Delivering Adjuvants and Antigens in Separate Nanoparticles Eliminates the Need of Physical Linkage for Effective Vaccination

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Abstract:

DNA rich in unmethylated CG motifs (CpGs) engage Toll-Like Receptor 9 (TLR-9) in endosomes and are well described stimulators of the innate and adaptive immune system. CpGs therefore can efficiently improve vaccines' immunogenicity. Packaging CpGs into nanoparticles, in particular into virus-like particles (VLPs), improves the pharmacological characteristics of CpGs as the protein shell protects them from DNase activity and delivers the oligomers to the endosomal compartments of professional antigen presenting cells (APCs). The current consensus in packaging and delivering CpGs in VLP-based vaccines is that both adjuvants and antigens should be kept in close proximity (i.e. physically linked) to ensure delivery of antigens and adjuvants to the same APCs. In the current study, we harness the draining properties of the lymphatic system and show that also non-linked VLPs are efficiently co-delivered to the same APCs in lymph nodes. Specifically, we have shown that CpGs can be packaged in one VLP and mixed with another VLP displaying the antigen prior to administration *in vivo*. Both VLPs efficiently reached the same draining lymph node where they were taken up and processed by the same APCs, namely dendritic cells and macrophages. This resulted in induction of specific CTLs producing cytokines and killing

target cells *in vivo* at levels seen when using VLPs containing both CpGs and chemically conjugated antigen. Thus, delivery of antigens and adjuvants in separate nanoparticles eliminates the need of physical conjugation and thus can be beneficial when designing precision medicine VLP-based vaccines or help to re-formulate existing VLP vaccines not naturally carrying immunostimulatory sequences.

Keywords: Oligonucleotides rich in non-methylated CG motifs (CpGs), Virus-Like Particles (VLPs), Toll-Like Receptor-9 (TLR-9), plasmacytoid dendritic cells (pDCs), conventional dendritic cells (cDCs) & Cytotoxic T-Lymphocytes (CTLs).

Introduction

Successful induction of protective T-cell responses begins with a small pool of naïve specific T-cells which proliferate and differentiate into a large population of effector T -cells acting to control infection (1). In order for this process to happen, several essential elements need to be in place including effective activation of the innate immune system in particular dendritic cells (DCs) (2, 3). Most adjuvants used are therefore capable of stimulating the innate immune system efficiently (4-6) resulting in improved function of APCs and increased production of pro-inflammatory cytokines. A classic example of such adjuvants are CpGs which are DNA rich in non-methylated CG motifs (7, 8). CpGs are present at high frequencies in prokaryotic DNA but are under-represented in mammalian DNA, where they usually are methylated. Presence of CpGs constitutes a Pathogen-Associated Molecular Pattern (PAMPs) indicating the presence of bacterial and other infections. Consequently, the immune system reacts with protective innate and subsequent adaptive immune responses to eliminate the pathogen (9). CpGs are recognized by a member of the evolutionary conserved family of Toll-Like Receptors, specifically Toll-Like Receptor 9 (TLR-9), which is expressed in the endosomes of a number of APCs (10, 11). Once taken up by APCs, CpGs reach the endosomal compartment and interact with TLR-9 (12). In humans, TLR-9 is mainly

expressed by plasmacytoid dendritic cells (pDCs) and B cells (10, 13), while in mice it is additionally expressed by monocytes, macrophages and conventional dendritic cells (cDCs) (14). There are several different types of CpGs exhibiting different properties. CpGs consisting of regular DNA (A-type CpGs) are potent inducers of interferon-alpha (INF- α) *in vitro* by stimulating pDCs but show little activity *in vivo*, as they are rapidly degraded by DNase (15). Packaging A-type CpGs into virus-like particles (VLPs) protects the DNA from degradation and enhances their activity *in vivo* (5). To enhance *in vivo* efficacy, CpGs have therefore been chemically stabilized using thioester bonds, resulting in B-type CpGs (16). Indeed, most potent *in vivo* are B-type CpGs with modified phosphorothioate backbone (17, 18). B-type CpGs have been tested in number of clinical trials where they have shown good adjuvant-activity and could significantly improve vaccine-efficacy by increasing their immunogenicity (3, 19, 20). Despite their favourable adjuvants activity, studies in mice have shown that free unmethylated CpG-motifs with a phosphorothioates backbone can induce intensive cellular activation and thus unfavourable systemic side effects such as splenomegaly or toxic shock (5, 21). In addition, their modified phosphorothioate-backbone increases their half-life and unspecific interactions with various proteins and cells *in vivo* (22, 23). We have previously shown that this obstacle can be overcome by packaging CpGs into VLPs, which essentially eliminates interaction of CpGs with cells and proteins other than professional APCs (5). This method of delivery also ensures effective activation of APCs that simultaneously present the VLPs derived antigens for effective induction of immune responses (24). Such CpG-loaded VLPs have been shown to induce potent immune responses in mice (5) as well as humans (3).

