CONTRIBUTION OF DEFECTIVE CYTOTOXICITY TO DEVELOPMENT OF CANINE HEMOPHAGOCYTIC HISTIOCYTIC SARCOMA

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CONTRIBUTION OF DEFECTIVE CYTOTOXICITY TO DEVELOPMENT OF CANINE HEMOPHAGOCYTIC HISTIOCYTIC SARCOMA

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University of Guelph, 2011

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Canine Hemophagocytic Histiocytic Sarcoma (CHHS) is an aggressive neoplasm of macrophages with local lymphocytic reaction. Similarities exist between CHHS and Familial Hemophagocytic Lymphohistiocytosis (FHL), a complex of histiocytic diseases in children, which is attributable to various defects in granule dependent killing (GDK). This led to the hypothesis that defective GDK compromises lymphocyte homeostasis and anti-tumor immunity which results in CHHS.

The sequence of canine perforin, a key effector molecule of GDK, was determined by RT-PCR and RACE. Genomic DNA from healthy and CHHS-affected dogs was sequenced and analyzed, but mutations with functional implications were not identified. Subsequently, tumor infiltrating lymphocytes (TIL) of CHHS were examined for GDK functionality. CHHS-TIL were compared to their functional counterparts in canine cutaneous histiocytoma (CCH), a benign histiocytic tumor in dogs, known to regress via lymphocytic reaction. To facilitate such comparison, functionality of CCH-TIL was studied by immunohistochemistry and confocal microscopy and quantified by image analysis.
applications. This provided novel insights regarding the physiology of TIL in tumor microenvironment and further characterizing CCH as a model for anti-tumor immunity.

The comparison revealed a clear, and highly significant structural difference in polarization and degranulation of CHHS-TIL which likely hampers GDK. This defect is similar to several variants of FHL, an association further supported by comparison of clinical and laboratory manifestations of CHHS and FHL. This study suggests that CHHS is a promising natural model for investigating the pathogenesis of FHL, for studying granule polarization and degranulation and assessing the role of TIL in anti-cancer immunity.
I express my deep and sincere gratitude to my advisor, Dr. Robert Jacobs. Thank you for your faith, trust, and encouragement and for endless support; for allowing me to pick the paths and lead this research as I envision it. Thank you for always being both critical and constructive and for teaching me the beauty and wisdom of simplicity.

Many thanks to my committee members: Dr. Darren Wood, Dr. Josepha DeLay, Dr. Brenda Coomber and Dr. Peter Moore for your advice and overall contribution throughout my work; special thanks goes to Dr. Moore for generously sharing DNA and tissue samples as well as knowledge and expertise regarding canine histiocytic diseases. Further special gratitude goes to Dr. DeLay and the excellent staff of AHL Histology, particularly Mrs. Susan Lapos who guided me through the witchcraft and spells of the world of immunohistochemistry.

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I am thankful for the Pet Trust foundation for generously funding this work. I am also thankful to the Bernese mountain dog breeders and owners who facilitated sample collections to this study, even in the most difficult times of mourning for their pet. I am hopeful that the work presented in this thesis will have beneficial implications on the health and wellbeing of dogs in general and particularly the Bernese mountain dog breed.

Finally, I thank my family, my parents Yoav and Ester Neta who taught me that all is possible, my dear husband, best friend and partner on the journey Ram Samocha, and our daughters Alma and Gille. Thank you for your faith, endless support and sacrifice. Thank you for bringing so much joy to my life; your love and support made it all possible and worthwhile.
DECLARATION OF WORK PERFORMED

All work reported in this thesis was performed by me with the following exceptions:

Rapid amplification of cDNA ends (RACE) attempting to identify the an un-translated exon of the perforin gene was done by Mrs. Shery X. Wen

The amino acids sequence of the immunogenic peptide which was utilized for the generation of the canine perforin antibody was selected by Dr. Paula Katavalos.

Peptide synthesis, animal immunizations, generation and purification of the antibody were done by Pacific Immunology ((Ramona, CA, USA))

Some of the tissue collection and nucleic acid extraction were performed by Dr. Peter Moore and his lab staff.

Dr. Feng Xu was involved and provided technical assistance for all of the confocal work.

Mr. William Sears was involved in all statistical analysis.

Mr. Ram Samocha was involved in Photoshop formatting of the figures in chapters 3 and 4
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<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>BMD</td>
<td>Bernese mountain dogs</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCH</td>
<td>Canine cutaneous histiocytoma</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster differentiating molecule</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>Colony-forming unit granulocyte macrophage</td>
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<tr>
<td>CG</td>
<td>Cytotoxic granules</td>
</tr>
<tr>
<td>CHD</td>
<td>Canine histiocytic diseases</td>
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<tr>
<td>CHHS</td>
<td>Canine hemophagocytic histiocytic sarcoma</td>
</tr>
<tr>
<td>CHS</td>
<td>Canine histiocytic sarcoma</td>
</tr>
<tr>
<td>CL</td>
<td>Cytolytic lymphocytes</td>
</tr>
<tr>
<td>CMLE</td>
<td>Conditional maximum likelihood estimate</td>
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<tr>
<td>CRH</td>
<td>Canine reactive histiocytosis</td>
</tr>
<tr>
<td>CS</td>
<td>Cutaneous histiocytosis</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic lymphocytes</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4, 6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Langerhans/dendritic cell</td>
</tr>
<tr>
<td>DHS</td>
<td>Disseminated histiocytic sarcoma</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulopathy</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>eCTL</td>
<td>Effector cytotoxic lymphocytes</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>ExPASy</td>
<td>Expert protein analysis system</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas-Fas ligand</td>
</tr>
<tr>
<td>FCR</td>
<td>Flat coated retriever</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>FHL</td>
<td>Familial hemophagocytic histiocytosis</td>
</tr>
<tr>
<td>FHLH</td>
<td>Familial Hemophagocytic lymphohistiocytosis</td>
</tr>
<tr>
<td>GDK</td>
<td>Granule dependent killing</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylated glycophaspatidylinositol</td>
</tr>
<tr>
<td>Grz</td>
<td>Granzymes</td>
</tr>
<tr>
<td>GrzB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hemotoxylin and eosin</td>
</tr>
<tr>
<td>HLH</td>
<td>Hemophagocytic lymphohistiocytosis</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box1 protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMHA</td>
<td>Immune mediated hemolytic anemia</td>
</tr>
<tr>
<td>IS</td>
<td>Immunological synapse</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LFOV</td>
<td>Large fields of view</td>
</tr>
<tr>
<td>LHS</td>
<td>Localized histiocytic sarcoma</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MAC-1</td>
<td>Macrophage adhesion molecule-1</td>
</tr>
<tr>
<td>MH</td>
<td>Malignant histiocytosis</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>MUE</td>
<td>Median unbiased estimate</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>Perf</td>
<td>Perforin</td>
</tr>
<tr>
<td>PMD</td>
<td>Piecemeal degranulation</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>PVDF</td>
<td>Immobilon P&lt;sup&gt;®&lt;/sup&gt; polyvinylidene fluoride</td>
</tr>
<tr>
<td>RACP</td>
<td>Rabbit anti canine Perf</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for glycation end products</td>
</tr>
<tr>
<td>RCH</td>
<td>Reactive cutaneous histiocytosis</td>
</tr>
<tr>
<td>RH</td>
<td>Reactive histiocytosis</td>
</tr>
<tr>
<td>RR</td>
<td>Rate ratios</td>
</tr>
<tr>
<td>RSH</td>
<td>Reactive systemic histiocytosis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RT PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH</td>
<td>Systemic histiocytosis</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SOTR</td>
<td>Stages of tumor regression</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocytes</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Trig</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TTB</td>
<td>Tris tween buffer</td>
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</table>
GENERAL INTRODUCTION

Histiocytes are cells of the immune-system. They are essential for antigen presentation and host defense. Histiocytic diseases in people and dogs cause pathologic infiltration of normal tissue with either reactive or neoplastic histiocytes of various histiocytic lineages. Accordingly, the classification scheme of histiocytic diseases in both human and veterinary pathology requires identification of the pathological lineage and the underlying pathological processes such as immune dysregulation and neoplasia.

Some breeds of dogs are prone to develop cancer or uncontrolled inflammation of histiocytes, collectively referred to as Canine Histiocytic Diseases (CHD). A wide array of phenotypic tools has been validated to facilitate classification of the different diseases in the CHD complex. Malignancies and immune-regulatory disorders of histiocytes are uncommon in the general canine population, but occur at exceedingly high prevalence in only a few breeds suggesting a genetic causation and a common underlying mechanism.

Canine Hemophagocytic Histiocytic Sarcoma (CHHS) is an aggressive variant of the CHD complex which manifests as both a neoplastic and an inflammatory disease. The duality of CHHS provides a valuable model to seek a fundamental mechanism responsible for the neoplastic and inflammatory manifestations of the CHD complex. CHHS shares similarity in clinical, histological and laboratory findings with a complex of genetic histiocytic diseases in children which are collectively referred to as Familial Hemophagocytic Lymphohistiocytosis (FHL).

FHL is a complex of fatal, genetic diseases of histiocytes that occur in young children and is strongly associated with consanguinity. Defective genes in children with
FHL encode for proteins that are involved in the effector mechanisms of the immune system, namely, in Granule dependent killing (GDK) which is employed by cytolytic lymphocytes (CL). The two main subsets of CL are the natural killer (NK) cells and the CD8+ cytotoxic lymphocytes (CTL) which serve as effectors of the innate and adoptive immune system, respectively. Functional CL kill malignant cells at early stages of cancer development, eliminate viral infection and terminate inflammatory processes. As demonstrated in children with FHL, lack of effective killing leads to detrimental consequences which include the development of perpetuating, fatal inflammation, increased susceptibility to cancer, and increased vulnerability to intracellular pathogens. FHL type 2, the most aggressive FHL variant, is associated with a mutation in the perforin (Perf) gene, the key cytotoxic mediator of GDK. Other FHL variants are associated with defects in various components of the cytotoxic apparatus including excretion and trafficking of cytotoxic granules.

Bernese Mountain Dogs (BMD) have the highest incidence of CHD. Malignancies of histiocytes are familial diseases in BMD and are a leading cause of mortality in the breed often at an extremely young age. BMD also show hereditary susceptibility to other types of cancers as well as to immune regulatory disorders of histiocytes and to borreliosis. The BMD breed’s demography, small founding population, pedigree barriers, and extensive use of popular subjects for reproduction have led to very high levels of consanguinity and unusually limited genetic pool in the current progeny. It is reasonably assumed that the genetic background of this breed accounts for its high morbidity and mortality, but a causative genetic locus has not been identified.
Given similarities in the clinical syndromes of BMD with CHHS and children with FHL, and the strong genetic background of CHHS in BMD, it is plausible that defective cytotoxic function also underlies CHHS. This mechanism may also account for other hereditary conditions affecting BMD. Demonstrating such a mechanism will provide a model that may account for the wide range of hereditary conditions causing such high morbidity and mortality in this breed.
CHAPTER 1.

LITERATURE REVIEW

Canine Histiocytic Diseases

Canine Histiocytic Diseases (CHD) constitute a heterogeneous group of diseases including immune regulatory disorders, and benign and malignant neoplasms. All subtypes of the CHD complex exhibit pathologic infiltration of normal tissue with histiocytes. However, despite this similarity, these conditions vary dramatically in their biological behavior and outcome. The classification of histiocytic diseases continues to cause a great deal of confusion in the human and veterinary literature. A possible source of this confusion is the delay between initial descriptions of these diseases and the availability of reliable markers that allow determination of the different lineage of the cells involved in each condition. In the last decade, there has been an immense advancement in the characterization of the different diseases of the CHD complex and a wide array of phenotypic tools is now available to facilitate the accurate diagnosis of these conditions. Similar to human pathology, the two key criteria for CHD classification are the type of underlying pathological process (neoplasia or immune regulatory disorder) and the origin and level of maturation of the pathogenic cell. Thus, a prerequisite to comprehend the complexity and nomenclature of the CHD complex is to become familiar with the different subsets of histiocytic lineages and their specific immunophenotypic characteristics.
**Histiocytes**

The term “histiocyte” refers to cells of the mononuclear phagocyte system (MPS) and represents a wide array of cells exhibiting variable morphological, functional and immunophenotypic characteristics. The MPS is divided into two major sub-categories; the monocyte/macrophage series and the Langerhans/dendritic cell (LC/DC) series. Cells of the MPS originate from common CD34+ bone marrow stem cells which give rise to lymphoid and myeloid progenitor cells. Lymphoid progenitors give rise to pluripotent precursors which may differentiate to thymic DC, and plasmacytoid DC. Interdigitating dendritic cells in T-cell rich regions of lymph nodes may originate directly from a pluripotent lymphoid precursor, but may also represent full maturation of homing LC subsequent to antigen presentation. Due to lack of canine-specific markers, DC of lymphoid origin have not yet been characterized in the dog and will not be further discussed. Myeloid progenitors give rise to the monocyte/macrophage series and to a committed precursor that differentiates to LC and to dermal and interstitial DC. Macrophages develop from a committed colony-forming unit granulocyte macrophage (CFU-GM) stem cell, which gives rise to pro-monocytes that further mature to monocytes and leave the bone marrow to circulate in peripheral blood. Circulating monocytes leave the bloodstream to enter tissues and complete their maturation to tissue macrophages according to the local cytokine milieu. Within tissue, macrophages may survive months and maintain proliferative capacity. LC also develop from a circulating mononuclear precursor, which originates from the bone marrow. Maturation of LC is initiated in the epidermis, but can only be completed in the T-cell rich region in the lymph nodes.
Despite some morphological similarities, susceptibility to similar growth stimulatory signals and several common surface antigens, macrophages/monocytes and LC/DC represent two distinct series which serve vastly different functionalities in innate and adaptive immunity. Macrophages and monocytes are competent antigen presenting cells (APC) but function primarily as professional phagocytes. Via phagocytosis, macrophages clear infectious agents as well as organic and inorganic waste. Serving as both professional APC and professional phagocytes, macrophages play a crucial role in defense mechanisms. Macrophages exhibit widespread tissue distribution and are particularly abundant in mucosal surfaces consistent with their function as gatekeepers of innate and adaptive immunity. Examples of fully differentiated end cells of the monocyte/macrophage series include splenic sinusoidal macrophages, pulmonary alveolar macrophages and Kupffer cells in the liver. LC and DC cells are poor phagocytes. The fundamental function of LC is antigen capture and processing. DC are the only APC that can activate naive lymphocytes, and therefore are essential for initiation of the adaptive immune response. LC, dermal and interstitial DC localize to epidermal surfaces, dermis and connective tissue, respectively.

Immunophenotyping of Histiocytes

Given the morphological similarity of the different histiocyte types, the availability of immunophenotyping techniques provides an efficient and reliable way to identify the origin of the pathogenic cell in the different histiocytic disorders. A great deal of effort has been dedicated to identifying and optimizing immunophenotypic profiling for the different histiocytic diseases in the dog. Similar to the approach in human pathology, the principal tactic is to target markers which define functionality and reflect specific localization of these cells.
Cluster Differentiating Molecule type 1 Family and the Major Histocompatibility Complex

Cluster Differentiating (CD) Molecule type 1 (CD1) and the Major Histocompatibility Complex (MHC) type 1 and 2 are the key molecules involved in processing and presentation of peptides, lipids and glycolipid antigens. In line with their fundamental role as APC, LC and DC express a rich array of MHC-2 and CD1a. Two major populations express high levels of CD1 and MHC 2 within the skin of the dog; LC and dermal DC. Markers that reflect specific localization of these cells are utilized to distinguish between these two populations. Conversely, macrophages which are less equipped to fulfill APC function exhibit weak to non-existent CD1 expression.

E-cadherin

E-cadherin is a calcium-dependent adhesion molecule which is expressed predominantly in epithelial tissue. Expression of E-cadherin on histiocytes is unique to LC cells, as it is dictated by their specific microenvironment reflecting interaction with neighboring keratinocytes.

Thy 1 (CD90)

Thy 1 is a heavily glycosylated glycophosphatidylinositol (GPI) anchored cell surface protein which was first identified on murine thymocytes and T-cells. Since initial characterization, expression of Thy 1 was found in other species including humans and dogs and in a wide variety of cells including: thymocytes, prothymocytes, neurons, mesenchymal stem cells, hematopoietic stem cells, NK cells and endothelium, particularly venules. When expressed by histiocytes, Thy 1 interacts with the leukocytes integrin Macrophage adhesion molecule-1 (MAC 1) (CD11b/CD18; alpha MB2) mediating adhesion of
mononuclear cells to endothelial cells and fibroblasts, and facilitating leukocyte homing and recruitment. Thus, within the context of histiocyte classification, combined expression of CD1 and Thy 1 is unique to dermal and interstitial dendritic cells, as it reflects their specific localization to connective tissue and strong association with post-capillary endothelial venules. Accordingly, in the dog, abundant Thy 1 expression was found on dermal DC and facilitates further distinction from epidermal LC.

Beta-Integrin Family: CD18/CD11a-d

CD18 is the beta subunit of the beta2-integrin complex. When utilized for flow cytometry, CD18 is considered a pan-leukocyte marker, as it is expressed on all leukocytes including lymphocytes, granulocytes and histiocytes. However, canine histiocytes and granulocytes express 10 fold more CD18 than lymphocytes. Subsequent to formalin fixation, the scant CD18 expression on lymphocytes is diminished to undetectable levels and cannot be regained by antigen retrieval methods. Therefore, for classification of fixed mononuclear cells in the face of negative expression of lymphoid differentiating markers, expression of CD18 is considered highly suggestive of histiocytic origin. The availability of an excellent canine antibody for formalin fixed paraffin embedded (FFPE) tissue makes CD18 a very popular antibody for diagnostic veterinary pathology. However, a few caveats with respect to this marker need to be considered. Firstly, although normal fixed B and T cells typically present as CD18 negative, it is not inconceivable that neoplastic lymphocytes may express CD18. Also, NK lymphocytes might exhibit CD18 immunoreactivity despite negative CD3 and CD79a expression. Furthermore, utilization of CD18 for distinction between the different histiocytic lineages relies on a more prominent expression on macrophages compared to DC. Correspondingly, strong CD18 expression
combined with weak expression of MHC-2 is supportive of macrophage origin while strong expression of MHC-2 with variable levels of CD18 expression is characteristic of DC origin.\textsuperscript{42, 43, 116} Such assessment is subjective and requires ample experience to yield reproducible results. In summary, CD18 is a good and reliable marker for the histiocytic lineages, but should not be used as a sole marker and should be interpreted with caution.

Expression of the alpha sub-unit of the integrin family is not specific for histiocytes, but can be used to distinguish different histiocyte lineages.\textsuperscript{99} CD11c is usually expressed by DC while macrophages typically express CD11b. CD11d is the latest recognized alpha subunit of the integrin family. The function of CD11d is not fully determined, however, it is a useful marker for immunophenotyping as it is expressed differently from other alpha integrins (CD11a-c). CD11d is a multi-ligand macrophage receptor expressed predominately on subsets of the myelomonocytic lineage and, particularly on macrophage foam cells and splenic red pulp macrophages.\textsuperscript{36} Its expression initially occurs early in bone marrow stem cells, decreases simultaneously with acquisition of other leukocyte integrins and re-emerges when peripheral blood monocytes differentiate to macrophage foam cells or to splenic red pulp macrophages.\textsuperscript{113} It has been shown recently that CD11d is involved in modulating macrophage adhesiveness and migratory capacity.\textsuperscript{165} As well, it has been shown that while low levels of CD11d in monocytes facilitates migration; up-regulation of CD11d promotes retention of macrophages at the site of inflammation.\textsuperscript{165}

Canine CD11d is expressed by macrophages in the splenic red pulp and bone marrow.\textsuperscript{104} Expression of CD11d in splenic red pulp macrophages is likely a consequence of their tissue origin as these molecules are also expressed on granular lymphocytes which are residents of splenic red pulp.
**Canine Histiocytic Diseases**

The Canine Histiocytic Diseases (CHD) complex has been sub-classified to five distinct entities reflecting different pathologic processes and pathogenic cell lineages. (Figure 1-1) These entities were defined based on clinical, histological and immunophenotypic characteristics. These include reactive cutaneous and systemic histiocytosis (RCH, RSH respectively), which are immune regulatory disorders of activated interstitial dendritic cells,\(^5\) CCH(CCH) a benign cutaneous neoplasm of LC;\(^1\) localized and disseminated histiocytic sarcoma (LHS, DHS respectively), highly malignant neoplasms of interstitial dendritic cells;\(^4\) and canine hemophagocytic histiocytic sarcoma (CHHS), classified as a malignant neoplasm of red pulp macrophages.\(^1\)

**Figure 1-1: Classification of Canine Histiocytic Diseases**

![Diagram of Canine Histiocytic Diseases]
Canine Reactive Histiocytosis

Immune regulatory disorders of activated dermal and/or interstitial DC

The term Canine Reactive Histiocytosis (CRH) refers to two distinct syndromes, cutaneous and systemic histiocytosis representing two clinical manifestations of reactive proliferation of activated interstitial dendritic cells.\(^5\)

**Cutaneous Histiocytosis (CH)** is a cutaneous and subcutaneous disease with no systemic expression. It is associated with a wide age range and has no specific breed or sex predilection.\(^5\)

**Systemic Histiocytosis (SH)** was initially described as a familial disease of BMD, but was later identified in several large canine breeds.\(^5\)

Cutaneous and subcutaneous lesions of cutaneous and systemic histiocytosis are identical and consist of non-pruritic non-painful cutaneous nodules. SH manifests with a wider lesion distribution which includes mucosal surfaces and visceral organs. Histologically, CH lesions consist of sheets of monomorphic morphologically unremarkable histiocytes extending from the dermal epidermal junction towards the deep dermis and often expanding to peripheral lymph nodes. SH consists of infiltration of parenchymal organs with similar well-differentiated histiocytes. SH lesions are angiocentric and are often associated with vaso-invasion, thrombosis and necrosis. Both CH and SH lesions are associated with a prominent reactive lymphocytic infiltrate.\(^5\)

The immunophenotypic profile of reactive histiocytosis is similar in both conditions and consists of strong expression of CD1a-c, CD11c and MHC-2 reflecting antigen presenting capacity of the proliferating population. Expression of Thy 1 reflects association with post-endothelial venules and expression of CD4 implies activation. Collectively, the
immunophenotypic profile of systemic and cutaneous histiocytosis is consistent with activated interstitial and dermal dendritic cells, respectively.

The etiology of SH is unknown, and to date, the initiating factors underlying the reactive histiocytic diseases complex in the dog are unclear. Bacterial and fungal cultures and special stains fail to identify infectious agents. Furthermore, remission of both CH and SH in response to immunosuppressive therapy suggests these are immune regulatory disorders. Drugs that have been most effective in controlling SH are associated with limiting expansion of T cells, suggesting that T cell activation is a key factor in the initiation and/or the acceleration of these disease processes.

**Canine Cutaneous Histiocytoma**

*Benign, self-limiting tumor of LC*

Canine Cutaneous Histiocytoma (CCH) is a common benign neoplasm of LC. It typically presents as a solitary, rapidly growing nodule, often located on the head or limbs. CCH affects mostly (but not exclusively) young dogs and has no specific breed predilection. Histologically, CCH lesions consist of sheets of proliferating LC originating in the epidermis and progressing towards the deep dermis. Immunophenotypically, CCH tumor cells resemble LC and are characterized by strong and consistent expression of CD1a, CD1b, CD1c and CD11c, MHC-2 and E-cadherin. Variable expression of other adhesion molecules (CD11b, CD44, CD54, CD49 and the intercellular adhesion molecule (ICAM) 1) distinguishes CCH tumor cells from resting epidermal LC and suggests an activated phenotype. It has been shown recently that E-cadherin expression can be detected on different cutaneous round cell tumors including plasma cell tumor, epitheliotropic
lymphoma and mast cell tumor. Overall, CCH rarely poses a diagnostic challenge and seldom requires immunophenotypic characterization. Initial characterization of E-cadherin in CCH was not applied for diagnostic purposes, but to recognize LC as the pathogenic histiocyte in CCH.

Although characterized by rapid growth and high mitotic activity, CCH is indisputably a benign tumor. It is characterized by circumscribed, non-invasive growth; it does not metastasize, and it undergoes spontaneous immune mediated regression. Malignant and/or non-regressing variants of LC tumors are reported to be highly aggressive and appear to be overrepresented in Shar-pei and Shar-pei crosses. However, these syndromes are not fully characterized. Malignant manifestation of cutaneous histiocytoma may provide a promising natural disease model for LC histiocytosis, a relatively common histiocytic disease in people.

**Canine Disseminated and Localized Histiocytic Sarcoma- Non Phagocytic Variant**

**Aggressive sarcoma of interstitial dendritic cells**

The term Canine Histiocytic Sarcoma (CHS) is typically used to describe the non-hemophagocytic variant of histiocyte malignancies. CHS is subcategorised based on clinical presentation to localized or disseminated diseases, both of which show strong predilection for BMD, Rottweilers, and Flat Coated Retrievers (FCR) with the former being exceedingly overrepresented.

**Disseminated Histiocytic Sarcoma (DHS)** is an aggressive multisystem neoplasm which is characterized by multiple lesions occurring simultaneously in different visceral
organs. The most commonly targeted organs are spleen, liver, lung, bone marrow, lymph nodes and the central nervous system.

**Localized Histiocytic Sarcoma (LHS)** manifests as rapidly growing, locally invasive tumors, typically occurring on the limbs. These tumors originate in the subcutis and rapidly metastasize to draining lymph nodes. Visceral manifestation of LHS is reported, but occurs less commonly.

