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Comparative evaluation of the protective efficacy of two formulations of a recombinant *Chlamydia abortus* subunit candidate vaccine in a mouse model

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Abstract

Chlamydia abortus (*C. abortus*) is the causative agent of ovine enzootic abortion (OEA) and poses a zoonotic risk to pregnant women. Current live attenuated 1B vaccines are efficacious but cause disease in vaccinated animals and inactivated vaccines are only marginally protective. We tested the ability of a new *C. abortus* subunit vaccine candidate based on the conserved and immunogenic polymorphic membrane protein D (Pmp18D) formulated in CpG1826+FL (Fms-like tyrosine kinase 3 Ligand; Flt3L) or *Vibrio cholerae* ghosts (VCG) to induce innate and cross protective immunity against genital *C. abortus* infection. We found that delivery of rPmp18D with VCG was more effective than with CpG+FL in up-regulating the expression of molecules critically involved in T cell activation and differentiation, including MHC II, CD40, CD80, and CD86, activation of TLRs and NLRP3 inflammasome engagement, and secretion of IL-1β and TNF-α but not IL-10 and IL-4. rVCG-Pmp18D-immunized mice elicited more robust antigen-specific IFN-γ IgA and IgG2c antibody responses compared to CpG+FL-delivered rPmp18D. Based on the number of mice with positive vaginal cultures, length of vaginal shedding, and number of inclusion forming units recovered following challenge with the heterologous *C. abortus* strain B577, vaccine delivery with VCG induced superior protective immunity than delivery with a combination of CpG1826 and FL, a nasal DC-targeting adjuvant. These results demonstrate that the ability of VCG to enhance protective immunity against genital *C. abortus* infection is superior to that of CpG+FL adjuvants.

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Conflict of interest

The authors declare that they have no competing interests.

Keywords

Chlamydia abortus; Pmp18D; vaccine delivery; cross protection; immunity

1. Introduction

Chlamydia abortus is the causative agent of ovine enzootic abortion (OEA) in sheep, goats, pigs and cattle leading to considerable economic losses worldwide and poses a zoonotic risk to pregnant women [1, 2]. The disease, contracted through ingestion or inhalation of *C. abortus*-infected material [3, 4] is characterized by acute placentitis with abortion occurring before the end of gestation. Natural infection often remains latent until the animal becomes pregnant, after which the organism invades the placenta, multiplies and eventually causes abortion [5, 6]. Zoonotic infections are also frequently asymptomatic leading to the development of complications, including severe septicemia, spontaneous fetal abortion, preterm labor or stillbirth [7, 8]. Thus, a vaccine capable of safely protecting against infection would be the most effective strategy to control infections and the resulting complications.

Although the current live attenuated 1B vaccines are efficacious, they have been implicated in cases of abortion [9]. The vaccines are expensive and hazardous to produce, labor-intensive, and challenging to manufacture in large quantities. Moreover, it is impossible to distinguish infected from vaccinated animals by serology alone [10], making it difficult to monitor vaccination practices. These vaccines are temperature attenuated at 39 °C, making them unsuitable for use in pigs. Also, inactivated and DNA vaccines although promising in principle, are only marginally protective [11], calling for alternative vaccine development strategies.

A number of potential *C. abortus* vaccine antigens have been predicted, including a unique family of polymorphic membrane proteins (Pmps) consisting of 18 pmp genes [12] that resemble autotransporters of the type V secretion system [13, 14]. The Pmp18D is a highly conserved and immunogenic outer membrane protein that is expressed throughout the chlamydial developmental cycle, plays an important role in pathogenesis and is a diagnostic and vaccine target [13, 14].

A subunit vaccine approach would require an effective delivery system to induce optimal protective immunity. In this respect, the *Vibrio cholerae* ghost (VCG) platform has been shown to be an effective carrier and delivery system for cloned antigens [15–17]. VCG are empty bacterial cell envelopes devoid of cytoplasmic contents and cholera toxin and are produced by genetic inactivation of *V. cholerae* cells, involving the controlled expression of cloned bacteriophage PhiX174 lysis gene *E*. The resulting bacterial ghosts share the functional and antigenic determinants of the envelope with their living counterparts [15]. CpG motif, the agonist of Toll-like receptor (TLR) 9, is a well-known stimulator of Th1 immune response [18] and the Fms-like tyrosine kinase 3 Ligand (Flt3L; FL) for Flt3 receptor on antigen presenting cells (APCs) is a safe and effective dendritic cell (DC)-targeting adjuvant [19]. CpG and FL delivered intranasally as a combined DC-targeting mucosal adjuvant elicited enhanced immune responses to co-delivered antigens [19, 20]. In

this study, we compared the immunomodulatory effect of VCG with CpG/FL adjuvants by evaluating their ability to induce the DC expression of MHC II and costimulatory molecules, innate immunity (assessed by TLR engagement) and production of cytokines in *in vitro* cultures. We then compared the ability of the adjuvants to enhance the protective immunity induced by *C. abortus* Pmp18D against heterologous challenge in a mouse model of genital infection. Our results demonstrated that incubation of DCs with Pmp18D+VCG induced enhanced secretion of proinflammatory cytokines and expression of MHC II and co-stimulatory molecules involved in DC maturation and activation compared with CpG/FL. Co-stimulation with VCG also induced higher TLR engagement, Th1-inducing capacity and cross-protective ability of Pmp18D than CpG/FL.

2. Materials and Method

2.1. Chlamydia stocks, antigens and animals

Stock preparations of *C. abortus* strain P16 and strain B577 (Dr. Bernhard Kaltenboeck, Auburn University, Alabama) were generated by propagating elementary bodies (EBs) in BGMK cells as previously described [21] and stored at −70°C. *C. abortus* antigen was prepared by UV-inactivation of EBs for 3 h. Purified Fms-like tyrosine kinase 3 (Flt3) ligand (FL) was obtained from R&D Systems, Minneapolis, MN and CpG 1826 ODN was obtained from InvivoGen, San Diego, CA.

Female C57BL/6 mice (aged 6 to 8 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the animal facility of Morehouse School of Medicine and studies were performed in compliance with institutional IACUC and Federal guidelines.

2.2. Construction of vaccine vectors and purification of recombinant Pmp18D (rPmp18D)

A 1317 bp N-terminal Pmp18D fragment was obtained from the genomic DNA of *C. abortus* strain P16 by PCR and inserted into vector pSTV66 using restriction sites incorporated into the primer sets. The resultant plasmid was designated pST-18D.

This N-terminal fragment was also inserted into vector pET-32a to generate plasmid pET-18D and expressed in *E. coli* BL21 (DE3). rPmp18D was purified by the Ni-NTA Purification System (Invitrogen, California, USA) according to the manufacturer's instructions. Endotoxin was removed using Detoxi-Gel™ (Thermo, Illinois, USA) and determined to be < 0.05 EU/mg protein using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo, Illinois, USA). Concentration of protein was calculated using the Pierce™ BCA Protein Assay Kit (Thermo, Illinois), adjusted to 500 μg/ml and stored at −80 °C. Protein expression was detected by SDS-PAGE and immunoblotting analysis was performed as previously described [16] using purified rabbit anti-Pmp18D polyclonal antibody.

2.3. Production of rVCG vaccines

Recombinant VCG expressing Pmp18D (rVCG-Pmp18D) were produced by gene *E*-mediated lysis essentially as described previously [22]. An rVCG construct expressing

glycoprotein D from HSV-2 (rVCG-gD2) was also produced and used as antigen control. Lyophilized ghost preparations were stored at room temperature until used.

2.4. Determination of Toll-Like Receptor (TLR) engagement and co-stimulatory molecule expression

Dendritic cells were generated from bone marrow (BMDC) by a standard procedure described previously [23]. Purified dendritic cells (5×10^5 cells/ml) were cultured for 24 h with Pmp18D plus 100 μg of VCG or CpG 1826 (10 μg) and Flt3L (FL; 150 ng). Harvested cells were stained with monoclonal antibodies conjugated with either PE- or FITC (PharMingen, San Diego, CA) against DC surface markers, TLRs and NLR/P3 and analyzed by flow cytometry on a FACScan Flow Cytometer (Becton-Dickinson, CA). Examined DC markers included markers for DC maturation and those critically involved in activation of innate immune signaling as well as T cell activation and differentiation. Controls were incubated with isotype-matched irrelevant antibodies. Marker expression was assessed on gated CD11c cells.

2.5. Analysis of cytokine profile of antigen-pulsed DCs

Culture supernatants of DCs pulsed with rVCG vaccine constructs for 48 h were assayed for IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p70), IFN-γ and TNF-α by cytokine ELISA as described below.

2.6. Immunization, challenge and analysis of protective immunity

Mice (10–12 mice/group) were immunized intranasally (IN) with 20 μl PBS containing 1.5 mg of lyophilized rVCG-Pmp18D or rVCG-gD2 or rPmp18D (10 μg) with or without CpG 1826 (10 μg) and FL (150 ng) per mouse on days 0, 14 and 28. All immunizations were administered while under ketamine (75 mg/kg Ketaset, Zoetis, Florham Park, NJ)/xylazine (15 mg/kg Anased, Lloyd, Shenandoah, IA) anesthesia. Serum and vaginal wash samples were obtained 2 weeks after the second immunization and 2 and 4 weeks after the last immunization and stored at −80°C until analyzed. Three weeks after the last immunization, animals were challenged intravaginally with 10^6 IFUs of the heterologous *C. abortus* strain B577 to assess cross protection and the level of infection was assessed as described previously [16]. Experiments were repeated to contain 10–12 mice per group for immunogenicity studies and 8 mice/group for challenge studies.

