ABSTRACT

ROSS, PETER. Initial Studies for the Characterization of a Major Histocompatibility Complex Class I Restricted, Peptide-Specific Cytotoxic T Lymphocyte Response in Dogs. (Under the direction of Dr. Paul R. Hess.)

Classical Major Histocompatibility Complex (MHC) class I molecules bind short, cytosolic-origin peptides for scrutiny by T-cell receptors (TCR) on cytotoxic T lymphocytes (CTL), which aid in the control and elimination of intracellular pathogens. These MHC genes are highly polymorphic, with each allele having an inherent specificity to bind peptides with particular amino acid residues at certain positions. A diverse repertoire of CTL specific for particular peptide:MHC complexes (pMHC), is generated through random rearrangement, including nucleotide addition and deletion, of the TCR genes. When a TCR interacts with the pMHC for which it is specific for, the CTL is triggered to destroy the pMHC bearing cell. This specificity of both peptide binding to the MHC in combination with the TCR for particular pMHCs allows for CTL responses to be directed against cells in a peptide-specific and MHC allele restricted manner. In mice and humans, the study of peptide binding to the MHC and peptide-specific CTL responses have led to many advances in the study and treatment of infections, tumors, transplantation, and some autoimmune diseases. Unfortunately, little effort has been aimed at the characterization of these interactions in veterinary organisms, limiting the ability to develop and implement better treatments for diseases affecting these species.

The purpose of this body of work was to characterize the peptide binding specificity for an MHC allele in the dog allowing for the identification of the first peptide-specific CTL

response in this species. Previous studies suggest that the dog is relatively unique among vertebrate species in that the species may possess a single classical MHC class I locus, Dog Leukocyte Antigen-88 (DLA-88). Therefore, we sought to (1) identify DLA-88 allelic frequency within popular dog breeds, (2) determine the peptide binding motif for a prevalent allele allowing for the prediction of MHC binding peptides from pathogenic proteins, (3) create an assay to validate MHC association of peptides predicted to bind, and (4) identify a DLA-88-restricted, peptide-specific CTL response.

Using sequence-based typing, we established that DLA-88*50801 is a prevalent allele within the Golden Retriever breed. To identify the binding motif of this allele, bound peptides were eluted and sequenced by tandem mass spectrometry (MS/MS) revealing both conserved residues and variation in bound peptide length. *In silico* molecular modeling was utilized to confirm each of the conserved residues within the binding motif of DLA-88*50801: X-AVILFM-AVILFM-$X_{(5-8)}$-KR. Based upon previous work that had identified antigen-specific CTL responses directed against the whole hemagglutinin protein of canine distemper virus (CDV-HA), we predicted several CDV-HA derived peptides with theoretical capability to bind DLA-88*50801 in order to identify a peptide-specific CTL response. Using the DLA-88*50801 binding assay developed here, we demonstrate that motif-based prediction identifies genuine MHC binding peptides. Despite finding multiple CDV-HA peptides capable of binding this MHC allele, no CTL response, as measured by interferon-γ secretion, was detected among cells from a CDV immune dog cultured with peptide-pulsed target cells using any of these peptides. The inability to stimulate a DLA-88*50801 CDV-HA antigen-specific response may indicate that CDV-specific CTL responses restricted by

this allele are directed against another viral protein. While the ultimate objective of this project – defining the first anti-viral peptide-specific CTL response in the dog – was not achieved, this work has nonetheless resulted in the development of valuable and useful tools and information that should facilitate the antigen discovery process in the near furture. The identification of a viral peptide-specific CTL response will allow for better understanding of the canine anti-viral immune response which may ultimately lead to advances in veterinary care.

Initial Studies for the Characterization of a Major Histocompatibility Complex Class I Restricted, Peptide-Specific Cytotoxic T Lymphocyte Response in Dogs

by
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DEDICATION

To Duncan. My wonderful dog who kept me company while I lived alone for my first three years in Raleigh.

To my wife. I realize that I can be difficult at times and she ensures that my priorities are where they should be.

To Murphy Hess. The brave soul who was subjected to multiple blood draws for the sole purpose to complete this project.

BIOGRAPHY

Peter Ross was born in Temple, Texas but was predominantly raised in Fairmont, West Virginia where his parents Lou Ann and Michael Morais still live near. He has one older brother, Andrew, and one younger sister, Lindsey. Since his childhood, Peter has been involved in multiple sports from baseball, soccer, basketball, track and field, and football. His love of sports has only grown more intense as he has gotten older.

Peter graduated from East Fairmont High School in 2004 as one of the class valedictorians. For the majority of his life, Peter had wished to be a Veterinarian so he enrolled at nearby West Virginia University in the fall of 2004 to pursue a degree in Animal and Nutritional Sciences. He had never seriously thought of a career in industry until a graduate student taught biochemistry course during the spring of his junior year. This course was taught by explaining the seminal experiments that provided the evidence for the topics that were being discussed in class. He credits the teaching style of this graduate student for both exposing him to research science and igniting a desire to pursue a career in the field. Peter graduated from WVU in the spring of 2008 and enrolled in the Immunology graduate program that fall in Raleigh, NC. Peter spent most of his first year rotating through various labs but ultimately joined Dr. Paul Hess's lab in the spring of 2009 where his research focus was on the canine major histocompatibility complex class I molecule, Dog Leukocyte Antigen-88.

In May of 2011, Peter married his college sweetheart Laura whom he met while attending WVU. They currently live in Raleigh, NC with their two amazing dogs Duncan and Buffy.

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I thank my family and friends for supporting me with love and companionship during my graduate school career. I believe all of you played a major part in raising me. Lastly, I would like to thank my wonderful wife for her constant love and understanding.

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Chapter 1: Literature review and project outline

The primary function of $CD8^+$ cytotoxic T lymphocytes (CTL) is the elimination of intracellular pathogens such as viruses and some bacteria by T-cell receptor (TCR) recognition of antigenic peptides in associated with major histocompatibility complex (MHC) class I proteins on the target cell's surface (1, 2). The MHC molecule forms a heterotrimeric peptide:MHC complex (pMHC) composed of beta-2 microglobulin (β2m), the MHC heavy chain, and a short peptide fragment that could conceivably originate from any protein being produced within that cell (3). When the presented peptide is of non-self origin, the cell displaying the pMHC is targeted for destruction by the responding CTL. The study of the TCR:pMHC interaction responsible for CTL recognition has led to multiple advances in the study of tumor immunology, vaccination, and autoimmune diseases such as type I diabetes mellitus and multiple sclerosis (4-8). Unfortunately, the majority of studies have been confined to murine and human models and therefore the clinical benefits have also been restricted to these species. The present study was conducted to gain knowledge on canine pMHC complexes and identify specific CTL responses in the dog, in order to develop better veterinary therapeutics for treating cancer and infectious diseases. In order to better understand the importance of the pMHC interaction, this chapter will first discuss the key discoveries regarding the MHC in mice and humans followed by a synopsis of the current information in dogs.

Major histocompatibility complex

It has been suggested that the MHC genomic region has coevolved with the adaptive immune system in jawed fish (9). Many immunologically relevant genes are encoded in this region including the MHC class I and class II genes, the transporters associated with antigen processing (TAP), and proteasome subunits (10-14). The following sections will describe many important discoveries that helped to determine how these genes are involved in shaping and directing CTL activity.

When it was discovered that previously sensitized lymphoid cells were specifically cytotoxic for cells bearing the sensitizing antigen (15), questions remained about what target cell molecules were responsible for this recogntion. In 1974, Zinkernagel and Doherty discovered that CTL responses directed against lymphocytic choriomeningitis virus (LCMV) were restricted by MHC class I genes in an allospecific manner, that is, lysis was observed only when effector and target cells shared at least one MHC class I allele (16). These findings ultimately led to the Nobel Prize-winning "altered self" hypothesis which states that CTL continuously monitor the MHC class I proteins of self origin and only eliminate cells expressing versions of the molecule that have been structurally "altered" by infection (17). However substantial evidence supporting this theory was not available until 1978, when a study using murine chimeras to ascertain whether the host or T cell MHC genotype was the critical factor in restricting Vaccinia virus-specific responses. The results using bone marrow chimeras showed that T cells were educated to respond to infections only in the context of the MHC alleles that were expressed in the host's thymus (18). However, if donor splenic

cells, containing mature T cells, were used to generate the chimeras, T-cell recognition of both donor MHC alleles was maintained. Together, this information demonstrated that T cells are educated during their development in the thymus to recognize foreign antigens in the context of the "self" MHC molecules that are expressed during development.

If CTLs are educated to recognize self MHC molecules yet only respond against "altered" MHC molecules, the question remained – In what way are MHC class I molecules altered by infection? Using the influenza infection model, it was shown that when primed CTLs were incubated with target cells expressing a single viral protein, in this case nucleoprotein (NP) or hemagglutinin (HA), CTL lysis was still observed (19). The identification of NP specific lysis challenged the idea that CTLs recognized infected cells through recognition of intact foreign proteins at the cell surface, as NP is not a membrane protein and therefore would not be present in its native conformation at this site. Therefore, further research sought to determine how NP was recognized by the responding CTL. The answer came when a pair of studies by Townsend et al. discovered that a short peptide fragment of the NP protein is ultimately responsible for sensitizing targets for CTL lysis. The first of these studies utilized a series of plasmids encoding either full-length or truncated versions of the NP gene to transfect target cells for NP-specific CTL (20). Using this model, the same degree of specific lysis was obtained, whether target cell expressed whole antigen or a fragment of the gene. Furthermore, different fragments of NP were required for recognition by CTL restricted by different MHC alleles suggesting that each MHC allele was altered in a unique manner. The second study utilized synthetic peptides corresponding to

even shorter regions of the NP protein to sensitize target cells *in vitro* prior to co-incubation with NP-specific CTLs (21). The results indicated that clonal CTL responses were directed against short peptides, which were hypothesized to be the breakdown products of the intact protein, in the context of a single MHC class I allele. Ultimately, the H2-D^b restricted NP peptide found in this study would become the first immunodominant epitope discovered.

Following the discovery that CTLs possess dual specificity for self MHC and non-self peptide, great efforts were aimed at discovering the mechanism for antigenic peptide presentation for CTL recognition. Three findings led to the hypothesis that an intracellular protein degradation and processing pathway existed: 1) intact proteins could not sensitize target cells for lysis when added to the culture medium, 2) short peptides derived from the intact protein added to the culture medium could sensitize cells for lysis, and 3) when the whole protein was synthesized by the target cell, it could be targeted for lysis (22). Using inhibitors of a macromolecular complex known as the proteasome that degrades intracellular proteins (23), Rock et al. demonstrated that degradation of protein antigens was necessary for CTL recognition (24). In this study, cells were treated with proteasome inhibitors prior to electroporation with either intact ovalbumin (OVA) protein or an OVA-derived peptide capable of being recognized by CTL in the context of H2-D^b, SIINFEKL (25). When these cells were then used as targets for OVA-specific CTL, the cells electroporated with intact OVA were not susceptible to lysis while CTL were still capable of recognizing the SIINFEKL peptide bearing targets, indicating that protein breakdown is required for CTL recognition. Nevertheless, the molecular machinery required for transporting these peptides

from the cytosol to the cell membrane where CTL recognition occurs remained elusive until the discovery of the TAP genes (26). The encoded proteins from these genes form a heterodimeric complex that transport short peptides into the endoplasmic reticulum (ER) where peptide-free MHC class I molecules are present in close proximity (27). Once an MHC molecule has bound a suitable peptide, the MHC dissociates from the TAP complex and is exported to the cell surface for CTL scrutiny (28).

Classical and nonclassical MHC

In the previous section, the MHC class I molecules discussed were those of the classical or class Ia family. Classical MHC I genes 1) are expressed across a wide variety of tissue types, 2) are highly polymorphic, and 3) their products restrict conventional αβ TCR expressing $CD8^+$ T cells (29). If an MHC class I gene does not meet these criteria, it is designated as a nonclassical or class Ib gene.

The vast majority of nonclassical class I genes are actually pseudogenes incapable of producing functional protein as the result of frameshift mutations, premature stop codons, or other expression defects (30). Those nonclassical genes that are expressed appear to fulfill niche roles or serve as immunologic backup mechanisms allowing for the clearance of pathogens that attempt to evade the adaptive immune response. For example, the gene encoding the CD1 molecule is a nonclassical gene exhibiting limited polymorphism (31). The CD1 protein aids in the clearance of infection by binding and presenting lipid antigens to both invariant natural killer T cells (iNKT) and γδ TCR expressing T cells (32, 33). These T-

cell subsets are capable of producing cytokines and inducing apoptosis of infected cells very rapidly after TCR stimulation (33-35), thus participating in concert with the innate immune response to control infection while the adaptive immune response is readied for action (36).

Another class Ib molecule, human leukocyte antigen (HLA) –E, binds a restricted set of peptide epitopes derived from the leader peptides of the classical MHC molecules and acts as an inhibitory ligand for natural killer (NK) cells (37). The downregulation of class Ia molecules by certain pathogens as a method of immune evasion will concurrently result in reduction of HLA-E expression due to the loss of peptide ligand (38). The loss of HLA-E surface expression sensitizes the infected cell to NK lysis, thus serving as a backup mechanism for the elimination of infected cells (39).

The MHC class I-related molecules (MIC) -A and –B are nonclassical proteins involved with cell maintenance. These molecules are expressed at the cell surface without the association of β2m or bound peptide, are upregulated following cellular stress, and serve as activating receptors for γδ T cells (40). By this mechanism, stressed cells that may be more susceptible to infection can be eliminated prior to an actual infection occurring (41, 42). Additionally, MIC molecules play a role in tumors evading immunosurveillance, the normal process of the immune system that monitors and eliminates neoplasias throughout the body (43, 44). When MIC-A or –B are secreted by tumors as soluble proteins, the recognition receptors on effector cells are downregulated allowing the tumor to evade this arm of the immune system (45).

Although the classical MHC class I genes have received more attention over the years and are indispensible for adaptive immunity, expressed nonclassical MHC genes also play important roles. Typically, these genes are recognized by relatively invariant receptors, in comparison to αβ TCRs for classical MHC class I genes, and provide redundancy checkpoints that ensure proper immunity is established during the course of infection. These checkpoints may be necessary for a sufficiently protective adaptive immune response as many functional homologs have been found across mice and humans (46, 47).

CTL epitope prediction

Immunodominance

After the establishment of dual specificity for pMHC complexes by responding CTL, several studies suggested that CTL responses were directed against a surprisingly small set of peptides out of all theoretically possible peptides that could originate from a given pathogenic protein. Moreover, the recognized peptides were defined in an MHC class I allele-specific manner (48-50). A report in 1990 demonstrated that a single antigenic peptide determinant was responsible for essentially all of the CTL response directed against a pathogen. In this study, Van Bleek and Nathenson utilized ^{51}Cr release assays to measure the lytic capacity of polyclonal vesicular stomatitis virus (VSV) specific CTLs. They found that complete inhibition of lysis of VSV nucleocapsid protein expressing target cells could be achieved by the addition of cold targets labeled with a single VSV nucleocapsid protein

derived peptide (51). This phenomenon of CTL responses against a given pathogen being directed against a single or very few pathogen-derived peptide(s) is known as immunodominance.

There are many factors that contribute to immunodominance. The processing and presentation of peptides from whole proteins is a complex process involving many steps including protein degradation by the proteasome and transport of peptides into the ER by the TAP complex. By 1997, it was appreciated that these processes were involved in shaping immunodominance (52). However, determining all of the contributing factors as well as each factor's influence on restricting the immunodominant repertoire has proven to be more difficult. Some of the most informative data concerning this issue has come from studies using the Vaccinia virus (VV) model. VV genes theoretically encode for more than 115,000 peptides that are 9 or 10 amino acids (AA) long, the peptide length generally preferred for MHC binding, yet only 15 immunodominant epitopes have been discovered in the context of HLA-A*0201 – a reduction factor slightly less than 8,000 (53, 54). Assarsson et al. sought to determine the relative contribution of individual antigen processing and presentation processes that result in this drastic level of restriction for the VV CTL response (54). In order to eliminate the possibility that a particular pathogenic protein was not expressed in a sufficient quantity to elicit a CTL response, they reduced their analysis to include only 102 VV antigenic proteins, containing approximately 70,000 9- and 10- AA long peptides, for which CTL responses had been previously detected in the context of any MHC allele. As the CTL response is directed against a pMHC complex, the first objective of this study was to

determine the number of VV peptides capable of forming a suitable complex by incubating radiolabeled peptides with HLA-A*0201 molecules and measuring the amount of peptide that bound to the MHC. They found only about 2.5% - 3% of all possible peptides were capable of forming pMHC complexes. To determine the impact antigen processing had on restricting immunodominance, HLA-A*0201 transgenic (Tg) mice were immunized with VV derived, MHC binding peptides. Following peptide immunization, specific CTL responses were detectable against peptide-loaded target cells for approximately half of all binding peptides. However, when these peptide-specific CTL were tested for their ability to recognize VV infected cells, only about 15% remained capable of responding, suggesting that the remaining 85% of peptides recognized by CTL were not naturally processed. Together, these findings indicate that MHC binding, availability of a CTL with the appropriate TCR, and antigen processing all contribute to immunodominance. However, these factors do not fully explain the complete 8,000-fold restriction observed in VV suggesting that additional uncharacterized factors exist (54). A diagram showing the restrictive potential of each step in antigen processing and presentation for shaping immunodominance is provided in Figure 1.

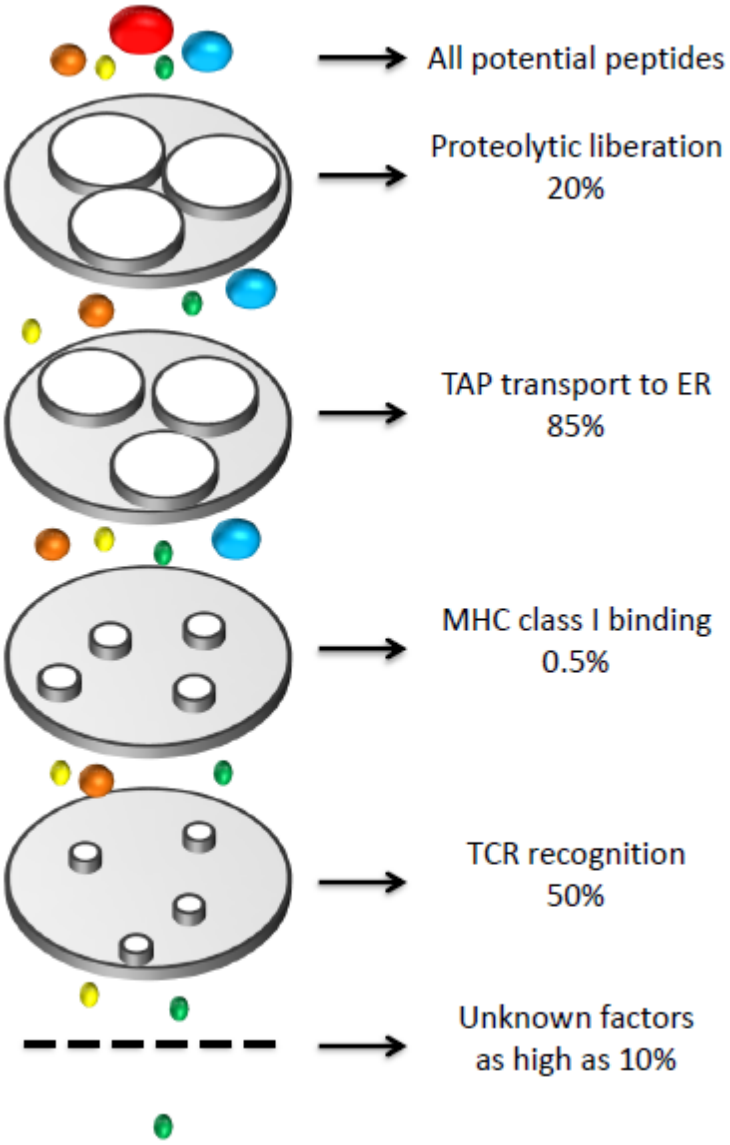

Figure 1
. Diagram of factors contributing to immunodominance. All potential foreign peptides from a pathogen go through a series of cellular processes that act as "filters" effectively narrowing the number of peptides recognized by CTL until only an immunodominant epitope(s) remains. Values accompanying each "filter" indicate the percentage of peptides capable of passing the current step out of the total available pool that have passed all previous steps. Diagram adapted from (55).

Determining what factors made several VV peptides subdominant epitopes, naturally processed peptides capable of eliciting a CTL response but not found during natural infection (56), was also studied as these epitopes can be protective in infections where the immunodominant epitope is absent or modified (57, 58). Factors such as peptide binding affinity for MHC, peptide off-rates from MHC, and TCR avidity for pMHC complexes were not found to have a significant correlation with immunodominant peptide selection (54). At this time, immunodominance cannot be fully explained as the factors that have been identified do not account for the complete level of restriction observed during the course of a natural infection; but, it is likely that immune regulation by other cellular subsets, such as T regulatory cells, plays a role (59).

Characteristics of peptide binding to MHC

To date, the largest single restriction element in immunodominance that has been identified is the ability for a peptide to bind the MHC (54, 55). This finding can be justified by taking into consideration the three-dimensional structure of MHC molecules. The MHC heavy chain forms three globular extracellular domains called α1, α2, and α3. The site for peptide binding, commonly called the "peptide binding pocket," is situated between the α1 and α2 domains and is essentially a groove comprised of two alpha helices on the sides with eight beta sheets forming the floor (60). Although MHC class I molecules are highly polymorphic, there are key binding characteristics within the peptide binding site that help give all classical MHC molecules an inherent specificity for binding peptides. First, there are

two clusters of residues responsible for binding the amino and carboxyl termini of the peptide backbone in a side chain-independent manner, termed the A and F pockets, respectively (61-63). In addition to the A and F pockets, some polymorphic residues of the MHC heavy chain have been shown to interact with the peptide backbone (64, 65). While the precise location of these internal peptide backbone interactions is variable between alleles, the common function is to increase the inherent specificity for peptides. Importantly, these interactions with the peptide backbone impart a polarity to the bound peptides ensuring that every peptide is bound in an amino to carboxyl orientation.

As mentioned previously, MHC class I is a highly polymorphic gene. Most of these polymorphisms are located within the α1 and α2 domains that form the peptide binding pocket. Moreover, many of the polymorphisms occur at positions within the molecule where the AA side chains are oriented towards the peptide binding pocket (63). This feature grants peptide specificity to different MHC alleles through the formation of the B, C, D, and E pockets (named sequentially between the A and F pockets) along the central part of the binding groove that are responsible for stabilizing the internal residues of the bound peptide (63). In contrast to the conserved binding of the A and F pockets, the polymorphism in the central pockets results in variable binding characteristics across different MHC allelic products. For example: a negatively charged pocket is created in HLA-A*6801 by a polymorphism at position 74 that is not present in HLA-A2 (66); polymorphisms at position 9 and 97 create a shallow, polar pocket in H2-D^b that is flat and hydrophobic in H2-K^b (67);

and a deep hydrophobic pocket with negatively charged residues residing in the bottom is found in HLA-B27 (68).

Allele-specific binding motifs

The peptide binding pocket allows for an inherent general specificity for peptides across all MHC class I molecules through the A and F pockets as well as allele-specific binding preferences through the polymorphisms found in the B, C, D, and E pockets. For instance, the hydrophobic pocket in HLA-B27 has been found to exclusively bind R residues at position 2 (P2) of the bound peptide (a designation of P# will henceforth indicate the residue found in sequential number from amino to carboxyl termini for the bound peptide) (68). However, many alleles do not restrict peptide binding to a single AA, but rather to a restricted set of peptides based on the chemical binding characteristics (hydrophobic, polar, charged, etc) or molecular size (69) of their side chains. When a position of the bound peptide sequence is found to be restricted by an allele, this position is called an "anchor", due to the preference for a specific AA at that position to facilitate MHC binding. All the anchor positions for a given allele are collectively called the binding motif. These motifs are highly allele-specific, as differences can be observed even between very similar alleles (70).

