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Vaccine 22 (2004) 1732–1743
Vaccine
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Influence of polypeptide size and intracellular sorting on the induction of epitope-specific CTL responses by DNA vaccines in a mouse model

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Abstract

We have analysed the influence of size, intracellular localisation, and sorting of various human immunodeficiency virus type 1 (HIV-1)-derived Gag and Env polypeptides containing well defined $H2^d$-restricted cytotoxic T lymphocyte (CTL) epitopes on the induction of a humoral and cellular immune response after DNA vaccination. Thus, expression vectors were generated based on RNA- and codon-optimised genes encoding (i) budding competent full-length Gag, (ii) a myristylation defect mutant $GagMyr^-$, (iii) the isolated p24 capsid moiety of Gag as well as variants of these proteins, which were C-terminally fused HIV gp120-derived V3 epitope (R10I), respectively. These constructs were compared to different minitopes each encoding one of the $H2^d$-restricted Gag epitopes A9I and E10F or the V3 epitope R10I that were directly linked to the C-terminus of an Ad2-E3 protein-derived ER signal peptide.

Immunological evaluation of these constructs in BALB/c mice revealed that both, the budding competent as well as the intracellular Gag proteins were—irrespective of their molecular weights—equally efficient in the priming of Gag-specific humoral and cellular immune responses. In addition, the capacity of these constructs to stimulate Gag-specific humoral as well as H2-K^d and H2-L^d restricted cellular immune responses was not influenced by C-terminal fusion of the immunodominant H2-D^d restricted V3 epitope. Chimeric GagV3 polyproteins encoding all three major CTL epitopes within a continuous polyprotein were more efficient to stimulate epitope-specific cellular immune responses than the selected minitopes. In addition, the minitopes failed to induce epitope-specific antibody responses. These results clearly show the advantages of complex polypeptides over minitopes regarding the induction of strong humoral and cellular immune responses.

Keywords: Polypeptide; Intracellular sorting; CTL responses

1. Introduction

The development of a prophylactic and therapeutic human immunodeficiency virus type 1 (HIV-1) vaccine remains one of the most desirable objectives of research aimed in controlling the current AIDS epidemic.

Over the last years, a variety of reports clearly indicated that cellular effectors of the immune system including cytotoxic T lymphocytes (CTL) are of key importance in controlling HIV infection [1–4]. A strong antiviral cytotoxic activity has been shown to correlate timely with the clearance of viremia in primary infection [5,6]. Furthermore, Gag-specific T helper cell and CTL responses seem to correlate inversely with the viral load [7,8]. In addition, evidence from our own studies and others in long-term non-progressing (LTNP) individuals suggests that the quality rather than breadth of epitope recognition by the individuals CTLs may contribute to a long lasting control of viral replication [4,9]. Thus, Gag derived immunogens, if presented properly, may turn out to be valuable compounds in future HIV candidate vaccines [10,11].

The direct injection of naked DNA has been shown to be a promising approach to induce humoral and cellular immune responses (for reviews see [12–14]). Plasmid DNA immunisation reveals some potential advantages compared to traditional protein vaccination due to the priming of strong T helper 1 (Th1) and CTL responses, the prolonged antigen expression, and the long-lived effector activity [12,15]. It is common sense that bone-marrow derived professional APCs are critical for the induction of immune responses after plasmid DNA immunisation [16,17]. Such APCs have been suggested to be directly transfected leading to endogenous protein synthesis and engagement of the MHC class I and II processing and presentation pathway [18,19]. Alternatively, in the context of ‘cross-priming’ APC may incorporate and process extracellular proteins, which have been produced and secreted by muscle cells [20–22]. This contrasts a previous dogma stating that exogenous proteins are

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doi:10.1016/j.vaccine.2004.01.035

almost exclusively degraded via the MHC class II processing and presentation pathway. Indeed, for some polypeptides, such as HIV Gag-based virus-like particles [23–25], alternative pathways have been revealed, supporting the MHC class I-restricted epitope presentation from exogenous proteins.

In order to elucidate the influence of the size and intracellular trafficking of selected immunogens on immune activation, we compared three different classes of RNA- and codon-optimised Gag derived DNA vaccine constructs for their capability to induce specific humoral and cellular immune responses: (i) a non-modified Gag that is able to bud from the cell membrane in form of virus-like particles (VLP) and thus supports cross-priming events, (ii) a myristylation deficient and—per definition—budding incompetent Gag and (iii) the cytoplasmic p24 capsid protein. Furthermore, a potential impact of cellular protein sorting and presentation in the presence of a fused, immunodominant and envelope derived CTL epitope on the induction of Gag-specific CTL responses should be determined. For that purpose, chimeric Gag/Env vaccine constructs harbouring an immunodominant H2-D^d-restricted V3-loop-derived epitope were generated and analysed in the BALB/c mouse model. Furthermore, in an attempt to modulate loading of processed peptides on MHC class I molecules, ER-targeting minitope expression units have been constructed and compared to the above complex polypeptides regarding their capacity to induce humoral and epitope-specific cellular immune responses.

2. Material and methods

2.1. Plasmid constructs

The construction of the syngag-expression vector pCsyngag, encoding a codon-optimised HIV *gag* gene has been previously described in detail [26]. The myristylation deficient syngag-expression construct pCsyngagMyr$^-$ (Fig. 1A) was generated from pCsyngag by replacing the glycine at position 2 with alanine using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California), and the mutagenesis primers glyala-mut_f (5′-GGC GCC AGC ATG GCC GCC AGG GCC AG-3′) and glyala-mut_r (5′-CTG GCC CTG GCG GCC ATG CTG GCG GC-3′). The codon-optimised coding sequence of the 24 kDa capsid protein (synp24) was amplified by PCR from pCsyngag by using the primers synp24_f (5′-gct cgc tcG AAT TCG CC GCC ACC ATG CCC ATC GTG CAG AAC ATC CAG G-3′) and synp24_r (5′-gct cgc aGG ATC CTC ATC ACA GCA CCC TGG CCT TGT GGC CGG-3′). Thereby, an optimal Kozak-sequence (5′-GCC GCC ACC-3′) [27] and a start codon were added 5′ to the coding regions.

