Effects of cyclosporine and dexamethasone on canine T cell expression of interleukin-2 and interferon-gamma

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1. Introduction

Glucocorticoids and cyclosporine are commonly used as immunosuppressive agents in both veterinary and human medicine. Glucocorticoids act through binding the glucocorticoid receptor in cell cytoplasm, and then translocating to the nucleus to modulate the activity of glucocorticoid response elements. Glucocorticoids alter the expression of various cytokines and cell processes in lymphocytes and other immune cells, and affect both innate and adaptive immune responses (Czock et al., 2005). Glucocorticoids also inhibit the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), independent of their effects on glucocorticoid response elements (Czock et al., 2005). The primary mechanism of action of cyclosporine is inhibition of the enzyme calcineurin, which in turn blocks activation of the transcription factor nuclear factor of activated T cells (NFAT) and decreases production of pro-inflammatory cytokines like IL-2, IL-4, IFN-γ, and TNF-α (Fruman et al., 1992; Rao et al., 1997). A combination of glucocorticoids and cyclosporine leads to combined inhibition of NF-κB, AP-1, and NFAT, thereby blocking the three transcription factors triggered after T cell receptor binding (Murphy, 2008), and causing an additive or synergistic impact on T cell activity (Ferron and Jusko, 1998; Freed et al., 1988).

Abbreviations: AP-1, activator protein 1; GOI, gene of interest; MFI, mean fluorescence intensity; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; PMA, phorbol 12-myristate 13-acetate; qRT-PCR, quantitative reverse transcription polymerase chain reaction; R-PE, R-phycoerythrin

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Pharmacodynamic monitoring of levels of the cytokines produced by T cells has been explored in human transplantation medicine as a means of identifying the biological effects of a given immunosuppressive regimen on a patient. Pharmacodynamic monitoring allows for individualized immunosuppressive therapy, and such monitoring has been able to associate very low T cell cytokine expression with potential for infection and malignancy, and high residual T cell cytokine expression with inadequate immunosuppression and the potential for transplant rejection (Sommerer et al., 2008a; b; Sommerer et al., 2006). Immunologic assays are also beginning to be explored in veterinary medicine to assess the effects of cyclosporine and other immunosuppressant medications on lymphocyte responses in dogs and cats. Most of this work has been done in \textit{in vitro} drug incubation studies (Fellman et al., 2011; Kobayashi et al., 2007; Kuga et al., 2008; Kyles et al., 2000; Nafe et al., 2014), but our laboratory has also evaluated T cell cytokine production \textit{ex vivo} in dogs receiving oral cyclosporine (Archer et al., 2011; Fellman et al., 2016; Riggs et al., 2019).

In human medicine, measurement of T cell production of NFAT-regulated cytokines is considered useful for monitoring calcineurin inhibitor therapy after transplantation, even in the presence of other co-administered immunosuppressive drugs. However, some of these other agents (especially glucocorticoids) are also known to affect T cell cytokine expression and could influence the results of pharmacodynamic assays. In fact, glucocorticoid-mediated T cell suppression has been shown to be associated with reduced cytokine levels, including reduced levels of IL-2 (Almawi et al., 1991). Since cyclosporine is frequently used concurrently with glucocorticoids, it is important to determine what effects glucocorticoids may have on any proposed biomarker assay used for pharmacodynamic monitoring of cyclosporine therapy.

Different methods have been used for T cell cytokine evaluation in both animals and humans, but few studies compare the results found with techniques reporting different measures of cytokine expression, such as protein expression with flow cytometry, and relative gene expression with quantitative reverse transcription polymerase chain reaction (qRT-PCR). The authors are only aware of one study comparing different measures of expression in the context of immunosuppressive pharmacodynamics, which found the two methods of measurement to provide comparable results in cynomolgus monkeys (Flores et al., 2004).

The present study investigates the \textit{in vitro} effects of exposure to cyclosporine and dexamethasone on T cell cytokines measured using both flow cytometry and qRT-PCR in a whole blood assay. Goals of this study were to explore the possible effects of concurrent glucocorticoid administration on assays intended for cyclosporine monitoring in dogs given cyclosporine, and to compare the responses of the two different measures of cytokine expression across a range of drug concentrations. Samples from three dogs were also used to determine the time of maximal cytokine expression after activation to provide technique development information for subsequent drug effect studies.

