

ABSTRACT

NEMEC, PAIGE SCARLETT. Identifying Canine Cancer/Testis Antigens for Use in Hematologic Cancer Immunotherapies. (Under the direction of Dr. Paul Hess).

Development of immunotherapies for chemoresistant cancers is desperately needed for patients of various species. Studying spontaneous cancer in the dog offers a suitable model for the advancement of cancer immunotherapy treatments for both human and canine medicine. Vaccines and adoptive cell therapy (ACT) are two immunotherapies that have recently shown promising results in cancer patients. However, the high cost and time-consuming protocols of ACT make this therapy unfeasible for most pet owners. Vaccines are a more practical treatment-option due to their relatively low cost, quick and easy administration, and low-risk for toxicities. Nevertheless, to develop advanced immunotherapy treatments, identification of tumor associated antigens (TAA) and their major-histocompatibility complex classical class I (MHC-I)-restricted epitopes, which are recognized by cytotoxic CD8⁺ T cells, need to be elucidated. Many of these epitopes have been found in humans, however, the canine is significantly running behind.

In order to pursue immunotherapy in the dog, as both a patient and pre-clinical model for human medicine, we sought to identify canine CD8⁺ T cell TAA epitopes in Boxer's and Golden Retrievers-- two popular, inbred dogs with increased risk of the highly chemoresistant hematologic cancer, T cell lymphoma (TCL). T cell epitope discovery can be assisted by determination of binding motifs of common dog leukocyte antigen (DLA) class Ia allotypes. Boxer's and Golden Retrievers have restricted MHC diversity resulting in high prevalence of the DLA-88*034:01 and DLA-88*508:01 class one alleles in each breed, respectively. The peptide binding motif is only known for the latter allele, so we sought to identify the motif of DLA-88*034:01 to assist peptide prediction from tumor antigens. To determine the motif of this allele, we sequenced bound peptides from DLA-88*034:01-FLAG complexes by liquid

chromatography-tandem mass spectrometry (LC-MS/MS). Indeed, we identified an allele specific motif that prefers 9-mer peptides with the amino acid preferences: basic at position (P)1; hydrophobic at P2; acidic at P4; histidine at P6; and phenylalanine at P9.

With two prevalent, breed-dominant MHC allotype peptide binding motifs, we were able to search for potential canine TAA epitopes. We were specifically interested in identifying cancer/testis antigens (CTAs) which have proven to be successful targets in human cancer immunotherapy treatments. CTAs are appealing due to their expression being limited to immunoprivileged germ line tissues such as the testis and/or ovary, high and frequent expression in multiple cancer types, and use in as an “off-the-shelf” treatment. We found DLA-88*508:01-restricted peptides that mapped back to 5 human orthologue TAAs: IGF2BP3, AKR1E2, SPECC1, TPX2 and NT5C1B. To identify these as canine CTAs and to establish their utility as an immunotherapy target, we probed normal and malignant canine tissues for their expression. NT5C1B proved to be the most ideal target due to its limited expression in normal tissues and its expression in over half of the TCL samples we probed. During our CTA search in the literature we also identified CCTA-4 as a possible immunotherapy target.

Once allele-restricted TAA peptides are established, it is important that their potential to activate CD8⁺ cytotoxic T lymphocytes (CTL), the immune cell responsible for tumor killing, be determined. As a positive control, we infected the canine cell line, DH82, with CDV and obtained allele restricted CDV gene products. These gene products were then tested in a number of CTL assays, but we were unable to identify a viral CD8⁺ epitope CTL --something that has not yet been done in the dog. Although identifying an anti-viral peptide specific CTL was not achieved, the information and tools gained from this work will allow for the advancement of antigen discovery in the dog and potentially lead to a successful cancer vaccine.

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Identifying Canine Cancer/Testis Antigens for use in Hematologic Cancer Immunotherapies

by
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DEDICATION

My dissertation is dedicated to my wonderful friends and family who provided me support in every form and always believed in me. Also to those who have been directly or indirectly impacted by cancer in any way –we are working towards a cure!

BIOGRAPHY

Paige Nemec was born in Garfield Heights, OH, but raised in the rural town, Mantua, OH by her parents, Gary and Geri Nemec with her younger brother, Conner and younger sister, Sydney. Paige has always been quite active and played soccer, softball, volleyball, ran track and wrestled growing up. However, soccer and wrestling proved to be her two core sports and she became the first female in Ohio to qualify and win a match at the Ohio High School State Wrestling Tournament and went on to play NCAA Division II soccer at Lake Erie College. As a female in the male-dominated sport of wrestling, Paige had to overcome many obstacles to break-through the stereotype. She believes this taught her that perseverance, dedication and hard-work, pays off and has ultimately shaped who she is today.

Paige has always had a love for science and pursued a Bachelor's degree in Chemistry, with minors in Biology and Mathematics at Lake Erie College. While at Lake Erie, Paige had the fortunate opportunity of interning at The Lubrizol Corporation, as a synthetic chemist intern. Her mentors at this internship inspired her to pursue a PhD; she desired the depth of knowledge and the level of thinking they displayed. Although Paige enjoyed her chemistry education, which can be attributed to her wonderful mentor, Dr. Jonathan Tedesco, she found a passion in biology and more specifically immunology. After taking a number of biology classes with Dr. Deborah Schulman, she decided to switch paths and pursue a PhD in biomedical sciences.

In 2014, Paige enrolled in the NCSU Comparative Biomedical Sciences Program where she joined Dr. Paul Hess's Lab to pursue research in identifying canine cancer antigens for use in cancer immunotherapies. Although Paige does not have a dog of her own, she often dog-sits and has many step-dog children she feels are like her own. Paige hopes to help both humans and her canine family with the research she has performed at NCSU.

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CHAPTER 1: CANCER IMMUNOTHERAPY IN CANINES: PLAYING CATCH (UP) WITH HUMANS

Introduction

Immunotherapy is the current focus for treating patients with various cancers and is needed to complement traditional chemotherapy strategies that fail to eliminate tumor burden. These are typically metastatic solid tumors and hematologic malignancies such as lymphoma, leukemia and myeloma. The first immunotherapy strategy to eradicate cancer dates back to the 1890's by Dr. William Coley who treated patients with bacterial components and observed tumor regression in some patients. Coley developed this therapy after observing some patients who had previously been infected by *Streptococcus pyogenes* went into complete remission, although some patients experienced what is now known as a “cytokine storm”¹. Since then, the idea of whether immunotherapies can be effective without having serious off-target cytotoxicity has been highly debated. However, various strategies have seen clinical success and are currently used in human medicine.

Cancer is also a major problem for dogs too, with similar outcomes as humans, warranting the search for immunotherapies in this species. Many pet owners are on the lookout for other cancer therapy options, but the lack of immunological tools in the canine is a rate-limiting step we must overcome. Here, we provide evidence for the need of new cancer therapies in the dog, how immunotherapy can be successful, as evidenced in human medicine, and the tool-kit we are developing to make this possible.

Canine Cancer

Out of the close to 90 million pet dogs living in the US, around 6 million will be diagnosed with cancer annually^{2,3}. Although cancer affects all dogs, it is notable that certain

breeds, specifically those that are highly inbred, have a higher incidence of cancer related deaths than others. For instance, it has been observed that popular pure breeds such as Bernese Mountain Dogs, Boxers, and Golden Retrievers are in the top 5 dog breeds with the highest proportion of neoplastic causing deaths ⁴. Pure breeding introduces selective pressures and although may produce admirable phenotypic traits, undesirable genetic traits and diseases, such as cancer, are also inherited ^{5,6}.

Of the most common canine malignancies, hematological neoplasms are both frequently diagnosed and quite deadly for man's best friend, with 10-12% of all canine cancer deaths stemming from lymphoma, the most common hematological malignancy ⁷. Similar to humans, the standard care of treatment for dogs diagnosed with cancer is chemotherapy, with remission rates close to 90% ⁸. Although remission rates are high they are typically short lived. The prognosis is especially poor in dogs with highly chemoresistant hematological malignancies such as T-cell lymphoma (TCL) and histiocytic sarcoma (HS). In the overall canine population, T cell lymphoma (TCL) makes up 20-40% of diagnosed lymphomas, which are highly prevalent in many popular breeds such as Boxers and Retrievers and is more resistant to chemotherapy than the more common, B cell lymphoma ⁹⁻¹². Currently, the prognosis for dogs with TCLs undergoing standard chemotherapy with the drugs, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone (CHOP) is between 5-8 months, with only approximately 20% dogs diagnosed with TCL living beyond 2 years ^{10,12,13}. The prognosis for dogs diagnosed with HS is much worse; with two studies demonstrating a median survival rate of less than 200 days in dogs treated with chemotherapy ^{14,15}.

This chemoresistance has initiated the quest for improved cancer treatments. Although various targeted therapies have been developed for canine malignancies, efficacy has been

underwhelming in clinical trials^{16,17}. There have been a multitude of human cancer therapy studies demonstrating success with various cancer immunotherapies, but the tools for these studies have been well established¹⁸⁻²⁰. One such tool that is commonly used for the identification for CTL responses against tumors is a peptide-MHC tetramer²⁰⁻²⁴. Success with this tool depends on identifying a specific cancer antigen and its MHC-restricted immunogenic peptide. The peptide-MHC complex is then developed into a multimer which acts as magnet for epitope-specific T cells. The tetramer can be used as a tool for discovery of epitope-specific T cells or for detection of epitope-specific T cells during an immune response such as after bacterial and viral infection or after vaccination (such as a cancer vaccine)^{25,26}.

Unfortunately, an epitope-specific T-cell response nor a MHC class I or class II tetramer has not been identified for use with dog samples²⁶. This makes identifying a CTL to verify cancer vaccine efficiency in the dog very difficult. The goal of our studies was to develop the necessary tools to identify CTL in dogs after vaccination with a cancer antigen. In order to do this we needed to identify a TAA to target, determine a dog MHC allele(s) motif to analyze, search for peptides in the TAA that may bind to the specific MHC allele, verify peptide binding using an allele-specific cell line, and then determine peptide immunogenicity using a CTL assay. Once these pieces are put together, a canine epitope specific pMHC tetramer can be developed for use in detecting viral or tumor CTL responses.

Current Immunotherapies (Vaccines)

There is a growing list of immunotherapies that are currently used to treat patients with cancer. The T-cell growth factor antibody, Il-2, has demonstrated antitumor activity in renal cell carcinoma and melanoma, and although chance of toxicity in multiple organs is a concern, current research is focusing on concentrating the Il-2 activity to the tumor microenvironment.

Another ground-breaking immunotherapy strategy involves targeting the check-point receptors, CTLA-4 and PD-1 on T cells and PD-L1 on cancer cells. The commercialized CTLA-4 antibody, ipilimumab, works by depleting regulatory T cells (Tregs) in the tumor microenvironment and PD-L1 acts by binding to PD-1 on T cells to suppress effector T-cell function^{27,28}. The most recent breakthrough therapy that is currently approved by the FDA to treat B-cell lymphoma is the use of chimeric antigen receptor T (CAR-T) cells. This strategy works by engineering the T cells to recognize a specific antigen on the tumor while not being restricted to the MHC haplotype. However, the antigen must be expressed on the surface of the cell and not be present in critical cell or tissue types. Because it is possible to replace the functionality of B cells with exogenous immunoglobulin Gs (IgGs), current CAR therapies target CD19 and CD20, which destroy both normal and malignant B-cells²⁹. This functional replacement is not possible for T cells, so a more tumor specific antigen (TSA) is needed to be identified for this to occur. Secondly, most TSAs are not cell surface molecules and are presented via an MHC molecule and therefore need other strategies utilizing cytotoxic T cells such as adoptive cell transfer and vaccination.

Vaccination is an appealing immunotherapy because it is inexpensive, relatively non-toxic, and deliverable on an outpatient basis. This therapy is also attractive in settings with limited resources such as in third world countries and in veterinary medicine. However, demonstrating high efficacy remains a challenge for the numerous vaccines strategies that have been evaluated. Since Coley's toxin vaccination in 1943, many vaccination milestones have been achieved. In 1943, Dr. Ludwick Gross demonstrated 3-methylcholanthrene (MCA)-induced tumors were prevented when vaccinated with tumor cell suspension³⁰. This work was later shadowed in 1976 when Hewitt et al. challenged the idea that vaccines could be effective

against spontaneous cancers when his laboratory observed no tumor immunogenicity, although this critique was later questioned by a number of immunologists³¹⁻³³. The potential for tumor vaccination therapy became more widely accepted in 1982 when Van Pel and Boon demonstrated that the murine immune system can provide vaccine-induced protection against murine thymic leukemia³⁴. Many experiments in the years to follow led to two important concepts: that immune responses can be tumor-specific and that immunization can prevent tumor growth. Many critical lessons have been learned along the way, such as, the suppressive environment the tumor bears on the immune system and the need for higher specificity, or, tumor specific antigens^{35,36}. Rosenberg did just that in 1998 when he utilized a peptide based vaccine encoding two melanoma antigens, MART-1 and gp100, to successfully immunize 42% of the patients receiving the vaccine³⁷. This study also drew focus to the use of dendritic cells (DCs) for antigen delivery to prime and expand T cells and to target DCs for adjuvant activation for effective immune responses after vaccination. This led to a surge in vaccines targeting various melanoma antigens and other shared differentiation antigens. However, in 2004, a cancer immunotherapy update from Rosenberg et al. presented a dim view of cancer vaccines showing only a 2-3% objective response rate regardless of vaccine type³⁸. But Rosenberg remained hopeful and indicated that this was not a dead-end for vaccines, but rather, the approach needed to be modified. One of the biggest concerns for vaccine strategies at the time was if the right antigen was being targeted. Targeting the popular differentiation antigens resulted in low T-cell avidity and low number of antigen specific T cells.

Cancer Antigens (T cell recognized)

Cytotoxic T lymphocytes (CTLs) are responsible for immune surveillance of neoplastic cells by recognition of transformed cell's altered protein repertoire. This recognition is restricted

by the class I major histocompatibility complex (MHCI) allele and the peptide presented in the MHC binding groove³⁹. Self, viral and/or cancer proteins within a cell are cleaved via the proteasome which generates a peptide pool where MHC allele motif-restricted peptides will be delivered into the endoplasmic reticulum through the transporter associated with antigen processing (TAP) complex. A peptide with a matching motif will be loaded into the MHC complex, which is made up of the class I heavy chain and β_2m , and exported to the surface of the cell for surveillance by immune cells. Healthy cells with MHC complexes presenting self-peptides will not cause a CTL response due to the cognate T cell either being deleted or becoming a regulatory T cell during T-cell maturation therefore avoiding a potentially deadly autoimmune response⁴⁰. However, if a healthy cell becomes virally infected or oncogenic, foreign peptides will be displayed via the MHC class I and activate CD8+ T cells to kill the infected or cancerous cell. This defining property of CD8 effector T cells, is the basis of many current cancer treatments that target tumor associated antigens (TAAs).

TAAs can be divided into different classes such as tissue differentiation antigens, normal proteins overexpressed by cancer cells, viral antigens, tumor specific neo-antigens and tumor germline antigens (cancer-testis antigens- CTAs). Each one of these classes can be divided into shared and non-shared tumor antigens as well as low-tumor specificity or high-tumor specificity antigens^{41,42}. Each group of TAAs have pros and cons to be useful for immunotherapy treatment, but overall the ideal TAA should be frequently overexpressed in cancer cells, not expressed in normal tissues (except for immunoprivileged sites such as retinal and germline tissues), immunogenic, and expressed across many tumor types. The latter would not prevent a TAA from becoming a immunotherapeutic target, but is an ideal characteristic when it comes to treating a breadth of patients. A summary of these T-cell recognized TAAs is illustrated in Figure 1.

Furthermore, the Translational Research Working Group of the National Cancer Institute generated a list of criteria to categorize the top “ideal” tumor antigens. These criteria are as follows (with prioritization in descending order): therapeutic function, immunogenicity, role of the antigen in oncogenicity, specificity, expression level and percent-positive cells, stem cell expression, number of patients with antigen positive cancers, number of epitopes and cellular location of expression⁴³. Using these criteria, the NCBI determined a list of 75 tumor antigens they described as “ideal” cancer antigens based on the score given for each characteristic they evaluated. Some of these antigens will be described in the next sections and are also depicted in Table 1.