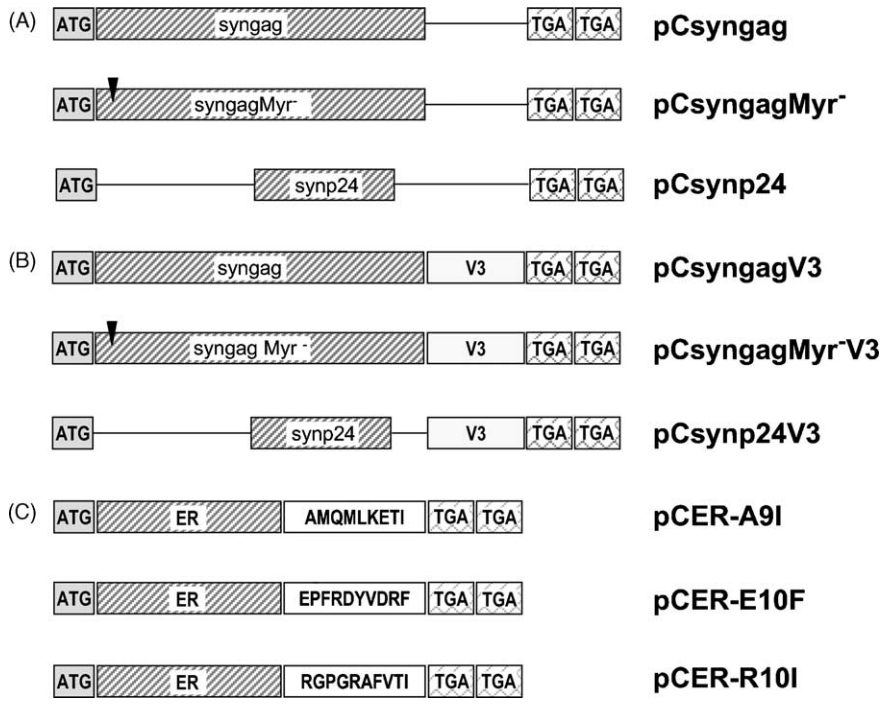

Fig. 1. Schematic representation of various gene constructs for the expression of various HIV-Gag- and Env-derived polypeptides. (A) Codon- and RNA-optimised synthetic genes for the expression of the complete Gag protein (syngag), a myristylation deficient variant of syngag (syngagMyr$^-$), and the p24(CA) moiety of the Gag polyprotein. (B) Illustration of various chimeric *gagV3* genes, including the above described *gag* genes, which were 3′ fused to the 36-mer V3-loop of HIV_{LAI} gp120 Env protein. The position of amino acid (Gly to Ala) exchange within *gag*, resulting in the Myr$^-$ phenotype is indicated by a triangle. (C) Illustration of three variant minitopes containing either the murine p24-specific CTL epitopes A9I or E10F or the V3-specific murine CTL-epitope R10I, which were each 5′ fused to an adenovirus Ad2/E3-derived ER-targeting sequence. The start-codons (ATG) and double stop-codons (TGATGA) are indicated.

The obtained 693 bp PCR fragment was digested with *Eco*RI/*Bam*HI and cloned into the linearised pCDNA3.1 vector. The resulting plasmid was entitled pCsynp24 (Fig. 1A). In order to fuse the 36-mer V3-loop of HIV gp120 3′ to syngag and syngagMyr$^-$, the stop-codon of the syngag-fragment pCR-F6 [26] was deleted by PCR mutagenesis using primers Δ_{Stop}_f (5′-CGA CCC CAG CAG CCA GGG ATC Cgg gag cgg cg-3′) and Δ_{Stop}_r (5′-cgc CGC tcc cGG ATC CCT GGC TGC TGG GGT CG-3′). Following a *Bgl*II/*Bam*HI digestion, the resulting fragment was introduced into pc-syngag (pCsyngagΔ_{Stop}) and pCsyngagMyr$^-$ (pc-syngagMyr$^-\Delta_{Stop}$). Equally pCsynp24Δ_{Stop} was generated by PCR-amplification of the p24 coding region from pCsyngag by using the primers synp24_f (5′-gct cgc tcG AAT TCG CC GCC ACC ATG CCC ATC GTG CAG AAC ATC CAG G-3′) and synp24_r2 (5′-gct cgc aGG ATC CCA GCA CCC TGG CCT TGT GGC CGG-3′). The 115 bp V3 region of the HIV-1_{LAI} *env* gene was generated synthetically by using the overlapping primers V3_f1 (5′-CAG GCC CAA CAA CAA CAC CAG GAA GAG CAT CAG AAT CCA GAG GGG CCC CGG CAG GGC CTT CG-3′), V3_r1 (5′-GCA GTG GGC CTG CCT CAT GTT GCC GAT CTT GCC GAT GGT CAC GAA GGC CCT GCC GGG GCC C-3′), V3_f2 (5′-cgg cAG ATC TAA CTG CAC CAG GCC-3′) and V3_r2 (5′-gcg atG GAT CCT CAG TTG CAG TGG GCC TGC-3′) and cloned subsequent to *Bgl*II/*Bam*HI digestion into the *Bam*HI linearised vectors pCsyngagΔ_{Stop}, pCsyngagMyr$^-\Delta_{Stop}$ and pCsynp24Δ_{Stop}. The resulting expression constructs were entitled pCsyngagV3, pCsyngagMyr$^-$V3 and pCsynp24V3 (Fig. 1B).

In addition, various minitopes were constructed expressing secreted forms of HIV p24(CA)-derived epitopes A9I (aa 197–205) and E10F (aa 291–300) or the HIV gp120-derived peptide R10I (aa318–327). Therefore, these $H2^d$-restricted epitopes were 5′ fused to the coding region of the adenoviral (Ad2) 19 kDa-E3 ER signal peptide and the genes of that fusion proteins were flanked 5′ by an optimised Kozac sequence and 3′ by two stop-codons. The minitopes were constructed using following synthetic oligonucleotides. For ER-A9I the primers ER-f1 (5′-cgc cac cAT GAG GTA CAT GAT TTT AGG CTT GCT CGC CCT TGC GGC AGT CTG CAG CGC TGC-3′), ER-gag1/r1 (5′-GGT CTC CTT CAG CAT CTG CAT GGC GGC AGC GCT GCA GAC TGC CGC AAG G-3′), ER-f2 (5′-gct cgc tcg aat tcg cca ccA TG-3′) and ER-gag1/r2 (5′-cgt cag gct cga gTC ATC AGA TGG TCT CCT TCA GC-3′) were used. ER-E10F was generated by using the primers ER-f1, ER-gag2/r1 (5′-CCT GTC CAC GTA GTC CCT GAA GGG CTC GGC AGC GCT GCA GAC TGC CGC AAG G-3′), ER-f2 and ER-gag2/r2 (5′-cgt cag gct cga gTC ATC AGA ACC TGT CC-3′), the primers ER-f1, ER-V3/r1 (5′-GGT CAC GAA GGC CCT GCC GGG GCC CCT GGC AGC GCT GCA GAC TGC CGC AAG G-3′), ER-f2 and ER-V3/r2 (5′-cgt cag gct cga gTC ATC AGA TGG TCA CGA AGG CCC TGC-3′) were used for the creation of ER-R10I. All minitope constructs were introduced into the *Eco*RI/*Xho*I linearised plasmid pCDNA3.1. The resulting plasmids were entitled pCER-A9I, pCER-E10F and pCER-R10I (Fig. 1C).

