1-1-2017

Development of a Pharmacodynamic Assay to Assess the Effect of Cyclosporine in the Canine Patient

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By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

August 2017

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Title of Study: Development of a pharmacodynamic assay to assess the effect of cyclosporine in the canine patient

Pages in Study 117

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Cyclosporine is used in veterinary medicine to treat a number of inflammatory and immune-mediated conditions, however firm oral dosing protocols have yet to be established in the dog. Traditionally a pharmacokinetic approach, through measurement of blood drug concentrations, has been the primary method of establishing if the given dose is effectively suppressing the immune system. However, there is some debate over how well blood drug concentrations correspond to immunosuppression, since individuals can vary in response to the same drug concentration. Our research group believes that a pharmacodynamic approach could alternatively be used to accurately determine cyclosporine dosages in individual patients since this will give a measurement of the immune system's response to the drug, rather than simply how the body is processing it. This method will give a more accurate assessment of the patient's immune system, and allow for better immunosuppressant therapy. The objective of this thesis was to develop a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay that could reliably predict patient outcome during cyclosporine treatment. This assay would essentially work as a diagnostic tool that clinicians can use to help determine if they were

using an appropriate cyclosporine dose for their patients. The assay measures cytokine expression of activated T cells, which are the target cell for the active metabolite of cyclosporine. Our objectives were achieved, firstly, through validation of the assay. Since this assay will be used by clinicians throughout the nation, we first established if shipping conditions affected the sample, and therefore assay results. Once the effect of sample storage time and temperature were determined, optimal sample collection timing was established. Finally, cytokine levels were measured in samples from clinical cases and healthy control dogs to examine the difference in cytokine expression between these two groups. An effective and reliable treatment method for cyclosporine has yet to be established in the dog; therefore the results of this thesis will lead to better therapeutic monitoring and more efficient use of cyclosporine therapy in canine patients.

DEDICATION

To my Dad, Mom, and brothers Ryan, Jason, and Jonathon for their support and encouragement all these years.

ACKNOWLEDGEMENTS

The hard work and dedication of many people helped to make this thesis possible. I would like to thank everyone who assisted with the copious benchtop work including Dr. Evangel Kummari, Dr. Lakshmi Narayanan, Dr. Santosh Kumar, Dr. Joyce Follows, and Charlie Mulligan. I also want to thank Dr. Robert Wills for all of his help with statistical analysis. Thank you also to my committee members Dr. Camillo Bulla, Dr. Todd Pharr, and Dr. Mark Lawrence for their support and guidance. I would like to express sincere gratitude to Dr. Andrew Mackin for his enthusiasm and never ending stream of ideas. I cannot thank Dr. Todd Archer enough for his patience, guidance, and mentorship these past 6 years. I could not have asked for a better advisor. And finally thank you to my vet school friends for all the coffee, encouragement, laughs, jokes, and late nights.

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CHAPTER I

LITERATURE REVIEW

The immune system is essential for the body to function properly and provides protection against disease. Both innate and adaptive immunity defend the body against pathogens like viruses, parasites, and bacteria. This system is like a double-edged sword, and if not tightly regulated, can become wildly destructive. Regulation is accomplished through a number of cell mediators and immune cell interactions, but when these components are able to escape tight regulation, autoimmune disease results. The pathogenesis of autoimmune diseases can be incredibly complex, involving both genetic and environmental factors, and these diseases can therefore often times be very difficult to treat. This is especially true in veterinary medicine, since most therapies have been developed for use in humans, and are not thoroughly evaluated in companion animal species. Thus the focus of this thesis is to develop a better understanding of the treatment of immune-mediated disease in canine patients, with a specific emphasis on the immunosuppressive drug cyclosporine.

Cyclosporine

Cyclosporine A (CsA) is a powerful immunosuppressive agent used in both human and veterinary medicine to prevent transplant rejection and to treat a variety of inflammatory and immune-mediated diseases. Its discovery provided a major breakthrough for transplant medicine, and it is still widely used today. Cyclosporine use

is also growing in the veterinary medical field, as it is employed to treat an increasing number of inflammatory and immune-based diseases.

Discovery and Synthesis

Cyclosporine was first discovered in the 1970s at the Sandoz Lab located in Switzerland, and involved the efforts of both F. Borel and H. Stähelin.[1] In 1958 the Sandoz company set up a screening system for antibiotics. At that time employees would regularly pick up random soil samples while traveling or on vacation, and bring back the samples to the laboratory for testing on the slight chance that they would contain a microorganism that could be developed into the next great antibiotic.[2] While on holiday in Norway, H.P. Frey picked up a soil sample intending to return it to the laboratory for testing, completely unaware that he held the key to the drug that would revolutionize transplant medicine.[2] In the meantime, another laboratory was being set up in the Sandoz company that experimented on immunosuppressive properties of test compounds under Drs. H. Stähelin and S. Lazary.[1] Frey's sample, number 24-556, was submitted to both the general screening program, as well as to Stähelin's laboratory for immunosuppressive testing after extraction.[1] Stähelin tested the extract on mice, observing its cytostatic activity and lack of cytotoxicity, and found significant immunosuppressive effects, while Borel performed a hemagglutinin test using mouse serum.[2] A second sample of 24-556 was submitted to Borel's laboratory, but upon testing produced disappointing results compared to the previous study. Despite its poor performance, however, the group decided to continue investigation into the compound.[1]

The original soil sample contained the saprophyte *Tolypocladium inflatum*, a pathogen of beetle larvae that produces many secondary metabolites with potential uses

within the medical and agricultural industries, including cyclosporine.[3] Borel characterized many of the immunosuppressive properties of cyclosporine in mice via skin and bone marrow transplantation studies. In these experiments, Borel showed that cyclosporine inhibits lymphocyte proliferation, suppresses cellular and humoral immunity, and has low myelotoxicity.[4] After Borel presented his findings at the British Society for Immunology in London in 1976, other laboratories began experimenting with the cyclosporine molecule in other animal species and humans, with exciting results.[1] Based on these experiments, researchers recommended clinical investigation of cyclosporine's potential for use in transplant therapy, but first absorption, distribution, and metabolism in healthy individuals needed to assessed.[5] There were a few issues initially with absorption in humans due to the lipophilic nature of cyclosporine, however once the correct vehicle was used, the problem resolved.[5]

In the late 70s, the first clinical investigations of cyclosporine in human patients began. In a study of 34 patients with organ allografts, the majority of organs were still viable, some after more than a year, using cyclosporine as an immunosuppressive agent.[6] Further studies confirmed that an ideal protocol would combine cyclosporine and glucocorticoids to maximize immunosuppression, while minimizing cyclosporine's toxic effects on the kidney, and avoiding infection.[5,7] In 1983 the FDA approved the use of cyclosporine for preventing organ rejection after transplantation in people.[8]

Cyclosporine is a cyclic polypeptide whose chemical structure was revealed using a combination of x-ray crystallography and chemical degradation.[9] After the usefulness of cyclosporine was determined in clinical trials, it was important to develop a method of

synthesizing the drug in mass quantities. Not only would this make the drug more widely available, but also it would cheapen its cost.

Cyclosporine Formulations

There are two distinct formulations available for oral cyclosporine in the dog. Sandimmune®, the original preparation, is a corn-oil based drug first introduced to the market in the 1980s. While considered the gold standard to prevent organ rejection in transplant medicine at the time, it displayed significant variation between patients in terms of absorption and oral bioavailability.[10-12] This led to the production of a new ultramicronized formulation named Neoral®. This product formed a microemulsion with gastrointestinal fluids after oral consumption, which evenly distributed the drug throughout the fluid, allowing better absorption.[13] Studies showed this new formulation displayed lower inter- and intra-individual variability, and achieved more predictable blood drug concentrations.[11,14] In 2003 the FDA approved a veterinary microemulsion formulation (Atopica®) for use in dogs. Since its approval, Atopica® has undergone multiple pharmacokinetic studies in dogs, and thus is the recommended formulation for use in canine patients.

Pharmacokinetics: Absorption, Distribution, Metabolism, and Excretion

Once ingested, cyclosporine is transported to, and then absorbed by, the epithelial cells of the small intestine. Cyclosporine is not well absorbed orally, and its bioavailability can differ greatly from one individual to the next. In dogs, bioavailability can range from 23% to as much as 45% and, in the presence of food, can be further reduced by 20%.[15] Diabetes, gastric motility issues, diarrhea, and liver disease can also

impact absorbability.[10,16] P-glycoprotein pumps within the intestinal cells can also diminish CsA levels by pumping the drug from the enterocyte cytoplasm back into the intestinal tract, although so far this has been shown to only be an issue in man and not dogs.[17,18]

After intestinal absorption, CsA enters the bloodstream, circulating throughout the body. Its high lipid solubility allows it to easily cross cell membranes.[10] The drug prefers red blood cells (RBCs) over plasma proteins at a ratio of 2:1.[10] While in the plasma ,cyclosporine is 80-90% protein bound, mostly to high density lipoprotein, and it typically distributes within RBCs; therefore a patient's RBC count and lipoprotein numbers can impact drug distribution.[10,16] Post-circulation, cyclosporine accumulates in the skin, liver, kidneys and fat, and will maintain sustained drug levels in these sites even after discontinuation of therapy.[10,19]

Metabolism of cyclosporine occurs mainly in the liver by way of the cytochrome P450 system, which alters CsA into a number of metabolites through hydroxylation or N-demethylation.[20] The rate of metabolization differs from species to species, but in the dog 70-100% of the drug is metabolized in the first 30 minutes.[20] The main cytochrome P450 enzymes responsible for cyclosporine metabolism are CYP3A4 and CYP3A5.[16] The predominant metabolites in the dog, in decreasing order of quantity, include 9 γ-hydroxylated metabolite, 4 N-desmethylated metabolite, 1-β-(8´) hydroxylated metabolite, and 1-β-1-ε-cyclized metabolite.[20] Total quantities of individual metabolites are known to vary from one species to another, with baboons and hamsters having the most comparable numbers to humans.[20] A number of factors can impact CsA metabolism, including liver disease, age, and other drugs.[10] Several drugs are known to inhibit CsA

metabolism, and one is often used purposefully to decrease oral doses while maintaining adequate blood levels. Ketoconazole tends to be the drug of choice for generating this effect in dogs, and works by inhibiting cytochrome P450. Ketoconazole has been shown to reduce CsA doses by as much as 75%, which can greatly reduce the cost of treatment for owners.[21]

The majority of cyclosporine metabolites are excreted through the bile, with an elimination half life time of about 9-12 hours in the dog.[15] Only a very small percentage of the drug's metabolites are excreted in the urine, and therefore kidney disease does not greatly impact drug excretion.[10,16,20]

Mechanism of Action

After the discovery of its immunosuppressive properties, much work went into unraveling cyclosporine's mechanism of action. Cyclosporine is a potent inhibitor of T lymphocytes. During a normal immune response, antigens are presented to T cell receptors. This binding results in a signaling cascade within the cell, leading to cytokine production, cell proliferation, and an overall enhancement of the immune response. T cells play a vital role in cell-mediated immunity, and are important for destroying infected and tumor cells,[22,23] maintaining immunological tolerance,[22,23] providing an immune memory response,[24] and assisting other white blood cells (WBCs) during an immune response.[25] It is essential for T cells to function properly because, when they become hyperactive, immune-mediated disease results, while reduced T cell function can lead to immune deficiencies and infection. Disease states resulting from overactive T cells are often treated using immunosuppressive agents like cyclosporine, which targets the abnormal immune cell response.

Cyclosporine works predominantly by inhibiting the activity of T lymphocytes. When an antigen complexed with the major histocompatibility complex (MHC) binds to a T cell receptor (TCR), a signaling cascade results. This binding results in a conformational change, making the receptor's immune receptor tyrosine activation motifs (ITAM) sequences, which reside in the cell cytoplasm, more accessible. This action recruits the tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase), which phosphorylates the ITAMs, thus activating them. Another cytosolic tyrosine kinase, ZAP70 (zeta-chain associated protein kinase 70 kDa) is also activated by Lck. ZAP70s phosphorylation results in the recruitment and activation of other proteins, including SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa). SLP-76 binds phospholipase C, which then cleaves PIP2 (phosphatidylinositol biphosphate) into two substrates, DAG (diacylglycerol) and IP3 (inositol triphosphate). Within the cytosol, IP3 binds to the endoplasmic reticulum, causing the release of calcium into the cell interior. The influx of calcium causes activation of the calcium-dependent intermediate messenger calmodulin, which subsequently binds and activates calcineurin, which is usually found in the cytosol of the cell. Activated calcineurin dephosphorylates regulatory sites on the transcription factor nuclear factor of activated lymphocytes (NFAT). NFAT then translocates to nucleus and induces transcription of certain cytokines including interleukin-4 (IL-4), tumor necrosis factor- α (TNF-α), interferon-γ (IFN-γ), and IL-2.[26]

Cyclosporine works by interfering with the T cell activation signaling cascade at the level of calcineurin. When cyclosporine enters the cell, it binds to the cytosolic protein cyclophilin, specifically cyclophilin A, since this is the main type of cyclophilin found in T cells. This complex of cyclosporine and cyclophilin inhibits calcineurin,

which normally activates NFAT. Since NFAT is thereby not dephosphorylated, and therefore does not localize to the nucleus, the resulting effect is decreased expression of cytokines controlled by NFAT. This leads to inhibition of proliferation and activation of both helper and cytotoxic T lymphocytes, and an overall decrease in the immune response.

Adverse Reactions

Cyclosporine has a relatively narrow therapeutic window, and has mostly concentration-dependent side effects. Side effects tend to be more common at higher doses, and are usually reversed once the drug is discontinued.[19] Gastrointestinal symptoms are the most common adverse reactions, and include vomiting, diarrhea, nausea, and decreased appetite. These can sometimes occur even at the low dosage used to treat atopic dermatitis in dogs.[19] Other reported side effects in dogs include gingival hyperplasia, excess shedding, papillomatosis, and hypertrichosis.[15] Although kidney function may be impaired in human patients receiving cyclosporine, this issue is not commonly reported in the veterinary literature. One study, using beagle dogs, kept them on a very high 45 mg/kg daily dosage for 12 months, and observed no change in the nephrotoxicity biomarker calbindin.[27] It is still not understood why dogs seem to be protected from nephrotoxicity due to cyclosporine, since humans and rats can undergo renal damage at much lower doses.

Cyclosporine should be used carefully in the presence of other drugs, since it is known to have reactions with some of them. Doxycycline, methylprednisolone, erythromycin, and grapefruit juice have all been shown to increase cyclosporine blood levels, while omeprazole and rifampicin are known to decrease them in humans.[19] In the

veterinary field azole antifungals such as ketoconazole, calcium channel blockers, and macrolide antibiotics are just some of the drugs known to increase blood levels when used in combination with cyclosporine.[15]

Contraindications for cyclosporine use include a previous diagnosis of malignant neoplasia in dogs, and uncontrolled infection or hypertension in man, since suppressing the immune system can worsen diseases of this nature.[15,19] Cyclosporine is known to increase production of transforming growth factor- beta (TGF-β) and vascular endothelial growth factor (VEGF), cytokines which are known to enhance tumor growth.[28] Cyclosporine also inhibits DNA repair, and has anti-apoptotic properties.[28] The combination of all of these effects can theoretically lead to cancer and encourage metastasis if neoplasia is already present. Cyclosporine can cause hypertension in man, however the exact mechanism is still not well understood. It is hypothesized that increased prostaglandin production and decreased excretion of sodium, water, and potassium contribute to hypertension.[29,30] Cyclosporine should also be used conservatively in veterinary patients suffering from renal or hepatic disease. Cyclosporine is able to cross the placental barrier, as well as enter the milk of lactating individuals, therefore it should not be used in pregnant or nursing patients.[19]

Pharmacokinetic Monitoring

Monitoring cyclosporine drug therapy is essential for a number of reasons: there is wide individual variability in CsA pharmacokinetics, a threshold drug concentration must be reached to minimize the risk of treatment failure, and toxicity must be avoided by ensuring trough concentrations are not over a safe limit.[10] Studies describing monitoring of CsA concentrations have been undertaken using a variety of blood sample

types as well as a number of different analysis techniques, and because of these variations it is often difficult to compare studies.

Blood, plasma, and serum have all been used as potential sample types for monitoring CsA concentrations. Whole blood drug levels seem to give the most consistent numbers compared to serum or plasma.[10] Easier sample collection as well as the ability to store heparinized samples before running the assay make the use of whole blood more convenient than plasma or serum.[10]

Radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) are the two most widely studied methods for measuring CsA concentrations. RIA is an extremely sensitive procedure which measures CsA concentrations through the use of radiolabeled drug and binding site competition principles.[31] There are a couple of issues with using RIA, however. Samples outside the range of the linear curve have been observed in patients with liver problems, leading to the need for sample dilution, and results in these patients can be misleading since ideally an undiluted sample should be used.[10] Furthermore, RIA is not very specific, since a number of CsA metabolites can cross-react during analysis. Cross-reactivity ranges anywhere from 4-32% depending on the metabolite in question, meaning that samples can be given a falsely high drug concentration, since not all metabolites actively suppress the immune system.[10] Cyclosporine concentrations measured by RIA have been found to be consistently higher compared to HPLC measured samples, likely due to CsA metabolites.[31] A number of CsA metabolites have little or no immunosuppressive properties, therefore a falsely high reading can make it appear that the patient is sufficiently immunosuppressed when, in reality, they require a higher dose.[10] HPLC is a more labor intensive method and requires

specialized equipment and experienced users, which are not available at all facilities. For HPLC, a series of columns and solvents are used to separate the sample into its component parts, allowing one to measure drug concentrations. This makes the method more specific than RIA, since there is no metabolite cross reaction.[10] HPLC is therefore the gold standard for measuring CSA blood concentrations, especially in patients with liver issues, since there is no metabolite cross reactivity.

More recently, liquid chromatography-mass spectroscopy (LC-MS) has been used to measure CsA concentrations.[32-35] One study even validated an assay that could be used in dried blood spots, allowing patients the convenience of taking blood samples at home.[33] While this technique is used in human patients and in research, it has yet to be validated for use in canine patients.

Once sample type and analysis method were perfected, the next step was to identify when to take the sample post-dosing. Initially, monitoring trough concentrations was suggested to avoid over dosage and potential liver and kidney damage. Trough samples would be taken just before the next dose of CsA was given, representing the lowest drug concentration achieved. There was much debate in transplantation medicine over ideal blood trough concentrations, with recommended target concentrations ranging anywhere from 100-500 ng/ml ,depending on the institution and the assay used.[36] Wide ranges in suggested target trough blood concentrations and poor correlation to clinical outcome lead scientists to search for a better marker of immunosuppression and toxicity. Kahan suggested using the area under the concentration-time kinetic curve, originally starting with a 12 hour time window.[37] This method, however, was quite impractical for use in patients, given the number of samples and processing time needed. Shorter time

frames were therefore investigated for area under the curve measurement. A 4 hour sampling technique proved useful,[38] however sample collection and preparation was still labor intensive, and the search for a better test still continued. Eventually, peak drug concentrations (2 hour post-dose sample) were found to correspond well with the risk of graft rejection.[39] Studies in man show that peak drug concentrations are reached 2 hours after oral dosing, and thus this is the recommended time for blood collection for therapeutic monitoring.[40,41] Similar sample collection times have been established in the dog as well.[42] A recent study in our own laboratory showed peak cyclosporine concentrations were reached 2 hours after drug administration, then fell rapidly thereafter to trough levels.[43] Measurement of a single peak drug concentration is much more convenient than measuring area under the curve, and provides physicians with a method for individualizing therapy that allows for CsA's wide inter-individual variability.

A similar search for ideal cyclosporine monitoring techniques has been embarked upon in veterinary medicine. Cyclosporine is used to treat a number of immune-mediated diseases in dogs, therefore having a monitoring protocol in place would be ideal, since CsA pharmacokinetics has been shown to be variable between individual canine patients. However, little work has been done in this area, and current blood concentration recommendations are mostly extrapolated from studies of kidney transplantations in dogs. As in human medicine, initial attempts to monitor CsA in dogs began using trough concentrations. The starting recommendation was to obtain a trough drug concentration of 500-600 ng/ml.[44] Veterinary medicine has mirrored its human counterpart, and now reference laboratories often recommend monitoring both trough and peak samples, although target peak levels have yet to be thoroughly evaluated in the dog. Target values

also vary depending on the laboratory and analysis method used. A new and more efficient test that reliably predicts clinical outcome would be highly beneficial for veterinary patients receiving CsA, since some of the diseases that this drug is used to treat are life threatening, sometimes necessitating rapid changes in dosing regimens in order to save the patient.

Pharmacodynamic Monitoring

While measuring blood cyclosporine concentrations was originally the primary means of assessing immunosuppression in patients, individuals vary widely in response to the same dose, and published target blood concentrations vary from one institution to the next. Dogs may even vary in immune response to the same cyclosporine blood concentration; therefore even though a patient may have achieved a therapeutic blood concentration according to certain standards, sufficient immunosuppression may not be reached due to individual variation in response to the drug. One way to address this problem is to take a pharmacodynamic approach to measuring cyclosporine's effect on the immune system, rather than solely a pharmacokinetic one. By measuring the drug's impact on the immune system, rather than merely how the body is processing the drug by measuring blood concentrations alone, immunosuppressive effects can be more reliably evaluated, and dosages can be better tailored to the individual patient. Such pharmacodynamic monitoring has already been successfully employed in human transplant medicine. Similar to pharmacokinetic monitoring, a number of different techniques, including quantitative reverse transcription polymerase chain reaction (qRT-PCR),[45,46] T cell proliferation assays,[47] calcineurin inhibition[48] and flow cytometry,[49,50] have all been explored as potential new assays. While pharmacodynamic monitoring

offers a great opportunity for individualizing drug therapy, a number of issues still need to be perfected, such as which biomarkers to test and how to assess them.

A number of studies have evaluated T cell production of cytokines, primarily IL-2 and INF-γ, in response to drug therapy, as a pharmacodynamic biomarker assay of cyclosporine's effects. IL-2 is mainly produced by activated T cells, and promotes B and T cell proliferation and differentiation.[51] IL-2 is known to be inhibited by exposure to cyclosporine both *in vitro* and *in vivo,* and this response has been studied in mice,[52] humans,[52,53] and dogs.[54] Minimal or no inhibition of IL-2 production has been correlated to acute organ rejection in transplant recipients receiving cyclosporine, and patients with high IL-2 levels prior to transplantation were more likely to reject the new organ.[50,55] Similar IL-2 levels were found between stable graft patients and healthy volunteers, while levels were higher in patients suffering acute rejection episodes.[56] IL-2 receptor expression has also been evaluated, and some studies have shown an increased number of IL-2 receptors in patients with cyclosporine-induced nephrotoxicity, and during rejection episodes.[56] However, cyclosporine does not directly influence IL-2 receptor expression, and measuring T cell cytokine production has been shown to be a more sensitive indicator of rejection.[56] Like IL-2, INF-γ is produced by T cells, but is mainly responsible for macrophage activation.[57] T cell INF-γ production is also used as a biomarker of cyclosporine therapy, and INF-γ has shown to decrease in expression with drug therapy, although this result has been variable between studies.[56] It has therefore been suggested that measurement of INF-γ may not be specific enough for evaluating cyclosporine therapy.[56] Other cytokines that have been investigated as potential biomarkers for

cyclosporine therapy include IL-4, IL-6, TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-4.

Initial pharmacokinetic monitoring of blood drug concentrations was cumbersome and time-consuming, and therefore alternative methods of measuring cyclosporine's effect were explored. In 1998 van den Berg et al. developed a flow cytometry-based clinical assay to monitor the immunosuppressive effect of drugs in human transplant recipients.[58] The assay measured the functional effect of cyclosporine via intracytoplasmic IL-2 production. A negative correlation was found between IL-2 production and cyclosporine blood concentration, suggesting that the test could be used to assess cyclosporine's cellular effects. A later study in lung transplant recipients showed similar results, and the authors suggested that this technique would be more useful than monitoring blood drug concentrations, and could be used to better tailor immunosuppressive therapy to the individual patient.[59] Similar protocols have been explored in the canine patient, although this type of assay has yet to be offered as a send out test for general practitioners using immunosuppressive agents.[43,54,60] While flow cytometric assays have the capacity to detect specific cell populations and therefore are more specific for T cell function, samples must be processed the same day of collection, which can be challenging to accomplish if multiple samples are received throughout a working day. Another limitation to flow cytometry is that it involves a time-consuming protocol which, once started, must be completed the same day. Unfortunately flow cytometry is impractical for use in a clinical setting in which samples may get submitted at inconvenient times.