VLPs have been utilized in many forms as an attractive vaccine platform since their first application in vaccinology in the form of the Hepatitis B vaccine followed more recently by the human papilloma virus (HPV) vaccine (25). Most VLP nanoparticles are about 20-30nm

in diameter and can be used efficiently to display antigens and to deliver adjuvants safely *in vivo* (26). VLPs are highly immunogenic vaccine platforms not only for B-cells but also for induction of protective T-cell responses. Their immunogenicity for B-cells is mainly derived from their rigid and repetitive surface, which may be viewed as a geometric Pathogen-Associated Structural Pattern (PASP), as essentially all viruses and many bacteria have such surfaces (27). Their immunogenicity for T-cells is mostly due to their ability to drain to lymph nodes and charge resident professional APCs and simultaneously activating them if loaded with CpGs or RNA (13, 28).

The current consensus when using CpGs as adjuvants with VLP-based vaccines is to keep both ingredients in close physical proximity (i.e. physically linked) to obtain optimal responses via systemic or mucosal administration (5, 11, 29). In previous studies using VLP-based vaccines and CpGs as adjuvants, both CTLs epitopes and CpGs were linked to the same VLP (5, 30), resulting in strong CTL responses in mice and humans (3, 5, 11). In the current study, we show that CpGs as adjuvants can be efficiently packaged in separate VLPs and mixed with the VLPs displaying the CTL epitopes prior to administration *in vivo*. This novel method generates comparable results to the standard method where adjuvants and CTL epitopes are linked to the same VLP. Thus, packaging adjuvants into carrier VLPs eliminates the need to physically linkage to the antigenic VLPs.

Results:

Efficient packaging of B-type CpGs into VLPs derived from bacteriophage Q β

We have previously shown that VLPs derived from the bacteriophage Q β spontaneously package RNA-derived from *E. coli*, which can be degraded by RNase and replaced by B-type CpGs which stably bind to positively charged amino acids inside the VLP shell. Q β -VLPs have pores of ~2nm in diameter which allow the diffusion of small proteins such as RNase to digest the naturally loaded RNA. The B-type CpGs 1668 used in this study can

also diffuse through the same pores and bind to arginine repeats present inside the capsid naturally binding the genomic phage RNA. Titration experiments were carried out to optimize B-type CpG packaging into the empty capsid Q β -VLPs (Fig. 1A). Packaging Q β -VLPs was most efficient with a relatively low concentration of CpGs (1.125 μ g/20 μ g Q β -VLPs) indicating that low concentrations apparently facilitate efficient diffusion of CpGs through Q β -VLP pores likely because of limited aggregation and precipitation of the adjuvant. The H-2D^b restricted peptide p33 derived from lymphocytic choriomeningitis virus (LCMV) was used as a model antigen in this study. As described previously, p33 peptide was extended by the addition of 3 amino acids GGC at the C-terminus and then chemically coupled to Q β -VLPs using SMPH (succinimidyl-6-("maleimidopropionamido) hexanoate bifunctional) for cross-linking (31) (Fig. 1B). The additional bands in the SDS-PAGE show efficient coupling of p33 to Q β -VLP monomers (Fig. 1C). Densitometric analysis indicates the average presence of about two p33 peptides per subunit, indicating that about 360 peptides are displayed per particle. As seen previously, immunization of mice with these VLPs resulted in the induction of CTL responses *in vivo* as assessed by tetramer-staining (5). As we planned to mix empty or RNA-loaded VLPs with CpG-loaded Q β -VLPs, we wanted to ensure that CpGs would not pass from loaded VLPs to empty VLPs. To address this question, we packaged CpGs as described above into empty Q β -VLPs, and incubated these packaged VLPs for 10 min, 1h or 2h with empty or RNA loaded Q β -VLPs coupled to p33 (Fig. 1D). The mixtures were run on agarose gels and stained with SYBR safe to visualize DNA and RNA and Coomassie Blue to visualize protein. The results clearly show that there is no CpG transfer from loaded Q β -VLPs to empty or RNA-loaded Q β -VLPs, as the different VLPs remained as distinct bands which did not change in intensity (Fig. 1D). As VLPs loaded with small amounts of CpGs migrate more slowly than properly loaded VLPs (Fig. 1A), the absence of such bands in (Fig. 1D) also excludes transfer of small amounts of CpGs.

B-type CpGs packaged in separate Q β -VLPs mixed with Q β -VLPs displaying the antigens reach the same draining lymph node and efficiently charge the same APCs in vivo