In order to maintain the ability to bind a diverse range of peptides for presentation to CTL, the binding motif for any MHC allele is not equally restrictive for all positions of the bound peptide sequence. In fact, many binding motifs have anchor sites near the termini of the bound peptide while the center of the peptide is generally allowed to vary in AA

composition (25). This variability is often accommodated by a kink or bulge conformation of the bound peptide to allow the backbone and sidechains of these residues to be oriented away from the MHC molecule (65, 68, 71). While this orientation facilitates MHC binding despite the variability of these residues, it also allows for direct recognition of these residues through the TCR of responding CTL. Studies have demonstrated that TCR recognition of pMHC complexes occurs in a highly peptide-specific manner as CTL recognition and function is abrogated when AA substitutions are made at non-anchor residues of known CTL epitopes (72, 73). This incredible peptide specificity of responding CTL can be explained by the manner in which the TCR binds the pMHC – forming stable interactions with both the bound peptide and the MHC molecule at the same time (74, 75).

CTL epitope prediction

As immunodominant epitopes recognized by CTL are dependent upon the MHC allele, these epitopes are shared between individuals expressing the same MHC allele allowing for the study of peptide-specific CTL responses in genetically similar animals or humans (76). The methods utilized in the discovery of the first immunodominant epitopes were extremely time consuming and laborious (discussed earlier in this chapter). Therefore, determining an effective method to quickly identify these epitopes for a variety of antigens and thus allow for the study of peptide-specific CTL responses against a broad array of pathogens has been an important objective. The prediction methods described to date are aimed at determining the peptides capable of fulfilling the requirements to pass one of the

restrictive factors contributing to immunodominance; however, there is currently no method designed to predict TCR availability (77).

Proteolytic liberation is one of the first molecular events that must occur for a peptide to be presented by class I molecules and contributes to immunodominance. Accordingly, a computer algorithm, called NetChop, has been developed to predict which peptides are liberated by the proteasome (78). One key advantage of this prediction method is the finding that the carboxyl termini of class I-bound peptides are generated directly by the proteasome (79). Unfortunately, the algorithm has not shown great sensitivity at predicting all proteasomal cleavage sites and has therefore not resulted in useful predictions of CTL epitopes as many potential epitopes are not identified (80, 81).

A second restrictive step in determining immunodominant epitopes is the ability of proteasome-liberated peptides to be transported into the ER by the TAP complex. Consequently, algorithms have been developed to predict CTL epitopes by determining peptides of the proper MHC binding length that are also capable of TAP transport (82). However, a major problem with this method is that many MHC binding peptides are subjected to amino terminal digestion post-ER transport (83, 84). Due to this finding, more recent attempts take into account the ability for amino terminus trimming in the ER by searching for TAP transportable peptides of all lengths (85). While these programs can accurately predict peptides that are transported by TAP, there are significant drawbacks to employing these methods for CTL epitope prediction. First, the singular impact of TAP transport on restricting immunodominance hierarchies is low in relation to other factors in

antigen processing (54). Second, employing these methods to predict CTL epitopes excludes peptides that may be processed through an alternative TAP-independent mechanism (86, 87). Therefore, TAP transport has not proven to be a useful method for CTL epitope prediction (88).

MHC class I binding constitutes the single most important immunodominance restriction factor (54). It is therefore not surprising that MHC binding motifs are the basis for a number of different CTL epitope prediction algorithms (89-92). As determining the binding motif of a given MHC allele is generally straightforward through the direct elution of bound peptides or peptide-binding assays (93, 94), applying this technique to a variety of MHC alleles is possible. Recently, more sophisticated motif-centric prediction techniques have been developed utilizing Artificial Neural Networks (ANN) (95). ANN methods utilize computer networks trained on large datasets of pMHC binding affinities to allow the prediction of MHC binding peptides from uncharacterized MHC protein sequences and have shown a remarkable ability to correctly predict binding peptides (96). The program NetMHCpan, a prediction method employing ANN, has been implemented to successfully predict not only MHC-binding peptides for alleles with unknown motifs, but also for MHC alleles from non-human, non-murine species (97).

Recent attempts have been made to better predict CTL epitopes by combining the predictive power of proteasomal liberation, TAP transport, and MHC binding into a single tool (98-101). While these methods have the ability to improve the predictive power over techniques that primarily employ MHC binding motifs in some situations, they suffer from

the same drawbacks as the TAP and proteasomal liberation algorithms. Until these combinatorial methods can be adequately verified, the best choice for predicting CTL epitopes remains the MHC binding motif centric algorithms (102).

Importantly, two principal problems impair the usefulness of using MHC binding motifs to predict pathogenic epitopes. The first is the false-positive rate or the rate at which peptides identified with the potential to bind the MHC are not genuinely capable of such binding. While this issue increases the temporal and financial strain of identifying an epitope by necessitating additional experiments to confirm peptide binding to the MHC (103, 104), the false-positive rate would not be exceptionally detrimental if the epitope was always included in the pool of peptides identified by these algorithms. However, using an MHC binding motif to predict binding peptides also results in false as some immunodominant epitopes do not match the defined binding motif. Since these non-canonical epitopes do not conform to the binding motif, a motif-centric prediction algorithm cannot reliably identify these peptides.

Non-canonical peptides

MHC-binding peptides that do not match the defined binding motif are called non-canonical peptides (105). Since their discovery, the ability of these peptides to be naturally processed and recognized by CTL has been confirmed (106-108). Often, non-canonical peptides are bound to the MHC through the stabilization of their side-chains in alternative binding pockets (107, 109) or avoiding the canonical peptide binding pockets all together

(108). Even though these peptides are typically of much lower affinity than their motif-matched counterparts (106, 110, 111), non-canonical peptides with high affinity have been described (109). It is likely that non-canonical peptides have a role in protective immunity as immunodominant CTL responses directed against these epitopes have been observed in some contexts (107, 112). While the frequency of non-canonical peptides bound to MHC class I molecules has not been accurately determined, the presence of such peptides likely contributes to a lower level of accuracy with which MHC binding prediction algorithms identify CTL epitopes (111).

Cytotoxic T cell mediated immunity in the dog

The ability to study peptide-specific CTL responses in dogs is limited by a lack of knowledge pertaining to the molecules involved in their function. This section will summarize the information that was available prior to performing the studies that are described in chapters 2-5.

The Dog Leukocyte Antigen complex

The genomic MHC region of dogs is called the Dog Leukocyte Antigen complex (DLA) and is located primarily on chromosome 12 (113). To date, six MHC class I-like loci have been described in this region while one other class I-like gene has been characterized that is encoded on chromosome 18 (114). Of the seven described class I loci, *DLA-12a*, *-53*, and *C1pg-26* are believed to be pseudogenes, as *-12a* and *-53* encode truncated proteins with

premature stop codons and *C1pg-26* contains multiple frameshift and insertion mutations that are atypical for class I genes, in addition to lacking an in-frame start codon (115). The four remaining genes, *DLA-12*, *DLA-64*, *DLA-79*, and *DLA-88*, all contain appropriate genetic elements that would allow for normal transcriptional/translational events to occur (115). Furthermore, mRNA transcripts have been found for these four genes using healthy canine peripheral blood lymphocytes (PBL). This has been accomplished through reverse transcriptase polymerase chain reaction (RT-PCR), in the case of *DLA-12*, *-64*, and *-88* (115), or ribonuclease protection assays, in the case of *DLA-79* (114). These findings indicate that there are at least four transcribed MHC class I genes in dogs.

As discussed earlier, there can be a wide array of functional differences between classical and nonclassical MHC class I genes. Therefore, an important goal following the identification of class I genes in the dog is the categorization of these genes as classical or nonclassical. For an MHC class I gene to be designated as classical it must be polymorphic, expressed in a wide variety of tissue types, and restrict conventional αβ T-cell responses (29). The expressed DLA gene for which the most definitive classification can be made is *DLA-79*. Although this gene is found to be expressed in a wide array of tissues, *DLA-79* also exhibits uncharacteristically low sequence homology to other class I genes, is encoded on a different chromosome than the genomic DLA region, and shows limited polymorphisms (114). With this information, *DLA-79* has been designated as a nonclassical MHC class I gene in dogs. The other expressed canine MHC class I loci, *DLA-12*, *-64*, and *-88*, cannot be as accurately assigned as classical or nonclassical genes at this time. A study in 1998 analyzed the relative

level of polymorphism each locus exhibits and presents the most informative data for the tentative designation of these loci (116). To perform this analysis, blood samples were taken from a panel of unrelated, mixed breed dogs to maximize the likelihood of identifying polymorphisms. Following genomic deoxyribonucleic acid (DNA) isolation, locus specific PCR was performed to amplify the α1 and α2 domains encoding the peptide binding pocket (where the most polymorphisms would be expected to occur) followed by cloning and sequencing of those amplimers. This revealed a very low level of polymorphism for both *DLA-12* (2 alleles from 18 individuals) and *DLA-64* (3 alleles from 20 individuals) while *DLA-88* exhibited a substantially higher level of polymorphism (29 alleles from 25 individuals) (116). Moreover, *DLA-88* heterozygosity was high (16 heterozygotes, 9 homozygotes). Based upon their polymorphism, *DLA-12* and *-64* have been categorized as nonclassical genes while *DLA-88* possesses the highest likelihood of being a classical MHC class I gene (116). Further analyses of *DLA-88* have revealed a total of 51 alleles originating from > 205 unrelated dogs (116-119).

Antigen-specific T-cell responses in dogs

The current state of studying specific T-cell responses in dogs is still at the whole antigen level. Several studies have successfully identified antigens recognized by both MHC class I restricted CTL and MHC class II restricted T helper cells in dogs. The few studies that have attempted to define class II peptide epitopes have done so by analyzing responses against pools of overlapping peptides comprising the entire open reading frame (ORF) of the

antigenic protein (120-123). At this time, this technique has not yet been used to determine an MHC class I restricted response. Using overlapping peptides to find peptide epitopes is costly to perform, but there is currently no other option available as no reliable prediction algorithm exists for use in dogs due, at least in part, to there being no known MHC binding motifs for any DLA molecule. A summary of the antigens for which a specific T-cell response has been characterized in dogs and the method of detection used to identify that response is provided in Table 1.

Table 1 Pathogens for which an antigen-specific T-cell response has been identified in dogs. Detection method abbreviations: CTL – cytotoxic assay, CE – cytokine expression, P – proliferation, DTH – delayed type hypersensitivity. ND, not determined

Pathogen	Antigen	Detection Method	MHC Restriction	Reference(s)
Canine Distemper Virus	Whole virus	CTL	ND	(124)
	Hemagglutinin	CE, CTL	ND	(125, 126)
	Fusion protein	P	ND	(123)
Canine Oral Papillomavirus	Whole virus	CE, P, DTH	ND	(122)
Adenoassociated Virus	Capsid protein	CE	ND	(120)
Canine Parvovirus	VP_2	P	II	(121)
	VP_1	P	II	(121)
Leishmania infantum	Whole pathogen	P, CE, DTH, CTL	ND	(127-131)
	LACK protein	P, CE	I and II	(132)

Project rationale and outline

As previously stated, the principal goal of this project was to characterize pMHC interactions in order to identify a peptide-specific CTL response in the dog. Unfortunately, the knowledge in the veterinary literature regarding canine MHC class I molecules did not provide the necessary information to pursue this goal directly, including such basic information as the frequency of class I alleles or means to predict peptide-MHC binding. Therefore, this project started out by studying the canine MHC prior to attempting to identify a CTL response at the minimal epitope level.

In order to study epitope-specific CTL responses across individuals, the CTL must be restricted by a common, shared MHC allele. Consequently, the most useful MHC allele to study in an outbred population, such as the dog, would be present in a large proportion of

individuals. In fact, one of the most highly studied MHC alleles in humans, *HLA-A2*, has a high prevalence worldwide (77). Although current data suggests that *DLA-88* may be the only classical MHC class I gene in dogs, no estimates of allelic prevalence have been made. Therefore, the first objective of this study was to identify a prevalent *DLA-88* allele and thus determine a candidate allele for further study. Dog breeds are subpopulations where allelic variability would be expected to show higher levels of restriction than the general population due to the inbreeding that occurred during breed creation. Analogous studies of allelic diversity at MHC class II loci have shown that this is indeed the case (133-135). Hence, the first objective was to determine the *DLA-88* allelic prevalence in several breeds, and ideally, identify a dominant allele. For this study, we chose the popular Golden Retriever and Boxer breeds.

Once a prevalent allele had been identified, the second objective was to identify the peptide binding motif, which would allow for the first predictions of MHC binding-peptides in dogs. The ability to predict such peptides would reduce the temporal and financial strain involved with finding a CTL epitope, as fewer peptides would require testing.

However as discussed earlier, motif-centric prediction algorithms suffer from a high false-positive rate. This shortcoming has been largely overcome in mice and humans through the development of assays that validate peptide-MHC binding, ensuring that only genuine MHC-binding peptides are tested for CTL reactivity (136-139). Therefore, the third objective of this study was to develop a cell-based tool to validate peptide-MHC binding of synthetic peptides to the prevalent *DLA-88* allele.

Lastly, the final objective was to identify a CTL epitope in the dog. To accomplish this, the peptide binding motif was used to predict peptides from canine distemper virus (CDV) with a high likelihood to bind the prevalent *DLA-88* allele. Following their prediction, the cell-based validation tool was used to confirm that the predicted peptides were, in fact, capable of MHC binding. Finally, PBL from a dog vaccinated against CDV and possessing the allele of interest were tested for CTL reactivity against the CDV peptides capable of MHC binding. If a single (or very few) CDV peptides capable of eliciting a CTL response can be identified, these will represent the first *DLA-88* restricted epitope(s) defined in the species. An outline of the project objectives and the chapters in which they will be discussed is provided in Figure 2.

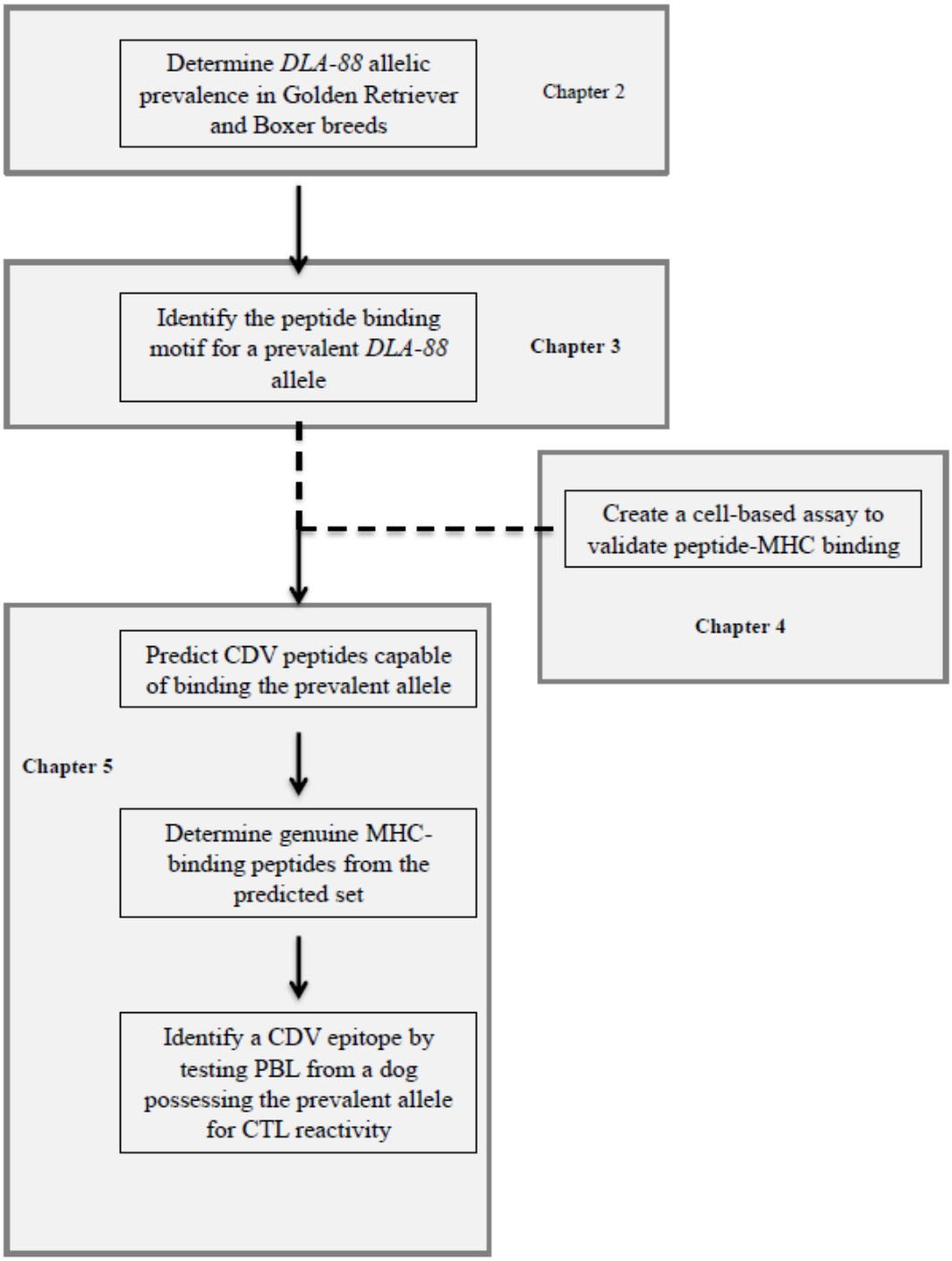

Figure 2 Diagram of the study objectives. Chapters where each objective will be discussed are noted.

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Chapter 2: Allelic diversity at the DLA-88 locus in Golden Retriever and Boxer breeds is limited

Rationale

Determining an individual's genotype for a polymorphic gene can be accomplished through several means. Sequence specific oligonucleotide probes can be used for the detection of alleles of known sequence (1). Single-stranded conformational polymorphism (SSCP) allows for alleles to be visualized on an agarose gel due to differences in migration patterns caused by intrastrand conformations formed by single-stranded DNA (2). Importantly, SSCP has a limited ability to identify single base pair substitutions and cannot unambiguously identify all sequence differences (3). In the case of *DLA-88*, many alleles differ in sequence by as little as a single base pair (4). Furthermore, the library of *DLA-88* alleles is likely not yet complete, meaning that the identification of new alleles is probable and therefore our techniques must ensure that these novel sequences are genuinely obtained from the *DLA-88* locus. For these reasons, we chose to employ sequence-based typing techniques. The major drawback of this method is the error rate of the DNA polymerase utilized during PCR amplification leading to incorrect sequence data (5); however, this concern has largely been eliminated with the availability of proofreading DNA polymerases. Using this method, we aim to characterize the prevalence of *DLA-88* alleles within two popular dog breeds as well as report any unknown alleles in these individuals.

Contributions

I performed all of the experiments and data analysis for this chapter unless otherwise specified in the following: Shannon entropy analysis was performed by Ben Vincent (co-author), the Swiss Model was generated by Ed Collins (co-author), Supplemental Figure 1 was generated by Paul Hess (co-author), and I was guided by Jeff Thorne in conducting the positive residue selection analysis.

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BRIEF COMMUNICATION

Allelic diversity at the *DLA-88* locus in Golden Retriever and Boxer breeds is limited

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Abstract

In the dog, previous analyses of major histocompatibility complex class I genes suggest a single polymorphic locus, dog leukocyte antigen *(DLA)-88*. While 51 alleles have been reported, estimates of prevalence have not been made. We hypothesized that, within a breed, *DLA-88* diversity would be restricted, and one or more dominant alleles could be identified. Accordingly, we determined allele usage in 47 Golden Retrievers and 39 Boxers. In each population, 10 alleles were found; 4 were shared. Seven novel alleles were identified. *DLA-88*05101* and **50801* predominated in Golden Retrievers, while most Boxers carried **03401*. In these breeds, *DLA-88* polymorphisms are limited and largely non-overlapping. The finding of highly prevalent alleles fulfills an important prerequisite for studying canine CD8+ T-cell responses.

Classical major histocompatibility complex (MHC) class I molecules are widely expressed, cell surface glycoproteins that regulate the development and function of CD8+ T-cell responses through the presentation of cytosolic-origin peptide epitopes. In part, these class I molecules are distinguished from their non-classical counterparts by extensive polymorphisms. In humans, for example, there are >1500 alleles at the human leukocyte antigen *(HLA)-A* locus, >2000 alleles at the *HLA-B* locus and >1000 alleles at the *HLA-C* locus (1). Allelic variation, which arises primarily by intralocus recombination or point mutation, is frequently concentrated in hypervariable regions (HVRs), where residues of the heavy chain α1 and α2 domains contact the bound peptide (2). Such variation can have important consequences. In a murine model, an H-2K allele with differences only in the peptide-binding determinants was associated with increased diversity of the corresponding cytotoxic T-cell repertoire and enhanced resistance to viral infection (3). Selection pressure from pathogens can act on allelic variation to maintain the polymorphism of classical MHC molecules (4). Not surprisingly, restricted MHC diversity can have devastating effects on population survival, as illustrated by the particularly dramatic example of transmissible facial tumors of Tasmanian devils (5).

In the domestic dog, seven class I loci have been identified in the dog leukocyte antigen (DLA) complex, and of these, four genes are transcribed: *DLA-12, -64, -88* (all on chromosome 12) and *-79* (on chromosome 18) (6, 7). The *DLA-88* locus appears to be the most polymorphic, with 51 published alleles identified from > 205 unrelated dogs (8–11). Variability at the other loci appears much more limited, with two, three and four alleles described to date for *DLA-12, -64* and *-79*, respectively, from samples obtained from 18–20 dogs (8).

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While there have been no large scale DLA class I sequencing efforts published so far, it is reasonable to estimate that the overall pool of diversity in dogs will be much smaller than in humans, given the potentially low number of founders, and the recency of domestication, which is too short an evolutionary time to permit the creation of new alleles in any significant number (12). With some estimations of as few as 50 to 100 founding wolves (13), the total number of class I alleles at the *DLA-88* locus may be no greater than several hundreds in the domesticated dog population. This supposition is consistent with class II data, with a total of only 100 reported alleles at the most variable locus, *DLA-DRB1,* obtained from >1600 dogs (14).

The polymorphisms of MHC molecules constitute a barrier to allotransplantation and also represent a substantial obstacle for studying antigen-specific T-cell responses across unrelated individuals. Advances in understanding cytotoxic CD8+ T-cell activity in autoimmune, infectious and neoplastic canine diseases, as well as with immunization or transplantation, could be expedited by defining frequently occurring class I alleles in subpopulations of dogs. Breeds are an obvious choice for performing such analyses, as one would expect allelic diversity to be low, due to the limited number of foundation stock as well as the use of inbreeding and overutilization of popular sires. Moreover, the easily recognized physical characteristics that define breed members could be a convenient means of identifying dogs that have a potentially high likelihood of sharing alleles or haplotypes. Indeed, intrabreed variation at several canine class II loci is quite limited (15–17). To date, however, no assessment of the prevalence of canine MHC class I alleles has been performed, while in humans, such information is readily available (18). In this investigation, we hypothesized that, within a breed, *DLA-88* polymorphisms would be restricted, and one or a few alleles would dominate the locus. To examine this prediction, we sought to determine allelic variation in cohorts of dogs from two popular breeds, Golden Retrievers and Boxers, using reverse transcription-polymerase chain reaction (RT-PCR) and sequence-based typing.

For the study, venous blood samples were obtained from unrelated adult dogs ($n = 65$) admitted to the North Carolina State University (NCSU) Veterinary Teaching Hospital (VTH), from volunteer donors ($n = 24$) recruited through the Clinical Studies Core of the NCSU Center for Comparative Medicine and Translational Medicine or from Beagles ($n = 2$) from the NCSU Laboratory Animal Resources unit. The blood collection protocol was approved by the NCSU Institutional Animal Care and Use Committee. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation ($400g \times 30$ min at room temperature) over a Histopaque 1.077 continuous density gradient (Sigma-Aldrich, St. Louis, MO). From lysates of PBMCs or the canine histiocytic cell line DH82 (ATCC CRL-10389), RNA was purified using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized by RT using an oligo$(dT)_{15}$ primer.