Quantitative reverse-transcriptase PCR (qRT-PCR) has also been explored for its potential use in pharmacodynamic monitoring of immunosuppressants. In 1993 Koutouby et al. developed a qRT-PCR based assay that, like flow cytometry assays, measured cyclosporine's effect via IL-2 expression.[61] The protocol utilized peripheral blood mononuclear cells, and found decreasing levels of IL-2 transcription with increasing levels of cyclosporine *in vitro*. While this technique does not directly measure the amount of IL-2 protein produced, mRNA levels are known to correlate to protein expression levels.[62,63] Although use of qRT-PCR can allow for samples to be stored post RNA collection, Koutouby's protocol took 24 hours to complete and therefore could not be efficiently used in a clinical setting. More recent advances in qRT-PCR made the technique faster, and protocols using whole blood sample were established, which shortened sample processing. Like flow cytometry, qRT-PCR has also been previously tested in dogs. One *in vitro* study showed that canine mononuclear cells had a concentration-dependent reduction in IL-2, IL-4, and IFN-γ mRNA expression when incubated with cyclosporine.[64] Another group demonstrated increased IL-2 and IFN- γ expression levels in tissue biopsies from dogs suffering from anal furunculosis.[65] Tissue IL-2 levels then dropped significantly after the initiation of cyclosporine therapy, the recommended treatment protocol for anal furunculosis.

Another approach to the pharmacodynamic monitoring of cyclosporine is to measure calcineurin enzyme activity. Calcineurin is an intracellular phosphatase that is inhibited by the CsA-cyclophilin complex in a concentration-dependent manner.[66] This inhibitory effect has been observed in whole blood, leukocytes, and peripheral blood mononuclear cells,[67-69] although whole blood samples are reported to be superior to the

others.[67] A number of assays have been developed, each of which has a different approach to quantifying calcineurin activity. Koefoed-Nielsen et al. developed an assay that estimates calcineurin activity by measuring its ability to dephosphorylate a radioactive peptide via liquid scintillation. Dealing with radioactive substances proved to be challenging, however, so more rapid and feasible methods were explored. Sellar et al. developed a spectrophotometric-based detection method, and although the convenience of whole blood sampling is lost, the protocol can be completed in one workday using standard laboratory equipment.[68]

Although a number of pharmacodynamic assays have been validated for use in human patients receiving cyclosporine, few such assays exist for veterinary patients, and most veterinarians rely on the measurement of blood drug concentrations to assess CsA efficacy. Lymphocyte proliferation[70] and qRT-PCR cytokine quantification[64] have been evaluated in dogs on topical or systemic cyclosporine, but as of yet no one has validated these assays for use in canine clinic patients receiving immunosuppressive doses of cyclosporine. As has been observed in humans, dogs have variable responses to cyclosporine therapy; therefore an assay that can accurately assess the drug's effect on the immune system would be helpful in tailoring immunosuppression therapy.

Immune-Mediated Diseases

Autoimmune diseases are the result of dysregulation of the body's immune system. This immune system is normally under tight regulation through a system of checks and balances involving a number of different cell types and inflammatory mediators. Dysregulation can occur as a result of a genetic predisposition, certain environmental factors, or both. A number of autoimmune diseases have been described in

veterinary medicine, and those in which cyclosporine is a recommended therapy will be further discussed, including immune-mediated hemolytic anemia (IMHA), immune-mediated thrombocytopenia (IMT), inflammatory bowel disease (IBD), atopic dermatitis, and anal furunculosis (AF).

Immune-Mediated Hemolytic Anemia (IMHA)

Immune-mediated hemolytic anemia (IMHA) is a type II hypersensitivity reaction and, in dogs, is one of the more common diseases of the immune system.[71,72] IMHA is characterized by antibody-mediated destruction of red blood cells, leading to all of the symptoms associated with anemia. The disease was first described in dogs in the 1960s[73] and, since then, much has been discovered concerning its etiology, clinical signs, diagnosis, and treatment. Yet despite our increased understanding of the condition, and the development of numerous therapeutic options, many patients with IMHA still have a relatively poor outcome.[71,72,74]

In the healthy animal, aged or damaged red blood cells are filtered out of the blood by the mononuclear phagocyte system (MPS).[71] The MPS contains both macrophages and monocytes dispersed in the blood and throughout tissues in the body, including the spleen, central nervous system, lymph nodes, and liver.[57] One of the functions of the MPS is to clear aging RBCs from the blood, which is accomplished by antibodies targeted to antigens present on the RBC membrane.[71] When a RBC reaches the end of its lifespan, its membrane becomes more rigid and fragile and eventually becomes coated with antibody.[75] The RBC is then targeted for ingestion by a macrophage either in circulation or in one of the organs of the MPS.[75] After ingestion by a macrophage, the

RBC is then broken down by lysosomal enzymes, so that iron and other cell components can be recycled by the body.[75]

In a dog suffering from IMHA, antibodies produced by the humoral immune system bind to antigens on RBCs of all ages, thereby targeting them for premature destruction by macrophages and, in some cases, also resulting in the activation of the complement cascade.[71,76] In some patients, RBC precursor cells of the bone marrow can be targeted for destruction as well.[71] Without an adequate number of functioning RBCs, oxygen cannot be adequately distributed throughout the body. RBC shortage has an impact on all of the body's systems, since oxygen is essential for every cell to function. One of the most common targets of anti-RBC autoantibodies is glycophorin, a transmembrane glycoprotein found on RBCs.[71] Antibodies can be of the IgG, IgM, or IgA isotype, and the specific isotype has been shown to be correlated to disease severity.[71] Since the IgM isotype is better at fixing complement, diseases associated with IgM tend to be more severe.[71]

There are two main types of IMHA, primary and secondary. In the more common primary or idiopathic IMHA, there is no underlying cause for the disorder.[71] Antibodies produced by the body target antigens on the RBC membrane. Secondary IMHA, in contrast, often results from an acquired immune response to a foreign antigen that is linked with a RBC membrane. Secondary IMHA can be caused by a variety of conditions, including infection, neoplastic disorders, and even the use of certain drugs.[71] Infections from viruses, bacteria, and protozoa have all been found to be associated with IMHA.[71] There is also thought to be an association between IMHA and vaccination, but a specific vaccine has yet to be identified, and the exact mechanism by which vaccination

causes IMHA has yet to be established.[71] There are also a few different forms of IMHA including peracute IMHA, acute IMHA, chronic IMHA, cold agglutinating IMHA, and pure red cell aplasia.[77] Acute IMHA is the most common form seen in dogs, while the chronic form tends to develop more in cats.[77]

Dogs of any age can develop IMHA, but typically symptoms emerge after the first year of life.[72,74] Usually anemia develops fairly quickly, and most dogs act lethargic, lose their appetite, and occasionally experience vomiting and diarrhea.[72] Upon physical examination, signs can include pale mucous membranes, jaundice, an enlarged spleen and liver, pigmented urine, fever, rapid breathing, and swollen lymph nodes.[71]

Certain breeds such as cocker spaniels, toy and miniature poodles, and old English sheepdogs are more susceptible to developing IMHA, suggesting a possible genetic component to the disease.[78] One of the main events in the development of the disease is when the MHC proteins recognize self or foreign proteins, therefore genes coding for MHC molecules are thought to be possible candidates for genetic predisposition to IMHA.[72] Females and castrated males have a higher frequency of IMHA[72,79] with the majority of cases occurring in middle-aged female dogs.[71]

Correct diagnosis of IMHA is essential for appropriate treatment, since a different therapeutic approach is taken depending on whether the patient is suffering from the primary or secondary type. The underlying cause needs to be addressed in secondary IMHA, while treatment for primary IMHA involves aggressive immunosuppressant therapy.[71] Typical hallmarks of IMHA include a positive Coomb's test, auto-agglutination, and spherocytosis.[77] To diagnose primary IMHA, slide agglutination or a direct Coomb's test are usually the first tools used, and show if anti-erythrocyte

antibodies are present.[72] Other diagnostic techniques for anti-RBC antibodies include flow cytometry, enzyme-linked immunosorbent assays, and a gel test.[72] With flow cytometry, one is able to detect and quantify IgG and IgM antibodies bound to RBCs.[77]

A variety of treatment options are available for patients diagnosed with IMHA, ranging from surgery (splenectomy) to immunosuppressant drug use. The ultimate goals of most therapies are to suppress antibody production and/or RBC lysis and opsonization.[76,80] Glucocorticoids are often the first drug of choice for IMHA treatment, in order to induce remission of the disease.[76] Prednisone, prednisolone and dexamethasone are the most common glucocorticoids used. Glucocorticoid mechanism of action includes suppression of the transcription of a number of pro-inflammatory cytokines, which helps to reduce the activity of macrophage RBC opsonization as well as the complement system.[76] Glucocorticoids also inhibit Fc (fragment crystallizable) receptors on the macrophage membrane, preventing the receptors from binding and opsonizing antibody-coated RBCs.[76] While glucocorticoids are often effective for inducing remission in IMHA patients, they are not ideal for long-term use to maintain remission due to the numerous side effects associated with their use.[76]

Once remission of IMHA has been induced, an immunosuppressant drug is usually prescribed to maintain remission and avoid relapse.[76] In severe cases, immunosuppressive agents can also be used during initial therapy. A number of agents have been used in IMHA treatment, including azathioprine, mycophenolate mofetil (MMF), leflunomide, and cyclosporine, each of which has a slightly different mechanism of action with their own advantages and disadvantages. Azathioprine inhibits purine synthesis, ultimately inhibiting cell activation and proliferation.[15] MMF also inhibits

purine synthesis via the *de novo* pathway, resulting in the suppression of T and B cell activity.[15] Leflunomide targets T cells by inhibiting pyrimidine synthesis and decreasing DNA and RNA synthesis.[15] Cyclosporine, as described previously, suppresses T cell NFAT-regulated cytokine production via inhibition of calcineurin.[55] Azathioprine and cyclosporine are the immunosuppressive agents most commonly used to treat canine IMHA.[71]

Human intravenous immunoglobulin (hIVIG) has also been used to treat IMHA. Like glucocorticoids, hIVIG works by reducing macrophage phagocytosis as well as complement activation.[76] HIVIG is typically used to treat severe cases of IMHA, given its rapid onset and effectiveness.[76]

When a dog is suffering from severe IMHA, and is not responding well to immunosuppressive therapy, another option to consider is splenectomy.[76] The spleen is responsible for filtering the blood, but also functions as a site for antibody production, and as a key organ in the MPS system that phagocytoses RBCs. Since the spleen is not essential for survival, splenectomy is a feasible option to induce and maintain IMHA remission.[76] However, in dogs in which IMHA is mediated by IgM autoantibodies, it less likely to be effective.[76]

Even though there are a number of therapeutic options available to treat IMHA patients, the survival rate is still relatively poor, with only 50% of patients surviving.[71] Most patients that do not survive die within the first two weeks after diagnosis.[72] Usually death results from hypercoagulable blood resulting in pulmonary thromboembolism or disseminated intravascular coagulation.[71] However, dogs that survive this initial phase typically have a good outcome in the long run.[76] While our understanding of IMHA has

improved and a number of treatment options have been developed, new therapies that target severe cases and more research is needed to improve patient survival.

Immune Mediated Thrombocytopenia (IMT)

Immune-mediated thrombocytopenia, often also referred to as idiopathic thrombocytopenic purpura (ITP), is an autoimmune disorder in which the body's defense system produces antibodies against platelet antigens, targeting platelets for destruction. This leads to a low platelet count, predisposing to excessive bleeding and, ultimately, death if not properly treated. IMT can be idiopathic,[81] or it can be triggered by a number of factors including parasites[82], neoplasia[83], bacterial or viral infection[84], or medications.[85] In idiopathic or primary IMT, disease begins with the attachment of autoantibodies to platelet surface antigens, or deposition of immune complexes on the platelet exterior.[86] In particular, the surface antigens GP (glycoprotein) IIb /IIIa or Ib/IX complexes have been found to be targets for autoantibodies in the dog.[87,88] T cells are largely responsible for ensuring the immune system doesn't attack self cells, and some studies have found either abnormal numbers or function of T cells in patients with IMT.[89-91] Gender, genetics, and environmental factors can all contribute to the pathogenesis of the disease.[92] Once bound by autoantibodies, the platelets are then targeted for destruction by macrophages in organs such as the spleen. The macrophages recognize the Fc receptor of the antibody bound to the platelet, and then destroy the platelet via phagocytosis.

IMT can be diagnosed in dogs of any age, but the condition has a predilection for females and certain breeds, including cocker spaniels, poodles, German shepherds and old English sheepdogs.[92] Typical presenting patient complaints include mucosal and

cutaneous hemorrhage, lethargy, reduced appetite, bloody diarrhea, and bruising.[92] Diagnosis involves attaining an accurate platelet count and blood smear evaluation. Antiplatelet autoantibody assays have been developed for the dog and can help facilitate a diagnosis. These assays can either detect antibodies in the serum that have the ability to bind platelets, or antibodies directly bound to the platelet surface. Assays for platelet-bound antibody are done via flow cytometry and have a high sensitivity, although they are prone to false positive results.[92,93] While a number of tests are available to aid in the diagnosis of IMT, typically it is a diagnosis of exclusion after ruling out all other possible causes of thrombocytopenia.

A number of therapeutic options are available to treat IMT. Glucocorticoids are the mainstay of most therapeutic regimens. Recovery is primarily due to inhibition of antibody-induced phagocytosis by macrophages.[94] Glucocorticoids specifically work by impairing Fc gamma receptor function, which leads to reduced binding of IgG coated platelets and reduced platelet destruction.[94] In dogs that don't respond well to steroid therapy, additional immunosuppressive drugs are then added. Medications can include vincristine, cyclophosphamide, azathioprine, MMF, danazol, cyclosporine, and hIVIG. Vincristine has been shown to be very efficacious in the treatment of IMT. Vincristine not only increases platelet numbers through stimulation of megakaryocyte breakdown, but also reduces platelet phagocytosis.[92,95] Splenectomy is another therapeutic option. During treatment, supportive care is very important and while, transfusions are not commonly required, in the event of uncontrolled hemorrhage, transfusion may be necessary.[92] The goal of treatment is to maintain a normal platelet count within the range of 60,000-200,000 platelets/µl after removal of all drugs.[92]

Atopic Dermatitis

Canine atopic dermatitis has been diagnosed since the early 1930s.[96] Atopic dermatitis is a type I hypersensitivity reaction involving IgE or IgG antibody.[77] While atopic dermatitis was first thought to be the result of inhaled allergens, years of research has shown it to be a complex, multifactorial disease in which genetic predisposition and IgE antibodies generated against environmental antigens are typical.[96] Approximately 10% of dogs are affected, with certain breeds such as Chinese Shar-Peis, Boxers, Dalmatians, Boston terriers, Labrador retrievers, and golden retrievers most commonly diagnosed.[77]

Although atopic dermatitis has been around for quite some time, it wasn't until recently that a wide scale study on its diagnosis was published.[97] Pruritus is the classical sign of atopy, typically affecting the ears, feet, face, or ventral abdomen,[77] although its presentation can differ depending on breed.[96] Pathogenesis involves a number of immune cells, with mast cells playing a key role. When exposed to an allergen, IgE binding to Fc receptors on mast cells leads to the release of preformed mediators such as histamine and cytokines, initiating an inflammatory response.[57] Mast cells tend to congregate in the skin around ears and paws, therefore it's no coincidence that these sites are the most commonly affected.[96] Increased numbers of both T helper and T cytotoxic cells have been found in atopic skin lesions.[98] Eventually the region is infiltrated by lymphocytes, dominated primarily by T_H1 cells, with progression to chronic inflammation.[96] IFN-γ, the predominant T_H1 cytokine, has been shown to be increased in these lesions.[99] Given the role that T cells play in the pathogenesis of atopic dermatitis, therapies targeting this component of the immune system, such as cyclosporine, should be relatively effective in

its treatment. The skin barrier itself is also an important component of the pathogenesis of the disease. In the normal healthy dog, the stratum corneum layer of the skin acts as a barrier against environmental allergens and transepidermal water loss. Studies have shown that atopic dogs have lower ceramide and epidermal lipid production[100,101], increased transepidermal water loss[100], and decreased filaggrin (a protein that plays a pivotal role in skin keratinization) production[102]. All of these contribute to reduced barrier function of the skin, allowing allergens to penetrate and generate an inflammatory response.

Diagnosis of atopic dermatitis is largely based on history and clinical signs and, like IMT and IMHA, atopy is also a diagnosis of exclusion.[96] Atopic dermatitis has a fairly high prevalence within the canine community, with approximately 10% of dogs suffering from the disease.[103] In his 2010 paper, Favrot listed certain criteria that should be met for a diagnosis of atopy, such as clinical signs that begin to occur before the dog reaches 3 years of age, pruritus that responds to glucocorticoids, involvement of feet and ears, and pruritus that occurs before the onset of skin lesions.[97] While these criteria can aid in the diagnosis, they are not all inclusive. Given the fact that certain breeds are more commonly affected than others, a genetic component to the disease is highly likely. Microarray analysis has shown differential gene expression in healthy dogs versus dogs with atopy, particularly those involved in skin barrier function, immune response, gene transcription, and programmed cell death.[104] Allergen testing is also an important component of diagnosis. Identifying potential allergens and subsequent avoidance can be quite helpful in controlling the disease in the long run.

Canine atopy is a lifelong disease, and typically requires lifelong treatment. The first line of defense is to determine the causative agent for flare ups, and to avoid future interaction.[105] Acute flare ups of atopic dermatitis are typically initially treated with topical glucocorticoids like hydrocortisone, or oral glucocorticoids.[105] Additional immunosuppressive agents like tacrolimus or cyclosporine are indicated in more chronic cases.[105] Cyclosporine was first approved for use in canine atopic dermatitis in 2002, and has been shown to be quite effective.[106-110] Other potential therapeutic options include oclacitinib maleate, interferons, commercial canine atopy diets, and antihistamines.[111]

Anal Furunculosis (AF)

Anal furunculosis (AF) is a disease characterized by chronic inflammatory and ulcerative lesions forming around the anus of the dog. Dogs suffering from the disease typically display difficulty defecating, painful ulceration around the anus, purulent sinus tracts, and reduced appetite.[77] Older dogs and those with a broad-based tail are at a greater risk of developing AF.[77] Anal furunculosis is similar to perianal Crohn's disease in man, and primarily affects German shepherds.[112] Other breeds that have been diagnosed with AF include Irish setters, Labrador retrievers, Old English sheepdogs, Jack Russell terriers, border collies, and English bulldogs, as well as various mixed breeds.[113,114]

In one study consisting of over 300 dogs, approximately 80% of AF cases were German shepherds,[113] which highly suggests a genetic component to the disease. Much work has gone into investigating potential genes involved in the pathogenesis of AF. Due to the fact that T cell specific cytokines have been shown to be increased in AF lesions[115] and the fact that the disease is highly responsive to the immunosuppressive agent

cyclosporine, its highly likely that the immune system plays a role in the pathogenesis of AF, especially CD4+ T cells.[116] T cells are activated once presented with antigen complexed with MHC class II. Investigations into the link between canine MHC genes and AF have found there to be a five-fold greater risk of developing AF in German shepherds carrying the DLA-DRB1*00101 allele.[116] However this allele is present in other breeds, and therefore cannot solely explain the development of disease.[116] In addition to MHC genes, researchers have also looked into pattern recognition receptors (PRRs), particularly toll-like receptors, of German shepherds, and their potential link to AF. Although multiple single nucleotide polymorphisms (SNPs) were found, the researchers concluded that PRRs are not implicated in AF pathogenesis.[117] Work regarding matrix metalloproteinase (MMP) expression has found there to be increased levels of MMP-9 and MMP-13 in AF lesions, implicating macrophages in its pathology.[118] Macrophage activity has been hypothesized to be due to IFN-γ production from T helper cells.[118] Expression of IFN-γ and IL-2 expression, both of which are primarily T_H1 cytokines, is known to be increased in AF lesions.[115] Although cytokine expression does not correlate with lesion severity, IL-2 has been shown to decrease with the use of cyclosporine.[65] In addition to the immune system genetic susceptibility of German shepherds, an anatomical predisposition has also been linked to AF. Budberg et al. found an increased density of apocrine sweat glands in dogs predisposed to developing AF.[119] While an immune component of the disease has been established, the pathogenesis of the AF likely involves multiple genes as well as an environmental component. The pathogenesis of AF is still not completely understood and much work still needs to be

done to have a better understanding of disease development, however investigation into its treatment appears to be much more productive.

Prior to the use of cyclosporine, AF management was frustrating and difficult for practitioners, and often included the use of surgery to either remove affected tissue or amputate the tail.[77] While there is evidence suggesting the beneficial use of immunosuppressive doses of prednisone,[120] cyclosporine appears to be the superior treatment. Cyclosporine has been shown to be very effective in treating AF.[114,121-123] Cyclosporine inhibits the activity of T helper cells, without affecting T suppressor cell function, reducing inflammation and allowing lesions to heal.[124] The cost of cyclosporine often inhibits its long-term use in patients, however using it in combination with ketoconazole slows its metabolism and prolongs its duration of effect, ultimately leading to lower costs for the owner and making its use a more feasible option.[114] Other immunosuppressive agents, such as tacrolimus and azathioprine, have also been shown to be effective therapeutic options for AF.[125,126]

Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) consists of a group of gastrointestinal diseases categorized based on location in the body and principal cell type.[77] Dogs are mainly affected by lymphocytic-plasmocytic enteritis (LPE), lymphocytic-plasmocytic colitis (LPC), and eosinophilic gastroenteritis (EGE) types, named according to predominant cell types.[127] There are also a number of specific IBD forms that commonly occur in certain breeds, like Boxers, Basenjis, Norwegian Lundehunds, Irish Setters and soft-coated Wheaten terriers. The entire gastrointestinal tract can be affected, but the proximal small intestine is the most commonly affected site in dogs.[128] While the cause of IBD is

unknown, a number of predisposing factors can help initiate or exacerbate disease, such as genetics, environment, infectious agents, parasites, dietary allergens, and drug-induced disease. Loss of mucosal tolerance to endogenous bacteria is known to be a crucial factor leading to the development of chronic inflammation in the gastrointestinal tract.[128] Studies with mice and humans have shown that problems with either the mucosal barrier, mucosal immune system, or endogenous microflora can lead to chronic stimulation of the immune system, ultimately leading to pathologic change and disease.[128] There is likely a similar pathogenesis in dogs.

Diagnosis of IBD can be a difficult undertaking, since one must rule out all other potential causes of the gastrointestinal symptoms, however a number of tools are available to diagnose and monitor IBD. Definitive diagnosis requires biopsy of the intestinal tract with a histological diagnosis provided by a pathologist. While in human medicine the most commonly used technique for monitoring is clinical scoring indices,[129] specifically the Crohn disease activity index,[130] in canine patients veterinarians most commonly use subjective assessment of clinical signs to determine treatment course efectiveness.[131] To date, two scoring indices have been developed to help manage canine IBD patients, the clinical IBD activity index (CIBDAI) and the canine chronic enteropathy clinical activity index (CCECAI). The CIBDAI evaluates six separate gastrointestinal signs including attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss. Each is scored on a range of 0-3, and then added together to give a final score. which is then used to classify the disease based on severity.[132] The CCECAI assesses serum albumin, ascites, peripheral edema, and pruritus, in addition to the previously mentioned signs, again scoring them from 0-3.[133]

This scoring system has shown to be useful in predicting negative outcome.[133] While these scoring systems have their use, most of the parameters are subjectively evaluated, and are therefore prone to certain errors. Abdominal ultrasound has been studied for IBD diagnosis, but in dogs evaluating intestinal wall thickness does not appear to be a useful management tool.[131] In human patients, a number of serological markers have been studied and proven useful in IBD management, therefore clinicians have searched for similar markers in canine patients. Some of the markers currently under investigation include C-reactive protein, albumin, cobalamin, folate, and perinuclear antineutrophilic cytoplasmic antibodies.[131] C-reactive protein (CRP) is an acute phase protein synthesized by the liver, and plasma levels of CRP are increased during inflammation. Measurement of CRP can be quite informative in human patients, since levels have been shown to be associated with disease activity, histologic inflammation, and even therapeutic efficacy.[134-137] Serum CRP levels have been shown to be increased in dogs with IBD,[132,138] but one study did not find a correlation between CRP levels and disease severity or histopathologic grade.[138] Although CRP increases in response to any inflammation and is not specifically associated with the gastrointestinal tract, measuring CRP can be useful in gauging response to therapy after an IBD diagnosis has been made.[131] Albumin can also be a useful diagnostic marker in IBD cases. Measurement of albumin is best used as a predictor of negative outcome, since dogs with a severe form of the disease will tend to lose protein through the gut.[133] While biomarkers of IBD have their use, none stand alone to establish an accurate diagnosis. Biomarkers are mainly helpful indicators of prognosis or treatment effectiveness.

