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Corresponding Author: Dr. Jose Carlos Gomez-Villamandos,

Corresponding Author's Institution: University of Cordoba

First Author: Verónica Molina

Order of Authors: Verónica Molina; María A Risalde; Pedro J Sánchez-Cordón; Miriam Pedrera; Fernando Romero-Palomo; Camilla Luzzago; Jose Carlos Gomez-Villamandos

Abstract: Bovine respiratory disease (BRD) is a complex infectious disease caused by several bacterial and viral pathogens, including bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1). Both viruses are important cattle pathogens that induce a broad immunosuppression on cell-mediated immune response on its own. To our knowledge, no studies have been made in cattle comparing the leukocyte population counts, including systemic changes in lymphocyte subpopulations, during dual viral respiratory infections in calves. Our aim was to evaluate quantitative changes in immunocompetent cells in healthy calves and calves with subclinical bovine viral diarrhea (BVD), both inoculated with BHV-1. Neutrophils and lymphocyte exhibited changes in behaviour which can contribute to the immunosuppression of BVDV, thus accounting for some of the inter-group differences. Unlike the others lymphocyte subpopulations studied, CD8+ T cell displayed an early depletion in BVDV inoculated calves that can promote greater dissemination of BHV-1 aggravating the course of the disease.

Suggested Reviewers: Jose Manuel Sánchez-Vizcaíno
jmvizcaino@visavet.ucm.es

Karl Stähl
karl.stahl@bvf.slu.se

Marisa Arias
arias@inia.es

Effect of infection with BHV-1 on peripheral blood leukocytes and lymphocyte subpopulations in calves with subclinical BVD

V. Molina[a], M. A. Risalde[a], P. J. Sánchez-Cordón[a], M. Pedrera[a], F. Romero-Palomo[a], C. Luzzago[b] and J. C. Gómez-Villamandos[a]*

[a] *Department of Comparative Pathology, Faculty of Veterinary, University of Córdoba-Agrifood Campus of International Excellence (ceiA3), Campus de Rabanales, 14014, Córdoba, Spain*

[b] *Department of Veterinary Pathology, Hygiene and Public Health, Faculty of Veterinary Medicine, Università di Milano, Via Celoria 10, 20133 Milano, Italy*

* Corresponding author. José C. Gómez-Villamandos: jcgomez@uco.es

Tel.: +34 957218162; fax: +34 957212018.

Abstract

Bovine respiratory disease (BRD) is a complex infectious disease caused by several bacterial and viral pathogens, including bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1). Both viruses are important cattle pathogens that induce a broad immunosuppression on cell-mediated immune response on its own. To our knowledge, no studies have been made in cattle comparing the leukocyte population counts, including systemic changes in lymphocyte subpopulations, during dual viral respiratory infections in calves. Our aim was to evaluate quantitative changes in immunocompetent cells in healthy calves and calves with subclinical bovine viral diarrhea (BVD), both inoculated with BHV-1. Neutrophils and lymphocyte exhibited changes in behaviour which can contribute to the immunosuppression of BVDV, thus accounting for some of the inter-group differences. Unlike the others lymphocyte subpopulations studied, CD8+ T cell displayed an early depletion in BVDV inoculated calves that can promote greater dissemination of BHV-1 aggravating the course of the disease.

Keywords

Bovine respiratory disease complex; bovine viral diarrhea virus; bovine herpesvirus type 1; immune response; flow cytometry; cell subsets

Abbreviations

BHV-1	bovine herpesvirus type 1
BHV-1.1	bovine herpesvirus type 1 and subtype 1
BVD	Bovine viral diarrhea
BRD	Bovine respiratory disease

BVDV	bovine viral diarrhea virus
BHV1 group	group of animals inoculated only with BHV-1.1
BVDV/BHV1 group	group of animals inoculated with BVDV and BHV-1.1
$TCID_{50}$	tissue culture infective dose 50%
dpi	days post-inoculation with BHV-1.1
hpi	hours post-inoculation with BHV-1.1
ºC	Celsius grades
Rt	room temperature

1. Introduction

Bovine respiratory disease (BRD) complex is a major cause of economic loss in the cattle industry, weakening the immune system and making the animal more susceptible to secondary infections. Several infectious agents have been associated with BRD, including bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1). Frequently, severe respiratory tract disease in cattle is associated with concurrent infections of these pathogens (Srikumaran et al., 2007). Although it is known that both viruses can colonize the respiratory tract on its own, their participation in the BRD is mainly due to their enhancer role in mixed infections derived from their significant immunosuppressive effect (Potgieter et al., 1984; Castrucci et al., 1992; Kelling, 2007).

