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Chimeric virus-like particles for the delivery of an inserted conserved influenza A-specific CTL epitope

Wan-Shoo Cheong[a], Jessica Reiseger[b], Stephen John Turner[c], Richard Boyd[b], Hans-Jürgen Netter[a,*]

[a] Department of Microbiology, Monash University, Clayton, Victoria, Australia
[b] Monash Immunology and Stem Cell Laboratory, Monash University, Clayton, Victoria, Australia
[c] Department of Microbiology and Immunology, The University of Melbourne, Victoria, Australia

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ABSTRACT

The small hepatitis B virus surface antigens (HBsAg-S) have the ability to self-assemble with host-derived lipids into empty non-infectious virus-like particles (VLPs). HBsAg-S VLPs are the sole component of the licensed hepatitis B vaccine, and they are a useful delivery platform for foreign epitopes. To develop VLPs capable of transporting foreign cytotoxic T lymphocyte (CTL) epitopes, HBsAg-S specific CTL epitopes at various sites were substituted with a conserved CTL epitope derived from the influenza matrix protein. Depending on the insertion site, the introduction of the MHC class I A2.1-restricted influenza epitope was compatible with the secretion competence of HBsAg-S indicating that chimeric VLPs were assembled. Immunizations of transgenic HHDII mice with chimeric VLPs induced anti-influenza CTL responses proving that the inserted foreign epitope can be correctly processed and cross-presented. Chimeric VLPs in the absence of adjuvant were able to induce memory T cell responses, which could be recalled by influenza virus infections in the mouse model system. The ability of chimeric HBsAg-S VLPs to induce anti-foreign CTL responses and also with the proven ability to induce humoral immune responses constitute a highly versatile platform for the delivery of selected multiple epitopes to target disease associated infectious agents.

1. Introduction

Envelope and capsid proteins from various viral agents can be synthesized as virus-like particles (VLPs) in the absence of nucleic acid. Due to their repetitive structure, VLPs are highly immunogenic and have the ability to induce anti-viral humoral and cellular immune responses (Jondal et al., 1996; Pumpens and Grens, 2001; Ruedl et al., 2002). As exogenous antigens, VLPs have the ability to enter the major histocompatibility complex (MHC) class I pathway in contrast to less structured, exogenous antigens, which are processed via the MHC class II pathway (Schirmbeck et al., 1995a, 1996). Normally, only endogenously synthesized antigens are efficiently presented via the MHC class I pathway inducing cytotoxic T lymphocyte (CTL) reactions. Because of the ability of exogenous VLPs to induce CTL reactions in the absence of viral replication and gene expression, they represent attractive tools for developing therapeutic vaccine approaches.

The small envelope protein (HBsAg-S) encoded by the human hepatitis B virus (HBV) has the capacity to self-assemble with host-derived lipids into VLPs. HBsAg-S VLPs are the sole antigenic component of one of the most successful vaccines (Bruss and Ganem, 1991; Maupas et al., 1981; Stirk et al., 1992; Szmuness et al., 1980). Research outcomes and clinical trials have shown that they are a successful delivery system for foreign B-cell epitopes or protein domains (Berkower et al., 2004; Bisht et al., 2002; Bojang, 2006; Delpeyroux et al., 1986, 1990; Eckhart et al., 1996; Michel et al., 2007; Netter et al., 2001; Neurath et al., 1989; Phogat et al., 2008; Pumpens et al., 2002). HBsAg-S VLPs containing foreign B-cell epitopes in the external hydrophilic loop (Delpeyroux et al., 1986; Eckhart et al., 1996; Netter et al., 2001,2003; Phogat et al., 2008) induced anti-foreign antibody responses. Immunizations also induced potentially neutralizing antibodies indicating that the inserted sequences have retained a native conformation (Delpeyroux et al., 1986; Vietheer et al., 2007). The preparation of the antigen and the mode of delivery are critical for the elicited immune response. In the presence of the adjuvant aluminium hydroxide, HBsAg-S VLPs lack the ability to induce a CTL response but induce a humoral response. In the absence of adjuvant, wild-type HBsAg-S VLPs primed $CD8^+$ CTL responses (Schirmbeck et al., 1996). Endogenous synthesis of modified HBsAg-S proteins after

* Corresponding author at: Department of Microbiology, Clayton Campus, Wellington Road, Monash University, Victoria 3800, Australia.
E-mail address: hans.netter@med.monash.edu.au (H.-J. Netter).

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genetic immunizations showed that the replacement of HBsAg-S specific CTL epitopes with foreign CTL epitopes allowed the correct processing of the inserted foreign sequences (Woo et al., 2006).

To determine whether exogenous HBsAg-S VLPs are able to induce an anti-foreign CTL response, HBsAg-S specific CTL epitopes were replaced with an influenza A derived CTL epitope. CTL responses have been reported to play an important role in the clearance of influenza virus infections in the mouse model (Doherty et al., 2006) but the protective importance of the cellular immunity in humans infected with influenza A virus has still to be established (Grebe et al., 2008). However, some investigations indicated that cross-reactive cellular immunity can at least contribute to protection against new influenza variants (McMichael et al., 1983; Powell et al., 2007). The induction of $CD8^+$ T-cell memory in mice can reduce the damage caused by a high virulent H7N7 influenza A virus (Christensen et al., 2000) supporting the view that the induction of an appropriate CTL immune response will limit viral growth and accelerate viral clearance. Vaccines inducing broad heterotypic immunity targeting conserved CTL-specific peptide antigens that vary little between various influenza A strains may constitute an important supplement to overcome the limitations given by antibody-based vaccines, which provide only protection if the infecting viruses express antigenically well-matched hemagglutinin antigens (Webster et al., 1982; Hikono et al., 2006). A large number of antigenic peptides that bind to MHC class I molecules have been identified. Some of the identified CTL epitopes showed a limited degree of variation, possibly functional constraints have limited the variability of these epitopes (Berkhoff et al., 2005; Gianfrani et al., 2000). CTLs against conserved epitopes located in the matrix (M) protein have been shown to confer protective immunity against influenza viruses of various subtypes (Kuwano et al., 1988; Ulmer et al., 1993). The M protein is remarkably conserved across different subtypes. The conserved MHC class I A2.1 restricted epitope "GILGFVFTL" ($M1_{58-66}$) of influenza virus was selected as a tool to determine to determine if HBsAg-S VLPs can deliver foreign CTL epitopes. The highly conserved property of $M1_{58-66}$ epitope in combination with other conserved epitopes may help to overcome the emergence of immune escape mutants (Berkhoff et al., 2006). Mutations in conserved CTL epitopes can interfere with viral fitness and therefore, a selected set of conserved CTL-epitopes may represent attractive viral vaccine components (Berkhoff et al., 2006; Fernandez et al., 2005; Sanchez-Merino et al., 2008; Neumann-Haefelin et al., 2007; Timm et al., 2004).

