Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com
ScienceDirect
Vaccine 24 (2006) 6356–6365
Vaccine
www.elsevier.com/locate/vaccine

Overlapping synthetic peptides as vaccines

Shisong Jiang [a,b,1], Ruijiang Song [a,b], Sergei Popov [a,b], Saied Mirshahidi [a,b], Ruth M. Ruprecht [a,b,*]

[a] *Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA 02115, USA*
[b] *Harvard Medical School, Boston, MA 02115, USA*

Received 16 December 2005; received in revised form 13 April 2006; accepted 20 April 2006
Available online 5 June 2006

Abstract

Several vaccine strategies aim to generate cell-mediated immunity (CMI) against microorganisms or tumors. While epitope-based vaccines offer advantages, knowledge of specific epitopes and frequency of major histocompatibility complex (MHC) alleles is required. Here we show that using promiscuous overlapping synthetic peptides (OSP) as immunogens generated peptide-specific CMI in all vaccinated outbred mice and in different strains of inbred mice; CMI responses also recognized viral proteins. OSP immunogens also induced CMI ex vivo in dendritic cell/T-cell cocultures involving cells from individuals with different HLA haplotypes. Thus, broad CMI was induced by OSP in different experimental settings, using different immunogens, without identifying either epitopes or MHC backgrounds of the vaccinees.

Keywords: Overlapping synthetic peptides; Cell-mediated immunity; Vaccine

1. Introduction

Viral infections remain a major public health concern, and the recent emergence of new human pathogens, including severe acute respiratory syndrome (SARS) and avian influenza viruses, reinforces the need to develop rapid methodologies to identify appropriate vaccines for the epidemics [1,2]. The identification of cancer antigens capable of stimulating CMI has inspired the development of anti-tumor vaccines; considerable progress has been made towards the development of preventative and therapeutic cancer vaccines [3–6]. The efficacy of vaccines based upon epitopic T-cell peptides in melanoma patients has been evaluated [7–10], and immunization with peptides or adoptive transfer of peptide-pulsed dendritic cells (DC) have generated some favorable results.

CMI is an important immune mechanism to contain infections caused by many viruses, such as herpes simplex virus (HSV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and human immunodeficiency virus (HIV) [11,12]. Both $CD4^+$ and $CD8^+$ T lymphocytes, which generally function as T helper (Th) or cytotoxic T lymphocytes (CTL), respectively, recognize viral antigens through interactions between T-cell receptors (TCR) and viral peptides displayed by host major histocompatibility complex (MHC) molecules. Unlike B cells, which react to the stimulation of intact antigenic proteins, CTL do not directly recognize such antigens [13,14]. To generate CTL responses, antigens need to be present in the cytoplasmic compartment of antigen-presenting cells (APC). There, antigens are degraded by proteasomes and aminopeptidases into peptides that subsequently bind to MHC class I molecules [15–17]. For the purpose of delivering antigens to the cytoplasmic compartment, most current candidate AIDS vaccines employ live attenuated vectors (e.g. canary pox, recombinant modified vaccinia virus Ankara (MVA) or adenovirus) or plasmid DNA encoding viral gene products [18,19]. These strategies are limited for the following reasons: for live vector vaccines, immune responses against

* Corresponding author. Tel.: +1 617 632 3719; fax: +1 617 632 3112.
E-mail address: ruth_ruprecht@dfci.harvard.edu (R.M. Ruprecht).
[1] Present address: MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, UK.

0264-410X/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2006.04.070

vector components may dominate the immune responses and thus dampen the immunogenicity of inserts [3,19]. Some live vectors may be problematic in immunocompromised individuals [20]. DNA vaccines alone, although they generated strong CTL responses in mice, were less effective in higher animals [21].

Epitope-based peptide vaccines and DNA vectors encoding peptides based upon known T-cell epitopes were also effective in inducing CTL responses [22,23]. Because of MHC restriction, the breadth of such responses may be narrow, which may favor viral escape variants. Using a DNA prime/MVA boost strategy, a single epitope vaccine was tested in rhesus monkeys [24]. Although this vaccine strategy generated strong CTL activity, it failed to protect the monkeys against SIV challenge [24]. To broaden CTL responses, multi-epitope vaccines are being developed by a number of groups [25–30]. One such multi-CTL epitope vaccine, which is intended for a DNA prime/MVA boost strategy, consists of a pool of CTL epitopes from HIV clade A Gag p24/p17. In mouse and monkey immunogenicity studies and subsequently in human clinical trials, specific CMI, as measured by interferon-γ (IFN-γ-specific enzyme-linked immunospot (ELISPOT) analysis, was induced [28,31–37]. Designing epitope-based vaccines for a general population is a labor-intensive process involving assessment of the frequency of MHC types and identification of T-cell epitopes [38–41]. Bearing in mind the number of MHC alleles within a population and the number of proteins encoded by a given virus, it would be difficult to identify most CTL epitopes for most MHC alleles. When coping with an emerging epidemic caused by a new microorganism, such an approach may be impractical.

Here we present a novel vaccine strategy utilizing promiscuous OSP to generate antigen-specific CMI in individual vaccine recipients with different MHC backgrounds. OSP represent sequential stretches of 15–20 amino acids (aa) of a known viral or tumor antigen (Fig. 1); a given peptide within the OSP pool overlaps with its neighboring peptides by several aa. Historically, OSP have been used to identify CTL and Th epitopes in vitro. OSP can contain both CTL and Th epitopes. As peptides, they may be bound directly by MHC molecules expressed on the cell surfaces or ingested and processed by APC. Furthermore, OSP are promiscuous in that they contain multiple epitopes that can bind to different MHC alleles. As such, OSP vaccines could be designed rapidly as neither epitopes nor MHC alleles need to be identified specifically; only the aa sequences of viral protein(s) need to be known.

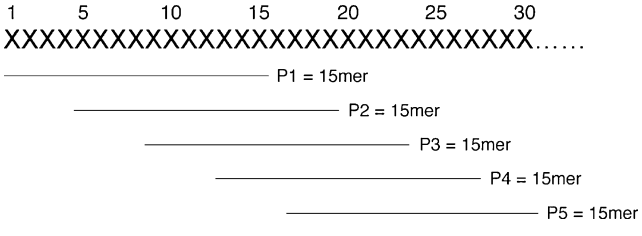

Fig. 1. Schematic map of overlapping synthetic peptides (OSP). The first 30 amino acids (aa) of a protein sequence are shown, in which X represents any one of 20 essential aa. Individual peptides (Pn) were made to be 15 aa in length (15 mer) and to overlap with their neighboring peptides by 11 aa.

