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BVDV permissiveness and lack of expression of co-stimulatory molecules on PBMCs from calves pre-infected with BVDV

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Abstract

Bovine viral diarrhea virus (BVDV) has been detected in peripheral blood mononuclear cells (PBMCs) of immunocompetent animals, not being clear whether the development of a specific humoral immune response can prevent BVDV infection. The aim of this study was to evaluate the ability of non-cytopathic BVDV to replicate and produce infectious virus in PBMCs from calves pre-infected with BVDV and to elucidate the immunomodulatory effect of BVDV on these cells in an *in vitro* model. Quantification of virus was by quantitative PCR, while its replicative capacity and shedding into the extracellular environment was evaluated by viral titration. Apoptosis was assessed by flow cytometry analysis of annexin V and propidium iodide, and by expression of caspase-3/7. Flow cytometry was used to analyze the expression of CD14/CD11b/CD80, CD4/CD8/CD25, MHC-I/MHC-II and B-B2 markers. Our results showed that PBMCs from cattle naturally infected with BVDV were more susceptible to *in vitro* BVDV infection and showed a more severe apoptosis response than those from naïve animals. Non-cytopathic BVDV *in vitro* infection also resulted in a lack of effect in the expression of antigen presentation surface markers. All these findings could be related to the immunosuppressive capacity of BVDV and the susceptibility of cattle to this infection.

Keywords: apoptosis; bovine viral diarrhea virus; immunological surface markers expression; peripheral blood mononuclear cells; virus titration.

1. Introduction

Bovine viral diarrhea virus (BVDV) is responsible for acute and persistent infection in cattle, as well as for establishing immunosuppressive mechanisms that favor secondary infections [1]. BVDV can lead to acute infection in susceptible immunocompetent cattle, persistent infection, or chronic infection in immune-privileged sites [2]. The most important consequences for herds occur when a non-cytopathic (ncp) BVDV strain infects a susceptible cow early in gestation; the fetus becomes immunotolerant to this infecting viral strain and remains persistently infected (PI), shedding the virus throughout its lifetime and serving as a reservoir of infection for naïve animals [3].

The immunosuppressive effect of BVDV is attributed to its ability to infect T and B lymphocytes and monocytes, the main components in percentage terms of peripheral blood mononuclear cell (PBMC) populations [4,5]. Infection of PBMCs disrupts the normal mechanisms of immune stimulation, leading to immune evasion and chronic infection [6]. In general, ncp BVDV strains induce large decreases in white blood cell counts [7,8], as well as the expression of major histocompatibility complex (MHC) type II and CD80/86 in antigen-presenting cells (APCs) [9,10,11]. Furthermore, T cell proliferation response in ncp BVDV infections is slower and less prolonged than in cytopathic (cp) BVDV [12,13,14].

There are also many studies measuring antibody neutralization and cross-neutralization of BVDV strains to prevent disease after vaccination [15]. An *in vitro* study demonstrated that lymphocytes in cattle that had been vaccinated against BVDV were less efficiently infected than in naïve animals [16]. However, BVDV antigens have been detected in PBMCs in seropositive animals with the presence of neutralizing antibodies [17,18] and an *in vitro* infection model demonstrated that PBMCs from immune animals were permissive to BVDV re-infection by homologous or heterologous strains [19].

A variety of mechanisms enabling evasion of the host immune response has been proposed to explain the immunosuppressive effect of BVDV in naïve animals, although so far no experimental studies have been performed to determine which specific mechanisms developed by BVDV predispose to re-infection in seropositive animals. The aims of this study were to evaluate the ability of ncp BVDV to replicate and produce infectious virus in PBMCs in calves pre-infected with BVDV and try to elucidate the immunomodulatory effect of BVDV on these cells that favors possible BVDV re-infection.

2. Materials and methods

2.1. Animals

Two dairy herds were selected based on the serological and virological BVDV results among herds participating in an infectious disease control program [20].

Sera and PBMCs were collected from eight heifers (10-12 months old; purebred Holstein–Friesian or mixed with other breeds) divided into two groups: i) the naïve group, four BVDV-seronegative animals from a herd negative for BVDV antibodies at two serological surveys performed at least 6 months apart; ii) the BVDV pre-infected group, comprising four BVDV-seropositive animals from a herd with ongoing infection caused by a PI animal with a BVDV-1 strain slaughtered 2 months earlier. The experimental protocol of animal handling was approved by the Italian Ministry of Health (protocol number 2006070977-003).

BVDV viremic status was tested by RT-PCR [21]. BVDV antibody status in serum was evaluated by competitive ELISA (Ingezim BVD Compac, Ingenasa) and virus neutralization (VN) tests against cp BVDV-1a (NADL strain, ATCC® VR-534™). For the VN, a Madin Darby Bovine Kidney cell line (MDBK, ATCC® CCL-22™) was used, maintained in complete culture medium [MEM supplemented with 10% of fetal calf serum

(FCS), L-glutamine 2mM, antibiotics and antifungal agents] (Euroclone). The VN titer for each serum was the highest dilution at which the virus was neutralized in 50% of the wells [22]. Animals were regarded as seronegative when no neutralization was observed at the lowest dilution (1:4).