To determine if HBsAg-S VLPs can be specifically designed to deliver a foreign CTL epitope, an influenza-specific CTL epitope was inserted into HBsAg-S at different locations. Vaccinations were performed in HHDII mice that are transgenic for human MHC class I complex (Firat et al., 2002). The efficient and cost-effective delivery of a designed set of CTL-epitopes may provide an essential supplement to overcome restrictions of antibody-based vaccines that are unable to neutralize viruses such as influenza A strains with variant hemagglutinin proteins.

2. Materials and methods

2.1. Vectors expressing modified HBsAg-S proteins

To minimize conformational changes that could affect the ability of HBsAg-S to form VLPs, HBsAg-S specific CTL epitopes were replaced with a CTL epitope derived from matrix 1 protein (M1) of influenza A virus (Chisari and Ferrari, 1995; Engelhard et al., 1991; Nayersina et al., 1993; Schirmbeck et al., 1995b, 2002). The constructs expressing the truncated HBsAg-S proteins with the CTL epitopes deleted were published [pDCTL100 (pΔFLL), pDCTL200 (pΔIPQ), and pDCTL300 (pΔGLS)] (Woo et al., 2006) and pDCTL400 (pΔSIL) (Chisari and Ferrari, 1995) (Table 1). The HBsAg-S specific cDNA inserts were isolated and cloned via the *Eco*RI restriction site into the pCI vector (Promega, Madison WI, USA) to generate the corresponding constructs, pCTL100, pCTL200, pCTL300 and pCTL400. At the deletion sites of the HBsAg-S specific CTL epitopes, a *Blp*I restriction site was introduced into the pCTL100, pCTL200, pCTL300 and pCTL400 constructs by site-directed mutagenesis according to the manufacturer's recommendations (QuickChange, Stratagene, La Jolla, USA). Site-directed mutagenesis was carried out by using primers 5′-GGACCTGCATGACTACCGGTCAAGGAACCTC-3′ and 5′-GAGGTTCCTTGACCGGTAGTCATGCAGGTCC-3′, resulting in p100/BlpI, p200/BlpI, p300/BlpI, and p400/BlpI, respectively. For the insertion of the DNA sequence encoding the influenza virus-specific A2.1-restricted CTL epitope NH_2-GILGFVFTL-COOH ($M1_{58-66}$) (Engelhard et al., 1991), two $M1_{58-66}$-specific complementary primers 5′-TTAGCGGCATCCTGGGCTTCGTGTTCACCCTGGC-3′ and 5′-TAAGCCAGGGTGAACACGAAGCCCAGGATGCCGC-3′ were designed, annealed to create double stranded (ds) DNA molecules with *Blp*I-specific overhangs at both ends, and then ligated into p100/BlpI, p200/BlpI, p300/BlpI, and p400/BlpI via the *Blp*I restriction site, generating p100/GIL, p200/GIL, p300/GIL, and p400/GIL.

2.2. Expression of recombinant HBsAg-S

The HEK 293T cell line (DuBridge et al., 1987) was supplied by Dr. Joseph Torresi, University of Melbourne. Cells were grown in tissue culture flasks (Sarstedt T75 or T175, Hannover, Germany) in the presence of DMEM (Dulbecco's Modified Eagles Medium; CSL, Victoria, Australia), supplemented with 10% foetal calf serum (FCS), 292 μg/ml GlutaMax-1 (Gibco BRL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, USA). Cultures were incubated in humidified CO_2 incubator at 37 °C, and transfected when grown in monolayer with FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche, Mannheim, Germany). After transfection, the VLP-producing cells were kept in FCS-free HEK293 medium (Gibco BRL, USA). Tissue culture fluid was collected at day 5 post-transfection and centrifuged at 3500 rpm for 15 min in a bench top centrifuge Biofuge Pico (Heraeus, Hanau, Germany) to remove cellular debris. The supernatant (25–28 ml) was transferred to a Beckman 50 Ultra-Clear centrifuge tube, and underlaid with

Table 1
Deleted HBsAg-S CTL epitopes in pCTL constructs.

Construct name	Amino acid (aa) position within HBsAg-S	HBsAg-S specific sequence removed	MHC class I restriction of removed HBsAg-S sequence	References
pCTL100	22–30	FLLTRILTI	Human A2.1	Chisari and Ferrari (1995)
pCTL200	28–39	IPQSLDSWWTSL	Mouse H $2L^d$	Schirmbeck et al. (1995b, 2002)
pCTL300	185–194	GLSPTVWLSV	Human A2.1	Nayersina et al. (1993)
pCTL400	207–216	SILSPFLPLL	Human A2.1	Chisari and Ferrari (1995)

The pCTL constructs refer to the pCI vector (Promega, Madison WI, USA), the truncated HBsAg-S inserts are derived from the pDCTL constructs (Woo et al., 2006) which are based on the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). The aa positions in HBsAg-S, the MHC class I restriction and the references are indicated. The pCTL100, 200, 300 and 400 constructs were used to substitute the removed HBsAg-S sequence with the $M1_{58-66}$ sequence.

5 ml 20% (w/v) sucrose in STE buffer (100 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA). Particles were pelleted by ultracentrifugation at 25,000 rpm for 16 h at 4 °C (Sorvall rotor AH629, Beckman L8-70M ultracentrifuge). The supernatants were discarded, then the pellets were resuspended in STE, quantified and stored at −70 °C. HBsAg-S was detected using the Monolisa HBsAg ULTRA (BioRad, Hercules, USA).

2.3. Animals

HHDII mice, which are transgenic for human MHC class I 2.1 were made available by Dr. Lemonnier, Institute Pasteur, France and bred at the Monash Animal Services (Firat et al., 2002; Himoudi et al., 2002). Male and female HHDII mice were used, each study group contained animals of both sexes, animals were age matched, and between 6 and 12 weeks of age.

2.4. Peptides

The peptide "GILGFVFTL" was synthesized by Mimotopes (Clayton, Australia) or Chemicon (Sydney, Australia) with at least 90% purity.

2.5. Immunization and restimulation of splenocytes

For genetic immunizations, HHDII mice were administered with 100 μg plasmid intramuscularly (i.m.) into musculus tibialis or intradermally (i.d.) into the ear lobe. VLPs (20 μg) were immunized subcutaneously (s.c.) at the tail base. As control, to prime an influenza-specific cellular immune response, the influenza virus A/Puerto Rico/8/34 (H1N1) (A/PR8) (1.5×10^7 pfu) was injected into the peritoneal cavity, which does not support productive viral infection. Ten days or 4 weeks after the injection, spleens were collected, and used for *in vitro* restimulation in CTL and ELISPOT assays. For *in vitro* restimulation, cell suspension was adjusted to 2×10^7 ml^{-1} and transferred into T25 or T75 depending on the total cell number available, then cultured with appropriate peptide (1 μg/ml) for 5 days at 37 °C in a CO_2 incubator.

