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RESEARCH ARTICLE

Exploring the dynamics of IL-6, TGF-β1, and CD8+ T cells in the canine transmissible venereal tumor: new perspectives

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ABSTRACT

Canine transmissible venereal tumor (CTVT) serves as a valuable model for studying tumor-immune system interactions due to its unique progression and regression phases. This study investigated the roles of IL-6, TGF-β1, and CD8+ T cells in CTVT progression and regression phases, evaluating their association with therapeutic response and cytological malignancy criteria. Samples from 25 untreated dogs were analyzed via cytology, confirmed via histopathology. Immunohistochemistry for IL-6 and TGF- β1, and CD8+ via flow cytometry. IL-6 and TGF-β1 expression was detected in 100% of tumor samples, with cytoplasmic localization scoring an intensity in 75% of the histological section showed positive staining. Interestingly, IL-6 was exclusively expressed by tumor cells in certain cases. CD8+ T cell density in tumors demonstrated a significant negative correlation (ρ=-0.65, $P<0.05$) with mitotic activity, suggesting an inverse relationship between cell proliferation and immune response. Therapeutic response was established at the 4th week of treatment for most cases, although some required up to ten weeks to achieve complete remission. Resistant cases were included in a second-line chemotherapy protocol with doxorubicin, reflecting the variability in tumor behavior. These findings propose a novel hypothesis regarding IL-6 production by neoplastic cells as a mechanism to suppress antigen presentation and immune activation. Further exploration of IL-6's dual role in tumor progression and regression may reveal potential therapeutic targets for enhancing immune response in CTVT and other cancers.

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INTRODUCTION

Canine Transmissible venereal tumor (CTVT) is a round cell neoplasm that affects the genital mucosa of both female and male dogs. It occurs in dogs of various age groups, primarily in young and sexually mature animals, and is transmitted through implantation in mucous membranes via coitus, biting, licking, scratching, or sniffing an infected animal (Ganguly *et al.*, 2016; Baez-Ortega *et al.,* 2019). CTVT serves as an excellent study model for cancer immunology due to its ability to evade immune defense mechanisms. Biologically, it typically demonstrates benign characteristics; however, it can also exhibit features of malignancy, including metastasis to organs such as the liver, kidneys, lymph nodes, brain, and heart (Ostrander *et al.*, 2016; Zhou *et al.*, 2023; Laissaoui *et al.*, 2024).

Experimentally, CTVT displays a predictable growth pattern that includes both progression and regression phases (Hsiao *et al.,* 2002). According to studies by Hsiao

et al. (2008), after implantation, neoplastic cells exhibit evasion mechanisms that is triggered in two ways. First, upon implantation, CTVT cells produce tumor growth factor β1 (TGF-β1), a cytokine that promotes the suppression of natural killer (NK) cells and prevents the infiltration of CD8+ lymphocytes. In the second mechanism, evasion occurs due to the absence of lymphocytes; thus, interleukin-6 (IL-6) is not produced by tumor-infiltrating lymphocytes (TILs) IFN-γ; consequently, major histocompatibility complexes I and II (MHC I and II) tumor cells (Hsiao *et al.,* 2008).

During the progression phase, CTVT suppresses IL-6 secretion, thereby inhibiting immune recognition regulated by MHC class I and II and NK cells. When the level of IL-6 secreted by TILs reaches a certain threshold, its activity begins simultaneously with that of IFN-γ, allowing the infiltration of lymphocytes such as CD8+ cells, which activates the expression of MHC class I and II on CTVT cells, initiating the regression phase. The transition from the progression phase to regression is marked by an increase in CD8+ lymphocytic infiltration and the induction of MHC expression. During regression, CD8+ cells gradually initiate the process of populating the tumor microenvironment and pro-inflammatory-producing cytokine IL-6 (de Hsiao *et al.,* 2008; de Sanctis *et al*., 2024)**.**

Some authors propose the concept of IL-6 antagonizing TGF-β1. In this context, in vivo, the IL-6 plasmid (pIL-6) was used in combination with the IL-15 plasmid to antagonize TGF-β1 and activate the cytotoxicity of T cells and NK cells. The results demonstrated that the combination of pIL-6/pIL-15 with intratumoral administration mediated by electroporation successfully regressed the tumor and decreased TGF-β1 levels, resulting in the successful regression of the neoplasm (Zhang *et al*., 2005; Chou *et al.,* 2009; Faro *et al*., 2023).