DHS and LHS differ in their clinical behavior and outcome. DHS and visceral LHS are consistently devastating diseases with rapid deterioration and poor response to all therapeutic attempts.\(^2, 4, 136\) Peripheral LHS shows a more favorable prognosis with some cases exhibiting no post-surgical reoccurrence or metastasis in response to wide surgical excision or amputation.\(^4\) Given the highly metastatic nature of visceral LHS, it may be difficult to distinguish metastatic LHS from DHS. Thus, it is difficult to avoid wondering whether DHS is truly a primary multicentric malignancy or whether it represents an end-stage manifestation of highly metastatic visceral LHS.

Despite differences in the clinical presentation and prognosis, LHS and DSH are characterized by identical morphological, histological and immunophenotypic features. These tumors are nodular, poorly demarcated and locally invasive with multiple areas of necrosis. Histologically the masses consist of highly pleomorphic, non-hemophagocytic histiocytes effacing normal tissue architecture. Cytological pleomorphism is a characteristic feature of both LHS and DHS. Cell morphology ranges from spindle to round, and the cells exhibit marked anisocytosis and anisokaryosis, occasional multinucleation, frequent features of nuclear gigantism and a high mitotic index. Admixed with the neoplastic histiocytes is a reactive lymphocytic infiltrate.
To date, the immunophenotypic profiles of DHS and LHS are identical. Tumor cells display a phenotype of interstitial DC, consistently expressing molecules involved in antigen presentation (CD1b, CD1c and MHC-2), as well as adhesion molecules (beta 2-integrin-CD11c and Intra-Cellular Adhesion Molecule (ICAM) I). Consistent negative expression of CD4 suggests that the cells comprising DHS and LHS are resting DC, different from the activated phenotype seen in cells of reactive histiocytosis. The lymphocytic infiltrate is dominated by CD8+ cytotoxic T cells, the effectors of a Th1 anti-tumor immune response.

The prevalence of DHS and LHS vary between the pre-disposed breeds. Benoit Hedan et al. have recently presented a thorough comparison between anatomical localization of HS in 45 FCR and 101 BMD. This study demonstrated significant variation between the two breeds. LHS was found to be seven times more frequent in the FCR than in the BMD (46.1% versus 6.5%) while DHS was approximately two-fold higher in the BMD than the FCR (50.8% versus 25.6%); 87% of BMD were presented with HS affecting one or more internal organs compared with 48% of FCR. Furthermore, peripheral manifestation of HS on the limbs was >10 times more frequent in the FCR than in the BMD (38.4% versus 3.2%). These results confirm previous studies which pointed towards breed-specific manifestations of HS and emphasize the value of investigating these diseases separately within the different pre-disposed breeds. Moreover, it was initially unclear whether a difference in biological behavior between peripheral LHS and visceral LHS or DSH are the result of delayed recognition due to differences in anatomical location or whether these truly represent variably aggressive diseases. The breed specific disease manifestation demonstrated by Benoit Hedan and others further support that DSH and peripheral LHS are separate entities. It is likely that specific immunophenotypic
characteristics of these conditions will be identified once a wider array of canine immunophenotypic tools becomes available.

**Canine Hemophagocytic Histiocytic Sarcoma (CHHS)**

Malignant sarcoma of splenic red pulp macrophages

Canine HHS is an aggressive sarcoma of macrophages which is associated with overwhelming systemic inflammation and severe haematological abnormalities. Similarly to DHS, CHHS is highly breed-specific and shows predilection for the same breeds, namely BMD, Rottweilers and FCR, with BMD being strikingly overrepresented. The age range of dogs affected with CHHS is wide (2.5-8.5 years) and there is no sex predilection. Clinical presentation of CHHS is different from that of the DHS. Dogs are presented for vague complaints of weakness and lethargy and are frequently febrile. Abnormal clinical and clinicopathologic findings include splenomegaly and/or hepatomegaly and mild to moderate regenerative anemia and/or thrombocytopenia. Mild prolongation of the prothrombin time (PT) is also reported as a characteristic feature. However, this change may be partly contributed to by over representation of BMD for which increased PT is reported as a breed-related biological variation. Common changes in the biochemistry profile of dogs with HHS include hypoalbuminemia and hypocholesterolemia. Hyperbilirubinemia may occur, but is not a consistent finding. Due to its clinical presentation, CHHS is often initially mistaken for immune mediated hemolytic anemia (IMHA) and/or Evans syndrome. Lack of agglutination and spherocytes in the peripheral blood smear and a negative Coomb’s test are used to distinguish cases of CHHS from those of IMHA. CHHS is an exceptionally
aggressive and rapidly progressive disease, with a range of 2-32 (mean 7.1) weeks from initial onset of clinical signs to death or euthanasia. 104

Lesions of CHHS are notably different from those of dendritic cell origin, grossly appearing as diffuse organomegaly (typically spleen and/or liver) with no distinct masses. Histologically, the lesions consist of massive expansion of erythrophagocytic histiocytes in sinusoids of splenic red pulp and in the bone marrow with secondary infiltration of hepatic sinusoids and the lungs. 47, 104 The lesions typically contain large foci of extra medullary hematopoiesis (EMH) and multiple areas of infarction and necrosis. CHHS tumor cells exhibit marked cytologic variability ranging from an atypical cell population showing marked anisocytosis, anisokaryosis, nuclear gigantism and a high mitotic index, to what appears to be well-differentiated, reactive macrophages with minimal mitotic activity. 104 These morphological variations occur between tissues (tumor cells in the spleen show more prominent features of malignancy compared with those of the bone marrow, but also between patients.) 104 Nevertheless, there is no documented association between cytological atypia and disease progression as indicated by weeks of survival. Similarly to all variants of CHD, CHHS tumors are also associated with a reactive lymphocytic infiltration.

The immunophenotypic profile of CHHS tumor cells includes dominant expression of MHC-2 and the leuko-integrin CD11d/CD18 with minimal, inconsistent expression of CD11c, CD11b and CD1c. 104 This profile is different from that of LHS and DHS, which dominantly express myeloid dendritic cell characteristics (CD1c+, CD11c+ and CD11d-) and further distinguishes CHHS as a separate entity. This profile resembles that of macrophages resident in splenic red pulp and bone marrow and is in agreement with their phagocytic nature and with the distribution of the lesions. 43, 104, 113, 165 The lymphocytic
infiltrate in CHHS consists of CD8+ CTL which, similarly to other CHD variants, may represent an anti-tumor immune response.

Notwithstanding the classification of CHHS as a neoplastic process, it is well established that fulminating systemic inflammation is a prominent and consistent component of CHHS which largely dictates the morbidity and fatality of this disease. Fingerprints of the inflammatory surge include pyrexia, striking hyperferritinemia, hypoalbuminemia, macrophage activation, erythrophagia and the development of disseminated intravascular coagulopathy (DIC) as a common lethal sequel process. Moreover, given the recently reported association between tissue inflammation and up-regulation of CD11d, it is likely that the extensive expression of Cd11d in CHHS is also reflective of the inflammatory component of this disease. CHHS is a rapidly devastating disease. It bears a grave prognosis and is not responsive to any of the currently applied chemotherapeutic approaches. Surprisingly, there has been no reported attempt to address the inflammatory component of this disease from a therapeutic perspective.

The Lymphocytic Reaction in CHD

Tumor Infiltrating Lymphocytes (TIL)

TIL are a consistent finding in all lesions of the CHD complex. Immunophenotypic characterization of these lymphocytes reveals dominance of CD8+ CTL. TIL are observed commonly in different neoplasms of people and animals. These infiltrates consist primarily of CD8+ CTL and reflect the response of the adaptive immune system to aberrations in the antigenic profile of neoplastic cells. Yet, despite the induction of specific anti-cancer immunity, the majority of tumors utilize multiple
mechanisms to inhibit TIL and escape anti-tumor immune response.\textsuperscript{57, 89, 133} Only rarely do TIL show effective cellular immunity reflected by tumor regression and enhanced long-term survival.\textsuperscript{12, 32, 61, 171} The prevalence of TIL in histiocytic diseases likely reflects sustained antigen presenting capacity in the proliferating cells as well as an ability to evoke an anti-tumor lymphocytic reaction. Yet, the only histiocytic tumor associated with productive anti-cancer immunity reflected by tumor regression is CCH.

Regression of CCH

CCH is one of a few naturally occurring tumors that display spontaneous immune mediated regression and hence serves as an example of effective cell mediated anti-tumor immunity. Regression of CCH is a dynamic process that involves changes in tissue morphology, cell composition and antigenic phenotype. Histologically, tumor regression is characterized by gradual infiltration by small, morphologically unremarkable lymphocytes commencing at the periphery and accumulating within the parenchyma of the tumors.\textsuperscript{34, 106} The infiltrate is initiated by CD4+ lymphocytes which are rapidly outnumbered by CD8+ CTL and though consistently dominated by T cells, is accompanied by a gradual increase in B cells and subtle penetration by macrophages.\textsuperscript{83} Initial progress in tumor infiltration and regression is associated with up regulation of Th1 cytokines; IL2, TNF-alpha and IFN-gamma, known to support cellular immunity, and is followed by increased expression of Inducible nitric oxide synthase (iNOS) mRNA indicating macrophage activity.\textsuperscript{83} These changes suggest a role for CD4+ lymphocytes as initiators of a Th1 anti-tumor immune response that likely involves stimulation of APC to prime CTL, and the requirement of non-specific effector cells like macrophages which contribute to cytotoxic activity by synthesis of reactive nitric oxide (NO) metabolites. It has been shown that the magnitude and the
distribution of the lymphocytic infiltration relates to the degree of tumor regression\textsuperscript{34, 106} and since CTL dominate the inflammatory response through most stages of regression they are presumed to be the main effectors of the process.

Tumor cells decrease in numbers throughout tumor regression and are eventually outnumbered by lymphocytes.\textsuperscript{83, 106} It has been suggested that the susceptibility of CCH to immune-mediated regression is attributable to the ability of the neoplastic LC to maintain functional capacity as potent stimulators of the immune response accompanied by a strong lymphocytic reaction.\textsuperscript{15} Immunophenotypic changes of the tumor cells include increased expression of MHC-1, MHC-2 and ICAM1\textsuperscript{16, 84, 120} and decreased expression of E-cadherin\textsuperscript{120} which results in weakening of the connection between LC and keratinocytes. Surprisingly, notwithstanding the presumed key role attributed to CTL in CCH regression, there was no increase in apoptotic cells during the progression of the immune mediated regression of this tumor.\textsuperscript{83} This unexpected finding may be attributable to rapid turnover of apoptotic cells, but may also imply that cell death in CCH regression is not mediated solely by induction of apoptosis.

**Genetics of Canine Histiocytic Diseases**

The high incidence of histiocytic disease in a small group of breeds generated interest in the possible hereditary nature of these conditions. In 1986, Moore and Rosin first described a familial presentation of malignant histiocytosis which included a series of 13 cases with disease characteristics similar to the syndrome that was later named HHS.\textsuperscript{127} In 1995, Padgett et al. analyzed inheritance of 127 BMD affected with what was referred to as “histiocytosis”. Inclusion criteria for the study were not specified thus it is difficult to
determine which types of histiocytic conditions were analyzed in this study. This group tracked pedigree data through several generations and demonstrated genealogical relation of affected dogs including multiple cases occurring in the same litter, multiple cases in offspring of given dams and sires and higher frequency of the disease among offspring of affected parents. The trait was concluded to be heritable, however, segregation analysis did not follow the pattern of Mendelian or sex-linked modes of inheritance and the calculated hereditary coefficient was low. Segregation of the disease was explained by neither fully recessive models nor by sex-linked inheritance. A multifactorial polygenic mode of inheritance was suggested. Similar results and final conclusions of polygenic inheritance were presented by Abadie & Hedan et al in 2009; however this group also was unclear regarding inclusion criteria. A retrospective study in the Swiss population of purebred BMD identified diseased dogs in all of the families which were analyzed, indicating little chance of eliminating these diseases by selective breeding.

**Bernese Mountain Dogs**

The BMD is one of four closely related mountain dogs which originated in the Swiss Alps. The Berner Sennenhund (in Swiss) or Bernese (in English), refers to the breed’s origin in the Berne region (canton Berne), and is a large working dog with a distinctive tricolour long coat. BMD were initially bred at the end of the 18th century. In 1907, breeders started to promote pure breeding of native dogs, fixed the characteristics of the breed and established the first BMD club with only six registered sires. By 1910, there were 107 typical subjects and in 1920 the breed was initially exported across Europe. In 1926, BMD were first introduced to the United States and in 1937 the breed achieved recognition.
by the American Kennel Club. Since then, the breed rapidly gained popularity in both Europe and North America with thousands of new registrations per year. Yet, despite considerable expansion of the breed worldwide the breeding pool of the BMD remained restricted, as sires are regularly imported from Europe to the US for breeding purposes. The history of the breed’s demography, a small founding population, pedigree barriers, and extensive use of popular sires dictated an unusually limited genetic pool in the current progeny. Striking data from multigenerational pedigree analysis of 327 BMD revealed a high consanguinity coefficient of 3.2 %. Moreover, it has been demonstrated that at each generation, only 5.5 % (± 0.3%) of sires and 13.2 % (± 0.3%) of dams were used for reproduction, with only 0.78% (± 0.1%) of sires and 3% (± 0.3%) of dams producing more than the half of next generation. Genetic homogeneity is reflected by a low rate of polymorphic single nucleotide polymorphisms (SNP), a low number of haplotypes within a linkage disequilibrium (LD) block and extremely low nucleotide heterozygosity (~15% while ~33% being an average value in pure breeds and ~66% for mixed breed dogs). Owing to cross-continent breeding programs, no significant geographical sub-structures of populations were created and the US and European subpopulations remained fairly genetically homogenous.

It appears that the limited genetic variability of BMD had detrimental consequences on the health and longevity of the breed. Several studies demonstrated an exceptionally high morbidity and mortality amongst BMD. A large-scale survey, which was done recently in Denmark on 2928 registered pure and mixed breed dogs, showed that BMD had the shortest lifespan with a median age of death of 7 years compared with 9-10 years in other purebreds and 11 years in mixed breed dogs. Cancer was found to be a leading
cause of death accounting for 34-42% of BMD mortalities. Predisposition to develop neoplasia was found to be higher than any other breed and three times higher than the overall population average. Correspondingly, an additional recent comprehensive survey, which analyzed mortality data of 350,000 insured Swedish dogs, also showed that BMD are at much higher risk of dying from neoplasia than the baseline breeds.\textsuperscript{50} Moreover, it has been shown that in BMD, fatality due to neoplasia occurs strikingly early, by four years of age, and earlier than any other breed.\textsuperscript{2, 25} Even those breeds which are extremely prone to neoplasia (e.g. boxers), deaths due to tumors start to increase only at six years of age.\textsuperscript{25} The most common neoplasms and main contributors to the high mortality are malignancies of histiocytic origin (CHS, and CHHS).\textsuperscript{25} Furthermore, it has been shown repeatedly that BMD affected with these fatal conditions are genealogically related.\textsuperscript{111} Histiocytic tumors are not the only cancer to which BMD are predisposed.\textsuperscript{25} BMD are more prone to develop mast cell tumors, lymphoma and melanoma.\textsuperscript{2, 25} Thus, although no specific mutation has been identified or associated with increased predilection to neoplasia, it is clear that a hereditary predisposition to neoplasia plays an important role in the high morbidity and mortality of BMD.

**Mechanism of Canine Histiocytic Diseases (CHD)**

Notwithstanding the comprehensive characterization of the clinical and pathological presentation of CHD complex, there has been very little progress towards the understanding of the pathophysiology and etiology of these conditions and the mechanisms that underlie these diseases remain enigmatic. Moreover, although there is unequivocal evidence for a genetic contribution to the development of both malignant and reactive CHD \textsuperscript{2}, no specific
causative locus has been identified in the affected breeds and the different heritable histiocytic diseases were never linked to each other on a mechanistic or genetic base.

Several groups have looked at potential contributing factors to elucidate the mechanism of histiocytic diseases in dogs and particularly in BMD. Soller et al. evaluated dysregulation of the receptor tyrosine kinases (Kit, Flt3) in CHS cell lines and tumor samples and concluded that dysregulation of Kit/SCF, Flt3/Flt3L, and Met/HGF signaling pathways is unlikely to occur and/or contribute to development of HS.\textsuperscript{137}

Zavodovskaya et al. scanned canine cytokine cDNA transcripts of TNF alpha, Interleukin (IL)-1 alpha and IL-1 beta and identified several missense mutations in coding sequences of Tumor Necrosis Factor (TNF) alpha, IL-1 alpha and IL-1 beta; some of which were identified in BMD.\textsuperscript{170} These results may reflect a genuine contribution to the development of histiocytic diseases. However, this study did not provide linkage between these SNPs and development of histiocytic diseases as dogs which participated in the study were neither sick nor monitored for future development of HS. Thus, there is no indication that these sequence aberrations have any clinical significance.

Evaluation of ferritin levels as a potential biomarker for CHS revealed that serum ferritin was significantly higher compared to other neoplastic diseases, but significantly lower compared to IMHA.\textsuperscript{58} Unfortunately, only a single case of CHHS was included in the study. However, the serum ferritin concentration in this single case was a prominent outlier with levels strikingly exceeding those reported in dogs with CHS and IMHA. Although this was only a single observation, it may be reflective of the fulminating inflammatory nature of CHHS and point towards a potential unique biomarker for this variant.
A recent molecular cytogenetic profiling of CHS in BMD and FCR identified DNA copy number aberrations in CHS and CHHS tumors of both breeds. A small subset of recurrent genomic imbalances demonstrated deletion of known tumor suppressor genes CDKN2A/B, RB1 and PTEN. However, the vast majority of recurrent copy number aberrations were associated with the tumor phenotype while only a minority was associated with the breed. Thus, these aberrations are not likely related to the inheritance of these conditions. Moreover, this study stresses the difference between peripheral and visceral histiocytic sarcoma and emphasizes the value of investigating the different CHS variants independently.

Additional recent studies employed quantitative real-time polymerase chain reaction (RT-PCR) and Immunohistochemistry (IHC) to evaluate expression of the receptor for advanced glycation end products (RAGE) and the high mobility group box1 protein (HMGB1) in histiocytic sarcoma. Both these markers were shown to be required for the maturation and migration of DCs and were therefore hypothesized to have a role in the progression of histiocytic diseases. Significant down-regulation of RAGE and HMGB1 in CHS tumor samples of variable tissue origin compared to their non-neoplastic counterparts was identified as a promoting factor in development of CHS. Given the potential effect of inflammation on expression of these markers, lack of clear distinction between CHHS and CHS cases was particularly problematic. Furthermore, an additional control group of reactive histiocytic disease was lacking. Such a control group would have been highly valuable, as it would allow delineation of the contribution of these changes to histiocytic inflammation and neoplasia independently.
Collectively, despite much effort there has been very little progress towards the understanding of the mechanism of CHD either for each disease independently or as a complex of disorders. A major limitation of these studies is the failure to use a consistent disease classification scheme, even though Moore et al have provided an extensive array of phenotypic tools to make such distinctions.\textsuperscript{4, 5, 106} Unfortunately, the term histiocytic sarcoma is often applied to describe the entire spectrum of CD18 immunopositive sarcomas resulting in collective analysis of all histiocytic malignancies, namely CHHS of macrophage origin and dendritic cell sarcoma (CHS).\textsuperscript{2, 47, 111, 132, 139, 154} Although it is highly conceivable that dendritic cell sarcoma (CHS) and CHHS are related on a mechanistic and/or genetic basis, until the underlying mechanisms of these diseases is uncovered or until a common causative genetic locus is identified, these diseases should be considered as separate entities.

Histiocytic Disease in People

Malignant Histiocytosis (MH)

Much of the confusion in the veterinary literature regarding canine histiocytic diseases, particularly CHHS and CHS, is attributable to the term MH. The name “MH of BMD” was initially offered to describe two cohorts of “CHHS-like” cases in an attempt to convey the duality of this disease accounting for both its inflammatory and neoplastic components, and to point towards similarity to MH in people.\textsuperscript{124, 127} However, since initial designation of this term, it has been used frequently and interchangeably for both CHHS and CHS.\textsuperscript{2, 66, 111, 139} This adds to the nomenclature confusion and misclassification of these diseases.
In people, the term MH refers to aggressive neoplasia of splenic red pulp macrophages (also called reticuloendothelial histiocytes) which, similarly to CHHS, presents with massive expansion of erythrophagocytic histiocytes, pyrexia, wasting, hepatosplenomegaly, and progressive cytopenias. The first study, which presented the MH entity in people in the early 1970s, was followed by a retrospective study in the 1990s. The follow-up study applied immunophenotypic and molecular techniques (assessing clonal gene rearrangement of T-cell receptor or immunoglobulin heavy chain) to re-evaluate the cases which were initially designated as MH. This retrospective study revealed that genuine malignancy of the reticuloendothelial histiocytes is exceedingly rare. Correspondingly, it was found that in the vast majority of MH cases, the proliferation of hemophagocytic histiocytes was reactive (versus neoplastic) and evoked by variable primary causes including occult lymphoid neoplasia, infectious diseases and a wide spectrum of genetic or acquired immune regulatory disorders. In dogs, lymphoid neoplasia has not been associated with MH (CHHS), but the presence of an underlying inherited or acquired immune regulatory disorder was never investigated. This question is particularly relevant given the strong predilection of BMD to immune regulatory disorders and the familial nature of CHHS. CHHS is classified as a neoplastic disease based on its metastatic nature. However, although many cases of CHHS exhibit features of malignancy and are indisputably neoplastic, it is difficult to ignore those cases that exhibit benign, well-differentiated morphology, but share a similarly aggressive clinical behaviour and outcome. It is therefore plausible that, similarly to MH in people, more than one underlying process contributes to the clinical manifestation of CHHS. In the current human literature all reactive, MH-like syndromes are collectively designated as hemophagocytic lymphohistiocytosis (HLH).
Familial Hemophagocytic Lymphohistiocytosis (FHL)

HLH is a severe immune regulatory disorder caused by uncontrolled proliferation of activated lymphocytes and histiocytes.\textsuperscript{78} HLH results from acquired and/or inherited immune deficiencies manifesting as impaired cellular immunity.\textsuperscript{138} Acquired HLH develops due to strong activation of the immune system subsequent to systemic infections, cancer or autoimmune disorders.\textsuperscript{78, 152} Primary, otherwise known as familial, HLH (FHL) constitutes a complex of autosomal recessive diseases which are strongly associated with consanguinity.\textsuperscript{173} Several manifestations of FHL are currently known. Overall, different underlying anomalies are associated with different levels of severity and with subtle variation of disease manifestation. Acute presentation of FHL occurs at infancy and is associated with striking hypercytokinemia, life threatening cytopenias and severe systemic inflammation.\textsuperscript{76} A later FHL onset occurs in older children and young adults and may present with a less detrimental inflammatory disease but with increased susceptibility to hematopoietic cancers.\textsuperscript{107} Defective genes in FHL encode proteins which are involved in GDK.\textsuperscript{173} GDK is the principal execution mechanism of cytotoxic lymphocytes, the main subsets of which are the CD8+ CTL and NK cells. Five types of FHL have been characterized; four of which (FHL types 2-5) have been assigned to specific causative loci.\textsuperscript{173} The most common and most aggressive FHL variant (FHL type 2) is attributable to a mutation in \textit{PRF1} which encodes Perf, the key cytolytic effector molecule.\textsuperscript{27, 78, 91, 156, 173} Other FHL variants are associated with mutations in genes encoding proteins which participate in the trafficking, priming and excretion of cytotoxic granules (\textit{UNC13D, STX11, RAB27A, and STXBP2}) and constitute the core of the GDK apparatus.\textsuperscript{108, 144, 173}
Cytotoxicity and Granule Dependent Killing (GDK)

Cell mediated cytotoxicity is a fundamental component of the immune system. It plays a critical role in protecting the organism against intracellular pathogens and cancer, and is pivotal for lymphocyte homeostasis. The main cytotoxic effectors of innate and adaptive immune systems are NK cells and CTL respectively. These lytic lymphocytes mediate cytotoxicity via both GDK and/or Fas-Fas ligand (FasL) pathways. In the GDK pathway, target cell recognition results in degranulation and release of stored cytocidal proteins, including Perf, granzymes that act in concert to achieve target cell death. Although controversy exists regarding the exact mechanism of the Perf lethal hit and its means of synergism with granzyme, it is well established that Perf permeabilizes target cell membranes, supports delivery of pro-apoptotic granzyme, and exposes target cells for potential osmotic lysis. The Fas-FasL pathway is considered the slow execution modality of cellular immunity. Binding of Fas, expressed by target cells, to FasL, expressed by cytotoxic effectors, results in initiation of caspase-dependent apoptosis and target cell death. Collectively, the FHL complex demonstrates the indispensability of GDK in sustaining the immune system within favorable, non-destructive boundaries and in maintaining effective cancer immune surveillance.

Perforin

Function and regulation of Perforin

Perforin (Perf) is the key effector molecule of the GDK pathway. It is a potent pore-forming protein which is uniquely synthesized in CL and stored along with granzyme in secretory lysosomal organelles referred to as cytolytic granules of NK cells and CTL.
target cell recognition and assembly of an immunological synapse (IS), cytolytic granules polarize and degranulate releasing Perf and GrzB to the sealed compartment of the IS. Subsequent to excretion, Perf monomers anchor to the plasma membrane lipid bilayer of target cells, and polymerizes to create trans-membrane channels\textsuperscript{157} This sequence of events results in permeabilization of target cell membranes which facilitates entry of pro-apoptotic granzymes to the target cell membrane \textsuperscript{27, 88, 141} while exposing the target cells to risk of osmotic lysis.