At the time of the study, naïve animals were confirmed to be free from both BVDV antigens and antibodies, while animals pre-infected with BVDV through exposure to the PI calf were confirmed to be BVDV antigen-free and VN antibody titers to BVDV-1 were >128. The leukocyte profile was analyzed with the Sysmex XT-2000iV hematology analyzer, using specific settings for bovine blood (Sysmex Corporation, Kobe, Japan). Total leukocyte and platelet counts were within the normal range for bovine (Table 1) [23].

Table 1. Mean ± standard deviation (n=4) of total leukocyte and platelet counts from the animals used in this study, and normal reference values.

(n x 103/µl)	BVDV pre-infected group	Naïve group	Normal values[1]
Lymphocytes	5.31	3.86	4.5 (2.5-7.5)
Monocytes	0.9	1.05	0.4 (0.02-0.8)
Neutrophils	1.98	1.55	2 (0.6-4)
Eosinophils	0.11	0.43	0.7 (0-2.4)
Basophils	0.10	0.12	0.05 (0-0.2)
Total leukocytes	8.40	7.00	8 (4-12)
Platelets	351	197.50	500 (100-800)

[1] Normal blood values for cattle (Schalm's veterinary hematology. 4th ed. Philadelphia: Lea & Febiger, 1986)

2.2. Virus

Ncp BVDV-1 was used for the *in vitro* infection, since it is the most prevalent genotype of BVDV [24]. Experiments were performed with the ncp BVDV-1 7443 strain (courtesy of the Institut für Virologie, Hanover, Germany), which had been used for *in vivo* infections [8,25,26].

The titer of the virus stock was determined by immunoperoxidase monolayer assay (IPMA) in MDBK cells, as described by Lucchini et al. [19]. Final ncp BVDV-1 strain 7443 stocks of $10^{4.6}$ tissue culture infectious dose 50%/µl ($TCID_{50}$/µl) were used to infect PBMCs. PBMCs were incubated at 37ºC for 2 hours with BVDV at a multiplicity of infection (m.o.i.) of 1, as laid down previously in other studies [4,19,27], **showing that PBMCs were efficiently infected with higher viral loads compared to 0.1 m.o.i.**

2.3 Cell separation, culture and infection of PBMCs

Blood collection was by jugular venipuncture using sterile bags containing CPDA-1, centrifuged at 1,200 x g for 30 min at room temperature (RT) and the buffy coat separated and resuspended 1:2 in PBS. The buffy coat was diluted 2:1 and layered onto the Histopaque-1077 (Sigma-Aldrich), then centrifuged at 1,200 x g for 30 min at RT before collection of the ring of PBMCs at the interface. Live cells were counted by the trypan blue dye exclusion test and resuspended in RPMI-1640 medium at $1x10^6$ cells/ml. Cells from each animal were separated into mock-infected control and BVDV-infected with ncp BVDV-1 at a m.o.i. of 1 for 2 hours at 37ºC. The inoculum was removed by washing the cells in RPMI-1640 medium to eliminate extracellular virus. Cells were resuspended in leukocyte culture medium [RPMI-1640, 1% L-glutamine, 25 mM Hepes and 10% FCS] (Euroclone) and incubated in 5% CO_2 at 37ºC for 18, 24 and 48 hours post-inoculation (hpi).

PBMCs were harvested at each time point, removing adherent cells after incubating for 10 min with cell-dissociation solution (Sigma-Aldrich). Supernatants were separated from cells by centrifugation at 400 x g for 10 min.

2.4. Virus assays in PBMCs infected with BVDV

2.4.1. Virus quantification at 18, 24 and 48 hpi

RNA extraction from cell culture supernatants (extracellular BVDV) and freeze-thawed PBMCs at -80ºC (intracellular BVDV) was performed using the QIAamp Viral RNA Mini Kit (QIAgen). The total RNA concentration in each sample was quantified with a NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies Inc.) and its purity assessed using the A260/A280 ratio. The RNA (1µg) was retrotranscribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories).

External standards were constructed to enable quantification of PCR products from ncp BVDV-1 strain 7443 [28], and consisted of plasmids containing inserts of the amplified BVDV gene sequences ranging from 1 to 10^8 copies of virus cDNA/µl.

Quantitative reactions were performed in 12µl of EvaGreen Supermix (Bio-Rad Laboratories) with 500 nM BVDV primers and 1µl cDNA, using the Eco Real-Time PCR thermal cycler system (Illumina Inc.). Each sample was tested in duplicate with the thermal profile set at 50 ºC for 2 min, 95 ºC for 7 min, followed by 40 cycles at 95 ºC for 10 s and at 60ºC for 30s. Reverse transcription controls were performed by omitting the reverse transcription reaction, and template controls by adding nuclease-free water. Copy numbers of BVDV RNA/µg were calculated by reference to the standard curves. Results were expressed in absolute copy numbers. The detection limit was 10 copies for BVDV plasmids.

PCR controls included genomic RNA isolated from BVDV-negative PBMCs and MDBK cells, as well as negative reagents, such as water.