While studies are showing the relationship between the expression of IL-6, TGF-β1, and CD8+ molecules and tumor regression and progression, there are also studies questioning this relationship and its causal relationship. The objective of this work was to clarify the controversial role of IL-6 and TGF-β1 in tumors and of CD8+ T cells in both the tumor and bloodstream during the phases of tumor regression and progression. Additionally, this study aimed to relate these findings to the therapeutic response and cytological criteria of patients with malignancies.

MATERIALS AND METHODS

Ethics approval and consent to participate: This study was performed in line with the principles of the Declaration of Helsinki. The research obtained approval from the Ethics Committee for Animal Use at the Faculty of Veterinary Medicine and Animal Science of São Paulo State University (ECAU-FMVZ/UNESP) under protocol 0081/2021. Informed and explicit consent was obtained from the owners of the animals included in the study.

Sample collection: Twenty-five untreated dogs, with no preference for breed, sex, or age, were selected. The sample size followed criteria established in Chiang *et al.* (2013) and studies by the group Alzate *et al.* (2019). After confirming the CTVT diagnosis through fine-needle aspiration cytology with a 24G needle, a biopsy was performed under multimodal general anesthesia as described by do Prado Duzanski *et al.* (2022). Samples were stored in PBS (pH 7.2) for cytometry and in 10% buffered formalin for 24 hours for H&E processing and immunophenotyping of IL-6 and TGF-β1. Additionally, 10mL of jugular blood was collected in EDTA tubes to assess CD8+ cells in the bloodstream.

Malignancy criteria and therapy response: The groups were established based on the analysis of cytomorphological subtypes as established by Flórez *et al.* (2012) and the tumor progression status according to Mukaratirwa *et al*., (2003). Similar research was conducted by Flórez *et al.* (2016), Duzanski *et al.* (2017) supported this categorization. With the simultaneous classification of subtypes, smears were analyzed for malignancy. Samples subjected to Giemsa staining were used for evaluation. Cellular characteristics were divided into general, cytoplasmic, nuclear, and nucleolar characteristics and assessed according to methods developed by other authors Duzanski *et al.* (2017) and Silveira *et al.* (2009).

Each criterion was quantified by counting 100 cells per field in a total of 10 fields, yielding 1000 cells analyzed per sample. The frequency of each cytomorphological feature was calculated as the total number of occurrences observed across these 10 fields. The mean count of each feature was then determined from the double-blind assessments, ensuring unbiased evaluation. This approach followed established methods from Flórez *et al.* (2012), Mukaratirwa *et al.* (2003), Duzanski *et al.* (2017).

The animals received an identical therapeutic protocol, consisting of weekly intravenous infusions of vincristine sulfate at a dose of 0.75mg/m² of body surface area, and were monitored throughout treatment to assess tumor response. CTVT is a neoplasm well-documented for achieving complete remission with chemotherapy (Amaral *et al.* 2007; Florez *et al.* 2012; Duzanski *et al.* 2017). The response was categorized as follows: complete response (CR) when total tumor regression was achieved within four weeks after the start of therapy, partial response (PR) when regression was less than 50%, and resistant (R) when there was no measurable regression.

Immunophenotyping for TGF-β1 and IL-6: Immunohistochemistry was employed to identify TGF-β1 and IL-6, following an adapted protocol. Positive and negative controls, as well as antibody dilutions, were established based on the manufacturer's recommendations and internal standardizations. For TGF-β1, the antibody (clone ORB214661) was sourced from Biorbyt™ and used at a 1:200 dilution, with canine lung tissue serving as the positive control. For IL-6, the antibody (clone ORB579133) was also from Biorbyt™, used at a 1:400 dilution, and canine tonsil tissue served as the positive control.