Shared vs. Non-shared TAAs

Shared antigens include differentiation antigens, normal proteins overexpressed by cancer cells, germline TAAs and viral antigens⁴⁴. Most shared cancer antigens targeted for immunotherapy today are ‘self’ antigens which is somewhat surprising and brings into question whether an effective immune response can be generated against self-peptides expressed in tumors. This is especially questionable when considering immune tolerance. Peripheral and central tolerance are two road blocks in immunotherapy utilization. Peripheral tolerance is an extrathymic process where TAA specific T cells become suppressive (regulatory T cells, Tregs), anergic or deleted in the periphery. This may be due to lymph node stromal cells (LNSCs) expressing tissue specific antigens (TSAs) or T-cell interaction with tolerogenic DCs which lack co-stimulatory signals or express programmed death ligands (PD-L)⁴⁵. These Tregs have a pro-tumor effect by suppressing CTLs in an antigen-specific manner⁴⁶.

Central tolerance is also a potential hindrance in TAA immunotherapies. Some TAA expression has been found in the thymus which results in deletion of T cells recognizing these

self-tissue-restricted antigens due to their expression via the autoimmune regulatory gene, AIRE. However, even with these obstacles, we know that the immune system is indeed able to mount an effective response because tumor infiltrating lymphocytes (TILs) have been isolated and demonstrated anti-tumor activity and used as adoptive cell transfer (ACT) immunotherapy in some cases^{47,48}.

Of the shared TAAs, viral antigens are the only non, self-antigens. Viral antigens are ideal targets because only infected cells present viral peptides on their surface and they are seen as foreign antigens, circumventing the issue of autoimmune toxicities and immune tolerance, respectively. The downside of these antigens is the low frequency of virally associated tumors, limiting the breadth of patients that can be treated with this type of immunotherapy⁴¹. However, vaccine development against viral antigens has seen great success in humans. One clear example is the Human Papilloma Virus (HPV) vaccine, which targets the major virus like particles in the oncogenic HPV strains⁴⁹. Epstein Barr Virus (EBV) is another virus that causes some cancers. It is responsible for ~10% of gastric carcinomas and Hodgkin's and Burkitts lymphoma⁵⁰.

Although a vaccine has yet to be licensed against, Phase 1 and II clinical trials for a vaccine against the viral antigen, gp350, demonstrated safety and efficacy in preventing development of infectious mononucleosis, which is induced by EBV. However, phase III clinical trials have yet to be performed due to the vaccine not preventing latent EBV infection⁵¹. Overall, shared TAAs have an advantage in a clinical setting because off-the-shelf therapies can be developed for a more rapid treatment turnaround time and their expression can be easily detected and monitored.

Non-shared antigens are mutated self-proteins, where neo-epitopes arise in a patient specific manner. Various immunotherapies have demonstrated effective anti-tumor responses to neo-epitopes^{52,53}. Neoantigens are formed due to mutations which lead to the generation of

peptides that are only found in the patient's specific tumor cells. These peptides are therefore not subject to central tolerance so will not only be seen as foreign peptides to CTLs, but also pose less risk for autoimmune toxicities. Neoantigens are more commonly identified in tumors with a high mutational burden, such as melanoma (UV exposure) and lung cancers (tobacco smoking and pollution inhalation)⁵⁴. However, neoantigens have been discovered in cancers associated with lower mutational burden such as lymphoma. These neo-antigens are typically mutated immunoglobulins that are recognized by autologous T cells⁵⁵⁻⁵⁷. Nevertheless, detecting neoantigens require a tumor tissue biopsy, genetic sequencing, and manufacturing, which is invasive, costly and time-intensive⁵³.

In the context of the dog, and highly metastatic tumors such as HS and TCL, a shared TAA is a particularly felicitous target for immunotherapy. This is due to the quicker turn-around time to administer treatment and potential lower cost for off-the shelf therapeutics. Because HS and TCL are typically late stage diagnoses, a quick treatment may mean the difference between disease free survival and death. Low-cost treatments are needed because many owners will opt not to treat their pet due to high treatment costs, sometimes with only limited efficacy and potential decrease in the quality of life in the patient⁵⁸.

Shared Self TAAs with Low Tumor Specificity

Tissue differentiation antigens and overexpressed self-antigens are both examples of shared TAAs with low tumor specificity. Tissue differentiation antigens are defined as antigens that are restricted to a specific tissue type in both normal and malignant cells. The first tissue differentiation antigens to be discovered were tyrosinase, MART-1 and gp-100⁵⁹⁻⁶¹. These antigens were derived from melanomas and are expressed in normal melanocytes and retinal cells. Although they have been used as targets in peptide and vector based vaccines,

immunological efficacy has remained low, even with the addition of various adjuvants and cytokines⁶²⁻⁶⁴. Furthermore, these antigens are not broadly expressed across tumors and more importantly, pose the risk for an autoimmune reaction due to their expression in somatic, healthy tissues. Yee et al. directly demonstrated this in a study where one patient who was treated with CD8⁺ T-cell clones specific for MART-1, developed inflammatory lesion that resulted in loss of cutaneous melanocytes. This was verified by characterizing the T-cell infiltrates in the skin lesion biopsy using MART-1 peptide-MHC (pMHC) tetramers which revealed that 28% of the CD8⁺ T cells were MART-1 specific and responsible for the melanocyte death⁶⁵. Due to the expression in normal tissues, tissue differentiation antigens are not ideal immunotherapy targets, but the rationale is that the expression in normal tissues is below the “threshold” in which CTL will recognize the antigen and therefore avoid autoimmune toxicities⁴⁴.

Another type of shared TAA that demonstrate low tumor specificity, are overexpressed self-antigens. These antigens face similar challenges to tissue differentiation antigens due to the fact that they are self-antigens that are expressed in normal tissues as well as cancerous tissues. However, some of these antigens such as HER-2/neu and p53, have demonstrated humoral and CTL responses after peptide vaccination, although clinical responses were not significant^{66,67}. HER-2/neu is an example of an overexpressed self-antigen that has been widely studied for clinical use in cancer immunotherapies. HER-2/neu is an attractive target because it is highly overexpressed across multiple tumor types, mainly of epithelial origin, with overexpression in 25% of invasive breast and ovarian cancers, ~45% of pancreatic cancers and up to 66% of gastrointestinal adenocarcinomas having been reported⁶⁸⁻⁷¹. In patients treated with HER-2-neu specific T cells or antibodies, autoimmune responses are absent, suggesting that basal expression of HER2-neu on normal tissues does not activate an immune response. Furthermore, it may be

that HER2-neu specific T cells recognize overexpressed subdominant epitopes since cognate T cells for the dominant epitope are most likely deleted in the thymus during development, thereby overcoming central tolerance^{72,73}.

Wilms' tumor gene (WT1) is an example of an overexpressed TAA found in hematological malignancies. WT1 is found across various cancer types such as acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) as well as a variety of solid cancers. Although not broadly expressed in normal tissues, WT1 is expressed in normal kidney and in germline tissues such as the gonads and ovaries, making this antigen blur lines with a germline TAA⁷⁴⁻⁷⁷. Initial phase I and II clinical trials demonstrated a WT1 peptide vaccine given to patients with AML caused no adverse effects, but efficacy was not determined to be clinically significant^{24,78}. Due to the high ranking of WT1 on the prioritized cancer antigens by the National Cancer Institute, more clinical trials are likely to be funded and immunotherapies against WT-1 developed⁷⁹.

So although TAAs that are expressed in normal tissues can still be potential immunotherapeutic targets, these antigens should be considered with precaution: an autoimmune response is possible and even if the response is non-fatal, the adverse effects are not ideal for the patient and in order to overcome tolerance (in most cases) and produce a significant clinical response more combination therapeutics need to be evaluated.

Shared Self-Tumor Antigens with High Tumor Specificity

Cancer/testis antigens (CTAs) are both self-proteins and highly tumor specific. CTA expression is limited to germline cells and during embryo development, but neoplastic cells aberrantly express CTAs due to epigenetic mechanisms⁸⁰. For reasons that are not yet clear, reactivation of these genes seems to contribute to many cancer characteristics^{81,82}. Most

identified CTAs, in which their function has been determined, are highly active during gametogenesis and serve to promote migration, meiosis, implantation and immortalization^{81,83}. Interestingly, these gamete associated functions mirror many of the properties of cancer cells such as transformation, invasion, aneuploidy and metastasis^{81,83-85}. Although these antigens are highly expressed in tissues such as the testis and ovary, these sites are immunologically privileged, meaning they lack classical MHC molecules and therefore avoid cytotoxic immune recognition^{86,87}. These unique properties make CTAs promising therapeutic targets for cancer immunotherapy.

The first human CTA to be discovered was in 1991 from a melanoma patient and was termed the melanoma antigen-1 (MAGE-A1). An autologous CTL responses was successfully elicited against MAGE-A1 and the researchers further demonstrated normal tissue expression was limited to the testis and placenta⁸⁸. Since then, 12 MAGE family members have been identified and all located on the X-chromosome⁸⁹. This led to an explosion of CTA discoveries and clinical trials looking to exploit the unique properties of these antigens. Although some clinical trials using MAGE as the therapeutic target initially seemed promising, neurological and cardiac on-target, off-tumor toxicities proved to be fatal in some patients^{90,91}. These toxicities have prompted researchers to seek out a more promising CTA target.

Another X-linked, well-studied CTA, is New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1). NY-ESO-1 was first discovered by Chen et al. using serological analysis of recombinant cDNA expression libraries (SEREX)^{92,93}. By generating a tumor cDNA library and combining autologous patient serum, researchers were able to analyze antibody responses against specific tumor antigens, in which they observed a high IgG titer indicating helper T-cell involvement. Furthermore, they were able to identify a CTL clone that specifically

lysed NY-ESO-1 transfected cells and verified peptide specific lysis using peptide pulsed T2 cells and analyzed chromium release from the NY-ESO-1 CTLs⁹⁴. Not only were humoral and cell-mediated responses to NY-ESO-1 recorded, but expression was limited to only the testis, ovary and tumor tissue, making this a clear target for immunotherapy treatment⁹². The refined expression of NY-ESO-1 to only reproductive tissues and its expression across many tumor types greatly reduces the potential for off-target effects and allows for a broader pool of patients eligible for NY-ESO-1 specific immunotherapy. Indeed there is an increasing number of clinical trials in humans using various immunotherapies targeting NY-ESO-1, with some vaccine strategies having encouraging results⁹⁵.

NY-ESO-1 and MAGE are arguably the most successful tumor CTA targets to date, but with improved immunological methods and increased knowledge of the CTAs, the search is still on for the “ideal” tumor antigen. Furthermore, with the emergence of different vaccine formulations, such as combination therapies with checkpoint inhibitors and next generation adjuvants to further promote a CTL response, the future is looking bright for cancer vaccines.

Cancer Vaccines in the Dog

Interestingly enough, dogs were the first species to receive a USDA- approved therapeutic vaccine to treat cancer. Oncept is a DNA vaccine that received full licensed approval to treat canine oral melanoma in 2007⁹⁶. The vaccine targets the human melanocyte protein tyrosinase and triggers an immune response in dogs, but due to its similarity to canine tyrosinase, targets this protein which is abundant in the dog’s melanoma tumor cells. But as discussed previously, because this is an overexpressed antigen normally found in somatic tissues, off-target toxicities are of concern. Furthermore, the efficacy of this vaccine remains controversial due to the lack of randomized clinical trial data that, unlike the FDA, the USDA does not require.

Multiple retrospective studies analyzing the clinical benefit of Oncept found no difference in progression-free survival, disease-free survival or median survival times in dogs receiving or not receiving the vaccine^{97,98}. There is a clear need for further development of vaccines in the dog.

Current clinical trials in the dog are determining the efficacy of autologous tumor vaccines for treatment of dogs with malignant tumors^{99,100}. The focal point behind this vaccine strategy is the mutational burden cancers naturally acquire which result in neoepitopes and are a natural source for immunogenic antigens, since the mutated antigen is non-self. This is not a new concept^{101,102}, but next generation sequencing has allowed for specific identification of these neoantigens and this approach has been demonstrated in human studies, but it has not been established whether private antigens are better than shared antigens^{53,103,104}.

Our focus is on developing immunotherapies, specifically vaccines, for dogs with hematologic malignancies, particularly PTCL and HS. Although neoantigen discovery is an attractive angle to employ, there are some issues in exclusively adopting this approach: 1) this is a more time consuming option than off-the shelf vaccine strategies (time most patients do not have to spare), 2) the cost may be beyond what owners are willing to pay, and 3) there is evidence that tumors such as lymphoma have very low mutational burden¹⁰⁵. Therefore, in formulating immunotherapy for canine PTCL and HS, we took a two-pronged approach, building tools that would allow us to hunt for both neoantigens and shared CTAs.

In humans, a DNA vaccine using peptide analogue sequences from the CTA, Per ARNT Sim domain containing 1 (PASD1), was successful at inducing epitope-specific T-cell responses against acute myeloid leukemia tumors in human leukocyte antigen (HLA)-A2.1 transgenic mice (HHD)¹⁰⁶. A similar study used a DNA fusion gene vaccine and found PASD1 epitope specific CTL in HHD mice¹⁰⁷. Other studies investigating PASD1 in hematologic malignancies found

epitope specific CTL and CD4 responses in diffuse large B-cell lymphoma patients¹⁰⁸.

Furthermore, a phase-II clinical trial evaluating a MAGE-A3 multi-peptide vaccine combined with Poly-IC as an adjuvant and granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance T-cell priming, showed promising results in multiple myeloma patients. MAGE-A specific CTL responses were detected in 88% of patients and 62% of patients demonstrated clinical responses (partial, near-complete and complete) at days +180¹⁰⁹. Some human CTAs have been identified and studied in canine solid tumors, but less success has been found in hematological malignancies¹¹⁰⁻¹¹². With increasing evidence that CTA vaccines can be successful in human hematologic malignancies, and expanding knowledge of CTA expression in canine cancers, developing a canine CTA vaccine against hematologic malignancies is an optimistic strategy.

Ultimately, both neoantigens and CTAs are promising candidates for use in canine cancer vaccines, which warrants an empiric approach such as a head-to-head vaccine trial to determine the best method.

Classical Class I MHC

Because we are primarily interested in CD8+ T-cell responses (CTLs) against tumors, it is necessary to understand the MHC class I molecules and peptides that drive those responses. Classical class I MHC gene are characterized as being highly polymorphic, expressed on the cell surface, having wide tissue distribution and control CD8+ T-cell responses¹¹³. In humans, there are three class I classical gene loci, human leukocyte antigen (HLA)-A, -B and -C, and each loci has over 1,000 different alleles where each allele varies in preference of peptide size and amino acids that will bind¹¹³. The outbred nature of humans has led to high polymorphism at each MHC loci and many heterozygote individuals. Therefore, the large diversity of peptide sets that will be

presented by any one pathogen decreases the likelihood it will spread within a population¹¹³. Because HLA alleles have been widely studied, allele frequency data is readily available and specific haplotypes have been associated with ethnicities as well as disease^{114,115}. Unfortunately, large studies of MHC prevalence data has not been found for the dog leukocyte antigen (DLA). However, multiple factors make this task less formidable: 1) dogs have only one known classical class I MHC allele, DLA-88, with only 73 alleles discovered to date and 2) due to the desire for purebred dogs in Western culture, the likelihood of one or two highly prevalent alleles to stand out within breeds is probable¹¹⁶⁻¹¹⁹. The high prevalence of an MHC allele within breeds increases the chances of any one dog in this breed will be eligible for peptide based vaccine study.

Developing a Canine Immunologic Tool-kit

To find the right antigens and peptides for a vaccine study, we needed to build some immunologic tools for the dog. First, we identified at risk canine populations for tumors we are interested in, and searched for prevalent DLA-88 alleles in these breeds¹²⁰. Secondly, we needed to develop a DLA-88-retrievable cell line, based on either PTCL or HS, so we could define the motif (needed for neoepitope prediction) and to look for CTA peptides. Finally, we needed to develop an assay that would allow us to verify peptide binding for DLA-88 motifs. We have previously accomplished these tasks for DLA-88*508:01, a prevalent allele in Golden Retrievers^{121,122}.

This project was designed to take divergent paths forward, and expand to a second DLA-88 allotype, and if possible, lead to a vaccine trial in dogs- these goals were achieved. Our first step was to identify an epitope-specific CTL in dogs, and formally establish DLA-88 as a classical restriction element, which has not yet been done- this work, using DLA-88*508:01, is

described in Chapter 2. Next, Chapter 3 will detail the pursuit of CTA-origin peptides in the DLA-88*508:01 allotype and assess their preliminary immunotherapeutic usefulness by defining expression across tumors and normal tissues. In Chapter 4, we define the motif of a second prevalent DLA-88 allotype, DLA-88*034:01, and search for peptide epitopes from the same CTAs. Chapter 5 will describe how we built a cell line to verify the motif experimentally, and viral peptides for cytotoxicity assays that will be used as a positive control in a vaccine trial. Finally, Chapter 6 will briefly mention the discovery of canine cancer/testis antigen (CCTA)-4 as a high value CTA target that will be used in an upcoming vaccine clinical trial.