2. Materials and methods

2.1. Peptides

The following peptides were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: OSP HIV Gag Consensus B (OSP-Gag_{conB}) consisted of a group of 123 peptides of 15 aa in length, with 11 aa overlaps between sequential peptides and spanning the entire HIV consensus B Gag sequence. Peptides were >80% pure. OSP HIV_{MN} Env (OSP-Env_{MN}) consisted of a series of 212 peptides comprising the HIV_{MN} Env region. Most of the peptides were 15 aa in length, with 11-aa overlaps between sequential peptides. Most peptides were >80% pure. OSP HIV Gag IIIB (OSP-Gag_{IIIB}) consisted of a series of 122 peptides of 15 aa in length, with 11-aa overlap between sequential peptides. OSP SIVmac239 Env (OSP-Env_{SIV}) consisted of a series of 87 peptides of 20 aa in length, with 10-aa overlaps between sequential peptides. Peptides were >80% pure. The P7G peptide (AMQMLKETI) and the envelope V3 peptide from HIV clade C (GPGQAFYAT) were synthesized at the Molecular Biology Core Facility of the Dana-Farber Cancer Institute (DFCI, Boston, MA). Both peptides were >97% pure.

2.2. Mice and immunization

Randomly bred, outbred NMRI mice (Taconic Farms, NY) were immunized subcutaneously (s.c.) with OSP-Gag_{conB} or OSP-Env_{MN}, at 5 μg of each individual peptide in 100 μl PBS per mouse together with MLP + TDM Adjuvant System (Sigma, St. Louis, MO) three times at 3-week intervals. Control groups were only given adjuvant or PBS. Another 3 week after the last boost, splenocytes were collected and IFN-γ-specific ELISPOT assays and intracellular staining for IFN-γ were performed.

Inbred mice BALB/c (H-2^d) and C57BL/6 (H-2^b) (Taconic Farms, NY) were immunized with OSP-Gag_{IIIB} at 5 μg of each individual peptide in 100 μl PBS per mouse together with MLP + TDM Adjuvant System (Sigma, St. Louis, MO). Control mice were only given adjuvant. Immunizations were given at weeks 0, 3, and 6. Three weeks after the last immunization, mice were sacrificed for CTL and T-helper cell proliferation assays.

Animal experiments were approved by the Animal Care and Use Committee at DFCI. The facilities at DFCI are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Procedures complied with the US Government Principles for

the Utilization and Care (Use) of Vertebrate Animals Use in Testing, Research, and Training, the Guide for the Care and Use of Laboratory Animals, the provisions of the Animal Welfare Act, and other applicable laws and regulations.

2.3. Isolation and differentiation of blood dendritic cells from normal human donors

Leukopacks were provided by anonymous, normal blood donors at the DFCI Blood Bank (Boston, MA). MHC tissue-typing was performed at Brigham and Women's Hospital (Boston, MA). DC were isolated and differentiated from peripheral blood mononuclear cells (PBMC), which were incubated in a plastic cell-culture flask for 2 h at 5% CO_2 and 37 °C. Adherent cells were collected and incubated in complete RPMI supplemented with interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Stem Cell Technology, Vancouver, Canada) (DC medium). An additional 2 ml of DC medium was added to the culture each day. On day 6, detached cells were collected and transferred into a new flask with fresh DC medium. DC were pulsed overnight with OSP-Env_{SIV}, followed by irradiation at 25 Grey (2500 rad) and used to generate CTL ex vivo by three stimulations of autologous PBL at weekly intervals. CTL assays were performed 1 week after the last stimulation.

2.4. Mouse CTL assay

Effector cells consisted of splenic mononuclear cells that were isolated from OSP- or adjuvant-only immunized mice and restimulated (2×10^6/ml) in vitro with 1 μM peptide for 7–10 days. Target cells were P815 cells (H-2^d, for BALB/c mice) or EL-4 cells (H-2^b, for B57BL/6 mice). Target cells were labeled with ^{51}Cr (70 μCi/2×10^6 cells; Perkin-Elmer, Boston, MA) and pulsed overnight with or without OSP-Gag_{IIIB} (1 μM), or infected overnight with vaccinia virus [two plaque forming units (pfu)/target cell] expressing HIV Gag_{IIIB} (NIH AIDS Research and Reference Reagent Program), or wild-type vaccinia virus (Therion, Cambridge, MA).

In the case of H-2^d restricted CTL, P7G (AMQMLKETI) [42], the known CTL epitope within HIV p24, was included to test if OSP-Gag_{IIIB} could generate P7G-specific (H-2^d restricted) CTL in BALB/c mice. A non-epitopic peptide, HIV clade C Env V3 peptide (GPGQAFYAT), was used as negative control.

Effector and target cells were cocultured at different ratios for 6 h, and cytolysis was determined by ^{51}Cr release. The percentage specific ^{51}Cr release was calculated as: $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton-X 100. Spontaneous release was determined from the target cells incubated without addition of effector cells.

2.5. CTL assays with human cells

Effector cells consisted of PBL stimulated with irradiated autologous DC that had been pulsed with or without OSP. Target cells were EBV-transformed, autologous B cell lines. These cells were labeled with ^{51}Cr (70 μCi/2×10^6 cells; Perkin-Elmer, Boston, MA) and pulsed overnight with or without OSP-SIV_{Env} (1 μM), or infected overnight with vaccinia virus (two plaque forming units (pfu)/target cell) expressing SIV *gag-pol-env*, or wild-type vaccinia virus (Therion). Effector and target cells were cocultured and the percentage specific ^{51}Cr release was calculated as mentioned above for mouse CTL assays.

2.6. Intracytoplasmic cytokine staining and flow cytometry analysis

Mouse splenocytes were cultured at 5×10^6 cells/ml with IL-2 (20 U/ml) with or without 1 μM OSP in 24-well culture plates for 6 h. Four hours before harvesting, cells were treated with Golgistop (BD PharMingen, San Diego, CA) according to the vendor's protocol. Splenocytes were then stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (BD PharMingen) or an immunoglobulin isotype control for 20 min. Splenocytes were then subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit (BD PharMingen) and FITC-conjugated anti-IFN-γ antibody (20 μg/ml) according to the manufacturer's instructions. Samples were acquired on an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA), and data were analyzed using Expo 32 software (Beckman Coulter).