The expression of cytokines TGF-β1 and IL-6 was quantified using a semi-quantitative method as described previously, in which the intensity of staining by antibodies was assessed at 4X and 10X magnification, dividing the tissue field into four quadrants and evaluating both the

intensity of staining and the distribution of expression within the tissue: 0 (absent), +1 (weak), +2 (moderate/high in 2/4 of the field), +3 (moderate/high in ¾ of the field), and +4 (moderate/high throughout the entire field); method adapted from McCarty (1986). The antigen distribution was assessed based on the following criteria: +1 (expression solely in tumor cells), +2 (expression in both tumor and epithelial cells), and +3 (expression in tumor, epithelial, and inflammatory cells).

Flow cytometry CD8+ T cells: Populations of T cells (CD8+) were analyzed in blood and tumor samples. Samples were acquired in the cytometer FACSCanto™ II [BD Biosciences, configuration 4:2–2 lasers (488nm Blue and Red 633nm) with FACSDiva™ software (BD Biosciences)]. A total of 20,000 events were read for blood analysis. Likewise, for the tumor, 20,000 total events were also read. The results were analyzed in the software FlowJo™, version vX.10.6 (Tree Stars Inc.) and expressed as percentage of positive cells for the markers in question.

Blood samples were placed in Histopaque™ 1077 (Sigma) solution in a ratio of 1:1 and centrifuged at 400g for 40min at 21°C. Subsequently, the mononuclear cells were transferred to fresh tubes and washed twice with cell culture medium (DMEM low glucose solution (Gibco) containing 100IU/mL penicillin and 250ng/mL amphotericin B) by centrifugation at 400g for 5min. Finally, the cells were resuspended in 1mL of culture medium for cell viability and quantity analysis by the Trypan Blue exclusion test in a Neubauer chamber (Strober, 2015).

Ex vivo samples of CTVT were subdivided into 3-5mm^3 fragments and submitted to enzymatic digestion with Collagenase IV (Sigma) in a water bath for 40min at 37.5°C. Subsequently, the digested tissue was transferred to a mesh filter coupled to a tube containing culture medium and washed twice with the same medium by centrifugation at 400g for 5min. Finally, tumor cells were resuspended and determined following the same methodology applied to the blood cells.

A minimum of approximately 2×10^5 viable cells were added to 1.5mL conical tubes and centrifuged at 400g for 20 seconds at 18°C in cytometry buffer (0.5% BSA in PBS). After discarding the supernatants, the anti-CD8+ antibody (Clone YCATE55.9, Monoclonal, 1:15 dilution, RPE-labeled; AbD Serotec™) and the respective isotype controls were added at appropriate concentrations to the pellets and incubated for 20 minutes at 4°C in the dark. At the end of the incubation, the samples were washed three times with 300µL of cytometry buffer solution (centrifugation at 1000 g for 20 seconds) and resuspended in 200µL of fixative solution (0.5% BSA in PBS with 2% formaldehyde) for analysis.

Statistical analysis: Regression analysis was conducted using generalized linear models by applying the GENMOD procedure in SAS™ software version 9.3, with gamma distribution and the log link function. The incidence of categorical variables was assessed using chi-square tests and Fisher's exact test. Multiple comparisons were analyzed using Tukey–Kramer adjusted p values ($P<0.05$). Correlation analysis was performed using the Spearman rank correlation method, as the study involved both continuous and categorical data and did not exhibit normality.

RESULTS

Among the tumors in the twenty-five selected patients, only tumors located in the genital region were observed. Regarding metastasis, in many patients, the neoplasm remained confined to the site of origin; metastatic processes were observed in only one patient (1/25). In this study, most patients exhibited chemotherapy-related side effects, including hematological (50%), dermatological (9%), and gastrointestinal (9%) effects. No side effects were reported in 32% of the patients. Table 1 provides a comprehensive summary of the cases included in this study, detailing the main variables analyzed, such as age, breed, sex, and histopathological features.

