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Pharmacodynamic Monitoring of Canine T-Cell Cytokine Responses to Oral Cyclosporine

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Background: Pharmacodynamic assays measure the immunosuppressive effects of cyclosporine on T-cells and offer an alternative assessment of efficacy in individual patients.

Objective: To assess the immunosuppressive effects of high and low dosage cyclosporine on canine T-cells and to develop a novel testing system for individualized dose adjustment.

Animals: Seven healthy female Walker hounds.

Methods: Experimental study using a paired comparison design. Flow cytometry was used to measure T-cell expression of IL-2, IL-4, and IFN-γ. Cytokine expression 8 days after oral administration of high and low dosages of cyclosporine was compared to baseline and washout values, respectively. The high dosage was initially 10 mg/kg q12h and was then adjusted to attain established immunosuppressive trough blood drug concentrations (>600 ng/mL). The low dosage was 5 mg/kg q24h.

Results: High dosage cyclosporine resulted in significant decreases in IL-2 and IFN-γ expression ($P = .0156$, $P = .0156$), but not IL-4 expression ($P = .2188$). Low dosage cyclosporine was associated with a significant decrease in IFN-γ expression ($P = .0156$), while IL-2 expression was not affected ($P = .1094$).

Conclusions and Clinical Importance: T-cell function is suppressed at trough blood drug concentrations exceeding 600 ng/mL, and is at least partially suppressed in some dogs at low dosages. Direct evaluation of T-cell function could be an effective, more sensitive alternative to measuring blood drug concentrations for monitoring immunosuppressive therapy.

Key words: Flow cytometry; Interferon-gamma; Interleukin-2; Interleukin-4.

Cyclosporine is a potent immunosuppressive drug with treatment applications in both human and veterinary medicine. Cyclosporine specifically targets T-cell function, ultimately inhibiting calcineurin within the cell.[1–4]

Inhibition of calcineurin prevents activation of nuclear factor of activated T-cells, which regulates the production of several important cytokines including interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α).[5,6] Decreased production of IL-2 is thought to be the main cause of the cyclosporine's immunosuppressive effects.[7–9]

Cyclosporine has been used to treat many inflammatory and immune-mediated diseases in the dog.[4,10–17] The lipophilic nature of cyclosporine affects drug bioavailability, which has made the use of the drug challenging to clinicians. The oral bioavailability of cyclosporine is highly unpredictable, with wide ranges of blood concentrations seen in dogs receiving similar dosages.[13,14,18–20] Because of this variability, therapeutic drug monitoring of cyclosporine blood concentrations is usually recommended if a favorable response is not initially achieved.[3,20,21] Interpretation of cyclosporine blood concentrations in the individual dog, however, is difficult. There is limited evidence in the veterinary literature correlating cyclosporine blood concentrations with clinical response for many inflammatory and immune-mediated diseases. Target therapeutic ranges are often anecdotal, and recommendations vary considerably among references with regards to desired blood cyclosporine concentrations.[20,22,23] Clinical response is often the most reliable means of assessing immunosuppression in treated animals. The relationship between clinical response and drug blood concentrations also appears to be highly variable, with a given cyclosporine concentration being effective in some animals but not others.[13,24] Additionally, for some conditions, positive clinical response is often apparent at drug concentrations well below those typically recommended for immunosuppression.[19] For this reason, while cyclosporine is marketed for the

Abbreviations:

HPLC	high-pressure liquid chromatography
IFN-γ	interferon-gamma
IL-2	interleukin-2
IL-4	interleukin-4
MFI	mean fluorescence intensity
mRNA	messenger ribonucleic acid
PBMC	peripheral blood mononuclear cell
QRT-PCR	quantitative reverse transcriptase polymerase chain reaction
TNF-α	tumor necrosis factor-alpha

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treatment of canine atopic dermatitis, therapeutic drug monitoring is not routinely recommended for this condition.[10,25,26] Based on the unpredictable relationship between blood drug concentrations and clinical response, there is clearly a need to find a better method for monitoring the immunosuppressive effects of cyclosporine, particularly in animals with life-threatening conditions.