The changes related to the immunosuppressive action of BVDV and BHV-1 include a transient leucopenia in calves characterized by lymphopenia (Griebel et al., 1987-1988; Müller-Doblies et al., 2004; Pedrera et al., 2009a,b; Risalde et al., 2011b; Raya et al., 2011) with decreases in absolute numbers of B lymphocytes (Bolin et al., 1985; Brewoo et al., 2007), T lymphocytes, mainly both CD4+ and CD8+, and γδ to

a lesser extent (Bolin et al., 1985; Griebel et al., 1987-1988; Winkler et al., 1999; Archambault et al., 2000; Chase et al., 2004), as well as monocytopenia (Glew et al., 2003; Van Drunen Little-van den Hurt, 2007; Pedrera et al., 2009b; Raya et al., 2011) and neutropenia (Filion et al., 1983; Chase et al., 2004; Pedrera et al., 2009a,b; Raya et al., 2011), with impairment of function in monocytes, neutrophils and lymphocytes (Filion et al., 1983; Griebel et al., 1987-1988; Brown et al., 1991; Potgieter, 1995; Tikoo et al., 1995; Glew et al., 2003; Chase et al., 2004). It is known that the cell-mediated immune response plays a key role in countering infection both in BVDV (Howard et al., 1992) and in BHV-1 (Babiuk et al., 1996; Van Drunen Littel-van den Hurk, 2007), since the humoral response is not sufficient to eliminate infected cells.

However, to our knowledge, no studies have been made comparing the leukocyte populations, including changes in lymphocyte subpopulations, during dual BVDV and BHV-1 viral respiratory infections in peripheral blood of calves. Parallel studies on kinetics of the cellular response may give valuable information for the understanding of the cell-mediated immune response during co-infections with these viral agents in BRD.

The aim of this study was to deepen in the systemic consequences after the immunosuppression associated to an acute BVDV infection, especially the exacerbate effect developed in co-infections with BHV-1 in cattle, such as has been described on histopathological studies (Potgieter et al., 1984; Castrucci et al., 1992). Therefore, we examine systemic changes in leukocyte counts and lymphocyte subpopulations appeared in apparently recovery calves of an experimental bovine viral diarrhea (BVD) and in healthy calves, both challenged with BHV-1.1.

2. Materials and methods

2.1. Experimental procedure

A detailed description of the experimental procedure is given in Risalde et al. (2011b). In brief, thirty male Friesian calves (8-9 months old) were housed in the Animal Experimental Centre of Cordoba University and had an adjustment period of one week before the experiment started, being controlled daily for clinical signs of disease. At the beginning of the experiment, the calves were randomly assigned to three different groups called according to the inoculation they were exposed to. Fourteen calves belonged to the BVDV/BHV1 group, twelve calves to the BHV1 group and four belonged to the negative control group.

The animals of BVDV/BHV1 group were infected by intranasal inoculation with 10 ml (5ml per nostril) of a suspension containing 10^5 tissue culture infective dose 50% ($TCID_{50}$)/ml of non-cytopathic BVDV genotype-1 strain 7443. Twelve days later, when the calves did not show neither clinical signs nor viremia against BVDV, the animals of both BVDV/BHV1 and BHV1 groups were challenged receiving an intranasal inoculation with a total of 2 ml (1ml per nostril) containing 10^7 $TCID_{50}$ of BHV-1 subtype 1 (BHV-1.1) virulent strain Iowa. The animals of the negative control group received 1 ml of tissue culture fluid free of viruses in each nostril. Clinical examinations were performed daily and EDTA blood samples were taken at 0, 6, 12 and 18 hours post-inoculation with BHV-1.1 (hpi), 1, 2, 4, 5, 7, 9 and 14 dpi days post-inoculation with BHV-1.1 (dpi).

2.2. Blood leukocyte counts

Total and differential leukocyte counts were done on EDTA-blood samples by electronic counting using a Cell-Dyn 3700 Hematology Analyzer (Abbott Diagnostics, Abbott Laboratories, Abbott Park, IL, USA).

2.3. Immunolabelling of lymphocyte differentiation antigens

A panel of primary monoclonal antibodies (mAbs) (VRMD, Pullman, WA) specific for bovine CD4+ T cells (CACT138A), CD8+ T cells (CACT80C), B cells (BAQ44A) and γδ T cells (CACTB6A) were used to examine lymphocyte subsets. FITC conjugate goat anti-mouse IgM (μ-chain specific) (Sigma-Aldrch, Saint Louis, Missouri, USA) and R-phycoerythrin conjugate goat anti-mouse IgG_1 (Invitrogen, San Diego, CA) were used as secondary Ab. Fresh EDTA blood samples were collected and stored at room temperature (Rt), performing the surface labelling within 1 hour after blood extraction, as follows. 100 μl of whole blood diluted 1:1 with PBS was incubated with 1 μl of each primary mAb enumerated above. Following 30 min of incubation at 4ºC in the dark, the cells were washed with 2 ml of PBS. Next, 200 μl of secondary Ab diluted 1:500 with PBS were added and the cells were incubated for 30 min at 4ºC in the dark. After labelling, erythrocytes were lysed, using 2 ml of lysing solution (FACS Lysing Solution 10X Concentrate, Becton Dickinson, San José, CA, USA) for 10 min at Rt. The lysing process was stopped by adding 2 ml of PBS. Finally, the cells were fixed adding 200 μl of formaldehyde buffered solution (CellFix 10X Concentrate, Becton Dickinson, San José, CA, USA) to each sample and were stored at 4ºC in the dark until flow cytometric analysis.