2.7. Purification of immune T cells

Four weeks after immunization, T cells were purified from the iliac lymph nodes (ILN) and spleens (SPL) of immunized mice using the gentleMACS Dissociator, Pan T Cell Isolation Kit II and the Midi magnetic bead-activated cell-sorting (MidiMACS) separator (Miltenyi Biotech, Auburn, CA). A separate pool of γ-irradiated (2000 rad) splenocytes prepared from naive animals was used as a source of antigen-presenting cells (APCs).

2.8. Detection of cytokine production by ELISA

The level of Th1/Th2 response was assessed by measuring the *Chlamydia*-specific IFN-γ IL-12, IL-4 and IL-10 cytokine production by ILN and splenic T cells as described

previously [16]. Briefly, purified T cells (10^6 cells/well) were cultured with APCs (2×10^5/well) with and without (control) *C. abortus* antigen (10 μg/ml) for 5 days and supernatants were assayed for cytokines using the Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software (Bio-Rad, Hercules, CA). The mean and SD of 5 replicate cultures were calculated. The experiment was repeated twice.

2.9. Measurement of T cell proliferation

Purified immune T cells were assessed for their ability to proliferate in response to *in vitro* restimulation in culture with chlamydial antigen as described previously [16] using the XTT Cell Viability Kit according to the manufacturer's instructions (Cell signaling, Boston, MA). After three days of *ex vivo* antigen-restimulation, XTT detection solution was added to the T cell mixture and the absorbance read at 450 nm. The stimulation index (SI), the ratio between stimulated and non-stimulated cells, was then calculated.

2.10. Determination of mucosal and systemic antibody levels

The amount of antigen-specific antibodies (IgG, IgG2c and IgA) in sera and vaginal washes of immunized mice was measured by a standard ELISA procedure described previously [24]. Results, generated simultaneously with a standard curve, display data sets corresponding to absorbance values as mean concentrations (ng/ml) ± SD and represent the mean values from two experiments each with five replicates.

2.11. Statistical analysis

Statistical analyses were performed with the GraphPad Prism package (GraphPad Software, Inc. La Jolla, CA, USA) on a PC computer. The statistical significance of the difference between two groups was evaluated by Student's t-test and between more than two groups by one-way ANOVA. Differences were considered to be significant at $p^* < 0.05$ or $p^{**} < 0.01$.

3. Results

3.1. VCG enhance the activation and maturation of BMDCs in vitro

DCs cultured for 24 h with Pmp18D in combination with VCG or CpG+FL were quantified for expression of MHC II and co-stimulatory molecules. The results showed that VCG enhanced the expression of MHC class II and CD80, CD86 and CD40 (Fig. 1A) significantly higher ($p<0.05$) than CpG+FL indicating that the ability of VCG to modulate the maturation and proliferation of DCs is superior to that of CpG+FL combination adjuvants.

3.2. VCG stimulate the induction of TLR engagement and DC cytokine secretion in vitro

We evaluated the ability of DCs pulsed with Pmp18D in combination with either VCG or CpG+FL to engage different TLRs leading to the production of proinflammatory cytokines in 48-h DC culture supernatants. Stimulation of DC with PmpD+VCG and rVCG-PmpD resulted in the upregulated expression of TLRs 2, 4 and 5, and NLRP3 that was significantly higher ($p<0.05$) than stimulation with rPmp18D or rPmp18D + CpG+FL (Fig. 1B). Also, significantly higher ($p<0.05$) levels of IL-1β, TNF-α IL-12p70 and IL-6 cytokines were

secreted by DCs pulsed with PmpD+VCG and rVCG-PmpD compared to those pulsed with rPmp18D with and without CpG+FL (Fig. 1C). However, all antigen combinations induced the secretion of only marginal levels of IL-4, indicating the induction of predominantly Th1-promoting cytokines.

3.3. Vaccination with rVCG-Pmp18D or rPmp18D elicits antigen-specific T cell responses

To examine specific Th1/Th2 cell responses induced by the vaccine candidates, T cells purified from the ILN and spleens of immunized mice 4 weeks postimmunization were analyzed for Th1/Th2 cytokine production upon restimulation with *C. abortus* antigen (Fig. 2). Significantly higher ($p< 0.05$) amounts of antigen-specific IFN-γ were produced by both systemic (Fig. 2A) and mucosal (Fig. 2B) immune T cells from rVCG-Pmp18D-immunized mice compared to those from rPmp18D with and without CpG/FL or rVCG-gD2-immunized mice. The results also showed the secretion of significantly lower ($p < 0.05$) levels of IL-4 compared to IFN-γ by T cells, indicating the induction of antigen-specific Th1-type cellular response (Fig. 2A & B).

3.4. Immunization with rVCG-Pmp18D and rPmp18D induced proliferation of immune T cells

Purified immune T cells from the SPL and ILN of rVCG-Pmp18D or rPmp18D-immunized mice were assessed for their ability to proliferate in response to *in vitro* restimulation in culture with *C. abortus* antigen by the XTT proliferation assay. Stimulation index (SI) values (the ratio between absorbance values of antigen-stimulated and non-stimulated cells) obtained after stimulation of T cells in the presence or absence of antigen were then analyzed. Fig. 3 shows mice immunized with rVCG-Pmp18D had significantly higher ($p< 0.05$) T cell proliferative responses compared to Pmp18D, rPmp18D+CpG/FL or VCG-gD2-immunized mice. Moreover, the magnitude of proliferation of splenic T cells was significantly higher ($p<0.05$) than that of the ILN T cells, indicating a potentially greater concentration of specific IFN-γ-responsive cells in systemic rather than mucosal tissues postimmunization.

3.5. Induction of antigen-specific antibody responses in mice immunized with rPmp18D and rVCG-Pmp18D

Specific antibody responses elicited after immunization were measured by titrating the serum and vaginal secretions of vaccinated and control mice against *C. abortus* antigen, using an ELISA assay. The results (Fig. 4) showed that the magnitude of antibody response was time dependent with the rVCG-Pmp18D vaccine showing an immunogenic advantage. In general rVCG-Pmp18D-immunized mice developed significantly higher ($P< 0.05$) antigen-specific total IgG (4A), IgG2c (4B) and IgA (4C) antibodies in both vaginal secretions and serum, compared to those immunized with rPmp18D with and without CpG/FL.

To determine if only two immunizations could induce significant antibody responses, levels of antibody were determined from serum and vaginal wash samples obtained 2 weeks after the second vaccine dose. The results showed high levels of antigen-specific IgG, IgG2c and

IgA antibody isotypes were elicited in serum and vaginal wash of immunized mice following prime boost immunization (Fig. 5).

3.6. Intranasal immunization with rVCG-Pmp18D and rPmp18D vaccines confers cross protection against heterologous genital C. abortus challenge infection

To determine if intranasal immunization could effectively prevent or decrease heterologous chlamydial shedding, immunized animals were challenged intravaginally with the heterologous *C. abortus* strain B577 three weeks after the last immunization and periodically monitored for number of chlamydial IFUs shed. The results showed that the rate of clearance of the infection by the rVCG-Pmp18D group was significantly higher ($P < 0.05$) compared to the other groups from day 3 to 15 post challenge. Mice immunized with the rVCG-Pmp18D vaccine, which cleared infection within 2 weeks (day 15) after challenge shed approximately 3-log lower chlamydial IFUs than the rPmp18D alone or controls (rVCG-gD2) and more than 2-log lower IFUs than the rPmp18D+Cp/FL-immunized mice (Fig. 6A). The results indicate that the level of cross protective immunity conferred by rVCG-Pmp18D against live infection is superior to that of rPmp18D administered with a combination of CpG/FL.

We further evaluated the number of mice in each group shedding *Chlamydia* at each time point. The number of mice (expressed as a percentage) shedding *Chlamydia* at each time point paralleled the efficacy data. By day 15-post challenge while none (0%) of the mice immunized with rVCG-Pmp18D shed bacteria, 60% of the mice immunized with rPmp18D co-delivered with CpG/FL still shed bacteria up to day 18 postchallenge (Fig. 6B). However the rVCG-gD2 control-immunized mice shed bacteria up to day 24 postchallenge (Fig. 6B).

4. Discussion

The current commercially available inactivated vaccines provide inadequate protection [25] and the live attenuated *C. abortus* vaccines, though protective, cause disease leading to abortion in sheep [9]. The finding that successful vaccination against OEA requires the induction of effector cells or cytokines that polarize the immune response towards a Th1-type response [26] suggests the choice of an appropriate adjuvant/delivery system capable of activating a Th1-type response. In previous reports, we showed that the novel VCG platform is a highly effective delivery system, enhancing significant immune responses and protection in the absence of supplementary adjuvants [17, 27]. However, the mechanisms associated with the increased immunity induced by VCG have not been clearly defined. The important role of innate immunity in primary infection by *C. abortus* has been demonstrated [28]. Innate immunity not only acts as a first line of defense against infection but leads to specific immunity through the recruitment of T-cell subsets and secretion of different cytokines [28]. The present study was undertaken to compare the immunomodulatory ability of VCG with that of an established Th1-promoting adjuvant, CpG in the induction of innate and adaptive immunity. We showed that rPmp18D plus VCG was more effective than CpG +FL in stimulating the activation of DCs to express the molecules critically involved in T cell activation and differentiation, including MHC II, CD40, CD80, and CD86; activation of innate immune signaling involving TLR2, TLR4, TLR5 and NLRP3 inflammasome

engagement and production of the proinflammatory cytokines, IL-1β and TNF-α. The results suggest that DC production of proinflammatory cytokines may involve the TLRs/MyD88 and inflammasome/caspase pathways. The optimal expression of surface activation markers and functional maturation of DCs influence the induction of a T cell response and the magnitude and pattern of cytokine response [29]. The secretion of proinflammatory cytokines not only triggers an inflammatory response through the recruitment of immune cells such as neutrophils and macrophages, but also activates adaptive immunity [30].

