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Impact of Codon Usage Modification on T Cell Immunogenicity and Longevity of HIV-1 Gag-Specific DNA Vaccines

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Key Words
HIV · Synthetic genes · DNA vaccine · T cell immunogenicity

Abstract
In this study, we analyzed the in vitro expression, potency and longevity of immune responses induced in a Balb/c mouse model by a synthetic HIV-1 *gag* gene exhibiting a codon usage that was adapted to that of highly expressed mammalian genes *(syngag).* In contrast to a vector containing the wild-type (wt) *gag* gene, the syngag construct enabled highly efficient Gag expression in both human and rodent cell lines in complete absence of Rev and Rev-responsive element. Immunization of Balb/c mice with the wt gag plasmid DNA induced only weak and inconsistent humoral immune responses. Mice vaccinated by syngag but not wt gag developed substantial and highly consistent Gag-specific antibody titers showing a clear T helper 1 polarization even with low doses of DNA. Moreover, vaccinated mice developed a strong Gag-specific cellular immune response, including cytotoxic T cells, which was not observed in wt gag-immunized animals. Both humoral and cellular immunity were efficient and lasted for more than 20 weeks. Furthermore, the induction of the humoral as well as the cellular immune response was independent of the immunization route (intramuscular or subcutaneous). These results clearly show the advantages of codon-optimized genes with respect to the expression and immunogenicity of plasmid DNA constructs, making them promising vaccine candidates for further studies.

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Introduction

Infection of humans by human immunodeficiency virus type-1 (HIV-1) leads in the vast majority of reported cases to immunodeficiency, severe clinical symptoms and death often within less than 10 years. The development of a prophylactic and therapeutic HIV-1 vaccine therefore remains one of the most desirable objectives of research aimed at controlling the current AIDS epidemic. Abundant clinical evidence suggests that, besides neutralizing antibodies, other effectors of the cellular immune response such as cytotoxic T lymphocytes (CTLs) may be of importance in controlling HIV-1 infection [1–3].

A strong antiviral cytotoxic activity has been shown to correlate temporally with the clearance of viremia in primary infection [3–6], and it could be further demonstrated that Gag-specific T helper cell and CTL responses

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correlates inversely with the viral load [7, 8]. In addition, a decline of Gag-specific CTL precursors was shown to coincide with a drop in CD4, increasing viral load and disease progression in chronically infected persons [9]. Furthermore, evidence from studies in long-term nonprogressing individuals suggests that the quality of epitope recognition by the individual's CTLs may account for a long-lasting control of viral replication [10, 11]. In this regard, Gag-derived immunogens seem to be very useful for vaccine development and immunotherapeutic interventions because the protein is relatively conserved among diverse HIV-1 subtypes, and broad cross-clade CTL recognition directed against Gag-specific targets has been documented [12–14].

Over the last decade, the direct injection of naked DNA has been shown to be a promising approach to inducing humoral and cellular immune responses [15–17]. There seem to be potential advantages of using plasmid DNA for immunization compared to traditional protein vaccination due to the induction of strong T helper 1 (Th1) and CTL responses, prolonged antigen expression and long-lived effector activity [17–19]. Plasmids expressing nonoptimized HIV-1-derived genes have recently been shown to induce humoral and cellular immune responses in rodents [16, 18], nonhuman primates [20–22] and in phase I studies in humans [23, 24]. However, in most of these studies, both the titers of induced antibodies and specific CTLs were transient and low. Several factors are suggested to be essential for the efficacy of a DNA expression vector, e.g. the quality of foreign gene expression unit, composition of DNA backbone and gene-regulatory elements [17]. It has also been shown that the route and method of immunization can be important modulators of DNA vaccination [17, 25].

We recently reported the construction of a synthetic *gag* gene exhibiting a codon usage that was adapted to that of highly expressed mammalian genes. This resulted in a Rev/Rev-responsive element (RRE)-independent, stable and increased expression of Gag and the induction of strong humoral and cellular immune responses following DNA immunization of mice compared to wild-type (wt) gag constructs [26–28].

In the present study, we compared the expression rates and immunogenicity of vectors containing a wt or a codon-optimized *gag* gene in different cell lines of mammalian origin. Furthermore, we characterized more closely the induction of immune responses by the superior synthetic DNA vaccine construct with respect to (1) dose dependency, (2) influence of the route of delivery and (3) the longevity of humoral and cellular immunity.

Materials and Methods

Plasmid Constructs

The construction and cloning of UTRgagRRE and the p-syngag plasmid has been described previously in detail [26]. The plasmid pCsRevsg25-GFP (termed p-Rev in this report), expressing Rev fused to green fluorescent protein (GFP), was kindly provided by Dr. Marcus Neumann (GSF, Munich, Germany).

Virus-Like Particles and Synthetic Peptides

Virus-like particles (VLPs) were produced as described in detail previously [29]. The p24(CA)-derived 9-mer peptide A9I (AMQML-KETI) and the 10-mer peptides E10F (EPFRDYVDRF) and R10I (RGPGRAFVTI) were purchased from Toplab (Martinsried, Germany). Recombinant p24 was purchased from Mikrogen (Munich, Germany).

Cell Lines and Transfections

The H-2^d mastocytoma cell line P815 (TIB 64) and the H-2^d B-lymphoma cell line A20 (TIB 208) were obtained from the American Type Culture Collection (Rockville, Md., USA). P815 and A20 cells were propagated in RPMI medium supplemented with 5% (v/v) heat-inactivated FCS, 50 μ*M* 2-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin. CHO (Chinese hamster ovary cells), H1299 (human lung carcinoma cells) and C2C12 (mouse muscle cells) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% FCS, 2 m*M* *L*-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. All mammalian cell lines were maintained in a humidified atmosphere with 7% CO_2 at 37°.

The cells were transfected by the calcium coprecipitation technique as described previously [26]. Briefly, 1.5×10^6 C2C12 cells or 3×10^6 H1299 or baby hamster kidney cells were seeded on 100-mm-diameter culture dishes, incubated for 24 h and then transfected with 45 μg of different Nucleobond AX (Macherey-Nagel, Düren, Germany) purified plasmid constructs. Sixteen hours after transfection, the cell culture supernatant was replaced by fresh medium. Cells and supernatants were harvested 48 h after transfection.

