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# Baculovirus capsid display in vaccination schemes: effect of a previous immunity against the vector on the cytotoxic response to delivered antigens

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## Abstract

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) infects lepidopteran invertebrates as natural hosts, although it also has been used as display vector for vaccine development. In this work, we evaluated the effectiveness of repetitive doses of AcMNPV-based vectors on the cytotoxic immune response specific to the capsid-displayed heterologous antigen ovalbumin (OVA). Our results demonstrate that baculovirus vectors induce a boosting effect in the cytotoxic immune response to OVA, making possible to recover the levels obtained in the primary response. Moreover, mice preimmunized with wild-type baculovirus showed a complete lack of antigen-specific CD8 cytotoxic T lymphocytes (CTLs) that may be related to the presence of antibodies directed to baculoviral surface proteins, particularly to GP64. However, baculovirus was able to induce the innate immune response in spite of a previous response against this vector, although some quantitative differences reflect a distinct activation of the immune cells in prime and boost. This is the first report in which the novel capsid display strategy is evaluated in prime-boost schemes to improve efficient CTL responses.

**Keywords** Baculovirus · Cytotoxic immune response · Repetitive doses · Capsid display

## Introduction

Baculovirus (BV) are dsDNA viruses that infect invertebrates as natural hosts. BV can enter mammalian cells but are unable to replicate in mammalian or other vertebrate cells, because insect transcription factors are required for early transcription and viral DNA replication. Furthermore, their low cytotoxicity in mammals makes this virus an excellent candidate for gene therapy and *vector*-based *vaccines* (Chen et al. 2011; Luo et al. 2013). The BV as a vaccine platform has been engineered in three mayor paths: genetic transduction, surface display, and capsid display of antigens. The field of application of BV includes vaccines for pathogens such as viruses, parasites, and bacteria as well as expression vectors for cancer treatment. The display of antigens on the surface of BV by fusing them with the transmembrane domain of glycoproteins of the envelope is a good strategy to achieve humoral responses (Tavarone et al. 2017). BV surface display has been successfully used in the development of vaccines for avian influenza virus (AIV) (Chen et al. 2013; Chen et al. 2010; Prabakaran and Kwang 2014; Prabakaran et al. 2010; Wu et al. 2009; Wu et al. 2011), enterovirus 71 (Kiener et al. 2013; Premanand et al. 2013), pseudorabies virus (Grabowska et al. 2009), *Plasmodium falciparum* (Iyori et al. 2013; Mizutani et al. 2014), *Plasmodium berghei* (Yoshida et al. 2009), and *Plasmodium yoelii* (Yoshida et al. 2010).

However, for several infectious diseases, the humoral responses are not enough to clear the pathogen and a strong cellular immune response becomes essential for this purpose.

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A CD8 T cell response is critically necessary to control several primary viral infections, such as HIV and hepatitis C, and also for the intracellular parasite *Mycobacterium tuberculosis* and the liver stage of plasmodia. Likewise, cancer therapies require CD8 T cell response in order to achieve a successful anti-tumoral response (Pinschewer 2017). Recombinant viral vectors can elicit potent cytotoxic T lymphocyte (CTL) responses by expressing heterologous antigens in the cytoplasm of dendritic cells, thus leading to an efficient T cell priming. However, vectored vaccines based on BV expressing antigens in its surface (surface display) fail to lead efficient CTL response (Tavarone et al. 2017).

Previously, we have characterized the BV capsid display approach as a novel alternative strategy to induce a T cell immune response. We have demonstrated that the specific transport of a heterologous antigen fused to VP39 in the capsid allowed MHCI presentation and CD8 T cell activation. In addition, only one injection of the recombinant BV induced an immune response strong enough to deal with a tumor challenge and rendered higher CTL responses than the transduction approach (Molinari et al. 2011; Tavarone et al. 2017).

Multiple immunizations are commonly required for a vaccine to be successful. By using BV surface display vectors, we observed that the homologous prime-boost strategies are effective amplifying humoral responses. However, a previous response against vector impairs the BV transduction efficiency making inappropriate homologous prime-boost regimens. Indeed, the BV capsid display approach seems to have particular features and differences in the mechanisms involved in eliciting cellular immune responses with respect to the classic transduction strategy. For this reason, it is worth testing its potential in prime-boost regimens.

