
http://researchrepository.murdoch.edu.au/10808/

Copyright: © 2012 Elsevier B.V.

It is posted here for your personal use. No further distribution is permitted.
Accepted Manuscript

Title: Flow Cytometric Analysis of Lymphocyte Subset Kinetics in Bali Cattle Experimentally Infected with Jembrana Disease Virus

Authors: I.W. Masa Tenaya, Kathy Heel, Philip A. Stumbles, Graham E. Wilcox

PII: S0165-2427(12)00206-1
DOI: doi:10.1016/j.vetimm.2012.06.013
Reference: VETIMM 8835

To appear in: VETIMM

Received date: 4-8-2011
Revised date: 24-4-2012
Accepted date: 11-6-2012


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Flow Cytometric Analysis of Lymphocyte Subset Kinetics in Bali Cattle

Experimentally Infected with Jembrana Disease Virus

I. W. Masa Tenaya a,1, Kathy Heel b, Philip A. Stumbles a* and Graham E. Wilcox a

a School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, W.A., 6150, Australia; b Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Crawley, W.A., 6009, Australia; 1 Present address: BPPH Wilayah VI, P.O. Box 3322, Denpasar, Bali, Indonesia

*Corresponding author: Tel + 61 8 9360 6201; Fax +61 8 9310 4144; e-mail; p.stumbles@murdoch.edu.au

Address correspondence and reprint requests to:

Dr P. A. Stumbles
School of Veterinary and Biomedical Sciences
Murdoch University
South St.
Murdoch, W.A., 6150
Australia
Abstract

Jembrana Disease Virus (JDV) is an unusual bovine lentivirus that causes an acute and sometimes fatal disease after a short incubation period in Bali cattle (*Bos javanicus*). The pathological changes occur primarily in lymphoid tissues, which feature proliferating lymphoblastoid-like cells predominantly throughout parafollicular (T-cell) areas, and atrophy of follicles (B-cell) areas. Five Bali cattle were experimentally infected with JDV and all developed typical clinical signs of Jembrana disease characterized by a transient febrile response, enlargement of superficial lymph nodes and a significant leukopenia. Flow cytometric analysis of PBMC during the acute (febrile) disease phase showed that the reduced number of lymphocytes was due to a significant decrease in both the proportion and absolute numbers of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T-cells or CD21<sup>+</sup> B-cells. At the end of the febrile phase, total numbers of both CD8<sup>+</sup> T-cells and CD21<sup>+</sup> B-cells increased significantly, while CD4<sup>+</sup> T-cell numbers remained below normal values, resulting in a significantly reduced CD4<sup>+</sup>:CD8<sup>+</sup> ratio. We speculate that the persistent depletion of CD4<sup>+</sup> T cells following JDV infection, through lack of CD4<sup>+</sup> T cell help to B cells, may explain the lack of production of JDV-specific antibodies for several weeks after recovery despite an increase in CD21<sup>+</sup> B cell numbers. Further, our previous data showing that IgG<sup>+</sup> plasma cells are targets for JDV infection, correlated with our current data demonstrating an increase in CD8<sup>+</sup> T cell numbers, supports the suggestion that anti-viral cytotoxic T cell or other cell-mediated immune responses may be critical in the recovery process, although this remains to be formally demonstrated for JDV.
Keywords
Jembrana Disease Virus; Lentivirus; Cattle; Flow cytometry; Lymphocyte subpopulations; Immunodeficiency

Abbreviations
BIV, bovine immunodeficiency virus; EDTA, ethylenediaminetetraacetic acid; EIAV, equine infectious anemia virus; FIV, Feline immunodeficiency virus; FACS, fluorescence-activated cell sorting; HIV, Human immunodeficiency virus; IgG, immunoglobulin G; JDV, Jembrana Disease Virus; mAb, monoclonal antibody; RNA, ribonucleic acid; SIV, Simian immunodeficiency virus.
Introduction