2.7. ELISPOT assay

ELISPOT assays were performed using ELISPOT kits from BioSource International (Camarillo, CA). Briefly, splenocytes were restimulated overnight with 1 μM OSP in the IFN-γ-precoated plates. Cells were discarded and biotinylated anti-IFN-y antibodies were added for 1 h at 37 °C followed by another hour of incubation at 37 °C of anti-biotin antibody labeled with enzyme. After color developed, spots were counted under a microscope. Results were expressed as SFU/10^6 cells.

2.8. Lymphocyte proliferation assay

Splenocytes were isolated and cultured at 2×10^6/ml in RPMI 1640 plus 15% FCS plus antibiotics in the presence of either HIV Gag_{IIIB} protein (15 μg/ml) (ImmunoDiagnostics Inc., Woburn, MA), HIV Env_{MN} gp120 (15 μg/ml) (ImmunoDiagnostics Inc.), OSP-Gag_{IIIB} (3 μg/ml), OSP-Env_{MN} (3 μg/ml) or OVA (15 μg/ml) for 5 days. Four hours before harvesting, cells were pulsed with 1 μCi/well of ^3H-thymidine. After cells were harvested, ^3H-thymidine incorporation was assessed using a β-counter (Beckman, Fuller-

ton, CA). Results are expressed as stimulation index (SI), i.e. the ratio of cpm of stimulated cells to cpm of cells grown in medium only.

3. Results

3.1. Vaccination with OSP induced CMI to peptides in outbred mice

First, we sought to determine if a set of OSP could generate CMI in genetically diverse mice. Outbred NMRI mice, generated from outbred Swiss mice by random breeding, provide a genetically diverse experimental group; these mice do not tolerate skin grafts from each other and express MHC molecules that are similar to H-2^q but differ between individuals of the same strain [43].

Outbred NMRI mice were immunized with OSP-Gag_{conB}, representing the consensus sequence of HIV clade B Gag, OSP-Env_{MN}, representing the aa sequence of HIV_{MN} gp120, adjuvant only, or phosphate buffered saline (PBS). Splenocytes were isolated from immunized mice and restimulated overnight in vitro with the two corresponding OSP pools. IFN-γ-specific ELISPOT assays showed that the number of spot-forming units (SFU) was higher in splenocytes from OSP-Gag_{conB}- or OSP-Env_{MN}-immunized mice when the cells were restimulated with the corresponding OSP compared to controls (Gag versus Env immunization, $P = 0.018$ and 0.0062 for Gag or Env restimulation, respectively, by Student's *t*-test) (Fig. 2(a) and (b)). SFU for individual mice of the two vaccine groups ranged between 70 and 290 SFU/10^6 splenocytes (OSP-Gag_{conB}-immunized group) and 0 and 48 SFU/10^6 cells (OSP-Env_{MN}-immunized group) after in vitro restimulation with OSP-Gag_{conB}; after restimulation with OSP-Env_{MN}, the numbers ranged between 0 and 25 SFU/10^6 cells (OSP-Gag_{conB}-immunized group) and 213 and 1210 SFU/10^6 cells (OSP-Env_{MN}-immunized group). The background, estimated as mean SFU + 2 standard deviations (S.D.) for the groups given PBS or adjuvant, was 13 SFU/10^6 cells (restimulation with OSP-Gag_{conB}) or 38 SFU/10^6 cells (restimulation with OSP-Env_{MN}). These data indicate that all five outbred mice in the two vaccine groups had generated specific CMI to their respective immunogens.

Next, intracellular staining of $CD4^+$ and $CD8^+$ T cells for IFN-γ was performed for splenocytes isolated from the same four groups of NMRI mice and restimulated in vitro with OSP-Gag_{conB}. The percentages of IFN-γ^+ cells in both the $CD8^+$ and $CD4^+$ populations from all OSP-Gag_{conB}-immunized mice were significantly higher when compared with those from mice immunized with OSP-Env_{MN} (Fig. 3(a) and (b)) ($P = 0.0002$ by Student's *t*-test). The percentages for $CD8^+$ IFN-γ^+ cells for individual mice of the two vaccine groups ranged between 2.7% and 4.01% (OSP-Gag_{conB}-immunized group) and 0.45% and 1.86% (OSP-Env_{MN}-immunized group). The background was 1.56%, calculated as the mean + 2 S.D. of $CD8^+$ IFN-γ^+ cells of individual animals given PBS or adjuvant. The percentages of $CD4^+$ IFN-γ^+ cells of individual mice in the two vaccine groups ranged between 4.66% and 5.95% (OSP-Gag_{conB}-immunized group) and 0.66 and 1.45 (OSP-Env_{MN}-immunized group); the background, calculated as above, was 0.77%. These data show that immunization with OSP induced specific T-cell responses, involving both $CD8^+$ and $CD4^+$ T cells, within each animal of a genetically diverse population of mice.

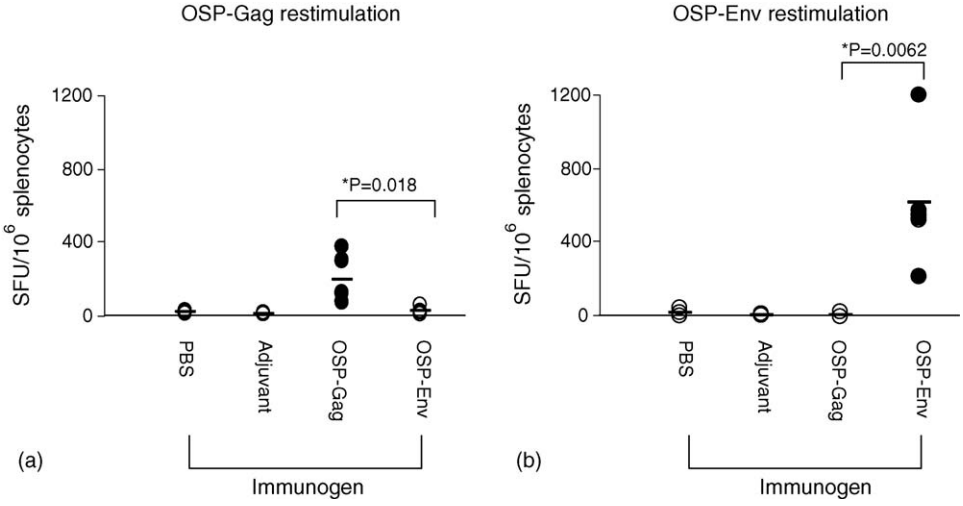

Fig. 2. IFN-γ-specific ELISPOTs of outbred NMRI mice. Four groups of five NMRI mice were immunized with OSP-Gag_{conB} (OSP-Gag), OSP-Env_{MN} (OSP-Env) (open circles), adjuvant, or phosphate-buffered saline (PBS) only. Splenocytes of individual mice were restimulated with either OSP-Gag (a: filled circles) or OSP-Env (b: filled circles) overnight. IFN-γ-secreting cells were measured by ELISPOT analysis; SFU, spot-forming units. *Indicates statistical significance in Student's *t*-test.