Pharmacodynamic assays evaluating selected biomarkers within the immune system are advocated in human medicine to help determine drug efficacy and make dosing recommendations.[27] For cyclosporine, these assays include quantitation of drug target enzymes (calcineurin), cytokines (IL-2 and IFN-γ), and markers of lymphocyte proliferation or activation (CD25 and CD95).[28–32] These biomarkers are involved in normal and pathological immune responses, so their inhibition serves as a quantitative, objective surrogate for inhibition of immune function. Pharmacodynamic monitoring of biomarkers of immunosuppression offers a more individualized approach to immunosuppressive therapy when blood concentrations do not correlate well with clinical response in dogs.

Pharmacodynamic monitoring of biomarkers such as cytokines that are indicative of immunosuppression has yet to be thoroughly explored in the dog. Our study uses flow cytometry to assess the effects of cyclosporine on canine T-cell production of 3 cytokines, IL-2, IL-4, and IFN-γ, that have potential utility as biomarkers of immunosuppression. These cytokines were evaluated at 2 drug dosages: a high dosage adjusted to attain trough blood concentrations that are expected to be reliably immunosuppressive, and the much lower dosage used to treat atopic dermatitis.

Materials and Methods

Dogs

Seven healthy intact female Walker hounds were used for the project. Health screening was performed before the study and included physical examination, complete blood count, serum biochemistry profile, urinalysis, fecal flotation, and heartworm testing. All animals were cared for according to guidelines approved by the Mississippi State University Institutional Animal Care and Use Committee. The animal facilities and program at Mississippi State University are accredited by the American Association for Accreditation of Laboratory Animal Care.

Study Design

Blood was drawn for baseline flow cytometric measurement of T-cell expression of the cytokines IL-2, IL-4, and IFN-γ in all dogs. Baseline samples were assessed in duplicate and averaged. Dogs were then given 2 different dosages of cyclosporine, a high dosage and then, 14 days after completion of the high dosage study, a much lower dosage. For the high dosage study, microemulsified cyclosporine[a] was administered at a starting oral dosage of 10 mg/kg every 12 hours. Dosages were adjusted to achieve a trough blood cyclosporine concentration (measured 12 hours after dosing) of at least 600 ng/mL, a trough concentration that has previously been established to attain sufficient immunosuppression to prevent organ rejection in canine transplant recipients.[23,24,33] One dog attained this trough concentration at the initial starting dosage, while the other six required dosage increases. On day 8 of cyclosporine administration, after trough drug concentrations of at least 600 ng/mL had been confirmed in all dogs, blood was drawn 8 hours after dosing and processed for repeat cytokine measurement. This time point was optimized in an earlier unpublished pilot study. Any cytokine not shown to be suppressed by high dosage cyclosporine was not subsequently analyzed in the low dosage cyclosporine phase of testing. Cyclosporine was then discontinued and, after a 2-week washout period, cytokine expression was again measured to ensure return to baseline levels before commencing the subsequent low dosage study. For the low dosage study, cyclosporine was administered at the labeled dosage for atopic dermatitis (5 mg/kg orally every 24 hours), and cytokine levels were again measured on day 8 of drug administration. Trough cyclosporine blood concentrations (measured at hour 24, immediately before the next dosage) were also measured by HPLC on day 8.

Cytokine Analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples collected from each dog utilizing density gradient centrifugation with Histopaque®-1077,[b] similar to a previously described method.[34] The isolated PBMC were reconstituted in complete media with an equal volume to that of the original blood sample in order to approximate the cell density present for each dog. RPMI 1640 medium[c] supplemented with 10% heat-inactivated fetal bovine serum,[d] GlutaMAX,[e] 1 mM sodium pyruvate,[f] 55 μM 2-mercaptoethanol,[g] 75 μg/mL gentamicin,[h] 2 mM HEPES, and 1 μL/mL MEM Amino Acids Solution without L-glutamine[i] was used for cell culture.