Perf is highly potent and potentially hazardous to membrane-bound organelles within host cells. Consequently, Perf is synthesized as an inactive precursor to prevent membrane damage as it passes through the endoplasmic reticulum and Golgi compartments. Within the cytolytic granules Perf is stored as the active peptide subsequent to post-translational modification. The highly acidic, calcium-poor environment within cytolytic granules precludes Perf polymerization and activation.\textsuperscript{149} Moreover, cytolytic granules are contain a high concentration of proteoglycans, which form inhibitory complexes with Perf at acidic pH.\textsuperscript{149} Thus, within the cytolytic granules, Perf can be safely cleaved resulting in its active form.\textsuperscript{149} Subsequent to secretion, the higher pH and calcium ion concentration of the extracellular compartment facilitates dissociation of Perf from the inhibitory proteoglycans and allows conformational changes. This results in exposure of the C2 domain, which enables calcium binding and polymerization within phospholipid head groups of the target cell membrane.

**Sequence and Structure of Perforin Gene**

Given its fundamental role in innate and adaptive immunity, specific regions of the Perf gene which bear functional implications, are highly conserved across species as diverse
as fish and primates.\textsuperscript{75} In people and mice, Perf is encoded by a single gene, which maps to chromosome 10.\textsuperscript{88} Gene structure and internal organization are similar in both species and consist of three exons and two introns, spanning approximately 7 kilobases (kb).\textsuperscript{87, 88, 167} Sequencing of the canine genome has resulted in mapping of Perf to chromosome 4. The predicted mRNA of canine Perf (XM_546148) consists of 1842 base pairs (bp) and codes for a protein of 613 amino acids (CanFam2.1 assembly, Broad Institute). Human Perf is a calcium-dependent, channel-forming protein of approximately 555 amino acids with a molecular weight of 62-67kDa.\textsuperscript{87, 88} Perf has several well-characterized domains. The main functional domains of the Perf gene share similarity to C9, the pore-forming effector molecule of the humoral immune system and the implementing component of the membrane attack complex of the complement system (MAC).\textsuperscript{167} An additional conserved domain is the calcium binding motif which is compatible with the C2 superfamily of calcium-binding proteins.\textsuperscript{157}

**Expression of Perforin**

Perf expression is cell-restricted and tightly regulated at both a transcriptional and translational level. Transcriptional regulation is attributed to a comprehensive set of cis-regulatory regions.\textsuperscript{118} The transcriptional factor Runx3 has a critical role in differentiation of cytotoxic lymphocytes as it regulates the expression of the three cardinal effectors of lytic lymphocytes: IFN-gamma, Perf and granzyme.\textsuperscript{40} Perf expression is associated with signals transduced from the T-cell receptor, the C-type lectin-like immunoreceptor of NK cells (NKG2D), and by interleukins; IL-2, IL-12, IL-15 and IL-21.\textsuperscript{9, 49} Perf expression correlates tightly with maturation of cytolytic effectors and with the gain of cytotoxic capacity.\textsuperscript{118} Accordingly, Perf is considered a valuable marker for peripheral differentiation of cytotoxic
lymphocytes and the most reliable predictor for GDK.\textsuperscript{118} This feature of Perf expression is utilized to monitor maturity and functionality of immune reactions.\textsuperscript{63, 68, 79, 118}

**The role of Perf in adaptive and innate immunity**

Perf functions in antimicrobial immunity and is essential for immune surveillance against cancer and for lymphocyte homeostasis.\textsuperscript{27, 91, 155, 172} Perf is a unique molecule in that there are no redundant pathways capable of duplicating its killing effectiveness. Accordingly, lack of Perf or expression of dysfunctional Perf is associated with severe consequences including loss of lymphocyte homeostasis, increased susceptibility to cancer, and reduced tolerance to intracellular pathogens.\textsuperscript{80, 91, 102, 150, 155} Indispensability of Perf is demonstrated in FHL 2 which is the most aggressive variant of the FHL complex.\textsuperscript{76, 78, 156} FHL 2 attributable to complete lack of Perf expression develop at early infancy (<6 month of age). The primary manifestation of FHL 2 is the immune regulatory disorder. Typically, the acute crisis associated with the immune dysregulation is severe and is eventually fatal unless bone marrow transplantation is successfully implemented.\textsuperscript{71} Conversely, increased susceptibility to cancer is typically associated with later onsets and a somewhat milder disease phenotype. The full array of Perf function is demonstrated in a strain of Perf deficient mice. These mice remain asymptomatic in a pathogen-free environment, but develop a fatal HLH-compatible syndrome subsequent to viral stimulation. Perf knockout mice also show increased susceptibility to viral and chemical tumorigenesis.\textsuperscript{150}
HYPOTHESIS AND OBJECTIVES

The general study hypothesis is that:

"DEFECTIVE CYTOTOXICITY IN BERNESE MOUNTAIN DOGS CONTRIBUTES TO DEVELOPMENT OF CANINE HEMOPHAGOCYTIC HISTIOCYTIC SARCOMA"

Major and specific study objectives:

The major objective of this thesis was to determine if pathophysiological similarities exist between CHHS in BMD and FHL of children. The specific objectives were formulated to assess the potential contribution of impaired lymphocyte cytotoxic capacity of BMD that may lead to the development of CHHS.

The first set of experiments focused on the canine perforin (Perf) gene as this is the most commonly defective gene in children with FHL. The first specific objective was to characterize and sequence the canine Perf gene in healthy (control) dogs and CHHS affected BMD.

The second objective was to assess the expression of Perf in CHHS. For this purpose, an antibody specific for canine Perf was generated. The ability to probe Perf in archived tissue facilitated the characterization of patterns of Perf expression. Investigation of Perf and Granzyme B (GrzB) expression in canine cutaneous histiocytoma (CCH) a benign, naturally regressing variant of the CHD complex outlined a model which unravels
the patterns of Perf and GrzB expression in an example of successful anti-tumor immune response.

The characterization of CCH as a model for successful anti-tumor immunity led to the approach for the third objective which focused on investigating functional aspects of GDK in lymphocytes infiltrating CHHS lesions. Immunofluorescence and confocal microscopy were employed to characterize and compare functional aspects of cytotoxicity in tumor infiltrating lymphocytes of CHH and CHHS. These included expression of Perf, but also other key components of the GDK apparatuses including formation of an immunological synapse (IS), T-cell receptor (TCR) signaling, and granule polarization and de-granulation. This last objective yielded the most exciting results and supported the initial hypothesis of this thesis as it demonstrated aberrations in GDK in lymphocytes of BMD affected with CHHS. These results suggest that CHHS of BMD may serve as a promising natural disease model for FHL of children.
CHAPTER 2.

STRUCTURE AND SEQUENCE VARIATION OF THE CANINE PERFORIN GENE

This chapter corresponds to the manuscript:


Abstract

Lymphocyte-mediated cytotoxicity is essential to control viral infections, limit lymphocyte expansion and activation, and survey for malignant cells. Humans with defects in lymphocyte cytotoxicity have reduced Perf function resulting in uncontrolled lymphocyte expansion, leading to excessive histiocyte activation and a hemophagocytic disorder. Dog breeds such as Bernese Mountain Dogs (BMD) have a high incidence of reactive and malignant diseases affecting histiocytes. This study addressed the hypothesis that changes in the Perf gene contribute to the development of hemophagocytic histiocytic sarcoma (HHS) in BMD. Canine Perf DNA was amplified and sequenced through multiple PCR assays from healthy and diseased dogs, and the gene structure determined by rapid amplification of cDNA ends. The coding component of the gene consists of 1679bp; with two exons of 536bp and 1143bp separated by an intron of 865bp. Gene configuration and location differ from that in other species although the coding sequence is highly conserved. Three silent single nucleotide polymorphisms (SNP) were identified. Analysis of their distribution indicated a consistent genotype among 6 middle-aged to older BMD without histiocytic
diseases. Among samples from 10 dogs with HHS and 10 without histiocytic diseases, SNPs occurred with variable frequency. It was concluded that changes in the amino acid sequence of Perf were not associated with HHS but that a constellation of SNP may characterize BMD without histiocytic disease. Investigation of more dogs is required to confirm a specific genotype. Future studies should focus on the potential contribution of reduced Perf expression and/or function to HHS in dogs.

**Introduction**

Cell-mediated cytotoxicity is an essential mechanism for both immune surveillance and immune regulation. Subsets of T-lymphocytes and NK cells are the cytotoxic effectors and can mediate cytotoxicity via the Perf/granzyme and the Fas/Fas ligand pathways.\(^{21,26}\) Human Perf is a calcium dependent, channel-forming protein of 555 amino acids (AA) with a molecular weight of 62-67KD.\(^{87,88}\) Perf has several well-characterized domains with high similarity to proteins of the complement system, in particular C9, which is part of the membrane attack complex (MAC),\(^{167}\) and with members of the C2 superfamily, which include calcium-binding proteins.\(^{87,88}\) Perf is synthesized as an inactive precursor and stored together with granzymes in membrane-bound cytoplasmic granules of lymphocytes. Upon recognition of MHC class I complex on target cells the granules polarize, move toward the point of contact, and merge with the cell membrane to release their contents into a small secretory cleft, referred to as the Immunological Synapse (IS).\(^{141}\) The IS is sealed from the external environment by tight membrane apposition of the cytotoxic and target cell to prevent leakage and to ensure high concentration of lytic proteins at the target area.\(^{140}\) In the presence of calcium ions,\(^{157}\) Perf monomers insert into the target cell membrane and polymerize to create
a transmembrane pore, which then results in osmotic lysis of the target cell and entry of granzymes into the cytosol to induce apoptosis via caspase-dependent and -independent mechanisms.\textsuperscript{27, 88, 141} Although this sequence of events was originally proposed for Perf entry and function, other routes also have been considered. Serglycin-bound granzymes are capable of entering the cytosol by endocytosis via the mannose 6-phosphate receptor.\textsuperscript{153} Based on this knowledge it has been suggested that Perf might be taken up by the same endosomes and to facilitate the cytoplasmic release of granzymes.\textsuperscript{59} The ability to induce Perf release by inoculation with lysosomotropic adenovirus further supported this hypothesis.\textsuperscript{59, 141} Nevertheless, some experimental results argued against a major role for the endocytic route of Perf cell entry. Firstly, sensitivity of Perf to low calcium concentration and low pH raised doubt regarding the ability of Perf to function at lysosomal conditions.\textsuperscript{157} Further, interference with endocytic activity of cells did not change target cell susceptibility to Perf-induced cell lysis.\textsuperscript{146}

Perf expression is cell-restricted and regulated by signals transduced from the T-cell receptor, the C-type lectin-like immunoreceptor of NK cells (NKG2D), and by interleukins (IL)-2, IL-12, IL-15 and IL-21.\textsuperscript{9, 49, 147} To limit non-specific cytotoxicity, production, storage and secretion of Perf by cytotoxic lymphocytes are tightly regulated.\textsuperscript{87, 155} Specific regions of Perf are conserved across species as diverse as fish and primates.\textsuperscript{75} In people and mice, Perf is encoded by a single gene, which maps to chromosome 10. Gene structure and internal organization are similar in both species and consist of three exons and two introns, spanning approximately 6KB.\textsuperscript{88} Sequencing of the canine genome has resulted in mapping of Perf to chromosome 4. This finding is in agreement with previous identification of synteny between regions on chromosome 4 of dogs with chromosome 10 in people.\textsuperscript{24}
Perf functions in antimicrobial immunity and is essential for immune surveillance against cancer, as shown by the markedly increased susceptibility of Perf knockout mice to viral and chemical carcinogenesis. Further, Perf is critical for limiting the expansion of antigen-specific CD8$^+$ T-cells. Hemophagocytic lymphohistiocytosis (HLH) in humans is associated with lack of Perf function and manifests with excessive lymphocyte proliferation and exaggerated macrophage activation resulting in extensive hemophagocytosis (Menasche, et al. 2005). Primary or genetic HLH may result from various mutations in the Perf gene resulting in reduced or altered protein function, or from defects in granule movement or extrusion proteins such as Munc 13-4 and syntaxin 11, respectively. Secondary or acquired HLH has been described in certain infectious, neoplastic and autoimmune diseases.

Hemophagocytic histiocytic sarcoma (HHS) in dogs is an aggressive neoplasm of macrophages with a strong inflammatory component, formerly called malignant histiocytosis. Clinical features of HHS such as pyrexia, cytopenia, hypoproteinemia, organomegaly and hemophagocytosis, are reminiscent of HLH in people. Further, strong breed predilection toward the disease in BMD, flat-coated retrievers (FCR) and Rottweilers suggests a genetic contribution. These same dog breeds are also prone to develop other inflammatory and neoplastic histiocytic diseases such as reactive histiocytosis (RH), a proliferation of activated interstitial dendritic cells (Affolter and Moore, 2000), and non-hemophagocytic histiocytic sarcoma, an aggressive neoplasm of interstitial dendritic cells. Lesion infiltration by CD8$^+$ lymphocytes is a consistent feature of canine histiocytic diseases, and may reflect anti-neoplastic responses or expansion of antigen-specific cytotoxic lymphocytes. Hence, we investigated the hypothesis that Perf abnormality may be a feature of canine histiocytic disorders.
Material and Methods

Samples

For RNA extraction, a sample of heparinized blood was obtained from a mixed breed dog. For genomic DNA (gDNA) extraction, samples of EDTA-anticoagulated blood and/or frozen tissue were obtained from 26 dogs that belonged to 3 groups: Group 1 consisted of 10 dogs with a histopathological diagnosis of HHS. This group included 5 BMD, 1 Golden Retriever, 2 Labrador Retrievers, 1 Standard Poodle and 1 FCR; 4 females, 3 males, and 3 dogs where gender was not recorded. Information about age was available for 8 dogs; median age was 6 years (range 5-11). Dogs were admitted as patients to the teaching hospital at the University of Guelph or the University of California, Davis. Group 2 were 10 purebred dogs from breeds without predilection to histiocytic disease. These dogs were admitted to the University of Guelph Veterinary Teaching Hospital for treatment of anal sac carcinoma, dermatitis, blastomycosis, gastrointestinal lymphoma, immune-mediated thrombocytopenia, diarrhea, hyperadrenocorticism, dental disease, or for castration. Group 3 was comprised of 6 BMD; 2 of these dogs were healthy breeding dogs and 4 were client-owned dogs with non-histiocytic diseases (hemangiopericytoma, myxosarcoma, lymphoma, foreign body intestinal obstruction). This group included 5 females and 1 male. The median age was 7.7 with a range of 5-14 years.
**Reverse transcriptase (RT) PCR and rapid amplification of cDNA ends (RACE)**

Peripheral blood mononuclear cells (PMBC) were isolated from heparinized whole blood by centrifugation over a Ficoll-Hypaque gradient. Total RNA was extracted from the recovered PMBC using an RNA extraction kit (Qiagen, Mississauga, ON) and reverse transcribed to cDNA (Invitrogen, Burlington, ON). Four forward and two reverse primers complementary to exons 2 and 3 of the predicted sequence (XM_546148) amplified four overlapping amplicons of the cDNA. Primers are listed in Table 2-1 labeled as F3, F4, F5, F8, R6, and R9; relative location of these primers on the gene is illustrated in Figure 1. The 3' end of exon 3 was obtained combining two of the forward primers (F3 and F4) with an additional reverse primer complementary to an untranslated region at the 3' end of exon 3 (R7). Primer combinations and conditions are summarized in Table 2-2.

For 5' RACE, RNA from PMBC was reverse transcribed with a gene-specific primer (GSP) to obtain a single stranded cDNA product. Through use of a reverse transcriptase enzyme with additional terminal deoxynucleotidyl transferase activity a synthetic oligonucleotide adaptor was incorporated at the 5' end of the cDNA (SMARTer RACE cDNA, Clontech, Mountainview, CA). PCR was carried out using a forward universal primer that anneals to the 5' oligonucleotide and a reverse GSP. Amplified products were separated in 1% agarose gels and bands of predicted size were excised. DNA was extracted and eluted (Qiagen), and then sequenced in both directions (Laboratory Services Division, Guelph, ON).
Gene structure

The final cDNA sequence was deposited in GenBank (FJ973622), and translated using Expert Protein Analysis System (ExPASy) software. Gene structure was determined by aligning the cDNA sequence with the gDNA of the canine Perf gene (NC_006586). The gene and intronic sequences were mapped in the canine genome with Basic Local Alignment Search Tool (BLAST).

Detection of SNP (SNP)

Genomic DNA was extracted from 200 µL of EDTA-anticoagulated whole blood and/or from frozen tissue with QIAamp DNA extraction kit (Qiagen) following instructions of the manufacturer. The DNA was eluted into 50 µL of buffer and stored at −20°C. Two additional primers with complete identity to intronic sequences of the canine Perf gene were designed (F1, R2); hence nine primers were used in different combinations (Tables 2-1 and 2-2) to amplify 6 overlapping fragments spanning the exons, as outlined in Figure 1. PCR was carried out with gDNA templates, primer combinations and conditions as summarized in Table 2-2. Amplified products were separated on 1% agarose gels and bands of the appropriate size were excised and sequenced in both directions.
Table 2-1: Primers for amplification of the canine perforin gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>CATGAAATCCCAGCAAGG</td>
<td>20,576 – 20,594</td>
</tr>
<tr>
<td>2R</td>
<td>TCCCCAGACCACCTCCAAGTTT</td>
<td>21,409 – 21,429</td>
</tr>
<tr>
<td>3F</td>
<td>AAGACCACCAGGACAGGTA</td>
<td>21,303 – 21,323</td>
</tr>
<tr>
<td>4F</td>
<td>AAGGCTGATGGGGAATGCTAA</td>
<td>22,179 – 22,200</td>
</tr>
<tr>
<td>5F</td>
<td>GCTTATCTCCAACCTACGGC</td>
<td>22,329 – 22,350</td>
</tr>
<tr>
<td>6R</td>
<td>TTCCGAAGTCCAGCCGC</td>
<td>23,076 – 23,094</td>
</tr>
<tr>
<td>7R</td>
<td>GTCTAATGGGGGTATGAAGAT</td>
<td>(+)76 – (+)97</td>
</tr>
</tbody>
</table>

a Location in reference to canine perforin gDNA (GenBank NC_006586)
b Located on the 3’ side of canine perforin gDNA.

Table 2-2: Amplification parameters for the canine perforin gene

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Amplification size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F, 2R</td>
<td>853</td>
<td>59.1</td>
</tr>
<tr>
<td>3F, 6R</td>
<td>1791</td>
<td>61.7</td>
</tr>
<tr>
<td>4F, 6R</td>
<td>915</td>
<td>61.7</td>
</tr>
<tr>
<td>5F, 6R</td>
<td>765</td>
<td>59.7</td>
</tr>
<tr>
<td>5F, 7R</td>
<td>1200</td>
<td>60.0</td>
</tr>
</tbody>
</table>

Figure 2-1. Schematic illustration of the structure of the predicted canine Perf gene (GenBank XM_546148) indicating the relative location of primers for amplification from cDNA and genomic templates.
Sequences from each animal were analyzed by alignment with the canine genome (Build 2.1, chromosome 4: 24,730,636 to 24,707,282 bp) and the Perf cDNA sequence as determined by RT-PCR and RACE. The alignments were performed with Vector NTI (Invitrogen) or Geneious (Biomatters, Auckland, NZ) software. Each electropherogram was visually assessed for adequate resolution and equivalent base signals. Sequence aberrations were accepted as SNP only when present in more than one of the overlapping amplicons. A site with a single peak differing from the reference gDNA sequence was interpreted as a homozygous mutation, and two approximately equally sized and overlapping base peaks were interpreted as a heterozygous mutation. If SNP were identified, the entire nucleotide sequence was translated with ExPASy and aligned (Geneious) with the predicted protein sequence to assess for changes in amino acids. Sequence analysis for presence of promoter binding sites was performed using web-based software UTRscan (http://www.ba.itb.cnr.it/BIG/UTRScan) (Heintzman and Ren, 2007). Phylogenetic trees were constructed with Geneious software.

**Statistical analysis**

All analyses were performed with Statistical Analysis Software (SAS Institute, Cary, NC). Initial Chi square testing was performed to assess whether the three mutations are significantly linked. To assess the relationship between presence of SNP and a diagnosis of HHS, Fisher's exact tests were performed. To estimate the odds of a dog with HHS having SNP in the Perf gene, the median unbiased estimate (MUE) and the conditional maximum likelihood estimate (CMLE) were calculated (Robinson, 2008). Significance was set at $p \leq 0.05$. 

43
Results

Samples

Samples of gDNA from 26 dogs were available for mutational analysis. Dog breeds with HHS (group 1) included those previously identified as having an increased risk of the disease, as well as two Labrador Retrievers and one Standard Poodle (Table 2-3). Dogs from breeds not known to be at risk of histiocytic diseases, and affected by illnesses other than HHS, included a wide range of breeds and conditions (group 2). BMD without HHS (group 3), were chosen to be of a similar age as dogs affected by HHS, and to be free of HHS at the time of examination. Three of these dogs had a neoplasm of non-histiocytic cell origin.

Gene structure

Initial alignment of the predicted cDNA with gDNA of canine Perf suggested that the gene structure was similar to that reported for people and mice: three exons separated by two introns. However, while the predicted first exon was similar in size to other species (133bp), the first intron was predicted to be exceptionally large (22,094bp). Further characterization of this large intron by sequence analysis identified a region of 1011bp with 77% identity to an untranslated region on human chromosome 10. This region flanks the 5’ end of the Perf gene and the 3’end of an ADAM metallopeptidase, a gene located upstream to Perf in both people and dogs. These findings suggested that either an evolutionary translocation event into intron 1 resulted in separation of exons 1 and 2 or that the predicted first exon is not part of the transcript. In order to establish the actual canine gene structure, mRNA of PMBC was reverse-transcribed and Perf was amplified. Primers (F3 and R6) were chosen to span the predicted second and third exons and applied to cDNA and gDNA templates (Fig.
1). The gDNA template yielded an amplicon that was 850-900bp larger than the product of the cDNA template, consistent with the predicted exon-intron structure for the region. In order to amplify the 3’ end of Perf transcript, a reverse primer complementary to genomic sequence ~20 bp downstream to the 3’ end (R7) was applied to a cDNA template. This PCR product yielded the stop codon followed by a short, presumably untranslated, 3’ sequence.

Attempts to amplify cDNA 5’ to the start codon in predicted exon 2 did not yield PCR products. Moreover, amplification with primers complementary to the first predicted exon did not yield a product. The most upstream product obtained by RT-PCR started at the ATG sequence (start codon) located at position 461 of the predicted exon 2. These results suggested that exon 1 of the predicted sequence was absent from the transcript. To further investigate this possibility, RACE was undertaken: 4 primers complementary to exon 2 and compatible with the universal RACE primer were designed and applied sequentially. None of these yielded a product that extended beyond the start codon in predicted exon 2. It was therefore concluded that the canine Perf cDNA is shorter than the predicted sequence (1679bp versus 1842bp) and does not include a 5’ untranslated region in the transcript. However, as in humans, rodents, and other species, the entire coding sequence for canine Perf resides in exons 2 and 3 (Fig. 2).

Sequences upstream of the Perf coding components in exons 2 and 3 have not been fully annotated for most species. In humans and rodents, exon 1 was shown to be part of the untranslated transcript, while in cattle exon 1 is only predicted to be part of the transcript and in horses exon 1 is not even predicted to be part of the transcript. Comparison of the putative exon 1 among human, bovine, canine, murine and rat showed marked variation in
length (ranging from 16bp in mice to 136bp in cattle) and less than 55% similarity. However, one short stretch of 28bp had 89% identity between human and bovine. This short sequence matched a canine intronic sequence 1500 bp upstream of the start codon in exon 2. Amplification from cDNA with a primer complementary to this sequence did not yield a product. This finding further supports that 1) the canine Perf transcript does not include a 5’ untranslated region; and 2) that a short conserved sequence previously part of an untranslated transcript has been retained in an intron. Promoter binding sites were not identified within this region.

Similarly to the other species, the translated sequence of canine Perf mRNA consisted of 555 AA with a membrane attack complex (MAC) and protein kinase C conserved region 2 (C2) domains. Identity of canine Perf with feline, human, bovine and murine Perf was 85%, 75%, 75% and 69%, respectively. Phylogenetic relationships are shown in Fig 3.

**Sequence analysis and SNP detection**

Five PCR assays overlapping by 400 to 900 bp resulted in at least duplicate coverage of Perf exons 2 and 3. These PCR assays were carried out on gDNA templates and yielded complete sequences of the coding region from all samples. Sequence analysis identified three SNP in dogs with HHS and in non-BMD controls presented with non-histiocytic diseases (Table 2-3). All of the SNP identified in controls were found in dogs with non-neoplastic diseases. Numbered in accordance with canine Perf cDNA (GenBank FJ973622), SNP were located at positions 457 (A replaced by C), 1200 (G replaced by A) and 1416 (G replaced by A). The relative frequency of the SNP among the different groups is summarized in Table 2-3. All mutations were silent, and did not change the encoded AA. SNP 457A/C and 1416 G/A, but not SNP 1200 G/A, had previously been identified as part
of the canine genome project (http://www.ncbi.nlm.nih.gov/SNP). When assessing the relationship between HHS status (present or absent) and the presence of SNP by Fisher's exact tests, estimates of the odds ratio were high (CMLE=4.368 and MUE=4.251) but the relationship was not significant ($p=0.163$, confidence interval = 0.635 to 40.662 [one-sided 95% confidence interval based on actual distribution]). Comparing dogs from groups 2 and 3 for a relationship between the presence of SNP and BMD breed status did not show a significant relationship ($p=0.234$), and the estimate of the odds ratio was low (MUE=0.236).
Figure 2-2: Schematic of human, mouse and canine Perf genes with location of SNP (*) indicated in the canine transcript.
Figure 2-3: Phylogenetic relationship of Perf from different species (unrooted tree).