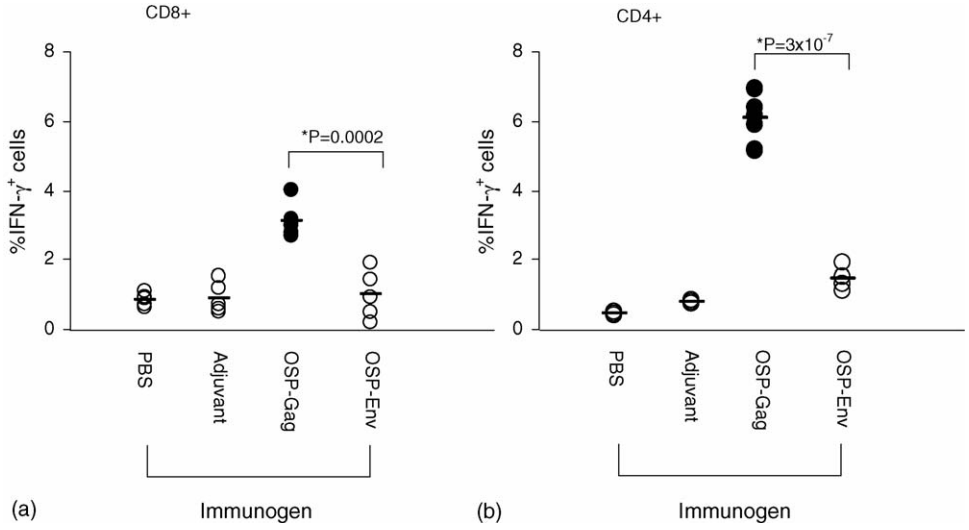

Fig. 3. OSP-Gag-specific intracellular IFN-γ staining of CD8+ and CD4+ cells. Four groups of five NMRI mice were immunized with OSP-Gag_{conB} (OSP-Gag) (dark circles), OSP-Env_{MN} (OSP-Env), adjuvant or PBS only. Splenocytes were restimulated with OSP-Gag for 6 h, stained with either anti-CD8 (a) or anti-CD4 antibodies (b) followed by staining with anti-IFN-γ antibodies. CD8 or CD4+IFN-γ+ cells were counted by flow cytometry. *Indicates statistical significance in Student's *t*-test.

3.2. Vaccination with OSP induced CMI to HIV Env protein in outbred mice

Next, we tested whether immunization with OSP could generate proliferative responses against an entire protein among the genetically diverse NMRI mice. Splenocytes were isolated from mice immunized with OSP-Env_{MN} and restimulated for 5 days in vitro with HIV_{MN} gp120 or HIV Gag protein. The splenocytes proliferated only when restimulated with Env but not with Gag (stimulation index 5.8 versus 1.9, $P = 0.0012$; Fig. 4). These results demonstrate that immunization with OSP generated specific proliferative immune responses to the whole HIV gp120.

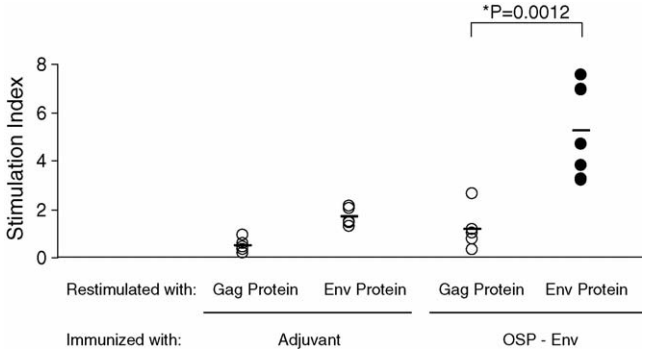

Fig. 4. OSP-Env-specific proliferative responses in outbred NMRI mice. Two groups of five outbred NMRI mice were immunized with OSP-Env or adjuvant only. Splenocytes were restimulated in vitro with Env or Gag (as negative control) proteins. Cell growth was measured by ^{3}H-thymidine incorporation. *Indicates statistical significance in Student's *t*-test.

3.3. Vaccination with OSP induced protein-specific CTL and proliferative responses in two inbred strains of mice

To test whether immunization with OSP could induce specific CTL responses to native viral proteins in mice with different MHC backgrounds, BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were immunized with OSP-Gag_{IIIB}. Splenocytes were restimulated with OSP-Gag_{IIIB} in vitro for 7 days, and ^{51}Cr release assays were performed to test for CTL activity. We observed specific lysis of target cells (P815 for BALB/c mice and EL-4 for C57BL/6 mice) infected with vaccinia virus (vv) expressing Gag_{IIIB} (vv-Gag) when compared with target cells infected with wild-type vv (Fig. 5(a) and (b)). Moreover, effector cells from BALB/c mice killed P815 cells (H-2^d) pulsed with peptide P7G, an H-$2K^d$-restricted epitope [42] (Fig. 5(c)). However, effector cells from immunized C57BL/6 did not lyse P7G-pulsed EL-4 cells (H-2^b) (Fig. 5(d)). These findings indicate that although effector cells from both mouse strains recognized and killed cells expressing the same Gag protein, the epitopes recognized by these effector cells differed, as P7G was only recognized by mice with the H-2^d background.