Cytokine expression levels in peripheral blood T-cells were analyzed as described previously.[35] Briefly, half of the isolated PBMC were activated with 12.5 ng/mL phorbol-12-myristate-13-acetate[j] and 0.8 μM ionomycin,[k] and the other half remained untreated. All cells were then incubated for 12 hours at 37°C in a 5% CO_2 incubator. Brefeldin A at a concentration of 1 μg/mL was added to each well with 2 hours remaining in the incubation to stop cytokine secretion from T-cells. Cells were then collected and washed with phosphate buffered saline. All samples were incubated with a fluorescein isothiocyanate-conjugated anti-CD3 monoclonal antibody[l] for 30 minutes at room temperature in the dark, and fixed and permeabilized using the BD Cytofix/Cytoperm Plus Kit[m] following the manufacturer's instructions. Cells were then incubated with appropriate antibodies for labeling of the cytokines of interest, either R-phycoerythrin-conjugated anti-bovine IL-4[n] (cross-reactive with canine IL-4[36]), R-phycoerythrin-conjugated anti-bovine IFN-γ[o] (cross-reactive with canine IFN-γ[36]), or biotinylated anti-canine IL-2,[p] for 30 minutes at room temperature in the dark. The anti-canine IL-2 antibody has been shown to be canine specific by in-house testing performed by the manufacturer. IL-2 samples had an additional 20-minute incubation with R-phycoerythrin-conjugated streptavidin[q] at room temperature in the dark. A final wash was applied to these samples, and all cells were then resuspended in phosphate buffered saline with 0.2% bovine serum albumin.

Cell staining was evaluated using a BD FACSCalibur Flow Cytometer and analyzed with CellQuest Pro software.[r] Forward scatter and side scatter were used to identify and gate lymphocytes based on their size and granularity. A 2nd gate selected CD3-positive cells. Cytokine levels were measured from cells that were located in both gates, and 10,000 total events per sample were collected. Mean fluorescence intensity (MFI) values with

single histogram statistics were used for assessment of cell staining. Negative controls included unactivated samples and isotype controls.

Cyclosporine Blood Concentrations

Blood cyclosporine concentrations were measured via HPLC, with the trough concentration taken 12 hours (high dose phase) or 24 hours (low dose phase) after the previous oral dose. Blood was collected into EDTA anticoagulant tubes, and analyzed within 24 hours using a modification of the HPLC assay used for therapeutic drug monitoring at the University of California at Davis (John D Patz, personal communication, 2008). Standard curves were made using blank EDTA anticoagulated whole blood and cyclosporine[s] at 0, 200, 400, 800, and 1,600 ng/mL. The extraction procedure used 2 mL of whole blood sample mixed with 6 mL of a protein precipitating solution consisting of 5% zinc sulfate, 20% acetonitrile, 30% methanol, and water. This solution also contained 400 ng/mL of cyclosporine D[t] as an internal standard. After vortexing and subsequent centrifugation, the supernatant was added to a prepared C18 solid phase extraction column.[u] After filtration of the sample by vacuum, the solid phase extraction column was washed with 5 mL of 50% acetonitrile followed by 1 mL of 100% methanol for elution of the drug. To the methanol eluent 200 μL of water was first added followed by 300 μL of hexane. This fluid was then vortexed and centrifuged with 200 μL of the aqueous layer extracted and placed in vials for subsequent HPLC analysis using a sample injection volume of 100 μL.