This study focused on the evaluation of induction of CTL response by BV capsid display in homologous and heterologous immunization schemes and the effect of the previous responses against the vector in adaptive immune response.

## Material and methods

### Cell lines and virus

BV were propagated in *Spodoptera frugiperda* insect cells (Sf9, Invitrogen, Argentina) grown in Excell medium culture (Sigma) at 27 °C. BV were collected by harvesting the supernatants and removing the cell debris by centrifugation (4000×*g*, 10 min, 8 °C). Virus titers were determined by an end-point dilution assay and converted to PFU/ml. Virus stocks were considered free of endotoxin (< 0.01 endotoxin U/ml) using the Limulus amebocyte lysate test (E-TOXATE, Sigma). A recombinant BV displaying 180 amino acids from ovalbumin (OVA), BVOVA, was constructed as previously described (Molinari et al. 2011). Briefly, a coding sequence for $OVA_{197–386}$ (spanning positions 197 to 386 of OVA) containing the immunodominant CD4 (ISQAVHAAHAEINEAGR) and CD8 (SIINFEKL) epitopes was cloned in frame to a second copy of VP39 under the polyedrin promoter to generate a capsid display vector (Fig. 1).

For some of the experiments, BVOVA was pretreated (before i.v. injection) with a mouse serum anti-BV at 1/100 dilution (for $1 \times 10^7$ pfu of BV), a mock mouse serum or a monoclonal antibody anti GP64 (V1 or V5 clone, Santa Cruz Biotechnology) at 0.2 μg of Mab for $1 \times 10^7$ pfu of BV for 30 min at 37 °C. The anti-BV serum was produced as follows: mice were immunized with $5 \times 10^7$ BV at 0 and 17 days and the serum was collected after 24 days. Both sera (BV and mock mouse serum) were treated for 30 min at 56 °C to inactivate complement previous performing the experiments.

A modified vaccinia Ankara virus (MVA) containing the OVA sequence under the control of the poxviral synthetic early/late promoter (pE/L) and the expression cassette for the GUS enzyme (MVAOVA) was used (Del Médico Zajac, unpublished). MVAOVA was propagated in a baby hamster kidney cell line (BHK- 21, ATCC CCL-10TM), and maintained in Dulbecco's modified medium supplemented with 2% fetal calf serum (FCS; Internegocios, Buenos Aires, Argentina).

Spleen cells were recovered from untreated C57BL/6 mice and collected in RPMI 10% FCS (Natocor Argentina) at 37 °C for functional assays.

### Mice

Six- to eight-week-old female C57BL/6 (H-2b) mice from Fundación Facultad de Ciencias Veterinarias (UNLP, La Plata, Argentina) were maintained in our animal facilities under specific pathogen-free conditions. All experiments were performed following internationally recognized guidelines with the approval of the Institutional Committee for Care and Use of Experimental Animals, CICUAE-CICVyA, INTA, Argentina (CICUAE-INTA 6/2014 y 58/2014). Isoflurane and $CO_2$ inhalation were used as anesthetic and the sacrifice method respectively to minimize mice suffering. Mice were injected via intravenous or intraperitoneal route.

### Cytokine detection assays

IL-6 and IFN-γ levels were measured in culture supernatants and serum samples by sandwich ELISA, following the manufacturer's instructions (BD Opt EIA Mouse IL-6 and IFN-γ AN-18, BD Biosciences, Argentina).

### In vivo killing assay

The in vivo killing assay was performed as previously described (Molinari et al. 2011). Briefly, naïve syngeneic splenocytes were pulsed or not with 10 μg/ml $OVA_{257–264}$ peptide (SIINFEKL) and labeled with a high or low