2.4.2. Virus titration at 18, 24 and 48 hpi

To evaluate the *in vitro* replication rate of BVDV in PBMCs, extracellular virus titers were determined by microtiter assay on 96-well plates. Briefly, quadruplicate 10-fold serial dilutions of the tested supernatants were made in MEM with a MDBK cell suspension of 1.5 x 10^4 cells/ml. The plate was incubated for 4 days at 37ºC with 5% CO_2, and an IPMA was

performed to detect the ncp BVDV strain, using monoclonal antibody 20.10.06 with cross-reactivity against the NS2/3 protein of BVDV (courtesy of Dr. E. Dubovi, Cornell University). Viral titers were calculated using the Reed-Muench method [22] and expressed as log10 $TCID_{50}$/µl.

Intracellular viral titers could not be calculated due to insufficient sample volume.

2.5. Flow cytometry

PBMCs from both groups were harvested at 18, 24 and 48 hpi, divided into aliquots of $2x10^5$ cells/100µl and incubated for 30 min at RT with optimally diluted mouse monoclonal antibodies (Table 2), including isotype-matched controls. For indirect labeling of B cells, cells were incubated with FITC-conjugated goat anti-mouse secondary antibody. After two washes, cells were fixed with 1% formaldehyde (CellFix 10x; Becton, Dickinson and Company) in PBS and kept in the dark at 4ºC until analysis. Cells ($1x10^4$ events) were analyzed on the FACSCalibur cytometry system (Becton, Dickinson and Company) and immunofluorescence staining was analyzed using Flowing Software (version 2.5.0). Results were expressed as the percentage or the mean fluorescence intensity (MFI geometric mean of the channel number) of the surface molecules stained on the gated cells. Three-, two- or single-color staining for leukocyte differentiation antigens was performed, as follows: CD14/CD11b/CD80, CD4/CD8/CD25, MHC-I/MHC-II and B-B2. **Gate strategy of the flow cytometry analysis of PBMCs is represented in the Supplementary Figure 1.**

Table 2. List of antibodies used for flow cytometry

*Clone-Fluorochrome	Specificity	Isotype	Source
TÜK4-AlexaFluor 647	anti-human CD14	IgG2a	Serotec
CC126-FITC	anti-bovine CD11b	IgG2b	Serotec
ILA159-RPE	anti-bovine CD80	IgG1	Serotec
CC8-FITC	anti-bovine CD4	IgG2a	Serotec
CC63-AlexaFluor 647	anti-bovine CD8	IgG2a	Serotec
ILA111-RPE	anti-bovine CD25	IgG1	Serotec
ILA88-FITC	anti-bovine MHC-I	IgG2a	Serotec
CC108-RPE	anti-bovine MHC-II	IgG1	Serotec
CC219-FITC	anti-bovine CD28	IgG1	Serotec
BAQ44A (unconjugated)	anti-bovine B cells (B-B2)	IgM	VMRD
Secondary Ab-FITC	goat anti-mouse IgM	(Secondary Ab)	Sigma-Aldrich

*All primary antibodies are monoclonal mouse antibodies.

2.6. Apoptosis assay

2.6.1. Measurement of PBMCs apoptosis by annexin V-FITC binding assay

Ca^{2+}-dependent binding of annexin V (AV)-FITC to phosphatidylserine was used to measure apoptosis in PBMCs. Dual staining with AV and propidium iodide (PrI) was used to discriminate between apoptotic and necrotic cells (MBL MEBCYTO Apoptosis kit). PBMCs were double-stained with AV and PrI diluted in binding buffer for 15 min at RT in the dark after 18, 24 and 48 hpi. The AV–/PrI– population represented live cells; the AV+/ PrI– population represented early apoptotic cells; the AV+/PrI+ population represented late-stage apoptotic and necrotic cells. PBMCs were analyzed on a FACSCalibur cytometer (Becton Dickinson Immunocytometry Systems) with excitation at 488 nm. A minimum of 2,000 events per sample were analyzed.

2.6.2. Measurement of apoptosis in PBMCs by caspase-3/7

Caspase-3 and -7 activity in PBMCs was detected at 18, 24 and 48 hpi using the Apo-ONE^{TM} detection assay (Promega Corporation), following the manufacturer's instructions.

Fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent (Thermo Electron Corporation) and was expressed as RFU (Relative Fluorescence Units).

2.7. Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 6.0. The non-parametric Mann–Whitney U test was used to analyze significant differences ($P<0.05$) between naïve and BVDV-pre-infected groups at the same time point (*), as well as between the *in vitro* BVDV-infected and (mock) control PBMCs (a: naïve animals; b: BVDV pre-infected animals).

3. Results

3.1. BVDV replication in PBMCs

BVDV RNA was not detected in the PBMCs or culture supernatants from the mock-inoculated samples of the two animal groups. There was scant detection of intracellular BVDV in PBMCs in both animal groups during this experiment, with slightly higher rates observed only at 48 hpi. Nonetheless, at 18 and 24 hpi, the quantity of BVDV RNA in the culture supernatants of PBMCs from pre-infected animals was significantly greater than in PBMCs from naïve animals ($P<0.0001$) (Figure 1).

The ability of BVDV to shed infectious virus in PBMCs to the extracellular environment was evaluated by measuring viral titers in the culture supernatants. Immunolabeling did not detect the presence of BVDV in MDBK cells treated with culture supernatants from mock-infected PBMCs. Throughout the experiment, PBMCs culture supernatants from both naïve and pre-infected groups showed consistently low extracellular BVDV titers, with a significant difference ($P<0.05$) between groups at 48 hpi (Figure 2).