- Numbers represent percentage of difference
Table 2-3: Single nucleotide polymorphisms in the perforin gene in dog with CHHS and other conditions

<table>
<thead>
<tr>
<th>Group</th>
<th>Breed</th>
<th>Age (median, range)</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>632 A/C</th>
<th>1375 G/A</th>
<th>1591 G/A</th>
<th>Number of dogs with genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(n=10)</td>
<td>BMD (4)</td>
<td>5.9 (5-11)</td>
<td>M (1)</td>
<td>HHS</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Labrador (1)</td>
<td></td>
<td>MN (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Standard poodle (1)</td>
<td></td>
<td>UK (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Golden retriever (1)</td>
<td></td>
<td>UK (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMD (1)</td>
<td>8</td>
<td>MN</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Labrador (1)</td>
<td>9</td>
<td>F</td>
<td>-/-</td>
<td>++</td>
<td>++</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2(n=10)</td>
<td>FCR (1)</td>
<td>UK</td>
<td>M</td>
<td>-/-</td>
<td>++</td>
<td>++</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pug, English Springer spaniel, Beagle, GSD, Chihuahua, Boxer, Dachshund, Bichon Frise, Shih Tzu, Jack Russell terrier</td>
<td>8.8 (0.5-14.2)</td>
<td>FN (5)</td>
<td>MN (3)</td>
<td>M (2)</td>
<td>Non-histiocytic disease</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
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<td></td>
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<td></td>
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<td></td>
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<td>++</td>
<td>++</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>BMD</td>
<td>7.7 (5.6-9.4)</td>
<td>FN (5)</td>
<td>M (1)</td>
<td>Non-histiocytic disease</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
</tr>
</tbody>
</table>

a BMD = Bernese mountain dog, FCR = Flat-coated retriever, GSD = German shepherd dog

b FN = female neutered, MN = male neutered, M = male, UK= unknown
c HHS = hemophagocytic histiocytic sarcoma
Discussion

Perf is a cytotoxic protein, indispensable for regulation of lymphocyte expansion during immune response and for immune surveillance against cancer (van den Broek, et al. 1996). Canine HHS is an aggressive neoplastic disease characterized by a prominent inflammatory component and strong breed predilection,\textsuperscript{104} the latter suggests a genetic contribution to the pathogenesis of the disease. A striking tendency of the same dog breeds affected with HHS to develop other malignant or inflammatory histiocytic diseases might imply heritable failure of cell-mediated cytotoxicity. Familial Hemophagocytic lymphohistiocytosis (FHLH) is a genetic immune regulatory disorder of human infants associated with reduced cytotoxic activity.\textsuperscript{78} Type 2 FHLH is the most common form of FHLH, and is associated with a wide range of mutations in the Perf gene.\textsuperscript{102, 173} Therefore, the goals of this study were to determine the structure of the Perf gene in dogs, and to assess whether mutations in Perf sequence may contribute to HHS in BMD.

Samples were chosen from dogs of similar ages to those affected with HHS. In order to increase the likelihood that BMD in group 3 (control BMD) would not develop HHS, only middle-aged to older dogs were chosen for this group. Although development of HHS subsequent to sampling could not definitively be excluded in these dogs, since the average age of BMD at death is only 7.5 years\textsuperscript{154}, this was considered unlikely. Only samples from dogs in North America were assessed, however, European and North American BMD populations are relatively genetically homogeneous as shown recently by a detailed analysis of SNP frequency on chromosome 1 in different populations.\textsuperscript{122}
Initial characterization of the structure of the canine Perf gene based on the predicted mRNA sequence suggested that the canine gene was approximately 10 fold larger than the human or mouse counterparts. This surprising size discrepancy was attributable exclusively to a large first intron in the canine gene. Exhaustive attempts to identify a putative transcribed first exon using RACE and sequence-based RT-PCR indicated that the canine transcript only includes exons 2 and 3, and not the far upstream located predicted first exon. In humans and rodents, the first exon of Perf codes for an untranslated region that is not part of the protein, and the coding sequence is confined to the second and third exons. In other species (equine and bovine) the first exon of Perf also is not part of the experimental or predicted transcript. Further, alignment of the coding and non-coding sequences showed high inter-species similarity between exons 2 and 3, but considerable variation in length and sequence for the regions upstream of exon 2. Thus, it was considered most likely that in the dog, like in some other species, regions 5’ to exon 2 are not transcribed. Further support for this conclusion resulted from identification of a sequence in human exon 1 similar to a region in the large intron separating the predicted exon 1 from exon 2 in dogs. Regions upstream of the Perf coding sequence are heterogeneous in length and sequence across species, and neither mutation associated with disease or regulatory sequences or SNP have hitherto been identified in this area (Zur Stadt, et al. 2006).

Three polymorphic sites were identified in the canine Perf cDNA. Two of the SNP (457A/C and 1416G/A) had previously been identified (rs24165117 and rs2411012, http://www.ncbi.nlm.nih.gov/SNP). None of the three mutations coded for a change in AA, hence they were unlikely to affect Perf function. Dogs in group 3 (control BMD) did not have any polymorphic sites, while different combinations of SNP were observed in
groups 1 (dogs with HHS) and 2 (non-predisposed breeds). Initial Chi square testing indicated that all three mutations were significantly linked. Although the relationship between HHS and presence of polymorphisms was not significant, estimates of the odds of SNP occurring in dogs with HHS were relatively high. Thus, these preliminary findings suggest that a distinct genotype may characterize BMD without histiocytic disease. Such a genotype may be part of an oligenic model of inheritance recently reported to account better for heritability of HS than an autosomal recessive pattern (Abadie et al. 2009). Hence, results of this study should be interpreted with caution given the small sample size, but should be considered in studies using more complex genetic analysis and larger samples.

**Conclusion**

The coding sequence for Perf in dogs is shorter than predicted from the canine genome sequence, but consistent with those in other species. Changes in the amino acid sequence of Perf were not associated with HHS in dogs, but silent gene mutations occurred at higher frequency in BMD with HHS than in BMD without HHS. To assess whether reduced Perf function contributes to histiocytic diseases in dogs will require measurement of gene and protein expression, and functional evaluation of lymphocyte cytotoxicity in dogs.
CHAPTER 3.

IN SITU CHARACTERIZATION OF CYTOTOXICITY IN TUMOR INFILTRATING LYMPHOCYTES OF CANINE CUTANOUS HISTIOCYTOMA

Abstract

Perferin (Perf), the cytolytic effector molecule of cellular immunity is a tightly regulated effector molecule. It is a valuable marker for peripheral differentiation of cytotoxic lymphocytes (CL) and the most reliable predictor for granule dependent killing. Canine Cutaneous Histiocytoma (CCH), a benign, naturally-regressing tumor, offers a unique model for successful immune-mediated tumor regression. The present study characterizes expression of Perf and granzyme B (GrzB) in CL participating in the successful CCH anti-tumor immune response. An antibody targeting a conserved region of canine Perf was generated. Simultaneous immunohistochemical probing linked Perf to CD3+ lymphocytes. Co-localization of Perf and CD63 confirmed sub-cellular localization of Perf to secretory lysosomes. Consistent expression of Perf and GrzB in tumor infiltrating lymphocytes (TIL) of CCH contrasted with lack of expression in resting lymphocytes. Perf expression was found in a minute fraction of TIL, markedly less than GrzB. Proportions of cells showing polarization of Perf and GrzB immunoreactive granules were high suggesting frequent engagement of CL in immunological synapses (IS). Predominance of GrzB+ polarizing cells versus Perf+ polarizing cells implied frequent assembly of Perf-deficient CL in IS. By probing Perf and tracing its expression in tissue we collected novel data about TIL within the tumor microenvironment. We provide in situ confirmation for the differential expression of the Perf and GrzB as well as support for the presence of Perf independent killing
mechanism during tumor rejection. Outlining morphologically identifiable parameters of productive anti-tumor immunity provides a model for comparison to commonly encountered, ineffective TIL which hampers the advancement of cancer immunotherapy.

**Introduction**

CL are the primary executors of cell mediated immunity; they play essential roles in protecting organisms against intracellular pathogens and neoplastic cells and in maintaining lymphocyte homeostasis. The two main subsets of CL, the NK cells and the cytotoxic CD8+ T lymphocytes (CTL) represent the killer effectors of the innate and adaptive immune systems, respectively. CL exert cytotoxicity directly via granule-dependent killing (GDK) and/or Fas-Fas ligand (FasL) pathways and indirectly by release of cytokines. The GDK is a carefully orchestrated highly potent cascade and is the only non-redundant killing pathway of CL. Upon recognition of a target cell, CL form an Immunological Synapse (IS), a three dimensional structure which is assembled to synchronize specific intercellular communication and targeted cell killing. Following formation of IS, CL undergo conformational changes that result in cytotoxic granule polarization, membrane fusion and release of cytotoxic effector molecules.

Perf and granzymes (Grz), the main cytolytic effectors of CL, are two distinct cytocidal proteins that function in concert to induce target cell death. Perf is a pore-forming protein that anchors and polymerizes in the plasma membrane lipid bilayer of target cells to create trans-membrane channels. The Grz are a family of serine proteases which activate programmed cell death via both caspase-dependent and independent pathways.
GrzA and GrzB are the major killing Grz in CL, with GrzB being the most potent and most tightly regulated.\textsuperscript{29} Perf is pivotal for the delivery of Grz to the cytosol of target cells.\textsuperscript{88} Moreover, while delivering pro-apoptotic granzyme, Perf disrupts the integrity of target cell membrane exposing it to osmotic lysis and further accentuating the lethal effect of GDK.\textsuperscript{48} Although controversy exists regarding the exact process by which Perf delivers Grz to the target cell’s cytosol, it is indisputable that, unlike the functional redundancy of the Grz family,\textsuperscript{125, 134} Perf is indispensable for GDK.\textsuperscript{88}

Tumor infiltrating lymphocytes (TIL) are commonly observed in different neoplasms. These infiltrates consist primarily of CD8+ CTL and reflect the response of the adaptive immune system to aberrations in the antigenic profile of neoplastic cells.\textsuperscript{89} Yet, despite the induction of specific anti-cancer immunity, the majority of tumors utilize multiple mechanisms to inhibit TIL and escape anti-tumor immune response.\textsuperscript{57, 89, 133} Only a minority of cancer patients benefit from the cytotoxic effects of TIL and show effective cellular immunity reflected by tumor regression and enhanced long-term survival.\textsuperscript{12, 32, 61, 171} The failure of TIL to elicit successful response hampers the development of effective immunotherapeutic strategies. These approaches, which are designed to exploit the cytotoxic potential of TIL, are the cornerstone of novel cancer treatment options.\textsuperscript{89}

Differentiation and maturation of effector cytotoxic lymphocytes (eCTL) is a tightly regulated process.\textsuperscript{29, 40, 118} During immune response, following initial engagement of TCR and co-stimulatory molecules, naive T cells differentiate into a heterogeneous mix of effector and memory cells.\textsuperscript{10, 29} In the second phase of activation, fully differentiated effector cells recognize specific target cells and rapidly execute GDK with no need for a co-stimulatory
signal. Being expressed solely in terminally differentiated eCTL Perf is considered a promising marker for discrimination of fully differentiated eCTL and for providing the most accurate insights regarding the evolution and attainment of the cellular immune response. Several studies have investigated the differentiation and maturation of cytotoxic effectors via the expression of these cytocidal molecules. In such studies Perf expression was consistently shown to be a marker of fully differentiated eCTL and the only reliable indicator of killing capacity. However, these studies documented the expression of Perf by employing either reverse transcriptase-polymerase chain reaction (RT-PCR) or via flow cytometry of peripheral blood lymphocytes (PBL) which is the most accessible tissue for analysis. More recent studies have demonstrated that Perf RNA expression long precedes the actual expression of Perf protein. As such, the level of Perf RNA might not be an accurate indicator of the expression of this dynamically expressed protein and cannot reliably predict killing capacity. Similarly, it is well established that local and systemic immune responses are not inevitably similar, making PBL insufficiently representative of processes that occur within the tumor microenvironment. Thus, studies measuring Perf expression in PBL by flow cytometry, although estimating protein expression, do not necessarily reflect the functionality of TIL.

CCH is a benign neoplasm of epidermal LC that occurs commonly in young dogs. It is one of the few naturally-occurring tumors that displays spontaneous immune-mediated regression associated with TIL and, therefore, serves as a unique example for effective CTL-mediated anti-tumor immunity. Histologically, tumor regression is characterized by gradual infiltration of mature lymphocytes which begins to appear at the tumor periphery and accumulates within the tumor proper. The infiltrate is initiated by influx of CD4+...
lymphocytes which are rapidly outnumbered by CD8+ CTL. The magnitude and distribution of the lymphocytic infiltrate relates to degree of tumor regression.\textsuperscript{83} Ample studies have described different aspects of CCH tumor regression in order to extrapolate principles of successful anti-cancer immunity. Collectively, these findings define CCH regression as a dynamic process that involves changes in tissue morphology, cell composition and antigenic phenotype compatible with a Th1 anti-tumor response as outlined in both human and canine immunology.\textsuperscript{16, 84, 120} However, although it is traditionally accepted that CCH tumor cells are eradicated by GDK, TIL of CCH have not been examined from a functional perspective.

In the present study we characterized the maturation and differentiation of TIL through the course of CCH regression as reflected by expression of the cytocidal effector molecules Perf and GrzB. Using a robust imaging technology, we scanned tissue sections via multi-dimensional image acquisition and recorded differential expression of CD3, GrzB and Perf in the lymphocytic infiltrate of CCH. By overcoming the challenges of probing Perf in formalin fixed tissue we collected novel data about the in-situ maturation and differentiation of eCTL in a tumor microenvironment. These data, obtained from a naturally-occurring tumor, bear translational implications for cancer immunotherapy.
Material and Methods

Samples

Canine Cutaneous Histiocytoma

A total of 17 formalin-fixed paraffin-embedded CCH biopsy blocks were collected. The biopsies were initially excised for diagnostic purposes, fixed in 10% natural buffered formalin and embedded in paraffin. Histological diagnosis of histiocytoma was made by light microscopy based on previously described criteria. Hemotoxylin and Eosin (H&E) stained sections were used to classify CCH to four groups representing stages of tumor regression (SOTR I-IV). Classification criteria were based on degree and pattern of lymphocyte infiltration as previously described. Briefly, SOTR I included tumors with minimal lymphocytic infiltration restricted to the deep dermal periphery of the tumor. In SOTR II, moderate lymphocytic infiltration was diffuse within the body of the tumor and nodular at the deep dermal periphery. SOTR III included tissue with marked nodular and diffuse infiltration, located both in the periphery and body of the tumor. SOTR IV included tumors at final stages of regression with TIL outnumbering the histiocytic tumor cell population.

Normal lymphoid-rich tissues

Tissue samples of spleen, lymph node and small intestine were collected from fresh carcasses from dogs euthanized for non-neoplastic or immune related conditions (n=6). Tissues were processed as described above.
Lymphoma of granular lymphocytes

Three tissue blocks of intestinal, cutaneous and splenic lymphoma of granular lymphocytes excised for diagnostic/therapeutic purposes were collected from the archive and processed similarly.

Primary antibodies

Rabbit anti canine Perf (RACP) antibody

Anti-serum for canine Perf was raised in rabbit against the synthetic 13-mer peptide DQKAQSGSHEVRC targeting a conserved epitope on canine Perf (GenBank: ACR55686.1 amino acids 488-510). Peptide synthesis, coupling to keyhole limpet hemocyanin, animal immunizations, blood collection, affinity purification and antibody titrations were performed at Pacific Immunology (Ramona, CA, USA). An additional panel of commercial monoclonal and polyclonal primary antibodies was used for Immunohistochemistry (IHC) and immunofluorescence probing. Antibodies, sources, dilutions and specific conditions for antigen retrieval are listed in Table 3-1.

Immunoblot analysis

Total protein was extracted from a normal canine spleen. Aliquots of splenic lysate were separated by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and electrophoretically transferred to Immobilon P SQ Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA USA). The blot was blocked overnight with 1% Bovine Serum Albumin (BSA) in Tris (0.1mM) pH 7.6 with 0.1% Tween buffer (TTB) and probed with RACP primary antibodies (1:300) for 4 h at room temperature. Following an overnight wash, the blot was incubated with goat anti-rabbit IgG
coupled to horseradish peroxidase (HRP) diluted 1:5000 in TTB buffer and incubated for 1h at room temperature. The membrane was then washed overnight. Bound secondary antibody was detected using the enhanced chemiluminescence (ECL) detection kit (Amersham ECL blocking detection reagents) according to the manufacturer's instructions and recorded on X-ray film (Amersham Hyperfilm™ ECL).

### Table 3-1: Monoclonal and polyclonal primary antibodies; sources, dilutions and specific conditions for antigen retrieval

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expected immunopositive cells</th>
<th>Primary antibody</th>
<th>Resource</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perforin</td>
<td>Cytotoxic T lymphocytes, Natural killer cells</td>
<td>Rabbit anti-canine perforin peptide polyclonal</td>
<td>Bienzle &amp; Neta UO Guelph, Protease K (DakoCytomation)</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>CD63/LAMP 3</td>
<td>All hematopoietic cells</td>
<td>Mouse anti-human monoclonal</td>
<td>Thermo scientific 19281</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>T cells</td>
<td>Rabbit anti-human polyclonal</td>
<td>Dako A0452</td>
<td>Heat treatment, 2.4 min in Biocare decloaking chamber (90°C ~80PSI) pH: 6</td>
<td>1:50</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>Cytotoxic T lymphocytes, Natural killer cells</td>
<td>Rabbit anti-human peptide polyclonal antibody</td>
<td>SPRING bioscience, E2580</td>
<td>Heat treatment, 2.4 min in Biocare decloaking chamber (90°C ~80PSI) pH: 9</td>
<td>1:250</td>
</tr>
<tr>
<td>CD79a</td>
<td>B-cells</td>
<td>Mouse anti-human monoclonal</td>
<td>Dako HM57a</td>
<td>Heat treatment, 2.4 min in Biocare decloaking chamber (90°C ~80PSI) pH: 9</td>
<td>1:25</td>
</tr>
</tbody>
</table>
Immunohistochemistry (IHC)

For Perf probing, sections (4μm) were deparaffinized in xylene, rehydrated in graded alcohol concentrations, and washed in TTB, pH 7.6. Slides were then treated with hydrogen peroxide (0.3% in distilled water /10 min) to block endogenous peroxidase. Following antigen retrieval (Table 3-1), tissues were blocked for 10 min with universal blocker (Biocare Medical Concord, CA USA) and then incubated at room temperature with the primary antibody (RACP) diluted in TTB. The sections were washed three times in buffer and incubated for 30 min with HRP-labelled secondary antibody (goat anti rabbit IgG Envision+ Dako, Carpentaria, CA, USA). Secondary antibody binding was detected with diaminobenzidine (DAB) chromogen (Biocare); slides were counterstained with haematoxylin (Dako), and dehydrated in graded alcohol concentrations and xylene. Negative control sections consisted of substitution of the primary antibody with normal rabbit serum.

To further classify Perf expression in lymphocytes, Perf was probed along with the cluster differentiating lymphoid markers CD3 and CD79a for T and B cells respectively. For simultaneous probing of Perf and CD3, we applied a polymer double staining kit specifically optimized to simultaneously detect two rabbit primary antibodies with negligible cross linking (Polink DS-RR, Golden bridge international Life Science, Mukilteo, WA, USA). Protocols for CD3 and Perf labeling were optimized independently. Perf staining was carried out as described above but subsequent to the DAB detection step the slides were not counterstained or dehydrated. Rather, slides were washed three times in distilled water and immersed in citrate buffer, pH 6.0. Antigen retrieval for CD3 was carried out by heat
treatment and slides were rapidly chilled to room temperature. Denaturing reagent (Polink) was applied for 10 minutes at room temperature. Slides were briefly reviewed using a light microscope to confirm resilience of DAB labeling of Perf immunoreactivity. Subsequently, slides were washed and incubated with anti CD3 primary antibody diluted in TTB for 1h at room temperature, washed three times in buffer and incubated for 30 min with alkaline phosphatase (ALP) conjugated secondary antibody (Goat anti rabbit IgG-Polink). Secondary antibody binding was visualized with Ferangi blue, ALP substrate/chromogen (Polink) and the slides were counterstained with Weigert's iron hematoxylin (Biocare) and coverslipped with aqueous mounting media (Biomeda, Foster City, CA). The following controls have been set to ensure specific antibody detection: Omission of primary antibodies (each one at a time), swapping order of secondary antibodies and detection reagents and omission of detection of the firstly applied primary antibody to ensure efficient denaturing of exposed rabbit epitopes upon detection of the second primary antibody (ruling out crosslink).

Omission of primary antibody ruled out non-specific binding to secondary antibodies and set the threshold for nonspecific reaction of the detection reagents (background). Swapping detection method for a single stain of antibodies yielded expected staining pattern for each epitope. Although both detection systems exhibited efficient visualization of both antibodies, swapping the order of detection system was not applicable due to the different antigen retrieval protocols and different resilience of the detection reaction. Perf immunoreactivity was undetectable subsequent to heat treatment and therefore had to be detected first. Conversely, the photolytic treatment did not affect CD3 detection but was not sufficient to expose the CD3 epitopes and therefore did not replace heat treatment.
Also, while the DAB reaction sustained through the CD3-targeted heat retrieval, Ferangi blue labeling shattered in the course of the heat treatment and, therefore, had to be applied last. Omission of detection step for antibody A (Perf) did not change pattern and distribution of the detected antibody B (CD3) ensuring efficient denaturing of all rabbit epitopes and lack of cross reactivity.

For simultaneous probing of Perf and CD79a, slides were processed similarly until blockage of endogenous hydrogen peroxidases was completed. Slides were then washed 3 times in TTB and immersed in ethylenediamine tetra-acetic acid (EDTA) buffer, pH 9.0 (Dako). Antigen retrieval was carried out by heat treatment and slides were rapidly chilled to room temperature. Universal block (Biocare) was applied for 10 min at room temperature and removed without washing. A mixture of CD79a (mouse anti human antibody) and RACP (dilutions of 1:200 and 1:50 respectively) was applied and slides were incubated for 1h at room temperature. Slides were washed three times in buffer and incubated for 30 min with ALP conjugated secondary antibody (Goat anti-Mouse IgG- Biocare). Secondary antibody probing mouse epitopes (CD79a) was visualized with Ferangi blue, ALP substrate. Slides were washed thoroughly with TTB and then incubated for an additional 30 min with HRP conjugated secondary antibody (goat anti rabbit IgG). Secondary antibody binding to rabbit epitopes (Perf/ RACP) was detected with DAB chromagen (Biocare). Slides were counterstained with Weigert's iron hematoxylin (Biocare), coverslipped with aqueous mounting media (BiOmeda) and reviewed by light microscopy to assess staining pattern and distribution.
Confocal microscopy

Slide processing and immunofluorescence probing

Immunohistochemical detection methods for all primary antibodies were optimized during preliminary experiments and are summarized in Table 3-1. Four sections (8µm) were processed for each case. Slides were immersed in xylene and rehydrated in graded alcohol concentrations (100, 95, and 70%). To minimize autofluorescence, subsequent to rehydration the slides were immersed for 1h in 70% ethanol supplemented with 0.25% NH₃ as previously described.¹⁸ Rehydration was resumed by immersion in 50% ethanol for 10 minutes after which the sections were transferred to TTB, pH 7.6. To accentuate antigen retrieval and penetration, the sections were treated with permeabilization solution (PBS, 0.02% Triton X, 0.2% Bovine Serum Albumin) for 1h at room temperature and then blocked with 10% normal goat serum for an additional hour. The sections were incubated at 4°C overnight with a mixture of primary antibodies. Monoclonal mouse anti CD63 was combined with RACP and with polyclonal rabbit anti GrzB to assess localization of Perf and GraB respectively to lysosomal granules. Slides were then washed 4 times for 15 min with PBS and then incubated for 2 h with a mixture of secondary antibodies: Alexa 488 goat anti rabbit and Alexa 594 goat anti mouse (each diluted 1:500) (Invitrogen Burlington, ON Canada). The sections were washed again and mounted with ProLong® Gold antifade reagent with 4, 6-Diamidino-2-Phenyindole (DAPI) for double stranded DNA staining (Invitrogen). The slides were cured overnight at 37°C, sealed with clear nail polish and stored at 4°C until imaging was conducted.
Confocal imaging

3D high resolution acquisition:

Tissues were scanned using an Olympus FLUOVIEW FV1000 laser confocal microscope using the 60x/1.4 Plan Apo Oil objective and the Olympus FV1000 software (Olympus America Inc Center Valley, PA, USA). Specimens were illuminated with 405 nm Diode Laser, 488 nm Multiline Argon Laser and 633 nm Helium Neon Laser. Confocal microscope settings were established for optimal resolution. To acquire images suitable for co-localization measurements, green and red channels were carefully attuned using the grey scale. To obtain a series of optical sectioning (z stack) a range of depth was set by determining an upper and lower threshold and a series of 0.3-0.5 µm sections was acquired. Images were projected as a transparency of all layers.

Consecutive acquisitions:

To obtain high resolution images at high magnification while capturing large fields of view (LFOV) we employed a high-speed spinning-disk confocal system mounted on a Zeiss Axiovert 200M Inverted Microscope (Yokogawa Calgary Alberta Canada). The specimens were illuminated with a 405nm solid state laser, 488nm multi-line Argon laser and 633 nm solid state lasers. Multiple recordings (tiles) were acquired by sequentially automated positioning of the stage of the microscope covering 100 slightly overlapping fields and locally imaging the specimen at 63X magnification (Plan-ApoChromat 63x/1.4 oil). Once the series of tiles has been acquired, automated recombination of tiles recorded was carried out by automatic stitching software (Metamorph Universal imaging, Downingtown, PA, USA).
Image analysis

Expression of CD3, GrzB and Perf was measured by counting the number of immunopositive cells of each marker. Further, proportions of GrzB and Perf expression were assessed using total CD3 count as the denominator which represents the entire pool of infiltrating T lymphocytes. Polarization of immunoreactive GrzB and Perf granules was assessed by calculating proportions of polarizing cells out of total count of immunopositive cells for each marker.

