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The localization of a heterologous displayed antigen in the baculovirus-budded virion determines the type and strength of induced adaptive immune response

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Abstract In the search of strategies of presentation of heterologous antigens to elicit humoral or cellular immune responses that modulate and properly potentiate each type of response, researchers have been studying baculovirus (BV) as vaccine vectors with promising results. For some years, several research groups explored different antigen presentation approaches using the BV AcNPV by expressing polypeptides on the surface of budded virions or by de novo synthesis of heterologous antigens by transduction of mammalian cells. In the case of expression on the surface of budded virions, for example, researchers have expressed polypeptides in peplomers as GP64 glycoprotein fusions or distributed throughout the entire surface by fusions to portions of the G protein of vesicular stomatitis virus, VSV. Recently, our group developed the strategy of cross-presentation of antigens by fusions of GP64 to the capsid protein VP39 (capsid display) for the generation of cytotoxic responses. While the different strategies showed to be effective in raising immune responses, the individuality of each analysis makes difficult the comparison of the results. Here, by comparing the different strategies, we show that localization of the model antigen ovalbumin (OVA) strongly determined the quality and intensity of the adaptive response to the heterologous antigen. Furthermore, surface display favored humoral responses, whereas capsid display favored cytotoxic responses. Finally, capsid display showed a much more efficient strategy to activate CD8-mediated responses than transduction. The incorporation of adjuvants in baculovirus formulations dramatically diminished the immunostimulatory properties of baculovirus.

Keywords Baculovirus · Surface display · Capsid display · Transduction

Introduction

Baculovirus (Family Baculoviridae) is a diverse group of double-stranded DNA viruses infecting lepidopterans. Two enveloped phenotypes can be recognized in the viral progeny: budded virus and occlusion-derived virus. These phenotypes have the rod-shaped nucleocapsids in common but differ in the envelope. The prototype virus of the family is *Autographa californica multiple nucleopolyedrovirus*, AcNPV, which has a 134-Kbp genome and is the most widely used vector for recombinant expression of proteins in insect cells and larvae (Rohrmann 2013).

Besides its use as eukaryotic expression vector, AcNPV was recently assessed as a carrier for the display of heterologous proteins or peptides on the surface of budded viral particles, an approach known as baculovirus display (Grabherr et al. 2001). Baculovirus display was originally developed as the eukaryotic counterpart of phage display, a strategy to produce display libraries of complex proteins enabling the selection of variants with a desired property, such as catalytic activity or binding affinity (Boublik et al. 1995). Baculovirus displaying heterologous antigens on their surface was successfully used as vaccine vectors to elicit immune responses against pathogens of different origins as parasites (Iyori et al. 2013; Yoshida et al. 2010) and viruses (Peralta et al. 2007; Prabakaran et al. 2011).

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The original strategy for displaying polypeptides on the surface of budded virions employed fusions to a second copy of GP64, the major envelope glycoprotein of the budded phenotype, thus resulting in a peplomeric distribution of the fusion proteins.

However, a later strategy employing fusions of these virions between the signal peptide of GP64 and the transmembrane and cytoplasmic domains of VSV-G gene exhibited higher densities of recombinant proteins, with a homogeneous distribution along the viral envelope (Chapple and Jones 2002).

Although AcNPV is unable to replicate in mammalian cells, it can reach the nucleus of a variety of cell linages and mediate the transcription of transgenes placed under the regulation of appropriate mammal promotors (Kost and Condreay 2002). This transduction strategy was originally designed as an alternative approach for gene therapy but was also employed to develop vaccine vectors against a variety of pathogens. Indeed, as translated antigens, these polypeptides can be processed in the cytoplasm of antigen presenting cells and, in turn, the processed peptides can be charged in major histocompatibility complex class I (MHC-I) molecules to elicit cytotoxic immune responses to antigen-expressing cells, in a way similar to DNA vaccines (Argilaguet et al. 2013; Cao et al. 2011).

A third approach to display foreign proteins on baculoviral particles consists in fusing polypeptides to a second copy of VP39, the major nucleocapsid protein of budded and occlusion-derived virions. Kukkonen et al. (2003) first proposed this strategy, known as capsid display, to augment the delivery of nucleocapsids to the nucleus of mammalian cells. Recently, our group demonstrated that peptides derived of ovalbumin (OVA) displayed on the capsid of AcNPV can be cross-presented by dendritic cells in MHC-I molecules, thus eliciting a potent cytotoxic immune response in mice, strong enough to reject the implantation of tumor cells expressing OVA (Molinari et al. 2011).

So far, baculovirus has been employed as vaccine vehicles for antigen presentation using these three different approaches: surface display, capsid display, and gene transduction. Despite the promising results exhibited by each strategy, it is hard to elucidate the performance of each one in terms of the ability of eliciting humoral and cellular immune responses, as well as of the influence of adjuvants in the baculovirus formulations.

Thus, the aim of this work is to compare head to head the different strategies of antigen presentation and the influence of different adjuvants in the development of humoral and cellular immune responses to the model antigen OVA.

Material and methods

Viruses and cell lines

Wild-type AcNPV was purchased to BD Biosciences (BD Argentina) as AcNPV wild-type virus high titer stock solution (Cat. No. 554744). AcNPV and recombinant baculovirus were grown in *Spodoptera frugiperda* Sf9 cells (ATCC) cultured at 27 °C in EX-CELL medium (Sigma) supplemented with an antibiotic–antimycotic solution (Gibco). For the preparation of viral stocks, Sf9 cells grown in 175-cm^2 flasks were infected at a multiplicity of infection (MOI) of 0.1 and, 4 days post-infection (dpi), the supernatants were collected. Virus titers were determined by an end-point dilution assay employing a GFP infection-induced cell line as previously described (Hopkins and Esposito 2009). Monolayers of BHK-21 and J774 cells were respectively grown in DMEM or RPMI culture medium supplemented with 10 % FCS in a 5 % CO_2 atmosphere.