3.2. Ability of BVDV to stimulate T-cell responses

There was no observable evidence that BVDV had a statistically significant down-regulatory effect on the expression of CD14+ or CD11b+, although there seemed to be a downregulated expression of co-stimulatory molecules (CD14+) CD80+ in the monocyte populations of animals pre-infected with BVDV compared with naïve animals (Figure 3).

After BVDV infection, no statistically significant differences were found in the percentages of T lymphocytes (CD4+ or CD8+ subpopulations), B-lymphocytes or in the expression of the CD25 activation marker in the PBMCs of the two animal groups, or in the same group over the whole study period (Figure 4).

No significant changes were observed for the MFI of MHC-I and MHC-II (Figure 4), or (CD14+) CD11b+, (CD14+) CD80+ and (CD4+) CD25+ (data not shown) in both groups.

3.3. Ability of BVDV to induce cell death

PBMCs from naïve and pre-infected animals displayed decreased cell viability (mean percentage of live cells) after 18 hpi, and an apoptotic effect was observed in up to 60% of PBMCs primary cultures at 48 hpi (data not shown). The percentage of early apoptotic cells was higher in naïve animals than in those pre-infected with BVDV. Nevertheless, the enzymatic activity of caspase-3/7, the executioner enzymes in the final pathway of apoptosis, was significantly higher ($P<0.05$) in BVDV- pre-infected animals at 48 hpi (Figure 5).

4. Discussion

PBMCs represent the main target for replication of BVDV in *in vitro* systems [4,5]. Our results showed that PBMCs from both naïve animals and those pre-infected with BVDV were efficiently infected *in vitro* with BVDV in the conditions tested. PBMCs from BVDV pre-infected animals were more susceptible to BVDV infection *in vitro* and presented a

higher ability to release infectious BVDV into the extracellular environment. This could represent a survival strategy of ncp BVDV strains, enabling the BVDV to be more readily disseminated and to persist in the host cell population [14]. However, our results did not agree with those reported by Lucchini et al. [19], who observed lower BVDV titers in PBMCs cultures from immunized animals than from naïve ones, although the immunized animals were incompletely protected against heterologous BVDV strains. These findings suggest the involvement of a cell-mediated immune response able to control the BVDV load, and also that re-infections should be considered for their potential impact on vaccination programs.

Maintenance of the percentage of monocytes in naïve and BVDV-pre-infected groups was consistent with other *in vitro* studies showing that infection with a ncp strain did not kill purified monocytes [6] or monocyte-enriched cell cultures [29]. These similarities have also been observed in the absence of significant changes in CD11b expression in purified cell cultures after BVDV infection [6], thus reducing the possibility that expression of this marker may be partly responsible for impairment of the immune response associated with BVDV. Analyses of CD80/86 expression during BVDV infection and its possible effect on APC functions have shown variable results, ranging from downregulation of gene expression *in vivo* [9] and *in vitro* [10,11] to no effect on surface expression [6]. In our study, while not significant, downregulation of CD80 was observed on monocytes from both BVDV-infected groups, suggesting that a higher infectious dose or prolonged exposure might induce more obvious changes. No significant changes in MHC-II were observed after ncp BVDV infection, which coincides with some studies carried out on monocytes [6], but contrasts with other ones that reported an inhibitory effect on MHC class II expression by ncp BVDV [10,11].

The lack of change in the percentage of T lymphocytes after *in vitro* BVDV infection contrasts with the significant decreases observed in *in vivo* ncp BVDV infections [8,30]. The absence of changes in our study could indicate that T lymphocytes do not proliferate in response to antigen presentation of the virus, which might be explained by the unchanged percentage of (CD4+) CD25+ subpopulation cells of both animal groups [31]. In the BVDV-seropositive calves, these results contrast with the CD25 upregulation observed in other trials following infection or vaccination [32,33].

The reported effects on circulating B-lymphocytes during *in vivo* infections with BVDV vary from a decreased number to no change [9,30], possibly due to differences in viral strains. No changes in the percentage of B lymphocytes were observed in this study, which contrasts with the affection in circulating B-cells or in lymphoid follicles in *in vivo* BVDV infections with the same strain [8,26]. These studies showed that activation of the initiator caspase-8 seems to play a major role in lymphocyte apoptosis, suggesting that this could be caused by an indirect mechanism mediated by pro-apoptotic cytokines released by macrophages [25]. In our study, PBMCs from the pre-infected group presented significant caspase-3/7 activation and expression after *in vitro* infection, which translated into an irreversible process of programmed cell death [34]. This suggests a possible association between BVDV-pre-infection and the apoptotic mechanisms by which this virus is able to induce an immunosuppressed state in susceptible cattle in order to facilitate viral infection [5,25].

5. Conclusions

Our results showed that PBMCs from naturally infected BVDV-immune cattle are susceptible to BVDV re-infection of even greater intensity than those from naïve animals. This, together with the more severe apoptotic effects in pre-infected animals and the lack of

effect in the expression of surface markers characteristic of antigen presentation could be related to the immunosuppressive effect of the ncp BVDV strain and the susceptibility of cattle to this infection. Further studies to clarify the role played by cytokines in BVDV-induced apoptosis are necessary in order to gain a more complete understanding of the pathogenesis of this disease.