2.2. VLPs, r-p24 and synthetic peptides

Virus-like particles (VLPs) were produced as described in detail previously [24]. Recombinant p24 was purchased from Mikrogen (München, Germany). The p24(CA)-derived 9-mer peptide A9I (AMQMLKETI) and the 10-mer peptide E10F (EPFRDYVDRF) as well as the gp120 V3-loop-derived peptide R10I (RGPGRAFVTI) were purchased from Toplab (Martinsried, Germany).

2.3. Cell lines and transfections

The $H2^d$ mastocytoma cell line P815 (TIB 64) and the $H2^d$ B-lymphoma cell line A20 (TIB 208) were obtained from the American Type Culture Collection Rockville, MD). P815 and A20 cells were propagated in RPMI medium supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 IU of penicillin per ml and 100 μg of streptomycin per ml. H1299 (human lung carcinoma cells) were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% FCS, 2 mM L-glutamine, 100 IU of penicillin and 100 μg of streptomycin per ml. All mammalian cells were maintained in a humidified atmosphere with 7% CO_2 at 37 °C.

Cells were transfected by the calcium co-precipitation technique as described previously [26]. Briefly, 3×10^6 H1299 cells were seeded into 100 mm-diameter culture dishes, incubated for 24 h and then transfected with 45 μg of indicated Nucleobond AX (Macherey-Nagel, Düren, Germany) purified plasmid constructs. At 16 h post-transfection, the cell culture supernatant was replaced by fresh medium and cells and supernatants were harvested 48 h after transfection.

2.4. Immunoblotting and p24 capture assay

Total cell lysates were prepared 48 h post-transfection using a triple detergent puffer system (RIPA) which was supplemented with a cocktail of protease inhibitors (Boehringer Complete^{TM} Mini Kit; Mannheim GmbH, Mannheim, Germany). The supernatant (10 ml) was cleared by centrifugation and subsequently pelleted through a 30% (w/v) sucrose cushion at 120,000 × *g* at 16 °C for 2.5 h. The pellet was resuspended in 50 μl PBS and subsequently analysed by immunoblotting using an anti-p24 mab (16/4/2) [28] as described previously in detail [29]. The protein content was determined using a commercial p24 capture assay (Abbott, Wiebaden, Germany).

2.5. 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 immunised with the indicated plasmid concentrations by intramuscular (i.m.) saline injection with 50 μl of plasmid DNA in separate sites of both tibialis anterior muscles, followed by i.m. booster immunisations with the same doses of plasmid DNA.

2.6. 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 previously in detail [29]. The reported titers correspond to the reciprocal of the highest serum dilution that gave a three times higher OD value than the corresponding dilution of a non-immune serum.

2.7. Determination of cytokines in supernatants

Spleens were recovered under sterile conditions from mice at day 5 after the booster injection and the obtained splenic single cell suspensions were seeded at 2×10^6 cells per ml in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), and 1% penicillin-streptomycin (Gibco BRL), in presence or absence of Gag protein, recombinat p24 or the indicated peptides. After 48 h of culture, cytokine levels were determined from the precleared supernatant using commercial ELISA assays following the manufacturer's instructions (Becton Dickinson).

2.8. CTL assay

Cytotoxic activity of $CD8^+$ T-cells was measured by a ^{51}Cr-release assay as described previously in detail [29]. 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 labelled target cells.

3. Results

3.1. RNA- and codon-optimised DNA vaccine constructs

The advantages of a synthetic, RNA- and codon-optimised, Gag encoding expression unit (pCsyngag) over DNA vaccine constructs relying on wild-type (wt) HIV *gag* genes displaying an unusual AT-rich codon usage have been previously described in much detail [26,29–32]. Briefly, the adaptation of the wt *gag* codon usage to that of highly expressed mammalian genes has been shown to mediate a Rev-independent and almost 100-fold increased expression of the Gag protein that nicely correlated with an enhanced immunogenicity in BALB/c mice. In order to analyse the influence of the localisation of well characterised CTL epitopes (i) within a given polypeptide and (ii) within a defined cellular compartment on the immunological outcome, several RNA- and codon-optimised HIV *gag*- and chimeric *gag*/*env*-derived immunogens have been constructed. These constructs encode a budding competent full-length Gag protein as well as two—per definition—cytoplasmatic variants, the myristylation defective $GagMyr^-$ and the p24 capsid moiety of the Gag protein, both of them lacking signals for the secretion from transfected or in vivo transduced cells (Fig. 1A). Furthermore, to determine the possible influence of a foreign immunodominant CTL epitope towards cellular sorting and induction of immune responses towards various T-cell epitopes within a given antigen, the Env-derived immunodominant epitope R10I ($H2\text{-}D^d$) was fused to the above Gag polypeptides naturally comprising p24 epitopes A9I ($H2\text{-}K^d$) and E10F ($H2\text{-}L^d$) to generate GagV3, $GagMyr^-V3$ and p24V3 (Fig. 1B). These three epitopes are presented to $CD8^+$ T cells via a non-overlapping set of $H\text{-}2^d$ alleles, D^d, L^d and K^d, respectively (Table 1). Lastly, various secretion competent minitopes were generated by fusing each of the sequences encoding the above minimal CTL epitopes to the DNA sequence encoding the adenovirus Ad2 E3 protein-derived signal peptide, respectively (Fig. 1C).

3.2. Expression and secretion of Gag and Gag/Env derived HIV polypetides

In order to characterise and to compare the protein expression and secretion rates of these immunogens in a mammalian cell culture system, H1299 cells were transiently transfected with the depicted Gag-, GagV3- and minitope-expression vectors. Consistent with our previous results, the transfection of the pCsyngag construct yielded high levels of Gag expression and secretion in form of immature virus-like particles (VLP). In contrast, the myristylation deficient Gag variant ($pCsyngagMyr^-$) displayed comparable intracellular Gag expression, but failed to sup-

Table 1
Depiction of murine CTL epitopes and the ER-targeting sequence used in this study

(A) CTL epitopes		MHC-I restriction
A9I	AMQMLKETI	$H2\text{-}K^d$
E10F	EPFRDYVDRF	$H2\text{-}L^d$
R10I	RGPGRAFVTI	$H2\text{-}D^d$
(B) ER-targeting sequence (Ad2/E3)		
MRYMILGLLALAAVCSAA		

(A) Amino acid sequence and MHC class I restriction of two Gag-specific (A9I, E10F) and a V3-specific murine CTL epitope (R10I). (B) Amino acid sequence of the used ER-targeting signal derived from the Ad2/E3 protein.