2. Materials and methods

2.1. Animals

Six adult Walker hounds, determined to be healthy based on physical examination, normal complete blood count, serum biochemistry, urinalysis, and negative heartworm status, were used as blood donors. They were housed in a university setting, and protocols approved by the Institutional Animal Care and Use Committee were followed.

2.2. Reagents

Complete media was prepared as previously described (Fellman et al., 2011). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO), and stock solutions of 1 mg/mL PMA and 1 mM ionomycin were made in 100% ethanol. Working solutions of cyclosporine (Bedford Laboratories, Bedford, OH) and dexamethasone (Bimeda, Irwindale, CA) were prepared daily and diluted with 0.9% sodium chloride. Brefeldin A, the BD Cytofix/Cytoperm™ Plus Kit, and BD Pharm Lyse™ were purchased from Becton Dickinson (San Jose, CA). SYTOX™ AADvanced™ Dead Cell Stain Kit was purchased from Invitrogen (Carlsbad, CA).

Antibodies used for flow cytometry were: FITC-conjugated monoclonal anti-dog CD3 (MCA1774F, Abd Serotec, Raleigh, NC), R-phycocerythrin (R-PE)-conjugated monoclonal anti-bovine IFN-γ (MCA1783PE, Abd Serotec), and biotinylated anti-canine IL-2 (BAF1815, R&D Systems, Minneapolis, MN). RPE-conjugated streptavidin (#60669, Anaspec, San Jose, CA) was used as a secondary label for IL-2. Isotype controls were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

For qRT-PCR, RNA was extracted using a QiAamp® RNA Blood Mini Kit, and on-column DNase treatment was performed with the RNase-Free DNase Set, both from Qiagen (Valencia, CA). Assays were performed using a SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit with Rox used as a reference dye (Invitrogen, Carlsbad, CA).

2.3. Blood collection and stimulation

Blood was collected from the jugular vein into heparinized blood tubes. After plating, samples were activated with 0.8 μM ionomycin and 12.5 ng/mL PMA. For flow cytometry, samples were diluted 1:9 with complete media, and brefeldin A was added one hour after activation at a final concentration of 1 μg/mL to stop cytokine secretion from T cells. For qRT-PCR analysis, heparinized whole blood was used.

2.4. In vitro assays

2.4.1. Incubation study

Blood from 3 dogs was activated with PMA and ionomycin and incubated at 37 °C and 5% CO₂ for 2, 4, 6, 8, 10 and 12 h to determine the optimal time for cytokine assessment. As described above, whole blood was used for qRT-PCR analysis, and diluted whole blood samples for flow cytometry.

2.4.2. Exposure to cyclosporine and dexamethasone

Prior to activation for the \textit{in vitro} drug effect assessment, blood samples from 6 dogs were incubated with various concentrations of cyclosporine (10, 100, 500, and 1000 ng/mL) and dexamethasone (10 ng/mL, 100 ng/mL, 1 μg/mL, and 10 μg/mL) for 1 h. Cyclosporine concentrations were based on previously established achievable blood concentrations after oral cyclosporine dosing, and concentrations found to suppress T cell indices \textit{in vitro} (Archer et al., 2011; Kobayashi et al., 2007). Dexamethasone concentrations encompassed established typical blood concentrations after oral dosing, and approached the higher concentrations tested by Nafe and others (Greco et al., 1993; Nafe et al., 2014; Toutain et al., 1983). Based on results from the prior incubation study, samples were then activated and incubated for five hours for qRT-PCR analysis, and seven hours for flow cytometry. An untreated activated sample was also included, and unactivated samples were run for each treatment condition as a control.

To ensure that altered cytokine expression was not simply due to drug-associated cell death, lymphocyte viability was assessed at a later date in samples from 3 dogs (1 hound and 2 Golden Retrievers), after incubation with all drug concentrations reported in this study. Leukocytes were stained using the SYTOX™ AADvanced™ Dead Cell Stain Kit after red blood cell lysis with BD Pharm Lyse™ and evaluated using flow cytometry.

2.5. Flow cytometry

Flow cytometry was performed similarly to the previously published method (Fellman et al., 2011), with modifications for a whole blood
assay, as briefly described below. After incubation, cells (350 μL per sample) were collected from the wells and immediately incubated with FITC-conjugated anti-CD3 antibody for 25 min at room temperature in the dark. Red blood cells were then lysed using BD Pharm Lyse™ according to the manufacturer’s instructions. Following lysis, cells were washed and then fixed and permeabilized using the BD Cytofix/Cytoperm™ Plus Kit and stained with anti-IL-2 and anti-IFN-γ antibodies as described previously (Archer et al., 2011; Fellman et al., 2016, 2011).