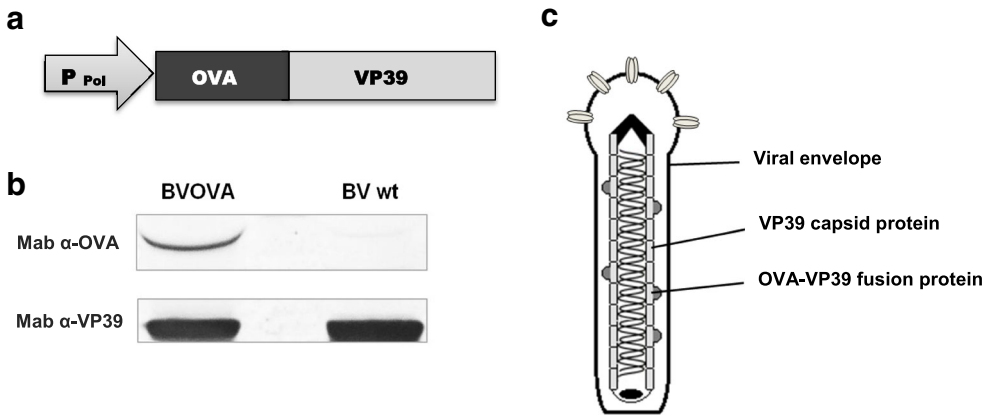


**Fig. 1** BVOVA capsid display. OVA (653–1222) sequence was cloned 5'of the VP39 sequence under the polyhedrin promoter (**a**). Western blot analysis of purified virions. OVAVP39 (MW64KDa) and VP39 (MW39KDa) proteins were detected with an anti-OVA polyclonal serum and anti-VP39 Mab, respectively (**b**). A schematic representation of the baculovirus capsid display strategy. The fusion partner (OVA) is incorporated into the viral capsid structure (**c**)

concentration (3 μM - 0.5 μM, respectively) of CFSE (Invitrogen, Argentina). Then, the $CFSE^{high}$- and $CFSE^{low}$-labeled cells were mixed at a 1:1 ratio, injected i.v. into immunized mice, and the CFSE+ cells remaining in the spleen were determined by flow cytometry after 20 h. Cytotoxicity was expressed as the percentage of lysis, calculated from $[1-(r_{control}/r_{immune})] \times 100$, where $r$ is given by the expression $\%CFSE^{low}/\%CFSE^{high}$ cells for immune and non-immunized (control) mice, respectively. The percentage of CTL inhibition was calculated from $100 - [1-(\%CTL_{treated}/\%CTL_{control})] \times 100$, where "treated" correspond to pre-BV immunization, BV serum, mock serum, or anti-GP64 Mab.

### Bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were prepared according to Inaba et al. (Inaba et al. 1992). Briefly, bone marrow cells were collected from the femurs and tibias of the mice and then cultured ($1 \times 10^6$ cells in a 60 mm plate) in complete RPMI medium supplemented with 10% of GM-CSF. Additional GM-CSF supernatant was added on days 3 and 7. Then, BMDCs were used at day 8–9.

For phenotypic marker analysis, BMDCs were cultivated for 18 h with BV or treated with Mab anti-GP64 antibody (V1) BV. Then, the cells were labeled using standard procedures with anti-CD11c (HL-3 clone, eBioscience), anti-CD86 (B7.2 clone, eBioscience), and anti-MHCII (I-A, M5/114.15.2 clone, eBioscience) and acquired in FACSCalubur flow cytometer and analyzed using CellQuest (BD Biosciences).

### Statistics

The results of all experiments are representative of at least two independent experiments and are expressed as mean ± SEM ($n = 4$). The statistical analysis was performed using one-way ANOVA analysis with Bonferroni posttest and Student's $t$ test with GraphPad Prism (La Jolla, CA). Values of $p < 0.05$ (*) or $p < 0.01$ (***) were considered significant.

## Results

### Induction of CTL immune response by BV capsid display in homologous and heterologous prime-boost regimes

In order to study the BV capsid display performance in prime-boost regimes, we first injected mice with BV carrying the model antigen OVA fused to VP39 in the capsid (BVOVA) and 15 days after we performed a boost with the same immunogen (homologous prime-boost) or PBS. We first measured the CTL response by means of a cytotoxic in vivo assay that allows to evaluate the LT-CD8 lytic activity. Seven days later, we evaluated the CTL-mediated response by injecting splenocytes charged with $OVA_{257–264}$ epitope. Lyses of these cells indicate LT-CD8 cytotoxic-specific response to $OVA_{257–264}$ epitope.