2.4. Flow cytometric analysis of lymphocyte subpopulations

The absolute number of each lymphocyte subpopulation reactive with each mAb was calculated using the total lymphocyte number and the proportion of the subpopulation given at the flow cytometric analysis at each time point. Indirect immunofluorescent stained cells were analyzed using a FACScan Flow Cytometer (Becton Dickinson Immunochemistry Systems, San José, Puerto Rico). The software used for data collection and analyses was CELLQuest (Becton Dickinson, San José, CA, USA). Twenty thousand events were collected from each sample. The following parameters were collected: forward light scatter (FSC), side light scatter (SSC), FITC fluorescence (FL1) and PE fluorescence (FL2). Lymphocytes were differentiated by their size (FSC) and granularity (SSC), and further evaluated for lymphocyte subpopulation by FL1 and FL2. The proportions of lymphocytes positive for each of the mAb were determined after subtraction of the corresponding control.

2.5. Statistical analyses

The values of total and differential leukocyte counts and absolute number of each lymphocyte subpopulation were assessed to calculate mean ± standard error. Duncan's Multiple Range Test was performed for BVDV/BHV1 and BHV1 groups to analyze significant differences of the values in each inoculated group at various time points (*). P values < 0.05 were considered significant. Non-paired Student's t-test was used to determine differences between both BVDV/BHV1 and BHV1 groups at the same time point (**). P values < 0.05 were considered significant. Statistical analysis software SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used for data analysis.

3. Results

3.1. Blood leukocyte count

In the BVDV/BHV1 group, the total leukocyte count (Figure 1) starts with significantly lower levels than BHV1 group and stayed in this way until 2 dpi. From this day, a significant increase in total cell count was observed, peaking at 5 dpi. Thereafter, the cell count decreased quickly until reach lowest levels at 9 dpi for this group, recovering values close to pre-inoculation levels the last day. However, in the BHV1 group a minor transient increase in leukocyte number lasted for 2 days after BHV-1.1 inoculation, which was followed by leucopenia between 4 and 9 dpi, remaining lower for the rest of the study compared with pre-inoculation values.

Parallel to leukocytes profile, the neutrophil count (Figure 1) was significantly different between both BVDV/BHV1 and BHV1 groups before BHV-1 inoculation (0 dpi) and stayed until 2 dpi. In the BVDV/BHV1 group, after a slight initial increase at 12 hpi, the neutrophils had a significantly pronounced peak at 4 and 5 dpi. After that, the cell count decreased quickly reaching the lowest level at 9 dpi, recovering the initial values on last day of the study. In the BHV1 group, after BHV-1 inoculation the neutrophil counts had a slight increase at 12 hpi and levelled out until the 2 dpi. However, from this day onward the neutrophil number underwent a significant drop at 4 dpi and remaining with similar low values until the end of the study.

In general, the dynamic of the lymphocyte population (Figure 2) was essentially the same in both BVDV/BHV1 and BHV1 groups after BHV-1.1 inoculation but with some differences. There are not significant differences in pre-inoculation levels between both inoculated groups. However, in the BVDV/BHV1 group, after a non significant slight increase at 6 hpi, the lymphocyte count had a

drop from 1 dpi, which was significantly more pronounced at 4 dpi, recovering values close to pre-inoculation towards the last days of the study. In the BHV1 group, the lymphocyte count decreased from BHV-1 inoculation and, although fluctuated somewhat, stayed low during the whole study period, reaching the lowest value on 5 dpi.

The kinetics of the monocytes is shown in Figure 2. In the BVDV/BHV1 group, the monocyte count did not change throughout the study. However, in the BHV1 group, the profile fluctuated somewhat, showing a descent between 2-4 dpi remaining at low levels until 9 dpi and overcoming from that time on the initial values at last day of the study.

In the negative control group, the total leukocyte count and the numbers of neutrophils, lymphocytes and monocytes (Figures 1-2) did not change throughout the study.

3.2. Lymphocyte subpopulations

In the BVDV/BHV1 group, the profile of the CD4+ and CD8+ kinetics (Figure 3) were essentially the same. In both lymphocyte subsets the levels decreased gradually from 1 to 4 dpi, being significantly the lower values of the study at this time point. Then the CD4+ and CD8+ cell numbers increased at 9 dpi, remaining close to pre-inoculation levels towards the end of the study.

In the BHV1 group, the CD4+ (Figure 3) values suffered a descent at 12 hpi and after a slight increase, levels dropped again at 4 dpi reaching the lowest values during the whole study period. After a short recovery at 7 dpi, the CD4+ values dropped and stayed lower than pre-inoculation levels during the rest of the study. The CD8+ subset had significant differences compared with BVDV/BHV1 group during

the first 18 hpi. In fact, the CD8+ subset remained with values close to pre-inoculation until this time point, and then the cell number showed a significant decrease and reached the lowest level at 4 dpi. Then the cell number increased transiently at 7 dpi, but the values remained low for the rest of the study compared with the pre-inoculation levels.

