



Evaluating the Efficacy of Metronomic Cyclophosphamide using Regulatory T Cell Populations and Markers of Angiogenesis in Canine Soft Tissue Sarcomas

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Abstract

Objective: The uninterrupted administration of low, oral doses of chemotherapy drugs (also known as metronomic chemotherapy) is thought to selectively target tumor angiogenesis as well as regulatory T cells (T_{reg}), both of which can facilitate tumor growth. This study was performed to determine whether evaluation of tumor growth, tumor angiogenesis and determination of T_{reg} numbers can be used to assess the efficacy of metronomic dosing of oral cyclophosphamide (CYC) in dogs with soft tissue sarcomas.

Procedure: Peripheral blood and tumor biopsies were obtained from five client-owned dogs with soft tissue sarcomas pre-treatment, and following 14 and 28 days of CYC therapy. Tumor size was measured in two dimensions using calipers at each sample collection. Patients then received local anesthesia and a core tumor sample was taken. These tissue biopsies were stained for endothelial cells using immunohistochemistry (IHC) and tumor microvessel density (MVD) was evaluated. Immunostaining and flow cytometry were performed on all blood samples to enumerate T_{reg} populations.

Results: Tumor size remained stable during the study period. Although tumor MVD and T_{reg} numbers tended to decrease, there were no significant differences in these parameters between pre-treatment and post-treatment time points.

Conclusions: Our results suggest that the doses of CYC utilized in this study were too low to significantly affect tumor growth over a 28 day period. Interestingly, there was a notable decrease in tumor MVD and T_{reg} cells between pre-treatment and day 14, both of which then increased by day 28. These trends suggest that metronomic CYC may be initially beneficial but its effects are not complete and are reversible.

Method

Inclusion criteria:

- Five dogs with measurable, histologically confirmed, spontaneously occurring soft tissue sarcoma, grade I or grade II, met the criteria for inclusion in this prospective clinical trial.

Exclusion criteria:

- Any diagnosed disease condition precluding sedation for biopsy or CYC therapy.
- Patients with metastatic tumors present.
- Patients receiving any radiation or chemotherapy within 3 weeks of enrollment into the study.
- No concurrent therapies including but not limited to non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids.

Treatment Schedule:

- Cyclophosphamide tablets compounded to 2.5 & 5.0 mg capsules by a professional compounding pharmacy.
- The first 2 dogs enrolled in this study received oral CYC at 10mg/m² per day for 4 weeks. Following interim analysis of the data, the next 3 dogs received oral CYC at 12.5 mg/m² daily for 4 weeks.

Assessment

- Physical exams, CBC, chemistry panel, urine analysis & pet quality of life questionnaires were performed at weeks 2 and 4 to assess toxicity.
- Blood samples, tumor size information and tumor biopsies were collected pre-treatment and post-treatment at days 14 and 28.
- Peripheral blood mononuclear cells (PBMCs) were stained with antibodies for cells expressing CD4⁺, CD8⁺ and CD4⁺FoxP3⁺ (T_{reg}) and analyzed using a Dako Cytomation CyAn flow cytometer.
- Tumor biopsies were immunostained with a cross-reactive human endothelial antibody to CD146. Tumor MVD was evaluated using AxioVision Release 4.6 imaging software.