Jembrana disease (JD) is an infectious disease of Bali cattle caused by Jembrana disease virus (JDV), a pathogenic lentivirus most closely related to Bovine Immunodeficiency Virus (BIV) (Chadwick et al., 1995). Infection of Bali cattle (Bos javanicus) with JDV causes an acute disease with clinical signs including fever, lethargy, anorexia and enlargement of the superficial lymph nodes beginning 5-12 days after infection that resolves within 17 days (Soeharsono et al., 1990; Soesanto et al., 1990; Wilcox et al., 1995). Hematological changes during the acute disease include leukopenia as a result of a lymphopenia, eosinopenia, neutropenia, thrombocytopenia, anemia, increased blood urea concentrations and diminished total plasma protein concentration (Soesanto et al., 1990). Gross pathological changes include vascular damage such as mild exudates and hemorrhages, but the most striking changes are lymphadenopathy and splenomegaly. Lymphoid tissues of all organs, particularly in the enlarged lymph nodes and spleen, feature proliferating lymphoblastoid-like cells predominantly throughout parafollicular (T-cell) areas, and atrophy of follicles (B-cell areas). A proliferative lymphoid infiltrate is also found in the parenchyma of most organs, particularly the liver and kidneys and an infiltrate containing proliferative macrophage-like cells is found in the lungs (Dharma et al., 1991).

In experimentally infected cattle, the case fatality rate was originally estimated to be 17% (Soeharsono et al., 1990) but was more recently reported to be 11.5% (Desport et al., 2009a). For naturally infected animals the case fatality rates vary: a mortality rate of 98.9% was reported during the first outbreak in 1962 however in more recent outbreaks mortality rates have varied from 20 – 37% in a new areas where JDV has not been previously reported (Ramachandran et al., 1996). During the acute phase, a high titer of virus (~10^{10} RNA genome copies/ml plasma) is present in the plasma, persisting at low levels for at least 25 months.
after recovery from the acute disease (Soeharsono et al., 1990; Stewart et al., 2005). The experimentally infected Bali cattle that survive the acute clinical disease do not develop any further clinical disease (Soeharsono et al., 1990; Soesanto et al., 1990) and resist re-infection (Soeharsono et al., 1990) but the immune mechanism responsible for recovery from the acute disease and continued immunity has not been defined. Although not formally proven, there is circumstantial evidence to suggest that neutralizing antibody does not play a major role in recovery as JDV-specific antibodies are not detectable until some weeks after recovery from the acute disease (Hartaningsih et al., 1994). This has lead to the hypothesis that cellular immune responses, presumably through IFNγ-mediated activation of viral-specific CD8+ cytotoxic T lymphocytes, play a critical role in recovery from JDV infection, as is the case for equine infectious anemia virus (EIAV) (Murakami et al., 1999) and HIV (Migueles and Connors, 2002), although again this remains to be formally proven for JDV.

Hyperplasia of T-cell areas and depletion of B-cell areas of lymphoid tissues during acute Jembrana disease is a hallmark of the disease (Dharma et al., 1991). Depletion of lymphocyte [the CD4+ T-cell and CD8+ T-cell] populations was observed histologically in JDV-infected Bali cattle and significant differences were found during acute illness in follicular compartments of lymph nodes (Dharma et al., 1994). The acute febrile phase of Jembrana disease is characterised by marked haematological changes that include leucopenia due to lymphopenia, eosinopenia, and a slight neutropenia and thrombocytopenia (Soesanto et al., 1990). The mechanism for the changes in lymphocyte populations in Jembrana disease remains unknown. It was suggested that the gradual depletion of CD4+ T-cells may have been due to infection of T-cells (Dharma et al., 1994). Although T-cells are the predominant target cell of some lentiviruses, including HIV (Alcami, 2004; Blankson et al., 2002; Brenchley et al., 2004; Clapham and McKnight, 2001), SIV (Brown et al., 2007; Mattapallil et al., 2005;
Picker, 2006) and FIV (Ackley et al., 1990), there is no evidence for infection of CD3+ T-cells or MAC387+ monocytes by JDV and the target virus infection was identified in lymphoid tissues as pleomorphic centroblast-like cells which were identified as IgG-containing cells, including plasma cells (Desport et al., 2009b).