2.6. CTL assay

The cell line EL4 S3 Rob HHD+ (HHDII+) was a gift from Dr. Andreas Suhrbier, Queensland Institute of Medical Research (QIMR) (Pascolo et al., 1997) and used as targets for the CTL assay. The cells were maintained in supplemented RPMI 1640 medium (JRH Biosciences, Lenexa, USA) in the presence of 400 μg/ml G418 (Gibco BRL, USA). Target cells (1.5×10^6) were sensitized at 37 °C for 1 1/2 hour with 1 μg/ml cognate peptide or incubated in the absence of peptide, and labelled with 100 μCi ^{51}Cr (MP Biomedicals, Irvine, USA). *In vitro* stimulated effector cells (splenocytes) were added at various effector to target (E:T) ratios in triplicate in 96-well microtiter plates (Nunc, Roskilde, Denmark). Controls included target cells but no effector cells (minimum ^{51}Cr release) and targets cells incubated with 10% sodium dodecyl sulfate (SDS) (maximum ^{51}Cr release). A volume of 100 μl of supernatant was collected from each well and transferred into disposable 1.2 ml microtitre tube (QSP, Petaluma, USA). The tubes were wax-sealed for counting in the gamma counter 1470 Wizard (LKB Wallac, Finland). The percentage specific lysis was calculated as indicated by Woo et al. (2006).

2.7. Murine IFNγ ELISPOT assay

To measure IFNγ levels in cell culture supernatants, Millipore MAIPS4510 plates were coated with 50 μl/well (8 μg/ml) IFNγ-specific capture monoclonal antibody (Mab) (BD Pharmingen, San Diego, USA) and incubated overnight at 4 °C. Splenocytes (10^6/50 μl) were then added to each well and stimulated in the presence of 20 μg/ml of cognate peptide or in the absence of peptide. As positive control, cells were incubated in medium containing 2 μg/ml concanavalin A (ConA) (Sigma, St. Louis MO, USA). Plates were cultured for 16–17 h at 37 °C, then incubated with biotinylated anti-cytokine monoclonal antibody (BD Pharmingen, San Diego, USA), followed by ExtraAvidin Alkaline phosphatase conjugates (BD Pharmingen, San Diego, USA). The presence of IFNγ-producing cells was visualised using Sigma Fast TM BCIP/NBT conjugate kit. Plates were dried and spots were quantitated with AID-Elispot Reader System (Autoimmun Diagnostika GmbH, Germany).

2.8. Viruses, infection of mice, and tissue sampling

A/PR8 and A/HKx31 virus stocks were grown in the allantoic cavity of embryonated chicken eggs at embryonic day 10, and stored in aliquots at −80 °C. Virus titres were determined as plaque-forming units on monolayers of Madin Darby canine kidney cells (Turner et al., 2005). The A/HKx31 (H3N2) influenza A virus is a laboratory-generated recombinant virus with the external surface components of A/Aichi/2/68 (H3N2) and the internal components of A/PR8 (H1N1) (Kilbourne, 1969). A/PR8 and A/HKx31 influenza viruses share the internal proteins (NP, NS1, NS2, M, PA, PB1, and PB2) but differ in their surface hemagglutinin (H) and neuraminidase (N) glycoproteins avoiding antibody-mediated cross-protection (Flynn et al., 1999). The strain A/HKx31 used for intranasal infections is of moderate virulence in mice (Reading et al., 1997; Tate et al., 2008; Webby et al., 2003) and intranasal infections with 10^4 pfu of A/HKx31 represent a non lethal dose to study the cellular immune response leading to viral clearance (Day et al., 2007; La Gruta et al., 2004; Turner et al., 2005). HHDII mice were primed intraperitoneally (i.p.) with each 1.5×10^7 pfu A/PR8 influenza virus or s.c. with each 20 μg modified VLPs to induce an anti-influenza immune response. Four weeks after immunization, mice were intranasally infected with a sublethal dose of 1×10^4 pfu A/HKx31 influenza virus. As control, non-immunized, naive mice were infected with the same amount of A/HKx31 virus. At different time points after A/HKx31 infection (day 3, 5 and 7), mice were humanely killed and spleens were collected.

2.9. Statistical analyses

Data were analyzed with the assistance of Dr. Aidan Sudbury at the School of Mathematical Sciences, Monash University. The statistical significance of differences between groups was calculated using Tukey simultaneous test and pair-wise comparisons or two ways ANOVA. P value <0.05 was considered significantly different.

3. Results

3.1. Cellular immune response after genetic immunization with recombinant HBsAg-S DNA encoding the influenza $M1_{58-66}$ epitope

To demonstrate that the foreign $M1_{58-66}$ epitope can be endogenously processed, plasmids expressing HBsAg-S with the influenza-specific sequence inserted at different sites (p100/GIL, p200/GIL, p300/GIL, and p400/GIL) (Table 1) were used to immunize HHDII mice. After 10 days, spleen cells were taken and assayed for the activity of $M1_{58-66}$ specific IFNγ-producing T cells. The Elispot assay was performed in triplicate by using samples from three mice and the mean values resulted in 150, 250 and 200 spots/10^6 cells for the constructs p200/GIL, p300/GIL and p400/GIL,

