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Relationship between the inflammatory infiltrate and the degree of differentiation of the canine cutaneous squamous cell carcinoma

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ABSTRACT

The inflammatory response may have pro or anti-neoplastic effects in tumors, depending on the histological type and malignancy level of the tumor. This study aimed to evaluate the profile of predominant inflammatory cells by immunohistochemistry in cutaneus squamous cell carcinoma (SCC) of dogs, comparing it with the degree of differentiation of the tumor. Twenty samples of SCC were analyzed. The tumors were histologically classified into two groups, differentiated SCC (SCCd=12) and undifferentiated SCC (SCCu=08). The tumor inflammatory infiltrate was determined by immunohistochemistry, in order to identify macrophages, lymphocytes and plasma cells. The comparison between groups, SCCd and SCCu, was not significant concerning the density of macrophages (P=1.0), T lymphocytes (P=0.335) and plasma cells (P=0.075). However, when comparing the inflammatory infiltrate in each group, the macrophages were the predominant cell type in both groups, a significant difference was found in the SCCd with plasma cells (P < 0.0001). In the SCCu the difference occurred with lymphocytes (P < 0.05) and plasma cells (P=0.0006). It could be concluded that the presence of inflammation in cases of SCC does not play a role in the differentiation of the neoplasm, since the inflammatory infiltrate was similar in both groups of SCC.

1. Introduction

Squamous cell carcinoma (SCC), also known as epidermoid carcinoma, is one of within the most frequent skin tumors, corresponding to 5% of skin tumors in dogs (Daleck, De Nardi, & Rodaski, 2007). Even though it may appear in any part of the body, SCC is most commonly observed in the head, abdomen, limbs and perineum (Conceição & Santos, 2010).

Microscopically, it can be observed that the cells present an increased hyperchromatic nuclei with aggregated chromatin. The nucleolus may or may not present variation in size, but is prominent. The histological pattern of SCC varies according to the differentiation degree of these cells. In the degree of differentiation tumors a formation of "keratin pearls" can be observed, these are depositions of keratin concentric lamellae at the center of nests of neoplastic cell cords. In less differentiated areas, keratinization of individual cells may occur, without the formation of keratin pearls. Cells may not produce keratin in more aggressive cases (Goldschmidt et al.,1998).

The participation of the immune system in the growth of tumors is still controversial. There are divergent studies regarding the participation of these cells in the suppression or in the development of neoplastic cells. Some studies have reported the contribution of inflammation to the development of cancer (Giercksky, 2001). The combination of immuno-tolerance mechanisms and evasion of the host immune system enable neoplastic cells to create a favorable environment for growth and tumor aggressiveness (Dunn, Old, & Schreiber, 2004; Satyam, Singh, Badjatia, Seth, & Sharma, 2011).

A significant infiltration of macrophages, lymphocytes and plasma cells can be observed in cases of mammary tumors in female dogs. Some studies have reported that the presence of these lymphocytes improve tumor prognosis, while in others it is believed that these cells favor the proliferation of neoplastic cells. The data regarding the role of the inflammatory cells in the development of the neoplastic cells is still uncertain (Damasceno et al., 2008).

Many studies performed in medicine and veterinary medicine highlight the importance of the immune response against neoplastic cells. On the other hand, studies evaluating an inflammatory response in skin tumors are scarce, regardless of the significant incidence of

these neoplasms in dogs. Current studies in dogs have a higher focus in mammary tumors in female dogs. Thus, the characterization of the inflammatory response in canine cutaneous squamous cell carcinoma is important, since it aims to contribute to the understanding of the complex biology of this tumor and with the prognosis and development of oncological immunotherapy studies. The objective of the present study was to evaluate the inflammatory cells profile which is predominant in cutaneous squamous cell carcinoma in dogs, using immunohistochemical analysis, and comparing it to the tumor's differentiation degree.

2. Material and methods

Twenty samples of canine cutaneous squamous cell carcinoma from the Department of Veterinary Pathology, College of Agricultural and Veterinary Sciences, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP, Jaboticabal, São Paulo State, Brazil) were analyzed. The samples after surgery were sent for histopathology. All surgical samples, were considered to be excisional and representative, because in each sample, when evaluated morphologically (hematoxylin and eosin) and by immunohistochemical techniques, different regions of the neoplasm were represented.

