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Live baculovirus acts as a strong B and T cell adjuvant for monomeric and oligomeric protein antigens

Suvi Heinimäki, Kirsi Tamminen, Maria Malm, Timo Vesikari, Vesna Blazevic*

Vaccine Research Center, University of Tampere, Finland

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ABSTRACT

Recombinant proteins produced by baculovirus (BV) expression systems contain residual BV after crude purification. We studied adjuvant effect of BV on antibody and T cell responses against two model antigens, monomeric ovalbumin (OVA) protein and oligomeric norovirus (NoV) virus-like particles (VLPs). BALB/c mice were immunized intradermally with OVA alone or OVA formulated with live or inactivated BV, and VLP formulations comprised of chromatographically purified NoV GII.4 VLPs alone or mixed with BV, or of crude purified VLPs containing BV impurities from expression system. Live BV improved immunogenicity of NoV VLPs, sparing VLP dose up to 10-fold. Moreover, soluble OVA protein induced IgG2a antibodies and T cell response only when co-administered with live BV. BV adjuvant effect was completely abrogated by removal or inactivation of BV. These findings support the usage of crude purified proteins containing residual BV as vaccine antigens.

1. Introduction

Numerous currently licensed vaccines are based on live attenuated or inactivated pathogens, but there is a tendency towards development of safer non-replicating subunit protein vaccines. Therefore, novel vaccines under development are often based on recombinant proteins. Production of recombinant proteins results in substantial amounts of impurities related to the expression technology utilized in the production process. Impurities derived from baculovirus (BV) expression system include live BV, baculoviral dsDNA genome, and baculoviral proteins, particularly envelope glycoprotein gp64 (Hervas-Stubbs et al., 2007; Huhti et al., 2013; Lappalainen et al., 2016).

At present, several recombinant protein vaccines based on BV expression technology are commercially available, including human vaccines against human papilloma virus (Cervarix®, GlaxoSmith*Kl*ine) and influenza virus (Flublok®, Protein Sciences), as well as veterinary vaccines against classical swine fever virus (Porcilis Pesti®, MSD Animal Health) and porcine circovirus type 2 (Porcilis® PCV, MSD Animal Health; CircoFLEX®, B. Ingelheim). Moreover, other potential vaccine candidates are in advanced stages of clinical trials, such as those directed against norovirus (NoV) (Vesikari and Blazevic, 2015; Atmar et al., 2016). Because of a strict regulatory control, substantial efforts are undertaken to purify proteins for use as human vaccine antigens. On the other hand, impurities related to BV expression system have commonly been associated with strong immunostimulatory effects. BV has been reported to possess adjuvant properties, promoting humoral and cellular immune responses against foreign antigens as well as activation of innate immune responses by inducing type I and II IFNs (Gronowski et al., 1999; Abe et al., 2005; Hervas-Stubbs et al., 2007; Suzuki et al., 2010). Moreover, wild-type BV has contributed to protection in mice from a lethal challenge of encephalomyocarditis virus (EMCV) (Gronowski et al., 1999), influenza virus H1N1 (Abe et al., 2003) and foot-and-mouth disease virus (FMDV) (Molinari et al., 2011; Quattrocchi et al., 2013), where protection was directly associated with immune responses elicited by BV.

In the history of human vaccines, the most successful in disease control and eradication have been those employing live attenuated viruses (e.g. polio, measles, yellow fever, influenza virus, rotavirus) (Minor, 2015). As these viruses can still infect cells and replicate to a certain limit, the live attenuated vaccines are able to induce fast and durable protective immunity. Instead, recombinant proteins are generally weakly immunogenic unless administered with an external adjuvant (Bachmann and Jennings, 2010; Josefsberg and Buckland, 2012). Due to the adjuvant and antiviral properties of BV described above, residual BV originating from the expression system could be considered as an adjuvant to improve immunogenicity of these proteins.

In the present study, we investigated adjuvant effect of BV on

* Correspondence to: Vaccine Research Center, University of Tampere, Biokatu 10, 33520 Tampere, Finland.
E-mail addresses: suvi.heinimaki@uta.fi (S. Heinimäki), kirsi.tamminen@uta.fi (K. Tamminen), maria.malm@uta.fi (M. Malm), timo.vesikari@uta.fi (T. Vesikari), vesna.blazevic@uta.fi (V. Blazevic).

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antibodies and T cell mediated immune responses against two different model antigens, a soluble monomeric ovalbumin (OVA) protein and NoV virus-like particles (VLPs). We demonstrate here that live BV promoted immunogenicity of both OVA and NoV VLPs, and the effect was abolished by virus inactivation.

2. Materials and methods

2.1. Antigenic formulations

2.1.1. Ovalbumin

Lyophilized OVA (Albumin from chicken egg white; Sigma) was dissolved at 2 mg/mL in phosphate-buffered saline (PBS; Lonza, Verviers, Belgium). The stock was sterilized with a 0.2 μm filter prior to use.

2.1.2. Production of mock BV

Live BVs were generated in Bac-to-Bac BV expression system (Invitrogen, Carlsbad, CA) in Sf9 insect cells and purified on sucrose gradients and ultracentrifugation as previously described (Huhti et al., 2013). The infectious BV titer of the mock BV, expressed as plaque-forming unit per mL (pfu/mL), was determined using a BacPAK™ Rapid Titer Kit (Clontech Laboratories, Mountain View, CA) according to the manufacturer's instructions.

The inactivated BV (IBV) was prepared from live BV by heat inactivation at 60 °C for 1 h. The inactivation of live BVs was confirmed by BacPAK™ Rapid Titer Kit.

2.1.3. Production of recombinant NoV VLPs

NoV GII.4 (Reference strain accession number AF080551) VLPs were produced in BV–insect cell expression system, as described in details elsewhere (Huhti et al., 2010). GII.4 VLPs were crudely purified on two discontinuous sucrose gradients and ultracentrifugation as described earlier (Huhti et al., 2010). In order to obtain highly purified proteins, the crude GII.4 VLPs were further purified using a combination of two-step anion exchange (HiTrap Q, GE Healthcare, Uppsala, Sweden) chromatography procedures (Huhti et al., 2013).