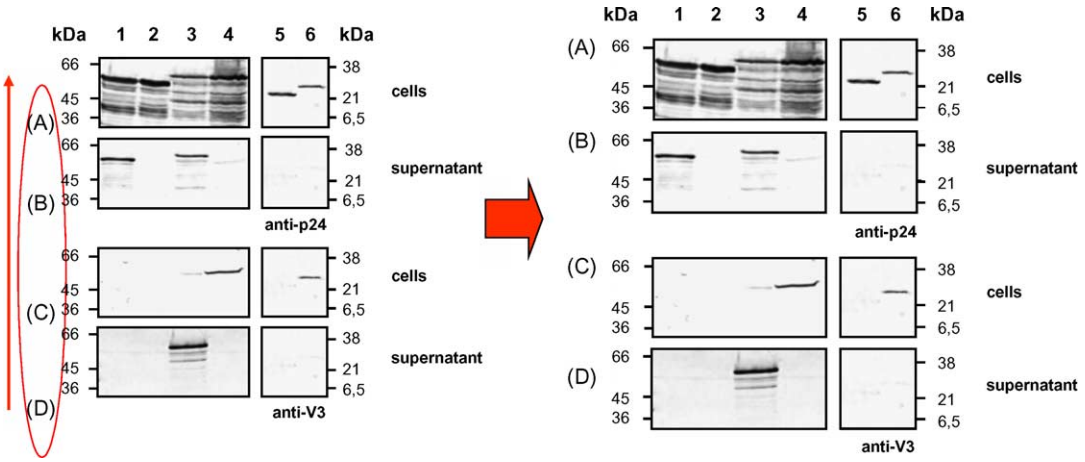

Fig. 2. Expression and secretion of various HIV polypeptides in mammalian cells. H1299 cells were transiently transfected by calcium phosphate precipitation with (1) pCsyngag, (2) pCsyngagMyr$^-$, (3) pCsyngagV3, (4) pCsyngagMyr$^-$V3, (5) pCsynp24 or the (6) pCsynp24V3 expression vector. Cells and supernatants were harvested 48 h post-transfection. (A and C) Cells were lysed and 100 μg of total protein were separated by a 12.5% (lanes 1–4) or a 15% (lanes 5 and 6) SDS-PAGE. (B and D) VLPs were concentrated from the cell culture supernatant by sedimentation through a 30% sucrose cushion. Pelleted antigens were dissolved in PBS and separated by 12.5% SDS-PAGE. Immunoblotting of separated antigens using either (A and B) a p24-specific antibody (16/4/2), or (C and D) a V3-specific monoclonal antibody (NAE 9305), respectively.

port the release of virus-like structures. Slightly reduced amounts of cell associated p24 capsid protein and almost no protein secretion was observed following transfection of H1299 cells with pCsynp24 (Fig. 2A and B). Furthermore, the expression and secretion of these Gag proteins was not significantly altered by C-terminal fusion of the gp120-derived V3-loop (Fig. 2A–D; lanes 3, 4 and 6). No antigen expression was detectable after transfection of cells with the minitopes (data not shown).

3.3. Humoral immune responses are induced independently from the cellular localisation of the encoded antigen

In order to determine the impact of polypeptide size, localisation and morphology on the magnitude and quality of humoral immune responses, groups of each five BALB/c mice were immunised i.m. with pCsyngag, pCsyngagMyr$^-$ and pCsynp24, respectively. Non-immunised mice and animals that received empty pCDNA3.1 plasmids served as negative controls. At weeks 1, 4 and 7 post-infection, sera were collected and analysed for HIV specific antibody responses and IgG1/IgG2a isotype distribution by ELISA (Fig. 3). All three constructs induced comparable titers of Gag-specific antibodies at all tested time points with mean peak levels of approximately 5×10^5 to 1×10^6 at week 7 post-immunisation. At least two-fold increased mean titers of Gag-specific antibodies were observed after immunisation with pCsyngagMyr$^-$ when compared to sera of mice that received pCsyngag and pCsynp24 vaccines that, however, did not reach statistic significance. In addition, a clear Th1 polarisation of Gag-specific antibody responses was induced by all vaccine constructs, as indicated by high titers of both IgG1 and IgG2a isotypes with a IgG1/IgG2a ratio <1 all time points post-immunisation.

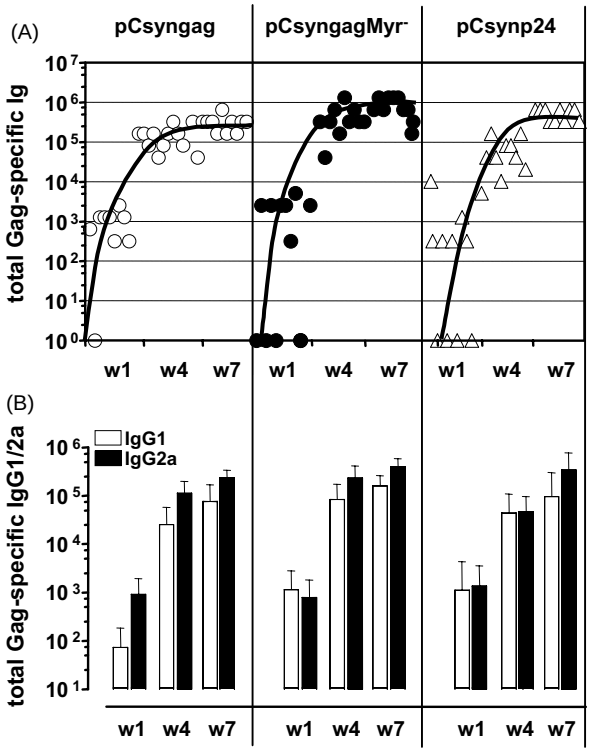

Fig. 3. Influence of the size and cellular localisation of various Gag antigens on the development of Gag-specific humoral immune responses. BALB/c mice were immunised i.m. with each 100 μg of plasmids pCsyngag, pCsyngagMyr$^-$ or pCsynp24. Mice were boostered at weeks 3 and 6 and bleeded at weeks 1, 4 and 7. (A) Each symbol represents the total Ig value of one individual mouse and (B) each bar represents the group mean and standard deviation ($n = 10$) for Gag-specific IgG1 and IgG2a isotypes. Values in (A) as well as (B) were determined by end-point dilution ELISA assay.

3.4. Localisation of the expressed antigen does not influence cellular immune responses

In order to compare the influence of antigen localisation on the stimulation of cellular immunity, groups of each five mice were immunised either with pCsyngag, expressing a budding competent Gag antigen or the two plasmids pCsyngagMyr$^-$ and pCsynp24 producing two intracellular variants of Gag. The antigen-specific cytokine secretion upon stimulation with recombinant proteins or peptides known to comprise CTL epitopes was used as a measure of either Gag-specific T helper cell subpopulations and cytotoxic T lymphocyte (CTL) responses. Splenocytes derived from all three experimental groups showed substantial IFN-γ release upon specific in vitro stimulation with both purified Gag and recombinant p24 antigens (Fig. 4A). Herein, the complete Gag protein, independent of its localisation, was superior to the p24 moiety to stimulate Gag-specific IFN-γ production. In addition, splenic cells of all groups of experimental mice showed IFN-γ production upon stimulation with the well characterised Gag CTL epitope A9I.