Since both cell-mediated and humoral immune effectors may control *C. abortus* immunity, we simultaneously investigated specific cellular and antibody responses in the serum and genital mucosa of immunized mice. The finding that vaccine delivery with VCG activated more robust local mucosal and systemic antigen-specific CD4+ T cell proliferation and IFN-γ production compared to CpG+FL confirms the results of the *in vitro* DC studies. Although the correlation between IFN-γ production and host immune control of *C. abortus* infection in sheep has been established [32, 33], endogenous IL-12 may not be required for resolution of *C. abortus* infection in mice [34]. Results from the present study confirm these reports. Although the relative importance of CD4+ T cells over other lymphocyte populations for host protection against OEA remains to be fully defined, in mice CD8+ T cells may play a role in the regulatory control of the CD4+ T-cell response and may have a direct cytotoxic or IFN-γ-mediated effect on infected cells [31]. Our results also show that IN immunization with the vaccines elicited significant systemic and local mucosal IgA and IgG2c antibody responses detectable in serum and vaginal lavage, with the rVCG-Pmp18D candidate showing an immunogenic advantage. Moreover, the magnitude of antigen-specific local mucosal and systemic antibodies elicited after a single booster immunization indicates that this vaccine may require no more than two doses to induce substantial immunity in the genital tract. It is well established that ewes seroconvert following *C. abortus* infection [35]. Although the role of antibody in protection against OEA once infection is established is debatable, antibody is likely to play a protective role against re-infection by either opsonizing EBs or preventing EB attachment to target cells [33].

Efficacy analyses of the vaccines against heterologous challenge infection with live *C. abortus* strain B577 showed significant reduction in vaginal shedding of bacteria in immunized mice compared to rVCG-gD2 controls. Comparison of the level of protection conferred by the vaccine candidates showed that rVCG-Pmp18D-immunized animals successfully resolved the genital challenge infection by day 15 postchallenge; these animals shed more than 2-log lower IFUs than the rPmp18D+CpG/FL-immunized mice and cleared infection 3 days earlier. Furthermore, by day 15-post challenge while none of the rVCG-Pmp18D-immunized mice shed bacteria, 100% of the rVCG-gD2-immunized mice still shed bacteria at this time point. The significant reduction in the number of recoverable *C. abortus* IFUs and shortening of the time taken to clear the challenge infection by rVCG-Pmp18D-immunized mice further underlines the advantage of the rVCG platform as a vaccine delivery system. These results are consistent with our previous reports indicating that delivery of subunit antigens in the context of VCG can generate efficient immunity in the absence of external adjuvants [15, 17, 24, 27] and confirms the superior immunomodulatory capacity of VCG compared to CpG and/or FL adjuvants. The results are significant

especially as subunit vaccines are often poorly immunogenic and require an adjuvant to function optimally.

In summary, we have demonstrated that the immunomodulatory capacity of VCG to enhance innate immunity and stimulate specific immune effectors that afforded cross protection in mice against heterologous challenge with live *C. abortus* is superior to that of CpG+FL adjuvants. Based on the number of mice with positive vaginal cultures, length of vaginal shedding, and number of *C. abortus* IFUs recovered, rVCG-Pmp18D elicited more robust cross protection than delivery of antigen with CpG1826 and FL adjuvant. A combination of CpG and FL delivered intranasally has been shown to be an effective DC-targeting mucosal adjuvant for co-delivered antigens [19, 20]. It is noteworthy that delivery of the rPmp18D with rVCG generated this substantial genital tract immunity in the absence of external adjuvants. These self-adjuvanting properties, coupled with the ease and low cost of production and absence of a cold chain requirement are invaluable for the rapid development and production of a cost-effective *C. abortus* vaccine for veterinary use. These data support further vaccine evaluation and testing for protection against OEA using a pregnant mouse model of *C. abortus* infection and in larger animals (sheep and pigs).

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Highlights

- The ability of recombinant *C. abortus* Pmp18D formulated in CpG+FL adjuvants or *Vibrio cholerae* ghosts (VCGs) to induce innate and cross protective immunity against genital *C. abortus* infection was evaluated.
- VCG-delivered rPmp18D was more effective than CpG+FL in stimulating the DC expression of molecules critically involved in T cell activation and differentiation, and activation of innate immune signaling involving TLRs and NLRP3 inflammasome.
- VCG-delivered rPmp18D elicited more robust antigen-specific cellular and humoral immune responses in mice compared to CpG+FL.
- Vaccine delivery with VCG induced superior cross-protective immunity than delivery with a combination of CpG1826 and FL.
- The vaccine delivery and immunomodulatory capacity of VCG is thus superior to that of CpG+FL adjuvants.

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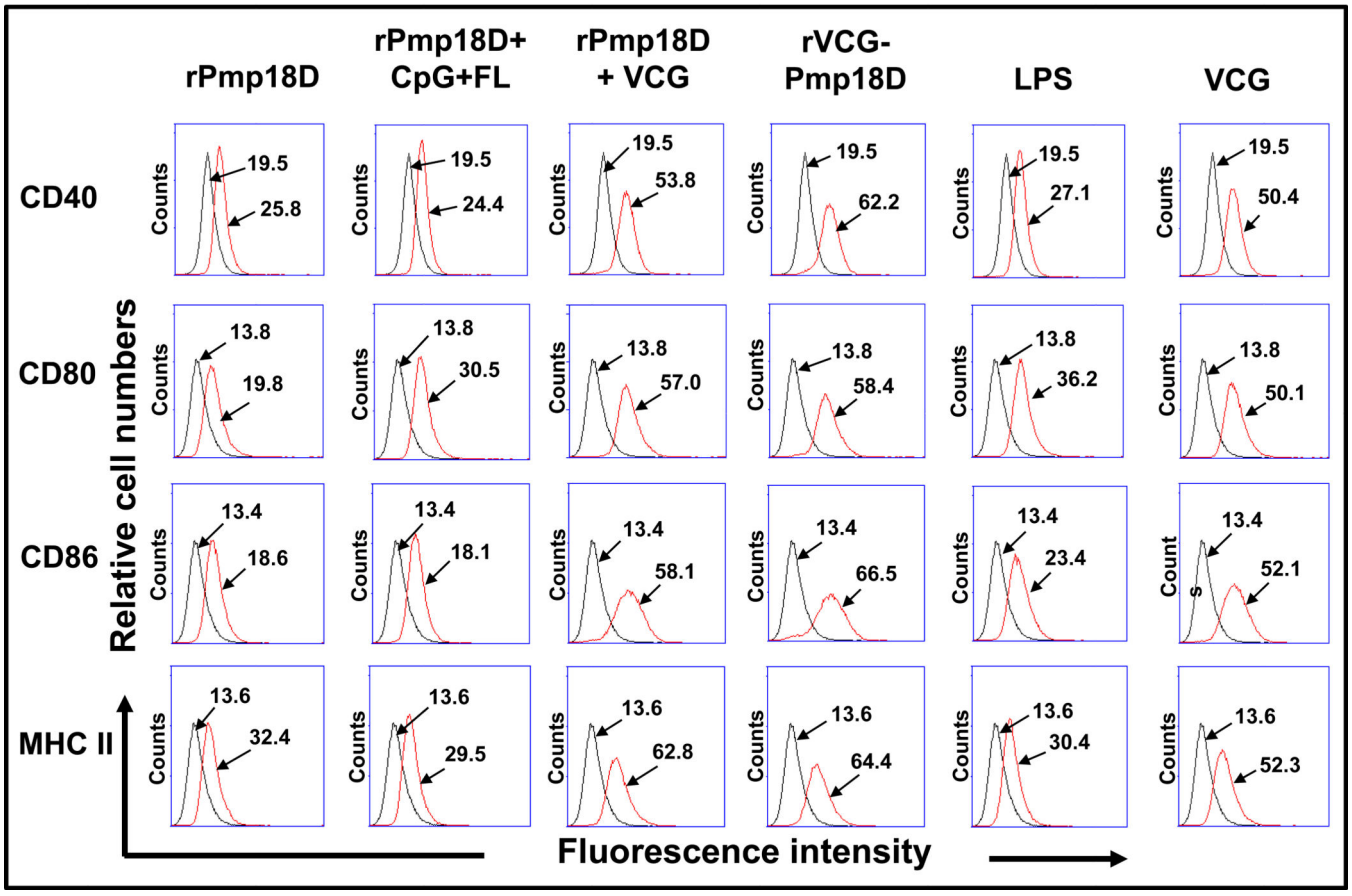