Although lymphopenia is a characteristic feature of Jembrana disease (Soesanto et al., 1990), changes in circulating lymphocyte subsets during the acute disease have remained uncharacterized. We report a flow cytometric analysis of the circulating CD4+ T cell, CD8+ T cell and CD21+ B cell populations during the febrile and early post-febrile phases undertaken to better understand the acute disease process associated with JDV infection. Our data indicate that shortly after the end of febrile periods (9 days after infection), CD8+ T cell numbers were elevated and this coincided with increased of INFγ expression. Given that antibodies were not detected until several weeks after the initial infections, this data may explain why the majority (80%) of experimentally JDV-infected animals survived and support the conclusion that CD8+ T cell-mediated protection is important in recovery from JDV infection.
Materials and Methods

Experimental animals and sample collection

Bali cattle used in the experimental studies were female, approximately 12 months of age and weighed 80-100 kg. They were obtained from Nusa Penida, a small island adjacent to Bali, where Jembrana disease has never been reported and where antibodies to JDV have not been detected (Hartaningsih et al., 1994). Cattle purchased from the island have consistently developed clinical signs of Jembrana disease when infected with JDV (Soeharsono et al., 1990). Animals for these experiments were transported to Bali island to the Disease Investigation Centre Region VI, Denpasar, Bali. On arrival, they were sprayed with insecticide, kept in an insect-proof house and given water and elephant grass (*Penecetum purpureum*) *ad libitum*. Prior to use, all cattle were pre-treated with a broad spectrum antibiotic (oxytetracycline) at a dose rate of 5 mg/kg bodyweight for 3 consecutive days, a broad spectrum anti-helmintic, and they were vaccinated against haemorrhagic septicaemia. Before inoculation with JDV, the absence of antibody to JDV was confirmed by ELISA test using a JDV recombinant CA antigen as described previously (Burkala et al., 1998).

Five Bali cattle were infected intravenously with an estimated 100 infectious doses of JDV contained in a 10% homogenate in Dulbecco’s modified Eagle’s medium of spleen which had been stored at −70 °C and previously prepared from an animal experimentally infected with JDV<Tab87> (Soeharsono et al., 1995). The febrile phase in the inoculated animals occurred from 5-11 days after infection. Blood samples were analysed prior to infection (day 0), at day 2 and daily from days 4 to 7 (febrile phase) and day 9 post-infection, and then finally at day 19 when the animals were euthanased for autopsy. Sterile EDTA-containing vacutainer tubes (Greiner Bio-One) were used to collect blood samples for recovery of lymphocytes.
Lymphocyte preparation

Peripheral blood mononuclear cells were isolated using Ficoll-Paque plus density separation (Amersham Biosciences, Australia) following the manufacturer’s instructions, then washed twice in FACS (fluorescence-activated cell sorting) buffer (Dulbecco’s phosphate-buffered saline [Thermo Scientific] supplemented with 5% heat inactivated FCS (Bovogen Biologicals, Australia) and 0.05% sodium azide (Sigma-Aldrich, Australia). The washed cells were then resuspended in FACS buffer and adjusted to a density of $1 \times 10^7$ cells/ml, and kept at 5°C until they were immunolabeled on the same day. Total white blood cells were counted using trypan blue staining and a haemocytometer. Absolute numbers lymphocyte subsets were calculated by multiplying the total lymphocyte count by the proportion of $\text{CD}4^+$, $\text{CD}8^+$ or $\text{CD}21^+$ cells as determined by flow cytometry (see below).

Antibodies and cellular markers

Lymphocyte subsets were labeled with 2.5 $\mu$g/ml mouse anti-bovine CD4 mAb (Serotec, UK), 5 $\mu$g/ml mouse anti-bovine CD8 mAb (Serotec, UK) or 20 $\mu$g/ml mouse anti-bovine CD21 mAb (Santa Cruz, USA) as a B-cell marker. An Alexa Fluor 488 (AF488) conjugated goat anti-mouse cross-absorbed secondary antibody (Invitrogen, Australia) was used to detect all reactive mAb antibodies (Table 1).

