7 IMMUNOHISTOCHEMICAL CHARACTERIZATION OF LYMPHOCYTE AND MYELOID CELL INFILTRATES IN SPIROCERCOSIS-INDUCED ESOPHAGEAL NODULES

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Immunohistochemical characterization of lymphocyte and myeloid cell infiltrates in spirocercosis-induced esophageal nodules

E. Dvir,[1] J. P. Schoeman,[1] S. J. Clift,[2] T. N. McNeilly[3] & R. J. Mellanby[4]

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[1]Department of Companion Animal Clinical Studies and [2]Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa, and [3]Moredun Research Institute, Midlothian, UK and [4]Royal (Dick) School of Veterinary Studies, Roslin Institute, Division of Veterinary Clinical Studies, University of

Edinburgh, Midlothian, UK

Correspondence: Eran Dvir, DVM, BVSc (hons), MMedVet (Med), Section of Small Animal Medicine, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa (e-mail: eran.dvir@up.ac.za).

7.1 Abstract

Spirocerca lupi is a nematode that infects the dog's esophagus and promotes the formation of an inflammatory fibroblastic nodule that progresses to sarcoma in approximately 25% of cases. Spirocercosis-associated esophageal sarcoma is an excellent and under-utilized spontaneous model of parasite-associated malignancy.

The inflammatory infiltrate of paraffin-embedded non-neoplastic esophageal nodules (n=46), neoplastic nodules (n=25) and normal esophagus (n=14) was examined by immunohistochemistry using MAC387 (myeloid cells), CD3 (T cells), Pax5 (B cells) and FoxP3 (T regulatory cells) antibodies. Myeloid cells predominated in 70% of nodules in pockets around the worms' migratory tracts and in necro-ulcerative areas in neoplastic cases. T cells predominated in 23% of cases with a focal or diffuse distribution, in the nodule periphery. No significant differences were observed between neoplastic and non-neoplastic stages. FoxP3+ cells were observed in low numbers, not significantly different from the controls.

The inflammation in spirocercosis is characterized by pockets of pus surrounded by organized lymphoid foci. There was no evidence of a local accumulation of FoxP3+ cells, unlike many previous studies which have reported an increase in Foxp3+ T cells in both malignancies and parasite infections. The triggering factor(s) driving the neoplastic transformation of the spirocercosis-associated chronic inflammatory nodule warrants further investigation.

Key words Spirocerca lupi, sarcoma, T regulatory cells, FoxP3, CD3, Pax5, MAC387

7.2 Introduction

Spirocerca lupi (S. lupi) is a nematode for which the dog is the final host (Bailey, 1972). In the dog the adult nematode resides in the esophagus which results in the formation of an esophageal nodule. Over time up to 25% of these nodules undergo neoplastic transformation (Dvir et al., 2001). Histologically the sarcoma has been classified as fibrosarcoma, osteosarcoma or anaplastic sarcoma (Ranen et al., 2008; Ranen et al., 2004). The different stages of the spirocercosis-induced esophageal nodule have recently been described (Dvir et al., 2010). It was proposed that non-neoplastic *S. lupi* nodules could be divided into 2 stages: an early inflammatory stage, where the nodule is characterized histologically by fibrocytes and abundant collagen, and a pre-neoplastic stage, where the nodule is characterized by the presence of activated fibroblasts (more mitoses and a greater proportion of fibroblasts that showed some degree of atypia) and reduced collagen. Both stages are characterized by lympho-plasmacytic inflammation. Finally the nodule develops into neoplastic sarcoma (Dvir et al., 2010). This study was the first to describe the high prevalence and severity of the lympho-plasmacytic infiltrates in *S. lupi*-induced nodules which have often previously been incorrectly classified as granulomas (Bailey, 1972). Neutrophils were also very common in the non-neoplastic cases, where they were distributed either diffusely or in purulent foci immediately adjacent to the worm tract(s) and their associated tissue debris. The neoplastic cases generally had less inflammation; the inflammation was predominantly suppurative and the foci of suppuration were typically confined to necro-ulcerative areas in the tumour.