The histological classification of squamous cell carcinoma was performed according to Goldschmidt and Hendrick (2002), and the samples were divided into two groups: Group SCCd (keratinized tumors, well differentiated and with formation of various keratin pearls, n=12) and Group SCCu (tumors with individual cell keratinization or no keratinization at all, poorly differentiated, n=8).

2.1. Histopathological analysis

The SCC samples were kept in paraffin packs and cut within a section thickness of 5 μm, and stained with hematoxylin and eosin, in order to be examined in light microscopy. The tumor organization pattern, the presence of mitosis figures, the intensity of the inflammatory response, the proportion of keratin pearls and the presence of fibrous stroma associated to the tumor were considered for a final classification of the tumors.

2.2. Immuno-histochemical analysis

Sections were embedded in paraffin, cut into 5 μm specimens and immunohistochemical analysis was performed, for immuno-detection of macrophages (mouse monoclonal antibody anti-macrophage, Serotec, MCA874G, cod. 1209, dilution 1:4000), T lymphocytes (rabbit polyclonal antibody, anti-CD3, Daki, cod. M7254, dilution 1:200) and plasma cells (rabbit polyclonal antibody, anti-CD138, Spring, cod. E4564, dilution 1:25). The sections were deparaffinized in a stove, at 60 °C, for one hour. They were then plated in absolute xylene (20 min), followed by soaking in decreasing ethylic alcohol solutions, until the tissue was hydrated in distilled water. In order to proceed with immunostaining of the referred antibodies, an antigenic recuperation by heat was performed (Pascal pressure chamber, Dako) in a sodium citrate buffer solution 10 mM (pH 6.0). Afterwards, the blocking of endogenous peroxidase with hydrogen peroxide 30 volumes (Merk) was performed, added to methyl alcohol, achieving a final solution with a concentration of 8%, for 30 min. The blocking of unspecific proteins was then carried out with a commercial product (Protein Block, Dako, cod. X0909), for 30 min, at room temperature. The incubation of primary antibodies for macrophages and T lymphocytes was performed

in a humid chamber, at 4 °C, for 18 h. For plasma cells, the incubation occurred in a humid chamber, at room temperature, for 18 h. The Streptavidin Biotin Peroxidase Complex kit (Kit LSAB, Dako, cod. K0690) was used as a secondary complex for macrophages and lymphocytes, and the Envision Dual Link System-HRP (Dako Cytomation, cod K406189-2) was used for immunostaining of plasma cells. Diaminobenzidine was the chromagen used (DAB, Dako, cod. S-3022-2) and the counterstaining was elaborated with Harris Hematoxylin. The negative control reaction was performed by replacing the primary antibody by anticorpo diluent (Dako Cytomation, reference S302283-2). The positive control reaction was done according to the manufacturer's recommendation.

Five microscope fields (Nikon Eclipse E200) were examined under a 40× objective lens, with an area of 0.197 mm^2, in order to determine the number of immunostained cells per animal for each antibody, of according to the protocol described by Moreira et al. (2010). The same areas of the tumor were evaluated for the different types of inflammatory cells and a mean was determined for each animal by antibody.

2.3. Statistical analysis

Non-parametric Kruskal-Wallis and Dunn Multiple Comparison tests, were used in order to compare the immunostaining of macrophages, lymphocytes and plasma cells in the groups, considering P < 0.05. The comparison between SCCd and SCCu groups, for the cell types, was evaluated by the Mann-Whitney non-parametric test. The analyses were developed in the Graphpad Prism program (version 4.00, 2003).

3. Results

The characteristics of the tumor malignancy allowed distribution of the samples into two groups (Table A.1). The presence of mitotic figures was predominant in the SCCu group, which presented the highest averages of mitotic figures. The variation in volume and cell shape (pleomorphism) was similar between the groups and the pattern of distribution of the tumor was compatible with the tumor's differentiation degree (Table A.1).

The tumor inflammatory infiltrate varied from discrete to moderate, mainly in deeper layers of the dermis, comprising macrophages, lymphocytes and plasma cells. Mast cells associated with this infiltrate were observed occasionally. In the superficial section, when the epidermis was ulcerated, degenerated neutrophils prevailed. In more differentiated tumor samples was noted little inflammatory infiltrate and in undifferentiated samples a higher inflammatory infiltrate associated with the tumor was observed. Desmoplasia around nests of neoplastic cells was frequently found, varying from moderate to severe intensity.