Immunoblotting and p24 Capture Assay

Total cell lysates were prepared 48 h after transfection using a triple-detergent buffer system (RIPA) which was supplemented with a cocktail of protease inhibitors (Complete™ Mini Kit, Boehringer Mannheim GmbH, Mannheim, Germany). Immunoblotting and p24 capture assay were performed as described previously in detail [27].

Vaccination of Mice

Female Balb/c mice (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions and injected at the age of 8–12 weeks. Mice were immunized with the indicated plasmid or VLP concentrations by intramuscular saline injection with 50 μl each in both tibialis anterior muscles or by subcutaneous saline injection with 200 μl at the base of tail, followed by indicated booster immunizations with the same doses.

Evaluation of Antibody Responses

Serum was recovered from mice by tail bleeding at the indicated time points after the booster injection. Anti-Gag antibodies were quantified by an end-point dilution ELISA assay (in duplicate) on

samples from individual animals as described before [27]. The reported titers correspond to the reciprocal of the highest serum dilution that gave an optical density value three times higher than the corresponding dilution of a nonimmune system.

Determination of Cytokines in Supernatants

Spleens were recovered under sterile conditions from mice 5 days after the booster injection and the obtained splenic single-cell suspensions were seeded at 2×10^6 cells/ml in RPMI-1640 medium containing 10% heat-inactivated FCS and 1% penicillin-streptomycin (Gibco), in the presence or absence of Gag protein (10 μg/ml). After 48 h of culture, cytokine levels were determined from the precleared supernatant using a commercial ELISA assay following the manufacturer's instructions (Becton Dickinson).

Intracellular IFN-γ Staining and FACS Analysis

IFN-γ expression by CD8+ cells was detected by intracellular staining. Splenocytes were stimulated with 10 μ*M* peptide in RPMI medium or medium alone as negative control for 6 h including brefeldin A (5 mg/ml) for the whole incubation time. Cells were harvested and transferred to U-bottomed microtiter plates (Greiner, Frickenhausen, Germany), washed twice in FACS buffer I [PBS without Mg^{2+}/Ca^{2+}, 1% FCS, 0.1% (w/v) sodium azide], incubated (10 min, 4°) with purified 2.4G2 antibody to block nonspecific binding of antibody to receptors for the invariant region of immunoglobulin (FcR), washed with staining buffer, surface stained with anti-CD4-FITC (catalog No. 553047, BD, Heidelberg, Germany) and anti-CD8-APC (catalog No. 553035, BD), washed twice with FACS buffer II [PBS without Mg^{2+}/Ca^{2+}, 0.1% (w/v) sodium azide], resuspended in 100 μl of Cytofix/Cytoperm (4% paraformaldehyde, 1% saponin) solution for 20 min at 4° and washed twice in 200 μl of Perm/Wash (PBS without Mg^{2+}/Ca^{2+}, 0.1% saponin) solution. Fixed and permeabilized cells were resuspended in 100 μl of Perm/Wash solution and stained for 25 min at 4° with anti-IFN-γ-phycoerythrin (catalog No. 554412, BD) or the corresponding phycoerythrin-conjugated rat IgG1 isotype control antibody. Cells were washed twice in Perm/Wash solution and suspended in FACS buffer I. Cells were analyzed by flow cytometry using a FACS Calibur and CellQuest software (BD). 3×10^4 CD8+ lymphocytes were analyzed.

ELISPOT Assay

96-well multiscreen MAHA-S45 plates (Millipore, Eschborn, Germany) were coated with 50 μl of anti-mouse IFN-γ antibody (catalog No. 554431, BD, Heidelberg, Germany; 1/500 PBS without Mg^{2+}/Ca^{2+}). After incubation overnight at 4°, wells were washed 6 times with blocking buffer (PBS without Mg^{2+}/Ca^{2+}, 10% FCS) and blocked with 200 μl of blocking buffer for 1 h at 37°. 100 μl containing 10^6 isolated spleen cells in RPMI medium and 100 μl of RPMI with 0.2 μg peptide or medium as negative control. After 24 h of incubation at 37°, plates were washed 6 times with washing buffer (PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20). Then, wells were incubated with 100 μl of biotinylated anti-mouse IFN-γ antibody (catalog No. 554410, BD; 1/500 in PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20, 10% FCS) at room temperature for 2 h and subsequently washed 6 times with washing buffer. Then, 100 μl of streptavidin-alkaline phosphatase (catalog No. 1089161, Roche, Mannheim, Germany; 1/5,000 in PBS without Mg^{2+}/Ca^{2+}, 0.05% Tween 20, 10% FCS) was added and incubated for 1 h at room temperature. Wells were washed 10 times with washing buffer. 50 μl of nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (catalog No. 49008620, Roche; diluted according to manufacturer's instructions) were added to each well. After incubation at room temperature for about 15–30 min, the reaction was stopped by discarding the substrate solution and washing the plates under running water. The plates were then air dried and colored spots were counted.

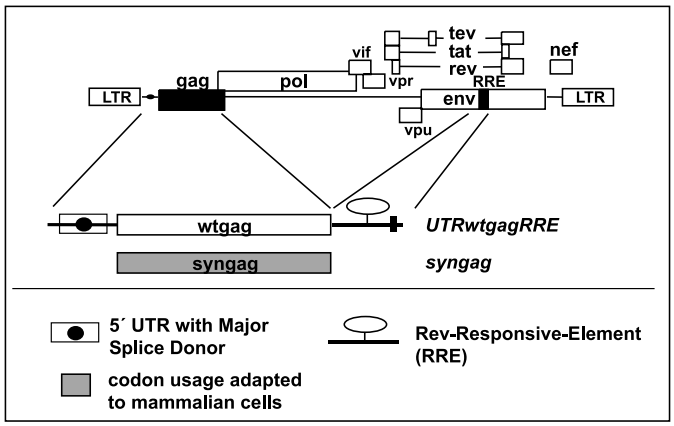

Fig. 1. Schematic representation of the wt and synthetic HIV *gag*-containing expression plasmids used. The wt gag reading frame was fused to the 5′ UTR containing a splice donor and the RRE containing a 3′ splice acceptor. LTR = Long terminal repeats.

CTL Assay

CTL assay by measuring ^{51}Cr release has been described before [27]. The percentage specific release was calculated as [(experimental release – spontaneous release)/(total release – spontaneous release)] × 100. Total counts were measured after adding 1% Triton X-100 to the labeled target cells.