The finding that *S. lupi* nodules have a marked lympho-plasmacytic infiltration is important since the association between chronic infection-induced inflammation and cancer is now well-described and is thought to be the mechanism responsible for up to 18% of global cancers (Vennervald and Polman, 2009). In terms of parasite-associated malignancies, three helminth infections have been classified as carcinogenic in humans, namely *Schistosoma haematobium, Clonorchis sinensis* and *Opisthorchis viverrini* and the presence of chronic inflammation induced by parasites or their deposition is considered a key element in their carcinogenesis (Vennervald and Polman, 2009). In dogs esophageal sarcoma (excluding leiomyosarcoma) is almost invariably associated with *S. lupi* infections, whereas in human oncogenic helminth-associated neoplasia the association is limited to only a few of the specific cancer cases (Herrera et al., 2005), making spirocercosis a highly attractive model to study the association between cancer, helminth infection and inflammation.

It is widely accepted that helminths and their antigens induce a Th2 response (Maizels et al., 2009), and although a Th2 response to the parasite is essential for the host to clear the infection, it is imperative that the immune response is well controlled. The Th2 response can be tightly controlled by CD4+ regulatory T cells (Tregs), which are characterized by the expression of CD25 and the intracellular forkhead box P3 (FoxP3) transcription factor, secretion of interleukin (IL)-10 and transforming growth factor β (TGFβ) (Maizels et al., 2009). While Tregs are essential in the prevention of autoimmune and allergic diseases via their inhibition of an autopathogenic immune response, induction of Tregs by helminths can facilitate long-lasting infection (Maizels et al., 2009). Similarly, Tregs can inhibit the anti-tumour immune response (Beyer and Schultze, 2006) and an increase in their number may facilitate tumour development. Numerous clinical studies on human patients with various types of

cancer have shown increased Tregs proportions in the peripheral blood, draining lymph nodes and within the tumours (Curiel et al., 2004; Heimberger et al., 2008; Liyanage et al., 2002; Wolf et al., 2003; Woo et al., 2001).

FoxP3+ Tregs can be identified in the dog using a cross-reactive, directly conjugated murine FoxP3 antibody (Biller et al., 2007). As in humans, tumour-bearing dogs were found to have an increased number and/or proportions of Tregs in the circulation (Biller et al., 2007; O'Neill et al., 2009; Tominaga et al., 2010), draining lymph nodes (Biller et al., 2007) and within the tumour (Tominaga et al., 2010). The fact that the role of Tregs is well described in both helminth infection and cancer may indicate a potential role in helminth-induced cancer such as spirocercosis. However, the role of Foxp3+ Tregs in helminth infections in dogs has not been investigated and the presence of FoxP3+ cells has not been examined by immunohistochemistry in canine tissue.

The primary objective of this study was to characterize the lymphocyte and myeloid infiltrate in *S. lupi* nodules by immunohistochemistry using antibodies against CD3 (T cells), Pax 5 (B cells) and MAC387 (myeloid cells) (Vanherberghen et al., 2009; Willmann et al., 2009). A secondary objective of the study was to investigate the prevalence of FoxP3+ Tregs in the *S. lupi* nodule by immunohistochemistry.

7.3 Materials and Methods

7.3.1 Case Selection

Seventy one formalin-fixed, paraffin-embedded *S. lupi*-induced esophageal nodules, collected between 1998-2009, were retrieved from the archives of the Section of Pathology, Faculty of Veterinary Science, University of Pretoria (retrospective study). The samples were collected during necropsy. In most cases, only one sample was

collected for diagnostic purposes. In the smaller non-neoplastic nodules, a transverse section was taken through the entire nodule. One 5µm-thick tissue section per block was stained with hematoxylin and eosin (H&E) for subsequent histological evaluation. Nodules were classified into neoplastic (n=25) and non-neoplastic (n=46) groups. Only one nodule was selected per dog for subsequent immunohistochemical analyses. If a dog had more than one nodule, the nodule that was most mature or advanced towards neoplastic transformation was selected. In the larger nodules multiple sections were taken and the most diagnostic section was selected.