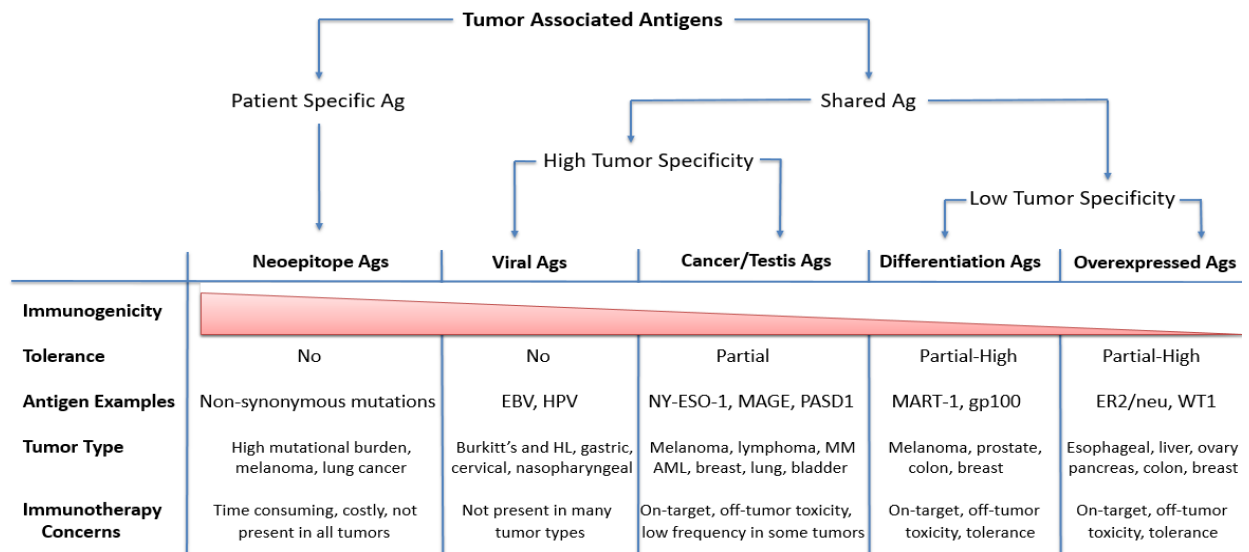


Figure 1.1. Summary of Tumor Associated Antigens (TAAs). TAAs can be divided between neoantigens and shared antigens. The neoantigen group consists of mutated antigen that the immune system sees as foreign, making them highly immunogenic. Only a few cancers have a high mutational burden, such as melanoma and lung cancer, and therefore neoepitopes are hard to target in all cancer types. They are also typically patient specific, increasing the cost and time for therapy against these antigens. Shared tumor antigens have the advantage of becoming targets for off-the-shelf therapies. However, due to tolerance mechanisms, their immunogenicity may not be as high. Viral antigens are seen as foreign, but only certain cancers are caused by viruses and so would be infective targets for most cancers. Cancer/testis antigens have seen success in human medicine with NY-ESO-1 and MAGE family targeted immunotherapies, but off tumor toxicities have increased awareness for more refined targets. Furthermore, differentiation and overexpressed antigens have surprisingly seen some success as immunotherapy targets, even with expression found in normal tissues and tolerance high. Each TAA has value as a immunotherapy target and further studies are need to expose their potential to decrease tumor burden and off-target toxicities.

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CHAPTER 2: DETERMINING CDV EPILOPE-SPECIFIC CD8⁺ CTL IN THE DOG

Abstract

Virally infected and cancerous cells are combated by cytotoxic CD8⁺ T lymphocyte (CTL) responses which are initiated by the interaction between a T-cell receptor (TCR) and an immunogenic peptide bound to class I major histocompatibility complexes (MHC I). In the dog, known CD8⁺ CTL responses have been limited to the protein level and specific epitopes have yet to be identified. Here we sought to determine the immunogenic peptide responsible for the CTL response against the Paramyxoviridae family viral pathogen, canine distemper virus (CDV). Most dogs are vaccinated against CDV and therefore should have a subset of CD8⁺ T cells ready to rapidly respond to secondary infection. The inbred nature of dogs has resulted in high prevalence of specific MHC class I alleles within breeds which is useful when studying MHC-restricted CD8⁺ T-cell responses. Our lab has previously determined that the MHC class I allele, DLA-88*508:01, is highly prevalent in Golden Retrievers (40%) and identified its peptide binding motif. This information allowed us to recognize DLA-88*508:01-restricted peptides from CDV and test for immunogenicity by monitoring intracellular interferon- gamma (IFN- γ) production by CD8⁺ T cells using flow cytometry and other means. Although a distinct CTL was not determined, the diverse self and viral peptides presented by DLA-88 allowed us to classify DLA-88 as a classical MHC class Ia gene by using a combination of mass spectrometry and cell-based binding assays. These methods can be extended to other MHC alleles and for discovery of processed and presented viral peptides for use in CTL assays, an important assay commonly used to determine vaccine efficacy.

Introduction

Tumor-Associated Antigen (TAA) discovery is important for developing cancer immunotherapies such as vaccines. Along with identifying new TAAs, there is a need to verify and monitor effective CTL responses elicited by CD8⁺ T cells to track vaccine efficacy. The gold standard to identify epitope-specific CTL responses is through peptide-MHC (pMHC) tetramer technology. This technology works by folding a specific (typically highly prevalent and disease associated) MHC allele heavy chain with β_2 -microglobulin (β_2m) along with the MHC allele-restricted peptide, followed by biotinylation and tetramerization of the complex to a fluorescently-coupled streptavidin¹. The specificity of the peptide allows for identification of a distinct T-cell clone. Garboczi and Wiley et al. were the first to describe this process using HLA-A2 and influenza viral peptides in 1992 and soon after Altman et al. demonstrated the use of the pMHC technology to identify HIV specific T- cells^{1,2}. This sparked the use of pMHCs to identify CTL from viruses and the potential for use against tumor antigens as well. The use of tetramers to identify antigen specific responses is now quite common at the clinical level to track vaccine efficacy³.

The use of tetramers is advantageous due to their ability to obtain CD8⁺ epitope specific T cells that may be physiologically exhausted. Moreover, they allow further expansion of low-number circulating epitope specific T cells. With the increase in TAA research, pMHC tetramers have been extended to identifying CTL against various tumor associated antigens. Well-established MHC-restricted epitopes are commonly used as a technical and experimental controls in CTL assays and are readily available in human, mice and non-human primate research. However, identification of MHC-restricted antigen epitopes are greatly lacking in other species, with the exception of bovine leukocyte antigen pMHC against the protozoa pathogen *Theileria*

*parva*⁴.

We are interested in developing a cancer vaccine for the dog and therefore will need a way to track efficacy of the vaccine. To do so, our goal is to establish a TAA epitope-specific pMHC tetramer in the dog. However, because an epitope specific CD8⁺ CTL response has not yet been documented in the dog, we first wanted to identify a positive control antigen to track CTL. Due to the ubiquitous nature of viral infections in the human population, viral epitopes are commonly used as positive CTL controls. In 2002, Currier et al. identified a pool of HLA-A and HLA-B-restricted viral peptides for use as a standard positive and quality control in CTL assays⁵. Identifying CTL against ubiquitous viruses such as influenza, HIV and cytomegalovirus (CMV) using tetramer technology are commonly used to track CTL in humans^{1,6,7}.

In the dog, no epitope controlling CD8⁺ T-cell responses have been reported and only a few CTL responses against entire proteins have been described: canine distemper virus (CDV), rabies virus, *Leishmania infantum*, autologous tumor cells, minor histocompatibility antigens (miHAgs) and the shared tumor antigen, HER-2/neu⁸⁻¹³. Dogs are not affected by ubiquitous viral infections like humans, making identifying a CTL against any one viral epitope a difficult task. However, the low levels of circulating viral CD8⁺ T cells are often boosted by canine vaccines. Dogs are commonly vaccinated against CDV, increasing the likelihood of identifying CDV epitope CTL¹⁴. Furthermore, a CTL response against the CDV protein, Hemagglutinin, has been documented, providing evidence as to where we might find an immunogenic epitope⁸. Moreover, CDV is the canine equivalent to the measles virus (MV) in humans, in which CTL responses have been found¹⁵. Finally, our DLA-88*508:01 specific cell line was easily infected with CDV, so choosing CDV as our model antigen was easily pursued.

Previously, we have identified canine CDV peptides restricted by DLA-88*508:01, a prevalent DLA allele in the popular dog breed, Golden Retrievers. A cell line was generated to express FLAG-tagged DLA-88*508:01 complexes in the parent canine histiocytic sarcoma cell line, DH82. The cell line was then infected with CDV (Onderspoort vaccine strain), expanded, lysed and immunoprecipitated (IP) to isolate DLA-88*508:01-restricted peptides. After mass spectrometry analysis, CDV peptides were identified¹⁶.

CDV encodes six genes: fusion (F), hemagglutinin (H), large polymerase (L), matrix (M), nucleoprotein (N), and phosphoprotein (P) and we found a total of 22 unique peptides from all six gene products. Some peptides were artifactual from the discovery process and intracellular proteolysis, so the list was further refined to 11 peptides that seemed “real” based on pMHC cell-surface stabilization assays. This assay utilizes the murine cell line, RMA-S, which has a defective transporter associated with antigen processing (TAP) protein. Impaired TAP prevents peptides from entering the endoplasmic reticulum (ER), which results in unstable and only transient expression of pMHC complexes on the cell surface¹⁷. The low level surface expression of endogenous MHC allows for the transfection of a specific MHC allele and for subsequent analysis of allele specific peptide binding. Using a MHC specific antibody, MHC complexes that are stabilized on the cell surface can be identified via flow cytometry. When a peptide is bound, the MHC complex is stabilized and allows for antibody staining, however, if no peptide binds in the MHC pocket, the complex destabilizes off the cell surface and MHC cannot be detected with an antibody. Eleven CDV peptides demonstrated MHC binding via flow cytometry and were considered non-artifact and restricted to DLA-88*508:01¹⁶.

The following peptides were verified as true binders: H₂₂₂₋₂₃₀, H₈₇₋₉₇, L₅₆₄₋₅₇₃, L₈₇₇₋₈₈₆, L₂₀₀₃₋₂₀₁₂, M₃₆₋₄₆, M₂₁₇₋₂₂₇, N₉₀₋₁₀₀, N₄₂₇₋₄₃₇, N₃₁₂₋₃₂₃ and V₁₂₉₋₁₃₉ (Supplemental table 1). Of these

11 candidates, the H peptides were the most appealing to test for immunogenicity due to CTL being demonstrated previously against the CDV-H protein⁸. However, tetramer staining against MV CTL has demonstrated immunogenicity against five or more different peptides, all at similar frequencies, so testing all 11 peptides was also warranted. Because developing tetramers for all 11 peptides would be costly and time consuming, we decided to identify immunogenicity using another commonly used method for identifying CTL –intracellular cytokine staining (ICS).

ICS is a well-established way of identifying stimulating peripheral blood mononuclear cells (PBMCs) using flow cytometry. When naïve CD8 T cells meet their cognate pMHC, they undergo clonal expansion and differentiate into effector and memory T cells. When stimulated a second time with the same antigen, effector and memory T cells release pro-inflammatory cytokines such as interferon gamma (IFN- γ), tumor necrosis factor- alpha (TNF- α) and interleukin-2 (IL-2) to clear virally infected or tumor cells¹⁸. ICS utilizes this CTL activity to identify T cells that have been activated by a certain antigen or peptide. After the cells have been stimulated with antigen, a golgi stop such as brefeldin A, is used to inhibit protein transport. The cytokine of interest can then be intracellularly detected via flow cytometry.¹⁹ We also stimulated T cells with PMA (20 ng/ml) and ionomycin (4 ug/ml), a protein kinase C activator and calcium ionophore, respectively, as a positive control for generating IFN- γ , to make sure we can detect it when it's being produced by T cells. Here we describe our results and challenges in identifying CDV DLA-88*508:01 epitope-specific CTL in the dog.

Materials and Methods

Cell culture and PBMC Isolation

Anticoagulated venous blood (~10 mL) was aseptically collected from a healthy Golden Retriever under a protocol approved by the NCSU Institutional Animal Care and Use

Committee. PBMCs were isolated by centrifugation (400 g x 30 min at room temperature) over a Ficoll-Paque™ PLUS 1.077 density gradient (GE Healthcare, Uppsala, Sweden). The harvested interface was washed twice with PBS, resuspended in RPMI 1640 containing 10% FBS, P/S and 2 mM L-glutamine (R-10 medium). Downstream use of the washed PBMCs varied depending on experiment (Table 1). The canine HS cell line DH82 (CRL-10389) was obtained from the ATCC, and grown in Dulbecco's Modified Eagle's Medium containing 15% FBS, penicillin (100 IU mL⁻¹)/streptomycin (100 µg mL⁻¹) (P/S). The BARC3 (RMA-S cells stably expressing DLA-88*508:01-FLAG) cell line had been previously generated and was cultured in RPMI media supplemented with 10% heat-inactivated FBS, P/S and 2 mM L-glutamine (R-10 medium). BARC3 cells were treated with mitomycin C (Sigma) at 50 µg/ml for 30 min at 37 °C and washed thoroughly 3 times to prevent transfer of drug to PBMC responder cells.

Restimulation of canine PBMCs with CDV (Peptide or Vaccine)

PBMCs were plated at various concentrations and in different flat-bottom tissue culture plates (depending on the experiment). All PBMCs were resuspended in R-10 media supplemented with IL-2 at concentrations of 5 or 10 U/ml. The peptides were in DMSO and added to cultures at either 5 µM for high affinity peptides or 10 µM for low affinity peptides. The live canary pox (CPV) virus vector was used in all three vaccines (CPV-H/F (CDV, hemagglutinin (H) and fusion (F) proteins) (Purevax Ferret Distemper Vaccine), CPV-R (rabies) (Purevax Rabies Vaccine), and CPV-FeLV (feline leukemia virus) (Purevax Recombinant Leukemia FeLV Vaccine)), and were obtained from the North Carolina State University College of Veterinary Pharmacy (Raleigh, NC). CPV-H/F infection of DH82 cells was added at 25 µl of vaccine to 1 ml of culture medium (flow data for optimized dose (high infection, minimal killing) is shown in Figure S1. Media was replaced with fresh R-10 media supplemented with Il-

2 (in some experiments II-7 also), every 2-3 days.

CTL: Intracellular Cytokine Staining

Peptide-pulsed or virally infected PBMCs, or DH82s, were used as target cells or peptides were added alone for the CTL assays. The cell based CTL assay used effector to target (E:T) ratios based on the number of responder cells were still viable after 1, 2 or 3 week restimulation with CDV antigen. Some responder cells were incubated with PMA (Sigma) (40 ng/ml) and ionomycin (Fisher Bioreagents) (4 µg/ml) as a positive control to detect cytokine production. The effector and target cells, (or just peptide) were incubated together for 6 hours at 37 °C, 5% CO₂. After the first hour, the golgi stop, brefeldin A (Biolegend) was added to culture at 1x from a 1000x stock. After 6 h, the cells were harvested, washed in FACS wash (PBS, 2% FBS, 2 mM EDTA, 0.02% sodium azide + 5% normal goat serum) and stained with the following antibodies, anti-canine CD8a (YCATE55.9-APC, eBioscience) at 1:50, anti-canine CD5 (YKIX32.2-PE, eBioscience) at 1:20 (positive control for flow gating) and incubated for 20 minutes at 4 °C in the dark. The cells were then fixed and permeabilized with the eBioscience Intracellular Fixation and Permeabilization Kit (Invitrogen). The cells were then intracellularly stained with either the canine cross-reactive anti-bovine-IFN-γ (PE, CC302, AbD Biotech) at 1:100 or isotype control, Armenian Hamster IgG Isotype Control PE (PE, eBio299arm, eBioscience) at 1:200 at 4 °C for 30 minutes in the dark. After 3 washes with the fix/perm buffer, the cells were analyzed via flow cytometry.

