

# Immunohistochemical Characterization and Quantification of Lymphocytes Infiltrating Bovine Lungs in East Coast Fever

Vallery M. J. Kessy, BVSc, MVM<sup>1</sup>

Jayro A. Matovelo, BVSc, PhD<sup>2\*</sup>

<sup>1</sup>*Veterinary Investigation Center Temeke  
Ministry of Livestock Development, Tanzania*

<sup>2</sup>*Department of Veterinary Pathology  
Sokoine University of Agriculture  
Morogoro, Tanzania*

\*Corresponding author (J. A. Matovelo): [matovelo@suanet.ac.tz](mailto:matovelo@suanet.ac.tz)

**KEY WORDS:** East Coast fever, immunohistochemistry, pathology, cattle

## ABSTRACT

This study was conducted to investigate the hypothesis that the lungs in East Coast fever are infiltrated by different subsets of lymphocytes that may be responsible in the pathogenesis of pulmonary edema. Four steers, 7 – 10 months old, were experimentally infected by subcutaneous injection with 0.5 ml of live *Theileria parva* sporozoites (Muguga stabilate 3087). Two others remained as uninfected controls. All six animals were clinically monitored daily before and after infection by screening peripheral blood smears, lymph node smears, rectal temperatures, lymph node enlargement, coughing, and dyspnea. Also, blood samples were taken at intervals of 3-4 days and analysed for total RBC and WBC counts, Hb, PCV, and differential leukocyte count. Each of the infected animals were humanely exsanguinated to death under general anaesthesia at the onset of dyspnea, together with one of the controls for sampling of lung tissues. In addition, two lung tissue samples were collected from two ECF naturally

infected cattle that were slaughtered during the advanced stage of the disease. The lung tissue samples from the experimentally and naturally infected animals were fixed in 4% neutral-buffered formaldehyde (pH 7.4) for 48 hours, and subjected to tissue processing procedures to obtain 4 µm thin sections that were stained routinely by H & E for histopathological examination and immunohistochemically by monoclonal antibodies labelled by streptavidin-biotin peroxidase complex to visualize lymphocyte subsets BoCD21,+ BoCD4,+ BoCD8,+ and BoWC1.+ Rectal temperatures started to increase above normal by day 8 post-infection while ECF was confirmed by lymph node smear examination by day 7 and dyspnea by day 15 post infection. It was demonstrated that the majority of lymphocyte subsets infiltrating the lungs of cattle infected with *Theileria parva* are BoCD4+ and BoCD8+ in the acute and advanced stages of ECF, respectively. The BoCD8+ T cells were about 11 times more than the rest of the other subsets in the naturally infected animals slaughtered in the advanced stages of ECF. The apparent higher mean number of both BoCD21+ and BoWC1+ during midway of the course of

infection suggests that these may have more roles to play in this stage than in the advanced stages of *Theileria parva* infection. Notwithstanding the mechanisms that attract these lymphocytes into the lungs, their presence indicates that they likely bring about local release of cytokines that contribute towards development of inflammation and pulmonary edema. It was concluded that any endeavour to develop ways to treat *Theileria parva* infection should study the specific cytokines released during ECF.

## INTRODUCTION

East Coast Fever (ECF) is a tick-borne disease caused by *Theileria parva* that inhabits the lymphocytes producing proliferation of infected and non-infected lymphocytes, enlargement of lymph nodes, and high morbidity and mortality rates in susceptible cattle.

Once inoculated by vector ticks (*Rhipicephalus appendiculatus*), the *T. parva* sporozoites infect and transform the host lymphocytes. Transformation of infected lymphocytes is believed to be accompanied by expression of different lymphokines and cytokines that play different roles in the host-parasite relationship (Kuby 1997). For example, Dobbelaere and Heussler (1999) showed that presence of *T. parva* in the host cell modulates the state of activation of a number of signal transduction pathways which lead to activation of transcription factors including nuclear factor- $\kappa$ B essential for the survival of infected cells hence the parasite.

Cytokines known to be expressed by lymphocytes in the course of ECF include: interleukin-2 (IL-2), gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ) (Shayan et al; 1999; Brown and Logan, 1986; DeMartin and Baldwin 1991; Prestos et al; 1992, Dobbelaere and Heussler 1999).

Apart from cytokines expressed by live lymphocytes, other vasoactive molecules are known to be released by disintegrating lymphocytes. These together with complement fragment C3a and C5 and fibrin degradation products are regarded to be vascular permeability mediators that induce occurrence

of pulmonary edema considered to be the immediate cause of death in ECF (Soulsby 1982; Jubb et al; 1992; Shitakha et al; 1983; Lawrence et al., 1994).

Although DeMartin and Baldwin (1991) indicated that *T. parva* infects both B and T lymphocytes, phenotypic studies of parasitized cells isolated from lymphoid tissues of infected cattle indicate that the substantial numbers of infected lymphocytes are T-lymphocytes (Emery et al. 1988).

There are no reports on the types of lymphocytes that infiltrate non-lymphoid organs in cattle infected with *T. parva*. The aim of the present study was, therefore, to identify and quantify the types of lymphocytes infiltrating the lungs during both experimental and natural ECF.

## MATERIALS AND METHODS

### Experimental Animals and Sampling

Four apparently healthy steers aged between 7 - 10 months were each infected by subcutaneous injection anterior to the left prescapular lymph node with 0.5 ml of inoculum of *Theileria parva* sporozoites (Muguga stabilize 3087) purchased from the International Livestock Research Institute (ILRI) Nairobi, Kenya. Two other similar steers were kept as uninfected control animals. Each of the four infected experimental animals was exsanguinated by severing the carotid artery under pentobarbitone sodium general anaesthesia at the onset of respiratory distress/dyspnea. The two non-infected control animals were also slaughtered by the same method at the same time when the infected animals were slaughtered.