Positive expression of IL-6 (Fig. 1) and TGF-β1 (Fig. 2) by tumor cells was observed in all patients. Both cytokines were cytoplasmic. The intensity of the labeling was scored as 3 (indicating more than 75% of the histological section showed positive staining) for both IL-6 and TGF-β1. In some cases, it was possible to observe positivity for IL-6 expression only in tumor cells while simultaneously observing no expression by immune system cells.

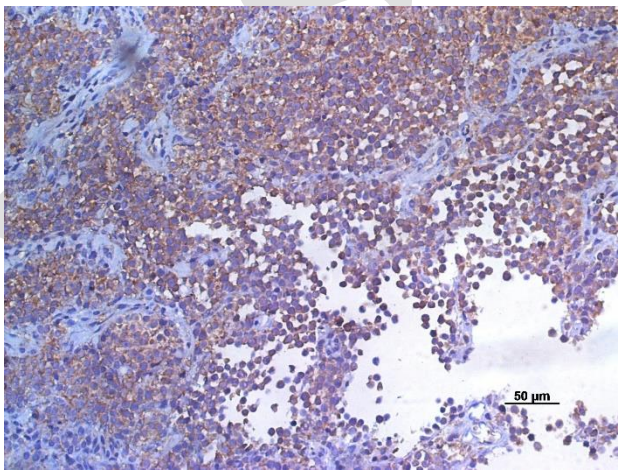

Fig. 1: Immunohistochemical reaction of IL-6 (Interleukin 6). Immunohistochemical reaction of IL-6 (Interleukin 6), highlighting the intense cytoplasmic expression by the neoplastic cells of CTVT and the absence of staining in the stromal connective tissue.

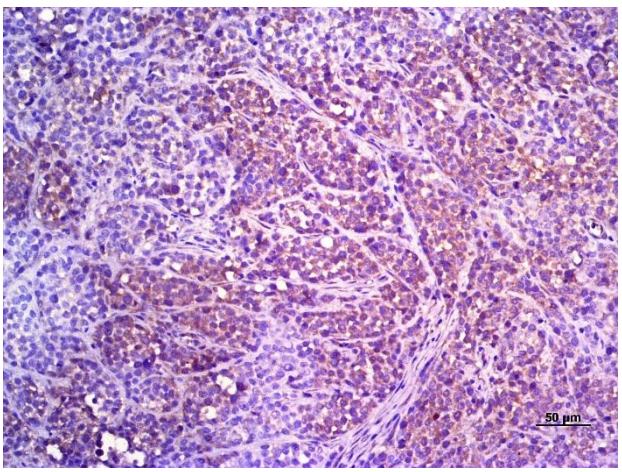

Fig. 2: Immunohistochemical reaction of TGF-β1(transforming growth factor beta 1). The immunohistochemical reaction of TGF-β1 shows intense cytoplasmic expression by the neoplastic cells of CTVT and the absence of staining in the stromal connective tissue.