We next sought to test if immunization with OSP generated proliferative responses to the whole viral protein in the two inbred mouse strains. Splenocytes from BALB/c and C57BL/6 mice immunized with OSP-Gag_{IIIB} were isolated and restimulated in vitro with either soluble Gag_{IIIB} protein or OSP-Gag_{IIIB}, and proliferative responses were measured. T cells from both mouse strains proliferated in response to both stimuli but not in response to the control protein ovalbumin (OVA) (Fig. 6(a) and (b)). These findings confirm that immunization with OSP induced immune responses that cross-reacted with native viral protein.

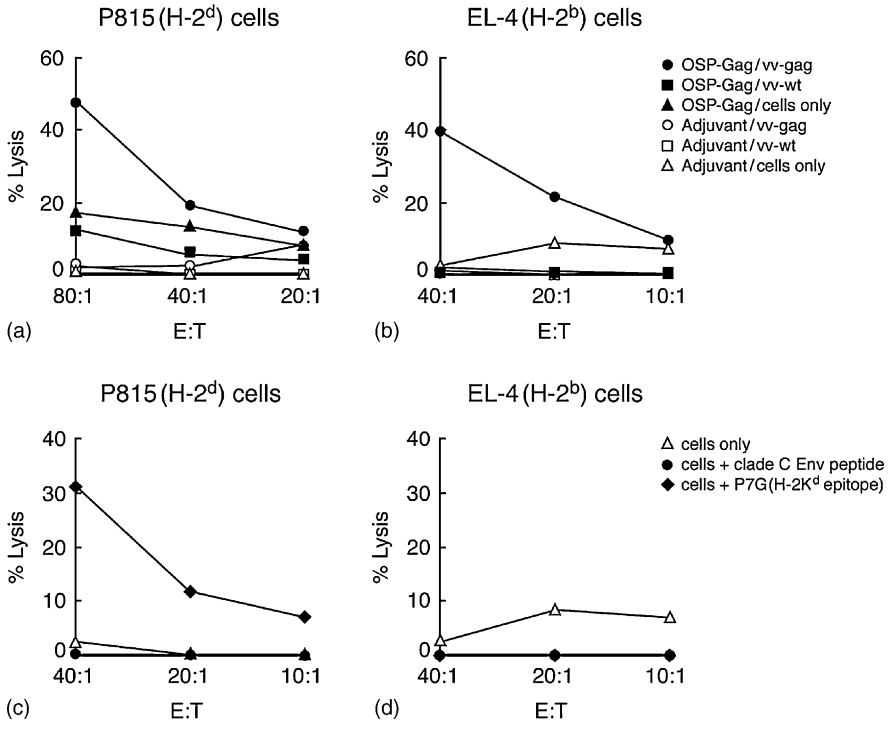

Fig. 5. Gag-specific CTL activity in inbred mouse strains. Five mice per group of BALB/c and C57BL/6 mice were immunized with OSP-Gag_{IIIB} (OSP-Gag). Pooled splenocytes were cocultured with target cells, P815 (a) or EL-4 (b), respectively, which had been infected with recombinant vaccinia virus expressing Gag (vv-gag), wild-type vaccinia virus (vv-wt) or treated with medium before cocultivation. Alternatively, P815 cells (c) and EL-4 cells (d) were also pulsed with peptide P7G, the known BALB/c HIV Gag_{IIIB} CTL epitope [42], or HIV clade C Env peptide. Specific lysis was measured by ^{51}Cr release.

3.4. Generation of specific CTL activity by OSP-pulsed human dendritic cell/T-cell cocultures ex vivo

Next, we sought to test if OSP could generate CMI in human cells of different MHC backgrounds ex vivo by stimulating human peripheral blood lymphocytes (PBL) with OSP-pulsed DC. Two leukopacks from different, anonymous donors (d#1 and d#2) were obtained and their MHC class I haplotypes [human leukocyte antigens (HLA)—A, B, C] were determined as the following:

- d#1: HLA-A (02, blank); B (08, 18); Bw4 (−,−); Bw6 (+,+); Cw (07, blank)
- d#2: HLA-A (11, 24); B (39, 51); Bw4 (−,+); Bw6 (+,−); Cw (07, 14).

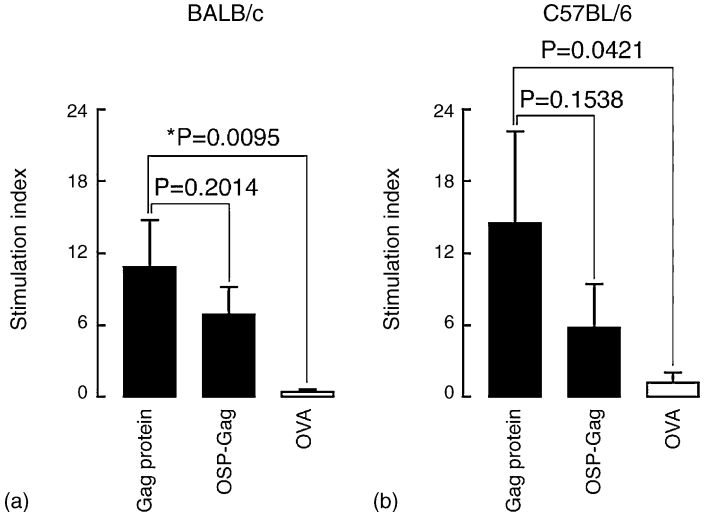

Fig. 6. Gag-specific proliferation in inbred mouse strains. Five mice per group of BALB/c and C57BL/6 mice were immunized with OSP-Gag_{IIIB} (OSP-Gag). Pooled splenocytes were restimulated in vitro with Gag protein, OSP-Gag or ovalbumin (OVA). Cell growth was measured by ^{3}H-thymidine incorporation. *Indicates statistical significance in Student's *t*-test. In (b) Statistical significance was not reached after Bonferroni correction for multiple comparisons.

