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Pharmacodynamic assessment of a panel of immunosuppressant drugs on *ex-vivo* canine T-lymphocyte proliferation

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INTRODUCTION

- A lack of understanding of specific immune defects underlying canine immune-mediated diseases hampers optimal treatment. Failure to tailor treatment to an individual's immune abnormality can result in lack of efficacy, added expense, and adverse effects.
- Development and validation of a small volume whole blood T-lymphocyte proliferation assay to test efficacy of suppression of lymphocytes using a panel of immunosuppressants would be a highly clinically relevant assay for patients with immune-mediated diseases.
- Our laboratory previously developed a pharmacodynamic flow cytometric assay to assess live, proliferating T-lymphocytes in the presence of mitogen and a panel of immunosuppressants at various concentrations. However, the earlier assay required approximately 30 mL of whole blood, which is not practical in clinical settings, especially in anemic patients.
- The goal of this study was to evaluate the *ex-vivo* suppression of canine T-lymphocyte specific proliferation by determining the 50% T-cell inhibitory concentration (IC_{50}) of dexamethasone, cyclosporine, mycophenolate and the active metabolite of leflunomide (A77 1726) utilizing a small volume whole blood flow cytometric assay.

HYPOTHESIS

- We hypothesized that a small volume whole blood flow cytometric assay would show dose-dependent suppression of T-lymphocyte proliferation in response to dexamethasone, cyclosporine, mycophenolic acid, and the active metabolite of leflunomide (A77 1726) in healthy dogs.

MATERIALS & METHODS

- **Dogs**: Whole blood samples from 6 healthy pet dogs of various ages, breeds, and genders were utilized for each assay.
- **Ex-vivo culture & incubation**: 125 µL of whole dog blood was incubated in RPMI culture media for 4 days in the presence (stimulated) or absence (unstimulated) of concanavalinA and lipopolysaccharide and varying concentrations of dexamethasone, cyclosporine, mycophenolic acid or A77 1726. The following concentrations were tested: dexamethasone (0.1, 1, 10, 100, 1,000, 10,000 µM), cyclosporine (0.2, 2, 10, 20, 30, 40, 80, and 200 ng/mL), mycophenolic acid (2, 5, 10, 20, 40, 80, 100, 200, 500 nM) and A77 1726 (1, 5, 10, 25, 50, and 200 µM).
- **Staining for viability, CD5 and Ki67**: Following incubation red blood cells were lysed and white blood cells were washed. All samples, except for unstained controls, were stained for 30 min at 4°C in the dark with a fixable viability dye eFluor-780 (eBioscience, San Diego, California, USA) to exclude dead cells. Cells were then stained with perCP-eFluor-710 labeled antibody (eBioscience, San Diego, California, USA) against canine CD5, a pan T-cell marker, for 15 min at 4°C in the dark to identify T-cells. All cells, including the unstained controls, were then incubated for 30 min at 4°C in cytofix/cytoperm solution (eBioscience, San Diego, California, USA). All cells except unstained controls were stained with anti-mouse/rat Ki67- FITC antibody (eBioscience, San Diego, California, USA) for 1 h. at 4°C in the dark to assess lymphocyte proliferation.
- **Flow cytometric analysis**: To quantify live, proliferating T-cells and assess suppressive drug action the Cyan ADP Flow Cytometer (Becton, Dickison and Company, Franklin Lakes, New Jersey, USA) was used to collect at least 20,000 gated events and Summit 5.2 Software (Beckman Coulter, Inc., Fullerton, California, USA) was used to analyze data. A plot of forward scatter (FSC) versus side scatter (SSC) identified all cells within the sample and a gate was applied to the lymphocyte population to exclude other cells. A plot of SSC versus eFluor-780 allowed for selection of live cells. Live lymphocytes were thus identified by these two gates and applied to a plot of eFluor-710 versus FITC allowing quantification of double-labeled CD5+Ki67+ (proliferating T-cells).
- **Calculating the IC_{50}**: The 50% inhibitory concentration, IC_{50}, was determined using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