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Declarations of interest

None.

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Figures

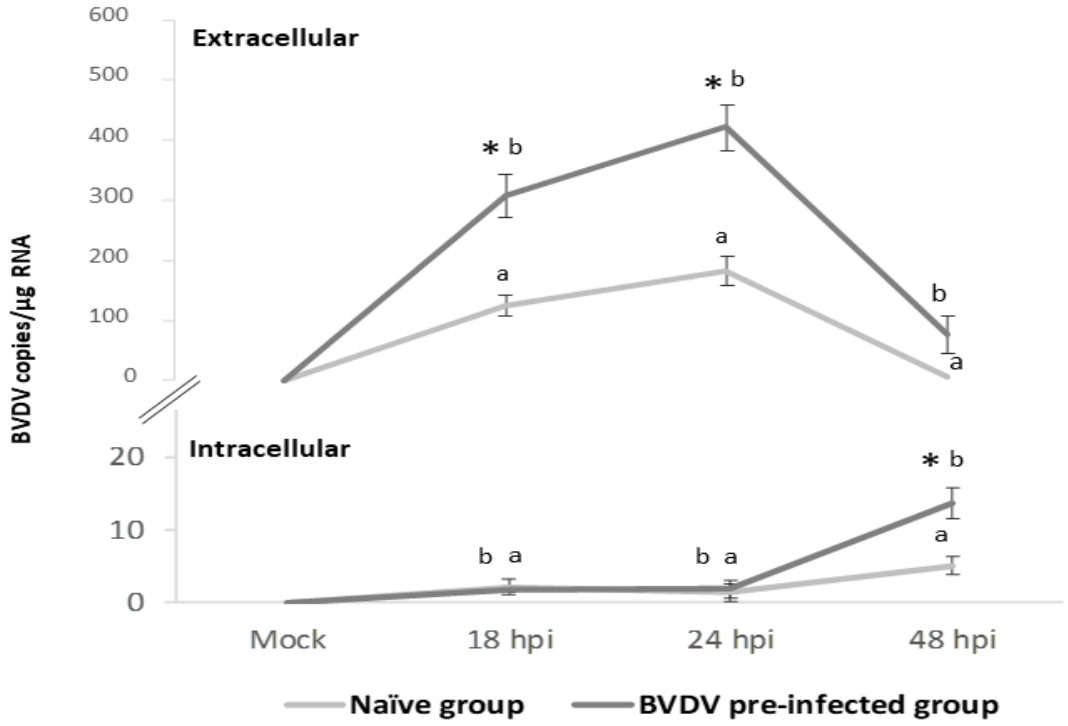
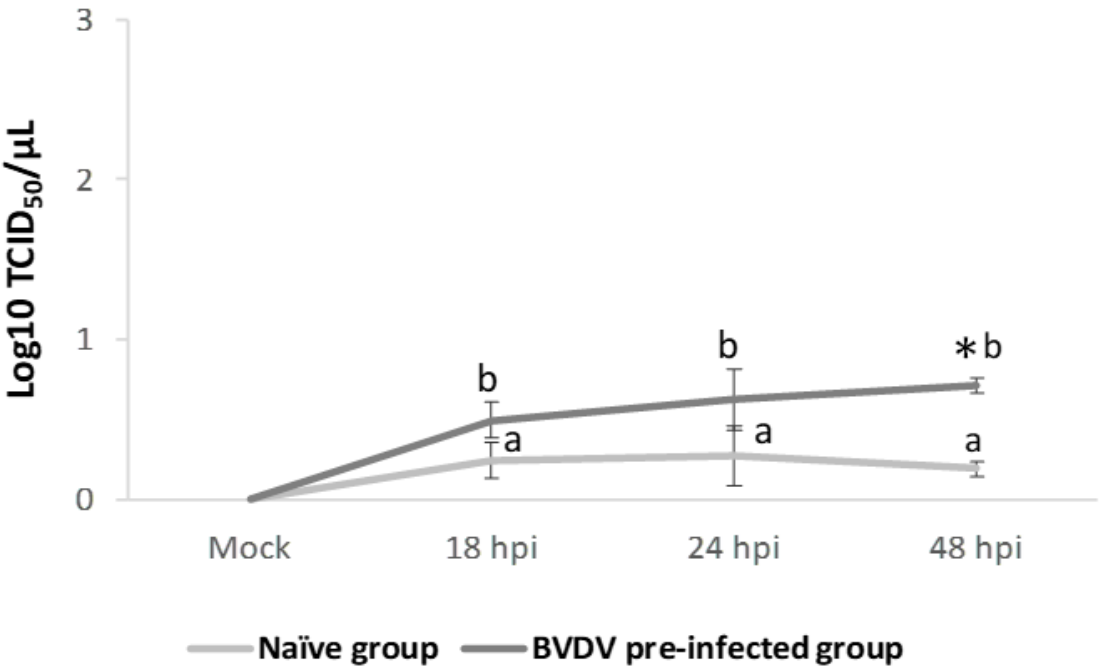


Figure 1. *BVDV replication in PBMCs.* Means (±SEM) of the absolute copy numbers of BVDV in lysated and freeze-thawed PBMCs, and in the supernatants from naïve and BVDV-pre-infected animals (n=4). Significant differences (P<0.05) between naïve and BVDV-pre-infected groups at the same time point (*) and between the *in vitro* BVDV-infected and control (mock) PBMCs (a: naïve animals; b: BVDV-pre-infected animals).