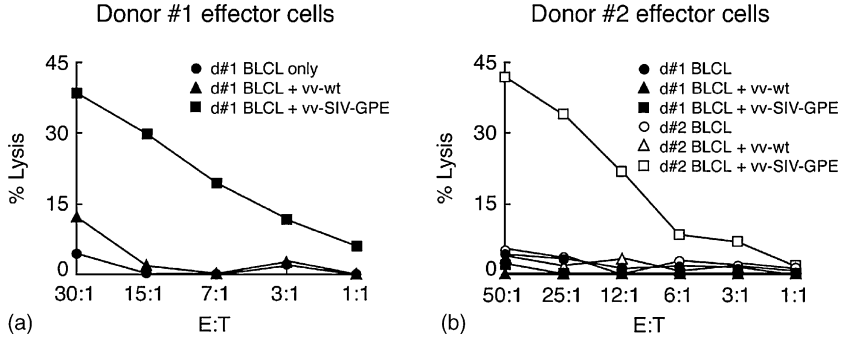

Fig. 7. SIV Env-specific CTL activity in human PBL after stimulation with OSP-pulsed DC. PBL of donor #1 (d#1, closed symbols) (a) or donor #2 (d#2, open symbols) (b) were stimulated with OSP-Env_{SIV}. Target cells (T, autologous B-lymphoblastoid cell lines, BLCL) were infected with recombinant vaccinia virus encoding SIV *gag*, *pol*, and *env* (vv-SIV-GPE), wild-type vaccinia virus (vv-wt) or treated with medium only before cocultivation with stimulated autologous effector cells (E) at various E:T ratios. Donor #2 effector cells were also mixed with donor #1 target cells infected with vv-SIV-GPE, vv-wt or medium only (b).

PBL were separated and cocultured for three rounds in vitro with irradiated autologous DC pulsed with or without OSP-SIV_{mac239} Env; chromium release assays were performed 1 week after the last stimulation. Effector cells from both d#1 and d#2 showed higher lysis of target cells (autologous B-lymphoblastoid cell lines (BLCL)) infected with vv expressing SIV-Gag/Pol/Env (vv-SIV-GPE) when compared to BLCL infected with wild-type vv (Fig. 7(a) and (b)). To determine if the lysis of d#1 and d#2 target cells was MHC restricted, effector cells from d#2 were cocultured with target cells from d#1. Chromium release assay result showed no recognition and killing between d#1 and d#2 (Fig. 6(b)). These results indicate that OSP-pulsed autologous DC were able to induce MHC-restricted, protein-specific CTL activity ex vivo in PBL.

4. Discussion

To be recognized by $CD4^+$ and $CD8^+$ T cells, antigenic proteins have to be degraded into peptide fragments, which contain the epitopes and which are bound to MHC class I or II molecules on the cell surface [15–17]. Early studies have shown that an intact viral protein detectable at the cell surface could not be recognized by CTL [13]; this was overcome by enhanced degradation of the antigen [14]. In a recent study [44], mice immunized with soluble antigen (HIV gp160) alone had neither detectable specific proliferative responses after stimulation with whole protein nor IFN-γ ELISPOTs in response to stimulation with recombinant, *env*-expressing vaccinia virus, indicating the weakness of cellular immune responses induced by soluble protein immunogens. In these vaccinated mice, moderate IFN-γ ELISPOT responses were only seen after specific stimulation with the dominant peptide epitope [44].

To bypass the constraints in the degradation efficiency of an intact, exogenous antigenic protein, we have tested the use of OSP as a vaccine strategy in the present study. OSP have long been used in assays in vitro to identify $CD4^+$ and $CD8^+$ T-cell epitopes. In our current proof-of-concept study, we have shown that OSP can be used as broadly active immunogens: immunization with OSP generated both specific $CD4^+$ and $CD8^+$ T-cell responses in outbred mice, and T cells proliferated also in response to restimulation with soluble, whole protein. In addition, OSP immunization stimulated specific CTL activity in different inbred mouse strains against vaccinia virus expressing the whole protein as well as an immunodominant epitope. These vaccinated animals also developed proliferative responses to OSP as well as native protein. Lastly, specific CMI could be induced by OSP immunization ex vivo using cocultivation of OSP-pulsed DC with autologous PBL from two different human donors. Thus, OSP were immunogenic in vaccines of different MHC backgrounds.

Several mechanisms of how OSP vaccination generated specific CTL in our experiments could have been operative. While CTL epitopes usually consist of peptides of 8–9 aa in length, peptides longer than 13 aa can also function in this capacity and bind to MHC class I molecules directly [45–53]. In contrast, peptides exceeding epitope length have been shown to enter cells, undergo processing by proteasomes or other enzymes and bind to MHC class I molecules [15,16,52,54]. Alternatively, the OSP immunogens in our experiments may have also been trimmed extracellularly by peptidases followed by direct binding to the MHC molecules on APC. We used OSP 15 or 20 aa long, which probably exceeded the length of conventional CTL epitopes. Based on the published reports [52,54], we postulate that the longer OSP underwent intracellular or extracellular processing. Indeed, we have indirect evidence that this may be the case. OSP-Gag_{IIIB}-immunized BALB/c mice mounted specific CTL activity against P7G, a known virus-specific epitope restricted by $H\text{-}2^d$ with the 9-aa sequence AMQMLKETI. OSP-Gag_{IIIB} included two 15-mer peptides that contained the P7G sequence, peptide 5033 (GHQAAMQMLKETINE) and peptide 5034 (AMQMLKETINEEAAE). It is likely that at least these two peptides were processed, bound to $H\text{-}2^d$ molecule and eventually stimulated virus-specific CTL. This might be an advantage over a single epitope-based vaccine because the chances of a specific epitope in the OSP to

be presented seem higher than in the single epitope-based vaccine.

Usually MHC class II molecules bind epitopes of 9–15 aa in length or longer. Contrary to MHC class I molecules for which epitope binding is confined to the cleft, MHC class II molecules can bind longer peptides the ends of which may protrude from the MHC binding cleft. However, peptides exceeding the MHC class II epitope length will be processed, either in a "cut/trim first, bind later" or "bind first, cut/trip later" model [55]. In our case, some OSP were 15 aa long and could be easily bound to MHC class II molecules to activate Th cells, which are important for maintaining CTL.

DC vaccines, which are formed by pulsing autologous DC ex vivo with various immunogens, such as tumor lysates, RNA or peptides, have great potential in priming and stimulating T cells. A number of clinical trials currently explore the safety and immunogenicity of DC vaccines [56]. It has been shown that addition of elements containing T-helper epitopes enhances the efficacy of DC-based vaccines [57]. Our OSP contained epitopes for both CD8 and CD4 T cells and therefore served as effective immunogens for the DC-based vaccine strategy we tested ex vivo. Our data showed that OSP-pulsed autologous human DC could prime CTL activity against a viral protein ex vivo in PBL. These immunogenicity experiments succeeded in cells of different HLA backgrounds, thus providing proof-of-concept data that such ex vivo immunogenicity strategies may eventually be developed in the future.

