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Assessment of canine immunity using computational and flow cytometric approaches

By

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Assessment of canine immunity using computational and flow cytometric approaches

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The Affymetrix GeneChip® Canine Genome 2.0 microarray is re-annotated using AgBase tools, up-to-date ID mapping and GO annotations associated with publicly available gene products updated on this array. This re-annotation makes the array more useful for researchers using the canine microarray for biological discovery. We use flow cytometry to determine if liposomal clodronate (LC) is an acceptable alternative to surgical splenectomy to facilitate detection of subclinical infection with *Babesia canis* in potential blood donor greyhounds. Our study shows that LC is not a reliable means of exposing babesiosis in greyhounds with a recent history of infection. We evaluate the effect of depletion of antigen presenting cells on regulatory T cells (Tregs) in dogs treated with LC by multi-color flow cytometry. We demonstrate that LC promotes increases in the $CD4^+CD25^+FOXP3^+$ Tregs affecting mostly the $CD4^+CD25^{low}FOXP3^+$ Tregs subset suggesting a role of monocytes in naïve T cell priming and differentiation into Tregs.

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CHAPTER I

INTRODUCTION

There is an ongoing movement to utilize high-throughput functional genomics

platforms to derive biologically significant information from lists of numerous identifiers

and accessions. In addition, there is a need for up-to-date accessions to facilitate data

availability and ease of use for various fields of research. The canine model, which is

used in several areas of medical research, has the potential to provide translational

information to promote medical research if data are readily available.

Babesia is an intracellular protozoan parasite that exploits red blood cells (RBCs).

It is the causative agent of babesiosis, which is not only considered an emerging disease

in the U.S., but is also a major concern in veterinary practice. Disease caused by *Babesia*

canis is commonly subclinical in greyhounds, which, in turn, become chronic carriers. As

the most common blood donors used in veterinary medicine, greyhounds must be

screened for *Babesia* to prevent a parasite transmission from blood donors to recipients

that could develop potentially fatal babesiosis. In the past, screening of potential blood

donors was performed using a surgical splenectomy to reveal circulating protozoa. As the

spleen is responsible for removing infected RBCs, absence of the spleen will lead to

observable parasitemia indicating that the animal is unfit to join the blood donor pool.

However, this invasive practice has dropped out of favor, because it leaves the animal

permanently immune-suppressed. Here, we explore the possibility of a medical splenectomy utilizing liposome encapsulated clodronate.

Liposomal clodronate (LC) is used to cause transient immune suppression in both research and clinical settings by depleting monocytes, macrophages and immature dendritic cells (DCs) via apoptosis. Here, greyhounds exposed to *Babesia* were treated with differing levels of LC and *Babesia* infected RBC numbers were evaluated via flow cytometric analysis over the course of treatment. We propose that LC treatment could be an alternative to surgical splenectomy for potential blood donors.

Also, for the first time, regulatory T cell (Tregs) numbers were evaluated in the absence of professional antigen presenting cells (APCs), monocytes, macrophages and immature DCs. Tregs are a subset of T cells defined as $CD4^+CD25^+FOXP3^+$, and that are critical for suppression and regulation of normal canine immune responses. Canine Tregs are poorly understood, but new staining techniques provide a key to unearthing much needed information on this critical cell type.

Three specific aims accomplished in this thesis research are as follows:

Functional Annotation of the Affymetrix GeneChip® Canine Genome 2.0 Microarray

Hypothesis: By re-annotating the Affymetrix Canine Genome 2.0 microarray, we will increase the number of annotations provided for the microarray as a whole and the quality of annotations provided for the array.

Objective: To improve the existing functional annotation of the Affymetrix Canine Genome 2.0 microarray.

To achieve this objective: A digital file including all Affymetrix probeset

identifiers on the microarray was re-annotated and updated using AgBase

tools. This updated information was made publicly available in a user-

friendly format at the Mississippi State University Agbase website.

Use of liposomal clodronate (LC) to facilitate detection of subclinical *Babesia canis* infection in potential blood donor greyhounds

Hypothesis: Transient immune suppression by liposomal clodronate (LC) will

facilitate detection of subclinical *Babesia canis* infection in potential

canine blood donors.

Objective: Evaluate the role of liposomal clodronate in the development of

parasite infection in erythrocytes of greyhounds with known exposure to

Babesia canis (PCR and anti-*Babesia* antibody positive) but without

evidence of productive *Babesia* infection in their red blood cells (flow

cytometry and blood smear negative).

To achieve this objective: Four greyhounds with a history of varying levels of

positivity for exposure/confirmed presence of *Babesia* were given a low,

medium, and high dose of liposomal clodronate. To evaluate the effect of

the drug, the levels of parasite-infected red blood cells in peripheral blood

was measured using specific single color flow cytometric analysis and

results analyzed by one-way ANOVA statistics.

The effects of selective LC-dependent removal of professional antigen presenting cells (APCs), monocytes, macrophages and immature dendritic cells (DCs) on the numbers and phenotypes of CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells

Hypothesis: Monocytes/macrophages and immature DCs are the major APC populations shaping protective adaptive immune responses in dogs, as has been demonstrated in humans and mice, and their impaired function leads to immune suppression through a conversion of naïve T cells into suppressive FOXP3-expressing Tregs.

Objective: Evaluate the role of professional APCs (monocytes/macrophages/DCs) in the generation of FOXP3-expressing regulatory T cells in normal dogs and dogs exposed to *Babesia canis*.

To achieve this objective: The numbers of Tregs in four greyhounds previously exposed to *Babesia canis* were assessed following the administration of low, medium, and high doses of liposomal clodronate. The numbers and phenotypes of Tregs and the levels of CD14$^+$ monocytes of four normal Walker hounds treated with the medium dose of LC, and Tregs were observed. For both studies, multiple color flow cytometric analysis was performed and the results were analyzed with one way ANOVA statistics.

CHAPTER II

REVIEW OF PERTINENT LITERATURE

Microarray and Gene Ontology

The use of microarrays in research generates a large amount of data that must be translated into biologically relevant information. In order to accomplish this, researchers use annotation, the practice of attaching biological information to sequences. This information can then be interpreted by researchers themselves or entered into modeling tools such as pathway analysis and Gene Ontology(GO) enrichment tools [1]. There are two components of annotation: structural annotation and functional annotation. Structural annotation is the identification of demarcation of the genomic boundaries of individual functional elements within the genome sequence and includes providing accessions and identifiers for commonly used public databases. This tends to occur as the genome sequence is assembled and continues as new information is acquired. Functional annotation associates a gene product's functional information with the gene product itself and is not commonly done during genome sequencing [1, 2]. GO has become the standard for functional annotation. It is a structured network consisting of defined terms and relationships between them that describe three attributes of gene products: molecular function, biological process, and cellular component [3]. These three independent ontologies are dynamic and while having a common language, they exist in a network that changes constantly as more information is acquired [4]. Using GO annotation,

researchers can take large amounts of data generated from microarrays and assign biological significance to their results based on the function, process, or component they are associated with. Researchers spend large amounts of time and money to annotate their singular dataset. In this project, we sought to re-annotate the Affymetrix Canine Gene Chip 2.0 microarray that was annotated in 2006. As information content of databases such as GO keeps changing, there is a need for re-annotation of microarrays and dissemination of this updated information to the public. The end result is improved ability to perform analysis of canine samples.

Liposomal Clodronate

Clodronate or dichloromethylene-bisphosphonate is being used in various types of treatments and experiments in many different fields of the scientific and medical communities [5]. When taken into professional phagocytes, including both DCs and monocytes/macrophages, clodronate is metabolized to adenosine 5' triphosphate with the end result being the lysis of the mitochondrial membrane within the host monocytes/macrophage, which leads to the induction of apoptosis [6, 43], therefore depleting the number of viable monocytes/macrophages and DCs that are available for immune response. A recent study, however, has argued that clodronate causes cell death via necrosis rather than apoptosis as previously reported [7]. If that is the case, the cytotoxicity of clodronate could very well cause adverse effects in local tissues when released at cell lysis.

When administered as a free pharmaceutical, bisphosphonates such as clodronate are considered to be "bone-seeking molecules" [7], and only very small amounts of the drug are taken up via phagocytosis. Thus, the resulting intracellular concentrations are

too small to induce the most often desired apoptosis [5]. In order to circumvent this, clodronate is often administered in the liposome encapsulated formation. This prolongs the amount of time the molecule spends in circulation and increases the chance of phagocytosis by professional phagocytes [7]. In instances when the immune response may be detrimental to the overall health of the patient, clodronate is one way that we may suppress both the innate and adaptive responses.

Liposomes are tiny vesicles composed of one or more concentric phospholipid bilayers, and they have been found to be efficient methods of delivering water soluble drugs to phagocytes [5]. Size and charge of the liposome are important characteristics to take into consideration when developing treatments or experiments. It has been found that the optimal liposome is negatively charged and has a size of 85±20nm. If too large, the particle runs the risk of inducing the production of pro-inflammatory cytokines such as IL-6, TGF-α, and IL-1β that can cause further damage when treating conditions such as autoimmune diseases. If too small, they can have no effect at all [7].

Liposomes are known to enhance phagocytosis in monocytes/macrophages, but there are at least two conditions that must be met in order for the encased clodronate to induce apoptosis in these cells. First, the phagocytosis of a liposome-encased clodronate is not enough to ensure apoptosis. There are many cell types that will take in this molecule, but the cells must also have the mechanisms required to lyse the liposomal membrane to release the clodronate contents. Second, the intracellular concentration of clodronate must reach a threshold. If the concentration is too low, apoptosis may not occur. In one study, activated monocytes were observed to have higher levels of phagocytosis and therefore had higher levels of associated apoptosis when compared to

resting or non-activated monocytes. Overall, the intracellular concentration of clodronate regulates the cytotoxic effects or degree of cell death achieved by a single dose of liposome-encased clodronate [5].

Many scientists are using liposome-encased clodronate to deplete the numbers of monocytes/ macrophages and DCs in the course of an immune response for many different types of experimental studies. LC has been used to provide controls for experiments evaluating the role of monocytes and macrophages in muscle injury [8] and even in diseases such as acute gout [9]. LC is being used to treat various autoimmune diseases due to its selective targeting of disease-related monocytes and macrophages [5], and it has been used in the study of dengue fever to prove that monocytes and macrophages are critical elements in controlling the virus [10]. In 2004, a study showed that by depleting monocytes and macrophages by administration of liposome-encased clodronate, hyperalgesia was decreased in part due to the decrease in pro-inflammatory mediators produced by those cell types [6]. However, when administering liposomal clodronate systemically, there is not a significant amount of monocytes/macrophage depletion in specific sites. In these cases where a specific tissue is being evaluated, direct local injections are effective in lowering monocyte/ macrophage numbers for several days. Depletion percentages for circulating monocytes/macrophages have been reported as high as 70%, and for certain populations of monocyte-derived tissue macrophages has been anywhere from 15-60% [6].