Statistical analysis

General approach

Data summarizing expression of CD3, GrzB and Perf in LFOV at corresponding sections of CCH were analyzed. Expression was measured by counts of cells in a given space (density) and by assessing proportions as described above. Two models were fitted according to the response types, counts and proportions reflective of a binomial measure. Transformations were applied at the outset based on the nature of the data; count data, using a log transformation, binomial data (reported as proportions) using a logit transformation. Results are reported as means with 95% confidence intervals (CI). Also reported are Rate Ratios (RR, also known as relative risks) for count data and Odds Ratios (OR) for proportion (binomial data) with 95% CI. Minimum level of statistical significance (p) was defined as 0.05.
Expression Density

To compare counts of CD3 GrzB and Perf in a given space (also referred to as density) across the different SOTR, a log-transformation was applied. After transformation, a standard 1-way ANOVA was conducted. Results are reported as means with 95% CI and Rate Ratios (RR, also known as relative risks) with CI when comparing between different markers.

Proportion of Expression

To assess the proportion of expression of the different cytolytic molecules in a given lymphocytic infiltrate the proportion of GrzB and Perf expression were compared using CD3 expression as a denominator. After applying a logit transform to accommodate the binomial responses, the data were analyzed using a general linear mixed model, with cell type (GrzB and Perf) as a fixed effect variable and case as a random blocking variable. This design is equivalent to a paired t-test. Again, results will be reported as proportions and OR (with 95% CI).

Proportion of Polarization

To compare proportions of polarization of GrzB and Perf across SOTRs, a logit transformation was applied to accommodate the binomial responses. The data were then analyzed using a general linear mixed model, with expression of GrzB or Perf as a fixed effect variable and as a random blocking variable. This design is equivalent to a paired t-test. Again, results will be reported as proportions and OR (with 95% CI).
Assessment of Models

To assess the Analysis of Variance (ANOVA) assumptions, comprehensive residual analyses were conducted. These included formally testing the residuals for normality using the four tests offered by SAS's Proc UNIVARIATE (Shapiro-Wilk, Kolmogorov-Smirnov, Anderson-Darling and Cramér-von Mises) and plotting the residuals against the predicted values and variables included in the model. Such analyses facilitate revealing outliers or other problems, such as unequal variances.

Results

Samples

Seventeen CCH biopsies representing different SOTR were retrieved and assigned to the following four groups based on magnitude and distribution of the lymphocytic infiltrate. (Figure 1) SOTR I: Minimal to absent lymphocytic infiltration (n=4), SOTR II: Moderate lymphocytic infiltration, diffuse within the body of the tumor and nodular at deep dermal periphery (n=4). SOTR III: Marked nodular and diffuse infiltration, in both the periphery and body of the tumor (n=4). SOTR VI: Numerous infiltrating lymphocytes are replacing mass and outnumbering the tumor cells (n=5).

RACP antibody probe Perf-compatible peptide in protein extraction of canine spleen.

Immunobloting of RACP detected a band at ~66kD on protein extract of canine spleen. The molecular weight of the band corresponds to published weights of human and murine Perf and to the predicted weight of canine Perf calculated by the Expert Protein Analysis System (ExPASy) web based software. 109 (Figure 2.A)
Perf expressing lymphocytes are detected in a small fraction of T cells infiltrating CCH but were undetectable in resting lymphocytes.

IHC analysis of Perf expression in CCH consistently identified a small group of Perf expressing lymphocytes among CCH TIL. Conversely, the majority of lymphocytes in the infiltrate lacked Perf immunoreactivity. Simultaneous probing of Perf and lymphoid cluster differentiation markers confirmed that Perf expression was restricted to CD3+ cells; however, only a small proportion of CD3+ cells express Perf. (Figure 2.B) No Perf expression was detected on CD79a+ lymphocytes. Perf was rarely and inconsistently detected in canine spleen and was repeatedly undetectable in all of the other evaluated lymphoid-rich tissues (mesenteric and submandibular lymph nodes, intestinal mucosa and thymus). Perf was also undetectable in all three cases of canine granular lymphoma despite strong characteristic (cytoplasmic, granular) expression of GrzB.

TIL in regressing CCH exhibit consistent differential expression of cytolytic molecules

To challenge the initial observation regarding the rarity of Perf expression and to further outline the differential expression of both cytolytic effectors we sought to collect corresponding quantitative data. The consecutive acquisition and multiple recording application facilitated collection of large sets of data for each marker. Accordingly, three Corresponding mosaic images recording expression of CD3, GrzB and Perf over LFOV (100 fields 63X) were generated for each case. Density of CD3 expression was significantly higher (P< 0.0001) than density of GrzB which was significantly higher than Perf density (P< 0.0001). These highly significant variations were consistent across all SOTR. (Figure 3.A) Mean density of CD3 was 1699 cells /LFOV (CI= 1218 to 2228). Mean Density of
GrzB was 365 cells /LFOV (CI= 226, 501) and Perf density was strikingly lower with mean of 61 cell/LFOV (CI=44, 84). RR’s reflecting comparison between markers were as follow: CD3: GrzB RR= 4.5671, P<0.001, (CI= 3.1646, 6.5912), CD3: Perf RR= 27.3846, P<0.001 (CI=18.8724, 39.7360), GrzB: Perf RR= 5.9961, P <0.001 (CI= 4.1256, 8.7146)

To evaluate the effect of SOTR on density of Perf and GrzB expression we used multiple pair-wise t tests while adjusting for multiple comparisons (i.e TUKEY test). Numerically GrzB density changed in a stepwise manner being lowest for SOTR I and highest in SOTR IV (Figure 3 B.1). SOTR IV for GrzB density was significantly higher than SOTR I (P=0.0079), SOTR I and II were not significantly different and SOTR III was marginally higher than SOTR I (P=0.05). The trend of change in Perf density across SOTRs was different (Figure 3 B.2). Numerically, SOTRs I, II, IV were similar; SOTR II was the lowest and SOTR III was the highest. SOTR III for Perf density was significantly higher than SOTR II (P=0.023). A simple effect was not identified. Consistent with the findings for the unadjusted density for Perf the ratio of CD3 to Perf was highest for SOTR III, RR=9.5997 (CI= 3.9664: 23.2333). When comparing proportions of Perf and GrzB using total CD3 as a denominator proportions of GrzB were significantly higher than those for perf (P<0.0001). The proportion of GrzB+ cells which reflects the subset of activated eCTL was 25.4% (CI= 15.971, 37.973). Proportions of Perf expressing cells was only 4.2% (CI= 2.776, 6.352) of the overall infiltrating CD3+ lymphocytes and 17.742% (CI=12.064, 25.328) of GrzB+ cells. No significant variation in proportions was associated with the different SOTRs. OR (GrzB: Perf) averaged over SOTR= 8.7077 (CI=5.36196, 14.1409).
Variation exists in the pattern of Perf and GrzB immunoreactivity

A high resolution 3 dimensional imaging of Perf expressing cells was conducted employing the Olympus FV1000 laser confocal microscope. Intriguingly, Perf and GrzB exhibited noteworthy variation in staining patterns. (Figure 4) For both markers, immunoreactivity was confined to the cytoplasm and exhibited a granular pattern. However, while Perf granules were coarse and somewhat indistinct (Figure 4.A) GrzB immunoreactive granules were small and sharply demarcated (Figure 4.B). Moreover, low numbers of Perf expressing cells displayed a non-granular, homogenous staining pattern. A large portion of Perf+ and GrzB+ cells exhibited unipolar granule distribution (polarization) while others displayed diffuse granule distribution. Differences were also noted in the patterns of granule polarization. GrzB granule polarization was tight and condensed (Figure 4.C.1) and was frequently associated with corresponding changes in nuclear morphology which included formation of a nuclear notch to which polarized granules localized. Conversely, Perf polarization (Figure 4.C.2.) was loose and less distinct and was rarely associated with corresponding nuclear changes.

Polarization of cytotoxic granules (CG) reflects active engagement of eCTL in an IS.\textsuperscript{17} Therefore, we measured and compared the percentage of Perf and GrzB polarization across the different SOTR types. (Figure 4.D) The proportions of polarizing Perf and GrzB reactivity were high but did not differ significantly (p=0.0713). However, given the marked and highly significant difference in the magnitude of expression of GrzB and Perf, variation in polarization across SOTR was analyzed separately for these two molecules. When looking at GrzB polarization alone, no significant difference was found across SOTR. Conversely,
we identified a significant effect of SOTR on Perf polarization ($P = 0.0319$). The largest mean of Perf polarization was detected in SOTR I SOTR II&III were similar while SOTR VI exhibited the lowest mean of polarization. The differences between SOTR I and IV remained significant following adjustment to multiple comparisons by applying the Tukey adjustment.

**Perf, but not GrzB co-localise with CD63, a marker of secretory lysosomes**

Given the unexpected variation in Perf and GrzB staining patterns we sought to determine the sub-cellular localization of these cytolytic effectors. (Figure 5) Cytotoxic granules (CG) are specialized excretory organelles also known as secretory lysosomes. These unique dual function organelles serve both as a degradative and as a secretory compartment and share immunophenotypic characteristics with conventional (non-secretory) lysosomes. As Perf and GrzB are traditionally known to localize to CG we hypothesized that both Perf and GrzB should exhibit a high degree of co-localisation with CD63, lysosomal integral membrane protein and an accepted marker of human and murine secretory lysosomes. However, while co-localization of Perf and CD63 was frequent between and within cells (Figure 5A, Figure 5C.1), GrzB and CD63 co-localization was rarely encountered (Figure 5.B Figure 5C.2). When found, co-localization of GrzB and CD63 was confined to coarse and indistinct granules that were somewhat reminiscent of characteristic Perf granules. Interestingly, Perf and CD63 co-localization was similarly high in cells that showed homogenous, non-granular staining pattern. Moreover, although the typical expression pattern of CD63 was granular as expected and, as previously documented, when associated with diffuse homogenous Perf expression the pattern for CD63 expression changed in concordance from granular to diffuse.
Figure 3-1: Photomicrographs of CCH biopsies, representing different stages of tumor regression (SOTR)

A: SOTR I, Minimal to absent lymphocytic infiltration. B: SOTR II, nodular lymphocytic aggregate is seen at the deep dermal periphery of the tumor. C: SOTR III Marked nodular and diffuse infiltration, in both the periphery and body of the tumor. D: SOTR IV, Final stage of regression; tumor cells are outnumbered by infiltrating lymphocytes. H&E, total magnification 50X. Bars represent 400 micron
Figure 3-2: Validation of rabbit anti canine Perf (RACP) antibody

A: Rabbit anti Canine Perforin (RACP) antibody probe Perf-compatible peptide on protein extract of canine spleen. Western blotting shows a band at ~66kD, corresponding to published weights of human and murine Perf, and to the predicted weight of canine Perf. B: RACP demonstrate granular cytoplasmic staining restricted to a subset of CD3+ lymphocytes. IHC of CD3+ cells (black arrow) show dark blue membranous staining (Ferangi Blue). Perf reactivity (white arrow) is seen as brown granular cytoplasmic staining (DAB chromagen) which is typically aggregated at one pole of the cells. Histiocytes are stained with haematoxylin nuclear counter stain and lack cytoplasmic staining (empty arrow). Occasionally, the staining pattern was suggestive of the presence of an immunological synapse; the magnified inset shows a CD3+ Perf-expressing cell stained with deep blue cytoplasmic staining (a) and a histiocyte stained with nuclear counter stained only (b) in close contact, with Perf immunoreactivity (c) highlighting their line of apposition suggesting assembly of an immunological synapse. Total magnification=630X. Bar represents 25 microns.
Figure 3-3: Differential expression CD3, GrzB and Perf

A

Differential expression CD3, GrzB and Perf

B

Expression of GrzB across SOTR

Expression of Perf across SOTR

C

Effect of SOTR on proportion of cells expressing cytolytic enzymes

Percentage of total CD3+ expressing GrzB+ and Perf+
Figure 3-3: Differential expression CD3, GrzB and Perf

Mean values are plotted and compared. Bars demonstrating the variability and skewness of the results confidence intervals (CI). Significant variations are indicated by asterisks.

A: Density of CD3, GrzB and Perf expression differ significantly across all SOTR. Density of CD3 is significantly higher than density of GrzB which is significantly higher than density of Perf.

B: Variation in density of Perf and GrzB across SOTRs: note different scales for GrzB (B.1) and Perf (B.2). B.1: GrzB density changed in a stepwise manner being lowest for SOTR I and highest in SOTR IV. SOTR IV for GrzB density is significantly higher than SOTR I, SOTR I and II are not significantly different and SOTR III is marginally higher than SOTR I (B.2). The trend of change in Perf density across SOTRs is different. Numerically SOTRs I, II and IV are similar; SOTR II is the lowest and SOTR III is the highest and is significantly higher than SOTR II.

C: Proportions of Perf and GrzB of the overall CD3 expressing cells differed significantly across all SOTR. Mean probability (presented as percentage) of GrzB+ is significantly higher than that of Perf+ across all SOTR. No significant variation in proportions is associated with the different SOTR. Proportions of polarizing Perf and GrzB reactivity are both high. Proportions of GrzB polarization is slightly higher but is not significantly different.

Figure 3-4: Variation exists in the pattern of Perf and GrzB immunoreactivity

Images represent multiple 0.3 micron optical sections exhibited in two channels and a merged illustration; DAPI nuclear staining (blue), Perf (Panel A) and GrzB (Panel B) (green). Layers are projected as a combined transparency to a 2D image. Scale bars represent 5 micron. Although consistently cytoplasmic, Perf immunoreactivity exhibits considerable morphological variation

A: Morphology of Perf expression ranges from diffuse homogenous (A.1) diffuse granular (A.2) and polarized granular (A.3,4) labeling. Perf immunoreactive granules are coarse and indistinct (A.2-4).

B: GrzB immunoreactivity is uniform and consists of identical pinpoint, sharply demarcated granules

C: Differences exist in the patterns of Perf and GrzB granule polarization

GrzB granule polarization is tight and condensed (C.1) and is frequently associated with corresponding changes in nuclear morphology. Changes in nuclear configuration manifested with formation of a nuclear notch (indicated by white arrow in the blue (DAPI) channel) to which polarized granules localize (merged image). Conversely, Perf polarization is loose and is not associated with corresponding changes in nuclear morphology. (C.2).

D: Proportions of Perf and GrzB polarization are not significantly different although there is a trend towards lower Perf polarization (p=0.07).

There is no significant difference in proportions of GrzB polarization between different SOTR (upper panel). However there is a subtle effect of SOTR on Perf polarization (P= 0.0319) (lower panel). The largest mean of Perf polarization is seen in SOTR I, means of SOTR II and III are similar while SOTR VI exhibited the lowest mean of polarization. The differences between SOTR I and IV remain significant following adjustment to multiple comparisons by applying the Tukey adjustment.
Figure 3-4: Variation exists in the pattern of Perf and GrzB immunoreactivity
Figure 3-5: CD63 co-localize with Perf but not with GrzB
**Figure 3- 5: CD63 co-localize with Perf but not with GrzB**

Images represent multiple 0.3 micron optical sections exhibited in two channels and a merged illustration; Perforin (A) and GrzB (B) (green) and CD63 (red). Layers are projected as a combined transparency to a 2D image. Bars represent 5 micron. Colocalization of markers is accepted when detected at least two 0.3 micron thick optical sections yield a bright yellow signal in the merged images.

A: CD63 immunoreactivity (red channel) exhibits granular staining pattern which aggregates in concordance to perforin polarization (green channel). CD63 reactivity in the background reflects excretory lysosomes in histiocytes and non perforin staining cells. In the merged channel, Perf show high levels of co-localization with CD63. Co-localization is similarly high across the different pattern of perforin immunoreactivity including homogenous (A.1) granular diffuse (A.2) and granular polarized (A.3-4) expression patterns.

B: GrzB (green channel) in its typical fine granular pattern exhibits minimal co-localization with CD63 (B. 1-2). Consistency of these findings is illustrated in a larger view (C) which captures multiple cells exhibiting similar pattern of high and low co-localization with CD63 of Perforin (1) and GrzB (2) expressing cells, respectively.
Discussion

CCH is a common, naturally occurring LC tumor in the domestic dog.\textsuperscript{106} CCH is known for its susceptibility to anti-cancer immune responses resulting in complete regression subsequent to a lymphocytic reaction.\textsuperscript{34, 83, 106} Regression of CCH is a dynamic process that is coupled with expansion of a CD8+ infiltrate. It has been suggested that the susceptibility of CCH to immune-mediated regression is attributable to the ability of the neoplastic LC to maintain functional capacity as potent stimulators of the immune response and their propensity to evoke a strong lymphocytic reaction.\textsuperscript{15} Correspondingly, despite being an ineffectual phenomenon in the vast majority of malignancies,\textsuperscript{57, 133, 135} in CCH, TIL serve as effective executors of tumor regression.

In the present study, we delineate the differential expression of GrzB and Perf in TIL of regressing CCH as markers of differentiation and maturation of eCTL. Our results show that consistent expression of Perf and GrzB occur in TIL of regressing CCH although these markers are undetectable in homing/resting lymphocytes. The lack of Perf and GrzB immunoreactivity in lymph nodes and intestinal mucosa of clinically healthy dogs is not surprising. These results fit well with previous studies which showed that expression of these cytolytic enzymes is linked to cell maturity.\textsuperscript{29} Moreover, it has been shown that naive human and murine CD8+ T cells express Perf mRNA but do not express Perf protein and lack cytotoxic capacity.\textsuperscript{29, 40, 118} These studies suggest that Perf protein expression is coordinated via posttranscriptional (translational)\textsuperscript{40, 118} regulation and therefore emphasize the advantage of IHC over RT-PCR for the investigation of functionally relevant Perf expression. Conversely, the inconsistency of Perf detection in spleen was somewhat
surprising as we expected NK cells, residents of splenic red-pulp, to consistently display Perf immunoreactivity. Being effectors of innate immunity, NK cells are required to spontaneously manifest cytotoxic activity with minimal sensitization. Therefore, unlike CTL which are subjected to diligent peripheral regulation, NK cells were traditionally assumed to circulate constitutively fortified with cytocidal proteins. However, there is ample evidence that, similarly to CTL, NK cells are subjected to peripheral functional maturation and that the accumulation of cytolytic enzymes is associated with a corresponding decrease in cell viability and proliferative potential. Furthermore, the presence of a key post-transcriptional regulatory step for Perf expression has been repeatedly demonstrated in both murine and human NK cells. It has been suggested that occult viral challenges may account for the alleged constitutive Perf and GrzB expression in NK cells. Accordingly, it has been demonstrated that once viral challenges are rigorously prevented, freshly isolated NK lack both Perf and granzyme protein expression despite abundant mRNA from these genes. The lack of Perf expression in granular lymphoma despite strong and typical GrzB expression is likely attributable the level of differentiation of the neoplastic clone. Clearly, the number of cases evaluated cannot be used to draw any conclusion regarding which clones are typically associated with granular lymphocyte malignancies in the dog. However, intriguingly, we previously showed the expression of Perf in cutaneous, splenic and intestinal lymphomas of cats. Reactivity of Perf in feline but not in canine granular lymphoma suggests sub-clonal variations between the tumor lineage origins between these two species.

When assessing the expression of cytolytic molecules in the lymphocytic reaction of regressing CCH we identified a consistent expression of both Perf and GrzB through all SOTR. When measured in a quantitative manner, Perf was expressed in a strikingly minute
fraction of TIL significantly lower than GrzB which by itself is expressed in small fraction of the overall T cells in the reaction. These findings agree with previous observations describing the phenotypic heterogeneity of lymphocytes during acute viral stimulation, graft rejection, and in the course of targeted autoimmunity. The variations demonstrated in our results are particularly distinct, as expression was assessed at post-transcriptional (translational) level. Differences in the dynamics of Perf and GrzB expression during CD8+ T cells activation have been documented in both murine models and humans. In mice, naive T cells exhibit a robust increase in expression of GrzB 2 days following sensitization while Perf mRNA decreases to below baseline and subsequently increases 4 to 8 days after sensitization. Stringent regulation of Perf expression is demonstrated not only by a late emergence but also by a short-lived peak. A marked decrease in the number of Perf (but not GrzB or FASL) expressing cells was documented at day 11 of tumor graft rejection and was credited to the net effect of activation induced cell death (AICD), and the elimination of tumor antigen resulting in decreased stimulation of de-novo Perf synthesis.

Assuming a key role for Perf in tumor cell eradication, we expected to identify statistically significant variations in Perf expression through the course of CCH regression. The experimental units in the present study are tissue sections from different dogs with CCH which are sub-classified according to SOTR. As each section represents a snapshot of the regression process, kinetics of eCTL differentiation cannot be extrapolated. Nonetheless, when expression data are averaged across the different SOTR it should represent, to a degree, the dynamics of the immune reaction. Looking at the data (Figure 3.B), Perf expression exhibits a notably different trend than the gradual increase in GrzB
expression as it develops with a late, short-lived peak as previously described.\(^{100}\) The lack of statistically significant SOTR effect may be attributable to low statistical power; however, it may also hint at the presence of autonomous overriding mechanisms that promote tumor cell elimination independently of Perf activity.

Further supporting an association between tumor regression and expansion of the lymphocytic infiltrate, we found ample direct and indirect evidence for the presence of activated eCTL in TIL of CCH. Infiltration of T cells within tumor parenchyma provides indirect evidence for successful priming and migration of antigen-specific T-cells and for lack of limiting stromal barrier.\(^{57}\) Large lymphocyte clusters within the body of the tumor in advanced SOTR (III and IV) likely reflect in situ proliferation of primed effectors. Lastly, the abundance of GrzB+ lymphocytes and the high proportion of cells which exhibit granule polarization indicate that copious activated, eCTL participate in the reaction and interact with target tumor cells. This finding may reflect a rapid turnover time. Thus, in view of this dynamic potent reaction, it appears unlikely that a minute, fixed fraction of Perf expressing effectors is the sole perpetrator of tumor regression.

Granule polarization of cytotoxic lymphocytes is a tightly orchestrated apparatus that is initiated subsequent to recognition of target cells by TCR of primed cytotoxic effector cells.\(^{46,141}\) Correspondingly, granule polarization can serve as a reliable morphological reflection of initial assembly or active engagement of eCTL in IS.\(^{17}\) We therefore examined the pattern of GrzB and Perf immunoreactivity in relation to SOTR, focusing on polarization of immunoreactive granules. Proportions of polarization were high for both markers and did not differ significantly amongst Perf and GrzB expressing cells. However, given the significant numerical advantage of polarized, GrzB+ cells over the total number of Perf +
cells, we concluded that a large fraction of GrzB-expressing cells are engaged in an IS despite lack of Perf expression. As GrzB requires Perf to attain efficient GDK, the role and outcome of these Perf deficient IS are yet to be determined. These could represent immature, unproductive engagement, or initial signaling events in the course of CTL activation and assembly of IS. Alternatively, these may represent functional IS executing a Perf-independent killing modality which may or may not involve GrzB activity.

Assembly of IS reflect intercellular communication of CL and target cells. CL utilize IS to facilitate targeted killing via both Perf-dependent and independent pathways. Moreover, as FasL in CL compartmentalizes to secretory lysosomal granules, FasL-mediated killing in CL is also attained via formation of IS and is subjected to similar trafficking and polarization regulation as Perf and Grz. Previous in vivo studies documented that two distinct types of cytotoxic effector cells exploiting different killing mechanisms evolve through the course of tumor graft rejection and recognized a corresponding switch from Perf-dependent to FasL-dependent killing. It is, therefore, conceivable that in the present study, Perf deficient IS represent FasL, granule dependent killing. It is noteworthy that there are no significant differences in the proportion of polarizing GrzB-expressing cells between the different SOTR. Conversely, proportions of Perf-positive polarized lymphocytes were significantly higher at initial stages of tumor regression where there is higher interaction between tumor and cytotoxic cells. These results further support the previous observation which suggested that Perf, but not GrzB and FasL expression is associated with changes in tumor antigenicity.

Lastly, and most unexpectedly, is the set of observations regarding variations in the pattern of GrzB and Perf immunoreactivity. These included variation in granule
morphology, density of polarization and associated changes in nuclear morphology. As RT-PCR or flow cytometry are the most commonly used techniques to evaluate Perf expression, there are only few studies describing IHC probing and visualization of Perf. Perf expression visualized by immunocytochemistry was described as punctate but the full array of staining patterns described in the present study was not reported. Immunocytochemistry is typically applied on cells under in vitro conditions, subsequent to induced activation. However, given the complexity of stimulatory and inhibitory interactions affecting lymphocytes in their natural microenvironment, in vitro studies may fail to capture the subtlety of physiological regulation as it is manifested in situ. Only few studies demonstrate Perf immunoreactivity in tissue specimens. In those, the morphology of Perf immunoreactivity was coarse granular and notably different from the characteristic pin-point pattern of GrzB immunoreactivity. More surprising was the striking discrepancy between the consistent co-localization of Perf and CD63 compared with the lack of such co-localization with GrzB. Collectively, these unexpected observations point towards the possibility of different sub-cellular compartmentalization of Perf and GrzB opposing a well-established dogma which localizes both GrzB and Perf to the same cytotoxic granules.

The CG consists of numerous small membrane vesicles and one or few electron dense cores enveloped together by a lipid bilayer. Electron microscopy localized GrzB to the electron dense core of murine CG while Perf was localized along with other granzymes (D, E and F) to the membrane vesicles. These variations in sub-granule compartmentalization of Perf and GrzB, if consistent in the dog, may account for the disparity in the pattern of Perf and GrzB immunoreactivity. Correspondingly, the pin-point, sharply demarcated GrzB particles likely represent the crystalloid core of CG while the coarse, indistinct Perf granules
may represent the membrane vesicles. CD63 is a tetraspanin known to localize to the limiting lipid bilayer of secretory lysosomes.\textsuperscript{121, 130} Sub-granule localization of CD63 in closer proximity to the presumed Perf-containing compartment may partly account for its co-localization with Perf but not GrzB.\textsuperscript{117} Moreover, CD63 has been associated with different forms of lysosomal granules exocytosis.\textsuperscript{94, 101} Thus, although the current study cannot provide an explanation for these unexpected morphological observations, we speculate that the variations in CD63 co-localization and configuration of granule polarization between Perf and GrzB may be attributable to differences in Perf and GrzB sub-granule localization and excretion modalities.