A VLP-trafficking experiment was carried out next to test whether Q β -VLPs loaded with CpGs mixed with separate Q β -VLPs coupled to antigen will reach the same draining lymph node and result in charging of the same APCs resident in the lymph node. To this end, we visualized the accumulation of both VLPs in the popliteal draining lymph nodes shortly after injection into mouse footpads. As shown in (Fig. 2A) Q β packaged with CpGs and labelled with Alexa Fluor 488 efficiently reached the popliteal draining lymph node in less than 10 minutes after injection in mice footpads. This was also observed for the other empty VLP, Q β ($\emptyset$)-p33 labelled with Alexa Fluor 594 (Fig. 2A). Thus, both types of VLPs reached the draining lymph nodes with very fast kinetics. Next, we tested by flow cytometry whether lymph node resident cells would take up both VLPs simultaneously and what cell types are mainly involved in this process. To this end, Q β -VLPs packaged with CpGs were labelled with Alexa Fluor 488 and mixed with Q β ($\emptyset$)-p33 or Q β (RNA)-p33 labelled with Alexa Fluor 647 as illustrated in (Fig. 2B). The mixed VLPs were then co-injected in mice footpads and the draining lymph nodes were analysed by flow cytometry 24h later. Different types of APCs efficiently took up VLPs and a large fraction of the cells were double positive for both VLPs, indicating that they were charged with both Q β -VLPs (Fig. 2C). As a significant fraction of lymph node resident APCs simultaneously took up both VLPs, this indicates that it may not be necessary to physically link antigens and adjuvants for co-delivery. Further analysis showed that the lymph node resident cDCs; CD8⁻CD11b⁺, CD8⁺CD11c⁺ and CD8⁺CD11c⁺, the monocyte-derived DCs CD11b⁺CD11c⁺ and macrophages CD11b⁺CDF4/80⁺ were predominantly taking up the labelled VLPs in the popliteal lymph node. As expected, B cells (CD45R/B220⁺) were very inefficient at taking up or binding VLPs (Fig. 2C). Next we analysed MHC class II expression on CD11c⁺ cells as a marker of

activation. The results revealed that MHC-II expression is strongly increased in those that took up both VLPs compared to CD11c⁺ cells from naïve mice, indicating their activation by the CpGs packaged in VLPs (Fig. 2D). Interestingly, cells that did not take up VLPs but resided in the VLP-draining lymph node also showed increased expression of MHC class II (Fig. 2D). This is consistent with the finding that TLR-mediated upregulation of activation markers is mediated by the soluble factor IFN-beta (32). To further study both VLPs co-localisation, immunohistostaining of popliteal lymph nodes was performed 24h after injection in mice footpads with Q β (CpGs) and Q β ($\emptyset$)-p33 labelled with two different colours. Sections were additionally stained against CD11b⁺ cells to visualize APCs. The results show that a significant number of CD11b⁺ cells within the paracortex and sub-capsular region took up both types of VLPs (Fig. 2E). The co-localization of both VLPs was further assessed by 2-photon microscopy 24h after injection in murine footpads. The results confirmed the simultaneous up-take of both VLPs by the same cell in the popliteal draining lymph node (Fig. 2F).

B-type CpGs packaged in Q β -VLPs and mixed with Q β -VLPs displaying p33 induce specific CD8⁺ T-cell responses in vivo

For optimal induction of CTL responses, DCs presenting the relevant antigen need to be activated by e.g. CpGs. The current consensus in vaccinology is therefore that CpGs need to be physically linked to VLPs as is the case for VLPs packed with CpGs and displaying peptide p33. As we have seen above that CpG-loaded and antigen-displaying Q β -VLPs are presented by the same APCs in draining lymph nodes, we tested next whether the same formulation can induce T-cell responses. To this end we produced seven groups of mixed and coupled vaccines, which were designed to also assess the adjuvants activity of CpGs and the bacterial RNA naturally packaged into the Q β -VLPs (Fig. 3A). Groups 1 Q β ($\emptyset$) and 2 Q β (RNA) represent control groups without antigen, Groups 3 Q β ($\emptyset$)-p33 and 4 Q β (RNA)-

p33 were not mixed with CpG-loaded VLPs to assess the importance of such adjuvants in enhancing T-cell response *in vivo*. In Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)] and Group 6 [Q β (RNA)-p33 + Q β (CpGs)], CpGs were packed in separate Q β -VLPs and mixed with other Q β -VLPs displaying p33 prior to administration *in vivo*. Finally, p33 coupled to CpGs-loaded Q β -VLPs served as positive control and reference. The different vaccine-formulations were tested *in vivo* to assess the ability of this adjuvant's delivery method to stimulate specific CD8⁺ T-cell responses in comparison with p33 coupled to the Q β -VLPs. C57BL/6 mice were immunized subcutaneously with 50 μ g of the vaccine formulations and seven days later spleens were collected for tetramer staining and intracellular cytokine analysis. We found that RNA encapsulated in Q β -VLPs did not strongly enhance immunogenicity of VLPs when compared to the empty Q β vaccine groups confirming earlier data (33). In addition, the specific CTL responses in Groups 3 Q β ($\emptyset$)-p33 and Group 4 Q β (RNA)-p33 was less than Group 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], Group 6 [Q β (RNA)-p33 + Q β (CpGs)] or Group 7 Q β (CpGs)-p33 indicating the importance of B-type CpGs as adjuvants in enhancing adaptive T-cell responses *in vivo*. Responses induced in Group 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)] were comparable to the response induced in Group 7 Q β (CpGs)-p33 demonstrating the efficiency of the novel method at inducing specific T-cell responses similar to the responses obtained with antigen coupled to CpG-loaded VLPs. In contrast, adding CpG-loaded VLPs to RNA-loaded VLPs coupled to peptide p33 Group 6, [Q β (RNA)-p33 + Q β (CpGs)] enhanced CTL responses relatively little over those induced by Group 4 Q β (RNA)-p33 (Fig. 3B). Intracellular cytokine staining experiments for INF- γ and TNF- α basically mirrored responses seen with tetramer staining, again showing that Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], and group 7 Q β (CpGs)-p33 performed best (Fig. 3C and 3D).