In 2005, a method utilizing LC's macrophage depletion characteristics was developed that allowed for the acceleration of alveolar macrophage reconstitution [11]. Also, LC has been used to decrease the instance of dissemination and brain invasion by

Cryptococcus neoformans. The causative agent of cryptococcosis, *Cryptococcus neoformans* is thought to use monocytes as a type of "Trojan horse" that can carry it to various organs including the spleen and lungs and even across the blood brain barrier [12]. Another study describes how the act of depleting macrophages can generally be both good and bad. It exacerbates early aortic lesions allowing for increase in collagen content which can cause a widening or growth to be observed. On the other hand, the depletion of macrophages does decrease the production of pro-inflammatory cytokines as previously stated [13].

All in all, encapsulation inside liposomes enhances the effectiveness of clodronate as an immune response inhibitor. Liposome-encased clodronate has widespread uses in both research and treatment, but should be selected for use on a case-by-case basis. It has both beneficial effects as well as adverse effects, and those must be weighed carefully.

Babesia

Babesia canis is an intracellular protozoan parasite carried by ticks, in particular *Rhipicephalus sanguineus* (the brown dog tick), which invades canine red blood cells (RBCs). Transmission from tick to canine host is complete after a 2-3 day feeding attachment by the tick [14, 15]. Once in the canine host circulatory system, the organism attaches to RBCs and is internalized via endocytosis [16]. Once in the cytoplasm of the host's RBCs, then the organism is free to reproduce via binary fission resulting in rupture of current RBC and infection of local RBCs [14].

It is known that *Rhipicephalus sanguineus* transmits the causative agent of canine babesiosis in greyhounds in the United States. *Babesia canis* subspecies *vogeli*, and disease caused by this protozoan has been recognized in greyhound kennels [14].

Greyhounds are the most commonly used blood donors in veterinary medicine, because a high percentage of the breed are universal blood donors, have high hematocrits, gentle dispositions, and easy to access jugular veins [17]. Greyhounds and their propensity for babesiosis causes concern in blood transfusion recipients, because a transfusion with *Babesia*-infected blood commonly leads to potentially devastating babesiosis. In donor recipients of *Babesia*-infected blood, severe babesiosis can lead to multiple organ dysfunction syndromes, septic shock, or even death [14, 18]. In puppies or adult dogs, babesiosis can often develop alongside a secondary disease. These cases are often characterized by fever, lethargy, anorexia and jaundice. Clinically, evidence may indicate hemolytic regenerative immune-mediated anemia, leukocytosis, and thrombocytopenia [19, 20, 14]. In greyhounds, it is often hard to diagnose babesiosis since it is most commonly a mild infection with non-specific clinical signs, and affected dogs can become chronic sub-clinical carriers [18].

Accepted methods for detection of babesiosis include microscopic identification of the organism on fresh blood smears, serologic testing using an IFA (indirect fluorescent antibody test), PCR (polymerase chain reaction) assay targeting the *Babesia* spp. small subunit ribosomal RNA (ribonucleic acid) gene in the blood, and flow cytometric evaluation of RBCs [14].There are limitations to some of these tests. Blood smears utilizing Giemsa stains are not sensitive tests for detecting *Babesia*, because it is hard to visualize the organism unless a moderate to high parasitemia is present [14, 21]. When running antibody tests such as IFA, it can be hard to distinguish active infection from one that has already cleared due to the persistence of antibodies within the host's immune system [18, 22]. Flow cytometry also relies on a detectable parasitemia. In

instances where tests are positive, donors must be rejected to prevent possible repercussions due to transfusions with infected blood. Although PCR is a definitive test for *Babesia canis,* this is not a technique most clinics have quick and easy access to, and PCR positivity alone does not permit quantitation of the number of infected RBCs. For this thesis research, the focus turned to flow cytometric analysis. Several methods for quantifying red blood cells infected with *Babesia* via flow cytometry have been successful: the use of HE (hydroethidine) in DMSO (dimethyl sulfoxide) for staining was utilized here [22, 23].

Regulatory T cells

Although they are well documented in human and murine research, little is known about the functional significance of regulatory T cells (Tregs) in the canine. However, it is apparent that Tregs are an important constituent of healthy function of the host immune system. Tregs may be either thymus-derived naturally occurring Tregs or Tregs that are induced in the periphery from conventional peripheral CD4^{+} T cells [24, 25]. Regulatory T cells are responsible for maintaining tolerance and preventing autoimmunity by retaining the ability to suppress the activation and function of effector T cells and antigen presenting cells (APCs) via their suppressive actions, which include both direct and indirect suppression. Direct suppression used against effector T cells can be considered an activation-dependent process, but requires cell-cell interactions in order actively suppress effector T cells [26]. On the other hand, indirect suppression, in evidence versus APCs, includes induction of the enzyme IDO (indoleamine 2,3-dioxygenase) which causes the development of a strong immunosuppressive agent and induces more Tregs [30].

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In normal systems, when activated or increased in numbers to a suitable concentration, Tregs can help to prevent autoimmune dysfunction of the host system [31, 33], or they may exacerbate any ongoing pathology [32, 34, 35, 36, 37, 38, 39]. Although more research is needed, studies have shown that a nuclear transcription factor FOXP3 (forkhead box P3 transcription factor) is necessary for the natural generation of naïve T cells into regulatory T cells with suppressive functions. Without FOXP3, the recognizable immunosuppressive function can be lost [27, 28, 29]. Currently, it has been shown that conventional T cells stimulated via antigen in the presence of essentials such as TGF-β, IL-10, and retinoic acid will be induced to Tregs [40, 41, 42].

There is still much research to be performed in order to comprehensively detail the function of Tregs and their role in the canine immune response, but studies are beginning to show that functions of Tregs in canines are similar to known functions described in human and murine models.

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CHAPTER III

FUNCTIONAL ANNOTATION OF THE AFFYMETRIX CANINE 2.0

MICROARRAY

Abstract

Researchers using high-throughput functional genomics platforms such as microarrays must be able to derive biologically relevant insights from the results, which are returned as lists of numerous identifiers and accessions. This is a problem due to the amount of time and resources needed to turn those accessions into applicable data. The lack of functional annotation associated with arrays is also a challenge for modeling array data. Despite an increase in the amount of canine genomics research, the annotation files provided for the Affymetrix GeneChip® Canine Genome 2.0 microarray contain functional annotations for only 14.5% of transcripts represented on this array. We re-annotated the Affymetrix canine array, providing updated database identifiers and new functional annotations. Here we linked the Affy probe IDs to identifiers from commonly used databases to create an ID mapping file which can be used to facilitate data sharing between differing databases. In addition, we linked these same gene products to existing functional annotation using the Gene Ontology (GO), and provided new GO annotation where none existed. After remapping, we identified 10,737 existing GO annotations for 5,127 of the newly mapped gene products represented on the array. In addition, we added 70,148 annotations for 16,966 gene products. Thus, we have provided annotations for

94% of this array, which represents a seven-fold increase in the number of gene products than were originally annotated. The resultant data is publicly available at the AgBase website (http://agbase.msstate.edu) and will be periodically updated during standard database updating procedures. The new data provided enables canine researchers to more efficiently functionally model their Affymetrix array datasets and translate array data into additional knowledge.

Background

The canine genome was initially released in July 2004 by the BROAD Institute's Dog Genome Project, and then a new version was released in May 2005 [4, 5]. A commonly used canine microarray, the Affymetrix GeneChip® Canine Genome 2.0 (GPL: 3738), with probes for over 18,000 canine mRNA/EST transcripts and over 20,000 non-redundant predicted genes, was designed to help obtain biologically relevant information from this genome (6). The identifier mappings and GO annotations associated with this array were last updated in 2006 [7].

The outdated or lack of functional annotations associated with microarrays hinders researchers who wish to model their array data results to obtain biologically relevant information. Thus, despite the importance of canine array research, only 5% of available datasets generated using this array have been published, while only 4% of available datasets have been published for all canine array platforms (based on Gene Expression Omnibus database, 2/2013), Figure 3.1. This represents thousands of dollars of research that has not yet been translated to biological relevance for biomedical and veterinary research. Moreover, these figures are likely to under-represent the magnitude of the problem, because not all datasets from canine array research will have been

submitted as researchers try to obtain annotations. However, re-annotation of arrays with the most up-to-date functional annotations can lead to significant changes in the interpretation of array datasets [8].
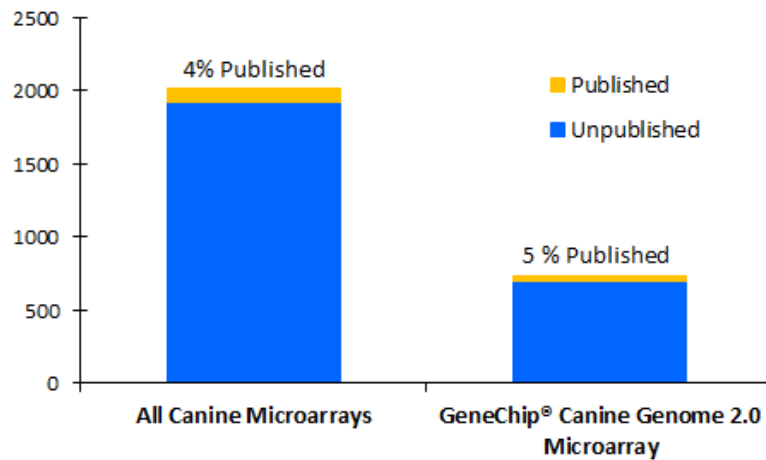
Figure 3.1   Estimation of data generated using canine assays.
Representative of canine datasets in GEO as of 2/2013.

Annotation is the practice of attaching biological information to sequences, and it is this information that is used not only by researchers but also by functional modeling tools such as pathways analysis and Gene Ontology (GO) enrichment analysis tools. There are two components of annotation: structural annotation and functional annotation. Structural annotation is the identification of individual functional elements within the genome sequence and includes providing accessions and identifiers for commonly used public databases. This is generally initiated as the genome sequence is assembled and continues on-going as new information is acquired. Functional information, alternatively, associates a gene product's functional information with the gene product itself and is not commonly performed during genome sequencing [1].

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GO has become the standard for functional annotation, and is a structured network consisting of defined terms and relationships between them that describe three attributes of gene products: Molecular Function, Biological Process, and Cellular Component [2]. These three independent ontologies are dynamic and while having a common language, they exist in a network that changes constantly as more information is acquired [3].

To make functional modeling in canine research more accessible for researchers, we re-annotated the largest and most frequently used microarray, the GeneChip® Canine Genome 2.0 Array from Affymetrix. This updated array annotation will be made publicly available at AgBase at http://www.agbase.msstate.edu.

