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T-Lymphocytic Infiltrate in Canine Mammary Tumours: Clinic and Prognostic Implications

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Abstract. *In recent years, considerable progress has been made in understanding the role of the immune system in tumour progression. However, in canine mammary tumours (CMT), the prognostic value of T-lymphocytes is not established. The aims of the present study were to characterize T-lymphocytic infiltrate in 57 canine mammary tumours (21 benign and 36 malign), by immunohistochemical detection of CD3 antigen, and to determine its association with several clinicopathological parameters and overall survival. CD3+ positive cells were counted in 10 high-power fields within the tumour (i.e. The tumour-infiltrating T-lymphocytes, TIL), in the peripheral area of the tumour and in the adnexal non-tumoural mammary gland. $CD3^+$ TILs were significantly more frequent in benign than in malignant tumours ($p<0.001$). Conversely, peripheral $CD3^+$ TILs were significantly more frequent in malignant than in benign neoplasias ($p<0.001$). For $CD3^+$ T-lymphocytes in the adnexal non-tumoural mammary gland, there was no statistical difference in their frequency between benign and malignant tumours. On survival analysis, there was a tendency towards an association of a higher number of $CD3^+$ TILs and a shorter overall survival ($p=0.08$). Interestingly for $CD3^+$ T-lymphocytes in the adnexal non-tumoural mammary gland, a statistically significant relationship was observed, with a higher number of lymphocytes conferring a reduced overall survival ($p=0.045$). Further studies will be required to better understand the biological implications of the current findings.*

Canine mammary gland tumours are amongst the most common neoplasms that affect female dogs (1), but despite their clinical relevance and high incidence, studies concerning the search for prognostic factors with potential therapeutic relevance are still necessary.

Several studies looked to possible roles of the inflammatory response in tumour behaviour both in veterinary and human medicine, however, the involvement of the immune system in the development of mammary cancer remains controversial (2).

In canines, the characterization of inflammatory cells associated with neoplasia has been reported for histiocytoma, transmissible venereal tumour, seminoma, papilloma, nasal carcinoma (3) and others by flow cytometry (4). In canine venereal sarcoma, the quantity of T-lymphocytes is higher in the group of tumors that show spontaneous regression or stable growth compared to those that exhibit progressive growth (5, 6). Likewise, in oral papilloma, the maximum number of T-cells infiltrating the tumour occurs during the period of rapid tumour regression (7) and in canine cutaneous histiocytoma, the lymphocytic infiltrate represents the morphological expression of an antitumor immune response, which correlates with observations of spontaneous regression "*in vivo*" (8, 9). In turn, in canine seminoma, the presence of T-lymphocytic infiltrate might explain the biological behaviour of these tumors, which that rarely metastasize, and the favourable prognosis often associated with them (10).

In human breast cancer, several studies suggest that certain types of inflammatory cells are not 'innocent bystanders' at breast tumour sites, but instead interact with tumour cells in the development and progression of the tumour itself (11-13). However, despite all the scientific evidence, the prognostic significance of T-lymphocytic infiltration during breast carcinogenesis is still subject to great debate (14-18). To our best knowledge, there is only one recent study that evaluates the presence and phenotype of tumour-infiltrating T-lymphocytes (TILs) in canine mammary neoplasms, but without prognostic information (19). Consequently, it is very important to analyze the impact of T-cells in the biological behaviour of these neoplasms. The aims of the present study were to characterize the T-lymphocytic infiltrate present in canine mammary tumours (benign and malignant) and to determine its association with clinicopathological parameters and with overall survival.

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Materials and Methods

Case selection. Fifty-seven canine mammary tumours (21 benign and 36 malignant) from a total of 57 female dogs, were obtained from the histopathological files of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, and included in this study. Case selection was based on the recruitment of the totality of cases seen in a period of one year (from January to December/2008). The cases that presented hyperplasia, dysplasia and mastitis were excluded. All the tumours were removed by regional mastectomy. The material was fixed in 10% neutral formalin and paraffin embedded. Sections (4 μm) were cut and stained with haematoxylin and eosin (HE) for histological examination.