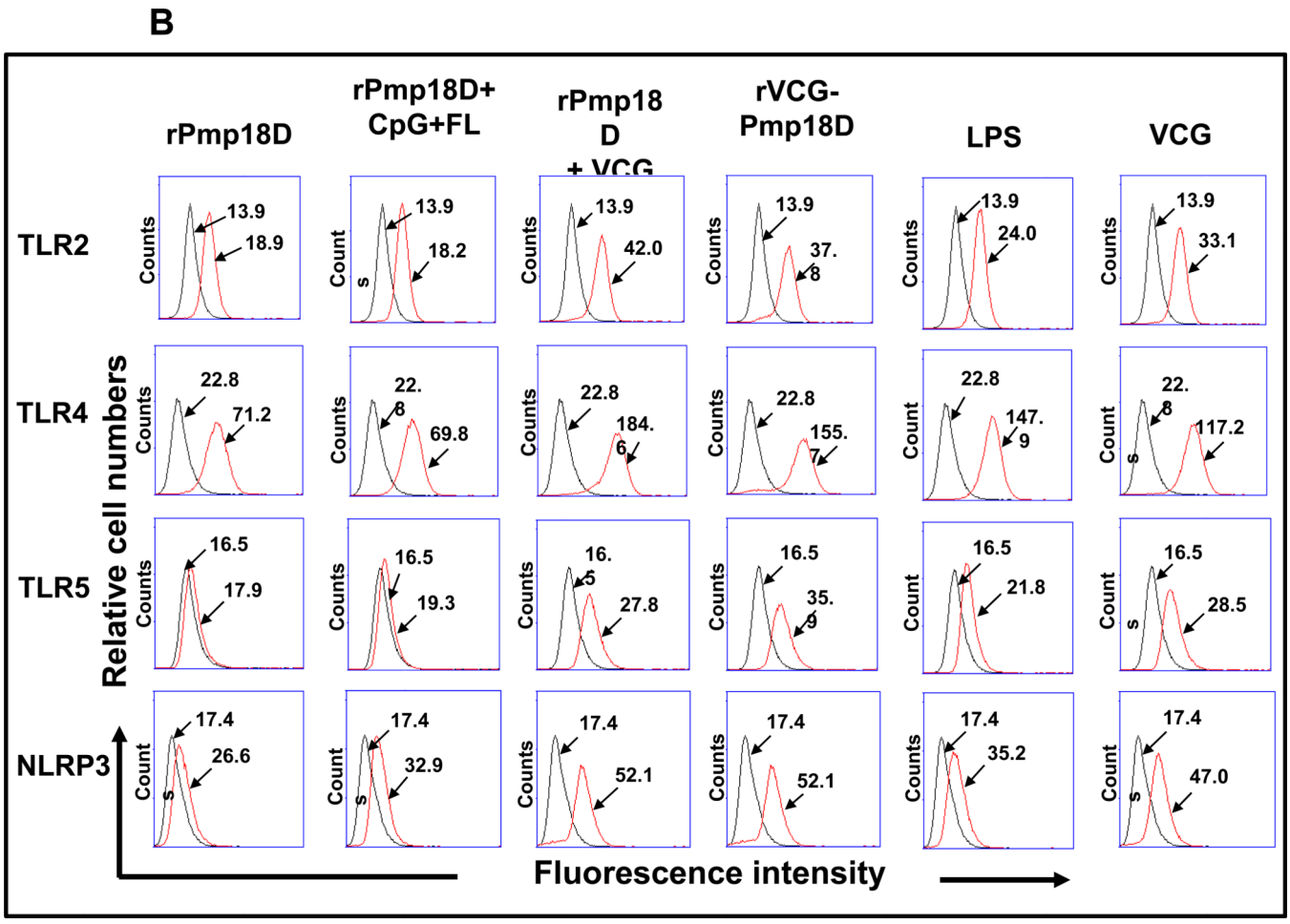

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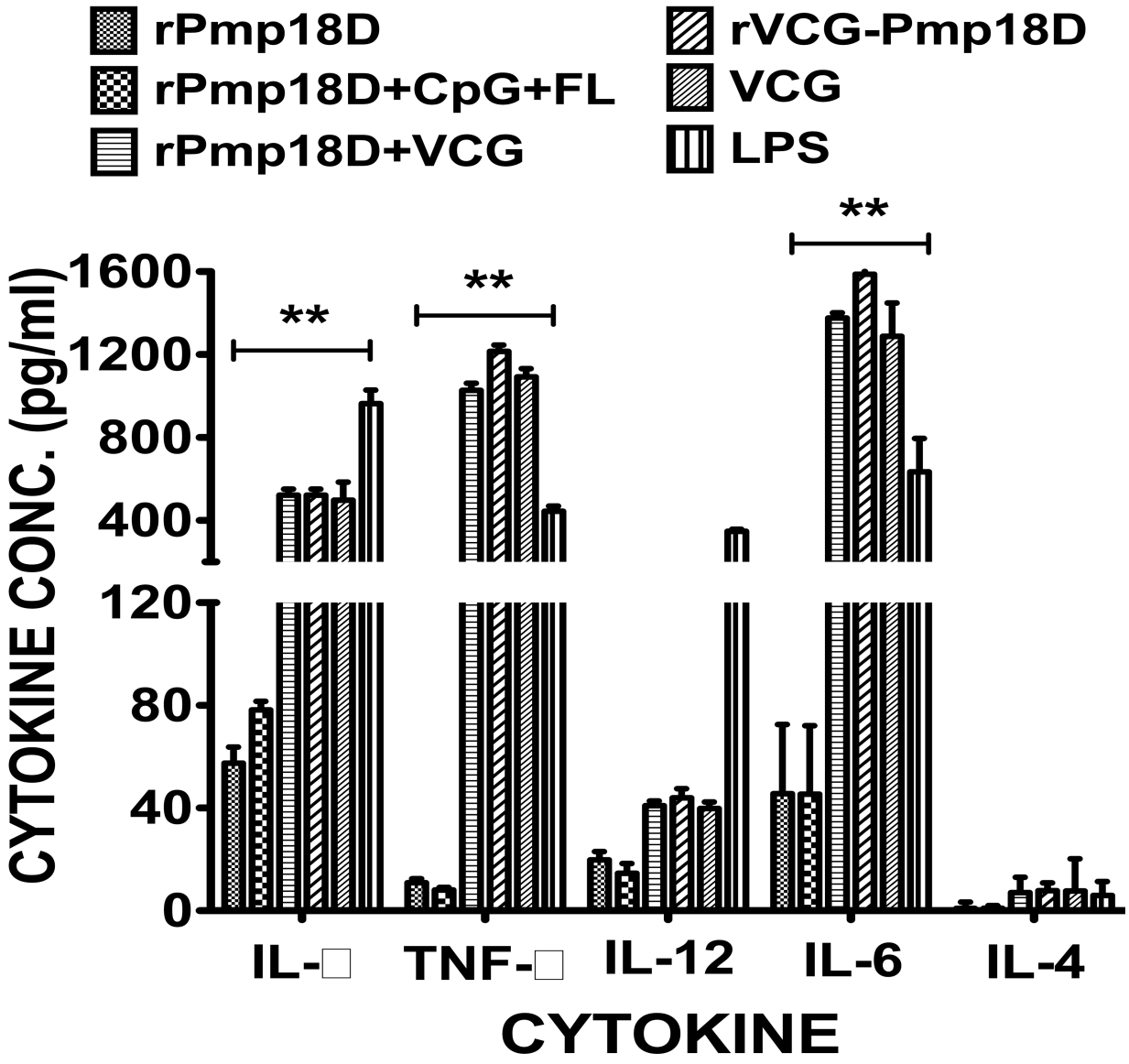

Figure 1.

Activation of DC co-stimulatory marker expression and TLR and NLRP3 engagement. BMDCs were isolated from mice by established procedures as described in the Materials and methods. Harvested cells were pulsed for 24 h with Pmp18D in combination with the adjuvants, stained with conjugated monoclonal antibodies against CD11C, CD40, CD80, CD86, 1Ab, TLRs 2, 4, 5, and NLRP3 inflammasome or isotype-matched controls, and quantified in triplicate by flow cytometry. Culture supernatants were collected and assayed for levels of Th1/Th2 promoting cytokines (IL-12, TNF-α IL-10 and IL-4) by cytokine ELISA using the Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. The concentration of the cytokines in each sample was obtained by extrapolation

from a standard calibration curve generated simultaneously. The data shows the mean fluorescence intensity of staining of cells expressing the indicated co-stimulatory marker (A) or TLR (B) after treatment with antigens Red curve) or culture media (Gray curve). Data is from one of two experiments with similar results. The levels of Th1/Th2-promoting cytokines produced by DCs after treatment with antigens are shown as the mean values (± S.D.) for triplicate cultures for each experiment (C). The results are from two independent experiments. Statistically significant differences between treatment groups was evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$).