Primers for the *DLA-88* gene were synthesized by Invitrogen (Carlsbad, CA): exon 1 forward 5′-CGGAGATGGAGGT GGTGA-3′ and exon 4 reverse 5′-GGTGGCGGGTCACACG-3′. Amplification of cDNA templates was performed using a high fidelity Advantage-HF 2 PCR Kit (Clontech, Mountain View, CA) on a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) programmed with the following cycling conditions: initial denaturation/hot start at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 20 *s*, annealing at 62°C for 30 s, extension at 68°C for 1 min and 15 *s*, succeeded by a final elongation step at 68°C for 5 min. All experiments included a negative control sample in which water was substituted for cDNA. Reaction products were separated by electrophoresis on a 1% agarose gel, excised, purified and ligated overnight at 4°C into a pGEM-T Easy Vector (Promega, Madison, WI). Transformed colonies of chemically competent *Escherichia coli* (GC10; Genesee Scientific, San Diego, CA) were selected by blue/white screening, and plasmids were purified by alkaline lysis (QIAprep Spin Miniprep Kit; Qiagen). Plasmids positive by *Eco*RI restriction digest were sequenced in both directions by Eurofins MWG Operon (Huntsville, AL) or Eton Bioscience Inc. (San Diego, CA), using T7 and SP6 primers. The obtained sequences were compared to all previously reported alleles of *DLA-12*, *-64*, *-79* and *-88* (8–11) using A plasmid Editor (ApE) software (19). For dogs with a single allele, a minimum of six colonies were sequenced; for heterozygotes, a minimum of two colonies were sequenced, if the alleles had been described previously. Sequences of novel alleles were determined from a minimum of six colonies, which were obtained from two or more different animals whenever possible, to insure that the observed allele was not due to PCR or sequencing error. Names for new alleles were assigned according to published conventions of the Comparative MHC Nomenclature Committee (20) by the curator (L. J. Kennedy) of the canine immunopolymorphism (IPD)-MHC database.

We designed primers to anneal to exons 1 and 4 of the DLA class I heavy chain to provide full-length sequence data for exons 2 and 3, which encode the α1 and α2 domains that contain the antigen recognition site (ARS) for the T-cell receptor. In genotyping *HLA-A*, *B* and *C* alleles, nucleotide sequences from exons 2 and 3 can unambiguously assign allelic identity in virtually all (>99.9%) cases (2), and accordingly, we used this approach in our study. Because of the high degree of homology between exons of the four transcribed MHC class I loci, it was not possible to produce individually specific forward and reverse primers for *DLA-88* amplification from cDNA. To circumvent this difficulty, the forward primer was designed to anneal to the *DLA-64* and *-88* loci, while the reverse primer was designed to anneal to the *DLA-12* and *-88* loci. To confirm the ability of the

primer pair to exclusively amplify transcripts from the *DLA-88* locus, we first tested cDNA from the canine cell line DH82, from which only a single *DLA-88* allele had been found previously (21); although unnamed, our analysis suggested that this sequence most likely represented *DLA-88*50801* (with 2 bp substitutions). Cloning of the ~650 bp amplimer yielded two sequences: *DLA-88*50801* and a putative new allele (now *DLA-88*05101*). Because the locus of origin of the second allele could not be definitively established by reference to *DLA-88* sequences in the canine IPD-MHC database, we repeated our evaluation of the primer set using cDNA from PBMCs isolated from a Beagle. As most reported alleles of *DLA-88* have been obtained from Beagles or Beagle mixes (9, 11, 22), the likelihood of identifying another novel *DLA-88* allele was presumed to be low. Indeed, only the previously reported allele *DLA-88*50201* was sequenced in 23 colonies from this dog, indicating that amplification from the other class I loci – *DLA-12*, *-64* and *-79* – was highly unlikely with our set of primers. To provide further support for this conclusion, we then amplified cDNAs from four additional Beagles; again, only established *DLA-88* alleles were identified in these analyses (*DLA-88*00401*, **00501*, **00601*, **50101* and **50201*).

We then sought to determine whether frequently shared alleles of *DLA-88* could be identified within a breed. To accomplish this objective, we compared nucleotide sequences for exons 2 and 3 from 47 Golden Retrievers. From this population, a total of 10 alleles were obtained; the relative frequencies are shown in Table 1. Four novel alleles – *DLA-88*01202*, **02101*, **03301* and **05101* –were found (Table 2). The most prevalent allele was *DLA-88*50801*. Together with *DLA-88*05101*, these two variants constituted 58% of the total allelic pool. When the frequency of occurrence was calculated on a per-dog basis, *DLA-88*05101* was the most commonly encountered allele (Table 1, right column); the difference in prevalence between these two measures is attributable to the greater number of homozygotes among dogs possessing *DLA-88*50801* ($n = 11$) than those with *DLA-88*05101* ($n = 4$). Of the Golden Retrievers carrying the remaining eight alleles, only four homozygotes were identified. Not surprisingly, the DH82 cell line, which was derived from a Golden Retriever in Ohio in the mid 1980s (23), possessed the two most common alleles of the breed.

To demonstrate that the finding of a few dominant class I alleles was not peculiar to Golden Retrievers, we performed the same analysis with another breed, the Boxer. Among these dogs ($n = 39$), 10 *DLA-88* alleles were again found; of these, only 4 were also common to Golden Retrievers (Table 1, bold face alleles). *DLA-88*03401* constituted 50% of alleles in the total pool (Table 1, middle column) and was present in 82% of Boxers (right column). Seven dogs were homozygous for *DLA-88*03401*, while for all other nine alleles, only seven homozygote animals were identified. Four of the alleles found in Boxers are new: *DLA-88*02803*, **02901*, **03201* and **05101* (Table 2).

Table 1 Prevalence of *DLA-88* alleles by breed

Golden Retriever ($n = 47$)		
Allele	Allelic frequency[a]	Phenotypic frequency[b]
00201	1.1	2.2
00501	9.6	17.4
00601	*1.1*	*2.2*
01202	5.3	8.7
02101	6.4	13
03301	2.1	4.4
03801	13.8	26.1
05101	27.7	47.8
50101	1.1	2.2
50801	31.9	40.4
Boxer ($n = 39$)		
Allele	Allelic Frequency	Phenotypic Frequency
00501	1.2	2.6
01201	4.7	10.3
02801	1.2	2.6
02803	12.9	34.4
02901	12.9	34.4
03201	2.4	2.6
03401	50.6	82.1
05101	2.4	5.1
50101	2.4	2.6
50801	9.4	17.9

DLA, dog leukocyte antigen. Alleles common to both breeds are shown in bold type.
[a]Numbers indicate percentage. Homozygote dogs were considered to possess two copies of the allele.
[b]The percentage of dogs with the indicated allele.

Interestingly, during our analysis of Boxers, we observed that one dog appeared to have three DLA-88 alleles. To rule out contamination as a possible source of this finding, RNA was isolated from a second blood sample from this dog; genotyping again revealed the same three alleles. Ultimately, 7 of the 39 Boxers (five males, two females) were observed to possess three different alleles. To our knowledge, none of these dogs had received a blood transfusion. Maternal

Table 2 Novel *DLA-88* alleles in the study populations

Alleles	Number of clones	Number of dogs	Breed	GenBank accession
05101	101	24	B; GR	HQ340121
02803	29	11	B	HQ340113
02901	23	11	B	HQ340112
02101	18	6	GR	HQ340114
01202	17	4	GR	HQ340115
03301	8	2	GR	HQ340117
03201	8	1	B	HQ340116

[a]B, Boxer; DLA, dog leukocyte antigen; GR, Golden Retriever.

microchimerism has been documented to result in the recovery of non-inherited HLA alleles from the PBMCs of healthy adults (24); however, this seems an unlikely cause, given the phenomenon was confined to Boxer dogs, and all of the individuals had the identical triplet combination: *DLA-88*02803*, **02901* and **03401* [note: in subsequent work, we have observed a few other combinations, but these always have contained either *DLA-88*02803* or **02901* or both (P. Ross, unpublished observations). Therefore, we also considered the possibility that our primer pair had amplified sequences from other class I loci. Given that *DLA-12*, *-64* and *-79* are minimally polymorphic, one would predict such off-target amplification to occur in a majority of the dogs, however, not simply in a small subset of one breed. Moreover, sequence analysis strongly supports *DLA-88* as the locus of origin for the two novel alleles, **02803* and **02901* (**03401* has been previously established), as pairwise identity of exons 2 and 3 of either allele with *DLA-88*00101* is 97%, but only 82% with *DLA-64* and 76% with *DLA-79*. The *DLA-12* and *-88* loci have far greater homology of exons 2 and 3, making this comparison much less useful; however, exon 1 sequences (encoding the leader peptide) are substantially disparate (6). Accordingly, we analysed exon 1 sequences from **02803* and **02901* and found 100% identity with *DLA-88*. Nor do any of the three alleles appear to be pseudogenes, as all had open reading frames when sequenced through the transmembrane domain (data not shown). It should be stated that other investigators also have found three *DLA-88* alleles specifically in dogs of the Boxer breed (Kennedy, personal communication, University of Manchester, Manchester, UK). Additionally, dogs possessing three *DQB1* alleles (with an analogous breed bias – Samoyeds) have been reported (14). For both loci – *DLA-88* and *-DQB1* – it is likely that this phenomenon is due to gene duplication, which has been found in the MHC of other species, such as the horse and cow (25, 26).

The alignment of the predicted amino acid sequences of the α1 and α2 domains of the seven novel alleles identified in Golden Retrievers and Boxers is shown in Figure 1. As expected, most of the amino acid variability is found in the defined HVRs (2). Outside of the HVRs, all amino acid differences identified in the new sequences, with the exceptions of pro 43, arg 50, glu 61 and glu 62 in *DLA-88*05101*, have been observed in at least one other allele. Of the 19 α1 and α2 residues in the β sheet of *HLA-A2* that interact with β-2 microglobulin (27), 16 are conserved across these canine alleles (Figure S1, *Supporting Information*). Similarly, all nine amino acids in the peptide-binding region that are conserved in human and mouse classical class I molecules (27) are present, as are the cysteine residues that form the disulfide linkage between the α2 helix (cys 164) and the floor of the binding groove (cys 101) (28). Additionally, all of these new alleles have exon 1 sequences that align with the *DLA-88* locus (data not shown). It is also worthy of note that, during this investigation, three additional sequences that were putative *DLA-88* alleles were found (Table S1, *Supporting Information*); however, we were unable to obtain a sufficient number of colonies to validate these sequences.

To investigate the relationship of the new alleles with those that have been previously reported (8–11), we created a phylogenetic tree of all nucleotide sequences (Figure 2), analogous to such analyses of class I alleles in other species (29–31). As expected, the new alleles (marked by arrows) followed branch points that led to tight clustering with other known *DLA-88* sequences. Further, these alleles are interspersed throughout the tree, consistent with the known creation histories for these relatively young (mid-to-late 19th century) breeds from disparate founders (wavy-coated Retriever, Tweed Water Spaniel, Irish setter, Bloodhound and the St. John's Water Dog for the Golden Retriever; the Bullenbeisser and the English bulldog for the Boxer) (32, 33).

Across species, a common characteristic of classical MHC class I molecules is the finding of positive selection of amino acid sites in the ARS (30, 31, 34, 35). We therefore evaluated all 58 *DLA-88* nucleotide sequences – the 7 alleles from this study and the 51 alleles previously described – for evidence of positive selection using Bayesian analysis. Five codons in the α1/α2 domains with a mean probability > 95% of being positively selected were identified: 73, 95, 114, 152 and 156. To assess the potential biologic relevance of these findings, we used the crystal structure of *HLA-A*1101* to generate a three-dimensional model of one highly prevalent canine allele, *DLA-88*03401*, which is depicted in Figure 3. From this modelling, it can be seen that all of the positively selected sites are predicted to occur in the ARS (specifically, the peptide-binding groove) of this class I molecule.

Bottlenecks in population size – in this case, due to breed founding some 50 to 75 generations ago – result in the loss of genetic variation at polymorphic loci, which is seen as decreased allele numbers and heterozygosity (36). For example, marked restrictions in class I polymorphisms due to selective breeding are evident in miniature swine (30). To compare the allelic variation present in our sample groups, we used the Shannon entropy as an index of diversity. The Shannon entropy is a member of the family of valid diversity indices that equally rates species richness and clonal dominance (37). Entropy was estimated with correction for unseen species and inference of confidence intervals (CI), as previously described by our group (38), which allows the statistical significance of entropy comparisons to be evaluated. Allelic diversity was not statistically different between Golden Retrievers (1.78, 95% CI 1.60–1.96) and Boxers (1.75, 95% CI 1.52–1.98); both populations were under-sampled for comprehensive discovery of all possible alleles (data not shown), as determined by the method of Egeland and Salas (39). Although the total number of alleles in the populations is unknown, this value can be estimated from the observed allele distributions. When this computation is performed using

Figure 1 Alignment of the predicted amino acid sequences of exons 2 and 3 (marked by red bars) for seven novel dog leukocyte antigen (*DLA*)-*88* alleles found in the study populations. Residues in light grey indicate identities with the reference sequence *DLA-88*00101* (top), while those in dark grey indicate differences. The three hypervariable regions are shown by the blue bars. For the naming of alleles, sequences with nucleotide differences that resulted in amino acid substitutions within any of the HVRs were designated as a new major type, (indicated by the first three digits), while sequences in which substitutions were confined to areas outside the HVRs were considered novel subtypes and numbered accordingly (last two digits).

a nonparametric estimator (40), as implemented in the software package SPADE (41), the calculated number of *DLA-88* alleles (and 95% CI) in the Golden Retriever and Boxer populations is 11.5 (10.1–25.1) and 10.3 (10.0–14.8), respectively. These values represent a level of allelic diversity at this classical MHC class I locus far less than that of humans and suggest that our experiment was successful in recovering the major alleles from each population.

The finding of limited intrabreed diversity at the *DLA-88* locus is not unexpected, as previous investigations of MHC class II allelic variation show analogous restrictiveness. For example, at the *DLA-DRB1* locus, only eight alleles were found in 31 Golden Retrievers, with three alleles (*DLA-DRB1*01201*, **01501* and **02001*) being dominant (14). Similarly, five *DLA-DRB1* alleles were observed in 41 Boxers; 71% shared the *DLA-DRB1*00401* allele. The magnitude of *DLA-88* restriction likely varies with breed. That Boxers have a single, dominant allele – *DLA-88*03401* – while Golden Retrievers do not is presumably the result of a less severe bottleneck in the latter breed (42). Therefore, breeds with very few founders, such as the Basenji, Doberman or Irish wolfhound, would be expected to carry few alleles. On the other hand, Beagles appear to have more class I variability. When the prevalence data from the 5 dogs in this study is combined with sequence-based typed Beagles in the literature ($n = 6$) (22) and ($n = 2$) (10), 10 different alleles (*DLA-88*00101*, **00401*, **00402*, **00501*, **00601*, **01602*, **04501*, **50101*, **50201* and **50801*) are observed in just 13 dogs. Interestingly, when diversity is calculated for the Beagle population, the CI of the entropy estimation crosses that of the Golden Retriever and Boxer populations, although the interval is quite wide. This observation might be explained by increased species richness (more alleles) in Beagles in the setting of less equally distributed alleles, but additional work would be necessary to confirm this hypothesis. As a second measure of decreased intrabreed genetic variation at the *DLA-88* locus, we also observed high levels of homozygosity in the sample dogs: 38.3% in Golden Retrievers; 35.9% in Boxers. While it is possible that our primer set failed to amplify some alleles, causing an overestimation of homozygosity, this seems less likely, as none of the 91 dogs in this study failed to return a *DLA-88* sequence. Moreover, our data are consistent with the range of MHC homozygosity (33–40%) measured at the *DLA-DRB1*, *-DQA1* and *-DQB1* loci in several studies (14, 15).

As one might anticipate, interbreed variability for *DLA-88* was high. Thus, of the 10 *DLA-88* alleles identified in each breed, only four were shared, reflecting the different founders and genetic separation of these breeds. Moreover, the frequency distribution of alleles was dissimilar. For example, the two most commonly occurring variants in Golden Retrievers, *DLA-88*05101* and **50801*, had a prevalence of only 9.4% and 2.4%, respectively, in Boxers, while *DLA-88*03401* was not observed in any Golden Retriever, despite being found in 82% of Boxers. Additionally, neither breed possessed two of the alleles that were present in our small sample of Beagles (*DLA-88*00402* and **50201*). These data suggest that the discovery of novel *DLA-88* alleles could be expedited by genotyping dogs from different breeds, particularly by obtaining samples across

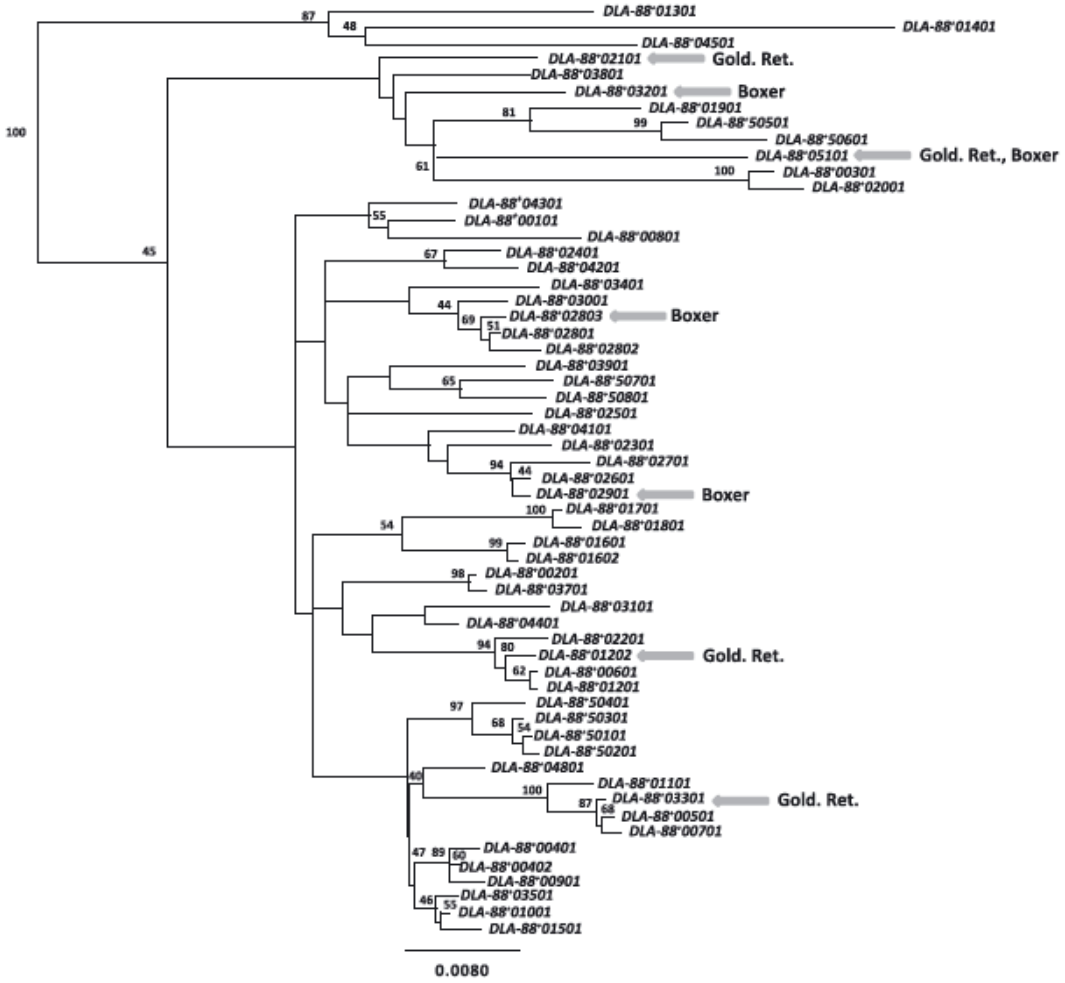

Figure 2 Phylogram of relationships between all known dog leukocyte antigen (*DLA*)-*88* alleles. The seven new alleles identified in Golden Retrievers (GR) and Boxers (B) are indicated by the arrows. The tree was constructed with Geneious v.5.1 (52) from the concatenated nucleotide sequences of exons 2 and 3 on the basis of Tamura-Nei genetic distances (53), using the neighbor-joining method (54) and was rooted by the inclusion of *HLA-A*0101* (GenBank AJ278305) as an outgroup. The numbers to the left of the internodes are the percentage of supporting bootstrap ($n = 1000$) replications; for clarity, only values ≥40 are shown. The units for the scale bar are the number of nucleotide substitutions per site.

genetic clusters (43), as has been done for canine class II (14).

The small sets of *DLA-88* polymorphisms found in comparatively large cohorts of dogs allowed us to estimate the potential total number of alleles in these breed populations, which, as described above, appears unlikely to exceed 25 in Golden Retrievers and 15 in Boxers. An important caveat to this conclusion is that many (but not all) of the animals evaluated in this study came from a relatively restricted geographic location in the southeastern United States. In humans, it is well established that for polymorphic genes, such as HLA class I and II, the prevalence of alleles can vary widely by region (18). Hence, by sampling from a limited area, we may have underestimated allelic diversity. Comparisons of variability at class II loci between European and North American dogs of the same breeds do reveal similar allele prevalence and dominant species (15, 16), but a few non-shared alleles are also observed. Thus, genotyping Golden Retrievers and Boxers from other regions ultimately will be useful to corroborate our findings. Nor could we strictly verify that all

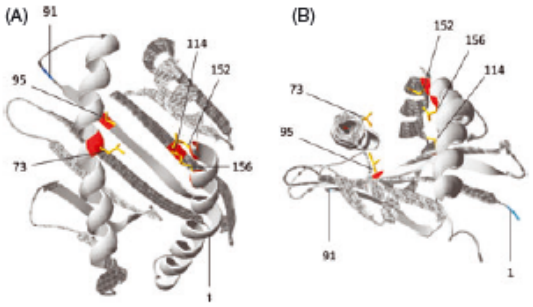

Figure 3 Schematic representation of dog leukocyte antigen (*DLA*)-*88*03401* (α1 and α2 domains) showing positively selected amino acid sites. Nucleotide sequences of exons 2 and 3 from all *DLA-88* alleles were aligned by codons and analysed in two parallel runs using the software program MrBayes 3.1.2 (55). In each run, 1.0×10^6 cycles were performed. Posterior probabilities for each amino acid site were analysed using the Tracer 1.5 program (56); codons were presumed to be under positive selection when posterior probability values exceeded 0.95. The burn-in value was set to the first 10% of cycles for each run. The proposed model is based on the high-resolution crystal structure of *HLA-A*1101* (57) and was generated using SWISS-MODEL (58–60). Views show onto (A) and along (B) the peptide-binding groove of the heavy chain (grey). Positively selected sites are indicated in red, with side chains shown in yellow. The first residues of the α1 domain (1) and α2 domain (91) are shown in teal.

dogs were unrelated. The majority of samples, however, were obtained from the NCSU-VTH, which is a large (>20,000 accessions per year) veterinary referral center for North Carolina, Virginia, South Carolina and Tennessee, and therefore, most dogs were very unlikely to be related. For Boxer samples obtained from a regional breed association ($n = 9$), for whom pedigrees were available, none of the dogs had a common sire or dam in >4 generations. Conversely, while all dogs in this study appeared to be purebred by visual inspection (performed by one of the authors, PRH), we did not verify lineage in all individuals, and undocumented outbreeding could have led to an overestimation of class I diversity.