After slaughter, a post-mortem examination was conducted paying particular attention to the appearance, size, shape, colour, and consistency and cut surface of the lungs and presence of fluid in the airways.

### Tissue preparation

Lung tissues from middle portions of right and left cranial and caudal lobes were taken within 10 minutes of slaughter, and immediately put in 4% neutral-buffered formaldehyde (NBF), pH 7.4, for 48 hours.

The formalin-fixed tissues were embedded in paraffin wax and sectioned (4 µm) and then mounted on microscope slides coated with chrome-gelatin. The sections were dried for 24 hours in an oven at 400 C, removed from the oven, and kept at room temperature until required for staining.

Half of the slides were stained with H & E and examined under light microscope, while the other half were subjected to streptavidin-biotin immunoperoxidase staining technique as described by Gutierrez, et al., (1999) with modifications as described in the following.

**Sampling from natural ECF infection**

Specimens of lungs, spleen and parotid lymph nodes were also collected from two ECF naturally infected animals that were slaughtered at the Morogoro Municipal abattoir. These animals were slaughtered at the life-threatening stage of clinical ECF that was characterized by low rectal temperatures (370 — 380C), severe respiratory distress, and discharge of frothy fluid from the nostrils. Impression smears from the spleen and lymph nodes were made and stained by Giemsa.

The lung specimens collected were handled as previously described with experimentally infected animals.

**Immunohistochemical staining**

The major lymphocyte subsets were immunohistochemically stained by the Strep AB Complex/Horse Radish Peroxidase (SABC) method.

Briefly, the tissue sections were deparaffinized in three sequential xylene washes, then rehydrated in ethanol baths, and subsequently brought to water. The tissue sections were then incubated in an antigen retrieval solution, in either 10 mM citrate buffer, pH 6.0 (APPENDIX IV) (Norton et al., 1994) or Tris-buffered saline solution as antigen retrieval solution (TBS-ARS), pH 8.2 (APPENDIX V) as indicated in Table 1 at temperature of 900C – 950C for 20 minutes.

The sections were cooled for 15 minutes, and then quenched with 0.3% H2O2 in TBS for 30 minutes at room temperature, and washed in Tris-buffered saline solution containing 0.25% Triton X-100 (TBS-T).

The sections were preincubated in a humidified chamber for 60 minutes with 10% normal rabbit serum (X0902, DAKO) in TBS-T, in which 1% Bovine Serum Albumen (Sigma A-4503) (BSA) and 0.5% full cream milk Nestle® was added in order to block non-specific binding sites and to reduce background staining.

A 100 µL of each of the mAb at optimal dilution recognizing B-cell and T-cell markers were used in the study (Table 1) was applied per section in a humidified chamber overnight at room temperature.

The sections were then washed with TBS-T to remove unbound antibodies and artifacts, and then incubated for 90 minutes at room temperature with biotinylated rabbit anti-mouse immunoglobulins (E0413, DAKO) diluted 1:500 in TBS-T containing 10% normal rabbit serum (X0902, DAKO),

**Table 1: Monoclonal antibodies used in the present study**

Monoclonal antibody	Ig isotype	Specificity	Reference	Dilution	Antigen retrieval solution
IL-A11	IgG2a	BoCD4; T cell subset	Baldwin <i>et al.</i> , 1986	1:100	Citrate buffer
IL-A65	IgG2a	BoCD21; B cells	Naessens <i>et al.</i> , 1990	1:100	Tris-buffered Saline
IL-A105	IgG2a	BoCD8; T cell subset	MacHugh <i>et al.</i> , 1993	1:100	Citrate buffer
IAH-CC15	IgG	BoWC1; γ/δ T cell sub set	Howard <i>et al.</i> , 1991	1:100	Citrate buffer

**Table 2.** The mean number of positive staining lymphocytes and unstained MNC per mm<sup>2</sup> in lung tissue of experimental and natural ECF cases

Type of infection Animal number	Number of leukocytic mononuclear cells (per mm <sup>2</sup> ) in lung tissue section.							
	Section stained by mAb IL-A65		Section stained by mAb IL-A11		Section stained by mAb IL-A105		Section stained by mAb IAH-CC15	
	CD21+	Unstained MNC	CD4+	Unstained MNC	CD8+	Unstained MNC	WC1+	Unstained MNC
Experimental infection								
21479	44	3338	45	2845	43	2607	25	2466
21481	39	3341	49	2796	44	2602	29	2387
21497	38	3362	49	2825	42	2651	28	2412
21498	47	3339	49	2802	39	2588	26	2303
Mean	42	3345	48	2817	42	2612	27	2392
s.e. ±	2.12	5.70	1.00	11.23	1.08	13.61	0.91	33.94
Natural infection								
21489	19	2842	11	3192	138	4299	13	2454
24902	17	2798	13	3124	146	4365	17	2336
Mean	18	2820	12	3180	142	4332	15	2395
s.e.±	±1.00	±22.00	±.00	±34.00	±4.00	±33.00	±2.00	±59.00

CD21+ = B-lymphocytes stained by monoclonal antibody IL-A65

CD4+ = Helper (TH) lymphocytes stained by monoclonal antibody IL-A11

CD8+ = Cytotoxic (TC) lymphocytes stained by monoclonal antibody IL-A105

WC1+ = Gamma-delta ( $\gamma/\delta$ ) lymphocytes stained by monoclonal antibody IAH-CC15

1% Bovine Serum Albumen (BSA). and 0.5% full cream NIDO milk.

The sections were washed four times with TBS-T, and then StreptABComplex/HRP (K 0377, DAKO) diluted 1:500 was applied for 90 minutes at room temperature in a humidified chamber and then rinsed with TBS-T.

