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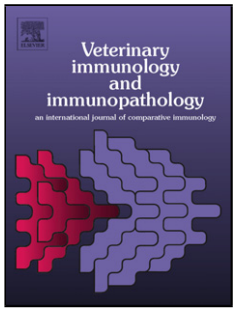

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Authors: I.W. Masa Tenaya, Kathy Heel, Philip A. Stumbles, Graham E. Wilcox

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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 $CD4^+$ T cells, but not $CD8^+$ T-cells or $CD21^+$ B-cells. At the end of the febrile phase, total numbers of both $CD8^+$ T-cells and $CD21^+$ B-cells increased significantly, while $CD4^+$ T-cell numbers remained below normal values, resulting in a significantly reduced $CD4^+$:$CD8^+$ ratio. We speculate that the persistent depletion of $CD4^+$ T cells following JDV infection, through lack of $CD4^+$ 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 $CD21^+$ B cell numbers. Further, our previous data showing that IgG^+ plasma cells are targets for JDV infection, correlated with our current data demonstrating an increase in $CD8^+$ 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.

144

145 ***Lymphocyte preparation***

146 Peripheral blood mononuclear cells were isolated using Ficoll-Paque plus density separation
147 (Amersham Biosciences, Australia) following the manufacturer's instructions, then washed
148 twice in FACS (fluorescence-activated cell sorting) buffer (Dulbecco's phosphate-buffered
149 saline [Thermo Scientific] supplemented with 5% heat inactivated FCS (Bovogen
150 Biologicals, Australia) and 0.05% sodium azide (Sigma-Aldrich, Australia). The washed cells
151 were then resuspended in FACS buffer and adjusted to a density of 1 x 10^7 cells/ml, and kept
152 at 5^{o}C until they were immunolabeled on the same day. Total white blood cells were counted
153 using trypan blue staining and a haemocytometer. Absolute numbers lymphocyte subsets
154 were calculated by multiplying the total lymphocyte count by the proportion of $CD4^+$, $CD8^+$
155 or $CD21^+$ cells as determined by flow cytometry (see below).

156

157 ***Antibodies and cellular markers***

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

163

164 ***Cell surface labeling of lymphocytes***

165 Due to Australian quarantine and logistical requirements, peripheral blood lymphocytes were
166 antibody-labeled and fixed on-site in Bali prior to transport to Australia for analysis by flow
167 cytometry at the University of Western Australia, using a previously validated protocol
168 (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

194 standard operating software on the FACSCalibur. Experimental data were analyzed and
195 population statistics calculated using FlowJo V7.2.5 (Tree Star Inc., USA) flow cytometric
196 analysis software.
197
198 ***Statistical analysis***
199 The absolute numbers of lymphocyte subsets were calculated by multiplying the percentage
200 of each lymphocyte subset obtained from flow cytometric analysis with the total lymphocyte
201 counts/ml, and were reported as a mean ± standard deviation (SD). A one-way ANOVA
202 (SPSS® 17.0) was used to assess group differences in the lymphocyte populations, while
203 differences between time points during infection were analyzed using Bonferroni's multiple
204 comparison. Significant differences in the mean values at different time points was analysed
205 by Tukey's HSD test. A value of $p<0.05$ was considered significant for all analyses.
206

206 **Results**

207

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

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

219

220 During the febrile and early recovery phases, the total number of $CD4^+$ T-cells decreased
221 significantly (Table 2 and Figure 5) and the percentage of $CD4^+$ T cells remained below
222 normal values until well after the febrile phase and into the early recovery phase (Figure 2).
223 Conversely, the total number of $CD8^+$ T-cells was reduced slightly during the febrile period
224 but increased significantly ($p<0.001$) above normal values in the early recovery phase (Table
225 2 and Figure 5). This corresponded to a slight decrease in the percentage of $CD8^+$ T cells
226 amongst PBMC during the febrile and early recovery phases compared to pre-infected cattle
227 (Figure 3).