Results

Codon Usage Optimization Enables Increased and Rev-Independent HIV-1 Gag Expression in vitro

The construction of the syngag expression vector p-syngag and the wt gag expression vector UTRwtgagRRE has been previously described in detail [26] (fig. 1). As already reported [27], Gag expression from wt gag without any cis-acting elements is extremely low or even undetectable after transfection of mammalian cells. By adding the authentic untranslated region (UTR) localized at the 5′ end of the HIV-1 *gag* gene, the resulting UTRwtgagRRE gene was expressed in human H1299, mouse C2C12 and hamster CHO cells (fig. 2). Cotransfection with the Rev expression vector p-Rev increased the Gag expression 7-fold in H1299 cells but had no significant influence on the expression in the rodent cell lines, sug-

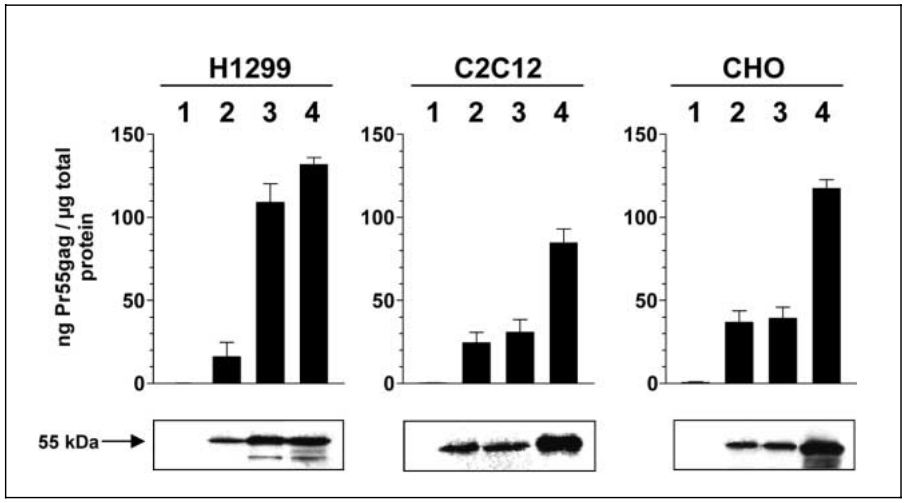

Fig. 2. Expression of HIV gag in different mammalian cell lines. H1299, C2C12 and CHO cells were transiently transfected by calcium phosphate precipitation with (1) pCDNA3, (2) UTRwtgagRRE, (3) UTRwtgagRRE + pRev and (4) syngag. Cells were harvested after 48 h, then 100 µg of total protein of cell lysates were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with a p24-specific antibody. The content of Gag protein in the cell lysates was measured by a p24 capture ELISA using purified Gag for standardization. Bars are the mean of triplicate determinations.

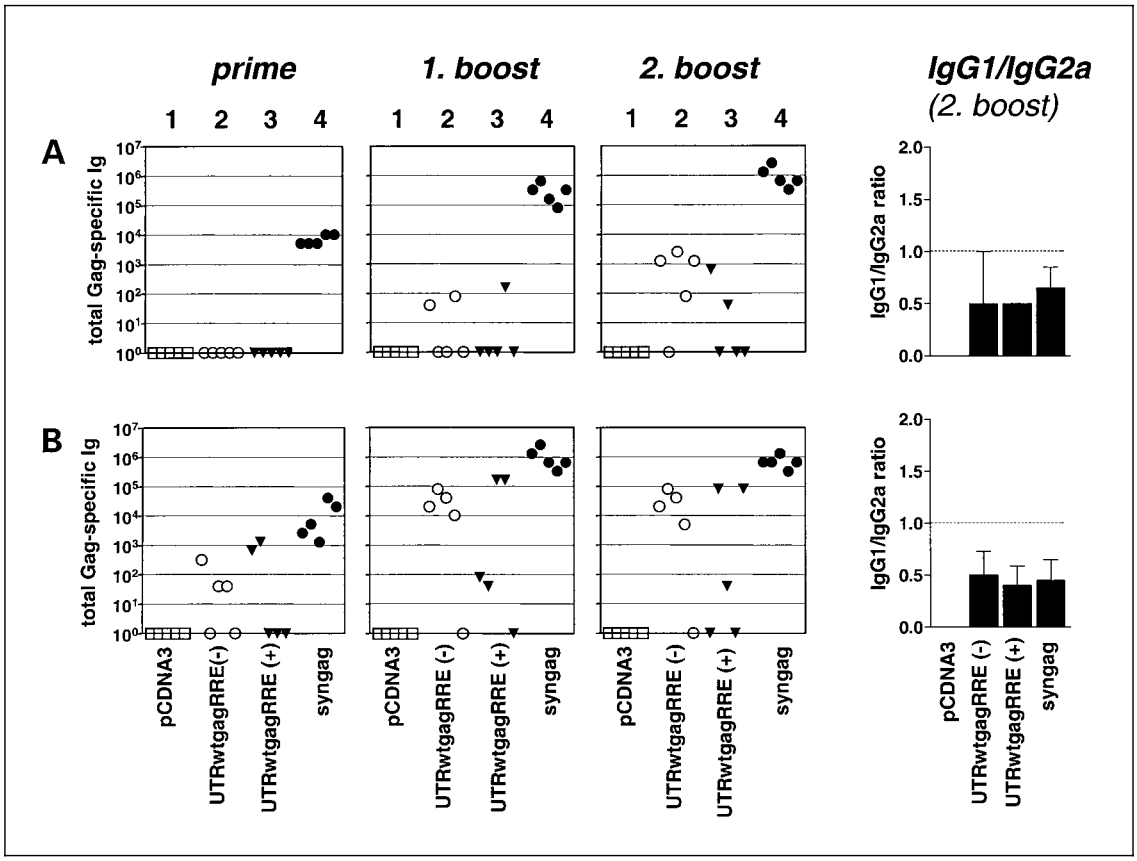

Fig. 3. Humoral immune response after intramuscular immunization of Balb/c mice. Mice were immunized and boostered with 20 µg (A) or 100 µg (B) of total plasmid DNA of (1) pCDNA33, (2) UTRwtgagRRE + pCDNA33 (2:1), (3) UTRwtgagRRE + pRev (2:1) and (4) syngag + pCDNA33 (2:1). Mice were boostered at weeks 3 and 6 and bled 1 week after the initial and booster immunizations. Each symbol represents the value of one mouse for anti-Gag-specific immunoglobulin antibodies as determined by end-point dilution ELISA assay. At the right, IgG1 to IgG2a ratios 1 week after the second booster immunization are shown. Bars represent the group mean (n = 5) for anti-Gag titers.