Mice immunized with a single dose of BVOVA showed approximately 50% specific lysis of target cells 1 week after vaccination. After 3 weeks, the percentage decreased to levels lower than 20%. However, a second dose of BVOVA was able to restore the levels of CTL to the primary response (Fig. 2a).

Nevertheless, considering that heterologous regimes with alternative vectors may elicit stronger responses, we assayed a heterologous prime-boost scheme of immunization by using a recombinant modified vaccinia Ankara virus that expressed OVA (MVAOVA) as booster. Mice immunized with BVOVA at day 0 received an injection with MVAOVA 15 days later. Ten days after the last immunization, boosted mice showed a significant increase in specific lysis of target cells compared to either mice immunized following a

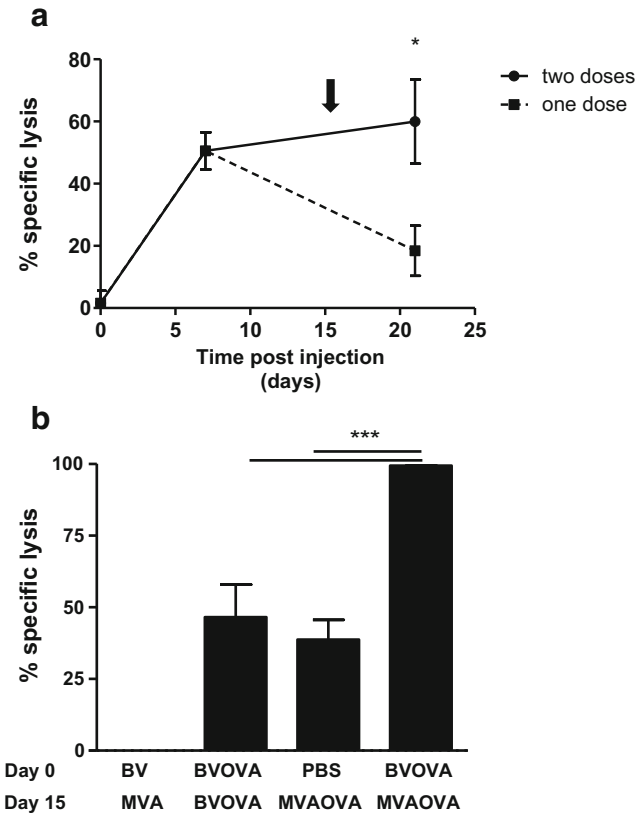


**Fig. 2** CTL response induced by different prime-boost schemes using BV capsid display. C57BL/6 mice were immunized with $1 \times 10^7$ PFU of BV or BVOVA or PBS by i.v. injection. Fifteen days later, mice received a second i.v. injection of $1 \times 10^7$ PFU of BV, BVOVA, or PBS (homologous prime-boost regime). Seven days after the last immunization, a SIINFEKL specific in vivo killing assay was performed. Arrow indicates immunization at 15 days. The comparison after 21 days was analyzed with student *t* test (**a**). C57BL/6 mice received a first i.v. injection of $1 \times 10^7$ PFU of BVOVA. Fifteen days later, mice received a second i.v. injection of either $1 \times 10^7$ PFU of BVOVA or MVAOVA, combined as shown in the figure (homologous and heterologous prime-boost regimes). Control mice were injected with $1 \times 10^7$ PFU of BV or MVA. Seven days after the last immunization, an in vivo killing assay was performed. ANOVA and Bonferroni posttest were applied (**b**). Values of $*p < 0.05$ or $***p < 0.01$ were considered significant

homologous scheme with BVOVA (BVOVA/BVOVA group) or mice receiving a single injection of MVAOVA (Fig. 2b).

Altogether, these results show that BV efficiently primed a CTL-mediated immune response to OVA and that a homologous boost was successful in recovering the initial levels. However, a heterologous boost with MVAOVA was more efficient and reinforced the CTL effect.

