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Tenaya, I.W., Heel, K., Stumbles, P.A. and Wilcox, G.E. (2012)
Flow cytometric analysis of lymphocyte subset kinetics in Bali cattle experimentally infected with Jembrana disease virus.
Veterinary Immunology and Immunopathology, 149 (3-4). pp. 167-176.

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

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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

Please cite this article as: Tenaya, I.W.M., Heel, K., Stumbles, P.A., Wilcox, G.E., Flow Cytometric Analysis of Lymphocyte Subset Kinetics in Bali Cattle Experimentally Infected with Jembrana Disease Virus, *Veterinary Immunology and Immunopathology* (2010), doi:10.1016/j.vetimm.2012.06.013

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1 **Flow Cytometric Analysis of Lymphocyte Subset Kinetics in Bali Cattle**
2 **Experimentally Infected with Jembrana Disease Virus**

3

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22 **Abstract**

23 Jembrana Disease Virus (JDV) is an unusual bovine lentivirus that causes an acute and
24 sometimes fatal disease after a short incubation period in Bali cattle (*Bos javanicus*). The
25 pathological changes occur primarily in lymphoid tissues, which feature proliferating
26 lymphoblastoid-like cells predominantly throughout parafollicular (T-cell) areas, and atrophy
27 of follicles (B-cell) areas. Five Bali cattle were experimentally infected with JDV and all
28 developed typical clinical signs of Jembrana disease characterized by a transient febrile
29 response, enlargement of superficial lymph nodes and a significant leukopenia. Flow
30 cytometric analysis of PBMC during the acute (febrile) disease phase showed that the
31 reduced number of lymphocytes was due to a significant decrease in both the proportion and
32 absolute numbers of $CD4^+$ T cells, but not $CD8^+$ T-cells or $CD21^+$ B-cells. At the end of the
33 febrile phase, total numbers of both $CD8^+$ T-cells and $CD21^+$ B-cells increased significantly,
34 while $CD4^+$ T-cell numbers remained below normal values, resulting in a significantly
35 reduced $CD4^+$:$CD8^+$ ratio. We speculate that the persistent depletion of $CD4^+$ T cells
36 following JDV infection, through lack of $CD4^+$ T cell help to B cells, may explain the lack of
37 production of JDV-specific antibodies for several weeks after recovery despite an increase in
38 $CD21^+$ B cell numbers. Further, our previous data showing that IgG^+ plasma cells are targets
39 for JDV infection, correlated with our current data demonstrating an increase in $CD8^+$ T cell
40 numbers, supports the suggestion that anti-viral cytotoxic T cell or other cell-mediated
41 immune responses may be critical in the recovery process, although this remains to be
42 formally demonstrated for JDV.

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43 **Keywords**

44 Jembrana Disease Virus; Lentivirus; Cattle; Flow cytometry; Lymphocyte subpopulations;

45 Immunodeficiency

46

47 **Abbreviations**

48 BIV, bovine immunodeficiency virus; EDTA, ethylenediaminetetraacetic acid; EIAV, equine

49 infectious anemia virus; FIV, Feline immunodeficiency virus**;** FACS, fluorescence-activated

50 cell sorting; HIV, Human immunodeficiency virus; IgG, immunoglobulin G; JDV, Jembrana

51 Disease Virus; mAb, monoclonal antibody; RNA, ribonucleic acid; SIV, Simian

52 immunodeficiency virus.

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53 **Introduction**

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

70

71 In experimentally infected cattle, the case fatality rate was originally estimated to be 17%
72 (Soeharsono et al., 1990) but was more recently reported to be 11.5% (Desport et al., 2009a).
73 For naturally infected animals the case fatality rates vary: a mortality rate of 98.9% was
74 reported during the first outbreak in 1962 however in more recent outbreaks mortality rates
75 have varied from 20 – 37% in a new areas where JDV has not been previously reported
76 (Ramachandran et al., 1996). During the acute phase, a high titer of virus (~10^{10}RNA genome
77 copies/ml plasma) is present in the plasma, persisting at low levels for at least 25 months