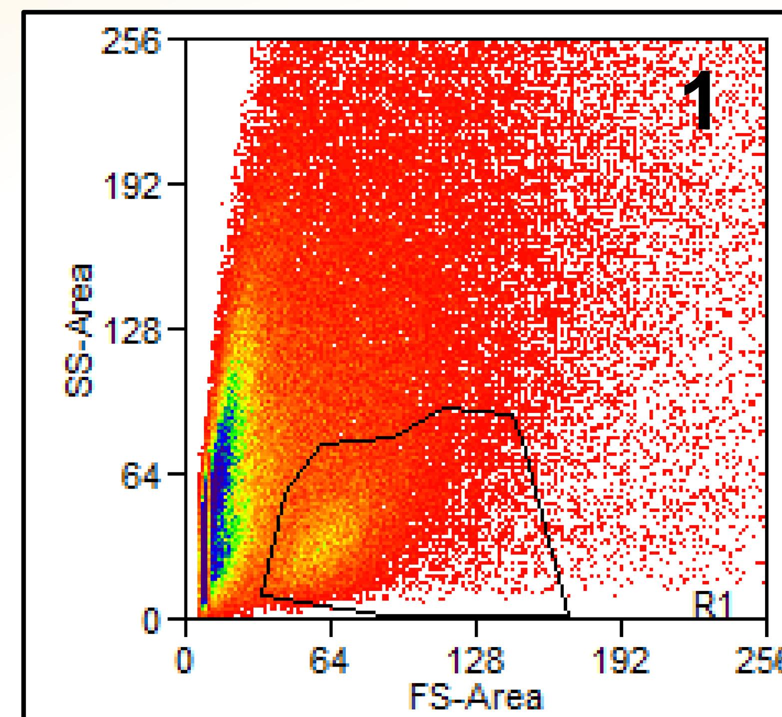

Figure 1. A plot of side scatter (SSC) vs. forward scatter (FSC) separates white blood cells based on complexity and size, respectively. All cells present in the sample are displayed as a dot. A gate (R1) has been placed around the population of lymphocytes in order to exclude cells that are not of interest.

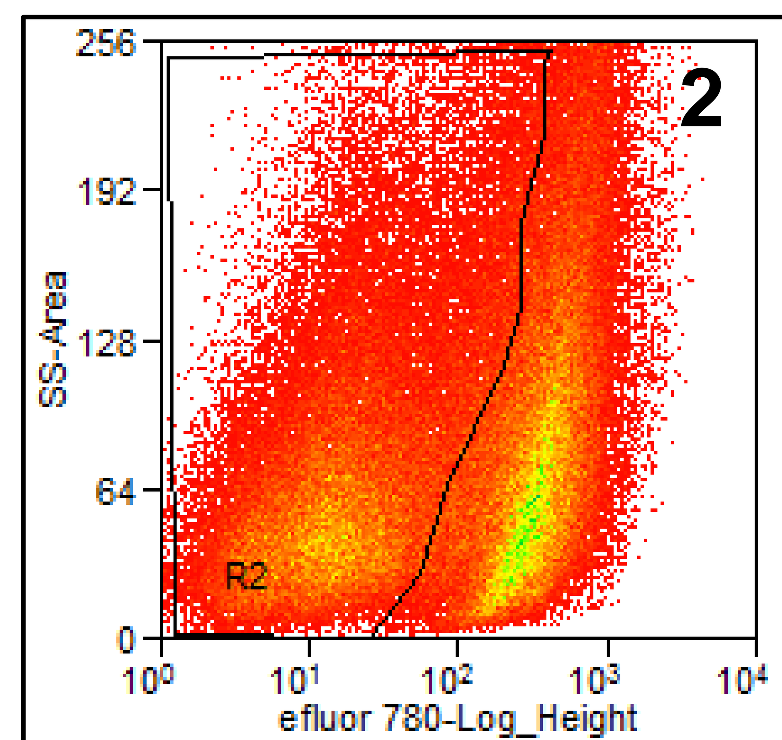

Figure 2. All cells are displayed on a plot of SSC vs. eFluor-780, the fixable viability dye. A gate (R2) contains the population of live cells. By subsequently applying this gate to the next plot, dead cells can be excluded.