Methods

Affymetrix Array Files and Array Statistics

The Affymetrix GeneChip® Canine Genome 2.0 (GEO platform accession GPL: 3738) has probes that represent 18,000 canine mRNA/EST transcripts and over 20,000 non-redundant predicted genes. We obtained canine array dataset numbers from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nih.gov/geo) using specific searches (Figure 3.1). The number of canine datasets submitted was obtained using the search criteria: dog[organism]. The number of those datasets that were linked to publications was obtained using the refined search criteria: dog[organism] AND "gds pubmed"[filter]. The number of datasets submitted from only the Affymetrix GeneChip® Canine Genome 2.0 array, was obtained using search criteria: GPL3738. The number of those datasets that were linked to publications was obtained by the refined search criteria: GPL3738 AND "gds pubmed"[filter].

Accession Mapping

Since different functional modeling tools often require different public database accessions or identifiers, accession mapping of Affy probe IDs to multiple public databases was done to provide an up-to-date, comprehensive cross-mapping file to support functional modeling. The ArrayIDer tool is available for public use and can be accessed via the AgBase website. This tool accepts numerous ID types, including Affymetrix probeset identifiers which result from the use of Affymetrix microarrays. A document is then generated by the tool which matches these probeset identifiers to a list of equivalent identifiers used by other publically available databases such as UniProtKB, Ensembl, RefSeq, and Entrez Gene. ID types preferred were UniProtKB (Universal Protein Knowledgebase) and RefSeq, with UniProtKB identifiers desired due to their high-quality manual annotations. Ensembl, RefSeq, and Entrez identifiers were mapped back to any corresponding UniProtKB and RefSeq protein identifiers using the publically available BioMart application provided by the Ensembl website [7]. All RefSeq protein identifiers were then checked against the National Center for Biotechnology Information (NCBI) database to update this list of identifiers with any revisions or deletions. Updated RefSeq protein identfiers were then mapped to UniProt identifiers using the publically available ID Mapping application [8]. These updated identifiers were compiled into a single ID Mapping file that is available online at the AgBase Array Annotation section. Figure 3.2 outlines the workflow of re-mapping and re-annotating the GeneChip® Canine Genome 2.0 microarray.

ID Mapping and Annotation
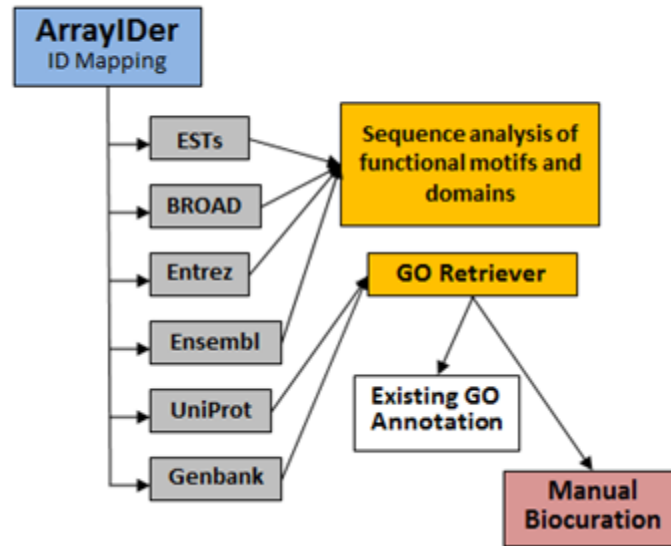
Figure 3.2   Workflow schematic showing the process of ID mapping and linking GO annotation.

Functional annotation begins by accession mapping through ArrayIDer which divides the input file into broad categories: ESTs, BROAD, Entrez, Ensembl, UniProKB, Genbank RefSeq. UniProtKB and Genbank RefSeq are sent through GORetriever which pulls existing GO annotations while the rest undergo manual biocuration. ESTs, BROAD, and Entrez accessions go through sequence analysis of functional motifs and domains.

GO Annotation

Since a key step in functional modeling is GO enrichment analysis, canine gene products from the ID Mapping file were linked to GO annotations. Currently, there is no dedicated GO annotation for dog gene products and the main source of existing GO annotation are the UniProtKB annotations provided by the EBI GOA Project [1]. We used the ID mapping file to identify any array probes that had a mapping to UniProtKB and used GORetriever [2] to get these annotations. Next we provided GO annotations for the remaining gene products represented on the array based on their type of database accession (which indicates the type of data available for the corresponding gene product).

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Expressed Sequence Tags (ESTs): Since there is no experimental functional data available for ESTs, these sequences can only be GO annotated based upon sequence analysis of functional motifs and domains [9]. EST sequences identified form the canine microarray were downloaded from the dbEST, prepared as FASTA files and this data was submitted to the InterProScan program [3]. The resulting InterPro identifiers were mapped to GO using the InterPro2GO mapping file [4]. These annotations are given the GO evidence code "Inferred from Electronic Annoation" (IEA) and provide broad functional information that can be used to predict function and support hypothesis testing.

BROAD: These identifiers are associated with the BROAD Institute of MIT and Harvard and the canine genome project. Retrieving annotations for this subset of identifiers is still currently ongoing. Updated annotations will be added to the publically available files as they are retrieved.

UniProtKB and RefSeq Identifiers: Using the GORetriever tool that is provided to the public by AgBase [2], all existing GO annotation for probes on the microarray associated with UniProtKB and RefSeq identifiers were separately annotated through critical analysis of experimental data of published literature using Gene Ontology Consortium (GOC) guidelines. The resulting compilation of updated GO annotations for all gene products associated with the Affymetrix GeneChip® Canine 2.0 microarray were compiled into a single file and will be made publicly available at the AgBase website in the Array Annotations section after quality check is done using GOC guidelines [6].

GAQ Score

The GO Annotation Quality (GAQ) Score [5] measures the breadth of GO annotation, the level of detail of the annotation, and the type of evidence used to make the

annotation to provide a quantitative measure of the overall quality of the GO annotations.

All updated anntoations retrieved were combined into a single file that was arranged into

the gene associated file format 2.0, and then uploaded into the AgBase tool in a delimited

form. Output form this tool provides a summary containing the total GAQ score, the

number of non-redundant gene products, and the mean GAQ.

Results and Discussion

Since functional annotation is continually changing as more data is added, to

derive biologically relevant functional information from arrays, scientists need ready

access to the most up-to-date annotation. Although microarrays are used to study canine

systems, only a small fraction of the data is published [Figure 3.1]; this lack of functional

annotation is hindering biological modeling of this data. For example, the Affymetrix

canine microarray was last annotated in May 2006, although it represents 36% of datasets

submitted in GEO. Our re-annotation provides increased amounts of functional

information and shows that the overall quality of this annotation has also increased.

Moreover, we provide annotated data in a format that will facilitate functional analysis

and make it publicly available for GO enrichment analysis tools.

The Affymetrix GeneChip® Canine Genome 2.0 microarray (GPL:3738) contains

over 18,000 probesets which represent over 20,000 gene products [10,11]. Prior to

remapping, the original 18,342 unique probeset identifiers from the array were mapped to

a total of 26,818 unique identifiers and accessions from UniProtKB, RefSeq, and

Ensembl which are commonly used public databases. Since each probeset is mapped to

its equivalent accession in each database, this ID mapping file creates a useful look up

table used to retrieve data from each of these databases. The identifiers represented on

this array were as follows: 6% UniProtKB, 55% RefSeq, 10% Ensembl, 1% Entrez Gene,

1% BROAD, and 27% ESTs [Figure 3.3]. From the small percentage of UniProtKB in

comparison to the larger percentage of the RefSeq and ESTs, we can see that high-quality

information linked to this array is limited. With the highest quality annotations being

UniProtKB, this initial mapping indicates a need for re-mapping and re-annotation with

the purpose of increasing the percentage of UniProtKB accessions. For example, the

majority of mapping is to RefSeq accessions. Of those 15,024 accessions, less than 1% of

the identifiers mapped to known proteins, designated NP. The remaining accessions were

mapped to predicted proteins, designated XP. Thus, less than 1% of those RefSeq

accessions have undergone manual biocuration to confirm or deny their predicted

function.
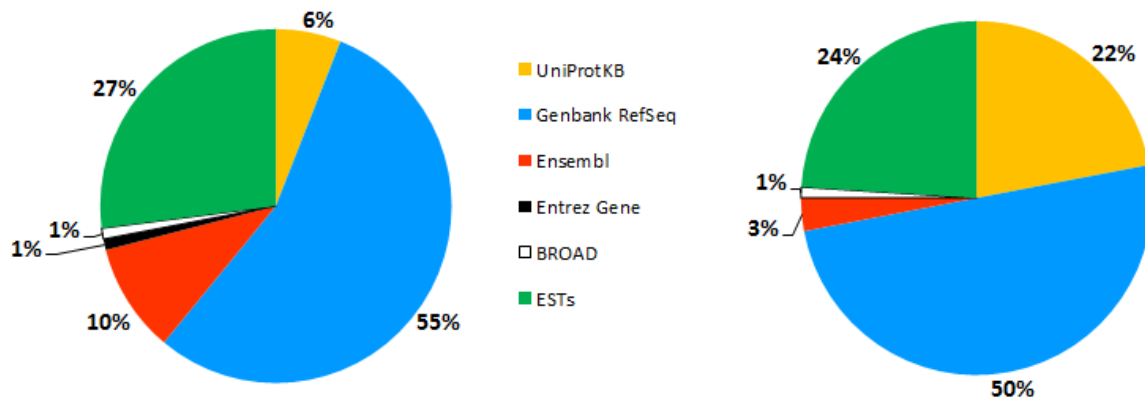
Figure 3.3   Breakdown of identifier types before and after re-mapping.

The increase in the number of UniProtKB indicates that the quality of the annotations
retrieved for the new ID mapping file will also increase.

After re-mapping, the probeset identifiers from the array were linked to a total of

29,909 unique identifiers and accessions. This increase in the number of identifiers

associated with this array is due to probeset identifiers mapping to more than one

identifier. The new breakdown of identifier types represented is as follows: 22% UniProtKB, 50% RefSeq, less than 3% Ensembl, 1% BROAD, and 24% ESTs [Figure 3.3]. As indicated previously, the higher the number of UniProtKB accessions, the higher the quality of total annotations associated with the array. UniProtKB accessions are the highest quality identifiers because of the biocuration the identifiers must undergo and the comprehensive nature of information linked to the identifiers. Although the majority of identifiers once again mapped to RefSeq accessions, as a result of the re-mapping, there was a marked improvement in the quality of annotations to be retrieved based on the increase in the percentage of UniProtKB identifiers. The complete results can be found at the AgBase website.