CpGs packaged in Q β -VLPs and mixed with Q β -VLPs displaying p33 induce lytic CTL responses *in vivo*

To examine the *in vivo* lytic activity of the generated p33 specific CD8⁺ T-cells, we performed an *in vivo* CFSE cytotoxicity assay where control and p33 pulsed cells from donor mice were labelled with low and high CFSE dye concentrations. The two labelled populations were mixed at a ratio 1:1 and injected into recipient mice vaccinated seven days earlier. The spleens of the recipient mice were harvested 4h later to analyse the percentages of both unpulsed CFSE^{low} and p33-pulsed CFSE^{high} cells. (Fig. 4A) shows examples of Groups 1 Q β ($\emptyset$), 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] & 7 Q β (CpGs)-p33. Lytic activity is seen in Groups 5, 6 & 7 while p33-pulsed cells were not deleted in the Group 1. Percentage of specific lysis was calculated and plotted as shown in (Fig. 4B). Groups 5, 6 & 7 showed the highest specific lytic activity demonstrating the ability of Q β -VLPs based vaccines at inducing lytic T-cells *in vivo*.

Discussion:

CpGs are considered potent stimulators of the innate immune system via binding to Toll-Like Receptor 9 (TLR-9). This engagement results in innate responses mainly characterised by upregulating costimulatory molecules and enhancing the production of cytokines and chemokines by APCs and B-cells (11). This initial innate response will later translate to effective adaptive immune responses in the presence of foreign target antigen. It has also been shown that synthetic CpGs are capable of improving the antigen presenting function of dendritic cells and macrophages, enhancing T-cell priming and activation of NK cell via secretion of cytokines (17). Therefore, CpGs can efficiently act as vaccine adjuvants (systemic or mucosal administration) for mounting faster, stronger and more long lasting immune responses (19). Previous studies have investigated the safety profile of CpGs *in vivo* and the results indicated that CpGs might predispose to toxic shock, increase auto-antibody formation as well as inflammation (34, 35). While results have been particularly pronounced in mice, there remains the concern of inducing anti-DNA antibodies in humans, in particular

if the CpGs are linked to surface exposed proteins. This obstacle can be overcome by packaging the synthetic CpGs into VLPs. This method not only increases the potency of the CpGs adjuvants by facilitating endosomal delivery in APCs but also avoids exposure of other cells than APCs to the packaged CpGs. In addition, B-cells cannot recognize packaged CpGs via their B-cell receptor, avoiding the induction of anti-DNA antibodies (5). Indeed, immunization with CpG-loaded VLPs results in strong and efficient CTL responses in the absence of toxicity in mice (5).

The current dogma for VLP-based vaccines is that both adjuvants and antigen should be coupled to the same VLP for optimal response (3, 5, 36). In this study, we show a novel strategy of delivering adjuvants utilizing VLPs, which is mainly based on packaging synthetic CpGs in separate VLP and mix them with the vaccine prior to administration *in vivo*. In this study, we have used B-type CpGs as a model adjuvant due to its favourable characteristics including being a small and single stranded oligonucleotide which can easily diffuse into the empty capsid Q β -VLPs through its 2 nm pores and bind to the internal positively charged arginine repeats (5, 28, 37). Stereomicroscopic images were generated to study the trafficking kinetics of two different VLPs in live mice. The results reveal that both VLPs efficiently reached the popliteal draining lymph node in less than 10 minutes which is surprisingly fast (38). DCs normally take up more than 50 VLPs upon immunization (39). It may be expected that both VLPs of a mixture would be taken simultaneously by the same cell. To address this, a trafficking experiment was performed to follow the drainage and uptake of VLP/CpGs and VLP/peptide after labelling each with specific fluorophore. The results demonstrated efficient drainage of both nanoparticles via the lymphatic system to the popliteal draining lymph node where they were taken up simultaneously by a number of different subsets of APCs, ensuring activation of the same cells that present the antigen. CD8⁺ CD11c⁺ and CD8⁻ CD11c⁺ are two known subsets of lymphoid resident DCs having

slightly different functions (40). Both subsets have the ability to up-take VLPs and process them for MHC-II presentation; however only the CD8⁺ subset, which is mainly located in the T cell zone of the lymph node, has the ability to cross present VLP-derived antigens to T-cells upon activation (39). An important point to keep in mind for future application in humans, is, however, that cDCs do not express TLR-9 and it is therefore important to also target pDCs in the lymph node and/or employ additional adjuvants, activating all DC-types. To confirm the up-take of both VLPs by the same cell, immunohistochemistry of cryosections of the popliteal lymph node and 2-photon microscopy imaging were carried out. The results corroborated the FCM findings and demonstrated the up-take of both VLPs by the same cell.