Cell surface labeling of lymphocytes

Due to Australian quarantine and logistical requirements, peripheral blood lymphocytes were antibody-labeled and fixed on-site in Bali prior to transport to Australia for analysis by flow cytometry at the University of Western Australia, using a previously validated protocol (Foster et al., 2007; Rocchi et al., 2007) with slight modification. Following lymphocyte
preparation, 1 ml of the lymphocyte suspension was incubated with 100 μl of primary antibody in FACS buffer for 30 minutes at 4°C, followed by 3 washes with FACS buffer (by centrifugation for 1 min at 479 g at 4°C). Secondary antibody (100 μl) diluted in FACS buffer was applied and incubated for 30 minutes at 4°C in the dark. The cell suspensions were then gently washed 3 times in FACS buffer, then washed once with PBS and the cells resuspended in 200 μl of fixation buffer (Dulbecco’s phosphate-buffered saline supplemented with 4% paraformaldehyde) for 5 minutes at 37°C. Finally, the cells were washed with 200 μl of ice-cold Dulbecco’s phosphate-buffered saline supplemented with 1% BSA (Sigma Aldrich, Australia) and resuspended in 1 ml of freezing medium (Dulbecco’s phosphate-buffered saline supplemented with 1% BSA and 10% dimethyl sulfoxide (Sigma Aldrich, Australia) before being transferred to freezing vials and then stored at -80°C. Samples were stored for up to 2 months in Bali prior to transport to Australia for analysis by flow cytometry.

Flow cytometric analysis

Prior to flow cytometric analysis, cryopreserved lymphocyte samples were thawed rapidly at 37°C in a water bath, then washed once with wash buffer (Dulbecco’s phosphate-buffered saline supplemented with 0.1% BSA) and resuspended in 1 ml of labeling buffer (Dulbecco’s phosphate-buffered saline supplemented with 10% heat-inactivated FCS and 0.1% sodium azide [Sigma Aldrich]). The immunolabeled samples were analyzed using a BD FACS Calibur flow cytometer (BD Bioscience, Australia) with a 488 nm excitation laser. Lymphocytes were gated in a forward scatter/side scatter plot (FSC-H vs SSC-H). A bandpass-specific filter (FL1, 530/30 nm) was used for Alexa Fluor 488 emission and acquired in the logarithmic scale. A minimum of 10,000 lymphocytes were examined per sample and an AF488 fluorescence histogram was used to compare the samples. Sample data were analyzed using BD CellQuest Pro V5.2 (BD Biosciences, Australia) which is the
standard operating software on the FACSCalibur. Experimental data were analyzed and population statistics calculated using FlowJo V7.2.5 (Tree Star Inc., USA) flow cytometric analysis software.

Statistical analysis
The absolute numbers of lymphocyte subsets were calculated by multiplying the percentage of each lymphocyte subset obtained from flow cytometric analysis with the total lymphocyte counts/ml, and were reported as a mean ± standard deviation (SD). A one-way ANOVA (SPSS® 17.0) was used to assess group differences in the lymphocyte populations, while differences between time points during infection were analyzed using Bonferroni’s multiple comparison. Significant differences in the mean values at different time points was analysed by Tukey’s HSD test. A value of $p<0.05$ was considered significant for all analyses.
Results

**Flow cytometric analyses of lymphocyte subset changes following JDV infection**

Prior to further analysis, sample preparation techniques were evaluated to assess the quality of lymphocytes in PBMC isolated using Ficoll-Paque at various time points following experimental JDV infection of cattle. All samples were shown to have high viabilities, with less than 5% dead cells as assessed by trypan blue staining (data not shown). Using flow cytometry gating for lymphocytes (Figure 1) and labeling for CD4+, CD8+ and CD21+ lymphocyte subsets (Figures 2 to 4), combined with total cell counts for assessment of absolute numbers of lymphocyte subsets, a significant differences in the total numbers of lymphocyte subsets was observed at the 3 major time points of JDV infection: pre-infection (day 0 prior to JDV infection), during the febrile phase (day 9) and during the immediate post-febrile phase (day 19).