The purity, identity and morphology of the crude and chromatographically purified NoV GII.4 VLPs were determined using previously published procedures (Huhti et al., 2013; Blazevic et al., 2016), such as SDS-PAGE followed by PageBlue™ staining (Thermo Fisher Scientific Inc., Rockford, IL) or immunoblotting with anti-BV gp64 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), Quant-it dsDNA Broad-Range Assay Kit (Invitrogen), Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD), BacPAK™ Rapid Titer Kit, and transmission electron microscopy (FEI Tecnai F12, Philips Electron Optics, Holland) after negative staining.

2.1.4. Synthetic peptides

A murine $CD4^+$ T cell epitope (^{323}ISQAVHAAHAEINEAGR^{339}, InvivoGen, San Diego, CA) derived from chicken OVA (McFarland et al., 1999) was used to test OVA-specific T cell responses by enzyme-linked immunospot (ELISPOT) interferon gamma (IFN-γ) assay. Rotavirus-specific 14-mer R6-1 peptide (^{289}RLSFQLMRPPNMTP^{302}) synthetized by Proimmune LTd. (Oxford, UK) was used as an irrelevant negative control peptide in the assays.

Seventy-six 18-mer overlapping peptides representing the entire 539 amino acid (aa) sequence of GII.4–1999 NoV VP1 were custom synthetized (Synpeptide Co. Ltd, Shanghai, China) and pooled (Malm et al., 2016b, 2016c). A complete peptide pool (named 99 pool) as well as an individual peptide 99-50 (^{344}TRAHKATVSTGSVHFTPK^{361}) corresponding to the previously identified murine 18-mer NoV-specific $CD4^+$ T cell epitope (Malm et al., 2016c) were used to test NoV-specific T cell responses by the ELISPOT.

2.2. Animal immunization and sample preparation

Female BALB/c OlaHsd mice, aged 6 weeks, were purchased from Envigo (Horst, the Netherlands). The mice were randomly divided into eight groups (Gr I–VIII), acclimatized under controlled specific pathogen-free conditions for one week prior to the start of the experiment, and maintained throughout the study period with food and water provided ad libitum. Animals (3–4 mice/experimental group) were immunized twice (at study weeks 0 and 3) intradermally (i.d.) at the base of the tail with 50 μl of different antigenic formulations, each formulation diluted in sterile PBS to contain the indicated dose of immunogen (Table 2). Control group received no antigen (PBS only). Immunizations were performed under general anesthesia induced with a mixture of Hypnorm® (VetaPharma Limited, Leeds, UK) and Dormicum® (Roche Pharma AG, Grenzach-Wyhlen, Germany).

To test the kinetics of the antibody responses in sera, tail blood samples (diluted 1:200 in PBS at the time of collection) were collected at study weeks 0 (pre-bleed, non-immune sera) and 2 or 3. Mice were sacrificed 35 days after the first immunization (at study week 5) by decapitation, when whole blood and lymphoid tissues were collected. Preparation of blood samples and a single-cell suspension from the spleen of each mouse was conducted according to the published procedures of our laboratory (Tamminen et al., 2012). All of the experimental procedures carried out were in accordance with the regulations and guidelines of the Finnish National Experiment Board (permission numbers ESLH-2009–06698/Ym-23 and ESAVI/4106/04.10.03/2012) and all efforts were made to minimize animal suffering. Animals were monitored for physical conditions throughout the experiment.

2.3. Detection of serum IgG and IgG subtypes by ELISA

2.3.1. OVA- and NoV GII.4-specific IgG, IgG1 and IgG2a responses

Sera of experimental mice were tested in ELISA for the presence of OVA- or NoV GII.4-specific IgG, IgG1 and IgG2a antibodies as described in detail elsewhere (Blazevic et al., 2011; Tamminen et al., 2012). Briefly, 96-well half-area polystyrene plates (Corning Inc, Corning, NY) were coated with OVA (200 ng/well for IgG, 100 ng/well for IgG subtypes) or GII.4 VLPs (20 ng/well). Serum samples at 1:200 dilution or serially diluted two-fold were added on the plates, and the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), IgG1 (Invitrogen) or IgG2a (Invitrogen) and SIGMA FAST OPD substrate (Sigma-Aldrich). Optical density (OD) values at 490 nm (OD_{490}) were measured by a microplate reader (Victor^2 1420; PerkinElmer, Waltham, MA). A sample was considered positive if the OD_{490} was above the cut-off value (mean OD_{490} of the control mice + 3 × SD) and $OD_{490} > 0.1$. The end-point titers were defined as the reciprocal of the highest dilution with an OD_{490} above the cut-off value. A titer of 100 was assigned to negative samples, being a half of the starting serum dilution.

2.3.2. BV-specific IgG response

Induction of BV-specific IgG response was evaluated by testing 1:200 diluted serum samples of individual experimental mice by ELISA as described above for OVA- and NoV-specific responses, but the microtiter plates were coated with mock BV (100 ng/well).

2.4. Antibody avidity assay

The avidity of OVA- and NoV-specific IgG antibodies was determined in 1:200 diluted serum samples according to the previously published avidity assay (Tamminen et al., 2012) using an extra urea incubation step to remove the low-avidity antibodies. Results were expressed as avidity index: (OD_{490} with urea/OD_{490} without urea) × 100%.

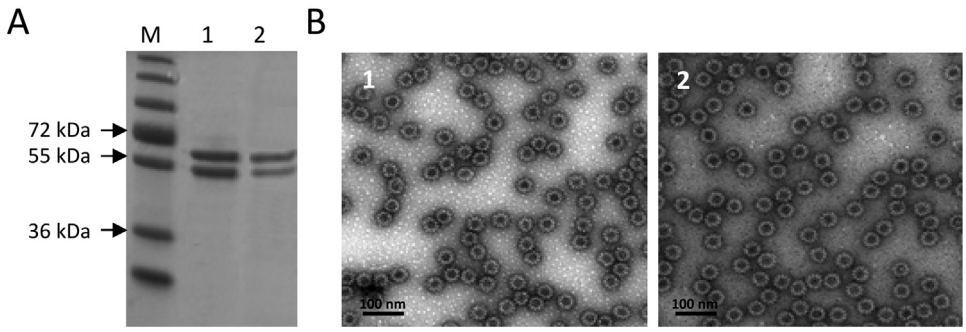

Fig. 1. Characterization of NoV GII.4 VLPs used for immunizations. (A) Purity and integrity analysis of crude (lane 1) and chromatographically purified (lane 2) NoV GII.4 VLPs with SDS-PAGE followed by PageBlue staining. Lane M, molecular weight marker. (**B**) Electron microscopy images of crude (panel 1) and chromatographically purified (panel 2) NoV GII.4 VLPs corresponding to the respective SDS-PAGE lanes 1 and 2 (A). Protein structures were examined after negative staining with 3% uranyl acetate. Images were observed at 18500 × magnification.