The present study characterizes in situ expression of cytolytic effectors in TIL which take part in regression of a naturally occurring tumor. The objective of this work was to typify features of lymphocytes participating in a successful anti-tumor immune response in the context of tumor microenvironment. Our results document consistent expression of the cytolytic molecules Perf and GrzB in primed TIL, which stand in stark contrast to lack of corresponding expression in resting and homing lymphocytes. Furthermore, we measured marked quantitative supremacy of GrzB over Perf expressing lymphocytes and identified evidence for the presence of a large subset of Perf deficient lymphocytes which are actively participating in assembly of IS. We, therefore, under \textit{in vivo} conditions, provide support for previous \textit{in vitro} observations: these include the differential expression of the cytolytic molecules and the presumed involvement of Perf-dependent and independent killing during tumor graft rejection. By delineating presenting features of a productive anti-tumor immune response, we offer a model that allows comparison to a more common presentation of ineffective TIL which hampers the advancement of cancer immunotherapy.
CHAPTER 4.

DEFECTIVE POLARIZATION AND DEGRANULATION OF CYTOTOXIC GRANULES IN CANINE HEMOPHAGOCYTIC HISTIOCYTIC SARcoma

Abstract

Canine Hemophagocytic Histiocytic Sarcoma (CHHS), an aggressive proliferative disease of macrophages in dogs has similar clinical and pathological presentation to Familial Hemophagocytic Lymphohistiocysis (FHL) in children. FHL constitutes a complex of inherited immune-deficiencies in humans which manifests as uncontrolled proliferation and activation of lymphocytes and histiocytes, resulting in hypercytokinemia and severe peripheral cytopenias. Defective genes in FHL encode proteins involved in granule-dependent killing (GDK), a fundamental apparatus of cellular immunity. CHHS is a familial disease in Bernese Mountain Dogs (BMD), a breed known for high consanguinity and further susceptibility to hematopoietic malignancies and immune-regulatory disorders. We hypothesized that, similar to FHL, defective GDK underlies CHHS. CHHS was critically defined from other diseases with similar clinical and pathological characteristics. Clinical data from 12 CHHS-affected BMD were collected and tissues sections from seven cases were analyzed to determine potential structural defects in interactions between T-cells and tumor cells. Immunofluorescence and three-dimensional imaging were applied to outline the microanatomy of intercellular communication between T cells and tumor cells and thus delineate key components of GDK. Formation of an immunological synapse (IS), T-cell receptor (TCR) signaling, granule polarization and de-granulation were evaluated. Cytotoxic Lymphocytes (CTL) infiltrate CHHS tumor parenchyma, form IS with tumor cells, and
exhibit intact TCR signal transduction manifested as ZAP70 phosphorylation and cytolytic enzyme expression. Conversely, polarization and de-granulation of cytotoxic granules were rarely detected in CHHS, suggesting a functional CTL defect. Thus, failure of cell-mediated target cell killing in the tumor microenvironment might be a component in the development of CHHS. The study herein provide data that CHHS is a promising natural model for investigating the pathogenesis of FHL as well as for studying granule polarization and degranulation including their role in the anti-cancer immune response.

Introduction

Hemophagocytic Lymphohistiocytosis (HLH) is a severe immune-regulatory disorder caused by uncontrolled proliferation of activated lymphocytes and histiocytes. ⁷⁸ HLH results from acquired and/or inherited immune deficiencies manifesting as impaired cellular immunity. ¹³⁽⁸⁾ Acquired HLH develops due to a strong activation of the immune system subsequent to systemic infections, cancer or autoimmune disorders. ⁷⁸, ¹⁵⁽²⁾ Primary (familial) HLH (FHL) constitutes a complex of autosomal recessive diseases which are strongly associated with consanguinity. Acute presentation of FHL occurs at infancy and is associated with striking hypercytokinemia, life threatening cytopenias and severe systemic inflammation. ⁷⁶ A later onset FHL occurs in older children and young adults and may present with a less detrimental inflammatory disease but increased susceptibility to cancer. ¹⁰⁽⁷⁾ Defective genes in FHL encode proteins which are involved in granule-dependent killing (GDK), ¹⁷⁽³⁾ the principal execution mechanism of cytotoxic lymphocytes (CL), the CD8+ cytotoxic T lymphocytes (CTL) and Natural killer (NK) cells. Five types of FHL have been characterized; four of them (FHL type 2-5) have been associated with specific causative
The most common and most aggressive variant (FHL type 2) is attributable to mutation in *PRF1* which encodes Perf (Perf), the key GDK effector molecule.\textsuperscript{27, 78, 91, 156, 173} Other FHL variants are associated with mutations in genes encoding proteins which participate in the trafficking, priming and secretion of cytotoxic granules (*UNC13D, STX11, RAB27A, and STXBP2*) and constitute the core of the GDK apparatus.\textsuperscript{108, 144, 173} GDK is a fundamental element of the immune system. It plays a critical role in protecting the organisms against intracellular pathogens and cancer and is pivotal for lymphocyte homeostasis.\textsuperscript{27, 46, 81} Collectively, the FHL complex demonstrates the indispensability of GDK in sustaining the immune system within favorable, non-destructive boundaries and in maintaining effective cancer immune surveillance.

CHHS is an aggressive proliferative disease of macrophages which exhibits similar clinical and pathological presentation to FHL.\textsuperscript{104} Like FHL, CHHS manifests with diffuse spleno- and/or hepatomegaly, severe, rapidly progressing cytopenias and fulminating systemic inflammation which often deteriorates to disseminated intravascular coagulopathy (DIC). The main target organs are similar to FLH and include splenic red pulp, bone marrow and liver.\textsuperscript{104} Laboratory abnormalities shared by CHHS and FHL include anemia, thrombocytopenia, and hypoalbuminemia. The histological hallmark of FHL and CHHS is similar and consists of massive proliferation of erythrophagocytic macrophages associated with a prominent infiltrate of CTL.\textsuperscript{104} CHHS is highly breed-specific and shows strong predilection for Bernese Mountain Dogs (BMD),\textsuperscript{2, 111, 115, 124, 127, 154} Rottweilers, and Flat Coated Retrievers\textsuperscript{36, 47} with BMD being the most over-represented. Moreover, although the specific genetic target is yet to be elucidated, CHHS has been classified as a familial disease of the BMD breed with polygenic mode of inheritance.\textsuperscript{2, 115, 124} Given the resemblance between...
HHS and FHL, and strong familial background of HHS we hypothesize that, similar to FHL defective cytotoxic function also underlies CHHS. We have previously attempted to link CHHS and FHL by investigating potential aberrations in the canine Perf gene,\(^{110}\) however, despite some sequence variations between healthy and affected BMD, no mutations bearing functional implications were identified.\(^{110}\) In the present study, we investigate functional aspects of GDK by imaging intercellular communication between CTL and tumor cells hypothesising that defects in GDK underlie CHHS.

In addition, a major limitation of some studies aimed at delineating the mechanisms of CHD is the failure to use a consistent disease classification scheme, even though Moore et al. have provided an extensive array of phenotypic tools to make such distinctions.\(^4, 104, 106\) To ensure a restricted disease classification, we applied a modified set of inclusion criteria which attempt to reconcile published CHHS diseases features with HLH 04, the revised HLH diagnostic guideline for human patients.\(^70\)

The current animal model for FHL is a strain of Perf knockout mice. These mice are asymptomatic in a pathogen-free environment but develop a severe HLH-like syndrome subsequent to viral stimulation.\(^14, 80, 82, 90, 150\) Thus, disease manifestation in the mouse model depends on pathogen exposure and is phenotypically specific to the Perf-dependent FHL variant (i.e. FHL 2). Consequently, the mouse model is far from ideal for studying the wide spectrum of primary HLH. Furthermore, a spontaneous large animal model for HLH is not yet available hence the significant of the BMD model suggested in this study.
Material and Methods

Case selection using a modified HLH04 guideline

Canine Hemophagocytic Histiocytic Sarcoma (CHHS)

Retrospective and prospective sample collection of CHHS was subjected to stringent selection criteria. Of 38 cases initially identified, 12 met inclusion criteria for further study. Of these, seven cases had formalin-fixed paraffin-embedded (FFPE) tissues blocks available for confocal analysis. Tissue blocks were sourced from the Ontario Veterinary College and the Animal Health Laboratory at the University of Guelph. Tissues were obtained for diagnostic purposes or during necropsy, fixed in 10% natural buffered formalin, and embedded in paraffin. Archived cases were selected from a pool of cases with a morphological diagnosis of CHSS or Malignant Histiocytosis (corresponding to previously used nomenclature) between 1999 and 2005 (n=23). Cases were also collected prospectively between 2006 and 2010. Prospective inclusion relied on suitable clinical criteria supported by hematological and cytopathological findings (n=15). Only dogs of pre-disposed breeds were included from both prospective and retrospective pools (n=12, n=12). When a CHHS diagnosis was made by cytopathology, tissues were collected during necropsy, after death or euthanasia, and subjected to similar protocols for processing. Only dogs for which necropsy had been approved were included (n=5). To minimize variation associated with tissue-specific artifacts and standardized immunofluorescence probing and confocal acquisition, only splenic tumors were evaluated. Thus only cases for which splenic tissue was available were included. To ensure consistent disease classification, all selected cases were subjected to further histopathological immunophenotypic evaluation according to the previously
described criteria. One confirmed case had to be excluded due to extensive autolysis and necrosis which hampered processing for immunofluorescent labeling and confocal imaging.

To help ensure that cases were of a single clinical entity and to facilitate comparison of CHHS and FHL, the scheme of inclusion criteria was set to correspond with HLH 04, the revised guideline for HLH diagnosis. The HLH 04 is a diagnostic checklist composed by the Histiocytic Society which outlines the updated diagnostic and therapeutic guidelines for HLH. HLH04 consists of 8 clinical, laboratory and histopathological criteria from which at least 5 should be fulfilled to establish FHL diagnosis. Detection of molecular or genetic abnormalities affecting immune response pathways is confirmatory for FHL diagnosis regardless of these criteria; a documented family history of FHL or consanguinity is considered highly supportive. HLH 04 criteria include: 1/Fever 2/organomegaly (spleen or liver), and 3/cytopenias affecting at least two lineages in the peripheral blood, 4/hemophagocytosis in bone marrow, spleen, or lymph nodes 5/hypertriglyceridemia and/or hypofibrinogenemia, 6/low or absent NK-cell activity, 7/hyperferritinemia, and 8/high levels of soluble IL-2 receptor (CD25). Some of these criteria are similar to the core characteristics of CHHS, however, not all these variables are available or routinely measured in dogs. Hypertriglyceridemia has not been directly linked to HHS and is not measured in the routine biochemistry profile for dogs. In HLH hypertriglyceridemia reflects cytokine-induced aberration in lipid metabolism. In CHHS, altered lipid metabolism manifests as hypocholesterolemia which is presumably also cytokine-induced. In septic dogs, serum TNF-alpha has been shown to correlate negatively with serum total cholesterol and positively with triglycerides (Trig). However, although serum Trig was significantly higher in septic dogs compared with healthy controls it was not sufficiently high to
consistently deviate from the reference range and present as hypertriglyceridemia. Thus, it has been suggested the hypocholesterolemia is a more reliable marker for an acute phase reaction in septicemic/endotoxemic dogs. Thus, assuming that similar mechanisms result in species-specific, manifestations of aberrant lipid metabolism, we sought a flexible criterion that would reflect any indication of defective lipid metabolism and thereby allowed either hypertriglyceridemic or hypocholesterolemic dogs to be included. We therefore modified the HLH guidelines to include six commonly encountered/routinely measured criteria in the dog, adding hypocholesterolemia as an alternative to hypertriglyceridemia. Accordingly, dogs presenting with at least four of the following six clinical, laboratory and histopathological findings were selected: 1/fever, 2/organomegaly (liver or spleen), 3/hyperferritinemia, 4/hypertriglyceremia and/or hypocholesterolemia, 5/hemophagocytosis.

Canine Cutaneous Histiocytoma

CHHS specimens were analyzed in comparison to Canine Cutaneous Histiocytoma (CCH), a common Langerhans cell tumor in the dog that is susceptible to immune mediated regression and serves as an established model of effective anti-tumor immunity. A total of 17 formalin-fixed paraffin-embedded CCH biopsy blocks were collected. The biopsies were excised for diagnostic purposes and processed similarly as described above. Histological diagnosis of histiocytoma was made by light microscopy based on previously described criteria. Seven cases were used for detailed 3 dimensional confocal imaging and all 17 cases were scanned using sequential acquisition for quantitative analysis.
Normal tissues

Tissue samples of spleen and skin were collected from fresh carcasses from dogs euthanized for non-neoplastic or non-immune related conditions (n=6). Tissues were processed as described above.

Immunohistochemistry

Primary antibodies

A panel of monoclonal and polyclonal primary antibodies was used for IHC and immunofluorescence probing. The sources and dilutions of antibodies, and the specific conditions for antigen retrieval are listed in Table 4-1.

Slide Processing

IHC was carried out as the final step of the case selection process and also served for preliminary optimization of detection methods prior to immunofluorescence and confocal microscopy imaging. Sections (4µm) were deparaffinized in xylene, rehydrated in graded alcohol concentrations, and washed in Tris (0.1mM) pH 7.6 with 0.1% Tween buffer (TTB), pH 7.6. Slides were then treated with hydrogen peroxide (0.3%/10 min) to block endogenous peroxidase. Following antigen retrieval (Table 4-1), tissues were blocked for 10 minutes with universal block (Biocare Medical Concord, CA USA), followed by incubation at room temperature with the primary antibody diluted in TTB as outlined in Table 4-1. The sections were washed three times in buffer and incubated for 30 min with HRP-conjugated secondary antibody (goat anti rabbit IgG Envision+ Dako, Carpentaria, CA, USA). Secondary antibody binding was detected with DAB chromagen (Biocare); slides were
counterstained with hematoxylin (Dako Cytomation), and dehydrated in graded alcohol concentration and xylene. Negative control sections were processed similarly but primary antibody was replaced with normal rabbit serum or murine IgG, according to the host in which the primary antibody was produced.
Table 4-1: Monoclonal and polyclonal primary antibodies; sources, dilutions and specific conditions for antigen retrieval

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Expected immunopositive cells</th>
<th>Primary antibody</th>
<th>Resource</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perforin</td>
<td>Cytotoxic T lymphocytes, Natural killer cells</td>
<td>Rabbit anti-canine (peptide) polyclonal</td>
<td>Bienzle &amp; Neta University of Guelph</td>
<td>Protease K (DakoCytomation) 5 min at room temperature</td>
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<tr>
<td>CD63/LAM P3</td>
<td>All hematopoietic cells</td>
<td>Mouse anti-human monoclonal</td>
<td>Thermo scientific 19281</td>
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<tr>
<td>CD3</td>
<td>T cells</td>
<td>Rabbit anti-human polyclonal</td>
<td>Dako A0452</td>
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<td>E cadherin</td>
<td>Langerhans cells</td>
<td>Purified mouse anti-human monoclonal</td>
<td>BD Transduction Laboratories 610181</td>
<td>Heat treatment, 2.4 min in Biocare decloaking chamber (90°C ~80PSI) pH: 6</td>
<td>1:100</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>Cytotoxic T lymphocytes, Natural killer cells</td>
<td>Rabbit anti-human peptide polyclonal antibody</td>
<td>SPRING bioscience, E2580</td>
<td></td>
<td>1:250</td>
</tr>
<tr>
<td>CD11d</td>
<td>Red pulp splenic macrophages, Granular lymphocytes in the spleen</td>
<td>Mouse anti-canine monoclonal</td>
<td>Moore UC Davis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD79a</td>
<td>B-cells</td>
<td>Mouse anti-human monoclonal</td>
<td>Dako HM57a</td>
<td>Heat treatment, 2.4 min in Biocare decloaking chamber (90°C ~80PSI) pH: 9</td>
<td>1:25</td>
</tr>
</tbody>
</table>
**Confocal microscopy**

**Slide processing and immunofluorescence probing**

Immune detection protocols for all primary antibodies were optimized during preliminary IHC experiments and are summarized in Table 4-1. Four sections (8µm) were processed for each case. Slides were immersed in xylene, and rehydrated in graded alcohol concentrations (100, 95, and 70%). To minimize formalin associated autofluorescence, subsequent to rehydration, the slides were immersed for 1h in 70% ethanol supplemented with 0.25% NH₃ as previously described.¹⁸ Rehydration was resumed by immersion in 50% ethanol for 10 min after which the sections were transferred to TTB, pH 7.6. To accentuate antigen retrieval and penetration, the sections were treated with permeabilization solution (PBS, 0.02% Triton X, 0.2% Bovine Serum Albumin) for 1h at room temperature and then blocked with 10% normal goat serum for an additional hour. The sections were incubated at 4°C overnight with one of the following primary antibodies: Polyclonal rabbit (PR) anti-phosphorylated zeta-chain-associated protein kinase 70 (pZAP70), PR anti-Perf (Perf), PR anti-granzyme B (GrzB) or with a mixture of primary antibodies at the following combinations: PR anti-CD3/Monoclonal mouse (MM) anti-E-cadherin, PR anti-CD3 / MM anti-CD11d, PR anti-GrzB/ MM anti-E-cadherin, PR anti-GrzB/ MM anti-CD11d, PR anti-CD3/ MM anti-CD63. Slides were then washed 4 times for 15 min with PBS and then incubated for 2h with a mixture of secondary antibodies: Alexa 488 goat anti-rabbit and Alexa 594 goat anti-mouse (each diluted 1:500) (Invitrogen Burlington, ON Canada). To minimize the bright autofluorescence of erythrocytes in splenic sections after fluorescence labelling, the slides were immersed for 30 min in 70% ethanol supplemented with 0.1%
Sudan Black B as previously described.\textsuperscript{18} The sections were then washed to remove excess Sudan Black B and mounted with ProLong\textsuperscript{®} Gold anti-fade reagent with 4, 6-Diamidino-2-Phenylindole (DAPI) for double stranded DNA staining (Invitrogen). The slides were cured overnight at 37\textdegree C, sealed with clear nail polish and stored at 4\textdegree C until imaging was conducted.

**Confocal imaging**

Three dimensional acquisitions:

Tissues were scanned using Olympus FLUOVIEV FV1000 laser confocal microscope with the 60x/1.4 Plan Apo Oil objective and the Olympus FV1000 software. Specimens were illuminated with 405 nm Diode Laser, 488 nm Multiline Argon Laser and 633 nm Helium Neon Laser. Confocal microscope settings were established for optimal resolution. For co-localization measurements, green and red channels were carefully attuned using the grey scale. To obtain a series of optical sectioning (z-stack) a range of depth was set by determining an upper and lower threshold and a series of 0.3-1.0 micron sections was acquired. Images are projected as a transparency of all layers. Assembly of IS was inferred when CD3 (a membrane marker of lymphocytes and an integral component of TCR) co-localized with CD11d or E-cadherin (membranous markers of CHHS and CCH respectively) in a minimum of two 0.5 micron sections as previously described.\textsuperscript{17} TCR signal transduction was assessed by probing pZAP70. Activation of effector CL was examined by probing the cytolytic enzymes GrzB and Perf. Immunofluorescence labeling of GrzB also allowed tracing the distribution of cytotoxic granules (CG). De-granulation of CG was confirmed by
co-localization with CD63, a lysosomal integral membrane protein and CD3 which reflected CD63 externalization.

Consecutive acquisitions:

To obtain high resolution images at high magnification while capturing large fields of view (LFOV) a high-speed spinning-disk confocal system mounted on a Zeiss Axiovert 200M Inverted Microscope (Yokogawa Calgary Alberta Canada) was employed. The specimens were illuminated with a 405 nm solid state laser, 488 nm multi-line Argon laser and 633 nm solid state lasers. Multiple recordings (tiles) were acquired by sequentially automated positioning of the stage of the microscope covering 100 slightly overlapping fields and locally imaging the specimen at 63X magnification (Plan-ApoChromat 63x/1.4 oil). Once the series of tiles has been acquired, automated recombination of tiles recorded was carried out by automatic stitching software (Metamorph Universal imaging, Downingtown, PA, USA).

Confocal analysis

Images obtained by consecutive acquisitions were analyzed to allow quantitative comparison of key characteristics of the lymphocytic infiltrate of CCH and CHHS. These included: Density and distribution of infiltrating CD3+, density of pZAP70, Perf and GrzB. Expression density was measured as a count of recorded events/LFOV. Also studied was the intracellular distribution of GrzB immunoreactive granules within GrzB+ cells. Subsequently, cells exhibiting polarized GrzB reactivity were counted and the proportion of cells exhibiting GrzB polarization was calculated out of the overall GrzB+ count. To assess
the distribution of CD3+ cells (referable to the degree of CTL penetration), the size of lymphocyte aggregates was measured and compared between CCH and CHHS lymphocytic infiltrate. Fluorescence signals, referred to as area of fluorescence (AOF) representing the number of fluorescent pixels, were measured for 800 individualized, CD3+ cells sampled in randomly assigned coordinates, and a median AOF of a single CD3+ cell was calculated. Subsequently, CD3 AOF was measured in a LFOV for all sections. Confluence of adhering CD3+ lymphocytes within aggregates generates a single AOF value. The median AOF value of individualized cells was used as a denominator for all recorded AOF values to facilitate estimation of numbers of cells comprising each AOF.

Statistical analysis

General approach

Data summarizing expression of CD3, GrzB, and Perf, pZAP70 and polarization of GrzB immunoreactive granules were analyzed. Two models were fitted according to the response types, counts and proportions reflective of a binomial measure as outlined above. Transformations were applied at the outset based on the nature of the data; count data, using a log transform, binomial data (reported as proportions) using a logit transform. Results are reported as means with 95% confidence intervals (CI). Rate Ratios (RR, also known as relative risks) for count data and Odds Ratio (OR) for proportion (binomial data) are also reported as means with 95% CI.
Expression density of CD3, pZAP70, GrzB and Perf in CCH and CHHS specimens

To compare density of CD3, pZAP70, GrzB and Perf between the two tumor types immunopositive cells in a given space unit (LFOV) were counted and a log-transform was applied. After transformation, a standard 1-way ANOVA was conducted. Results are reported as means with 95% confidence intervals (CI) and RR with CI when comparing between different markers.

Distribution of CD3+ lymphocytes in CCH and CHHS specimens

To calculate average and median AOF values of individualized CD3+ data were adjusted for normality using a natural log transform given the binomial nature of the data. Data representing size of lymphocyte aggregates were heavily skewed and normality could not be achieved by data transformation. To characterize the nature of aggregation of CCH verses CHHS the data were compared according to the following dichotomizing criteria: individualized cells vs. clusters composed of >1 cell, clusters composed of ≤ 4 cells vs. clusters composed of > 4 cells and clusters composed of ≤ 19 cells vs. more than 19 cells. For this analysis, given their binomial nature, the data were fitted to a generalized linear mixed model (GLIMMIX Procedure) leading to a logistic regression approach. GLIMMIX was used to account for sub sampling; the fixed effect was the tumor type (CCH vs. CHHS) and the random effect of case nested within tumor types. Results are reported as mean percentage of individualized/clustered cells with 95% CI. Tumor effect is reported as OR given the binomial nature of the data.
A summary statistic approach\(^{126}\) was applied to compare variation in cluster sizes. The summary statistics were skewness and standard deviation, computed for each case to become the observation in the analysis. Data were adjusted using a log transformation and a standard one way ANOVA was applied.

**The effect of tumor type and CD3 count on the expression of pZAP70 in CCH and CHHS**

To assess the effect of tumor type and CD3 counts on number of cells expressing pZAP70, a log transform was applied to normalize the pZAP70 data. The data were scaled to reflect the log of pZAP70 change per 100 CD3 cell and a general linear model (GLM) was applied.