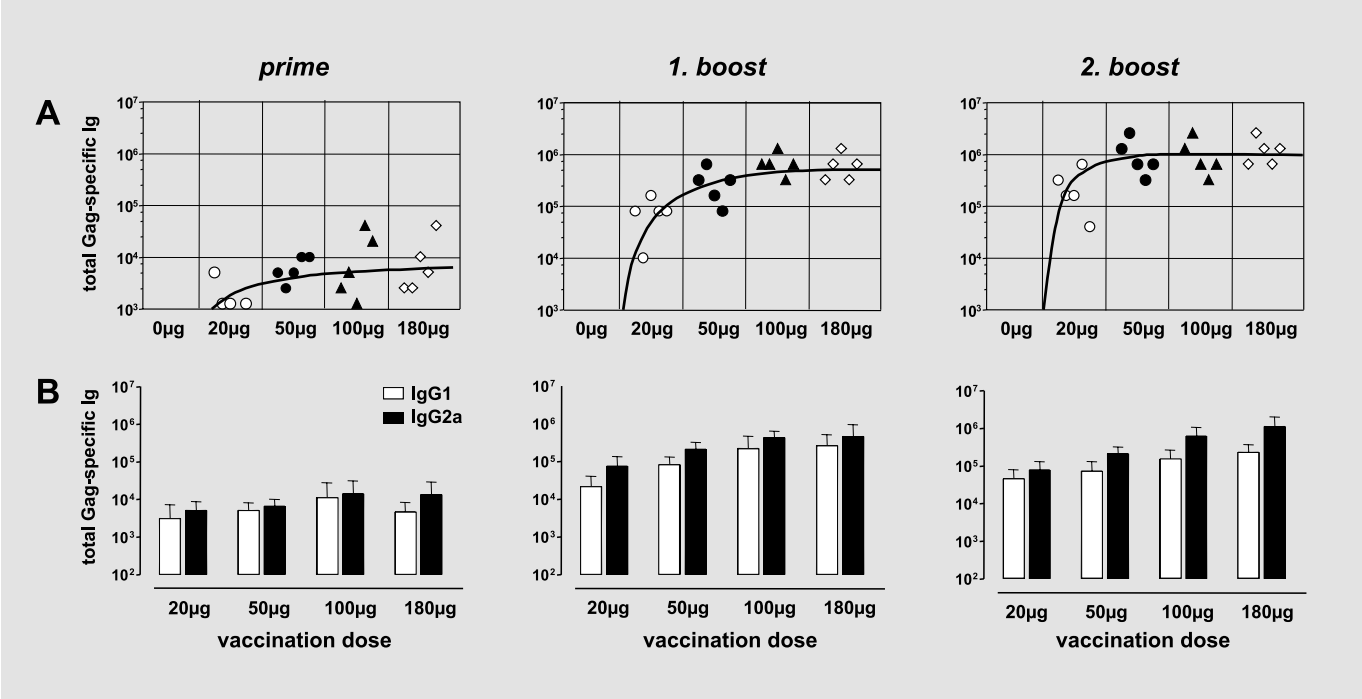

Fig. 4. Dose dependency of the humoral immune response after immunization with syngag plasmid DNA. Balb/c mice were intramuscularly immunized and boostered twice at weeks 3 and 6 with the indicated dose of syngag plasmid DNA. Anti-Gag-specific immunoglobulin titers (A) and IgG1 and IgG2a titers (B) were determined at weeks 2, 5 and 7. Bars in B represent the group mean (n = 5) for anti-Gag titers.

gesting that cell type-specific factors may contribute to the observed Rev responsiveness of UTRwtgagRRE. The highest expression rates were observed after transfection with the codon-optimized syngag vector in all three cell lines, with a 3- to 9-fold higher expression yield compared to the UTRwtgagRRE construct without Rev and a similar ratio in H1299 cells compared to the UTRwtgagRRE plus Rev. This strongly suggests that the adaptation of the gag codon usage to that of highly expressed mammalian genes allows highly efficient Gag expression independent of Rev and cis-acting regulatory elements.

Syngag Plasmid DNA Induces an Increased Humoral Immune Response

To compare the capacity of the different constructs to induce a humoral immune response, we immunized 5 Balb/c mice intramuscularly with 20 or 100 µg of a mix of p-UTRwtgagRRE/pCDNA3, p-UTRwtgagRRE/p-Rev or p-syngag/pCDNA3 and boosted them twice, at weeks 3 and 6. A control group was immunized with equal amounts of pCDNA3 plasmid DNA. Total immunoglobulin and IgG1/IgG2a titers were determined by ELISA (fig. 3). Vaccination with 20 µg of p-UTRwtgagRRE induced only low antibody titers, while using a dose of 100 µg increased the titers significantly, but some mice failed to develop an immune response. The coadministration of p-UTRwtgag and p-Rev neither induced higher nor more consistent antibody titers within the tested group of mice than p-UTRwtgagRRE alone. This finding is in agreement with our previous observation from expression studies (fig. 2), suggesting that Rev/RRE is not functional in rodent cells. Conversely, syngag plasmid DNA induced a substantial and in all mice comparable Gag-specific antibody response even with the low dose of 20 µg already after the initial immunization. Gag-specific antibody responses of all mice, irrespectively of the plasmid construct used, showed a clear Th1 polarization characterized by high titers of IgG2a, resulting in an IgG1/IgG2a ratio <1. No Gag-specific antibody response was detectable at any time point in the sera of control mice.