For negative tissue control purposes, 14 sections of normal distal third of dog esophagus were used. For 9 of the *S. lupi*-induced esophageal nodule cases (5 neoplastic and 4 non-neoplastic), the draining lymph nodes of the distal esophagus (bronchial) and remote lymph nodes (popliteal) were also collected. The entire lymph nodes were collected and a transverse section was fixed in paraffin. Lymph node was the positive tissue control for immunohistochemical labeling.

7.3.2 Immunohistochemical labelling of FoxP3, CD3, Pax5 and Myeloid/Histiocyte antigen MAC387

Four µm-thick serial sections were cut and mounted on Superfrost-Plus glass slides (Thermo), and dried overnight in an oven at 60ºC to enhance tissue adhesion. Following rehydration, antigen-retrieval was performed. For FoxP3, CD3 and Pax5 labeling, heat-induced epitope retrieval was performed by autoclaving at 121^{o}C for 10 minutes in 10mM citrate buffer pH 6.0. For MAC387 labelling, sections were pre-treated with Proteinase K (Dako) for 5 minutes at 25°C. The sections were washed twice in phosphate-buffered saline (PBS) and again in PBS containing 0.5% Tween 80 (PBST80) for 5 minutes. Endogenous peroxidase activity was quenched by incubating the tissue sections with 0.3% hydrogen peroxide in PBST80 for 20 minutes

at room temperature (RT). Following two washes in PBST80, slides were loaded into a Sequenza immunostaining centre (Thermo Scientific). Non-specific tissue antigens were blocked by incubation in 25% normal goat serum (NGS) in PBS/0.5% Tween 80 (PBS/T80) for 1 hr at RT prior to incubation overnight at 4°C with the following primary antibodies: 1:100 dilution of rat anti-mouse/rat FoxP3 monoclonal antibody (mAb) (FJK-16s, eBioscience, San Diego, CA, USA); 1:200 dilution of polyclonal rabbit anti-human CD3 antibody (Dako); 1:50 dilution of mouse anti-human Pax-5 mAb (clone 24, BD Biosciences). MAC 387 antibodies were incubated for 1 hour at 25ºC: 1:400 dilution of mouse anti-human Myeloid/Histiocyte Antigen mAb (clone MAC387, Dako). Control antibodies included: Rat IgG2a isotype control mAb (eBioscience), mouse anti-Border disease virus p125/p80 mAb VPM21 and purified rabbit immunoglobulin (Sigma-Aldrich), for rat, mouse and rabbit primary antibodies, respectively. All antibodies were diluted in PBS/T80 containing 10% normal goat serum (NGS).

Slides were washed twice in PBS and the appropriate secondary antibody (peroxidase-labelled anti-mouse or anti-rabbit EnVision™+ reagent, Dako) was applied to sections for 30 minutes at RT. After a final PBS wash, sections were incubated with 3,3'-diaminobenzidine (DAB) for 7.5 minutes at RT, washed in distilled water, counterstained with haematoxylin, dehydrated and mounted in Shandon synthetic mountant (Thermo Scientific).

7.3.3 Scoring of IHC labelling

Each nodule was scanned under the light microscope. The initial scan was done with a wide-angle lens at low power (x20) and the following data were recorded: The predominant inflammatory cell type, the distribution of the cell infiltrate (diffuse or focal/multifocal) and the location of the infiltrate within the nodule (peripheral,

central or both). CD3+ and Pax5+ cells tended to occur in a focal/multifocal distribution pattern in the sections and the foci of CD3+ and Pax5+ cells were counted in the most active x20 field (the field with the highest number of foci). CD3+ and Pax5+ infiltrates were subjectively scored 0-3 (Table 1). MAC387+ infiltrates were also scored 0-3; however, MAC387+ cells occurred more diffusely in sections, either evenly distributed or in patches and therefore the scoring system was slightly different (Table 2). Numbers of FoxP3+ cells were counted in 10 non-overlapping x400 fields (5 peripheral and 5 central fields per esophageal nodule using a 0.0625mm^2 graticule). In the normal esophagus control group and lymph nodes 5 non-overlapping x400 fields were counted. Counting was confined to CD3+ areas.

7.3.4 Statistical analyses

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc. CA, USA). The difference in prevalence and distribution of the different proportions of cell types was tested using the Chi square test. The differences between the scores of the different types of infiltrate were tested for significance between all groups using a Kruskal-Wallis Test, followed by Dunn's post-hoc test. *P* values of < 0.05 were considered significant.