Construction of recombinant baculovirus

To obtain the recombinant baculovirus BV-OVA_{sd}, we designed a DNA construct with a 543-bp sequence coding for the amino acids 198–379 of ovalbumin (GenBank: AH002466.2). This sequence is positioned downstream of a 114-bp sequence coding for the signal peptide of baculoviral GP64 protein (GenBank: M25420.1) and upstream of a sequence coding for the transmembrane and cytoplasmic domains of G glycoprotein from VSV (amino acids 442–511, GenBank: KU534946.1). This construct was cloned downstream of the polyhedrin promoter of pFastBacI plasmid (Invitrogen) to origin the transfer plasmid pSD-OVA.

For BV-OVA_{cd}, the construct consisted of a fusion of the 546-bp sequence coding for the amino acids 198–379 of ovalbumin sequence with its own ATG codon surrounded by a proper baculovirus late gene consensus sequence. This consensus sequence was located upstream of the coding sequence of baculoviral capsid protein VP39 (GenBank: M22978), under the polyhedrin promoter regulation of pFastBacI plasmid (Invitrogen). The resulting plasmid was named pCD-OVA (referred as pFBOVAcap in Molinari et al. 2011).

For BV-OVA_{cag}, the OVA sequence with its own ATG surrounded by a proper Kozak consensus was cloned under a CAG hybrid promoter consisting of the chicken β-actin promoter and a CMV-IE enhancer (Ikawa et al. 1995) in a pFastBacI vector lacking the polyhedron promoter. This plasmid was called pCAG-OVA.

The recombinant plasmids were used to produce recombinant baculoviruses using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Viral progenies propagated in Sf9 cells were concentrated by sucrose cushion ultracentrifugation, titrated by end-point dilution, and stored at 4 °C until use. The titers were expressed as infectious doses in tissue culture ($TCID_{50}$).

Western blot analysis

To analyze the presence of OVA protein in the outer envelope or in the inner capsid of recombinant baculovirus, we microcentrifuged 2 ml of 4 dpi supernatants of Sf9-infected

cells for 1 h to 14,000×*g* and resuspended the pellets in 1 ml of PBS or 1 ml of Triton X-100 lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1 % Triton X-100). After 30 min of incubation in ice, whole virions or nucleocapsids were pelleted by microcentrifugation for 1 h and pellets were resuspended in 1X Laemmli buffer (60 mM Tris–HCl pH 6.8, 10 % 2-mercaptoethanol, 1 % SDS, 0.002 % bromophenol blue, and 10 % glycerol) and boiled for 5 min. Fifteen microliters of each sample was resolved by SDS-12 % polyacrylamide gel electrophoresis. Proteins from the purified baculovirus were then transferred onto nitrocellulose membranes, and OVA and GP64 proteins were detected by using a rabbit anti-OVA serum or the Acv5 monoclonal antibody to GP64, an AP-labeled anti-mouse or anti-rabbit IgG goat antiserum (Dakkopats), and chemiluminescence (Thermo Scientific Pierce, USA).

Immunofluorescence assay

Mouse BALB/c monocyte macrophage J774 and BHK-21 cells were seeded at a density of 2×10^5 cells/well into a six-well tissue culture plate (BIOFIL, China) and transduced with BV-OVAcag or wild-type baculovirus at a MOI of 100 in PBS pH 7. After incubation for 24 h, the cells were fixed for 10 min with 2 % paraformaldehyde and incubated with a polyclonal anti-OVA rabbit serum. An Alexa 488-labeled anti-rabbit IgG was used as the second antibody. Fluorescent images were analyzed under an inverted fluorescence microscope (Nikon Ti Eclipse, Japan).

Animals, inoculations, and serum sampling

Six to 8-week-old female BALB/c or C57BL/6 mice were purchased from Fundacion Facultad de Ciencias Veterinarias (UNLP, La Plata, Argentina). For the evaluation of the humoral immune response, groups of five BALB/c mice were intraperitoneally injected three times, in intervals of 15 days, with 1×10^8 $TCID_{50}$ (300 μl) of the different baculoviruses. The baculoviruses were formulated by resuspending concentrated virions in PBS in the absence of adjuvant or by mixing equal volumes of viral suspensions with Incomplete Freund's adjuvant (Sigma), Montanide ESSAI IMS D 12802 VG PR (Seppic, France), and $Al(OH)_3$ (Rehydragel®, Chemtrade, Canada). Serum samples were taken the day before each vaccination. As a control, a group of mice received wild-type baculovirus.

For the evaluation of cytotoxic responses to OVA, groups of five C57BL/6 mice were immunized by a single intravenous injection of 1×10^8 $TCID_{50}$ of the different baculoviruses.

Serological tests

Serum samples of the different experiments were analyzed for the presence of total antibodies to OVA by a direct ELISA test. Briefly, Maxisorp plates (Thermo Scientific Nunc, USA) were coated overnight with a solution of 10 μg/ml of OVA protein (Sigma, USA) in carbonate/bicarbonate buffer pH 9.6 at 4 °C and blocked with a solution of 5 % skim milk in TBS-5 % equine serum. The serum samples were properly diluted twofold in TBS-1 % skim milk and incubated for 60 min at 37 °C in the Maxisorp plates; the plates were washed with TBS-0.1 % Tween 20 and subsequently incubated with an anti-mouse total immunoglobulins conjugated to peroxidase. Finally, the plates were revealed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent (BD, USA). Alternatively, Maxisorp plates were coated with wild-type budded baculovirus (1×10^7 $TCID_{50}$ per well); then, tenfold dilutions of sera were applied and total antibodies to baculovirus were revealed as previously described.

For IgG subclass determination, goat anti-mouse conjugated to HRP antibodies for each IgG subclass (Mouse Monoclonal Antibody Isotyping Reagents, Sigma) was employed. After extensive washing, 50 μl of TMB solution was used for color development and the reaction was finally stopped with 25 μl of a 2 N solution of sulfuric acid. Absorbance was measured at 450 nm (Multiskan Spectrum, Thermo Electron Corporation).

In vivo killing assay

Naïve splenocytes from C57BL/6 mice were pulsed with 10 mg/ml $OVA_{257–264}$ peptide (SIINFEKL) for 30 min at 37 °C, extensively washed, and finally labeled with 3 μM (high concentration) of carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen). Non-pulsed control splenocytes were labeled with 0.5 μM (low concentration) of CFSE. Then, 1×10^7 cells of populations $CFSE_{high}$ and $CFSE_{low}$ were mixed at a 1:1 ratio and intravenously injected into vaccinated mice. Numbers of CFSE+ cells remaining in the spleen after 20 h were determined by flow cytometry. Cytotoxicity was expressed as the percentage of lysis, calculated as $[1 - (r_{control} / r_{immune})] \times 100$, where r is given by the expression of $\%CFSE_{low}$ / $\%CFSE_{high}$ cells for vaccinated and non-vaccinated mice, respectively.