228

229 Due to the dramatic depletion of $CD4^+$ T-cells and significant increase in $CD8^+$ T-cells after
230 JDV infection, the $CD4^+$:$CD8^+$ T-cell ratio also decreased significantly ($p<0.05$) from 0.5:1 at

231 pre-infection to 0.25:1 and 0.01:1 during the febrile phase and post-febrile phase, respectively
232 (Table 3).
233
234 Total numbers of $CD21^+$ B-cells increased slightly during the febrile phase then increased
235 significantly (p<0.001) during the early recovery phase (Table 2 and Figure 5). This
236 corresponded with an increase in the percentage of $CD21^+$ B cells during the febrile and early
237 recovery phases (Figure 4).
238
239

239 **Discussion**

240 The nature of the response of Bali cattle to JDV infection, an acute disease process with a
241 short incubation period, a case fatality rate of about 17% and no recurrence of disease in
242 those animals that recover, is unusual for a lentivirus. The lack of any recurrence of disease in
243 animals that recover suggests the development of a strong protective immunity. The absence
244 of JDV-specific antibody until at least 5 weeks and not in most cattle until 11 weeks after
245 infection (Hartaningsih et al., 1994) implies that cell-mediated immune responses play a
246 major role in the recovery of the infected animals and probably in their continuing immunity.
247 The current study assessed the responses of peripheral blood lymphocyte subsets to JDV
248 infection in experimentally infected animals to gain insights into the kinetics of the
249 lymphocyte response following infection.
250
251 The use of flow cytometric analysis confirmed the previous report of the significant decrease
252 in $CD4^+$: $CD8^+$ T-cell ratio of lymphocytes in lymphoid tissues during the acute phase of
253 Jembrana disease but not during early post-febrile stages (Dharma et al., 1994). In this
254 current study, both $CD4^+$ and $CD8^+$ T-cells in peripheral blood significantly decreased during
255 the febrile phase compared to before infection, and this period corresponds to the duration of
256 the lymphopenia reported during the febrile phase of Jembrana disease (Soesanto et al.,
257 1990). The number of $CD8^+$ T-cells remained greater than $CD4^+$ T-cells during the febrile
258 phase but increased markedly during the post-febrile phase. Due to the significant increase of
259 $CD8^+$ T-cell numbers at the end of the febrile phase and a continuous depletion of $CD4^+$ T-
260 cells, this resulted in a dramatic decrease in the $CD4^+$:$CD8^+$ T-cell ratio during the post-
261 febrile phase.
262

263 The significant increase in the $CD8^+$ T-cell population after the febrile phase strongly
264 correlated with the expansion of $CD3^+$ T-cell numbers previously found in lymphoid tissues
265 during this stage (Desport et al., 2009a). This positive correlation may indicate that the
266 majority of the $CD3^+$ T-cells were $CD8^+$ T-cell subsets. Further, the increased population of
267 $CD8^+$ T-cells during JDV infection, in the absence of JDV-specific antibody until several
268 weeks after JDV-infection (Hartaningsih et al., 1994), provides additional support for the role
269 of these cells in the recovery from the acute disease process. Virus-specific $CD8^+$ cytotoxic
270 T-cells may play an important role in host defence against lentivirus infections (Levy, 1993;
271 Salk et al., 1993). The antiviral role of $CD8^+$ cytotoxic T lymphocytes has been considered to
272 be important in the inhibition of the progression of early EIAV infection before the
273 production of virus neutralizing antibody (Hammond et al., 1997; McGuire et al., 2004;
274 McGuire et al., 1994). It is also thought to be important in non-progressor HIV-infected
275 individuals (Cao et al., 1995; Migueles et al., 2002), and in controlling SIV replication and
276 protection against SIV challenge (Genesca et al., 2009; Genesca et al., 2008; Jin et al., 1999).
277 It is only in the transition to chronic infection that the impressive early potency of the
278 antiviral $CD8^+$ cytotoxic T-cells may wane (Pantaleo et al., 1997), which has been linked
279 with declining $CD8^+$ T cell function and the inability of the immune system to control viral
280 replication and spread of the virus (Migueles et al., 2002; Pantaleo et al., 1997; Zhang et al.,
281 2003). As with other lentivirus infections, at least during acute infections, the results of this
282 study tend to support the current hypothesis that virus-specific $CD8^+$ cytotoxic T
283 lymphocytes may play a crucial role in host defence against lentivirus infections (Levy,
284 1993), also supported by preliminary data showing elevated IFNγ mRNA during the recovery
285 phase (MT, PS, GW unpublished observations), however the specificity and anti-viral activity
286 of the $CD8^+$ T cell response for JDV remains to be determined.
287