In addition, all of these Gag-expression vectors were equally efficient to prime CTL activity against the A9I peptide as determined by a standard ^{51}Cr-release assay subsequent to an epitope-specific restimulation of 5-day mixed lymphocyte tumour cell culture (Fig. 4B). These data clearly indicate that the formation and release of VLPs from Gag expressing cells is no prerequisite for the priming of substantial cytotoxic T-cell responses.

3.5. C-terminal fusion of an immunodominant V3-loop-derived CTL epitope does influence Gag-specific cellular immune responses

In order to investigate a possible influence of immunodominant epitopes from other HIV reading frames on Gag-specific immune responses, expression vectors were generated, encoding a Gag protein derivative, which was C-terminally elongated by the immunodominant 36-mer V3-loop encoded by the HIV *env* gene. Comparative immunological testing of syngag and syngagV3 plasmids in BALB/c mice resulted in the induction of comparable Gag-specific total antibody, isotype and CTL responses (Fig. 5A–C), strongly indicating that the fusion of an highly immunogenic domain, such as the V3-loop, which comprises an immunodominant epitope, shows no modulatory effects on the immunological properties of the Gag protein.

However, pCsyngagV3 but not pCsyngag was sufficient to prime a strong V3-loop specific CTL response in addition to an unaltered anti-Gag CTL activity. These data indicate that the introduction of a foreign strong CTL epitope within the context of the Gag protein does not significantly alter the CTL priming by these chimeric antigens towards intrinsic Gag-epitopes, which have been described to be presented by different H2^d haplotypes. Furthermore, these data suggest

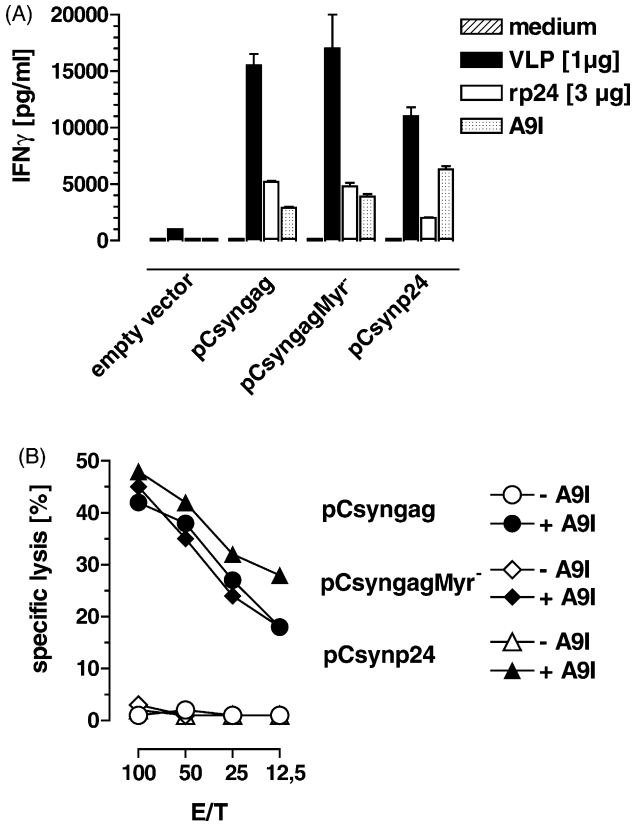

Fig. 4. Influence of the size and cellular localisation of various Gag antigens on the development of Gag-specific cellular immune responses. BALB/c mice were immunised i.m. with each 100 μg of plasmids pCsyngag, pCsyngagMyr$^-$ or pCsynp24. Mice immunised with 100 μg of empty vector served as negative controls. Mice were boostered at weeks 3 and 6 with the same plasmid concentration. Splenic cells were isolated 1 week after the second booster immunisation and tested for Gag-specific IFN-γ production (A) and CTL activity (B) upon specific restimulation. Therefore, single cell cultures of experimental mice were incubated (A) either with indicated recombinant proteins (1 μg VLPs or 3 μg rp24) or 10 μM of Gag-specific peptide (A9I). Non-stimulated spleenic cells served as negative controls. Supernatants were harvested after 48 h of stimulation and concentrations of IFN-γ were assayed by a commercial ELISA assay. The bars represent the mean values ± S.D. of triplicate cultures. (B) Splenic cells were restimulated with indicated peptide-pulsed A20 cells (irradiated with 20,000 rad) for 5 days. Cytotoxic response was read against P815 cells pulsed with the indicated peptide and, for negative control, with untreated P815 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).

that the priming towards these selected epitopes is neither influenced by polypeptide size nor by the antigen morphology or cellular localisation.

3.6. Minitopes induce substantial cellular but no humoral immune responses

Next, we analysed the possible influence of polypeptide size, morphology and localisation on the priming of CTL activities against three H2^d-restricted CTL epitopes (A9I, E10F within p24 and R10I within the V3-loop of gp120) by comparing the T-cell stimulatory properties of minitopes