7.4 Results

Myeloid cells predominated in 70% of cases, while T cells predominated in 23% of cases. In the remaining 7% of cases the number of T cells and myeloid cells were approximately equal. There was no difference in the proportion of myeloid and T cells between the neoplastic and non-neoplastic groups (p=0.27). When cells were present in normal esophageal sections they were diffusely scattered and myeloid and T cells tended to occur in equal proportions (Table 3). The inflammatory score of all

cell types was significantly higher ($p < 0.05$) in the spirocercosis groups compared to the control group, but was not different between the neoplastic and non-neoplastic groups (Table 4, 5 and 6).

Myeloid cells were most commonly confined to massive diffuse pockets around worm migratory tracts (Figure 1A) and to necro–ulcerative areas, the latter especially in neoplastic cases (Figure 1B). Most cases had massive diffuse areas that could not be counted. To a lesser extent, myeloid cells were diffusely scattered throughout the nodules (Table 4).

T cells occurred diffusely (Figure 1C) or in a focal/multifocal (Figure 1D) distribution pattern, predominantly at the periphery of the nodule (Table 5). The number of foci in the most active x20 field ranged from 0 to 18. B cells followed the same distribution within the nodule as T cells (Table 6), but there were fewer of them (Table 7) and they were more confined to focal/multifocal areas (Figure 1E).

FoxP3+ cells were detected in 30% of nodules (32% of neoplastic cases and 28% of the non-neoplastic cases), especially in T cell foci, but they were not observed in the normal esophagus. In most of the *S. lupi* cases where FoxP3+ cells were detected, the number of cells was very low (Table 8) and was not significantly different from the normal esophagus, where no FoxP3+ cells were detected. However, 3 cases (1 non-neoplastic and 2 neoplastic) contained a high power field with more than 10 FoxP3+ cells (up to 47 cells/0.0625mm^2 in a selected high power field; Figure 1F).

High numbers of FoxP3+ cells were observed in the lymph nodes (Table 9, Figure 1G), but no difference was observed between the bronchial and popliteal nodes and between the neoplastic-draining (86.44±34.39, mean±STD/0.0625mm^2) and non-neoplastic-draining nodes (85.95±54.55). These FoxP3+ cells were confined to CD3+ areas (Figure 1H).

7.5 Discussion

The current study revealed that the predominant inflammatory cells in *S. lupi* esophageal nodules are of myeloid lineage. These cells were identified by a MAC387 antibody, which does not enable differentiation between the different types of myeloid cells. However, based on the histological appearance, the vast majority of myeloid cells were neutrophils. These neutrophils formed pockets of pus around the worm, or they were confined to necro-ulcerative areas in the neoplastic nodules. Alternatively, neutrophils occurred diffusely throughout the nodules. The lymphocytic infiltrates had a prominent focal/multifocal distribution pattern (compared to the myeloid cells) and they were usually peripherally located within nodules. However, in the majority of cases, lymphocytes occurred in a mixed pattern; namely focal/multifocal and diffuse. The relative proportions of leukocytes within *S. lupi* nodules was different to our initial observations in H&E-stained sections (Dvir et al., 2010). This finding shows the importance of further identification and quantification of cells using IHC. There are two possible explanations for the observed difference. Firstly, in the current study plasma cells were not labeled, but plasma cell-rich foci in HE-stained sections would have been incorporated into the lympho-plasmacytic scoring in the previous study. Also, the focal/multifocal distribution pattern of the lympho-plasmacytic reaction, which frequently made it the predominant cell infiltrate in certain fields, may have biased our scoring over the whole slide in the previous study. We could also not demonstrate the difference in the inflammation score and composition of the cell infiltrate between neoplastic and non-neoplastic cases that we previously observed (Dvir et al., 2010).