288 It is unclear why $CD4^+$ T-cells were dramatically decreased despite the absence of infection
289 of T-cells by the virus, as shown in previous studies (Desport et al., 2009b) and again
290 confirmed in this study by immunohistochemistry (by co-staining lymphoid tissue with anti-
291 CD3 and anti-JDV capsid protein mAb) and by in situ hybridization for JDV RNA (data not
292 shown). A gradual depletion of $CD4^+$ T-cell subsets is associated with infection with the T-
293 cell tropic lentiviruses HIV (Alcami, 2004; Blankson et al., 2002; Brenchley et al., 2004;
294 Clapham and McKnight, 2001), SIV (Brown et al., 2007; Mattapallil et al., 2005; Picker,
295 2006) and FIV (Ackley et al., 1990). However, reduction of $CD4^+$ T-cell populations is not
296 always related to their infection by viruses. In EIAV infections, for example, both circulating
297 $CD4^+$ and $CD8^+$ T-cell subsets are reduced significantly during acute infection, although
298 mature macrophages and not T-cells are the main target cells of the virus (Cook et al., 2001;
299 Murakami et al., 1999; Oaks et al., 1998; Sellon et al., 1992). In EIAV infection, depletion of
300 the T-cell subsets is possibly an indirect effect of the virus infection or virus components
301 (Murakami et al., 1999). This so-called "bystander" depletion has also been observed in
302 uninfected $CD4^+$ T cells HIV infection, where induction of cell-surface pro-apoptotic
303 molecules such as Fas/FasL, viral proteins and undefined soluble mediators have been
304 implicated (Arokium et al., 2009; Ji et al., 2007; Holm and Gabuzda, 2005).
305
306 The population of $CD21^+$ B-cells increased prior to the febrile phase, indicating a transient
307 proliferation of B-cells or release of B-cells into the peripheral blood during this phase,
308 similar to that reported during BIV infection (Whetstone et al., 1997). The reason for this is
309 unknown but in HIV-1, a putative polyclonal B-cell stimulatory epitope has been found in the
310 carboxyl end of the envelope glycoprotein of the virus, specifically associated with Nef
311 (Chirmule et al., 1994). Tmx, an accessory protein of unknown function in bovine
312 lentiviruses, is expressed at a similar region of the genome as *nef*. (Chadwick et al., 1995;

313 Gonda et al., 1990). Nef is multifunctional, but mainly responsible for viral infectivity
314 (Brugger et al., 2007; Marsh, 1999; Qi and Aiken, 2008; Sol-Foulon et al., 2004). Although
315 the *nef* gene is not present in the bovine lentiviruses, they have a *tmx* gene in a similar
316 location to *nef*, and 2 unique genes *vpw* and *vpy* that seem to be analogous to the *vpr* and
317 *vpu/vpx* genes of primate lentiviruses (Garvey et al., 1990). Tmx may be involved in a
318 manner similar to Nef in the proliferation of B-cells *in vivo,* however this hypothesis would
319 need to be confirmed. During the febrile phase, there was a progressive reduction in the
320 numbers of $CD21^+$ B-cells which may be associated with replication of virus in these cells.
321

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.