An 1100 HPLC system[v] with degasser, quaternary pump, autoinjector, and diode array detector was used. The reverse phase column was a Phenomenex Luna 5u C18(2) with guard cartridge. The column was maintained at 75. A gradient mobile phase at 1 mL per minute was used consisting of acetonitrile (A) and water adjusted to pH 3.1 (B). The gradient was initially 65% A and 35% B that transitioned linearly over 5 minutes to 70% A and 30% B. This ratio was then held for 15 minutes. A 5 minute re-equilibration time followed each injection. Detection was at 200 nm. The retention time for cyclosporine was 4.2 minutes versus 5.6 minutes for cyclosporine D. The assay was linear over the standard curve range of 200–1,600 ng/mL with an r^2 of 0.9889. Based on the method described by Taylor, the limit of quantitation (LOQ) was 189.93 ng/mL and the limit of detection (LOD) was 56.97 ng/mL.[37] The assay had an average coefficient of variation of 6.7% (range 3.7–9.9%) and an average accuracy of 94.4% (range 92–98%).

Statistical Analysis

A paired comparison design was used in this study. The Wilcoxon signed rank test was used to compare the average baseline cytokine MFI values to the MFI values after treatment for each cytokine at the high dosage. The Wilcoxon signed rank test was also utilized to compare the average baseline cytokine MFI values to the MFI values after treatment for IL-2 and IFN-γ at the low dosage, to compare the washout cytokine MFI values to the MFI values after treatment for IL-2 and IFN-γ at the low dosage, and to compare the baseline cytokine MFI values to the washout cytokine MFI values for IL-2 and IFN-γ. Analyses were conducted for both cyclosporine dosages for IL-2 and INF-γ while only the effect of high dosage cyclosporine was analyzed for IL-4. The UNIVARIATE procedure in SAS for Windows® version 9.2[w] was used for statistical analysis. *P*-values $\leq .05$ were considered significant.

The data was analyzed to determine if MFI and cyclosporine concentration were correlated using PROC CORR (SAS for Windows® version 9.2[w]). A separate analysis was conducted for each cyclosporine dosage and cytokine combination.

Results

Cytokine Analysis

Expression of both IL-2 and IFN-γ decreased significantly from baseline values after administration of high dosage cyclosporine (for IL-2, $P = .0156$ and for IFN-γ, $P = .0156$), and cytokine expression decreased in all 7 dogs for both cytokines (Table 1). In contrast, IL-4 expression after administration of high dosage cyclosporine varied markedly between individual dogs, with no significant difference between baseline values and values after treatment ($P = .2188$). As a result of the inconsistent and nonsignificant changes in T-cell IL-4 expression in dogs administered cyclosporine at a high dosage, this cytokine was not evaluated in the subsequent low dose trial.

Expression of IFN-γ decreased significantly from baseline values ($P = .0156$) as well as washout values ($P = .0156$) after administration of low dosage cyclosporine, and cytokine expression decreased in all 7 dogs (Table 2). There was not a significant difference between the IFN-γ baseline values and the IFN-γ washout values ($P = .9375$). Expression of IL-2 at the low cyclosporine dosage had more variability in individual dogs compared to expression of IFN-γ at the same dosage, and compared to expression of IL-2 at the high cyclosporine dosage, with only 5 of 7 dogs showing moderately suppressed IL-2 expression on the lower dosage. Expression of IL-2 after administration of low dosage cyclosporine was not significantly different from baseline values ($P = .1094$) nor from washout values ($P = .6875$). IL-2 washout values were found to be significantly lower than the baseline IL-2 values ($P = .0469$).

Cyclosporine Blood Concentrations

The high cyclosporine dosage consisted of a starting dosage of 10 mg/kg every 12 hours, with the 12-hour doses adjusted upward as needed to ensure trough blood drug concentrations of at least 600 ng/mL. One dog achieved the minimum target trough concentration on the starting dose, while the other 6 dogs needed doses titrated up and had confirmed cyclosporine trough concentrations above 600 ng/mL by day 8. The low cyclosporine dosage was 5 mg/kg every 24 hours. After 8 days of dosing at the low cyclosporine dosage, blood was drawn for measurement of trough concentrations. Each trough concentration was drawn just before administration of the next cyclosporine dosage. Trough blood cyclosporine concentrations for high dosage cyclosporine ranged from 728 to 1,330 ng/mL, with a median value of 1,005 ng/mL (Table 1). Trough blood cyclosporine concentrations for low dosage cyclosporine ranged from below the level of detection (57 ng/mL) to 145 ng/mL, with all values being below the LOQ (190 ng/mL) (Table 2).