To evaluate the minimal dose inducing a substantial Gag-specific antibody response and to clarify the question of whether the antibody titers can be further enhanced by escalating plasmid DNA amounts, 5 Balb/c mice were

immunized intramuscularly with 20, 50, 100 or 180 μg of syngag plasmid DNA and boosted twice at weeks 3 and 6. Nonimmunized mice were used as controls. Total immunoglobulin and IgG1/IgG2a titers were determined by ELISA (fig. 4). Almost all mice showed substantial Gag-specific humoral immune responses 1 week after the initial immunization; after the first booster immunization, all mice within each group showed a consistent increase in Gag-specific antibody titers, whereas a second booster immunization revealed only a weak impact on overall Gag-specific titers. The first booster immunization increased the induced antibody titers by 50- to 100-fold, the second boost only 2- to 5-fold, underlining the necessity of at least one booster immunization to obtain substantial antibody titers. Optimal titers were obtained using DNA doses of 50–100 μg, reaching a plateau at ≥100 μg. Irrespective of the DNA dose used and the time point of bleeding, all mice showed a clearly Th1-polarized immune response (fig. 4).

Codon Usage Optimization Enables the Induction of Cellular Immune Responses

Balb/c mice were immunized and boostered twice intramuscularly with 100 μg of p-syngag or UTRwtgagRRE/Rev plasmid DNA. One week after the last booster immunization, splenic cells were prepared, specifically restimulated in a 5-day mixed lymphocyte tumor cell culture and tested for cytotoxic activity in a standard ^{51}Cr release assay. The p24(CA)-derived 9-mer and 10-mer peptides A9I (AMQMLKETI) and E10F (EPFRDYVDRF), known to be murine H-2^d-restricted p24 CTL epitopes [30, 31], were used for stimulation and readout; the 10-mer peptide R10I (RGPGRAFVTI), known to be a murine H-2^d-restricted V3/IIIB-specific CTL epitope [32, 33], was used as negative control peptide. In p-syngag-immunized mice, substantial numbers of Gag-specific CTLs were detectable after stimulation with the A9I peptide and, at a slightly reduced extent, after stimulation with E10F (fig. 5A). No CTL priming was observed using the V3/IIIB control peptide R10I, ensuring the specificity of the assay. In contrast, the mixture of the UTRwtgagRRE and Rev constructs did not induce a Gag-specific cellular immune response, thus underlining the superiority of the codon-optimized gene. Accordingly, in attempts to further characterize the immunogenicity of Gag-based DNA vaccines, we focussed on codon-optimized p-syngag. The induction of a strong cellular immune response was then characterized in more detail by measuring the specific induction of IFN-γ using different assays. For that purpose, spleen cells were stimulated with VLPs (1 or 5 μg), and supernatants were harvested and analyzed for their IFN-γ content after 48 h (fig. 5B). Stimulation with VLPs induced dramatically high IFN-γ titers (up to 17,000 pg/ml) with a relatively high background in pCDNA3-immunized mice, probably due to per se mitogenic properties of the VLPs. A very clear result without any background was seen after a more specific stimulation with the described panel of peptides; A9I again stimulated better than the weaker E10F epitope, whereas the control peptide R10I did not induce any IFN-γ at all. To assess Th2 differentiation, ELISA was performed from aliquots of the same cell culture supernatants to quantify the concentrations of secreted IL-4 and IL-5. In all groups of immunized and nonimmunized mice, no IL-4 or IL-5 secretion was detectable from the supernatants of specifically restimulated as well as nonstimulated splenocytes (data not shown). To quantify the number of IFN-γ-producing cells, an ELISPOT assay was performed; spleen cells were stimulated for 24 h with the most potent A9I peptide and thereafter analyzed. Significant Gag-specific numbers of IFN-γ spots were only detectable in the case of stimulated p-syngag-immunized mice (fig. 5C). Spleen cells were furthermore analyzed to quantify the number of CD8+ lymphocytes producing IFN-γ and were therefore stimulated with the A9I peptide for 6 h in the presence of brefeldin A, surface stained for CD4 and CD8 and thereafter labeled for intracellular IFN-γ. Analysis of the CD8+ lymphocytes showed a strong Gag-specific IFN-γ induction in p-syngag-immunized mice compared to control mice vaccinated with empty pCDNA3 vector (fig. 5D). There was no increase in IFN-γ production by other CD8+ cells detectable, underlining the specificity of the peptide stimulation.

Intramuscular and Subcutaneous Immunization Induce Comparable Immune Responses

Due to the fact that the route and method of DNA delivery are discussed as being important parameters concerning the aimed optimization of DNA vaccination protocols, we compared intramuscular and subcutaneous injection of p-syngag. Mice immunized and boostered twice developed substantial Gag-specific antibody titers with a clear Th1 polarization irrespective of the immunization route used (fig. 6). Nevertheless, subcutaneous immunization showed a delayed increase in antibody induction. However, after the second boost, there was no significant difference between the titers of intramuscularly and subcutaneously vaccinated mice. Furthermore, by both immunization routes, a substantial and comparable CTL activity was detectable in spleen cells isolated 1 week after