Myeloid cells and especially neutrophils play a major role in the innate local inflammatory response in the spirocercosis-induced nodule. Myeloid cells can have an

important role in cancer induction by generating proteases, free radical and nitrogen species that can cause oxidative damage to the DNA (Vennervald and Polman, 2009). They can also play a crucial role in establishing cytokine-induced tumour rejection (Di Carlo et al., 2001), and they also play a major part in endothelium-mediated lymphocyte trafficking and antigen presentation. Polymorphonuclear cells have shown both pro- and anti-inflammatory activities. They may participate in the switch to immune suppression by Th2 and Tregs through up-regulation of IL-10 (Di Carlo et al., 2001). More recently neutrophils have been shown to play a pivotal role in the regulation of the inflammatory response against cancer (Mattarollo and Smyth). For instance, neutrophils can be induced by serum amyloid A (SAA)1 to secrete IL-10 which induces suppression of immune surveillance (De Santo et al., 2010).

In the present study, T cells outnumbered B cells. To further differentiate between the different T cell types, especially into $CD4^+$ or $CD8^+$ cells, frozen sections (which were not available in this study) would be necessary. Based on the current knowledge of helminth-associated chronic inflammation these cells are likely to be Th2 CD4+ cells (Maizels et al., 2009). Th2 responses are generally correlated with suppressed cell mediated immune response and with enhanced tumour promotion and progression. B cell response is often associated with Th2 cell response and also with increased risk for neoplastic progression (de Visser et al., 2005; Shah et al., 2005; Tan and Coussens, 2007). Additionally, immunoglobulins and more specifically immune complexes are regarded as tumour promoting (Tan and Coussens, 2007). The humoral response in spirocercosis warrant further investigation for its role in the carcinogenesis in spirocercosis and also for the potential use of serology as a diagnostic tool in this disease.

This study reports for the first time an approach to the identification of FoxP3+ cells in excised diseased canine tissue. We hypothesized that Tregs will be present in high numbers in the spirocercosis-induced nodules and that their numbers will increase as the nodule progressed toward sarcoma, but although Foxp3+ cells were found in large numbers within CD3+ regions of lymph nodes, they were rarely observed in *S. lupi* associated esophageal nodules and when present, were usually in very small numbers. This is surprising considering the wide range of studies which have found increased numbers and proportions of Foxp3+ Tregs within tumours in humans (Carreras et al., 2006; Unitt et al., 2005; Xue et al., 2009) and murine models (Imai et al., 2007) including models of fibrosarcoma (Betts et al., 2007). The only other study to examine Tregs within canine tumours found similar results to the many other studies of human tumours and experimental cancer models. They reported that the percentage of FoxP3+ CD4+ cells in dogs with malignant melanoma was significantly increased in the blood compared with healthy control dogs, and the percentage of FoxP3+ CD4+ cells within tumours compared to blood was also significantly increased (Tominaga et al.). Therefore, this study clearly demonstrates that the developing dogma that Foxp3+ T cells are highly prevalent in tumour-associated inflammation is not universally true and emphasises that neoplastic transformation can still occur in the absence of immunosuppressive Foxp3+ T cells. It is in agreement with the canine literature on sarcoma (O'Neill et al., 2009), especially osteosarcoma (Rissetto et al., 2010). Interestingly, in humans with Ewing's sarcoma, there was also no infiltration of FoxP3+ cells into the tumours, whereas in patients with metastases, the number of FoxP3+ cells only increased in the bone marrow (Brinkrolf et al., 2009). The fact that a large number of positive cells were observed in a few cases, as well as in lymph nodes, but not in the iso- or tissue-controls, excludes technical error. Moreover, all

samples were fixed by the same method (formalin-fixed and paraffin-embedded) and the 9 positive controls (lymph nodes) originate from 9 of the study cases. Therefore, it seems feasible that there is a real difference in the immune response to sarcomas (especially in dogs), compared to other tumours, especially melanomas.