Unlike the others lymphocyte subpopulations studied, the γδ T subpopulation (Figure 4) showed pre-inoculation values significantly different between both inoculated groups, being much higher in the BVDV/BHV1 group. However, from 12 hpi, the dynamics in the BVDV/BHV1 and BHV1 groups were essentially the same where the values fluctuated somewhat for the rest of the study and had not significant differences among time points.

The B lymphocyte subset (Figure 4) presented a parallel profile in both BVDV/BHV1 and BHV1 groups characterized by a significant abrupt decrease from 1 dpi, dropping throughout the whole study period. From that time onward, the level of B cell counts in the BVDV/BHV1 group stayed mainly below BHV1 group values until the end of the study. Although in the BHV1 group was observed a transient increase on 9 dpi, it was followed by values close to zero at the last day.

The lymphocyte subpopulations analysed in the negative control group appeared without changes until the end of the experiment (Figures 3-4).

4. Discussion

This study evaluated quantitative changes in leukocytes and circulating lymphocyte subpopulations in healthy calves and calves with subclinical BVD, both inoculated with BHV-1, with a view to ascertaining the effect of BVDV on the response of these immunocompetent cells and on the development of BRD involving

247 a BHV-1 infection. Inter-group differences in the severity of clinical symptoms were
248 accompanied by differences in the viremia (Risalde et al., 2011b). Whilst leukocyte
249 populations such as monocytes displayed no significant alteration, lymphocyte
250 subpopulations and neutrophils exhibited changes in behaviour which probably
251 enhanced the immunosuppressive effect of BVDV, thus accounting for some of the
252 inter-group differences observed.

253 In BVDV-infected calves, leukocyte numbers are reported to return to
254 normal between 7 and 12 dpi (Wilhelmsen et al., 1990; Archambault et al., 2000;
255 Pedrera et al., 2009a,b). Here, at 0 dpi, i.e., 12 dpi BVDV calves inoculated with
256 BVDV displayed significantly lower leukocyte levels than healthy calves, though
257 neither viremia nor clinical symptoms were apparent (Risalde et al., 2011b); this
258 suggested a difference in status prior to inoculation of the secondary pathogen BHV-
259 1. Moreover, analysis of the various leukocyte populations indicated that this
260 difference was due mainly to a significant lower number of neutrophils, which
261 constitute the first line of cellular defence against invading pathogens (Paape et al.,
262 2003). Since lymphocytes and monocyte-macrophages are the primary target cells
263 for BVDV replication (Bruschke et al., 1998; Glew et al., 2003; Risalde et al.,
264 2011a), it was somewhat surprising that initial levels of these cell populations should
265 be similar in healthy and BVDV-infected calves prior to inoculation with BHV-1.

266 Following inoculation with BHV-1, a significant difference in leukocyte
267 counts was observed between the BVDV/BHV1 and BHV1 groups, mainly affecting
268 neurophils and, to a lesser extent, lymphocytes and monocytes. BHV1 is known to
269 induce a state of leukopenia associated with lymphopenia (Griebel et al., 1987,
270 1988), neutropenia (Filion et al., 1983) and monocytopenia (Van Drunen Littel-van
271 den Hurk, 2007). In the BHV1 group, leukocyte depletion was noted from 4 dpi.

Paradoxically, calves in the BVDV/BHV1 group – despite to present a lower leukocyte number, due probably to immunodepression induced by BVDV inoculation (Chase et al., 2004; Pedrera et al., 2009b) – displayed leukocytosis resulting from an increase in circulating neutrophils, with no significant change in monocyte numbers even though lymphopenia was more severe than in the BHV1 group. The neutropenia prompted by BHV-1 in the previously healthy group contrasted with the neutrophilia observed in the BVDV/BHV1 group which had initially exhibited BVDV-induced neutropenia. These findings could be indicating the continuing ability of calves with BVDV to react to infection by recruiting neutrophils. However, this response may be rendered less effective due to the inhibitory action of BVDV on neutrophil transendothelial migration (Brown et al., 1991; Potgieter, 1995; Glew et al., 2003). BVDV-induced sequestration of peripheral blood neutrophils would hinder their migration to the infection site and also ultimately give rise to neutrophilia; this effect may be enhanced by BHV-1 (Filion et al., 1983). However, the likelihood that BVDV might inhibit neutrophil migration is weakened by the fact that neutrophilia coincided here with increased evidence of inflammatory signs (Risalde et al., 2011b).