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78 after recovery from the acute disease (Soeharsono et al., 1990; Stewart et al., 2005). The
79 experimentally infected Bali cattle that survive the acute clinical disease do not develop any
80 further clinical disease (Soeharsono et al., 1990; Soesanto et al., 1990) and resist re-infection
81 (Soeharsono et al., 1990) but the immune mechanism responsible for recovery from the acute
82 disease and continued immunity has not been defined. Although not formally proven, there is
83 circumstantial evidence to suggest that neutralizing antibody does not play a major role in
84 recovery as JDV-specific antibodies are not detectable until some weeks after recovery from
85 the acute disease (Hartaningsih et al., 1994). This has lead to the hypothesis that cellular
86 immune responses, presumably through IFNγ-mediated activation of viral-specific $CD8^+$
87 cytotoxic T lymphocytes, play a critical role in recovery from JDV infection, as is the case
88 for equine infectious anemia virus (EIAV) (Murakami et al., 1999) and HIV (Migueles and
89 Connors, 2002), although again this remains to be formally proven for JDV.
90
91 Hyperplasia of T-cell areas and depletion of B-cell areas of lymphoid tissues during acute
92 Jembrana disease is a hallmark of the disease (Dharma et al., 1991). Depletion of lymphocyte
93 ~~[the $CD4^+$ T-cell and $CD8^+$ T-cell]~~ populations was observed histologically in JDV-infected
94 Bali cattle and significant differences were found during acute illness in follicular
95 compartments of lymph nodes (Dharma et al., 1994). The acute febrile phase of Jembrana
96 disease is characterised by marked haematological changes that include leucopenia due to
97 lymphopenia, eosinopenia, and a slight neutropenia and thrombocytopenia (Soesanto et al.,
98 1990). The mechanism for the changes in lymphocyte populations in Jembrana disease
99 remains unknown. It was suggested that the gradual depletion of $CD4^+$ T-cells may have been
100 due to infection of T-cells (Dharma et al., 1994). Although T-cells are the predominant target
101 cell of some lentiviruses, including HIV (Alcami, 2004; Blankson et al., 2002; Brenchley et
102 al., 2004; Clapham and McKnight, 2001), SIV (Brown et al., 2007; Mattapallil et al., 2005;

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103 Picker, 2006) and FIV (Ackley et al., 1990), there is no evidence for infection of $CD3^+$ T-
104 cells or $MAC387^+$ monocytes by JDV and the target virus infection was identified in
105 lymphoid tissues as pleomorphic centroblast-like cells which were identified as IgG-
106 containing cells, including plasma cells (Desport et al., 2009b).
107
108 Although lymphopenia is a characteristic feature of Jembrana disease (Soesanto et al., 1990),
109 changes in circulating lymphocyte subsets during the acute disease have remained
110 uncharacterized. We report a flow cytometric analysis of the circulating $CD4^+$ T cell, $CD8^+$ T
111 cell and $CD21^+$ B cell populations during the febrile and early post-febrile phases undertaken
112 to better understand the acute disease process associated with JDV infection. Our data
113 indicate that shortly after the end of febrile periods (9 days after infection), $CD8^+$ T cell
114 numbers were elevated and this coincided with increased of INFγ expression. Given that
115 antibodies were not detected until several weeks after the initial infections, this data may
116 explain why the majority (80%) of experimentally JDV-infected animals survived and
117 support the conclusion that $CD8^+$ T cell-mediated protection is important in recovery from
118 JDV infection.
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119 **Materials and Methods**

120

121 ***Experimental animals and sample collection***

122 Bali cattle used in the experimental studies were female, approximately 12 months of age and
123 weighed 80-100 kg. They were obtained from Nusa Penida, a small island adjacent to Bali,
124 where Jembrana disease has never been reported and where antibodies to JDV have not been
125 detected (Hartaningsih et al., 1994). Cattle purchased from the island have consistently
126 developed clinical signs of Jembrana disease when infected with JDV (Soeharsono et al.,
127 1990). Animals for these experiments were transported to Bali island to the Disease
128 Investigation Centre Region VI, Denpasar, Bali. On arrival, they were sprayed with
129 insecticide, kept in an insect-proof house and given water and elephant grass (*Penecetum*
130 *purpureum*) *ad libitum*. Prior to use, all cattle were pre-treated with a broad spectrum
131 antibiotic (oxytetracycline) at a dose rate of 5 mg/kg bodyweight for 3 consecutive days, a
132 broad spectrum anti-helmintic, and they were vaccinated against haemorrhagic septicaemia.
133 Before inoculation with JDV, the absence of antibody to JDV was confirmed by ELISA test
134 using a JDV recombinant CA antigen as described previously (Burkala et al., 1998).