During the febrile and early recovery phases, the total number of CD4+ T-cells decreased significantly (Table 2 and Figure 5) and the percentage of CD4+ T cells remained below normal values until well after the febrile phase and into the early recovery phase (Figure 2). Conversely, the total number of CD8+ T-cells was reduced slightly during the febrile period but increased significantly ($p<0.001$) above normal values in the early recovery phase (Table 2 and Figure 5). This corresponded to a slight decrease in the percentage of CD8+ T cells amongst PBMC during the febrile and early recovery phases compared to pre-infected cattle (Figure 3).

Due to the dramatic depletion of CD4+ T-cells and significant increase in CD8+ T-cells after JDV infection, the CD4+:CD8+ T-cell ratio also decreased significantly ($p<0.05$) from 0.5:1 at
pre-infection to 0.25:1 and 0.01:1 during the febrile phase and post-febrile phase, respectively (Table 3).

Total numbers of CD21$^+$ B-cells increased slightly during the febrile phase then increased significantly ($p<0.001$) during the early recovery phase (Table 2 and Figure 5). This corresponded with an increase in the percentage of CD21$^+$ B cells during the febrile and early recovery phases (Figure 4).
Discussion

The nature of the response of Bali cattle to JDV infection, an acute disease process with a short incubation period, a case fatality rate of about 17% and no recurrence of disease in those animals that recover, is unusual for a lentivirus. The lack of any recurrence of disease in animals that recover suggests the development of a strong protective immunity. The absence of JDV-specific antibody until at least 5 weeks and not in most cattle until 11 weeks after infection (Hartaningsih et al., 1994) implies that cell-mediated immune responses play a major role in the recovery of the infected animals and probably in their continuing immunity.

The current study assessed the responses of peripheral blood lymphocyte subsets to JDV infection in experimentally infected animals to gain insights into the kinetics of the lymphocyte response following infection.