IFN-γ detection assay

Levels of IFN-γ were measured in culture supernatants of splenocytes from vaccinated C57BL/6 mice incubated in 96-well flat-bottomed plates (1×10^6 cells/well) and re-stimulated with SIINFEKL peptide by sandwich ELISA, according to the manufacturer's instructions (BD Biosciences). XMG1.2 clone was used to capture and to detect IFN-γ. Concentrations of mouse IFN-γ in the samples were determined from a standard curve.

Statistical analysis

One-way ANOVA and Bonferroni post-test were used as statistical analyses to compare the humoral- and cell-mediated immune responses obtained from the different treatments. For these tests, we used GraphPad Prism (La Jolla, CA). *P* values of <0.05 were considered statistically significant.

Ethics statement

All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our Institutional Experimentation Animal Committee (procedure nos. 6/2014, 37/2015, 41/2015) approved the animal handling and experimental procedures. All animal procedures were performed under isoflurane anesthesia. Sacrifice was carried out by CO_2 inhalation. Maximum efforts were made to minimize mice suffering.

Results

Construction of the baculovirus vectors

In the "Material and methods" section, we describe the construction of recombinant baculovirus BV-OVA$_{sd}$, BV-OVA$_{cd}$, and BV-OVA$_{cag}$. Figure 1 displays a schematic representation of each vector. We first determined whether OVA polypeptide fused to the amino terminus of VSG-G or fused to VP39 was displayed on the surface of BV-OVA$_{sd}$ or on the capsid of BV-OVA$_{cd}$, as expected. For this purpose, we used a nonionic detergent or PBS to treat the purified budded virions and then re-pelleted and analyzed the fraction solutions by immunoblotting using an anti-OVA polyclonal antiserum.

Whole virions displayed proteins of the predicted sizes of approximately 35 and 60.3 kDa and only the band of 60.3 kDa was present in the detergent-treated nucleocapsids. These results demonstrated that OVA-G localized in the membrane, whereas OVA-VP39 was present in the capsid (Fig. 2a, upper panel). As a control of proper fractionation of nucleocapsids and envelopes, we also assessed the presence of GP64 glycoprotein (Fig. 2a, lower panel). As expected, no OVA product was detected in Sf9 cells infected with BV-OVAcag. This result is predictable because the promoter driving the expression of OVA in BV-OVAcag is a mammalian promoter that cannot be recognized by insect transcriptional factors (data not shown).

To test the ability of BV-OVA$_{cag}$ to express the OVA protein in mammalian cells, we transduced BHK-21 and J774 cells (subpanels b and c of Fig. 2, respectively) with BV-OVA$_{cag}$ or wild-type baculovirus. The BV-OVA$_{cag}$-transduced cells clearly displayed a fluorescent signal, whereas the cells transduced with wild-type AcNPV showed no fluorescence. These results demonstrated that BV-OVA$_{cag}$ can efficiently transduce professional and nonprofessional presenting cells and that OVA gene is actively transcribed from CAG promoter.

Humoral immune response induced by recombinant baculovirus

We also compared the humoral immune responses specific to OVA polypeptide elicited by BV-OVA$_{sd}$, BV-OVA$_{cd}$, and BV-

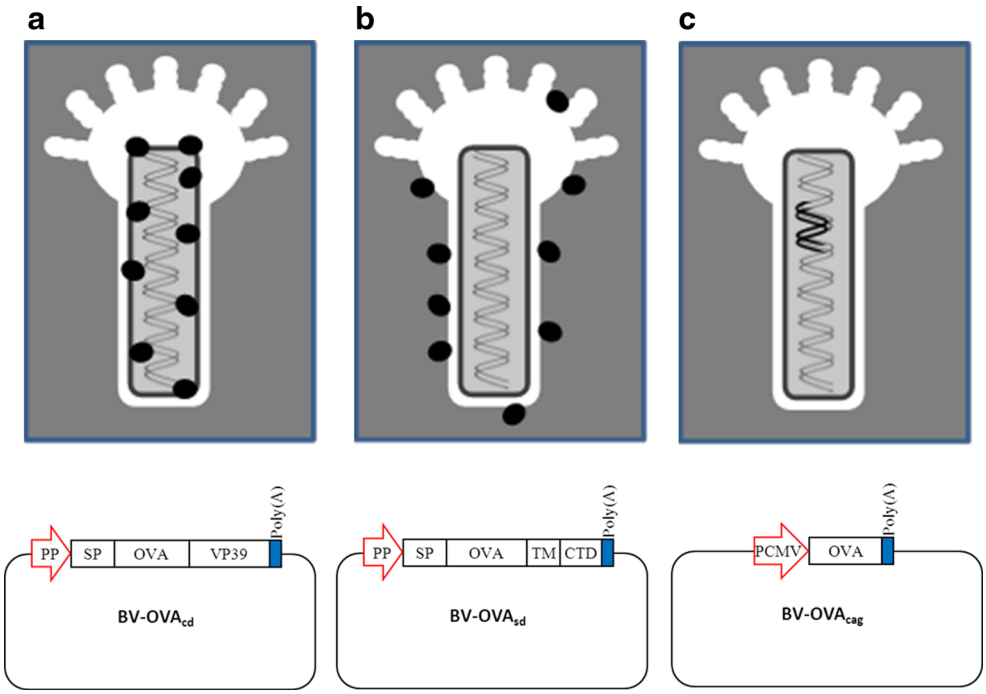

Fig. 1 Schematic representation of the structure of BV-OVAsd, BV-OVAcd, and BV-OVAcag. **a** The OVAcd gene cassette consisted of the OVA sequence fused to VP39 gene under the control of the polyhedrin promoter (*PP*). **b** The OVAsd gene cassette consisted of the GP64 signal sequence (*SP*), the OVA gene (coding for aa 198 to 379) fused to the TM and CTD domains of VSV-G gene (VSV-G 4401-4613), and a poly(A) sequence, which was driven by the PP. **c** OVAcag expression cassette was driven by the CAG promoter. OVA protein is depicted as *black circles* and OVA coding sequence as a *black segment* of a double helix of DNA