The restricted intrabreed diversity at the *DLA-88* locus found in this investigation suggests that it may be practical to study antigen-restricted CD8+ T-cell responses by screening dogs drawn from these easily recognizable subsets for highly prevalent allele usage. There are also well-documented associations of breeds with predispositions to particular diseases, including those modulated or mediated by the immune system – infectious diseases, cancer and autoimmunity – and ultimately, it may be possible to correlate specific *DLA-88* variants with susceptibility or resistance, as has been accomplished with canine class II alleles (44–50). Lastly, peripheral hematopoietic stem cell transplantation is becoming an increasingly utilized treatment for canine lymphoma (51), and the limited diversity within breeds will undoubtedly facilitate haplotype matching to advance the use of allogeneic donors for this therapy.

Conflict of interest

The authors have declared no conflicting interests.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Conservation of amino acids in the α1 and α2 domains between *HLA-A2* (top sequence; GenBank accession AAB16923.1) and seven novel *DLA-88* alleles in the β-2 microglobulin (b2m) and peptide-binding (pb) sites, and at two conserved cysteine (cc) residues. Light grey residues indicate identities, while those in dark grey indicate differences from the human reference sequence.

Table S1. Putative *DLA-88* alleles.

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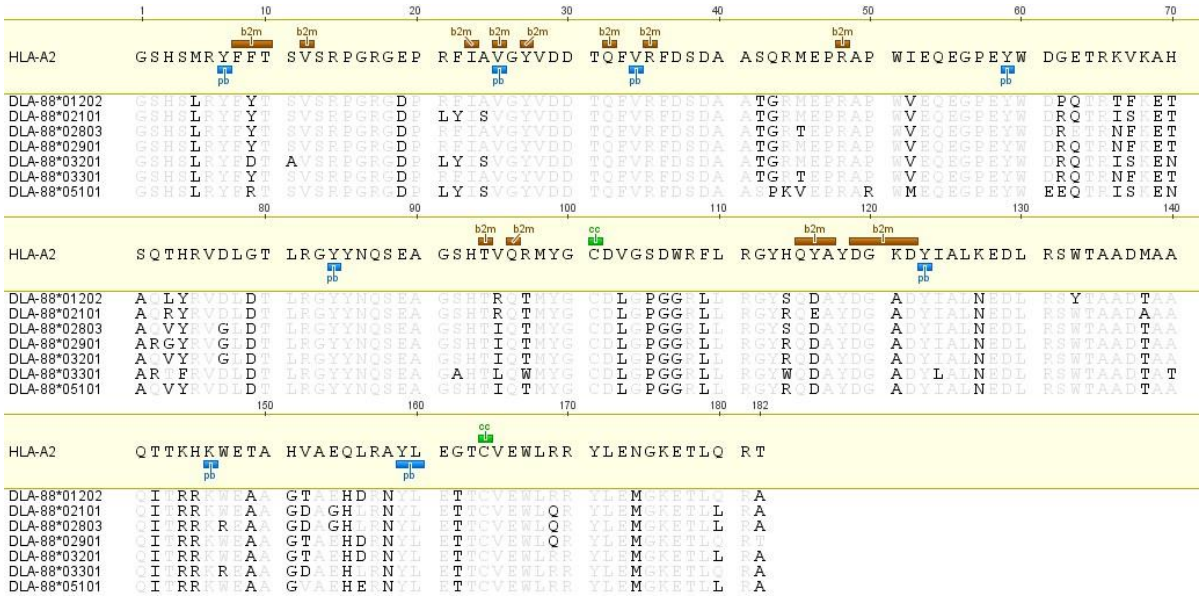

Conservation of amino acids in the a1 and a2 domains between *HLA-A2* (top sequence; GenBank accession AAB16923.1) and seven novel *DLA-88* alleles in the b-2 microglobulin (b2m) and peptide binding (pb) sites, and at two conserved cysteine (cc) residues. Light gray residues indicate identities, while those in dark gray indicate differences from the human reference sequence.

Supplemental Figure 1

Supplemental Figure 1

Supplemental Table 1 Putative *DLA-88* alleles[a]. [a]The H, J, and L sequences had a very high percentage pairwise identity with *DLA-88*00101*: 96.4, 97.1 and 97.3%, respectively (the mean percentage pairwise identity across all 58 alleles is 95.7%). None contained stop codons, and all maintained conservation of cys 104 and cys 164, and the □-2 microglobulin and peptide binding site residues. Leader sequences (exon 1) aligned with *DLA-88*. Sequences H and J were obtained from two different dogs with malignancies that were not included in the study populations. Sequence L was similar to *DLA-88*01601* (4 bp differences) and **01602* (5 bp differences). This putative allele was found in four Golden Retrievers; however, despite sequencing 57 clones from these dogs, only 4 clones could be obtained.
[b]B, Boxer; GR, Golden Retriever

Sequence	Number of clones	Number of dogs	Breed[b]	GenBank accession
H	2	1	B	HQ340119
J	1	1	GR	HQ340120
L	4	4	GR	HQ340122

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Chapter 3: The canine MHC class I allele DLA-88*50801 presents variable length peptides with a conserved binding motif
(Submitted to Journal of Immunology)

Running Title: Determining the binding motif of DLA-88*50801

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Keywords: Cytotoxic T cells

Antigens/Peptides/Epitopes

MHC

Rationale

Determining MHC binding motifs has traditionally been accomplished by sequencing the pool of bound peptides using a technique known as Edman degradation (1-3). This technique identifies the frequency with which particular amino acids occur at each position of the peptide sequences (4). More recently, tandem mass spectrometry has been utilized to identify the binding motifs of MHC molecules (5-8). The advantages of this technique over Edman degradation is that individual peptide sequences are returned, allowing for a more precise analysis to be performed. Additionally, if multiple length peptides are bound by the MHC being analyzed, residue conservations towards the carboxy terminus of the peptides, which may not have been observed with Edman degradation, can be detected (9). For example: if both 9- and 10-mer peptides are bound by a given MHC allele and the carboxyl terminus is restricted to D residues, Edman degradation will not accurately report all of the D residues at the terminus but rather their presence at both P9 and P10, possibly leading to incorrect conclusions to be drawn. Consequently, we utilized tandem mass spectrometry to conduct our analysis of the peptide binding motif for DLA-88*50801.

Contribution

I performed all of the experiments and data analysis for this chapter unless otherwise specified in the following: the Swiss Models were generated by Ed Collins (co-author), the *in vitro* pMHC complexes and circular dichroism experiments were conducted with substantial aid from Keith Miller (co-author), and I was guided by Jeff Thorne in conducting the statistical analysis.

Abstract

$CD8^+$ T cells are important for immunosurveillance of malignant and infected cells. Greater understanding of the role of $CD8^+$ T cells in controlling disease could be elucidated from large animal models, such as the dog, but MHC class I peptide binding specificities are unknown, precluding such studies. Therefore, we sought to determine the peptide binding characteristics of the prevalent canine MHC class I allele DLA-88*50801. Peptides eluted from affinity-purified MHC complexes were subjected to liquid-chromatography and tandem mass spectrometry to determine sequences, revealing 9 – 12 amino acid long peptides, with a bimodal distribution of lengths. Sequence alignment and *in silico* modeling of peptide:MHC complexes revealed peptide binding preferences at positions P2, P3, and P9 as *HLA-A*6801*. Recombinant DLA-88*50801 complexes were thermodynamically stabilized when folded with motif-matched peptides. These results define the first canine class I peptide binding motif, opening the door for the study of specific $CD8^+$ T-cell responses in dogs.

Introduction

Clinical applications of immunotherapy are an active area of investigation (10, 11). In some settings, immune control of tumors can lead to an elimination or equilibrium state, which is critically mediated, in part, by $CD8^+$ T cells (12) that recognize the presentation of peptide antigens by MHC class I molecules. Determination of tumor epitopes that elicit a specific $CD8^+$ T cell response has led to clinically successful immunotherapy, such as peptide vaccines and adoptive tumor-infiltrating lymphocyte therapy in haplotype-restricted patients (13, 14). Unfortunately, developing such therapies from murine tumor immunology studies, which are based on tumors arising from carcinogen injection, implantation, or transgenic expression of oncogenes, is problematic since these models are not readily translatable to human medicine. Hence, there is an increasing call for the use of large animal models with spontaneously developing tumors (15). The dog is particularly attractive, as many cancers affecting dogs also affect humans (16). However, use of this model is limited as little is known about MHC class I in the species. To date, evidence suggests that the dog is relatively unique among vertebrate species in that only one highly polymorphic locus exists, *Dog Leukocyte Antigen-88* (*DLA-88*).

The Golden Retriever breed has incredible potential for studying T-cell responses to spontaneous tumors due to its popularity and high lifetime risk of developing cancer (17). We have recently reported on two class I alleles in this breed that should have adequate prevalence ($\geq$40%) to allow for the identification of immunodominant $CD8^+$ T-cell responses

(18). In this study, we have determined the peptide binding motif of DLA-88*50801, a prevalent allele within Golden Retrievers, which may pave the way for epitope prediction and testing, and ultimately, the study of specific anti-tumor $CD8^+$ T-cell responses in the dog.

Materials and Methods

Preparation of plasmid constructs

To create a C-terminal FLAG-labeled *DLA-88*50801* construct, the nucleotide sequence of *DLA-88*50801*, from the translational start codon through position 350 glutamine (genbank accession #JQ733514), was first determined using DLA88-F and DLA88-R primers (Table 1) with DH82 cDNA template. This PCR-derived amplimer was ligated into a modified pcDNA3 vector that had a FLAG-epitope sequence (5'-GATTACAAGGATGACGACGATAAGTAA-3') directly downstream of the NotI restriction enzyme site in the multiple cloning region (gift from Jeff Yoder, NCSU). The *DLA-88*50801* coding sequence from the start of exon 2 through position 278 proline was codon optimized to minimize negative regulatory elements and generated with a ribosome binding site and Shine-Delgarno sequence by direct DNA synthesis (Genscript), and ligated into the pLM1 vector for bacterial expression (19).

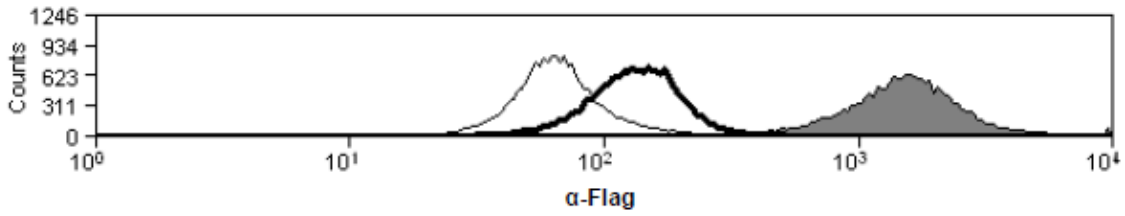
Table 1 PCR primers used in this study

Primer	Sequence 5'-3'
DLA88-F	GGAATTCCGGAGATGGAGGTGGTGA
DLA88-R	ATTAAGCGGCCGCCCTGGGCACTGTCATCGCGT
B2m-F1	CCAGTCTCCGGCGCTCTGC
B2m-R1	ACTGGCCCAGATAGGTCAAGCA
B2m-F2	CGGGATCCAGGAGGAATTTAAAATGGTGCAGCATCCCCCA
B2m-R2	CCCAAGCTTCTATTATCAGTTGTCTCGGTCCCA

Canine beta-2-microglobulin (β2m) (genbank accession #JQ733515) was cloned and sequenced (MWG Operon) using DH82 cDNA as PCR template and B2m-F1 and B2m-R1 primers (Table 1). Further amplification with B2m-F2 and B2m-R2 primers removed the leader peptide for bacterial protein expression as had been done previously for human β2m expression (20) and observed in mature canine β2m protein (21). This amplimer was ligated into the pLM1 vector for bacterial expression (19).

*Generation of a stable cell line expressing a C-terminal FLAG labeled DLA-88*50801 molecule*

The *DLA-88*50801*-FLAG construct was transfected into DH82 cells (22) (ATCC CRL-10389) utilizing Lipofectamine 2000 (Invitrogen) which underwent G418 selection (400µg/ml) one day post-transfection. Clones were isolated by limiting dilution and screened for intracellular FLAG expression (M2; Sigma) by flow cytometry after cell permeabilization. Clone 9-15 was used throughout this study (Supplemental Fig. 1).



Supplemental Figure 1. Generation of a stable clone expressing *DLA-88*50801* with a C-terminal FLAG tag. Intracellular FLAG expression was assessed by flow cytometry using α-FLAG antibody (M2; Sigma). Thin line – DH82 unstained; thick line – DH82 stained; shaded – 9-15 clone stained. Experiment was performed in duplicate

Cell surface DLA-88 protein isolation and Western blot

Cell surface proteins were isolated from DH82 and 9-15 cells by surface biotinylation using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific) according to the manufacturer's protocol. The background signal of the assay was established using unbiotinylated 9-15 cells. Samples were analyzed via Western blotting using α-FLAG antibody (M2; Sigma) followed by staining with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

*Isolation of DLA-88*50801 bound peptides*

DLA-88*50801 bound peptides were isolated essentially as previously described (6). Briefly, 8 x 10^9 9-15 cells were lysed (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% NP-40, and 1% protease inhibitor cocktail (Sigma)) and DLA-88*50801 complexes were immunoprecipitated using ANTI-FLAG M2 Affinity Gel (Sigma). MHC bound peptides were acid-eluted, filtered, purified using Hydrophilic-Lipophilic-Balanced reversed-phase cartridges (Waters), and concentrated by lyophilization to ~100µl.

Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) Analysis

Peptides were sequenced using a Waters Synapt G2 HDMS QToF mass spectrometer. The LC-MS/MS analyses were conducted at the Duke Proteomics Core Facility using a 5 µm

Symmetry C_{18} 180 µm I.D. X 20 mm column. Mass spectra were processed with Mascot Distiller (Matrix Science) and were then submitted to Mascot searches (Matrix Science) against a SwissProt_mammal database appended with reverse entries at 5 ppm precursor frequency and 0.03 Da product ion mass tolerances with no enzyme rules selected. Searched spectra were imported into Scaffold v3.0 (Proteome Software) and scoring thresholds were set to yield a peptide false discovery rate of 1.4% (implemented by the PeptideProphet algorithm) based on decoy database searches (23).

In silico *homology models of peptide DLA-88*50801 complexes*

Homology models of DLA-88*50801 were based upon the most homologous human MHC class I allele with a solved crystal structure, HLA-A*6801 (1TMC), as the starting model. Protein sequence corresponding to exons 2 and 3 were submitted to the Swiss-Model server (24) and threaded onto the peptide-binding domain of *HLA-A*6801*. The canine peptides (K9 and K11) were manually fit to the model using coot software (25) and energy minimized utilizing the YASARA server (26).

*Preparation of folded peptide/DLA-88*50801 complexes*

DLA-88*50801 MHC heavy chain and β2m proteins were produced in BL21 (DE3) E. coli (Genesee Scientific) as inclusion bodies and purified. Peptide:DLA-88*50801 complexes were folded and purified as previously described for murine peptide:MHC

complex (pMHC) monomers with synthetic peptides (27). The purity of prepared complexes was analyzed by SDS-PAGE.

Measurement of thermal stability by circular dichroism (CD)

Purified pMHC monomers were buffer exchanged in 10 mM KH_2/K_2HPO_4, pH 7.5 and concentrated. Melting curves were obtained by measuring the change in CD signal at 218 nM as a function of temperature from 5 to 94°C on a Chirascan plus (Applied Photophysics Ltd.). The T_m values were calculated based on a two-state denaturation model with corrections for pre- and post-transition linear changes as a function of temperature (28, 29).

Statistical Analysis

Variability in MHC bound peptide length between two alleles was evaluated using a modified Wilcoxon rank sum test. First, each eluted peptide was given a value equal to its length. To account for differences in the dominant peptide length for each allele, the median length value was subtracted from each individual peptide, respective to the MHC allele. These adjusted values were then squared and subjected to a Wilcoxon rank sum test using the R software package (30).

Results and Discussion

The identification of potential epitopes that could generate $CD8^+$ T-cell responses is aided greatly by computer algorithms that use MHC class I peptide-binding motifs to predict peptides with MHC binding potential from pathogenic proteins (31); however, to date, no such motifs have been described in dogs. Therefore, we sought to determine the binding motif of a classical *DLA* molecule that is prevalent within a popular dog breed that has a high lifetime risk of cancer (17-18).

DLA-88 is expressed at the cell surface

In the dog, four transcribed MHC class I loci exist (*DLA-12*, *-64*, *-79*, and *-88*), but only *DLA-88* is considered classical, as it is the sole highly polymorphic locus with 58 published alleles (18, 32-37). It is not known, however, whether the *DLA-88* gene product is expressed at the cell surface, which is necessary for TCR recognition; only the surface expression of the non-classical DLA-79 molecule has been confirmed (33). To determine if DLA-88 is present at the cell surface, we biotinylated surface proteins of 9-15 cells and assayed avidin-purified cell lysate for the FLAG epitope by Western blotting. As shown in Fig. 1, there was an approximate six-fold increase in signal compared to background, demonstrating the presence of DLA-88 on the surface of 9-15 cells. To corroborate this finding, co-localization of MHC class I and FLAG expression was demonstrated by confocal

microscopy on 9-15 cells (Supplemental Fig. 2). Together, these results indicate that DLA-88 is expressed on the surface of canine cells.

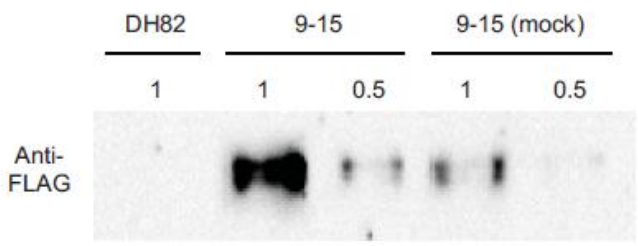
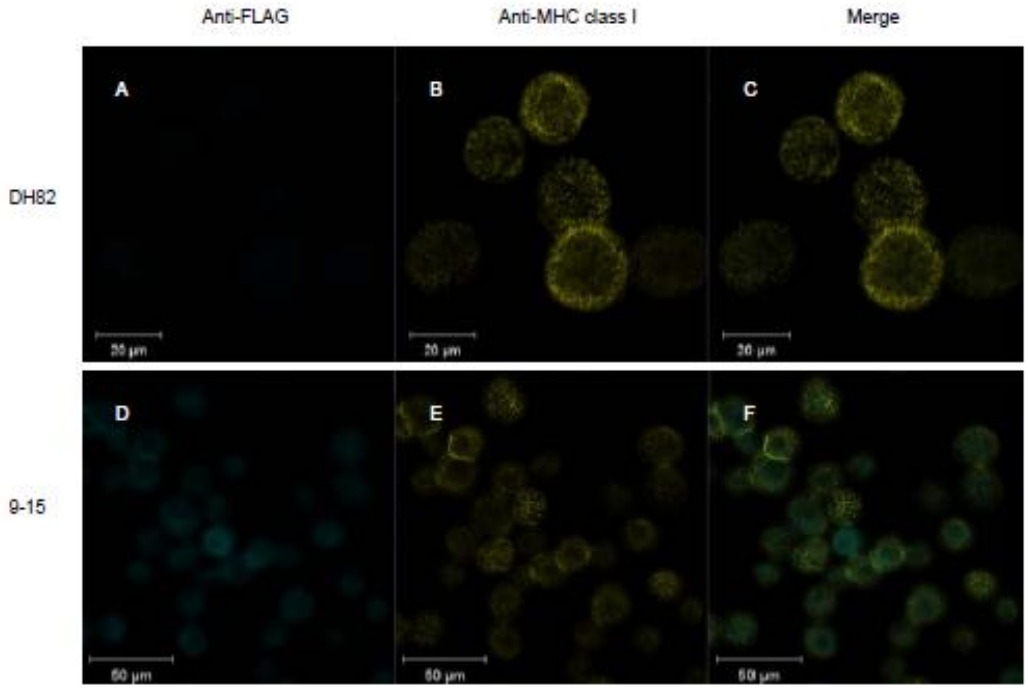


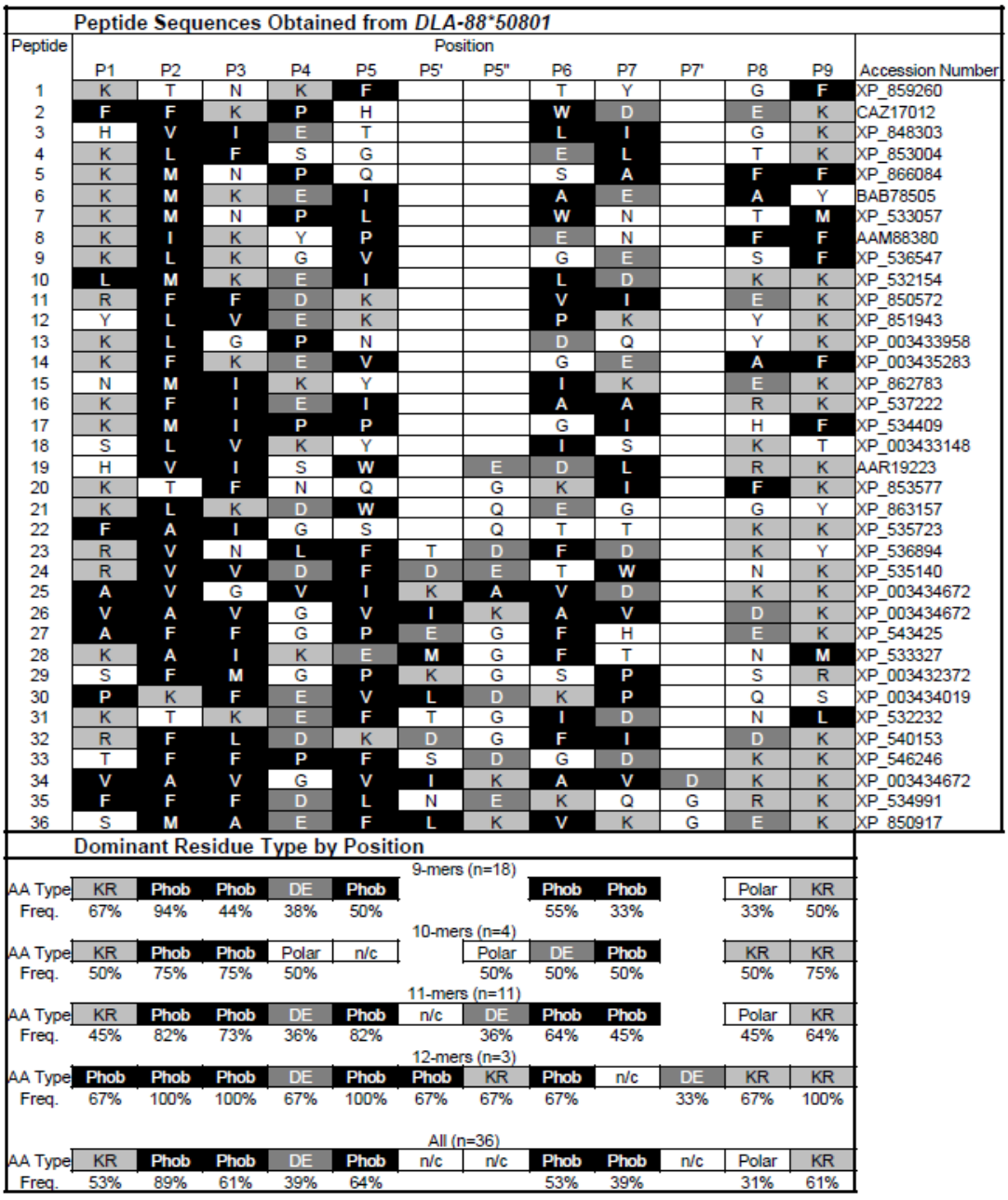
Figure 1 DLA-88 is expressed on the surface of canine cells. Surface proteins of DH82 and 9-15 cells were isolated and analyzed for FLAG epitope expression via Western blot. The 9-15 mock sample shows background signal of the assay as surface proteins were not biotinylated prior to avidin purification as described in methods. Numbers indicate relative concentration of protein loaded for Western blot analysis. Representative image from two independent experiments.