135

136 Five Bali cattle were infected intravenously with an estimated 100 infectious doses of JDV
137 contained in a 10% homogenate in Dulbecco's modified Eagle's medium of spleen which had
138 been stored at −70 °C and previously prepared from an animal experimentally infected with
139 JDV_{Tab87} (Soeharsono et al., 1995). The febrile phase in the inoculated animals occurred from
140 5-11 days after infection. Blood samples were analysed prior to infection (day 0), at day 2
141 and daily from days 4 to 7 (febrile phase) and day 9 post-infection, and then finally at day 19
142 when the animals were euthanased for autopsy. Sterile EDTA-containing vacutainer tubes
143 (Greiner Bio-One) were used to collect blood samples for recovery of lymphocytes.

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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°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

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169 preparation, 1 ml of the lymphocyte suspension was incubated with 100 μl of primary
170 antibody in FACS buffer for 30 minutes at 4°C, followed by 3 washes with FACS buffer (by
171 centrifugation for 1 min at 479 *g* at 4°C). Secondary antibody (100 μl) diluted in FACS buffer
172 was applied and incubated for 30 minutes at 4°C in the dark. The cell suspensions were then
173 gently washed 3 times in FACS buffer, then washed once with PBS and the cells resuspended
174 in 200 μl of fixation buffer (Dulbecco's phosphate-buffered saline supplemented with 4%
175 paraformaldehyde) for 5 minutes at 37°C. Finally, the cells were washed with 200 μl of ice-
176 cold Dulbecco's phosphate-buffered saline supplemented with 1% BSA (Sigma Aldrich,
177 Australia) and resuspended in 1 ml of freezing medium (Dulbecco's phosphate-buffered
178 saline supplemented with 1% BSA and 10% dimethyl sulfoxide (Sigma Aldrich, Australia)
179 before being transferred to freezing vials and then stored at -80°C. Samples were stored for
180 up to 2 months in Bali prior to transport to Australia for analysis by flow cytometry.
181
182 ***Flow cytometric analysis***
183 Prior to flow cytometric analysis, cryopreserved lymphocyte samples were thawed rapidly at
184 37°C in a water bath, then washed once with wash buffer (Dulbecco's phosphate-buffered
185 saline supplemented with 0.1% BSA) and resuspended in 1 ml of labeling buffer (Dulbecco's
186 phosphate-buffered saline supplemented with 10% heat-inactivated FCS and 0.1% sodium
187 azide [Sigma Aldrich]). The immunolabeled samples were analyzed using a BD FACS
188 Calibur flow cytometer (BD Bioscience, Australia) with a 488 nm excitation laser.
189 Lymphocytes were gated in a forward scatter/side scatter plot (FSC-H vs SSC-H). A
190 bandpass-specific filter (FL1, 530/30 nm) was used for Alexa Fluor 488 emission and
191 acquired in the logarithmic scale. A minimum of 10,000 lymphocytes were examined per
192 sample and an AF488 fluorescence histogram was used to compare the samples. Sample data
193 were analyzed using BD CellQuest Pro V5.2 (BD Biosciences, Australia) which is the

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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.
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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

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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
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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.

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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;

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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.
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321 **Conclusions**

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

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

339

340 **Funding sources**

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

343

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484 **Figure Captions**

485

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

493

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

501

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

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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.

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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

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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 ($p<0.05$).

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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