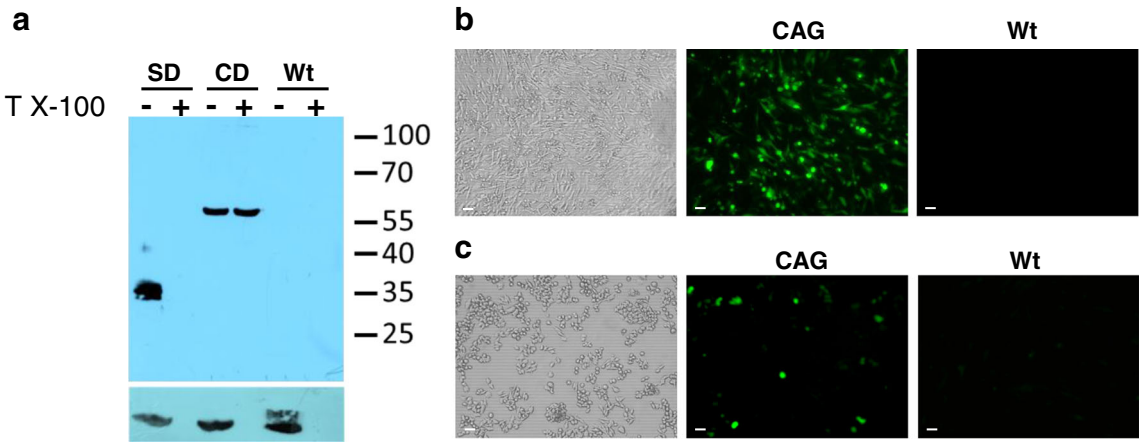

Fig. 2 Expression and localization of OVA. **a** Immunoblot analysis of recombinant budded virions using an anti-OVA polyclonal antibody. Whole budded virions were collected from supernatants of Sf9-infected cells, pelleted, and dissolved in SDS lysis buffer (−). Alternatively, pellets from microcentrifugation were treated with 1 % Triton X-100 lysis buffer to dissolve the envelope and nucleocapsids were re-pelleted and subjected to immunoblotting (+). Presence of OVA protein (*upper panel*) on virions or nucleocapsids obtained by detergent treatment was examined for BV-OVAsd (*lanes 1 and 2*), BV-OVAcd (*lanes 3 and 4*), and AcNPV-WT (*lanes 5 and 6*). As a control of fractionation, samples were additionally assessed for GP64 presence (*lower panel*). **b**, **c** Immunofluorescent staining of BHK-21 and J774 cells transduced with BV-OVAcag. BHK-21 and J774 cells were transduced with BV-OVAcag or BV-wt at an MOI of 10. At 24 h post-transfection, cells were fixed for 10 min with 2 % paraformaldehyde and processed for an indirect immunofluorescence assay using a rabbit antibody directed against OVA. Bound antibodies were detected with an Alexa 488-labeled anti-rabbit IgG and observed with fluorescence microscopy (*green*). Original magnification, ×40. *Bar* = 100 μm

OVA_{cag}. For this purpose, BALB/c mice were intraperitoneally injected three times with 1×10^8 $TCID_{50}$ of each baculovirus or with the same dose of wild-type baculovirus as a control. No adjuvants were used in vaccine formulations. Fourteen days after each inoculation, serum samples were taken and total antibodies to OVA were measured by direct ELISA. Figure 3a shows that 14 days after the primary immunization, specific OVA antibodies could be detected in all animals immunized with $BV\text{-}OVA_{sd}$. Furthermore, these antibodies increased after boosters in samples taken at days 29 and 44 after the primary dose. Specific antibodies against OVA were undetectable in sera from mice vaccinated with $BV\text{-}OVA_{cd}$, $BV\text{-}OVA_{cag}$, and wild-type baculovirus. All mice showed similar anti-baculovirus vector titers, which showed that the different constructions were similar in mass and immunogenic potential (Fig. 3b).

The assessment of IgG isotypes of antibodies to OVA elicited by the complete scheme of immunization with $BV\text{-}OVA_{sd}$ showed an evident marked Th-1 profile, with a predominance of the IgG2a isotype. However, IgG1, IgG2b, and IgG3 immunoglobulins were also present. Antibodies directed to virus vector after $BV\text{-}OVA_{sd}$ vaccination displayed a more balanced profile (Fig. 4c, d). Similar results were obtained for isotype profiles to the virus vector for $BV\text{-}OVA_{cd}$, $BV\text{-}OVA_{cag}$, and wild-type baculovirus (data not shown).

Therefore, the results demonstrated that presentation of the heterologous antigen OVA in the surface of budded virions is the best strategy to elicit a strong humoral immune response to heterologous antigen, with a bias to a Th-1 profile.

Cellular immune response induced by recombinant baculovirus

We then evaluated the cellular immune response elicited by each vector by measuring the percentage of specific lysis mediated by cytotoxic T lymphocytes (CTLs) specific to the SIINFEKL peptide present in the OVA sequence. For this purpose, we performed an in vivo killing assay using CFSE-labeled target cells. C57BL/6 mice were intravenously immunized with 1×10^8 $TCID_{50}$ of $BV\text{-}OVA_{sd}$, $BV\text{-}OVA_{cd}$, $BV\text{-}OVA_{cag}$, and wild-type baculovirus, and 7 days post-vaccination, specific CTL response was measured.

Figure 4a shows that injection of $BV\text{-}OVA_{cd}$ elicited a strong CTL response to the OVA peptide, whereas $BV\text{-}OVA_{sd}$ only elicited a mild CTL response (about 97 and 12 % of specific lysis, respectively). $BV\text{-}OVA_{cag}$ failed to mount a detectable CTL response at the dose assessed and, as expected, no specific lysis was observed in control mice vaccinated with wild-type baculovirus.

The strong CTL response to $OVA_{257–264}$ epitope was associated with a high release of IFN-γ from spleen cells after in vitro restimulation with SIINFEKL peptide (Fig. 4b). As $OVA_{257–264}$ epitope is known to bind MHC-I molecules of C57BL/6 mice, CD8 T lymphocytes seem to be the cells responsible for IFN-γ production.