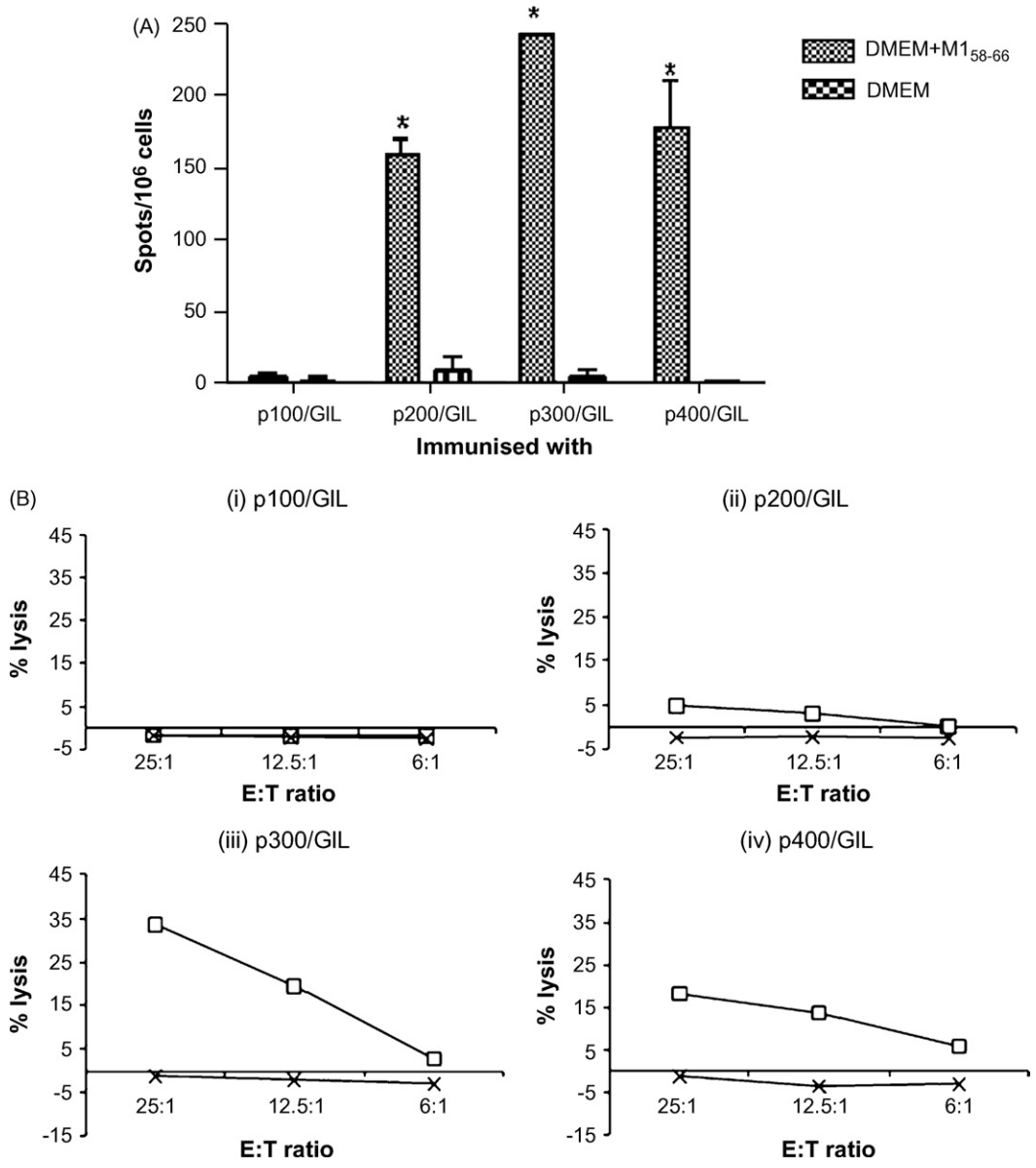

Fig. 1. Assays to determine the cellular immune response of HHDII mice immunized with plasmids encoding chimeric HBsAg-S proteins containing $M1_{58-66}$ sequence at different locations (Table 1). (A) Elispot assay to measure IFNγ-producing cells. Splenocytes derived from HHDII mice immunized with p100/GIL p200/GIL, p300/GIL and p400/GIL plasmids were incubated overnight in the presence (DMEM+$M1_{58-66}$) or absence (DMEM) of $M1_{58-66}$ peptide. Histogram bars represent means and standard deviations of the data obtained from three mice/group. $^{*}P < 0.001$ (DMEM+$M1_{58-66}$, vs. DMEM) (ANOVA). (B) Measurement of cytolytic activity by ^{51}chromium release assay. Effector cells derived from mice immunized with p100/GIL (i), p200/GIL (ii), p300/GIL (iii), and p400/GIL (iv) were mixed with target cells at different ratio (E:T) as indicated (*x*-axis). Percentage cytotoxicity (% lysis, *y*-axis) provided by effectors restimulated in the absence (×) and presence (□) of $M1_{58-66}$ peptide was measured using HHDII+ target cells. Each value represents the mean of three measurements, the variability for the three measurements was less than 10%.

respectively. The difference to the unstimulated negative controls was highly significant ($P < 0.001$) (Fig. 1A). The construct p100/GIL did not induce a detectable anti-$M1_{58-66}$ immune response most likely due to inappropriate expression of the chimeric protein or inefficient processing of $M1_{58-66}$ (Fig. 1A). To evaluate the cytolytic activity, splenocytes derived from three mice/group were combined and *in vitro* restimulated with $M1_{58-66}$ peptide for 5 days, then assessed in a CTL assay (Fig. 1B). In accordance with the Elispot outcomes, the effector cells derived from mice immunized with plasmids p300/GIL and p400/GIL were able to induce lysis of $M1_{58-66}$-pulsed HHDII+ target cells, no lysis was detected in samples derived from mice immunized with p100/GIL (Fig. 1B). DNA immunization with p200/GIL did not result in detectable cytolytic activity possibly the effector memory cells were not efficiently expanded (Fig. 1B). The results indicated that the MHC class I A2.1 restricted $M1_{58-66}$-sequence was properly processed and efficiently presented in the context of p300/GIL and p400/GIL constructs. These data are consistent with the findings that foreign MHC class I H-2^{d} restricted CTL-epitopes inserted into the HBsAg-S sequence can be successfully processed by DNA vaccinations (Woo et al., 2006) indicating that foreign epitopes with different MHC class I restriction can be delivered by HBsAg-S. Therefore, they may be suitable as templates for the efficient delivery of multiple epitopes.

3.2. Expression of modified VLPs

For the development of a protein vaccination strategy, truncated and modified HBsAg-S proteins were expressed in HEK293T cells and the cell culture supernatant assayed for the presence of HBsAg-S specific activity. Functional HBsAg-S proteins are

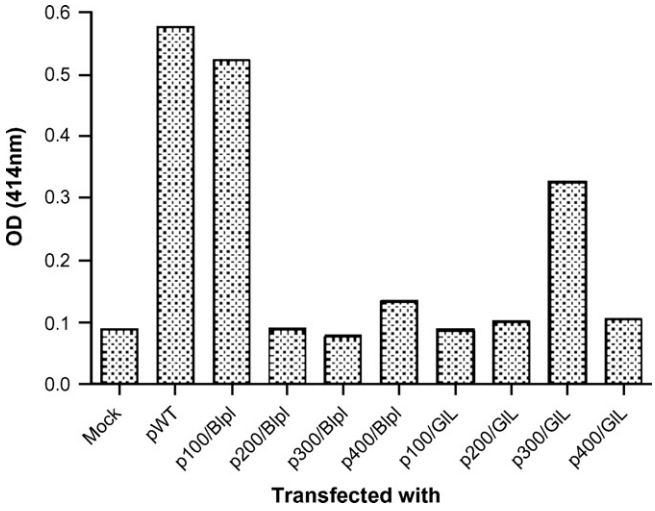

Fig. 2. HBsAg-specific ELISA (Monolisa, BioRad) to detect HBsAg-S VLPs in the cell culture supernatant. HEK293T cells were transfected with plasmids as indicated (*y*-axis). Negative control: transfection with empty pcDNA3 vector (Mock); positive control: transfection with pWT to express wild-type HBsAg-S. Statistical analysis was not performed as only one measurement was taken.