Supplemental Figure 2. DLA-88*50801 and FLAG expression co-localize at the cell surface of 9-15 cells. DH82 (A – C) and 9-15 (D – F) cells were surface stained for MHC class I (H58A; VMRD) followed by intracellular staining for the FLAG epitope (M2; Sigma). Samples were then immobilized on slides with ProLong Gold (Invitrogen) and co-localization of staining was analyzed using the Zen program (Zeiss) with a Zeiss Axio Observer Z1 microscope. Co-localization was consistently observed across all images taken in the z plane in 9-15 cells while none was observed in DH82 cells. (A and D) intracellular FLAG; (B and E) surface MHC class I; (C and F) merged image. Representative images from two independent experiments.

*Determination of the DLA-88*50801 binding motif*

Classical MHC class I alleles differ in their peptide binding preferences (38-41). To determine the binding motif of DLA-88*50801, we obtained the sequences of naturally processed and presented peptides to infer the conserved residues. As no DLA-88 allele specific antibodies were available, DLA-88*50801 molecules were affinity-purified from 9-15 cell lysate via the FLAG epitope tag. The MHC bound peptides were eluted, purified, and subjected to LC-MS/MS to determine individual sequences. A total of 37 peptide sequences of canine origin, ranging from 9 – 12 amino acids in length, were obtained; one was derived from β2m, representing a likely contaminant of the isolation process, and therefore was excluded from further analysis. To evaluate residue conservation at each position across the remaining peptides, amino acids were divided into four groups based upon chemical characteristics: hydrophobic (A, V, I, L, F, W, M, and P), charged-acidic (D and E), charged-basic (K and R), and polar (S, T, C, N, Q, Y, and H), with glycine (G) remaining unassigned. To assess residue preferences across all sequences, independent of length, "gap" positions (P5', P5", and P7') were inserted (Fig. 2) in a manner to maximize the finding of amino acid conservation. This allowed for all peptides to be aligned at both termini as the binding interactions between the bound peptide and MHC heavy chain are generally conserved at these sites (42, 43). The alignment was then analyzed for conservation (>60%) of the defined amino acid groups by each position of the bound peptide sequence, revealing that P2, P3, and P5 preferred hydrophobic residues, while P9 preferred a charged-basic residue.



Peptide Sequences Obtained from *DLA-88*50801*

Peptide	Position												
	P1	P2	P3	P4	P5	P5'	P5"	P6	P7	P7'	P8	P9	Accession Number
1	K	T	N	K	F			T	Y		G	F	XP_859260
2	F	F	K	P	H			W	D		E	K	CAZ17012
3	H	V	I	E	T			L	I		G	K	XP_848303
4	K	L	F	S	G			E	L		T	K	XP_853004
5	K	M	N	P	Q			S	A		F	F	XP_866084
6	K	M	K	E	I			A	E		A	Y	BAB78505
7	K	M	N	P	L			W	N		T	M	XP_533057
8	K	I	K	Y	P			E	N		F	F	AAM88380
9	K	L	K	G	V			G	E		S	F	XP_536547
10	L	M	K	E	I			L	D		K	K	XP_532154
11	R	F	F	D	K			V	I		E	K	XP_850572
12	Y	L	V	E	K			P	K		Y	K	XP_851943
13	K	L	G	P	N			D	Q		Y	K	XP_003433958
14	K	F	K	E	V			G	E		A	F	XP_003435283
15	N	M	I	K	Y			I	K		E	K	XP_862783
16	K	F	I	E	I			A	A		R	K	XP_537222
17	K	M	I	P	P			G	I		H	F	XP_534409
18	S	L	V	K	Y			I	S		K	T	XP_003433148
19	H	V	I	S	W		E	D	L		R	K	AAR19223
20	K	T	F	N	Q		G	K	I		F	K	XP_853577
21	K	L	K	D	W		Q	E	G		G	Y	XP_863157
22	F	A	I	G	S		Q	T	T		K	K	XP_535723
23	R	V	N	L	F	T	D	F	D		K	Y	XP_536894
24	R	V	V	D	F	D	E	T	W		N	K	XP_535140
25	A	V	G	V	I	K	A	V	D		K	K	XP_003434672
26	V	A	V	G	V	I	K	A	V		D	K	XP_003434672
27	A	F	F	G	P	E	G	F	H		E	K	XP_543425
28	K	A	I	K	E	M	G	F	T		N	M	XP_533327
29	S	F	M	G	P	K	G	S	P		S	R	XP_003432372
30	P	K	F	E	V	L	D	K	P		Q	S	XP_003434019
31	K	T	K	E	F	T	G	I	D		N	L	XP_532232
32	R	F	L	D	K	D	G	F	I		D	K	XP_540153
33	T	F	F	P	F	S	D	G	D		K	K	XP_546246
34	V	A	V	G	V	I	K	A	V	D	K	K	XP_003434672
35	F	F	F	D	L	N	E	K	Q	G	R	K	XP_534991
36	S	M	A	E	F	L	K	V	K	G	E	K	XP_850917

Dominant Residue Type by Position

9-mers (n=18)													
AA Type	KR	Phob	Phob	DE	Phob			Phob	Phob		Polar	KR	
Freq.	67%	94%	44%	38%	50%			55%	33%		33%	50%	
10-mers (n=4)													
AA Type	KR	Phob	Phob	Polar	n/c		Polar	DE	Phob		KR	KR	
Freq.	50%	75%	75%	50%			50%	50%	50%		50%	75%	
11-mers (n=11)													
AA Type	KR	Phob	Phob	DE	Phob	n/c	DE	Phob	Phob		Polar	KR	
Freq.	45%	82%	73%	36%	82%		36%	64%	45%		45%	64%	
12-mers (n=3)													
AA Type	Phob	Phob	Phob	DE	Phob	Phob	KR	Phob	n/c	DE	KR	KR	
Freq.	67%	100%	100%	67%	100%	67%	67%	67%		33%	67%	100%	
All (n=36)													
AA Type	KR	Phob	Phob	DE	Phob	n/c	n/c	Phob	Phob	n/c	Polar	KR	
Freq.	53%	89%	61%	39%	64%			53%	39%		31%	61%	

Figure. 2 Amino acid residue conservation by position of 36 endogenously bound peptides eluted from DLA-88*50801. Amino acids were divided into four groups based on chemical characteristics (hydrophobic (Phob), charged acidic, charged basic, or polar). The N- and C-termini of all sequences were aligned by the addition of "gap" positions in the longer peptides that were placed in a manner to maximize the finding of residue conservation at all positions. n/c, no consensus.

Due to the relatively small number of sequences obtained from the MS/MS analysis, defining the binding motif solely by frequency analysis may lead to the identification of an incorrect binding motif. To further support the binding motif, we created *in silico* molecular models of the canine pMHC complex to evaluate potential binding interactions between the heavy chain and amino acids at positions P2, P3, P5, and P9. These models were based upon the most homologous human class I allele with a solved crystal structure, HLA-A*6801 (40), using representative peptides of the most commonly observed lengths from our MS/MS analysis: the 9-mer KLFSGELTK ("K-9"; Fig. 3a) and the 11-mer RFLDKDGFIDK ("K-11"; Fig. 3b). At P2, this analysis shows that, although the side chain orientation differs between the two models, the P2 residue of each peptide (Fig. 3a ii and 3b ii) is able to form stabilizing interactions in the MHC binding pocket (the K-9 L is surrounded by Y7, V34, T45, E63, V67, and Y101 while E63 makes hydrogen bonds with the P1-P2 peptide-bond nitrogen; and, the K-11 F interacts with Y9), suggesting that this is a genuine preference of the DLA-88*50801 allele. Analysis of the interaction partners available near the P3 residues of the K-9 and K-11 peptides, F and L respectively, can neither confirm nor deny the putative preference for a hydrophobic amino acid at this site. At P5, the peptide side chain of K-11 is oriented away from the binding pocket, indicating that an MHC dependent residue preference at this position is unlikely. Further, the amino acids in the binding groove in close proximity to P5 are non-aliphatic (K66, E69, T70, Q158, and Y162), creating a polar environment that would oppose the interaction of hydrophobic residues. The C-terminal lysine of each model resides in a conserved binding pocket (Fig. 3a iv and 3b iv), where the positive charge could

interact with either the D77 or D118 residues of the DLA-88*50801 heavy chain. Therefore, based on both sequence and modeling analyses, we propose that the binding motif of DLA-88*50801 is X-AVILFM-AVILFM-$X_{(5-8)}$-KR.

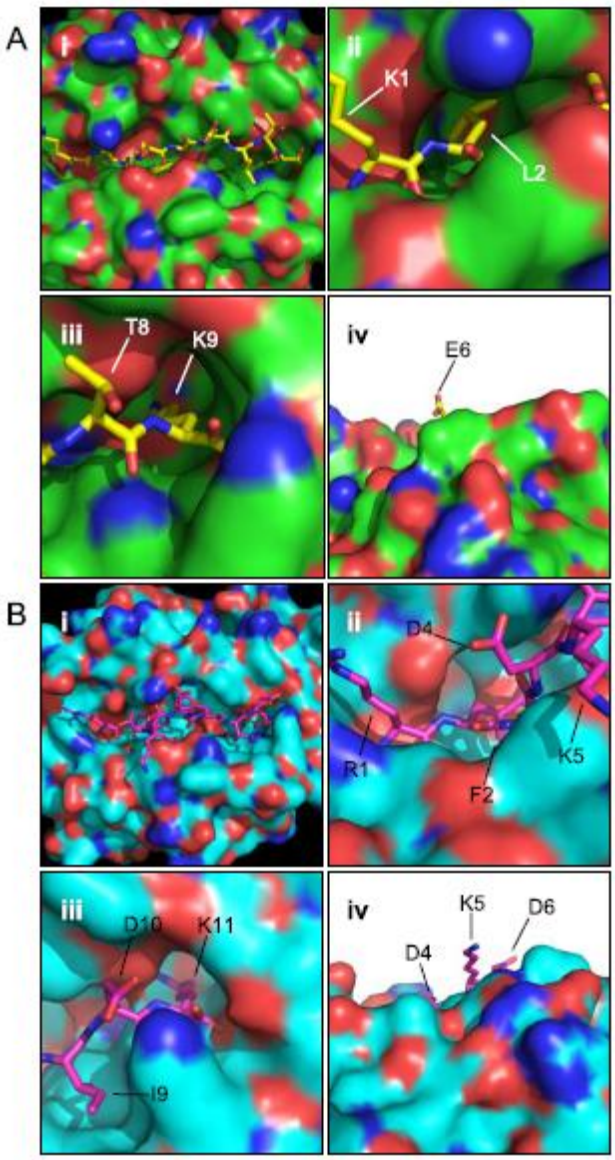


Figure 3 Molecular modeling of two different-length self peptides bound to DLA-88*50801. (A) Depiction of K-9, a nine amino acid long peptide (KLFSGELTK – sequence 4 in Fig 2); and (B) K-11, an eleven amino acid long peptide (RFLDKDGFIDK – sequence 32 in Fig 2) bound to DLA-88*50801. **I** Top down view of the modeled structure. Zoom views of P2 (**ii**) and C-terminii (**iii**) and surrounding residues of the peptide binding groove of DLA-88*50801. **Iv** Side views of the pMHC complexes; the panel in (B) iv shows a greater number of amino acid side chains protruding upwards from the longer K-11 peptide.

*Confirmation of the DLA-88*50801 binding motif*

To confirm the predicted DLA-88*50801 binding motif, recombinant pMHC complexes were prepared *in vitro* with peptides obtained from our analysis (Fig. 2) or a known H2-K^d restricted CTL epitope that did not match the putative binding motif (44). After purification of the complexes, the thermal stability was measured by circular dichroism as a function of temperature which correlates with the free energy of peptide binding to the MHC (45, 46). Thermal denaturation curves were generated by plotting the fraction of unfolded pMHC against temperature to calculate T_m values (Fig. 4). Complexes folded with motif-matched peptides (K-9 and K-11) had higher T_m values than that of the complex prepared with the motif-mismatched peptide, HA – a known H2-K^d restricted CTL epitope. The CD curves of both K-9/DLA-88 and K-11/DLA-88 complexes were smooth with single sharp transitions, consistent with a simple, one state cooperative unfolding transition. However, both HA/DLA-88 and protein purified in the absence of exogenous peptide had more complex spectra, indicating not only a lower T_m but also a set of transitions which can be correlated with the denaturation of unstable complexes followed by the unfolding of protein aggregates formed during the thermal denaturation process (~62°C for HA and ~55°C for no peptide). Collectively, this data suggests that DLA-88*50801 is stabilized by motif-matched peptides. Moreover, this data supports the finding that this MHC class I allele can bind both 9 and 11 amino acid long peptides well.

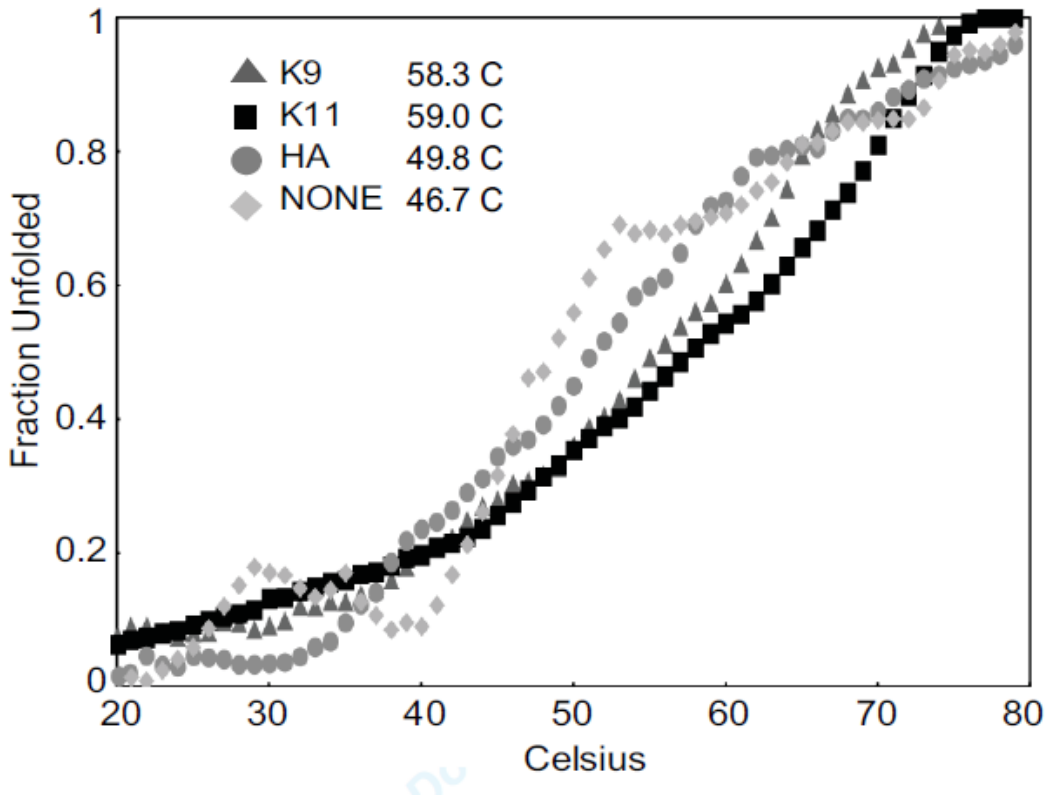


Figure 4 DLA-88*50801 is thermodynamically stabilized *in vitro* by motif-matched peptides as measured by CD. Folding of purified recombinant DLA-88*50801 and β2m proteins was performed with the addition of either: HA (IYSTVASSL), K-9 (KLFSGELTK), K-11 (RFLDKDGFIDK), or no peptides. Calculated T_m values for each preparation are listed by the chart key. Denaturation curves are from one (no peptide) or the average of two independent experiments (HA, K-9, and K-11).

Data from this study provides additional corroboration of *DLA-88* as a classical MHC class I locus in the dog. We show for the first time that DLA-88 is expressed at the cell surface, an essential characteristic of classical complexes. Moreover, the peptides eluted from DLA-88 appear to be derived from many different self proteins (Fig. 2), which is in contrast to the peptides that are generally bound by non-classical MHC class I loci (Reviewed in (47)).

The binding motif of DLA-88*50801 was found to have residue preferences at three positions; P2, P3, and P9. Notably, none of the obtained sequences were found to contain either W or P residues at P2 or P3, while all other hydrophobic residues were observed in at least one sequence. The molecular models support the exclusion of these residues at these positions from the binding motif based upon their structures, W is likely too large for the B binding pocket and P would force the peptide backbone to bend altering the conformation of the entire peptide.

In addition to residue specificity, class I alleles tend to have a length preference. For example, H2-K^b binds 96.2% 8-mers; H2-D^b binds 89.9% 9-mers; HLA-B7 binds 80% 9-mers; and HLA-A2 binds 100% 9-mers (48-50). Interestingly, two length species dominate the spectra of eluted peptides in DLA-88*50801 with both 9-mer and 11-mer peptides occurring at relatively high frequency, 50% and 30% respectively. The probability distribution of these peptides also contained more length variability than those bound by H2-D^b (48), with a p-value less than 2.2e-16. Further, this range in peptide length binding is similar to what has been observed with the homologous human MHC class I allele, *HLA-A*6801*, which can bind peptides ranging from 9 – 11 amino acids in length (40).

Not surprisingly, the binding motifs of DLA-88*50801 and HLA-A*6801 are remarkably similar, with the exception of threonine at P2 in *HLA-A*6801* (40). Nonetheless, three out of the four sequences obtained in this study which lack a hydrophobic residue at P2 have a threonine, indicating that this amino acid is likely also permissible. This finding serves as a reminder that MHC class I motifs reflect a binding preference, not a mandatory

requirement, for a particular peptide sequence to bind as many non-canonical peptides have been identified (51).

The defined peptide binding motif is supported by the finding that recombinant DLA-88*50801 pMHC complexes were thermodynamically stabilized by motif-matched peptides. Although the *HLA-A*6801* MHC can form a highly unstable complex when purified in the absence of exogenously added peptide (Collins EJ, unpublished observation), several lines of evidence indicate the purification of pMHC complexes in the presence of K-9 and K-11 peptides in this study: (i) a higher yield was retained in the K-9 and K-11 complex preparations as compared to the HA and no peptide controls following buffer exchange (data not shown), (ii) thermal denaturation curves for the K9 and K11 folds lacked complexity and fit well to a single unfolding transition, and (iii) the T_m was increased with K-9 and K-11 which has been shown to be related to peptide binding affinity in human and mouse pMHC complexes (28, 52). While HA provided a modest increase in thermal stability of the MHC complex over no peptide, K9 and K11 both stabilized the complex to a greater extent. We therefore conclude that 9- and 11-mer motif-matched peptides bind to and stabilize the DLA-88*50801 molecule.

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Footnotes

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Abbreviations

DLA	Dog leukocyte antigen
β2m	Beta-2-microglobulin
pMHC	peptide:MHC complex
CD	Circular dichroism
LC-MS/MS	Liquid chromatography tandem mass spectrometry

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Chapter 4: Development of a cell-based assay for the detection of canine MHC class I binding peptides: Unlocking epitope identification in the dog
(Submitted to Veterinary Immunology and Immunopathology)

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Rationale

A variety of techniques have been used to validate binding ability of peptides that have been predicted to associate with a given MHC molecule. Radioisotope or fluorochrome labeled peptides have been used to measure peptide binding by either ligand exchange of natural pMHC complexes or *in vitro* folding of recombinant MHC molecules (1-3). Other methods have measured the formation of pMHC complexes directly using enzyme linked immunosorbent assays or chromatography (4, 5). Importantly, the costliness and temporal demand of expressing and purifying recombinant MHC molecules makes these techniques

less desirable than cell-based assays for our use. Accordingly, we sought to create a cell-based tool analogous to the T2 and RMA-S cell lines that have proven useful for detecting peptide binding to the MHC in humans and mice, respectively (6, 7). These cell lines lack the ability to load peptides into the binding groove resulting in minimal surface MHC expression. When peptides are added to the culture medium of these cells, the MHC is stabilized at the cell surface by only those peptides capable of binding the MHC molecule allowing for the determination of MHC-peptide binding. Once created, a cell line has the obvious benefits of low maintenance cost as well as a renewing supply of MHC molecules to perform peptide binding validations.

Contribution

I performed all of the experiments and data analysis for this chapter.

Abstract

The establishment of immunodominant peptide epitopes for pathogens allows for more precise and informative study of CTL-mediated immunity than pathogen-specific responses. Unfortunately, the large number of potential peptides within an antigenic protein makes the experimental evaluation of all possible peptides difficult. Most immunodominant epitopes bind with high affinity to the MHC and in mice and humans the previously published identification of allele-specific binding motifs allows for the prediction of binding peptides from pathogenic proteins. Although peptide-binding motifs are useful for finding such peptides, many peptides not genuinely capable of MHC binding are also identified. Therefore, validating the MHC binding potential of predicted epitopes through the development of "peptide stabilization assays," using cells lacking the ability to naturally load peptides into the MHC, has further assisted immunodominant epitope identification; however, these techniques are unavailable in companion animal species. Previously, we determined the peptide-binding motif of the canine MHC class I allele Dog Leukocyte Antigen (DLA)-88*50801, allowing for epitope prediction in dogs. Herein, we describe the development of a cell-based assay to validate peptide binding to this allele using RMA-S cells stably transfected with DLA-88*50801. Moreover, our results suggest that this technique may have utility for other DLA-88 alleles facilitating the study of peptide-specific CTL in dogs.

Introduction

$CD8^+$ CTL function as the body's surveillance system against intracellular pathogens through the recognition of short, foreign-origin peptides displayed within the binding groove of class I MHC molecules. CTLs also play a beneficial role in the removal of malignant cells, as well as a pathogenic role in the perpetuation of several autoimmune diseases, such as type 1 diabetes mellitus and multiple sclerosis (8, 9). When the TCR binds to its specific peptide:MHC (pMHC) partner, the T cell is triggered to kill the infected cell. Studies designed to better understand the immunodominant responses of $CD8^+$ T cell populations are generally aimed at the discovery of peptide epitopes that can bind to the presenting class I molecules, thus driving the proliferation and differentiation of these cytotoxic cells as the availability of a suitable TCR for a given pMHC is one of the least restrictive factors in these responses (10). Once identified, these epitopes can be used in a number of assays of T cell function, including flow cytometry-based intracellular cytokine and/or tetramer detection, *in vitro* and *in vivo* cytotoxicity measurements, and ELISPOT or ELISA cytokine quantification. While a multitude of MHC-binding peptides have been identified in humans and mice, rarely have such epitopes been found in companion animal species (11).

Given the enormous diversity of peptides within most antigenic proteins, testing all possible peptides to find one or two immunodominant epitopes is extremely resource-intensive and therefore methods have been employed to rationally reduce the number of peptides to a level that is feasibly testable. MHC peptide-binding motifs allow for the

prediction of peptides within an antigen that are capable of MHC binding. However, the validation of putative class I-binding peptides is necessary as many non-binding peptides are also predicted by this technique. The combination of epitope prediction and validation can drastically reduce the total number of peptides considered for CTL recognition. For example: out of 10,660 total 9- and 10-mer peptides within 18 proteins in Vaccinia virus, only 1,657 were identified as potential HLA-A*0201 binders by motif analysis, of which only 263 were found to be legitimate MHC binding peptides (10). In mice and humans, validation of MHC binding peptides has been greatly facilitated by the development of immortal cell lines lacking the ability to naturally load peptides in the MHC binding groove. Without peptides, class I molecules are structurally unstable, and retained only transiently on the cell surface after trafficking from the endoplasmic reticulum. In this manner, surface expression of class I molecules is low-to-absent on both the human, T2, and murine, RMA-S, cell lines (6, 7). In RMA-S cells, this is due to a point mutation in the peptide transporter *TAP2* gene (12) that leads to a defective protein while T2 cells have a homozygous deletion of the class II region of the MHC where the TAP genes reside (13). What makes these cells so valuable for epitope study is that the addition of exogenous peptides capable of binding class I molecules to the culture medium results in MHC stabilization and prolonged surface expression. T2 and RMA-S provide *in vitro* "peptide stabilization assays" (14), which can be used to confirm MHC binders among peptides that were identified by a computer algorithm that searches for amino acid sequences matching consensus motifs (15). In addition, non-binding peptides are eliminated from further analysis if they do not stabilize MHC expression in T2 or RMA-S

cells (16). Furthermore, the degree of surface stabilization reflects the affinity of a given peptide for the class I molecule, which has important implications for the relative immunodominance of the $CD8^+$ T cell clonotype responding to that epitope. For example, a weak binder may engender no detectable T cell activity during the course of a natural infection. Typically, 15-20% of predicted peptides bind with an affinity less than 500 nM – the affinity cut-off below which most genuine epitopes are contained (10).