The gold standard for IBD diagnosis still continues to be intestinal biopsy histopathology, collected via endoscopy or laparotomy, to determine degree and type of inflammation.[139] Although collection of biopsies can be an invasive and expensive procedure, histopathology is useful in determining degree of inflammation and predominant cell type, which aids in the diagnosis and development of treatment plans. While endoscopy and histopathology has been the gold standard for a number of years, it wasn't until recently that a set of standards on morphologic change and inflammation evaluation was published. Again, a scoring system is used, which then correlates to severity of pathologic change in the sample.[140] However, to date, this scoring system has not been validated, and a couple of studies have shown that histopathologic improvement does not always correlate with clinical improvement.[133,141]

A number of treatment options are available for IBD, and like the previously discussed diseases, clinicians usually begin with the use of glucocorticoids, progressing to other immunosuppressive agents as severity of the disease increases. In addition to pharmacologic manipulation, dietary changes, antibiotics and probiotics are also added to the treatment regime. In mild to moderate cases of IBD, dietary modification may be the only change needed, since the reaction may be due to antigens in the food.[142] However, this is more commonly the case in cats rather than dogs.[143] Suggested diets typically contain a single protein source, and carbohydrates and proteins that the animal has not yet been exposed to.[143] The addition of omega fatty acids can help to alleviate intestinal inflammation, and use of glutamine will aid in maintaining villous integrity.[143] If dietary change alone is not effective, the addition of probiotics may provide some benefit, but again the combination of glucocorticoids and probiotics is only recommended for milder

cases. Probiotics alter inflammatory cytokine production,[144] reduce pathogen growth through production of antimicrobial metabolites,[145,146] and stimulate phagocyte activity.[145] Another option is to use an immunomodulatory agent, with β-glucans being the agent most commonly used.[143] β-glucans are a component of the cell wall of most fungal species and, in IBD cases, they function by acting as antigens, which stimulates both an innate and adaptive immune response, eventually leading to its suppression.[147] However, this type of therapy should only be reserved for milder cases, since human studies have shown gastrointestinal disease to worsen with the use β-glucans in more severe cases.[143] Topical nonsteroidal anti-inflammatory drugs (NSAIDs) like sulfasalazine are sometimes used in IBD cases restricted to the large intestine. Other NSAIDs that can also be used include olsalazine and mesalamine.[77] These agents act as an anti-inflammatory drugs through the suppression of inflammatory cytokines, prostaglandins, and leukotrienes, and are helpful for treating mild to moderate cases.[143]

While the previously mentioned therapies have their uses, the most effective treatment for IBD is immunosuppressive drug therapy. Glucocorticoids are the most common agent used for both large and small intestine IBD cases.[77] Prednisolone is the drug most often chosen by practitioners, and treatment is usually long-term.[143] When glucocorticoids fail or the side effects become too much to handle, the next step is often addition of another immunosuppressive agent, such as azathioprine, chlorambucil, or cyclosporine. In humans, cyclosporine is used in steroid-refractory cases of Crohn's disease as a last resort before colectomy, and functions by regulating pro-inflammatory cytokine production.[148] Cyclosporine probably works similarly in canine IBD patients. One study in IBD dogs treated with cyclosporine found significant improvement in

clinical signs, reduced clinical activity score, and reduced T cell populations in intestinal biopsies, and concluded that cyclosporine is an effective treatment for steroid refractory cases.[149] Since it is not advisable for patients to be on immunosuppressants for life, especially high doses that may be associated with significant side effects, once disease remission has been maintained for a certain period of time tapering of drug therapy is recommended. However, if a relapse occurs, immunosuppressant therapy should be immediately reinstated until clinical signs are under control.[143] Although a number of options are available for treatment of IBD, the overarching goal of any therapy is to decrease gastrointestinal signs and inflammation, and to promote weight gain as well as a healthy appetite.

Project Summary

Cyclosporine is used in veterinary medicine to treat an increasing number of immune-mediated conditions, although the only disease cyclosporine is currently approved for is atopic dermatitis in dogs and cats. Despite its widespread use, firm oral dosing protocols have yet to be established in the dog for the range of other diseases that the drug is used to treat, such as IMHA, IMT, IBD, and anal furunculosis. Currently, a pharmacokinetic assay is the primary laboratory means of assessing cyclosporine efficacy, through measurement of blood drug concentrations. The blood drug concentration is assumed to correlate to level of immunosuppression, however there is considerable debate regarding this assumption, given that individuals can vary in response to the same drug concentration. Our research group believes that, by taking a pharmacodynamic approach to monitoring the effect of cyclosporine on the immune system, we can better measure the drug's impact, and develop more individualized drug

regimens. Using this approach, the immune system's response to the drug will be measured, rather than how the body is processing the drug. To address this issue, our laboratory has evaluated the immunosuppressive effects of CsA on its target T cells, and has developed a pharmacodynamic qRT-PCR based assay for use in dogs that allows adjustment of drug dosage based on immunosuppressive effect.

Our laboratory has done some previous work in this field, which set the foundation for the development of our current assay. Our initial study looked at activated T cell expression of a number of cytokines, including IL-2, IFN- γ, and IL-4, using flow cytometry.[54] Peripheral blood mononuclear cells (PBMC) were incubated with cyclosporine, and a decrease in cytokine expression in a concentration- and time-dependent manner was observed. Surface markers CD25 and CD95 were also evaluated, and were also suppressed with the addition of cyclosporine. Next, an *in vivo* study was performed in healthy Walker hounds given a high and low dose of cyclosporine.[150] The same cytokines were studied via flow cytometry. Similar results to the previous study were found for IL-2 and IFN-γ, but not IL-4. These results suggested that IL-2 and IFN-γ could be used as potential biomarkers for monitoring cyclosporine's effectiveness. There was also some preliminary work done using qRT-PCR instead of flow cytometry, which showed the two assays to be comparable methods for measuring the effects of cyclosporine on T cells.

The overarching goal of our research was to develop a diagnostic tool that can reliably predict outcome when a canine patient is on a cyclosporine treatment regime. Clinicians would then be able to use the assay to allow more efficient and effective treatment of canine patients. The main objective for this thesis was to develop and

evaluate a robust qRT-PCR assay that could be used to accurately assess cyclosporine's effect on the canine immune system. We hypothesized that T cell cytokine production would be a reflection of the immunosuppressive effects of cyclosporine, and that measurement of cytokine expression could be used to determine the effectiveness of cyclosporine therapy. To address this, we first validated a qRT-PCR assay for measuring activated T cell cytokine production, specifically IL-2 and IFN-γ, for use in dogs. Next, the assay was tested with clinical patients with conditions such as IMHA, IMT, IBD, atopic dermatitis and anal furunculosis, in order to establish pre-treatment cytokine levels and the ideal timing of sample collection post drug therapy. The project concluded with an evaluation of the relationship between IL-2 and IFN-γ expression and clinical outcome, in order to determine how well the assay works in a clinical context.

References

1. Heusler K, Pletscher A. The controversial early history of cyclosporin. Swiss Medicine Weekly 2001;131:299-302.

2. Borel JF, Kis ZL, Beveridge T. The Search for Anti-Inflammatory Drugs. Birkhäuser Boston; 1995;27-63.

3. Bushley KE, Raja R, Jaiswal P, Cumbie JS, Nnogaki M, Boyd AE, Owensby CA, Knaus BJ, Elser J, Miller D, Di Y, McPhail KL, Spatafora JW. The genome of *Tolypocladium inflatum*: Evolution, Organization, and Expression of the Cyclosporin Biosynthetic Gene Cluster. PLoS Genetics 2013;9.

4. Borel JF, Feurer C, Gubler HU, Stähelin H. Biological effects of cyclosporin A: A new antilymphocytic agent. Agents and Actions 1976;6:458-475.

5. Borel JF. History of the discovery of cyclosporin and of its early pharmacological development. Wiener klinische Wochenschrift 2002;114:433-437.

6. Calne RY, Rolles K, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Henderson RG, Aziz S, Lewis P. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. Lancet 1979;2:1033-1036.

7. Starzl TE, Klintmalm GB, Weil R, Porter KA, Iwatsuki S, Schroter GP, Fernandez-Bueno C, MacHugh N . Cyclosporin A and steroid therapy in sixty-six cadaver kidney recipients. Surgery, Gynecology, and Obstetrics 1981;153:486-494.

8. Stähelin HF. The history of cyclosporin A (Sandimmune) revisited: Another point of view. Experientia 1996;52:5-13.

9. Wenger R. Synthesis of cyclosporine. Total syntheses of 'cyclosporin A' and 'cyclosporin h', two fungal metabolites isolated from the species Tolypocladium inflatum GAMS. Helvetica Chimica Acta 1984;67:502-525.

10. Ptachcinski RJ, Venkataramanan R, Burckart GJ. Clinical pharmacokinetics of cyclosporin. Clinical Pharmacokinetics 1986;11:107-132.

11. Kovarik JM, Mueller EA, van Bree JB, Tetzloff W, Kutz K. Reduced inter- and intraindividual variability in cyclosporine pharmacokinetics from a microemulsion formulation. Journal of Pharmaceutical Sciences 1994;83:444-446.

12. Archer TM, Boothe DM, Langston VC, Fellman CL, Lunsford KV, Mackin AJ. Oral cyclosporine treatment in dogs: A review of the literature. Journal of Veterinary Internal Medicine 2014;28:1-20.

13. Kovarik J, Mueller EA, Niese D. Clinical development of a cyclosporine microemulsion in transplantation. Therapeutic Drug Monitoring 1996;18:429-434.

14. Mueller EA, Kovarik JM, van Bree JB, Tetzloff W, Grevel J, Kutz K. Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation. Pharmaceutical Research 1994;11:301-304.

15. Plumb DC. Plumb's Veterinary Drug Handbook 6th ed. PharmaVet Inc.; 2008;5.

16. Tedesco D, Haragsim L. Cyclosporine: a review. Journal of Transplantation 2012:230386.

17. Lown KS, Mayo RR, Leichtman AB, Hsaio HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, Watkins PB. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. Clinical Pharmacology and Therapeutics 1997;62:248-260.

18. Mealey KL, Waiting D, Raunig DL, Schmidt KR, Nelson FR. Oral bioavailability of P-glycoprotein substrate drugs do not differ between ABCB1-1Delta and ABCB1 wild type dogs. Journal of Veterinary Pharmacology and Therapeutics 33:453-460.

19. Robson D. Review of the pharmacokinetics, interactions and adverse reactions of cyclosporine in people, dogs and cats. The Veterinary Record 2003;152:739-748.

20. Whalen RD, Tata PN, Burckart GJ, Venkataramanan R. Species differences in the hepatic and intestinal metabolism of cyclosporine. Xenobiotica 1999;29:3-9.

21. Dahlinger J, Gregory C, Bea J. Effect of ketoconazole on cyclosporine dose in healthy dogs. Veterinary Surgery 1998;27:64-68.

22. Brigl M, Brenner MB. CD1: Antigen presentation and T cell function. Annual Review of Immunology 2004;22:817-890.

23. Jiang H, Chess, L. An integrated view of suppressor T cell subsets in immunoregulation. Journal of Clinical Investigation 2004;114:1198-1208

24. Luo L, Sun Z, Cheng H, Luo G. Memory T-cell-specific therapeutics attenuate allograft rejection via mediation of alloreactivity in memory cells. Immunology Letters 2012;148:53-58.

25. Moss RB, Moll T, El-Kalay M, Kohne C, Soo Hoo W, Encinas J, Carlo DJ. Th1/Th2 cells in inflammatory disease states: Therapeutic implications. Expert Opinion on Biological Therapy 2004;4:1887-1896.

26. Marks F, Klingmuller U, Muller-Decker K. Cellular Signal Processing. New York: Garland Science; 2009.

27. Aicher L, Wahl D, Arce A, Grenet O, Steiner S. New insights into cyclosporine A nephrotoxicity by proteome analysis. Electrophoresis 1998;19:1998-2003.

28. Durnian J, Stewart RMK, Tatham R, Batterbury M, Kaye SB. Cyclosporin-A associated malignancy. Clinical Ophthamology 2007;1:421-430.

29. Kutkuhn B, Hollenbeck M, Heering P, Koch M, Voiculescu A, Reinhard T, Grabensee B. Development of insulin resistance and elevated blood pressure during therapy with cyclosporine A. Blood Pressure 1997;6:13-17.

30. Charnick SB, Nedelman JR, Chang CT, Hwang DS, Jin J, Moore MA, Wong R, Meligeni J. Description of blood pressure changes in patients beginning cyclosporin A therapy. Therapeutic Drug Monitoring 1997;19:17-24.

31. Donatsch P, Abisch E, Homberger M, Trabar R, Trapp M, Voges R. A radioimmunoassay to measure cyclosporin A in plasma and serum samples. Journal of Immunoassay 1981;2:19-31.

32. Bogusz MJ, Enazi EA, Hassan H, Abdel-Jawaad J, Ruwaily JA, Tufail MA. Simultaneous LC-MS-MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure. Journal of Chromatography 2007;850:471-480.

33. Sadilkova K, Busby B, Dickerson JA, Rutledge JC, Jack RM. Clinical validation and implementation of a multiplexed immunosuppressant assay in dried blood spots by LC-MS/MS. Clinical Chimica Acta 2013;421:152-156.

34. Bouquie R, Deslandes G, Renaud C, Daily E, Jolliet P. Validation and application of a fast semi-automated whole blood extraction for LC-MS/MS simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus - application to high throughput routine therapeutic drug monitoring. Analytical Methods 2013;5:5079-5088.

35. Koster RA, Dijkers ECF, Uges DRA. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood. Therapeutic Drug Monitoring 2009;31:116-125.

36. Critterio F. Adavances in cyclosporine therapy: therapeutic drug monitoring of cyclosporine: Evolution of the therapeutic drug monitoring of cyclosporine. Transplantation Proceedings 2004;36:S420-S425.

37. Kahan BD, Grevel J. Optimization of cyclosporine therapy in renal transplantation by pharmacokinetic strategy. Transplantation 1988;46:631-644.

38. Amante AJ, Kahan BD. Abbreviated AUC strategy for monitoring cyclosporine microemulsion therapy in the immediate posttransplant period. Transplantation Proceedings 1996;28:2162-2163.

39. Perner, F. Cyclosporine microemulsion (NeoralA) absorption profiling and sparse-sample predictors during the first 3 months after renal transplantation. American Journal of Transplantation 2002;2:148-156.

40. Cantarovich M, Barkun J, Besner JG, Metrakos P, Alpert E, Deschenes M, Aalamian Z, Tchervenkov JI. Cyclosporine peak levels provide a better correlation with the area-under-the-curve than trough levels in liver transplant patients treated with neoral. Transplantation Proceedings 1998;30:1462-1463.

41. Cantarovich M, Besner JG, Barkun JS, Elstein E, Loertscher R. Two-hour cyclosporine level determination is the appropriate tool to monitor Neoral therapy. Clinical Transplantion 1998;12:243-249.

42. Guaguere E, Steffan J, Olivry T. Cyclosporin A: a new drug in the field of canine dermatology. Veterinary Dermatology 2004;15:61-74.

43. Fellman C, Archer TM, Stokes JV, Wills RW, Lunsford KV, Mackin AJ. Effects of oral cyclosporine on canine T-cell expression of IL-2 and IFN-gamma across a 12-h dosing interval. Journal of Veterinary Pharmacology and Therapeutics 2016;39:237-244.

44. Mathews KA, Holmberg DL. Kidney transplantation in dogs with naturally occurring end-stage renal disease. Journal of the American Animal Hospital Association 2000;36:475.

45. Billing H, Giese T, Sommerer C, Zeier M, Feneburg R, Meuer S, Tönshoff B. Pharmacodynamic monitoring of cyclosporine A by NFAT-regulated gene expression and the relationship with infectious complications in pediatric renal transplant recipients. Pediatric Transplantation 2010;14:844-851.

46. Kuzuya T, Kobayashi T, Katayama A, Nagasaka T, Miwa Y, Uchida K, Nakao A, Yamada K. Evaluation of interleukin-2 mRNA in whole blood as a parameter for monitoring cyclosporine pharmacodynamics. Biological and Pharmaceutical Bulletin 2009;32:604-608.

47. Kurata Y, Kuzuya T, Miwa Y, Iwasaki K, Haneda M, Amioka K, Yamada K, Watarai Y, Katayama A, Uchida K, Kobayashi T. Clinical relevance of post-transplant pharmacodynamic analysis of cyclosporine in renal transplantation. International Immunopharmacology 2014;22:384-391.

48. Karamperis N, Koefoed-Nielsen PB, Brahe P, Højskov C, Egfjord M, Poulsen JH, Jørgensen KH. Correlations between calcineurin phosphatase inihibition and cyclosporine metabolites concentrations in kidney tranplant recipients: implications for immunoassays. Basic & Clinical Pharmacology and Toxicology 2006;98:569-574.

49. Dieterlen MT, Eberhardt K, Tarnok A, Bittner HB, Barten MJ. Flow cytometry-based pharmacodynamic monitoring after organ transplantation. Methods in Cell Biology 2011;103:267-284.

50. Boleslawski E, Conti F, Sanquer S, Podevin P, Chouzenoux S, Batteux F, Houssin D, Weill B, Calmus Y. Defective inhibition of peripheral CD8+ T cell IL-2 production by anti-calcineurin drugs during acute liver allograft rejection. Transplantation 2004;77:1815-1820.

51. Whittington R, Faulds D. Interleukin-2. Drugs 1993;46:446-514.

52. Randak C, Brabletz T, Hergenröther M, Sobotta I, Serfling E. Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. The EMBO Journal 1990;9:2529-2536.

53. Nishiyama S, Manabe N, Kubota Y, Ohnishi H, Kitanaka A, Tokuda M, Taminato T, Ishida T, Takahara J, Tanaka T. Cyclosporin A inhibits the early phase of NF-kappaB/RelA activation induced by CD28 costimulatory signaling to reduce IL-2 expression in human peripheral T cells. International Immunopharmacology 2005;5:699-710.

54. Fellman CL, Stokes JV, Archer TM, Pinchuk LM, Lunsford KV, Mackin AJ. Cyclosporine A affects the in vitro expression of T cell activation-related molecules and cytokines in dogs. Veterinary Immunology and Immunopathology 2011;140:175-80

55. Stein CM, Murray JJ, Wood AJ. Inhibition of stimulated interleukin-2 production in whole blood: a practical measure of cyclosporine effect. Clinical Chemistry 1999;45:1477-1484.

56. Awni WM. Pharmacodynamic monitoring of cyclosporin. Clinical Pharmacokinetics 1992;23:428-448.

57. Vinay K, Fausto N, Aster JC. Robbins and Cotran Pathologic Basis of Disease, 8th ed. Philadelphia: Saunders; 2010.

58. van den Berg AP, Twilhaar WN, van Son WJ, van der Bij W, Klompmaker IJ, Slooff MJ, The TH, de Leij LH. Quantification of immunosuppression by flow cytometric measurement of intracellular cytokine synthesis. Transplant International 1998;11 Suppl 1:S318-321.

59. Hodge G, Hodge S, Reynolds P, Holmes M. Intracellular cytokines in blood T cells in lung transplant patients--a more relevant indicator of immunosuppression than drug levels. Clinical and Experimental Immunology 2005;139:159-164.

60. Nafe L, Dodam JR, Carol RR. In-vitro immunosuppression of canine T-lymphocyte-specific proliferation with dexamethasone, cyclosporine, and the active metabolites of azathioprine and leflunomide in a flow-cytometric assay. Canadian Journal of Veterinary Research 2014;78:168-175.

61. Koutouby R, Zucker C, Zucker K, Burke G, Nery J, Roth D, Esquenazi V, Miller J. Molecular monitoring of the immunosuppressive effects of cyclosporine in renal transplant patients by using a quantitative polymerase chain reaction. Human Immunology 1993;36:227-234.

62. Barten MJ, Tarnok A, Garbade J, Bittner HB, Dhein S, Mohr FW, Gummert JF. Pharmacodynamics of T-cell function for monitoring immunosuppression. Cell Proliferation 2007;40:50-63.

63. Giese T, Zeier M, Meuer S. Analysis of NFAT-regulated gene expression in vivo: A novel perspective for optimal individualized doses of calcineurin inhibitors. Nephrology, Dialysis, Transplantation 2004;19:55-60.

64. Kobayashi T, Momoi Y, Iwasaki T. Cyclosporine A inhibits the mRNA expressions of IL-2, IL-4 and IFN-gamma, but not TNF-alpha, in canine mononuclear cells. Journal of Veterinary Medical Science 2007;69:887-892.

65. Tivers MS, Catchpole B, Gregory SP, House AK. Interleukin-2 and interferon-gamma mRNA expression in canine anal furunculosis lesions and the effect of ciclosporin therapy. Veterinary Immunology and Immunopathology 2008;125:31-36.

66. van Rossum HH, de Fijiter JW, van Pelt J. Pharmacodynamic monitoring of calcineurin inhibition therapy: Principles, performance, and perspectives. Therapeutic Drug Monitoring 2010;32:3-10.

67. Koefoed-Nielsen PB, Karamperis N, Jørgensen KA. Validation of the calcineurin phosphatase assay. Clinical Chemistry 2004;50:2331-2337.

68. Sellar KJ, van Rossu, HH, Romijn FM, Smit NM, de Fijiter JW, van Pelt J. Spectrophotometric assay for calcineurin activity in leukocytes isolated from human blood. Analytical Biochemistry 2006;358:104-110.

69. Millán O, Brunet M, Campistol JM, Faura A, Rojo I, Vidal E, Jiménez O, Vives J, Oppenheimer F, Martorell J. Pharmacodynamic approach to immunosuppressive therapies using calcineurin inhibitors and mycophenolate mofetil. Clinical Chemistry 2003;49:1891-1899.

70. Gilger BC, Andrews J, Wilkie DA, Wyman M, Lairmore MD. Cellular immunity in dogs with keratoconjunctivitis sicca before and after treatment with topical 2% cyclosporine. Veterinary Immunology and Immunopathology 1995;49:199-208.

71. Balch A, Mackin A. Canine Immune-mediated Hemolytic Anemia: Pathophysiology, Clinical Signs, and Diagnosis. Compendium Continuing Education for Veterinarians 2007:217-225

72. Piek C. Canine idiopathic immune-mediated hemolytic anemia: A review with recommendations for future research. Veterinary Quarterly 2011;31:129-141.

73. Lewis M, Schwartz RS, Gilmore CE. Autoimmune diseases in domestic animals. Annals of the New York Academy of Sciences 1965;124:178-200.

74. Reimer ME, Troy GC, Warnick LD. Immune-mediated hemolytic anemia: 70 cases (1988-1996). Journal of the American Animal Hospital Association 1999;35:384-391.

75. Reece W. Functional Anatomy and Physiology of Domestic Animals, 3rd ed. Ames: Blackwell Publishing; 2006.

76. Al-Ghazlat S. Immunosuppressive therapy for canine immune-mediated hemolytic anemia. Compendium Continuing Education for Veterinarians 2009;31:33-41.