secretion-competent and assemble into VLPs. Recombinant plasmids encoding wild-type HBsAg-S, truncated HBsAg-S proteins (p100/BlpI, p200/BlpI, p300/BlpI and p400/BlpI) or HBsAg-S proteins with the $M1_{58-66}$ epitope (p100/GIL, p200/GIL, p300/GIL and p400/GIL) were transfected into HEK293T cells. The cell culture supernatant was harvested at day 5 post-transfection and the presence of HBsAg-S was determined by an HBsAg-specific ELISA test (Fig. 2). Removal of HBsAg-S specific CTL epitopes abolished the ability of the p200/BlpI, p300/BlpI, and p400/BlpI constructs to synthesize efficiently secretion-competent HBsAg-S VLPs. The construct p100/BlpI expressed efficiently secretion-competent HBsAg-S proteins indicating that this location may be less sensitive with respect to modifications. However, inserting the $M1_{58-66}$ cDNA sequence into the *Blp*I site of p100/BlpI resulting in the construct p100/GIL interfered with secretion competence. The HBsAg-S protein 300/BlpI regained secretion competence after introduction of the $M1_{58-66}$ sequence indicating that a foreign sequence can reinstate the functionality of HBsAg-S (approx. 109 ng/ml) (Fig. 2). The lower amount of HBsAg-S 300/GIL in the cell culture supernatant compared to wild-type HBsAg-S (approx. 191 ng/ml) may be due to impaired secretion and/or could be an underestimation due to the loss of HBsAg-S specific antigenicity. The availability of 300/GIL VLPs allowed the investigation whether the modified VLPs applied as exogenous antigens can be correctly processed and induce an anti-$M1_{58-66}$ cellular immune response.

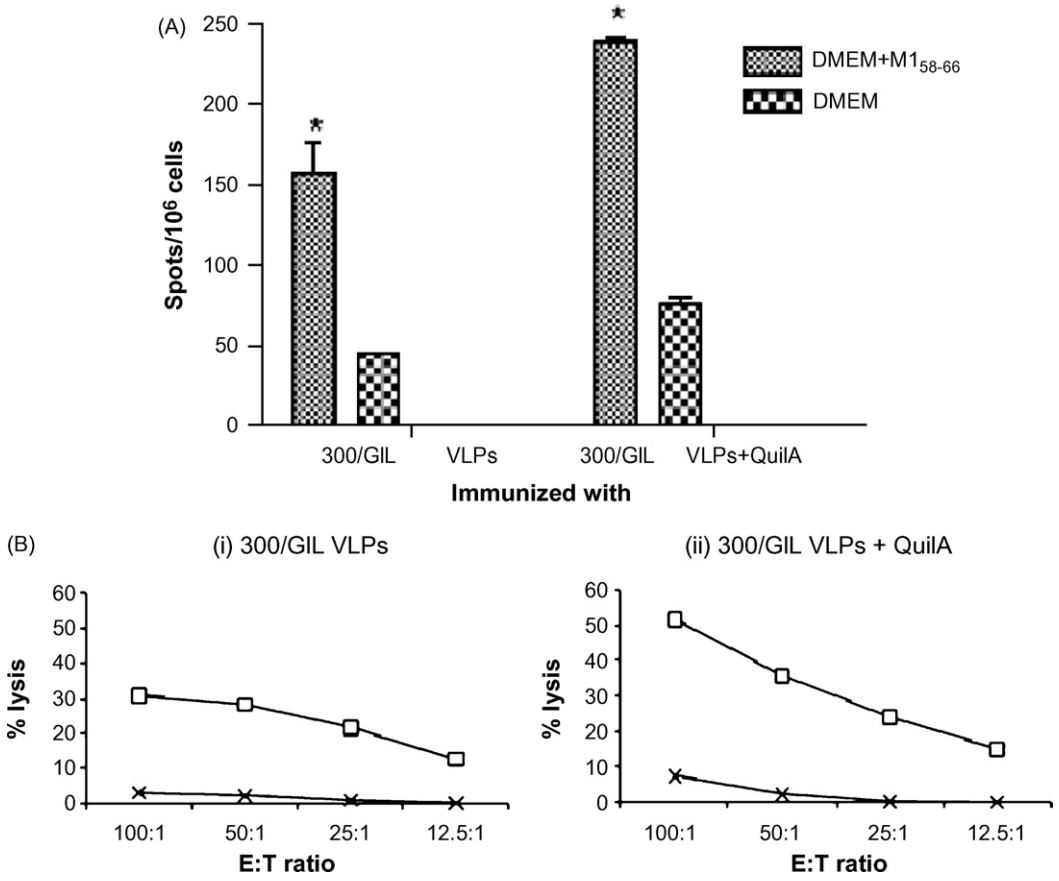

Fig. 3. 300/GIL VLP immunization and determination of the immune response 10 days after immunization. (A) Elispot assay to measure IFNγ-producing cells. Splenocytes derived from HHDII mice immunized with 300/GIL VLPs in the presence and absence of QuilA were incubated overnight in the presence or absence of $M1_{58-66}$ peptide. Histogram bars represent means and standard deviations of samples derived from three mice. $^{*}P < 0.01$ (DMEM+$M1_{58-66}$ vs. DMEM), $P = 0.025$ (VLPs vs. VLPs + QuilA) (ANOVA). (B) Measurement of cytolytic activity by ^{51}chromium release assay. Percentage cytotoxicity obtained by splenocytes (effectors) restimulated in the absence (×) and presence (□) of $M1_{58-66}$ peptide was measured using HHDII+ target cells. Effectors derived from HHDII mice immunized with 300/GIL VLPs (i) and 300/GIL VLPs in the presence of QuilA (ii) were mixed with different effector to target ratios (E:T) as indicated (*x*-axis). Each value represents the mean of three mice in triplicates. The values vary less than 10% within one group.