CTL: Target Killing

Target cells, CDV-infected DH82s or PBMCs, were stained with CFSE (CellTrace, C34570, Invitrogen) (0.25 µM) and placed at 37 °C, 5% CO₂ in a humid incubator for 20 minutes. The “green” target cells were then cultured with effector cells for 6 hours. The cell

death marker, 7AAD, was then added to the cells. Some cells were microwaved as a positive control for cell killing. The cells were not washed after 7-AAD addition and then they were analyzed by flow cytometry. CTL was measured by the number of CFSE⁺7AAD⁺ cells in each group.

T-Cell Cloning

After PBMC isolation for a DLA-88*508:01 positive dog as described previously, the cells were placed in a 6 well plate and incubated at 37 °C, 5% CO₂ for 2 h. The non-adherent and adherent were separated into two wells and the adherent PBMCs were infected with CPV-H/F and incubated as previously described, overnight. The following day, the infected adherent and non-adherent responder cells were mixed together at a ~2:1, responder to stimulator (R:S). Fresh media supplemented with IL-2 was added every 2-3 days. This process was repeated once more on day 7, except the responder cells were stained with CFSE, before being added to the infected autologous adherent PBMCs and incubated at 37 °C, 5% CO₂, overnight. The next day, the cells were stained with anti-canine CD8a (YCATE55.9-APC, eBioscience) antibody as previously described and DAPI was added to gate out dead cells. Cells were gated on CD8⁺CFSE^{lo} cells and subsequently single cell plated in three 96-well round-bottom plates (Supplemental Figure 2). The left over cells were pooled and placed into one well and further single cell sorted by limiting dilution. CPV-H/F infected autologous PBMCs were irradiated (30 Gray) and added to the sorted cells as a source of antigen every week. Unfortunately, we were unable to establish a T-cell clone due to high cell death and no detection of a proliferating clone after week 5.

Results and Discussion

To test the immunogenicity of the CDV peptides, we first identified a previously CDV vaccinated, DLA-88*508:01 positive dog whose T cells we could use as effector cells in a

cytotoxicity assay. Although the vaccinated dog should have circulating CDV specific CD8⁺ T cells, they are still rare, so to increase the likelihood of detecting the CDV-CTL, we restimulated canine PBMCs before the CTL assay. We used multiple strategies for the restimulation and CTL stage (summarized in Table 1).

Before we attempted to find a CTL from one of the CDV DLA-88*058:01-restricted peptides, we first wanted to establish that CTL was produced against whole CDV (experiment 1, Table 1). To accomplish this, we infected MHC-matched DH82 cells with the Onderspoort strain of CDV --the strain dogs are vaccinated against and is how we originally discovered the class I presented CDV peptides—and UV-irradiated the cells before stimulating PBMCs from a MHC-matched dog. Also, we UV-irradiated the infected DH82 cells to kill the virus and prevent the live CDV from transferring to responders in culture²⁰. However, we were unable to identify a CTL response in experiment 1 (Table 1). Interestingly, it has been shown that irradiation of APCs can cause release of Th1 response-suppressing molecules such as Il-10 and Il-12 antagonists^{21,22}. Although we thoroughly washed CDV-infected DH82 cells after irradiation, before adding to responder cells, it cannot be ruled out that potential immune suppressive molecules and/or UV-irradiated induced variables prevented IFN- γ production by effector cells. This may be an explanation for the lack of CTL response found in experiment #1, but unlikely since UV-irradiated PBMCs are commonly used in CTL assays, although the exact mechanism behind this technique is elusive. Therefore, we used peptide-pulsed cells for stimulators in the next experiments.

Moreover, we thought autologous adherent PBMCs may be better at providing any co-stimulatory signals needed to augment a CTL response over an immortal cancer cell line, so used adherent PBMCs pulsed with peptide as stimulators. APCs are able to cross-present exogenous

peptides to CD8+ T cells, so we hypothesized that the APCs in the PBMC population would be able to present peptides from the culture to responder cells, so we pulsed PBMCs with CDV peptide pools, high and low affinity peptides. We previously predicted affinity of each CDV peptide. We divided the peptides into two pools based on affinity to reduce competition of binding that may result in masking of low-affinity peptides. However, even when adding peptides alone or using peptide-pulsed DH82 cells in the CTL assays (experiments 2 and 3, Table 1), we could not detect measurable CTL by ICS.

Next, we decided to use BARC3 cells as stimulators (experiment #4) because they are immortalized cells that are abundant and more obtainable than autologous PBMCs. Furthermore, we have identified peptide-binding to the cell surface by MHC stabilization assays. However, we got similar results from our CTL assay (experiment #5, Table 1). This may have been due to lack of co-stimulatory molecules, such as B7-1, on BARC3 cells, which has been shown to be necessary for effector and memory T-cell activation²³. Transfecting BARC3 cells with the B7-1 gene may be an approach to consider in the future that may resolve this shortcoming.

We thought that ICS may not be sensitive enough so we decided to analyze target cell lysis instead. Measuring chromium (⁵¹Cr) release of target cells after culture with effector cells is the gold standard for measuring CTL, but due to the toxic nature and unavailability of ⁵¹Cr, we pursued a flow cytometry based assay instead (experiments #6-9, Table 1). While pursuing these experiments, Dow et al. published a paper demonstrating PD-L1 is expressed on DH82 cells and we hypothesized this may have prevented CTL responses during restimulation. PD-1 is expressed on T cells and acts as a co-stimulatory inhibitor molecule when is bound by its ligand, PD-L1, and therefore this interaction is a regulator of peripheral tolerance²⁴. Other inhibitory molecules may be expressed on this cell line, but due to lack of canine specific reagents, this

question remains unknown. We then only used DH82 cells during the 6-hour CTL after this because we thought the time frame was too brief for PD-L1 to suppress CTL. However, we again did not identify a CTL response, so we may have underestimated the effect of PD-L1 on T-cell suppression and did not use the DH82's after experiment #8.

During this time, we also thought failure to detect CTL may be due to two possible scenarios: our probable CDV peptides are not immunogenic epitopes in DLA-88*508:01, or this dog was not individually responsive to them (vaccine failure). Also, although the dog had been vaccinated multiple times, there is variation from patient to patient in immune responses⁹ and given the old age of the dog (12 years of age), the aged immune system may have not given a very robust response²⁵.

Neither possibility seemed likely. Assarsson et al. has shown that in general, that 50% of peptides have an available corresponding TCR for response²⁶, and with two class I-binding H peptides, one should be recognized. However, both could be wrong, so we went backwards to try to identify whole viral antigen CTL. CTL against the CDV H protein has previously been observed and so we sought to use H antigen in our next experiments. Use of recombinant H protein is a possibility, but it is costly and technically challenging to successfully get stimulators and targets to cross-present antigen from entire protein. A simpler method is to infect stimulators and targets with virus. We already ruled out using live CDV vaccine, which may kill responder cells and introduce additional variables to the assay, so an alternative route was needed. To circumvent these difficulties, we used a canarypox virus (CPV) that expressed Onderstepoort H and F (CPV-H/F), but is replication incompetent. We determined the optimal dose of CPV-H/F that would infect DH82 cells (Supplemental Figure 1) We then used this model in various CTL experiments (experiments #6-10). Fortunately, there are parallel CPV viruses expressing the

rabies glycoprotein or feline leukemia virus FeLV p27 that we used as negative controls for our targets. Surprisingly, we observed potential “real” CTL in some wells against rabies, however, replicate wells did not confirm this phenomenon (experiment #10, Figure 1).

We were puzzled by the lack of CTL from our CDV stimulated responder cells. The testable possibility is that H is a subdominant response in dogs (or potentially just this particular dog), and that dominant epitopes lie in other gene products (there are 7 total in CDV). In humans, responses have been found against the fusion (F) and nucleoprotein (N) proteins in measles virus (MV), the human equivalent of CDV²⁷⁻³⁰. There are no CPV vaccines expressing other CDV genes, making it difficult to directly analyze and identify non-H or F epitope CTL. Due to these difficulties, we decided to add peptides directly to responders.

Peptide vaccine development and *in vitro* CTL studies have demonstrated that adding peptide alone can induce an immune response and various mechanisms have been proposed for this phenomena³¹⁻³³. For this reason we used the minimal epitopes that we found by MS and confirmed presentation via MHC stabilization assays, indicating they may be legitimate targets for CTL, and pulsed autologous PBMCs directly with peptide. We did use this approach early on in experiment #3, however, this time we narrowed down our peptide pools in experiments #11-13 in order to exclude the possibility of peptide binding competition limiting an immunodominant peptide from binding and, to increase the concentration of a single peptide being presented by APCs within the PBMC population. The only potential CTL we observed in these assays was in experiment #11, where there was a ~0.15% increase in CD8⁺IFN- γ ⁺ cells in response to the CDV matrix peptide (M₃₆₋₄₆), a high affinity, DLA-88*508:01 binding peptide. Although percentages as low as 0.1% have been concluded as a positive result, we felt that because this was only one data point, we needed reproduction of these results to truly confirm

the validity of this conclusion (Figure 2)³⁴.

Unfortunately, there were many moving parts in these experiments, and it is possible, that our restimulation and target conditions were suboptimal in a way we didn't know, and only a finite number of combinations can be tested. Time and costs were also limitations to the combinations we could have tested, so we also tried a different strategy, determining whether we could generate a T-cell line or clone against these epitopes. It has only been accomplished once in the dog—Rimmelzwaan et al. isolated CD4 T cell clones against canine parovirus—so we based our methodology on this experiment and techniques that have been performed in humans and in bovines for *Theileria parva*^{4,35}. One method we used was to sort proliferating CD8⁺ T cells (CFSE^{lo}) after restimulation with CPV-H over a 4 week period, but keeping the primary cells viable proved to be a difficult task (Supplemental Figure 2). One reason for this was lack of reliable canine specific CD3 or CD28 antibodies. These are co-stimulatory molecules that are typically needed to keep long-term cultured primary T cells viable. We also added irradiated PBMCs and various cytokines to the PBMC culture. Nonetheless, we still observed high cell death, even with the consistent addition of Il-2, an important cytokine for T-cell survival and homeostasis³⁶.

Interestingly, some experiments indicate IL-2 promotes regulatory T-cell (Treg) differentiation and will suppress CTL activity and there is conflicting evidence on the use of low vs. high concentrations of IL-2 to prime CTL^{37,38}. CTL and expansion of epitope-specific CD8⁺ T cells have been reported using Il-2 concentrations ranging from 5 U/ml to 1000 U/ml^{30,39}. Nanan et al. demonstrated measles epitope-specific CD8⁺ CTL responses could be obtained from in-vitro peptide stimulation with use of Il-2 addition at 5 U/ml³⁰. Because we were attempting to identify the canine equivalent, we consistently used 5-10 U/ml concentrations of Il-2 in our

experiments. There is also evidence that the cytokine, Il-7, is important for expansion of memory T cells and reduced cell death *in vitro*, and has been used in CTL assays, so to increase cell viability and help augment a CTL response we added Il-7 in some experiments⁴⁰⁻⁴³. However, in our hands, addition of Il-7 did not seem to provide an advantage of cell survival and expansion. Some studies have added the cytokines Il-15 and Il-21 for enhancement of effector cell differentiation and cytotoxic T-cell activation, so experimenting with more cytokines and cytokine combinations may help boost T-cell proliferation and production of IFN- γ ^{44,45}.

Despite failing to identify an immunogenic CDV peptide, we did demonstrate that DLA-88 processes and presents diverse self and viral peptides indicating it is a classical MHC class Ia gene. Furthermore, we identified CDV peptides that are restricted to DLA-88*508:01 using mass spectrometry and cell-based binding assays. This method in identifying a MHC motif and allele-restricted peptides can be applied to any MHC allele of interest.

Table 1. Summary of CTL Experiments Performed.

| Exp. # | Stimulator | Number/length of stimulation | Target | Epitopes | Control | Output | Result |
|--------|--|------------------------------|---|---|--------------------------------|-----------------------------|----------------------------------|
| 1 | CDV infected DH82 cells | 1 x 6 days | CDV infected DH82 cells | Not specific | Uninfected DH82 cells | IFN- γ (IC) | No CTL |
| 2 | Peptide-pulsed PBMCs* | 1 x 6 days | Peptide pulsed DH82 cells | High and low affinity peptide pool** | K11*** | IFN- γ (IC) | No CTL |
| 3 | Peptide pulsed PBMCs* | 1 x 6 days | Peptide only | High and low affinity peptide pool** | K11*** | IFN- γ (IC) | No CTL |
| 4 | Peptide-pulsed BARC3 cells | 1 x 6 days | Peptide-pulsed BARC3s | High and low affinity peptide pool** | K11*** | IFN- γ (IC) | No CTL |
| 5 | Peptide-pulsed BARC3 cells | 1 x 6 days | Peptide only | High and low affinity peptide pool** | K11*** | IFN- γ (IC) | No CTL |
| 6 | CPV-H/F infected DH82 cells | 1 x 6 days | Peptide pulsed DH82s | H ₈₇₋₉₇ and H ₂₂₂₋₂₃₀ | CPV-R [±] and K11*** | Target killing [§] | No CTL |
| 7 | CPV-H/F [±] PBMCs | 1 x 6 days | CPV-H/F [±] infected DH82s | Not specific | CPV-R [±] | Target killing [§] | No CTL |
| 8 | CPV-H/F [±] PBMCs | 1 x 6 days | CPV-H/F [±] infected DH82s | Not specific | CPV-FLV [±] | Target killing [§] | No CTL |
| 9 | CPV- H/F [±] PBMCs* | 1 x 6 days | CPV- H/F infected PBMCs* | Not specific | CPV-FLV [±] | Target killing [§] | No CTL |
| 10 | CPV- H/F and CPV-R [±] infected PBMCs | 2 x 6 days | CPV-H/F and CPV-R [±] infected PBMCs | Not specific | CPV-H/F and CPV-R [±] | IFN- γ (IC) | Possible Rabies CTL |
| 11 | Peptide-pulsed PBMCs | 2 x 6 days | Peptide-pulsed PBMCs | H ₈₇₋₉₇ , H ₂₂₂₋₂₃₀ and M ₃₆₋₄₆ , M ₂₁₇₋₂₂₇ | K11*** | IFN- γ (IC) | Possible CDV- M ₃₆₋₄₆ |
| 12 | Peptide-pulsed PBMCs | 3 x 6 days | Peptide only | M ₃₆₋₄₆ and pool of L ₂₀₀₃₋₂₀₁₂ , N ₉₀₋₁₀₀ , N ₃₁₂₋₃₂₃ , V ₁₂₉₋₁₃₉ | K11*** | IFN- γ (IC) | No CTL |
| 13 | Peptide-pulsed PBMCs | 2 x 6 days | Peptide only | M ₃₆₋₄₆ , H ₈₇₋₉₇ , H ₂₂₂₋₂₃₀ | K11*** | IFN- γ (IC) | No CTL |

All PBMCs are autologous and * indicates adherent PBMCs were used

*High affinity peptide pool: H87-97, L2003-2012, M217-227, M36-46, N90-100 and low affinity peptide pool: H222-230, L564-573, L877-886, N427-437, V129-139, F169-178

*** K11= self-peptide (DLA-88*508:01)

±Canarypox virus (CPV)-H(Hemagglutinin)/F (Fusion), CPV-R (Rabies), CPV-FeLV (Feline Leukemia Virus)

§Target killing analyzed via flow cytometry measuring CSFE⁺7AAD⁺ cells

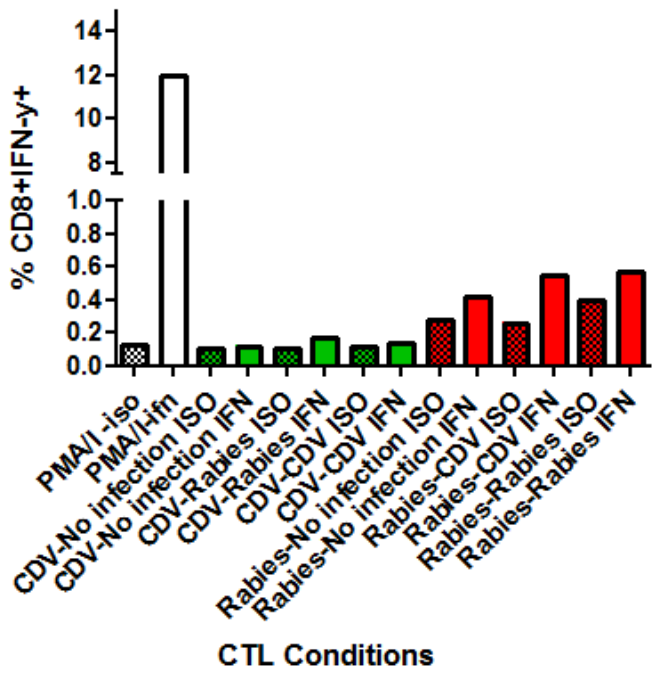


Figure 2.1. Potential rabies antigen CTL response. PBMCs were infected with either CPV-H/F, CPV-R or not-infected for 24 h then responder cells (autologous PBMCs) were added and restimulated for 6 days. This process was repeated for a second restimulation period of 6 days. Autologous PBMCs were obtained on day 13 and infected with either CPV-H/F or CPV-R and used as target cells. The 2 week-long restimulated responder cells were added to the target cells at a 2:1, E:T ratio for a 6 hour period. After 1 hour, brefeldin A was added. After 6 hours, CD8⁺ cells were identified for IFN- γ production using flow cytometry. ISO, isotype.