Samples were assayed using the FACScalibur™ Flow Cytometer (Becton Dickinson) and analyzed with CellQuest™ Pro software (Becton Dickinson). Lymphocytes were identified using forward scatter and side scatter, and 10,000 events were collected per sample. An additional gate was placed around CD3+ cells to include T cells and exclude B cells. Cytokine staining was evaluated for CD3+ lymphocytes by measuring mean fluorescence intensity (MFI) with single histogram statistics. Negative controls included unactivated samples and isotype controls. For viability assessment, lymphocytes were identified using forward scatter and side scatter and 5,000 lymphocytes were collected per sample.

2.6. RNA isolation and qRT-PCR analysis

After incubation, total RNA was isolated from 1 mL heparinized whole blood as previously described (Riggs et al., 2013). Cytokine gene expression analysis was performed similarly to the previously described method, with the modifications described below. Briefly, a 20 μL reaction volume was used with 30 ng template and 200 nM of each primer. Samples were run on a Stratagene™ Mx3005P Real-Time PCR system, and Stratagene™ MxPro QPCR software, version 4.10 (Agilent Technologies, Santa Clara, CA) was used for analysis. Experimental samples were run in triplicate, with non-template controls run in duplicate. Relative gene expression was calculated with mean Ct values using the 2−ΔΔCt method, where: ΔΔCt = (CtGOI − Ctnorm)treated − (CtGOI − Ctnorm)untreated, and GOI is the gene of interest (IL-2 and IFN-γ) and norm is the normalizer gene (GAPDH) (Livak and Schmittgen, 2001).

2.7. Statistical analysis

Mean fluorescence intensity (MFI) data were analyzed for flow cytometry, and the percent reduction in cytokine gene expression relative to untreated sample expression was evaluated for qRT-PCR. Following visual assessment of quantile-quantile plots of the data (PROC UNIVARIATE, SAS for Windows 9.3, SAS Institute, Inc., Cary, NC, USA), the data were judged to not be normally distributed. Consequently, non-parametric methods of analysis that accounted for the hierarchical structure of the data were utilized (Brunner and Puri, 2001; Shah and Madden, 2004). For each outcome (flow cytometry: IL-2, IFN-γ; qRT-PCR: IL-2, IFN-γ), the data were ranked and then analysis of variance type statistics were obtained through PROC MIXED (SAS for Windows 9.3, SAS Institute, Inc., Cary, NC, USA) by using the ANOVAF option and the MIVQUE0 estimation method for the covariance parameters and a REPEATED statement specifying dog identity as the subject and an unstructured covariance structure. Either cyclosporine or dexamethasone concentration was included in the models as a fixed effect. Differences in least square means with the SIMULATE adjustment of P values were used for multiple comparisons of each of the four concentration levels to the untreated sample. To allow method comparison using the same outcome, percent reduction in cytokine expression relative to untreated levels was calculated for flow cytometry to parallel the results found with qRT-PCR. The effect of method (flow cytometry vs qRT-PCR), drug concentration, and their interaction on percent reduction in cytokine expression was then assessed. If the interaction term was significant, differences in least square means with the SIMULATE adjustment of P values were used to make comparisons between the two methods at each of the drug concentrations. A P value of less than 0.05 was considered to be significant for all analyses.

3. Results

3.1. Incubation study

Results of the blood sample incubation study are shown in Fig. 1. For both methods, cytokine production after PMA and ionomycin activation was similar for the three dogs tested. For flow cytometry (Fig. 1 A and B), both IL-2 and IFN-γ MFI increased to reach a maximum at either 6 or 8 h, and then decreased with longer testing intervals, except for 1 dog that had its highest IFN-γ level at 4 h. Quantitative RT-PCR results (Fig. 1 C and D) are shown as the ΔΔCt value between the 2, 4, 6, 8, 10, and 12-h incubation value and unstimulated cytokine production. Cytokine production was assessed visually and found to increase substantially from 2 h to 4 h for both IL-2 and IFN-γ, and mildly more at 6 h, but remained at a similar expression level thereafter. To allow the greatest potential to find a difference in expression and the most stable expression level, the time cytokines reached an approximate maximum was selected. Seven hours of activation was recommended for flow cytometry, and five hours for qRT-PCR. These incubation times were used in the following experiments.
3.2. Cyclosporine and dexamethasone treatment