77. Merck Veterinary Manual, 10th ed. Whitehouse Station: Merck & Co; 2010.

78. Norris C. Immune-Mediated Hemolytic Anemia and Immune-Mediated Thrombocytopenia. In: Textbook of Veterinary Internal Medicine Client Information Series WB Sauders; 2000.

79. Carr AP, Panciera DL, Kidd L. Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. Journal of Veterinary Internal Medicine 2002;16:504-509.

80. Allison AC. Immunosuppressive drugs: the first 50 years and a glance forward. Immunopharmacology 2000;47:63-83.

81. Putsche JC, Kohn B. Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). Journal of the American Animal Hospital Association 2008;44:250-257.

82. Gould SM, McInnes EL. Immune-mediated thrombocytopenia associated with Angiostrongylus vasorum infection in a dog. Journal of Small Animal Practice 1999;40:227-232.

83. Graff EC, Spangler EA, Smith A, Denhere M, Brauss M. Hematologic findings predictive of bone marrow disease in dogs with multicentric large-cell lymphoma. Veterinary Clinical Pathology 2014;43:505-512.

84. Axthelm MK, Krakowa S. Canine distemper virus-induced thrombocytopenia. American Journal of Veterinary Research 1987;48:1269-1275.

85. Handagama PJ, Feldman BF. Drug-induced thrombocytopenia. Veterinary Research Communications 1986;10:1-20.

86. McMillan R, Tani P, Mason D. The demonstration of antibody binding to platelet-associated antigens in patients with immune-mediated thrombocytopenic purpura. Blood 1980;56:993-995.

87. Lewis DC, Meyers KM. Studies of platelet-bound and serum platelet-bindable immunoglobulins in dogs with idiopathic thrombocytopenic purpura. Experimental Hematology 1996;24:696-701.

88. McMillan R. Autoantibodies and autoantigens in chronic immune thrombocytopenic purpura. Seminars in Hematology 2000;37:239-248.

89. Quagliata F, Karpatkin, S. Impaired lymphocyte transformation and capping in autoimmune thrombocytopenic purpura. Blood 1979;53:341-349.

90. Semple JW, Bruce S, Freedman J. Suppressed natural killer cell activity in patients with chronic autoimmune thrmbocytopenic purpura. American Journal of Hematology 1991;37:258-262.

91. Garcia-Sarez J, Prieto A, Reyes E, Manzano L, Merino JL, Alvarez-Mon M. The clinical outcome of autoimmune thrombocytopenic purpura patients is related to their T cell immunodeficiency. British Journal of Haematology 1993;84:464-470.

92. Lewis DC, Meyers KM. Canine idiopathic thrombocytopenic purpura. Journal of Veterinary Internal Medicine 1996;10:207-218.

93. Liu XG, Li JL, Qin P, Ren J, Ma SH, Sun L, Shi Y, Ji XB, Zhu YY, Ma DX, Guo CS, Du X, Hou M, Peng J. Determination of platelet-bound glycoprotein-specific autoantibodies by flow cytometric immunobead assay in primary immune thrombocytopenia. European Journal of Haematology 2011;86:339-346.

94. Ruiz P, Gomez F, King M, Lopez R, Darby C, Schreiber AD. In vivo glucocorticoid modulation of guinea pig splenic macrophage Fc gamma receptors. Journal of Clinical Investigation 1991;88:149-157.

95. Park H, Kim J, Song K, Seo K. Application of vincristine-loaded platelet therapy in three dogs with refractory immune-mediated thrombocytopenia. Journal of Veterinary Science 2015;16:127-130.

96. Nuttal T, Uri M, Halliwell R. Canine atopic dermatitis- what have we learned? Veterinary Record 2013;172:201-207.

97. Favrot C, Steffan J, Seewald W, Picco F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Veterinary Dermatology 2010;21:63-69.

98. Sinke JD, Thepen T, Bihari IC, Rutten VP, Willemse T. Immunophenotyping of skin-infiltrating T-cell subsets in dogs with atopic dermatitis. Veterinary Immunology and Immunopathology 1997;57:13-23.

99. Nuttall TJ, Knight PA, McAleese SM, Lamb JR, Hill PB. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical and Experimental Allergy 2002;32:789-795.

100. Shimada K, Yoon J, Yoshihara T, Iwasaki T, Nishifuji K. Increased transepidermal water loss and decreased ceramide content in lesional and non-lesional skin of dogs with atopic dermatitis. Veterinary Dermatology 2009;20:541-546.

101. Reiter LV, Torres SM, Wertz PW. Characterization and quantification of ceramides in the non-lesional skin of canine patients with atopic dermatitis compared with controls. Veterinary Dermatology 2009;20:260-266.

102. Marsella R, Samuelson D, Harrington L. Immunohistochemical evaluation of filaggrin polyclonal antibody in atopic and normal beagles. Veterinary Dermatology 2009;20:547-554.

103. Hillier A, Griffin CE. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. Veterinary Immunology and Immunopathology 2001;81:147-151.

104. Merryman-Simpson AE, Wood SH, Fretwell N, Jones PG, McLauren WM, McEwan NA, Clements DN, Carter SD, Ollier WE, Nuttall T. Gene (mRNA) expression in canine atopic dermatitis: microarray analysis. Veterinary Dermatology 2008;19:59-66.

105. Olivry T, DeBoer DJ, Favrot C, Jackson HA, Mueller RS, Nuttall T, Prélaud P. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. Veterinary Dermatology 21:233-248.

106. Radowicz SN, Power HT. Long-term use of cyclosporine in the treatment of canine atopic dermatitis. Veterinary Dermatology 2005;16:81-86.

107. Saridomichelakis M, Olivry T. Review: An update on the treatment of canine atopic dermatitis. The Veterinary Journal 2016;207:29-37.

108. Feld D, Marsella R, Ahrens K. A Prospective, Double-blinded, Controlled Pilot Study to Evaluate the Effects of Cyclosporine (Atopica) on Skin Barrier Function in Canine Atopic Dermatitis by Measurement of Trans-Epidermal Water Loss. International Journal of Applied Research in Veterinary Medicine 2014;12:18-23.

109. Steffan J, Favrot C, Mueller R. A systematic review and meta-analysis of the efficacy and safety of cyclosporin for the treatment of atopic dermatitis in dogs. Veterinary Dermatology 2006;17:3-16.

110. Kim H, Park HM. Effect of cyclosporine in canine atopic dermatitis: Safety, clinical evaluation, and mechanism studies. Pakistan Veterinary Journal 2016;36:194-198.

111. Olivry T, Bizikova P. A systematic review of randomized controlled trials for prevention or treatment of atopic dermatitis in dogs: 2008-2011 update. Veterinary Dermatology 2013;24:97-e26.

112. Massey J, Short AD, Catchpole B, House A, Day MJ, Lohi H, Ollier WER, Kennedy L.J. Genetics of canine anal furunculosis in the German shepherd dog. Immunogenetics 2014.

113. Day MJ, Weaver BMQ. Pathology of surgically resected tissue from 305 cases of anal furunculosis in the dog. Journal of Small Animal Practice 1992;33:583-589.

114. Patricelli AJ, Hardie RJ, McAnulty JE. Cyclosporine and ketoconazole for the treatment of perianal fistulas in dogs. Journal of the American Veterinary Medical Association 2002;220:1009-1016.

115. House A, Gregory SP, Catchpole B. Expression of cytokine mRNA in canine anal furunculosis lesions. The Veterinary Record 2003;153:354-358.

116. Kennedy LJ, O'Neill T, House A, Barnes A, Kyostila K, Innes J, Fretwell N, Day MJ, Catchpole B, Lohi H, Ollier WER. Risk of anal furnuculosis in German shepherd dogs is associated with the major histocompatibility complex. Tissue Antigens 2007;71:51-56.

117. House AK, Binns MM, Gregory SP, Catchpole B. Analysis of NOD1, NOD2, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 genes in anal furunculosis of German shepherd dogs. Tissue Antigens 2008;73:250-254.

118. House AK, Catchpole B, Gregory SP. Matrix metalloproteinase mRNA expression in canine anal furunculosis lesions. Veterinary Immunology and Immunopathology 2007;115:68-75.

119. Budsberg SC, Spurgeon TL, Liggit HD. Anatomic predisposition to perianal fistulae formation in the German shepherd dog. American Journal of Veterinary Research 1985;46:1468-1472.

120. Harkin KR, Walshaw R, Mullaney TP. Association of perianal fistula and colitis in the German shepherd dog: response to high-dose prednisone and dietary therapy. Journal of the American Animal Hospital Association 1996;32:515-520.

121. Mathews KA, Sukhiani HR. Randomized controlled trial of cyclosporine for treatment of perianal fistulas in dogs. Journal of the American Veterinary Medical Association 1997;211:1249-1253.

122. Mathews KA, Ayres SA, Tano CA, Riley SM, Sukhiani HR, Adams C. Cyclosporin treatment of perianal fistulas in dogs. Canadian Veterinary Journal 1997;38:39-41.

123. Griffiths LG, Sullivan M, Borland WW. Cyclosporin as the sole treatment for anal furunculosis: preliminary results. Journal of Small Animal Practice 1999;40:569-572.

124. Hawthorne AB, Hawkey CJ. Immunosuppressive drugs in inflammatory bowel disease. A review of their mechanisms of efficacy and place in therapy. Drugs 1989;38:267-288.

125. Stanley BJ. Long-term prospective evaluation of topically applied 0.1% tacrolimus ointment for treatment of perianal sinuses in dogs. Journal of the American Veterinary Medical Association 2009;35:397-404.

126. Tisdall PLC, Hunt GB, Beck JA. Management of perianal fistulae in five dogs using azathioprine and metronidazole prior to surgery. Australian Veterinary Journal 1999;77:374-378.

127. Kolodziejska-Sawerska A, Rychlik A, Depta A, Wdowiak M, Nowicki M. Cytokines in canine inflammatory bowel disease. Polish Journal of Veterinary Science 2013;16:165-171.

128. German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. Journal of Veterinary Internal Medicine 2003;17:8-20.

129. Hodgson HJ, Bhatti M. Assessment of disease activity in ulcerative colitis and Chrohn's disease. Inflammatory Bowel Disease 1995;1:117-134.

130. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Chrohn's disease activity index. Gastroenterology 1976;70:439-444.

131. Collins MT. Canine inflammatory bowel disease: current and prospective biomarkers for diagnosis and management. Compendium Continuing Education for Veterinarians 2013;35:E5.

132. Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, Eckersall PD, Benson TJ, Evans R. A scoring index for disease activity in canine inflammatory bowel disease. Journal of Veterinary Internal Medicine 2003;17:291-297.

133. Allenspach K, Wieland B, Grone A, Gaschen F. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. Journal of Veterinary Internal Medicine 2007;21:700-708.

134. Solem CA, Loftus EV, Tremaine WJ, Harmsen WS, Zinsmeister AR, Sandborn WJ. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. Inflammatory Bowel Disease 2005;11:707-712.

135. Vermeire S, Van Assche G, Rutgeerts P. C-reactive protein as a marker for inflammatory bowel disease. Inflammatory Bowel Disease 2004;10:661-665.

136. Kiss LS, Szamosi T, Molnar T, Miheller P, Lakatos L, Vincze A, Palatka K, Barta Z, Gasztonyi B, Salamon, Horvath G, Tóth GT, Farkas K, Banai J, Tulassay Z, Nagy F, Szenes M, Veres G, Lovasz BD, Vegh Z, Golovics PA, Szathmari M, Papp M, Lakatos PL. Early clinical remission and normalization of CRP are the strongest predictors of efficacy, mucosal healing, and dose escalation during the first year of adalimumab therapy in Crohn's disease. Alimentary Pharmacology and Therapeutics 2011;34:911-922.

137. Lee KM, Jeen YT, Cho JY, Lee CK, Koo JS, Park DI, Im JP, Park SJ, Kim YS, Kim TO, Lee SH, Jang BI, Kim JW, Park YS, Kim ES, Choi CH, Kim HJ. Efficacy, safety, and predictors of response to infliximab therapy for ulcerative colitis: a Korean multicenter retrospective study. Journal of Gastroenterology and Hepatology 2013;28:1829-1833.

138. McCann TM, Ridyard AE, Else RW, Simpson JW. Evaluation of disease activity markers in dogs with idiopathic inflammatory bowel disease. Journal of Small Animal Practice 2007;48:620-625.

139. Hall EJ. Clinical laboratory evaluation of small intestinal function. The Veterinary Clinics of North America Small Animal Practice 1999;29:441-469.

140. Washabau RJ, Day MJ, Willard MD, Hall EJ, Jergens AE, Mansell J, Minami T, Bilzer TW. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. Journal of Veterinary Internal Medicine 2010;24:10-26.

141. Craven M, Simpson JW, Ridyard AE, Chandler ML. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). Journal of Small Animal Practice 2004;47:336-342.

142. Munster M, Horauf A, Bilzer T. Assessment of disease severity and outcome of dietary, antibiotic, and immunosuppressive interventions by use of the canine IBD activity index in 21 dogs with chronic inflammatory bowel disease. Berlin and Munich Veterinary Weekly 2006;119:493-505.

143. Malewska K, Rychlik A, Nieradka R, Kander M. Treatment of inflammatory bowel disease (IBD) in dogs and cats. Polish Journal of Veterinary Science 2011;14:165-171.

144. Ashraf R, Vasiljevic T, Smith SC, Donkor ON. Effect of cell-surface components and metabolites of lactic acid bacteria and probiotic organisms on cytokine production and induction of CD25 expression in human peripheral mononuclear cells. Journal of Dairy Science 2014;97:2542-2558.

145. Chrzastowska M, Kander M, Depta A. Prospects for the use of probiotic bacteria in the treatment of gastrointestinal diseases in dogs. Polish Journal of Veterinary Science 2009;12:279-284.

146. Mitsuyama K, Toyobaga M, Sata M. Intestinal microflora as a therapeutic target in inflammatory bowel disease. Journal of Gastroenterology 2002;37:73-77.

147. Chen J, Seviour R. Medicinal importance of fungal beta-(1-->3), (1-->6)-glucans. Mycological Research 2007;111:635-652.

148. Steiner S, Daniel C, Fischer A, Atreya I, Hirschmann S, Waldner M, Neumann H, Neurtah M, Atreya R, Weigmann B. Cyclosporine a regulates pro-inflammatory cytokine production in ulcerative colitis. Archivum Immunologiae et Therapiae Experimentalis 2015;63:53-63.

149. Allenspach K, Rufenacht S, Sauter S, Gröne A, Steffan J, Strehlau G, Gaschen F. Pharmacokinetics and clinical efficacy of cyclosporine treatment of dogs with steroid-refractory inflammatory bowel disease. Journal of Veterinary Internal Medicine 2006;20:239-244.

150. Archer TM, Fellman CL, Stokes JV, Pinchuk LM, Lunsford KV, Pruett SB, Langston VC, Mackin AJ. Pharmacodynamic monitoring of canine T-cell cytokine responses to oral cyclosporine. Journal of Veterinary Internal Medicine 2011;25:1391-1397.

CHAPTER II

ANALYTICAL VALIDATION OF A QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAY FOR EVALUATION OF T-CELL TARGETED IMMUNOSUPPRESSIVE THERAPY IN THE DOG

Previously published in Veterinary Immunology and Immunopathology December 2013 by Riggs C, Archer T, Fellman C, Figueiredo AS, Follows J, Stokes J, Wills R, Mackin A, and Bulla C.

Abstract

Cyclosporine is an immunosuppressive agent that inhibits T-cell function by decreasing production of cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ). In dogs, there is currently no reliable analytical method for determining effective cyclosporine dosages in individual patients. Our laboratory has developed a quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) assay that measures IL-2 and IFN-γ gene expression, with the goal of quantifying immunosuppression in dogs treated with cyclosporine. This study focuses on analytical validation of our assay, and on the effects of sample storage conditions on cyclosporine-exposed samples. Blood from healthy dogs was exposed to a typical post-treatment blood concentration of cyclosporine (500 ng/ml) for 1 hour, and then stored for 0, 24, and 48 hours at both room temperature

and 4°C. The study was then repeated using a cyclosporine concentration of 75 ng/ml, with sample storage for 0, 24, and 48 hours at 4°C. Cytokine gene expression was measured using RT-qPCR, and assay efficiency and inter- and intra-assay variability were determined. Storage for up to 48 hours did not significantly alter results compared to samples that were processed immediately. Validation studies showed our assay to be highly efficient and reproducible and robust enough to be feasible under standard practice submission conditions.

Introduction

Normal T-cell function is essential for destruction of diseased cells, maintenance of immunological tolerance, provision of an immune memory response and facilitation of the involvement of other white blood cells in immune responses.[1-4] Immunomodulators, including immunosuppressive agents, can be used to target T-cells and thereby modify both cell-mediated and humoral immune responses.

Cyclosporine is an effective immunosuppressive agent used in both human and veterinary medicine to prevent transplant rejection and treat inflammatory and immune-mediated diseases such as atopy, inflammatory bowel disease, immune-mediated hemolytic anemia, and anal furunculosis.[5-8] Cyclosporine is a potent T-cell inhibitor that works by binding to intracellular cyclophilin, resulting in a cyclosporine-cyclophilin complex that inhibits calcineurin. Calcineurin is required by the T-cell to produce nuclear factor of activated T-cells (NFAT)-regulated cytokines, such as IL-2 and IFN-γ, that help activate T-cells and stimulate lymphocyte proliferation and differentiation.[9] The presence of cyclosporine within the T-cell serves to suppress cytokine production and ultimately T-cell function.[10-12]

An ideal cyclosporine dosing protocol has not been established in the dog. While measurement of blood cyclosporine concentrations is the standard method of monitoring therapy, published target blood concentrations vary widely from one reference laboratory to the next. Individual dogs may even vary in immune response to the same blood cyclosporine concentration.[10] Pharmacodynamic assessment of the actual biological effects of cyclosporine on T-cell function would be preferable to simple monitoring of blood drug concentrations, but a clinically validated tool to assess cyclosporine pharmacodynamics in the dog has not been established. As an important step towards development of a suitable pharmacodynamic assay, we evaluated a RT-qPCR assay of NFAT-regulated cytokine gene expression. Our objectives were to perform analytical validation of the assay using established MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines,[28] and to determine the effects of sample storage conditions on cyclosporine-exposed samples when blood was incubated with cyclosporine at concentrations comparable to levels that are therapeutically feasible in dogs at standard dose rates.

Materials and Methods

Blood Sample Collection and Storage Study

Two different concentrations of cyclosporine were evaluated in our study. We initially evaluated the effects of duration of storage at two different storage temperatures on assay results in samples from 10 dogs when samples were exposed to a concentration of cyclosporine that is considered to be therapeutic (500 ng/mL). In a subsequent smaller study, we evaluated the effects of storage duration at a single storage temperature on

assay results in samples from four dogs when samples were exposed to a concentration of cyclosporine that is considered to be subtherapeutic (75 ng/mL).

Ten healthy intact female Walker hounds were used. In our initial study, 30 mL of blood was collected from the jugular vein using a Vacutainer collection system and then fractionated in 10 aliquots of 3 mL using heparinized tubes. Five of the 3 mL samples of whole blood were then exposed to cyclosporine at a concentration of 500 ng/mL (Sigma, St. Louis, MO, USA Cat no. C1832). One sample was processed via addition of activators (PMA, ionomycin) immediately post-collection and after 1 hour of cyclosporine exposure, while additional tubes (4) were stored at room temperature or 4°C for either 24 or 48 hours prior to activation. As a control, 5 untreated (no cyclosporine addition) samples were included, with one sample processed via addition of activators immediately post-collection, and the 4 additional tubes stored at the same time and temperature combinations as the cyclosporine-treated samples.

A subsequent smaller storage study was then completed using a lower drug concentration and a single storage temperature. Twenty milliliters of blood was collected from the jugular vein from four healthy intact female Walker hounds. Blood was then fractionated into six aliquots of 3 mL using heparinized tubes. Half of the 3 mL samples of whole blood were then exposed to cyclosporine at a concentration of 75 ng/mL (Sigma, St. Louis, MO, USA Cat no. C1832). After a 1 hour incubation period, one sample was processed via addition of activators immediately, while additional tubes were stored at 4°C for either 24 or 48 hours prior to activation. As a control, an untreated sample was included at all time points.

T-cell Activation

Whole blood samples were activated using a combination of PMA (12.5 ng/mL) (Sigma, St.Louis, MO, USA Cat no. P8139-1MG) and ionomycin (0.8 µM) (Sigma, St. Louis, MO, USA Cat no. I0634-1MG). Samples were then incubated for 5 hours at 37°C with 5% CO_2.

RNA Isolation and Quality Assessment

Total RNA was isolated from 1 mL of heparinized whole blood using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA Cat. No. 52304) according to the manufacturer's instructions and then stored at -80°C until use. An on-column DNase (27.27 Kunitz units) treatment (Qiagen, Valencia, CA, USA Cat. No. 79254) was done for all samples to remove genomic DNA. RNA was quantified using a Nanodrop ND-1000 spectrophotometer using the ND-1000 V3.3.0 software.

RNA quality was assessed using the Agilent RNA 6000 Bioanalyzer. Twelve samples were analyzed, and an RNA integrity number (RIN) was determined for each.

Cytokine Gene Expression Quantification

Cytokine RT-qPCR results were analyzed by comparing cyclosporine-treated samples to untreated controls across the various times and temperatures. IL-2 and IFN-γ genes and reference gene GAPDH expression were quantified via RT-qPCR using a SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR kit with Rox used as a reference dye (Invitrogen, Grand Island, NY, USA Cat no. 11736-059). Primers for IL-2, IFN-γ, and GAPDH were based on reported GenBank nucleotide sequences as previously

published by Kobayashi et al, and are shown in Table 2.1.[13] All reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, NY, USA Cat no. 4351106) using 7500 software v2.0.6 for analysis. The RT-qPCR was performed in a 20µL final volume containing 0.75ng/µL of template and 200nM of each primer. Thermal cycling parameters were as follows: 50°C for 3 min, 95°C for 5 min, then 40 cycles of 95°C for 15 sec and 60°C for 30 sec followed by a melting analysis that comprises 95°C for 15 sec, 60°C for 1 min, after which the ramp speed decreases from 1.667°C/sec to 0.01667°C/sec and data is collected continuously until it reaches 95°C, temperature is then held for 30 sec and finally 60°C for 15 sec. All samples were run in triplicate while non-template controls were run in duplicate. A no reverse-transcriptase control, using GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA Cat no. A6001) following the manufacturer's instructions, was also included in one run to ensure there was no contaminating genomic DNA. To calculate the relative change in gene expression for all samples, the $2^{-\Delta\Delta Ct}$ method was employed using GAPDH as a reference gene where $\Delta\Delta Ct = (Ct_{GOI} - Ct_{norm})_{treated} - (Ct_{GOI} - Ct_{norm})_{untreated}$ where GOI is the gene of interest and norm is the reference gene.[14] Cytokine gene expression was presented as a percentage for each time and temperature combination where the untreated control sample represented 100% gene expression for IL-2 and IFN-γ.

Table 2.1 Sequences of primer sets used in the qRT-PCR assay

Primer Set		Primer Sequence (5' - 3')	GenBank Accession Number
GAPDH	Forward	AACTCCCTCAAGATTGTCAGCAA	AB038240
	Reverse	CATGGATGACTTTGGCTAGAGGA	
IL-2	Forward	CCTCAACTCCTGCCACAATGT	U28141
	Reverse	TGCGACAAGTACAAGCGTCAGT	
IFN-γ	Forward	GCATTCCAGTTGCTGCCTACT	AF126247
	Reverse	ACCAGGCATGAGAAGAAATGCT	

Primers used published by Kobayashi et al., 2007

Validation of qRT-PCR Assay

In Vitro Transcription

The PCR products of the three amplified genes were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA Cat no. A9281), cloned into pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA) and propagated into chemically competent DH5α premade Z-competent *Escherichia coli* cells (Zymo Research, Irvine, CA, USA Cat no. T3007). Purified plasmid DNAs (PureLink HiPure plasmid miniprep kit, Invitrogen, Grand Island, NY, USA Cat no. K2100-02) were sent for sequencing (Eurofins MWG Operon, Huntsville, Alabama, USA). The plasmid quantification was assessed spectrophotometrically and the number of molecules was determined on the basis of plasmid size and corresponding DNA mass. One microgram of plasmids previously linearized with restriction enzyme *SpeI* (New England BioLabs, Ipswich, MA, USA Cat no. R0133S) were used for the synthesis of the

recombinant transcripts using MAXIscript T7 *in vitro* transcription kit (Invitrogen, Grand Island, NY, USA Cat no. AM1312).