Compared to CpGs, the bacterial RNA encapsulated in Q β -VLPs did not show strong increase of the immunogenicity of the nanoparticle. The inefficient CTL responses induced by RNA loaded VLPs is not due to degradation of RNA before cellular uptake, as RNA loaded VLPs induce equally strong IgG and IgA responses as CpG-loaded VLPs, (in contrast to empty VLPs and dependent on Myd88), demonstrating the ability of RNA in VLPs to activate TLR7/8 *in vivo* (41).

Furthermore, *in vivo* immunization using Q β -VLPs cross-linked to p33 without administration CpGs failed to produce a significant specific T-cell population, mirroring the importance of activating TLR-9 in APCs for enhancing adaptive immune responses.

When induction of T cell responses was assessed for mixed vaccine formulations, they induced CTL responses at similar efficiencies as obtained with standard conjugate vaccine. Intra-cellular cytokine staining confirmed significant increase in the levels of INF- γ and TNF- α in the groups where the Q β -VLPs packaged with B-type CpGs was mixed with Q β -VLPs displaying p33 -either with or without RNA- and responses were comparable to the levels obtained p33 coupled to CpG-loaded VLPs. *In vivo* cytotoxicity assays were carried

out to assess the lytic activity of the induced CD8⁺ cells by measuring lysis of peptide pulsed CFSE-labelled adoptively transferred splenocytes. [Qβ(Ø)-p33+Qβ(CpGs)] and [Qβ(RNA)-p33+Qβ(CpGs)] induced similar lysis as the standard Qβ(CpGs)-p33 vaccine. Thus, conjugated and non-conjugated vaccine preparations induced CTL responses in a similar range. The specific lysis of p33 pulsed cells in the other groups was not more than what was seen in the control group, and only low frequencies of tetramer⁺ CD8⁺ T cells was induced by these formulations.

Our results have interesting potential implications for autoimmunity and cancer immunology. Naturally occurring exosomes or debris from dying cells may behave like our empty, antigen-loaded VLPs during infection. Specifically, exosomes and cell debris may be taken up by host APCs leading to presentation of self-antigens. If the APCs simultaneously take up viral particles or bacteria during an infection, they may become activated in a way similar to the APCs activated by the CpG-loaded VLPs in the present manuscript. Hence, in analogy, this may result in specific T cell responses against the self-antigens, facilitating autoimmunity. On the other hand, it may be sufficient to inject CpG-loaded VLPs into local tumors to induce anti-tumor immunity, as local APCs may be charged with tumor-derived antigens leading to induction of tumor specific T cells subsequent to the activation of the APCs.

This novel method of delivering CpG adjuvants in separate VLPs can be beneficial in several ways. First in case of designing and developing personalized VLP-based vaccines as required for precision vaccines, it will be helpful to have a single “adjuvant” VLP which can be formulated with the different patient-specific antigen bearing VLPs. It will also allow to easily adjuvant VLPs which do not naturally carry RNA or DNA, such as HPV VLPs, Influenza-derived VLPs or Hepatitis B surface Ag VLPs. Hence, the here described VLP formulation of simply mixing adjuvants and antigen carrying VLPs will facilitate the

development of precision vaccines as well as help to re-formulate not naturally adjuvant VLP vaccines.

Materials and Methods:

Generation of Q β -VLPs

The process of producing, purifying and characterizing Q β -VLPs was previously discussed and described in details in (31, 42, 43).

Generation of Q β (RNA)-p33 vaccines

Q β (RNA)-VLPs with natural bacterial RNA was derivatised using SMPH succinimidyl-6-(X-maleimidopropionamido) hexanoate (Thermo SCIENTIFIC) for 1 hour at RT. Excess linker was removed using amicon centrifuge tubes 100kDa MWCU. A modified version of P33 peptide with the addition of (GCC) to the C-terminus of the peptide to facilitate its coupling to the derivatised Q β (RNA)-VLPs was purchased from (Pepscan PRESTO) and dissolved in DMSO in a 10-fold excess over Q β -VLPs monomers. The mixture was incubated for 2h at RT and the excess peptide was dialyzed.

Generation of Q β ($\emptyset$)-p33 vaccines

Natural bacterial RNA was digested using RNase A (Merck) (1.2 mg/ml) for (3mg/ml Q β) for 3h at 37°C. Digestion of RNA was confirmed using 1% agarose gel and SYBR safe dye. Q β ($\emptyset$)-VLPs was derivatised using SMPH succinimidyl-6-(X-maleimidopropionamido) hexanoate (Thermo SCIENTIFIC) for 1 hour at RT. Excess linker was removed using amicon centrifuge tubes 100kDa MWCU. A modified version of P33 peptide with the addition of (GCC) to the C-terminus of the peptide to facilitate its coupling to the derivatised Q β was purchased from (Pepscan PRESTO) and dissolved in DMSO in a 10-fold excess over Q β -VLPs monomers. The mixture was incubated for 2h at RT and the excess peptide was dialyzed.