The immunodetection of macrophages (Fig. A.1.A/A.1.B) and lymphocytes (Fig. A.1.C/A.1.D) was observed in the cytoplasm of cells and for plasma cells (Fig. A.1.E/A.1.F) this occurred in the cytoplasmic membrane in SCC samples.

The comparison between groups (SCCd and SCCu) was not significant (Fig. A.2) concerning the density of macrophages (P=1.0), T lymphocytes (P=0.335) and plasma cells (P=0.075). However, when comparing the predominant cellular profile within each group (Fig. A.2), a significant predominance of macrophages in relation to the other types of cells was observed in SCCd (P < 0.001) and in SCCu (P=0.0006). However, the SCCu group had a greater involvement of lymphocytes associated with macrophage (Fig. A.2.B).

4. Discussion

In the present study, when comparing the inflammatory infiltrate in each group, it was observed that macrophages were the predominant cell type in both squamous cell carcinoma groups. This present study differs from reports in horses, that were observed to have an intense local immune response, represented by numerous CD3+T lymphocytes, B cells and IgG-secreting plasma cells, that were associated with equine SCC from the early stages of tumor growth. This immune response was not correlated with the histological grade or invasiveness (Pérez, Mozos, Martín, & Day, 1999), but studies performed with breast cancer in women found that the high expression of macrophages associated to the tumor was correlated to a poor prognosis (Lee, Happerfield, Borbrow, & Millis, 1997; Leek et al., 1996). The extensive infiltration of these macrophages was already reported and showed to be associated to high malignancy tumors, large tumors and necrosis focal points (Lee et al., 1997).

The role of macrophages in the inflammatory infiltrate associated to SCC was not clear, since they were more evident in the deeper areas of the tumor and not in places where there was epidermis ulceration. In the literature, differentiated populations of macrophages may have anti or pro-tumor action, because they produce different cytokines profiles and express different surface receptors. Several factors related to the tumor's microenvironment may activate macrophages and direct them to a M1 or M2 profile, according to published studies (Yuan, Chen, & Yang, 2008). Tumors tend to have a predominance of macrophages with M2 phenotype, induced by the microenvironment cytokines profile, which are generally anti-inflammatory, such as IL-10. These macrophages would be inefficient in activating a cytotoxic type immune response, which is efficient against tumor cells. Therefore, the M2 would stimulate angiogenesis and changes in the extracellular matrix, interesting aspects for the invasion and metastasis in more aggressive tumors (Guruvayoorappan, 2008). In the present study, the antibody used for immunostaining of macrophages was MCA874G, which has affinity in binding to calprotectin, a protein present in young macrophages. The same is used as a marker for inflammatory diseases and participates in processes such as tumorigenesis, apoptosis and cellular homeostasis, as well as responses mediated by Th1 cells in immune and immune-pathological reactions (Dhas, Bhat, & Gane, 2012). It would be important to evaluate in future studies what is the predominant phenotype of macrophages present in SCC, in order to understand better their more invasive biological behavior.

The second most commonly found cell type in SCC in the present study were lymphocytes. They did not show significant differences between groups SCCd and SCCu, but it had greater immunodetection in SCCd group (Fig. A.2.D). Some studies highlight the presence of lymphocytes associated to tumor inflammatory infiltrate, which may indicate a protective response or a cytotoxic activity against the tumor. Currently, there are studies with neoplasias in humans that report the presence of T cells acting in favor of malignant tumor development (Nzula, Going, & Stott, 2003). Malignant neoplasias seem to suppress antitumor response of Th1 lymphocytes in tissues (Satyam et al., 2011). This anergy of T lymphocytes may also be related to the deficiency of MHC-1 molecules in the tumor's microenvironment, induced by some immuno-suppressor cytokines, such as IL-10 (Th2 response). These aspects facilitate tumor progression (Matsuda et al., 1994). Furthermore, in future studies, it would also be interesting to evaluate the different types of T lymphocytes (CD4 and CD8, Treg), as well as pro and anti-tumor cytokines, in order to better elucidate the activity of these lymphocytes in the tumor's microenvironment.

Plasma cells had a low number in both SCC groups. Their activation takes place by the interaction between cells presenting antigen and TCD4+lymphocytes, which in a standard response would lead to the production of antibodies by activated plasma cells. The profile of Th1 cytokines is one of the activators of this cell type (Murphy, Travers, & Walport, 2008).