Limit of Detection and qRT-PCR Efficiency

The limit of detection (LOD) of all 3 RT-qPCR assays was determined in triplicate using 10-fold serial dilutions of recombinant transcripts representing 10^1 to 10^6 copies of RNA per reaction.

Assay efficiency was assessed using five 10-fold serial dilutions run in triplicate of total RNA isolated from one healthy Walker hound. The slope of the resulting curve was used to calculate assay efficiency using the following equation: Efficiency = $-1+10^{(-1/slope)}$

Inter-assay and Intra-assay Variation

Inter-assay variation was determined by running one sample in triplicate on nine different days. Intra-assay variation was calculated using the mean and standard deviation of C_t values for a reaction run in triplicate. This was replicated on nine different plates, all using the same RNA sample, and the coefficients of variation (CV) calculated for each run were averaged together. For all measurements, mean value, standard deviation, and CV were calculated for the threshold cycle (C_t) values.

Statistical Methods

For the storage study, the data were visually assessed for normality using the UNIVARIATE procedure in SAS for Windows 9.3 (SAS Institute, Inc., Cary, NC) for both the IL-2 and IFN-γ outcomes. Each outcome was found to be approximately normally distributed. A mixed model repeated measures analysis was conducted for each

out-come using the MIXED procedure. Separate models were assessed for each storage temperature and treatment combination. Time was included in the models as a fixed effect. The repeated measures of samples taken from the same dog over time were accounted for in a repeated statement using a first order autoregressive covariance structure. A random statement with dog as the random effect was used to account for between-dog variation. Differences in least square means with Dunnett adjustment of p-values were used for comparisons of the 24 h and 48 h samples to the 0 h cytokine gene expression levels if time was found to be a significant fixed effect. An alpha level of 0.05 was used to determine significance in all analyses.

Results

RNA Quality

RNA quality was assessed using samples from a variety of storage time and temperature combinations. RIN values had an average of 8.26 and a range of 7.60-8.60 where a RIN value of 1 represents degraded RNA and a value of 10 represents high quality intact RNA.

Specificity and Sensitivity

Specificity was assured by melt-curve analysis demonstrating one amplification peak and sequencing of the amplified products.

Amplified RT-qPCR products that were sent for sequencing were confirmed to be canine IL-2, IFN-γ and GAPDH sequences using the basic local alignment search tool (BLAST).

Regression lines of five 10-fold serial dilutions of total RNA (dilution vs. C_t) had slopes of -3.51 for GAPDH, -3.58 for IL-2, and -3.58 for IFN- γ (Table 2.2). The resulting amplification efficiencies were 92.86% for GAPDH, 90.33% for IL-2, and 90.27% for IFN-γ (Table 2.2).

The limit of detection of the assays was determined using 10-fold serial dilutions of recombinant transcripts and was found to be 1000 copies of a single RNA transcript for IL-2, IFN- γ, and GAPDH.

Table 2.2 Assay amplification efficiency, slopes of regression lines, and inter-assay and intra-assay variation for reference gene and cytokine genes

Gene	Efficiency (%)	Amplification Factor	Slope	Inter-assay Variation		Intra-assay Variation		
				CV (%)	SD (C_t)	CV (%)	SD (C_t)	SD Range
GAPDH	92.86	1.93	-3.51	7.07	1.22	0.24	0.04	0.015 - 0.091
IL-2	90.33	1.90	-3.58	6.97	2.13	0.33	0.098	0.037 - 0.244
IFN-γ	90.27	1.90	-3.58	4.73	1.49	0.88	0.277	0.039 - 0.382

Assay efficiency for reference gene and cytokine genes determined using five tenfold dilutions (10^1- 10^6) of total RNA isolated from a healthy intact female Walker hound. Inter-assay variation determined by running one sample in triplicate on nine different days. CV was calculated using the mean and standard deviation of C_t values for one sample run on nine different days. Intra-assay variation was calculated using the mean and standard deviation of C_t values for a reaction run in triplicate. This was replicated on nine different plates all using the same RNA sample, and the CVs calculated for each run were averaged together. (CV = coefficient of variation, SD = standard deviation)

Cytokine Gene Expression

Combined cytokine gene expression data for all 10 dogs in our initial therapeutic cyclosporine concentration study is shown in Figure 2.1. The data for all 10 animals at each time and temperature combination were pooled to determine the medians and first and third quartiles. Only one cyclosporine-treated sample was not suppressed below 50% of untreated cytokine gene expression. Cytokine gene expression data for all four dogs in

our subsequent subtherapeutic cyclosporine concentration study is shown in Figure 2. Adequate RNA was isolated from all samples in our initial 10 dog study after storage for 24 hours, at both 4°C and room temperature, while at 48 hours adequate RNA was isolated from all 4°C samples, with several room temperature samples having insufficient RNA extracted for gene analysis. Adequate RNA was isolated from all samples in our subsequent four dog study at 4°C.

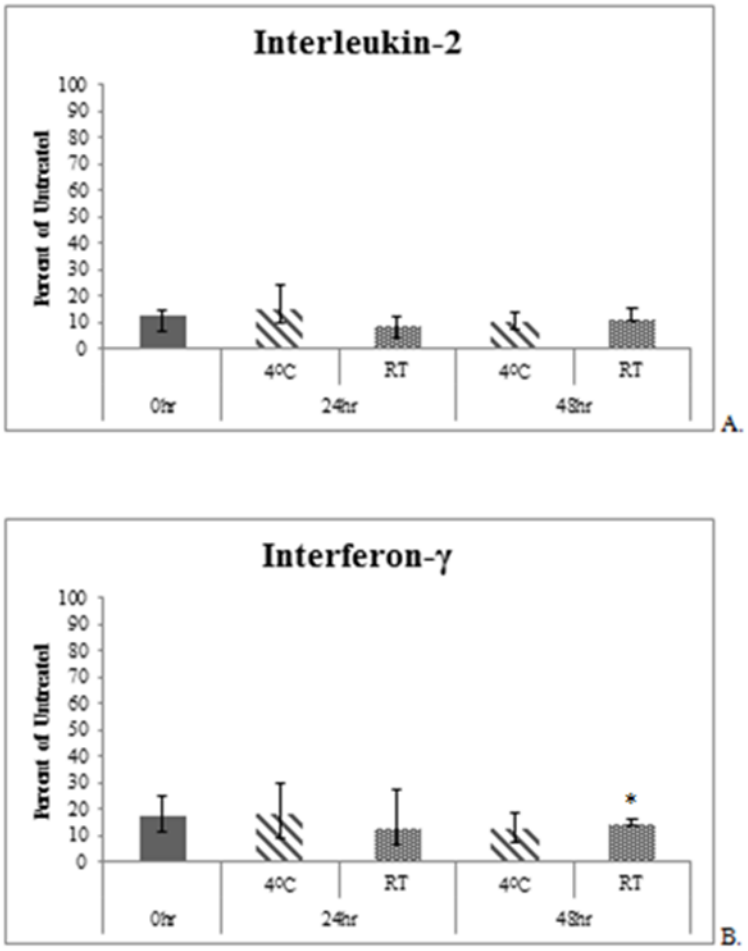

Figure 2.1 Cytokine gene expression in samples exposed to 500 ng/mL cyclosporine as a percentage of expression in unexposed samples

Figures presented as median, first and third quartiles (n = 10 dogs except for 48 hour samples at room temperature, where n = 5 dogs). A IL-2. B IFN-γ.

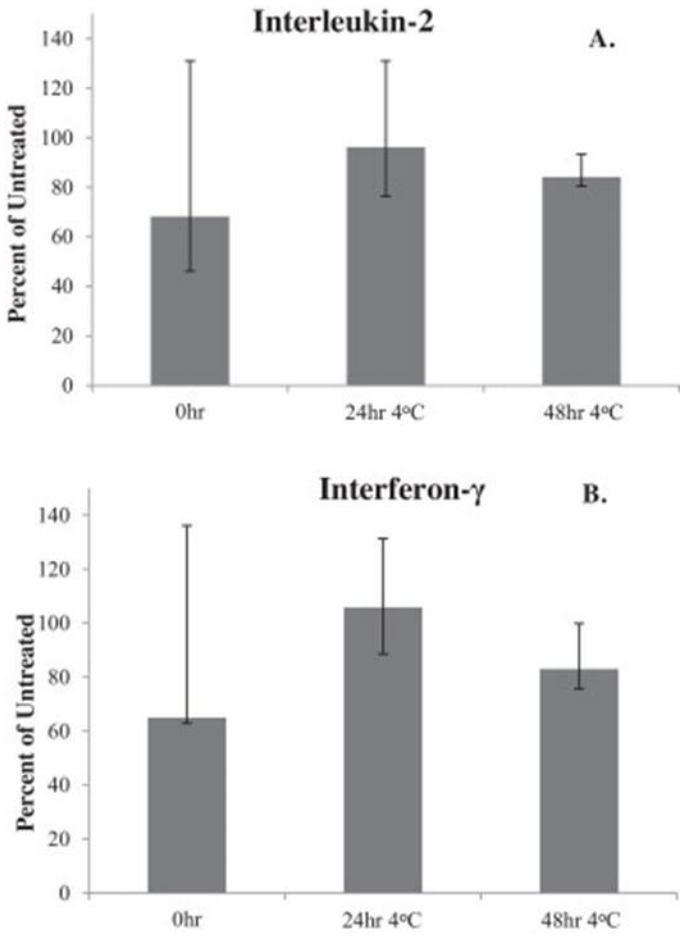

Figure 2.2 Cytokine gene expression in samples exposed to 75 ng/mL cyclosporine as a percentage of expression in unexposed samples

Figures presented as median, first and third quartiles (n = 4 dogs). A IL-2. B IFN-γ.

Inter-assay and Intra-assay Variation

Inter-assay variation for GAPDH, IL-2, and IFN- γ had a CV of 7.07%, 6.97%, and 4.73%, and a SD of 1.22, 2.13, and 1.49 C_t, and intra-assay variation had a CV of 0.24%, 0.33%, and 0.88%, and a SD of 0.040, 0.098, and 0.277 C_t respectively (Table 2.2).

Discussion

Optimal cyclosporine treatment protocols have not been established for dogs, in part due to individual to individual variability in immunosuppressive effects despite comparable blood concentrations.[10] A poor correlation between cyclosporine blood concentrations and clinical response has been demonstrated with both canine atopy and inflammatory bowel disease.[15,16] Cyclosporine dosing in veterinary cases is therefore often empirically adjusted based solely on clinical response.

Cyclosporine blood concentrations similarly do not always predict rejection in human transplant medicine, and this problem has prompted the development of pharmacodynamic assays of the drug's biological effects.[17-22] While such assays have proven to be valuable for dosing humans, a practical veterinary counterpart has yet to be developed.

Previous work in our laboratory evaluated flow cytometry as a method to measure T-cell cytokine production in dogs in response to oral cyclosporine, and documented suppressed levels of IL-2 and IFN-γ.[10] Flow cytometry requires immediate sample processing, however, and results can also be influenced by daily variations in machine setup. Quantitative RT-PCR offers an appealing alternative, as samples are potentially stable enough to allow standard submission from external sources, and extracted RNA can also be frozen and saved for batch analysis.[13,23,24]

In this study, we analytically validated a RT-qPCR panel of NFAT-regulated cytokine gene expression designed to measure the effect of drugs such as cyclosporine on T-cell function. Specifically, we evaluated assay repeatability, precision, and accuracy as well sample stability under different storage conditions. Our results suggest that our assay

is analytically sensitive and specific, and that blood samples can be kept for up to 48 hours at 4°C, and 24 hours at 4°C or room temperature without loss of interpretable results. Storage at room temperature for 48 hours was less optimal because of a diminishment in the amount of RNA that can be extracted.

In the initial storage portion of our study, we demonstrated consistently reduced cytokine gene expression in response to an *in vitro* model where whole blood was incubated with cyclosporine at concentrations comparable to clinically relevant blood levels (Figure 1). Under a range of storage times and temperatures, gene expression following incubation with cyclosporine was suppressed below 50% of the degree of expression in untreated samples in all but one sample. The majority of samples suppressed to 25% or less, which is a similar degree of cytokine suppression seen in other studies evaluating cyclosporine at a concentration of 500 ng/mL.[25,26] We speculate the single result which suppressed by less than 50% was a laboratory error, since other samples collected from the same animal at the same time and run in parallel exhibited more pronounced suppression of gene expression. Changes in the degree of suppression of gene expression were not seen over time despite exposure to cyclosporine for up to 48 hours, suggesting that samples from dogs receiving cyclosporine could be mailed by veterinarians under standard handling conditions without loss of assay validity, despite the presence of cyclosporine in the submitted sample.

Following our initial storage study, we conducted a smaller study using a lower concentration of cyclosporine to ensure that prolonged drug exposure over 1-2 days of storage did not cause a progressive decrease in cytokine gene expression that could be misinterpreted as evidence of therapeutic efficacy. We conducted this second study at

4°C only because, based on our initial study, we determined that 4°C was the preferred temperature for sample submission without excessive loss of extractable RNA. Compared to exposure to a cyclosporine concentration of 500 ng/mL, exposure of samples to 75 ng/mL of cyclosporine caused a less consistent reduction in cytokine gene expression. Importantly, however, sample storage did not increase the degree of cyclosporine-mediated suppression of gene expression (Figure 2.2).

In order to validate our assays, amplification efficiency, LOD, and inter- and intra-assay variability were determined. For PCR, amplification efficiency documents how well the target is amplified by the assay, and amplification efficiencies between 90 and 110% are generally considered acceptable.[27] Our assay efficiencies fell with this range, confirming adequate performance. The LOD assesses the sensitivity of an assay, and has a minimum theoretical limit of three copies per PCR reaction.[28] The LOD for our assay was determined to be 1000 copies, similar to LODs reported by other groups developing similar assays.[29] Although our LOD is higher than ideal, our target genes are highly expressed, and typical samples will have sufficient cells with ample gene expression to ensure a much higher copy number. We therefore expect that the impact of a higher than optimal LOD on the clinical applicability of our assay will be minimal. In fact, LOD values lower than those obtained in our study have been shown to be vulnerable to the Monte-Carlo effect, which can result in inaccurate data.[30] The repeatability and reproducibility of our assay was evaluated by calculating intra-assay and inter-assay variation. For quantitative assays, a CV value greater than 20% is thought to result in a significant loss of precision.[30] For our assays, CV values for intra- and inter-

assay variability were less than 10%, suggesting a high level of repeatability and reproducibility.

In conclusion, our RT-qPCR assays of NFAT-regulated cytokine gene expression appear to be sensitive, efficient, precise and robust enough to permit evaluation of the effects of cyclosporine on T-cell function in dogs. Furthermore, samples analyzed using our assays provide stable results for 24-48 hours under standard handling conditions, and can therefore be routinely shipped without loss of sample viability.

References

1. Brigl M, Brenner, M.B. CD1: Antigen presentation and T cell function. Annual Review of Immunology 2004;22:817-890.

2. Jiang H, Chess, L. An integrated view of suppressor T cell subsets in immunoregulation. Journal of Clinical Investigation 2004;114:1198-1208.

3. Luo L, Sun, Z., Cheng, H., Luo, G. Memory T-cell-specific therapeutics attenuate allograft rejection via mediation of alloreactivity in memory cells. Immunology Letters 2012;148:53-58.

4. Moss RB, Moll, T., El-Kalay M., Kohne, C., Soo Hoo, W., Encinas, J., Carlo, D.J. Th1/Th2 cells in inflammatory disease states: Therapeutic implications. Expert Opinion on Biological Therapy 2004;4:1887-1896.

5. Barten MJ, Tarnok A, Garbade J, Bittner HB, Dhein S, Mohr FW, Gummert JF. Pharmacodynamics of T-cell function for monitoring immunosuppression. Cell Proliferation 2007;40:50-63.

6. Daigle JC. More economical use of cyclosporine through combination drug therapy. Journal of the American Animal Hospital Association 2002;38:205-208.

7. Jensen SA, Dalhoff KP. Cyclosporine therapeutic drug monitoring. Transplantation Proceedings 2001;33:3003-3005.

8. Plumb DC. Plumb's Veterinary Drug Handbook. 6th ed. PharmaVet Inc.; 2008;5.

9. Kumar Vinay AKA, Nelson Fausto, Jon C. Aster. Robbins and Cotran Pathologic Basis of Disease, 8th ed. Philadelphia: Saunders; 2010.

10. Archer TM, Fellman, C.L., Stokes, J.V., Pinchuk, L.M., Lunsford, K.V., Pruett, S.B., Langston, V.C., Mackin, A.J. Pharmacodynamic monitoring of canine T-cell cytokine responses to oral cyclosporine. Journal of Veterinary Internal Medicine 2011;25:1391-1397.

11. Fellman CL, Stokes JV, Archer TM, Pinchuk LM, Lunsford KV, Mackin AJ. Cyclosporine A affects the in vitro expression of T cell activation-related molecules and cytokines in dogs. Veterinary Immunology and Immunopathology 2011

12. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. Annual Review of Immunology 1997;15:707-747.

13. Kobayashi T, Momoi Y, Iwasaki T. Cyclosporine A inhibits the mRNA expressions of IL-2, IL-4 and IFN-gamma, but not TNF-alpha, in canine mononuclear cells. Journal of Veterinary Medical Science 2007;69:887-892.

14. Livak KJ, Schmittgen, T.D. Analysis of relative gene expression data using real time quantitative PCR and the 2ΔΔCT method. Methods 2001;25:402-408.

15. Allenspach K, Rufenacht S, Sauter S, Grone A, Steffan J, Strehlau G, and Gaschen F. Pharmacokinetics and clinical efficacy of cyclosporine treatment of dogs with steroid-refractory inflammatory bowel disease. Journal of Veterinary Internal Medicine 2006;20:239-244.

16. Steffan J, Strehlau G, Maurer M, Rohlfs A. Cyclosporin A pharmacokinetics and efficacy in the treatment of atopic dermatitis in dogs. Journal of Veterinary Pharmacology and Therapeutics 2004;27:231-238.

17. Zucker C, Zucker K, Asthana D, Carreno M, Viciana AL, Ruiz P, Esquenazi V, Nery J, Burke G, Miller J. Longitudinal induced IL-2 mRNA monitoring in renal transplant patients immunosuppressed with cyclosporine and in unmodified canine renal transplant rejection. Human Immunology 1996;45:1-12.

18. Caruso R, Perico N, Cattaneo D, Piccinini G, Bonazzola S, Remuzzi G, Gaspari F. Whole-blood calcineurin activity is not predicted by cyclosporine blood concentration in renal transplant recipients. Clinical Chemistry 2001;47:1679-1687.

19. Brunet M, Campistol JM, Millan O, Vidal E, Esforzado N, Rojo I, Jiménez O, Oppenheimer F, Corbella J, Martorell J. Pharmacokinetic and pharmacodynamic correlations of cyclosporine therapy in stable renal transplant patients: evaluation of long-term target C(2). International Immunopharmacology 2003;3:987-999.

20. Härtel C HH, Schlenke P, Fricke L, Schumacher N, Kirchner H, Müller-Steinhardt M. Individual variability in cyclosporin A sensitivity: the assessment of functional measures on CD28-mediated costimulation of human whole blood T lymphocytes. Journal of Interferon and Cytokine Research 2003;23:91-99

21. Barten MJ, Gummert JF. Biomarkers in Transplantation Medicine: Prediction of Pharmacodynamic Drug Effects. Transfusion Medicine and Hemotherapy 2007:182-187.

22. Sommerer C. HW, Enk A., Meuer S., Zeier M., Giese T. Pharmacodynamic immune monitoring of NFAT-regulated genes predicts skin cancer in elderly long-term renal transplant recipients. Clinical Transplantation 2008;22:549-554.

23. Hartel C, Fricke L, Schumacher N, Kirchner H, Müller-Steinhardt M. Delayed cytokine mRNA expression kinetics after T-lymphocyte costimulation: a quantitative measure of the efficacy of cyclosporin A-based immunosuppression. Clinical Chemistry 2002;48:2225-2231.

24. Maeda S, Ohno, K., Nakamura, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., Goto-Koshino, Y., Fujino, Y., Tsujimoto, H. Quantification of chemokine and chemokine receptor gene expression in duodenal mucosa of dogs with inflammatory bowel disease. Veterinary Immunology and Immunopathology 2011:290-298.

25. Kuzuya T, Kobayashi T, Katayama A, Nagasaka T, Miwa Y, Uchida K, Nakao A, Yamada K. Evaluation of interleukin-2 mRNA in whole blood as a parameter for monitoring cyclosporine pharmacodynamics. Biological and Pharmaceutical Bulletin 2009;32:604-608.

26. Konstandin MH, Sommerer C, Doesch A. Pharmacodynamic cyclosporine A-monitoring: relation of gene expression in lymphocytes to cyclosporine blood levels in cardiac allograft recipients. Transplant International 2007;20:1036-1043.

27. Taylor S, Dijkman G, Alsarraj M, Nguyen M. A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. Methods 2010;50:S1-5.

28. Bustin S, Garson J, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl M, Shipley G, Vandesompele J, and Wittwer C. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 2009;55:611-622.

29. Spackman E SD, Myers TJ, Bulaga LL, Garber LP, Perdue ML, Lohman K, Daum LT, Suarez DL. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology 2002;40:3256-3260.

30. Karrer, E., Hogenhout, S., Bennett, A., Bostock, R., Martineau, B., Gilchrist, D., Alexander, D. In situ isolation of mRNA from individual plant cells: Creation of cell-specific cDNA libraries. Proceedings of the National Academy of Science USA 1995;92:3814-3818.

31. Reed GF, Lynn F., and Meade, B.D. Use of Coefficient of Variation in Assessing Variability of Quantitative Assays. Clinical and Diagnostic Laboratory Immunology 2002;9:1235-1239.

CHAPTER III

ALTERATIONS IN ACTIVATED T-CELL CYTOKINE EXPRESSION IN HEALTHY DOGS OVER 7 DAYS OF DAILY DOSING WITH ORAL CYCLOSPORINE

Abstract

Cyclosporine is a powerful T-cell inhibitor used in the treatment of immune-mediated and inflammatory diseases in the dog. There is limited information on how to best monitor patients on cyclosporine therapy. Currently, pharmacokinetic assays that measure the concentration of cyclosporine in the blood are used to assess if the dose is effective, however target blood drug concentrations have not been shown to reliably correlate with suppression of T-cell function in the dog. In human transplant recipients, therapeutic drug monitoring has shifted from a pharmacokinetic to a pharmacodynamic based approach. Our laboratory has validated a qRT-PCR assay to measure the pharmacodynamic effects of cyclosporine in the dog. In this study, activated T-cell expression of IL-2 and IFN-γ was measured using qRT-PCR daily for 7 consecutive days in 8 healthy Walker hounds receiving cyclosporine at a dosage of 10mg/kg every 12 hours. Cytokine production was found to be markedly decreased within 24 hours after the initiation of cyclosporine. Based on these results, cyclosporine causes a rapid drop in T-cell cytokine production that is sustained with continued dosing in healthy dogs.

Introduction

Cyclosporine is a potent immunosuppressive agent used to treat an increasing number of immune-mediated conditions in the veterinary field. Despite its popularity, ideal oral cyclosporine dosing protocols for systemic immunosuppression have yet to be established for the dog. Cyclosporine is a cyclic polypeptide that inhibits T-cell function by targeting the intracellular phosphatase calcineurin, leading to reduced transcription of nuclear factor of activated T cells (NFAT) regulated cytokines such as interleukin-2 (IL-2) and interferon-gamma (IFN-γ). These cytokines have been shown to be increased in many immune-mediated and inflammatory conditions, including atopy,[1] immune-mediated hemolytic anemia (IMHA),[2] and anal furunculosis.[3-5] Cyclosporine-induced reduction in the transcription of cytokines under the control of NFAT leads to inhibition of T-cell function and an overall decrease in humoral and cell-mediated immune responses.