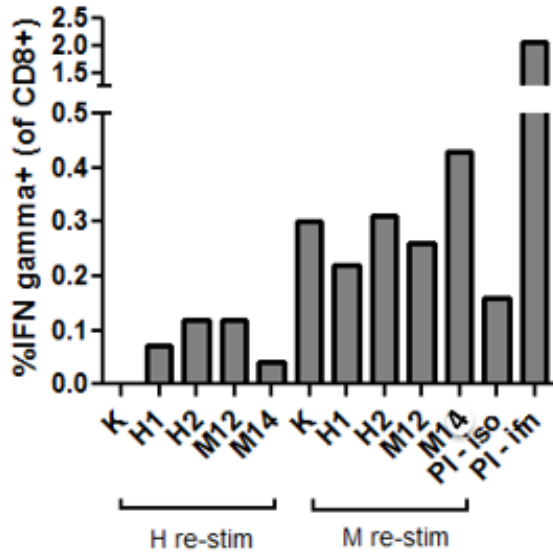
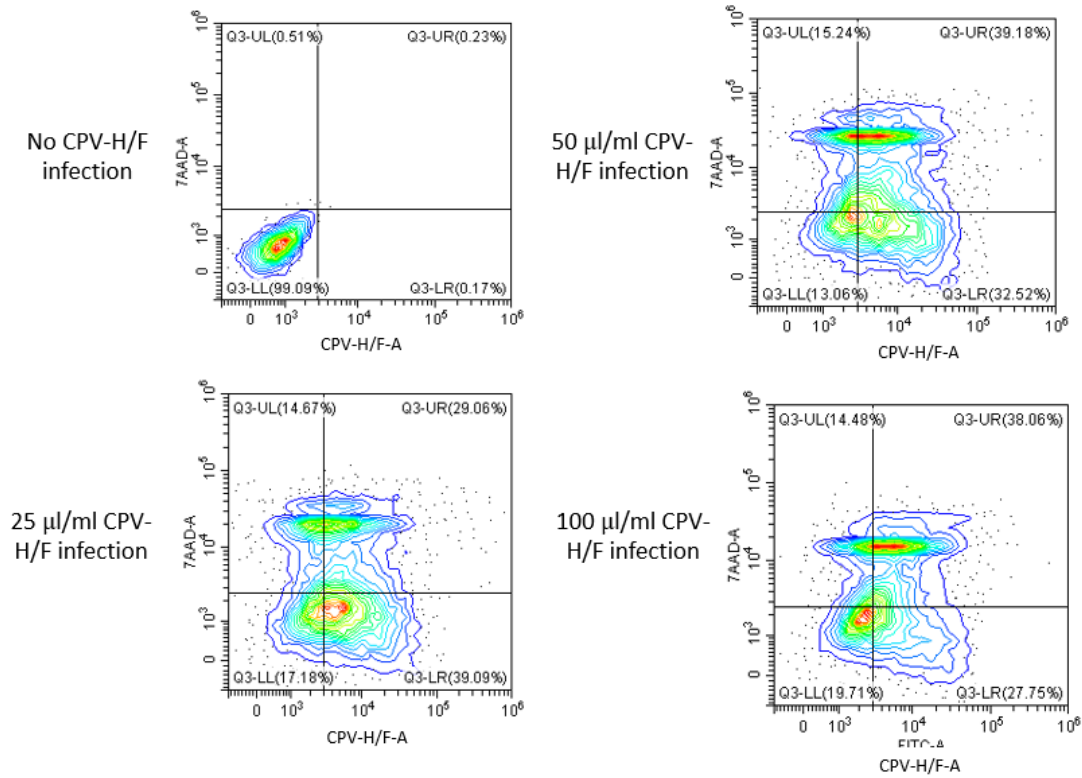
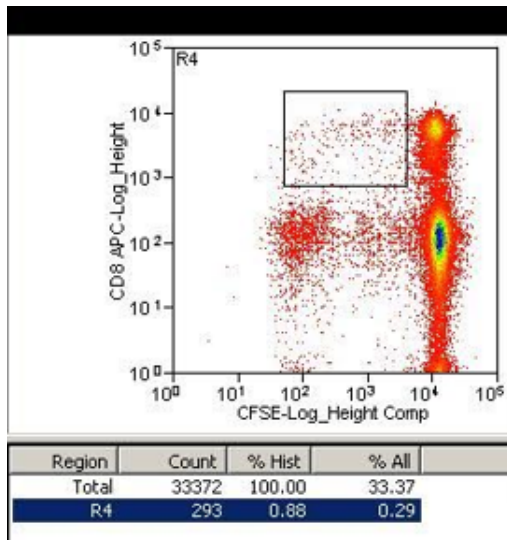


Figure 2.2. IFN- γ production by canine CD8⁺ T cells after stimulation with CDV H and M peptides ex vivo. from a DLA-88*508:01 GR were plated in a 6 well tissue culture plate (1.5×10^7 cells) and primed with either pooled H1 (high affinity, $10 \mu\text{M}$) and H11 (low affinity, $5 \mu\text{M}$) peptides or pooled M12 (low affinity, $5 \mu\text{M}$) and M14 peptides ($10 \mu\text{M}$) and cultured for 7 days in a humidified, 5% CO₂, 37 °C incubator. Media supplemented with IL-2 (5 U/ml) was changed every 2-3 days. On day 7, PBMCs from the same dog were thawed and irradiated and added to each well (2×10^6 cells). On day 15, PBMCs (6×10^5 cells) from the same GR were incubated overnight with individual peptides, H1, H11, M12, M14 or K (self-peptide) in a 12-well plate at the same concentrations as previously stated. On day 16, the two week-long H and M peptide pool stimulated PBMCs were harvested, washed and each group combined with the 24 h stimulated PBMCs at a 3:1, E:T ratio, for 6 hours. Some cells were pooled for PMA and ionomycin addition as a positive control. After the first hour, brefeldin A was added to the cultures. After 6 h of restimulation, the cells were stained with CD8 and intracellular IFN- γ antibodies and analyzed via flow cytometry.



Supplemental Figure 2.1. Determining optimal dose for CPV-H/F infection of DH82 cells. We added doses ranging from 0-100 µl/ml of live CDV (Onderstepoort strain) to DH82 cells and stained with CDV polyclonal antibody conjugated to FITC and stained these cells with 7-AAD to identify dead cells. Cell in Q3-LR indicate live, CDV-infected cells.



Supplemental Figure 2.2 Cell sorting of CD8⁺CFSE^{lo} effector cells after stimulation with the CPV-H/F vaccine. Cells within the black box were single cell sorted into three 96-well round bottom plates in attempt to obtain a CDV specific T-cell clone.

Supplemental Table S2.1. CDV peptides used in restimulation and CTL assays.

| Peptide Pool #1: Low Affinity | | | Sample # |
|--------------------------------------|---|----------------------|-----------------|
| Hemagglutinin (H) | <u>A</u> ISDG <u>V</u> Y <u>G</u> K | H ₂₂₂₋₂₃₀ | 1 |
| Large polymerase (L) | ISNGIGKY <u>F</u> K | L ₅₆₄₋₅₇₃ | 4 |
| | <u>K</u> AIEKGFDRY | L ₈₇₇₋₈₈₆ | 5 |
| Nucleocapsid (N) | <u>V</u> ANQQPPT <u>I</u> N <u>K</u> | N ₄₂₇₋₄₃₇ | 17 |
| V (V) | <u>L</u> <u>V</u> <u>V</u> PAGAVSN <u>R</u> | V ₁₂₉₋₁₃₉ | 19 |
| Fusion | KLMPNVSLI | F ₁₆₉₋₁₇₇ | 26 |

| Peptide Pool # 2: High Affinity | | | Sample # |
|--|--------------------------------------|------------------------|-----------------|
| Hemagglutinin (H) | <u>Q</u> VIDVLT <u>P</u> LF <u>K</u> | H ₈₇₋₉₇ | 11 |
| Large polymerase (L) | <u>S</u> LHGPPFH <u>A</u> K | L ₂₀₀₃₋₂₀₁₂ | 6 |
| Matrix (M) | <u>F</u> MVHIGNFS <u>R</u> K | M ₂₁₇₋₂₂₇ | 12 |
| | <u>R</u> VIDPGLGDR <u>K</u> | M ₃₆₋₄₆ | 14 |
| Nucleocapsid (N) | <u>R</u> IIDDPDVS <u>I</u> K | N ₉₀₋₁₀₀ | 15 |
| | <u>Y</u> MVILENSVQ <u>N</u> K | N ₃₁₂₋₃₂₃ | 23 |

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CHAPTER 3: CANCER-TESTIS ANTIGENS IN CANINE HISTIOCYTIC SARCOMA AND OTHER MALIGNANCIES

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Abstract

Cancer-testis antigens (CTAs) are a category of self-proteins aberrantly expressed in diverse malignancies, mostly solid tumors, due to epigenetic de-repression. Normally expressed only in fetal or gametogenic tissues, CTAs are tantalizing immunotherapy targets, since autoimmunity risks appear minimal. Few prevalent CTAs have been identified in human hematologic cancers, and just two in their veterinary counterparts. We sought to discover new CTAs in canine hematologic cancers such as histiocytic sarcoma (HS) and lymphoma to foster immunotherapy development. To accomplish this, the ligandome binding the dog leukocyte antigen (DLA)-88*508:01 class I allele overexpressed in an HS line was searched by mass spectrometry to identify possible CTA-derived peptides, which could serve as CD8⁺ T-cell epitopes. Twenty-two peptides mapped to 5 human CTAs and 12 additional proteins with CTA characteristics. Expression of five promising candidates was then evaluated in tumor and normal tissue by quantitative and end-point RT-PCR. An ortholog of the established CTA, IGF2BP3, had unexpectedly high expression in peripheral blood mononuclear cells (PBMCs). Four other testis-enhanced proteins were also assessed. AKR1E2, SPECC1 and TPX2 were expressed

variably in HS and T-cell lymphoma biopsies, but also at high levels in critical tissues, including kidney, brain and marrow, diminishing their utility. A more tissue-restricted candidate, NT5C1B, was detected in T-cell lymphomas, but also at low levels in some normal dog tissues.

Interestingly, treatment with a demethylating agent induced NT5C1B expression in lymphoma cells, but not PBMCs, suggesting that such pharmacologic manipulation could expand the therapeutic window and value of this CTA in dogs.

Introduction

Adaptive immunotherapy for cancer can have dramatic results when conventional treatments fail.¹ Widespread implementation of such treatment can be promoted by explicit identification of tumor-specific antigens (TSAs). Uncharacterized antigen mixtures administered as active-specific immunotherapy (i.e., vaccines containing tumor cells or their constituents) have mostly been ineffective, regardless of the modification, delivery system or adjuvant. While adoptive cell therapy (ACT) using *ex vivo*-expanded, autologous tumor-infiltrating lymphocytes (TILs) that recognize unknown antigens can result in durable anti-tumor responses in human malignant melanoma, TILs are not available in all malignancies and patients, and are often ineffective in tumor types other than melanoma.² To circumvent these limitations, genes encoding $\alpha\beta$ T-cell receptors (TCR) and chimeric antigen receptors (CAR) that target established TSAs can be introduced into normal T cells to re-direct their cytotoxicity against cancer cells, and upon transfer, these engineered T cells can eradicate chemoresistant tumors.^{3,4}

Not surprisingly, then, the discovery and value assessment of TSAs is an important aspect in advancing adaptive immunotherapy development, whatever the form. Human cancer antigens have been prioritized according to nine criteria, including immunogenicity, role in oncogenicity, specificity, expression level and percentage of TSA-positive cells, and the number

of patients with TSA-positive cancers.⁵ No TSA is ideal, of course, and valuations shift, depending on immunotherapeutic use. For example, vaccination with melanoma differentiation antigens such as gp100 and MART1 have been associated with measurable but very low objective response rates (ORRs; ~3%) and minimal adverse effects.⁶ Targeting gp100 and MART1 by TCR gene transfer improves the ORR, up to 30%, but autoimmune toxicity becomes severe, dimming the utility of this category of TSAs in ACT, a much more potent therapy.⁷ For the purposes of vaccination, attention has now largely turned to mutation-origin neoantigens, where off-tumor expression and tolerance are minimal.^{8,9} However, the private nature of mutation-origin neoantigens makes them currently unsuitable for ACT. For TCR and CAR development, which is a time- and resource-intensive process, antigens shared between patients are needed.

For cancers of non-viral origin, the most well-studied category of shared TSAs with limited-to-negligible somatic tissue expression are cancer-testis antigens (CTAs), which include MAGE, the first identified tumor antigen.¹⁰ The CTA category comprises hundreds of diverse, mostly intracellular antigens that are expressed during fetal development, silenced in normal adult tissues, except germ cells, and re-expressed in various tumors through epigenetic alterations. In fact, the CTAs MAGE A3 and NY-ESO-1 are the only TSAs of self-origin in the top 10 of the National Cancer Institute's prioritized antigen list with no known non-gametogenic tissue expression.^{5,11} The therapeutic usefulness of such CTAs has been compellingly demonstrated. Administration of T cells bearing a high-affinity TCR against a human leukocyte antigen (HLA) class I-restricted peptide from NY-ESO-1 resulted in ORRs in patients with synovial cell sarcoma and melanoma, without apparent toxicities.¹² At present, 26 clinical trials of ACT against this CTA are actively recruiting human patients with various cancers.¹³

In dogs, immunotherapy is a desperately-needed therapeutic option for malignant round cell tumors, such as disseminated histiocytic sarcoma (HS) and high-grade lymphoma, where inherent and acquired chemoresistance ultimately dooms almost all afflicted patients. Accordingly, using HS as a model hematopoietic cancer, we sought to determine whether any proteins whose tissue expression patterns approximated human CTAs could be found. Specifically, liquid chromatography-tandem mass spectrometry was utilized to profile the non-mutant ligandome of a classical dog leukocyte antigen (DLA)-88 class I allele expressed in representative canine HS cell line, and peptides were mapped back to source proteins. The expression pattern of promising candidates in tumor specimens and normal tissues was subsequently assessed by reverse-transcription PCR to estimate the value of these TSAs as targets of cytotoxic T cells.

Materials and Methods

2.1 Tissue acquisition and processing

Frozen canine tumor samples were purchased from the Pfizer-Canine Comparative Oncology & Genomics Consortium (Frederick, MD, USA; pulmonary adenocarcinomas; histiocytic sarcomas; fibrosarcomas; peripheral nerve sheath tumors) and the NCSU-CVM Clinical Studies Core Tissue Bank (malignant mesothelioma; gastric adenocarcinoma; hepatocellular carcinoma; cecal gastrointestinal stromal tumor). Frozen lymph node biopsy samples in TRIzol from dogs with high-grade peripheral T-cell lymphoma and B-cell lymphoma were kindly provided by Dr. Steve Suter. An oral malignant melanoma sample in RNAlater (ThermoFisher Scientific, Waltham, MA) was a gift of Dr. Marlene Hauck. Normal tissues were collected immediately post-mortem from healthy young adult Beagles ($n=2$; lymph nodes only) and hound-mix dogs ($n=3$; diverse tissues) that were maintained and euthanized by NCSU-CVM

Laboratory Animal Resources for reasons unrelated to this study. Normal testis was also acquired as discarded tissue after routine elective castration of a healthy young adult mixed-breed dog at NCSU. Samples were placed in 5-fold excess RNAlater in individual cryovials overnight at 4°C, and then stored at -80°C. Peripheral blood mononuclear cells (PBMCs) from normal and leukemic dogs were isolated from leftover EDTA-anticoagulated blood samples in the NCSU Clinical Pathology Laboratory, after complete blood count analyses had been performed.