B-type CpGs packaging into Q β -VLPs

B-type CpGs (5'-TCC ATG ACG TTC CTG ATG CT-3') (20 mer) with phosphorothiate bonds were synthesised by (iba). Q β was solubilized in 20mM HEPES. Bacterial RNA was digested using RNase A (Merck) (1.2 mg/ml) for (3mg/ml Q β) for 3h at 37°C. Digestion of RNA was confirmed using 1% agarose gel and SYBR safe dye. Packaging of B-type CpGs in Q β -VLPs was optimised using different concentration of B-type CpGs. 1.125 μ g/20 μ g VLPs in a volume of 25 μ l gave the best packaging. Larger amounts/volumes were used for vaccine preparation. The packaging of CpGs was confirmed also using 1% agarose gel, while Q β -VLPs protein was detected using coomassie blue stain.

Measuring specific CD8⁺ response using tetramer staining

C57BL/6 mice (9-12 weeks old; Harlan) were used to test cytotoxic T-cell specific CD8⁺ response using tetramer staining by flow cytometry. P33 tetramer was purchased from TCMetrix designed using H2D^b allele, p33 (KAVYNFATM) and APC fluorochrome. Mice were injected subcutaneously with 50 μ g Q β -VLPs developed vaccines, seven days later spleens were collected and stained using anti-Fc block, live/dead, CD3, CD8 and tetramers for analysis (all from eBioscience).

Measuring INF- γ and TNF- α using intracellular cytokine analysis

C57BL/6 mice (9-12 weeks old; Harlan) were used to test INF- γ and TNF- α using intracellular cytokine analysis protocol. The spleen was collected seven days after subcutaneous injection, (2X10⁶ cells) were collected and treated with 1 μ g/ml p33, Monensin (Sigma-Aldrich) (1:1000) and Brefeldin A (Sigma-Aldrich) (1:1000) for 6h at 37°C. The cells were then processed for surface staining using Fc block and anti CD8⁺ (eBioscience). Fixation and permeabilization were carried later for intra-cellular cytokine staining using anti INF- γ and TNF- α (all from eBioscience), reading was done by flow cytometry.

Trafficking experiment

Q β ($\emptyset$)-p33 and Q β (RNA)-p33 were cross-linked to p33 using SMPH cross-linker (Thermo SCIENTIFIC) and labelled with Alexa Fluor 647 according to the manufacturer's instructions (Thermo Fisher SCIENTIFIC). B-type CpGs was packaged in separate Q β -VLP as explained earlier and labelled with Alexa Fluor 488 according to the manufacturer's instructions (Thermo Fisher SCIENTIFIC). C57BL/6 mice (9-12 weeks old; Harlan) were injected with 25 μ g of each Q β ($\emptyset$)-p33 or Q β (RNA)-p33 and Q β (CpGs) in footpads under isoflurane anaesthesia. Popliteal lymph nodes were collected 24h later and processed for analysis with flow cytometry after treatment with collagenase D (Roche) and DNase I (Boehringer) in 5% FSC containing DMEM, for 30 min at 37°C. Cells were stained for anti- CD11b, CD11c, CDF4/80, CD45R/B220 and MHC-I (all from eBioscience), Acqua Live/Dead dye (Life Tech).

Immunohistochemistry

Mice were injected with Q β (B-type CpGs) labelled with Alexa Fluor 488 and Q β ($\emptyset$)-p33 labelled with Alexa Fluor 647, 24h after injection the popliteal lymph nodes were collected, embedded in Tissue-Tek O.C.T compound (Sakura) and snap frozen in liquid nitrogen. The lymph node tissues were cut into 8 μ m sections and allowed to dry overnight prior to staining. The sections were fixed with 100% acetone and air dried. They were pre-wet in PBS for 5min then blocked with 1%BSA + normal mouse serum for 20min. The sections were stained then with CD11b+ antibodies for 60min. Washing was carried out later in PBS and the sections were mounted by adding one drop of Fluoromount G solution. The sections were imaged on a Zeiss Axio Imager.A2 microscope, using the Plan-NEOFLUAR 20x/0,5 objective (Zeiss).

Stereomicroscopic Imaging of Popliteal LNs

Anesthetized C57BL/6 mice (9-12 weeks old; Harlan) were prepared for imaging by shaving the right leg, skin and adipose tissue was then removed to expose the popliteal lymph node as described in (44). Mice were then stabilized on a customized imaging platform. Bright field

illumination images were acquired to allocate the lymph node. Q β (CpGs) labelled with Alexa Fluor 488 or Q β ($\emptyset$)-p33 labelled with Alexa Fluor 594 were prepared in PBS and prior to imaging 25 μ g of either VLPs was injected in the mice right footpad to visualize the trafficking of the VLPs using fluorescent light illumination and a CCD camera (Nikon).