In the original annotation file (released in 2006), the Affymetrix annotation file provided with the canine array linked the probesets with 15,822 GO annotations for 2,608 gene products represented on the array. After re-mapping these identifiers, we linked the updated probeset with 10,737 GO annotations representing 5,127 gene products. This decrease in the amount of GO annotations is partly due to the new set of gene products associated with the array and partly because of revisions to the GO annotations (as functional annotations are improved, some previous annotation may be deleted or revised). Since our remapping of the array probesets decreased the initial functional annotation associated with these gene products, we next provided additional GO annotation for these gene products.

Since the EBI GOA Project provides GO annotations for UniprotKB proteins and AgBase biocurators provide GO annotation for Genbank(RefSeq) proteins with no equivalent UniProtKB entry, we found that there were already 41,155 existing GO

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annotations for UniProtKB accessions and 69,821 existing GO annotations for Genbank(RefSeq) proteins. Moreover, AgBase biocurators manually added detailed, experimental base GO annotations from published papers, providing another 321 GO annotations for 44 gene products (this work is ongoing). Since 98% of the Genbank(RefSeq) proteins are designated as "XP" or "unknown proteins" (that is, there is no direct evidence that these proteins are translated, but rather they are predicted based on EST and homology to other known mammalian proteins), it is unlikely that these proteins will have any direct experimental evidence as to their function. Thus, we focus our manual biocuration on the 1053 UniProtKB proteins that currently have no GO and the 97 "known" Genbank(RefSeq) proteins (designated with "NP" prefix).

Likewise, EST sequences also have no functional literature. However, because ESTs are typically short sequences, they cannot be reliably linked to orthologous genes in other species. Providing a first pass functional annotation for these sequences requires an analysis of functional motifs and domains to identify sequences with conserved function [9]. Using this approach, we provided 22,006 annotations for 7,291 ESTs on the array that had no equivalent mRNA or proteins sequence. However, 99.2% of the gene products were annotated as ND or "no data". While this may be considered as a "null" result, it does alert researchers to the fact that there is currently no functional data available for these transcripts; further functional classification must await improved sequence annotation. However, these initial GO annotations can be added to the AgBase database in order to perform periodical updates to add more functional information as it becomes available.

Overall, we added 70,148 GO annotations for 16,966 gene products. This represents an increase from an initial 14% of the gene products represented on the array associated with functional annotation to 94% of the gene products represented on this array having some GO annotations. This represents almost a seven-fold increase in the number of gene products than were initially annotated [Figure 3.4]. Moreover, analysis of the GO quality (using the AgBase GAQ Score tool) mirrors this data in indicating an increase in quality of mean GAQ score from the initial 87.4% to 90.4%.

Original Array Gene Products with GO — 14% / 86%
Gene Products with GO after Re-Mapping of IDs — 28% / 72%
Final Gene Products with GO — 6% / 94%

With GO Annotations   Without GO Annotations
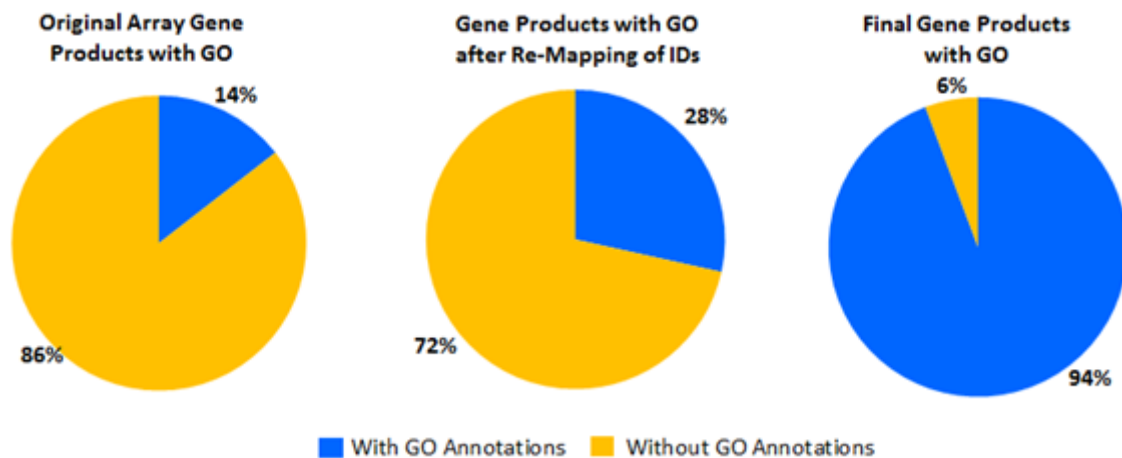
Figure 3.4   Gene products represented on the array that are linked to GO annotation.

Of the 18,000 gene products represented, the original annotation file available with the Affymetrix GeneChip® Canine Genome 2.0 microarray only provided GO annotations for 14% of the entire array. After re-mapping and re-annotation, we have provided GO annotations for 94% of all gene products represented on this array.

Conclusion

By re-analyzing and updating the annotation data associated with the Affy canine array we were able to provide a comprehensive list of public database accessions that the probes represent and to associate these gene products with GO annotation to facilitate functional modeling. In doing so, we have not only updated the canine gene products

represented on the array to reflect the newest genome release, but we have also improved

the functional annotation linked with these products by almost seven-fold. Since 95% of

the canine datasets produced from this array (and submitted to GEO as of 2/2013) remain

unpublished, our goal is to facilitate functional modeling of data produced by this and

other canine arrays. By making this data publicly available we expect to facilitate the use

of canine functional genomic data to provide insights into canine biology, rather than to

generate long lists of transcripts. With the advent of new sequencing technologies that are

producing even larger and more complex transcriptome data sets, it is becoming

increasingly important that functional annotation is provided for those gene products

which have functional data available.

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CHAPTER IV

USE OF LIPOSOMAL CLODRONATE (LC) TO FACILITATE DETECTION OF

SUBCLINICAL INFECTION WITH BABESIA CANIS IN POTENTIAL

BLOOD DONOR GREYHOUNDS

Background

An emerging disease in the U.S., babesiosis has become a particular problem in

greyhound kennels. As greyhounds, often retired from the racetrack, are the most

common breed to be used as blood donors, babesiosis has a critical impact on the use of

blood donors in veterinary medicine [3]. In greyhounds, babesiosis is most commonly

associated with *Babesia canis vogeli*, a protozoan parasite of red blood cells (RBCs) that

is carried by the brown dog tick, *Rhipicephalus sanguineus* [1]. It is thought to take 2-3

days of attachment by the tick for transmission of the sporozoite stages of the organism to

occur [4].

Although babesiosis is usually subclinical in greyhounds, it can cause serious

illness, even fatalities, in dogs inadvertently transfused with infected blood [5, 6].

Identification of donors with occult *Babesia* infection is therefore essential for

transfusion safety. Techniques utilized to detect the presence of *Babesia* include direct

microscopic observance of blood smears, IFA (indirect fluorescent antibody) serology,

PCR (polymerase chain reaction) [2, 6], and counting infected cells using a laser-based

cell counter such as flow cytometry [7, 8]. Each method has its own draw backs. For

example, the blood smear depends on active parasitemia, but in greyhounds the infection is commonly subclinical with animals being chronic carriers [6]. The organism therefore might not show up when using only direct microscopy. This is also a draw back with detection via flow cytometry. When running antibody tests, it is hard to prove whether a positive test is the result of a current infection or an infection already cleared by the host's immune system. PCR, on the other hand, is a definitive test for *Babesia* infection, but it is not a technique that most clinics have readily available. It does not allow for quantitation of the number of infected RBCs.

In the past, deciding whether or not an animal could become a blood donor was as easy as surgically removing the spleen. Since the spleen is required to help fight infection with blood-borne pathogens, splenectomy would allow for a flare-up of any blood borne parasite. This highly invasive procedure is no longer recommended, and an alternative is needed. In this study, we sought to evaluate the use of liposomal clodronate (LC) to facilitate the detection of subclinical infection with *Babesia canis* in potential blood donor greyhounds.

Liposomal clodronate has been shown to deplete macrophages and monocytes by induction of apoptosis via lysis of the mitochondrial membrane [9,10]. This results in an overall transient immune suppression that loses effect when the drug is no longer present in therapeutic levels. Since macrophages are needed to recognize and remove *Babesia*-infected RBCs from circulation, it has been proposed that LC may 'unveil' hidden babesiosis in infected greyhounds. The main objective of this study was to determine if LC would reveal any sequestered organisms in dogs with a history of exposure to *Babesia canis*

Methods

Dogs

Four healthy *Babesia canis*-exposed adult greyhounds and one Walker hound (used as a non-treated control) was used for this study. Health was confirmed based on physical examination, blood smear, complete blood count (CBC), serum biochemistry, and urinalysis. The greyhounds had a history of being variably positive for *Babesia canis* infection: one was IFA negative/PCR low positive, another was IFA and PCR negative but came from the same source as the other affected dogs and was housed and transported with them, another was IFA positive/ PCR high positive, and a final dog was IFA negative/ PCR positive. Greyhound results also varied between IFA/PCR testing dates. At the time of administration of LC, all dogs had become negative on every test for *Babesia canis* for at least three months. All animals were cared for according to guidelines approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC), and were housed in a university setting under standard conditions. The Mississippi State University animal facilities and program are accredited by the American Association for Accreditation of Laboratory Animal Care.

Treatment

Liposome clodronate (LC) (dichloromethylenedisphosphonic acid disodium salt, SIGMA, encapsulated into liposomes at Colorado State University) was administered to four greyhounds at low (0.5 ml/kg), medium (1 ml/kg), and high (2 ml/kg) doses via slow intravenous infusion at a constant rate into an indwelling peripheral venous catheter over a 90-minute period, using an infusion pump.

Study Design

A schematic of the study design is shown in Figure 4.1. Briefly, the study began

with baseline monitoring for levels of infected RBCs in greyhounds prior to treatment

with LC and subsequent assessment of levels of infected RBCs in LC-treated dogs
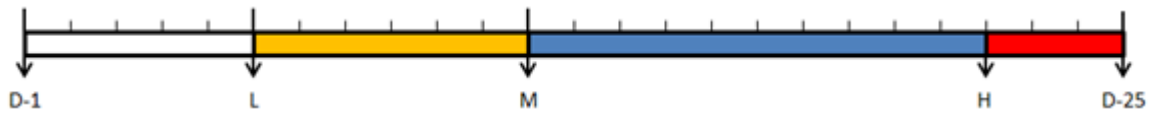
(Figure 4.1).

Figure 4.1   LC treatment timeline.

Arrows L (yellow), M (blue), H (red) indicate the low, medium, and high dose
treatments. Each dash indicates a RBC assessment time point (three times a week).
(D) represents the number of RBC assessment time points in the study.

Reagents

BD Pharm Lyse™ (10X) lysing solution (BD Biosciences) was used to remove

red blood cells from the white blood samples collected in EDTA tubes. Hydroethidine

(HE) or dihydroethidium (Invitrogen D1168) in DMSO (Sigma D2650) at 1:2000 was

used to stain any nuclear components within RBCs for analysis by flow cytometry.