The objective of this study was to generate a cell line to be used in a "peptide stabilization assay" for Dog Leukocyte Antigen (DLA)-88, the only classical MHC class I locus described in dogs to date (17, 18). To accomplish this goal, the DLA-88*50801 allele was stably transfected into RMA-S, similar to what has been described previously for other exogenous MHC alleles (19-21) cells and a clone [designated Bispecies Antigen Recognition Cells clone 3 (BARC3)] was generated, characterized, and verified. This clone exhibits similar characteristics as its murine predecessor allowing for the validation of predicted DLA-88*50801-binding peptides as genuine binders. Herein, we show the detection of surface peptide:MHC (pMHC) complexes in a DLA-88 specific manner with little to no contribution from the murine MHC molecules. Moreover, we show that DLA-88*50801 forms a complex with murine β2m, suggesting that modification of RMA-S cells could be utilized for any DLA-88 allele of interest. The validation of this methodology should constitute a valuable addition to the toolkit for studying $CD8^+$ T-cell responses in the dog.

Materials and methods

Cell culture and cloning of BARC3 cells

The murine lymphoma cell line, RMA-S, was cultured in RPMI medium supplemented with L-glutamine and 10% FBS. A plasmid encoding DLA-88*50801 with a FLAG epitope tag at the carboxyl terminus was previously created by our lab (Ross P and Hess PR manuscript submitted, The canine MHC class I allele DLA-88*50801 presents variable length peptides with a conserved binding motif; Chapter 3) and transfected into RMA-S cells using Lipofectamine 2000 (Invitrogen). Selection was performed using G418 (1 mg/ml) and individual clones were isolated by limiting dilution and screened by intracellular staining and flow cytometry using the anti-FLAG Ab M2 (Sigma). Clone 3 (BARC3) was used throughout this study.

MHC stabilization assays

RMA-S and BARC3 cells were cultured in flat bottom 96-well tissue culture plates at 1×10^5 cells per well overnight at 27° C in order to accumulate MHC molecules at the cell surface, similar to what has been described previously (7). Accumulated MHC molecules were peptide loaded by adding 100 µg/ml DMSO solubilized (unless otherwise indicated) K9 (KLFSGELTK), K11 (RFLDKDGFIDK), (Peptide 2.0) or NP396 (FQPQNGQFI) peptides (Genscript) to the overnight cultures followed by 5 hours of culture at 37° C to allow most empty MHC molecules to be removed from the cell surface. When stability of specific

pMHC complexes was to be assessed, peptide loaded BARC3 cells were washed in PBS to remove any free peptide and incubated in the presence of 5 µg/ml Brefeldin A (BFA) (Biolegend) for the indicated length of time.

Flow cytometry analysis

For staining, cells were washed with FACS buffer (PBS with 2% FBS and 0.1NaN_3) prior to incubation with the applicable primary or secondary Ab for 15 min at 4° C in 96-well round-bottom polypropylene plates. The following primary unconjugated mAbs were used in this study at optimized concentrations: anti-canine MHC class I (H58A, VMRD; 3F10, Ancell), anti-H2-D^b (28-14-8; eBioscience), anti-$K^{b/d}$ (34-1-2S; eBioscience), and anti-murine β2m (S19.8; BD Pharmingen). For all experiments, the secondary Ab was AlexaFluor 647-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) with background staining established by omission of primary Ab. All flow cytometry list mode data was collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star) using forward versus side scatter to gate on live cells. Graphical presentation and analysis of the data was performed using Prism 5 (GraphPad).

Results and discussion

Characterization of the BARC3 cell line

To establish a method capable of specifically detecting DLA-88 in the presence of murine H2 MHC molecules, we first sought to obtain an anti-MHC class I Ab previously used for the detection of DLA-88 (Ross P and Hess PR manuscript submitted) that does not detect H2. RMA-S cells accumulate MHC molecules on the cell surface when cultured at 27° C overnight (7). Therefore, to test the specificity of the anti-MHC class I Ab H58A for murine H2 molecules, RMA-S cells were cultured overnight at 27° C prior to staining and flow cytometry analysis. Culture of RMA-S cells overnight at 27° C resulted in the increase of both K^b and D^b (as detected by K^b and D^b specific Abs 34-1-2S and 28-14-8, respectively; eBioscience) while no increase in H58A staining was observed (Figure 1A). Therefore, the H58A does not detect the H2 MHC molecules endogenous to the RMA-S cell line.

To create the canine "peptide stabilization assay," a plasmid encoding a FLAG-epitope tagged version of DLA-88*50801 was transfected into RMA-S cells. Individual clones were then isolated by limiting dilution and screened for intracellular FLAG expression (Figure 1B shows BARC3, the clone used throughout this study). In order to ensure DLA-88 surface expression, BARC3 cells were cultured overnight at 27° C resulting in the upregulation of DLA-88 (Figure 1C). To characterize the length of time required for downregulation of empty MHC molecules, BARC3 cells were cultured at 27° C overnight followed by increasing lengths of time at 37° C (Figure 1C and D). Five hours of 37° C

culture was sufficient to remove approximately 85% of DLA-88 from the cell surface. Together, these results indicate that empty DLA-88*50801 molecules are upregulated on the surface of BARC3 cells following culture at 27° C, downregulated by further culture at 37° C, and can be specifically detected using the anti-MHC class I Ab H58A.

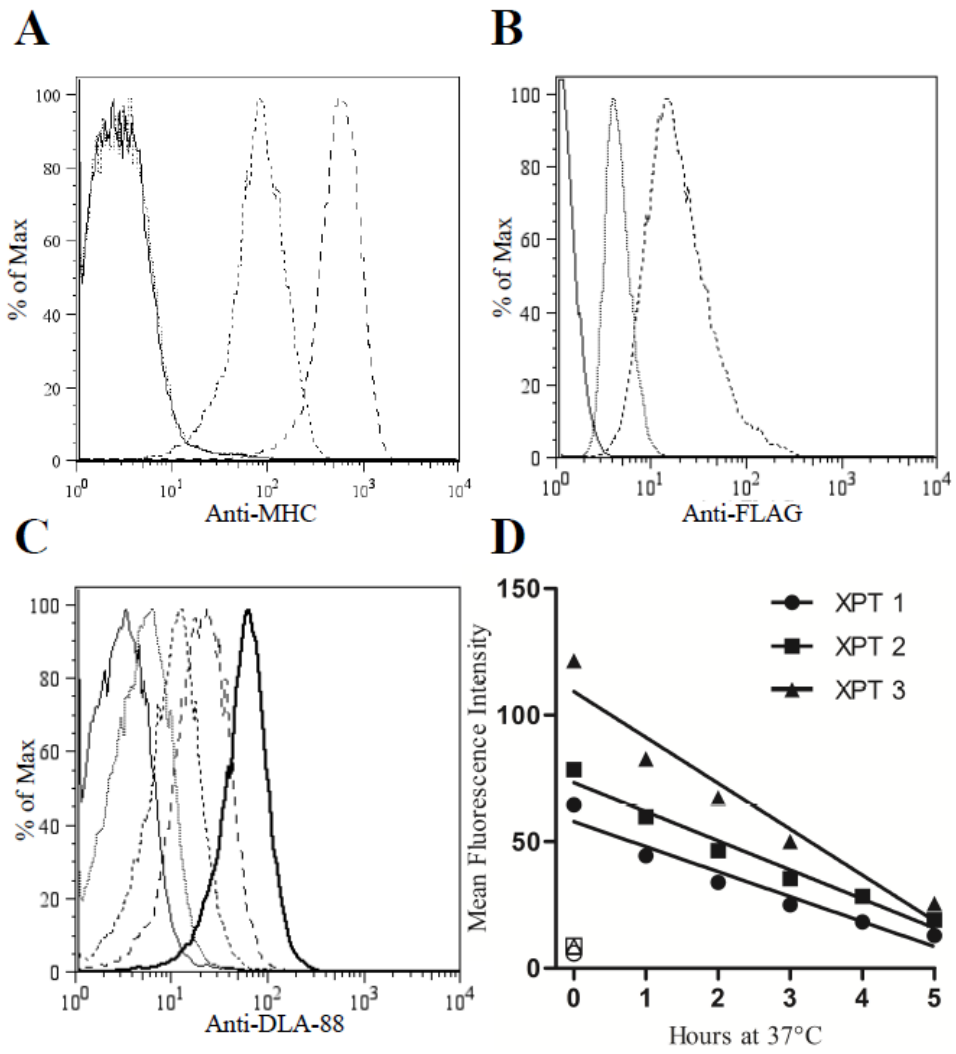

Figure 1. DLA-88*50801 is upregulated at the cell surface of BARC3 cells following culture at 27° C and can be specifically detected using the anti-MHC class I antibody H58A. (A) H2-D^b (dashed) and H2-K^b (long dashed) are upregulated on RMA-S cells following overnight 27° C culture, yet no increase in H58A staining is observed (dotted) as determined by flow cytometry analysis. The solid line indicates cells stained with the secondary antibody only. (B) BARC3 cells stably express DLA-88*50801. RMA-S (dotted) and BARC3 (dashed) cells were assessed for FLAG epitope expression by intracellular staining following overnight culture at 27° C. The solid line shows unstained BARC3 cells. (C) Cold culture induced DLA-88*50801 is downregulated on the cell surface of BARC3 cells following culture at 37° C. BARC3 cells were cultured at 27° C overnight followed by 5 (dashed), 3 (long dashed), or 0 (bold) hours culture at 37° C prior to staining and flow cytometry analysis. The dotted line indicates cells that were maintained at 37° C for the duration of the assay, while the solid line indicates cells maintained at 27° C and stained with secondary antibody only. (D) Graphical representation of three independent experiments (XPT) of C. Linear regression of each repetition is shown. Open icons indicate samples that were maintained at 37° C for the duration of the assay. Data from B, C, and D is representative of three independent experiments.

Validation of the canine "peptide stabilization assay"

When exogenous peptides capable of binding H2-K^b or D^b are added to the culture medium of RMA-S cells, surface expression of the respective MHC molecule is stabilized through the formation of pMHC complexes, where the degree of stabilization indicates the relative affinity of each peptide (14). We have previously characterized two DLA-88*50801 binding peptides, K9 and K11 (RossP and HessPR manuscript submitted, The canine MHC class I allele DLA-88*50801 presents variable length peptides with a conserved binding motif; Chapter 3), by *in vitro* folding of recombinant pMHC complexes. To determine the efficacy of performing a canine "peptide stabilization assay," BARC3 cells were cultured overnight at 27° C in the presence of exogenously added peptides followed by 5 hours of culture at 37° C. As expected, both K9 and K11 showed a dose-dependent stabilization of surface DLA-88 while the known H2-D^b binding epitope, NP396 (22), did not (Figure 2A and C). To confirm that this result was not confined to the NP396 peptide, an additional H2-D^b binding peptide, NP366 (23, 24), was tested and also failed to stabilize DLA-88 (data not shown). To further ensure the specificity of the H58A Ab, untransfected RMA-S cells were cultured with K9, K11, and NP396. No increase in H58A staining was observed regardless of peptide treatment (data not shown). Importantly, the NP396 peptide was capable of stabilizing H2-D^b on RMA-S cells, mean fluorescence intensity of 217 with NP396 versus 14 without peptide, when measured with an anti-H2-D^b Ab (clone 28-14-8; eBioscience). Another anti-MHC class I Ab, 3F10, recapitulated the results obtained with H58A (Figure 2B).

Our previous study indicated that both K9 and K11 peptides stabilized the pMHC complex approximately the same amount as measured by circular dichroism of *in vitro* folded complexes (Ross P and Hess PR manuscript submitted, Chapter 3), yet here K11 treatment resulted in relatively more DLA-88 at the cell surface, indicated by greater mean fluorescence intensity values than K9 labeled cells. This finding could be due to either higher MHC affinity, resulting in greater MHC stabilization prior to the 5 hour 37° C culture, or a longer half life ($t_{1/2}$), resulting in less pMHC loss over time. The addition of brefeldin A to the culture medium of peptide loaded T2 cells has been used to measure the off-rates of various peptides from the MHC (25). Therefore, we sought to determine the $t_{1/2}$ of K9 and K11 pMHC complexes using this method. This analysis revealed that the $t_{1/2}$ of K11 pMHC complexes was indeed longer than that of K9 complexes (Figure 2D). This finding led us to conclude that the increased DLA-88 surface stabilization by K11 is likely due to a longer $t_{1/2}$ when compared to K9. Together, these results indicate that the addition of DLA-88*50801 binding peptides to the culture medium of BARC3 cells stabilizes surface expression of DLA-88*50801. Further, the murine MHC molecules present in these cells do not interfere with the efficacy of the DLA-88 specific "peptide stabilization assay."

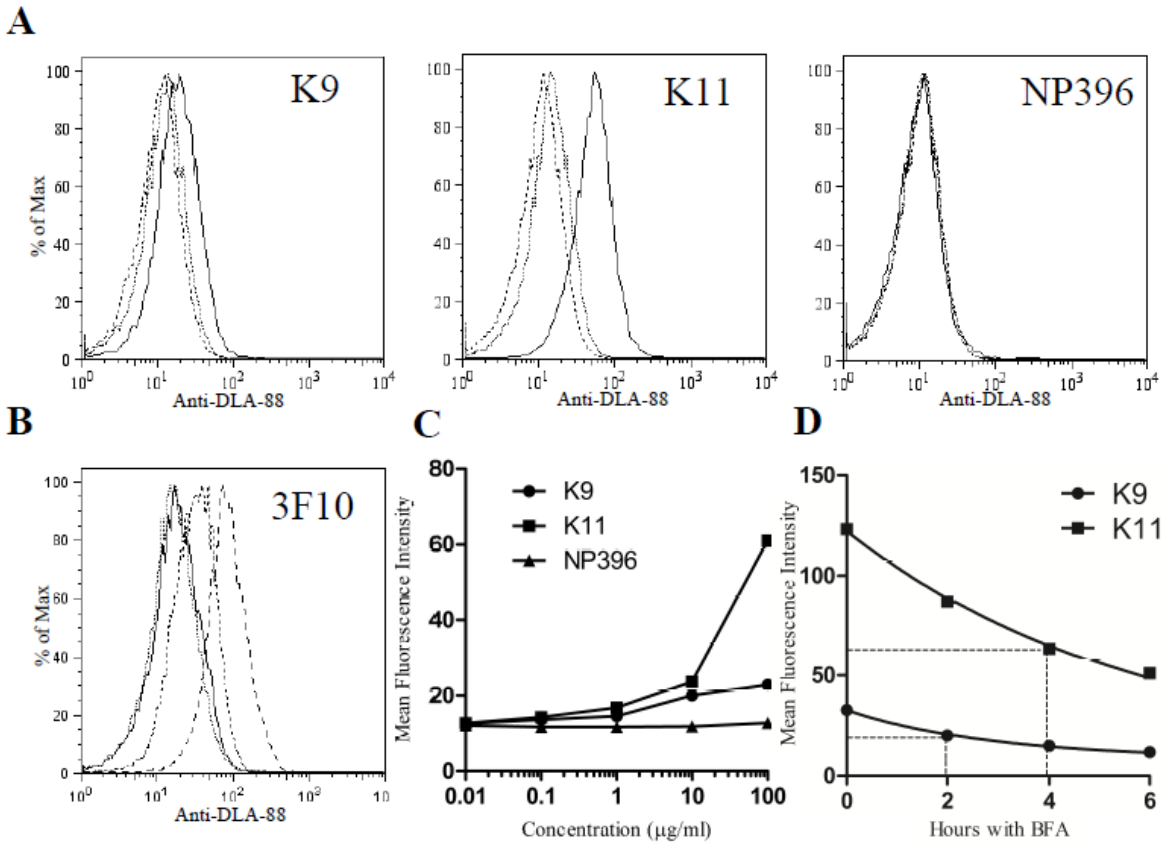

Figure 2. DLA-88*50801 is stabilized on the surface of BARC3 cells by the addition of DLA-88*50801 binding peptides. (A) K9 and K11 peptides stabilize DLA-88*50801 on the cell surface while the H2-D^b binding NP396 epitope does not. BARC 3 cells were cultured in the presence of 100 (solid), 1 (dotted), or 0.01 (dashed) µg/ml of the indicated peptide. (B) An alternative anti-MHC class I antibody, 3F10, confirms the results obtained using H58A. BARC3 cells were cultured with 100 µg/ml of NP396 (dotted), K9 (dashed), K11 (long dashed), or no peptide (solid). (C) Graphical representation of data shown in (A). (D) Dissociation of K9 and K11 peptides from pMHC complexes occurs at different rates. BARC3 cells were cultured in the presence of indicated peptide overnight at 27° C followed by 5 hours at 37° C to allow dissociation of unstable complexes. After washing with PBS to remove unbound peptide, brefeldin A was added to the 37° C culture and cells were harvested at the indicated time points. The dissociation half-life ($t_{1/2}$ – shown by dotted lines) was calculated by non-linear regression, using a one phase exponential decay model for curve fitting. In the first experiment, the $t_{1/2}$ for K9 was 1.9 h (95% CI 1.2 – 3.2 h), and 6.0 h for K11 (95% CI 1.7 – 2.4 h). In the second experiment (shown in graph), the $t_{1/2}$ for K9 was 2.0 h (95% CI 1.7 – 2.4 h), and 4.0 h for K11 (95% CI 3.3 – 5.0 h). Data are representative of two (B and D) or three (A and C) independent experiments.

DLA-88 interacts with murine β2m

Our results suggest that DLA-88 is able to form a complex with murine β2m. To test this hypothesis directly, we incubated RMA-S or BARC3 cells with various peptides in serum-free media and measured surface β2m expression by flow cytometry. Serum-free media was used because bovine β2m from FBS can stabilize MHC in some systems (26). As expected, RMA-S cells incubated with NP396 showed an increase in β2m surface expression over the no peptide control (Table 1). However, addition of the K11 peptide was capable of increasing β2m expression on BARC3 cells but not on RMA-S cells (Table 1). These data indicate that DLA-88*50801 can form a heterotrimeric pMHC complex with murine β2m.

Table 1 Surface expression of murine β2m of RMA-S and BARC3 cells as measured by flow cytometry. * Numbers indicate mean fluorescence intensity of live cells. ND – not determined. Results are representative of three independent experiments

	Peptide Treatment		
Cell Line	None	NP396	K11
RMA-S	80	240	91
BARC3	161	ND	281

This study outlines the development of a canine "peptide stabilization assay" for the validation of peptide binding to DLA-88*50801 using BARC3 cells – a modification of RMA-S cells. The assay described here is faster, higher throughput, and more cost-effective than *in vitro* folding and thermal stability measurement for determining DLA-88*50801 peptide binding (Ross P and Hess PR, manuscript submitted, The canine MHC class I allele DLA-88*50801 presents variable length peptides with a conserved binding motif; Chapter 3).

This protocol should allow for screening relatively large peptide libraries and may also prove useful for evaluating CTL recognition of specific pMHC (28).

Most interaction sites between the MHC heavy chain and β2m are conserved across not only DLA-88 alleles but also across species. The results of this study indicate that DLA-88*50801 is able to form a pMHC complex with murine β2m. Together, this suggests that analogous cell lines could be created with any DLA-88 allele of interest and would not require the simultaneous expression of canine β2m. Therefore, RMA-S cells transfected with DLA alleles can be used to perform canine "peptide stabilization assays" and should aid in the discovery of pathogenic CTL epitopes, allowing for the study of peptide-specific CTL immunity in the species.

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Chapter 5: Attempted determination of a DLA-88*50801 restricted, peptide-specific CTL response

Rationale

The previous chapters focused on development of the tools necessary for detecting a peptide-specific CTL response in the dog. In Chapter 2, we found that DLA-88*50801 was a prevalent MHC class I allele within the Golden Retriever breed. We described the discovery of the peptide binding motif of DLA-88*50801 in chapter 3, enabling the prediction of peptides capable of binding to and being presented by this canine MHC allele. Finally, a cell-based assay capable of validating peptide MHC binding was developed and described in Chapter 4. The combination of these findings will allow for the prediction and detection of the first peptide-specific CTL response in the dog. This chapter will document the application of this information for determining a DLA-88*50801 restricted CTL response against the hemagglutinin protein of canine distemper virus.

Contribution

I performed all of the experiments and data analysis for this chapter.

Abstract

Many peptide-specific cytotoxic T lymphocyte (CTL) responses have been described that are directed against a litany of pathogenic and oncogenic antigens. However, the study of CTL responses has been largely confined to mice and humans with comparatively little effort aimed at describing these responses in veterinary species, such as the dog. Previously, we identified the peptide binding motif of a prevalent MHC class I allele in Golden Retrievers, Dog Leukocyte Antigen-88*50801 (DLA) and developed a peptide stabilization assay for use with this allele. Together, these findings allow for the prediction and validation of MHC binding peptides from foreign proteins for the first time in the species. Here, we aim to implement these tools to identify a peptide-specific CTL response in dogs. To accomplish this, we have predicted DLA-88*50801 binding peptides from the canine distemper virus hemagglutinin protein, confirmed the MHC binding of the predicted set, and attempted to identify a CTL response directed against the peptides capable of MHC binding. This study describes the first documented attempt to determine a $CD8^+$ CTL epitope in dogs.

Introduction

The primary function of $CD8^+$ cytotoxic T lymphocytes (CTL) is the elimination of infected cells through the recognition of foreign peptides bound within the binding groove of major histocompatibility complex (MHC) class I molecules on the target cell's surface through the CTL's T-cell receptor (TCR). This response is highly specific with a given TCR typically capable of efficiently recognizing only a single peptide within the context of the restricting MHC allele (1, 2). While theoretically any pathogenic peptide able to bind the MHC could be recognized by an appropriate TCR bearing CTL, usually CTL responses are directed against a single or very few peptides from a given pathogen within the context of an MHC allele, a finding known as immunodominance (3, 4). This phenomenon has allowed for the study of peptide-specific CTL responses across individuals that share MHC class I alleles (5), immune regulation of tumors (6), and the perpetuation of autoimmune diseases (7, 8).

Unfortunately, the available information on the MHC is largely confined to mice and humans. Therefore the potential clinical applications of this information are limited (9, 10). Expanding our knowledge on the MHC to veterinary species, such as the dog, would allow for better veterinary care and would enable the use of these animal models for research (11, 12). Nevertheless, the current information on the canine MHC, designated Dog Leukocyte Antigen (DLA), is limited. Four full-length transcribed class I genes have been identified DLA-12, -64, -79, and -88 (13). Of these, only DLA-79 (14) and -88 (Chapter 3) have been

demonstrated to be expressed at the cell surface. However, DLA-88 is the only locus that possesses a substantial level of polymorphism with a total of 58 published alleles (15-19). Based upon these criteria, DLA-88 is currently thought to be the only classical MHC class I gene in the dog (15). Here, we sought to identify the first peptide-specific CTL response restricted by DLA-88*50801 using previously developed peptide prediction (Chapter 3) and MHC binding methodologies (Chapter 4). The demonstration that DLA-88 restricts conventional CTL responses would allow for the definitive characterization of this locus as a classical MHC gene (20).

