virus-like particles presenting chimeric envelope glycoproteins induce cytotoxic T-cells and neutralizing antibodies. Virology 1997;235:26–39.
[34] zur Megede J, Chen MC, Doe B, Schaefer M, Greer CE, Selby M, et al. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. J Virol 2000;74:2628–35.
[35] Bojak A, Wild J, Deml L, Wagner R. Impact of codon usage modification on T-cell immunogenicity and longevity of HIV-1 gag-specific DNA vaccines. Intervirology 2002;45:275–86.
[36] Liu WJ, Gao F, Zhao KN, Zhao W, Fernando GJ, Thomas R, et al. Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. Virology 2002;301:43–52.
[37] Belyakov IM, Earl P, Dzutsev A, Kuznetsov VA, Lemon M, Wyatt LS, et al. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc Natl Acad Sci USA 2003;100:9458–63.
[38] Firat H, Tourdot S, Ureta-Vidal A, Scardino A, Suhrbier A, Buseyne F, et al. Design of a polyepitope construct for the induction of HLA-A0201-restricted HIV 1-specific CTL responses using HLA-A*0201 transgenic, H-2 class IKO mice. Eur J Immunol 2001;31:3064–74.
[39] Ureta-Vidal A, Firat H, Perarnau B, Lemonnier FA. Phenotypical and functional characterization of the CD8+ T-cell repertoire of HLA-A2.1 transgenic* H-2KbnullDbnull double knockout mice. J Immunol 1999;163:2555–60.
[40] Goulder PJR, Altfeld MA, Rosenberg ES, Nguyen T, Tang YH, Eldridge RL, et al. Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. J Exp Med 2001;193:181–93.
[41] Koenig S, Conley AJ, Brewah YA, Jones GM, Leath S, Boots LJ, et al. Transfer of Hiv-1-Specific Cytotoxic T-Lymphocytes to an Aids Patient Leads to Selection for Mutant Hiv Variants and Subsequent Disease Progression. Nat Med 1995;1:330–6.
[42] Loirat D, Lemonnier FA, Michel ML. Multiepitopic HLA-A*0201-restricted immune response against hepatitis B surface antigen after DNA-based immunization. J Immunol 2000;165:4748–55.