An ideal oral dosing protocol for the commencement of systemic immunosuppressive dosages of cyclosporine in the dog has not been established. While measurement of blood cyclosporine levels was the first established method of estimating the degree of immunosuppression in canine clinical patients, dogs have been shown to vary markedly in T-cell response to the same cyclosporine blood concentration.[6] Although canine patients may achieve blood drug concentrations within a targeted range, sufficient immunosuppression may not be achieved in some dogs due to individual variations in response to cyclosporine. To address this issue, we have developed and validated a quantitative reverse transcription polymerase chain reaction (qRT-PCR) based assay that assesses the effects of cyclosporine therapy on T cells in dogs via measurement

of the Type 1 T helper (Th1) cytokines IL-2 and IFN-γ.[7] Utilizing a pharmacodynamic approach to measure the impact of cyclosporine on T-cell function facilitates a more targeted assessment of drug effects on the immune status of the patient, and dosage recommendations and adjustments can be made based on immunosuppressive effects rather than target drug concentrations. In this current study, we use the qRT-PCR assay to determine the timing of onset of peak suppression of T-cell function in dogs commencing oral cyclosporine.

In this investigation, activated T-cell cytokine levels were assessed daily in healthy Walker hounds treated with immunosuppressive doses of cyclosporine for 7 straight days. The study was designed to facilitate determination of the ideal timing of sample collection for pharmacodynamic monitoring after the commencement of cyclosporine therapy in canine patients. We hypothesized that a downward trend of cytokine production would be observed over the 7 day treatment course, and that maximal suppression of T-cell function would be attained before the end of the study period.

Materials and Methods

Experiment and animal care protocols were approved by Mississippi State University Institutional Animal Care and Use Committee. Mississippi State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Blood Sample Collection

Eight Walker hounds, members of a healthy research dog colony, were dosed every 12 hours with 10 mg/kg of oral modified cyclosporine (Atopica, Greenfield, IN, USA) for 7 days. Approximately 3 ml of blood was collected from the jugular vein daily for 7 days, 2 hours after morning dosing, using a needle and syringe. Samples were immediately transferred into a heparin tube. Dogs were fed twice daily, and not given their morning meal until after blood collection. Prior to commencement of drug administration, a pre-study sample was collected in the same fashion to serve as our baseline for comparison purposes.

Cyclosporine Blood Concentrations

Whole blood was collected into EDTA anticoagulant tubes 2 hours after oral dosing on Day 7 of cyclosporine treatment for all 8 dogs, and shipped on ice to the Auburn University Veterinary Clinical Pharmacology Laboratory for analysis. Cyclosporine concentrations were measured using a previously described method.[8] Briefly, the Siemens (New York, NY, USA) Cyclosporine Immunoassay® (CSA) and the Siemens Cyclosporine Extended Range Immunoassay® (CSAE) were used to measure cyclosporine concentrations in canine whole blood samples containing EDTA. All samples were analyzed with a Siemens (New York, NY, USA) Dimension Xpand Plus® general chemistry analyzer.

T-Cell Activation

Phorbol myristate acetate (12.5 ng/mL) (Sigma, St.Louis, MO, USA Cat no. P8139-1MG) and ionomycin (0.8 µM) (Sigma, St. Louis, MO, USA Cat no. I0634-

1MG) were used to activate whole blood samples as previously published by Riggs et al.[7] All samples were then incubated for 5 hours at 37°C containing 5% CO_2.

RNA Isolation

RNA was isolated using the protocol previously published by Riggs et al.[7] Total RNA was isolated from 1 mL of heparinized whole blood using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA Cat. No. 52304) following the manufacturer's instructions. All samples underwent an on-column DNase (27.27 Kunitz units) treatment (Qiagen, Valencia, CA, USA Cat. No. 79254). Post collection samples were stored at -80°C until use. A Nanodrop ND-1000 spectrophotometer using ND-1000 V3.3.0 software (NanoDrop Technologies, Wilmington DE, USA) was used to determine RNA concentration.

Cytokine Gene Expression Quantification

The results of cytokine qRT-PCR data were evaluated by comparing daily cyclosporine treatment samples to a pre-treatment baseline sample using protocols previously published by Riggs et al.[7] A SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR kit using Rox used as a reference dye (Invitrogen, Grand Island, NY, USA Cat no. 11736-059) was used to quantify expression of housekeeping gene GAPDH and genes of interest IL-2 and IFN-γ. Primers used in the reaction were based on GenBank nucleotide sequences as previously reported by Kobayashi et al.[9] All reactions were run on a Stratagene Mx3500P Multiplex Quantitative PCR system (Agilent Technologies) using Mx Pro software for analysis. The RT-qPCR reaction was performed with a final volume of 20µL containing a total of 30ng of template RNA and 200nM of

each primer. The following thermal cycling parameters were used: 50°C for 3 min, 95°C for 5 min, then 40 cycles of 95°C for 15 sec and 60°C for 30 sec followed by a melting analysis that comprises 95°C for 15 sec, 60°C for 1 min, after which the ramp speed decreases from 1.667°C/sec to 0.01667°C/sec and data is collected continuously until it reaches 95°C, temperature is then held for 30 sec and finally 60°C for 15 sec. All samples were run in triplicate, while non-template controls were run in duplicate. ΔCt values of treatment days were compared to ΔCt value of a pre-treatment sample where $\Delta Ct = Ct_{GOI} - Ct_{norm}$ in which GOI is the gene of interest and norm is the reference gene. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative change in expression using GAPDH as a reference gene where $\Delta\Delta Ct = (Ct_{GOI} - Ct_{norm})_{treated} - (Ct_{GOI} - Ct_{norm})_{pre\text{-}treatment}$ where GOI is the gene of interest and norm is the reference gene.[10] Cytokine gene expression was presented as a percentage where the pre-treatment baseline sample represented 100% gene expression for IL-2 and IFN-γ.

Statistical Analysis

The change in IL-2 ΔCt and IFN-γ ΔCt values in response to activation in pre-treatment samples was calculated by first subtracting the activated value from the un-activated value for each dog for each cytokine. The distributions of these changes were then assessed using PROC UNIVARIATE in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). The change in IL-2 ΔCt due to activation of pretreatment samples was found to be normally distributed while the change in IFN-γ ΔCt was not. A one sample t test and a Wilcoxon Signed Rank test using PROC UNIVARIATE were conducted to determine if the changes due to activation were significant for IL-2 ΔCt and IFN-γ ΔCt,

respectively. The effect of day on each of IL-2 ΔCt and IFN-γ ΔCt were assessed by mixed model analysis using PROC MIXED, SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA). Separate models were made for each cytokine. For each model, day was included as the fixed effect and dog identity was included as a random effect. The effect of repeated measures of each dog over time was accounted for using a repeated statement with an autoregressive one correlation structure. If the effect of day was found to be significant, pairwise comparisons were made between the activated pretreatment value and each of the values for Day 1 to Day 7 using Dunnett's adjustment for multiple comparisons of least squares means. The distribution of the conditional residuals was evaluated for each mixed model to ensure the assumptions of the statistical method had been met. An alpha level of 0.05 was used to determine statistical significance.

Results

Cyclosporine Blood Concentrations

Day 7 peak (2 hours post-administration) blood cyclosporine concentrations are presented in Table 3.1. Peak drug concentrations ranged from 1,671 ng/ml to 3,052 ng/ml, with a median of 2,363ng/ml.

Table 3.1 Cyclosporine Blood Concentrations

Dog	Cyclosporine blood level (ng/mL)
1	2,545*
2	2,678*
3	1,671
4	2,182*
5	1,694
6	3,052*
7	1,949
8	2,983*

Peak (two hour post-administration) blood concentrations of cyclosporine in 8 dogs after 7 days of oral modified cyclosporine at 10mg/kg every 12 hours. Results labeled * indicate values were obtained by dilution.

qRT-PCR

Quantitative RT-PCR results are presented in Figures 3.1 and 3.2 for IL-2 (A) and IFN-γ (B). In Figure 3.1, ΔCt values are presented. A statistically significant increase in ΔCt value, which indicates decreased cytokine expression, was observed for pre-treatment un-activated samples and all days of cyclosporine treatment for both IL-2 and IFN-γ (p-value <0.0001). Cytokine expression is presented as a percentage of an activated pre-treatment sample taken prior to any drug administration in Figure 3.2. This shows the significant decrease in IL-2(A) and IFN-γ(B) levels induced by cyclosporine therapy. Statistical analysis was completed using ΔCt values. In Figure 3.2 an activated pre-treatment baseline sample represents 100% cytokine production (not represented in the figure). An un-activated baseline sample was significantly decreased for both IL-2 and IFN-γ expression compared to the pre-treatment activated baseline sample, indicating adequate activation of samples (p-value <0.0001). A significant decrease in both IL-2 and IFN-γ RNA production, compared to pre-treatment, was observed after only 24 hours of

cyclosporine therapy (P-value <0.0001). All subsequent days of treatment were found to have a statistically significant decrease in ΔCt value, compared to pre-treatment, in both IL-2 and IFN-γ expression (p-value <0.0001). Minimal variation was seen in cytokine production over the 7 days of cyclosporine administration.

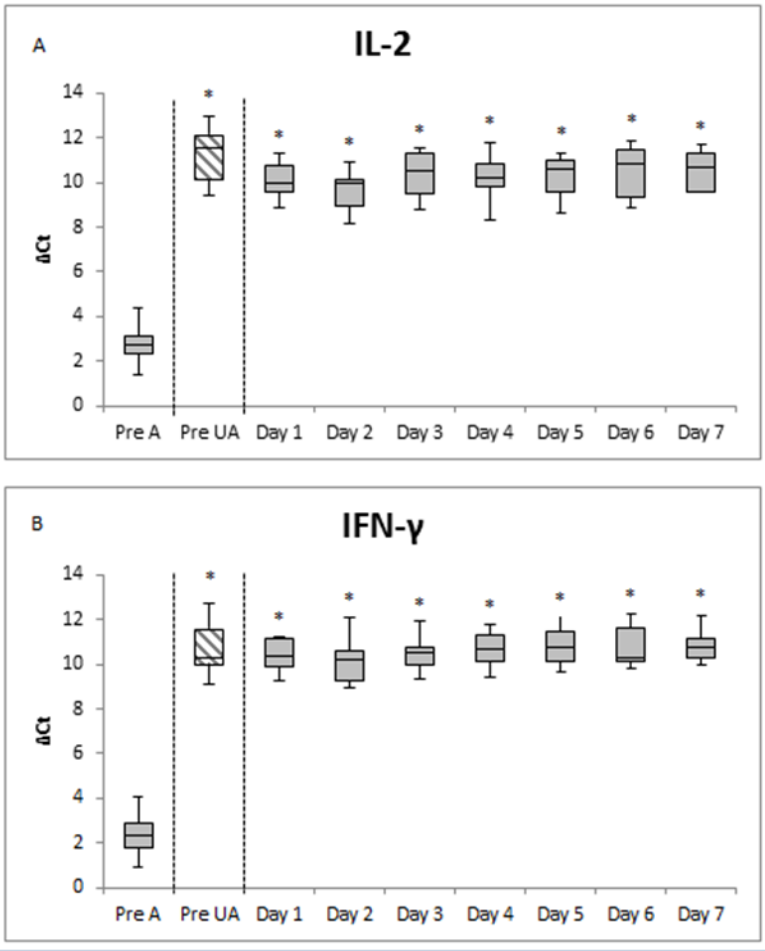

Figure 3.1 ΔCt Values for Interleukin-2 and Interferon-γ

Box plots of whole blood IL-2 (A) and IFN-γ (B) RNA expression for 8 healthy Walker hounds treated for 7 days with 10mg/kg cyclosporine every 12 hours. Expression is presented as ΔCt values where $\Delta Ct = Ct_{GOI} - Ct_{norm}$ in which GOI is the gene of interest and norm is the reference gene. The line within each box denotes the median, box edges represent the first and third quartiles, and whiskers extend to maximum and minimum values. Asterisks represent samples with a statistically significant change in ΔCt (p-value < 0.0001) compared to an activated pre-treatment sample. RT-qPCR was also performed without activation (un-activated) on pre-treatment samples for comparison with activated samples to ensure that adequate activation was achieved. (IL-2 = interleukin-2, IFN-γ = interferon-gamma, Pre A = pre-treatment activated, Pre UA = pre-treatment un-activated)

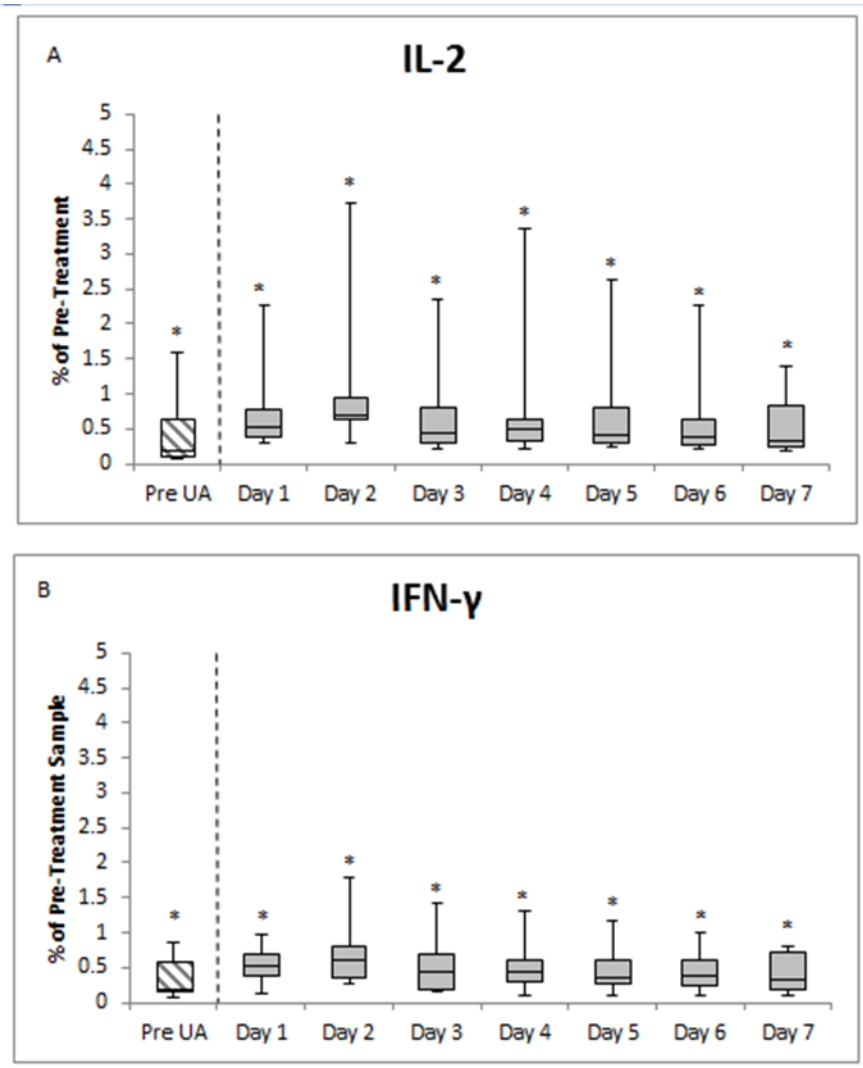

Figure 3.2 Percent of Pre-Treatment for Interleukin-2 and Interferon-γ

Box plots of whole blood IL-2 (A) and IFN-γ (B) RNA expression presented as a percentage of an activated pre-treatment baseline sample, in which the pre-treatment sample represents 100% cytokine production, for 8 healthy Walker hounds treated for 7 days with 10mg/kg cyclosporine every 12 hours. The line within each box denotes the median, box edges represent the first and third quartiles, and whiskers extend to maximum and minimum values. All samples were suppressed below 5% for IL-2 (A) and below 3% for IFN-γ (B) compared to an activated pre-treatment sample. Asterisks represent samples with a statistically significant change in ΔCt (p-value < 0.0001) compared to an activated pre-treatment sample. RT-qPCR was also performed without activation (un-activated) on pre-treatment samples for comparison with activated samples to ensure that adequate activation was achieved. (IL-2 = interleukin-2, IFN-γ = interferon-gamma, Pre UA = pre-treatment un-activated)

Discussion

Cyclosporine is used to treat a number of immune-mediated diseases in the dog, and until recently therapeutic monitoring has standardly consisted of measuring blood drug concentrations. A qRT-PCR based assay to assess activated T-cell cytokine production in canine patients undergoing cyclosporine therapy is now available, and has shown promise for use in clinical cases. However, ideal timing of sample collection for the qRT-PCR assay after commencement of cyclosporine therapy has not been established. A previous study demonstrated marked suppression of cytokine production after 7 days of therapy, but no testing occurred before the Day 7 testing. Thus, no study has been performed to show the daily effects of cyclosporine on T cells over the initial dosing period. The goal of this study was to determine ideal timing for sample collection after the initiation of cyclosporine treatment.

For this study, 8 healthy Walker hounds were given modified cyclosporine orally at a dose rate of 10mg/kg every 12 hours for 7 days. This dose rate has previously been established to reliably inhibit T-cell function in dogs.[8] Blood was collected 2 hours after oral dosing each day, and activated T-cell IL-2 and IFN-γ mRNA expression levels were measured via qRT-PCR. Two hours post-dosing has previously been established to reflect peak post-administration suppression of cytokine expression.[7,11] Cytokine production was observed to dropped dramatically within the first day of cyclosporine administration. By Day 1, median IL-2 expression had dropped to 0.53% of pre-treatment values (range 0.30% to 2.26%), and median IFN-γ expression had dropped to 0.40% of pre-treatment values (range 0.12% to 0.96%). This response is approximately 100 fold greater than the half maximal inhibitory concentration (IC_{50}) that is standardly used in pharmacodynamic

assays to measure of the effectiveness of a drug at inhibiting a specific biological function. A significant decrease in both IL-2 and IFN-γ expression, compared with pre-treatment values, was also observed in all subsequent days of therapy. A low degree of variability, as evidenced by narrow ranges between minimum and maximum values, was observed throughout the 7 days of the study. The results of this study suggest that activated T-cell IL-2 and IFN-γ RNA production is markedly decreased within 24 hours after the initiation of cyclosporine administration in healthy dogs, and that this effect is sustained throughout one week of drug administration.

Therapeutic drug monitoring (TDM) in dogs on immunosuppressive doses of cyclosporine is important for a number of reasons. Firstly, there is wide individual-to-individual variability in cyclosporine pharmacokinetics, and the same dose used in two different patients may not reach the same target blood concentrations. Secondly, a threshold drug concentration must be achieved to minimize the risk of treatment failure and, thirdly, toxicity must be avoided by ensuring trough concentrations are within a safe range.[12] Currently, TDM of canine patients on cyclosporine is often accomplished through measuring peak or trough blood drug concentrations collected 2 hours or 12-24 hours, respectively, after drug administration.[11,13] Doses are then adjusted until target therapeutic blood drug concentrations are achieved. Little research is available in dogs to support establishment of ideal target peak cyclosporine concentrations, and most recommendations for systemic immunosuppression are extrapolated from trough concentrations in renal transplant studies that utilize dogs.[3] This pharmacokinetic approach to TDM, while providing useful information, primarily provides a measurement of how the body is processing the drug, and does not necessarily reflect cyclosporine's

immunosuppressive effects. In fact, previous studies in dogs have established that, at comparable blood cyclosporine levels, activated T-cell expression of NFAT-regulated cytokines can be highly variable, thus further emphasizing the need for an individualized pharmacodynamic approach to clinical patients.[14]

Peak cyclosporine blood concentrations are presented in Table 3.1 for all 8 dogs included in the study. The average drug concentration for the group was 2,344ng/mL which is significantly higher than recommended target concentrations (800-1400ng/mL) provided by Auburn University Clinical Pharmacology Laboratory, but comparable to peak cyclosporine concentrations reported in previous studies using the same drug dose rate.[11] Previously, trough cyclosporine concentrations of 500ng/mL have been recommended in order to achieve adequate immunosuppression.[15] The relatively high peak drug concentrations could account for the significant decrease in cytokine expression observed in this study. There was significant variation in peak blood concentrations within the 8 dogs, with a range of 1,671 to 3,052ng/mL, representing a nearly 90% difference between dogs. This observation demonstrates the importance of monitoring dogs on cyclosporine therapy, since blood drug concentrations can vary significantly between dogs receiving similar oral dosages of cyclosporine. This variability could be the result of a number of factors, including individual variability in drug absorption and/or excretion mechanisms. This variation in blood drug concentrations, however, was not reflected in the cytokine assay, as cytokine expression swiftly decreased in all dogs after only 24 hours of cyclosporine administration and remained markedly suppressed for the remainder of the study. The consistency of the cytokine decrease could due to poor assay sensitivity for detecting minor changes in drug

concentrations at such high blood cyclosporine concentrations, or could indicate that cytokine expression is maximally suppressed with the given drug concentrations.

Previous research with dogs has shown a marked decrease in activated T cell IL-2 and IFN-γ expression with cyclosporine therapy. For TDM, a practical pharmacodynamic assay that measures actual T-cell responses to cyclosporine offers an attractive alternative to measuring blood drug concentrations in canine patients receiving cyclosporine.[4,8,9,11,16] A validated assay for dogs on cyclosporine therapy that assesses activated T-cell expression of NFAT-regulated cytokines has not been available until recently,[7] and there is still limited data on ideal timing for sample collection for monitoring therapy with this method. In this study, a significant decrease in cytokine production was observed after only 24 hours of cyclosporine therapy in healthy dogs. This trend was maintained on subsequent days of treatment over 7 consecutive days. Based on these results, TDM using the RT-qPCR assay can be commenced as early one day after commencing cyclosporine therapy.

Additional research is needed to assess the applicability of the qRT-PCR assay for clinical cases. Firstly, the dose rate used in this current study, 10 mg/kg twice daily, is relatively high. Based on observational clinical experience with samples submitted through our laboratory over the past few years, this dose rate markedly suppresses T-cell function in most (but not all) canine patients, and a lower oral dose rate of between 5 to 8 mg/kg twice daily is sufficient to adequately suppress T-cell function in most patients. We chose the dosage of 10 mg/kg to ensure reliable suppression of T-cell function for the purposes of our study, but it is possible that lower dose rates may take longer to effect. Secondly, anecdotal observations in a limited number of samples obtained over the first

week of cyclosporine therapy in several canine clinic patients suffering from severe immune-mediated diseases such as IMHA have suggested that maximal decreases in T-cell function may be delayed for several days compared to healthy dogs. These preliminary observations suggest that more time may be needed to adequately suppress cytokine production in clinical canine patients suffering from severe immune-mediated or inflammatory diseases. Further work is therefore warranted in clinical patients to best determine ideal timing for sample collection following commencement of cyclosporine therapy.

In conclusion rapid and sustained suppression of IL-2 and IFN-γ expression is observed after only 24 hours of cyclosporine administration in healthy dogs. As this assay is intended for use in clinical cases of immune-mediated and inflammatory-based diseases in which cyclosporine is used for treatment, the rapid decrease in cytokine expression may not be reflective of immune system response in these types of patients. Further research is therefore necessary in clinically affected animals to determine ideal sample collection timing after starting cyclosporine therapy.

Acknowledgements

This study was funded by the Dr. Hugh G. Ward Endowment. We would like to thank Dr. Dawn Boothe and the Auburn Clinical Pharmacology Laboratory for assistance with blood cyclosporine concentrations.

Conflict of Interest

The authors are affiliated with the Mississippi State University Pharmacodynamic Laboratory, which provides the assay evaluated in this study as a commercial service to veterinarians.

References

1. Nuttall TJ, Knight PA, McAleese SM, Lamb JR, Hill PB. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical and Experimental Allergy 2002;32:789-795.

2. Kjelgaard-Hansen M, Goggs, R., Wiinberg, B., Chan, D.L. Use of serum concentrations of interleukin-18 and monocyte chemoattractant protein-1 as prognostic indicators in primary immune-mediated hemolytic anemia in dogs. Journal of Veterinary Internal Medicine 2011;25:76-82.