Cyclosporine caused a concentration-dependent decrease in IL-2 and IFN-γ expression as measured with both flow cytometry (Fig. 2) and qRT-PCR (Fig. 3). At 10 ng/mL cyclosporine, no significant difference was identified for either cytokine with either method relative to untreated control samples \( (P \geq 0.21) \). By 100 ng/mL cyclosporine, however, both IL-2 and IFN-γ expression were significantly reduced for both methods \( (P \leq 0.01) \). No additional suppression of cytokine production was noted at higher concentrations of cyclosporine using flow cytometry \( (Fig. 2A, 2B, P \geq 0.16) \), while significantly greater suppression of both cytokines was noted at 500 ng/mL than 100 ng/mL with qRT-PCR \( (Fig. 3A, 3B, P \leq 0.025) \). No additional suppression, however, occurred with an increase to 1000 ng/mL \( (P \geq 0.81) \).

For dexamethasone, results from flow cytometry and qRT-PCR were not as closely related as they were for cyclosporine. IL-2 was not significantly suppressed with any dexamethasone concentration as measured by flow cytometry \( (Fig. 2C, P \geq 0.095) \), while qRT-PCR showed low but uniform significant reduction in gene expression at all concentrations tested \( (Fig. 3C, P \leq 0.037) \). Suppression of IFN-γ was concentration-dependent after treatment with dexamethasone, and reached maximum levels at 100 ng/mL for both methods \( (Figs. 2D and 3D) \). Overall, dexamethasone caused more cytokine suppression than cyclosporine at lower concentrations, but cyclosporine caused more cytokine suppression than dexamethasone at higher concentrations.

The interaction between method and cyclosporine concentration on the percent reduction in cytokine expression was significant for both IL2 and IFN-\( \gamma \) \( (P < 0.01) \). There was no difference between flow cytometry and qRT-PCR for cyclosporine at 10 or 100 ng/mL \( (P \geq 0.11) \), while at 500 and 1000 ng/mL, qRT-PCR showed a significantly greater degree of suppression of both IL-2 and IFN-\( \gamma \) than flow cytometry \( (P < 0.01) \). For dexamethasone, there was significantly greater suppression with qRT-PCR relative to flow cytometry for both IL-2 and IFN-\( \gamma \) \( (P < 0.01) \) irrespective of drug concentration.

Good lymphocyte viability \( (> 95\%) \) was confirmed on samples from all 3 dogs evaluated at all the drug concentrations reported in this study (data not shown).

4. Discussion

This study documents in vitro responses to exposure to two commonly used immunosuppressive medications, cyclosporine and dexamethasone, using canine whole blood assays. Both drugs are known to reduce the expression of pro-inflammatory cytokines \( (Arya et al., 1984; Giese et al., 2004; Grabstein et al., 1986; Yoshimura and Kahan, 1985) \), and this effect was reflected in the present study. Both cyclosporine and dexamethasone reduced the gene expression of IL-2 and IFN-\( \gamma \). Cyclosporine caused similar suppression of both cytokines, and caused a greater inhibition at high drug concentrations than did dexamethasone. Dexamethasone, in contrast, caused greater suppression of IFN-\( \gamma \) than IL-2. Flow cytometry was used to confirm effects on protein production, and documented marked suppression of both IL-2 and IFN-\( \gamma \) for cyclosporine, but only IFN-\( \gamma \) was significantly decreased after dexamethasone treatment. Our study also confirmed that reduced cytokine expression occurred in the presence of viable cells and was therefore not simply a manifestation of drug-induced cell death.

While flow cytometry cytokine suppression plateaued at cyclosporine concentrations greater than 100 ng/mL, qRT-PCR was notable in
reflecting significantly greater suppression at 500 ng/mL than 100 ng/mL. This is important because 100 ng/mL is at the very low end of published target blood cyclosporine concentrations in dogs, and concentrations higher than this are frequently achieved with oral drug dosing (Boothe, 2012). Although being sensitive to the effects of low drug concentrations is important for a biomarker, the ability to discriminate greater degrees of immunosuppression at higher blood concentrations is likely to further increase the utility of the assay, since the intent of the assay is not only to document the effects of cyclosporine on T cells, but to also identify patients with excessive immunosuppression that may be at risk of secondary infections.