Results

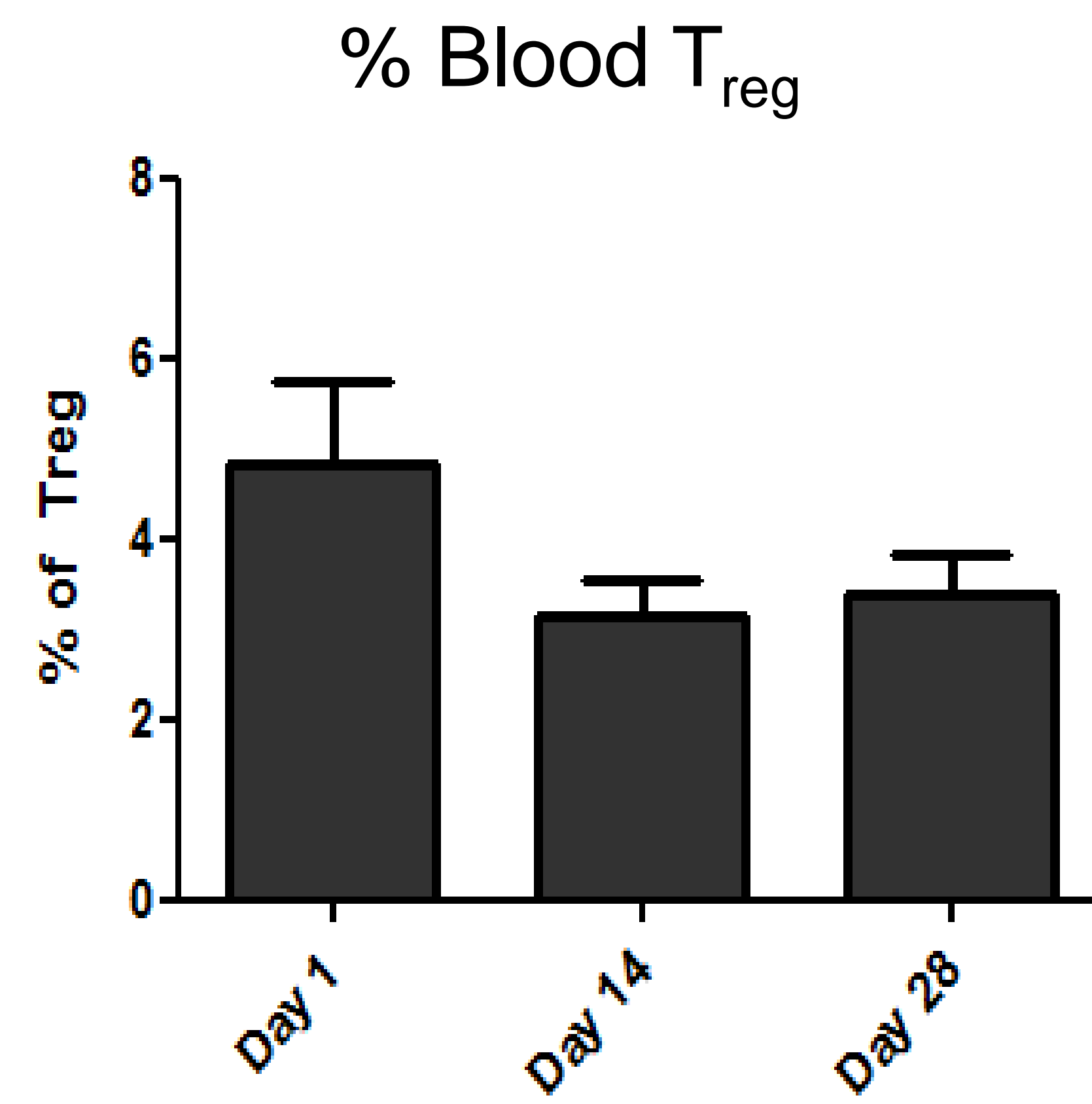


Figure 1.1 Effects of metronomic CYC on regulatory T cell populations. Peripheral blood and tumor draining lymph node aspirates were taken pre-treatment and during CYC treatments at days 14 and 28. PBMC were combined with cross-reactive antibodies for mouse regulatory T cells. Data were compared using 1-way ANOVA and no significant decreases ($P < 0.05$) were observed.