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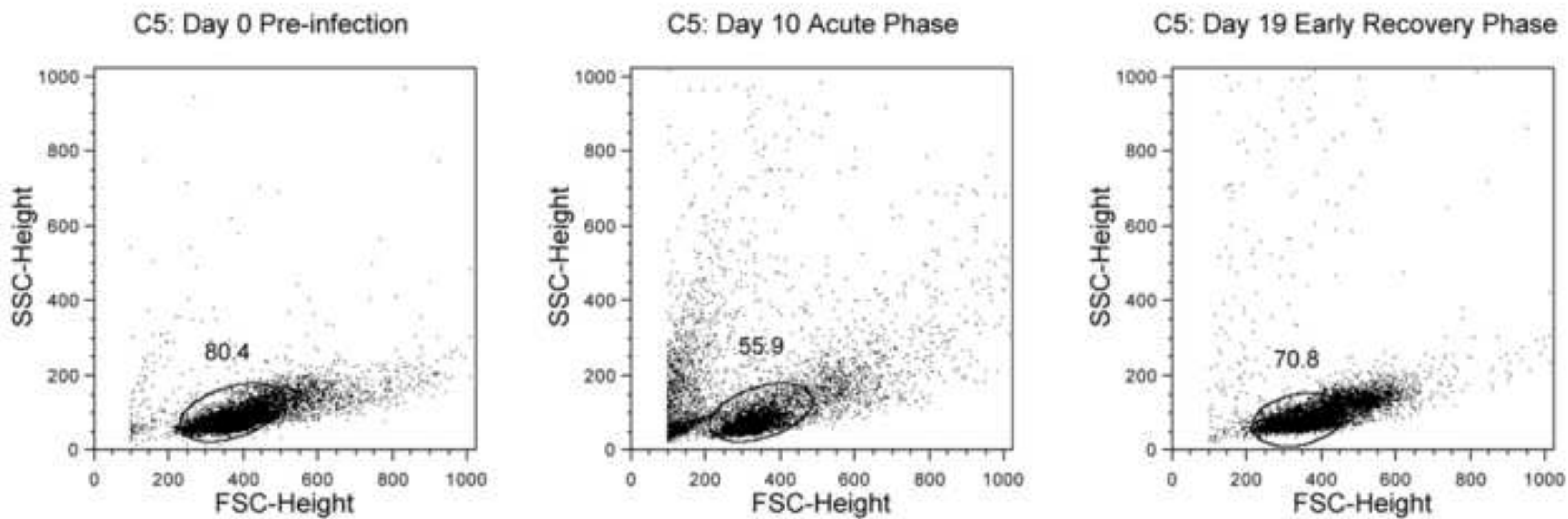

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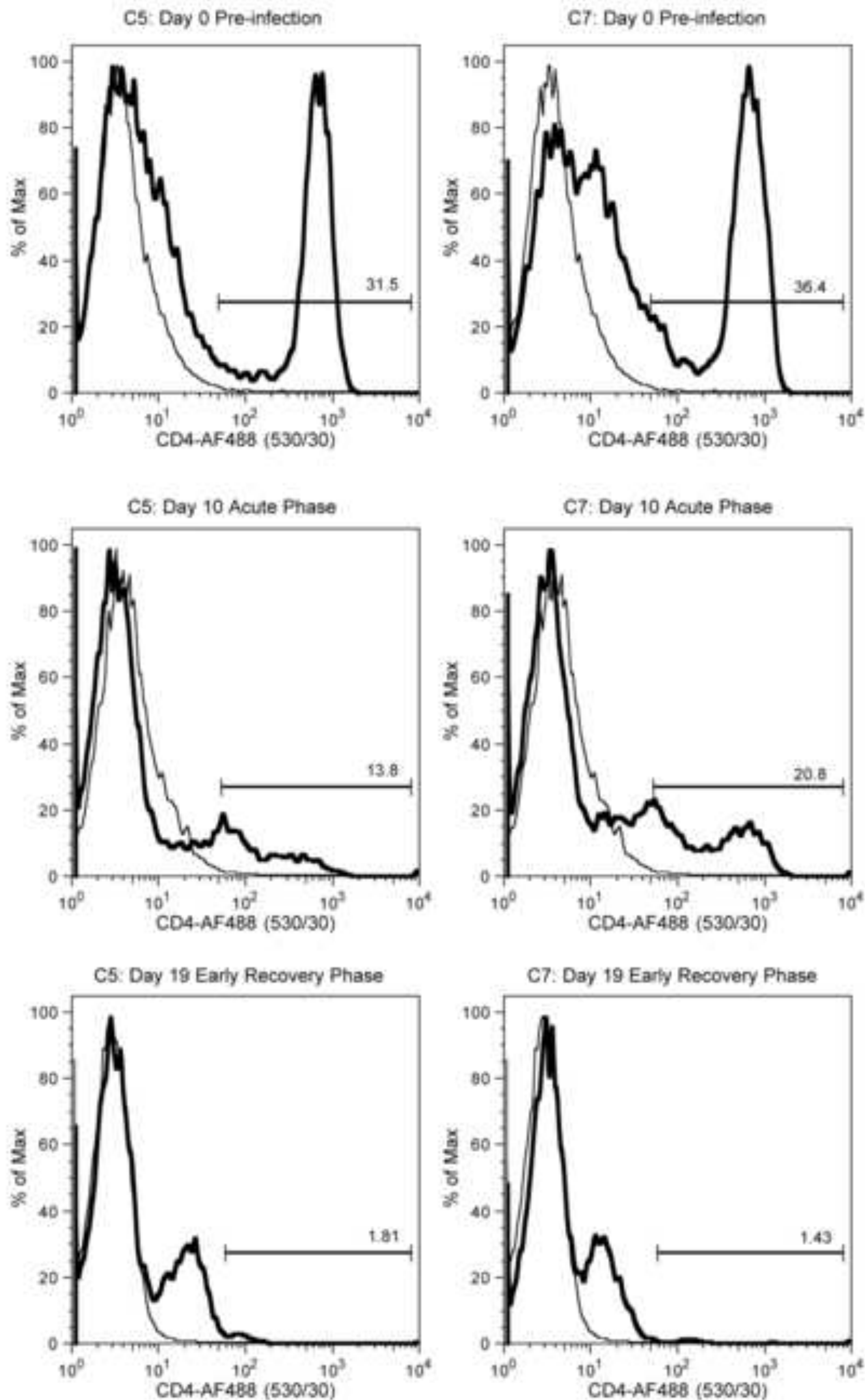