BHV-1 viremia of varying duration has been reported in infection involving highly-virulent strains, and also in very young animals (Castrucci et al., 1992; Kaashoek et al., 1996). On the other hand, BHV-1 replication has been confined to the surface of airway mucosae in animals over 3 months old (van Drunen Littel-van den Hurk, 2007). In calves with around 9 month-old, BHV-1.1 was not detected from blood in calves infected only with BHV-1.1, in contrast to calves co-infected with BVDV and BHV-1.1 in which BHV-1.1 was confirmed from 4 dpi until the end of the study; that it could be indicating a failure of anti-BHV1 mechanisms in calves

297 inoculated with BVDV. This impairment of antiviral mechanisms appears to have
298 induced a reactivation of BVDV in these animals, since at the time of BHV-1
299 inoculation calves in the co-infected BVDV/BHV1 calves did not exhibit BVDV
300 viremia, and after BHV-1.1 inoculation, BVDV reappeared (Risalde et al., 2011b). In
301 fact, previous works report transitory viremia until 12-14 dpi in BVD (Wilhelmsen et
302 al., 1990; Collen et al., 2002; Brackenbury et al., 2003; Kelling, 2007; Pedrera et al.,
303 2009b). A key role in the impairment of antiviral capacity may be played by
304 circulating T lymphocyte subpopulations (CD4+, CD8+ and γδ) and B cells,
305 involved among others in the cell-mediated immune response, which are known to be
306 capable of interacting with infected cells (Srikumaran et al., 2007).
307 Analysis of lymphocyte subpopulations showed that CD4+ T cell levels were
308 similar in both groups prior to inoculation with BHV-1, reflecting normal values in
309 contrast to the characteristic CD4+ T cell depletion induced by BVDV (Howard et
310 al., 1992; Collen et al., 2002). Following inoculation with BHV-1, both groups
311 displayed a slight decline in CD4+ T cell numbers from 6 hpi, being the lowest
312 counts recorded at 4 dpi. Despite this decline, numbers in both groups had returned to
313 normal levels by the end of the study. This would suggest that BVDV does not
314 impair the regenerative capacity of circulating CD4+ T cells, changes in CD4+
315 lymphocyte levels in response to BHV-1 being similar in healthy calves and calves
316 with subclinical BVD. The similarity in CD4+ lymphocyte kinetics indicates that the
317 changes observed were induced probably by BHV-1, which can infect specifically
318 this subpopulation (Babiuk et al., 1996; Winkler et al., 1999; Van Drunen Littel-van
319 den Hurk, 2007).
320 Although CD8+ T cell levels were similar prior to BHV-1 inoculation,
321 significant differences in cell counts between the BVDV/BHV1 group and the BHV1

group were apparent from 6 hpi. The constant decline observed in the BVDV/BHV1 group was not seen in the BHV1 group, suggesting that it was attributable not to infection of these cells by BHV-1 but rather to BVDV-induced migration of CD8+ T cells to the infection site (Winkler et al., 1999; Van Drunen Littel-van den Hurk, 2007). Between 1 and 7 dpi, CD8+ T cell counts behaved in a similar manner in the two groups, the most marked depletion being observed at 4 dpi. The decline in numbers was more pronounced in the BVDV/BHV1 group, where the virus was not contained, being BHV-1 detected in the blood of BVDV/BHV1 calves from 4 dpi (Risalde et al., 2011b). These results may be indicate an impairment of the cytotoxic action of CD8+ T cells as a defence against the cell-to-cell dissemination characteristic of BHV-1 prior to blood-borne dissemination (Van Drunen Little-van den Hurt, 2007). CD8+ T cell depletion from the start of the study, together with delayed production of IFNγ compared to healthy calves, would favour the development of BHV-1 viremia as well as the reactivation of BVDV and its persistence in target organs, aggravating the lesions associated with both diseases (Risalde et al., 2011b).

Numbers of γδ lymphocytes were higher in the BVDV/BHV1 group prior to infection with BHV-1. This initial difference might have affected the response to secondary infection, although the systemic role of this lymphocyte subpopulation in countering pathogenic agents has not yet been clearly established. Amadori et al. (1995) note that in BHV-1 infection inhibition of virus replication is among the antiviral activities attributed to circulating γδ T cells. In the present study, γδ T cells reached normal levels in the BVDV/BHV1 group at 6 hpi. Thereafter, and throughout the study, kinetics and cell counts remained similar in both groups. This would suggest that, although circulating γδ T cell levels were initially higher in the

BVDV/BHV1 group, these cells did not play a major systemic role in containing BHV-1.1 dissemination, since viremia was detected in this group, but not in the BHV1 group (Risalde et al., 2011b).

Reports regarding changes in circulating B lymphocyte counts following BVDV infection vary; some authors have noted a decline (Brewoo et al., 2007), others have observed no effect (Archambault et al., 2000), whilst still others have recorded a transitory increase (Brodersen and Kelling, 1999). BVDV exerts its greatest effect on thymic and follicular B lymphocytes in lymph nodes and Peyer's patches (Brodersen and Kelling, 1999; Pedrera et al., 2009a,b; Raya et al., 2011; Risalde et al., 2011b). Although pre-inoculation counts were similar in both groups, B lymphocyte counts in the BVDV/BHV1 group were lower than in the BHV1 group from 1 dpi onwards, possibly reflecting the reduced ability of depleted lymphoid organs to produce B cells. Brewoo et al. (2007) report that, in addition to prompting a severe reduction in the number of cirulating B lymphocytes, BVDV often results in the suppression of the functional activities of these cells. However, in the BVDV/BHV1 group anti-BVDV Ab were detected from 4 dpi BHV1 (i.e. 16 dpi BVDV) (Risalde et al., 2011b), being a delayed response, a finding also reported by other authors (Wilhelmsen et al., 1990; Archambault et al., 2000; Müller-Doblies et al., 2004, Pedrera et al., 2009b), while neutralizing Ab to BHV-1 were detected in both groups from 14 dpi BHV1 (Risalde et al., 2011b); other authors (Kaashoek et al., 1996; OIE, 2010) have noted a BHV-1 specific humoral immune response at around 7-10 dpi. Here, a strong Ab response was observed following inoculation with BHV-1 (Risalde et al., 2011b), suggesting that despite the sharp drop in B cell numbers in both groups, functional capacity was not diminished; similar findings are reported by Filion et al. (1983).