The possible role of Tregs in the pathogenesis of spirocercosis-induced sarcoma is especially intriguing due to the well-documented role of Tregs in helminth infection. In chronic helminth infection, and spirocercosis-induced inflammation is, indeed, chronic, Tregs reduce the intensity of the infection (Maizels et al., 2009). There is evidence that the increased Tregs response facilitate long lasting chronic inflammation that reduce auto-immunity and allergy in infected subjects (Wilson and Maizels, 2004). This notion is part of the proposed mechanism of what is known as the "hygiene hypothesis" that describes the association between of helminth infection and low incidence of autoimmunity (Maizels, 2009). The Tregs-induced increased "self tolerance" may reduce anti-tumour immunity and this could potentially be the link between spirocercosis and tumour formation. It appears, however, that although FoxP3+ cells were circulating in lymphatics around *S. lupi* nodules, "homing" into the nodules did not take place. The low number of FoxP3+ cells does not entirely preclude their potential role in local or systemic immune inhibition in spirocercosis but functionality assays are required. However, it is important to acknowledge that although FoxP3 is the gold standard marker of murine Tregs (Rouse, 2007), there are many types of Tregs that do not express FoxP3, for example the widely described Tr1 cells (Rouse, 2007). These cells also regulate the immune response through secretion of IL-10 and TGFβ and it is possible that they are involved in immunoregulation in spirocercosis.

One weakness of the current study is that tissue sampling was not standardized. Unfortunately, this is the reality when utilizing clinical cases, especially in a retrospective study. The cell counting was also limited to a single section. However, because this is primarily a descriptive study, we believe the results are valid. Moreover, in the search for Tregs, we tried to augment the chances for finding them by limiting the count to areas with high CD3+ cells presence (based on the lymph node findings and pilot observations) and yet we met with limited success. Therefore, the lack of FoxP3+ cells in most of the *S. lupi* nodules seems reliable. The study also provide unique *in situ* morphologic picture of the FoxP3+ infiltrate, which no dog study has reported. The key question in spirocercosis remains: What is the trigger for the transformation from the chronic inflammatory, fibroblastic nodule to sarcoma? This transformation may be triggered by the inflammatory response or, alternatively, via worm excretory / secretory (ES) products. Recent studies have shown that ES products from *Opisthorchis viverrini*, a helminth that induces cholangiocarcinoma in humans, increased fibroblast cell proliferation in cell cultures (Thuwajit et al., 2004). However, the theory of stimulation of cells in the nodule by the worm does not completely exclude the inflammatory mediation hypothesis, because other studies have shown that *Opisthorchis viverrini* ES products up-regulate the expression of TGFβ, which may represent an indirect carcinogenic effect via immunosuppression (Thuwajit et al., 2006). Many studies have elucidated the role played by helminth ES products in the modulation of the immune response, especially via the inhibition of innate cell functions and induction of a Th2 response (Hewitson et al., 2009). Such mechanisms clearly warrant further investigation if we are to understand the pathogenesis of *S. lupi*-induced sarcoma.

7.6 Tables

Table 1

Scoring system for CD3+ and Pax5+ infiltrates

score	Infiltrate intensity (x400 fields)	Number of foci (x20 fields)
0	scant or absent	0
1	positive cells evident but not in all fields	≤ 1
2	positive cells present in all fields but markedly fewer in number than other inflammatory cells	≤ 3
3	Positive cells predominant	≥4

Table 2

Scoring system for MAC387+ infiltrates

score	Infiltrate intensity (x400 fields)
0	scant or absent
1	positive cells evident but not in all fields
2	positive cells present in all fields but markedly fewer in number than other inflammatory cells
3	Positive cells predominant

Table 3

Leukocyte prevalence in the different groups

Group	Predominantly MAC387	Predominantly CD3	Equal CD3 and MAC387
Neoplastic	72% (18/25)	24% (6/25)	4% (1/25)
Non-neoplastic	70 % (32/46)	21.5% (10/46)	8.5% (4/46)
Normal esophagus	43% (6/14)	50% (7/14)	7% (1/14)

Table 4

Nodule distribution and score of MAC387+ cells

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Score	
	Even	Patchy	Mixed	Peripheral	Central	Both		Mean	Median
Neoplastic	88% (22/25)	4% (1/25)	8% (2/25	60% (15/25)	8% (2/25)	32% (8/46)	0/46	2.04 ± 0.98	2 (1-3)
Non-neoplastic	98% (45/46)	2% (1/46)	0/46	6% (3/46)	22% (10/46)	72% (33/46)	0/46	2.37 ± 0.9	3 (1-3)
Normal esophagus	50% (7/14)	1/14 (7%)	0/14	NA	NA	NA	43% (6/14)	0.57 ± 0.51*	1 (0-1)