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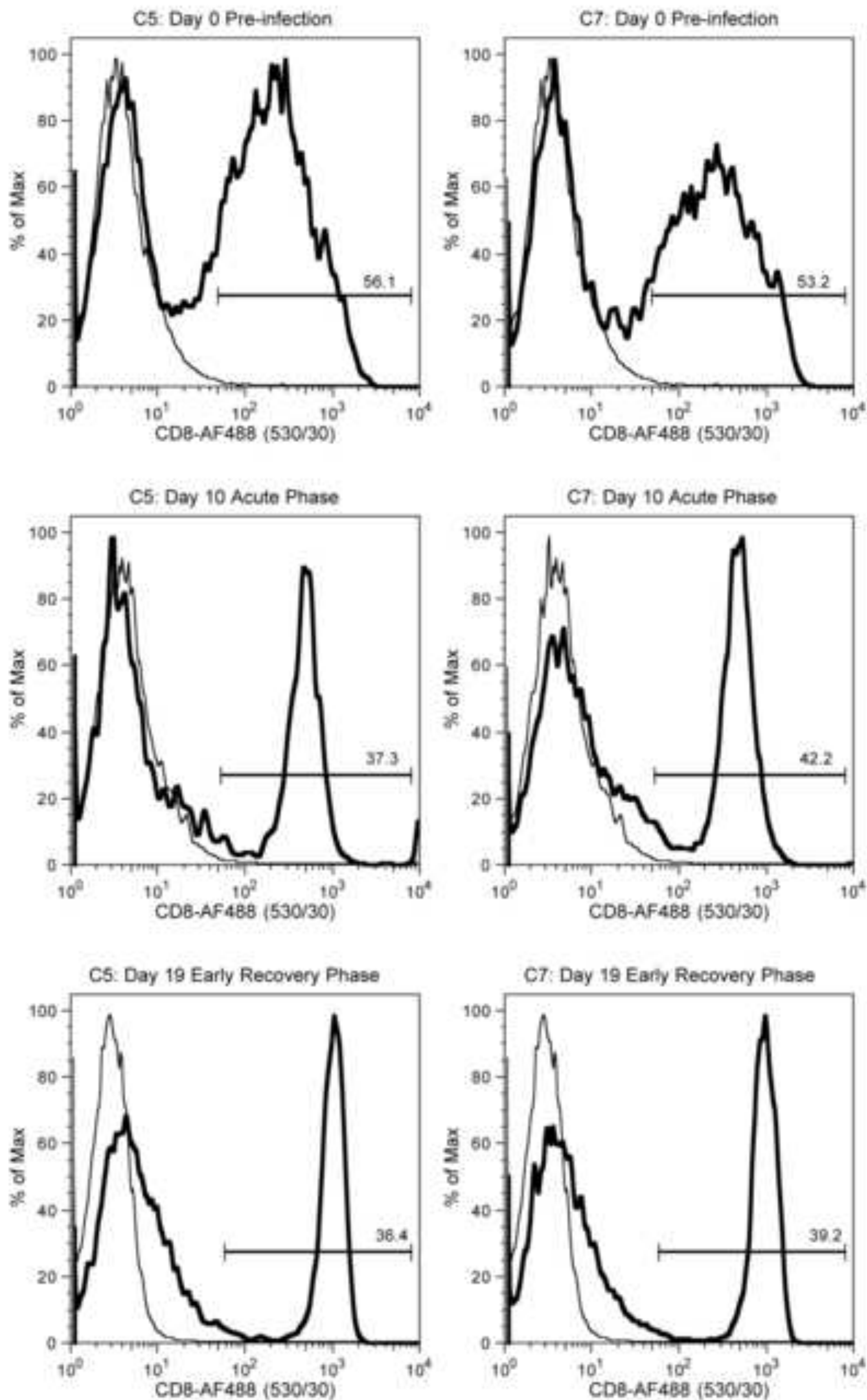