Clinicopathological evaluation. Tumours were classified according to the World Health Organization (WHO) criteria for canine mammary neoplasms (20). Malignant tumours were graded in accordance with the method proposed by Elston and Ellis (21) based on the assessment of three morphological features: i) the degree of tubule formation; ii) nuclear pleomorphism; and iii) mitotic index. Each of these features was scored on a scale of I to III (slight, moderate or marked degree). In brief, tubular formation was considered as I when the tumour had more than 75% of tubules; II when the tumour had 10 to 75% of tubules and III when the tumour presented <10% of tubular formation. For nuclear pleomorphism, grade I was considered when the nuclei were small, regular and uniform; II for moderate nuclear size and variation and III when there was marked nuclear variation. The mitotic index was calculated by counting all mitoses in 10 high power fields (HPF; magnification of ×400) using a Zeiss Axiolab microscope (field diameter 0.575 mm, field area 0.260 mm^2) in order to ensure equivalence with assessments made by Elston and Ellis (21). Mitotic index I was considered when the number of mitoses was between 0 and 8, II when the number of mitoses was between 9 and 18, and III when the number of mitoses was equal or greater to 19, as previously described (22). The histological grade was based on the total score: grade I, well-differentiated (3-5 points); grade II, moderately differentiated carcinoma (6-7 points); and grade III, poorly differentiated carcinoma (8-9 points).

Each tumour was assessed for size (T1 <3 cm; T2 ≥3 and <5 cm; T3 ≥5 cm), skin ulceration, tumour histological type, presence of necrosis, mitotic index, nuclear grade, histological grade of malignancy, tumour invasiveness (locally invasive or vessel invasion), regional lymph node involvement and presence of distant metastasis at the time of diagnosis.

Immunohistochemical analysis. For immunohistochemistry, 3 μm sections were prepared and a streptavidin–biotin–peroxidase complex method was used with a commercial detection system (Ultra Vision Detection System, Lab Vision Corporation, Cheshire, UK) following the manufacturer's instructions. Antigen retrieval was carried out by microwave treatment in 0.01 M citrate buffer, pH 6.0. Sections were heated three times (5 min each) at 750 W, and the evaporated buffer was replaced with distilled water after each heating session. After cooling at room temperature (approximately 20 min), the sections were immersed in 3% H_2O_2 for 30 min to block endogenous peroxidase activity. All slides were then incubated with a blocking serum (Lab Vision®) for 10 min and then incubated with the specific antibody. As a specific antibody, polyclonal antibody against CD3 (Dako®) was used with dilution 1:50 in phosphate-buffered saline (PBS), incubated for 2 h at room temperature. After incubation, tissue sections were rinsed in PBS and then incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (Lab Vision). Subsequently, the colour was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) at 0.05% with 0.01% H_2O_2 (30%), and slides were counterstained with Gill's haematoxylin, dehydrated and mounted for evaluation by light microscopy. The primary antibody was replaced with PBS for negative controls and positive controls consisted of sections of canine lymph nodes.

Immunoreactivity evaluation. Positivity was indicated by the presence of a brown labelling pattern in the cytoplasm or in the cytoplasmatic membrane of lymphoid cells. Positive T-lymphocytes were evaluated in the tumour (TIL), in the periphery of the tumour and in the adnexal non-tumoural mammary gland, when present (n=25). The periphery of the tumour was considered as the marginal edge of the tumour, external to the connective capsule. When tumours did not present this structure, the periphery was considered as the most proximal external region of the tumour, constituted mostly by the surrounding stroma. The adnexal non-tumoural mammary gland was considered as the region constituted by an apparently normal mammary gland without visible morphological alterations, adjacent to the tumours.

The three regions with the most intense and homogeneous positivity of each of the counting areas were selected. In these regions, we all labelled cells were counted, evaluating a total of 10 HPFs (×400) following a previously described method (23).

Follow-up data. After surgical excision of the tumours, follow-up was carried out in the 36 animals with malignant mammary tumours, for a mean follow-up period of 18 months (minimum 4, maximum 26 months). Overall survival (OS) was defined as the period between surgery and natural death due to the tumour or euthanasia in advanced stages of the disease (confirmed at necropsy).