## Influence of the immune response against BV vector on the cytotoxic response induced by capsid display recombinants

The differences in CTL response observed between the animals boosted with BV or MVA could be due to the presence of an immune response against the BV vector elicited after the primary immunization. Thus, we decided to study the effect of pre-existent immunity against BV on the CTL-mediated response against OVA displayed in the BV capsid. For this purpose, we first injected mice with BV-wt or PBS and 15 days after with a boost of BVOVA. As expected, mice vaccinated with PBS (day 0) and BVOVA (day 15) showed 80% of specific lysis. By contrast, this specific CTL response was completely abolished in mice immunized with BV-wt (day 0) and BVOVA (day 15). Thus, a previous injection with BV completely abrogates the OVA-specific cytotoxic response (Fig. 3a).

We subsequently evaluated whether the humoral immunity against BV could be involved in the disability of induction of CTL by BVOVA. For this purpose, we immunized mice with BVOVA pretreated either with an anti-BV serum or a mock serum (sera complement had been inactivated) (Fig. 3b). In addition, we used a neutralizing monoclonal antibody directed to GP64 baculoviral surface protein (V1) or a non-neutralizing control (V5) (Fig. 3c). Seven days after the immunization, we performed an in vivo CTL assay to evaluate the LT-CD8 lytic activity. These results showed a complete lack of lytic activity after treatment with the anti-BV serum and after neutralization with V1. Thus, we demonstrated that neutralizing antibodies against GP64 impair the ability of BVOVA to induce a de novo anti-OVA CTL response.

## Influence of previous response against BV vector in the induction of innate immunity

The activation of the innate immune response is essential for the development of an efficient adaptive immune response. With this in mind, we studied if the pre-existing immune response against BV vector affects the induction of cytokines, as key effector molecules in the innate immune system. To evaluate this, we measured the presence of pro-inflammatory and inflammatory cytokines in serum samples taken shortly (6 h) after one or two BV-wt immunizations. IL-6 levels were similar after the first and second immunization; meanwhile, IFN-γ levels were higher after the second dose. Thus, both pro-inflammatory and inflammatory cytokine induction was not abolished by an anti-BV pre-existent immune response (Fig. 4a, b). Interestingly, the mice that were administrated with two doses of BV displayed significantly higher levels of IFN-γ compared to one-dose BV-injected mice.

The dendritic cell is a professional presenting cell and has a pivotal role between innate and adaptive immune system. In particular, the priming of a cellular immune response against a specific antigen needs the activation of dendritic cells (DCs) and the presentation of the antigen in the context of MHC I molecules. Therefore, we studied if pre-existing immunity against BV interferes with DC maturation. To accomplish that, we performed assays with bone marrow dendritic cells