3.3. T cell response after VLP immunization

To test whether chimeric VLPs can induce an anti-$M1_{58-66}$ cellular immune response, HHDII mice were immunized s.c. with 300/GIL VLPs, splenocytes were harvested 10 days after immunization and the number of IFNγ-secreting cells was determined by an Elispot assay. $M1_{58-66}$-specific IFNγ-producing cells could be detected in mice immunized in the presence and absence of QuilA adjuvant (Fig. 3A). The difference compared to the negative control was highly significant ($P < 0.01$). The results obtained by 300/GIL VLP immunizations with or without QuilA showed a significant difference after stimulation with the $M1_{58-66}$ peptide ($P = 0.025$) indicating that QuilA has an adjuvanting effect for the establishment of an effector T cell response. These data were consistent with the measurement of the anti-$M1_{58-66}$ specific cytolytic activity (Fig. 3B) indicating that the chimeric VLPs are capable of inducing a cellular immune response against the foreign antigen. To test whether the VLPs are able to establish a $M1_{58-66}$-specific core memory response, HHDII mice were immunized and 4 weeks later the anti-$M1_{58-66}$ response tested. As control, influenza A/PR8 was injected i.p. to prime an anti-influenza immune response. Spleens derived from three mice/group were assayed for the presence of $M1_{58-66}$-specific IFNγ producing cells showing that mice immunized with A/PR8 influenza virus and 300/GIL VLPs had high numbers of IFNγ-producing cells after *in vitro* stimulation. The difference between $M1_{58-66}$ *in vitro* stimulated cells and unstimulated cells was highly significant ($P < 0.001$) (Fig. 4A). Also, splenocytes derived from the HHDII mice primed with A/PR8 virions and 300/GIL VLPs were restimulated for 5 days in vitro, and analyzed for cytolytic activity resulting in ≥40% lysis at different E:T ratios of $M1_{58-66}$-pulsed HHDII+ target cells (Fig. 4B). The outcomes of both assays also showed that immunizations with 300/GIL VLPs

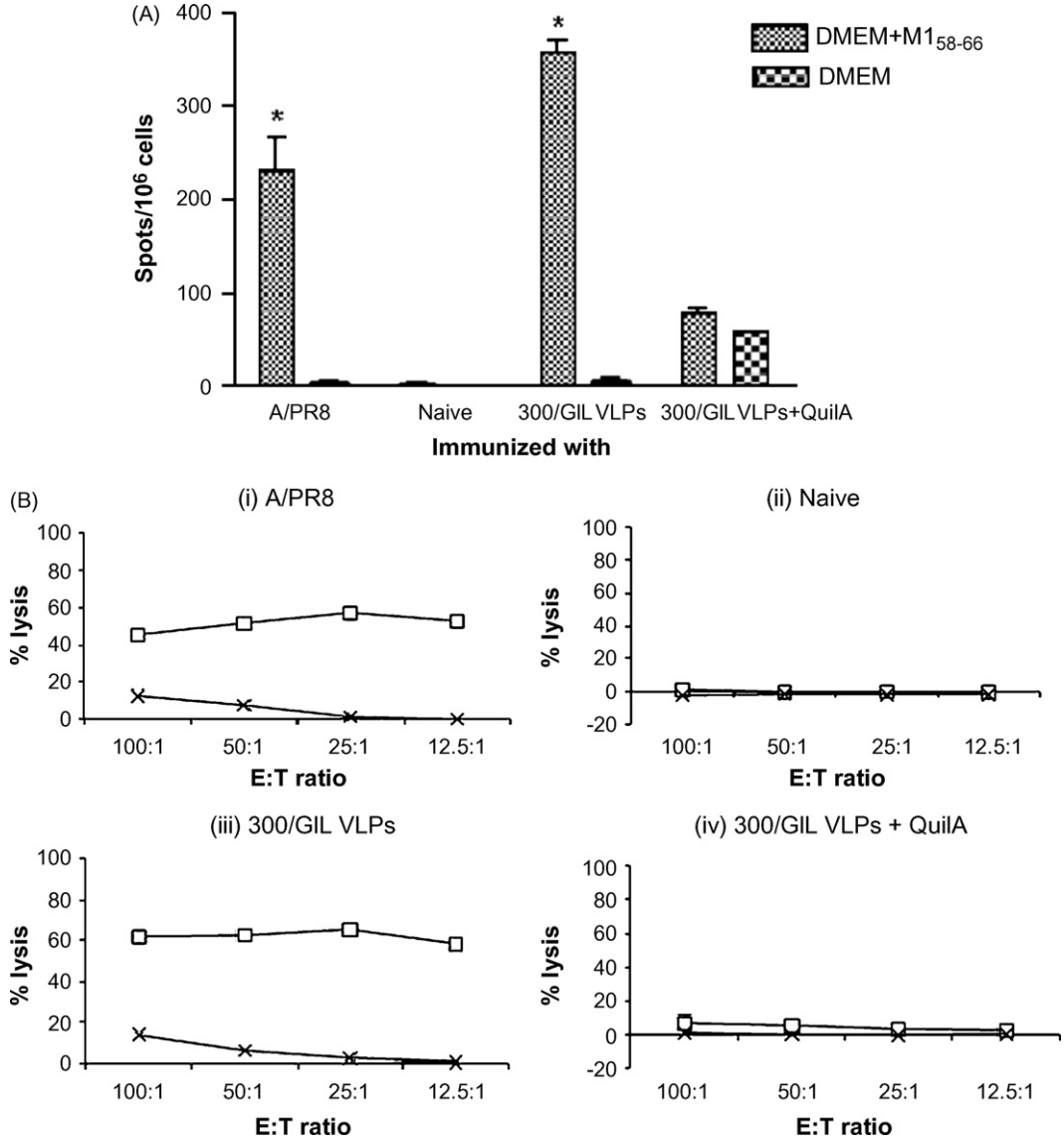

Fig. 4. 300/GIL VLP immunization and determination of the immune response 4 weeks after immunization. (A) Elispot assay to measure the number of IFNγ-producing cells. Splenocytes derived from naive HHDII mice, and from mice immunized with A/PR8 virions or 300/GIL VLPs in the presence or absence of QuilA were incubated overnight in the presence or absence of $M1_{58-66}$ peptide. Histogram bars represent means and standard deviations of three mice/group. $^*P < 0.001$ (DMEM+$M1_{58-66}$ vs. DMEM) (ANOVA). (B) Measurement of *in vitro* cytolytic activity 4 weeks after immunization. Effectors derived from naive HHDII mice (ii) and mice immunized with A/PR8 (i), 300/GIL VLPs (iii), and 300/GIL VLPs in the presence of QuilA (iv) were mixed with target at different ratio (E:T) as indicated (*x*-axis). Percentage lysis provided by effectors restimulated in the absence (×) and presence (□) of $M1_{58-66}$ peptide was measured using HHDII+ target cells. Each value represents the mean of three mice in triplicates. The values vary less than 10% within one group.

in the presence of QuilA did not establish a memory response (Fig. 4). Immunizations in the presence of QuilA showed no significant difference for stimulated and unstimulated IFNγ-producing cells ($P > 0.05$) and no lysis of target cells was detected in the CTL assay after restimulation (Fig. 4). The experiments were repeated twice with similar outcomes (data not shown). Similar to the priming approach with A/PR8 virions, modified VLPs could establish a memory T cell response specific for the $M1_{58-66}$ sequence. The presence of the adjuvant QuilA interfered with establishing a long lasting memory response probably by providing an inappropriate priming environment in the HHDII mouse model.