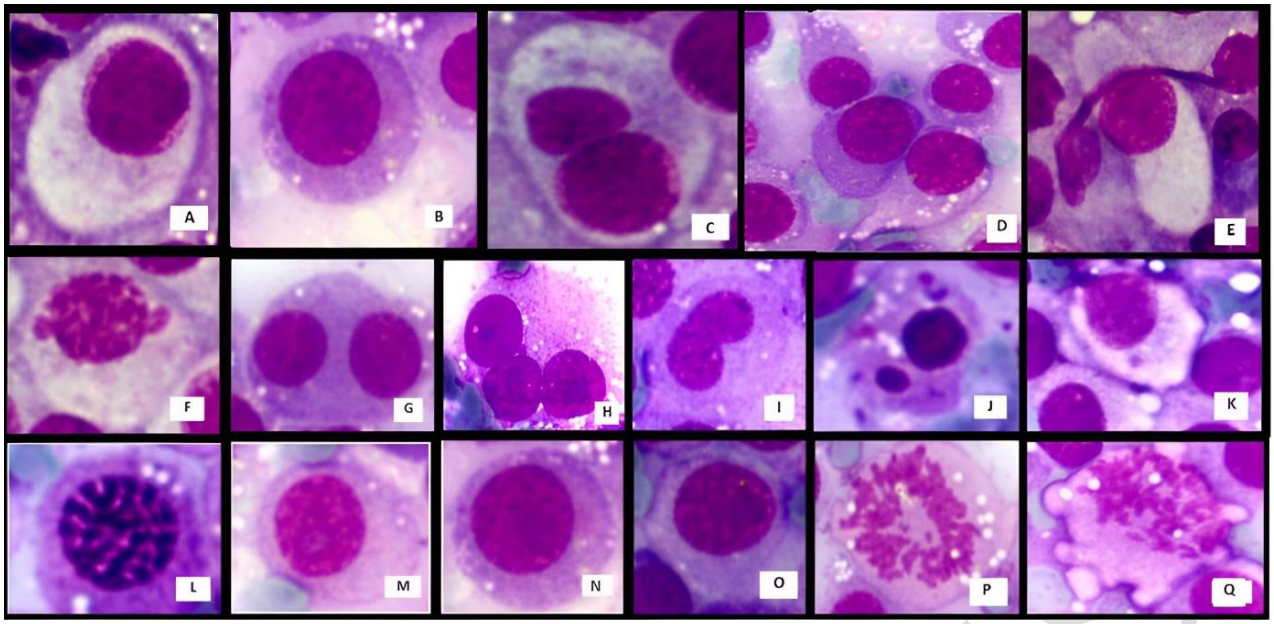

Fig. 3: Microphotographs of cytological criteria of CTVT. A: Plasmacytoid subtype; B: Lymphocytoid subtype; C: Cannibalism; D: Tadpole cell; E: Spindle cell; F: Nuclear budding; G: Binucleation; H: Multinucleation; I: Reniform nucleus; J: Apoptotic body; K: Micronucleation; L: Coarse chromatin; M: Loose chromatin; N: Dense chromatin; O: Granular chromatin; P: Mitosis; Q: Aberrant mitosis (400X).

Table 1: Comprehensive summary of the cases included in this study

Case	ID	breed	Sex	Age (Years)	Subtype	Fase
1	206880	Mixed	female	>2<6	Mixed	Progressive
2	212125	Mixed	male	>2<6	Mixed	Progressive
3	212578	Mixed	female	>2<6	Mixed	Progressive
4	213120	Mixed	female	>2<6	Plasmacytoid	Regressive
5	213404	Mixed	male	>2<6	Plasmacytoid	Progressive
6	213339	Mixed	female	>2<6	Plasmacytoid	Progressive
7	214123	Mixed	male	>2<6	Plasmacytoid	Progressive
8	214195	Mixed	male	>2<6	Plasmacytoid	Progressive
9	217085	Mixed	male	>2<6	Lymphocytoid	Regressive
10	215648	Mixed	male	>6	Mixed	Progressive
11	216136	Mixed	male	>2<6	Lymphocytoid	Progressive
12	216631	Mixed	female	>2<6	Lymphocytoid	Progressive
13	217052	Mixed	male	>2<6	Lymphocytoid	Progressive
14	217407	Mixed	male	>2<6	Lymphocytoid	Progressive
15	217405	Mixed	male	>2<6	Plasmacytoid	Progressive
16	217370	Mixed	male	>6	Lymphocytoid	Progressive
17	217747	Mixed	female	>2<6	Mixed	Progressive
18	217784	Mixed	male	>6	Plasmacytoid	Regressive
19	218434	Mixed	female	>2<6	Mixed	Progressive
20	201601	Mixed	female	>2<6	Lymphocytoid	Progressive
21	201602	Mixed	female	>2<6	Lymphocytoid	Progressive
22	201603	Mixed	female	>2<6	Plasmacytoid	Progressive
23	201604	Mixed	male	>2<6	Lymphocytoid	Progressive
24	201607	Mixed	male	>2<6	Plasmacytoid	Progressive
25	201605	Mixed	female	>2<6	Plasmacytoid	Progressive