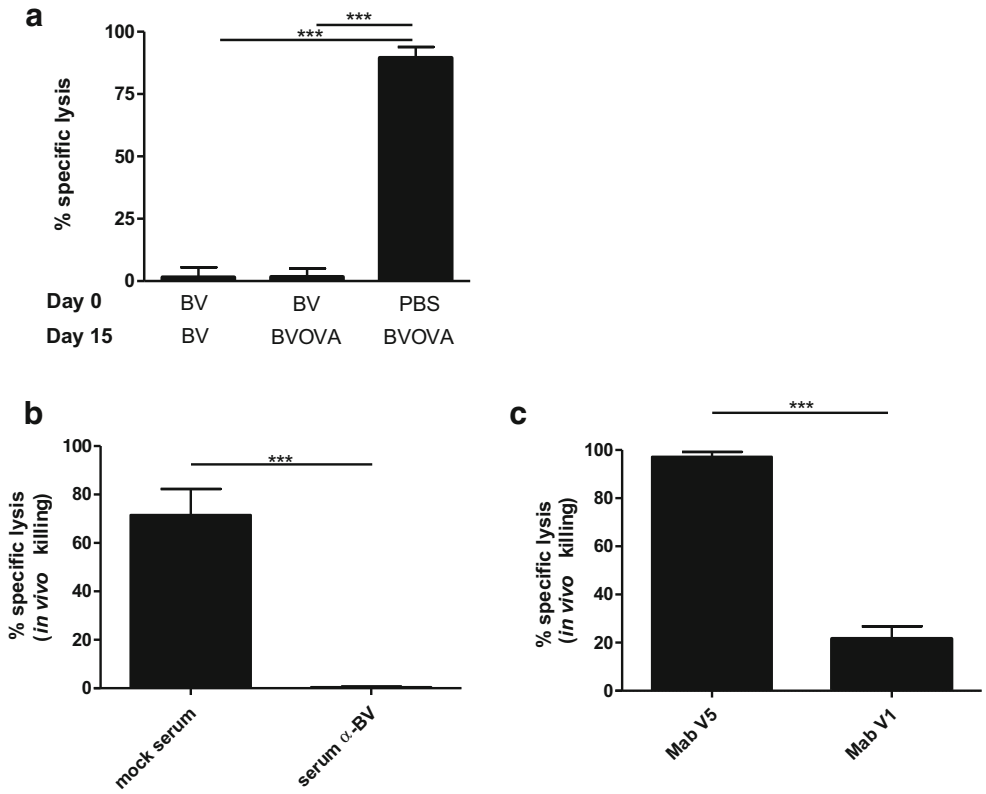


**Fig. 3** Influence of the humoral immune response against BV vector on the cytotoxic response induced by capsid display. C57BL/6 mice were immunized by a first i.v. injection of $5 \times 10^7$ PFU of BV or PBS. Fifteen days later, mice received a second i.v. injection of $5 \times 10^7$ PFU of BV or BVOVA, combined as shown in the figure. Seven days after the last immunization, a SIINFEKL specific in vivo killing assay was performed. ANOVA and Bonferroni posttest were applied (**a**). BVOVA was treated before i.v. injection with anti-BV serum or mock serum and 7 days after immunization, an in vivo killing assay was performed (**b**). Also, BVOVA was incubated with the neutralizing Mab α-gp64 (V1) and non-neutralizing Mab α-gp64 (V5) before mice injection. At 7 days post-injection, an in vivo killing assay was performed (**c**). In **b**, **c**, a student *t* test was applied. Values of $*p < 0.05$ or $***p < 0.01$ were considered significant

differentiated in vitro that were incubated with BV-wt alone or BV-wt pre-treated with the neutralizing monoclonal antibody V1. After 18 h, we analyzed the percentage of $CD11c^+$ cells expressing high quantities of CD86 or MHC II by flow cytometry (Fig. 4c). Surprisingly, although all the assessed parameters were slightly lower than in the treatment with BV-wt alone, the BV-wt pre-treated with the neutralizing antibody induced the maturation of the BMDCs. This was evidenced by the proportion of $CD86^{hi}$ and $MHCII^{hi}$ populations.

These results indicate that BV pre-existing immunity does not affect the induction of IL-6 and IFN-γ by the viral vector. In addition, DCs were able to mature under the stimulus of BV even when neutralized with antibodies.

## Discussion

The display in the capsid of baculovirus as fusions to VP39 has been recently described as a new strategy to present foreign antigens with a particular efficiency for allowing MHC I antigen presentation by professional cells and induction of CTL responses (Molinari et al. 2011; Tavarone et al. 2017). Moreover, this approach significantly reduced the amount of antigen required for obtaining a high immune response and favored the cross-presentation pathway (Molinari et al. 2011).

In order to investigate different strategies to optimize CTL responses using BV vectors, in this study we evaluated the novel BV capsid display both in homologous and heterologous vaccination schemes. In addition, the effect of pre-existing response against BV was considered.

BV has been used as vaccine vector in homologous prime-boost regimens for the induction of humoral immunity showing promising results. Indeed, Sim et al. (2016) reported that in a hemagglutinin BV surface display approach, the booster immunization significantly improves HA-specific IgG and IgA response in serum or bronchoalveolar lavage fluid of mice, respectively (Sim et al. 2016). Other groups demonstrated a homologous booster effect in the humoral immune response with a recombinant BV constructed following a transduction design under CMV promoter (Gwon et al. 2016). Ye et al. obtained similar results using both display and transduction strategies for the capsid protein of porcine circovirus type