Peroxidase activity in the sections was visualised using 0.01% H<sub>2</sub>O<sub>2</sub> in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate-chromogenic solution for Horse Radish Peroxidase (HRP) in 50mM TBS for 5 - 15 minutes at room temperature in a humidified chamber.

The sections were counterstained with Mayer's haematoxylin for 10 - 20 seconds (depending on the extent of color intensity by the DAB reaction), rinsed with distilled water, dehydrated in alcohol, and then cleared in xylene before being mounted with DPX. The slides were dried in an oven

at 37°C overnight before viewing under light microscope (Olympus BHT/BH-2 microscope) and selected photomicrographs recorded in ProFoto 100 film.

For the negative tissue control incubations (Pinkus, 1982; O'Leary, 2001), tissue specimen from lung of control animals were fixed and embedded in the same manner as the infected tissue specimens, and then stained by the same primary antibodies.

### Counting of Positively Stained B- and T-lymphocytes Subsets

Immunostained lymphocytes in sections of lungs from cattle experimentally and naturally infected with *Theileria parva* were counted under the light microscope at X100 objective in the interalveolar interstitium. This location was purposefully selected in order to avoid peribronchial areas that normally have subepithelial diffuse or dense unencapsulated lymphatic tissues/lymph nodules that are associated with the bronchi

**Table 3.** Mean number and ratio between positive lymphocytes and negative MNC in bovine lungs with ECF

Type of MNC	Number of MNC (Per mm <sup>2</sup> )		Ratios of positive to negative MNC	
	Infection type			
	Experimental	Natural	Experimental	Natural
BoCD21+	42	18	1	1
Negative MNC	3345	2820	76	156
BoCD4+	48	12	1	1
Negative MNC	2817	3158	58	262
BoCD8+	42	142	1	1
Negative MNC	2612	4332	62	31

and bronchioles (Banks, 1986; Slauson and Cooper, 1990; Jubb et al., 1992).

The numerical density and location of lymphocyte populations in the tissue sections were assessed microscopically by counting the positively stained cells in 30 chosen fields of 0.01105 mm.<sup>2</sup> The final count for each field was calculated as the mean of the 30 fields counted and expressed as the number of cells per mm.<sup>2</sup>

The counting of lymphocytes was conducted as follows. Using the X-Y translational mechanical stage of the microscope, the specimen slide was moved in both the X-axis (right and left) and Y-axis (back and forth) directions (2-dimensional) in order to examine the entire microscope slide in a systematic and uniformly random to sample 30 fields for each mAb. The basic stepping length in either direction was one division of the graduated locator marks positioned on the mechanical portion of the stage. All of the BoCD4+, BoCD8+, BoWC1+ and BoCD21+ cells were considered for counting if and when they displayed a brown stained cytoplasm and unambiguously visible nucleus, and also whether the cells were completely or partly inside the field area. Statistical analysis

Student two sample t-test (Kirkwood, 1988) employing software Statistix 7.0 was used to determine the significance difference (\*P<0.05) in mean numbers lymphocyte cell types infiltrating lung tissue sections between artificial and natural ECF infections.

## RESULTS

### Immunohistochemical Visualization of Lymphocytes Infiltrating the Lungs

The mean number of leukocytes expressing the BoCD4+, BoCD8+, BoWC1+, and BoCD21+ molecules, and the mean number of unstained mononuclear cells (MNC) in lungs of cattle experimentally and naturally infected with *T. parva* are shown in Table 2. Positive cells in tissue sections were seen having golden-brown staining cytoplasm.

There were no positive staining cells in the lung sections of the control animals. The unstained MNC included lymphocytes and macrophages.

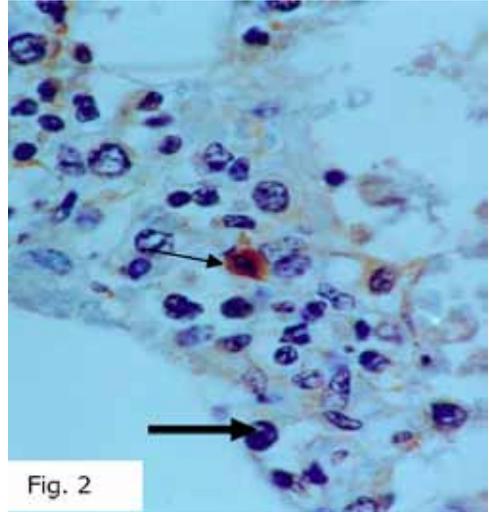
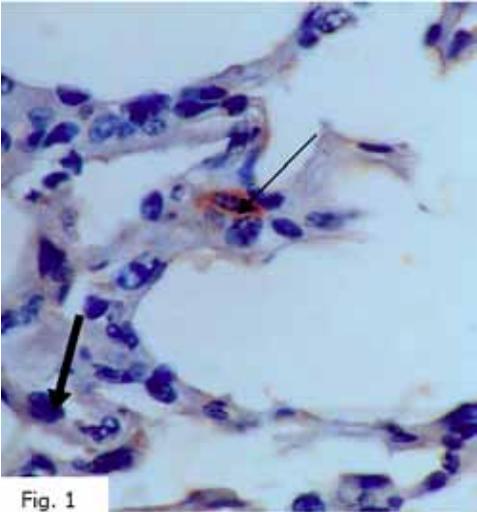
There was no significant difference (\*P<0.05) between the number of positive staining cells in the experimentally infected and naturally infected animals. Similarly, the number of unstained MNC in the two groups did not differ significantly (\*P<0.05) from each other.

The mean numbers of positive B- lymphocytes (BoCD21+) and T-lymphocytes (BoCD4+, BoCD8+, and BoWC1+) and the ratios between these positive lymphocytes and negative MNC in lungs of cattle experimentally and naturally infected with *Theileria parva* are shown in Table 3. The photomicrographs of the same are shown in Figures 1 - 8.