2.5. NoV blocking assay

To examine the ability of serum antibodies to block the binding of NoV VLPs to a cellular histo-blood group antigen (HBGA) receptor, pig gastric mucin (PGM) type III (Sigma Chemicals) was used as a source of HBGAs according to the published procedure (Lindesmith et al., 2012) with slight modifications (Malm et al., 2017). Group-wise pooled two-fold serum dilutions (starting at 1:50) were pre-incubated for 1 h at 37 °C with 0.1 μg/mL of NoV GII.4 VLPs before plating on 96-microwell plates coated with 2.5 μg/mL of PGM. Maximum binding was determined with NoV VLPs without serum. Bound VLPs were detected with human NoV antiserum, an anti-human IgG-HRP (Novex) and Sigma FAST OPD substrate, followed by determination of OD_{490} readings with a microplate reader (Victor^2 1420). Blocking index (%) was calculated as follows: 100% − [OD_{490} (wells with serum)/ OD_{490} (wells without serum, maximum binding) × 100%]. Results were expressed as the blocking titer 90 (BT90), the reciprocal of the highest serum dilution blocking ≥90% of the VLPs binding to the HBGAs.

2.6. Cell-mediated immune responses

2.6.1. NoV and OVA-specific ELISPOT IFN-γ

Antigen-specific T cell responses were analyzed using a slightly modified ELISPOT IFN-γ assay (Tamminen et al., 2013). Group-wise pooled splenocytes (0.2×10^6 cells/well) were plated on Multiscreen HTS-IP filter plates (Millipore, Billerica. MA) coated with a monoclonal anti-mouse IFN-γ antibody (Mabtech AB, Nacka Strand, Sweden) at 5 μg/mL. For detection of OVA-specific IFN-γ producing cells, cell were stimulated in duplicates with OVA peptide (0.2, 1, 2, and 4 μg/mL) or R6-1 peptide (negative control, 4 μg/mL). To detect NoV-specific IFN-γ producing cells, cells were stimulated in duplicates with GII.4–99 peptide pool (2 μg/mL), GII.4 99-50 peptide (4 μg/mL) or OVA peptide (negative control, 4 μg/mL). Background control (culture medium; RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine; all from Sigma-Aldrich) and cell viability control (10 μg/mL T cell mitogen Concanavalin A; Sigma-Aldrich) were tested in each assay. After overnight incubation at 37 °C, IFN-γ secretion was detected with biotinylated anti-mouse IFN-γ monoclonal antibody and alkaline phosphatase -conjugated streptavidin (both from Mabtech AB). The spots developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate) substrate (Mabtech AB) were counted by ImmunoSpot® automatic CTL analyzer (CTL-Europe GmbH, Bonn, Germany). The results were expressed as mean spot-forming cells (SFC)/10^6 splenocytes of duplicate wells. An increase of at least twice above the negative control peptide SFC counts was considered as a positive result.

2.6.2. BV-specific ELISPOT IFN-γ

BV-specific T cell responses were assessed by ELISPOT IFN-γ assay as described above, except group-wise pooled splenocytes (1×10^5 cells/well) of immunized and control mice were stimulated with mock BV (1×10^4–10^6 pfu/well).

2.7. Statistical analyses

The Mann-Whitney *U*-test and Kruskal-Wallis test were employed to determine the statistical differences between the non-parametric observations of two or more independent groups. All analyses were conducted by IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY), Version 23.0. The statistically significant difference was defined as $p \le 0.05$.

3. Results

3.1. Characterization of NoV VLPs used for immunization

Crude and chromatographically purified NoV GII.4 VLPs were characterized for purity, identity and morphology prior to use for immunizing animals. Fig. 1A shows SDS-PAGE, where NoV capsid protein appeared as a doublet band, typical for the capsid GII.4 protein (Huhti et al., 2010), in both VLP preparations. Instead, a faint band corresponding to the size of the BV envelope gp64 protein, was observed only in the crude preparation (Fig. 1A). The presence of gp64 in the crude purified VLPs was confirmed by immunoblotting (Table 1) with anti-BV gp64. Furthermore, the presence of BV was further tested by determining the infectious BV titers (Table 1). Although crude VLPs are produced by the standard methods as described above, different production lots have inconsistent quantity of BV (data not show). Moreover, crude and chromatographically

Table 1
Specifications and analysis of impurities related to the expression system in the crude purified and chromatographically purified NoV GII.4 VLPs.

Specification	Crude GII.4 VLPs	Pure GII.4 VLPs
Morphology[a]	VLPs (~38 nm)	VLPs (~38 nm)
Infectious BV (pfu/mL)[b]	10^7	0
BV gp64[c]	+	–
Total DNA (ng dsDNA/10 μg protein)[d]	< 30	< 10
Endotoxin (EU/10 μg protein)[e]	< 0.1	< 0.1

[a] Electron microscopy after negative staining.
[b] BacPAK™ Rapid Titer Kit.
[c] SDS-PAGE followed by immmunoblotting with anti-BV gp64.
[d] Quant-it dsDNA Broad-Range Assay Kit.
[e] Limulus Amebocyte Lysate Assay.

Table 2
Antigenic formulations used for immunization of experimental mice groups and body weights.