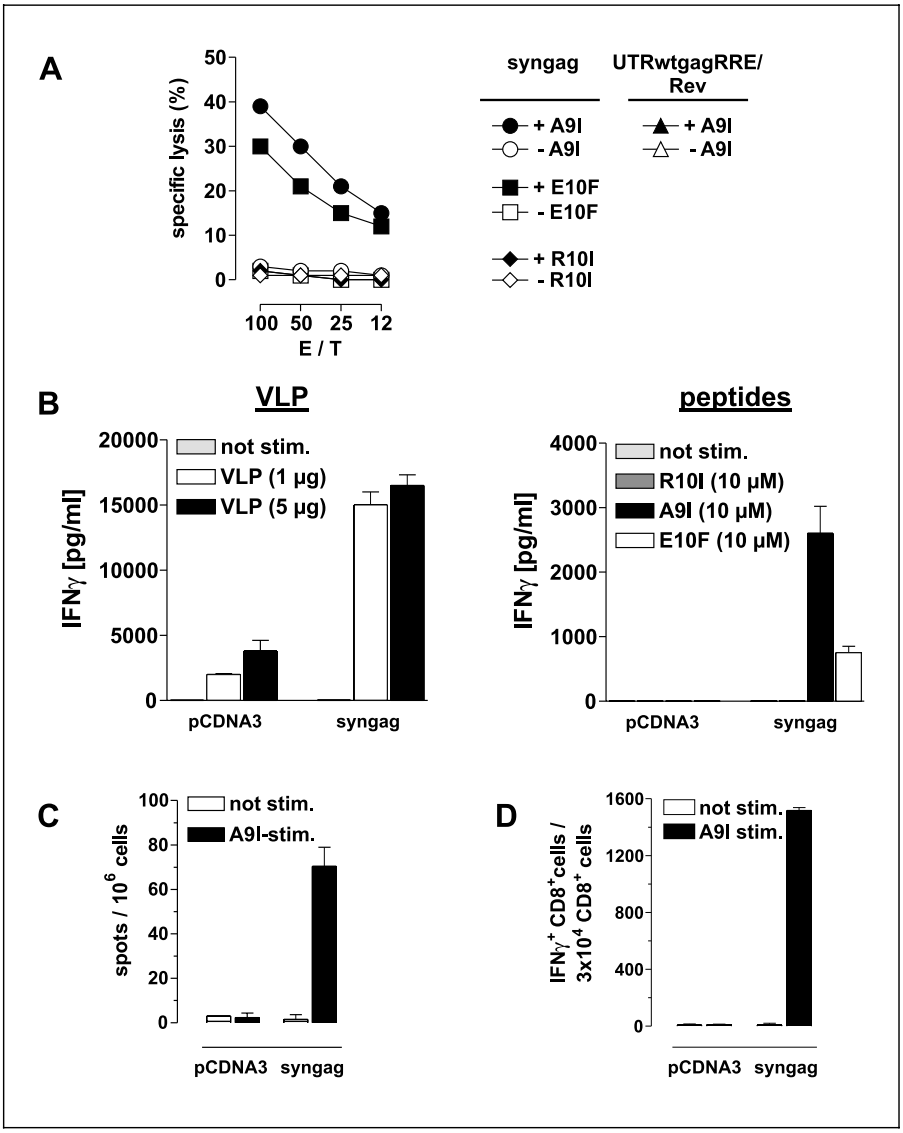

Fig. 5. Cellular immune responses after immunization with syngag plasmid DNA. Balb/c mice were intramuscularly immunized and boostered with 100 µg of syngag or UTRwtgagRRE/Rev plasmid DNA. One week after the second booster, immunized spleen cells were isolated and tested for Gag-specific CTL activity (A) and IFN-γ production (B–D). A Spleen cells were stimulated with A20 cells pulsed with the indicated peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic response was read against P815 cells pulsed with the indicated peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. The data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio. B Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with VLPs or the indicated peptides; unstimulated spleen cells served as negative controls. After 48 h, supernatants were harvested and tested for IFN-γ by a commercial ELISA. The bars represent the mean values of triplicate cultures. C Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with 10 µ*M* A9I peptide. After 24 h, IFN-γ production was determined using a commercial ELISPOT assay. The bars represent the mean values of triplicate cultures. D Spleen cells of mice immunized with pCDNA3 or with syngag plasmid DNA were stimulated with 10 µ*M* A9I peptide for 6 h in the presence of brefeldin A; spleen cells not stimulated with peptide served as negative controls. Cells were surface stained for CD8 and intracellularly stained for IFN-γ. 30,000 CD8+ cells of gated lymphocytes were tested for IFN-γ production.

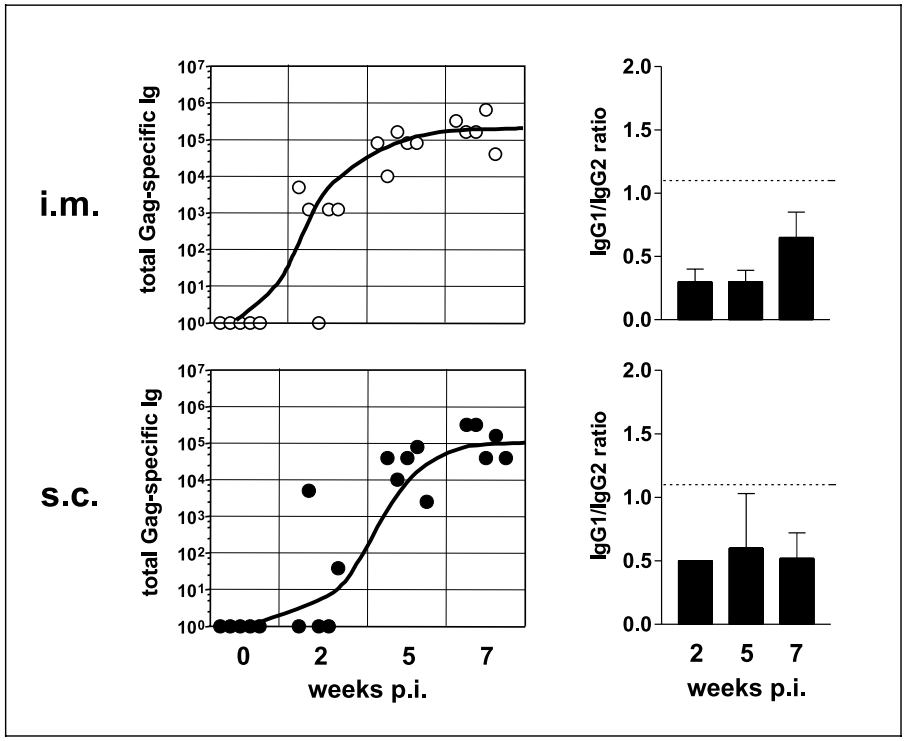

Fig. 6. Influence of immunization route on the Gag-specific humoral immune response. Balb/c mice were immunized with 50 μg of syngag plasmid DNA either by intramuscular (i.m.) or subcutaneous (s.c.) immunization, boostered twice at weeks 3 and 6 and then bled at weeks 2, 5 and 7. Each symbol represents the value of one mouse, while each bar represents the mean values of one group (n = 5) for anti-Gag-specific antibodies as determined by endpoint dilution ELISA assay. p.i. = Postimmunization.

the second booster immunization and tested in a standard ^{51}Cr release assay against A9I peptide-pulsed P815 cells (fig. 7). Conversely, IFN-γ release into cell supernatant after 48 h of stimulation with VLPs (5 or 10 μg) or recombinant p24 protein revealed a significant reduction in the case of subcutaneous immunized mice compared to intramuscular injection. Thus, aside from cytokine release, both immunization routes induce comparable humoral and cellular immune responses.