Cell Preparation

White Blood Cells (as positive controls)

To remove red blood cells (RBCs), whole blood samples collected in EDTA were

incubated with BD Pharm Lyse ™ lysing buffer for 15 minutes in the dark at room

temperature, gently vortexing every 5 minutes. The remaining cell populations were

washed, incubated with a 1:2000 hydroethidine solution for 30 minutes at 37°C, then

were washed and analyzed by flow cytometry.

<u>Red Blood Cells</u>

Whole blood samples collected in EDTA were washed and buffy coats were discarded. RBCs were incubated with a 1:2000 hydroethidine solution for 30 minutes at 37° C, then were washed and the level of HE inclusion was analyzed by flow cytometry.

Flow Cytometry

Canine RBCs were gated based on their relative size and granularity using forward and side scatters (FSC and SSC, respectively) with FACSCalibur Flow Cytometer (Becton Dickinson). Data were analyzed using FlowJo 7.6.4 Software (Tree Star, Inc.). The staining resulting from inclusion of HE into the RBC was analyzed by using single histogram statistics (Figure 4.2).
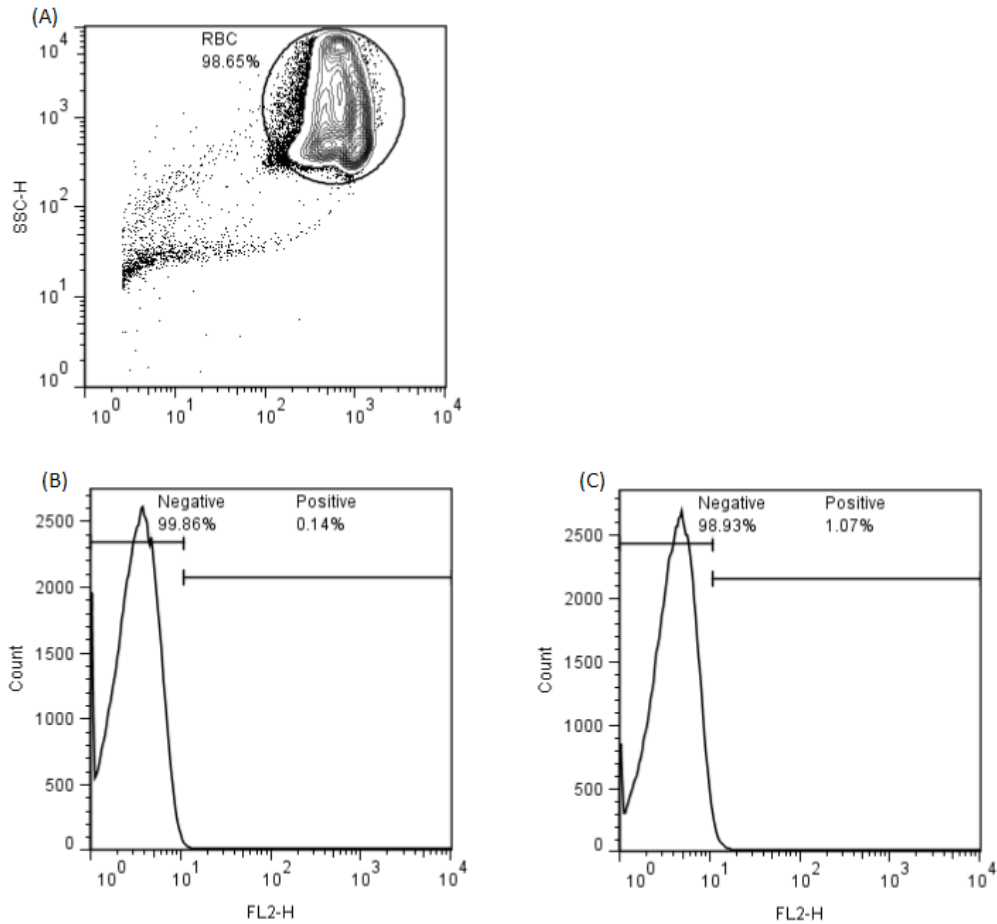
Figure 4.2   Assessment of RBCs exhibiting HE inclusion by flow cytometry.

Canine red blood cells were gated using forward and side scatters (A). RBCs stained by inclusion of HE into the nuclei were analyzed by using single color histogram statistics (B-negative control; C-experimental sample).

Statistical Analysis

RBCs exhibiting inclusion of HE as a specific population was expressed as a percentage of the total RBC numbers. Then, data was subjected to a one-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison post hoc test and are presented as means + SD. The level of significance for all tests of effects was set at $P < 0.05$.

Results and Discussion

Many canine vector-borne diseases can cause serious, even life-threatening clinical conditions in dogs, with a number also having significant zoonotic potential and affecting human populations [1, 11]. Since greyhounds are one of the most common breeds utilized as blood donors in veterinary medicine, accurate and efficient detection of canine vector borne diseases such as babesiosis is essential for preventing potentially devastating transfusions of infected blood. Here we used flow cytometry to determine the level of *Babesia canis* infection in greyhounds treated with LC.

Consistent baseline negative status on assays for *Babesia* upon repeat testing was confirmed over a 2 week period by a single IFA, flow cytometry and PCR approximately every second day and daily smear examination (data not shown). Flow cytometry and blood smear examinations (modified Wright's stain) were performed at MSU, and serology (IFA) and PCR were performed at the North Carolina State University (NCSU) Vector Borne Disease Diagnostic Laboratory. The 4 greyhounds were then given intravenous liposomal clodronate at a single low dose of 0.5 ml/kg. *Babesia* status was monitored over a 2 week period post-treatment by daily smear examination, and by flow cytometry and PCR approximately every second day. The same study design was then repeated at a medium liposomal clodronate dose of 1 ml/kg and then, finally, at a high liposomal clodronate dose of 2 ml/kg except that, after the final dose of liposomal clodronate, *Babesia* status was monitored for an extended post-treatment period of 4 weeks by daily smear examination, by flow cytometry and PCR every second day, and by repeat serology. There were minor numerical non-significant increases in the numbers of HE$^+$ RBCs at various time points in greyhounds exposed to all three doses of LC (Figure

4.3). Interestingly, a marked increase in the numbers of HE[+] RBCs was found in an

unrelated canine patient with increased numbers of reticulocytes by CBC (Figure 4.4)

suggesting that the LC-related increases in greyhounds could be due to increased

numbers of reticulocytes, although daily CBCs performed on the 4 greyhounds did not

support this speculation. Based on lack of evidence of *Babesia* infection by blood smear

examination, PCR or serology at any point during the study period (data not shown), we

conclude that LC does not appear to be a reliable means of exposing occult babesiosis in

greyhounds with a recent history of harboring the organism. To investigate if the

numerical non-significant increases in HE[+] RBCs numbers were due to the effect of LC,

RBCs from 3 healthy Walker hounds (see Methods, Chapter 5) exposed to the medium

dose of LC were assessed by flow cytometric analysis. There were no significant

differences in the numbers of HE[+] RBCs in the LC-treated animals compared to the non-

treated controls (data not shown) suggesting that the observed numerical increases in the

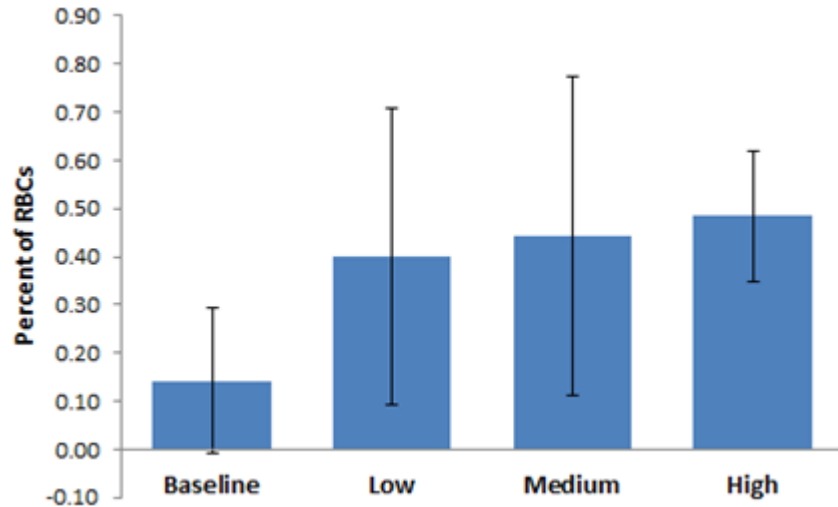numbers of HE+ RBCs were not LC-related.

Figure 4.3   Effect of LC exposure on numbers of RBCs exhibiting inclusion of HE in greyhounds in *Babesia canis*-exposed greyhounds.

RBCs from 4 healthy but *Babesia*-exposed greyhounds treated with low (0.5 ml/kg), medium (1 ml/kg), and high (2ml/kg) doses of LC were assessed by single color flow cytometry approach. Data are expressed as a mean % of total RBCs. No statistical significance indicated.
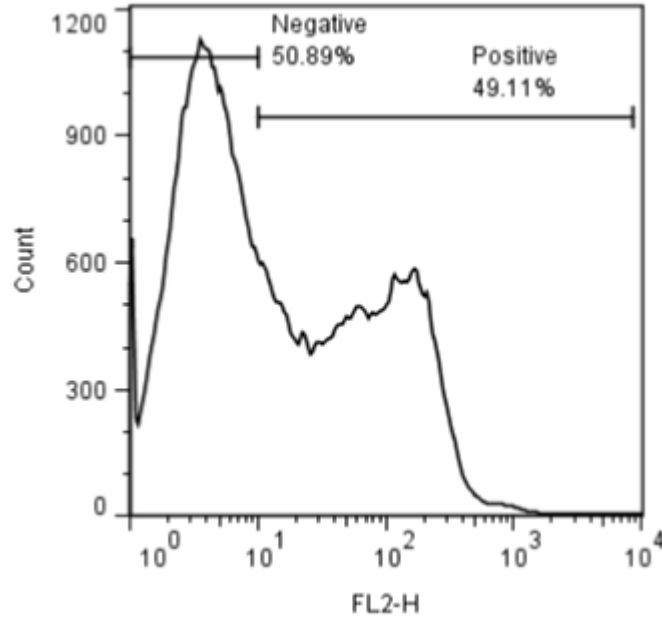

Figure 4.4   Flow cytometric analysis of RBC HE incorporation in the canine clinical patient diagnosed with reticulocytosis.