Thus, the results showed that the capsid display strategy was much more efficient to mount a specific CTL response that the surface displays and transduction strategies.

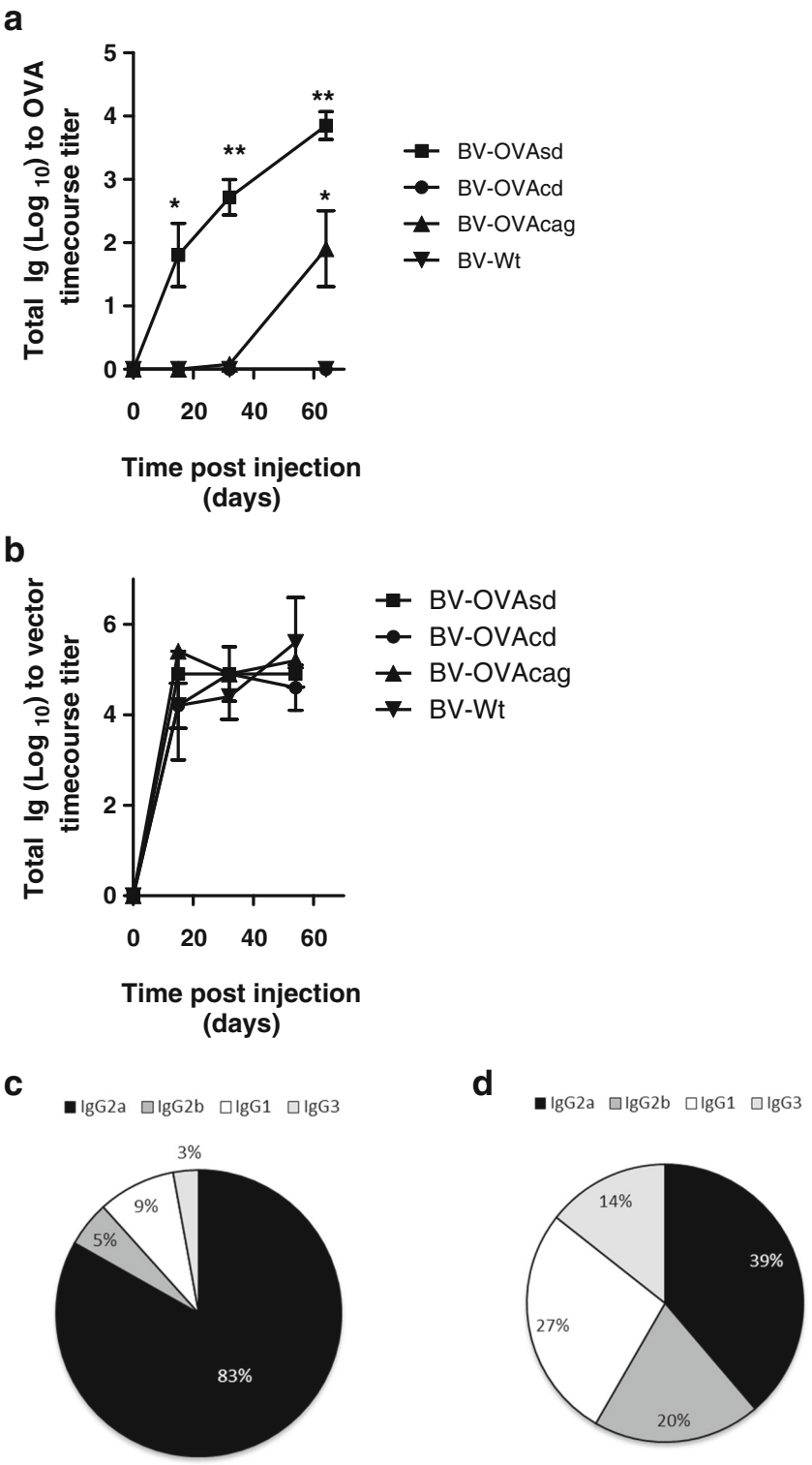

Fig. 3 Antibody profile elicited by the different presentation strategies. Sera from mice vaccinated at days 0, 15, and 30 with BV-OVAsd, BV-OVAcd, and BV-OVAcag were obtained at days 14, 29, and 60 and assessed by direct ELISA for total antibodies to OVA (**a**) or AcNPV (**b**). Wild-type baculoviruses were used as a control. The ratio of IgG isotypes elicited after vaccination with BV-OVAsd was calculated for antibodies OVA-specific (**c**) or baculovirus-specific (**d**)

Influence of adjuvants in humoral and cellular immune responses elicited by baculovirus vectors

Finally, we assessed the influence of different adjuvants to improve humoral and cellular immune responses specific to OVA. With this in mind, we formulated the different baculovirus vectors including $Al(OH)_3$, incomplete Freund's adjuvant, or Montanide 802. The formulations included 1×10^8 $TCID_{50}$ of BV-OVA_{sd}. Groups of six BALB/c mice were intraperitoneally injected three times with the vaccine formulations. As a control, another group received the same dose of wild-type baculovirus.