In this study, nuclear and cytoplasmic malignancy criteria were quantified across the 25 cases of CTVT cytology. These included mitosis (μ=2.89), binucleation (μ=2.13), multinucleation (μ=0.22), signet-ring cells (μ=0.217), macrokaryosis (μ=1.63), lymphoglandular corpuscles (μ=2.87), spindle cells (μ=3.76), tadpole-shaped cells (μ=21.22), plasma membrane thickening (μ=1.54), cannibalism (μ=0.43), emperipolesis (μ=0.043), cytoplasmic projections (μ=43.91), hyperchromasia (μ=39.26), nuclear vacuoles (μ=4.61), rhinoform nuclei (μ=5.24), nuclear budding (μ=5.26), nuclear lobulation (μ=0.93), micronucleation (μ=0.33), and perinuclear halo (μ=0.91). The most frequently observed malignancy criterion across the 25 cases was cytoplasmic projections (μ=44), present in 100% of cases (25/25) The most common morphological criteria are represented in Fig. 3.

The expression of both cytokines, IL-6, and TGF-β1, did not show a significant correlation (P>0.05) with these cytohistological criteria, growth phase, or cell subtype. However, it is noteworthy that a significant negative correlation was observed between the mitosis cytohistological criterion and the number of CD8+ cells in the tumor, suggesting a potential relationship between cell proliferation and immune response in this neoplasm.

DISCUSSION

The literature describes various side effects of vincristine in dogs, including vomiting, depression, alopecia, skin ulceration, and hematological toxicity such as leukopenia (Punchkande *et al.*, 2022; Nazer *et al.*, 2023). In this research, hematological toxicity and gastrointestinal signs were observed in less than 10% of the cases in the studied dogs. Despite the low incidence, these effects have been documented previously, aligning with the findings reported in the literature.

The immunoexpression of the cytokines IL-6 and TGF-β1 was observed in 100% of the patients, regardless of whether they were in the progression or regression phase. Contrary to the findings of Hsiao *et al.* (2002; 2008), which reported increased expression of IL-6 during the progression phase and TGF-β1 during the regression phase, our analysis did not reveal statistical evidence supporting the preferential expression of either cytokine based on the phase of the process.

It is important to emphasize that the cited studies were conducted on experimentally transplanted tumors, whereas our investigation exclusively focused on animals with naturally implanted tumors. This methodological difference may significantly influence the results, as the characteristics and interactions of the tumor microenvironment, cellular adaptation, and host immune response differ inherently between experimental models and spontaneous tumors (Hsiao *et al.*, 2008; Tiwari *et al.*, 2023).

In this context, the observed results may be attributed to the specific characteristics of the tumor microenvironment in naturally implanted animals, where both cytokines were highly expressed during both the progression and regression phases. These cytokines may exert distinct functions depending on the specific dynamics between the tumor and the host immune system (Zhou *et al*., 2023).

The results of this study contrast with the findings of Tiwari *et al.* (2021), who investigated IL-6 expression using RT-PCR and reported overexpression of this cytokine during the progressive phase and low expression during the regressive phase. It is important to note that, similar to our study, Tiwari *et al.* (2021) included animals with naturally implanted tumors; however, differences in sample selection and methodologies were evident. While their study included 12 animals, our research comprised a total of 25. Moreover, their investigation evaluated IL-6 expression in both progressive tumors and regression induced by vincristine treatment, whereas our study included 22 progressive tumors and 3 naturally regressed tumors without treatment.

These methodological differences, along with the sample size and the statistical model employed, may influence the results obtained and their interpretation. The inclusion of data from tumors undergoing regression induced by treatment might have introduced differential dynamics in IL-6 expression patterns due to the direct effects of vincristine. In contrast, our evaluation reflects the baseline expression of the cytokine in the natural state of the tumor prior to any therapeutic intervention. This contrast highlights the importance of carefully considering the effects of the statistical model and experimental conditions when comparing studies investigating the role of IL-6 in the tumor microenvironment of CTVT (Zhou *et al*., 2023)

Given the results regarding the expression of IL-6, a new hypothesis is that during the progression phase of CTVT, cells produce IL-6 via an antagonistic mechanism to suppress the production of IL-6 by TILs, as illustrated in Fig. 1. The exclusive expression of IL-6 is observed in neoplastic cells, which may result in the absence of the major histocompatibility complex (MHC), which is normally induced by IL-6, leading to decreased antigen presentation (Mihara *et al.,* 2012; Tanaka *et al.,* 2014).