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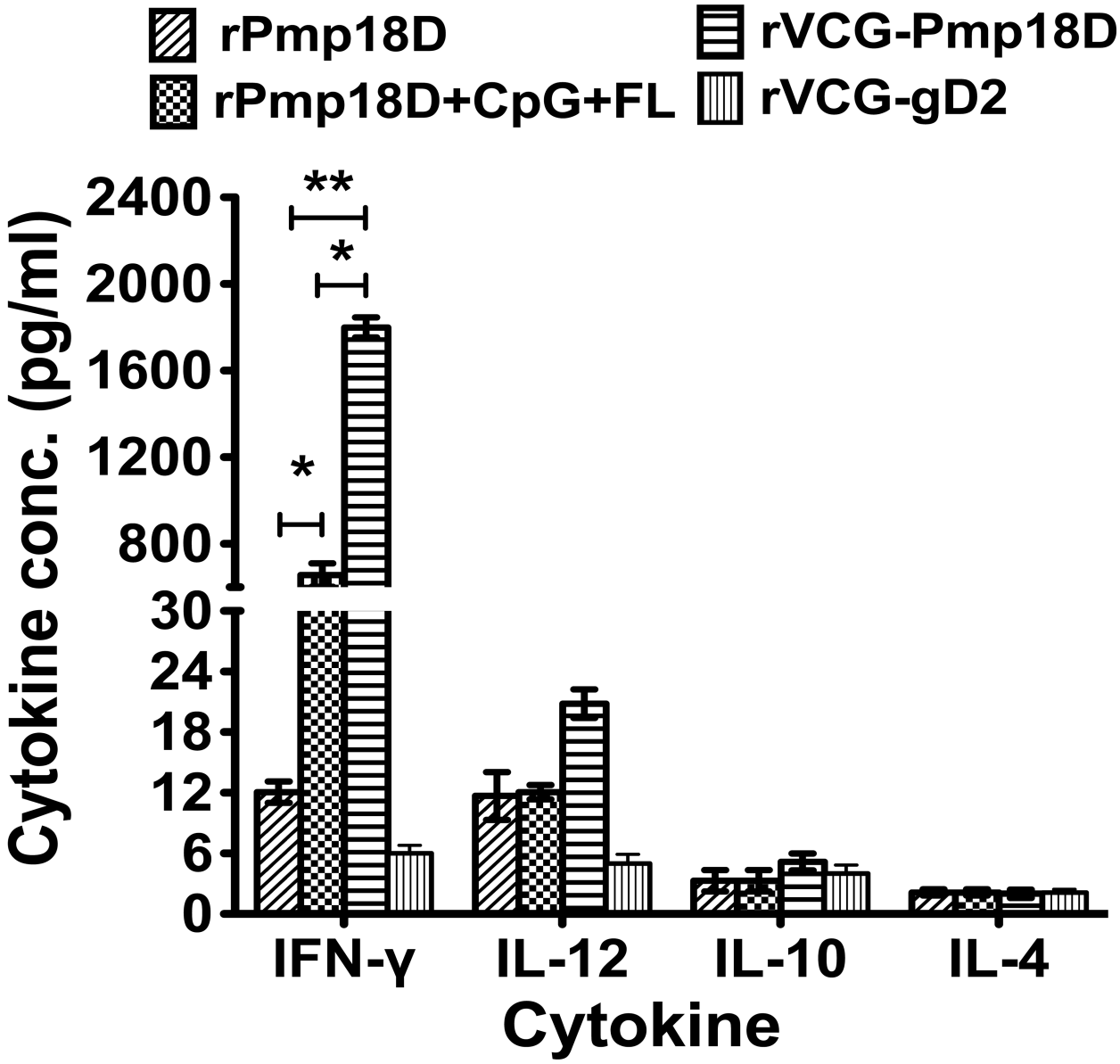

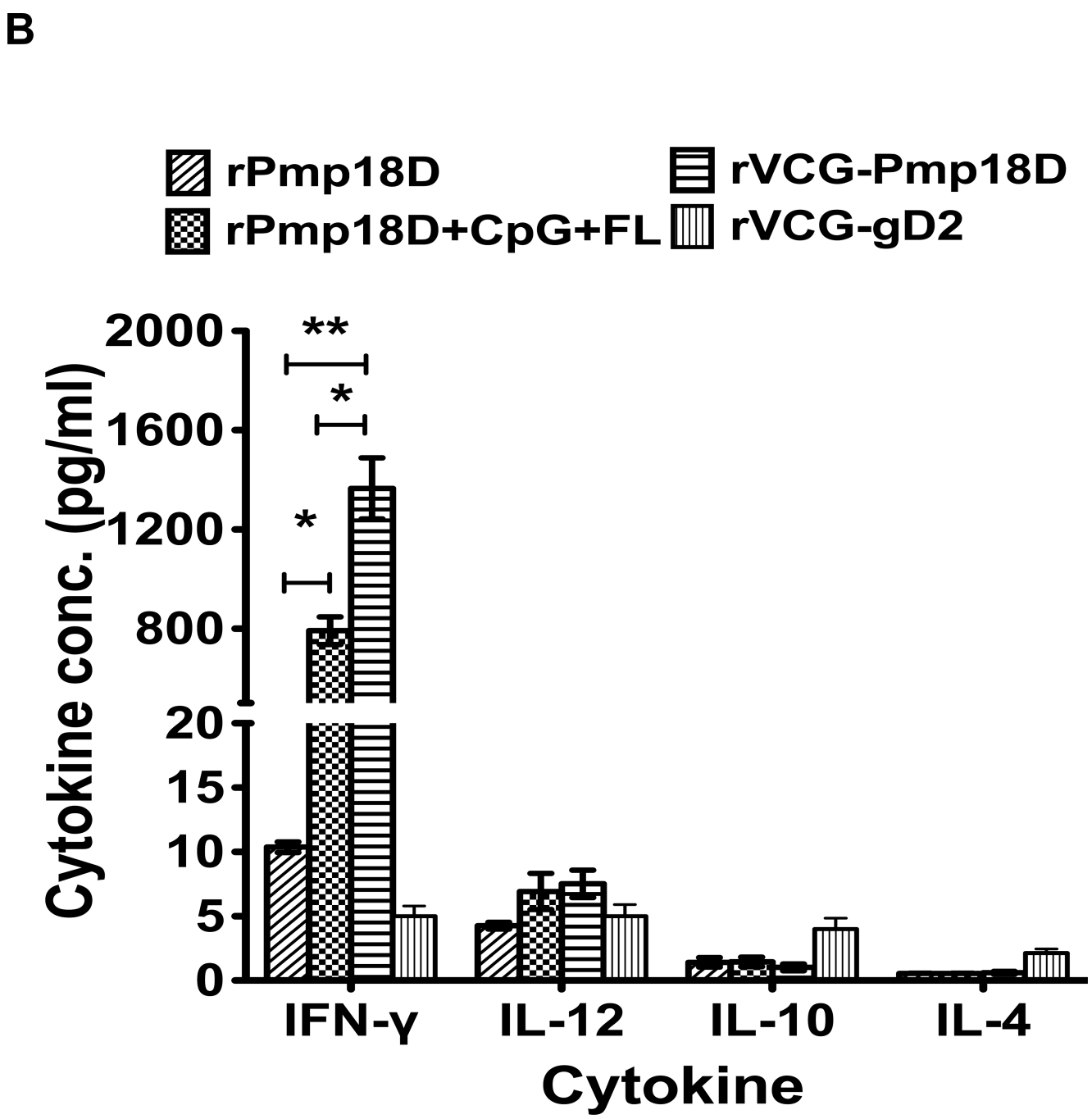

Figure 2.

C. abortus-specific genital mucosal and systemic cytokine responses. Groups of mice were immunized IN and boosted twice as described in the materials and methods section. At week 4 postimmunization, T cells were purified from the SPL and ILNs of immunized mice and controls and restimulated *in vitro* with *C. abortus*. The amount of systemic (A) and local mucosal (B) Th1 (IFN-γ and Th2 (IL-4) as well as IL-12 and IL-10 cytokines contained in supernatants of culture-stimulated cells was measured using Bio-Plex cytokine assay kit in combination with the Bio-Plex Manager software. The concentration of the cytokines in

each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (± S.D.) for triplicate cultures for each experiment. The cultures without antigen did not contain detectable levels of cytokine and so the data were excluded from the results. The results are from two independent experiments for a total of 10–12 mice. Significant differences between Th1 and Th2 cytokines (IFN-γ and IL-4) were evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$).