494

495 **Figure 2.** Ability of *BVDV in PBMCs to produce infectious virus in the extracellular*
496 *environment.* Means (±SEM) of the log10 $TCID_{50}$/ml of infectious BVDV in the culture
497 supernatants (n=4). Significant differences (P<0.05) between naïve and BVDV-pre-infected
498 groups at the same time point (*), and between *in vitro* BVDV-infected and (mock) control
499 PBMCs (a: naïve animals; b: BVDV pre-infected animals).

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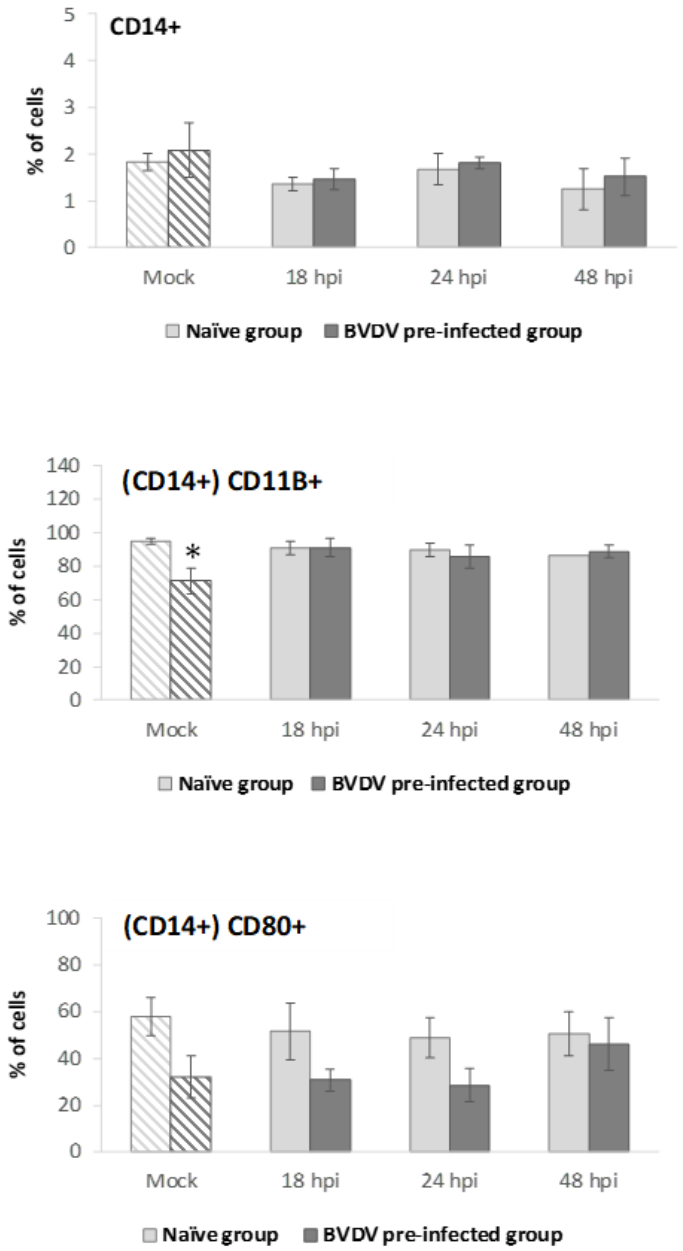
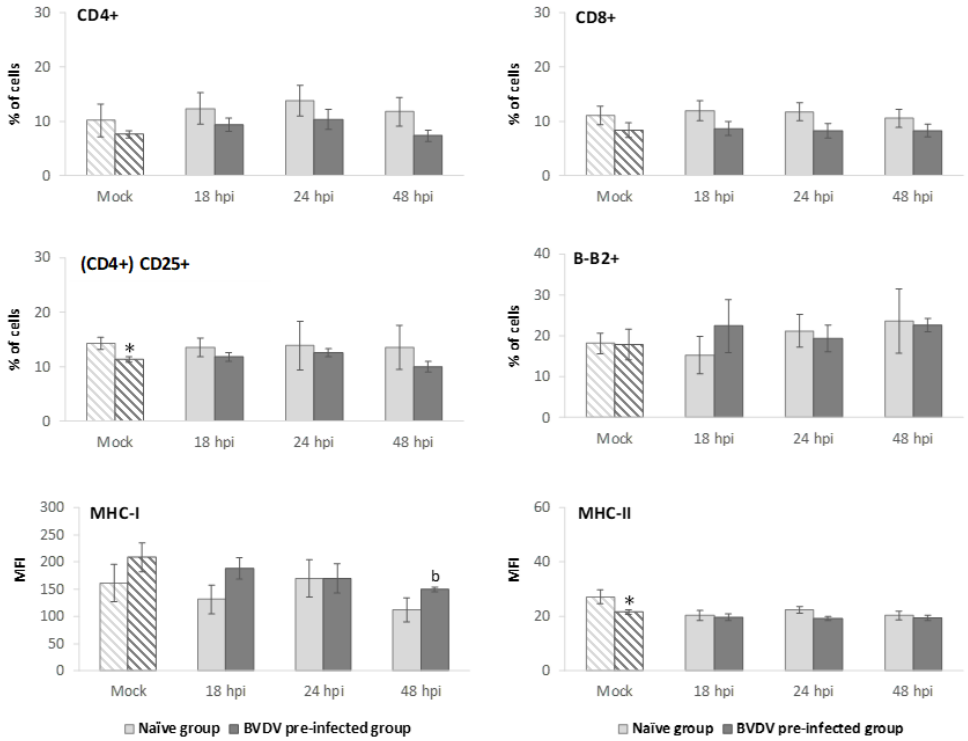