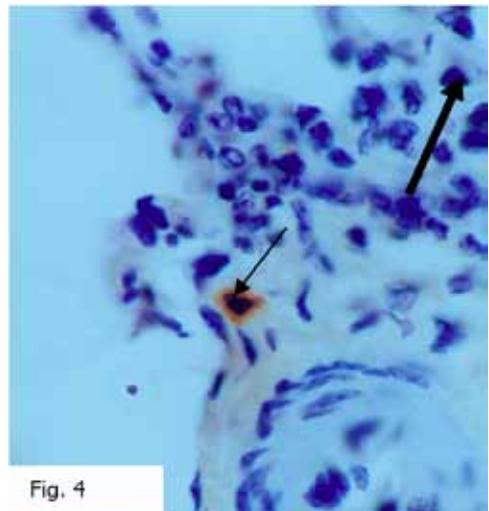
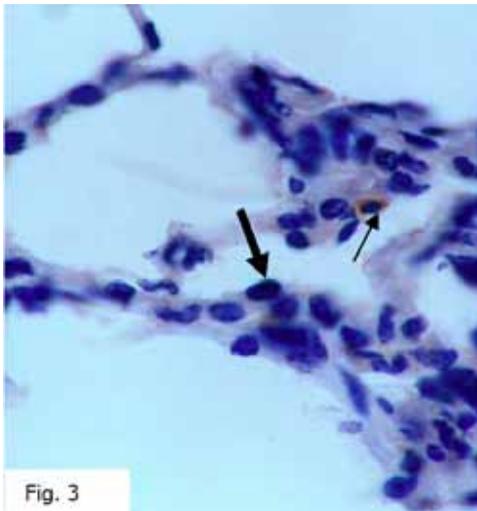
From Table 3, it is observed that there are more:

- BoCD21+ B-lymphocytes in

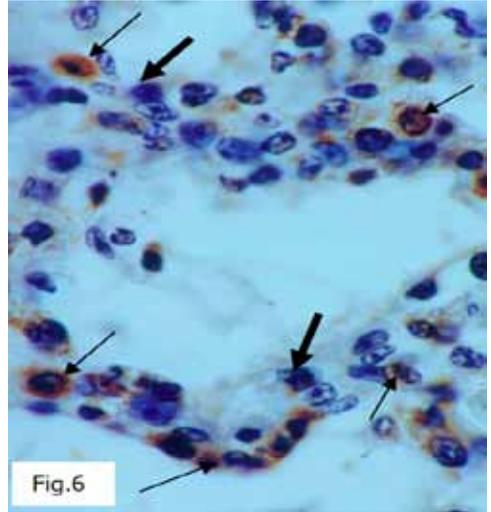
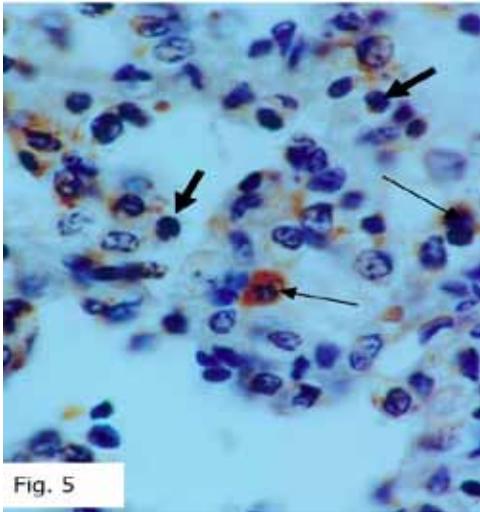
**Figure 1 (artificially infected animals) and Figure 2 (naturally infected):** Immunostained formalin-fixed paraffin-embedded bovine lung section with mAb IL-A65 to BoCD21 after pre-treatment with TBS-AR and SABC method. Positive cell (arrow) in interalveolar tissue shows brown stained cytoplasm, and negative MNC (thick arrow). X 1000.



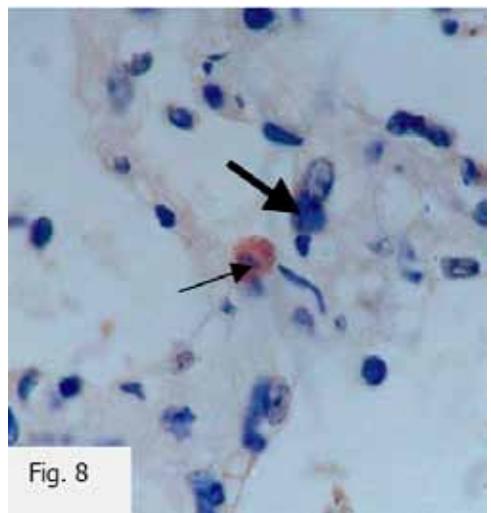
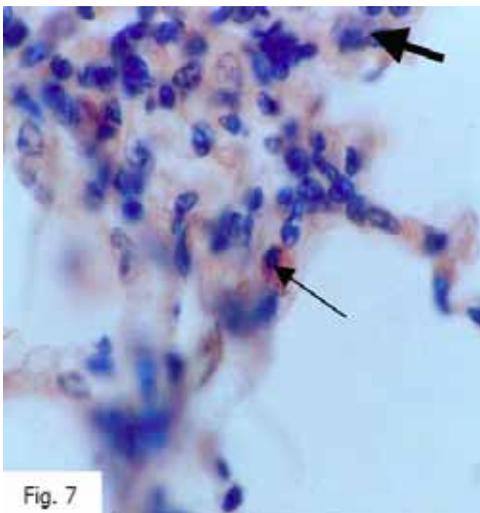
**Figure 3 (artificially infected animals) and Figure 4 (naturally infected):** Immunostained formalin-fixed paraffin-embedded bovine lung section with mAb IL-A11 to BoCD4 after pre-treatment with citrate buffer and SABC method. Positive cell (arrow) in interalveolar tissue shows brown stained cytoplasm, and negative MNC (thick arrow). X 1000.