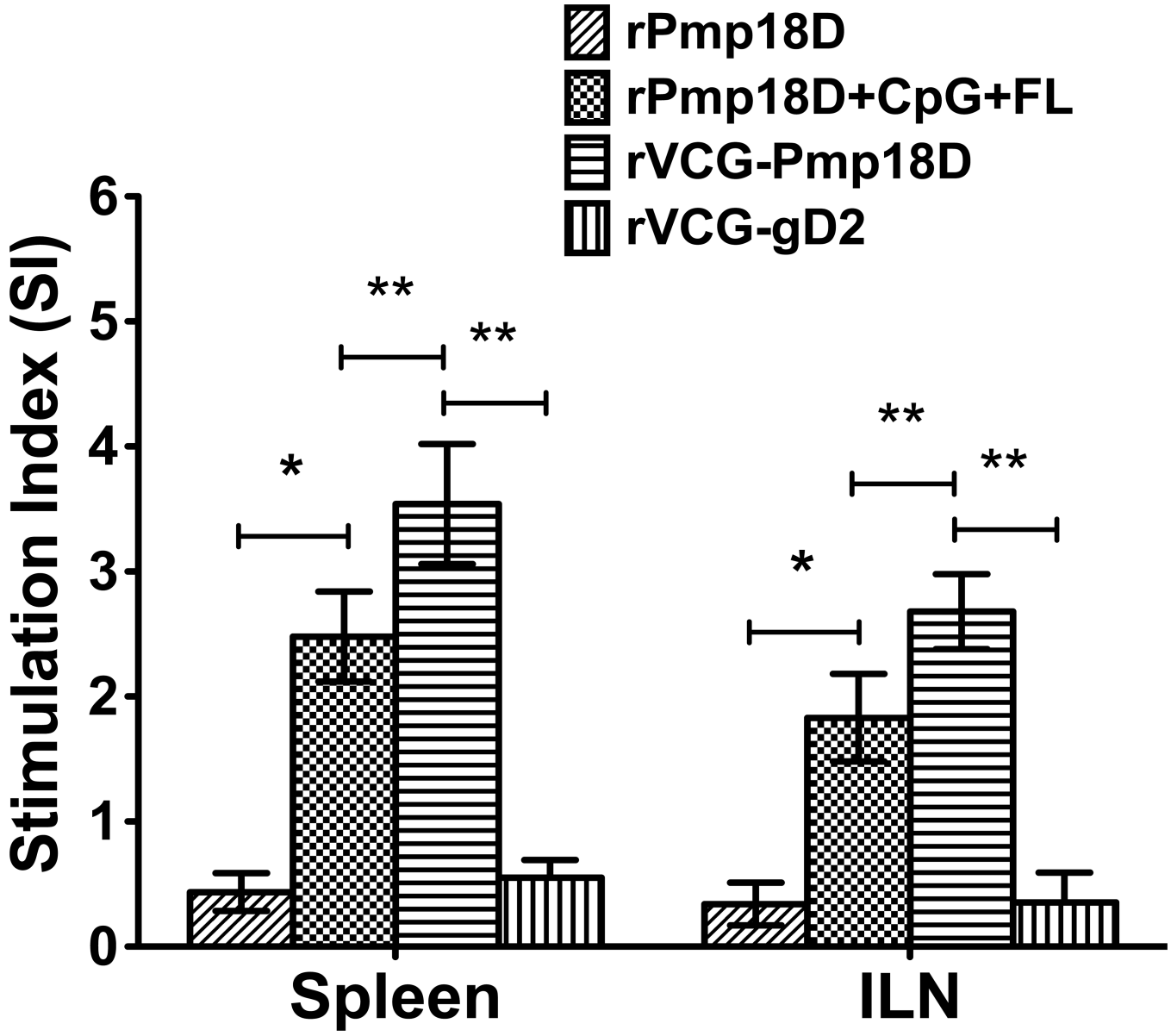

Figure 3.

C. abortus-specific proliferative responses. Four weeks after the last boost, ILNs and splenic CD4+ T cells from groups of IN immunized mice were restimulated *in vitro* with *C. abortus* antigen for 3 days. The antigen-specific proliferative response was determined using the XTT Cell Viability Kit (Cell signaling, Boston, MA); incorporation was detected by addition of XTT detection solution and the absorbance was read at 450 nm. Results are expressed as the stimulation index (SI), the ratio between absorbance values of stimulated and non-stimulated cells and the bars represent the mean and S.D. of six replicates from two independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$).

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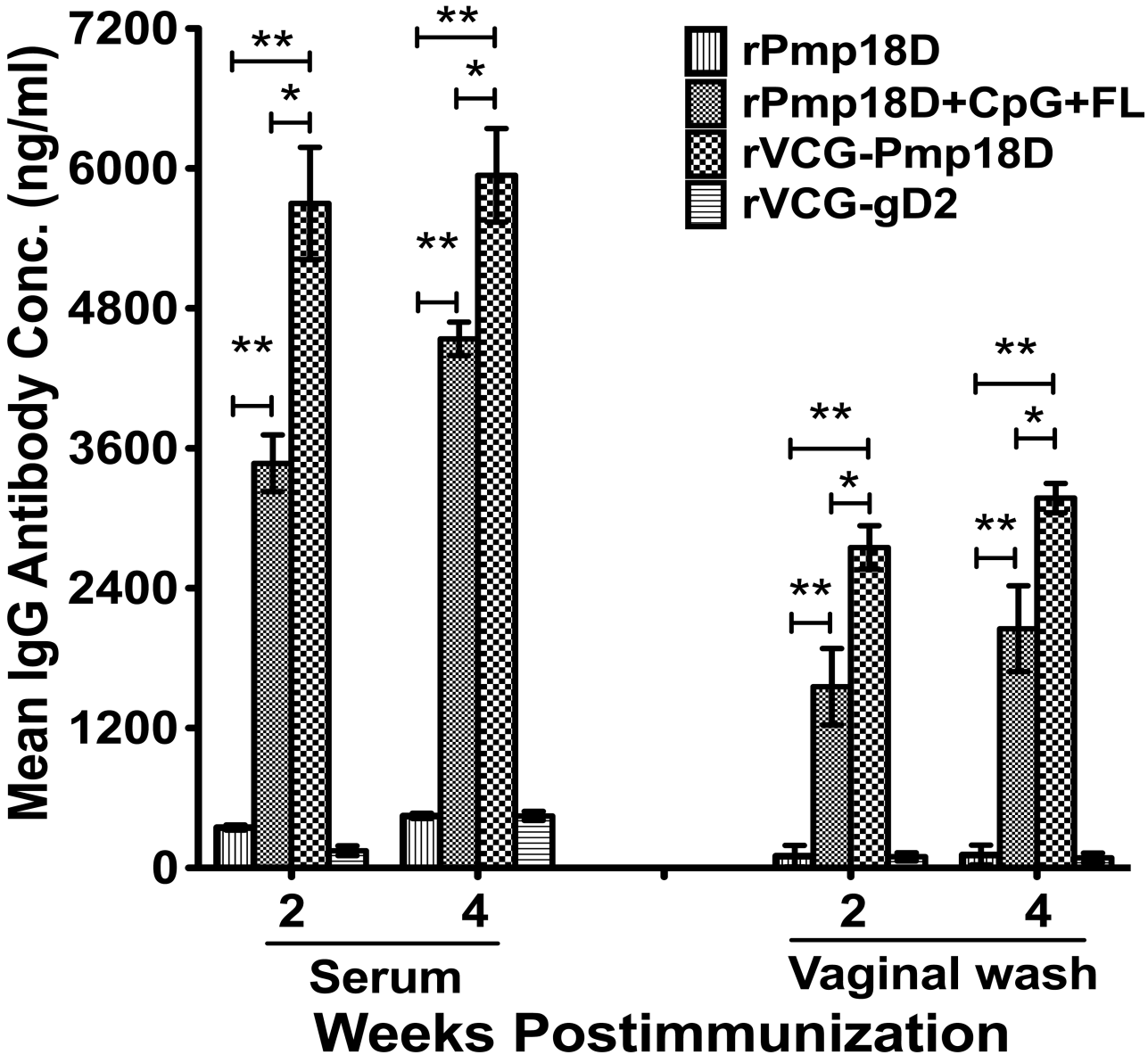

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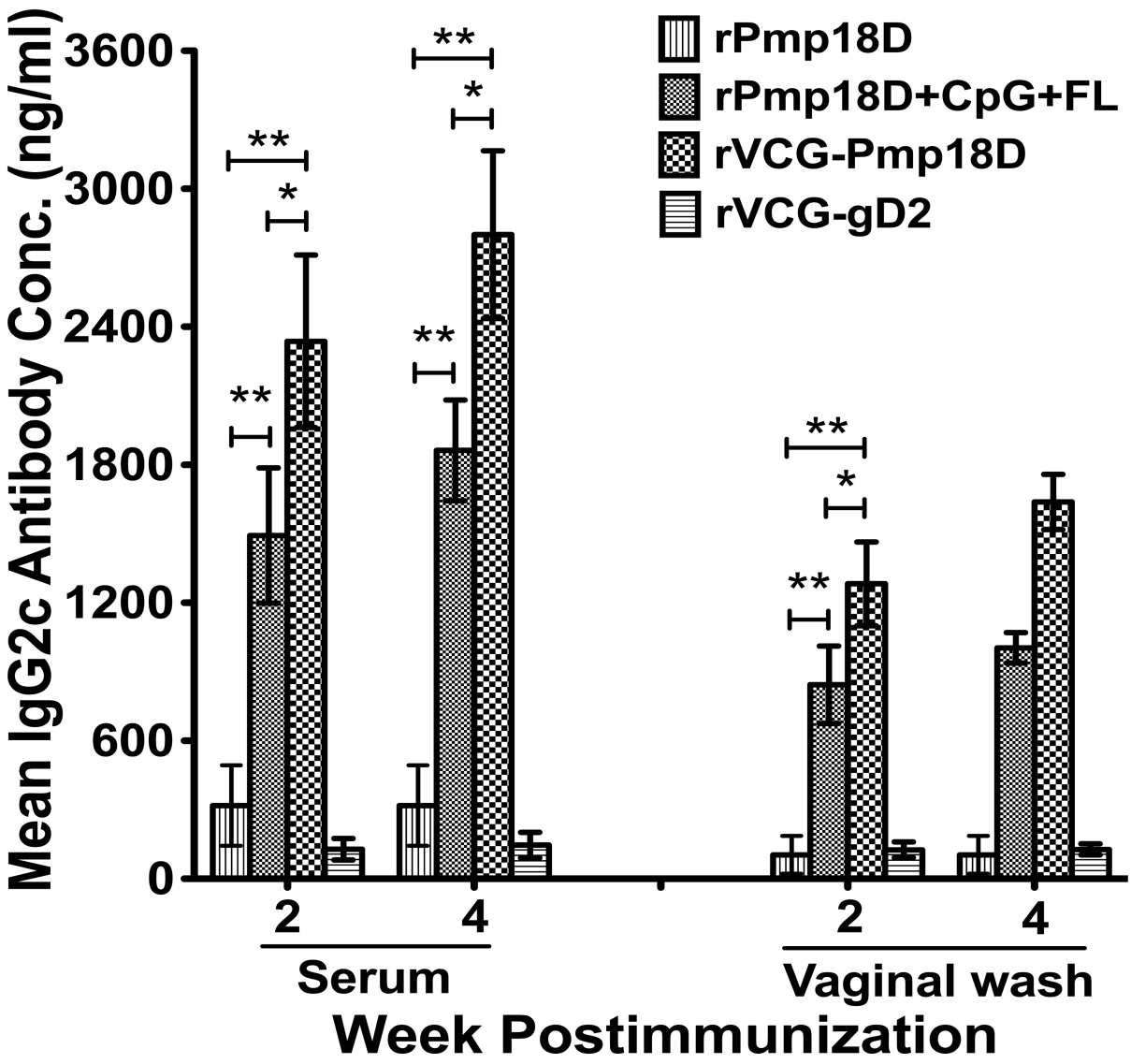