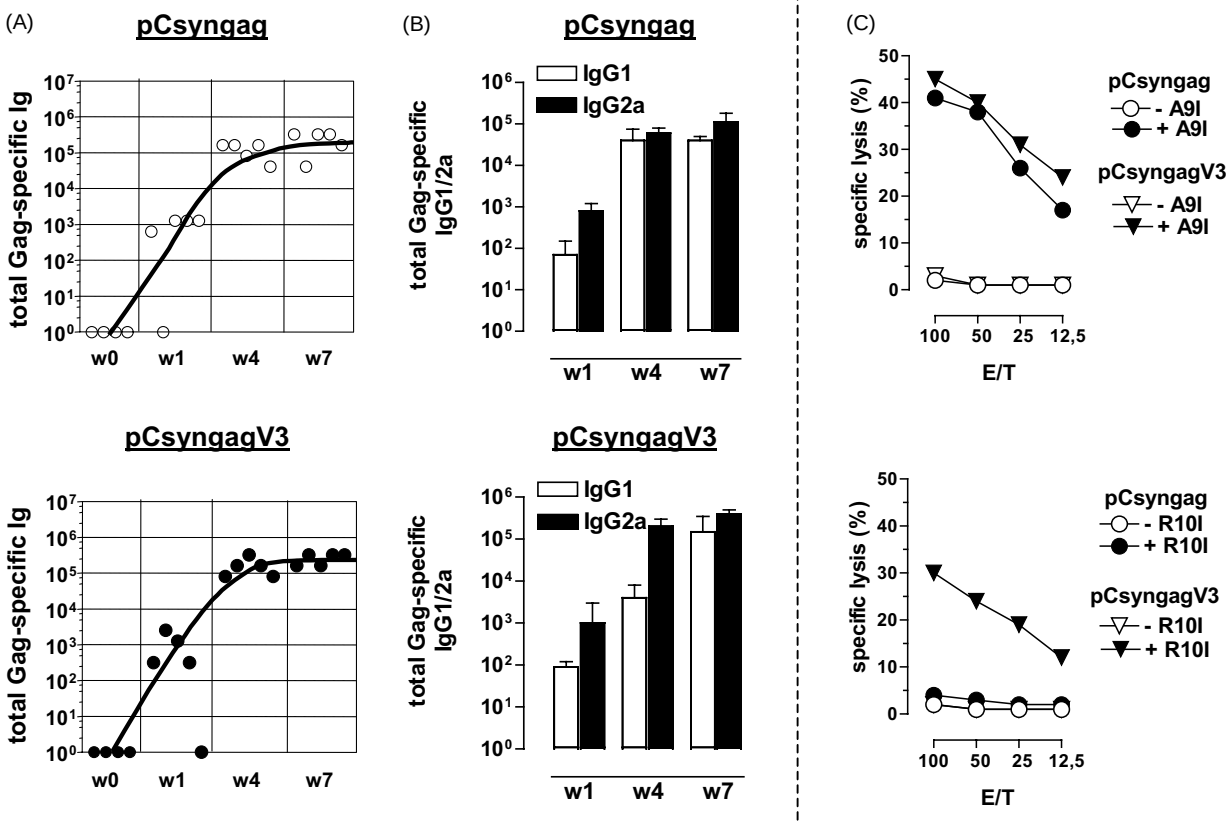

Fig. 5. Influence of the V3-loop on induction of Gag-specific humoral and cellular immune response induced by *gag* or chimeric *gagV3* expression vectors. BALB/c mice were immunised i.m. with 100 μg of either a syngag or a syngagV3 expression vector and boostered at weeks 3 and 6 with the same dose of plasmid DNA. The titers of anti-Gag-specific total Ig were determined at weeks 0, 1, 4 and 7. Each symbol in (A) represents the value of total Ig of one individual mouse, each bar in (B) the group mean ± S.D. ($n = 5$) of anti-Gag IgG1 and IgG2a isotypes as determined by end-point dilution ELISA assay. (C) Splenic cells were isolated 1 week after the second booster immunisation and tested for Gag- and V3-specific CTL activity. Spleen cells were stimulated with peptide-pulsed A20 cells (irradiated with 20,000 rad) for 5 days. Cytotoxic response was read against peptide-labelled P815 cells and, for negative control, untreated P815 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).

with that of the chimeric syngagV3 and syngagMyr$^-$V3 constructs. Within the minitopes, the minimal CTL epitopes were fused to an Ad2-E3 protein-derived signal peptide, allowing direct access of the epitope to the ER, respectively (Fig. 1C).

Immunisation experiments in BALB/c mice confirmed the capacity of the chimeric GagV3 polypeptides expressed by the DNA vaccine to induce substantial anti-Gag-specific antibody titers (Fig. 6A and B) even after the first booster immunisation. The measured isotype profile of Gag- and Env-specific antibodies indicated a clear Th1 polarisation of the induced immune response at all determined time points post-infection (data not shown). In contrast, the minitopes failed to induce any measurable Gag-specific (Fig. 6A and B) and Env-specific (data not shown) humoral immune responses.

Plasmids expressing both variants of full-length chimeric GagV3 polyproteins stimulated the generation of cytolytic responses against all tested Gag- and V3-loop-derived H2^d-restricted CTL epitopes at comparable magnitudes and independent of the cellular localisation of the encoded antigen (Fig. 7A and B). For comparison, only two out of three minitopes were able to induce substantial CTL responses against the encoded epitope (Fig. 7C and D). However, these cytolytic activities were notably lower than those induced by the more complex GagV3 protein encoding DNA vaccines. For the minitopes, the highest CTL reactivity was generated by pCER-R10I encoding the immunodominant V3-epitope, whereas only a weak but significant CTL activity towards the A9I epitope within p24 was stimulated by pCER-A9I. The minitope pCER-E10F failed to stimulate epitope-specific CTL, although the epitope per se has been shown to act as a potent CTL immunogen when used in the autologous context of Gag polypeptides. These data were confirmed by determining the IFN-γ release after specific in vitro restimulation of splenocytes with either soluble p24 or a nonameric CTL peptide representing the minimal CTL epitope as stimulator (Fig. 8). All complex GagV3

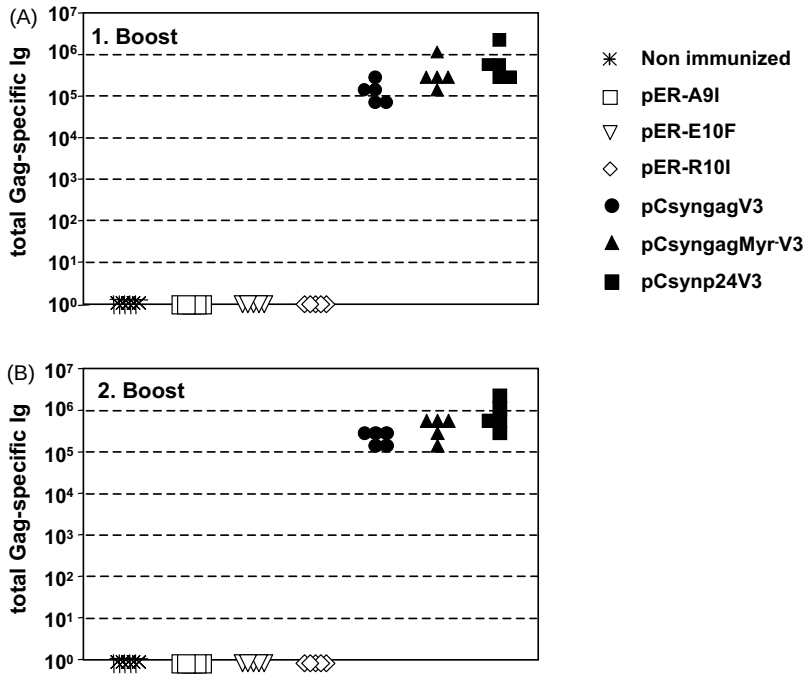

Fig. 6. Comparison of Gag-specific humoral immune response induced by expression vectors expressing either minitopes or Gag-derived polypeptides. BALB/c mice were immunised i.m. with 100 μg of plasmids pER-A9I, pER-E10F, pER-R10I, pCsyngagV3, pCsyngagMyr$^-$V3 or pCsynp24V3 and boostered at weeks 3 and 6 with the same type of immunogen. Bleedings were taken at weeks 4 and 7 and assayed for the titers of total anti-Gag Ig antibodies (A and B). Each symbol represents the total Ig value of one individual mouse as determined by end-point dilution ELISA assay.

expressing DNA vaccine constructs but only minigen pCER-A9I stimulated immunogen-specific T cells producing high amounts of IFN-γ upon stimulation with soluble p24. In addition, IFN-γ production in response to stimulation with A9I, E10F, and R10I peptides was observed from all splenocytes immunised with plasmids encoding GagV3 polypeptides. In contrast only minitopes pCER-A9I and pCER-R10I were sufficient to prime weak T-cell activity against the autologous epitope.