3. Archer TM, Boothe DM, Langston VC, Fellman CL, Lunsford KV, Mackin AJ. Oral cyclosporine treatment in dogs: A review of the literature. Journal of Veterinary Internal Medicine 2014;28:1-20.

4. Tivers MS, Catchpole B, Gregory SP, House AK. Interleukin-2 and interferon-gamma mRNA expression in canine anal furunculosis lesions and the effect of ciclosporin therapy. Veterinary Immunology and Immunopathology 2008;125:31-36.

5. House A, Gregory SP, Catchpole B. Expression of cytokine mRNA in canine anal furunculosis lesions. Veterinary Record 2003;153:354-358.

6. Archer TM, Fellman CL, Stokes JV, Pinchuk LM, Lunsford KV, Pruett SB, Langston VC, Mackin AJ. Pharmacodynamic monitoring of canine T-cell cytokine responses to oral cyclosporine. Journal of Veterinary Internal Medicine 2011;25:1391-1397.

7. Riggs C, Archer T, Fellman C, Figueiredo AS, Follows J, Stokes J, Wills R, Mackin A, Bulla C. Analytical validation of a quantitative reverse transcriptase polymerase chain reaction assay for evaluation of T-cell targeted immunosuppressive therapy in the dog. Veterinary Immunology and Immunopathology 2013;156:229-234.

8. Fellman C, Archer TM, Stokes JV, Wills RW, Lunsford KV, Mackin AJ. Effects of oral cyclosporine on canine T-cell expression of IL-2 andIFN-gamma across a 12-h dosing interval. Journal of Veterinary Pharmacology and Therapeutics 2016;39:237-244.

9. Kobayashi T, Momoi Y, Iwasaki T. Cyclosporine A inhibits the mRNA expressions of IL-2, IL-4 and IFN-gamma, but not TNF-alpha, in canine mononuclear cells. Journal of Veterinary Medical Science 2007;69:887-892.

10. Livak KJ, Schmittgen, T.D. Analysis of relative gene expression data using real time quantitative PCR and the 2ΔΔCT method. Methods 2001;25:402-408.

11. Fellman CL, Archer TM, Stokes JV, Wills RW, Lunsford KV, Mackin AJ. Effects of oral cyclosporine on canine T-cell expression of IL-2 and IFN-gamma across a 12-h dosing interval. Journal of Veterinary Pharmacology and Therapeutics 2016;39:237-244.

12. Ptachcinski RJ, Venkataramanan, R., Burckart, G.J. Clinical pharmacokinetics of cyclosporin. Clinical Pharmacokinetics 1986;11:107-132.

13. Guaguere E, Steffan J, Olivry T. Cyclosporin A: a new drug in the field of canine dermatology. Veterinary Dermatology 2004;15:61-74.

14. Archer TM, Fellman CL, Stokes JV, Pinchuk LM, Lunsford KV, Pruett SB, Langston VC, Mackin AJ. Pharmacodynamic monitoring of canine T-cell cytokine responses to oral cyclosporine. Journal of Veterinary Internal Medicine 2011;25:1391-1397.

15. Daigle JC. More economical use of cyclosporine through combination drug therapy. Journal of the American Animal Hospital Association 2002;38:205-208.

16. Fellman CL, Stokes JV, Archer TM, Pinchuk LM, Lunsford KV, Mackin AJ. Cyclosporine A affects the in vitro expression of T cell activation-related molecules and cytokines in dogs. Veterinary Immunology and Immunopathology 2011 140:175-180.

CHAPTER IV

ACTIVATED WHOLE BLOOD CYTOKINE EXPRESSION IN HEALTHY DOGS COMPARED TO DOGS DIAGNOSED WITH INFLAMMATORY BOWEL DISEASE OR IMMUNE-MEDIATED HEMOLYTIC ANEMIA

Abstract

Immune-mediated diseases in dogs can be difficult to manage. Cyclosporine is an immunosuppressive agent being used with an increased frequency for treatment of a growing number of inflammatory and immune-based diseases in veterinary medicine. Cyclosporine is a potent T-cell inhibitor and, until recently, an objective assessment of its effects on target cells was not available. A PCR-based pharmacodynamic assay which measures cytokine production is now available to assist with determining ideal dosing protocols for canine patients with diseases such as immune-mediated hemolytic anemia (IMHA) and inflammatory bowel disease (IBD). In this study, expression of the T-cell cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) cytokine were determined via qRT-PCR, after an activation step, in canine patients diagnosed with either IMHA or IBD before receiving cyclosporine therapy, and compared to cytokine expression in healthy dogs. Both IL-2 and IFN-γ expression were found to be significantly decreased in IMHA patients compared to the other two groups. A significant difference was not observed between healthy dogs and those diagnosed with IBD. This finding did not support our original hypothesis that cytokine production would be increased in dogs with immune-

mediated diseases due to the pro-inflammatory nature of the disease pathogenesis. We suspect our finding may have been due to the effects of other immunosuppressant agents, since the only criteria for inclusion in the study population was that cyclosporine could not have been used for treatment.

Introduction

The immune system includes a complex organization of cells, antibodies and pro-inflammatory molecules with specific functions which are in place to protect the body from disease. Occasionally, however, these complex protective mechanisms can lead to the immune-mediated destruction of host tissues after a breakdown in self-tolerance. The cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) are pro-inflammatory molecules produced by lymphocytes within the immune system, primarily T cells, with IL-2 serving to promote B and T cell proliferation,[1] and IFN-γ serving to activate macrophages.[2] In the human literature, IL-2 and/or IFN-γ levels are increased in many immune-mediated conditions, including inflammatory bowel disease (IBD),[3,4] atopic dermatitis,[5,6] autoimmune hemolytic anemia (AIHA),[7,8] and immune-mediated thrombocytopenia (IMT).[9] This trend is paralleled in dogs, with IL-2 and/or IFN-γ levels increased in atopy,[10] immune-mediated hemolytic anemia (IMHA),[11,12] and anal furunculosis.[13,14] In contrast, IL-2 levels in cases of canine IBD are reported to vary, with some studies demonstrating increased levels,[15] some studies revealing decreased levels,[16] and other studies showing no significant change at all.[17]

Cyclosporine is a powerful immunosuppressive agent used in the field of veterinary medicine to treat an increasing number of inflammatory and immune-mediated diseases. Over the past few years, the Pharmacodynamic Laboratory at the Mississippi

State University College of Veterinary Medicine (MSU-CVM) has been developing useful biomarkers for monitoring cyclosporine therapy in canine clinical patients. Cyclosporine is a calcineurin inhibitor that serves to reduce T-cell production of nuclear factor of activated T cell (NFAT) related cytokines such as IL-2 and IFN-γ. Recently, we validated an assay that measures activated whole blood expression of the RNA coding for the cytokines IL-2 and IFN-γ via quantitative reverse transcription polymerase chain reaction (qRT-PCR) in healthy dogs,[18] and are now using this assay in clinical patients to assess the effectiveness of cyclosporine therapy, and to adjust doses accordingly.

This current study was undertaken in order to determine NFAT-related cytokine levels in canine patients with either IMHA or IBD before commencing cyclosporine therapy, and compare these to cytokine levels found in normal healthy dogs. The inflammatory bowel diseases are a group of gastrointestinal disorders characterized by inflammatory cell infiltrate of the gastrointestinal tract.[19] While the exact causes of IBD are not precisely determined, there is strong suspicion of underlying immune-mediated pathology.[20] IMHA is a type II hypersensitivity reaction in which antibodies target red blood cells for destruction. Compared to IBD, which is primarily localized to the gastrointestinal tract, IMHA is considered more of a systemic disease, since red blood cells distributed throughout the body are vulnerable to destruction. Given the inflammatory nature of both IBD and IMHA, we hypothesized that unactivated and activated T-cell production of IL-2 and IFN-γ would be higher in dogs with these diseases compared to normal dogs. Given the more systemic nature of IMHA, we also suspected that NFAT-regulated cytokine expression would be higher in dogs with IMHA compared to those suffering from IBD. Both IBD and IMHA in dogs are commonly

treated with cyclosporine. Since baseline (pre-treatment levels) of cytokine expression have the potential to have an impact on levels following treatment with cyclosporine, our study was designed to determine if baseline NFAT-regulated cytokine expression was different in dogs with IMHA or IBD compared to normal dogs.

Materials and Methods

Patient Selection

Nine adult female Walker hounds, part of a research dog colony, were used for the healthy control treatment group. Clinical patients with either IBD or IMHA were recruited for this study over a period of 3 years. Dogs diagnosed with IBD or IMHA were either recruited from MSU-CVM Small Animal Internal Medicine service, or from other boarded veterinary internal medicine specialists throughout North America. Samples received from specialists outside of MSU-CVM were shipped to the Pharmacodynamic Laboratory overnight on ice. The effect of shipping and handling on sample analysis was addressed in a previous study, and provided samples were processed within 2 days of collection and stored and transported on ice, shipping and handling was found to have no significant impact on results.[18] All subjects included in the study had yet to receive a dose of cyclosporine. Informed owner consent was obtained for all patients before inclusion in the study. IBD cases were confirmed via endoscopic biopsy of the gastrointestinal tract with histopathology evaluated by a board certified pathologist. IMHA cases were diagnosed based on standard clinical criteria with no evidence of secondary causes of anemia identified.

Blood Sample Collection

Approximately 3mL of whole blood was collected from each dog from the jugular vein with a needle and syringe and immediately inserted into a heparin tube to prevent coagulation. Samples were stored at 4^{O}C or shipped on ice before cell activation.

T-Cell Activation

Whole blood samples were activated using the protocol previously described by Riggs et al., with the reagents phorbol myristate acetate (PMA; 12.5 ng/mL) (Sigma, St.Louis, MO, USA Cat no. P8139-1MG) and ionomycin (0.8 µM) (Sigma, St. Louis, MO, USA Cat no. I0634-1MG).[18] Samples were then incubated for 5 hours at 37°C with 5% CO_2.

RNA Isolation

Isolation of total RNA from samples (both activated and unactivated) was completed using the protocol previously described by Riggs et al.[18] A QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA Cat. No. 52304) was used per manufacturer's instructions. An on-column DNase (27.27 Kunitz units) treatment (Qiagen, Valencia, CA, USA Cat. No. 79254) removed potential contaminating DNA. To measure RNA concentration, a Nanodrop ND-1000 spectrophotometer using the ND-1000 V3.3.0 software (NanoDrop Technologies, Wilmington DE, USA) was used. Samples were stored at -80°C until PCR analysis.

Cytokine Gene Expression Quantification

Expression of cytokines of interest was assessed in healthy dogs, and in those diagnosed with either IBD or IMHA. IL-2 and IFN-γ levels were measured with qRT-PCR. GAPDH was included in the study as the reference gene. Primers used for the genes included in the study were based on previously reported nucleotide sequences by Kobayashi et al.[21] A SuperScript™ III Platinum® SYBR® Green One-Step RT-qPCR kit containing the reference dye ROX (Invitrogen, Grand Island, NY, USA Cat no. 11736-059) was used to quantify GAPDH, IL-2, and IFN-γ expression. The reactions were analyzed with a Stratagene Mx3500P Multiplex Quantitative PCR system (Agilent Technologies) using Mx Pro software for analysis. All reactions had a final volume of 20µL containing a total of 15ng of template RNA and 200nM of each primer. Thermal cycling parameters were followed as previously published by Riggs et al.[18] All samples were run in triplicate while non-template controls were run in duplicate. To compare gene expression, ΔCt values were determined for each individual dog using GAPDH as a reference gene where $\Delta Ct = Ct_{GOI} - Ct_{norm.}$ GOI is the gene of interest and norm is the reference gene. The difference between gene expression in activated and unactivated samples for each group was also calculated as the ΔΔCt, where ΔΔCt = ΔCt Unactivated - ΔCt Activated. The Ct value represents the cycle number at which sample fluorescence reaches a threshold level, therefore samples with a lower Ct value require fewer cycles to for the target nucleotides to be detected, and consequently have higher gene expression compared to a sample with a higher Ct value.

Statistical Analysis

The effects of activation, group (normal, IBD, IMHA), and their interaction on IL-2 ΔCt and IFN-γ ΔCt were tested in separate linear mixed models using PROC MIXED in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). Dog identity was included in the models as a random effect. Separate linear models were also fit to determine if there were differences among the three treatment groups for IL-2 ΔΔCt and IFN-γ ΔΔCt using PROC MIXED. Differences in least squares means with the simulate adjustment for multiple comparisons were determined for significant main effects or interaction terms. Diagnostic plots of residuals for each outcome were assessed to ensure the assumptions of the statistical method had been met. An alpha level of 0.05 was used in determining the significance of results.

Results

Patient Profiles

Age, sex, and breed of dogs included in the study are presented in Table 4.1.

Nine healthy female Walker hounds from a research colony were used for the control group. The group had a median age of 4 years with a range of 2-9 years. All were intact females (n=9).

Nine patients were recruited into the IBD group, with a median age of 6 years, ranging from 21 months to 13 years. Within this group, 4 dogs were neutered males, 1 was an intact male, and 4 were spayed females. Breeds represented included Yorkshire terrier (n=2), dachshund (n=1), Shih Tzu (n=1), rat terrier (n=1), beagle (n=1), pit bull terrier (n=1), goldendoodle (n=1), and boxer (n=1). Cases were confirmed via endoscopic biopsy of the gastrointestinal tract with histopathology evaluated by a board

certified pathologist. As per study requirements, none of these patients had received any cyclosporine, however some had received other immunosuppressive agents, including prednisone (n=2), azathioprine (n=1), or zonisamide (n=1).

Nine patients were recruited into the IMHA group, with a median age of 7 years, ranging from 3 to 8 years. Within this group, there were 3 neutered males, 1 intact male, and 5 spayed females. Breeds represented included dachshund (n=2), Newfoundland (n=1), Maltese (n=1), pit bull terrier (n=1), cattle dog cross (n=1), beagle (n=1), Doberman pinscher (n=1), and miniature schnauzer (n=1). Diagnosis was made by the presence of evidence of an immune-mediated process against red blood cells (either spherocytosis, slide agglutination positive, or Coomb's test positive) as well as no other evidence of a disease process causing the anemia. As per study requirements, no patients in this group had received any cyclosporine, however some were already receiving other immunosuppressive agents, including prednisolone (n=1), dexamethasone (n=1), and/or azathioprine (n=1).

Table 4.1 Case Profiles

Diagnosis	Age (years)		Sex				Number of Breeds Represented
	Median	Range	MN	MI	FS	FI	
IBD	6	1-13	4	1	4	0	8
IMHA	7	3-8	3	1	0	5	8
Healthy	4	2-9	0	0	0	9	1

Profiles of cases (n = 9 per treatment group) selected for study denoting age, sex, and breeds included. (IBD = inflammatory bowel disease, IMHA = immune-mediated hemolytic anemia, MN = male neutered, MI = male intact, FS = female spayed, FI = female intact).

Interleukin-2

Interleukin-2 RNA expression quantified via qRT-PCR for all three groups is presented in Figure 4.1 (A). P-values for comparison between group ΔCt values are

presented in Table 4.2. A statistically significant difference was found between activated and unactivated samples for all groups (p-value < 0.0001), indicating adequate activation of all samples. Activated samples had a significantly higher expression level of IL-2, denoted by a lower ΔCt value. Patients diagnosed with IMHA were found to have a significantly lower IL-2 production (higher ΔCt value) in activated samples compared to healthy dogs (p-value 0.046) and those with IBD (p-value < 0.0005). This same difference was observed for unactivated samples as well for both healthy (p-value 0.0006) and IBD (p-value 0.0029) group comparisons. No significant difference was found between healthy dogs and those with IBD for activated samples (p-value = 1) nor for unactivated samples (p-value < 0.0789).

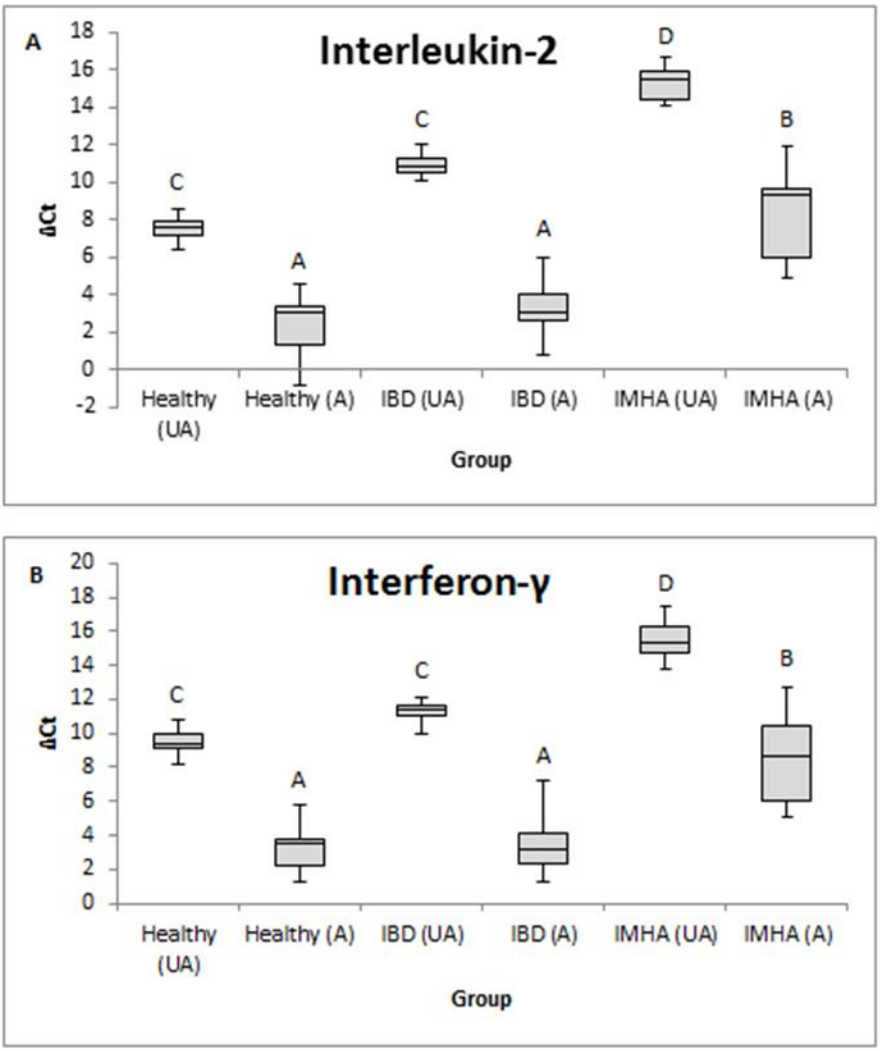

Figure 4.1 Interleukin-2 and Interferon-γ ΔC_T

Box and whisker plots of IL-2 (A) and IFN-γ (B) RNA expression from canine whole blood presented as ΔCt value where ΔCt = Ct IL2- Ct GAPDH in 9 dogs grouped as either healthy or diagnosed with IBD or IMHA. A lower ΔCt represents a higher level of gene expression. Lines within each box represent the median, box bottom and top represent the first and third quartiles respectively, and whiskers range to maximum and minimum values obtained. In groups labeled with the same letter, a statistically significant difference was not found (p-value >0.05). (UA = unactivated, A = activated, IBD = inflammatory bowel disease, IMHA = immune-mediated hemolytic anemia, IL-2 = interleukin-2).

Interferon-γ

Interferon-γ RNA expression measured by qRT-PCR for all three groups is presented in Figure 4.1 (B). P-values for comparison between group ΔCt values are presented in Table 4.2. A statistically significant difference was found between activated and unactivated samples for all groups (p-value <0.0001), indicating adequate activation of all samples. All activated samples had a lower ΔCt value, indicating increased level of expression of IFN-γ in relation to unactivated samples. Dogs in the IMHA group had the lowest IFN-γ production (highest ΔCt value) compared to the IBD group (p-value 0.0010) and healthy dogs (p-value 0.0406). No difference was seen between healthy dogs and those with IBD (p-value = 0.9965).

Table 4.2 ΔC_T P-values

Comparison of ΔC_T	P-value			
	IL-2		IFN-γ	
	Act	Un-Act	Act	Un-Act
Healthy v IBD	1	0.0789	0.9965	0.9965
Healthy V IMHA	0.0456	0.0006	0.0406	0.0406
IBD v IMHA	0.0005	0.0029	0.0010	0.0010

P-values for ΔC_T comparison between treatment groups. An alpha level of 0.05 was used in determining the significance of results. (UA = unactivated, A = activated, IBD = inflammatory bowel disease, IMHA = immune-mediated hemolytic anemia, IL-2 = interleukin-2, IFN-γ = interferon-γ)

Differences Between Activated and Unactivated Samples

Figure 4.2 depicts the difference between activated and unactivated samples for each group, measured as ΔΔCt. A higher ΔΔCt value indicates a larger difference between activated and unactivated samples. Dogs with IBD were found to have a higher difference between activated and unactivated samples compared to the healthy control group for IL-2 (p-value = 0.0279). No difference was found when comparing healthy

dogs to those with IMHA (p-value = 0.0875) or when comparing dogs with IBD to those with IMHA (p-value 0.8521). A significant difference was not found for IFN-γ across the three groups (Figure 4.2) (p-value = 0.4218).

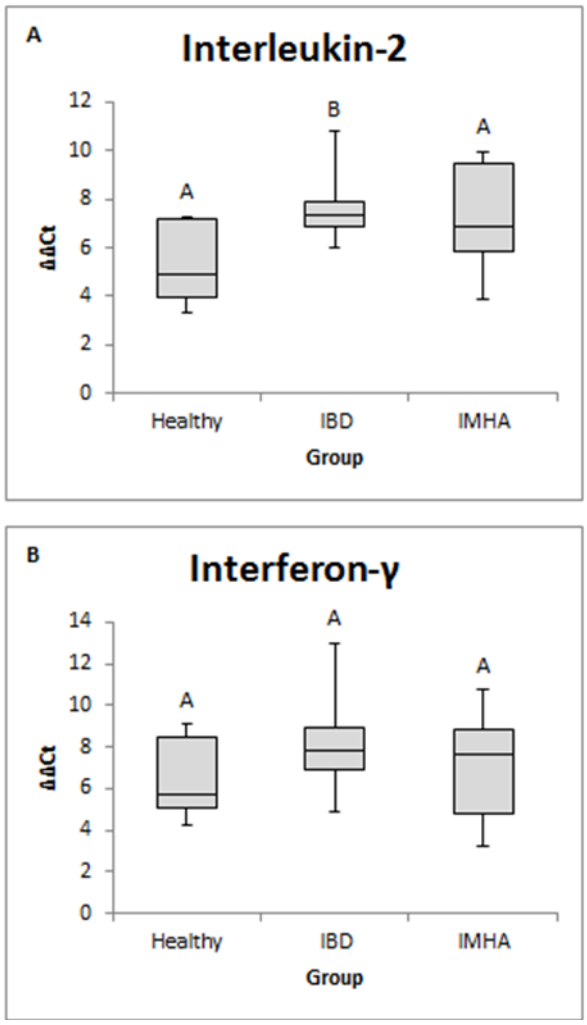

Figure 4.2 Interleukin-2 and Interferon-γ $\Delta\Delta C_T$

Box and whisker plots of difference between activated and unactivated IL-2 (A) and IFN-γ (B) expression from canine whole blood presented as ΔΔCt value where ΔΔCt = ΔCt UA - ΔCt A in 9 dogs grouped as either healthy or diagnosed with IBD or IMHA. A higher ΔΔCt value indicates a larger difference between activated and unactivated samples. Lines within each box represent the median, box bottom and top represent the first and third quartiles respectively, and whiskers range to maximum and minimum values obtained. Treatments that share the same letter are not significantly different. No difference was found between treatment groups for IFN-γ (p-value = 0.42). (UA = unactivated, A = activated, IBD = inflammatory bowel disease, IMHA = immune-mediated hemolytic anemia, IL-2 = interleukin-2, IFN-γ = interferon-γ)

Discussion

In this study, unactivated and activated whole blood expression of the RNA coding for the NFAT regulated cytokines IL-2 and IFN-γ was compared between healthy dogs and dogs diagnosed with either IMHA or IBD prior to the onset of cyclosporine therapy. IBD and IMHA were selected so that cytokine expression could be compared between a relatively localized immune-mediated disease process (IBD) and a process that would be expected to affect the patient on a more systemic level (IMHA).