Currently, there are no reports on the existence of IL-6 isoforms. However, their expression in tumor cells raises a new question about whether this tumor cytokine exhibits pleiotropic, antagonistic, or synergistic effects via immunovigilance mechanisms. This observation provides a fascinating perspective that requires detailed exploration to understand the impact of IL-6 on the dynamics of the tumor microenvironment and its interaction with the immune system. This contrast highlights the complexity of the underlying mechanisms and underscores the relevance of complementary methodological approaches for a comprehensive understanding of the phenomenon in question.

Tanaka *et al.* (2014) described this negative feedback related to IL-6 in dendritic cells. Once recently synthesized and internalized in the cell, it binds to intracellular receptors (IL-6RA), resulting in a regulatory mechanism. Remarkably, our study appears to be the first to hypothesize the existence of this "loop" mechanism in CTVT, highlighting the importance of further investigating the role of IL-6 in this specific context.

Recent research utilizing various methodologies, including Mendelian analyses, has indicated that elevated levels of IL-6 are associated with various neoplasms, such as breast, colorectal, and gastric carcinoma. These levels have been investigated both systemically and in the tumor microenvironment, with tumor-associated macrophages and fibroblasts being identified as the main sources of IL-6 production (Cui *et al.*, 2024; Ghofrani-Shahpar *et al.*, 2024; Hu *et al.,* 2024).

Therefore, a more in-depth understanding of the molecular mechanisms underlying tumor progression is essential. Moreover, these findings suggest that tumor cells could serve as valuable models to study the processes of carcinogenesis and immune evasion. The presence of IL-6 in tumor cells raises the question of whether this cytokine, derived from tumors, exhibits structural or functional similarities to endogenous IL-6. Investigating these comparisons could provide key insights into the cellular signaling pathways involved in tumor progression and immune regulation.

Our findings, which demonstrated significant expression of TGF-β1 during the regression phase and IL-6 expression in the tumor microenvironment, align with previous studies in canine tumors like CTVT. However, unlike Tiwari *et al.* (2021), who observed systemic changes, our results were obtained from tissue samples, focusing on the local tumor microenvironment rather than systemic responses. This distinction highlights the importance of evaluating cytokine expression and immune mechanisms at the tumor site, as these localized interactions can provide more specific insights into tumor progression and immune evasion.

Our methodology, using immunohistochemistry (IHC) to examine cytokine expression, differs from previous studies that employed PCR-based techniques to assess systemic mRNA expression. Future studies should utilize more precise methods, such as PCR or mRNA evaluation in tumor tissue, to further elucidate the role of TGF-β1 and IL-6 in the tumor microenvironment. Such approaches could enhance our understanding of the tumor biology and immune response, supporting the development of targeted therapies. This research highlights the complexity of cytokine interactions in the microenvironment and suggests that tumor cytokines may exhibit differences in their activity compared to endogenous cytokines, which could have important implications for tumor progression and therapeutic response.

The three protein subtypes of TGF-β1 are described; although they share a highly conserved amino acid sequence, they exhibit similar functions in many contexts but also show differences in terms of tissue expression, regulation, and specific biological activity. This research evaluated only the TGF-β1 subtype, serving as a basis for future investigations to assess the two remaining isoforms to enhance the understanding of this mechanism (Massagué *et al.,* 2023).

The expression of TGF-β1 in the regression phase of CTVT may be related to immune system evasion through the allograft mechanism. This implies that as the

tumor regresses, the neoplastic cells at this time may produce the cytokine TGF-β1 to transplant themselves to another tissue, thus preventing death. This phenomenon could be a mechanism of tumor escape, allowing tumor cells to persist and spread, even during the regression of the primary tumor (Lu *et al.,* 2011). Therefore, this cytokine plays an important role in the escape mechanism of tumors implanted in other animals since the neoplasm enters the regression phase. However, further studies are needed to confirm this potential escape mechanism.