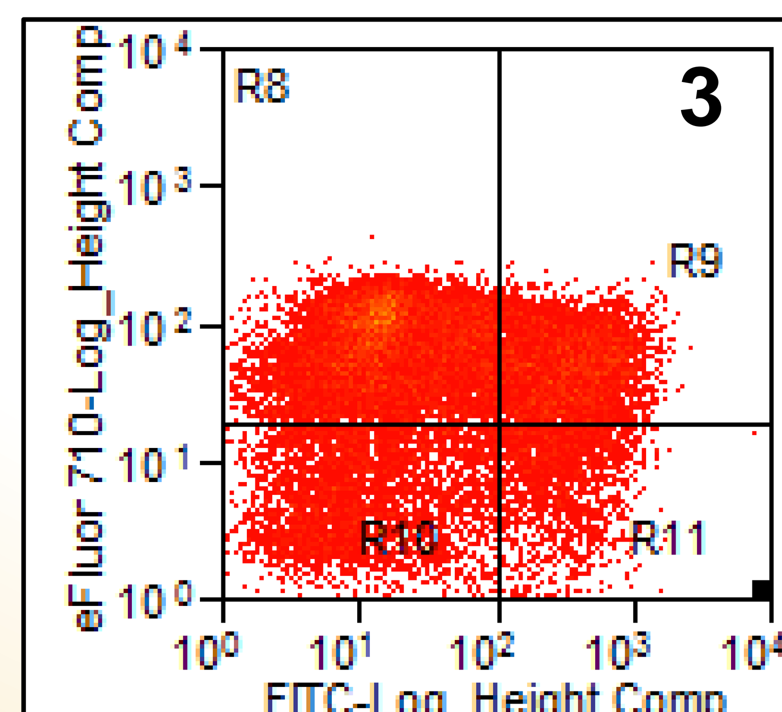

Figure 3. The gates R1 (lymphocytes) and R2 (live cells) are applied to a plot of CD5-perCP-eFluor-710 (T-cells) versus Ki67- FITC (proliferating cells). Cells in R9 represent the population that is double positive for CD5 and Ki67, i.e., proliferating T-cells.