4. Discussion

The major objective of the presented study was to determine the influence of polypeptide size, morphology, intracellular localisation and sorting on the immunogenicity of several HIV-1 Gag and Env-based candidate DNA vaccines. Herein, we focused primarily to the induction of cell-mediated immune responses towards previously described CTL epitopes with known H2^d haplotype restriction.

Our data suggest that the molecular weights—within the size limits of the tested Gag-derived polypeptides—as well as the morphology (soluble versus particulate) of the analysed immunogens do not seem to have any impact on the strength of the induced humoral and cellular immune responses. In fact, both Gag- and p24-expression vectors were able to stimulate comparable H2^d-restricted CTL responses against two previously described epitopes within the p24(CA)-moiety of Gag (A9I, E10F). Thus, the efficacy of epitope processing and MHC class I-restricted presentation seems not to be majorly influenced by the size of the epitope-bearing polypeptide. However, as the molecular weights of the analysed proteins differed at best by a factor of two, our findings do not exclude the possibility that polypeptides with drastically divergent size may be processed and presented with different kinetics and efficacy resulting in a modified immunological outcome.

Furthermore, contrasting previous observations by Qiu et al. [33], the localisation and trafficking of the Gag-derived immunogens do not significantly influence the strength and kinetics of humoral and cellular immune activation. Whereas expression of the myristylated full-length Gag protein leads to an efficient targeting of the Gag molecules to the inner leaflet of the host cell membrane supporting the assembly of polyvalent lipoprotein aggregates to be released from the plasma membrane by budding [34,35], myristylation deficient Gag proteins and the p24 capsid moiety of Gag have been suggested to remain primarily in the cytoplasm [36,33]. Accordingly, the induction of Gag-specific antibodies provides indirect evidence that full-length Gag as well as the—per definition—cytoplasmic derivatives thereof, GagMyr$^-$ and p24, are in vivo released from cells in a way, which allows almost identical engagement of Gag-specific T helper and B cells. Especially in the light of our in vitro findings monitoring the strict intracellular adherence of p24 and the myristylation deficient full-length Gag (GagMyr$^-$) within transfected mammalian cells, the molecular mechanisms underlying the strong in vivo

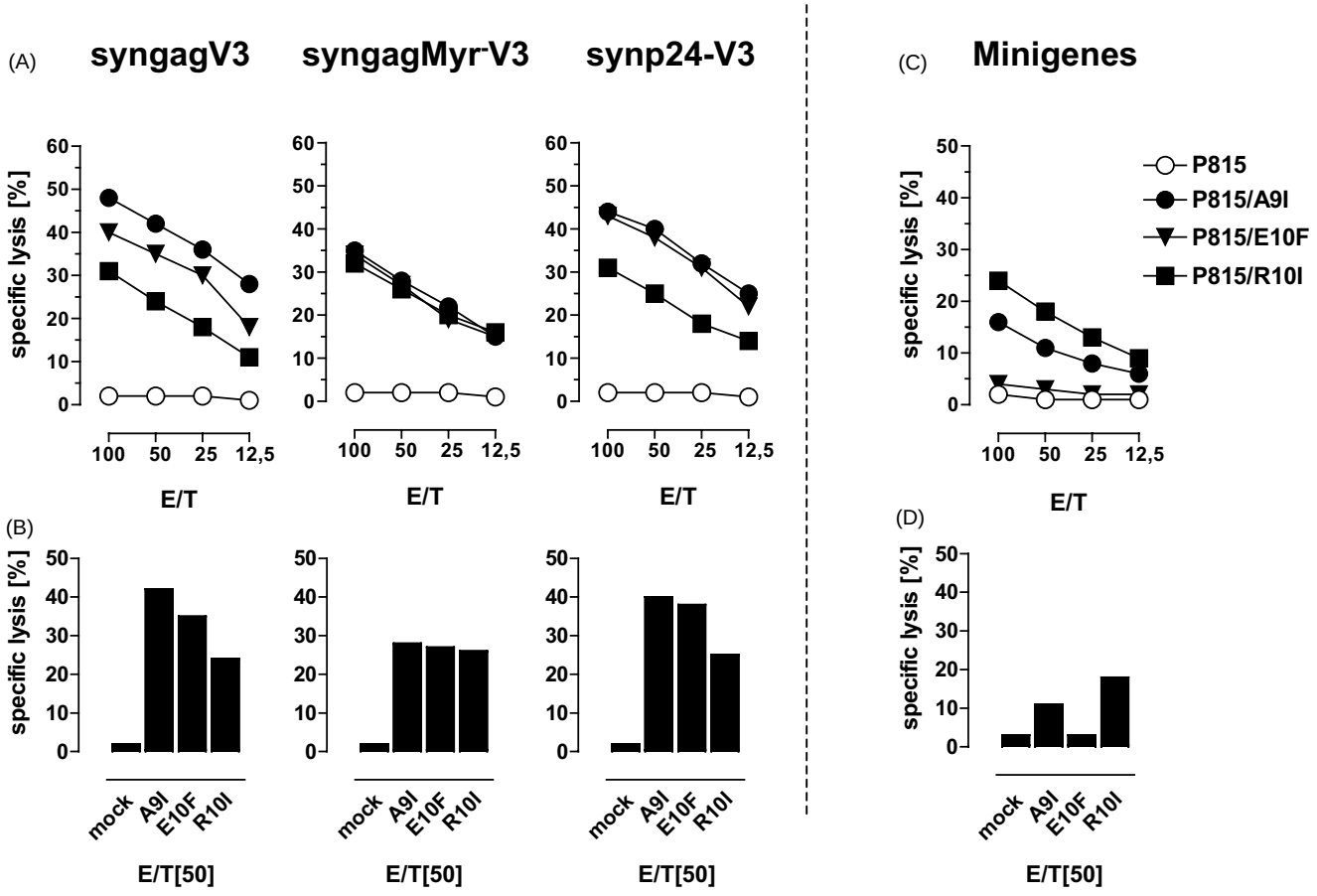

Fig. 7. Comparative analysis of Gag-specific cellular immune response induced by expression vectors expressing either various variants of Gag polypeptides or minigenes. BALB/c mice were immunised i.m. with 100 μg of the plasmids pCsyngagV3, pCsyngagMyr⁻V3, pCsynp24V3, pER-A9I, pER-E10F or pER-R10I and boostered at weeks 3 and 6 after the primary injection. Splenic cells were isolated 1 week after the second booster immunisation and assayed for CTL activity. Spleen cells were restimulated for 5 days with A20 cells pulsed with the indicated peptides (irradiated with 20,000 rad). Cytotoxic response was read against peptide-pulsed P815 cells and, for negative control, with untreated P815 target cells in an standard ^{51}Cr-release assay. (A) and (C) show the percentage of specific lysis for each effector/target (*E*/*T*) ratio, (B) and (D) show the comparison of values at an *E*/*T* of 50. The data shown are the mean values of triplicate cultures with a standard error less than 15% of the mean.