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CHAPTER V

SELECTIVE LC-DEPENDENT REMOVAL OF PROFESSIONAL ANTIGEN PRESENTING CELLS, MONOCYTES/MACROPHAGES, AND IMMATURE DENDRITIC CELLS PROMOTES INCREASES IN CD4$^+$CD25$^+$FOXP3$^+$ REGULATORY T CELLS

Abstract

A critical component of host immune systems, regulatory T cells (Tregs) have been described in detail in humans and mice. Although some researchers have begun to study canine Tregs, comprehensive functional information has not yet been produced. We evaluated the effect of depletion of professional antigen presenting cells (APCs) on the levels and phenotypes of regulatory T cells (Tregs) in dogs treated with liposomal clodronate (LC) by multi-color flow cytometric analysis. We demonstrate that numbers of Tregs increased after administration of various LC doses in greyhounds, and the same effect was found in Walker hounds by using the medium LC dose. Our study shows a correlation between levels of CD14$^+$ monocytes and Tregs in dogs treated with LC. These data demonstrate that, as in humans, the population of CD4$^+$CD25^{high} T cells most reliably identified as the highly enriched FOXP3$^+$ Tregs in dogs. In addition, we defined the CD4$^+$CD25^{low}FOXP3$^+$ cells as the major regulatory T cell subset affected by LC exposure suggesting the role of monocytes in naïve T cell priming and differentiation into Tregs.

Introduction

An important aspect of the host immune system, regulatory T cells (Tregs) are a known $CD4^+CD25^+FOXP3^+$ subset of T cells which are responsible for maintaining tolerance by suppression of natural immune responses [1]. These cells have been proven to develop normally, and they can also be induced to develop as antigen specific Tregs from conventional peripheral $CD4^+$ T cells [2].

Although there is very little known about the functional significance of regulatory T cells in the canine, Tregs are an important constituent of healthy function in the host immune system. In normal systems when activated or increased in numbers to a suitable concentration, Tregs can help to prevent autoimmune dysfunction of the host [3], or they may only exacerbate a current problem [4]. Although more research is needed, studies have shown that FOXP3 (forkhead box P3 transcription factor) is necessary for the generation of naïve T cells into regulatory T cells with suppressive functions. Without FOXP3, the recognizable immunosuppressive function can be lost [5].

Monocytes and macrophages are professional antigen presenting cells (APCs) that express multiple phagocytic and signaling pattern recognition receptors (PRRs) that sense and bind pathogen-associated molecular patterns (PAMPs) and produce cytokines [6,7,8]. Recent studies show that monocytes and macrophages are not limited to presenting antigens to effector T cells and stimulating and shaping T cell-mediated immune responses; they also prime naïve T cells, thus initiating adaptive immune responses [6,7,8,9,10].

Liposomes have been proven to increase the effectiveness of certain drugs by being used as drug carriers and allowing the liposome to follow its natural course,

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undergoing phagocytosis. Clodronate (dichloromethylene bisphosphate), a small hydrophilic molecule which functions to induce apoptosis via lysis of the mitochondrial membrane of host monocytes, dendritic cells, and macrophages, is one such drug [11]. The overall effect on the immune system of administering liposomal clodronate is a transient immune suppression which may be either detrimental or helpful to the host. It can be seen in the depletion of the number of viable circulating monocytes and macrophages [12]. Liposome encapsulated clodronate (liposomal clodronate, LC) is currently being used in research in a variety of approaches that include: to selectively deplete monocytes and macrophages to provide controls for evaluating muscle injury [13]; to prove the role of the immune cells in dengue fever [14]; to accelerate alveolar macrophage reconstitution [15]; to decrease dissemination and brain invasion of *Cryptococcus neoformans*;[16], and to treat diseases such as gout and various autoimmune diseases [17].

Current researchers in canine immunology utilize the specialized depletion tactics made possible by LC, but have also begun to explore the unknown concerning regulatory T cells.

In the current study, we assessed the levels and phenotypes of $CD4^+CD25^+FOXP3^+$ regulatory T cells in greyhounds and Walker hounds treated with different doses of LC.

Materials and Methods

Dogs

Healthy adult greyhounds and Walker hounds were used for this study. Health was confirmed based on physical examination, complete blood count (CBC), serum

biochemistry, and urinalysis. CBC and serum biochemistry were completed by the Mississippi State University College of Veterinary Medicine Diagnostic Laboratory Services (CVM-DLS).All animals were cared for according to guidelines approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC), and were housed in a university setting under standard conditions. The Mississippi State University animal facilities and program are accredited by the American Association for Accreditation of Laboratory Animal Care.

Treatment

Liposome clodronate (LC) (dichloromethylenedisphosphonic acid disodium salt, SIGMA, encapsulated into liposomes at Colorado State University) was administered to four greyhounds at low (0.5 ml/kg), medium (1 ml/kg), and high (2 ml/kg) doses via slow intravenous infusion at a constant rate into an indwelling peripheral venous catheter over a 90-minute period, using an infusion pump.

In the Walker hound study, LC (Encapsula NanoSciences in Nashville, TN) was administered to three Walker hounds as previously described at a single dose of 1 ml/kg. In both studies, control dogs received no treatment.

Study Design

The design of the greyhound study is shown in Figure 5.1. Briefly, the study was divided into baseline monitoring of Tregs in greyhounds prior to their treatment with different doses of LC and the assessment of levels of regulatory T cells in LC-treated dogs (Figure 5.1). Similarly to the greyhound study, the Walker hound study consisted of the baseline and after the treatment monitoring of Tregs. However, the experimental

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animals were exposed only to the medium dose of LC, and their peripheral blood

monocytes levels as well as total cell blood counts were determined in addition to Tregs.
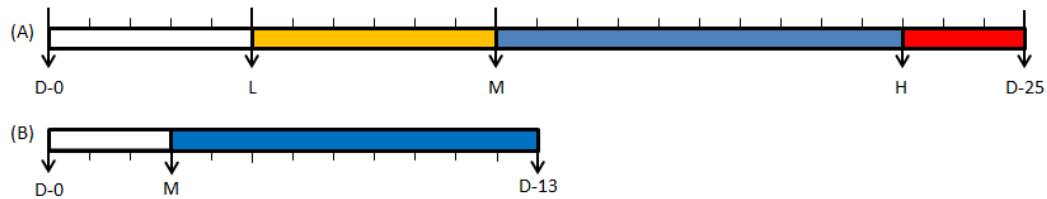

Figure 5.1   LC treatment timeline.

Arrows L, M, H indicate the low, medium, and high dose treatments. The dashes indicate
Treg assessment time points. (D-0/25/13) represents the number of assessment time
points in the study. (A) represents the greyhound study. (B) represents the Walker hound
study.

Reagents and Antibodies

BD Pharm Lyse™ (10X) lysing solution (BD Biosciences) was used to remove

red blood cells from the white blood samples collected in EDTA tubes.

Fluorescein -conjugated mouse anti-canine CD14 mAbs (LS-C43762, Lifespan

Biosciences, Inc.) were used to stain monocytes. Fluorescein -conjugated rat anti-canine

CD4 (LS-C127352, Lifespan Biosciences, Inc), Phycoerythrin (PE)-conjugated mouse

anti-canine CD25 (P4A10) and the FOXP3 Staining Buffer Set (including

Fixation/Permeabilization Diluent, Fixation/Permeabilization Concentrate, 10X

Permeabilization Buffer) and allophycocyanin (APC)-conjugated rat anti-canine FOXP3

mAbs (FJK-16s) (all from eBioscience Inc.) were used to stain regulatory T cells.

Cell Preparation

Monocytes

Whole blood samples collected in EDTA were incubated with Fluorescein-conjugated anti-CD14 mAbs for 30 minutes in the dark at 4°C. To lyse and remove red blood cells, samples were incubated with BD Pharm Lyse ™ lysing buffer for 15 minutes in the dark at room temperature, gently vortexing every 5 minutes. The resulting cell populations were washed and analyzed by flow cytometry.

Regulatory T cells

Whole blood samples collected in EDTA were incubated with Fluorescein-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs for 30 minutes in the dark at 4°C. To remove red blood cells, samples were incubated with BD Pharm Lyse ™ lysing buffer for 15 minutes in the dark at room temperature, gently vortexing every 5 minutes. Red blood cells were removed, and the remaining cell populations were washed and stained with anti-FOXP3 staining buffer set following the manufacturer's instructions. Briefly, cells were incubated with fixation/permeabilization solution for 30 minutes in the dark at 4°C, washed twice with permeabilization buffer followed by incubation with APC-conjugated anti-FOXP3 mAbs for 30 minutes in the dark at 4°C. After a single wash, cells were then analyzed by flow cytometry.

Flow Cytometry

Red blood cell depleted canine cells were gated based on their relative size and granularity using forward and side scatters (FSC and SSC, respectively) with FACSCalibur Flow Cytometer (Becton Dickinson). Immunofluorescent stainings were

analyzed using FlowJo 7.6.4 Software (Tree Star, Inc.). The CD14 immunofluorescent

staining in canine monocytes was analyzed by using single histogram statistics (Figure

5.2). A three-color analysis was performed to assess the FOXP3 staining by gating on

CD4+CD25+ double positive T cells and analyzed by using single histogram statistics

(Figure 5.3). In addition, the intensity of the CD25 fluorescence in the CD4+FOXP3+

cells was assessed by using dot plots with multiple gate statistics (Figure 5.4).
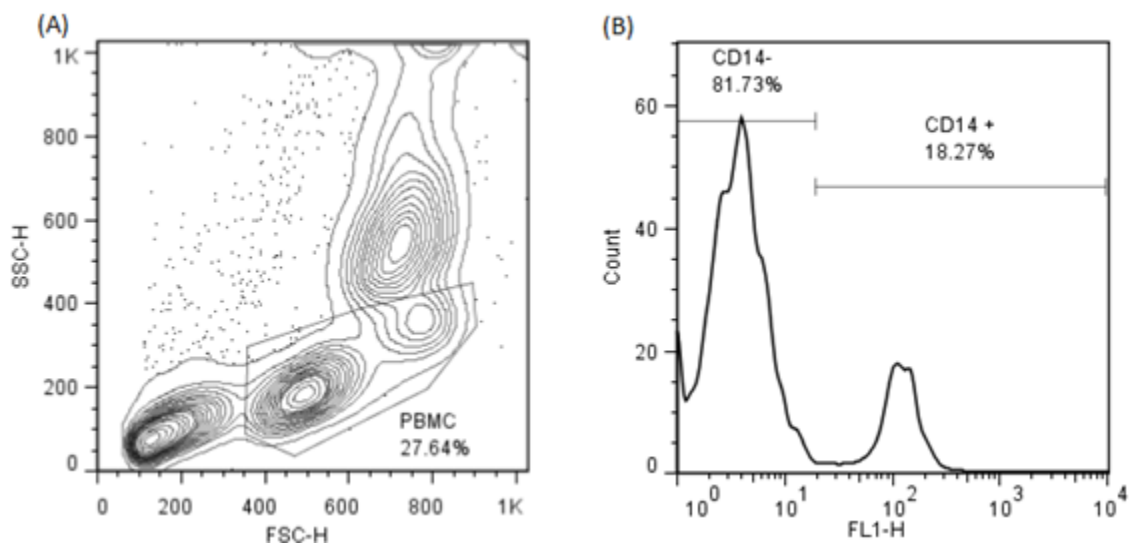

Figure 5.2   Assessment of CD14$^+$ monocytes by flow cytometry.