**The effect of tumor type on polarization of GrzB immunoreactivity**

Cells exhibiting polarized distribution of GrzB immunoreactive granules were counted over a LFOV. Polarized cells were calculated as a proportion of the overall GrzB+ cells. Data were transformed using a logit transformation compared between the CCH and the CHHS tumor types using the MIXED Procedure. The MIXED Procedure was used to account for sub-sampling; the fixed effect was the tumor type (CCH vs. CHHS) and the random effect of case nested within tumor type. Results are reported as means with 95% confidence intervals (CI) and OR with CI reflecting the tumor effect on polarization. Results are reported as means with 95% confidence intervals (CI) and Rate Ratios with CI when comparing between different markers.
Assessment of Models

To assess the ANOVA assumptions, comprehensive residual analyses were conducted. These included formally testing the residuals for normality using the four tests offered by SAS's Proc UNIVARIATE (Shapiro-Wilk, Kolmogorov-Smirnov, Anderson-Darling and Cramér-von Mises) and plotting the residuals against the predicted values and variables included in the model. Such analyses facilitated revealing outliers or other problems, such as unequal variances.
Results

Case selection

A flow chart representing the selection process CHHS cases is outlined in Figure 4-1. Selected cases and relevant clinical data are listed in Table 4-2. Eight cases were confirmed by histopathology and IHC. Three of the confirmed cases originated from the retrospective pool and five were collected prospectively. All selected cases were BMD. Although all three predisposed breeds were considered acceptable and were initially represented, only BMD fit the complete selection process adopted in this study. Dogs ranged in age from to three to 10y (median =5y). There were seven females (five spayed and two intact) and a single neutered male. Over-representation of females is not necessarily reflective of female predilection as three of four BMD which were not included due to unavailability of tissue were males. Anemia and hemophagocytosis were the most common findings and were found in all cases (n=12). Thrombocytopenia was found in 11 of 12 dogs. Dog number 4 was not thrombocytopenic but had a platelet count at the low end of normal. Eleven dogs exhibited enlargement of spleen (n=9) or liver (n=2) at initial physical exam. For dog number 2, the spleen was not enlarged by palpation and appeared mottled but not enlarged by ultrasound imaging. Histopathology of the spleen of this dog revealed modest tumor expansion involving only low numbers of sinusoids. Ferritin and triglycerides are not routinely measured and could therefore be obtained only for dogs which were selected prospectively. All confirmed cases for which ferritin could be measured exhibited strikingly high levels ranging from 8000 to 44173 ng/dl corresponding to 10 to 55.2 times the upper reference limit for dogs (800 ng/dl). Moreover, three additional BMD dogs which matched
clinical and cytopathological criteria, but were excluded as tissue was unavailable (dogs number 9, 10 and 12) also exhibited strikingly high ferritin levels ranging from 5000 to 38279 ng/dl. Four of five confirmed cases for which cholesterol was measured exhibited hypocholesterolemia. Dog number 8 had serum cholesterol within normal range but increased levels serum Trig. This was the only dog for which serum-Trig was measured (n=4) which exhibited hypertriglyceridemia. Overall, five of six confirmed cases for which relevant data were available exhibited altered lipid metabolism. Only 7 of the 23 retrospectively collected cases fit the inclusion criteria. Of those, 4 cases which matched inclusion criteria were excluded based incompatible immunophenotypic profile which was compatible with Dendritic cell sarcoma (n=2) and granular lymphoma (n=2). Conversely, all prospectively selected dogs of the listed breeds and for which tissue was available were included in the study.

Diagnosis of CHHS

In the present study, the combination of history, clinical presentation, cytopathology and laboratory data allowed highly specific classification of CHHS. Accordingly, initial cytopathological diagnosis of all prospectively selected cases was confirmed by auxiliary histopathological and immunophenotypic evaluation. Conversely, histopathology, when applied by different pathologists, possibly with incomplete clinical background information, was found to be more prone to misclassification. Consequently, initial histopathological diagnosis was found to be erroneous in 50% of the prospectively selected cases. The concluding histopathological evaluation applied in this study focused on specific CHHS characteristics previously suggested; namely the unique diffuse sinusoidal distribution of CHHS tumor cells. Following this approach, histopathology facilitated correct
identification of all CHHS cases independently of IHC. Nevertheless, we found that IHC was helpful to confidently distinguish CHHS from other similar splenic round cell tumors. The lymphoid cluster differentiating markers CD3 and CD79a had not disclosed cases of misdiagnosed T cell or B cell lymphoma. In the presence of negative CD3 and CD79 and positive CD18 was considered supportive of a histiocytic cell origin but was neither sufficient to distinguish DCS nor rule out NK cell lymphoma from CHHS. CD11d is the antibody of choice for the diagnosis of CHHS and detection of CD11d+ tumor cells expanding splenic sinusoids and migrating along liver sinusoids is considered to be the hallmark of CHHS. ¹⁰⁴ (Figure 4-2) However, CD11d is not unique to CHHS tumor cells as it is also expressed by normal macrophages and granular lymphocytes that are resident in splenic red-pulp but not hepatic Kupffer cells. ⁴₃, ¹⁰⁴ IHC for CD11d should therefore be used with caution in the spleen, and in conjunction with histopathology. The combination of CD11d and GrzB allowed distinction between red pulp splenic macrophages of CHHS (Cd11+, GrzB -) and splenic granular lymphoma (CD11d+ GrzB+). From the prospectively collected cases which matched the clinical inclusion criteria one case exhibited an immunophenotypic profile of CD18+ CD3- CD11d- and GrzB- and nodular tumor growth consistent with a splenic DCS. Two additional cases were excluded based on an immunophenotypic profile of CD18+, CD3-, CD11d + and GrzB+ consistent with granular lymphoma, likely of NK cell origin given the negative CD3 results. In all three cases, initial doubt regarding the CHHS diagnosis was raised during the final histopathological evaluation based on atypical distribution and morphology of tumor cells.
Tumor infiltrating lymphocytes (TIL) of CCH and CHHS differ in density and distribution:

The lymphocytic infiltrate of CCH and CHHS differ from the typical lymphocytic population of the affected tissues (skin and spleen, respectively). In both tumor types the CD3+ infiltrate appeared similar in magnitude and distribution to the lymphocytic infiltrate recognized by morphology on the H&E sections. Accordingly, in both tumor types the lymphocytic infiltrates were dominated by CD3+ cells. This was compatible with previous reports which described dominance of CD8+ T-cells in both tumor types \(^{104}\) and with the expected lymphocytic infiltrate in an established cellular reaction. Density of CD3 expression (referable to number of positively stained cells in a LFOV) was significantly different between the two tumor types (p=0.0003). The mean CD3 expression density in CCH was 1741.6 cells/LFOV (CI=1303.2, 2327.5) significantly higher than that of CHHS which was 521.2 (CI= 324.6, 837). RR of CD3 expression in CCH vs. CHHS = 3.3 (CI=1.9, 5.8) Figure 4-3 (A&D).

Detection of T-cell infiltrates within tumor cell regions suggests that T cells have been primed and successfully migrated to distal tumor tissue. Some tumors evade anti-cancer immunity by limiting permeation of primed CTL effectors and thus hampering interaction between tumor cells and effector CTL. Accordingly, it has been demonstrated that density and location of TIL within the tumor are key factors in the productivity of the immune response.\(^{57,61}\) We therefore, evaluated the degree of infiltration of CD3+ lymphocytes within tumor parenchyma, assuming that dispersed distribution provides indirect evidence for lack of tumor parenchymal barrier. The experimental values used for this analysis were the complete set of automated measurement of CD3+ AOF signals for each case in a LFOV.
These values represented either individualized cells or confluent clusters of lymphocytes which were giving a single signal.

Based on 800 discrete CD3+ cells, the mean and median AOF values for individualized CD3+ were 1319 and 1432 pixels, respectively. The 95% tolerance interval, which estimates the normal range, is 577-3016 (computed with 95% confidence). Given the skewness of the data, the median value was chosen to serve as a denominator to facilitate estimation of the number of cells comprising each AOF signal. The vast majority of TIL in CHHS penetrated the tumor as individualized cells. Accordingly, only 5.7% (CI=3.8, 8.6) of the lymphocytes in CHHS comprised confluent aggregates consisting of a minimum of 2 cells. Conversely, the probability of infiltrating CCH lymphocytes constituting confluent clusters $\geq$ 1 cell was significantly higher (p<0.0001) and estimated at 20.95% (CI=16.86, 25.73). OR for clusters $\geq$ 1 cells in CCH vs. CHHS=4.366 (CI=2.6261, 7.259) (Figure 4-3B). Results were similar when comparing the proportion of larger aggregates comprised of $\geq$ 4 and $\geq$ 19 cells. The estimated probability of lymphocytes to compose confluent aggregate $\geq$ 4 cells in CHHS tumor was 0.71% (CI=0.4, 1.25), significantly lower (p<.0001) than in CCH which was estimated at 6.65% (CI= 5.07, 8.69). OR, CHHS vs. CHH =9.966 (CI=5.243, 18.944). The calculated probability of lymphocytes to appear as large confluent aggregates ($\geq$ 19 cells) in CHHS tumor was 0.073% (CI=0.016, 0.33), significantly lower (p=0.0051) than CCH which was 0.856 % (CI=0.45, 1.6). OR (CCH vs. CHHS) was calculated at 11.825 (CI=2.304, 60.691) OR= 11.825 (CI =2.304, 60.691). Clusters $\geq$ 20 cells were analyzed as well but probabilities turned out negligible. Skewness and standard deviation (SD) of the CD3 AOF data were measured (adjusted with log transform). CHHS skewness
and SD were found to be significantly lower than that of CCH (p= 0.0186) and (p=0.0003), respectively.

**TIL form immunological synapses with tumor cells in both CCH and CHHS tumor types:**

T cells and tumor cells were labeled simultaneously to study their in-situ interaction. An anti-CD3 antibody was used to label lymphocytes while antibodies targeting E-cadherin and CD11d were employed to label LC and macrophages in CCH and CHHS, respectively. E-cadherin is expressed on Langerhans CCH tumor cells, but may also be expressed on other epidermal-associated leukocytes like lymphocytes, mast cells and plasma cells. In conjunction with morphological characteristics, E-cadherin immunopositive cells were confidently recognized as histiocytes and were therefore calcified as Langerhans tumor cells. CD3+ lymphocytes were consistently negative for E-cadherin expression. Lack of E-cadherin expression on CCH TIL further supports a distal (non-epidermal) origin of these lymphocytes emphasizing the reactive nature of the infiltrate. CD11d is a marker of splenic red pulp macrophages and as such it is expressed on both tumor cells and normal splenic macrophages. In this case, as well, morphology and distribution of tumor cells was highly characteristic and facilitated identification.

Synapses of CD3+ and tumor cells were imaged in all samples studied for both CCH and CHHS tumor types. Synapsing contacts were visualized as bright yellow signals representing co-localization of CD3 with E-cadherin or CD11d in CCH (Figure 4A) and CHHS (and 4B), respectively. A consistent morphological dissimilarity was noted in the distribution of CD3 immunoreactivity of synapsing lymphocytes in CCH and CHHS. In
CCH, CD3 immunoreactivity was clumped and aggregated towards the synapsing surfaces. Conversely, synaptic lymphocytes in CHHS exhibited homogeneous circumferential distribution of CD3 immunoreactivity.

_TIL in CCH and CHHS exhibit intact TCR signalling and express cytolytic enzymes reflective of an activated effector phenotype:_

Lymphocytes expressing pZAP70 were identified in both CHHS and CCH tumor specimens, indicating that in both tumor types TCR engagement and downstream signal transduction were productive (Figure 3C). The mean count for pZAP70 in CCH, not adjusting for any other variables, was 1086.2 events/LFOV (CI=587.6, 2008.0). For CHHS the estimate median was 100.2 l (CI= 14.24, 705.4). To assess the actual tumor effect on pZAP70 expression the model had to account for the strong association between the ZAP70 peptide and the CD3 complex and consequently to consider the intrinsic differences in CD3 density between the two tumor types. A log transformation was applied to normalize the pZAP 70 data. Changes of log pZAP70 were assessed per 100 CD3 cell increase. There was no interaction between the explanatory variables of tumor type and CD3. Numerically, the expression density of pZAP70 in CCH was found to be 1.77 times higher than in CHHS for every given CD3 count (CI=0.4625, 6.772). However, despite the relatively high $R^2$ (0.4) there was no significant effect of either tumor type ($p= 0.3611$) or CD3 ($p= 0.2615$) on the expression density of pZAP70. Despite the fact that tumor effect on pZAP70 reached significance when examined independent from CD3, it was considered biologically meaningless. With regard to CD3, while accounting for tumor effect, CD3 counts had no effect on pZAP70 likely because CD3 and tumor type are highly related. CD3’s exclusive
effect on pZAP70 was significant (P=0.047) as expected. The CD3/pZAP70 interaction, without accounting for tumor effect, reflects an increase of 3.918% in pZAP70 for every 100 cell increase in CD3 expression.

*Expression of cytolytic enzyme is not statistically different between TIL of CCH and CHHS*

The cytolytic enzymes Perf and GrzB are markers of activated, fully differentiated CTL and NK cells.\(^{29, 72}\) Thus, the expression of these effector molecules is reflective of activation and maturation of CTL. Expression of GrzB and Perf was documented by confocal microscopy in both CCH and CHHS specimens. Despite significant differences in density of CD3+ lymphocytes neither expression of GrzB nor that of Perf was affected by the tumor type *(Figure 3D)*. The mean of GrzB expression in CCH and CHHS was 399.135 (CI=265.159, 603.079) and 167.225 (CI=52.960, 528.03), respectively. Although CCH cases exhibited higher GrzB expression the difference was not statistically significant (P=0.1513). Perf expression was also not statistically different between the two tumor types (P=0.8654). For CCH, mean Perf expression was estimated at 86.836 CI (43.499, 93.682). For CHHS, Perf expression was estimated at 56.230 with exceptionally wide CI (12.4492, 253.980). Lack of statistical significance may reflect insufficient statistical power given the low sample size. However, considering the highly significant difference in density of the CD3 infiltrate, the expression of GrzB and Perf in a sub-set of CD3+ lymphocytes was expected to be markedly different even if only due to the strong effect of tumor type on the magnitude of the infiltrate (similar to pZAP70).
Synapsing lymphocytes in CCH exhibit distinct polarization of cytotoxic granules which is lacking in their CHHS counterparts

Being a key cytolytic enzyme, GrzB was used to trace the trafficking and distribution of cytotoxic granules upon interaction of CTL with tumor cells. Imaging of GrzB immunoreactivity in CCH specimens revealed that lymphocytes which are in close contact with tumor cells often exhibited tight polarization of GrzB immunoreactive granules towards tumor cells. Granule polarization was also associated with corresponding conformational changes in the lymphocyte nuclei which included formation of a distinct nuclear notch to which polarized granules have localized. Quantitative analysis of polarizing GrzB+ cells estimated proportions of GrzB polarization in CCH at 86.9% (CI=75.87, 93.35). Conversely in CHHS specimens in the vast majority of GrzB+ cells the distribution of cytolytic granules remained dispersed as the proportion of polarizing cells was estimated only at 8.7% (CI=3.0, 22.5). This difference was highly significant (p<0.0001) and was associated with exceptionally high odds ratio (OR) of 69.79 (17.86, 272.8) (Figure 5D).

Degranulation of cytotoxic granules

Cytotoxic granules are specialized secretory lysosomes that function as degradative and excretory organelles and share immunophenotypic characteristics with conventional (non-secretory) lysosomes. To evaluate degranulation capacity of cytotoxic granules in CTL, CCH and CHHS specimens were simultaneously labeled for CD3 and CD63, a lysosomal integral membrane protein. Next, we documented externalization of CD63 based on co-localization with membranous CD3. The typical pattern and distribution of CD63 immunoreactivity was fine, granular and primarily cytoplasmic, compatible with the expected distribution of secretory and conventional lysosomes. Low numbers of cells exhibiting co-localization of
CD63 and CD3 were documented in each of the CCH specimens (Figure 6). Similar cells could not be detected in CHHS specimens. Moreover, cytoplasmic CD63 immunoreactivity of CCH, CTL was notably clumped interfacing the internal contour of the limiting cell membrane. Conversely, CD63 immunoreactivity in CHHS specimens was distributed homogenously within the cell cytoplasm and did not exhibit any tendency to aggregate along the cell boundaries.
Figure 4-1: Algorithm for case selection

**RETROSPECTIVE CASE COLLECTION**
1999-2005
Morphological Diagnosis CHHS/ MH
n=23

**PROSPECTIVE SAMPLE COLLECTION**
2006-2010
Hematological & Cytological Diagnosis
n=15

**Pre- Disposed Breeds**
n=12
BMD (n=5), FCR (3), Rottw (3) Lab (1)

**Pre- Disposed Breeds**
n=12
BMD (n=9), Rottw (n=2), FCR (n=1)

**HLH 04 compatibility**
Relevant Clinical & Clinicopathological Presentation
n=7

**HLH 04 compatibility**
Relevant Clinical & Clinicopathological Presentation
n=9

**Tissue Availability**
n=7

**Tissue Availability**
Owner’s Consent to Autopsy
n=5

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**Dendritic cell Sarcoma**
Nodular Tumor Growth
CD18+ CD11d-, GrzB +CD3-CD79-n=2 (FCR, Rottw)

**CHHS**
Diffuse Sinusoidal Tumor Growth
CD18+ CD11d+, GrzB - CD3-CD79-n=3 (BMD)

**Granular Lymphoma**
Diffuse Tumor Growth
CD18+ CD11d+, GrzB +CD3-CD79-n=2 (BMD, Lab)

**n= 12**
Auxiliary Histopathological Review
Focusing on the unique Tumor cell distribution in CHHS

**Immunophenotypic characterization**
CD18, CD3, CD79a CD11d GrzB

**CHHS**
Diffuse Sinusoidal Tumor Growth
CD18+ CD11d+, GrzB - CD3-CD79-n=5 (BMD)

---

BMD: Bernese mountain dog
FCR: Flat coated retrievers
Table 4-2: Selected cases according to a modified HLH 04 guideline

Clinical and laboratory data from 12 selected cases. To be included, cases were required to fulfill a minimum of four of six clinical and laboratory criteria. Each met criterion is color coded and may consist of more than one element. **Part A:** cases confirmed by IHC and utilized for confocal work (with the exception of case number 8). **Part B:** cases diagnosed by cytology for which tissue was not available.

<table>
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<th>Breed</th>
<th>Age (Years)</th>
<th>Sex</th>
<th>Fever</th>
<th>OrgMeg</th>
<th>Ferritin (nmol/L)</th>
<th>Chole/Trig (mmol/L)</th>
<th>HCT (L/L)</th>
<th>Plt (10^9/L)</th>
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</tr>
</thead>
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Reference range: 80-800 3.6-10.2 0.12-1.3 0.36-0.56 117-748

**Abbreviations:** **BMD** = Bernese mountain dog, **MN**=male neutered, **F**= Female spayed, **Y**=yes, **N**=No, **HCT** = Hematocrit, **Plt** = Platelet count, **Chole**= Cholesterol, **NA**=not available, **Trig**=Triglycerides, **OrgMeg**=Organomegaly, **Hemo**= Hemophagocytosis, **IHC** = Immunohistochemistry

* Tissue was available for IHC to confirmed CHHS diagnosis but was not suitable for confocal imaging and analysis due to profound autolysis
Figure 4-2: Spleen (A-D) and liver (E) of CHHS-affected dogs
Figure 4-2: Spleen (A-D) and liver (E) of CHHS-affected dogs

**A**: H&E, spleen (dog number 7); erythrophagocytic histiocytes in red pulp cords ranging in level of cytological atypia. Bar=20 micron

**Inset 1**: Well differentiated macrophages with intracytoplasmic erythrocytes.

**Inset 2**: Atypical erythrophagocytic tumor cell in mitosis (in the middle). Two small morphologically unremarkable lymphocytes are (black arrow head) and rubricytes (black arrow) are also present. Bar=20 micron

**Inset 3**: Atypical erythrophagocytic tumor engulfing hemosiderin, recently phagocytized erythrocytes and a rubricyte.

**B**: H&E, Spleen (dog number 3); cytologically atypical tumor cells (black arrow head) expanding a red pulp sinus. Large numbers of rubricytes (black arrow) reflect the extra medullary hematopoiesis that is associated with this tumor.

**C**: CD11d/ hematoxylin counterstain, (dog number 3) CD11+ tumor cells in a red pulp sinus

**D**: CD11d/ hematoxylin counterstain (dog number 5) CD11+ tumor cells mixed with large numbers of small morphologically unremarkable lymphocytes

**E**: CD11d/ hematoxylin counterstain (dog number 5) CD11+ tumor cells are seen in liver sinuses
A: Two mosaic images illustrating CD3 expression in CCH and CHHS:

The images are comprised of 100 slightly overlapping, fields obtained by confocal consecutive acquisition (Yokogawa) at 63 X magnifications and stitched to a LFOV (Metamorphe); magnified insets represent 9 fields. The images display an overlay of the blue (DAPI nuclear counter stain) and green (CD3) channels and provide a representative example for difference in density and distribution of CD3+ lymphocytes in CCH and CHHS.
B: Bar chart comparing tendency towards lymphocyte aggregation in lymphocytic infiltrate of CHHS and CCH.

Percentage of aggregating cells in CCH and CHHS are plotted. Bars represent 95% confidence intervals (CI). Dotted grey section represents percentage of lymphocytes which are found in confluent aggregates comprised of two cells or more. White sections represent percentage of lymphocytes which are found as individualized cell. CHHS show significantly lower tendency towards aggregation compared with CCH with less than 6% of the cells comprising confluent clusters (P<0.001). OR for clusters ≥1 cells in CCH vs. CHHS=4.366.

C: Expression of pZAP70 in CCH and CHHS

The images display an overlay of the blue (DAPI nuclear counter stain) and Magenta (pZAP70) channels.
D: Bar chart illustrating variation in expression density of CD3, GrzB and Perf in CCH and CHHS.

Mean values of CD3, GrzB and Perf immunopositive cells in LFOV are plotted and compared. Bars represent 95% confidence intervals (CI). Significant differences are indicated. Differential expression of CD3, GrzB and Perf is consistent across the two tumor types. Expression density of CD3 is significantly higher in CCH compared with CHHS. Expression of GrzB and Perf are numerically higher in CCH but are not statistically significant. Expression density of Perf exhibits exceptionally wide confidence intervals due to high internal variation of the data.
Figure 4-4: Confocal Images of CD3+ Cells forming synapse with tumor cells (Panel A) and CHHS (Panel B).
Figure 4-4: Confocal images of CD3+ cells forming synapse with tumor cells of CCH (Panel A) and CHHS (Panel B).

The images consist of stacks of 0.5-1 micron optical sections projected to 2D image displayed in three channels and a merged illustration which represents the overlay of all channels: DAPI nuclear staining (blue), CD3+ T cells (green), and E-cadherin or CD11d (red) for tumor cells of CCH (A) and CHHS (B) respectively. Scale bar =5 micron (DAPI channel). Synapsing contacts are defined as areas where co-localization of both markers occurs between two cells in a minimum of two 0.5 micron thick optical sections giving a bright yellow signal in the merged images (white arrows). A multi-plan lateral view of the perpendicular plane of the area of contact is also depicted to allow tracing the contingency of the co-localization signal. Three representative synapses are shown for each tumor type. Similar synapses were documented in all cases studied.

Figure 4-5: Distribution of Granzyme B (GrzB) immunopositive granules in cytotoxic lymphocytes (CTL) synapsing with CCH (A) and CHHS (B) tumor cells.

The images consist of stacks of 0.5-1.0 micron optical sections projected to 2D image displayed in three channels and a merged illustration which represents the overlay of all channels. DAPI nuclear staining (blue), GrzB (green), and E-cadherin or CD11d (red) for CCH (A) and CHHS (B) tumor cells respectively. Scale bar =5 micron (DAPI channel).

Images A and B show GrzB+ cells interacting with CCH and CHHS tumor cells respectively. A.1: All GrzB+ cells show tight polarization of immunoreactive granules towards CCH tumor cells. B: GrzB immunoreactive granules in CHHS are homogenously dispersed around the circumference of immunopositive lymphocytes. Images A.1 and B.1 capture multiple GrzB+ cells demonstrating the consistency of each pattern. Images A.2, B.2 and B.3 illustrates a higher magnification of two GrzB+ lymphocytes in close contact with CCH (A.2) and CHHS (B2&3) tumors cells; note the close interaction between GrzB+ cells and tumor cells in both tumor types.

Panel C: Association between nuclear morphology and granule distribution. C.1: Lymphocytes exhibit characteristic nuclear indentation toward CCH tumor cells while GrzB immunoreactive granules localized to these nuclear notches. C.2: Nuclei of CTL of CHHS lack similar indentation and GrzB+ granules are dispersed. Corresponding line profiles of pixel distribution further demonstrate the difference in distribution of GrzB immunoreactivity. Red arrows point towards nuclear notches in CCH lymphocytes in the images and their corresponding line profile illustrations.

D. Bar chart representing quantitative analysis of polarizing GrzB+ cells in 17 CCH and 7 CHHS tumors. The proportions of polarizing GrzB+ cells for CCH and CHHS are plotted. Bars demonstrating the variability and skewness of the results represent 95% confidence intervals (CI). Probability of polarization in CCH is 87% strikingly higher and significantly different (P<.0001) from CHHS in which the probability of polarization is 9%. Exceptionally high odds ratio (OR) of 69.79 (CI=17.86, 272.8) demonstrate the dramatic effect of tumor type on probability of polarization which is evident despite a relatively small sample size.
Figure 4-5: Distribution of Granzyme B (GrzB) immunopositive granules in cytotoxic lymphocytes (CTL) synapsing with CCH (A) and CHHS (B) tumor cells
C

1. DAPI
   - DAPI
   - DAPI/GrzB

2. DAPI
   - DAPI
   - DAPI/GrzB

D

Polarization of Cytotoxic Granules

- Polarized
- Diffuse

P<0.00001
OR= 69.79

CCH (n=17)  CHHS (n=7)
Probability of Polarization (%)
Figure 4-6: Examining externalization of the lysosomal membrane integral protein CD63, to assess degranulation in CCH and CHHS

Images represent multiple 0.5 micron sections projected in 2D images and exhibited in two different channels CD3+ T cells (green), CD63 (red) and a merged illustration. Scale bar= 5 microns (Green channel)

Panel A(1-3):

Translocation of CD63 immunoreactivity to outer cell membrane is indicative of degranulation of lysosomal granules in CCH TIL. CD63 externalization is confirmed by the consequent co-localization of CD63 (red) with membranous CD3 (green) which results in a bright yellow signal in the merged illustration. Panel 1 and 2 shows full CD63 externalization while panel 3 shows a more subtle appearance (white arrow). Note the aggregation of CD63 reactivity which interfaces the internal contour of the limiting cell membrane
Panel B (1, 2):

CTL in CHHS labeled with CD3 and CD63. CD63 reactivity (red) is evenly distributed in the cytoplasm of CD3+ cells and neither aggregation nor externalization is recognized.
Discussion
In the present study, a stringent disease classification scheme was applied to ensure consistency in diagnosis of CHHS. In dogs, different malignancies apart from CHHS manifest as splenic round cell tumors. These include splenic B, T or NK cell lymphoma and dendritic cell sarcoma (DCS), either as a localized splenic tumor or as part of a disseminated disease. Splenic round cell tumors may present similarly to CHHS both clinically and histopathologically. Moreover, these tumors often evoke local reactive hemophagocytosis in splenic macrophages which further makes distinction from CHHS difficult. From a hematological perspective CHHS can be mistaken with immune mediated hematological diseases like immune mediated hemolytic anemia (IMHA) and Evans syndrome. However, dogs with CHHS are negative on Coomb’s testing.