2.2 Culturing and treatment of cell lines

The canine HS cell line DH82 (CRL-10389) and the T-cell lymphoma line OSW (PTA-9116) were obtained from the ATCC, and were grown in Dulbecco's Modified Eagle's Medium containing 15% FBS, penicillin (100 IU mL⁻¹)/streptomycin (100 µg mL⁻¹) (P/S) and RPMI 1640 containing 20% FBS, P/S and 2 mM L-glutamine, respectively. The cell clones 9-15 (DH82 cells stably expressing DLA-88*508:01-FLAG) and BARC3 (RMA-S cells stably expressing DLA-88*508:01-FLAG) had been previously generated and were cultured as described.^{14,15} In some experiments, cell lines and PBMCs were cultured with R-10 medium containing 15 µM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich, St. Louis, MO, USA), diluted from a 200 mM stock solution prepared in DMSO and stored at -80°C. The medium and 5-aza-dC were replaced every 12 hours for the 72-hour incubation period. All cells were grown under standard incubator conditions (37°C; humidified 5% CO₂ atmosphere), unless otherwise stated.

2.3 Isolation and sequencing of DLA-88*508:01-bound peptides

The mass spectral data used here are from a previously reported study¹⁴ and additional peptide isolation experiments. Large-scale culture and weekly harvests of the 9-15 clone, affinity isolation of peptide-DLA-88*508:01-FLAG from 9-15 lysates, acid elution of bound peptides,

and additional peptide clean-up and concentration were performed as described.¹⁴ Eluates were analyzed by LC-MS/MS, using a nanoACQUITY UPLC system (Waters) and Thermo Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). The software program Mascot 2.5.1 (Matrix Science; matrixscience.com) was used to search mass spectral data against a modified *Canis lupus familiaris* database, and peptide and proteins were identified using the program Scaffold (Proteome Software; proteomesoftware.com).

2.4 Generation and phenotypic evaluation of blood-derived dendritic cells (DCs)

Dendritic cells were prepared as previously described.¹⁶ Anticoagulated venous blood (~10 mL) was aseptically collected from a healthy Golden Retriever under a protocol approved by the NCSU Institutional Animal Care and Use Committee. PBMCs were isolated by centrifugation (400 g x 30 min at room temperature) over a Ficoll-PaqueTM PLUS 1.077 density gradient (GE Healthcare, Uppsala, Sweden). The harvested interface was washed twice with PBS, resuspended in RPMI 1640 containing 10% FBS, P/S and 2 mM L-glutamine (R-10 medium), and plated at 1.2×10^7 cells per well in a 6-well plate. Non-adherent cells (~70% of input) were removed after 2 hours by gentle pipetting with PBS, and R-10 containing recombinant canine IL-4 (50 ng mL⁻¹; Kingfisher Biotech, St. Paul, MN, USA) and GM-CSF (33 ng mL⁻¹; R&D Systems, Minneapolis, MN, USA) was added (2 mL per well). Fresh medium and cytokines were added on days 3 and 6. DCs were harvested for use by pipetting on day 7. To assess surface expression of CD11c, 10^5 cells per well in a 96-well plate were washed twice with FACS buffer (PBS containing 2% FBS, 2 mM EDTA and 0.02% sodium azide) and then incubated with an anti-canine CD11c monoclonal antibody (mAb; clone CA11.6A1, Novus Biologicals, Littleton, CO, USA) at a 1:6 v:v ratio for 30 minutes at 4 °C. As a negative control to establish background fluorescence, the primary mAb was omitted. After two washings with

FACS buffer, cells were stained with a phycoerythrin-conjugated goat anti-mouse IgG Ab (Bio-Rad, Hercules, CA, USA) for 30 minutes at 4°C, washed again, and analyzed immediately on a Cytotflex flow cytometer (Beckman Coulter, Indianapolis, IN, USA). List mode data was analyzed with CytExpert software. Viable cells were discriminated using forward and side scatter gating. To obtain photomicrographs, an air-dried spread of day 7 DCs concentrated by centrifugation (400 g x 6 min) were stained with Wright-Giemsa on an Aerospray Pro Stainer, and photographed with a Nikon DS-Fi2 camera through an Olympus BX41 microscope.

2.5 Isolation of RNA and preparation of cDNA

Thawed tissue samples were homogenized in TRI Reagent (Zymo Research, Irvine, CA, USA) using a mini-bead beater (Biospec Products, Bartlesville, OK, USA). For cell samples, $1-5 \times 10^6$ cells were processed in 500 μ L TRI Reagent. Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions, re-suspended in nuclease-free H₂O and stored at -80°C. RNA concentrations >100 ng/ μ L, with 260 nm/280 nm and 260 nm/230 nm absorbance ratios >1.8 and >1.0, respectively, as determined by NanoDrop 2000 spectrophotometer, were judged to be the minimum quality for analysis. Prior to cDNA synthesis, samples were DNase-treated using a Turbo DNA-free Kit (ThermoFisher Scientific). DNase-free RNA (2.5 μ g) was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), performed according to the manufacturer's instructions, on a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY, USA), using the following reaction times: priming: 5 minutes at 25°C; reverse transcription: 30 minutes at 42°C; reverse transcriptase (RT) inactivation: 5 minutes at 85°C. Control samples were prepared in parallel by omitting RT from the reaction mixtures. The cDNA was subsequently cooled to 4°C and stored at -80°C.

2.6 PCR amplification of normal and tumor tissue cDNA

Oligonucleotide primers (Supplemental Table S1) for PCR amplification were designed with NCBI/Primer-BLAST, and synthesized by Invitrogen (Carlsbad, CA, USA) or Integrated DNA Technologies (Coralville, IA, USA). Quantitative gene expression was performed on a LightCycler 480 System (Roche, Indianapolis, IN, USA) using iQ SYBR Green (Bio-Rad) and the cycling conditions listed in Supplemental Table S2. Reaction efficiency curves were constructed using dilutions of cDNA from gene-positive samples (i.e., DH82 cells and/or testis) and for all genes, efficiency values exceeded 1.85. The ribosomal proteins L8 and L32 (RPL8; RPL32) genes served as reference genes, using previously published primer pairs.¹⁷ Because both yielded similar quantitative data in multiple qPCR analyses, RPL32 was omitted from later experiments. Thawed cDNA samples were diluted 1:10 in nuclease-free H₂O prior to qPCR. Reactions were run in duplicate wells, and included a no-RT control (for each tissue) and a no-template control (for each gene). Testis cDNA served as a positive control in all experiments. Amplification of the intended genes was verified by Sanger sequencing of reaction products (not shown). Data was analyzed using the LightCycler 480 software, v. 1.51.62. C_q values >35.00 were considered non-detectable. Expression levels of each gene relative to the reference gene was calculated by the $2^{-\Delta\Delta CT}$ method ($2^{Cq(\text{reference})-Cq(\text{target})}$), using Microsoft Excel, and the data was displayed graphically with Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Endpoint RT-PCR amplification was performed with a Hotstar HiFidelity Polymerase Kit with Q solution (Qiagen, Germantown, MD, USA) in 25 μ L reaction volumes with 1 μ L cDNA and 1 μ M primers, using a Mastercycler Pro running cycling program 2 (Supplemental Table S2). In all experiments, water was substituted for cDNA template as a negative control. Reaction products (5 μ l) were electrophoresed on a 2% agarose gel containing GelRed Nucleic Acid Stain

(Biotium, Hayward, CA USA) at 130V for 30 minutes, and visualized and photographed using an Epi Chemi II Darkroom (Ultra-Violet Products, Upland, CA, USA). A 100 bp (Novagen Perfect DNA 100 bp, Millipore/Sigma, Burlington, MA, USA) or mid-range (Fisher exACTGene; ThermoFisher Scientific) ladder was included on each gel as a molecular weight reference. Gel pictures in the figures were cropped and re-sized to provide a uniform appearance, but no other image manipulation was performed. Verification of the integrity of each tissue cDNA was performed by obtaining a single bright band of the correct length following RT-PCR amplification of RPL8, employing the same primer pair as in the qPCR assays (not shown).

2.7 Cloning and sequencing of NT5C1B

The RT-PCR products from DH82 cells, testis and brain were gel-extracted, isolated using the Zymoclean Gel DNA Recovery Kit (Zymogen), and TA-cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Plasmid DNA isolated from transformed *E. coli* clones (G10 Chemical Competent Cells, Genesee Scientific, San Diego, CA, USA) were screened by *EcoRI* restriction digest. Positive plasmids were sequenced using standard T7 and SP6 primers by the NCSU Genomics Science Laboratory. Sequences were aligned to the canine NT5C1B gene with Geneious software (Biomatters, Auckland, NZ).

2.8 Assessment of CTA-derived peptide binding to DLA-88*508:01

Peptides were synthesized by Peptide 2.0 (Chantilly, VA, USA), reconstituted in DMSO and stored as stock solutions at -80°C. A peptide-MHCI surface stabilization was performed as previously described.^{14,15} Briefly, BARC3 cells (10^5 per 100 μ L) were cultured in R-10 containing 100 μ M peptide overnight at 27°C, and then incubated for an additional 5 hours at 37°C prior to staining with a primary murine anti-MHCI mAb (H58A; Monoclonal Antibody Center, Washington State University, Pullman, WA, USA), followed by washing and secondary

incubation with an Alexa Fluor 647-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Peptide-independent MHCI staining of BARC3 cells at 27 and 37°C was performed to validate assay performance. The canine self-peptide *K-11* (RFLDKDGFIDK) served as a positive binding control. Data was acquired on a modified FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA). A semi-quantitative binding score was computed from the mean fluorescence intensity (MFI), using the formula: $(MFI_X - MFI_{no\ pep@37C}) / (MFI_{no\ pep@27C} - MFI_{no\ pep@37C})$, where X represents the tested peptide. The motif-negative viral peptide NP₃₉₆₋₂₀₄ (FQPQNGQFI; GenScript, Piscataway, NJ, USA), an established non-binder, was used to compute the assay's lower limit of detection, defined as the mean binding score plus 2 standard deviations.

Results

3.1 Proteins with enhanced testis expression identified in an HS cell line by LC-MS/MS

To investigate the possible presentation of HS-associated CTA peptides by the canine classical MHC class I molecule, DLA-88, we used a cell clone (9-15) derived from DH82 cells stably transfected with a FLAG epitope-tagged DLA-88*508:01 heavy chain.¹⁸ DLA-88*508:01 was used as a model allele because it is carried by ~40% of Golden Retrievers¹⁹, a breed with an increased risk of HS, lymphoma and other malignancies.²⁰ Peptides eluted from DLA-88*508:01 complexes isolated by FLAG immunoprecipitation of 9-15 cell lysates were identified by LC-MS/MS.¹⁸ From 5 independent analyses, we found peptides derived from 17 self-proteins that appeared likely to have increased expression in canine testis relative to somatic tissues, which could qualify one or more of them as legitimate CTAs.

3.2 Human CTA orthologs found in canine HS specimens

Six peptides representing 5 predicted canine proteins with orthologs listed in a public database of human CTAs²¹ were recovered (Table 1). Within this group, IGF2BP3 (Insulin-Like Growth Factor 2 mRNA Binding Protein 3; also known as KH Domain-Containing Protein Overexpressed In Cancer [KOC] or IGF2 mRNA-Binding Protein 3 [IMP-3]), originally discovered in a melanoma cell line²², appeared to be the most promising member. In humans, the expression of IGF2BP3 appears largely restricted to the fetal tissues, ovary, testis, brain and placenta, and to tumors of diverse origin, including lymphomas (data from The Human Protein Atlas [HPA], www.proteinatlas.org, v.16.1²³). IGF2BP3 is immunogenic in cancer patients, inducing spontaneous antibody responses and T-cell reactivity in tumor tissue, regional lymph nodes and blood.²⁴⁻²⁶ Further, minimal epitopes restricted by two Human Leukocyte Antigen (HLA)-A alleles have been identified^{27,28}, and peptide vaccination elicits strong cytotoxic T lymphocyte (CTL) responses associated with prolonged survival in patients with squamous cell carcinoma.²⁹⁻³¹

As a first step in evaluating the potential utility of this CTA as a TSA in dogs, we used qPCR to investigate the relative expression of IGF2BP3 mRNA in selected normal tissues, which is unknown. Expressed sequence tags (ESTs) can be helpful in predicting expression³², but no canine IGF2BP3 ESTs have been reported (ESTs were searched in the NLM/NCBI database using the BLASTN 2.7.1 program.³³). Figure 1A shows that, using primer pair 1, IGF2BP3 mRNA was found in DH82 cells, but was unexpectedly low in testis and high in PBMCs. The expression seemed discordant with human and murine datasets showing very restricted distribution of IGF2BP3 mRNA and protein (EMBL-EBI Expression Atlas [EEEE], (<http://www.ebi.ac.uk/gxa>)³⁴, although two RT-PCR studies reported “weak expression” in

human leukocytes³⁵, and expression in normal adult mouse brain, heart, lung, ovary and several other tissues (without providing data).³⁶ We sought to confirm or refute our initial findings using different primers (Supplemental Table 1, pair 2), and obtained similar results (Figure 1B).

IGF2BP3 mRNA was surprisingly high in canine lung. While message was also found in all 8 HS biopsy specimens that we tested (not shown; range 0.17 – 1.1), the high expression in a number of somatic tissues suggested that IGF2BP3 may not be a useful TSA to target in dogs.

The IGF2BP3 peptide that we originally recovered by LC-MS/MS, LLVPTQFVGAIIGK, is an improbable binder of DLA-88*508:01, which strongly prefers 9- to 12-mers.¹⁸ An LC-MS/MS analysis performed subsequent to the qPCR tissue survey did yield a DLA-88*508:01 motif-matched nonamer peptide originating from IGF2BP3, but given the unlikely utility of this CTA, class I binding was not verified experimentally.

Sperm-associated antigens (SPAG) are also potential immunotherapeutic targets³⁷, and SPAG6 is overexpressed in pediatric acute myeloid leukemia.³⁸ However, this particular CTA appears to also be normally expressed in human brain, trachea, lung³⁷, and in murine lymphocytes³⁹, so was not pursued further.

3.3 Additional proteins from DH82 cells with enhanced testis expression of mRNA

In addition to the 5 human CTAs, our LC-MS/MS datasets contained peptides from 12 additional predicted proteins (Table 1) whose human orthologs appear to have largely tissue-restricted (testis or testis/brain) expression, according to public gene expression databases: HPA, EEEA and the UniProt Knowledgebase, <http://www.uniprot.org/uniprot>⁴⁰. Eight were represented by at least one peptide that matched the DLA-88*508:01 binding motif and were considered eligible targets of CTL restricted by that allele. Of those, one was a homolog of a scantily-characterized open reading frame (C30H15orf39), and three (ATXN2L; CASC1; ZNF570) were considered

likely to have mixed tissue expression based on human data, so these four were not evaluated. Peptides from the remaining four gene products (AKR1E2; SPECC1; TPX2; NT5C1B) were synthesized and tested for class I binding in a peptide-MHC surface stabilization assay.¹⁵ All bound DLA-88*508:01 to varying degrees (Table 1; Supplemental Figure 1A) and were therefore considered potentially immunogenic.

The enzyme AKR1E2 (aldo-keto reductase family 1 member E2; also known as Human Testis-Specific Protein [HTSP]) is a member of a large superfamily that is a purported testis-restricted protein^{41,42}, and also expressed in a small percentage of human carcinomas and melanomas. Analysis of 7 HS biopsies showed moderate expression in all samples, ranging between 1.8 – 6.4% of testis (Supplemental Figure 1B). However, it is also evident AKR1E2 is expressed in a number of canine somatic tissues; in fact, renal expression was equivalent to testis. Human CTAs can be classified according to several overlapping schemes. One system designates CTAs as either 1) testis-restricted (testis only, \pm placenta); 2) testis/brain-restricted; and 3) testis-selective.⁴³ Another approach segregates these genes based on reverse transcription (RT)-PCR profiling into 1) testis-restricted; 2) tissue-restricted (testis and ≤ 2 non-gametogenic tissues); 3) differentially-expressed (testis and ≤ 6 [of 13 standard] non-gametogenic tissues); and 4) ubiquitously expressed CTAs.⁴⁴ By this latter metric, canine AKR1E2 presumably falls into the last category, and hence, is not promising as a tumor-discriminating target for CTL responses.