There are few studies performed in dogs that evaluate the different subpopulations of leucocytes associated to the SCC inflammatory infiltrate. The intricate relationship between tumor cells and host cells require further studies, in order to understand the influence of leucocyte infiltrate on the progression of malignant neoplasia, such as the SCC case. Increased the understanding of this interaction between neoplasia and host cells will allow the development of specific therapies against this type of neoplasia.

5. Conclusion

It could be concluded that the presence of inflammation in cases of SCC in this study does not play a role in the control of differentiation of the neoplasm, since macrophages were the type of inflammatory cell most commonly associated to the tumor in both the differentiated and as undifferentiated group.

Conflict of interest

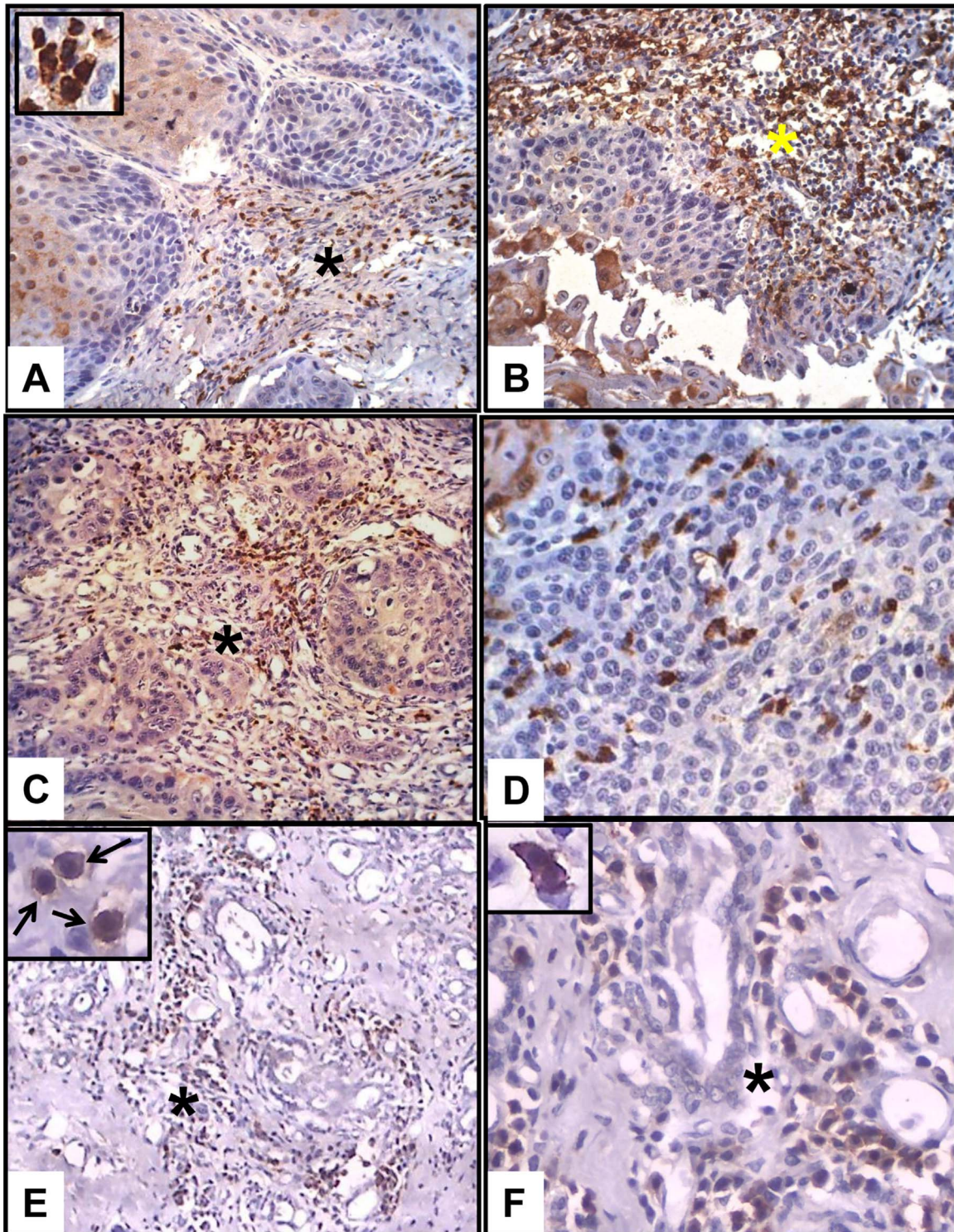The authors declare that they have no conflict of interest.