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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).

509

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

517

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

525

525 **Table 1.** Primary and secondary antibodies used for flow cytometric analysis of
526 lymphocytes from cattle infected with JDV.

Antibody	Source	Isotype/clone	Cat./Lot No
Primary antibody			
Mouse anti-bovine CD4	Serotec	IgG2a/CC8	MCA1653G
Mouse anti-bovine CD8	Serotec	IgG2a/CC63	MCA1653G
Mouse anti-bovine CD21	Santa Cruz	IgG2b/CC51	SC-101835
Secondary antibody			
Goat anti-mouse	Invitrogen	Alexa Fluor 488	A-11029

527
528

528 **Table 2.** Comparison of lymphocyte subsets during 3 major phases after JDV infection.

Lymphocyte population	Mean absolute number cells/ml ± SD		
	Pre-infection	Febrile phase	Post-febrile phase
T-helper cells, $CD4^+$	2418 ±277[a]	176± 171[b]	45 ±10 [b]
Cytotoxic T-cells, $CD8^+$	1210±206 [b]	768±489[b]	3187±601 [a]
B-cells, $CD21^+$	1799 ± 404 [b]	2065±823[b]	4225 ± 841 [a]

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

530 (p<0.05).

531

532

532 **Table 3.** Changes in $CD4^+$:$CD8^+$ T-cell ratio during the course of JDV infection.