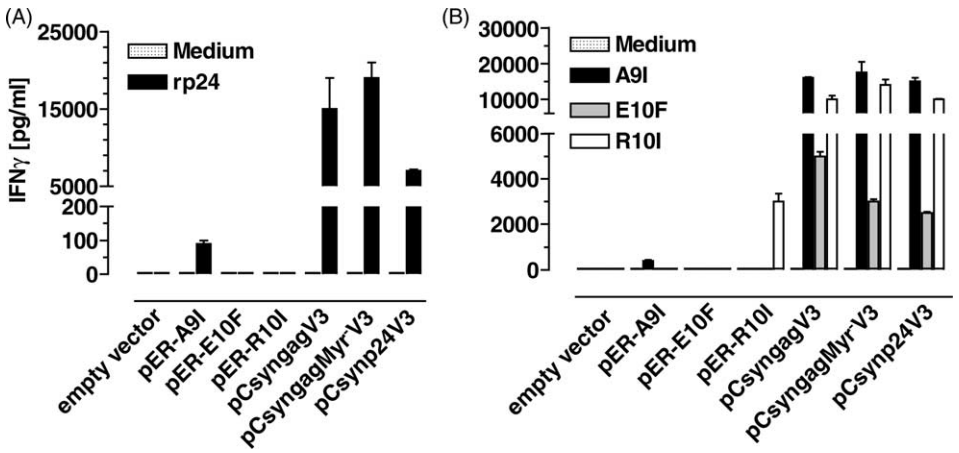

Fig. 8. Comparative analysis of Gag-specific IFN-γ response induced by minitopes in comparison to full-length constructs. BALB/c mice were immunised as described in Fig. 8. Spleen cells were isolated at day 7 after the second booster immunisation and tested for Gag-specific IFN-γ production upon specific restimulation. Therefore, splenic cells were incubated with (A) 3 μg recombinant p24 protein or (B) 10 μM Gag-specific (A9I, E10F) or V3-specific (R10I) peptides. Unstimulated spleen cells served as negative controls. Supernatants were harvested after 48 h of stimulation and tested for the levels of IFN-γ by a commercial ELISA assay. The bars represent the mean values ± S.D. of triplicate cultures.

activation of Gag-specific antibody responses are yet not clear. Possibly, GagMyr$^-$ and p24 may be released from the antigen producing cells by permeance, or apoptosis or necrosis in consequence of antigen production or immune recognition. Alternatively, the intramuscular needle injection of a considerable volume of plasmid DNA may unspecifically support necrosis of muscle tissue followed inflammation. Both possibilities may lead to the generation and uptake of apoptotic vesicles or free antigen by professional antigen presenting cells and thus contribute to an enhancement of antigen-specific humoral and cellular immune responses. This hypothesis would be in accordance with the observations by others reporting that the induction of apoptosis significantly promotes the stimulation of immune responses towards per definition cytoplasmic antigens [37,38]. Accordingly, there is clear evidence suggesting that the induction of CTL and IFN-γ producing T cells may be supported in all cases—and independent from our in vitro findings demonstrating cytoplasmic location of GagMyr$^-$ and p24—by cross-priming events via antigens in vivo [21,39]. Due to the leakyness of transduced cells regarding the release of otherwise cytosolic antigens, genetic strategies that aim to target Gag-derived antigens to the cytosolic compartment or, in the contrary, supporting the release from cells are not suited to quantify the contribution of cross-priming to the generation of cell mediated immune responses.

The C-terminal fusion of the immunodominant H2-D^d MHC class I-restricted V3 epitope derived from the third variable loop of the HIV-1 external glycoprotein gp120 [40] resulted in the expression of fusion proteins that were almost equally expressed in transfected mammalian cells and maintained their in vitro characteristic regarding particle release or cytoplasmic restriction. In all cases, irrespective of the size and biochemical properties of such Gag fusions, the addition of this immunodominant epitope did not influence the capacity of these antigens to stimulate CTL activation towards two H2-K^d and H2-L^d restricted CTL epitopes located within p24. Thus, knowing that the analysed CTL epitopes are presented via different MHC class I haplotypes, respectively, we conclude that CTL induction in that case is neither limited by epitope processing, nor by transport of these peptides into the ER. A competition of the two Gag derived epitopes with the immunodominant V3 epitope was not observed. These results strongly support recent findings by others demonstrating the feasibility of multiepitope DNA vaccines to elicit broad CTL responses against multiple HI-viral epitopes [41,42]. This is of particular interest in the light of previous vaccination studies in non-human primates demonstrating that single viral epitope-specific CTL responses may not be sufficient to block infection with pathogenic SIV [43,44]. However, previous studies of others and us [9,45,4] provided clear evidence that the quality in addition to the breadth of CD8$^+$ T-cell responses may play a central role during the long-term control of viral replication in vivo.

Furthermore, our investigations clearly demonstrate the superiority of polypeptide vaccines over minitopes regarding the induction of epitope-specific humoral and cellular immune responses. Successful immunisation using minigens was reported primarily in 1996 [46]. Recent studies by others have shown that a fusion of an immunogenic peptide to an ER translocation signal increases the potency to stimulate CD8$^+$ cytotoxic T cells [46–48]. Targeting the CTL epitope to the ER may enhance antigen presentation by allowing the peptide to directly enter the ER, bypassing proteosomal degradation and/or TAP-mediated translocation. In addition—by providing a defined proteolytic cleavage site—the ER signal peptide may enhance the immunogenicity of the minitopes by facilitating the exact generation of desired CTL epitope within the ER of a transfected cell. In our experiments, we showed an efficient induction a cellular immune response by administering minitopes containing the p24-epitope A9I and the V3-epitope R10I. However, we failed to induce CTL towards the p24-epitope E10F. On the other hand, correct processing and MHC class I presentation of epitope E10F was observed from all Gag and p24 polypeptides expressed by DNA vectors. This discrepancy may be explained either by an insufficient or unprecise cleavage between the signal peptide and CTL epitope or the destruction of the minitope-produced CTL epitope by degradation of the translocated peptide by persistent proteases.

In sum, the presented data provide evidence that the group specific antigen per se—irrespective of its size or genetic modifications influencing the secretion of antigen in vitro—represents a strong immunogen for the induction of T cells if administered in form of an optimised DNA vaccine. The breadth of immune responses could be expanded by addition of a strong and immunodominant H2-D^d restricted CTL epitope without altering the immune response towards the pre-existing CTL epitopes within the Gag protein. Based on these findings, more complex, Gag-based RNA and codon-optimised DNA vaccines are currently being analysed both in mice and non-human primates.

Acknowledgements

This work was supported by EU Grant Nos. QLRT-PL-01321, QLK2-CT-01316 and the Bavarian Research Foundation “Forimmun (488/02)” to RW.

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