Experimental group	Immunogen	Injection dose	Start weight (g)[a]	Termination weight (g)[b]
I	OVA	45 μg	17.9 ± 0.3	21.0 ± 0.8
II	OVA + BV	45 μg + 10^7 pfu	17.4 ± 1.1	20.6 ± 1.2
III	OVA + IBV	45 μg + 10^3 pfu	17.9 ± 1.4	21.4 ± 1.8
IV	GII.4 VLPs	1 μg	17.9 ± 0.9	20.5 ± 1.0
V	GII.4 VLPs	10 μg	17.5 ± 0.4	20.5 ± 0.5
VI	GII.4 VLPs + BV	1 μg + 10^7 pfu	17.3 ± 4.3	21.1 ± 3.9
VII	Crude GII.4 VLPs	1 μg (10^5 pfu)	17.2 ± 1.0	20.8 ± 2.1
VIII	PBS	–	17.8 ± 0.6	20.7 ± 0.5

[a] Mean weight +/− SD per experimental group of mice at study week 0 (prior to immunization).
[b] Mean weight +/− SD per experimental group of mice at study week 5.

purified NoV GII.4 VLPs were morphologically similar as indicated by EM analysis (Fig. 1B). Hence, the chromatographic purification did not affect morphology or integrity of VLPs, but removed impurities related to the BV expression system, as virtually no traces of dsDNA genome, live BV or baculoviral proteins were detected in the pure VLP preparations (Table 1).

3.2. Assessment of physical condition

Regardless of the antigenic formulation used for immunization, all animals remained healthy and gained weight during the study period (Table 2). No difference in body weight between the experimental groups was observed (p=0.98).

3.3. Enhancement of OVA-specific serum antibody responses by BV

Possible influence of BV on OVA-specific immune responses was investigated by immunizing the experimental mice on a two-dose schedule with 45 μg of OVA alone or together with live BV or IBV at an interval of three weeks (Table 2). Two immunizations of each formulation elicited considerable level of total anti-OVA IgG antibodies (Fig. 2A). Similar IgG responses with end-point titers of 12800 were induced, when OVA was administered alone or together with IBV. Instead, co-administration of OVA with live BV resulted in 16-fold increase in the IgG titer (end-point titer 204800). No OVA-specific antibodies were detected in sera of control mice.

Determination of OVA-specific IgG subtype IgG1 and IgG2a titers, representing Th2- and Th1-type responses, revealed induction of IgG1 antibodies by each OVA formulation (Fig. 2B) but induction of significant IgG2a antibodies only by the OVA formulations containing live BV (Fig. 2C). Co-administration of OVA with BV generated ≥16-fold higher levels of IgG1 (end-point titer 409600) compared to administration of OVA alone or together with IBV. No IgG2a was detected after immunization of mice with OVA alone while formulation with IBV induced very low level of IgG2a, probably due to incomplete inactivation of BV (Table 2) containing 10^3 pfu. In contrast, combination of OVA and live BV induced very high anti-OVA IgG2a antibodies (end-point titer 102400).

Serum samples from individual mice were further assayed for the avidity of OVA-specific IgG antibodies. Immunization with OVA or combination of OVA and IBV induced IgG antibodies with low avidity (respective avidity indices 21.0 ± 5.8% and 10.4 ± 1.3%), but inclusion of BV in the formulation elevated the avidity of antibodies (avidity index 47.5 ± 8.8%) (Fig. 2D). No statistical difference (p=0.083) was detected in the avidity indices between the groups immunized with OVA alone and a combination of OVA and BV, but the avidity was significantly greater (p=0.021) in mice co-administered with OVA and BV compared to the mice co-administered with OVA and IBV.

3.4. Induction of OVA-specific T cell responses by live BV

The difference in induction of Th1 responses by OVA formulations with or without BV was further studied measuring Th1-type cytokine IFN-γ production from the splenocytes of immunized mice (Fig. 3). Cells from the mice receiving OVA and BV responded with considerable IFN-γ release to *ex vivo* stimulation with the 17-mer OVA-specific peptide, representing a $CD4^+$ T cell epitope (Fig. 3). On the contrary, immunization with OVA alone or together with IBV did not induce OVA-specific IFN-γ production by T cells (p=0.020 for both) (Fig. 3). No response to the negative control peptide R6-1 was detected in any of the study groups (data not shown).

3.5. Improved immunogenicity of NoV VLPs by BV

To examine effect of BV on NoV GII.4-specific humoral immune responses, mice were immunized twice with 1 or 10 μg doses of pure NoV GII.4 VLPs, 1 μg dose of pure VLPs combined with BV or 1 μg dose of crude-purified VLPs containing BV (Table 2). After the first immunization, 1 μg of pure VLPs induced significantly lower IgG response in comparison with other VLP formulations (p=0.003), whereas 10 μg dose of pure VLPs as well as both VLP formulations containing BV resulted in similar responses (p=0.221) at study week 2 or 3 (Fig. 4A). Two immunizations of each antigenic formulation containing NoV GII.4 VLPs elicited high level of total anti-GII.4 IgG antibodies at week 5 (Fig. 4A). Control mice remained negative for GII.4-specific total IgG during the study period (Fig. 4A).

Determination of IgG subtype titers showed induction of both Th2- and Th1-type response by each GII.4 VLP formulation. Similar IgG1 responses were elicited, irrespective of the presence or absence of BV (Fig. 4B). Instead, administration of the GII.4 VLPs with BV resulted in 8-fold higher IgG2a titers (end-point titers of 204800) compared with administration of 1 or 10 μg doses of pure VLPs (end-point titers of 25600) (Fig. 4C). No GII.4-specific IgG subtype antibodies were detected in sera of control mice (Fig. 4B and C).

3.6. Effect of BV on functionality of GII.4-specific antibodies

Testing of individual immune sera for the avidity of GII.4-specific IgG antibodies indicated, that immunization with 1 μg of pure VLPs induced antibodies with a considerably lower (p=0.029) avidity (avidity index 18.4 ± 7.9%) as compared with antibodies induced with a combination of VLPs and BV (67.2 ± 10.3%) or crude-purified VLPs with residual BV (60.9 ± 3.9%) (Fig. 5A). No statistical difference (p=0.624) was observed between the groups of mice immunized with 10 μg of pure VLPs (avidity index 48.7 ± 8.9%) and the two VLP formulations containing BV (Fig. 5A).