Acknowledgements

Appendix A

see Figs. A.1 and A.2.

Appendices

see Table A.1.

Fig. A.1. Photomicrographs of canine cutaneous squamous cell carcinoma. (A) Note in the SCCd (20× objective lens) and (B) in SCCu (40× objective lens) the cytoplasmatic immunostaining of macrophages (anti-MCA874G) in inflammation associated with the tumor (*/insight). (C) In the SCCd (20× objective lens) and (D) in SCCu (40× objective lens), the immunodetection of T lymphocytes (anti-CD3) was observed in the inflammatory infiltrate of the tumor (*). The positive immunostaining in membrane of plasma cells (anti-CD138) was observed in inflammatory infiltrate of the tumor, in SCCd (E/10× objective lens) and (F) in SCCu (F/40× objective lens) (*/insight – arrows/40× objective lens). Streptavidin Biotin Peroxidase Complex.
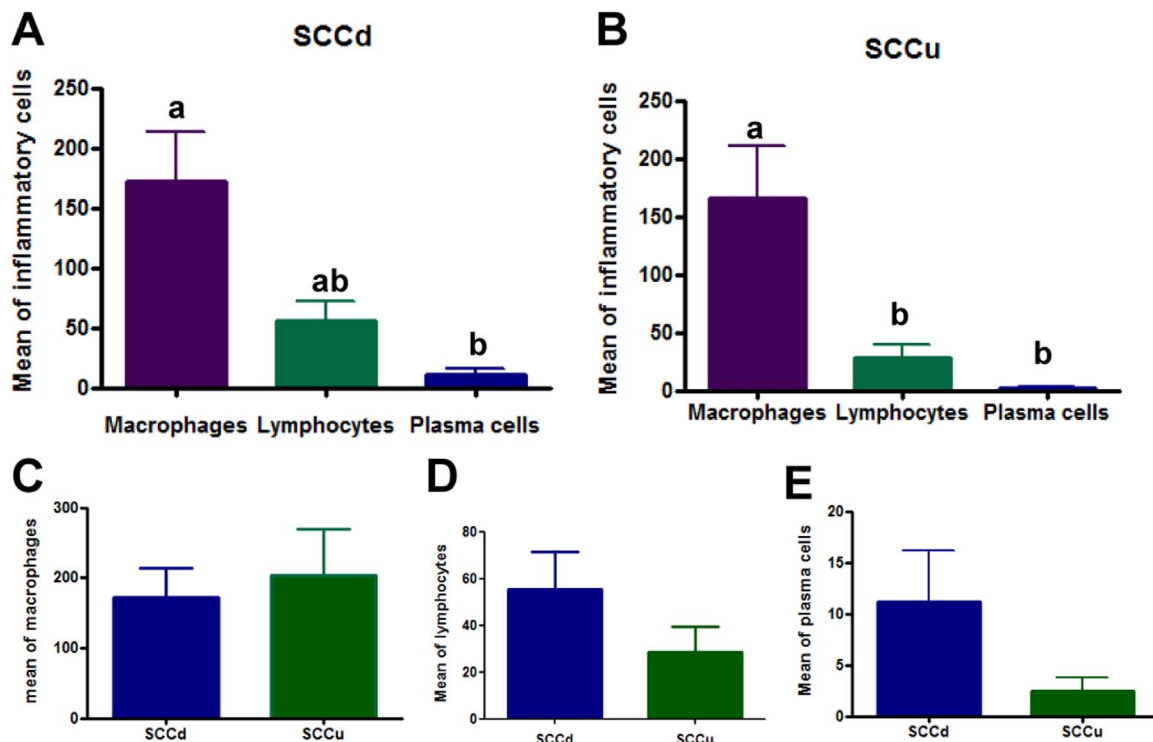
Fig. A.2. Predominant profile of inflammatory cells in dogs with cutaneous squamous cell carcinoma. (A) Note that in both the differentiated group (SCCd) and (B) in the undifferentiated group (SCCu), there has been a predominance of macrophages in the tumor infiltrate. Kruskal-Wallis test and Dunn test ($p < 0.05$). Note the comparison between groups, SCCd and SCCu, was not significant concerning the density of macrophages (C), T lymphocytes (D) and plasma cells (E). Mann-Whitney test ($p > 0.05$).