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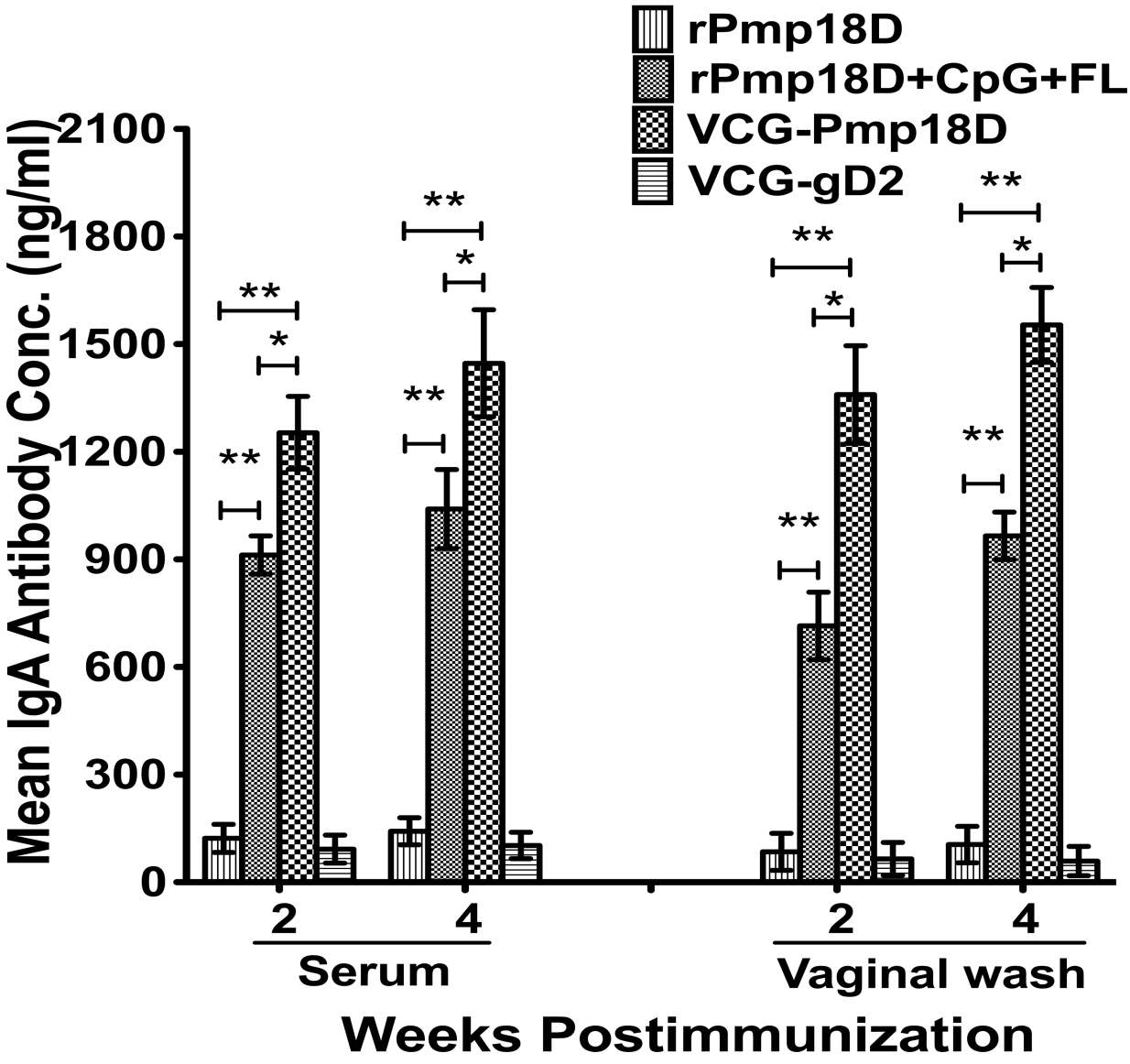

Figure 4.

Antigen-specific total IgG, IgG2c and IgA antibody responses induced after three vaccine doses. Groups of mice were immunized IN three times, 2 weeks apart as described above. Serum and vaginal lavage samples were obtained at 2 and 4 weeks after the third immunization and pooled for each group. An antibody ELISA was used to assess the concentration of total IgG (A), IgG2c (B) and IgA (C) antibodies elicited in serum (systemic) and genital lavage (mucosal) samples. Results generated simultaneously with a standard curve, display data sets corresponding to absorbance values as mean concentrations (ng/ml) ± SD of triplicate cultures for each experiment. The results are from two

independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$).

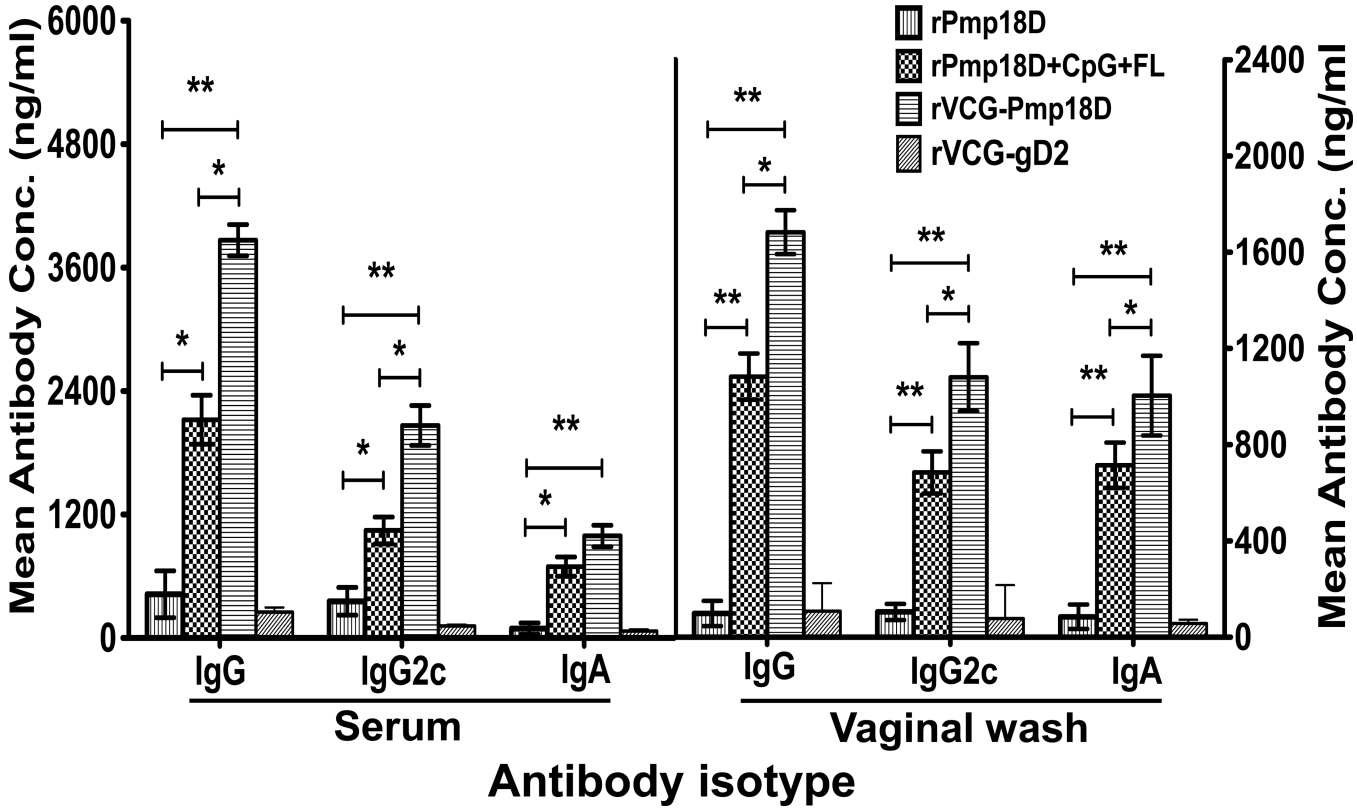

Figure 5.