*The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 5

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Foci number		Score	
	Diffuse	Focal / multifocal	Mixed	Peripheral	Central	Both		Mean	Median†	Mean	Median
Neoplastic	24% (6/25)	12% (3/25)	48% (12/25)	48% (12/25)	0	36% (9/25)	16% (4/25)	3.56 ± 4.69	1 (0-16)	1.56 ± 1,21	1 (0-3)
Non-neoplastic	52% (24/46)	13% (6/46)	33% (15/46)	48% (22/46)	4.5% (2/46)	45.5.5% (21/46)	2% (1/46)	2.68 ± 4.04	1 (0-18)	1.78 ± 0.94	2 (0-3)
Normal esophagus	43% (6/14)	0/14	0/14	NA	NA	NA	57% (8/14)	0	0	0.43 ± 0.51*	0 (0-1)

Nodule distribution and score of CD3+ cells

† 2 cases in the non-neoplastic group had focally extensive areas of cell infiltrate that could not be counted

* The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 6

Nodule distribution and score of Pax5+ cells

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Number of foci		Score	
	Diffuse	Focal / multifocal	Mixed	Peripheral	Central	Both		Mean	Median	Mean	Median
Neoplastic	8% (2/25)	20% (5/25)	24% 6/25	44% (11/25)	0/25	8% (2/25)	48% (12/25)	2.44 ± 3.91	1 (0-16)	0.96 ± 1.14	1 (0-3)
Non-neoplastic	28% (13/46)	33% (15/46)	13% (6/46)	45.5% (21/46)	4.5% (2/46)	24% (11/46)	26% (12/46)	1.8 ± 2.62	0 (0-1)	1.15 ± 1.07	1 (0-3)
Normal esophagus	0/14	0/14	0/14	NA	NA	NA	100% (14/14)	0	0	0*	0

* The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 7

Lymphocyte prevalence in the different study groups

Group	Predominantly CD3	Predominantly Pax5	Equal prevalence
Neoplastic	80% (20/25)	0/25	20%, 5/25
Non-neoplastic	78% (36/46)	4.5% (2/46)	17.5%, 8/46
Normal esophagus	100% (14/14)	0/14	0/14

Table 8

The number of FoxP3+ cells per 0.0625mm^2 in the different groups

Group	FoxP3+ cells			
	Peripheral		Central	
	Mean±STD	Median (range)	Mean±STD	Median (range)
Neoplastic	0.73±3.65	0 (0-3)	0.13±0.39	0 (0-2.2)
Non-neoplastic	0.69±1.19	0 (0-4.2)	1.34±5.55	0 (0-27.8)
Normal esophagus	Mean:0±0, Median: 0			