Table 1. Flow cytometric measurement (mean fluorescence intensity or MFI) of activated T-cell expression of interleukin-2 (IL-2), IL-4, and interferon-gamma (IFN-γ) before (baseline) and after (day 8) administration of high dosage oral cyclosporine (starting dose 10 mg/kg orally twice daily, adjusted to attain trough levels >600 ng/mL). Accompanying trough blood cyclosporine concentrations during the administration of high dosage cyclosporine are shown.

	High Dosage Cyclosporine									
	IL-2 (MFI)			IL-4 (MFI)			IFN-γ (MFI)			Cyclosporine trough concentration (ng/mL)
	Baseline	Post-treatment	Δ from baseline	Baseline	Post-treatment	Δ from baseline	Baseline	Post-treatment	Δ from baseline	
Median	60	28.9	− 36.4	8.33	11.02	3.03	29.9	12.9	−19.7	1,005
Range	(51.1–84.5)	(23.6–34.1)	(−54.3–−22.3)	(6.29–10.62)	(7.63–11.81)	(−0.41–4.73)	(19.1–36.3)	(6.9–14.2)	(−23.3–−7)	(728–1,330)

Table 2. Flow cytometric measurement (mean fluorescence intensity or MFI) of activated T-cell expression of interleukin-2 (IL-2) and interferon-gamma (IFN-γ) before (baseline), after washout (washout), and after (day 8) administration of low dosage oral cyclosporine (5 mg/kg orally once daily). Accompanying trough blood cyclosporine concentrations during the administration of low dosage cyclosporine (BDL, below detectable limit) are shown.

	Low Dosage Cyclosporine										
	IL-2 (MFI)					IFN-γ (MFI)					
	Baseline	Washout	Post-treatment	Δ from baseline	Δ from washout	Baseline	Washout	Post-treatment	Δ from baseline	Δ from washout	Cyclosporine trough concentration (ng/ml)
Median	60	54.8	53.9	−14.5	−1.6	29.9	28.1	23.4	−6.2	−6.6	63
Range	(51.1–84.5)	(47.1–78.4)	(45.5–65.3)	(−30.6–12.9)	(−24.5–7.9)	(19.1–36.3)	(18.9–50.6)	(14.1–25.5)	(−14.1–−4.4)	(−27.2–−3.4)	(BDL to 145)

Cyclosporine Blood Concentration and Cytokine Correlation

IL-2 MFI was not significantly correlated with cyclosporine concentration for either high dosage ($r = -0.16$, $P = .7317$) or low dosage ($r = -0.22$, $P = .6293$) cyclosporine administration. Similarly, IFN-γ MFI was not significantly correlated with cyclosporine concentration for either high dosage ($r = -0.41$, $P = .3619$) or low dosage ($r = -0.13$, $P = .7858$) cyclosporine administration.

Discussion

Our study has established that activated T-cell expression of 2 cytokines, IL-2 and IFN-γ, is reliably suppressed in dogs receiving cyclosporine at established immunosuppressive dosages, and that these cytokines are therefore strong candidates for development as biomarkers of immunosuppression for subsequent pharmacodynamic assays. There is clearly a need to develop assays that have the potential to more accurately predict the immunosuppressive effects of cyclosporine in the individual dog, and that allow dosage adjustments that improve clinical outcomes. Such assays have the potential to provide individualized therapy in dogs suffering from severe and life-threatening immune-mediated diseases, as blood concentrations do not always correspond to clinical response.[19]