**Figure 5 (artificially infected) and Figure 6 (naturally infected):** Immunostained formalin-fixed paraffin-embedded bovine lung section with mAb IL-A105 to BoCD8 after pre-treatment with citrate buffer and SABC method. Numerous scattered positive cells (arrows) in interalveolar tissue show brown stained cytoplasm, and negative MNC (thick arrows). X 1000.



**Figure 6 (artificially infected) and Figure 7 (naturally infected):** Immunostained formalin-fixed paraffin-embedded bovine lung section with mAb IAH-CC15 to BoWC1 after pre-treatment with citrate buffer and SABC method. Positive cell (arrow) in interalveolar tissue shows brown stained cytoplasm, and negative MNC (thick arrow). X 1000.



the lung sections of experimentally infected animals than in the naturally infected animals.

- BoCD4+ T-lymphocytes in the lung sections of experimentally infected animals than in the naturally infected animals.
- BoCD8+ T-lymphocytes in the lung sections of naturally infected animals than in the experimentally infected animals.
- BoWC1+ T-lymphocytes in the lung sections of experimentally infected animals than in the naturally infected animals.

The ratios of lymphocytes infiltrating bovine lung tissue sections during ECF in experimental and natural infection are shown in Tables 4 and 5 below.

It can be seen from Tables 4 and 5 that in the experimental *Theileria parva* infection, the ratios of lymphocytes infiltrating bovine lungs range from 1:2 and 1:1. BoCD4+ and BoCD8+ lymphocytes appear to be in the same ratio. BoCD21+ lymphocytes are slightly more than BoCD4+ lymphocytes

by about one and one-half times. BoCD4+ lymphocytes are about two times more than the BoWC1+ lymphocytes. BoWC1+ lymphocytes are about one and one-half times more than BoCD8+ lymphocytes. BoCD21+ lymphocytes are about one and one-quarter times more than BoCD8+ lymphocytes. BoWC1+ lymphocytes are about two times more than BoCD21+ lymphocytes.

This study has shown that lymphocyte immunophenotypes infiltrating lung tissues of cattle infected with *T. parva*, the majorities of lymphocytes are of BoCD4+ at the onset of respiratory distress in experimental infection, followed by BoCD8+ and BoCD21+, and then by BoWC1+ lymphocytes

It is observed from Tables 4 and 5 that BoCD8+ lymphocytes infiltrating bovine lungs in the natural *Theileria parva* infection are about 8 times more than the BoCD4+ lymphocytes, BoCD21+ lymphocytes are about 5 times more than the BoCD8+ lymphocytes, and BoWC1+ lymphocytes are about 9 times more than BoCD8+ lymphocytes;

**Table 4.** Ratios of lymphocyte types infiltrating bovine lungs with ECF

Type of infection	Ratios of lymphocyte type to one another per mm <sup>2</sup>					
	BoCD4+: BoCD8+	BoCD4+: BoWC1+	BoCD4+: BoCD21+	BoCD8+: BoWC1+	BoCD8+: BoCD21+	BoWC1+: BoCD21+
Experimental	1:1.07	1:0.56	1:1.36	1:1.56	1:1.27	1:0.64
Natural	1:8.45	1:1.25	1:0.59	1:9.47	1:5.03	1:0.83

**Table 5.** Relative ratios of lymphocyte types infiltrating bovine lungs with ECF

Type of infection	Relative ratios of lymphocyte type to one another, per mm <sup>2</sup>			
	BoWC1+	BoCD4+	BoCD8+	BoCD21+
<sup>a</sup> Experimental	27	48	42	42
	(1:1)	(1:1.8)	(1:1.6)	(1:1.6)
<sup>b</sup> Natural	15	12	142	18
	(1:1.3)	(1:1)	(1:11.8)	(1:1.5)

<sup>a</sup> The denominator used to compute ratio is the lowest number of cells/mm<sup>2</sup> in the experimental infection i.e. 27.

<sup>b</sup> The denominator used to compute ratio is the lowest number of cells/mm<sup>2</sup> in the natural infection i.e. 12.

BoCD4+ lymphocytes are about 2 times more than the BoCD21+ lymphocytes; BoWC1+ lymphocytes are about one and one-quarter times more than BoCD4+ lymphocytes; BoCD21+ and BoWC1+ lymphocytes appear to be in the same ratio.

This study has shown that in lymphocyte immunophenotypes infiltrating lung tissues of cattle infected with *T. parva*, the majority of lymphocytes are of BoCD8+ at the terminal stages of natural infection and followed by BoCD21+, BoWC1+ and then by BoCD4+ lymphocytes.

## DISCUSSION

The results of immunohistochemical staining in this study have demonstrated that the majority of lymphocyte immunophenotypes infiltrating lungs of cattle experimentally infected with *T. parva* are of BoCD4+ lymphocytes at the onset of respiratory distress (ie, day 15 post-infection the stage when the animals were sampled) and followed by BoCD8+ and BoCD21+ and then by BoWC1+ lymphocytes.

The study has also shown that the majority of lymphocyte immunophenotypes infiltrating lungs in the naturally infected cattle at the advanced stages of infection are BoCD8+ lymphocytes followed by BoCD21+, BoWC1+, and then by BoCD4+ lymphocytes.