Statistical analysis. The statistical software SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) version 12.0 was used for statistical analysis. Associations between $CD3^+$ T-lymphocyte expression and categorical variables were studied with the application of ANOVA tests. The Bonferroni *post hoc* test for multiple comparisons of means was also carried out, when its use was appropriate.

Survival curves were generated by the Kaplan–Meier method and the survival rates were compared using the Breslow test. The cut-off values considered correspond to the mean values in the group of malignant tumours for each of the variables considered. All values are expressed as means±S.E. In all statistical comparisons, $p<0.05$ was accepted as denoting significant difference.

Results

Tumours. Benign tumours (n=21) were classified as simple (n=7) and complex adenomas (n=5), and benign mixed mammary tumours (n=9). Malignant tumours (n=36) were diagnosed as complex carcinomas (n=19), solid carcinomas (n=7), tubulopapillary carcinomas (n=8) and carcinosarcomas (n=2).

Table I. *Frequency of CD3+ T-lymphocytes in benign and malignant canine mammary tumours.*

Location	Group	n	Mean	S.E	*p*-Value
Intratumoral	Benign	18	611	80.944	<0.001
	Malignant	36	256	37.704	
Peripheral	Benign	14	21	2.369	<0.001
	Malignant	29	170	19.205	
Adnexal mammary gland	Benign	14	170	44.509	0.124
	Malignant	25	107	16.683	

n, Number of samples; S.E, standard error.

T-Lymphocyte immunostaining. CD3 immunostaining was always observed in the cytoplasm or in the cytoplasmatic membrane of T-lymphocytes in a diffuse and homogeneous pattern. T-Lymphocytes tend to contact closely with neoplastic cells and sometimes accumulate around the walls of the veins that drain the tumour. However, although we verified the presence of perilobular and perivascular T-lymphocyte clusters, these were less frequent than diffuse inflammation, which emerged as the predominant pattern of infiltration.

As shown in Table I, mean values for the presence of intratumoural positive TILs in benign tumours were higher than those of their malignant counterparts, the difference being statistically significant. The presence of $CD3^+$ T-lymphocytes at the periphery of the tumour was also statistically associated with tumoural behaviour but in an inverse manner (malignant tumours had a higher number of T-lymphocytes than benign neoplasias).

Clinicopathological characteristics and T-lymphocytes infiltrate in malignant tumours. $CD3^+$ TILs were statistically significantly associated with the tumour histological type and tumour invasiveness. Solid carcinoma and tumours with vessel invasion presented the highest TIL positivity (Table II).

T-Lymphocytes in the periphery of the tumour were observed and evaluated in only 29 out of the 36 cases included. In the remaining 7 cases (2 complex carcinomas, 3 solid carcinomas and 2 carcinosarcomas), this area was not suitable for the correct evaluation of the positive T-lymphocytes due to the antigen retrieval treatment. The presence of T-lymphocytes in the tumour periphery was statistically significantly associated with histological type and mitotic index. Solid carcinoma and tumours with high mitotic index had the highest positivity for T-lymphocytes in this counting area (Table II).

In the present study, it was possible to evaluate the T-lymphocytic infiltrate in the non-tumoural mammary gland adjacent to the tumour in 25 cases. Clinical features such as tumour size, histological grade of malignancy, tumour invasiveness, presence of lymph node involvement and presence of distant metastasis at the time of diagnosis were positively and statistically associated with the presence of T-lymphocytic infiltrate in the adnexal non-tumoural mammary gland. All of these results are summarized in Table II.