Our study utilized flow cytometry to evaluate T-cell expression of cytokines to quantitate the biologic effects of cyclosporine on T-cells in healthy dogs. Many human studies include flow cytometric analysis of T-cell cytokine and surface molecule expression as biomarkers when investigating the immunosuppressive effects of drugs such as cyclosporine.[28,38–40] Few such studies are found in the veterinary literature, though one study did demonstrate suppression of lymphocyte proliferation via flow cytometry after the use of topical cyclosporine for the treatment of keratoconjunctivitis sicca in dogs.[41] Cytokine analysis in veterinary immunosuppression research more commonly utilizes quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assays to measure cellular messenger ribonucleic acid (mRNA) expression.[42–46] One recent study investigating the effects of cyclosporine on canine mononuclear cells in vitro, for example, showed a concentration-dependent reduction in IL-2, IL-4, and IFN-γ mRNA expression via QRT-PCR.[43] Another recent study evaluated cellular IL-2 and IFN-γ mRNA expression within lesional biopsies from German Shepherd Dogs with anal furunculosis before and after therapy with cyclosporine, and demonstrated a significant reduction in IL-2 expression after treatment.[42] Previous in vitro work in our laboratory established the utility of flow cytometric techniques for monitoring the effects of cyclosporine on activated T-cell expression of cytokines and surface molecules,[35] and we therefore elected to use this method for our current study. This study has established that activated T-cell expression of the cytokines IL-2 and IFN-γ shows promise as a potential biomarker of immunosuppression for pharmacodynamic monitoring.

Although activated T-cell expression of IL-2 and IFN-γ was reliably suppressed by high dosage cyclosporine therapy in the dogs in our study, expression of IL-4 was not similarly affected. We included IL-4 in our high dosage study because previous work in our laboratory showed that activated T-cell expression of IL-4 was suppressed by in vitro exposure to cyclosporine,[35] and because IL-4 was used in human studies as a biomarker of immunosuppression.[28] Because IL-4 did not reliably suppress during the high dosage cyclosporine phase of our current study, we concluded that further exploration of IL-4 as a potential biomarker was not warranted, and elected not to include this cytokine in the low dosage phase of our study. Failure of T-cell expression of IL-4 to suppress after administration of oral cyclosporine may be because of a dog-specific difference in in vivo responses compared to in vitro responses. One in vivo study of cyclosporine in dogs showed a drug-associated reduction in IL-2 but not in antibody (IgA, IgG, and IgM) production, a presumed Type 2 helper T-cell-dependent response that utilizes IL-4 for upregulation.[47] Although the researchers did not directly measure IL-4, they concluded that cyclosporine had a negligible effect of on humoral immunity.[47]

In our study, we used 2 extremes of cyclosporine dosing, a high dosage adjusted upward as needed to meet target trough blood concentrations of 600 ng/mL, and a much lower fixed dosage. We elected to use our high dosage protocol in order to be as certain as possible that the treated dogs were reliably immunosuppressed, and thereby establish the degree of suppression of cytokine biomarkers associated with immunosuppression. With the high dosage protocol, activated T-cell expression of IL-2 and INF-γ was significantly reduced, consistent with findings in pharmacodynamic studies in people.[28,29,48] The lower fixed cyclosporine dosage utilized in our study is the labeled dosage approved by the US Food and Drug Administration for the treatment of canine atopy.[24] The atopy dosage of cyclosporine has not been previously definitively documented to cause significant suppression of the canine immune system, and whether or not this low dosage of cyclosporine can cause clinically relevant immunosuppression remains controversial among veterinary dermatologists. Individual dogs on the atopy dosage of cyclosporine long term have, however, been anecdotally reported to develop secondary infections, suggesting the possibility that even this low dosage can sometimes cause immunosuppression. Our study provides preliminary supportive evidence that low dosages of cyclosporine may have immunosuppressive effects in dogs, in that even the atopy dose used in our low dose phase was associated with a significant decrease in activated T-cell expression of IFN-γ. Remarkably, T-cell expression of both IFN-γ and IL-2 was observed to decrease in individual dogs even when trough blood cyclosporine concentrations were far below published target trough concentrations and were below the LOQ,