5. Conclusion

Developed cell-mediated immune response against BHV-1, in animals previously infected with BVDV, results less effective in containing the spread of virus and clarification of the BHV-1 and BVDV. The failure of the antiviral mechanisms developed against BHV-1 in the calves infected with BVDV could be partially due to the significant changes observed in the CD8+ T lymphocyte subpopulation compared with healthy animals. This coupled with significant numerical changes found in the neutrophil population, may contribute to poor cell-mediated immune response against viral secondary infections with BHV-1, showing the synergy of BVDV and BHV-1 on viremia, but also on clinical symptoms, in the animals of the BVDV/BHV1 group, predisposing to the development of inflammatory processes.

Conflict of interest statement

The authors do not have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Figure captions

Figure 1. Mean number ($x10^9l^{-1}$) ± standard error of total leukocyte and neutrophil counts in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). ***** Significant differences ($p < 0.05$) within each inoculated group at various time points. ****** Significant differences ($p < 0.05$) between inoculated groups at the same time point. (dpi BHV-1.1, means days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-inoculation with BHV-1.1).

Figure 2. Mean number ($x10^9l^{-1}$) ± standard error of lymphocyte and monocyte counts in blood samples from calves experimentally inoculated with bovine herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea virus and bovine herpesvirus-1 (BVDV/BHV1 group). ***** Significant differences ($p < 0.05$) within each inoculated group at various time points. ****** Significant differences ($p < 0.05$) between inoculated groups at the same time point. (dpi BHV-1.1, means

565 days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h,
566 means hours post-inoculation with BHV-1.1).
567
568 **Figure 3.** Mean ± standard error absolute numbers ($x10^9l^{-1}$) of CD4+ and CD8+
569 lymphocytes in blood samples from experimentally inoculated with bovine
570 herpesvirus-1 (BHV1 group) versus calves inoculated with both bovine viral diarrhea
571 virus and bovine herpesvirus-1 (BVDV/BHV1 group). *****Significant differences ($p <$
572 0.05) within each inoculated group at various time points. ******Significant differences
573 ($p < 0.05$) between inoculated groups at the same time point. (dpi BHV-1.1, means
574 days post-inoculation with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h,
575 means hours post-inoculation with BHV-1.1).
576
577 **Figure 4.** Mean ± standard error absolute numbers ($x10^9l^{-1}$) of γδ and B lymphocytes
578 in blood samples from experimentally inoculated with bovine herpesvirus-1 (BHV1
579 group) versus calves inoculated with both bovine viral diarrhea virus and bovine
580 herpesvirus-1 (BVDV/BHV1 group). *****Significant differences ($p < 0.05$) within each
581 inoculated group at various time points. ******Significant differences ($p < 0.05$) between
582 inoculated groups at the same time point. (dpi BHV-1.1, means days post-inoculation
583 with BHV-1.1; 0, include BHV-1.1 pre-inoculation values; h, means hours post-
584 inoculation with BHV-1.1).
585