Immunophenotypic characterization of the lymphocytes that infiltrate lungs during ECF suggested that BoCD4+ and BoCD8+ lymphocytes play a dominant role in the local immune response in ECF, while B (CD21+) and BoWC1+ ( $\gamma/\delta$ ) cells play a lesser role.

The results in this study showed higher mean number of BoCD4+ lymphocytes during the midway of the course of ECF infection than in the advanced stage of *Theileria parva* infection. These results suggest that BoCD4+ lymphocytes may have more roles to play in the midway than in the advanced stage of *Theileria parva* infection.

The presence of large numbers of T cells particularly BoCD4+ T cells and

macrophages in the lungs points to a direct, local recognition of antigens that requires presence of accessory cells exposing MHC II molecules and capable of antigen presentation.

The high number of BoCD4+ T cells that was observed at the onset of dyspnea in the experimentally infected animals indicated that satisfactory degree of cell-mediated immunity was elicited to control the infection. It is known from other studies, that BoCD4+ T cells release cytokines, particularly TNF- $\beta$  and IFN- $\gamma$ , which are responsible for Th1-type response essential for controlling intracellular pathogens such as *Theileria parva* (Taracha et al., 1998). In addition, BoCD4+ T cells are known to produce macrophage-activating cytokines such as IFN- $\gamma$ . Such activated macrophages produce mediators such as NO, which destroy the intracellular schizont (Ahmed and Mehlhorn, 1999). The TNF- $\beta$  and IFN- $\gamma$  cytokines in the case of ECF recruit BoCD8+ T cells, which are cytotoxic T cells (CTL). The CTL secrete molecules that destroy the lymphoblasts containing schizonts before they can release fresh crop of merozoites able to infect other lymphocytes (Taracha et al., 1998).

The results of this study have also shown that BoCD4+ lymphocytes decreased, while the BoCD8+ lymphocytes increased in the advanced stages of the disease as observed in the cases of natural infection when compared to experimentally infected animals that were slaughtered at day 15 post infection at onset of respiratory distress.

The results in this study showed higher mean number of BoCD8+ lymphocytes in the advanced stage than in the midway of the course of *Theileria parva* infection. The apparent higher numbers of BoCD8+ lymphocytes in the advanced stage of ECF infection suggests that they may have more roles to play during the advanced stage than in the midway of the course of *Theileria parva* infection.

The increase in BoCD8+ lymphocytes in comparison to BoCD4+ T cells in the advanced stages of the disease suggests an

adequate degree of cell-mediated immunity in an attempt to control the infection.

In addition, BoCD8+ lymphocytes are known to be responsible for the release of cytokines, particularly IFN- $\gamma$ , which is responsible for Th-1 type response. Also the CTL cells secrete TNF- $\beta$  that is directly cytotoxic to the infected cells. It is also shown that IFN- $\gamma$  and TNF- $\gamma$  are synergistic in promoting NO synthesis that is required in the control of infection, therefore increasing the capacity to fight the infection (Ahmed and Mehlhorn, 1999).

The results in this study showed higher mean number of BoCD21+ lymphocytes during the midway than in the advanced stages of *Theileria parva* infection. The apparent higher mean number of B-lymphocytes during midway of the course of infection indicates that they may have more roles to play in this stage than in the advanced stages of *Theileria parva* infection.

The scarcity of B cells in the lymphocytic infiltrates in the lungs during the terminal stages of ECF suggests that the local humoral response is of little importance as is with other intracellular pathogens (Su et al., 1997; Yang and Brunham, 1998). Perhaps, B cells as antigen-presenting cells would process the schizont antigens and present them to Th1 helper cells leading to further production of Th1 cytokines.

The results in this study showed higher mean number of BoWC1+ lymphocytes during the midway than in the advanced stage of *Theileria parva* infection. The apparent higher numbers of BoWC1+ lymphocytes during the midway of the disease course suggests that in this stage they may have more roles to play than in the advanced stage of *Theileria parva* infection. This is in agreement with the results that they may have a significant role in control of the disease before the more specific cytotoxic BoCD8+ cells are recruited in substantial numbers to combat the infection (McKeever, 2001).

It has been shown in many studies, especially in mice lacking either  $\alpha/\beta$  or  $\gamma/\delta$

T cells,  $\gamma/\delta$  T cells can contribute to immune competence, but they do so in a way that is distinct from  $\alpha/\beta$  T cells. It is also evident that  $\gamma/\delta$  T cells may not recognize antigen the same way as do  $\alpha/\beta$  T cells.

Chien et al., (1996) showed that  $\gamma/\delta$  T cell recognition of three murine protein antigens and the Herpes virus glycoprotein gI does not require antigen processing and that the proteins are recognized directly. They suggested that pathogens, damaged tissues, or even B and T cells can be recognized directly, and cellular immune responses can be initiated without a requirement for antigen degradation or specialized APCs, therefore giving  $\gamma/\delta$  T cell greater flexibility than the more classical type of  $\alpha/\beta$  T cell-mediated immunity.

Moreover, Daubenberger et al., (1999) investigating the responses to primary infection with *T. parva* in young cattle which have the predominance of  $\gamma/\delta$  T cells (about 50% of T cells in the peripheral blood and lymphoid organs) provided evidence that bovine  $\gamma/\delta$  T cells participate in the early phase of an immune response against *T. parva*. These responses are MHC-unrestricted and cross-reactive between a broad range of different parasite stocks.

The high mean numbers of unstained LMNC observed in the interstitium of alveolar parenchyma in the lungs included lymphocytes and macrophages. Among the unstained lymphocytes there may be included lymphocytes that should have stained but did not because of failure to express the anticipated surface marker.

Notwithstanding, the mechanisms that attract these lymphocytes into the lungs during *Theileria parva* infection, their presence in the lungs suggests that locally they release cytokines that contribute towards development of inflammation and pulmonary edema. This would happen even before the lymphocytes start to disintegrate during the course of the infection.