3.4. Recall of anti-$M1_{58-66}$ memory response by infection with influenza A/HKx31 virus in the mouse model

For the development of a potent vaccine, a prolonged immune response is required to provide protection against a subsequent infection. As unadjuvanted 300/GIL VLPs induce anti-$M1_{58-66}$ specific IFNγ effector and memory CTL responses after 4 weeks post-priming (Fig. 4), it was investigated whether an influenza A infection in the mouse model can recall VLP-induced $M1_{58-66}$-specific memory T-cell responses. HHDII mice were primed s.c. with 300/GIL VLPs or i.p. with influenza A/PR8 virus, and then 4 weeks after immunization, influenza A virus A/HKx31 (H3N2) was administered intranasally to induce an infection. As control, naive (non-immunized) mice were infected with A/HKx31 and the immune response monitored. On days 3, 5 and 7 after A/HKx31 challenge, spleen cells were harvested, and the kinetic of the T cell responses of infected naive and immunized mice was assessed. The cellular immune response following the A/HKx31 virus challenge was evaluated using Elispot and CTL assay. On day 3 after A/HKx31 challenge, splenocytes from mice immunized with A/PR8 had at average 153 spots/10^6 cells. IFNγ-producing cells were not detected in samples derived from mice immunized with 300/GIL VLPs or in naive mice (Fig. 5). On day 5 after A/HKx31 challenge, splenocytes derived from mice immunized with A/PR8 and 300/GIL VLPs contained an average number of 129 and 75 $M1_{58-66}$-specific IFNγ-producing cells/10^6 total cells, respectively. The differences between these two groups were not statistically significant ($P > 0.05$). IFNγ-producing cells were not identified in naive mice indicating that the A/HKx31 infection has not mounted a detectable primary immune response on day 5 after infection. On day 7 after A/HKx31 challenge, the Elispot assay with splenocytes derived from A/PR8- and 300/GIL VLP-immunized mice resulted in high numbers of average 352 and 302 IFNγ-producing lymphocytes/10^6 cells (Fig. 5A). Naive mice infected with A/HKx31 showed also detectable levels of 144 IFNγ-producing cells/10^6 cells representing the primary immune response against the A/HKx31 influenza virus infection. There are no significant differences between the A/PR8-, and 300/GIL VLPs-immunized groups ($P > 0.05$), the difference of both groups to the naive group is highly significant ($P < 0.001$). These results indicated that modified VLPs can establish a memory T cell response, which can be recalled after A/HKx31 infection as shown by the presence of IFNγ-producing cells at day 5 post-challenge. Parallel to the Elispot assays, splenocytes taken at the days 3, 5 and 7 post-A/HKx31 challenge were *in vitro* stimulated and analyzed for their lytic capability. No CTL activity was detected at days 3 and 5 (Fig. 5B), at day 7 post-challenge, splenocytes derived from A/PR8- and 300/GIL VLP-primed mice were able to lyse $M1_{58-66}$-pulsed target cells with 10% and 20% lysis, respectively. Naive mice that were exposed to A/HKx31 did not show CTL activity at early time points post-infection (days 3, 5 and 7) but a primary response was induced due to the identification of IFNγ-producing cells (Fig. 5B). Tetramer staining of the $M1_{58-66}$-specific CD8+ T cell population harvested from the spleens on days 3, 5 and 7 of A/PR8- or 300/GIL VLP-immunized mice after challenge with the A/HKx31 virus indicted that $M1_{58-66}$-specific T cells could be expanded but the overall number of CD8+ T cells was low with approximately 10,000 cells (0.23%) and 7730 cells (0.17%), respectively. In conclusion, immunization with the

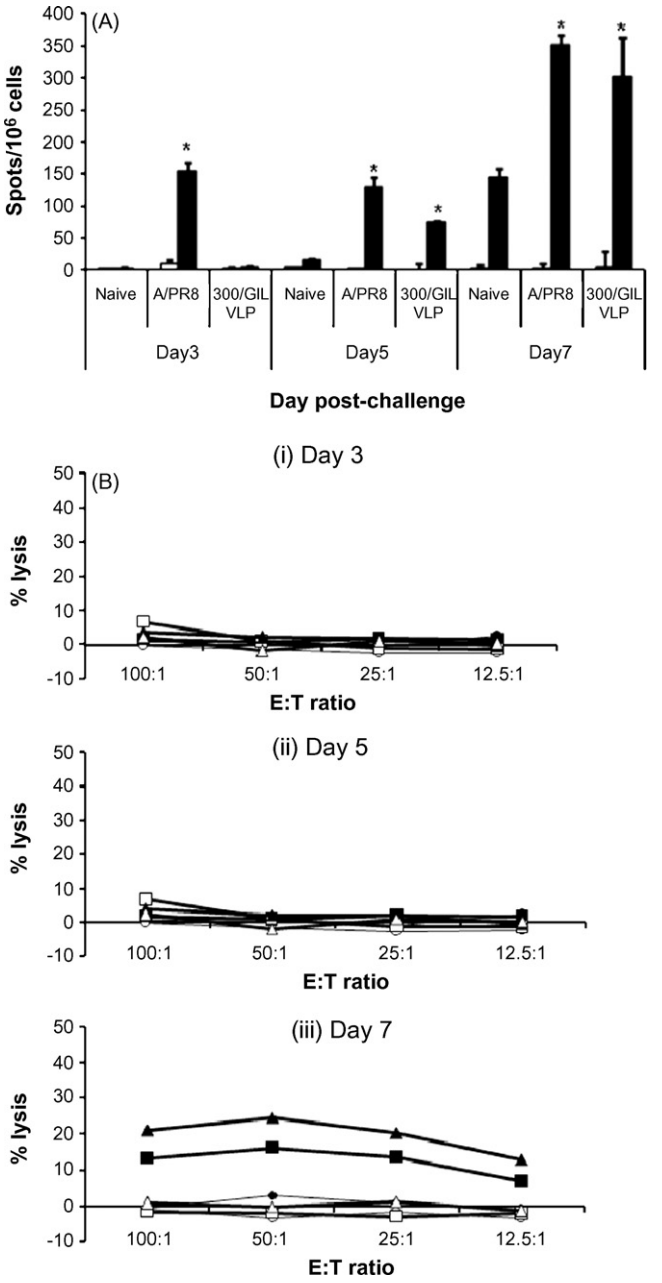

Fig. 5. Recall of anti-$M1_{58-66}$ memory response by influenza infection in a mouse model. Mice immunized with A/PR8 (H1N1) or 300/GIL VLPs were challenged with A/HKx31 (H3N2) influenza virus 4 weeks after immunization. (A) Elispot assay to measure IFNγ-producing cells. Splenocytes were collected on day 3, 5 and 7 post-challenge and incubated overnight in the presence (black) or absence (white) of $M1_{58-66}$ peptide. Naive mice were not immunized but infected with A/HKx31. Histogram bars represent means and standard deviations of three mice. $^*P < 0.001$ (PR8, 300/GIL VLPs vs. naive) (ANOVA). (B) Measurement of cytolytic activity in CTL assay. Splenocytes derived from HHDII mice immunized with A/PR8 (square), 300/GIL VLPs (triangle) and naive (oval) mice were collected on day 3 (i), 5 (ii) and 7 (iii) post-A/HKx31 challenge, then mixed with target cells at different ratios (E:T) as indicated (*x*-axis). Using HHDII+ target cells, percentage cytotoxicity provided by splenocytes restimulated in the absence (open shapes) and presence (closed shape) of $M1_{58-66}$ peptide was measured. Percentage of lysis (*y*-axis) calculated was mean values of three measurements. The values within each group vary less than 10%.

chimeric VLPs could induce a memory response, which could be recalled by an influenza A/HKx31 infection in a mouse model indicating that the VLPs are a potent delivery tool for inserted foreign epitopes.