Figure 1

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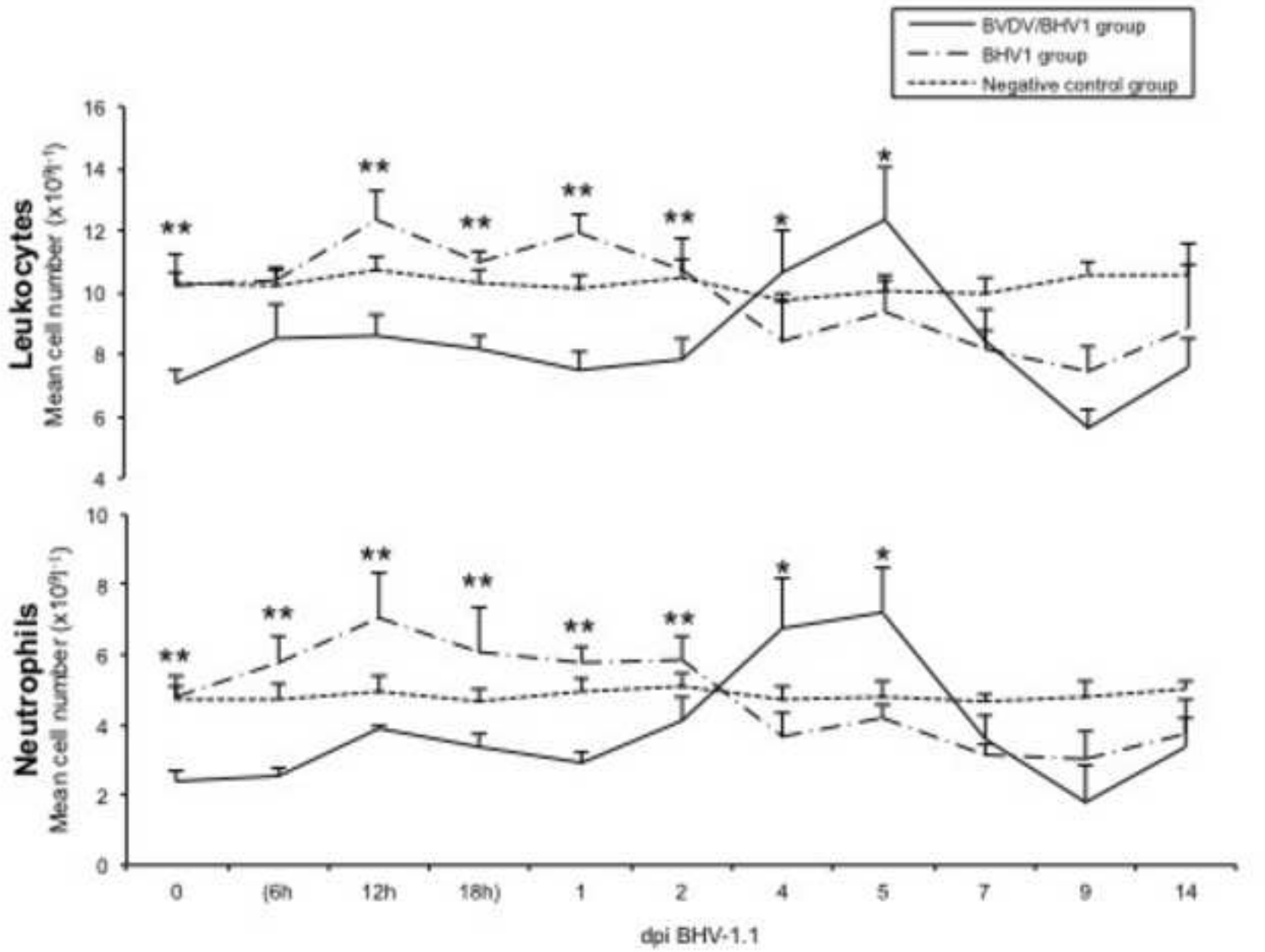

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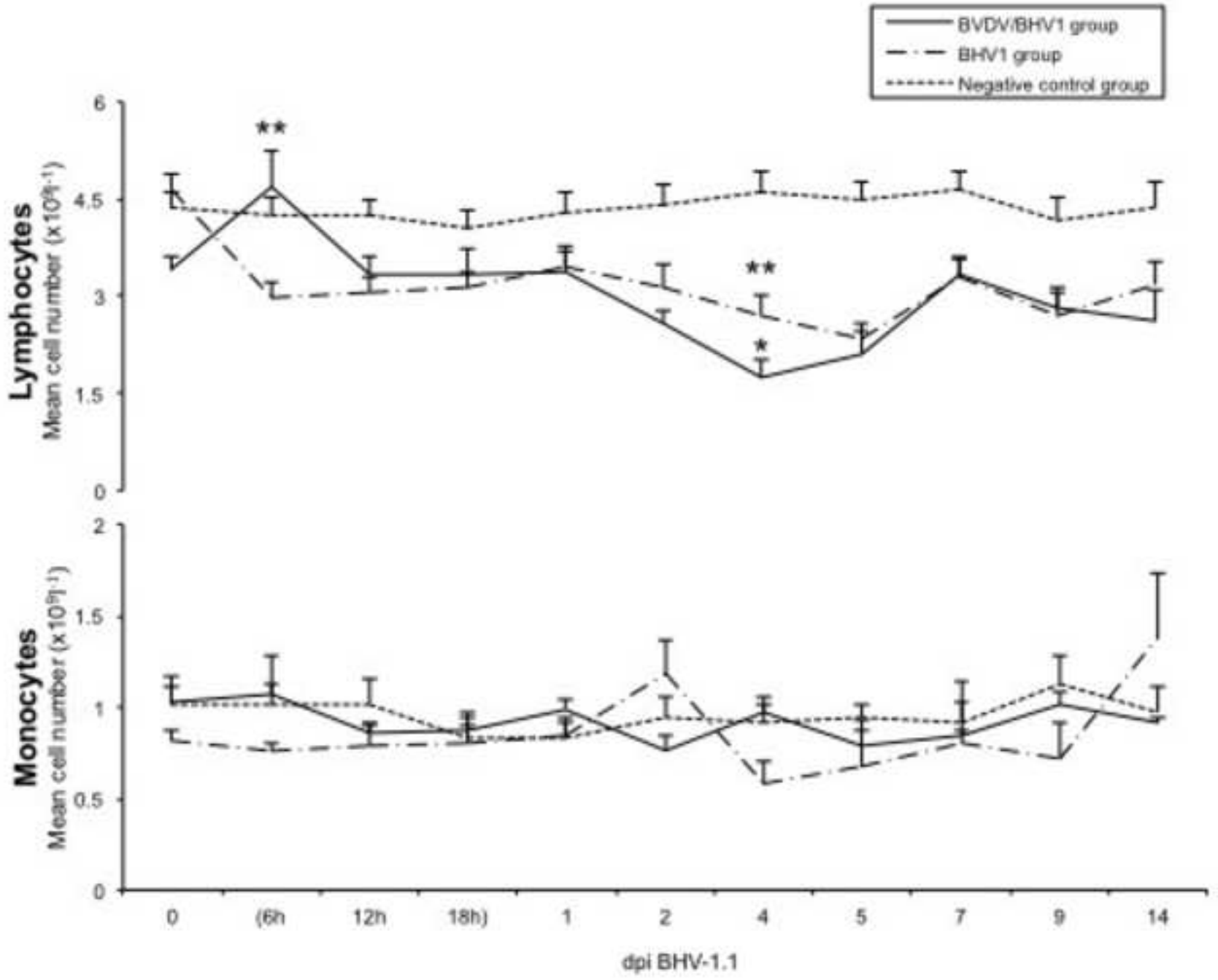