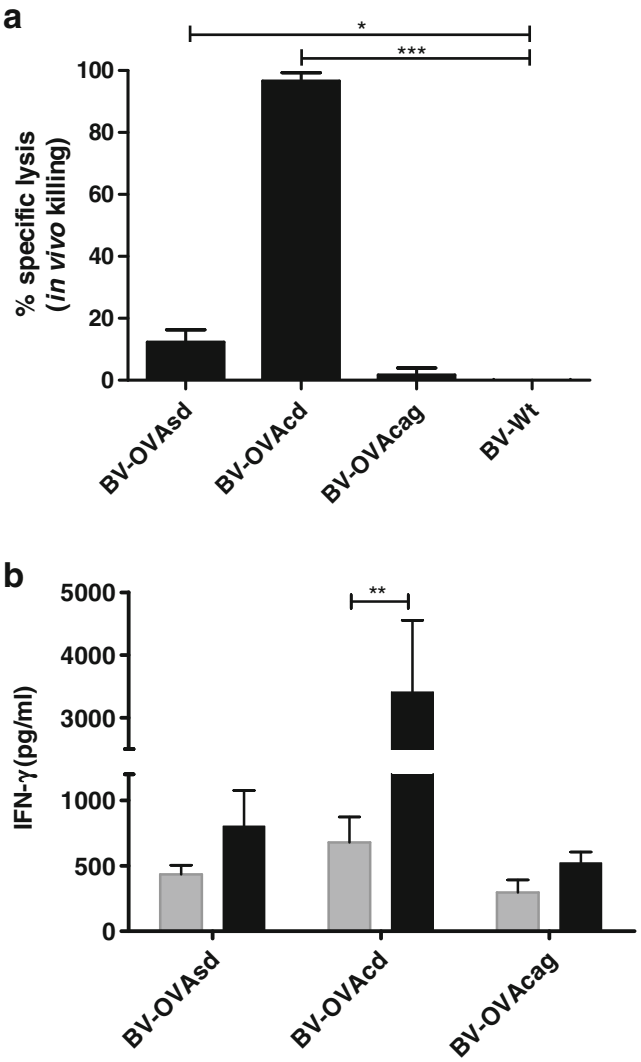

Fig. 4 Cellular immune response elicited by the different presentation strategies. OVA-specific cellular immune responses of mice vaccinated with BV-OVAsd, BV-OVAcd, BV-OVAcag, and BV-wt were assessed at 7 days by a cytotoxic in vivo assay (**a**) as described in the "Material and methods" section and by direct ELISA for IFN-γ in supernatants of splenocytes stimulated with OVA (0.1 μg/ml) (**b**). Values are expressed as percentage of specific lysis. *$p < 0.05$, ***$p < 0.001$

Kinetics of antibodies to OVA of the different formulations were quite similar, with Montanide 802 and Freund's reaching the higher titers. Moreover, Montanide 802 responded earlier than the other treatments. BV-OVA$_{sd}$ was able to induce antibodies to OVA even in the absence of any adjuvant (Fig. 5a, left panel). As expected, no anti-OVA antibodies were elicited in mice vaccinated with wild-type baculovirus. All the treatments were similar in inducing antibodies to the baculovirus vector (Fig. 5a, right panel).

To evaluate the impact on the cytotoxic response to OVA, we intraperitoneally vaccinated C57BL/6 mice with the vaccine formulations involving BV-OVA$_{cd}$ formulated with the same adjuvants. Surprisingly, all the adjuvants completely abolished the induction of a cytotoxic response by the baculoviral vector (Fig. 5b).

Altogether, the results show that adjuvants have very different effects on the ability of baculovirus to elicit humoral or cellular responses to a displayed heterologous antigen.

Discussion

After more than 30 years of baculovirus development and an enormous experience in the production of simple and complex recombinant proteins employing the BEVS technology, research in vaccinology involving recombinant baculovirus is now recognized as a powerful tool. Several advantages make baculovirus an ideal vaccine vector: the simplicity of manipulation, the possibility of scaling-up for industrial production, the availability of plasmid vectors for different antigen localization allowing capsid display or surface display, the ability of budded virions to transduce a broad spectrum of mammalian cells, and the strong impact of baculovirus in the innate immune system of mammals (Abe et al. 2005; Abe and Matsuura 2010).

Using AcNPV for the display of antigens on the surface of budded virions or their expression from mammal promoters, several researchers have developed experimental vaccines with the ability to elicit humoral responses against different pathogens (revised in (Lin et al. 2014)). Indeed, the recognition of heterologous epitopes in the baculoviral surface by B lymphocytes produces antibodies against the displayed heterologous polypeptide and, in turn, the expansion of B clones after collaboration with T lymphocytes. However, to date, the humoral response to antigens expressed de novo by the transduction strategy is somewhat difficult to explain. B lymphocytes encounter large particulate antigens in secondary lymphoid organs bound to the surface of macrophages, dendritic cells, or follicular dendritic cells in a non-processed form and this interaction triggers the formation of the immunological synapse (Yuseff et al. 2013). Endocyted particles are then processed, and derived peptides are presented to $CD4^+$ T lymphocytes in the context of major histocompatibility complex class II (MHC-II) molecules.

Baculovirus vectors displaying several copies of heterologous antigens serve both as particles able to be tethered to specialized cells that present the antigen to B cells as well as carriers to mature dendritic cells (owing its pathogen-associated molecular patterns) able to activate CD4+ cells recognizing antigenic peptides loaded on MHC-II molecules at dendritic cell surface. In contrast, non-secreted antigens synthesized de novo from adequate promoters in professional antigen presenting cells are loaded on MHC-I molecules. Although, in a lesser extent, part of the processed peptides could be charged in MHC-II molecules by a nonconventional mechanism, only traces of native, non-processed antigens