4. Discussion

For the development of VLPs capable to activate precursor CTLs against selected foreign epitopes, well-characterized HBsAg-S specific CTL epitopes at different locations were replaced with the MHC class I restricted influenza-specific $M1_{58-66}$ epitope (Table 1). Replacing HBsAg-S specific sequences with an influenza-specific epitope was performed to facilitate processing and presentation of the foreign epitope and also to preserve the correct HBsAg-S conformation, which is required for VLP formation. The sequence pattern surrounding the C-terminus of an epitope can influence the effective cleavage by proteasomes and hence the substitution of characterized epitopes should retain the cleavage sites and facilitate presentation via MHC class I molecules (Nielsen et al., 2005). Vaccination with DNA showed that the endogenous expression of the modified HBsAg-S results in the correct processing of the inserted MHC class I A2.1 restricted $M1_{58-66}$ epitope as evidenced by the $M1_{58-66}$ specific IFNγ-response and lysis of target cells. This is consistent with the finding that the insertion of H-2^d restricted epitopes resulted in protective cellular immune responses against both respiratory syncytial virus and human papilloma virus epitopes (Woo et al., 2006). To avoid the potential risks of DNA recombination, chimeric HBsAg-S VLPs were developed and their potential to deliver the foreign CTL epitope via the exogenous pathway was determined. The replacement of the HBsAg-S specific CTL epitope "GLSPTVWLSV" with the influenza A $M1_{58-66}$ epitope allowed the formation of secretion-competent VLPs. Chimeric 300/GIL VLPs were efficiently produced in the cell culture system, and therefore available for studies related to protein immunizations. Immunizations in the presence and absence of the adjuvant QuilA established an effector response as measured 10 days after immunization (Fig. 3). VLPs were able to be cross-presented inducing a Th1-type immune response as indicated by the presence of IFNγ-producing T-cells after stimulation by $M1_{58-66}$ peptide. Also, cytolytic activity was detected confirming the induction of a Th1-type response (Fig. 3).

The main feature in vaccine development against virus infection is the ability of the vaccine to induce a prolonged memory response. Hence, the potential of 300/GIL VLP immunization to sustain a memory T cell response over 4 weeks was investigated. Results showed that immunization with unadjuvanted VLPs were sufficient to induce a cellular memory response. The immunization and infection studies with influenza A virus in the mouse model suggests that 300/GIL VLPs induce the generation of memory T-cells, which are established in the early antigen driven phase. Studies in a C57Bl/6 mouse model showed that the memory T cells contract in the spleen in the 3 weeks after the primary infection representing a cell population, which is stable for more than 1 year (Kedzierska et al., 2007, 2006; Flynn et al., 1999). Surprisingly, the presence of the QuilA adjuvant interfered with the establishment of a memory T cell immune response (Fig. 4) possibly due to CTL exhaustion. Infection studies have shown that an exposure with a high number of viruses exhausted antiviral cytotoxic effector T cells, and in vitro restimulatable precursor CTLs disappeared after day 12 post-infection (Moskophidis et al., 1993). Similarly, the low overall number of $CD8^+$ CTLs in HHDII mice may have facilitated T cell exhaustion in the presence of the potent QuilA adjuvant (Firat et al., 2002). Alternatively, the absence or presence of QuilA may have resulted in different priming environments in the HHDII mouse model. The presence of QuilA may have contributed to a differentiation profile of the responding $CD8^+$ T cells, which interfered with the maturation of memory T cells possibly due to the presence of inappropriate inflammatory conditions. Similarly, the use of CpG oligonucleotides as adjuvant does not necessarily promote the establishment of memory responses. Vaccination studies with peptide-loaded dendritic cells (DCs) generated memory $CD8^+$ T cells, however the presence of CpG oligonucleotides as inducers of inflammation compromised the development of memory $CD8^+$ T cells (Badovinac et al., 2005). Also, the context of epitope presentation can influence the quality of the memory response. Vaccinations with lipopeptides in the absence of inflammatory inducers resulted in the establishment of influenza A specific cellular immune memory (Day et al., 2007). Immunization studies using lipopeptides, which were applied intranasally (Day et al., 2007; Deliyannis et al., 2006) allowed the development of memory $CD8^+$ T cells in the lung tissue. Lung-resident memory $CD8^+$ T cells could be recalled by influenza infections and represented potentially early mediators of pulmonary protection.

To prove whether memory $CD8^+$ T cells established by subcutaneous injections of modified VLPs can be recalled by a infection, mice were challenged with an intranasal injection of influenza A virus. In contrast to the mice primed with A/PR8 virions or GIL/300 VLPs, naive mice showed a delayed primary effector response to A/HKx31 challenge. In the primed mice, memory $CD8^+$ T cells could be recalled after an influenza A infection, and they may be able to confer protection at the site of viral infection. Despite the detection of $M1_{58-66}$-specific $CD8^+$ CTL response in mice primed with 300/GIL VLPs or A/PR8 virions (Fig. 5), no significant viral clearance was observed in vaccinated mice compared to unvaccinated mice (data not shown). A strong contributing factor opposing viral clearance was most likely the low $CD8^+$ T cell number present in HHDII mice as measured by the tetramer staining. Nevertheless, this study has shown that modified VLPs as exogenous antigens have the potential to induce cellular immune responses against the inserted foreign CTL epitope. They are highly effective in establishing a memory immune response, which can be recalled during an influenza infection. Woo et al. (2006) have shown by DNA vaccination that modified HBsAg-S proteins are able to induce protective cellular immune responses directed against human papilloma virus and respiratory syncytial virus (RSV)-specific epitopes, and therefore HBsAg-S VLPs can be designed to target various disease relevant epitopes. The use of HBsAg-S proteins with multiple and different foreign epitopes may result in designed VLPs that are able to provide heterotypic immunity against various influenza strains.

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