2-Photon Microscopy Imaging

C57BL/6 mice (9-12 weeks old; Harlan) were immunized with Q β (CpGs) labelled with Alexa Fluor 488 and Q β ($\emptyset$)-p33 labelled with Alexa Fluor 594 in the right footpad, and 24h later right popliteal lymph node was surgically exposed as above. The labelled VLPs were then excited using a TrimScope 2PM system (LaVision Biotec) equipped with a tunable Ti:Sa Laser (Spectraphysics) set to 840 nm excitation between 0 – 80 μ m below the LN surface identified by SHG signal from the collagen capsule. Emitted light and second harmonic signals were detected through 447/55-nm, 525/50-nm, and 593/40-nm with nondescanned detectors as described in (45).

In vivo Cytotoxic Assay using CFSE dye

C57BL/6 mice (9-12 weeks old; Harlan) were purchased and immunized with 50 μ g subcutaneous injections. 7 days after immunization target cells spleenocytes were collected from naïve mice and divided into 2 groups after lysing of RBCs, the first group (1×10^7 cells) was labelled with low concentration CFSE dye (Thermo Fisher SCIENTIFIC) and kept unpulsed while the other group (1×10^7 cells) was labelled with high concentration CFSE and pulsed with 1 μ M p33 peptide. The 2 prepared target cells were then mixed and each immunized mouse received (2×10^7 cells) from the mixed populations via i.v. route injection. 4h later the spleens were collected and the samples were processed for analysis using flow cytometry. The specific lysis of each vaccine was calculated using the following formula: Ratio = (CFSE^{high}/CFSE^{low}) while the % specific lysis was calculated as $100 \times (1 - \text{CFSE}^{\text{high}} \text{ pulsed} / \text{CFSE}^{\text{low}} \text{ unpulsed})$.

All procedures on animals were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986 and approved by the UK Home Office and the local ethical review committee for Clinical Medicine, University of Oxford.

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Figure 1: *Efficient packaging of B-type CpGs into VLPs derived from bacteriophage Q β*

A, Q β -VLPs loaded with different concentrations of B-type CpGs were analysed on agarose gels stained with either SYBR safe (top: staining RNA and DNA) and Coomassie Blue (bottom: staining protein), Lane 1: Q β +RNase, Lane 2: 9 μ g CpGs/20 μ gQ β , Lane 3: 4.5 μ g CpGs/20 μ gQ β , Lane 4: 2.25 μ g CpGs/20 μ gQ β , Lane 5: 1.125 μ g CpGs/20 μ gQ β , Lane 6: 0.56 μ g CpGs/20 μ gQ β , Lane 7: 0.28 μ g CpGs/20 μ gQ β and Lane 8: 0.0156 μ g CpGs/20 μ gQ β . B, Schematic diagram illustrating p33 coupling process to Q β -VLPs using SMPH bifunctional cross-linker. C, SDS-PAGE stained with coomassie stain, Lane 1: protein marker, Lane 2: Q β -VLPs 14kD, Lane 3: Q β -VLPs after RNA digestion using RNaseA, Lane 4: Q β (CpGs)-p33 after packaging with CpGs and derivatization for coupling to p33 using SMPH bifunctional cross-linker; arrows indicate Q β -VLP monomers and dimers (which are formed by SMPH cross-linking of 2 monomers) with coupled p33 peptides. D, Agarose gels stained with either SYBR safe (top: staining RNA and DNA) and Coomassie Blue

(bottom: staining protein) confirming stability of B-type CpGs packaging into empty capsid Q β -VLPs. Q β (CpGs) was mixed with Q β ($\emptyset$)-p33 for 10min, 1h & 2h as shown in Lanes 2, 3, 4 & 5 or with Q β (RNA)-p33 for 30min, 1h & 2h as shown in Lanes 6, 7, 8 & 9.

Figure 2: B-type CpGs packaged in separate Q β -VLPs carrier mixed with Q β -VLPs displaying the antigens reach the same draining lymph node and efficiently charge the same APCs in vivo. A, Stereomicroscopic images of popliteal draining LN in live mice illustrating the accumulation of Q β (CpGs) labelled with Alexa Fluor 488 and Q β ($\emptyset$)-p33 labelled with Alexa Fluor 594 at 10min post injection. From the top: bright field image of LN (identified by arrowheads), middle: fluorescent images prior to injection and last: 10 min post injection taken with appropriate fluorescent filter sets. B, injection scheme: Q β ($\emptyset$)-p33 or Q β (RNA)-p33 labelled with Alexa Fluor 647 mixed with Q β (CpGs) labelled with Alexa Fluor 488. C, Mice were injected into foot pads as described in B and cell subsets in draining lymph nodes were assessed by flow cytometry, frequencies of double positive cells within the different subsets are plotted (3 mice per group), one representative of 3 similar experiments is shown. D, histogram count of MHC-II expression gated on CD11c⁺ cells in naïve and vaccinated groups with [Q β (CpGs)+Q β ($\emptyset$)-p33] or [Q β (CpGs)+Q β (RNA)-p33]. The histogram count was obtained for the 3 subsets (red: the double negative subset in naïve mice, blue: the double negative subset in vaccinated mice, orange: double positive subset in vaccinated mice). E, immunohistostaining of popliteal lymph nodes cryosections 24h after vaccination with Q β (CpGs) labelled with Alexa Fluor 488 and Q β ($\emptyset$)-p33 labelled with Alexa Fluor 647, the cryosections were probed with antibodies detecting CD11b⁺ cells demonstrating the uptake and colocalization of both VLPs. F, 2-Photon Microscopy-generated maximum intensity projections of popliteal draining LN in live mice illustrating the colocalization of both VLPs -Q β (CpGs) labelled with Alexa Fluor 488 (green) and Q β ($\emptyset$)-p33 labelled with Alexa Fluor 647 (red), 24h post injection in C57BL/6 mice footpads. Collagen fibres are visualized by second harmonic generation (SHG, blue). 1: overview image. scale bar, 30 μ m, 2-4. Magnified image from indicated area showing green and SHG (blue) signal (2) and red and SHG signal (3), as well as the overlay (4). Arrowheads identify exemplary green and red double-positive structures, presumably dendritic cells. scale bar, 20 μ m.