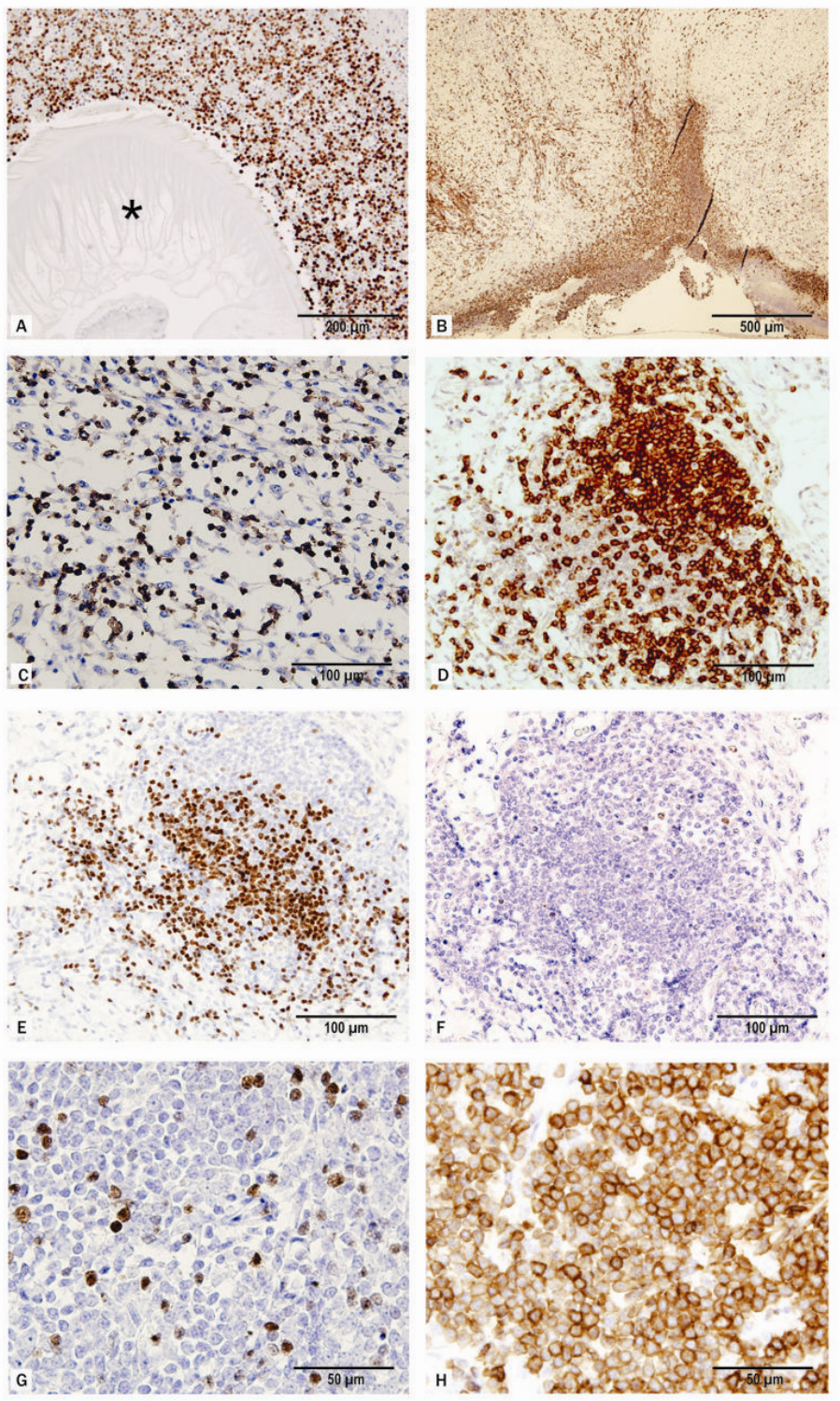
Table 9

Number of T regulatory cells per 0.0625mm^2 in the lymph nodes of the different groups

Group	Bronchial lymph nodes (5 fields)		Popliteal lymph nodes	
	Mean±STD	Median	Mean±STD	Median (range)
Neoplastic (n=5)	86.44±34.39	97 (38-130)	91.5±23.59	84.5 (65-112)
Non-neoplastic (n=4)	85.95±54.55	81 (33-158)	108.35±35.8	100 (74-156)

7.7 Figure Legends

Figure 1



A: MAC387+ leukocytes, predominantly neutrophils, around a *Spirocerca lupi* parasite (asterisk) in a non-neoplastic esophageal nodule.

B: MAC387+ leukocytes, predominantly neutrophils, in an extensive area of ulceration in a *Spirocerca lupi*-induced esophageal osteosarcoma.

C: Diffuse distribution of CD3+ T lymphocytes in a *Spirocerca lupi*-induced esophageal undifferentiated sarcoma.

D: Focal/nodular distribution of CD3+ T lymphocytes in a *Spirocerca lupi*-induced esophageal undifferentiated sarcoma.

E: Pax5+ B lymphocytes in the same lymphoid focus at the periphery of Spirocerca *lupi*-induced esophageal undifferentiated sarcoma as is shown in D.

F: FoxP3+ cells in the same lymphoid focus at the periphery of *Spirocerca lupi*-induced esophageal undifferentiated sarcoma as is shown in D and E.

G: FoxP3+ cells in a bronchial lymph node, draining the distal esophageal osteosarcoma referred to in figure D.

H: CD3+ T lymphocytes in the same area of bronchial lymph node as shown in figure G.