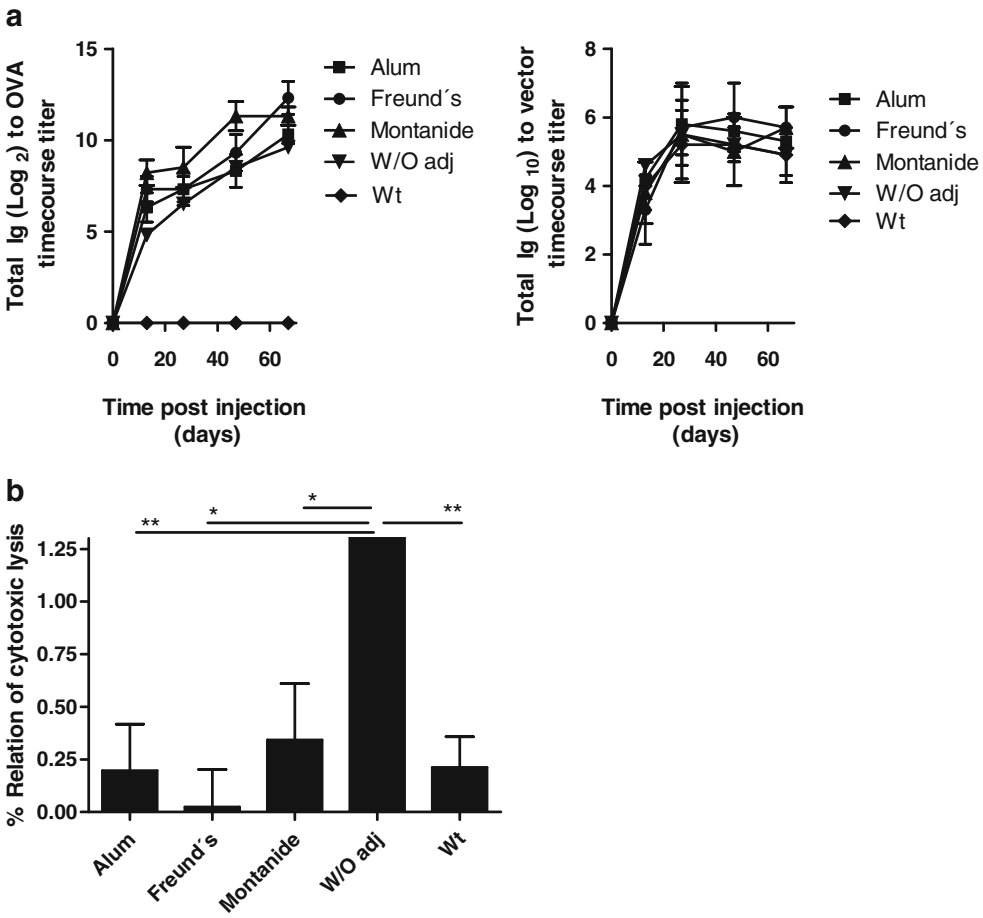

Fig. 5 Effect of adjuvants in humoral and cellular immune responses induced by BV-OVAsd and BV-OVAcd. Sera from mice vaccinated with BV-OVAsd in Alum, Freund's, Montanide, and without adjuvant (*W/O adj*) were assessed by direct ELISA for total antibodies to OVA (**a**, *left panel*) or AcNPV (**a**, *right panel*). Wild-type baculoviruses were used as a control. Specific cellular immune response of mice vaccinated with BV-OVAcd with Alum, Freund's, Montanide or W/O adj, and BV-wt were assessed at 7 days by a cytotoxic in vivo assay and expressed as the ratio of each treatment with CTL mean value of W/O adj group (**b**). $*p < 0.05$, $**p < 0.01$

escaping from transduced cells would be available for B cell recognition (Thiele et al. 2015).

In accordance with this, BV-OVA$_{sd}$, but not BV-OVA$_{cd}$, elicited antibodies to OVA at the doses assessed. Low antibody levels against OVA were evident in BV-OVA$_{cag}$-vaccinated mice after the second booster, despite similar constructions were reported to elicit humoral responses against the heterologous antigen, even though doses one or two orders of magnitude higher were employed (Cao et al. 2011; Fang et al. 2010).

Thus, our results together with evidence from researchers using surface display (Mlambo et al. 2010; Xu et al. 2011) demonstrate that the higher antibody levels to the heterologous antigen are achieved when the antigen is displayed on the surface of budded virions. The inclusion of adjuvants in the vaccine formulation did not augment substantially the antibody titers to OVA. This result reinforces the pieces of evidence showing that baculovirus has strong adjuvant properties per se.

In consonance with the cytokine profile induced by AcNPV, with high levels of IFN-γ, the immunoglobulin isotypes to OVA reflected a Th-1-biased profile (Yoshida et al. 2010). A broad spectrum of Ig isotypes was elicited against the baculoviral vector, but antibodies to OVA were mostly of IgG2a subclass. Thus, vaccine schedules based on eliciting neutralizing antibodies to pathogens that do not replicate in myeloid cells or to those that could be neutralized by antibody-dependent cell-mediated cytotoxicity responses related to Th1-Ig could benefit of baculovirus vectors. However, special attention should be paid to the vaccine design to pathogens that could benefit from antibody-dependent enhancement (ADE) (Rodrigo et al. 2009).

Several authors have used the transduction strategy to induce cellular immune responses because the non-secreted antigen transcribed in cells transduced by a baculovirus vector is processed in the proteasome. Therefore, the resulting peptides are presented on MHC-I molecules and this presentation leads to the activation of CD8$^+$ T cells. The assessment of CTL to OVA demonstrated that the capsid display strategy was the more efficient approach for inducing cytotoxic responses. In BV-OVA$_{cd}$, OVA antigen is a component of the capsid of budded virions. In fact, this localization impedes its recognition by B cells but favors that OVA reaches the cytoplasm of infected antigen presenting cells, particularly dendritic cells. This cytoplasmic localization allows presentation of baculovirus antigens on MHC-I molecules once nucleocapsids leave the endosomes.

Previously, we have shown that dendritic cells are able to cross-present the SIINFEKL peptide derived from OVA to

$CD8^+$ lymphocytes (Molinari et al. 2011) and that baculoviruses strongly activate dendritic cell maturation. Because BV-OVA_{cd} and BV-OVA_{cag} were inoculated in similar quantities, cytosolic presentation seems to be a more efficient mechanism for $CD8^+$ activation than the classic mechanism of de novo synthesized polypeptides. The low CTL responses in BV-OVA_{sd} could be explained by the localization in the viral particle. Indeed, antigens attached to the surface remain in the endosome membrane once GP64 mediates membrane fusion, whereas antigens on the capsid reach the cytoplasm. In the cytoplasm, antigens can be processed by the proteasome and in turn can be presented in MHC-I molecules by antigen presenting cells. The antigens that remain in the endosome could undergo recycling and the content therefore could be sorted to either proteasomes and/or lysosomes and, in turn, could be cross-presented in MHC-I molecules with a lower efficiency than through the cytosolic pathway. Altogether, BV-OVAcd, but not BV-OVAsd, could function as a direct delivery of the antigen into the cytoplasm and could exploit the classical cytosolic MHC-I pathway (Moron et al. 2004). Future experiments are needed to clarify this mechanism.

The addition of adjuvants completely abrogated the capacity of the experimental vaccines to mount CTL responses, thus suggesting the importance of viral integrity to mediate this presentation.

Altogether, our results demonstrate that antigen localization is essential to elicit humoral or cellular responses to heterologous antigen vectorized by budded AcNPV. However, adjuvants are irrelevant or even detrimental to specific immune responses.