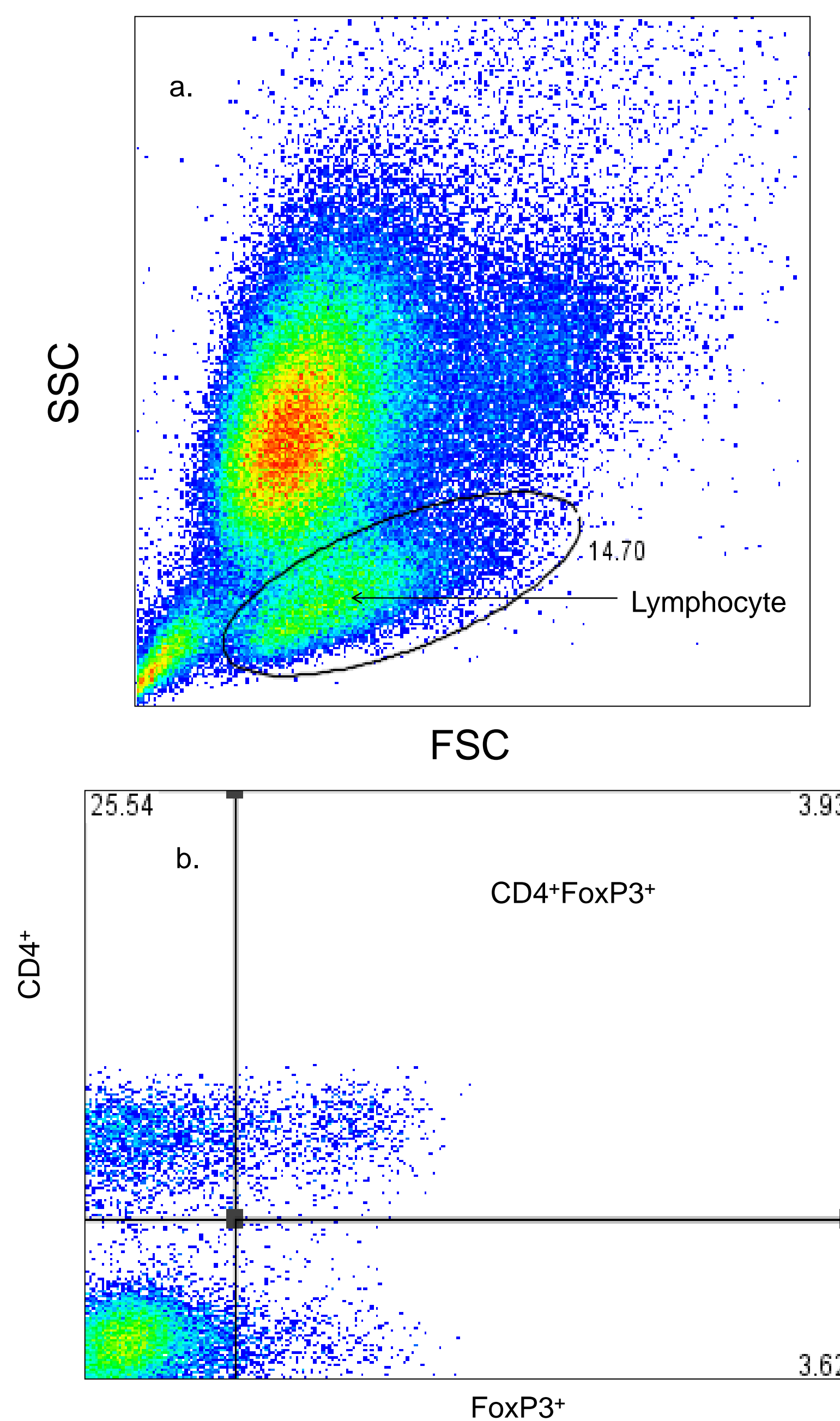


Figure 1.2 Flow cytometric analysis of PBMC. a) Flow cytometry showing the gating technique to identify lymphocyte populations from other circulating cells. b) PBMCs were stained using lymphocyte specific CD4, CD8 & intracellular FoxP3 antibodies to delineate T lymphocytes from other circulating cells. Further analysis was performed to identify CD4⁺FoxP3⁺ cells (T_{reg}) within the already identified CD4⁺ & CD8⁺ lymphocyte population.

Results (Continued)