Figure 3: B-type CpGs packaged in Q β -VLPs and mixed with Q β -VLPs displaying p33 induce specific CD8⁺ T-cell responses in vivo. A, Seven vaccine groups; from the left Group1 Q β ($\emptyset$) after digesting the bacterial RNA, Group 2 Q β (RNA) containing the bacterial RNA, Group 3 Q β ($\emptyset$) coupled to p33 using SMPH cross-linker, Group 4 Q β (RNA) coupled to p33 using SMPH cross-linker, Group 5 Q β ($\emptyset$) coupled to p33 using SMPH cross-linker and mixed with Q β (CpGs), Group 6 Q β (RNA) coupled to p33 using SMPH cross-linker and mixed with Q β (CpGs), Group 7 represents the standard VLP-based vaccine where Q β packaged with CpGs and coupled to p33 using SMPH cross-linker (Q β (CpGs)-p33). B, Flow cytometry data indicating the frequency of CD8⁺ Tetramer⁺ cells in each Group. Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] & 7 Q β (CpGs)-p33 showed a significant increase when compared with Groups 1 Q β ($\emptyset$), 2 Q β (RNA), 3 Q β ($\emptyset$)-p33 and 4 Q β (RNA)-p33. Group 3 vs Group 6 did not show significant difference. No significant difference was detected when comparing Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)] and 6 [Q β (RNA)-p33 + Q β (CpGs)] to Group 7 Q β (CpGs)-p33. For detailed statistical analysis refer to supplementary table 1. C, Flow Cytometry data indicating the frequency of CD8⁺ INF- γ ⁺ cells in each Group. Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] & 7 Q β (CpGs)-p33 showed a significant increase in frequency of INF- γ ⁺ in CD8⁺ cells when compared with Group 1 Q β ($\emptyset$), Group 2 Q β (RNA) and Group 4 Q β (RNA)-p33 only. The difference between Group 3 Q β ($\emptyset$)-p33 and Groups 5, 6 & 7 was not significant (ns). No significant difference was detected when comparing Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)] and 6 [Q β (RNA)-p33 + Q β (CpGs)] to Group 7 Q β (CpGs)-p33. For detailed statistical analysis refer to supplementary table 2. D, Flow Cytometry data indicating the frequency of CD8⁺ TNF- α ⁺ cells in each Group. Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] and 7 Q β (CpGs)-p33 showed a significant increase in frequency of TNF- α ⁺ in CD8⁺ cells when compared with Group 1 Q β ($\emptyset$), Group 2 Q β (RNA) and Group 4 Q β (RNA)-p33. The difference between Group 3 Q β ($\emptyset$)-p33 and Groups 5, 6 & 7 was not significant (ns). No significant difference was detected when comparing Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)] and 6 [Q β (RNA)-p33 + Q β (CpGs)] to Group 7 Q β (CpGs)-p33. For detailed statistical analysis refer to supplementary table 3. (3 mice per group), one representative of 3 similar experiments is shown.

Figure 4: B-type CpGs packaged in Q β -VLPs and mixed with Q β -VLPs displaying p33 induce lytic CTL responses in vivo. A, flow cytometry data indicating the frequency of unpulsed CFSE^{low} and p33-pulsed CFSE^{high} cells in Groups: 1 Q β ($\emptyset$), 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] and 7 Q β (CpGs)-p33. B, % of specific lysis in each developed group using the following formula $100 \times (1 - \text{CFSE}^{\text{high}} \text{ pulsed} / \text{CFSE}^{\text{low}} \text{ unpulsed})$. Groups 5 [Q β ($\emptyset$)-p33 + Q β (CpGs)], 6 [Q β (RNA)-p33 + Q β (CpGs)] and 7 Q β (CpGs)-p33 showed a significant specific lytic activity when compared with Groups 1 Q β ($\emptyset$), 2 Q β (RNA), 3 Q β ($\emptyset$)-p33 and 4 Q β (RNA)-p33. For detailed statistical analysis refer to supplementary table 4. (3 mice per group), one representative of 3 similar experiments is shown.

Figure 1.

Figure 2.

E

F

Figure 3.

Figure 4.

Delivering Adjuvants and Antigens in Separate Nanoparticles Eliminates the Need of Physical Linkage for Effective Vaccination

Novel Method