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

533

534

Figure 1

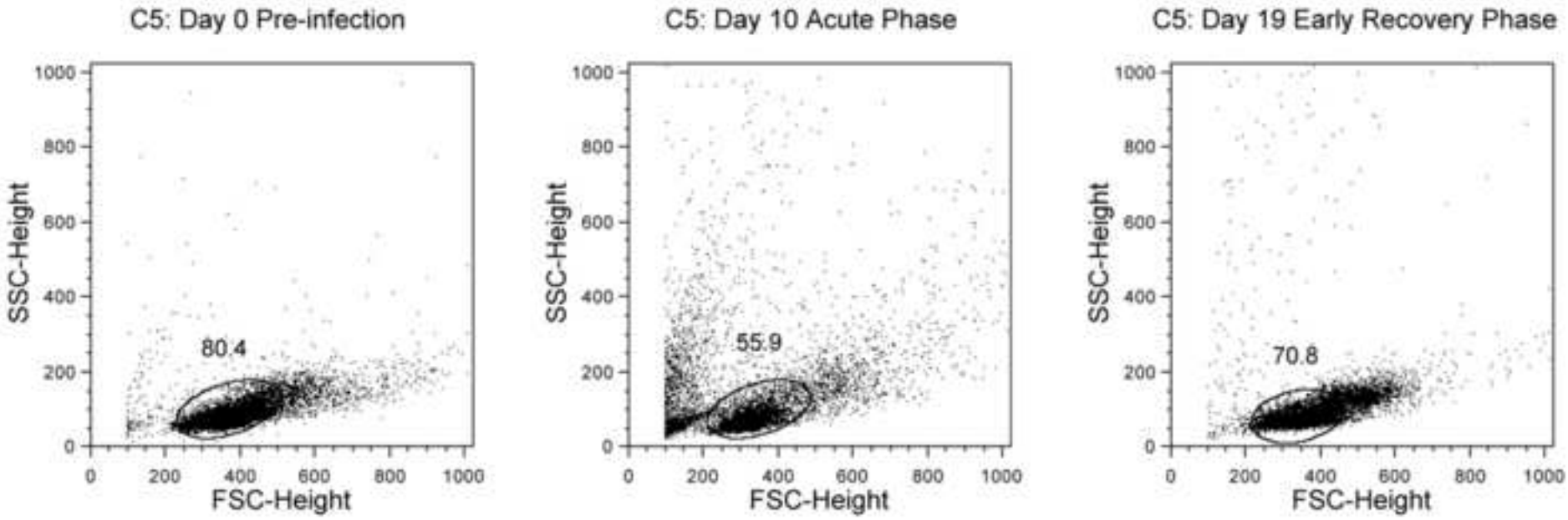


Figure 2

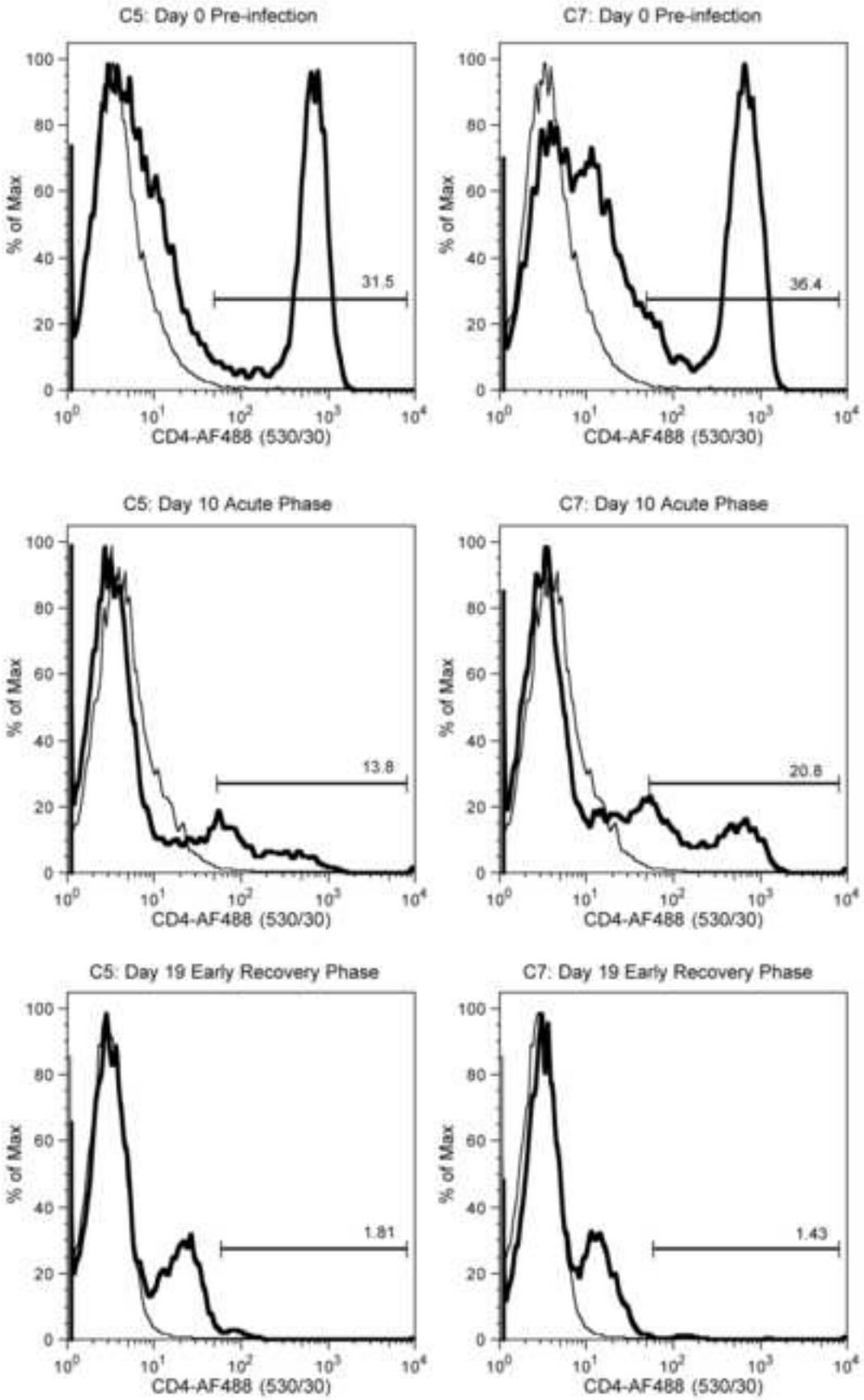


Figure 3