The identification of peptide-specific CTL responses by predicting pathogen-derived peptides capable of being presented by MHC molecules has proven to be successful in both mice and veterinary species (21, 22), but requires substantial preliminary work to identify the binding motif for the MHC allele of interest (Chapter 3). Despite this initial time and work investment, there are a variety of factors that make this approach more desirable than its alternatives. Defining a CTL epitope by expressing a single pathogenic gene, followed by fragments of this gene, in target cells requires the generation of multiple transfectants, as was done for the initial CTL epitope discovery (23). On the other hand, using overlapping peptides can overcome the initial temporal demand but is costly to perform as every encoded peptide for the pathogen or gene of interest must be obtained. While both of these methods have been implemented successfully (23, 24), arguably the largest drawback of these methods is that they impart only a limited insight towards the identification of a CTL epitope from another pathogen. To predict CTL epitopes, an MHC allele's binding motif need only

be identified once in order to be applied towards the identification of CTL epitopes for any number of pathogens within the context of that allele.

We focused this study on the hemagglutinin protein from canine distemper virus (CDV-HA). This antigen is the only pathogenic protein for which an antigen-specific CTL response has been identified in dogs (25). Moreover, previous studies have identified this response in a total of four individual dogs of unknown DLA-88 genotype indicating that CDV-HA may contain epitopes for several DLA-88 alleles (25, 26). Additionally, most dogs are routinely vaccinated against CDV with a live viral vaccine (27). This type of vaccine is capable of eliciting a potent CTL response, increasing the likelihood that a peptide-specific response could be identified in an immunized individual (28-30).

Materials and methods

Isolation and cloning of the CDV-HA gene

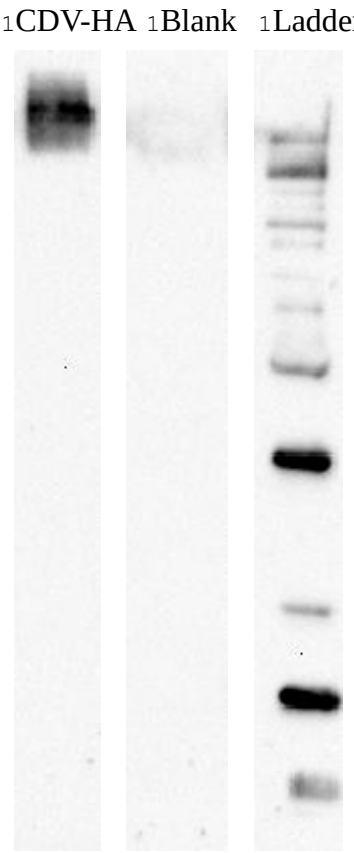
To determine the sequence of the hemagglutinin (HA) gene in the vaccine strain of CDV that is in use at the North Carolina State University College of Veterinary Medicine (NCSU-CVM) where the dog used in this study was immunized, RNA was extracted from reconstituted NobiVac1 DAPPV vaccine using the QIAamp Viral RNA mini kit (Qiagen) according to the manufacturer's protocol, and cDNA was synthesized by reverse transcription using random primers. PCR amplification of CDV-HA from template cDNA was performed using the forward 5' – ATGCTCCCCTACCAAGACAAG – 3' and reverse primers 5' – ATGTGTATCATCATACTGTCAG – 3' with the following cycling conditions: initial 5 minute denaturation at 95 °C followed by 33 cycles of denaturation at 94 °C for 15 seconds, annealing at 57.7 °C for 1 minute, and elongation at 72 °C for 2 minutes, followed by a final elongation at 72 °C for 10 minutes. Resulting amplimers were ligated into the pGEM-T Easy Vector (Promega), cloned, and sequenced (MWG Operon). Further amplification of this plasmid with the forward 5' – CGGCGGAAGCTTACTTAGGGCTCAGGTAGT – 3' and reverse primers 5' – ATTAAGCGGCCGCAACGGTTACATGAGAATCTT – 3' was performed to insert the CDV-HA gene into a pcDNA3 mammalian expression construct with a FLAG epitope tag directly downstream of the NotI restriction enzyme site (gift from Jeff Yoder).

*DLA-88*50801 peptide stabilization assay*

CDV-HA derived peptides predicted to bind DLA-88*50801 were assessed for genuine DLA-88*50801 binding using the Bispecies Antigen Recognition Cell (BARC3) peptide stabilization assay as described in chapter 4. Briefly, 1 – 3 mgs of each peptide (Mimotopes) was solubilized in 100 µl Dimethyl Sulfoxide (DMSO). BARC3 cells were cultured overnight at 27 °C in 96-well flat-bottom plates at a concentration of 1 x 10^6/ml in media containing 1% volume reconstituted peptide or 100 µg/ml of the control K11 peptide (RFLDKDGFIDK; Peptide 2.0). Following 5 hours of further culture at 37 °C to allow empty MHC molecules to be down regulated from the cell surface, the relative amount of peptide-stabilized DLA-88*50801 molecules was assessed by flow cytometry to measure the peptide's ability to bind this MHC allele.

Generation of DH82-HA cell line

The canine histiocytic cell line, DH82 (31) (ATCC CRL-10389), was transfected with the CDV-HA mammalian expression construct using Lipofectamine 2000 (Invitrogen). One day post-transfection, cells were placed under 800 µg/ml G418 antibiotic selection. One week later, G418 concentration was reduced to 200 µg/ml and clones were isolated by limiting dilution. Expression of the CDV-HA protein was confirmed by Western blot using the M2 anti-FLAG Ab (Sigma) (Supplemental Figure 1). The stable clone used in this study will be designated DH82-HA.



Supplemental Figure 1 Confirmation of FLAG epitope expression in CDV-HA cells. The expected size of CDV-HA is approximately 70 kDa. FLAG epitope expression was visualized around 200 kDa; however, trimerization of HA proteins has been observed with influenza HA (32, 33).

Peripheral blood lymphocyte isolation

Blood samples from a previously CDV-immunized Golden Retriever homozygous for DLA-88*50801 were collected by the North Carolina State University Veterinary Teaching Hospital (NCSU-VTH). Peripheral blood lymphocytes (PBL) were isolated by centrifugation (400 x g for 30 min) using Histopaque 1.077 density gradient (Sigma-Aldrich). Contaminating red blood cells were removed using a pH 7.2 lysis buffer comprised of 0.15 M NH_4Cl, 1 M $KHCO_3$, and 0.1 mM EDTA. PBL were washed several times in PBS prior to use in further assays.

CTL stimulation assay

To assess peptide-specific CTL responses, BARC3 cells were pulsed with pools of CDV-HA derived peptides for which DLA-88*50801 binding had been confirmed (each peptide comprising 1% total media volume – importantly, the additional DMSO concentration did not adversely affect cell viability) overnight at 27 °C. The following day, the peptide-pulsed BARC3 cells were treated with 50 µg/ml mitomycin C for 30 min to inhibit further growth. Following multiple PBS washes to remove the mitomycin C, the peptide pulsed BARC3 were co-cultured with freshly isolated PBL at a 1:10 ratio in the presence of 20 U/ml recombinant human IL-2 for 4 days at 37 °C in 24-well plates (primary stimulation). On day 5, stimulated PBL were isolated over Histopaque 1.077 gradient and restimulated with freshly prepared peptide pulsed BARC3 cells (at approximately a 2:1 ratio), and/or phorbol myristate acetate (PMA) (40 ng/ml) and Ionomycin (4 µg/ml) as a

positive control, for 6 hours (secondary stimulation) in the presence of brefeldin A (5 - 40 µg/ml). Following the secondary stimulation, PBL were assessed for $CD8^+$ interferon-γ (IFN-γ) production by intracellular staining and flow cytometry. To ensure that CDV-HA specific CTL are present and detectable in the PBL preparations, we substituted CDV-HA transfected DH82-HA cells for peptide pulsed BARC3 cells during both primary and secondary stimulations.

Cell culture

All cell culture was performed at 37 °C in a humidified incubator with 5 % CO_2 unless otherwise indicated. BARC3 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with L-glutamine (2mM), 10 % FBS, and 400 µg/ml G418. DH82 cells were maintained in DH82 media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% FBS. DH82-HA cells were cultured in DH82 media supplemented with 200 µg/ml G418. Canine PBL were cultured in PBL media: RPMI supplemented with L-glutamine (2mM), 10 % FBS, and 550 µM 2-mercaptoethanol. All PBL co-cultures for CTL stimulation assays were performed in PBL media.

Flow cytometry and antibodies

For staining, cells were washed with FACS buffer (PBS with 2% FBS and 0.1 NaN_3) prior to incubation with the applicable primary or secondary Ab for 15 min at 4° C in 95 well round bottom polypropylene plates. Surface MHC stabilization was assessed with the anti-

canine MHC class I Ab (H58A, VMRD) in combination with the secondary AlexaFluor 647-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) at optimized concentrations. Cell surface CD8 and Thy1.2 expression was assessed using rat anti-dog CD8 (MCA1039; Abd Serotec) and anti-mouse Thy1.2 (48-0902-80; eBioscience), respectively. Intracellular IFN-γ expression was assessed using the mouse anti-bovine IFN-γ Ab (MCA1783; Abd Serotec) with irrelevant background intracellular staining being established using the Armenian hamster IgG isotype control (eBio299Arm; eBioscience). All flow cytometry list mode data was collected using a FACSCalibur (BD Biosciences) or LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star) using forward versus side scatter to gate on live cells.

Results and discussion

*Prediction of CDV-HA derived, DLA-88*50801 binding peptides*

In this study, our ultimate goal is to characterize a peptide-specific CTL response directed against CDV-HA in a previously immunized, DLA-88*50801 homozygous dog. However, CDV-HA possesses a high level of genetic variability across different virus isolates (34). As a CTL response generated by vaccination can only be specific for the peptides encoded by the vaccine strain of virus, we sought to determine the sequence of CDV-HA in the NobiVac1 DAPPV vaccine in use at the NCSU-CVM. To accomplish this, viral RNA was isolated and reverse transcribed, because CDV is a negative strand RNA virus, prior to performing PCR to amplify the CDV-HA gene. This amplimer was cloned and sequenced and the inferred AA translation is provided in Figure 1.

```
     -          -          -          -          -          -          -          -          -          -
     1          1          2          3          4          5          6          7          8          9
                                                1          1          1          1          1          1
  1 - MLSYQDKVGA FYKDNARANS TKLSLVTEEH GGRRPPYLLF VLLILLVGIL ALLAITGVRF HQVSTSNMEF SRLLKEDMEK SEAVHHQVID VLTPLFKIIG
101 - DEIGLRLPQK LNEIKQFILQ KTNFFNPNRE FDFRDLHWCI NPPSKVKVNF TNYCESIGIR KAIASAANPI RLSALSGGRS DIFPPHRCSG ATTSVGKVFP
201 - LSVSLSMSLI SRASEIINML TAISDGVYGK TYLLVPDDIE REFDTQEIRV FEIGFIKRWL NDMPLLQTTN YMVLPEDSKA KVCTIAVGEL TLASLCVEES
301 - TVLLYHDSSG SQDGILVVTL GIFGATPMDH IEKVIPVAHP SMEKIHITNH RGFIKDSIAT WMVPALASDK QEEQKGCLES ACQRKTYPMC NQTSWEPFGG
401 - RQLPSYGRLT LPLDASVDLQ LNISFTHGPV ILNGDGMDYY ESPLLNSGWL TIPPKNGTIF GLINKAGRGD QFTVIPHVLT FAPRASSGNC YLPIQTSQII
501 - DRDVLIESNL VVLPTQSFRY VIATYDISRS DHAIVYYVYD PIRTISYTHP FRLTTKGRPD FLRIECFVWD DNLWCHQFYR FEANIANSTT SVENLVRMRF
601 - SCNR
```

Figure 1 Translation of the CDV-HA nucleotide sequence.

As discussed previously, the combination of peptide prediction and MHC binding validation techniques have proven to be valuable in the identification of CTL epitopes from pathogenic proteins (35-37). The most useful prediction method to date is MHC binding, due to this being the most restrictive factor contributing to immunodominance hierarchies that has been identified (38). However, predicting peptides capable of MHC binding is error prone and can result in the identification of many peptides not genuinely capable of binding the MHC, known as false positives, necessitating the confirmation of pMHC formation using MHC binding validation methodologies (39). The classic peptide stabilization assay using immortal cell lines lacking the ability to naturally load peptides into the MHC complex has shown utility to separate true binding peptides from false positives (40-42). Once identified, labeling of these cell lines with the pathogen-derived, MHC binding peptides has been successfully employed for the identification of CTL epitopes (43, 44). Accordingly, we sought to employ this strategy to identify a DLA-88*50801 restricted, CDV-HA CTL epitope in the dog.

In an effort to maximize the identification of CDV-HA derived, DLA-88*50801 binding peptides, three peptide MHC binding prediction methods were utilized. First, the DLA-88*50801 binding motif determined in Chapter 3 was used. Second, an artificial neural network (ANN) algorithm, netMHCpan, which has been successfully employed for the identification of MHC binding peptides from non-murine, non-human MHC alleles, was utilized (45). To increase specificity of this algorithm, the protein sequences of both full-length DLA-88*50801 and CDV-HA (Figure 1) were uploaded to the server as templates for the MHC backbone and antigenic protein, respectively. Lastly, the SYFPEITHI database

was used with the motif of the closest human MHC homolog, HLA-A*6801 (46). Importantly, only 9- and 11- mer peptide predictions were made as these were the most commonly observed lengths of naturally bound ligands (Chapter 3). For the 11-mer predictions, only the defined motif described in Chapter 3 and netMHCpan were used as SYFPEITHI was unable to predict any peptides of this length. Of note, netMHC pan may have a limited ability to accurately predict 11-mer peptides as the K11 peptide (RFLDKDGFIDK), a known binding peptide (Chapters 3 and 4), was characterized as a non-binder by this algorithm. The known 9-mer binding peptide K9 (KLFSGELTK) was predicted to bind.

Following the prediction of DLA-88*50801 binding CDV-HA peptides using all three methods, we further revised our prediction set by excluding peptides with major deviations from the binding trends observed for naturally processed self peptides as described in Chapter 3. In general, a predicted peptide was excluded if it contained two or more residues not matching the defined motif. Other excluded peptides contained a proline at P3, which would have a profound impact on the conformation of the bound peptide, or a glutamate at P1, a position which usually contained a positive charge and where no negatively charged AA was observed (Chapter 3). The complete list of 41 predicted peptides not excluded based upon these criteria are shown in Table 1.

Table 1 CDV-HA derived peptides predicted to bind DLA-88*50801. Each peptide has been given a number in order to easily refer back to this list throughout this chapter. Abbreviations for prediction methods are: NMP – netMHCpan, SYF – SYFPEITHI, and MOT – DLA-88*50801 binding motif from Chapter 3.

Peptide #	Sequence	Prediction Method(s)	Position in CDV-HA
1	GTIFGLINK	NMP, SYF, MOT	457-465
2	SLSMSLISR	NMP, SYF	204-212
3	SVSLSMSLISR	NMP	202-212
4	IIGDEIGLR	NMP, SYF, MOT	98-106
5	VIATYDISR	NMP, SYF, MOT	521-529
6	TTSVENLVRMR	NMP	589-599
7	AISDGVYGK	NMP, SYF	222-230
8	LTAISDGVYGK	NMP, MOT	220-230
9	TTSVENLVR	NMP, SYF	589-597
10	NLWCHQFYR	NMP, SYF	572-580
11	RVFEIGFIK	NMP, SYF, MOT	249-258
12	KTNFFNPNR	NMP, SYF	121-129
13	KTNFFNPNREF	NMP	121-131
14	TISYTHPFR	NMP, SYF	544-552
15	RTISYTHPF	NMP	543-551
16	RTISYTHPFRL	NMP	543-553
17	VVLPTQSFR	NMP, SYF. MOT	511-519
18	NLVVLPTQSFR	NMP, MOT	509-519
19	LVVLPTQSFRY	NMP	510-520
20	STSNMEFSR	NMP, SYF	64-72

Table 1 Continued

Peptide #	Sequence	Prediction Method(s)	Position in CDV-HA
21	SIATWMVPA	NMP, SYF	357-365
22	SIATWMVPALA	NMP	357-367
23	KVFPLSVSL	NMP, SYF, MOT	197-205
24	KVFPLSVSLSM	NMP	197-207
25	NMLTAISDGVY	NMP	218-228
26	LLVPDDIER	NMP, SYF, MOT	233-241
27	RLSALSGGR	NMP, SYF	171-179
28	YMVLPEDSK	NMP	271-279
29	YMVLPEDSKAK	NMP, MOT	271-281
30	AIVYYVYDPIR	NMP, MOT	533-543
31	IVYYVYDPIRT	NMP	534-544
32	QVIDVLTPL	NMP	87-95
33	QVIDVLTPLFK	NMP, MOT	87-97
34	ALLAITGVR	NMP, SYF, MOT	51-59
35	ILALLAITGVR	NMP, MOT	49-59
36	ITNHRGFIK	NMP, SYF	347-355
37	KLNEIKQFI	NMP, MOT	110-118
38	LLVGILALL	NMP, SYF	45-53
39	KAIASAANPIR	NMP, MOT	161-171
40	RLLKEDMEK	NMP, MOT	72-80
41	FILQKTNFF	NMP	117-125

*Validation of DLA-88*50801 binding for the predicted set of CDV-HA peptides*

To determine which of the CDV-HA peptides in our prediction set were genuinely capable of binding DLA-88*50801, the BARC3 peptide stabilization assay described in Chapter 4 was utilized. Briefly, each peptide was added to BARC3 cells individually and incubated overnight at 27 °C. The following day, cells were incubated for 5 hours at 37 °C to allow empty MHC molecules to be removed from the cell surface prior to anti-DLA-88 staining and flow cytometry analysis. This assay determined that 17 of the 41 predicted peptides bound and stabilized DLA-88*50801 on the surface of BARC3 cells. The 17 confirmed binders, along with the relative level of DLA-88 stabilization, are provided in Table 2.

Not surprisingly, individual peptides stabilized DLA-88*50801 to varying degrees. This finding could be due to differences in MHC affinity or off-rate between peptides; however, we hypothesize that this finding is actually the result of differences in concentration between peptides because the concentration of each individual peptide may differ by as much as a factor of three due to the format in which they were purchased. The potential difference in individual peptide concentration inhibits our ability to calculate peptide affinities or off-rates. Due to these complications, the relative hierarchy of peptide stabilization (Table 2) may not be accurate.

Table 2 Predicted peptides from CDV-HA that are capable of stabilizing DLA-88*50801 as measured by BARC3 peptide stabilization assay. Individual peptides were pulsed onto BARC3 cells as described in materials and methods and stabilized DLA-88 was measured by flow cytometry. A peptide was considered an MHC binder if DLA-88 signal intensity was increased approximately 50% over background. Numbers accompanying the peptide sequence refer to the number given in Table 1. No peptide indicates the background signal of the assay. The K11 peptide is a self-origin peptide known to bind DLA-88*50801 and was included as a positive control for the assay.

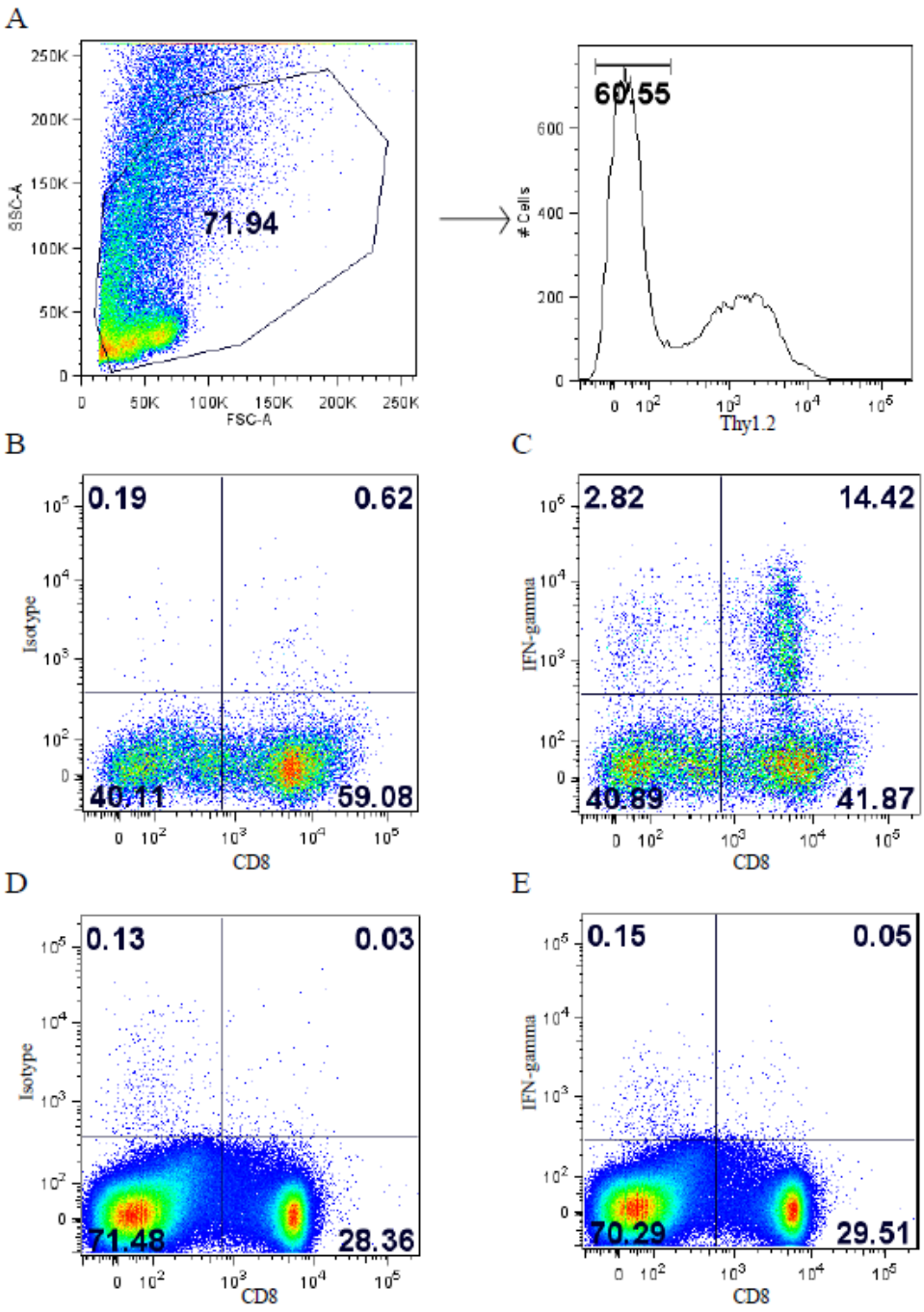
Peptide	DLA-88 Mean Fluorescence Intensity
None	24
K11	106
SIATWMVPA (21)	35
SLSMSLISR (2)	35
KVFPLSVSL (23)	40
LLVPDDIER (26)	40
YMVLPEDSK (28)	43
GTIFGLINK (1)	48
LVVLPTQSFRY (19)	49
RVFEIGFIK (11)	53
QVIDVLTPL (32)	53
KVFPLSVSLSM (24)	54
VIATYDISR (5)	55
ITNHRGFIK (36)	57
SIATWMVPALA (22)	58
KAIASAANPIR (39)	62
RTISYTHPFRL (16)	71
KLNEIKQFI (37)	73
ILALLAITGVR (35)	349

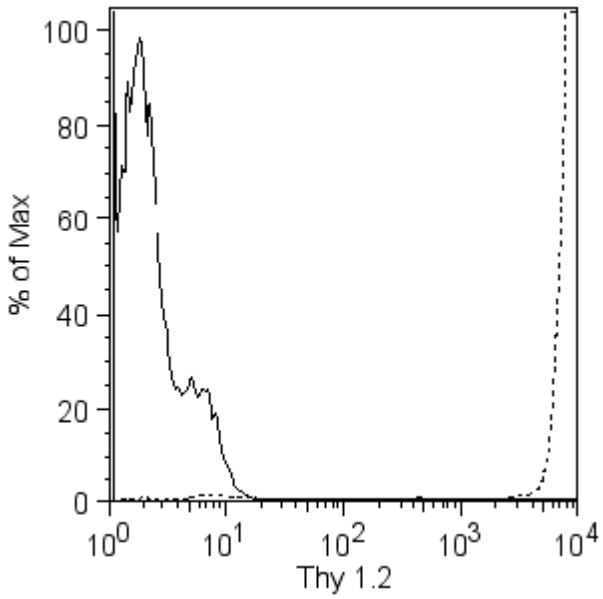
Attempting to characterize a peptide-specific CDV-HA CTL response

To identify the first peptide-specific CTL response in dogs, PBL were isolated from a DLA-88*50801 homozygous Golden Retriever that had been previously immunized against CDV. The PBL were stimulated with CDV-HA peptide labeled BARC3 cells and the frequency of interferon-γ (IFN-γ) producing $CD8^+$ events was evaluated. To minimize the number of blood samples needed and decrease the number of individual sample wells required, BARC3 cells were pulsed with pools of the DLA-88*50801 binding CDV-HA peptides to serve as stimulators. This strategy allowed for a greater number of PBL to be stimulated with each peptide pool, theoretically increasing the frequency of CTL capable of responding against one of the peptides. The peptide pools were divided based upon relative level of DLA-88 stabilization in order to minimize competitive binding (peptide 35 was pulsed alone as it stabilized MHC to a far greater extent than any other peptide; pool 1 – peptides 11, 16, 22, 36, 37, and 39; pool 2 – peptides 1, 5, 19, 24, and 32; and pool 3 - peptides 2, 4, 21, 23, 26, and 28). The isolated PBL were then incubated with the peptide pulsed stimulators at a 10:1 ratio for four days (primary stimulation). On day five, the PBL were restimulated with freshly prepared BARC3 cells pulsed with the appropriate peptide pool at approximately a 2:1 ratio for six hours in the presence of brefeldin A (secondary stimulation) and assayed for $CD8^+$ IFN-γ production by flow cytometry. It should be noted that this stimulation and detection protocol is similar to those that have been employed for the successful detection of peptide-specific responses in other species (43, 47) as well as a CDV-HA antigen-specific response in the dog (26).