The functionality of the GII-4-specific antibodies was further examined by measuring the blocking potential of the pooled immune sera, where PGM was employed as the HBGA source for GII.4 VLP binding. Each VLP formulation induced antibodies able to block ≥90% of the VLP binding (Fig. 5B). However, 4-fold higher blocking titers BT90 were observed in mice immunized with VLPs combined with BV or crude-purified VLPs containing BV, compared with mice immunized with 1 μg dose of pure VLPs (Fig. 5B). Moreover, administration with 10 μg dose of pure VLPs resulted in antibodies with 2-fold greater BT90 as compared with antibodies induced with lower dose of pure VLPs (Fig. 5B).

3.7. Enhancement of GII.4-specific T cell responses by BV

Induction of T cell responses by NoV GII.4 VLP formulations was

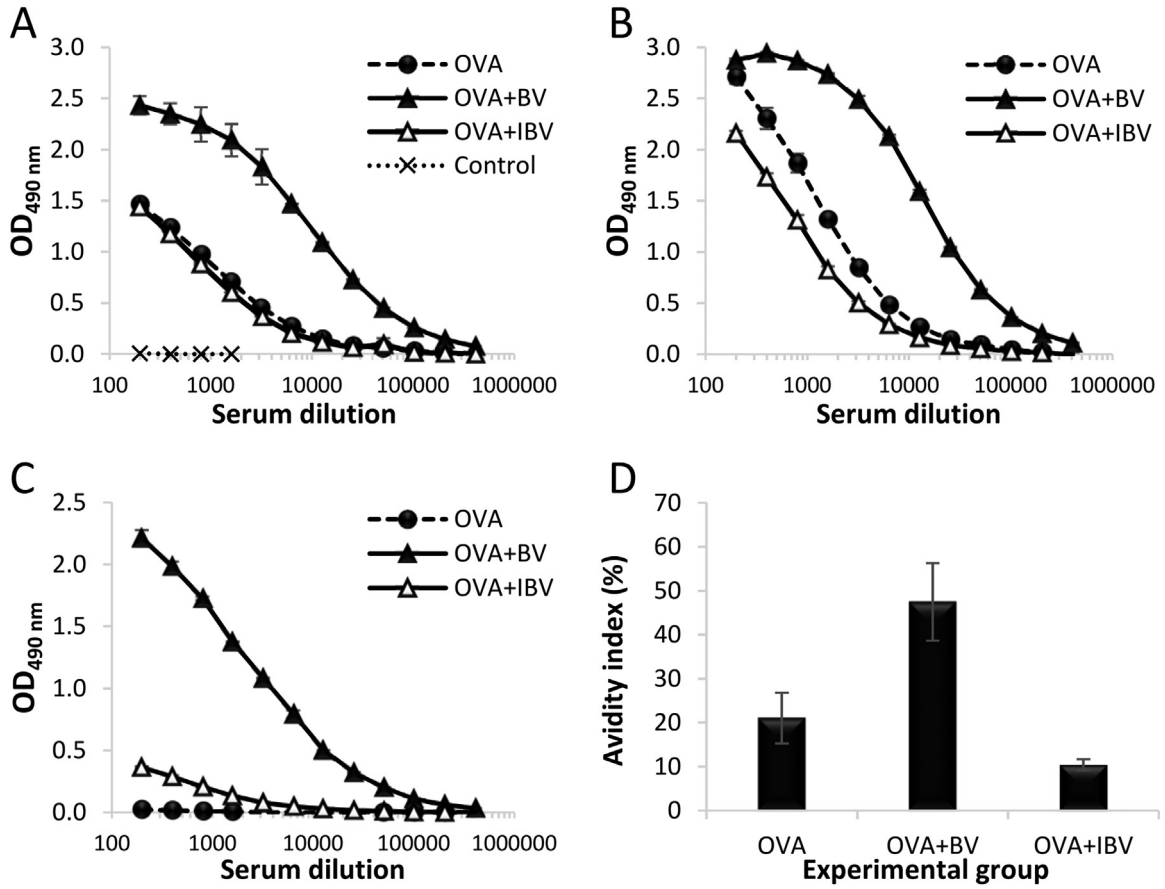

Fig. 2. *OVA-specific serum IgG responses.* End-point titrations of anti-OVA IgG (A), IgG1 (B) and IgG2a (C) antibodies of group-wise pooled termination sera of mice immunized with OVA alone or formulated with BV. Control mice received PBS only. Mean titration curves with standard errors of duplicate wells are shown. (D) Mean avidity indices (%) with standard error of the means of serum OVA-specific IgG antibodies.

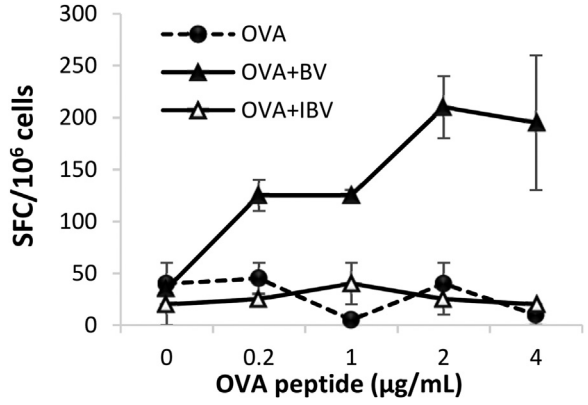

Fig. 3. *OVA-specific T cell responses.* Different concentrations of an OVA-specific peptide were employed to stimulate IFN-γ production from the group-wise pooled splenocytes of mice immunized with OVA alone or together with BV or IBV. Mean IFN-γ spot-forming cells (SFC)/10^6 splenocytes of duplicate wells with standard errors of the means are shown.

characterized by analyzing Th1-type cytokine IFN-γ production from the splenocytes of experimental mice. Splenocytes from the mice receiving 1 µg of pure GII.4 VLPs did not produce IFN-γ in response to any stimulation (Fig. 6). Instead, immunization of mice with all other VLP formulations elicited a robust IFN-γ response, when stimulated with the 18-mer 99-50 peptide representing a NoV-specific $CD4^+$ T cell epitope, or with 99 peptide pool representing the entire GII.4–1999 NoV VP1 (Fig. 6 and data not shown). Ten µg dose of pure VLPs induced similar quantities of IFN-γ secreting cells to 1 µg of VLPs combined with BV (p=0.08) as well as 1 µg of crude-purified VLPs containing BV (p=0.885). Negative control OVA peptide stimulated no IFN-γ production by the cells from any of the experimental groups (Fig. 6).