Immune Responses Induced by p-syngag Are Long Lasting

In order to analyze the progression and longevity of humoral and cellular immune responses, we immunized Balb/c mice with 50 μg of syngag DNA plasmid and boosted them twice. At weeks 2, 5, 7 and 20 after the initial immunization, mice were bled and tested for Gag-specific immunoglobulins. Antibody titers reached their maximum at week 7 and decreased only slightly until week 20 (fig. 8A). Throughout the whole observation period, the antibody response showed a clear Th1 polarization (fig. 8B). Spleen cells isolated at weeks 7 and 20 after the initial plasmid DNA injection and tested in a standard ^{51}Cr release assay against A9I peptide-pulsed P815 cells showed substantial CTL activity (fig. 8C). The lysis capability was reduced only slightly by week 20 compared to week 7. These results clearly demonstrate that vaccination with p-syngag efficiently induces long-lasting humoral as well as cellular immune responses in mice.

Discussion

As we and others were able to show previously, the use of wt gag DNA plasmids for immunization studies is highly limited both for reasons of safety and efficacy. This is primarily due to the Rev/RRE dependency of late HIV-1 gene expression including the wt gag gene. In the absence of Rev, wt gag mRNA is unstable and characterized by a short nuclear half-life, both of which account for a weak or even undetectable Gag expression. Accordingly, previous attempts to use wt gag for plasmid DNA vaccination induced only insufficient or no immunogenicity. We were recently able to show that the Rev-dependent expression from the wt gag gene is the result of a concerted action of multiple cis-acting sequences, involving intragenic AU-rich sequence clusters and the 5′ UTR including the major splice donor site as well as a 3′ RRE [26]. Although the coadministration of p-Rev with UTRwtgagRRE can enhance the expression of Gag in human H1299 cells, there

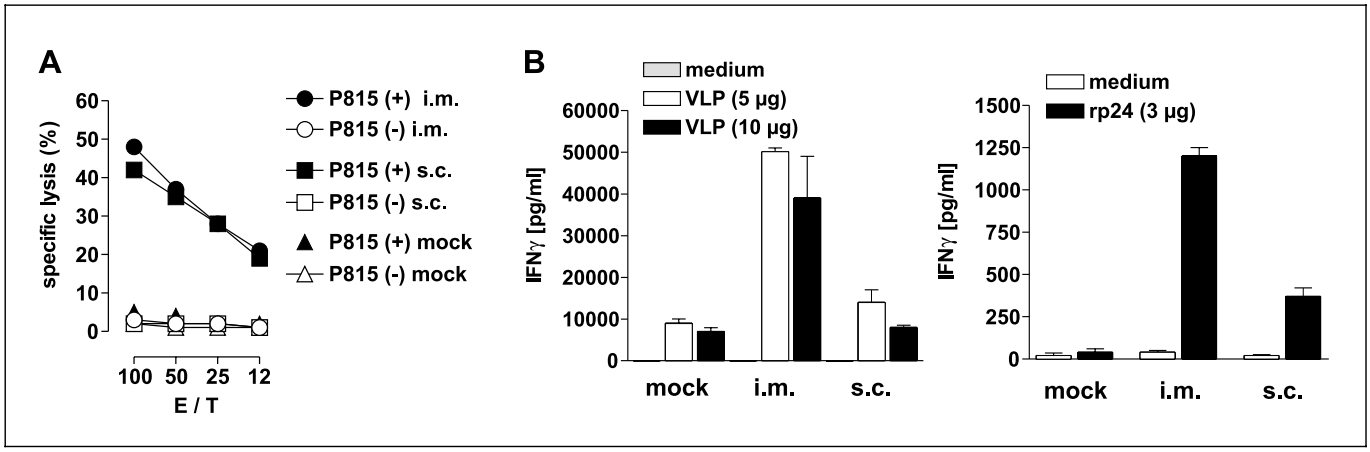

Fig. 7. Influence of immunization route on the Gag-specific cellular immune response. Balb/c mice were immunized with 50 µg of syngag plasmid DNA either by intramuscular (i.m.) or subcutaneous (s.c.) immunization and boostered twice at weeks 3 and 6. One week after the second booster immunization, spleen cells were isolated and tested for Gag-specific CTL activity (A) and IFN-γ production (B). A Spleen cells of immunized and nonimmunized mice were stimulated with A20 cells pulsed with the A9I peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic response was read against P815 cells pulsed with A9I peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. Data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio. B Spleen cells of immunized and nonimmunized mice were stimulated with VLPs or recombinant p24; unstimulated spleen cells served as negative controls. After 48 h, supernatants were harvested and tested for IFN-γ by a commercial ELISA. The bars represent the mean values of triplicate cultures.

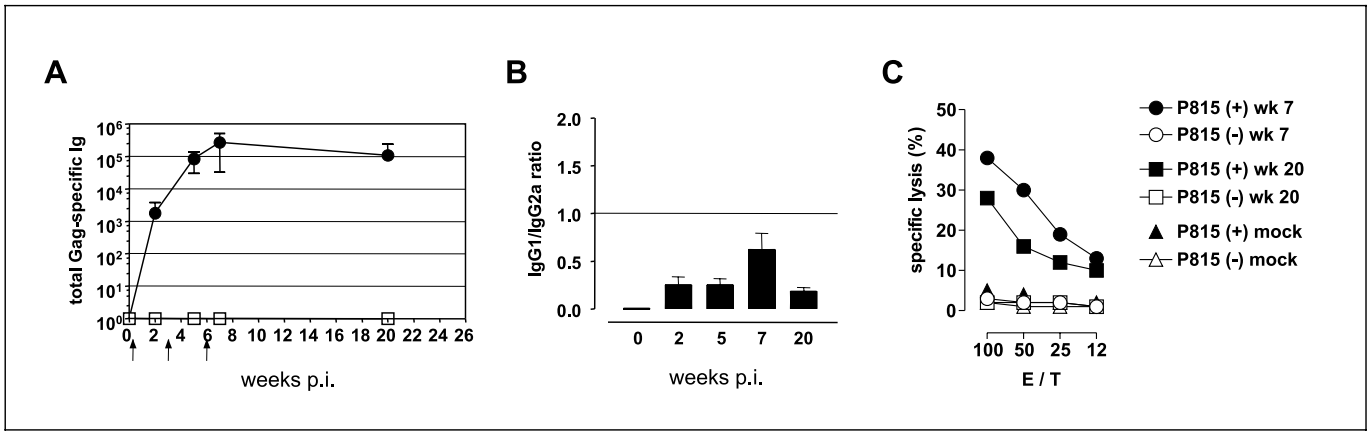

Fig. 8. Kinetics and strength of humoral and cellular immune response induced by intramuscular immunization with syngag plasmid DNA. Balb/c mice were immunized with 50 µg of syngag plasmid DNA and boostered twice at weeks 3 and 6 (time points indicated by arrows). Anti-Gag-specific immunoglobulin titers (A) and IgG1 and IgG2a titers (B) of immunized and nonimmunized mice were determined at the indicated time points. Symbols and bars represent the group mean (n = 5) for anti-Gag titers. p.i. = Postimmunization. C Spleen cells of immunized and nonimmunized mice were stimulated with A20 cells pulsed with the A9I peptide (irradiated with 20,000 rads) for 5 days. Cytotoxic responses were read against P815 cells pulsed with A9I peptide and untreated P815-negative target cells in a standard ^{51}Cr release assay. The data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean. E/T = Effector-target ratio.