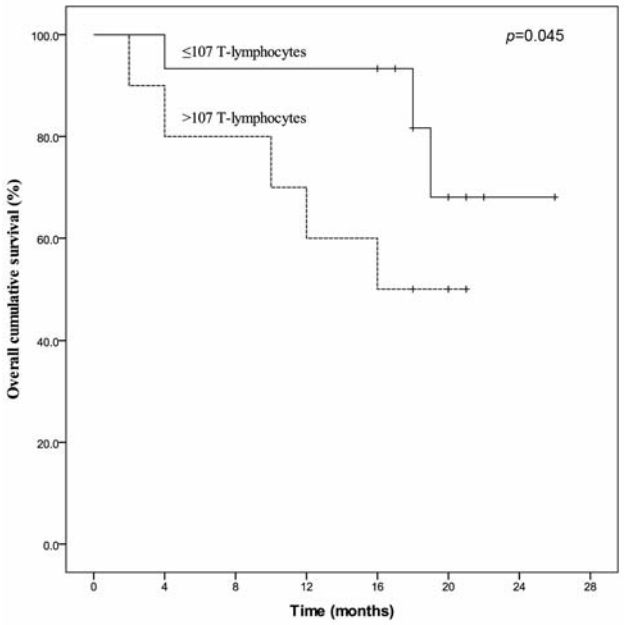

Figure 1. *Relationship between the presence of CD3+ T-lymphocytes in adnexal mammary gland and the overall survival in female dogs with malignant mammary tumours.*

Follow-up study. All cases had available follow-up data. In order to analyse the prognostic value of the presence of $CD3^+$ T-lymphocytes, we established its relationship with OS at 18 months (Table III).

Animals with benign tumours (n=21) remained alive. Among the animals with malignant neoplasms (n=36), 13 cases died of metastatic disease, confirmed at necropsy.

$CD3^+$ TIL and the $CD3^+$ T-lymphocytes in the tumour periphery were not statistically associated with the overall survival (p=0.08 and p=0.5 respectively). Interestingly, the presence of $CD3^+$ T-lymphocytes in the adnexal non-tumoral mammary gland was a significant predictor of OS (p=0,045) (Figure 1).

Discussion

The role of the T-lymphocytic infiltrate in cancer tumourigenesis remains controversial. In human medicine, some studies (in breast, oropharynx and cervical cancer) describe a relationship between high T-lymphocytic infiltrate, the smallest tumour diameter, absence of lymph node

Table II. *Relationship between the presence of CD3+ T-lymphocytes and clinicopathological parameters in malignant canine mammary tumours.*

Clinicopathological parameter	Intratumoral				Peripheral				Adnexal mammary gland			
	n	Mean	SE	*p*-Value	n	Mean	SE	*p*-Value	n	Mean	SE	*p*-Value
Tumour size												
T1 <3 cm	11	170	31.584	0.327	10	164	28.992	0.930	9	100^{ab}	21.378	0.017
T2 ≥3 cm and <5 cm	15	289	68.736		10	165	27.667		12	79^{a}	16.774	
T3 ≥5 cm	10	300	79.720		9	181	46.138		4	209^{b}	61.369	
Necrosis												
Absent	11	297	71.392	0.470	10	167	26.778	0.938	9	109	21.995	0.940
Present	25	237	44.774		19	171	26.206		16	106	23.462	
Ulceration												
Absent	10	341	81.051	0.163	7	142	53.218	0.422	7	139	46.339	0.240
Present	26	223	41.112		22	178	19.369		18	95	14.782	
Histological type												
C. tubulopapillary	8	255^{ab}	73.966	**0.014**	8	172	28.146	**0.034**	7	116	22.041	0.191
C. solid	7	469^{a}	89.761		4	287	80.069		2	223	156.500	
C. complex	19	166^{b}	42.445		17	141	20.072		14	90	18.653	
Carcinosarcoma	2	364^{ab}	129.00		-	-	-		2	76	23.500	
Mitotic index												
I	23	243	40.063	0.631	17	130^{a}	16.556	**0.008**	17	101	16.118	0.199
II	8	233	100.751		7	185^{ab}	42.907		5	81	25.846	
III	5	347	134.377		5	282^{b}	52.705		3	186	100.165	
Nuclear pleomorphism												
I	-	-	-	0.362	-	-	-	0.590	-	-	-	0.611
II	29	239	38.590		25	174	22		21	111	19.402	
III	7	327	113.186		4	143	32		4	87	24.107	
Histological grade of malignancy+												
I	18	210	44.331	0.355	16	152	21.145	0.595	14	90	17.558	**0.001**
II	13	275	62.349		8	185	30.444		10	104	19.048	
III	5	371	153.993		5	200	80.170		1	379	-	
Tumour invasiveness												
Locally invasive	22	191	39.228	**0.028**	19	176	18.724	0.658	17	80	12.086	**0.016**
Vessel invasion	14	358	67.945		10	158	44.394		8	164	39.959	
Lymph node involvement												
N_0	24	218	44.248	0.164	20	179	18.000	0.484	19	89	13.023	**0.047**
N_+	12	336	67.966		9	149	48.715		6	165	52.331	
Metastasis												
M_0	32	253	40.737	0.819	26	168	17.529	0.821	22	93	12.778	**0.016**
M_1	4	281	108.811		3	183	128.56		3	213	94.439	