Table A.1

Malignancy characteristics of differentiated (SCCd) and undifferentiated squamous cell carcinoma (SCCu) in dogs.

Malignancy criteria	Groups	
	SCCd	SCCu
Pleomorphism[*]	46% accentuated	33% accentuated
	54% moderate	67% moderate
Tumor organization pattern	85% nests	17% nests
	15% cords	83% cords
Mitosis figures averages (10 CGA)	19.5	36.8

CGA=high-power fields (40× objectives lens).

[*] Predominant scores in tumor.

References

Conceição, L. G., & Santos, R. L. (2010). Sistema Tegumentar. , in: Santos, R. L., & Alessi, A. C. (Eds.). (2010). Patologia Veterinária . 1 ed. Roca: São Paulo, 454–455.

Daleck, C. R., De Nardi, A. B., & Rodaski, S. (2007). *Neoplasias de Pele. Oncologia em Cães e Gatos* (1 ed.) Roca: São Paulo, 258–262.

Damasceno, K. A., Ribeiro, L. G. R., Costa Neto, J. M., Toribio, J. M. M. L., Silva, N. S., Aguiar, P. H. P. Estrela-Lima, A. (2008). *Caracterização da Resposta Inflamatória nos Carcinomas Mamários de Cadelas* Gramado: COMBRAVET- Congresso Brasileiro de Veterinária, 24–41.

Dhas, D. B. B., Bhat, B. V., & Gane, D. B. (2012). Role of calprotectin in infection and inflammation. *Current Pediatric Research*, 16, 83–94.

Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annual Review of Immunology*, 22, 329–360.

Giercksky, K. E. (2001). COX-2 inhibition and prevention of câncer. *Best Practice & Research Clinical Gastroenterology*, 15, 821–833.

Goldschmidt, M. H., Dunstan, R. W., Stannard, A. A., Vontcharner, C., Walder, E. J., & Yager, J. A. (1998). *Histological classification of epithelial and melanocytic tumors of the skin of domestic* (2 ed.) Washington: Armed Forces Institute of Pathology, 105 .

Goldschmidt, M. H., & Hendrick, M. J. (2002). Tumors of the skin and soft tissues. , in: Meuten, D. J. (Ed.). (2002). Tumors in domestic animals . 4 ed. Iowa: Iowa State Press, 45–117.

Guruvayoorappan, C. (2008). Tumor versus tumor-associated macrophage: how hot is the link? *Integrative Cancer Therapies*, 7, 90–95.

Lee, A. H. S., Happerfield, L. C., Borbrow, L. G., & Millis, R. R. (1997). Angiogenesis and inflammation in invasive carcinoma of the breast. *Journal of Clinical Pathology*, 50, 669–673.

Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., & Harris, A. L. (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Research*, 50, 4625–4629.

Matsuda, M., Salazar, F., Petersson, M., Masucci, G., Hansson, J., Pisa, P. Kiessling, R. (1994). Interleukin 10 pretreatment protects target cells from tumor- and allo-specific cytotoxic T cells and downregulates HLA class I expression. *Journal of Experimental Medicine*, 180, 2371–2376.

Moreira, P. R. R., Vieira, L. M., Andrade, M. M. C., Bandarra, M. B., Machado, G. F., Munari, D. P. et al. (2010). Immune response pattern of the popliteal lymph nodes of dogs with visceral leishmaniasis. *Parasitology Research*, 107, 605–613.

Murphy, K., Travers, P., & Walport, M. (2008). *Janeway's immunobiology* (7 ed.) New York: Garland Science, 887.

Nzula, S., Going, J. J., & Stott, D. I. (2003). The role of B lymphocytes in breast cancer: a review and current status. *Cancer Therapeutics*, 1, 353–362.

Pérez, J., Mozos, E., Martín, M. P., & Day, M. J. (1999). Immunohistochemical study of the inflammatory infiltrate associated with equine squamous cell carcinoma. *Journal of Comparative Pathology*, 121, 385–397.

Satyam, A., Singh, P., Badjatia, N., Seth, A., & Sharma, A. (2011). A disproportion of TH1/TH2 cytokines with predominance of TH2 in urothelial carcinoma of bladder. *Urologic Oncology*, 29, 58–65.

Yuan, A., Chen, J. J. W., & Yang, P. C. (2008). Pathophysiology of tumor associated macrophages. *Advances in Clinical Chemistry*, 45, 199–223.