Another possibility is the effect of the cytokines IL-6 and TGF-β1 on immunosuppression. There are reports of the influence of these cytokines on the heterogeneous action of T lymphocytes, leading to the conversion of conventional T lymphocytes into regulatory T lymphocytes, which cause immunosuppression in canine lymphoma (Munhoz *et al.,* 2016). Therefore, IL-6 and TGF-β1 may still be associated with mechanisms of immunosuppression in the tumor microenvironment by stimulating an increase in Treg lymphocytes (Mahaki *et al.,* 2021); however, further research is needed in this direction to unravel this mechanism.

In this research, the CD8+ marker did not demonstrate a relationship with the clinical phase, therapy response, or cytohistological criteria. Studies suggest that an increase in CD8+T cells in the CTVT microenvironment is associated with tumor regression (Siddle *et al.,* 2015). The increase in CD8+ cells may have indirectly influenced the decrease in mitotic count. As observed in this study, there is an inversely proportional relationship between CD8+T cells and mitosis, which is a criterion for tumor progression in neoplasms (Kiupel *et al.,* 2011; Dutra *et al.,* 2008; Cardoso *et al.,* 2023; Jankowska *et al.,* 2024). The quantification of mitotic figures may have the potential, as in the Kiupel *et al*. (2011) grading system for mast cell tumors, to predict the behavior of CTVT economically and rapidly; in this case, the clinical phase and to avoid excessive use of chemotherapy (Kiupel *et al.,* 2011; Mason *et al.,* 2014).

Cytokines IL-6 and TGF-β1 play integral roles in immune response mechanisms and tissue repair. The intensity and distribution scores for both cytokines observed in this study indicate that the expression of IL-6 and TGF-β1 in tumor tissue was consistently above +3 for intensity and +1 for distribution. A score of +3 corresponds to moderate to strong intensity, indicating significant and consistent expression of these cytokines within tumor tissue. A score of +1 corresponds to expression solely in tumor cells, highlighting the localized presence of IL-6 and TGF-β1 within the tumor microenvironment. These findings suggest that neoplastic cells may impair, through mechanisms that remain unclear, the secretion of IL-6 and TGF-β1 by immune response cells, fostering an environment conducive to immune evasion (Lu *et al*., 2011; Massagué *et al*., 2023). Additionally, the quantification of mitotic figures in CTVT provides indirect information about the expression of CD8+ in the tumor microenvironment, which can be a way, based on studies already described in the literature, to predict the clinical stage and contribute economically and quickly to obtaining information about the patient's oncological condition (Mason *et al.,* 2014).

Conclusions: This study highlights the critical roles of cytokines IL-6 and TGF-β1 in the tumor microenvironment of CTVT, demonstrating their robust and consistent expression across both progressive and regressive phases of the disease. The findings suggest significant immune evasion mechanisms, including altered cytokine secretion by immune cells and the promotion of an immunosuppressive microenvironment. Furthermore, discrepancies with previous studies underscore the impact of methodological factors, such as sample size, experimental models, and analytical techniques, on cytokine expression patterns and tumor behavior. The inverse correlation observed between CD8+ T cell density and mitotic count underscores the intricate interactions governing tumor progression, while the quantification of mitotic figures emerges as a practical and cost-effective biomarker for prognostic assessment.

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Author's contributions: Conceptualization, FC (Fernando Carmona Dinau) and LM (Luis Mauricio Montoya); Methodology, CG (Carlos Mario González Zambrano), LM and FC; Software, CG and FC; Validation, LM, NS (Noeme Sousa Rocha); Formal Analysis, CG, FC, LM; Investigation, CG, FC, LM; Resources, NS; Data Curation, RO (Rogerio Antonio de Oliveira); Writing–Original Draft Preparation; Writing– Review & Editing, CG, FC, JJ (Juliana Jurado) and LM; Visualization, LM NS; Supervision, NS; Project Administration, NS.

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