The use of flow cytometric analysis confirmed the previous report of the significant decrease in CD4⁻: CD8⁻ T-cell ratio of lymphocytes in lymphoid tissues during the acute phase of Jembrana disease but not during early post-febrile stages (Dharma et al., 1994). In this current study, both CD4⁺ and CD8⁺ T-cells in peripheral blood significantly decreased during the febrile phase compared to before infection, and this period corresponds to the duration of the lymphopenia reported during the febrile phase of Jembrana disease (Soesanto et al., 1990). The number of CD8⁺ T-cells remained greater than CD4⁺ T-cells during the febrile phase but increased markedly during the post-febrile phase. Due to the significant increase of CD8⁺ T-cell numbers at the end of the febrile phase and a continuous depletion of CD4⁺ T-cells, this resulted in a dramatic decrease in the CD4⁺:CD8⁺ T-cell ratio during the post-febrile phase.
The significant increase in the CD8\(^+\) T-cell population after the febrile phase strongly correlated with the expansion of CD3\(^+\) T-cell numbers previously found in lymphoid tissues during this stage (Desport et al., 2009a). This positive correlation may indicate that the majority of the CD3\(^+\) T-cells were CD8\(^+\) T-cell subsets. Further, the increased population of CD8\(^+\) T-cells during JDV infection, in the absence of JDV-specific antibody until several weeks after JDV-infection (Hartaningsih et al., 1994), provides additional support for the role of these cells in the recovery from the acute disease process. Virus-specific CD8\(^+\) cytotoxic T-cells may play an important role in host defence against lentivirus infections (Levy, 1993; Salk et al., 1993). The antiviral role of CD8\(^+\) cytotoxic T lymphocytes has been considered to be important in the inhibition of the progression of early EIAV infection before the production of virus neutralizing antibody (Hammond et al., 1997; McGuire et al., 2004; McGuire et al., 1994). It is also thought to be important in non-progressor HIV-infected individuals (Cao et al., 1995; Migueles et al., 2002), and in controlling SIV replication and protection against SIV challenge (Genesca et al., 2009; Genesca et al., 2008; Jin et al., 1999). It is only in the transition to chronic infection that the impressive early potency of the antiviral CD8\(^+\) cytotoxic T-cells may wane (Pantaleo et al., 1997), which has been linked with declining CD8\(^+\) T cell function and the inability of the immune system to control viral replication and spread of the virus (Migueles et al., 2002; Pantaleo et al., 1997; Zhang et al., 2003). As with other lentivirus infections, at least during acute infections, the results of this study tend to support the current hypothesis that virus-specific CD8\(^+\) cytotoxic T lymphocytes may play a crucial role in host defence against lentivirus infections (Levy, 1993), also supported by preliminary data showing elevated IFN\(\gamma\) mRNA during the recovery phase (MT, PS, GW unpublished observations), however the specificity and anti-viral activity of the CD8\(^+\) T cell response for JDV remains to be determined.
It is unclear why CD4$^+$ T-cells were dramatically decreased despite the absence of infection of T-cells by the virus, as shown in previous studies (Desport et al., 2009b) and again confirmed in this study by immunohistochemistry (by co-staining lymphoid tissue with anti-CD3 and anti-JDV capsid protein mAb) and by in situ hybridization for JDV RNA (data not shown). A gradual depletion of CD4$^+$ T-cell subsets is associated with infection with the T-cell tropic lentiviruses HIV (Alcami, 2004; Blankson et al., 2002; Brenchley et al., 2004; Clapham and McKnight, 2001), SIV (Brown et al., 2007; Mattapallil et al., 2005; Picker, 2006) and FIV (Ackley et al., 1990). However, reduction of CD4$^+$ T-cell populations is not always related to their infection by viruses. In EIAV infections, for example, both circulating CD4$^+$ and CD8$^+$ T-cell subsets are reduced significantly during acute infection, although mature macrophages and not T-cells are the main target cells of the virus (Cook et al., 2001; Murakami et al., 1999; Oaks et al., 1998; Sellon et al., 1992). In EIAV infection, depletion of the T-cell subsets is possibly an indirect effect of the virus infection or virus components (Murakami et al., 1999). This so-called “bystander” depletion has also been observed in uninfected CD4$^+$ T cells HIV infection, where induction of cell-surface pro-apoptotic molecules such as Fas/FasL, viral proteins and undefined soluble mediators have been implicated (Arokium et al., 2009; Ji et al., 2007; Holm and Gabuzda, 2005).

The population of CD21$^+$ B-cells increased prior to the febrile phase, indicating a transient proliferation of B-cells or release of B-cells into the peripheral blood during this phase, similar to that reported during BIV infection (Whetstone et al., 1997). The reason for this is unknown but in HIV-1, a putative polyclonal B-cell stimulatory epitope has been found in the carboxyl end of the envelope glycoprotein of the virus, specifically associated with Nef (Chirmule et al., 1994). Tmx, an accessory protein of unknown function in bovine lentiviruses, is expressed at a similar region of the genome as nef. (Chadwick et al., 1995;
Gonda et al., 1990). Nef is multifunctional, but mainly responsible for viral infectivity (Brugger et al., 2007; Marsh, 1999; Qi and Aiken, 2008; Sol-Foulon et al., 2004). Although the nef gene is not present in the bovine lentiviruses, they have a tmx gene in a similar location to nef, and 2 unique genes vpw and vpy that seem to be analogous to the vpr and vpu/vpx genes of primate lentiviruses (Garvey et al., 1990). Tmx may be involved in a manner similar to Nef in the proliferation of B-cells *in vivo*, however this hypothesis would need to be confirmed. During the febrile phase, there was a progressive reduction in the numbers of CD21⁺ B-cells which may be associated with replication of virus in these cells.
Conclusions