Similarly to FHL, the diagnosis of CHHS is a stepwise process which requires integration of clinical and laboratory data with cytology, histopathology and IHC findings. The histopathological hallmark of CHHS is massive diffuse splenic enlargement which is attributable to accumulation of CD11d+, erythrophagocytic macrophages within red pulp sinusoids. In the case series presented here, clinical data in combination with hematology, biochemistry and cytology pointed towards a diagnosis of CHHS later confirmed by histopathology. Overall, 5 of 8 cases selected for inclusion originated from a prospective pool collected over 4 years. Exclusion of cases from the prospective pool occurred only due to non-availability of tissue or incompatible breeds. Not a single case was excluded due to misclassification and all cases adequately met the inclusion criteria. Conversely, retrospective case collection spanning over a longer time period (6y) yielded only 3 suitable cases. Most exclusions from the retrospective pool were attributed either to failure to meet adequate
number of inclusion criteria or due to misclassification. This discrepancy between the prospective and retrospective pools reflects several issues regarding the customary diagnostic approach to CHHS. Dogs with CHHS are typically critically ill and present with a myriad of hematologic problems and coagulopathies. Consequently, clinicians often favor an initial minimally-invasive diagnostic approach which includes biochemistry, hematology, abdominal ultrasound and cytological assessment of spleen and/or bone marrow. As this set of data allows the diagnosis of CHHS to be made with a fair degree of confidence, owners often decline further diagnostics, such as histopathology. Consequently, availability of tissue for these cases largely depends on the owner’s consent to necropsy. Thus, a large portion of CHHS affected dogs may not be represented in archives and are therefore underrepresented in retrospective studies. Dogs which were initially diagnosed by histopathology often lacked the typical hematological and clinical presentation and may be erroneously classified. Lesions that need to be histologically distinguished from CHHS are splenic DCS and splenic granular lymphoma; both are large round cell tumors which often evoke local hemophagocytosis and may have similar clinical presentations. A major source of confusion is the use of CD18 immunoreactivity and detectable hemophagocytosis as key supporting criteria for CHHS diagnosis despite the fact that neither is specific for CHHS. Thus, although having been introduced in 2006, neither the discriminating value of CD11d and tumor cell distribution, nor the clinical and hematological data are routinely integrated with histopathology in the diagnosis of CHHS. Moreover, confusion between disseminated DCS and malignant histiocytosis adds to the misclassification of CHHS. Undoubtedly, the combination of careful histopathological evaluation with an appropriate IHC panel facilitates a better diagnosis of CHHS. The IHC panel should include CD3, CD79a, CD18, and
CD11d as previously recommended\textsuperscript{104}, with the addition of GrzB to ensure discrimination of splenic granular lymphoma from CHHS.

The HLH 04-based inclusion criteria are the basis for the FHL and CHHS comparison. Criteria 1 - 5 of the HLH04 are the most typical findings of FHL. These, criteria which are also routinely evaluated in the dogs proved to be key characteristics of CHHS. Hyperferritinemia has been recently introduced to the HLH 04 guideline\textsuperscript{7} and was recently suggested as a promising marker for CHHS\textsuperscript{58}. Ferritin is an acute phase protein secreted by hepatocytes, Kupffer cells and macrophages (particularly iron-laden macrophages)\textsuperscript{159, 163}. It is a sensitive yet non-specific marker of acute and chronic inflammation which is elevated in a wide range of inflammatory and neoplastic conditions in both people and dogs\textsuperscript{7, 159}. In children with FHL ferritin is secreted by excessively activated macrophages and reaches remarkably high levels which are unique to this condition\textsuperscript{7, 41, 70, 160}. In the present study evaluation of serum ferritin concentrations was limited to cases for which serum was available. Of those, all dogs (9 of 9) for which CHHS was confirmed (or suggested by cytology) exhibited strikingly high serum ferritin concentrations, markedly higher than those reported for other inflammatory and neoplastic conditions including imitators of CHHS such as DCS and IMHA\textsuperscript{58}. These results agree with the utility of ferritin as a valuable CHHS marker while further highlighting CHHS similarity to FHL. The value of ferritin as a biomarker for CHHS was most evident in dog 2. This dog was likely early in the disease process as suggested by subtle tumor infiltration showed markedly elevated ferritin level. Although a larger number of affected dogs and controls need to be tested, this observation suggests that ferritin may serve as a sensitive marker for early detection of CHHS.
Since CHHS and FHL are similar clinically and pathologically, the next step was to link these conditions on a mechanistic basis. The study hypothesis proposed that defects in lytic capacity of TIL of CHHS hamper immune regression of this tumor and evoke a systemic inflammatory response which dictates the clinical presentation and the lethality of this disease. To assess functionality of CHHS TIL, we evaluated critical steps of the immune reaction as manifested within the tumor microenvironment adopting a previously applied approach. Dysfunctionality of TIL is common, as many tumors utilize multiple mechanisms to escape anti-tumor immunity. The challenge was therefore to localize the defect in lytic capacity and seek some distinction between CTL dysfunctionality that is attributable to tumor evasion from that which reflect an intrinsic defect in host immunity, namely in GDK. The effective anti-tumor reaction of CCH provided a basis for comparison.

Analysis of density and distribution of the lymphocytic infiltrate was used to rule-out tumor barrier as the underlying cause for the ineffective TIL in CHHS. Collectively, these results indicate that lymphocytes in CHHS freely infiltrate the tumor and are effectively dispersed within the tumor parenchyma. Lymphocytes of CCH showed stronger tendency towards aggregation compared with CHHS. To rationalize this finding it is suggested that aggregation of lymphocytes in CCH may not represent failure to permeate the tumor but may reflect clonal expansion of activated effectors that occurs in CCH but is lacking in CHHS. This may represent a local, tumor-induced inhibition, which may also account for the significant difference in density of the infiltrate between the two tumor types. Alternatively, these results may reflect intrinsic effect of the host-tissue (skin vs. spleen) on the lymphocytic infiltration.
Despite much larger lymphocytic infiltrates in CCH, we confirmed dispersed permeation of lymphocytes in CHHS, permitting CTL/tumor cell interaction. Systematic examination of different components of this interaction revealed that lymphocytes from both tumor types form synapses with tumor cells and displayed intact TCR signalling to the level of ZAP70 phosphorylation. ZAP 70 is a tyrosine kinase which is associated with the zeta chain of the CD3 complex of the TCR. Phosphorylation of ZAP70 represents the downstream outcome of TCR engagement and confirms that the extracellular binding event has been translated into an intracellular response which resulted in T-cell activation.

When accounting for the significant differences in density of the lymphocytic reaction, there was no tumor effect on pZAP70 density suggesting that efficient TCR signaling is not significantly different between the two tumor types. The effect of CD3 density on pZAP70 density was significant but subtle likely since only the phosphorylated peptide was measured.

Detectable expression of Perf confirmed the finding of a normal Perf gene in CHHS-affected BMD. Moreover, expression of Perf and GrzB provided evidence for the activation and complete maturation of cytolytic effector lymphocytes. The lack of a significant effect of tumor type on expression of cytolytic enzymes was unexpected given the vast discrepancy in density of CD3+ infiltrate between the two tumor types. Contribution of activated NK cells in the splenic tumors (CHHS) could account for some superfluous expression of Perf and GrzB in CHHS but it could not likely account for all of it as the lymphocytic infiltrates of both tumor types were clearly dominated by T cells. The high number of cells expressing Perf and GrzB in a relatively modest lymphocytic infiltrate implies that high proportions of the CTL lymphocytes in CHHS exhibit a phenotype of fully differentiated, primed effectors. Excessive, yet ineffective activation of CTL is one of the
key proposed mechanisms for the development of FHL. The presence of abundant activated
CTL effectors in the dysfunctional TIL of CHHS further links CHHS to FHL on a
mechanistic basis.

The finding that cytotoxic granules in CTL in CHHS rarely polarize despite being
closely adjacent to tumor cells and the dramatic discrepancy in proportions of polarization
between CHHS and CCH are striking. We demonstrated that the sub-cellular events which
are conditional for granule polarization, namely TCR engagement and signalling, have been
met. Therefore, and given strong evidence for excessive CTL activation, we propose that
ineffective polarization of cytotoxic granules is a cause for the defective lytic capacity of TIL
in CHHS.

Assessment of de-granulation of CG was based on the fact that CD63 translocation to
cell surface and merge to the outer cell-membrane in the course of CG exocytosis\textsuperscript{94, 117, 162}. A
similar approach to assess leukocyte degranulation was applied by flow-cytometry
demonstrating externalization of lysosomal associated membrane proteins\textsuperscript{8, 19, 94, 95, 114}. De-
granulation events are short-lived and therefore seldom observed. Thus quantification of de-
granulation events was not feasible but qualitative assessment consistently revealed de-
granulation events in all of the studied CHH cases while none was detected in CHHS. It
was speculated that lack of de-granulation in CHHS is likely a downstream effect as granule
polarization is prerequisite for successful de-granulation. Failure of granule exocytosis is the
underlying cause for most of FHL variants.\textsuperscript{28, 39, 108, 173} Rab27 regulates the distal detachment
of lytic granule from microtubules after polarization.\textsuperscript{102, 173} In FHL type 4, lytic granules fail
to dock to plasma membrane due to absence of functional Rab27, and exocytosis is
hampered.\textsuperscript{65} In FHL types 3 and 5, deficiency in members of the Munc-family (Munc 13-4
and 18, respectively) results in failure of polarized cytotoxic granules to fuse to plasma cell membrane and leads to a similar consequence. Collectively, all of the recognized FHL-related defects in granule exocytosis are localized downstream in the pathway subsequent to granule polarization. However, the current spectrum of recognized mutations accounts for only 50% of the diagnosed FHL cases. Those FHL cases lacking an identified molecular defect but meeting the HAL 04 criteria, exhibit reduced NK activity and impaired degranulation of CG (recognized by reduced externalization of LAMP). Several genes have been linked specifically to regulation of CG polarization. Amongst those, protein kinase C delta (PKC delta) and adaptor protein 3 (AP-3) which exhibit specific and non-redundant association with CG polarization, are promising candidates for both CHHS and the unresolved FHL cases.

The findings in this study suggest that CHHS in BMD could be a reliable model of FHL in human. The BMD is a large working dog and a common pet worldwide. BMDs were initially bred at the end of the 18th century with a founder population of 6 registered sires. Presently, despite growing popularity, the breeding pool of the BMD remains exceedingly restricted and consanguinity is common.

BMDs are particularly susceptible to proliferative diseases of histiocytes including: CHHS, dendritic cell sarcoma (DSC) and systemic histiocytosis, a severe immune regulatory disorder of histiocytes. The BMD breed’s demography, small founding population, pedigree barriers, and extensive use of popular sires dictated a limited genetic pool in the current progeny. Genetic homogeneity of this breed mimics isolated human populations and thus offers an exceptional model for characterization of complex genetic diseases, especially those known to be associated with consanguinity.
The current model for FHL is a strain of Perf deficient mice \(^8\). These mice remain asymptomatic in a pathogen-free environment but develop a fatal FHL-like syndrome subsequent to viral infection. In concordance with Perf’s role in cancer immune surveillance, Perf knockout mice also show increased susceptibility to tumorigenesis \(^15\). However, the murine model does not provide a good clinical match to all variants of FHL. Therefore, further characterization of this unique, natural disease model may contribute immensely to the understanding of both the human and canine diseases and promote research bearing translational implications for both species.
GENERAL DISCUSSION

The work presented in this thesis focuses on canine histiocytic diseases from a comparative, mechanistic perspective; investigating possible associations between CHHS and an aggressive familial histiocytic disease in children. Specifically, it addresses the potential contribution of defective cell-mediated cytotoxicity to development of CHHS.

In children, certain histiocytic diseases have a genetic origin and share similar presenting features with CHHS. The underlying cause for these diseases in children is abnormal cytotoxic function attributed to mutations in either the Perf gene or other genes involved in the granule depended cytotoxic pathway that is associated with Perf processing and excretion.

The decision to initially focus on Perf rose from the non-redundant role of Perf in GDK and the frequent involvement of mutations in the Perf gene in development of FHL. The mRNA sequence of canine Perf, which was available in the NCBI database, was predicted from the canine genome sequence and was not a result of direct sequencing. Thus, preliminary sequencing of the canine Perf mRNA was warranted. The mRNA transcripts of human and murine Perf are comprised of three exons. The coding sequence in these species is confined to the second and third exons while the first exon consists of a transcribed yet untranslated sequence. The transcript of Perf in the dog was found to be shorter than the predicted sequence in the NCBI data but consistent with those in other species showing 79% sequence similarity to the coding exons of the human gene. Diligent effort to identify a putative transcribed first exon using RACE and sequence-based RT-PCR was unrewarding suggesting that the canine transcript only includes two coding exons (exons
2 and 3 of the predicted mRNA sequence) and not the far, upstream-located first exon suggested by the predicted NCBI sequence. Regions upstream of the Perf coding sequence are heterogeneous in length and sequence across species. The first non-coding exon of Perf shows considerable inter-species variation in length and sequence for the regions upstream of exon 2. Conversely, exons 2 and 3 are highly conserved across species. Thus, it was considered most likely that, in the dog as in some other species (equine and bovine), regions upstream to exon 2 are not transcribed. Sequence variations in the non-coding region of human Perf are neither reported nor associated with aberrant Perf expression and FHL development (Zur Stadt, et al. 2006). Thus, although the 5' un-translated regulatory sequence of canine Perf was not elucidated, the confirmed coding sequence was considered sufficient to address the initial study hypothesis.

Nucleotide sequencing of samples from CHHS-affected dogs and control dogs revealed nucleotide polymorphism in both study and control groups. Changes in the amino acid sequence of Perf were not associated with CHHS in dogs, but silent gene mutations occurred at significantly higher frequency in BMD with CHHS than in BMD without CHHS. The significance of this finding is not clear given the small sample size (n=10 sick BMD, n=6 healthy BMD) and should be further assessed in a larger cohort of sick and healthy BMD. One of the challenges was finding healthy BMD controls, and being unable to verify whether these dogs would develop histiocytic diseases in the future. The approach taken to minimize this problem was to include only older BMD as controls and limit the age of healthy controls to 5 years. Nonetheless, as only silent mutations were identified, a mechanistic association between these SNPs and development of CHHS was highly unlikely, thereby rejecting the initial working hypothesis.
The next set of experiments was initially designed to assess whether reduced or defective Perf expression contributes to CHHS. To investigate Perf expression in-situ, an antibody targeting a conserved region of the canine Perf was generated. Its specificity was assessed by immunoblot analysis and an IHC protocol was optimized to probe Perf in FFPE tissue. The initial working hypothesis for the second study objective was that lymphocytes infiltrating CHHS lack Perf expression. The assumption (which was later concluded to be imprecise) was that these lymphocytes are reflective of the host ability or failure to express Perf. Accordingly, the initial objective of the IHC experiments was to prove lack of Perf expression in CHHS specimens. Consequently, a prerequisite condition was to identify a reliable positive control for Perf expression, namely, a tissue in which Perf expression can be consistently and reliably demonstrated by IHC. Meticulous evaluation of Perf expression in different lymphoid-rich tissues showed that Perf was rarely and inconsistently detected in canine spleen and was repeatedly undetectable in all of the other evaluated lymphoid-rich tissues. Conversely, Perf expression was consistently detected in a minute fraction of TIL in regressing CCH tumors. The scarce and sporadic expression of Perf outlined by the preliminary IHC experiments hampered the initial study plan that aimed to prove lack expression in CHHS. Given the scarce and poorly characterized expression of Perf, it became clear that demonstrating lack of expression in CHHS specimens will have limited value to assess the host ability to express this protein.

The limitations described above lead us to modify the second objective from studying Perf expression in CHHS to a more general investigation of the nature, distribution and expression patterns of Perf. The uniquely consistent expression of Perf in TIL of CCH
stimulated specific interest in the successful anti-tumor immune response which facilitates the regression of CCH.

A set of novel observations reported in chapter 2 includes the wide range of Perf expression patterns and the striking variations in the pattern of GrzB and Perf immunoreactivity. Persistent co-localization of Perf and CD63 confirmed that despite highly heterogeneous expression patterns, the sub-cellular compartmentalization of Perf to secretory lysosomal granules is consistent. Perf expression has rarely been demonstrated in situ by IHC as it is typically studied by RT-PCR and flow cytometry. Less commonly, Perf expression is examined by cytochemistry of cells under in vitro conditions and subsequent to induced activation. Consequently, there are scarce references for comparison of Perf expression in-situ. The wide array of Perf staining patterns identified in this study likely results from the various physiological interactions which affect lymphocytes in their natural microenvironment. Variation in expression pattern of Perf and GrzB, and the striking discrepancy in colocalization of these effectors with CD63, reflect the difference in sub-organelle compartmentalization of Perf and GrzB and imply a possible difference in excretion mechanisms of these effectors.

CD63 has been associated with piecemeal degranulation (PMD), a secretory mechanism mediated by transport vesicles. PMD was proposed for the differential release of eosinophil granule-proteins independent of granule trafficking and polarization. According to this model, membrane bound secretory compartments coated with CD63 bud from the granule surface and migrate independently to merge with the cell surface. Furthermore, while in classical exocytosis granules release their entire content following
granule fusion; it has been shown that PMD allows preferential excretion of selected proteins, suggesting that a distinct cellular mechanism may be involved in mobilization of specific proteins.\textsuperscript{94, 101} PMD was never described or proposed in CL. Clearly, the self-directed, autonomous excretion, suggested by the PMD model is more appropriate for the volatile, nature of eosinophil excretion then to the explicitly targeted and highly regulated excretion of CL.\textsuperscript{74} However, when reviewing the present results from a purely morphological perspective, PMD should be considered. It is therefore possible, that in the dog, while GrzB excretion is restricted to granule exocytosis, Perf may migrate independently of the granule trafficking and polarization apparatuses as the cargo of membrane vesicles in PMD. The present study cannot provide sufficient data to support this proposal. A follow-up prospective study investigating subgranular protein fractionation of cells isolated from fresh tissue would test this hypothesis and unravel the mechanism behind these surprising morphological observations.

The characterization of TIL in regressing CCH outlines characteristic features of an effective anti-tumor immune reaction. This model could be utilized in the future to study the more frequent phenomenon of ineffective TIL. Specifically in the present work, TIL of CCH were compared to their CHHS counterparts. Accordingly, the third chapter presents detailed comparisons of TIL in CCH and CHHS specimens focusing on a spectrum of functional aspects of GDK which included (amongst others) the original question regarding Perf expression.

Hyper-reactive CD8+ cells play a critical role in the pathophysiology of FHL. Lymphocytes in FHL lesions not only fail to eliminate, but essentially evoke the expansion
of, hyper-reactive histiocytes and are the inducers of the clinical syndrome. Accordingly, when investigating TIL of CHHS, the question becomes more complex as the objective was to demonstrate both activation of CTL effectors and failure of GDK.

The results presented in the third chapter do indicate both excessive CTL activation and paralysis of the GDK apparatus. Lymphocytes in CHHS were seen interacting with tumor cells and forming what appeared to be a synaptic conjugates. This confirms that subcellular events, which are conditional for granule polarization, have occurred and points towards a defect in the mechanism of cytotoxic granule polarization. Expression of cytolytic enzymes was not significantly different between CCH and CHHS. However, given the significantly smaller number of lymphocytes in the CHHS infiltrate, expression of Perf and GrzB was considered excessive. As expression of GrzB and Perf was previously associated with CTL activation, excessive Perf and GrzB expression was interpreted as a reflection of CTL activation. In light of these findings, the stark difference in polarization of cytotoxic granules likely indicates an intrinsic defect in CTL rather than reflecting lymphocyte quiescence due to lack of tumor cell recognition.

In children, although the most common FHL variant (FHL2) is associated with mutations in the Perf gene, all of the other FHL variants and, consequently, the majority of FHL patients present with failure in granule exocytosis resulting from various underlying causes. From a clinical perspective, CHHS is less compatible with FHL2. It is characterized by a relatively later onset and, in association with general susceptibility to other cancers, both of which are more characteristic of FHL variants attributable to defective exocytosis (FHL 3-5).
Collectively, all of the recognized FHL-related defects in granule exocytosis are localized downstream in the pathway subsequent to granule polarization \(^{78,173}\). Yet, FHL patients designated currently as FHL1, lack identifiable molecular defects, but exhibit reduced NK activity and impaired degranulation of CG (recognized by reduced externalization of CD63) \(^{108,173}\). It is possible that the mechanism proposed in the BMD model corresponds to the unresolved mechanism underlying FHL1.

**Challenges in Experimental Design**

The rarity and the devastating nature of CHHS only permitted a research project largely based on archived tissues. This posed various challenges in experimental design. The decision to pursue this study, despite its limitations, stems from its potential ability to provide novel insights regarding the physiology of TIL within the intact microenvironment of naturally occurring tumors.

The scarce and inconsistent expression of Perf in spleen of healthy dogs hampered sequencing of protein extracted from the SDS-PAGE. Thus, despite reproducible appearance of a prominent band of the expected size on the blot, probing of Perf was not confirmed by sequencing. Given the IHC results, it is likely that similar processing of protein lysate of regressing CCH would have yielded a larger quantity of Perf which may have been sufficient for sequencing. However, although CCH is very common in veterinary practice, dogs with CCH are typically handled "in house" and are rarely referred to teaching hospitals. Thus, obtaining freshly frozen CCH tissue, as required for protein work, was not feasible despite diligent effort. The IHC experiments, which demonstrated simultaneous expression of Perf and CD3 and colocalization of Perf and CD63 as demonstrated by
confocal microscopy, provide further confidence regarding the specificity of RACP antibody. To ensure that the scarce expression of perforin is not a reflection of poor sensitivity of the RACP antibody, both IHC and western blotting were repeated with a commercial Perf antibody. This antibody which previously exhibited sensitive detection of perforin in feline granular lymphoma yielded similar results to the RACP.109

Given the high susceptibility of BMD to various diseases and the wide age range in which CHHS and other malignancies may develop in this breed, an additional challenge of this study was finding healthy BMD controls. More specifically, it was difficult to confirm that dogs which are considered healthy will not develop CHHS (or other types of cancer) later in life. The approach taken to minimize this problem was to include only older BMD (> 5 years) as healthy controls (given an average lifespan of BMD was recently reported to be 7 years2, 25, 111). This approach was practical but did not provide a perfect solution. For example, two cases which were initially chosen as healthy controls had to be retrospectively excluded subsequent to development of lymphoma and dendritic cell sarcoma. Also, although most CHHS-affected BMD were younger than 5 years of age, few cases presented at a relatively old age (up to 10 years) further emphasizing the problem in assigning BMD as "healthy" controls even at advanced age.

The best way to approach these challenges in the future may be to design a prospective study over a longer period of time such that a cohort of young and healthy BMD would be followed throughout their lives. Monitoring dogs as they age facilitates adequate sample collection prior to the onset of disease. Analysis of the data when the study is terminated will allow segregation of those characteristics associated with the development of disease.
Significance of the Results and Future Direction

The study herein provides initial data to suggest that cellular immune deficiency contributes to the development of CHHS and suggests BMD as a useful naturally occurring animal model of FHL. The current animal model for FHL is a strain of Perf knockout mice which require viral stimulation to develop a severe HLH-like syndrome \[14, 80, 82, 90, 150\]. The requirement for viral stimulation makes the mouse model far from ideal for studying primary HLH. Further, these mice mostly exhibit characteristics similar to the FHL 2 variant which is Perf dependent and may not fully represent the entire FHL spectrum. A spontaneous large animal model for HLH is not yet available hence the significant of the BMD model suggested in this study.

The significance of the study from a veterinary perspective is that for the first time a comprehensive mechanism is offered to link different canine histiocytic diseases on a mechanistic basis. Furthermore, a defect in GDK, with the potential consequence of decreased cellular immunity, suggests a common pathophysiological mechanism to the different hereditary conditions that devastate the BMD breed. These include familial susceptibility to immune regulatory disorders and malignant histiocytic diseases as well as predilection to various hematopoietic malignancies and Borreliosis.

From a mechanistic perspective, it possible that decreased cellular immunity accounts for other histiocytic diseases and for the multiple hereditary conditions which contribute to the high morbidity/mortality of BMD. Clearly, the data provided in this study suggest, but are insufficient to prove, this proposed mechanism. Follow up studies will need to focus on reproducing and expanding these findings on lymphocytic infiltrates of other
histiocytic diseases such as reactive histiocytosis and dendritic cell sarcoma. Another critical follow-up will be to assess lymphocytes away from the tumor microenvironment either by isolation from a fresh tumor or by investigation of peripheral blood lymphocytes. This will reduce the likely present tumor effect on these lymphocytes and allow more specific conclusions about the inherited deficiencies in the host (BMD).

To establish the novel observation reported in this study, future work will need to support and reproduce these findings by in vitro experiments. Such experiments which were hampered in the current study due to unavailability of fresh samples might be facilitated by prospective sample collection prior to disease development. Assuming a genetic background to CHHS such approach (sampling prior to development of a clinical diseases) should not have significant implications for the results. The alleged association between lack of granule polarization and defective cytotoxicity should be challenged by in vitro functional cytotoxic assays. Screening for potential target genes can be facilitated by RNA microarray and comparative proteomic profiling of lymphocytes in healthy and diseased BMD. Also indicated is sequencing of genes which exhibit specific and non-redundant association to CG polarization such as PKC delta and adaptor protein 3 (AP-3) as well as global testing of cellular immunity in BMD.
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