We next investigated SPECC1 (Sperm Antigen With Calponin Homology And Coiled-Coil Domains 1; also known as Cytokinesis and Spindle Organization B [CYTSB]), identified from a very strongly DLA-88*508:01-binding peptide. In the dog, a single EST, from testis, has been reported (GenBank CX988153.1). Northern blot analyses have shown negligible expression

in normal human tissues, including the thymus, but high transcript levels in testis and some tumor cell lines.⁴⁵ There are just a handful of other publications tying SPECC1 to cancer, as a novel fusion partner with platelet-derived growth factor receptor- β in myelomonocytic leukemia⁴⁶, and as a pro-apoptotic gene in head-and-neck carcinoma cell lines.^{47,48} SPECC1 mRNA was found in DH82 cells (Figure 2A) and in 9 of 10 histiocytic biopsy samples (Figure 2B). Using previously established criteria of weak ($>0.1\%$ of testis), moderate ($>1.0\%$ of testis) and strong ($>10\%$ of testis) expression⁴⁹, moderate expression was seen in 5/10 (50%), and strong expression was observed in 4/10 (40%) specimens. SPECC1 message was also detected at moderate or higher levels in canine lung adenocarcinomas and various sarcomas (Supplemental Figure 1C), but was negative ($n=1$), weak ($n=4$; 0.34, 0.42, 0.44, 0.52% of testis) or moderate ($n=4$; 1.1, 1.1, 1.6, 8.0% of testis) in PTCL biopsies (not shown). By comparison, in those assays, no message was found in 3/4 normal lymph nodes included as controls; 1 node had weak SPECC1 expression in 1 of 2 replicate wells (the other was not detectable). An accompanying spleen sample was negative, as were PBMCs. Because dendritic cells (DCs) are the parent of most canine histiocytic malignancies, but normally present at very low frequencies in blood, we enriched PBMCs for DCs by 7-day incubation with GM-CSF and IL-4, yielding $\sim 50\%$ CD11c+ cells with characteristic morphology (Supplemental Figure 1D), but found no SPECC1 expression. Given these encouraging results, the distribution of SPECC1 in other normal somatic tissues was investigated further, revealing strong expression in the central nervous system (CNS), bone marrow and urinary bladder, and moderate expression in pituitary gland, small and large intestine, and skeletal muscle (Figure 2C, left). The brain and marrow findings were confirmed in a partial assessment of a second dog (Figure 2C, right).

Two separate LC-MS/MS analyses yielded a total of three DLA-88*508:01-binding peptides (Table 1) derived from TPX2 (TPX2, Microtubule Nucleation Factor; also known as Differentially Expressed in Cancerous and Non-Cancerous Lung Cells [DIL] 2; or Targeting Protein for Xklp2). Originally found by mRNA differential display in human lung carcinoma lines, TPX2 was shown to be expressed in fetal, but not adult, lung tissues.⁵⁰ Data from human atlases indicated mostly weak, mixed expression in normal tissues, with substantial enhancement in testis. All evaluated malignancies in the HPA contained small numbers of TPX2-positive cells. HLA-restricted TPX2 peptides pulsed on DCs have elicited CTL responses.⁵¹

As a key regulator of Aurora-A kinase, which controls spindle assembly and function during mitosis^{52,53}, TPX2 expression in the DH82 line (peptides, and mRNA [Figure 3C]) might simply result from the fact that, at any given time, a substantial fraction of cultured cells are undergoing division. Similarly robust expression might not be observed in clinical HS specimens. Analysis of biopsies by qPCR, however, showed strong and moderate expression in 8 and 2 samples, respectively (Figure 3A). Message levels in HS were generally much higher than in samples of various other solid tumor types (Supplemental Table 3). TPX2 mRNA was found in 12 of 12 PTCL samples, with strong expression in half, and moderate expression in the remainder (Figure 3B), as well as in the T-cell lymphoma line, OSW (Figure 3C). To gauge the extent to which TPX2 expression might be restricted to testis and tumors in the dog, we again evaluated a panel of normal canine tissues. Blood-derived, DC-enriched cultures only yielded weak TPX2 expression (<1% of testis; not shown), and as seen in Figure 3D (dog #1), most somatic tissues, including lymph nodes, were negative or weakly positive. However, brain and bone marrow strongly expressed TPX2 (14.7 and 15.9% of testis, respectively). Similar findings

were observed in selected tissues from additional dogs, although the brain was negative in dog #3. Interestingly, ESTs for TPX2 have only been derived from canine heart and thymic tissues.

While the strong expression of these two testis-enhanced genes across multiple canine HS (SPECC1 and TPX2) and PTCL (TPX2) samples initially made their products attractive as candidate rejection antigens for these hematologic malignancies, the wider distribution of their mRNAs in critical tissues of the dog, most notably brain and bone marrow, would appear to dim prospects of their development for T-cell immunotherapy.

Finally, we evaluated NT5C1B (5'-Nucleotidase, Cytosolic IB). The human ortholog is also known as AIRP (Autoimmune Infertility-Related Protein). One report shows NT5C1B expression in diverse non-gametogenic murine and human tissues by RT-PCR⁵⁴, while other datasets from humans show testis-restricted mRNA expression.

By qPCR with primer pair 1, NT5C1B expression was seen in canine testis, but not in somatic tissues, nor in the DH82 cell line or selected HS or PTCL biopsy samples. Cloning and sequencing of the qPCR amplicon confirmed the NT5C1B target. To potentially improve detection, we tested a second set of qPCR primers (pair 2). In this next round of analyses, 0 of 8 HS specimens (HS114 had a negligible signal, 0.02% of testis) and 1 of 12 PTCL specimens (PTCL7 showed weak expression, 0.2% of testis) were positive (Figure 4A, B). We also assayed for NT5C1B message in PBMCs of patients with overt leukemias (1 PTCL; 1 undifferentiated) and in B-cell lymphoma nodal biopsies ($n=3$), but no signal was detected. In humans, expression by immunohistochemical staining is observed in melanomas, gliomas and diverse carcinomas (highest expression, gastric and pancreatic; most frequently positive, colorectal [HPA]), but there was essentially no message in a small panel of canine solid tumors (Supplemental Table 3). We also saw no NT5C1B mRNA in selected somatic tissues from 3 normal dogs (Figure 4C),

except for negligible expression in a single brain sample (0.03% of testis), and in 1 kidney sample (1 well was 34 cycles, the other >35 [not detectable]). As with primer pair 1, no expression was detected in DH82 cDNA (Figure 4A), despite the fact that an NTC51B-derived peptide had been retrieved from these cells in two separate isolations and LC-MS/MS analyses. A possible explanation is the well-established discordance between mRNA and protein expression: high protein concentrations can result from high translation rates and protein stability, despite minimal transcription.⁵⁵ In that case, our qPCR may not have been sufficiently sensitive for NTC51B detection; however, a robust signal was always returned from testis. Accordingly, samples were re-probed for NTC51B mRNA by endpoint RT-PCR, using two different primer sets (pairs 3, 4; Supplemental Table 1). Expression was observed in testes and DH82, but not in liver (Figure 4D). The HS biopsy samples were then evaluated over multiple assays, yielding weak bands in most samples, but signals were inconsistent across reactions (not shown). HS114, the sample that generated a negligible signal by qPCR (Figure 4A) was most consistent, with 6 of 9 PCRs producing a band. No NTC51B was amplified from cDNA isolated from a PBMC-derived DC culture. NTC51B message was detectable in 8 of 13 PTCL biopsies, with bright bands in 2 samples, and faint bands in the remaining 6 (Figure 4E). Encouragingly, no bands were observed when multiple somatic tissues from dog #1 were assayed (not shown). To verify these results, similar tissues from dog #2 and #3 were evaluated, and most were negative; however, in some repetitions (not shown), bands were intermittently seen in brain, lung and kidney (4 of 5 PCRs each) and bone marrow, pituitary gland, heart and spleen (2 of 5 PCRs each). Sequencing of the product from brain returned NTC51B, ruling out an off-target amplification as an alternate explanation for the observed band. Overall, the endpoint RT-PCR mirrored the qPCR data, showing that there is low NTC51B message expressed in canine HS and

PTCL, as well as in some normal tissues of some dogs. In reviewing available canine EST data, there are 14 NT5C1B cDNA clones from testis, but also 2 clones from kidney, consistent with our findings showing transcripts in this organ. Interestingly, the latter two clones (GenBank DN331931.1; DN330130.1) are splice variants, both retaining a 136-bp fragment of the 3' end of intron 6, followed by exons 7 and 9; exon 8 is skipped. In the 5 ESTs from testis that cover this region, as well as the sequence that we obtained, exon 8 is expressed. If consistent, this difference could be exploitable for immunotherapy: that is, should splice variants in tumors (and testis) yield peptides not encoded by NT5C1B transcripts in normal somatic tissues, then the corresponding CTL would pose no risk for autoimmunity. However, sequencing of an NT5C1B amplicon from brain confirmed expression of exon 8, ruling out this possibility.

3.4 A DNA demethylation agent induces *de novo* expression of NT5C1B in tumor cells

Since NT5C1B is expressed variably-to-negligibly in somatic tissues of the dog, it might still be a valuable target if expression was markedly higher in PTCL, but subjectively, that did not appear to be the case. The band intensity in normal tissues, when present (not shown), was similar to the less-bright bands of most PTCL samples (Fig. 4E, excluding samples PTCL3 and PTCL7). A pharmacologic means to magnify this difference could be useful to develop NT5C1B as a TSA. The DNA demethylating agent 5-aza-dC has been shown to induce or enhance expression of human CTAs in tumor cell lines, which can persist for weeks following removal of the drug.^{56,57} Treatment with 5-aza-dC induced expression of NT5C1B (Fig. 5D) in the canine T-cell lymphoma line OSW, but not in normal PBMCs, showing that this agent could be a valuable adjuvant to NT5-directed immunotherapy in dogs, analogous to combination therapies being evaluated for human cancer patients (reviewed in Chiappinelli *et. al*⁵⁸).

Discussion

In this study we sought to identify CTAs in canine hematopoietic malignancies (HS and lymphoma) as potential targets for T-cell immunotherapy. In our approach, the discovery process was positioned downstream, at the level of the MHC class I-presented peptide, to insure that any CTA that was found was not only present as protein, but also potentially immunogenic: that is, processed and available for CD8⁺ T-cell recognition. The limitation of this method is that individual tumors could not be probed for CTA-derived peptides, because specific antibodies for DLA-88 immunoprecipitation are lacking. Hence, positive findings in DH82 cells had to be extended to patient biopsy samples by RT-PCR analysis. Nonetheless, a powerful advantage of direct class I ligandome searching is that peptides from any high-value CTA can immediately be synthesized to isolate the cognate TCR, by T-cell cloning or peptide-MHC tetramer sorting, for ACT development.

We identified 22 peptides that mapped to 17 proteins with characteristics of CTAs. While a large number of CTAs have been identified in human tumors, few have been reported in veterinary oncology. Testis-enhanced TSAs were recently reported in canine mammary gland tumors.⁵⁹ The CTAs MAGE and MAGE-2 have been found in feline, but not canine, lymphomas.⁶⁰⁻⁶³ The testis-enhanced TRAF-interacting protein (TRAIP) gene was shown to be overexpressed 2.5-fold in a canine T-cell lymphoma xenograft, when compared to normal lymph nodes.⁶⁴ However, a pioneer proteomics study in canine lymphoma did not report any CTAs.⁶⁵

To assess the potential therapeutic worth of CTA candidates identified by MS, three criteria, similar to those used to assign relative value to human antigens^{5,66}, were considered: 1) a previous connection (i.e., human or veterinary literature) tying the protein to cancer; 2) expression across multiple patient tumor samples; and 3) lack of normal tissue expression, with

this last category carrying the greatest weight. Unfortunately, none of the CTAs that we found exhibit the pristine absent somatic tissue expression that typifies high-value human CTAs such as MAGE A3 and NY-ESO-1. This characteristic likely reflects the fact that the CTAs that we identified were all single-copy genes, distributed across autosomes (Table 1). In humans, a slight majority of CTAs originate on chromosome X, particularly those in multigene families, such as MAGE and SSX. Transcription of these X-linked CTAs in somatic tissues appears to be more tightly regulated than that of their non-X counterparts. Thus, of the 39 human CTAs that are highly testis-restricted (i.e., no mRNA expression other than testis and placenta), 35 originate on the X-chromosome.⁴³ Why no X-linked CTAs were recovered here is unknown, but may reflect the insensitivity of the mass spectrometric approach or peculiarities of the DH82 cell line. Interestingly, Chen *et al.* observed MAGE expression via immunohistochemistry in histiocytic proliferative disease biopsy specimens⁶⁰, but to date we have not identified MAGE peptides in DH82, even when a different DLA-88 allele, *034:01, has been immunoprecipitated (in 3 independent experiments; Nemeč & Hess, manuscript under review). An additional explanation for this dearth is that the canine genome appears to lack definitive orthologs of many of the human X-linked CTAs, which are evolutionarily young genes, with many family members arising after the divergence of Laurasiatherian mammals.^{67,68}

Expression of some CTAs in transformed cells may represent an epiphenomenon of altered genomic methylation or acquisition of stem-like properties, but in general, CTAs are thought to contribute to oncogenesis⁶⁹, presumably to a degree sufficient to offset the increased immunogenicity and susceptibility to immunoediting (expression of CTAs can be associated with CD8+ T-cell signatures in tumors^{70,71}). The role of X-linked CTAs in oncogenesis is generally far less clear-cut than for single-copy autosomal CTAs, increasing the relative value of

these latter antigens, despite their less-restricted tissue expression. For example, IGF2BP3 is an RNA-binding protein that post-transcriptionally regulates multiple transcripts, including IGF2, CD44, MMP9, cyclins D1, D3 and G1, and the oncogenes MYC and CDK6. Consequently, IGF2BP3 overexpression in B-acute lymphoblastic lymphoma increases proliferation and decreases apoptosis.^{72,73} IGF2BP3 also promotes stemness and chemoresistance^{74,75}, and facilitates NK cell immunoevasion.⁷⁶ As one might therefore expect, IGF2BP3 expression in a wide variety of human tumors positively correlates with increased metastatic/invasive behavior and a poor prognosis.⁷² While the multifactorial oncogenicity of IGF2BP3 would seem to make it a particularly valuable immunotherapeutic target, some human expression data⁷⁷ and our findings would suggest that this CTA is not well-suited for ACT in either species, given the severe toxicities associated with the use of other overexpressed antigens for this purpose.⁶⁶ On the other hand, inhibiting the function of this or other oncogenic CTAs in hematologic malignancies by pharmacologic means may be an important, under-investigated therapeutic approach.⁷³

Of course, some testis-enhanced, autosomal-encoded proteins may have functions in the few other somatic tissues where they are expressed that would preclude safe inhibition. For example, we observed high SPECC1 message in CNS tissue. When SPECC1 is knocked out in mice, decreased avoidance learning results⁷⁸, so pharmacologic inhibition could have adverse higher-order behavioral effects in addition to anti-tumor benefits. Cancer-testis antigens with similar expression patterns have been considered to belong to a distinct subgroup known as cancer-testis-brain antigens, which includes other gene products that we recovered: CTNNA2, DLGAP2, DLGAP3, DPYSL5 and KIF5C. It is now recognized, however, that some of these proteins are more appropriately considered brain differentiation antigens, with concurrent high

expression in testis.⁴⁹ Interestingly, our mass spectrometric analyses identified peptides representing many proteins (~60) that are exclusively- or highly-expressed, or enriched, in the CNS (for example: PACSIN1, NTM, and BASP1, respectively), which possibly indicates a microglial origin of DH82. This cell line also expresses a number of other putatively tissue-restricted proteins, including 11 from striated muscle (e.g., PYGM) and 3 from adrenal tissue (e.g., MGARP). Not unexpectedly, various leukocyte-associated proteins were also found, including CD14, CD18, CD29, CD44, CD56, CD88, DOCK2, FERMT3 and SIGLEC10, complement and chemokine receptors, and classical and non-classical MHC molecules.