The present study has clearly demonstrated dramatic changes in the population of T-cell subsets and B-cells during the course of Jembrana disease. A striking finding was that, during the febrile and early recovery phases, the total number of CD4$^+$ T-cells decreased significantly and the percentage of CD4$^+$ T cells remained below normal values until well after the febrile phase and into the early recovery phase. CD21$^+$ B-cells, possibly mature JDV-specific B-cells that host viral replication, increased in peripheral blood prior to the onset of the febrile phase and then declined in numbers and this decline corresponded to the decrease in numbers of these cells in tissues during the febrile phase of the disease. CD8$^+$ T-cell numbers increased during the acute disease and may well play a role in the recovery process before the production of anti-viral antibody.
Acknowledgements

We would like to express our gratitude to the staff of the BPPH Wilayah VI, Indonesia, for assistance in conducting the animal experiments. The authors acknowledge the facilities, scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments.

Funding sources

This study was funded by the Australian Centre for International Agricultural Research, Canberra.
References


Ji, J., Chen, J.J., Braciale, V.L, Cloyd, M.W., 2007, Apoptosis induced in HIV-1-exposed, resting CD4+ T cells subsequent to signaling through homing receptors is Fas/Fas ligand-mediated. J Leukoc Biol 81, 297-305.


Figure Captions

Figure 1. Representative forward scatter and side scatter gating of PBMC used to identify lymphocytes and their subsets by flow cytometry. Peripheral blood was isolated from cattle on day 0 (pre-infection) and at days 10 (acute phase) and 19 (recovery phase) following experimental JDV infection and PBMC isolated by Ficoll-Paque density separation. Following lymphocyte subset labeling (see following figures), cell suspensions were analysed by flow cytometry and FSC and SSC gates set as indicated for subsequent lymphocyte analysis. One typical representative of 5 cattle is shown for each time point.

Figure 2. Flow cytometric analysis of CD4$^+$ T-cells following JDV infection. PBMC were isolated at days 0, 10 and 19 post-infection and labeled with an anti-CD4-AF488 mAb. Cells were gated by flow cytometry for lymphocytes as described in Figure 1, and fluorescence histograms shown for representative cattle C5 (left) and C7 (right) demonstrating a significant reduction in the percentage proportion of CD4$^+$ T-cells from pre-infection through the acute and early recovery phases. For each histogram, CD4-AF488 immunolabeled samples (thick black line) are compared to a non-labeled sample (thin black line).

Figure 3. Flow cytometric analysis of CD8$^+$ T-cells following JDV infection. PBMC were isolated at days 0, 10 and 19 post-infection and labeled with an anti-CD8-AF488 mAb. Cells were gated by flow cytometry for lymphocytes as described in Figure 1, and fluorescence histograms shown for representative cattle C5 (left) and C7 (right), demonstrating a minor decrease in the percentage proportion of CD8$^+$ T-cells from pre-infection through the acute and early recovery phases. For each histogram, CD8-AF488 immunolabeled samples (thick black line) are compared to a non-labeled sample (thin black line).
Figure 4. Flow cytometric analysis of CD21$^+$ B-cells following JDV infection. PBMC were isolated at days 0, 10 and 19 post-infection and labeled with an anti-CD21-AF488 mAb. Lymphocytes were gated as described in Figure 1, and fluorescence histograms shown for representative cattle C5 (left) and C7 (right) demonstrating an increase in the percentage proportion of CD21$^+$ B-cells from pre-infection through the acute and early recovery phases. For each histogram, CD21-AF488 immunolabeled samples (thick black line) are compared to a non-labeled sample (thin black line).