n, Number of samples; SE, standard error; C, carcinoma; + according to Elston and Ellis21. Mean values with different superscript letters denote statistically significant differences for each item considered according to Bonferoni *post hoc* test ($p<0.001$).

involvement and absence of distant metastasis (14, 24-27). Other researchers indicate that inflammatory responses mediated by T-lymphocytes in invasive breast cancer are closely associated with poor tumour differentiation and cancer progression (13).

In veterinary medicine, there is one recent study that evaluated the presence and phenotype of TILs in canine mammary tumours (19), but the prognostic impact of immune responses in canine mammary cancer has not been reported yet. For other types of neoplasm (cutaneous histiocytoma and transmissible venereal tumour), T-lymphocytes appear to be involved in tumour regression (5, 6, 8).

Our results showed that the number of intratumoural T-lymphocytes was higher in benign tumours with respect to their malignant counterparts. The present results are in agreement with a study performed for human prostate cancer (23); according to this study, the presence of a dense immune cell infiltrate in benign lesions might indicate that these lesions have the necessary immunogenicity to recruit cells of the immune system. The initial response to damaged cells is thus dependent on cell-mediated immunity, whereas the decrease in density of immune cells in malignant tumours reflects immunosuppression, and the development of these tumours was associated with a defect in the same kind of

Table III. *Relationship between the number of CD3+ T-lymphocytes and overall survival at 18 months in 36 female dogs with malignant mammary tumours.*

Variable	n	Cumulative survival (%)	*p*-value
Intratumoral CD3+ T-lymphocytes			
≤256	18	71.97	0.08
>256	18	41.67	
Peripheral CD3+ T-lymphocytes			
≤170	15	57.14	0.50
>170	14	71.43	
CD3+ T-lymphocytes in adnexal mammary gland			
≤107	18	81.67	0.045
>107	7	50.00	

n, Number of samples.

immunity. In our opinion, this mechanism may be present in canine mammary tumours, justifying the higher values of T-lymphocytes associated with benign lesions.

With regard to peripheral areas, malignant tumours had a higher number of $CD3^+$ T-lymphocytes than benign tumours. The variation of the inflammatory responses involves protection against tumour development or promotion of tumour development and progression (15). This could be justified by the polarity of the responses of different subsets of lymphocytes in tumours, as $CD4^+$ T-lymphocytes, for primary sites of cancer and/or their distant metastases (15) and the imbalance of the normal ratio of T-lymphocytes (28). However, in the present study, we considered the T-lymphocytes independently of their subset (cytotoxic T-cells, T-helper cells, T-regulatory cells). Further studies are needed in order to clarify their individual role in neoplastic disease.

In malignant tumours, we observed a marked variation in the lymphocytic infiltrate amongst different tumour samples, from a small number of T-lymphocytes to high numbers. This observation has already been described in human breast cancer (12, 29) and seems to indicate that the presence of a malignant tumour in the canine mammary gland does not always lead to an influx of large numbers of lymphocytes since in some tumours the number of T-lymphocytes was scarce.