In addition to the findings that vasoactive substances released during disintegration of lymphocytes activate, the comple-

ment cascade and subsequent formation of anaphylatoxins (C3a and C5a) in the lungs are responsible for the development of pulmonary oedema (Shitakha et al. 1983; Lawrence et al. 1994) and the possibility of local cytokine release in the lungs that can modulate vascular permeability causing microvascular leakage. It would therefore appear that multiple molecules, including cytokines, participate in the development of pulmonary oedema.

While the infiltration of lymphocytes into lung tissue is part of a wider dissemination of white blood cells into parenchymatous organs in ECF, the presence of leukocytic mononuclear cells in lung parenchyma seems to occur at the same time with edema and disintegration of the cells.

Lymphocytes are known for direct and indirect production of pro-inflammatory cytokines some of which are known to have vasoactive influences like IL-1 and TNF- $\alpha$  (Wewers et al., 1997) and the fact that disintegrating lymphocytes release lysosomal substances whose destructive effects on the tissue cells may cascade into vascular changes. It seems lymphocyte presence in the lungs are responsible for the edema in the lungs as seen in the sections.

The absence of similar changes in other parenchymatous organs, eg, kidneys and liver is probably due to differences in the compactness of cells between organs.

## CONCLUSION

As long as *Theileria parva* infection there is a production and or consumption of cytokines including IFN- $\gamma$  (DeMartini and Baldwin, 1991; Ahmed et al., 1999; Taracha, 1998), IL-2 (Dobbelaere et al., 1988; DeMartini and Baldwin, 1991) and TNF- $\alpha$  (Taracha, 1998) in association with lymphocyte transformation and proliferation that occurs before lymphocyte destruction, the presence of such lymphocytes (presumably immunocompetent) in lungs points to local cytokine production and or consumption that could lead to microvascular leakage.

Previous studies using vascular permea-

bility markers have demonstrated pulmonary vascular permeability changes associated with ECF (Matovelo, et al., 2000; Gwamaka, 2001). They have also demonstrated heavy infiltration of leukocytic mononuclear cells, especially lymphocytes, in parenchymatous organs. They have shown that some of the infiltrating lymphocytes have been in different stages of disintegration and others entire disintegration. This has ushered in the view that lymphocytes in lungs secrete cytokines and other mediators of inflammation when such cells disintegrate.

The present study has demonstrated and characterised major lymphocyte subpopulations in the lung by the use of immunohistochemistry. The cells characterised are known to secrete various cytokines that directly or indirectly are linked to increase of vascular permeability.

Therefore, further studies are recommended to demonstrate the presence of mediators of vascular permeability changes in situ in lungs. Further studies will also be useful in the area of deciphering the peak time for leukopenia and infiltration in the parenchymatous organs. – the decline in WBC counts after day 8 is either due to infiltration into parenchymatous organs or lympholysis or both.

This study is yet another contribution to the pathogenetic information that is useful in optimisation of regimes in management of ECF and particularly ECF associated pulmonary edema in cattle.

## REFERENCES

1. Ahmed, J. S. and Mehlhorn, H: Review: the cellular basis of the immunity to and immunopathogenesis of tropical theileriosis. *Parasitology Research* 1999; 85:539-549.
2. Ahmed, J. S.; Schnitger, L.; and Mehlhorn, H: Review: Theileria schizonts induce fundamental alteration on their host cells. *Parasitology Research* 1999; 85:527-538.
3. Baldwin, C. L.; Teale, A. J.; Naessens, J. G.; Goddeeris, B. M. MacHugh, N. and Morrison, W. I: Characterization of a subset of bovine T lymphocytes that express BoT4 by monoclonal antibodies and function: similarity to lymphocytes defined by human T4 and murine L3T4. *Journal of Immunology* 1986; 136:4385-4391.
4. Banks, W. J: Applied Veterinary Histology, 2nd