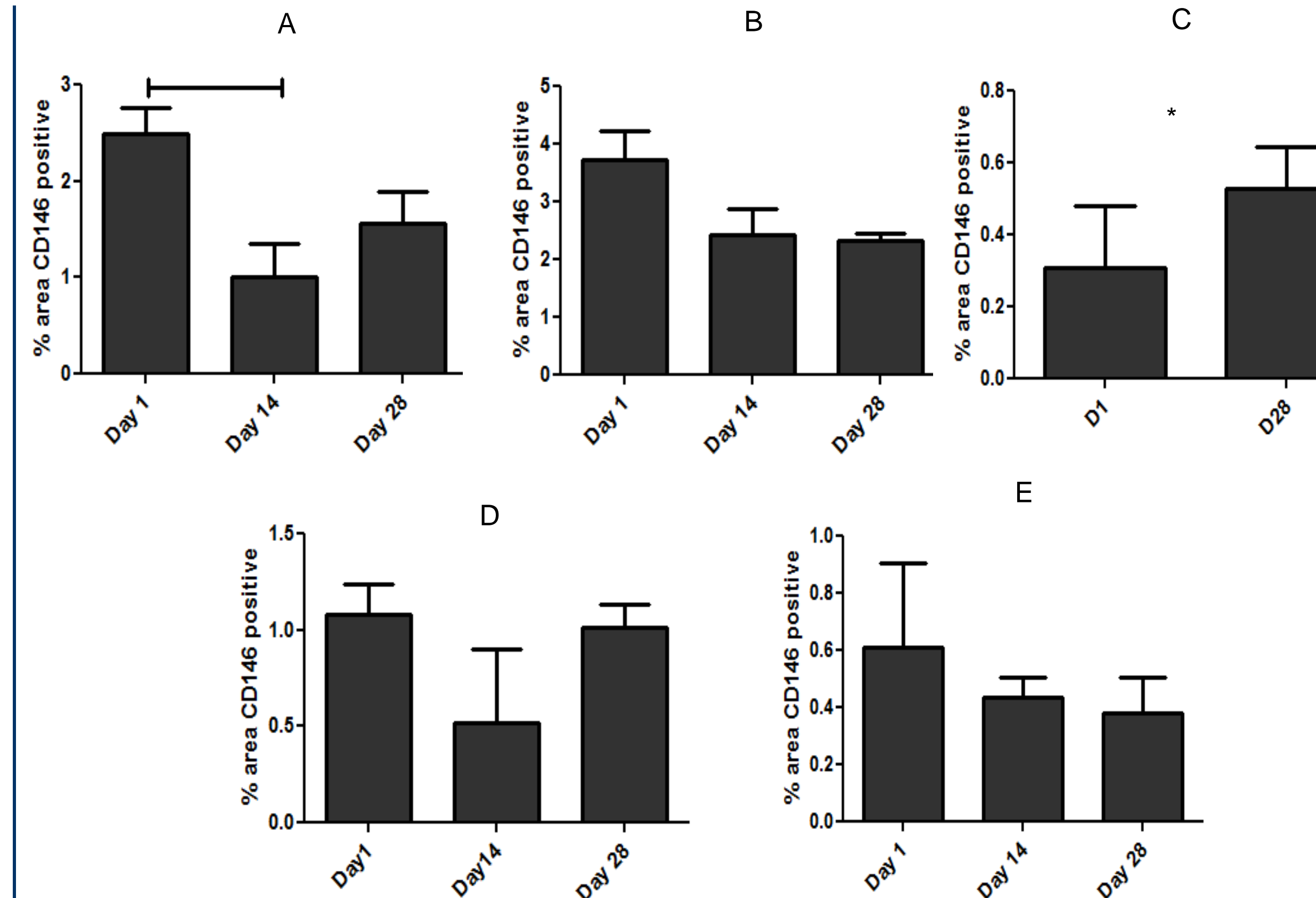


Figure 2.1 Effects of metronomic CYC on tumor MVD in dogs with soft tissue sarcoma. Tumor biopsies were obtained from five dogs (A-E) pre-treatment, at 14 days and again at 28 days after starting CYC therapy. Tumor MVD was evaluated using IHC with a cross-reactive CD146 antibody to human endothelial cells. Pre- and post-treatment tumor MVDs were compared by a 1-way ANOVA and significant differences ($P < 0.05$) are noted with a perpendicular bar. The asterisk denotes a biopsy at day 14 that did not include tumor cells and thus could not be evaluated.