To analyze the data from this assay, a gating scheme was employed that selected live cells based on forward and side scatter but excluded any Thy 1.2^+ cells (Figure 2A). This ensured that our analysis of CD8 and IFN-γ was performed only on the canine PBL as BARC3 cells are Thy 1.2^+ but the anti-mouse Thy1.2 specific antibody does not detect canine PBL (Supplemental Figure 2). As a positive control, primary and secondary stimulations were performed with unpulsed BARC3 cells with the addition of PMA and ionomycin during the secondary stimulation. Analysis of this sample with an isotype control Ab revealed a very low level of background signal (Figure 2B) while staining with the IFN-γ Ab resulted in a robust population of $CD8^+$ IFN-γ producing cells (Figure 2C). However, the PBL stimulated with the various peptide pool pulsed BARC3 cells resulted in no detectable $CD8^+$/IFN-γ^+ population indicating that a peptide-specific CTL response was not detectable using this assay (Figure 3C and D).

Figure 2 Failure to identify a peptide-specific $CD8^+$ IFN-γ response in PBL stimulated by BARC3 cells pulsed with CDV-HA peptide pools. PBL isolated from a DLA-88*50801 homozygous dog previously immunized against CDV were stimulated with BARC3 cells labeled with the CDV-HA peptide pools. The frequency of $CD8^+$/IFN-γ^+ cells was then assessed by flow cytometry. (A) Gating scheme used for data analysis. Only Thy 1.2^- cells were analyzed from a liberal forward versus side scatter population to remove the Thy 1.2^+ BARC3 cells from analysis. (B and C) PBL stimulated with unpulsed BARC3, PMA, and ionomycin as a positive control. (D and E) Representative images from one of the peptide pool stimulations (pool 3). (B and D) Isotype control. (C and E) IFN-γ. Numbers indicate percentages.


A
SSC-A
FSC-A
71.94
Cells
Thy1.2
60.55
B
Isotype
CD8
0.19
0.62
40.11
59.08
C
IFN-gamma
CD8
2.82
14.42
40.89
41.87
D
Isotype
CD8
0.13
0.03
71.48
28.36
E
IFN-gamma
CD8
0.15
0.05
70.29
29.51

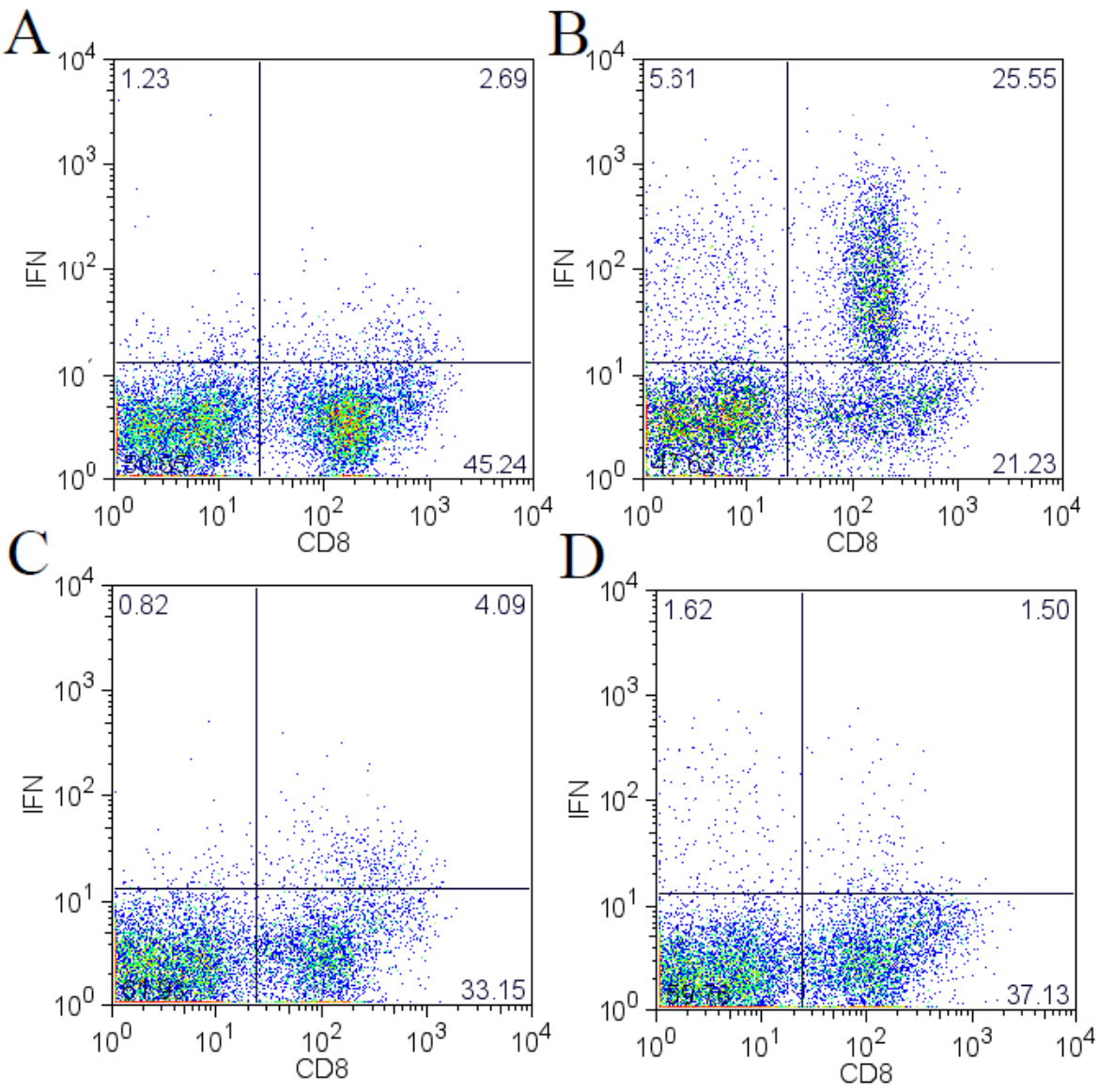


Supplemental Figure 2 Thy1.2 is expressed by BARC3 cells but not by canine PBL. Cultured BARC3 cells (dashed line) and canine PBL (solid line) were assessed for Thy1.2 expression by flow cytometry. Only the BARC3 cells were found to express Thy1.2.

Attempted confirmation of a CDV-HA antigen-specific response

As previous CDV-HA antigen-specific CTL responses in dogs were identified in individuals of unknown DLA-88 genotype and after failing to identify a CDV-HA peptide-specific CTL response within our predicted set of peptides, we sought to confirm the presence of a CDV-HA antigen-specific response in the previously immunized, DLA-88*50801 homozygous dog (25-26). The identification of an antigen-specific response would suggest that either the peptide stimulation/detection protocol used here is ineffective or that the CDV-HA epitope was not included in our prediction set; however, the failure to identify an antigen-specific response would suggest that the anti-CDV CTL response is not directed against an HA-derived peptide in the context of DLA-88*50801. To address this question, the DH82 cell line, which endogenously expresses the DLA-88*50801 allele (19), was stably transfected with a plasmid encoding the full length CDV-HA gene. These DH82-HA cells were then used as stimulators in a CTL stimulation assay analogous to those performed for the peptide pools. Unexpectedly, DH82 cells possess the ability to suppress $CD8^+$ IFN-γ production when stimulated by PMA and ionomycin. As seen in figure 3A and B, PBL are capable of a robust $CD8^+$ IFN-γ response when stimulated with PMA and ionomycin in the absence of DH82 cells. However, when DH82 cells are present, the $CD8^+$ IFN-γ response is dramatically reduced (figure 3C and D). This finding eliminated our ability to use the DH82-HA cells as stimulators and therefore we are presently unable to confirm a CDV-HA antigen-specific response in this dog.

Figure 3 Confirmation of a CDV-HA antigen-specific response is complicated by an apparent suppressive ability of DH82 cells. PBL from a CDV immunized, DLA-88*50801 homozygous dog were stimulated with PMA and ionomycin and $CD8^+$ IFN-γ production was assessed by flow cytometry. (A and B) PBL stimulated with PMA and Ionomycin. (C and D) PBL stimulated with PMA and Ionomycin in the presence of DH82 cells. (A and C) Isotype control. (B and D) IFN-γ.


A
1.23
2.69
45.24
IFN
CD8
B
5.81
25.55
21.23
IFN
CD8
C
0.82
4.09
33.15
IFN
CD8
D
1.62
1.50
37.13
IFN
CD8

The aim of this study was to identify the first, peptide-specific CTL response in dogs, and therefore demonstrate that DLA-88 restricts $CD8^+$ T cells, allowing for the definitive designation of this locus as a classical MHC class I gene. Using several algorithms, we predicted 41 peptides of the CDV-HA protein and confirmed that 17 were genuinely capable of MHC binding. Unfortunately, no peptide-specific CTL responses against any of these 17 peptides were detected. Moreover, we were unable to confirm a DLA-88*50801 restricted, CDV-HA antigen-specific response due to an unexpected ability of DH82 cells to inhibit $CD8^+$ IFN-γ production. Due to these results, we are unable to definitively characterize DLA-88 as a MHC class Ia gene.

Our failure to detect a CDV-HA specific CTL response could be explained by a potential suppressive mechanism by the DH82 cells themselves. Tumors can employ a variety of mechanisms to evade the immune system is through immunosuppression (reviewed in 48). Since DH82 cells were originally created from a histiocytic tumor (31), it is possible that they have retained some suppressive function. Indeed, tumorgenic cell lines derived from humans and mice (49, 50), as well as the dog (51), have been found to possess immunoregulatory properties. While no prior study suggests that DH82 cells are suppressive, subsequent studies could be performed to determine if an immunosuppressive mechanism is employed by DH82 cells. By incubating PBL with or without DH82 cells during the primary and/or secondary stimulation prior to PMA/Ionomycin stimulation, one could determine if the DH82 cells are in fact suppressive and during which stimulation they must be present in order to exert this effect. If a suppressive mechanism is confirmed, one could determine if this effect required cell contact or was mediated by some soluble factor by

repeating this culture/stimulation in a transwell system where both cell groups share the same media but cannot make cell-to-cell contact.

A variety of other possibilities could also explain our current inability to identify a DLA-88*50801 restricted, peptide-specific response. First, it is possible that the CDV-HA CTL epitope was not contained within our predicted peptide pool. It is important to note that only 9- and 11- mer peptides were included in this analysis, yet both 10- and 12- mer peptides are capable of binding this allele (Chapter 3). It is also possible that the CDV-HA epitope is a non-canonical peptide preventing our prediction methods from identifying this peptide (52-54). Second, the *in vitro* peptide-specific CTL stimulation prior to the IFN-γ assay may have been ineffective. Typically one includes a control peptide known to elicit a response to ensure that the stimulation protocol is effective and moreover that peptide-specific CTL are detectable (44, 55). However, no peptide-specific response is currently known in the species. PMA and ionomycin served as the positive control for IFN-γ detection, but does not provide information on the efficacy of stimulation (56-58). Lastly, although CDV-HA is the only pathogenic protein for which a CTL response has been demonstrated in dogs, the studies describing these responses were conducted using four individual animals (two beagles, two of unknown breed) of which the DLA-88 genotype was not determined (25, 26). As epitopes are defined in the context of the presenting MHC allele, it remains possible that CDV-specific CTL responses in DLA-88*50801 individuals are not directed against CDV-HA.

Importantly, this study has further confirmed the results from Chapters 3 and 4. The DLA-88*50801 binding motif identified binding peptides with a relatively low false positive

rate (8/17 motif predicted peptides were genuinely capable of MHC binding; tables 1 and 2). However, motif prediction also failed to identify 9 of 17 peptides capable of MHC binding. This finding implies that our binding motif may be too rigid in its definition of which residues are required at each anchor position, as peptides with non-motif residues at anchor positions were found to be MHC binders (notably, 8/17 true binding peptides did not have a positively charged residue at the carboxy terminus, Table 2). Redefining the motif of DLA-88*50801 more loosely may lead to the identification of a larger number of genuine MHC binding peptides but also may increase the false positive rate. In addition, the BARC3 peptide stabilization assay provided a useful method for the validation of MHC binding for the predicted peptides. Together, these results indicate that the coupling of a modified motif-based epitope prediction method and the BARC3 peptide stabilization assay might aid in the discovery of a peptide-specific, DLA-88*50801 restricted CTL response despite the failure to identify one in these studies.

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Chapter 6: Dissertation summary and future directions

Prior to these studies, Dog Leukocyte Antigen-88 (DLA-88) was believed to be the only classical MHC class I locus in dogs as it represented the only expressed class I gene that also possessed a significant level of polymorphism with 51 described alleles (1-5). However, in addition to being polymorphic, classical MHC class I genes must be expressed across many tissue types and restrict conventional αβ T-cell responses, neither of which have been described for DLA-88 (6). Accordingly, the present studies were undertaken to develop research methodologies for the characterization of a DLA-88 peptide-specific $CD8^+$ T-cell response and therefore provide information to further support the designation of this locus as a class Ia gene.

The studies described in Chapter 2 were designed to identify a DLA-88 allele that was found in high enough prevalence to warrant further study. To accomplish this, we determined the DLA-88 genotype of individuals from two dog breeds, Golden Retrievers and Boxers, by sequence-based typing. We hypothesized that inbreeding during breed creation may restrict the number of alleles present within each population as has been reported for class II loci (7-9). The results of this study indicated that relatively low interbreed, but high intrabreed, DLA-88 allelic diversity existed. Thus, we identified several candidate alleles for further study that were highly prevalent within a population. Ultimately, we focused our further efforts on DLA-88*50801 due to the large number of homozygous dogs identified and therefore CTL responses would potentially be restricted by a single MHC allele in these individuals.

Next, we sought to determine the peptide binding motif of DLA-88*50801 and thereby create a tool for the prediction of peptides capable of binding this allele from any pathogenic protein. To achieve this goal, peptides were eluted and isolated from affinity purified DLA-88*50801 molecules and sequenced by MS/MS. This analysis returned 36 sequences ranging from 9 to 12 AA in length. Overall prevalence of each AA category at each position of the bound peptide sequence was then assessed when the 36 sequences were aligned at both termini. Additional confirmation of the MHC peptide binding preferences was obtained by generating *in silico* molecular models of the pMHC and examining the binding interactions between the bound peptide and the MHC backbone. These analyses revealed that DLA-88*50801 was capable of binding multiple length peptides conforming to a X-AVILFM-AVILFM-$X_{(5-8)}$-KR motif. In addition, this study reported the surface expression of DLA-88 for the first time.

Although the determination of the peptide binding motif for an MHC allele allows for the prediction of binding peptides, motif-centric peptide binding prediction methods often identify many peptides that are not genuinely capable of binding (10). In mice, an effective method for the confirmation of peptide binding to the MHC is the RMA-S cell line in a peptide stabilization assay (11, 12). This cell line lacks the ability to naturally load peptides into the MHC and therefore lacks surface expression under normal circumstances; however, the addition of MHC binding peptides to the culture media stabilizes pMHC complexes which can then be measured by flow cytometry. In Chapter 4, we sought to adapt RMA-S cells for use in a DLA-88*50801 peptide stabilization assay by creating a stable line expressing this canine allele, as has been done previously for human and rat MHC alleles

(13, 14). The cells we established, Bispecies Antigen Recognition Cells 3 (BARC3), bind peptides K9 and K11. We then demonstrated that the BARC3 assay can be used to confirm peptide binding to the DLA-88*50801 molecule.

The studies described in Chapter 5 aimed to implement the DLA-88*50801 binding motif to predict peptides from the pathogenic protein CDV-HA that are able to bind in the DLA-88*50801 peptide binding groove, validate the binding of these peptides using the BARC3 peptide stabilization assay, and identify a peptide-specific CTL response restricted by the DLA-88*50801 allele. Out of the 41 predicted peptides from CDV-HA, we were able to confirm the binding ability of 17 using the BARC3 assay. To identify a peptide-specific CTL response, PBL from a CDV vaccinated, DLA-88*50801 homozygous dog were stimulated with BARC3 cells labeled with subsets of the 17 binding peptides and $CD8^+$ IFN-γ production was assessed by flow cytometry. However, we were unable to detect a response against any of the peptides tested in this study. This finding warranted the confirmation of a CDV-HA antigen-specific response in this animal. To address this question, we sought to analyze $CD8^+$ IFN-γ production stimulated by DH82-HA cells which endogenously express the *50801 allele and were transfected to express the CDV-HA protein. However, due to an unexpected suppression of IFN-γ production by DH82 cells, we were unable to utilize the DH82-HA cells to confirm the antigen-specific response.

Together, the data presented here has provided much needed information for the study of CTL responses in dogs. We have found that DLA-88 allelic diversity is different between breeds. This finding may make it possible to correlate specific DLA-88 alleles with susceptibility or resistance to a variety of diseases that particular breeds are predisposed to,

akin to findings for DLA class II molecules (15-17). Additionally, this study has developed valuable tools allowing for the prediction and validation of DLA-88*50801 binding peptides in the binding motif and BARC3 cell line respectively. Ultimately, this information will be exceptionally helpful in the determination of a peptide-specific CTL response despite the failure to identify one here.

Future Directions

Having failed to identify a DLA-88*50801 restricted, peptide-specific CTL response in this study, this section will outline several experimental strategies for discovering one in future work.

- It is possible that the CTL epitope was not contained within our predicted set of peptides or that the epitope is 10 or 12 AA in length. Less stringent peptide predictions could be made to expand the peptide pool to include those with weaker predicted binding ability for experimental testing. This strategy should identify more peptides capable of MHC binding. Perhaps one of these previously unidentified peptides will be recognized by responding CTL. Prior to implementing this strategy, it is necessary to detect a CDV-HA antigen-specific response. This could be accomplished through stimulation of PBL with a canine cell line co-expressing DLA-88*50801 and CDV-HA that does not inhibit IFN-γ responses.

- Identification of DLA-88*50801 bound CDV-HA peptides could be determined directly through the techniques employed in Chapter 3. Stable transfection of 9-15 cells with CDV-HA prior to DLA-88*50801 isolation and MS/MS sequencing of eluted peptides may result in the identification of naturally processed CDV-HA peptides. This technique has the advantage of being able to identify non-canonical peptides yet suffers from potential financial constraints on the amount of material required to submit for MS/MS analysis. Confirmation of a CDV-HA antigen-specific response would be prudent prior to implementing this strategy.

- Although RMA-S cells are capable of both presenting peptide-stabilized MHC molecules to stimulate a memory $CD8^+$ response (18) and generating primary CTL responses (19, 20), the stimulation protocol used here may be inadequate for CTL detection. In a previous study that focused on evaluating canine CTL, the investigators supplemented the culture media with IL-4 and IL-7 in addition to IL-2 which may aid in the differentiation and proliferation of T cells, theoretically increasing the frequency of IFN-γ^+ cells (21). Additionally, over expression of the co-stimulatory molecule B7 by RMA-S cells has been shown to potently stimulate CTL responses (22). This strategy may be useful to increase amount of IFN-γ each CTL produces. Alternatively, greater sensitivity might be achieved using a different method of detection, for example by evaluating proliferation of canine CDV-specific T cells, (using thymidine incorporation or CFSE dilution) or by directly measuring the ability of CDV-specific CTL to lyse infected target cells (using Cr or europium release). While all of these methods have

been utilized to measure CTL stimulation, currently there has not been a study aimed at determining which of these methods may be the most sensitive. However, until a CTL epitope is identified, determination of optimal experimental conditions for evaluation of additional peptides remains elusive as the absence of a response may be due to the use of a peptides that do not function as CTL epitopes.

- Perhaps the most assured method, but possibly the most costly and labor intensive, is to perform a study analogous to the original CTL epitope discoveries in mice (23-25). This strategy would involve cloning and expressing each CDV gene in a DLA-88*50801 positive cell. The maximal CTL response generated by virally infected cells would then be compared with single gene expressing cells. Following the determination of the target antigen, expressing short fragments of this gene in target cells would identify a relatively small region of the protein encoding the CTL epitope. Individual peptides from this region could then be tested for CTL recognition in order to isolate the DLA-88*50801 CDV epitope. Alternatively, this method could be utilized prior to the implementation of any of the above strategies to identify which gene product(s) are recognized by responding CTL.

- Previous studies performed on the CDV-related measles virus (MV) would suggest that the identification of a CDV-HA specific CTL response in the dog is possible. Previous studies have demonstrated that MV immune mice and humans possess strong MV-HA specific CTL responses (26-28). Moreover, these CTL have been restimulated *in vitro*

with cells constitutively expressing the MV-HA antigen and subsequently tested for peptide specificity analogous to our study (26), demonstrating that this procedure can be fruitful with a closely related virus. However, the murine studies conducted routine vaccinations much more frequently than is feasibly possible to do in the veterinary setting with dogs (26, 27). This reduced vaccination schedule may lead to a lowered CDV-HA specific CTL precursor frequency which may require a much longer *in vitro* restimulation protocol in order to detect. However, in measles seropositive humans it has been demonstrated that MV-HA specific CTL can be detected using an elongated *in vitro* restimulation procedure (28). Ultimately, while these studies would suggest that a CDV-HA specific CTL response should be identifiable in the dog, it is difficult to ensure that our stimulation and detection protocol is sufficient without a positive control peptide-specific restimulation.

The studies presented here extend our previously limited understanding of the canine adaptive immune system by demonstrating that MHC class I molecules are present at the cell surface and bind short peptides derived from a diverse set of proteins conforming to an overall sequence motif. This motif allows for the prediction of MHC class I binding peptides. Together, this work demonstrates that many of the discoveries that have been made regarding murine and human classical MHC genes also apply to the canine system. Based upon the similarities that have been demonstrated here, subsequent work is likely to establish that the DLA-88 locus restricts canine CTL responses in an allele and peptide-specific manner.

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