and in 2 instances, below the level of detection by HPLC. That some dogs showed substantial decreases in T-cell cytokine production despite having extremely low trough drug concentrations suggests that therapeutic drug monitoring of trough blood cyclosporine concentrations as a means of predicting immunosuppression in individual dogs is of questionable reliability. Our low dosage study demonstrated that blood drug concentrations and T-cell suppression were both highly variable among individual dogs treated with the same oral dosage of cyclosporine, supporting the proposition that, in a clinical setting, pharmacodynamic assays may be needed in order to determine individual patient responses to immunosuppressive therapy. Comparison of the cyclosporine concentrations and MFI values for each cytokine did not demonstrate a correlation between the two measures at either dosage. This further calls into question the utility of blood drug concentrations and supports the need for a more individualized, patient specific approach when monitoring cyclosporine therapy in dogs.

One weakness of our study is that, because of the lack of a cross-over design, all dogs received high cyclosporine dosages before subsequently receiving low dosages. A possible residual drug effect after high dosage cyclosporine could have played a role in our IL-2 analysis because a statistically significant reduction in values was seen between original baseline and washout values. This effect was not appreciated with the analysis of the IFN-γ data, a difference that could be because of a possible prolonged post-treatment effect of cyclosporine on the expression of IL-2 but not IFN-γ.

Our study was performed in healthy research dogs, with demonstration of suppression of T-cell expression of cytokines in response to oral dosing of cyclosporine. Clinical dosing recommendations cannot be made at this time based on our assay, because we do not as yet know whether cytokine expression corresponds with clinical efficacy in dogs as it does in humans. Our study only incorporated 2 extreme dosages of cyclosporine, and did not evaluate alterations in activated T-cell expression of cytokines associated with the mid-range cyclosporine dosages that are often used for treating inflammatory and immune-mediated diseases in the dog. Performing similar analyses at cyclosporine dosages between the 2 dosages used in our study would further clarify the potential clinical utility of flow cytometric measurement of T-cell cytokine expression as a biomarker of immunosuppression. Finally, clinical studies in clinical patients at dosages approximating those typically used for immunosuppressive therapy will help determine this assay's ability to predict immunosuppression and allow for dose adjustments in the individual dog.

Footnotes

[a] Atopica, Novartis Animal Health, Basel, Switzerland
[b] Histopaque®-1077, Sigma-Aldrich, St. Louis, MO
[c] RPMI 1640 Medium, 21870-084, Invitrogen, Carlsbad, CA
[d] 10% Heat-inactivated fetal bovine serum, 10438-026, Invitrogen
[e] GlutaMAX, 35050, Invitrogen
[f] Sodium Pyruvate, 11360, Invitrogen
[g] 2-mercaptoethanol, 21985-023, Invitrogen
[h] Gentamicin, 15750-060, Invitrogen
[i] MEM Amino Acids Solution without L-glutamine, 11130, Invitrogen
[j] Phorbol-12-myristate-13-acetate, P-8139, Sigma-Aldrich
[k] Ionomycin, I-0634, Sigma-Aldrich
[l] Anti-canine CD3 antibody, MCA1774F, AbD Serotec, Raleigh, NC
[m] BD Cytofix/Cytoperm Plus Kit, Becton Dickinson, San Jose, CA
[n] Anti-bovine IL-4 antibody, MCA1820PE, AbD Serotec
[o] Anti-bovine IFN-γ antibody, MCA1783PE, AbD Serotec
[p] Biotinylated anti-canine IL-2, BAF1815, R&D Systems, Minneapolis, MN
[q] Streptavidin, #60669, Anaspec, San Jose, CA
[r] FACSCalibur Flow Cytometer and CellQuest Pro software, Becton Dickinson
[s] Cyclosporine, Sigma-Aldrich
[t] Cyclosporine D, Novartis Pharmaceuticals, East Hanover, NJ
[u] Varian Bond Elut 1 cc 100mg solid phase extraction column, Varian Incorporated, Walnut Creek, CA
[v] Agilent 1100 HPLC system, Agilent Technologies, Santa Clara, CA
[w] SAS for Windows® version 9.2, SAS Institute Inc., Cary, NC

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