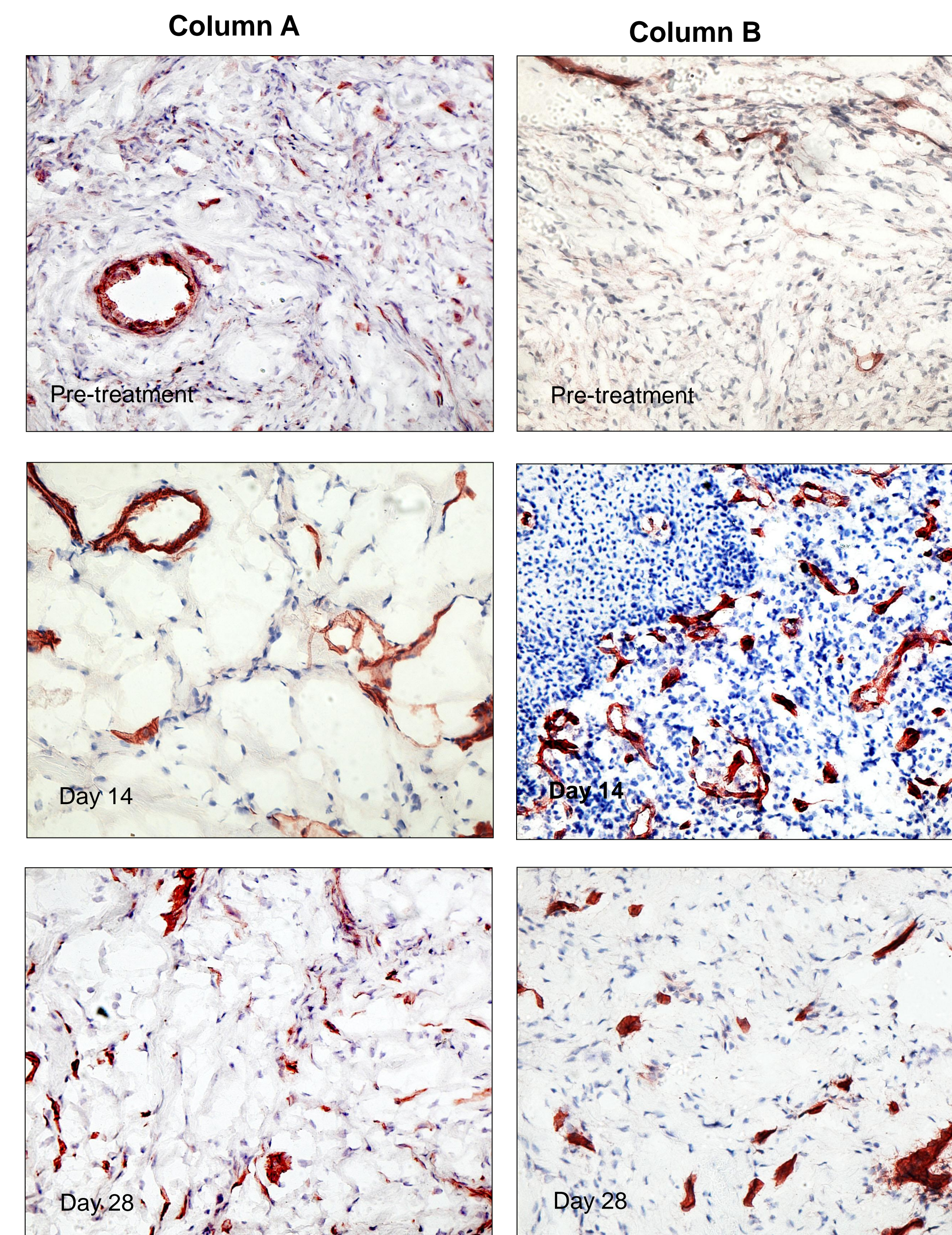


Figure 2.2 Evaluation of tumor MVD using immunohistochemistry. Tumor biopsies were collected and stained as described above in 2.1. Column A and Column B are slides from 2 dogs showing the 3 different time points. Although tumor MVD tended to decrease over time, these differences were not significant.

Conclusions

Metronomic chemotherapy is a promising route of therapy for both human and canine cancer patients. Selectively targeting pathways that are pro-tumor is the major benefit of this approach but other possible benefits also include low toxicity, low cost, decreased physical stress and ease of administration for the patient and owner. Many challenges exist in developing effective protocols such as determination of optimal dosing schedules and drug combinations and identification of relevant tumor biomarkers.

In this study we sought to determine the effects of metronomic dosing of CYC on T_{reg} and tumor MVD in dogs with soft tissue sarcoma. At the current dosages, CYC administration was not associated with significant decreases in regulatory T cell populations, tumor angiogenesis or tumor growth. Interestingly, however, treatment did cause notable decreases in both T_{reg} cells and tumor MVD between pre-treatment and day 4 in 4 of 5 patients. In 2 of these patients T_{reg} cells and tumor MVD then increased between the day 14 and day 28 time points. This may suggest that, although metronomic CYC initially decreases T_{reg} and tumor vasculature, its effects are not complete and are likely overcome by the tumor. We also found that tumor growth was either absent or very minimal in all patients. Therefore, metronomic CYC therapy may inhibit tumor progression through other mechanisms than those investigated here.

Finally, physical examinations, complete blood counts, chemistry panels, urine analysis and pet quality of life questionnaires were performed at each time point to evaluate the possible toxicity of treatment. No adverse clinical reactions were noted, suggesting that metronomic dosing of CYC is a safe form of treatment for dogs with soft tissue sarcoma.

Our future studies will investigate whether alterations in the dosage of CYC or incorporation of other agents such as anti-inflammatory drugs increases treatment efficacy. In addition we will continue to evaluate T_{reg} and tumor MVD as well as other potential tumor biomarkers such as tumor infiltrating macrophages and lymphocytes for their utility in monitoring metronomic chemotherapy.

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