One of the potential advantages of OSP vaccines is the high number of epitopes included. In theory, OSP immunogens should include all possible epitopes of the corresponding full-length protein, including epitopes for both $CD4^+$ and $CD8^+$ T cells. Indeed, our results have shown that OSP vaccination induced both $CD4^+$ and $CD8^+$ T-cell responses in all outbred mice in our trial.

The other advantage is that OSP vaccines are promiscuous as they contain all peptide sequences of their corresponding protein, including epitopes for different MHC alleles. Consequently, these vaccines have broad immunogenicity in vaccine recipients with diverse genetic backgrounds, as our results in outbred and different strains of inbred mice have shown. Similar to epitope-based vaccines, OSP immunogens contain only "viral elements," or in the case of cancer vaccines, "oncogene elements", such that generation of non-related immunity, e.g. immunity against vectors, can be avoided. In fact, we have generated specific cellular immunity against a novel cellular oncogene in mice using the OSP vaccination approach. In a pilot experiment, vaccinated mice had a statistically significant delay in tumor growth compared to controls (Mirshahidi et al., unpublished data). Furthermore, OSP are not expected to pose any safety risks, in contrast to concerns that might arise with some live vaccine vectors. Thus, preclinical and clinical evaluations can be conducted with a single mixture of OSP for a given clinical indication.

OSP vaccines have also advantages of other peptide vaccines such as: they can be made in large quantities; they are stable and easily transportable. However, lowering the production cost of OSP vaccines remains a challenge for the future.

In summary, we have demonstrated that the use of OSP can overcome the need to identify individual epitopes and to match MHC types. Moreover, OSP generated both CTL and Th responses in a broad population of vaccine recipients. Thus, the OSP vaccines hold promise as a novel CMI-based vaccine strategy against viral infections and possibly also malignancies.

Acknowledgements

We thank Dr. Glenn Dranoff (Dana-Farber Cancer Institute) for his critical reading of this manuscript and Barbara Fanning and Susan Sharp for their assistance in the preparation of this manuscript. Supported by National Institutes of Health grants RO1 AI43839 and PO1 AI48240 to R.M.R. and Center for AIDS Research core grant IP30 28691 awarded to the Dana-Farber Cancer Institute as support for the Institute's AIDS research efforts and by the Claudia Adams Barr Program in Innovative Basic Cancer Research to S.M.

References

[1] Pearson H, Clarke T, Abbott A, Knight J, Cyranoski D. SARS: what have we learned? Nature 2003;424(6945):121–6.
[2] Normile D. Avian influenza. Evidence points to migratory birds in H5N1 spread. Science 2006;311(5765):1225.
[3] Berzofsky JA, Terabe M, Oh S, Belyakov IM, Ahlers JD, Janik JE, et al. Progress on new vaccine strategies for the immunotherapy and prevention of cancer. J Clin Invest 2004;113(11):1515–25.
[4] Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. Science 2004;305(5681):200–5.
[5] Pardoll DM. Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol 2002;2(4):227–38.
[6] Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature 2001;411(6835):380–4.
[7] Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4(3):321–7.
[8] Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4(3):328–32.
[9] Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 2001;61(17):6451–8.
[10] Palucka AK, Dhodapkar MV, Paczesny S, Burkeholder S, Wittkowski KM, Steinman RM, et al. Single injection of CD34+ progenitor-derived dendritic cell vaccine can lead to induction of T-cell immunity in patients with stage IV melanoma. J Immunother 2003;26(5):432–9.
[11] Gandhi RTW. B. D. Immunologic control of HIV-1. Annu Rev Med 2002;53:149–72.
[12] McMichael AJ. HIV vaccines. Annu Rev Immunol 2006;24:227–55.
[13] Coupar BE, Andrew ME, Both GW, Boyle DB. Temporal regulation of influenza hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. Eur J Immunol 1986;16(12):1479–87.