In our series, the $CD3^+$ intratumoural T-lymphocytes ($CD3^+$ TILs) were statistically significantly associated with tumour histological type. Solid carcinomas presented the highest frequency of $CD3^+$ TILs, and these results are in agreement with one recent study in canine mammary tumours (19). This probably reflects the involvement of T-lymphocytes in canine mammary tumourigenesis, but more studies are needed to clarify these findings. TILs have also been statistically associated with tumour invasiveness. Tumours with vessel invasion presented the highest values of T-lymphocytes in the intratumoural area. Contrary to the results of our series, one work on squamous cell carcinomas of the oral mucosa in humans revealed that the quantitative predominance of the inflammatory infiltrate was shown to be directly related to the large areas of invasion into the tissues underlying the tumour parenchyma (30). However, the fact that they are very different tumours makes it difficult to establish comparisons.

The number of $CD3^+$ T-lymphocytes in the periphery of the tumour was statistically associated with tumour histological type and once again solid carcinomas presented the highest values. $CD3^+$ T-lymphocytes in the tumour periphery also revealed a statistically significant association with a high mitotic index. Contrary to the results obtained in our work, one study about canine transmissible venereal tumour, performed by Pérez *et al*. (5), suggests that a higher frequency of $CD3^+$ T-lymphocytes is associated with a lower mitotic index. However, once again, the fact that they are very different tumours makes it difficult to establish comparisons.

The higher frequency of $CD3^+$ T-lymphocytes in the adnexal non-tumoural mammary gland in our study, revealed a statistical association with tumour size, histological grade of malignancy, tumour invasiveness, presence of lymph node involvement and presence of distant metastases at the time of diagnosis (see Table II). To our best knowledge, there are no studies in human or veterinary medicine that assesses the presence of T-lymphocytes in the adnexal non-tumoural mammary gland. Our results lead us to believe that the inflammatory microenvironment, rich in T-lymphocytes, in the adnexal non-tumoural mammary gland has a prominent role in the progression of the cancer. However, due to the lack of other studies describing this finding, both in human and veterinary medicine, we cannot exclude the hypothesis that this constitutes a part of epiphenomena not related to tumour progression. Consequently more studies are necessary to clarify this subject.

In the present series, considering the relationship with the OS for $CD3^+$ TILs, it is noteworthy that although the difference between groups did not reach statistical significance ($p=0.08$) there was a tendency towards a higher number of $CD3^+$ TILs and a shorter OS. Further studies, with a large series of tumours and with long-term follow-up periods are necessary in order to clarify the present results.

$CD3^+$ T-lymphocytes in the adnexal non-tumoural mammary gland presented a statistically significant association with overall survival ($p=0.045$). The reason why only the presence of $CD3^+$ T-lymphocytes in adnexal non-tumoural mammary gland appears to have prognostic significance, and a statistically significant relationship with a larger number of parameters that reflect the aggressiveness of the tumour, remains unclear. However, a possible mechanism might be related to the effects of some

chemokines (such as CCL2 and CCL5) produced by both leukocytes and tumour cells themselves (31-33). The expression of inflammatory chemokine CCL2 was detected in human breast tumour cells, whereas normal mammary epithelial cells in proximity to the tumour cells did not express this chemokine (31, 34, 35). Concomitantly with increased monocyte migration, it has been recently indicated that CCL2 reduces some functions of T-lymphocytes in the tumour (31, 36). In turn, the chemokine CCL5 was also expressed in large amounts in tumour cells, whereas epithelial cells and normal ductal epithelial cells that constitute benign breast lesions in the vicinity of the tumour cells were practically not evident (31, 32, 37). This chemokine may therefore have the ability to down-regulate T-lymphocyte migration (in unidentified ways) in breast tumours (31, 37). It is possible that a similar mechanism might be present in canine mammary tumours. The results of our study lead us to believe that the pro-tumourigenic immune responses of $CD3^+$ T-lymphocytes in adnexal non-tumoural mammary gland may be the starting point for the development and expansion of tumour cells.

In accordance with other authors (38), we believe that canine mammary neoplastic tissue presents a 'Darwinian' microenvironment that selects for the type and extent of inflammation most favorable to tumour growth and progression.

In conclusion, our results showed that the presence of $CD3^+$ TILs were related to tumoural behaviour and revealed a tendency to be related to a shorter OS in canine malignant mammary tumours. $CD3^+$ T-lymphocytes in adnexal non-tumoural mammary gland were also implicated in tumour progression and survival. Further studies will be required to better understand the biological implications of the current findings.

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