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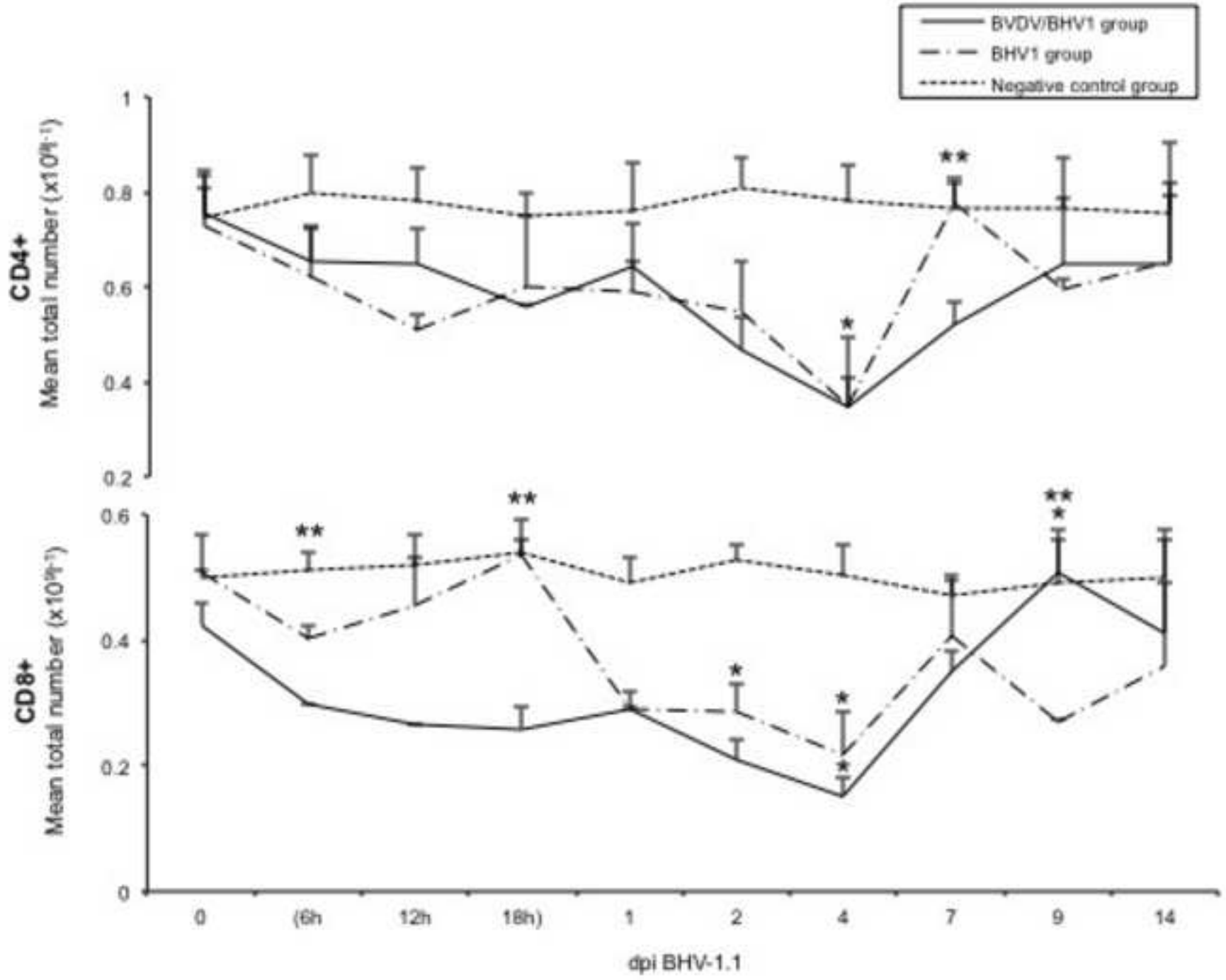

Figure 4

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