RBC- depleted canine cells were gated based on their relative size and granularity using
forward and side scatters (A). The CD14 immunofluorescent staining in canine
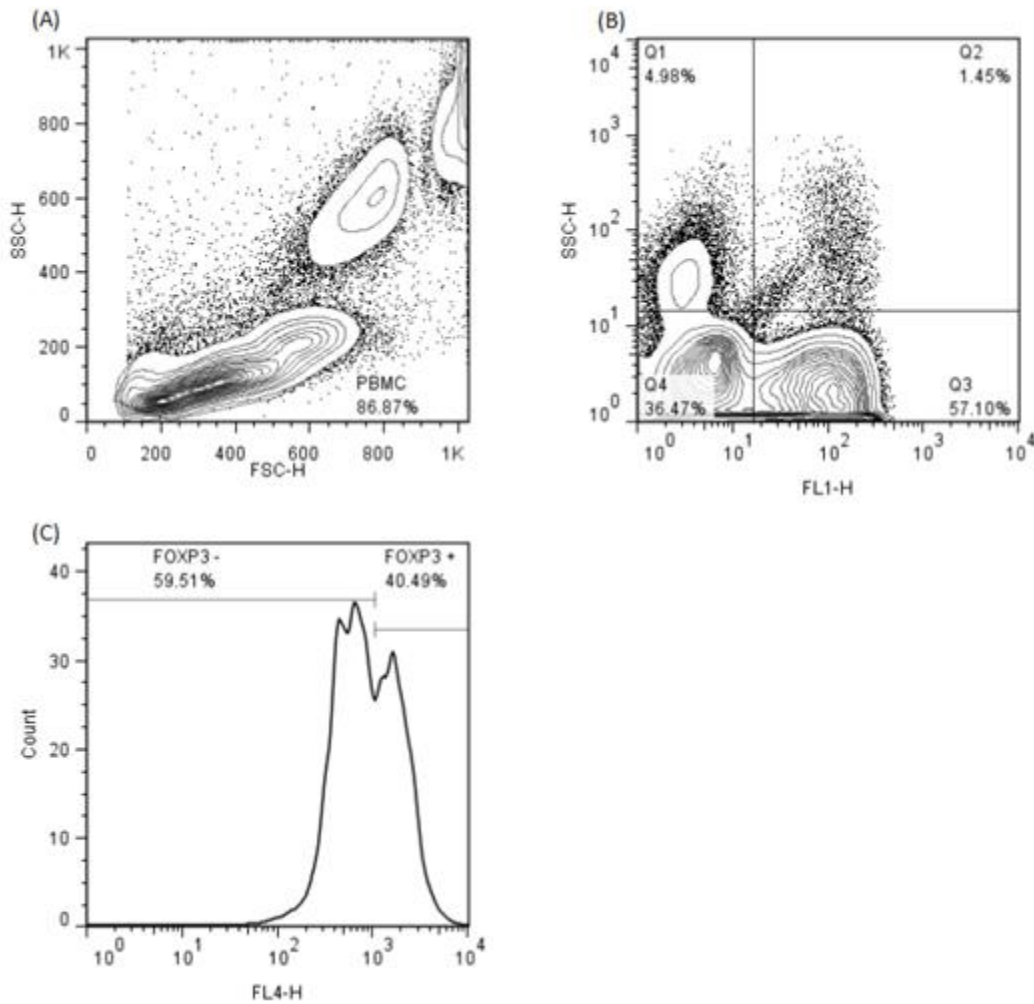monocytes was analyzed by using single histogram statistics (B).

Figure 5.3   Identification of CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells by three color flow cytometry approach.

RBC- depleted canine cells were gated based on their relative size and granularity using forward and side scatters (A). Two color analysis for the CD4+CD25+ T cells was performed by using dot plots with quadrant statisics (B). The FOXP3 staining intensity was analyzed by using single histogram statistics (C).
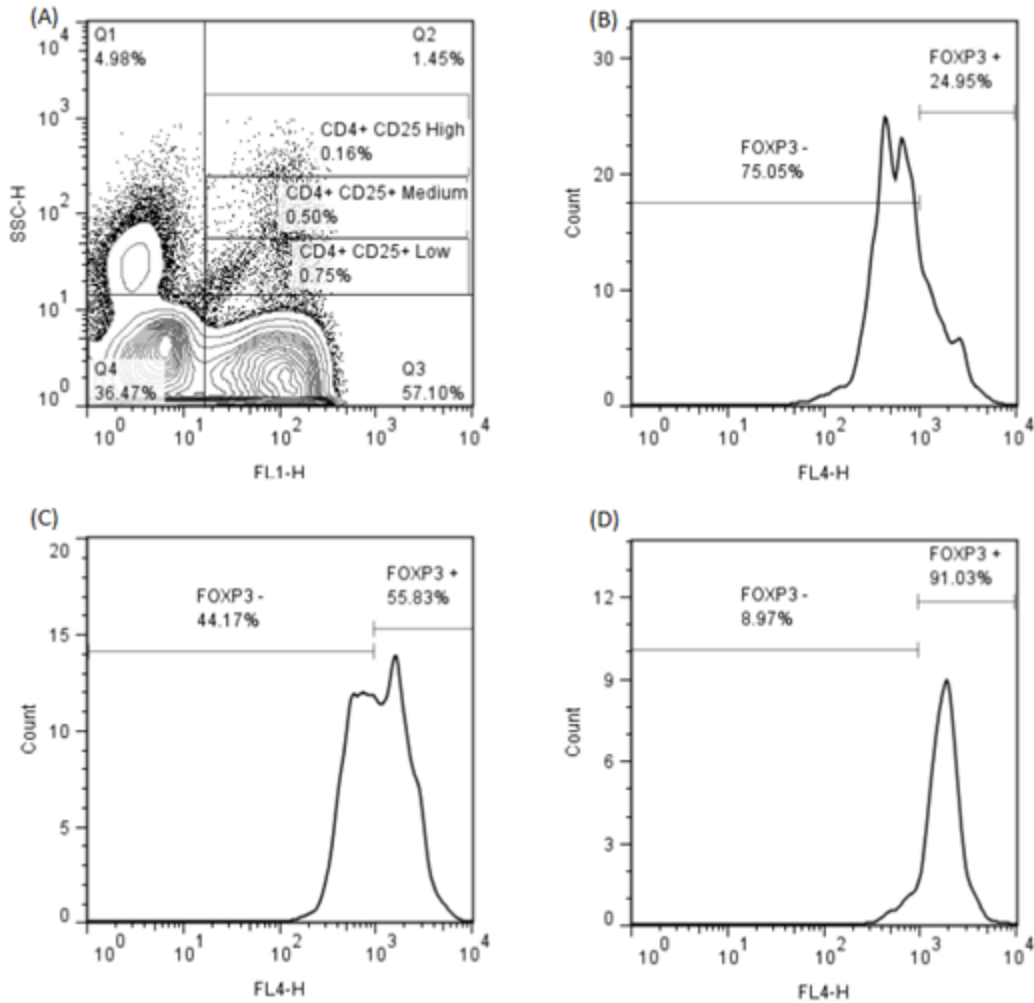
Figure 5.4   FOXP3 expression in peripheral blood of CD25^{low}, CD25^{medium}, and CD25^{high} CD4$^+$ T cells.

The CD4+ T cells were gated based on the brightness of CD25 staining (A). FOXP3 histograms of T cells of low, medium, and high CD25 fluorescence intensity (B,C,D, respectively).

Statistical Analysis

Regulatory T cell marker-specific populations were expressed as a percentage of the total lymphocyte numbers or as absolute cell numbers. CD14+ monocyte populations were expressed as a percentage of PBMC. Then, data was subjected to a one-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison post hoc

test and are presented as means + SD. The level of significance for all tests of effects was set at P<0.05.

Results

Liposomal clodronate exposure promotes increases in the levels of CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells in greyhounds

To characterize effects of the monocyte depletion on the levels of Tregs in dogs, we treated 4 healthy greyhounds with different doses of LC and assessed the levels of peripheral blood Tregs by flow cytometry. Percentage of CD4$^+$CD25$^+$FOXP3$^+$ Tregs significantly increased in greyhounds treated with low and medium doses of LC (Figure 5.5). Although the percentage of Tregs in dogs treated with high dose of LC increased, these increases were non-significant (Figure 5.5).
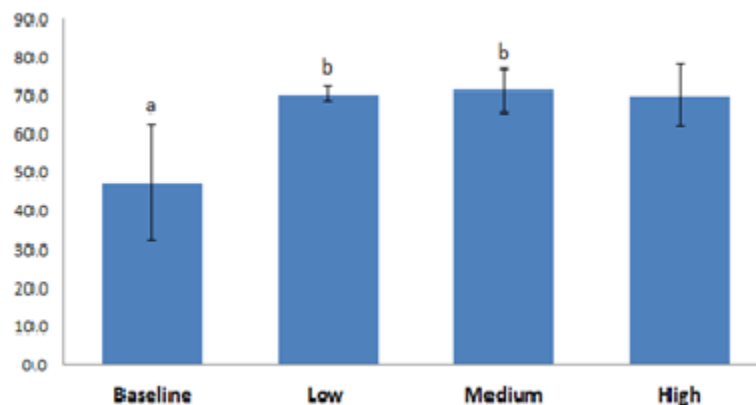

Figure 5.5   LC exposure promotes increases in regulatory T cell populations in dogs.

Regulatory T cells from 4 healthy greyhounds exposed to low (0.5 ml/kg), medium (1 ml/kg), and high (2 ml/kg) doses of LC were assessed by three color flow cytometry approach. Data are expressed as a mean % Tregs of total PBMC. (a b) indicates treatment differences (P<0.05).