Antigen-specific total IgG, IgG2c and IgA antibody responses induced after two immunizations. Groups of mice were immunized IN three times, 2 weeks apart and serum and vaginal lavage samples were obtained 2 weeks after the second immunization and pooled for each group. An antibody ELISA was used to assess the amount of antibodies elicited in serum and genital lavage samples. Results generated simultaneously with a standard curve, display data sets corresponding to absorbance values as mean concentrations (ng/ml) ± SD of triplicate cultures for each experiment. The results are from two independent experiments. Significant differences between experimental groups were evaluated at ($p^* < 0.05$) or ($p^{**} < 0.01$).

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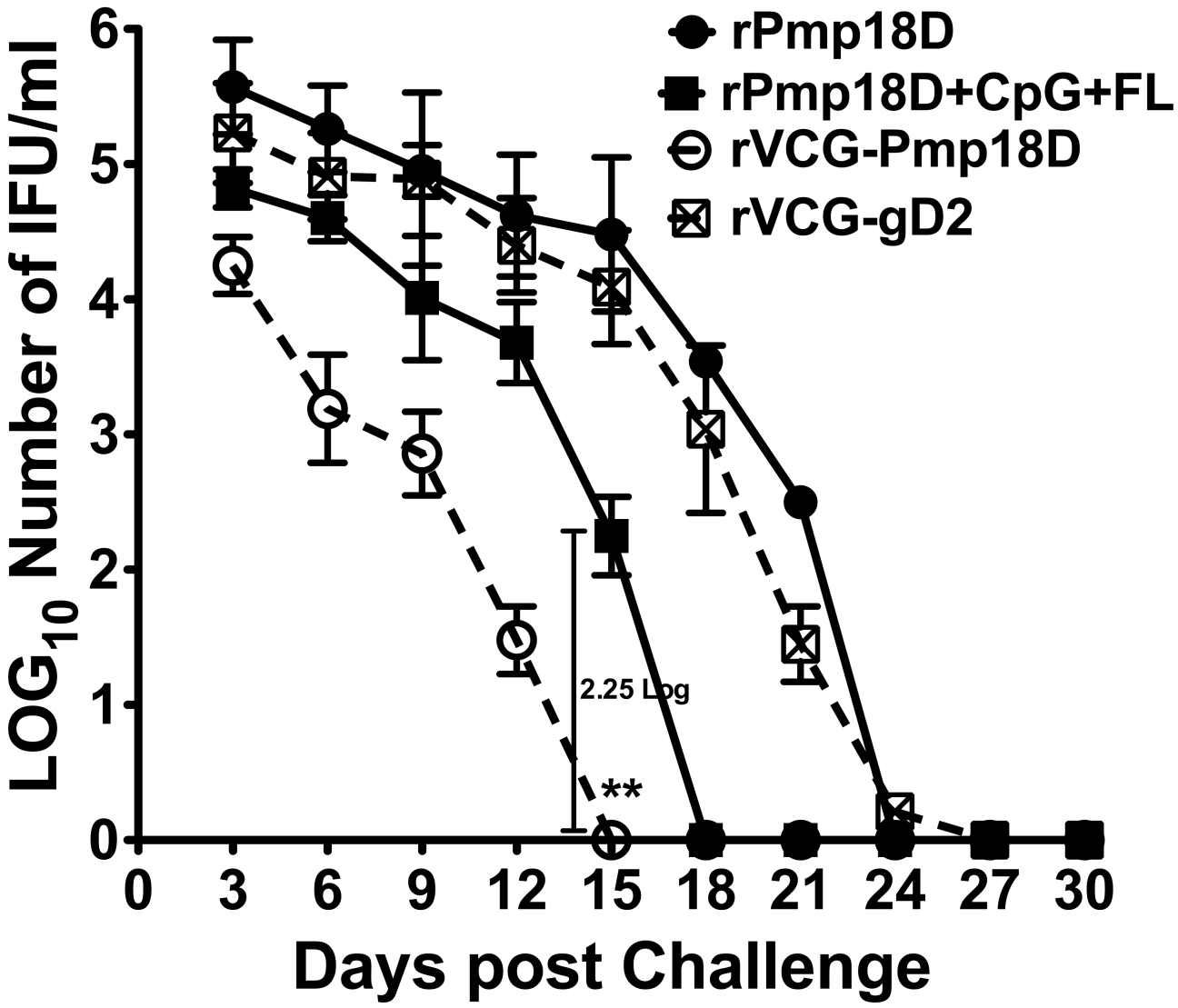

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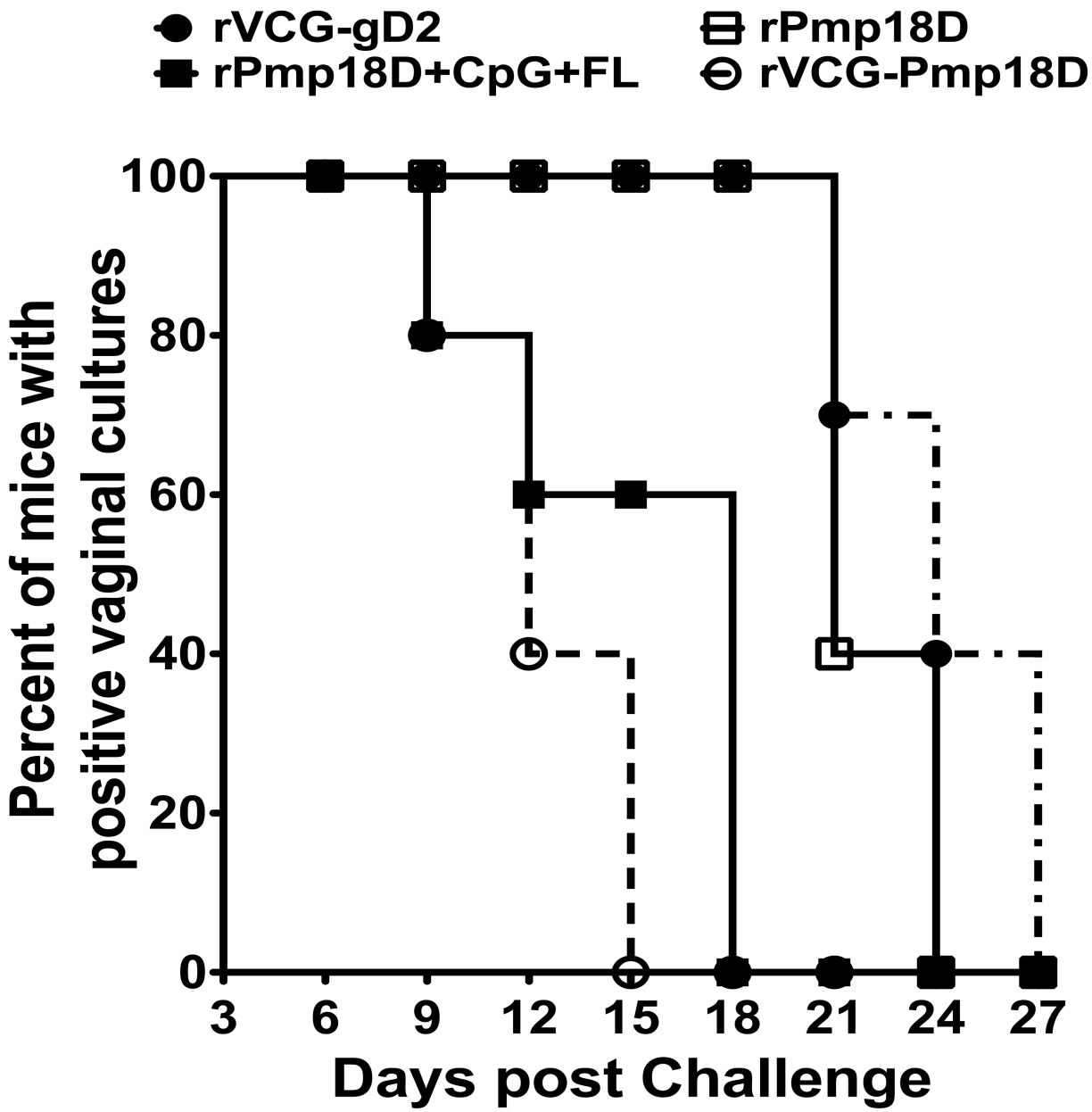

Figure 6.

Number of *C. abortus* IFUs recovered from vaginal swabs following heterologous intravaginal challenge. Groups of mice immunized IN as described above were challenged intravaginally with 1.0×10^6 IFU of live heterologous *C. abortus* strain B577 three weeks after the last immunization. One week prior to challenge, mice were administered Depo Provera to stabilize the estrous cycle and facilitate a productive infection. Infections were monitored by cervicovaginal swabbing of individual animals every 3 days and *Chlamydia* was isolated from swabs in tissue culture and enumerated. The data show (A) the mean

recoverable IFUs expressed as $\log_{10}$ IFU/ml ± S.D. and (B) the number (%) of mice with positive vaginal cultures at different time points. Differences between experimental groups were compared by Student's *t* test at $p < 0.05$. The experiment was repeated to contain 8 mice per group.