[14] Townsend A, Bastin J, Gould K, Brownlee G, Andrew M, Coupar B, et al. Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. J Exp Med 1988;168(4):1211–24.
[15] Rock KL, York IA, Saric T, Goldberg AL. Protein degradation and the generation of MHC class I-presented peptides. Adv Immunol 2002;80:1–70.
[16] Kessler BM, Glas R, Ploegh HL. MHC class I antigen processing regulated by cytosolic proteolysis-short cuts that alter peptide generation. Mol Immunol 2002;39(3/4):171–9.
[17] Townsend AR, Gotch FM, Davey J. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. Cell 1985;42(2):457–67.
[18] Peiperl L. HIV vaccines in development, 2004. http://chi.ucsf.edu/vaccines.
[19] McMichael AJ, Hanke T. HIV vaccines 1983–2003. Nat Med 2003; 9(7):874–80.
[20] NIAID. Virus vectors for AIDS vaccines, 1998.
[21] Doria-Rose NA, Ohlen C, Polacino P, Pierce CC, Hensel MT, Kuller L, et al. Multigene DNA priming-boosting vaccines protect macaques from acute $CD4^+$-T-cell depletion after simian-human immunodeficiency virus SHIV89.6P mucosal challenge. J Virol 2003;77(21):11563–77.
[22] Hanke T, Samuel RV, Blanchard TJ, Neumann VC, Allen TM, Boyson JE, et al. Effective induction of simian immunodeficiency virus-specific cytotoxic T lymphocytes in macaques by using a multiepitope gene and DNA prime-modified vaccinia virus Ankara boost vaccination regimen. J Virol 1999;73(9):7524–32.
[23] Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr Opin Immunol 2003;15(4):461–70.
[24] Allen TM, Jing P, Calore B, Horton H, O'Connor DH, Hanke T, et al. Effects of cytotoxic T lymphocytes (CTL) directed against a single simian immunodeficiency virus (SIV) Gag CTL epitope on the course of SIVmac239 infection. J Virol 2002;76(20):10507–11.
[25] Wilson CC, McKinney D, Anders M, MaWhinney S, Forster J, Crimi C, et al. Development of a DNA vaccine designed to induce cytotoxic T lymphocyte responses to multiple conserved epitopes in HIV-1. J Immunol 2003;171(10):5611–23.
[26] Le TT, Drane D, Malliaros J, Cox JC, Rothel L, Pearse M, et al. Cytotoxic T cell polyepitope vaccines delivered by ISCOMs. Vaccine 2001;19(32):4669–75.
[27] Belyakov IM, Derby MA, Ahlers JD, Kelsall BL, Earl P, Moss B, et al. Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc Natl Acad Sci USA 1998;95(4):1709–14.
[28] 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(9):951–5.
[29] Mortara L, Gras-Masse H, Rommens C, Venet A, Guillet JG, Bourgault-Villada I. Type 1 CD4(+) T-cell help is required for induction of antipeptide multispecific cytotoxic T lymphocytes by a lipopeptidic vaccine in rhesus macaques. J Virol 1999;73(5):4447–51.
[30] Andrieu M, Desoutter JF, Loing E, Gaston J, Hanau D, Guillet JG, et al. Two human immunodeficiency virus vaccinal lipopeptides follow different cross-presentation pathways in human dendritic cells. J Virol 2003;77(2):1564–70.
[31] Hanke T, Blanchard TJ, Schneider J, Ogg GS, Tan R, Becker M, et al. Immunogenicities of intravenous and intramuscular administrations of modified vaccinia virus Ankara-based multi-CTL epitope vaccine for human immunodeficiency virus type 1 in mice. J Gen Virol 1998;79(Pt 1):83–90.
[32] Hanke T, Blanchard TJ, Schneider J, Hannan CM, Becker M, Gilbert SC, et al. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. Vaccine 1998;16(5):439–45.
[33] Hanke T, Schneider J, Gilbert SC, Hill AV, McMichael A. DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum*: immunogenicity in mice. Vaccine 1998;16(4):426–35.
[34] Hanke T, Neumann VC, Blanchard TJ, Sweeney P, Hill AV, Smith GL, et al. Effective induction of HIV-specific CTL by multi-epitope using gene gun in a combined vaccination regime. Vaccine 1999;17(6):589–96.
[35] Hanke T, McMichael A. Pre-clinical development of a multi-CTL epitope-based DNA prime MVA boost vaccine for AIDS. Immunol Lett 1999;66(1–3):177–81.
[36] Mwau M, Cebere I, Sutton J, Chikoti P, Winstone N, Wee EG, et al. A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans. J Gen Virol 2004;85(Pt 4):911–9.
[37] Peter K, Men Y, Pantaleo G, Gander B, Corradin G. Induction of a cytotoxic T-cell response to HIV-1 proteins with short synthetic peptides and human compatible adjuvants. Vaccine 2001;19(30):4121–9.
[38] Paschen A, Eichmuller S, Schadendorf D. Identification of tumor antigens and T-cell epitopes, and its clinical application. Cancer Immunol Immunother 2004;53(3):196–203.
[39] Singh-Jasuja H, Emmerich NP, Rammensee HG. The Tubingen approach: identification, selection, and validation of tumor-associated HLA peptides for cancer therapy. Cancer Immunol Immunother 2004;53(3):187–95.
[40] Novitsky V, Smith UR, Gilbert P, McLane MF, Chigwedere P, Williamson C, et al. Human immunodeficiency virus type 1 subtype C molecular phylogeny: consensus sequence for an AIDS vaccine design? J Virol 2002;76(11):5435–51.
[41] Ertl HC, Dietzschold B, Gore M, Otvos Jr L, Larson JK, Wunner WH, et al. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J Virol 1989;63(7):2885–92.
[42] Doe B, Walker CM. HIV-1 p24 Gag-specific cytotoxic T-lymphocyte responses in mice. Aids 1996;10(7):793–4.
[43] Costagliola S, Rodien P, Many MC, Ludgate M, Vassart G. Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. J Immunol 1998;160(3):1458–65.
[44] Rasmussen RA, Ong H, Kittel C, Ruprecht CR, Ferrantelli F, Hu S, et al. DNA prime/protein boost immunization against HIV clade C: safety and immunogenicity in mice. Vaccine 2006;24(13):2324–32.
[45] Urban RG, Chicz RM, Lane WS, Strominger JL, Rehm A, Kenter MJ, et al. A subset of HLA-B27 molecules contains peptides much longer than nonamers. Proc Natl Acad Sci USA 1994;91(4):1534–8.
[46] Gromme M, Uytdehaag FG, Janssen H, Calafat J, van Binnendijk RS, Kenter MJ, et al. Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci USA 1999;96(18):10326–31.
[47] Horig H, Young AC, Papadopoulos NJ, DiLorenzo TP, Nathenson SG. Binding of longer peptides to the H-2Kb heterodimer is restricted to peptides extended at their C terminus: refinement of the inherent MHC class I peptide binding criteria. J Immunol 1999;163(8):4434–41.
[48] Stryhn A, Pedersen LO, Holm A, Buus S. Longer peptide can be accommodated in the MHC class I binding site by a protrusion mechanism. Eur J Immunol 2000;30(11):3089–99.
[49] Speir JA, Stevens J, Joly E, Butcher GW, Wilson IA. Two different, highly exposed, bulged structures for an unusually long peptide bound to rat MHC class I RT1-Aa. Immunity 2001;14(1):81–92.
[50] Jiang S, Borthwick NJ, Morrison P, Gao GF, Steward MW. Virus-specific CTL responses induced by an H-2K(d)-restricted, motif-negative 15-mer peptide from the fusion protein of respiratory syncytial virus. J Gen Virol 2002;83(Pt 2):429–38.
[51] Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 1991;351(6324):290–6.
[52] Gnjatic S, Atanackovic D, Matsuo M, Jager E, Lee SY, Valmori D, et al. Cross-presentation of HLA class I epitopes from exoge-

nous NY-ESO-1 polypeptides by non-professional APCs. J Immunol 2003;170(3):1191–6.
[53] Rock KL. A new foreign policy: MHC class I molecules monitor the outside world. Immunol Today 1996;17(3):131–7.
[54] Seifert U, Maranon C, Shmueli A, Desoutter JF, Wesoloski L, Janek K, et al. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. Nat Immunol 2003;4(4):375–9.
[55] Sercarz EE, Maverakis E. Mhc-guided processing: binding of large antigen fragments. Nat Rev Immunol 2003;3(8):621–9.
[56] Ridgway D. The first 1000 dendritic cell vaccines. Cancer Invest 2003;21(6):873–86.
[57] Shimizu K, Thomas EK, Giedlin M, Mule JJ. Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein. Cancer Res 2001;61(6):2618–24.