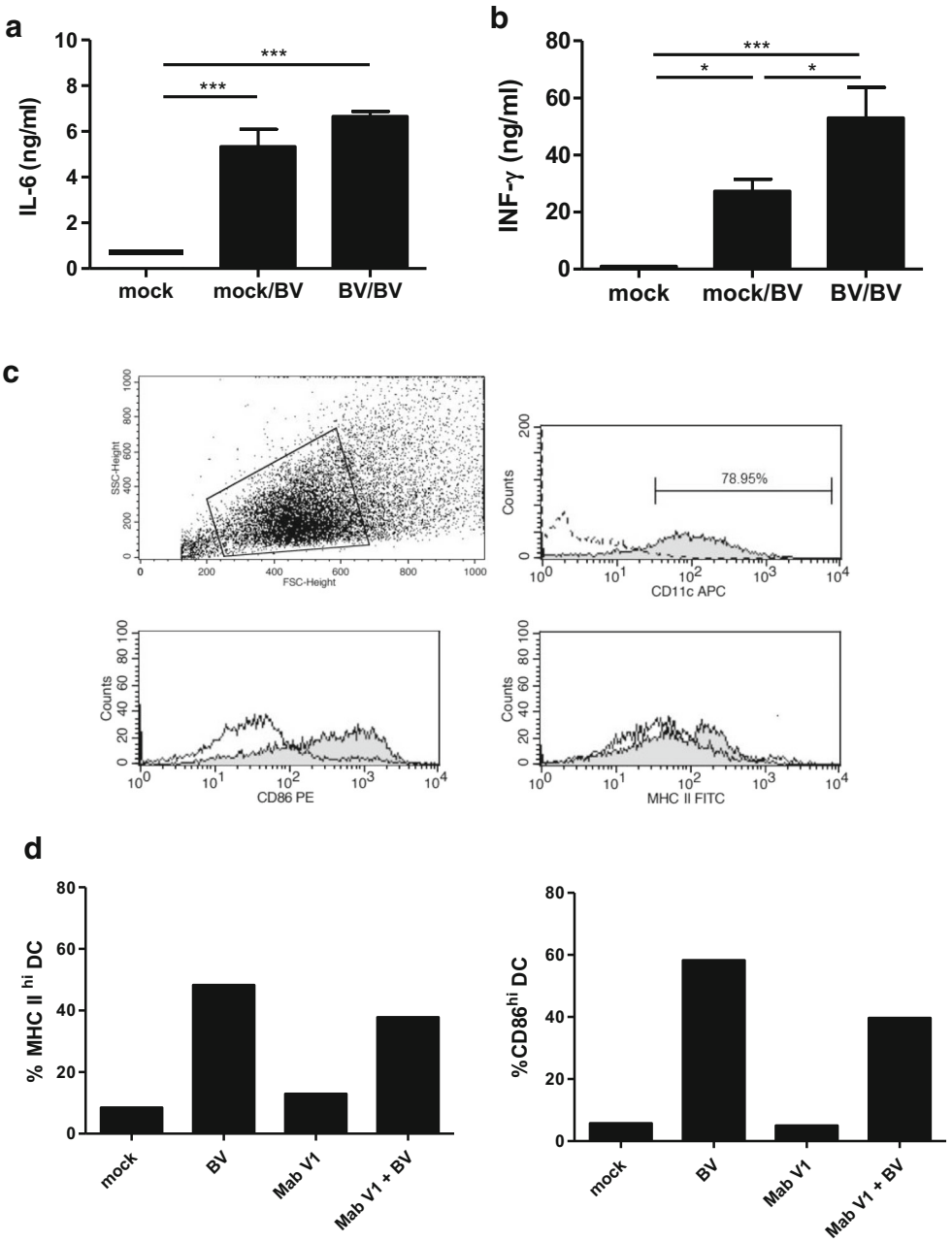


**Fig. 4** Effect of two doses and BV neutralization on the induction of BV innate immune response. C57BL/6 mice were i.v. injected with $5 \times 10^7$ PFU of BV or with a supernatant from uninfected insect cells on day 0. On day 15, all mice received a second injection with $5 \times 10^7$ PFU of BV. An additional group of untreated mice was injected with supernatants of uninfected insect on day 15 as control (**a**, **b**). Six hours after the last injection, plasma samples were collected and assayed for IL-6 (**a**) and IFN-γ (**b**) by ELISA. ANOVA and Bonferroni post test were applied (**a**, **b**). BMDCs were incubated with BV, BV neutralized with Mab α-gp64 (V1) or Mab α-gp64 (V1) alone, as control, for 18 h. A gating strategy of CD11c expression on cells was employed and phenotypic markers CD86 and MHCII were analyzed on $CD11c^+$ population by flow cytometry (**c**). These results are representative of three independent experiments and are expressed as percentage of $MHCII^{hi}$ or $CD86^{hi}$ (**d**). Values of $*p < 0.05$ or $***p < 0.01$ were considered significant