Effects of LC treatment on the peripheral blood regulatory T cell and monocyte fluctuations in Walker hounds

To further investigate possible mechanisms of LC-dependent regulatory T cell fluctuations, we assessed the levels of $CD4^+CD25^+FOXP3^+$ Tregs and CD14+ monocytes in Walker hounds challenged with medium dose LC. As expected, LC treatment promoted decreases in $CD14^+$ monocyte numbers in all experimental dogs after 24hrs of treatment, followed by significant increases during compensatory period (4-7 days post treatment) and declining to initial baseline levels after 8-11 days of LC challenge (Figure 5.6, C). In general, the LC-dependent Tregs fluctuations were similar to the changes in the levels of $CD14^+$ monocytes. However in contrast to the monocyte levels, the percentage of Tregs after 24hrs of treatment showed only slight non-significant decrease (Figure 5.6, A). Next, we evaluated the absolute numbers of Tregs in the peripheral blood of Walker hounds before and after LC treatment. Figure 5.6, B demonstrates a significant decrease in the Treg numbers after 24hrs of LC treatment, an expected compensatory increase and a significant recovery of Tregs during the normalization period.
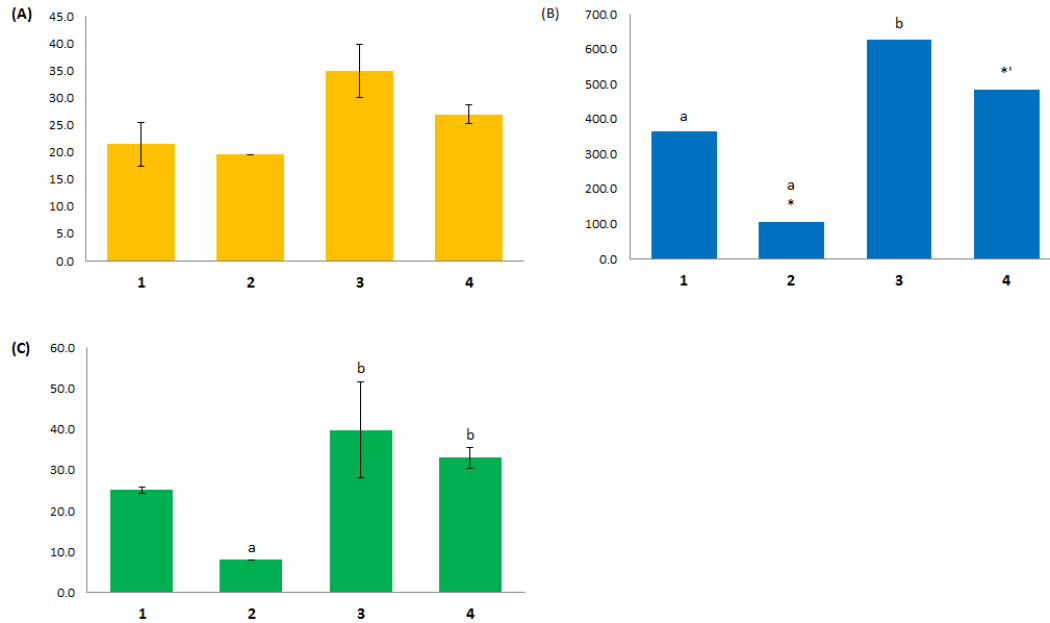
Figure 5.6   Effects of LC treatment on the levels of CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells and CD14$^+$ monocytes in peripheral blood of Walker hounds.

Regulatory T cells and monocytes from 3 healthy dogs exposed to medium dose of LC (1 ml/kg) were assessed by three and one color flow cytometry analysis, respectively. (A) % Tregs of total lymphocytes; (B) absolute numbers of Tregs; (C) % monocytes of total PBMC; (1) Baseline levels[prior to treatment]; (2) Initial response to LC [24 hours post treatment]; (3) Compensatory response to LC [4-7 days post treatment]; (4) Normalization [8-11 days post treatment]. Data are expressed as a mean % of total PBMC (A,C) or as mean absolute cell counts (B). (a b *) indicates group differences (P<0.005).

CD4$^+$CD25^{low}FOXP3$^+$ are the major regulatory T cell subset affected by LC exposure

Previously reported data in humans demonstrated that although CD4$^+$CD25$^+$ T cells contain Tregs, other cells such as recently activated pathogenic T cells may also fall in this phenotypic subset [29]. The top 2% CD4$^+$CD25^{bright} T cells most reliably identified a highly enriched FOXP3$^+$ Tregs. To identify the population of Tregs selectively targeted by LC we applied multiple gate statistics for the assessment of CD25 fluorescence intensity in CD4$^+$CD25$^+$FOXP3$^+$ T cells (Figure 5.7). There was a

numerical decline in % of Tregs expressing low, medium and high levels of CD25 two

days after treatment, followed by an increase, which was significant only in

$CD4^+CD25^{low}$ $FOXP3^+$ Tregs. The levels of Tregs in all three populations with

differential expression of CD25 after two weeks of LC exposure were comparable with
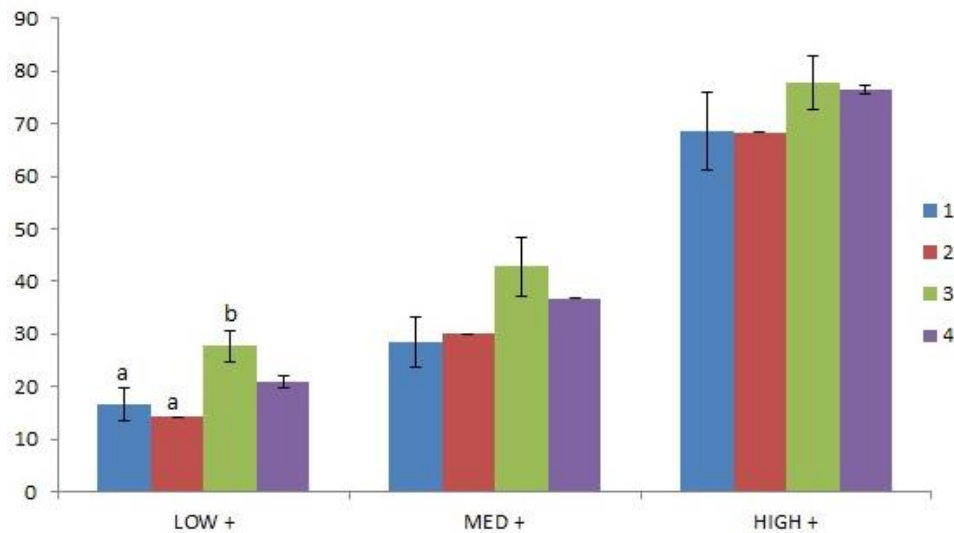
their levels prior to the LC treatment.

Figure 5.7   LC exposure promotes significant increases in the most recently activated
$CD4^+CD25^{low}FOXP3^+$ Tregs.

$CD4^+FOXP3^+$ T cells from the dogs exposed to LC were gated based on brightness of
CD25 staining resulting in separation into low, medium, and high expressers of CD25.
(1) Baseline levels [prior to treatment]; (2) Initial response to LC [24 hours post
treatment]; (3) Compensatory response to LC [4-7 days post treatment]; (4)
Normalization [8-11 days post treatment]. Data are expressed as mean % of total Tregs.
(a b) indicates group differences.

Discussion

Adequately described in humans and mice, regulatory T cells

($CD4^+CD25^+FOXP3^+$ T cells) are an essential requirement for the healthy function of the

mammalian immune system as they function to modify or inhibit effector cells. This

makes them a double edged sword. When functioning properly, they help to maintain homeostasis within the immune response, but if malfunctioning or absent, they can impede beneficial immune responses, and systemic and/or organ-specific autoimmunity can result [19].

Little is known about the functional purpose and clinical relevance of Tregs in canines, but results from recent studies have provided initial phenotypic and functional characterizations of Tregs within the canine system [20, 21, 22]. Current research objectives include understanding Tregs across a wide range of disease models. The results of current studies indicate that increased numbers of Tregs are associated with disease consequence [22, 23, 24, 25, 26, 27]. This study expands previous research to include new breeds of healthy dogs, while observing variation between numbers of Tregs in greyhounds and Walker hounds.

In the current study, the overall effect of the differing doses of LC on the experimental dogs was assessed during the greyhound study. As previously mentioned, LC has many uses in the treatment of autoimmune diseases as well as research [13, 14, 15, 16, 17, 18]. The goal in using LC was to transiently knock out the immune system by initiating a period of monocyte/macrophage/DC depletion, thus allowing us to evaluate the levels of decrease or generation of regulatory T cells over the defined treatment period.

For each dog, a similar pattern was observed in the levels of Tregs in response to each dose (low, medium, high). The overall health of the dogs also varied. At the low and medium dose, no clinical signs or negative effects were witnessed in the dogs. While at the high dose, dogs developed clinical signs including transient fever, diarrhea, clear

nasal discharge, and general malaise. To this end, the medium dose (1 mg/kg) was chosen

to be utilized in the Walker hound study. This dose allowed resultant changes in Treg

levels to be observed while avoiding the development of clinical signs.

For the first time, this study evaluated numbers of canine regulatory T cells in the

absence of professional antigen presenting cells (APCs), monocytes, and immature DCs

by utilizing LC. In the greyhound study, the number of Tregs after a visible profound

decline (during the initial response phase, 24 hrs post treatment) significantly increased

when dogs were treated with the low and medium dose, and while the high dose also

resulted in increase in Tregs, no significance was observed.

The relationships between monocytes/macrophages and Tregs within the canine

model are unknown. Recent studies have expanded the role of monocytes and

macrophages beyond presenting antigens to effector T cells and stimulating T cell

mediated immune responses. They also are responsible for initiating adaptive immunity

via priming of naïve T cells, triggering the generation of new Tregs, a critical subset of T

cells [6,7,8]. Therefore, in the Walker hound study, both monocyte and Treg numbers

were assessed simultaneously. The significant decreases in monocyte numbers in all

experimental dogs 24 hrs after treatment was expected and reflects the expected

mechanism of action of LC. When the bisphosphonate clodronate is incorporated within

liposomes, phagocytosis by monocytes/macrophages and immature DCs is enhanced

[28]. Lysis of the mitochondrial membrane of host cells by LC induces apoptosis

resulting in the transient depletion of monocytes [11]. At the same time, absolute Treg

numbers were also evaluated showing significant compensatory gains as well as the

significant recovery of Treg numbers during the normalization period. In theory, these

compensatory gains reflect initiation of adaptive immune response by production of new Tregs via activation from naïve T cells in the periphery.

As a part of the Walker hound study, we produced data that mirrors information reported in human Tregs that, although canine $CD4^+CD25^+$ T cells contain Tregs, other cells such as recently activated pathogenic T cells may also fall in this phenotypic subset. The top 2% $CD4^+CD25^{bright}$ T cells most reliably identified a highly enriched $FOXP3^+$ Tregs in dogs. We report that $CD4^+CD25^{low}FOXP3^+$ is the major regulatory T cell subset affected by LC exposure, showing the significantly increased numbers after 4-7 days of LC-treatment. These increases suggest the active recruitment and generation of Tregs from naïve $CD4^+$ peripheral blood T cells.

In conclusion, further research is essential in creating a working understanding of canine $CD4^+CD25^+FOXP3^+$ Tregs, and the role of professional APCs monocytes and DCs in their generation. Tregs are a critical subset of immune cells which are produced during T cell development in the thymus and can be induced in the periphery from naïve T cells to provide antigen specific inhibition in cases of infection or disease.

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