While our current study documented that qRT-PCR was more sensitive than flow cytometry for detecting the progressing effects of higher cyclosporine concentrations on T cell function in vitro, another recent study by our laboratory identified that, when cyclosporine is administered orally to dogs, flow cytometry but not qRT-PCR detected subtle differences in cytokine expression levels across the dosing interval (Fellman et al., 2016). This finding underscores the need for confirmation of in vitro findings with subsequent oral dosing studies in dogs. Further study at additional drug doses is needed to determine the optimal method for cytokine evaluation during oral cyclosporine therapy in dogs.

In this study, a statistical comparison of the degree of reduction in marker expression between flow cytometry and qRT-PCR was performed. Though often similar, differences in results for flow cytometry and qRT-PCR can occur due to differences in post-translational modification (Gerez et al., 1995; Khabar and Young, 2007). Although the clinical consequences of decreased protein production relative to decreased gene expression have not been established, and an argument can be made that decreased protein expression is ultimately the more relevant endpoint, the greater magnitude of reduction at higher drug concentrations identified for cytokine gene expression compared to protein production is potentially valuable from an analytical standpoint. Reductions of lesser magnitude are at greater risk of being confounded by biological and analytical variability. In this sense, the 90% decrease in gene expression identified at high cyclosporine concentrations is appealing. Quantitative RT-PCR also carries the advantage of enabling storage of RNA after extraction, thereby allowing samples collected on different days to be run on the same plate to further reduce analytical variability.

One strength of the described techniques is the use of whole blood and diluted whole blood assays. Cyclosporine is known to distribute into the different whole blood components and to bind to non-target sites, and thus effective inhibitory drug concentrations for pharmacodynamic measures are generally lower when tested in isolated PBMC than in whole blood, because in whole blood not as much free cyclosporine is available to affect T cells. This effect can sometimes lead to marked differences in effective inhibitory drug concentrations (Batiuk et al., 1996; Stein et al., 1999).

Although an insufficient number of drug concentrations were tested to precisely identify the IC50 in this study, cytokine expression approached maximal suppression at 100 ng/mL cyclosporine when measured using flow cytometry, and gene expression was approximately 50% suppressed at this concentration. Nafe and others reported an IC50 for proliferation for cyclosporine of 15.8 ± 2.3 ng/mL in canine PBMC stimulated with ConA (Nafe et al., 2014). Although an IC50 was not calculated, visual inspection of cytokine mRNA expression plots by Kobayashi and others also suggest approximately 50% inhibition of IL-

**Fig. 3.** Cytokine gene expression after in vitro exposure to varying concentrations of cyclosporine and dexamethasone assessed using qRT-PCR. Cytokine gene expression measured using qRT-PCR in whole blood after treatment with cyclosporine (3A, 3B) or dexamethasone (3C, 3D). Samples from six dogs were activated with PMA and ionomycin and incubated with cyclosporine (10, 100, 500, 1000 ng/mL) and dexamethasone (10 ng/mL, 100 ng/mL, 1 μg/mL, 10 μg/mL). Gene expression results are shown as the percent expression relative to untreated samples. The box plots demonstrate the median (line) and interquartile range (IQR, box), and the whiskers span all data points within 1.5 IQR of the nearer quartile. Outliers are indicated by an asterisk (*). Letters are used to indicate a significant difference from untreated values. Treatments that do not share a letter are significantly different (P < 0.05).
2. IL-4, and IFN-γ mRNA expression in canine PBMC treated with 10 ng/mL of cyclosporine (Kobayashi et al., 2007). These data agree with the previously mentioned tendency for IC₅₀ values to be lower in PBMC than whole blood, but interestingly, the values are much lower than most IC₅₀ values for lymphocyte proliferation or cytokine expression in whole blood or PBMC in humans. Most human studies report cyclosporine IC₅₀ values of 200–500 ng/mL depending on the parameter studied (Barten et al., 2001; Flores et al., 2004; Kuzuya et al., 2009; Piekoszewski et al., 1994; Stein et al., 1999).