3.8. Induction of BV-specific antibodies and T cell responses

Induction of BV-specific IgG response was evaluated in serum samples of mice immunized with OVA formulations (groups I-III, Table 2). As expected, addition of live BV or IBV to the OVA formulation induced BV-specific responses (p=0.015). No BV response was elicited immunizing with OVA alone, but considerable levels of BV-specific antibodies were induced, when OVA was co-administered with BV or IBV (Fig. 7A). No difference in antibody levels was noted whether OVA was administered with BV or IBV (p=0.149).

Induction of BV-specific T cell responses was studied measuring IFN-γ production from the splenocytes of the experimental groups. Cells from the mice receiving OVA alone did not produce IFN-γ in response to stimulation with BV (Fig. 7B). Instead, immunizations of mice with formulations containing live BV elicited high IFN-γ response (Fig. 7B). Moreover, low levels of IFN-γ were induced by co-administration of OVA with IBV, which were substantially lower compared with the responses induced by live BV formulation (p=0.019). No BV-specific IFN-γ response was detected by the cells of negative control mice (Fig. 7B).

4. Discussion

Several reports have shown live BV to have strong adjuvant properties, promoting adaptive immune responses against co-administered antigen and activation of innate immunity (Abe et al., 2003;

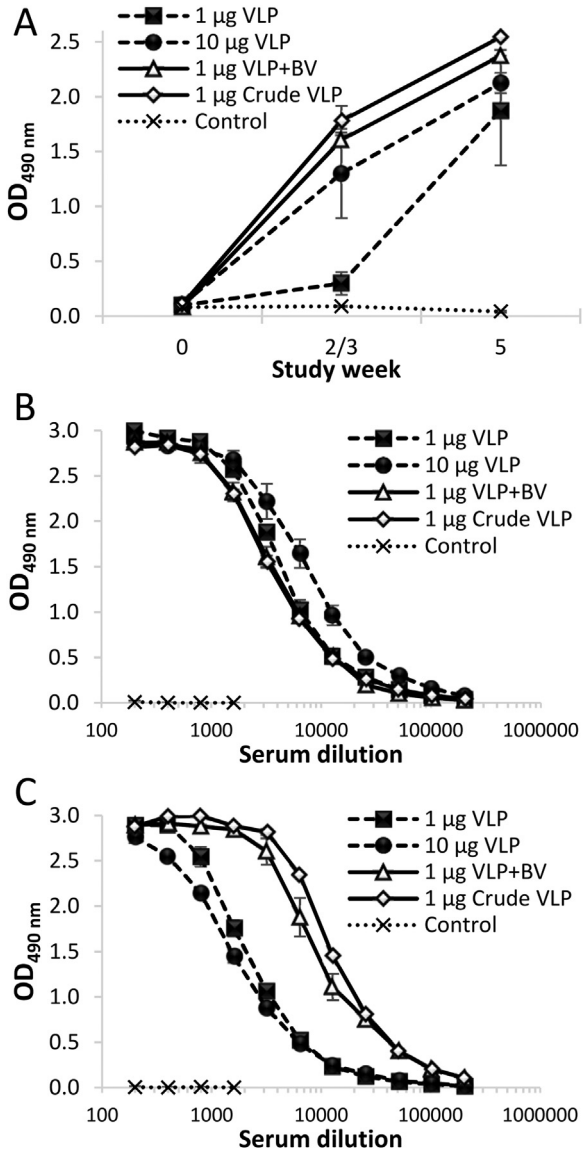

Fig. 4. *NoV-specific serum IgG responses.* (A) Kinetics of NoV GII.4-specific total IgG antibodies in serum samples of mice immunized with 1 or 10 µg of pure NoV GII.4 VLPs, 1 µg of pure VLPs combined with BV or 1 µg of crude-purified VLPs containing BV at weeks 0 and 3. Control mice received PBS only. Group mean OD_{490} values with standard error of the means of tail blood samples collected at study weeks 0 (pre-immune sera) and 2 or 3 as well as termination sera at week 5 are shown. End-point titrations of anti-GII.4 IgG1 (B) and IgG2a (C) subtype antibodies of group-wise (4 mice/group) pooled termination sera. Mean titration curves with standard errors of duplicate wells of up to 2 independent experiments are shown.

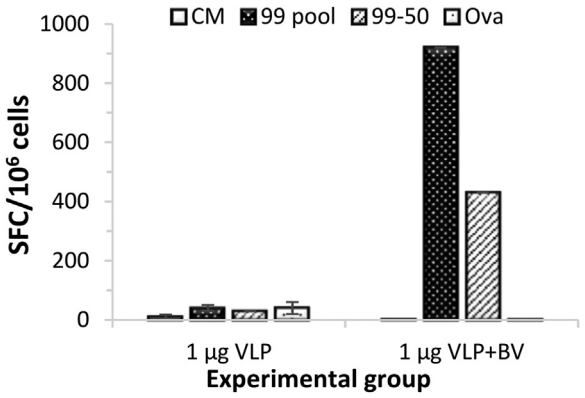

Fig. 6. *NoV GII.4-specific T cell responses.* NoV GII.4–1999 –specific 99 peptide pool and a single 99-50 peptide were used to stimulate IFN-γ production from the group-wise pooled splenocytes of mice immunized with 1 µg of pure NoV GII.4 VLPs or 1 µg of pure VLPs combined with BV. Culture medium (CM) as well as an OVA peptide were used as negative controls. Mean IFN-γ spot-forming cells (SFC)/10^6 splenocytes of duplicate wells with standard errors of the means are shown.