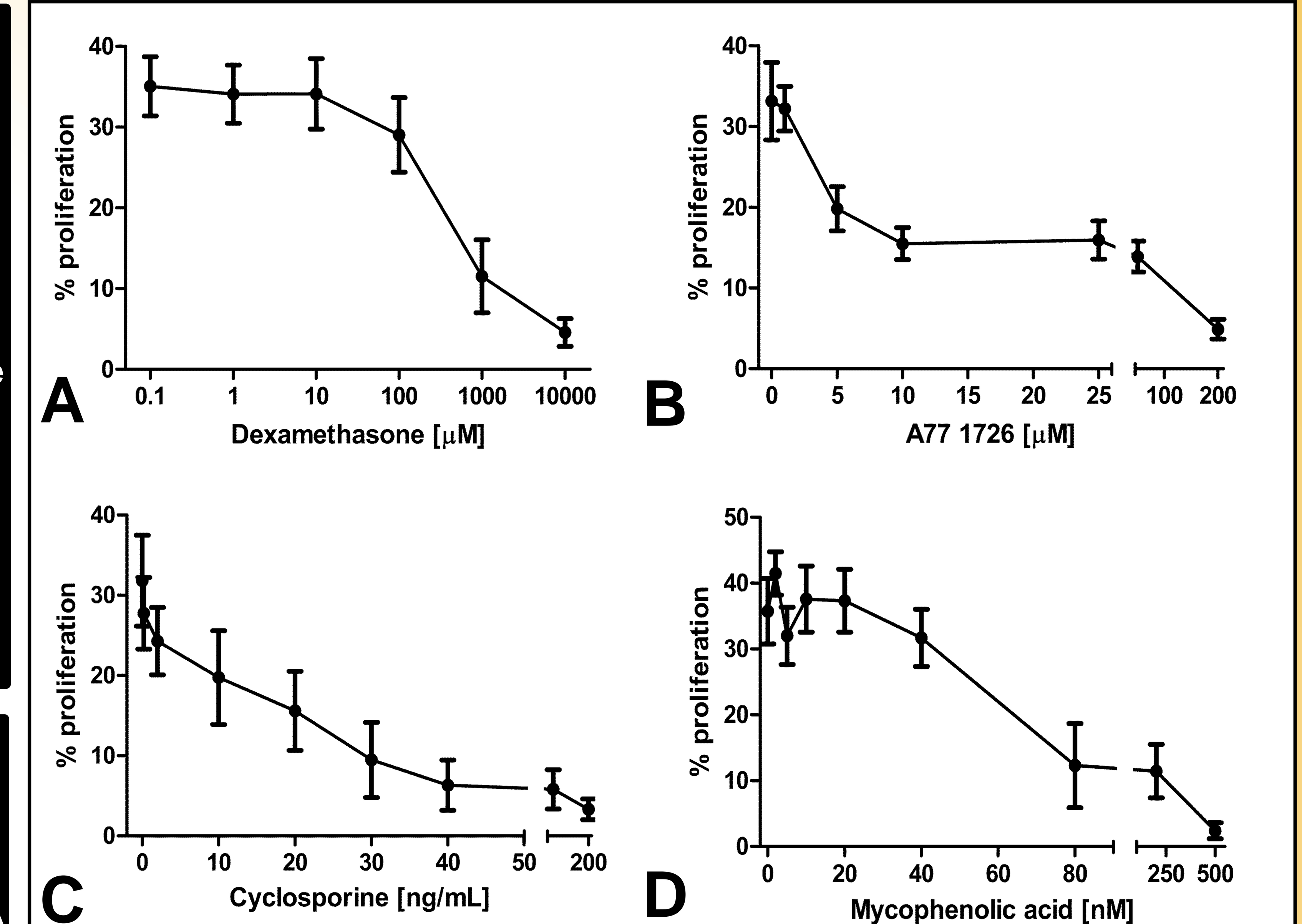

Figure 4. Percent proliferation vs. concentration of drug: The graphs show the mean ± SEM for each concentration. **A)** The mean ± SD IC_{50} for dexamethasone was 394.8 ± 871 µM. **B)** The mean ± SD IC_{50} for A77 1726 was 3.746± 6.8 µM. **C)** The mean ± SD IC_{50} for cyclosporine was 18.89± 36.2 ng/mL. **D)** The mean ± SD IC_{50} for mycophenolic acid was 106.3±157.7 nM.

FUTURE DIRECTIONS

- In the future this assay may be applied to pet dogs with spontaneous immune-mediated disease to help tailor individual treatment regimens.

CONCLUSIONS

- **The flow cytometric assay successfully allowed determination of percentages of proliferating T lymphocytes and calculation of the 50% inhibitory concentration (IC_{50}). Inhibition of T-lymphocyte proliferation by the panel of immunosuppressants was shown in a dose-dependent manner, with marked variability among dogs.**
- **The mean ± SD IC_{50} was 394.8 ± 871 µM for dexamethasone, 18.89± 36.2 ng/mL for cyclosporine, 106.3±157.7 nM for mycophenolic acid, and 3.746± 6.8 µM for A77 1726.**
- **Results support the use of this assay for detecting efficacy of individual immunosuppressants to diminish T lymphocyte proliferation.**

ACKNOWLEDGEMENTS

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