501

502 **Figure 3.** *Effect of BVDV on monocytes and cell surface markers expression.* Means (±SEM)
503 of the percentage of monocytes (CD14+) expressing CD11b or CD80 in BVDV-infected
504 PBMCs from naïve and pre-infected field cattle (n=4). Significant differences ($P<0.05$)
505 between naïve and BVDV-pre-infected groups at the same time point (*) and between the *in*
506 *vitro* BVDV-infected and (mock) control PBMCs (a: naïve animals; b: BVDV-pre-infected
507 animals).

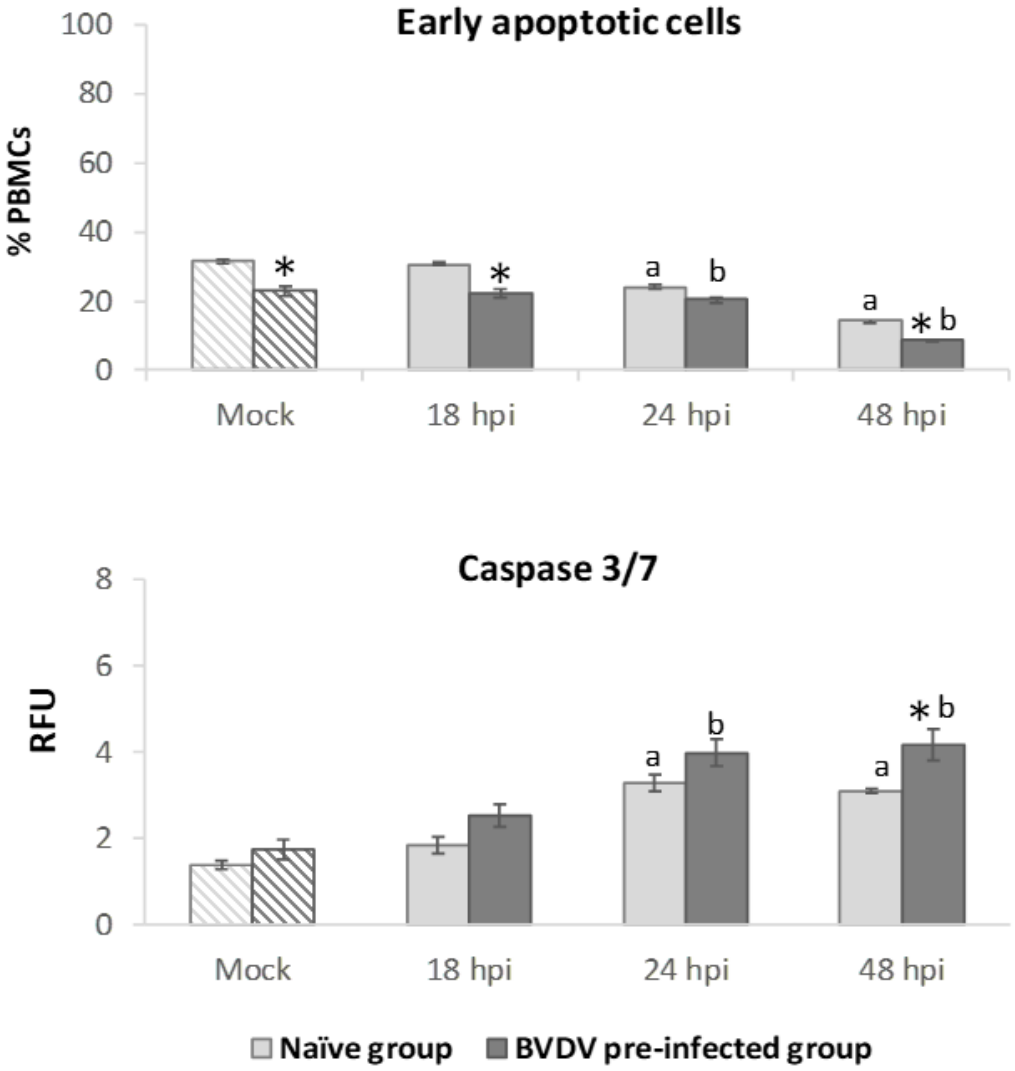
508



509

510 **Figure 4.** *Effect of BVDV on lymphocytes and cell surface markers expression.* Means
511 (±SEM) of the percentages of lymphocytes (CD4+ or CD8+ subpopulations) and B-
512 lymphocytes, as well as expression of different cell surface markers in BVDV-infected
513 PBMCs from naïve and pre-infected field cattle (n=4). Significant differences ($P<0.05$)
514 between naïve and BVDV- pre-infected groups at the same time point (*), and between the *in*
515 *vitro* BVDV-infected and control (mock) PBMCs (a: naïve animals; b: BVDV pre-infected
516 animals).