Hervas-Stubbs et al., 2007; Molinari et al., 2011; Margine et al., 2012; Quattrocchi et al., 2013). The adjuvant and antiviral properties of BV make it an interesting tool to be considered in improvement of immunological responses to vaccine antigens. This study was designed to investigate adjuvant effect of BV on adaptive immune responses against two different model antigens, monomeric OVA protein and oligomeric NoV VLPs. We found significant differences between the immune responses triggered by these proteins alone or formulated with BV in terms of antigen-specific antibody kinetics and titers, IgG antibody subtypes, induction of T cell responses, and importantly, functionality of the induced antibody responses. The differences were clearly associated with the presence of live BV in the preparations and not the structural or morphological differences (Fig. 1), as removal and inactivation of BV abrogated the responses.

The exact mechanism through which BV exerts its adjuvant behavior is not fully elucidated. Accordingly, mannose-binding residues in gp64 have been proposed to interact with the mannose receptor expressed on macrophages and dendritic cells (DCs) (Abe et al., 2003). Although mannose receptor plays an important role in host defense and induction of innate immunity, several evidence contradicts gp64 as the main factor in the stimulatory activities of BV (Abe et al., 2005; Hervas-Stubbs et al., 2007). Instead, gp64-mediated uptake of BVs and recognition of unmethylated CpG sequences within baculoviral DNA by Toll-like receptor 9 (TLR9)/MyD88 dependent and independent pathways have been suggested to be necessary for the activation of innate immune responses (Abe et al., 2005, 2009). BV is a potent inducer of IFN-α and IFN-β (Gronowski et al., 1999; Abe et al., 2005; Hervas-Stubbs et al., 2007) but also other cytokines of relevance, most notably IFN-γ (Suzuki et al., 2010). Adjuvant activity is primarily mediated by the production of IFN-α and IFN-β by immune cells (Abe et al., 2005;

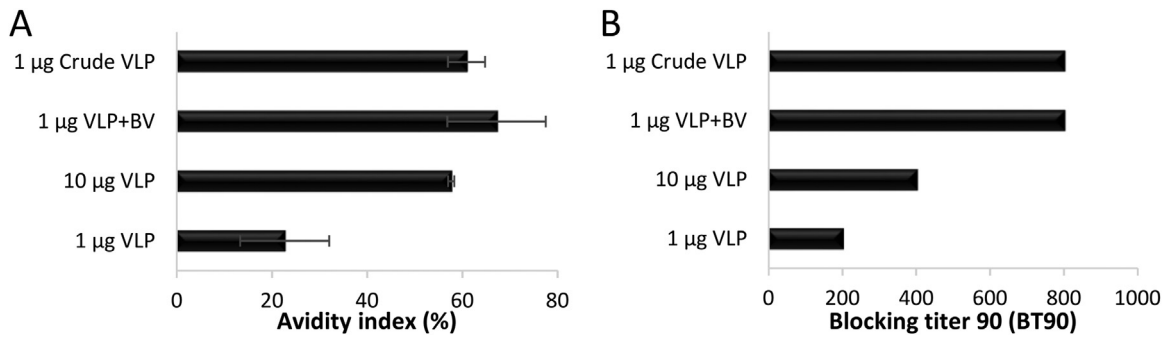

Fig. 5. *Avidity and blocking antibody titers of mice immunized with NoV GII.4 VLP formulations.* (A) Mean avidity indices (%) with standard error of the means of GII.4-specific IgG antibodies in termination sera of immunized groups. (B) Antibody titers of group-wise pooled sera blocking ≥90% (BT90) of the VLPs binding to the HBGAs.

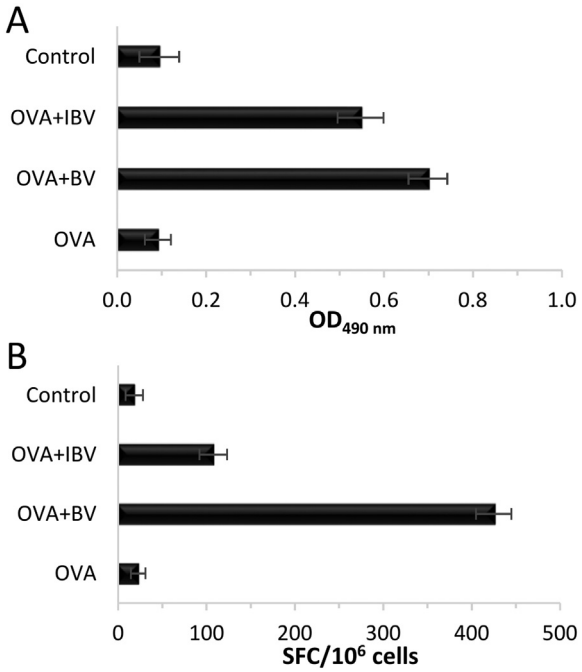

Fig. 7. *BV-specific antibodies and T cell responses.* (A) BV-specific serum IgG responses in mice immunized with OVA alone or together with BV or IBV. Control mice received PBS only. Shown are mean OD_{490} values with standard errors of the means of termination sera. (B) BV-specific T cell responses induced in mice immunized with different OVA formulations. BV was used to stimulate IFN-γ production from the group-wise pooled splenocytes of immunized and control mice. Mean IFN-γ spot-forming cells (SFC)/10^6 splenocytes of duplicate wells of 1–3 independent experiments with standard errors of the means are shown.

Hervas-Stubbs et al., 2007), probably due to the ability of type I IFNs to improve B cell as well as T cell responses (Le Bon et al., 2001, 2003; Deal et al., 2013).

The current study demonstrates that live BV has desirable features attributed to classical adjuvants, which work to spare the dose of the antigen and improve the immune responses. We detected a considerably higher immunogenicity of both antigens, OVA and NoV VLPs, when formulated with BV. Importantly, BV was able to spare the dose of the NoV VLPs used for immunization. In addition, BV acted to promote both Th1- and Th2-type responses without skewing the overall immune response in any particular direction. However, BV could be considered a potent Th1-type adjuvant, as OVA protein was capable of inducing antigen-specific IgG2a immunoglobulin subtype only when co-administered with BV. Consistent with this observation, Margine and co-workers (Margine et al., 2012) reported, that residual BV in antigenic formulations biases the isotype distribution of the antibody response towards the IgG2a immunoglobulin subtype, suggesting stimulation of cell-mediated responses. In here, the BV adjuvant effect was strongly affected by the removal or inactivation of BVs, indicating that the adjuvant properties were due to the presence of live BV. This is not surprising, as BV inactivation has been shown to completely abrogate immunopotentiation effect, partly due to abolished production of type I IFNs (Hervas-Stubbs et al., 2007), which are generated in response to live viruses (Goodbourn et al., 2000).