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Figure 4

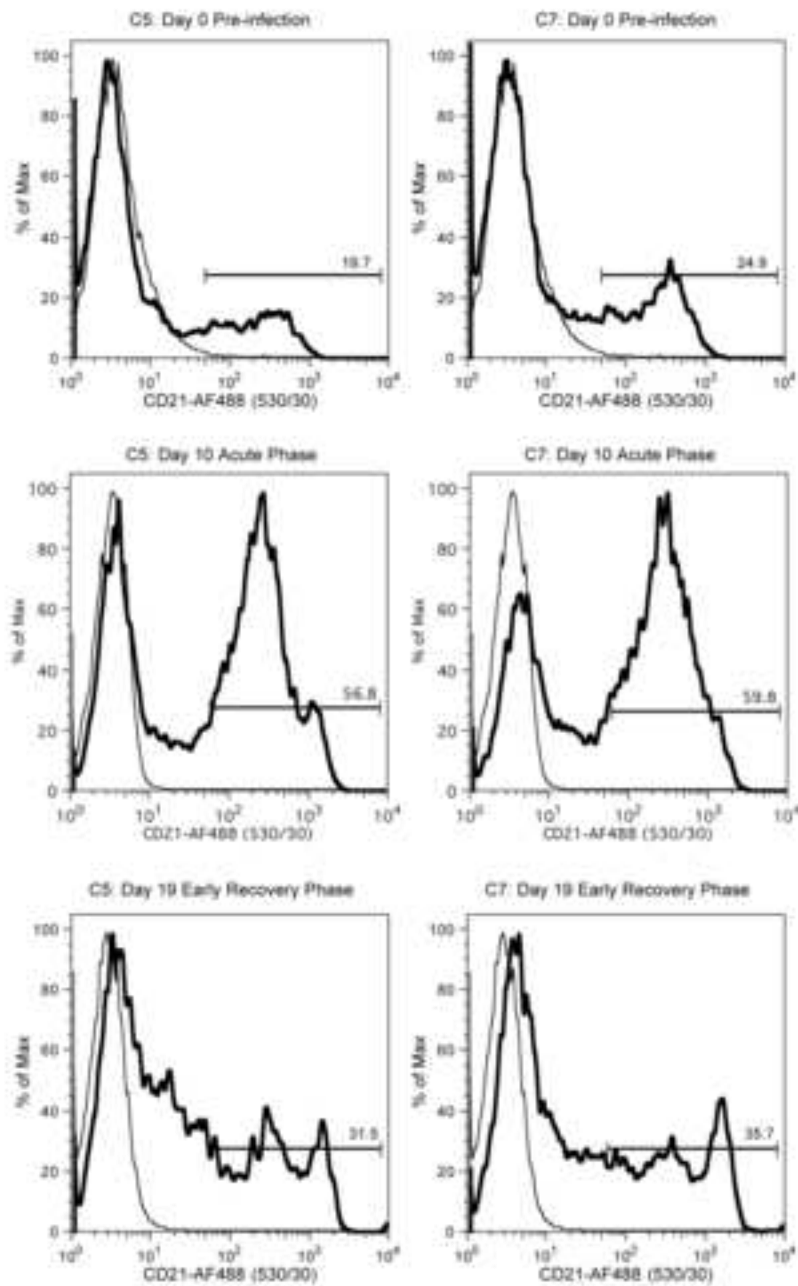

Figure 5