It is unclear whether the apparently greater lymphocyte responsiveness to cyclosporine in dogs is due to differences in assay performance, or to differences in species susceptibility to immunosuppressive effects. Genuine differences in species T cell susceptibility to cyclosporine may have important clinical ramifications, and could result from factors such as differences in cellular cypholin A levels, calcineurin activity, or P-glycoprotein function. Published target blood cyclosporine concentrations are similar in dogs, cats, and humans, despite the fact that little work has been done to correlate cyclosporine blood concentration with clinical response in veterinary medicine (Case et al., 2007; de Jonge et al., 2009; Hopper et al., 2012). Differences in T cell susceptibility could, potentially, indicate a need for different target concentrations in dogs than in humans.

The immune-modulating effects of dexamethasone and other steroids have also been investigated extensively in vitro. One study comparing the effects of cyclosporine and methylprednisolone on human T cells documented that both cyclosporine and methylprednisolone inhibited IL-2 expression to a greater extent than lymphocyte proliferation, and reported an additive, but not synergistic, effect of the drugs in combination (Freed et al., 1988). Other studies have documented cyclosporine to be less effective than prednisolone at inhibiting whole blood lymphocyte proliferation, but noted a synergistic effect when the drugs were used in combination (Ferron and Jusko, 1998; Ferron et al., 1998). One study reported dexamethasone at 39 ng/mL significantly inhibited PBMC proliferation in humans (Corrigan et al., 1991). In a study of lymphocyte proliferation by Nafe and others, however, a much higher dexamethasone IC₅₀ value of 1.36 ± 0.75 mg/mL (3460 ± 1900 μM) was reported for canine PBMC (Nafe et al., 2014).

In the present study, 10 ng/mL, 100 ng/mL, 1 μg/mL and 10 μg/mL dexamethasone were used. Uniform but fairly minimal suppression of IL-2 was identified with qRT-PCR, while no effect on IL-2 production was identified with flow cytometry. Dexamethasone induced greater suppression of IFN-γ compared to IL-2. Suppression was concentration dependent with both methods of measuring cytokine expression, but flow cytometry did not attain a median 50% reduction in IFN-γ expression, while median IFN-γ gene expression results were less than 50% at all concentrations tested. The concentrations evaluated in this study include and exceed typical blood dexamethasone concentrations (Greco et al., 1993; Toutain et al., 1983).

Although decreased expression of cytokines including IL-2 and IFN-γ is recognized to be a primary and well-documented effect of glucocorticoids (Arya et al., 1984; Gillis et al., 1979; Grabstein et al., 1986; Reem and Yeh, 1984), recent studies by two different groups documented a lack of effect of in vitro exposure to methylprednisolone on human whole blood IL-2 mRNA expression (Kuzuya et al., 2009), and of methylprednisolone on human whole blood IL-2 cytokine production by flow cytometry (Bohler et al., 2007). Our study shows that cyclosporine causes a greater degree of T cell cytokine suppression at high concentrations than dexamethasone, which is expected given that the specific mechanism of action of cyclosporine involves inhibition of NFAT-regulated cytokines. The minimal suppression of IL-2 following incubation with dexamethasone deserves further study, however, and suggests that IL-2 has the potential to be a fairly specific biomarker for cyclosporine in dogs receiving concurrent glucocorticoids.

One limitation of the current study is the small number of dogs tested, which may have limited the ability to identify significant differences between treatment groups. The single breed of dog tested is also a limitation, though Walker hounds are expected to be representative of dogs in general. Finally, assays performed in vitro may not reflect drug effects in the body. Therefore, further confirmation of these effects in both healthy and sick dogs is warranted.

In conclusion, this study provides an in vitro comparison of the effects of cyclosporine and dexamethasone on whole blood T cell cytokine assays in dogs. Cyclosporine caused concentration-dependent suppression of IL-2 and IFN-γ, with qRT-PCR better able to detect additional suppression at higher drug concentrations. Dexamethasone caused mild suppression of IL-2 as measured with qRT-PCR, but IL-2 levels were not significantly affected when measured by flow cytometry. In contrast, dexamethasone did cause concentration-dependent suppression of IFN-γ, and produced a significantly greater degree of suppression when measured by qRT-PCR compared to flow cytometry. This study determined that optimal incubation times for cytokine analysis in dogs are approximately five hours with PMA and ionomycin for qRT-PCR, and seven hours for flow cytometry. Further work is needed to determine the analytical method with the greatest utility for reflecting the immunosuppression achieved by cyclosporine and glucocorticoids in vivo, but our results suggest that cytokine monitoring may be a viable method for monitoring the effects of immunosuppressive drugs in dogs.

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