- Edition, William & Wilkins, Baltimore, USA. 1986:330–347.
5. Brown, W. C. and Logan, K. S: Bovine T-cells clones infected with *Theileria parva* produce a factor with IL2-like activity. *Parasitology* 1986; 8:189–92.
  6. Chien, Y.; Jore, R. and Crowley, M. P: Recognition by  $\gamma/\delta$  T cells. *Annual Review of Immunology* 1996; 14:511–532.
  7. Daubenberger, C. A.; Taracha, E. L. N.; Gaidulis, L.; Davis, W. C. and McKeever, D. J: Bovine  $\gamma/\delta$  T-cell responses to the intracellular protozoan parasite *Theileria parva*. *Infection and Immunity* 1999; 67:2241–2249.
  8. DeMartin, J. C. and Baldwin, C. L: Effects of gamma interferon, tumor necrosis factor alpha, and interleukin-2 on infection and proliferation of *Theileria parva*-infected bovine lymphoblasts and production of interferon by parasitized cells. *Infection and Immunity* 1991; 59:4540–4546.
  9. Dobbelaere, D. and Heussler, V: Transformation of leukocytes by *Theileria parva* and *Theileria annulata*. *Annual Reviews Microbiology* 1999; 53:1–42.
  10. Dobbelaere, D. A.; Coquerelle, T. M.; Roditi, I. J.; Eichhorn, M. and Williams, R. O: *Theileria parva* infection induces autocrine growth of bovine lymphocytes. *Proceedings of National Academy of Science, USA* 1988; 85:4730–4734.
  11. Emery, D. L.; MacHugh, N. D. and Morrison, W. I: *Theileria parva* (Muguga) infects bovine T lymphocytes in vivo and induces co-expression of BoT4 and BoT8. *Parasite Immunology* 1988; 10:379–391.
  12. Gutierrez, M.; Forster, F. I.; McConnell, S. A.; Cassidy, J. P.; Pollock, J. M. and Bryson, D. G: The detection of CD2+, CD4+, CD8+, WC1+T lymphocytes, B cells and macrophages in fixed and paraffin-embedded bovine tissue using a range of antigen recovery and signal amplification technique. *Veterinary Immunology and Immunopathology* 1999; 71:321–334.
  13. Gwamaka, M: The effect of corticosteroids and antihistaminic drugs on the development of pulmonary edema in East Coast fever. Unpublished Dissertation for Award of MVM degree at Sokoine University of Agriculture, Morogoro, Tanzania; 2001:68–81.
  14. Howard, C. J.; Morrison, W. I.; Bensaid, A.; Davis, W.; Eskra, L.; Gerdes, J.; Hadam, M.; Hurley, D.; Leibold, W.; Letteson, J. J: Summary of workshop findings for leukocyte antigens of cattle. *Veterinary Immunology and Immunopathology* 1991; 27:21–27.
  15. Jubb, K. V. F.; Kennedy, P. C. and Palmer, N. (). *Pathology of Domestic Animals*. 5th Edition, Academic press. San Diego, 1992; 2:539–699.
  16. Jubb, K. V. F.; Kennedy, P. C. and Palmer, N: *Pathology of Domestic Animals*. 5th Edition, Academic press. San Diego, 1992; 3:251–255.
  17. Kirkwood, B. R: *Essentials of Medical Statistics*. Oxford Blackwell Scientific Publications, London; 1988:147–152.
  18. Kuby, J: *Immunology*. 3rd Edition. New York. W. H. Freeman; 1997.
  19. Lawrence, J. A.; de Vos A. J. and Irvin, A. D. (). East Coast fever. In: *Infectious disease of livestock with special reference to southern Africa*. (Edited by Coetzer J. A. W., Thomson, G. R and Tustin, R. C.) Oxford University press, Cape Town, 1994; 1:309–325.
  20. MacHugh, N. D.; Taracha, E. L. and Toye, P. G: Reactivity of workshop antibodies on L cell and COS cell transfectants expressing bovine CD antigens. *Veterinary Immunology and Immunopathology* 1993; 39:61–67.
  21. Matovelo, J. A., Mwamengele, G. L. M. and Mbasasa, G. K: The suppressive effects of dexamethasone on the development of pulmonary oedema in East Coast fever. *Tropical Animal Health and Production* 2000; 32(6):353–356.
  22. McKeever, D. J: Cellular immunity against *Theileria parva* and its influence on parasite diversity. *Research in Veterinary Science* 2001; 70:77–81.
  23. Naessens, J.; Newson, J.; McHugh, N.; Howard, J.; Parsons, K. and Jones, B: Characterisation of a bovine leucocyte differentiation antigen of 145,000 MW restricted to B-lymphocytes. *Immunology* 1990; 69:525–530.
  24. Norton, A. J.; Jordan, S. and Yeomans, P: Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. *Journal of Pathology* 1994; 173:371–379.
  25. O’Leary, T. J: Standardization in Immunohistochemistry. *Applied Immunohistochemistry and Molecular Morphology* 2001; 9:3–8.
  26. Pinder, M.; Withey, K. S. and Roelants, G. E: *Theileria parva* parasites transform a subpopulation of T lymphocytes. *Journal of Immunology* 1981; 127:389–390.
  27. Pinkus, G. S: Diagnostic Immunocytochemistry of paraffin-embedded tissues. *Human Pathology* 1982; 13(5):411–415.
  28. Preston, P. M.; Brown, C. G. and Richardson, W: Cytokines inhibit the development of trophozoite-infected cells of *Theileria annulata* and *Theileria parva* but enhance the proliferation of macroschizont-infected cell lines. *Parasite Immunology* 1992; 14(2):125–141.
  29. Shayan, P., Schop, B., Conze, G., Schein, E. and Ahmed, J. S: Is interleukin 2 necessary for the autocrine proliferation of *Theileria* infected bovine cells? *Parasitology Research* 1999; 85:409–412.
  30. Shitakha, V. M.; Musoke, A. J.; Ramasamy, R. and Buscher, G: Complement activation and fibrinolysis during infection with *Theileria parva* (East coast fever) in cattle. *Veterinary Immunology and Immunopathology* 1983; 4:361–373.
  31. Slauson, D. O. and Cooper, B. J: *Mechanisms of Disease: A textbook of Comparative General Pathology*, 2nd Edition, Williams & Wilkins, Baltimore, USA; 1990.
  32. Soulsby, E. J. L: *Helminthes, Arthropods, and Protozoa of Domestic Animals*, 7th Edition. Bailliere Tindall, London; 1982.
  33. Su, H.; Feilzer, K.; Caldwell, H. D. and Morrison,

- R. P: Chlamydia trachomatis genital tract infection of antibody-deficient gene knockout mice. *Infection and Immunity* 1997; 65:1993–1999.
34. Taracha, E. L.; Awino, E. and McKeever, D. J: Distinct CD4<sup>+</sup> T-cell helper requirements in Theileria parva-immune and naïve bovine CTL precursors. *Journal of Immunology* 1998; 159:4539–4545.
35. Wewers, M. D.; Gadek, J. E. and Marsh, C. B: Pro-inflammatory cytokines. In: *The lung: scientific foundations*. Edited by Crystal, R. G.; West, J. B.; Weibel, E. R. and Barnes, P. J. Lippincott-Raven, Philadelphia; 1997:117-132.
36. Yang, X. and Brunham, R. C: Gene knockout B cell-deficient mice demonstrate that B cells play an important role in the initiation of T cell responses to Chlamydia trachomatis mouse pneumonitis lung infection. *Journal of Immunology* 1998; 161:1439–1446.