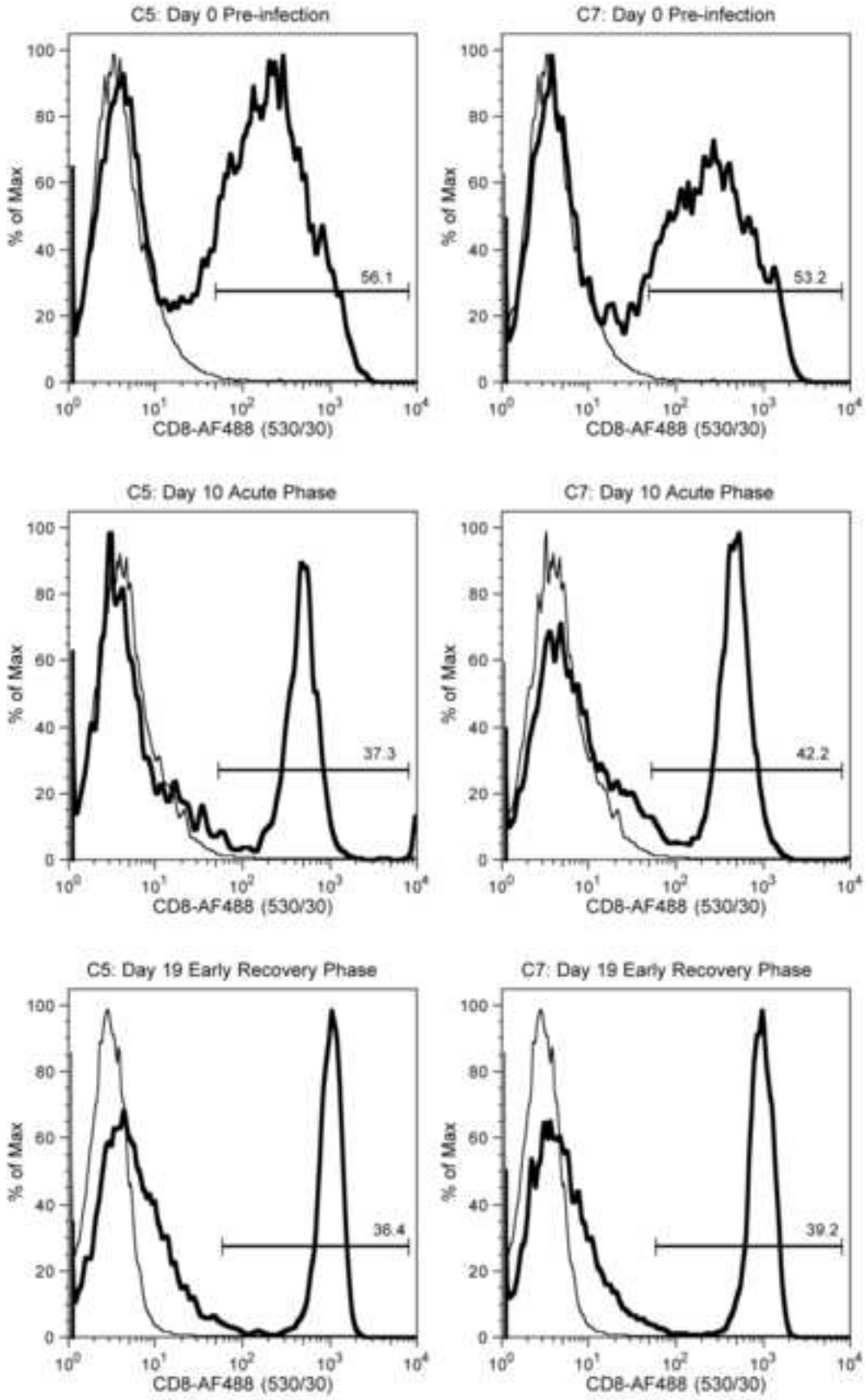
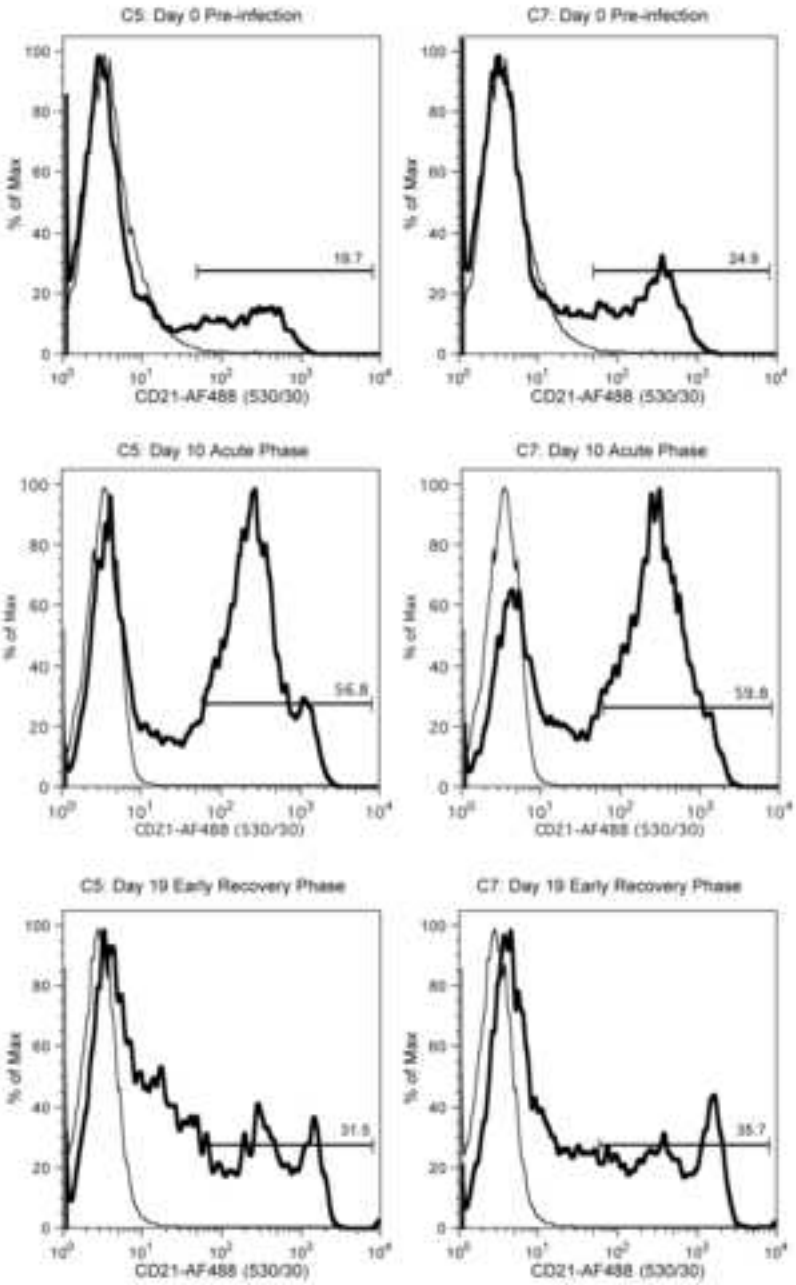