is no detectable effect in rodent cell lines, especially in mouse cells. This suggests that a lack of specific cellular [34, 35] and viral factors [36, 37] may be responsible for defects in Gag protein production and the lack of virion assembly in murine cells [27]. Hence, it was not surprising that a mixture of p-UTRwtgagRRE and p-Rev did not induce better humoral immune responses than the UTRwtgagRRE construct alone. However, suggesting that the coadministration of Rev could be successful in humans, there is still a limitation in use due to the high-risk potential of regulatory viral proteins and cis-acting sites within 5′ UTR that entirely overlap the HIV-1 RNA packaging signal [38]. Moreover, a multicomponent plasmid DNA containing gagpol and rev, for example, would be expected to have a highly reduced transfection capability due its size. The utilization of synthetic genes with a codon usage adapted to that of highly expressed genes of mammals represents an effective means to bypass complex viral regulation and, at the same time, to increase immunogenicity and safety profiles of current HIV-specific DNA vaccines. Accordingly, the resulting syngag construct was shown to be expressed independently of Rev/RRE, probably due to the destruction of inhibitory AT-rich sequences of wt gag, and its expression therefore was dramatically enhanced in vitro [26, 28].

Moreover, modification of codon usage within the gag gene resulted in an efficient induction not only of humoral immune responses but also in the efficient priming of Gag-specific CTLs. In contrast, p-UTRwtgagRRE with or without p-Rev was only capable of inducing weak and inconsistent antibody responses and no CTL activity. The improved immunogenicity of *syngag*- compared to *wt gag*-based vaccine vectors obviously correlated with the more efficient in vitro Gag protein expression observed in cultures of murine C2C12 cells. Alternative explanations suggesting that CpG islets randomly generated by increasing the overall CpG content within the synthetic gene could be excluded. Single point mutations destroying CpG motifs without altering the amino acid sequence did not alter the immunogenicity of the tested gag candidate DNA vaccines [27].

Even doses as low as 20 μg per intramuscular injection were sufficient to induce high and consistent antibody titers after a single booster immunization. These humoral responses displayed a clear Th1 bias characterized by an IgG1 to IgG2a ratio clearly less than 1. Furthermore, an initial vaccination with 100 μg of p-syngag was sufficient to induce Gag-specific antibody titers of about 1,000; further booster immunizations increased those titers to >1,000,000. Mice immunized and boostered twice with 100 μg of p-syngag developed strong cytotoxic activity against two known p24 CTL epitopes in Balb/c mice. This CTL response was highly Gag specific, as shown by the missing activity against target cells pulsed with an irrelevant peptide. Furthermore, spleen cells of p-syngag-immunized mice produced high amounts of IFN-γ, the key mediator of Th1-biased cellular immunity, after Gag-specific stimulation, verified by intracellular IFN-γ staining, ELISPOT assays and analysis of cell supernatants. A substantial release of Th2-associated cytokines like IL-4 or IL-5 was not detectable (data not shown).

A number of studies have shown that the method of DNA administration can influence both the strength and nature of immune responses [17]. In the present study, the capacity to induce an efficient immune response was comparable between intramuscular and subcutaneous vaccination. Both routes of plasmid DNA delivery induced a Th1-biased immune response with mostly IgG2a isotypes and comparable titers of Gag-specific immunoglobulins, although the increase in titer was slightly delayed in the case of subcutaneous immunization. The induced cellular immune response measured by ^{51}Cr release assay was comparably high between both groups, although spleen cells of subcutaneously vaccinated mice showed significantly decreased IFN-γ release.

It is worth noting that intramuscular vaccination with p-syngag induces both long-lasting humoral and cellular immune responses. Antibody titers as well as the cytotoxic capacity were nearly unchanged even after 5 months, thus underlining the induction of prolonged memory cell responses.

In sum, these findings confirm and considerably extend data of several groups clearly demonstrating that vaccine vectors exploiting codon optimization of genes to enhance expression yields elicit enhanced humoral and cellular responses compared to vector constructs encoding the corresponding wt gene [39–41]. Due to the obvious advantages of synthetic genes regarding safety profiles and immunogenicity, many current protocols to prevent or treat infectious diseases such as HIV infections or malaria rely on optimized DNA sequences. The advantages of synthetic genes are utilized not only in the context of DNA vaccines. Accordingly, optimized DNA sequences are exploited to foster expression of, for example, viral or prarasitic antigens in cell culture or to enhance the immunogenicity and safety of viral and bacterial vectors such as recombinant vaccinia viruses (e.g. modified vaccinia virus Ankara), alpha viruses (Semliki Forest virus, Venezuelan equine encephalitis virus) or recombinant salmonella or listeria. Of note, one of the most promi-

nent HIV vaccine projects worldwide (EuroVac, www.eurovac.net) utilizes synthetic genes encoding gagpolnef and env polygenes derived from a primary Asian clade C-based HIV-1 isolate for antigen delivery via the above-mentioned plasmid, viral and bacterial vectors. The strain underlying the engineered gene constructs has been selected carefully from amongst now several hundred virus isolates and is representative for the clade C HIV-1 epidemic in China [42]. Preclinical efficacy trials have already proven the potential of the above-mentioned DNA candidate vaccines in a prime boost strategy together with some of the mentioned delivery systems in a nonhuman primate SHIV 89.6P challenge model [in preparation]. Also, based on these promising results, clinical phase I trials in Europe comparing different prime boost strategies in combination with synthetic gene-based DNA vaccines are scheduled for the first quarter of 2003.

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