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Compliance with ethical standards

Ethical approval This article does not contain studies with human participants or animals performed by any of authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

Abe T, Matsuura Y (2010) Host innate immune responses induced by baculovirus in mammals. Curr Gene Ther 10:226–231

Abe T, Hemmi H, Miyamoto H, Moriishi K, Tamura S, Takaku H, Akira S, Matsuura Y (2005) Involvement of the Toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. J Virol 79:2847–2858

Argilaguet JM, Perez-Martin E, Lopez S, Goethe M, Escribano JM, Giesow K, Keil GM, Rodriguez F (2013) BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus. Antivir Res 98:61–65. doi:10.1016/j.antiviral.2013.02.005

Boublik Y, Di Bonito P, Jones IM (1995) Eukaryotic virus display: engineering the major surface glycoprotein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. Biotechnology (N Y) 13:1079–1084

Cao Y, Lu Z, Sun P, Fu Y, Tian F, Hao X, Bao H, Liu X, Liu Z (2011) A pseudotype baculovirus expressing the capsid protein of foot-and-mouth disease virus and a T-cell immunogen shows enhanced immunogenicity in mice. Virol J 8:77. doi:10.1186/1743-422X-8-77

Chapple SD, Jones IM (2002) Non-polar distribution of green fluorescent protein on the surface of *Autographa californica* nucleopolyhedrovirus using a heterologous membrane anchor. J Biotechnol 95:269–275

Fang R, Feng H, Nie H, Wang L, Tu P, Song Q, Zhou Y, Zhao J (2010) Construction and immunogenicity of pseudotype baculovirus expressing *Toxoplasma gondii* SAG1 protein in BALB/c mice model. Vaccine 28:1803–1807. doi:10.1016/j.vaccine.2009.12.005

Grabherr R, Ernst W, Oker-Blom C, Jones I (2001) Developments in the use of baculoviruses for the surface display of complex eukaryotic proteins. Trends Biotechnol 19:231–236

Hopkins R, Esposito D (2009) A rapid method for titrating baculovirus stocks using the Sf-9 easy titer cell line. BioTechniques 47:785–788. doi:10.2144/000113238

Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M (1995) A rapid and non-invasive selection of transgenic embryos before implantation using green fluorescent protein (GFP). FEBS Lett 375:125–128

Iyori M, Nakaya H, Inagaki K, Pichyangkul S, Yamamoto DS, Kawasaki M, Kwak K, Mizukoshi M, Goto Y, Matsuoka H, Matsumoto M, Yoshida S (2013) Protective efficacy of baculovirus dual expression system vaccine expressing *Plasmodium falciparum* circumsporozoite protein. PLoS One 8:e70819. doi:10.1371/journal.pone.0070819

Kost TA, Condreay JP (2002) Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol 20:173–180

Kukkonen SP, Airenne KJ, Marjomaki V, Laitinen OH, Lehtolainen P, Kankaanpaa P, Mahonen AJ, Raty JK, Nordlund HR, Oker-Blom C, Kulomaa MS, Yla-Herttuala S (2003) Baculovirus capsid display: a novel tool for transduction imaging. Mol Ther 8:853–862

Lin SY, Chung YC, Hu YC (2014) Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 13:1501–1521. doi:10.1586/14760584.2014.951637

Mlambo G, Kumar N, Yoshida S (2010) Functional immunogenicity of baculovirus expressing Pfs25, a human malaria transmission-blocking vaccine candidate antigen. Vaccine 28:7025–7029. doi:10.1016/j.vaccine.2010.08.022

Molinari P, Crespo MI, Gravisaco MJ, Taboga O, Moron G (2011) Baculovirus capsid display potentiates OVA cytotoxic and innate immune responses. PLoS One 6:e24108. doi:10.1371/journal.pone.0024108

Moron G, Dadaglio G, Leclerc C (2004) New tools for antigen delivery to the MHC class I pathway. Trends Immunol 25:92–97. doi:10.1016/j.it.2003.11.008

Peralta A, Molinari P, Conte-Grand D, Calamante G, Taboga O (2007) A chimeric baculovirus displaying bovine herpesvirus-1 (BHV-1) glycoprotein D on its surface and their immunological properties. Appl Microbiol Biotechnol 75:407–414

Prabakaran M, Meng T, He F, Yunrui T, Qiang J, Lin RT, Kwang J (2011) Subcutaneous immunization with baculovirus surface-displayed hemagglutinin of pandemic H1N1 Influenza A virus induces

protective immunity in mice. Clin Vaccine Immunol 18:1582–1585. doi:10.1128/CVI.05114-11

Rodrigo WW, Block OK, Lane C, Sukupolvi-Petty S, Goncalvez AP, Johnson S, Diamond MS, Lai CJ, Rose RC, Jin X, Schlesinger JJ (2009) Dengue virus neutralization is modulated by IgG antibody subclass and Fcgamma receptor subtype. Virology 394:175–182. doi:10.1016/j.virol.2009.09.024

Rohrmann GF (2013) Baculovirus molecular biology. 3rd edn., Bethesda (MD)

Thiele F, Tao S, Zhang Y, Muschaweckh A, Zollmann T, Protzer U, Abele R, Drexler I (2015) Modified vaccinia virus Ankara-infected dendritic cells present CD4+ T-cell epitopes by endogenous major histocompatibility complex class II presentation pathways. J Virol 89: 2698–2709. doi:10.1128/JVI.03244-14

Xu XG, Wang ZS, Zhang Q, Li ZC, Zhao HN, Li W, Tong DW, Liu HJ (2011) Baculovirus surface display of E envelope glycoprotein of Japanese encephalitis virus and its immunogenicity of the displayed proteins in mouse and swine models. Vaccine 29:636–643. doi:10.1016/j.vaccine.2010.11.045

Yoshida S, Araki H, Yokomine T (2010) Baculovirus-based nasal drop vaccine confers complete protection against malaria by natural boosting of vaccine-induced antibodies in mice. Infect Immun 78: 595–602. doi:10.1128/IAI.00877-09

Yuseff MI, Pierobon P, Reversat A, Lennon-Dumenil AM (2013) How B cells capture, process and present antigens: a crucial role for cell polarity. Nat Rev Immunol 13:475–486. doi:10.1038/nri3469