Figure 4



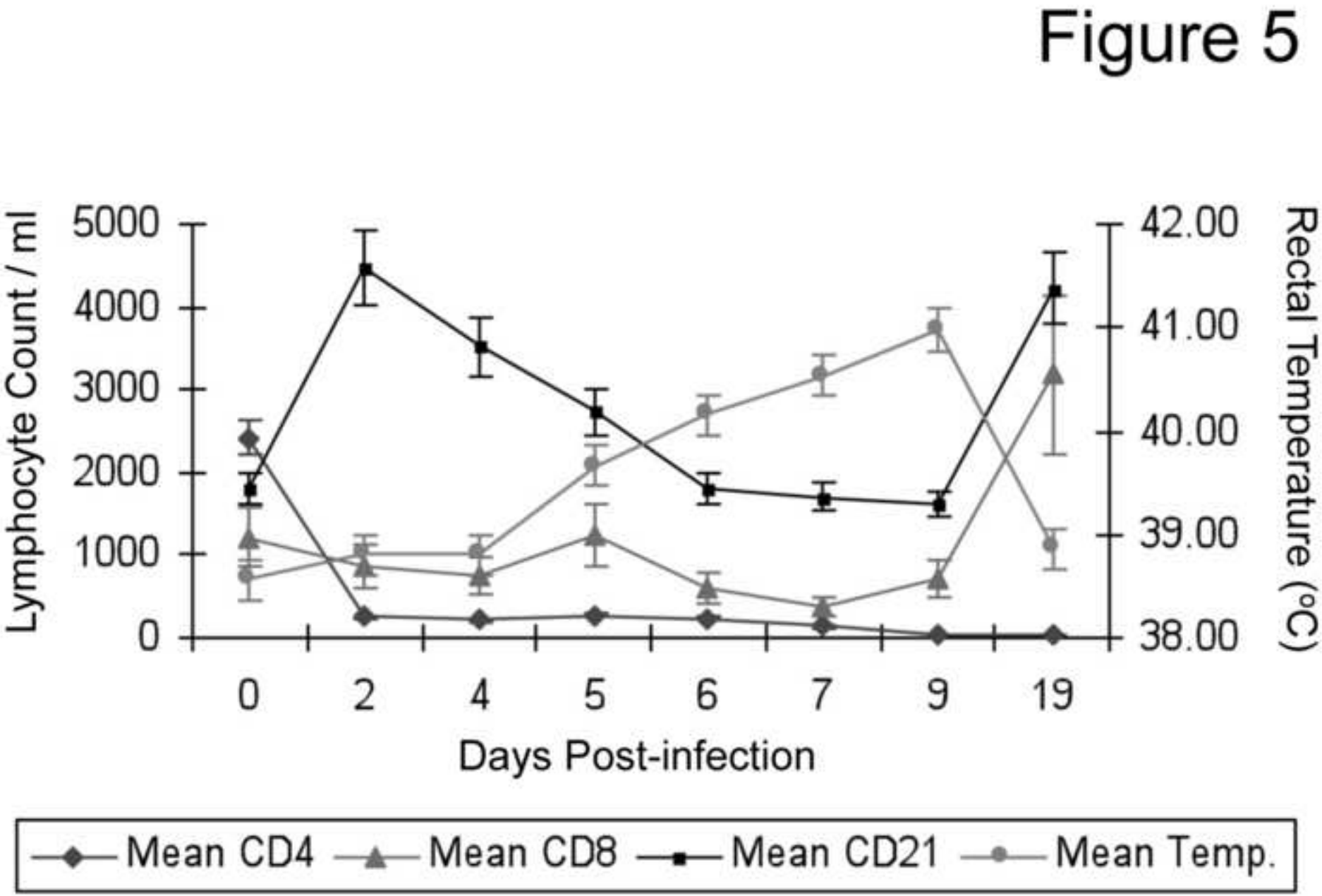


Figure 5
Lymphocyte Count / ml
5000
4000
3000
2000
1000
0
Rectal Temperature (°C)
42.00
41.00
40.00
39.00
38.00
0
2
4
5
6
7
9
19
Days Post-infection
Mean CD4
Mean CD8
Mean CD21
Mean Temp.