Antibodies with high avidity have been shown to promote efficient virus neutralization (Rockx et al., 2005; Puschnik et al., 2013). These antibodies have also been associated with protection from some viral infections, including vesicular stomatitis virus (VSV) (Salmi, 1991; Bachmann et al., 1997). Moreover, previous data by our group demonstrated that children with high-avidity anti-NoV IgG antibodies had fewer NoV infections than children with low-avidity antibodies (Nurminen et al., 2011). The present study shows that BV was able to induce functionally efficient humoral response, as live BV increased the avidity of serum IgG antibodies against co-administered OVA protein and NoV VLPs. Furthermore, NoV has been shown to use HBGAs, complex carbohydrates found on red blood cells, mucosal epithelial cells and as secreted free antigens in body fluids, as cellular attachment factors or receptors (Harrington et al., 2002; Marionneau et al., 2002; Huang et al., 2003). Anti-NoV antibodies which block binding of NoV VLPs to the HBGAs are considered correlate of protection against NoV infection (Harrington et al., 2002; Reeck et al., 2010; Nurminen et al., 2011; Malm et al., 2014). Our results demonstrate that administration of GII.4 VLPs in a combination with BV considerably increased the blocking potential of these antibodies.

Although the present study did not explore the induction of innate immunity, we hypothesize that following i.d. immunization BV is taken up by immature DC in the skin, and induces maturation and activation of these antigen presenting cells (APC) as previously demonstrated (Molinari et al., 2011), upregulating co-stimulatory molecules as well as production of pro-inflammatory cytokines (Hervas-Stubbs et al., 2007; Suzuki et al., 2010; Molinari et al., 2011; Quattrocchi et al., 2013). In concordance with these findings, we have recently shown that residual BV in the crude purified protein preparations induces TNF-α production by monocytes/macrophages in vitro (Malm et al., 2016a). TNF-α triggers the recruitment and activation of APCs at the injection site and facilitates migration of APCs to lymph nodes (Stoitzner et al., 1999), thus improving the antigen uptake and presentation to T cells.

Previous reports have demonstrated that BV enhances T cell responses against co-administered antigens (Hervas-Stubbs et al., 2007; Margine et al., 2012). Thus, after observing the adjuvant effect of BV on IgG2a antibody production against both antigens, we investigated antigen-specific IFN-γ cytokine production, a hallmark of Th1 cell immunity. BV promoted generation of T cell responses against co-delivered antigens, as addressed by considerable secretion of IFN-γ by splenocytes upon stimulation with OVA and NoV GII.4 derived peptides corresponding to the immunodominant $CD4^+$ T cell epitopes, as well as a peptide pool covering the entire sequence of NoV VP1. Similarly to the antibody response, BV inactivation abolished the capacity of BVs to induce OVA-specific T cell response, suggesting that induction of T cell response is also dependent on the presence of live BV particles. Cells of mice immunized with formulations containing live BV, but not IBV, produced high amount of IFN-γ upon ex vivo stimulation with BV. This indicates activation of T cells in lymphoid tissues by BV, which is in agreement with previous observations by others (Strauss et al., 2007; Molinari et al., 2011). Paracrine secretion of cytokines by BV-specific T cells is of a significance as these cytokines drive proliferation and differentiation of co-delivered antigen primed lymphocytes.

BV has a pronounced adjuvant effect on T cell responses to NoV, although it also improves functionality of the antibodies in terms of avidity and blocking/neutralization. However, more pronounced B and T cell adjuvant effect was observed to co-delivered OVA antigen, which is not surprising due to the less immunogenic nature of soluble monomeric proteins in comparison to the more immunogenic VLPs (Bachmann and Jennings, 2010). Superior immunogenicity of particles or protein aggregates over soluble proteins is strongly linked to the multivalent organization of antigen presented on particles (Bachmann et al., 1993; Ghosh et al., 2002). Unlike soluble proteins, the larger structures within the size range of ~40 nm are efficiently internalized by APCs (Fifis et al., 2004), and thus carried to the lymphoid organs and presented to T cells.

In conclusion, the presence of live BV in antigenic formulations strengthened both antibody and T cell immune responses to these proteins, further sparing the dose of the antigen. Therefore, it is tempting to speculate, that crude purified proteins could be excellent candidates for veterinary vaccines. For instance, a vaccine containing crude purified avian influenza antigens could be used to vaccinate wild birds and domestic poultry to limit the potential spread of pandemic influenza viruses, extremely harmful to human population (Noh et al.,

2016; Pushko et al., 2017). High quantities of the vaccine stocks could be produced relatively easy and at a low cost, without the need for time consuming and costly chromatographic purification steps. The residual BV in crude purified protein preparations would alleviate the need for addition of external adjuvants to the vaccine formulation because of the intrinsic immunostimulatory effect of live BV. Significant advantages of BV include its inherent inability to replicate in vertebrate cells (Tjia et al., 1983; Brusca et al., 1986), thus eliminating the need for inactivation, as well as low cytotoxicity and absence of pre-existing immunity (Shoji et al., 1997; Strauss et al., 2007). Although there is a high concern by regulatory agencies about contaminating viruses in human vaccines, BV could be considered as an adjuvant in veterinary vaccines due to their less stringent regulatory requirements (Adams, 2015). Because it is difficult to ensure the constant level of BV in crude protein preparations, the activity of BV in a vaccine formulation should be evaluated by determining the range of BV concentrations (e.g. adding exact amounts of BV into the pure VLPs) exerting adjuvant effect in carefully designed preclinical animal experiments, and thus, each crude vaccine batch needs to be tested to meet the concentration range. Since live BVs in vaccine formulations may provoke reactogenicity and raise safety concerns, further studies are needed to ascertain the reactogenicity, toxicity and safety of BV containing vaccine preparations in relevant animal models.

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