Figure 5. Lymphocyte subset changes related to the febrile response following JDV infection. PBMC were isolated at the indicated time points following JDV infection, labeled with AF-488 conjugated mAbs to CD4, CD8 and CD21 and the percentage proportion of lymphocyte subsets analysed by flow cytometry as described in Figures 1 to 4 above. This percentage data was then converted to a total lymphocyte count per ml of blood as described in Materials and Methods and plotted against rectal temperatures measured at the corresponding time points. Data presented are means of values from 5 animals ± SD.
Table 1. Primary and secondary antibodies used for flow cytometric analysis of lymphocytes from cattle infected with JDV.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Isotype/clone</th>
<th>Cat./Lot No</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse anti-bovine CD4</td>
<td>Serotec</td>
<td>IgG2a/CC8</td>
<td>MCA1653G</td>
</tr>
<tr>
<td>Mouse anti-bovine CD8</td>
<td>Serotec</td>
<td>IgG2a/CC63</td>
<td>MCA1653G</td>
</tr>
<tr>
<td>Mouse anti-bovine CD21</td>
<td>Santa Cruz</td>
<td>IgG2b/CC51</td>
<td>SC-101835</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>Invitrogen</td>
<td>Alexa Fluor 488</td>
<td>A-11029</td>
</tr>
</tbody>
</table>
Table 2. Comparison of lymphocyte subsets during 3 major phases after JDV infection.

<table>
<thead>
<tr>
<th>Lymphocyte population</th>
<th>Mean absolute number cells/ml ± SD</th>
<th>Pre-infection</th>
<th>Febrile phase</th>
<th>Post-febrile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-helper cells, CD4⁺</td>
<td>2418 ±277a</td>
<td>176± 171b</td>
<td>45 ±10b</td>
<td></td>
</tr>
<tr>
<td>Cytotoxic T-cells, CD8⁺</td>
<td>1210±206b</td>
<td>768±489b</td>
<td>3187±601a</td>
<td></td>
</tr>
<tr>
<td>B-cells, CD21⁺</td>
<td>1799 ± 404b</td>
<td>2065±823b</td>
<td>4225 ± 841a</td>
<td></td>
</tr>
</tbody>
</table>

Means in a row with different superscripts are significantly different by Tukey’s HSD

(p<0.05).
Table 3. Changes in CD4⁺:CD8⁺ T-cell ratio during the course of JDV infection.

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Mean CD4⁺ (number/ml)</th>
<th>SD</th>
<th>Mean CD8⁺ (number/ml)</th>
<th>SD</th>
<th>CD4⁺:CD8⁺ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2418</td>
<td>713</td>
<td>1210</td>
<td>14</td>
<td>0.5:1</td>
</tr>
<tr>
<td>2</td>
<td>248</td>
<td>11</td>
<td>869</td>
<td>12</td>
<td>0.28:1</td>
</tr>
<tr>
<td>4</td>
<td>217</td>
<td>8</td>
<td>740</td>
<td>12</td>
<td>0.29:1</td>
</tr>
<tr>
<td>5</td>
<td>274</td>
<td>24</td>
<td>1236</td>
<td>31</td>
<td>0.22:1</td>
</tr>
<tr>
<td>6</td>
<td>229</td>
<td>18</td>
<td>605</td>
<td>18</td>
<td>0.38:1</td>
</tr>
<tr>
<td>7</td>
<td>138</td>
<td>11</td>
<td>367</td>
<td>11</td>
<td>0.37:1</td>
</tr>
<tr>
<td>9</td>
<td>28</td>
<td>0.4</td>
<td>709</td>
<td>21</td>
<td>0.04:1</td>
</tr>
<tr>
<td>19</td>
<td>36</td>
<td>0.2</td>
<td>3187</td>
<td>32</td>
<td>0.01:1</td>
</tr>
</tbody>
</table>
Figure 1

C5: Day 0 Pre-infection

C5: Day 10 Acute Phase

C5: Day 19 Early Recovery Phase
Figure 2

C5: Day 0 Pre-infection

C7: Day 0 Pre-infection

C5: Day 10 Acute Phase

C7: Day 10 Acute Phase

C5: Day 19 Early Recovery Phase

C7: Day 19 Early Recovery Phase
Figure 3

C5: Day 0 Pre-infection

C7: Day 0 Pre-infection

C5: Day 10 Acute Phase

C7: Day 10 Acute Phase

C5: Day 19 Early Recovery Phase

C7: Day 19 Early Recovery Phase