2 (Ye et al. 2013). Thus, the humoral response seems to be successfully boosted by BV-vectorized vaccines.

Although BV prime-boost schemes have been widely exploited in vaccine applications, the effectiveness of the repetitive use of BV as a cytotoxic vaccine vector using the novel capsid display strategy has not been studied. Here, we demonstrated that the administration of a second dose of BVOVA recovered the levels of cytotoxic immune response observed after the first immunization, indicating a boosting effect. Thus, when the primary response reaches protective levels, this strategy would be good enough. On the other hand, in previous studies, we detected an amplification of the primary humoral immune response by using a surface display strategy in a homologous scheme (Tavarone et al. 2017). These observations suggest that the effectiveness of homologous prime-boost schemes is not the same in humoral and cellular immune responses.

For weak immunogenic antigens, the primary response must be heightened to develop an adequate global response. To assess the possibility to further improve the cytotoxic immune response induced by BV carrying OVA in the capsid, we used an MVA vector expressing OVA as booster in a heterologous scheme. This strategy conducted to a solid anti-OVA CTL response demonstrating that heterologous regimens seem to be a better choice when an increment in the cytotoxic level is needed.

When we injected BVOVA in mice preimmunized with wild-type BV, the immune system failed to elicit a CTL response specific to OVA. The immunological mechanisms and the antigen-presenting cells involved in the activation of memory CD8 or naïve CD8 cells are different (Guermonprez et al. 2002). Cellular and humoral immune components induced in primary immune response induced by BV or BVOVA injection could be involved in this abrogation. Antibodies directed to proteins in the surface of budded virions are the most probable cause of the lack of specific cytotoxicity, as no CTL to OVA was observed after BVOVA preincubation with a serum of a BV-immunized mice or GP64-specific monoclonal antibody.

The innate immunity has a pivotal role in adaptive immune response and the impact of BV on this response may not be the same in prime and boost immunizations. For example, BV is inactivated by complement triggered by classical and alternative pathways (Hoare et al. 2005). Indeed, high levels of anti-BV antibodies are produced and consequently, an inhibition of innate mechanisms could be expected after the second dose. Contrary to these expectations, high levels of IFN-$\gamma$ and IL-6 were present in sera of vaccinated mice and dendritic cells displayed phenotypic maturation markers after the exposure with neutralized BV. However, the maturation of these cells was slightly lower than those activated by non-neutralized BV and the IFN-gamma production was higher. These results indicate differences in the activation of the innate immune cells with or without a previous response against the vector. Indeed, naïve CD8 T cells need a strong maturation profile of dendritic cells to be activated. On the other hand, for the OVA-specific CD8 T cell recall, other antigen-presenting cells such as macrophages and B cells could be involved. Altogether, these considerations could explain the recovery of the primary cytotoxic response when BVOVA is used for homologous prime-boost regimens and the lack of priming in the context of preexistent immune response anti-BV. Further experiments are needed to clarify this point.

Overall, our results show that BV capsid display allows an efficient prime of CD8 memory cells and that this strategy is able to boost cytotoxic responses in homologous schemes. However, heterologous regimes are more successful increasing the cytotoxic levels reached in the primary immune response. Moreover, BV fail to activate naïve CD8 T cells responses if a previous humoral immunity against BV had been previously elicited. This indicates that, in order to induce cytotoxic immunity to different antigens using BV capsid display, simultaneous vaccination with recombinant BVs could be applied to overcome this limitation.

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## Compliance with ethical standards

**Ethical statement** All applicable international and national guidelines for the care and use of mice were followed.

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

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