517



518

519 **Figure 5.** *BVDV activation of induced cell death.* Means (±SEM) of the cell death
520 percentages of BVDV-infected PBMCs from naïve and pre-infected field cattle using the
521 annexin V-FITC binding assay, and caspase-3/7 activity by colorimetric assay (relative
522 fluorescence expressed as RFU -Relative Fluorescence Units-) (n=4). Significant differences
523 ($P<0.05$) between naïve and BVDV pre-infected groups at the same time point (*), and
524 between the *in vitro* BVDV-infected and (mock) control PBMCs (a: naïve animals; b: BVDV
525 pre-infected animals).

526

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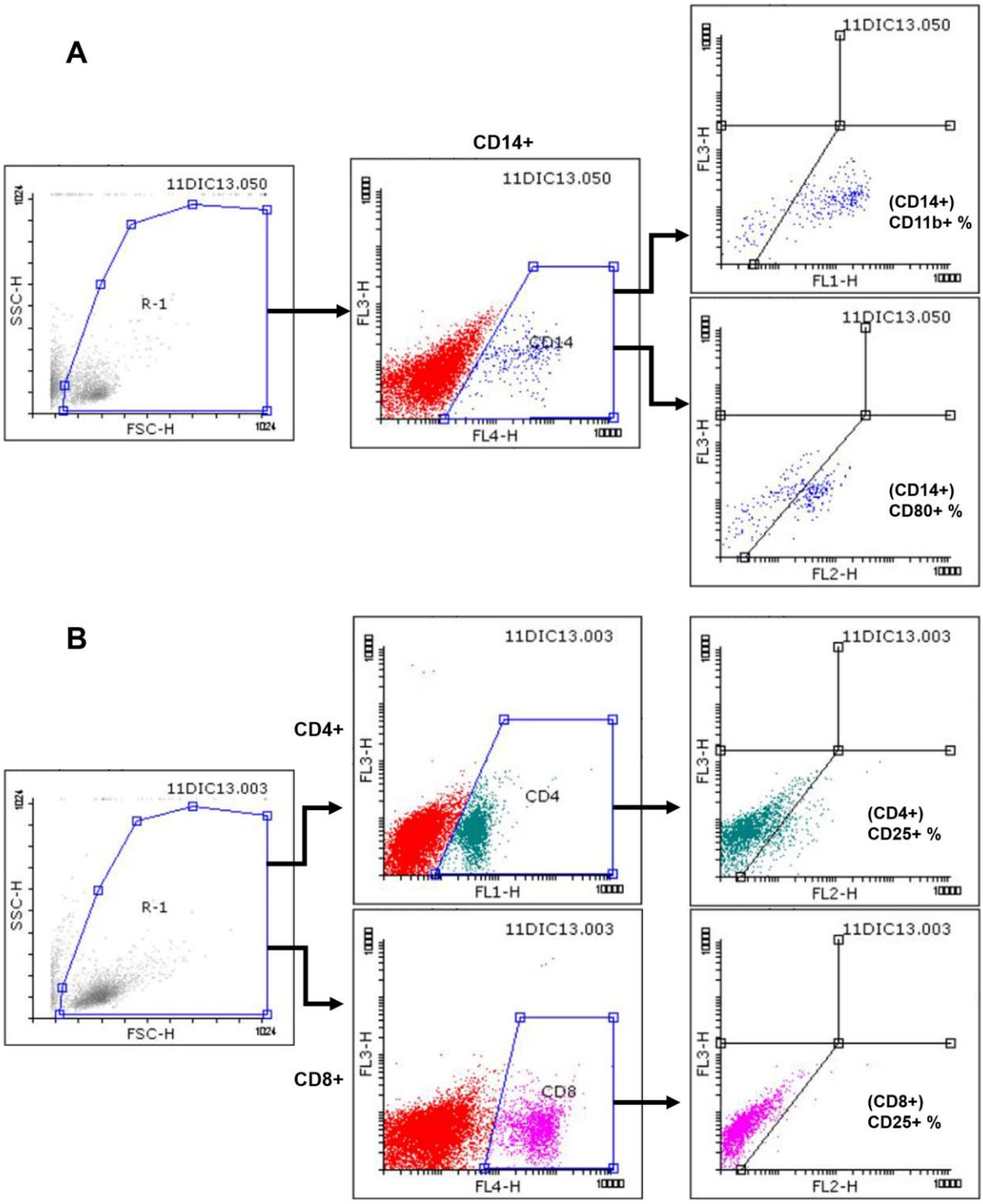

528

529 **Supplementary Figure 1.** Gate strategy of the flow cytometry analysis of PBMCs. **A)**

530 *Monocytes.* FL1 (FITC-CD11b), FL4 (AlexaFluor647-CD14), FL2 (RPE-CD80). FL3 (No

531 stain). **B)** *T lymphocytes.* FL1 (FITC-CD4), FL4 (AlexaFluor647-CD8), FL2 (RPE-CD25).

532 FL3 (No stain).

533