IBD is characterized by chronic inflammation of the gastrointestinal tract. While it is a relatively localized disease compared to IMHA, systemic biomarkers of inflammation, such as C-reactive protein (CRP), are known to be increased in canine IBD patients. C-reactive protein is a positive acute phase protein produced by the liver in response to inflammation. Assays that measure CRP concentration are already in use for monitoring canine IBD progression and response to treatment. It is therefore reasonable to assume that T cells in the systemic circulation in dogs with IBD would be more reactive, and thus have higher cytokine production, compared to clinically normal dogs. Previous research on NFAT-regulated cytokine production in canine patients with IBD has yielded mixed results. The majority of experiments quantified cytokines from intestinal biopsy samples and thus reflect cytokine expression at a local level. No study has focused on activated whole blood samples, which are more reflective of systemic T-cell function. Some studies of local gastrointestinal cytokines have found increased cytokine production,[15] some studies have found decreased production,[16] and other studies have found minimal change[17] when compared to a healthy control population. Peters et al. found no difference in local IL-2 or IFN- γ expression when compared to a control

population. However, the study population included both patients with IBD and patients with antibiotic-responsive diarrhea, and the control group used included dogs undergoing endoscopy for gastroesophageal disease or euthanized for other clinical reasons, and therefore could potentially have had high cytokine levels compared to normal healthy animals. In a more recent study by Jergens et al., decreased local IL-2 and IFN- γ levels were found in dogs with small intestinal IBD when compared to control dogs. This study had a relatively large sample population (n=48) compared to previous reports, and evaluated both small intestinal and colonic biopsy samples. However one limitation to the study was the use of semi-quantitative RT-PCR, which is known to be less sensitive and not as accurate as real-time RT-PCR.[22] None of the previously described studies measured cytokine expression of activated T cells in dogs with IBD. T-cell activation can help to unmask cytokine expression differences that may not be as apparent in unactivated samples, since activation causes maximal cytokine expression. In our study, utilizing activation with PMA and ionomycin in order to maximize T-cell cytokine production, a significant difference in ΔC_T value was not found between patients diagnosed with IBD and healthy control dogs for either IL-2 or IFN-γ expression. IBD is a relatively localized disease, therefore systemic cytokine levels may not be affected since our assay measures whole blood cytokine expression levels, and may be more reflective of systemic inflammatory responses. Although none of the tested patients had received cyclosporine, other immunosuppressive agents the patients received included prednisone (n=2), azathioprine (n=1), or zonisamide (n=1), and this may also have had an effect on cytokine production. Another explanation for the observed lack of difference between normal dogs and dogs with IBD may be related to disease duration in patients

included in the study. Cytokine patterns are known to change with time in human Crohn's disease[23] therefore it is possible that the IBD patients included in this study were in the early phase of disease, and would have cytokine levels more comparable to a healthy animal.

IMHA is a type II hypersensitivity reaction in which the immune system targets red blood cells for destruction. Previous studies measuring IL-2 production in IMHA patients have found increased levels of NFAT-regulated cytokines such as IL-2.[7,8,11,12] Abnormal cytokine levels in cases of IMHA have been reported in both human and veterinary literature. A report by Fagiolo and Toerenzi found increased IL-2 in both activated and unactivated supernatant samples from humans with autoimmune hemolytic anemia (AIHA) prior to any pharmacological therapy.[8] Comparatively, IFN-γ levels were not statistically different when compared to a healthy control group. Studies in canine patients have yielded similar results,[12,24] although none have analyzed activated whole blood samples, which as previously mentioned maximizes T cell cytokine production. One study by Johnson et al. analyzed serum cytokine levels via Luminex multiplex bead assay, and found elevated expression levels of T cell cytokines in both IMHA and sepsis patients. IFN-γ levels were not evaluated in this study. Due to the limited power of the study, however, individual cytokine levels could not be compared between study groups. A more recent report by Swann et al. used qRT-PCR to measure IFN-γ and chemiluminescent assays to measure serum IL-2 in dogs with IMHA. Dogs with IMHA had not received any immunosuppressive therapy, and were found to have significantly higher IL-2, but no difference in IFN-γ production, compared to healthy control animals. In our study, in contrast, cytokine production was found to be significantly reduced

within activated samples from dogs with IMHA compared to the healthy dog population or the IBD group. This finding is unexpected, since our original hypothesis was that baseline cytokine production would be increased in patients with a systemic disease as IMHA when compared to a healthy control group. We theorize that the observed trend was in part due to the criteria for case selection. For inclusion in this study, cases were only excluded if cyclosporine had been started, however use of other immunosuppressive agents was allowed. The assay used in this study has been shown to primarily reflect the pharmacodynamic effects of cyclosporine since it measures the drug's primary molecular targets and, in human medicine, this assay is typically considered to be unaffected by the presence of other immunosuppressive drugs. The effects of steroids and other immunosuppressive agents such as azathioprine on this specific assay, however, have yet to be fully explored in dogs. It is possible that the other immunosuppressive medications administered to some of the IMHA patients prior to cyclosporine had an effect on cytokine production, leading to the observed decrease seen in this group as compared to the normal dog population. Further research in this area is therefore warranted to explore the potential effects of drugs other than cyclosporine on our activated whole blood cytokine expression assay. Another possible explanation for the observed trend could be due to difference in circulating T-cell number between healthy dogs and those with IMHA. IMHA patients typically present with lymphopenia and patients included in the study had a lymphocyte count ranging from 226-535/μL (n=5) which was below the reference range of 1000-1400/ul. It is possible that the low lymphocyte count contributed to the reduced IL-2 and IFN-γ expression observed in this study as higher cytokine expression levels would be expected with an increasing lymphocyte count.

When differences between activated and unactivated samples were compared between the study groups, for IL-2 expression healthy dogs were found to have a smaller change following activation compared to dogs with IBD. No difference was found between IBD and IMHA study populations or between healthy dogs and IMHA groups. Our results suggest that T cells in dogs with IBD have a higher potential for activation compared to normal dogs. We suspect that the lack of the same detectable effect in dogs with IMHA may be due to the effects of other immunosuppressive drugs already being used for treatment, leading to reduced T cell susceptibility to activation or is the result of lymphopenia since a low circulating T-cell number would not be able to produce as many pro-inflammatory cytokines. Preliminary observations in our laboratory in clinical samples from dogs that have already started cyclosporine tends to mirror this trend, as dogs on lower cyclosporine doses have a tendency to have a greater difference between activated and unactivated samples compared to dogs on high cyclosporine doses, in which there is maximal suppression of T cells and thus minimal difference between activated and unactivated IL-2 expression. While this effect is expected in dogs receiving cyclosporine, it was unexpected in dogs receiving glucocorticoids and other immunosuppressive agents, and warrants further exploration. This same trend was not observed with IFN-γ, since no difference was detected between study group ΔΔCt values.

The assay used in this study is currently being used in clinical patients to monitor immunosuppression in patients on cyclosporine. Whole blood sample IL-2 expression collected after starting cyclosporine treatment is compared to a baseline or pre-treatment sample when such a sample is available. The decrease in IL-2 reflected in ΔΔCt values is used as a marker of immune system suppression in the patient, to determine if

cyclosporine is exhibiting its maximal effect. Recommendations on adjusting cyclosporine dosage are then made based on assay interpretation. The pathogenesis of disease and pharmacokinetics of cyclosporine are both considered with assay interpretation. For example, many cases of canine atopy receiving cyclosporine display relatively low levels of cytokine suppression using the assay, even though clinically patients are responding well to therapy. This is most likely due to concentration of cyclosporine in the skin, with locally high levels of the drug suppressing cytokine production, a phenomenon which would not be adequately reflected in a more systemic assay. A similar pattern is seen with cases of IBD, and we suspect this is also due to a local effect of cyclosporine at the level of the gastrointestinal tract, an effect that is not reflected in our assay.

Since obtaining a pre-treatment sample can be difficult, our assay can also use unactivated samples from the same patient during treatment for comparison. Activating T cells as part of our assay allows for maximum cytokine production and, since effective cyclosporine treatment markedly suppresses cytokine production, even in activated cells, activation serves to highlight the effects of the drug. Comparing unactivated and activated samples post-treatment, rather than pre- and post-treatment samples from the same patient, eliminates the need for pre-treatment samples. Elimination of the need for a pre-treatment sample is more convenient for veterinarians, who often don't have the opportunity to collect samples prior to therapy. Additionally, variations in baseline assay results seen with some diseases prior therapy, as was seen with the IMHA patients in this study, has the potential to have less impact on final result if pre-treatment samples are not used to calculate post-treatment T-cell responses.

Our study was the first study to use a PCR-based technique on activated samples to assess the effect of diseases such as IMHA or IBD on T-cell cytokine production. Although our assay measured the RNA coding for NFAT-regulated cytokines, and not actual protein levels, results from previous research comparing this technique to those that measure cytokine amounts have comparable results.[25] The use of qRT-PCR eliminates the challenge of finding canine antibodies which are not as widely available, compared to primers, which can be designed for potentially any gene of interest. This technique also does not require immediate analysis, as RNA can be stored for batch analysis.

In conclusion, our study found decreased expression of the NFAT-regulated cytokines IL-2 and IFN-γ in canine patients with IMHA compared to a healthy control group. These results are in contrast to previous studies which observed increased IL-2 expression in IMHA study populations although, since our study is the first to add an activation step, results may not be directly comparable. Dogs diagnosed with IBD were found to have cytokine levels similar to the healthy control population. Our results are complicated by the fact that some patients with IBD or IMHA received immunosuppressive agents other than cyclosporine. More research is needed to determine the effects of immunosuppressive agents other than cyclosporine on activated T-cell cytokine production in normal dogs, and in dogs with immune-mediated diseases. This study addressed two immune-mediated diseases, but there are a number of other immune-mediated conditions in veterinary medicine in which cyclosporine is prescribed, including atopy, immune-mediated thrombocytopenia, and anal furunculosis. Further research into cytokine profiles of patients with these other conditions is also warranted.

Acknowledgements

This study was funded by the Dr. Hugh G. Ward Endowment.

References

1. Whittington R, Faulds, D. Interleukin-2. Drugs 1993;46:446-514.

2. Kumar Vinay AKA, Nelson Fausto, Jon C. Aster. Robbins and Cotran Pathologic Basis of Disease, 8th ed. Philadelphia: Saunders; 2010.

3. Niessner M, Volk, B.A. Altered Th1/Th2 cytokine profiles in the intestinal mucosa of patients with inflammatory bowel disease as assessed by quantitative reversed transcribed polymerase chain reaction (RT-PCR). Clinical and Experimental Immunology 1995;101:428-435.

4. Breese E, Braegger CP, Corrigan CJ, Walker-Smith JA, MacDonald TT. Interleukin-2 and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. Immunology 1993;78:127-131.

5. Szczepański M, Kaczmarski M. The level of interleukine-2 (IL-2) in blood serum in children with food sensitive atopic dermatitis. Roczniki Akademii Medycznej W Białymstoku 1995;40:692-695.

6. Werfel T, Morita A., Grewe M, Renz H, Wahn U, Krutmann J, Kapp A. Allergen specificity of skin-infiltrating T cells is not restricted to a type-2 cytokine pattern in chronic skin lesions of atopic dermatitis. Journal of Investigative Dermatology 1996;107:871-876.

7. Fagiolo E, Vigevani F, Pozzetto U. High cytokine serum levels in patients with autoimmune hemolytic anemia. Immunological Investigations 1994;23:449-456.

8. Fagiolo E, Toriani-Terenzi C. Th1 and Th2 cytokine modulation by IL-10/IL-12 imbalance in autoimmune haemolytic anemia (AIHA). Autoimmunity 2002;35:39-44.

9. Rocha AM, Souza C, Rocha GA, de Melo FF, Clementino NC, Marino MC, Bozzi A, Silva ML, Martins Filho OA, Queiroz DM. The levels of IL-17A and of cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia. Haematologica 2011;96:1560-1564.

10. Nuttall TJ, Knight PA, McAleese SM, Lamb JR, Hill PB. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical and Experimental Allergy 2002;32:789-795.

11. Kjelgaard-Hansen M, Goggs R, Wiinberg B, Chan DL. Use of serum concentrations of interleukin-18 and monocyte chemoattractant protein-1 as prognostic indicators in primary immune-mediated hemolytic anemia in dogs. Journal of Veterinary Internal Medicine 2011;25:76-82.

12. Johnson V, Burgess B, Morley P, Bragg R, Avery A, Dow S. Comparison of cytokine responses between dogs with sepsis and dogs with IMHA. Veterinary Immunology and Immunopathology 2016;180:15-20.

13. Tivers MS, Catchpole B, Gregory SP, House AK. Interleukin-2 and interferon-gamma mRNA expression in canine anal furunculosis lesions and the effect of ciclosporin therapy. Veterinary Immunology and Immunopathology 2008;125:31-36.

14. House A, Gregory SP, Catchpole B. Expression of cytokine mRNA in canine anal furunculosis lesions. Veterinary Records 2003;153:354-358.

15. Ridyard AE, Nuttal TJ, Else RW, Simpson JW, Miller HRP. Evaluation of Th1, Th2 and immunosuppressive cytokine mRNA expression within the colonic mucosa of dogs with idiopathic lymphocytic-plasmacytic colitis. Veterinary Immunopathology 2002;86:205-214.

16. Jergens AE, Sonea IM, O'Connor AM, Kauffman LK, Grozdanic SD, Ackermann MR, Evans RB. Intestinal cytokine mRNA expression in canine inflammatory bowel disease: a meta-analysis with critical appraisal. Comparative Medicine 2009;59:153-162.

17. Peters IR, Helps CR, Calvers EL, Hall EJ, Day MJ. Cytokine mRNA quantification in duodenal mucosa from dogs with chronic enteropathies by real-time reverse transcriptase polymerase chain reaction. Journal of Veterinary Internal Medicine 2005;19:644-653.

18. Riggs C, Archer T, Fellman C, Figueiredo AS, Follows J, Stokes J, Wills R, Mackin A, Bulla C. Analytical validation of a quantitative reverse transcriptase polymerase chain reaction assay for evaluation of T-cell targeted immunosuppressive therapy in the dog. Veterinary Immunology and Immunopathology 2013;156:229-234.

19. Guilford WG. Strombeck's Small Animal Gastroenterology, 3 ed. Philadelphia, PA: WB Saunders; 1996.

20. German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. Journal of Veterinary Internal Medicine 2003;17:8-20.

21. Kobayashi T, Momoi Y, Iwasaki T. Cyclosporine A inhibits the mRNA expressions of IL-2, IL-4 and IFN-gamma, but not TNF-alpha, in canine mononuclear cells. Journal of Veterinary Medical Science 2007;69:887-892.

22. Bustin S, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR--a perspective. Journal of Molecular Endocrinology 2005;34:597-601.

23. Desreumaux P, Brandt E, Gambiez L, Emilie D, Geboes K, Klein O, Ectors N, Cortot A, Capron M, Colombel JF. Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease. Gastroenterology 1997;113:118-126.

24. Swann J, Woods K, Wu Y, Glanemann B, Garden OA. Characterisation of the Immunophenotype of Dogs with Primary Immune-Mediated Haemolytic Anemia. PLoS ONE 2016;11.

25. Flores MG, Zhang S, Ha A, Holm B, Reitz BA, Morris RE, Borie DC. In vitro evaluation of the effects of candidate immunosuppressive drugs: flow cytometry and quantitative real-time PCR as two independent and correlated read-outs. Journal of Immunological Methods 2004;289:123-135.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Inflammatory or immune-mediated diseases in dogs often require chronic or lifelong treatment with immunosuppressive agents. Such conditions can also be headaches to manage for both owner and veterinarian, since it can be challenging to determine the perfect combination of drugs needed to quell the immune system. Cyclosporine has been used to treat a number of immune-mediated diseases in veterinary medicine; however it is difficult to predict patient responses to the drug since not all animals are adequately suppressed with the same oral dose rate. In this dissertation, a pharmacodynamic assay was developed that allows clinicians to determine if the given dose of cyclosporine is appropriate for the individual canine patient. The assay utilizes qRT-PCR to quantify activated T-cell production of interleukin-2 (IL-2) and interferon-γ (IFN-γ), and the suppression of these cytokines is used to assess patient responses to cyclosporine. The assay was validated for use in clinical patients by first analyzing the effect of shipping and handling on samples. A seven day study in healthy dogs was then used to determine ideal timing of sample collection for pharmacodynamic monitoring after commencement of therapy. Finally, pre-treatment levels of cytokine expression in dogs with several common immune-mediated diseases were evaluated, in order to determine whether pre-existing disease affected cytokine levels.

As our laboratory has gathered more samples and data associated with using our assay, our group realized the need to modify the way we approached data analysis. The assay was originally developed and validated to measure two cytokines, IL-2 and IFN-γ, both of which are known to be suppressed by cyclosporine. We found that, when quantifying these in clinical cases, IFN-γ tended to be quite variable and did not correlate well clinically with the case outcome and reported response to therapy, while IL-2 tended to provide more predictable results. This led to the laboratory moving to using only IL-2 for analysis. We also initially hypothesized that a pre-treatment sample would be required to adequately determine the level of suppression for a given patient, and would need to be available for comparison with a post-treatment sample collected after the start of cyclosporine therapy. However, we found that we could get consistently similar interpretation of final results by comparing patient post-treatment results to a healthy dog control sample. This made coordination of sample collection much easier for practicing clinicians, who tended to forget to take a blood sample before starting therapy. Our initial data interpretation involved assessing $\Delta\Delta C_T$, which takes into account either results from a pre-treatment sample or a healthy control dog in order to compare cytokine production with and without exposure to cyclosporine. However, we found that this did not correlate well with the reported clinical case outcome, and thus shifted our focus to ΔC_T, which does not require a pre-treatment sample. Samples are instead compared to a healthy control dog run on the same plate. We also now compare the change in ΔC_T value between an activated and unactivated sample and analyze the change in activation in the clinical patient receiving cyclosporine compared to a dog with a normal functioning immune system. Based on the change in activation, patients are categorized into a certain

subcategory which denotes the amount of suppression observed in our assay. Subsequent treatment recommendations take into account not only the patient's assay results, but also clinical signs, cyclosporine dose, and other immunosuppressive agents on board. This approach truly allows for individual tailoring of drug therapy. Over the several years of offering this assay, and consulting with veterinarians regarding results, our laboratory has gradually developed the clinical experience needed to generate target levels of cytokine expression that correlate with clinical outcome.

Development of the assay was not without its challenges. There are many components that must fit together in order for the assay to function. As mentioned before, we initially believed that a pre-treatment sample would need to be included for proper analysis, however we quickly determined that collection of these samples was difficult for the busy clinician, and therefore we adapted our analysis to fit their needs by analyzing samples by their change in activation. Another issue encountered was activator quality. All samples are activated upon arrival to the laboratory. Our analysis assesses the change in activation within samples; therefore activators must be working appropriately for an accurate interpretation to be made. Occasionally, unexpectedly poor activation of both patients and control dogs could be traced back to poor activator quality. This issue led to the initiation of quality control measures including running samples from a healthy control dog as well as non-template control wells on every plate to ensure all components of the assay, including activators, were working correctly.

While the development of our assay has assisted veterinarians with patient management, there are still a number of questions yet to answer. The functionality of the assay has yet to be explored for agents other than cyclosporine. Although the mechanism

used in this assay is reported to be specific for cyclosporine and other calcineurin inhibitors, such as tacrolimus, it may be worth exploring the assay in patients on other immunosuppressants. Many patients receiving cyclosporine are also receiving concurrent glucocorticoids and other immunosuppressive agents and, although the concurrent use of other drugs is considered to have minimal impact on cytokine expression results in human patients, an understanding of the impact of other drugs on the assay in dogs would assist with interpretation of results. Similar assays could also be developed that are specific for other immunosuppressive drug mechanisms of action and that quantify different cytokines or other molecules. Our laboratory has to date focused on IL-2 and IFN-γ, both of which are considered pro-inflammatory. Assays that also include anti-inflammatory cytokines such as IL-10 may provide further information regarding the relationship between cytokine expression and clinical outcome. In comparison to cyclosporine other immunosuppressants are not as well studied in canine patients. Despite this fact, these drugs are being increasingly substituted for cyclosporine in cases of immune-mediated disease, most likely due to high cost associated with cyclosporine use. It is very possible that these immunosuppressant drugs exhibit a degree of variability in individual patients that is similar to cyclosporine, and that this variability simply has yet to be discovered due to the lack of relevant pharmacokinetic and pharmacodynamic studies in dogs. More research on the use of these other immunosuppressant drugs in dogs will likely show similar individual-to-individual variation, and encourage the development of drug-specific assays.

The assay developed in this thesis was specifically designed to be used in canine patients, but cyclosporine is also used in feline cases of allergic dermatitis and as an

immunosuppressant, especially after kidney transplants (which are more commonly performed in cats). Although pharmacokinetic assays are available that measure whole blood cyclosporine concentrations in cats, a pharmacodynamic assay has yet to be developed in this species. Access to such an assay that assists with determining ideal dosing protocols could help clinicians better manage their feline patients.

Analysis of samples from canine patients with different diseases has led us to modify our recommendations. For certain localized diseases like atopy, typically low suppression of IL-2 is observed, even though the patients' skin disease is clinically well controlled. We hypothesize that this is due to the fact that cyclosporine concentrates in the skin and is able to keep disease under control due to a local effect, and therefore our laboratory only recommends a change in dose if clinical signs are not adequately controlled. A similar scenario has been encountered with dogs suffering from inflammatory bowel disease (IBD). As with atopy cases, we suspect there is a local immunosuppressive effect at the level of the gut in dogs with IBD receiving cyclosporine. Since the assay measures the effects of cyclosporine on circulating T cells, it may not give an accurate portrayal of local disease control, particularly in tissues where cyclosporine is known to concentrate, such as the skin.

The greatest strength of our assay is that it is tailored to the individual patient. Dosage adjustments in cyclosporine are recommended based on IL-2 expression, and are therefore targeted in the specific mechanism of action of the drug. If the patient has marked IL-2 suppression, but the disease is still not well controlled, it is recommended to try an additional or alternative immunosuppressive agent, since cyclosporine is already exhibiting its maximal effect. Additionally, marked IL-2 suppression is associated with a

greater risk of secondary infection, and clinicians are advised to be vigilant for signs of infection. In contrast, if the patient has low to moderate IL-2 suppression, and the disease is not well controlled, increased cyclosporine dose rates are recommended. This assay is specifically targeted to cyclosporine's mechanism of action and indicates if cyclosporine is at the correct dose for the patient. This may not necessarily mean that the immune system is adequately suppressed in these individuals as other mechanisms could be at play leading to immune-mediated disease. We suspect that a marked reduction in pro-inflammatory cytokines like IL-2 and IFN-γ would indicate that the immune response has been decreased, but this effect has yet to be proved experimentally.

Use of this assay has also allowed us to identify dogs with altered cyclosporine disposition. Using this assay, we were able to identify a dog with a profound reduction in IL-2 expression despite a relatively low dose of cyclosporine. Subsequent investigation into the case led to the discovery that the dog was heterozygous for the MDR1 gene mutation. The MDR1 (or ABCB1) gene codes for the drug transporter P-glycoprotein, for which cyclosporine is a known substrate and, amongst other functions, P-glycoprotein serves as an efflux pump that pumps drugs such as cyclosporine out of lymphocytes. Heterozygous dogs are known to exhibit reduced P-glycoprotein function, and therefore we suspect that this led to increased intracellular levels of cyclosporine within T cells, causing a high degree of suppression of IL-2 production despite a relatively low dose of cyclosporine. This is just one example of the advantages of using a pharmacodynamic-based assay, since this effect may not have been noticed if only pharmacokinetic data was used for monitoring.

The overall objective for this dissertation research was to develop an assay that provides valuable information for clinicians and their patients receiving cyclosporine therapy. Initial results with clinical cases has been rewarding, and further investigation and statistical analysis correlating case outcome to assay results is warranted. The work in our laboratory also forges the way for the development of other assays for individualized drug therapy in the canine patient.