As mentioned, similar brain expression of TPX2 would appear to rule out safe immune targeting in those canine hematologic cancers in which it is highly expressed; however, this protein could still have other value in veterinary oncology. Similar to IGF2BP3, TPX2 has been implicated as a gene significantly associated with progression of numerous, diverse human carcinomas and intracranial tumors. TPX2 is a quantifiable indicator of cell proliferation in mantle cell lymphoma that can be correlated with overall survival⁷⁹, and could have similar utility in gauging prognosis in lymphomas of dogs. Additionally, TPX2 might serve as a biomarker for lymphomas that are resistant to Aurora-A specific inhibitors.⁸⁰ Given its apparent absence in canine PBMCs, TPX2 conceivably could be used to measure MRD in canine lymphoma. The CTAs MAGE and SPAG6, a protein we identified in DH82, have been shown to be useful for this purpose in human hematologic cancers.^{38,81}

By no means should the findings reported here be considered definitive. A commonality of all CTA discovery studies is that quite different conclusions regarding the expression of gene candidates in normal and cancerous tissues are often reached. In qPCR analyses, this discordance can occur even when the same tissue panels are probed.⁸² Not surprisingly, there are some

discrepancies between our findings and canine data deposited in the NCI OncoGenomics database⁸³ regarding the tissue expression of the five gene products profiled here. Also, as we and others have shown, there are also variations in expression in tissues obtained from different animals. Moreover, in many instances, there is disagreement between CTA mRNA and protein expression. For instance, NY-ESO-1 mRNA has been detected in liver and pancreas, but protein expression has never been documented in these tissues.⁴⁴ Thus, the next, higher-level evaluation of a promising canine CTA such as NT5C1B will require analyzing protein expression with a validated anti-canine antibody. Additionally, different mRNA and protein isoforms of CTAs are possible, which could serve as another source of tissue-specific peptides. For example, there are 5 predicted isoforms (X) of NT5C1B. One (X2) results from an alternate transcription start site, and another (X3) has a 183-bp deletion (supported by EST BM537000.1). The remaining 5' EST clones support isoforms X1, X4 and X5. The single 3' EST supports X1, X2 and X3. Evaluating whether such isoforms are differentially expressed by tissue or tumor type would certainly be needed prior to ultimately assigning a value to NT5C1B.

Finally, in most CTA-positive tumors, only a fraction of malignant cells typically produce the protein, which means that not all cells will be susceptible to T-cell killing. Such heterogeneity may reflect the fact that, in some cancers, CTA re-expression is a characteristic of a stem cell subpopulation.⁸⁴ This obstacle to immunotherapy could potentially be mitigated by treatments that simultaneously target multiple CTAs, such as a polyvalent vaccine. Also, as we observed, 5-aza-dC can also de-repress CTA expression, which has been shown to sensitize treated cell lines to lysis by CD8⁺ T cells.⁸⁵ Importantly, this agent can also enhance the homogeneity of CTA expression within tumors⁸⁶, and additionally, increase MHC class I expression at the cell surface.^{87,88} For these reasons, it seems apparent that co-treatment with

demethylating agents will be an obligatory component to any successful immunotherapy that targets CTAs in humans or dogs.

Conclusion

This study shows that a reverse antigen discovery process – from peptide to protein to mRNA – can be a fruitful means for finding shared TSAs, such as CTAs. Expanding this approach by surveying the MHC class I ligandome of other cell lines representing common and currently incurable hematopoietic (lymphoma) and solid (osteosarcoma; hemangiosarcoma) cancers to foster ACT development seems warranted.

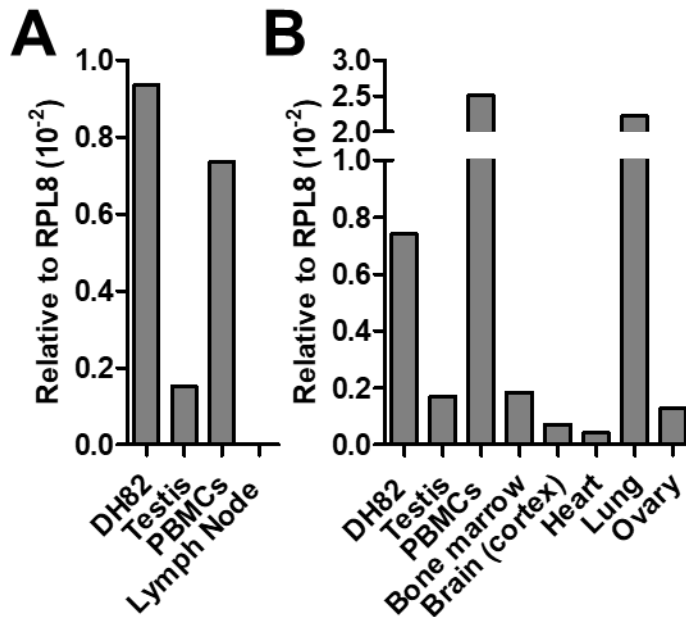


Figure 3.1. Expression of canine IGF2BP3 message (relative to RPL8) in DH82 cells and selected tissues, as determined by qPCR analysis, using two different pairs of primers. A, IGF2BP3 (primer pair 1) is more highly expressed in PBMCs than in the reference tissue, testis. B, IGF2BP3 (primer pair 2) is highly expressed in PBMCs and lung, and at detectable levels in diverse somatic tissues. Data represents multiple experiments.

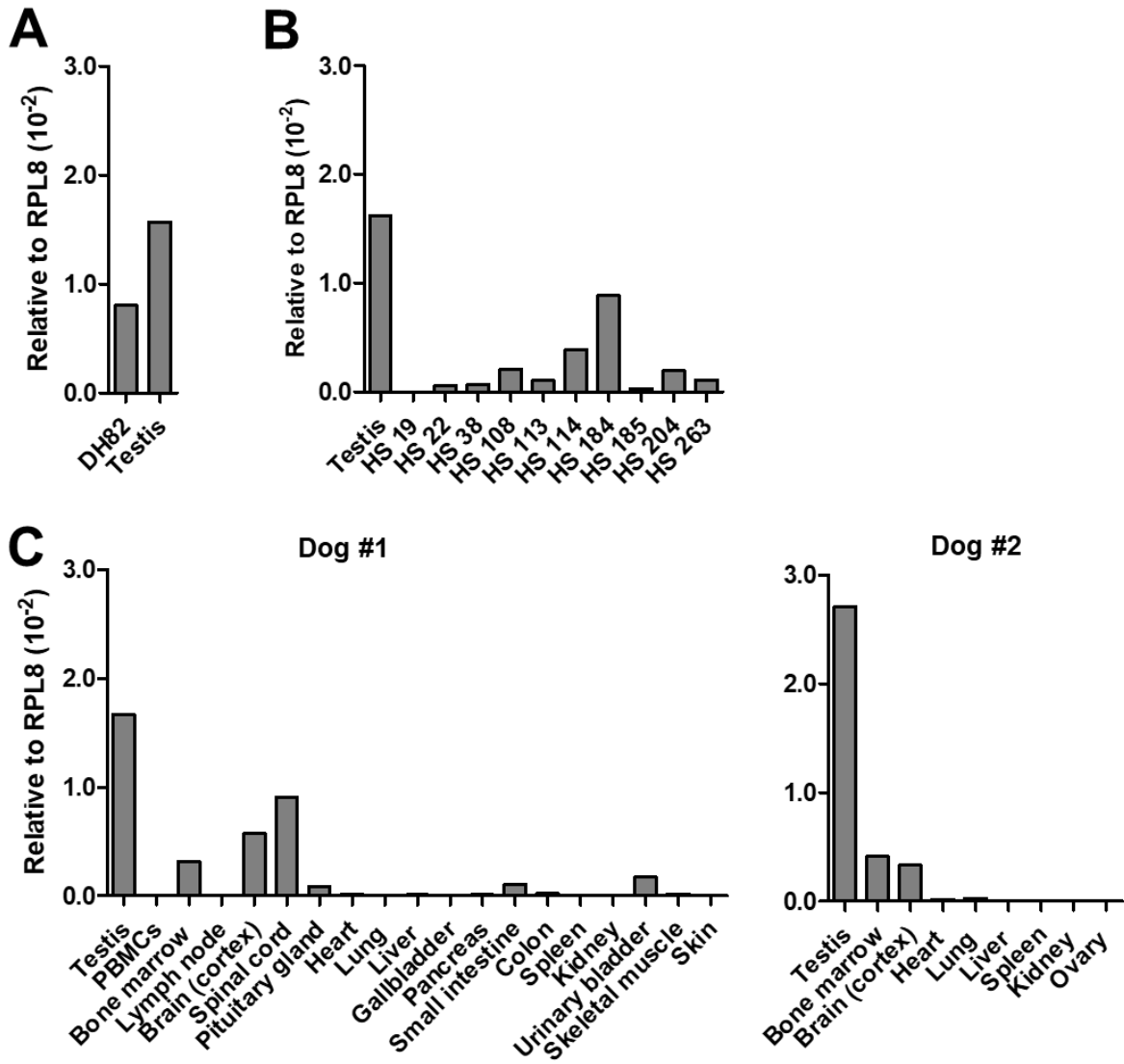


Figure 3.2. Expression of SPECC1 message (relative to RPL8) in DH82 cells and the indicated canine tissues and tumors, as determined by qPCR analysis. A, SPECC1 mRNA is found in DH82 cells and normal testis. B, SPECC1 is expressed in most HS biopsy specimens. C, SPECC1 is strongly expressed in canine bone marrow, brain and spinal cord. Data represents multiple experiments.

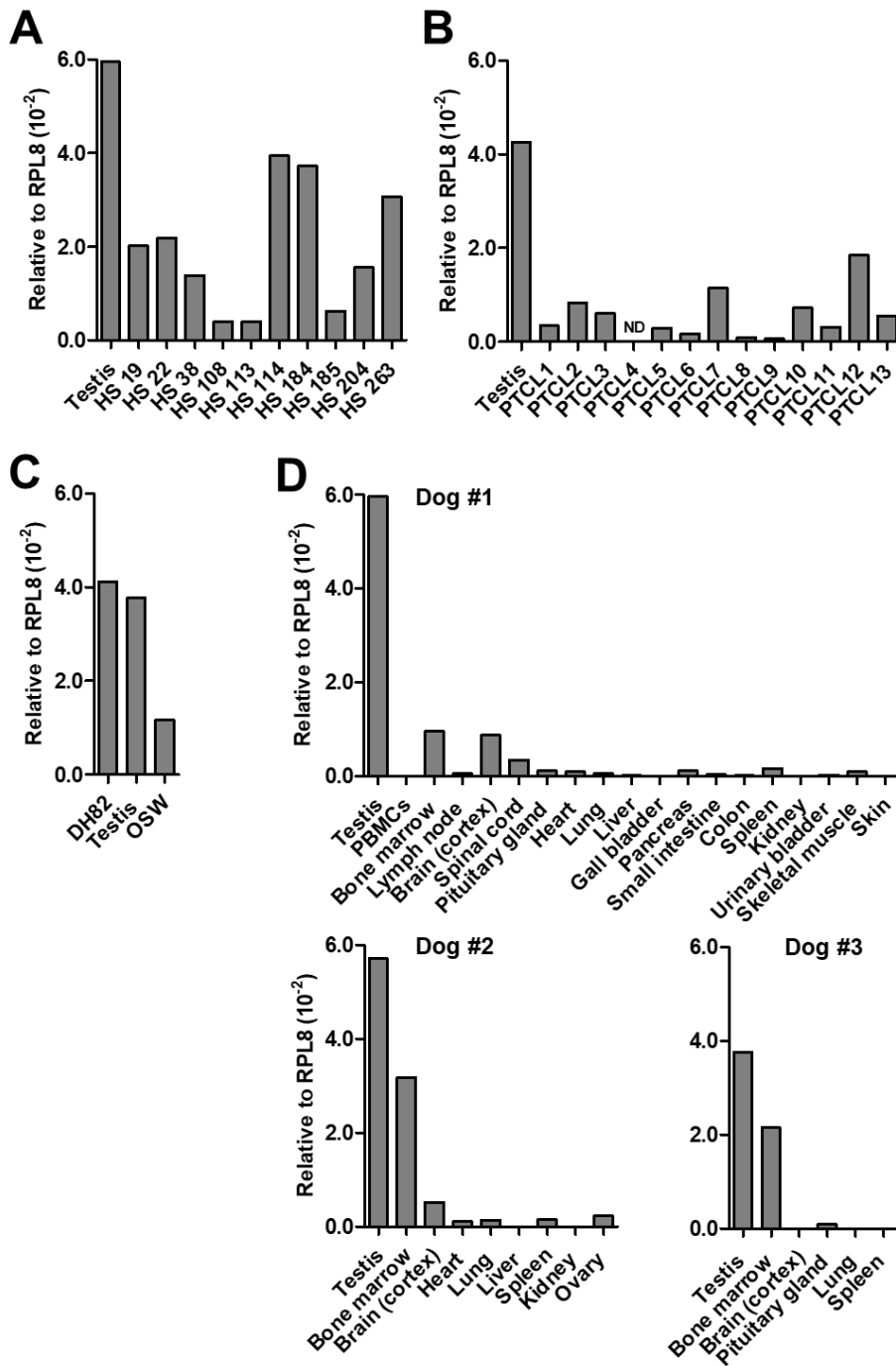


Figure 3.3. Expression of TPX2 message (relative to RPL8) in DH82 cells and the indicated canine tissues and tumors, as determined by qPCR analysis. A, TPX2 is strongly expressed in most HS biopsy specimens. B, TPX2 is expressed in most PTCL lymph node specimens; ND, not done. C, TPX2 is expressed in DH82 cells and the canine T-cell lymphoma line OSW. D. TPX2 is expressed strongly but variably in canine bone marrow and brain, and is weakly-to-minimally expressed in most other non-gametogenic tissues. Data represents multiple experiments.

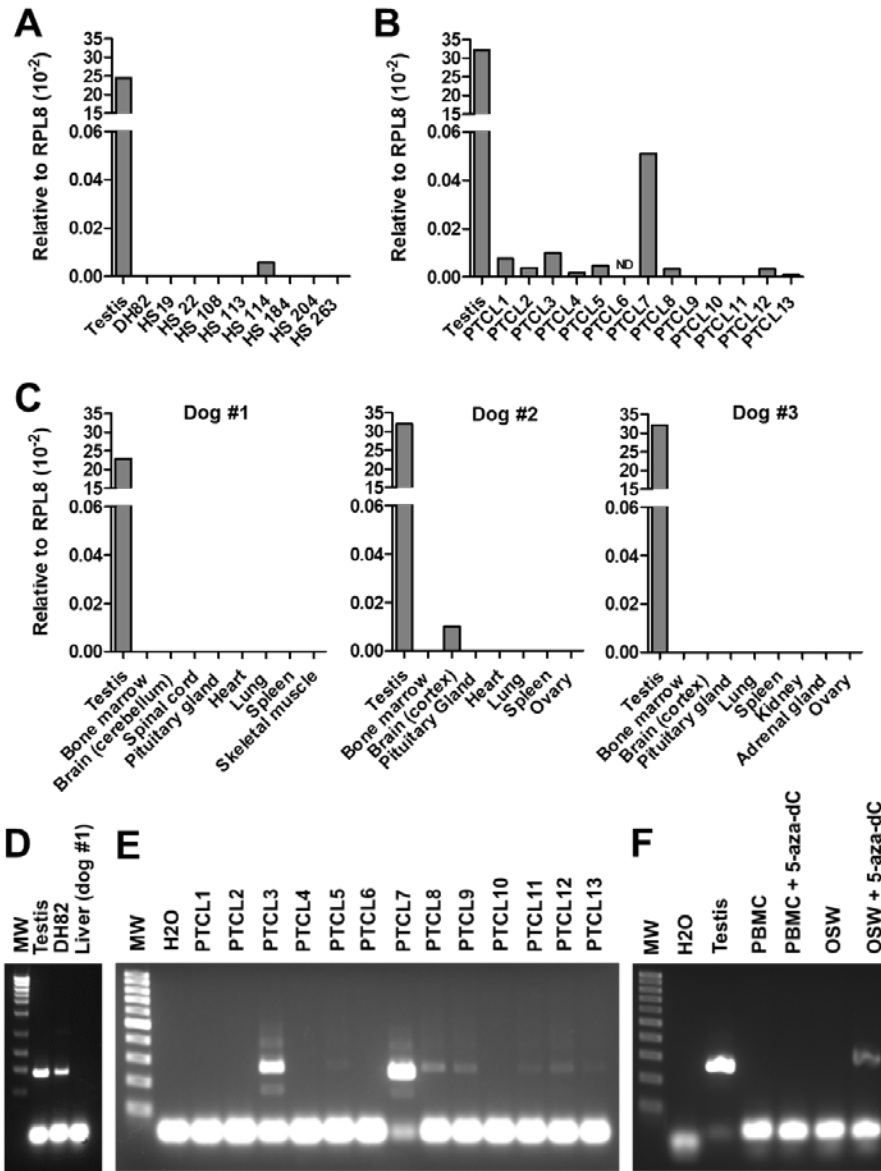
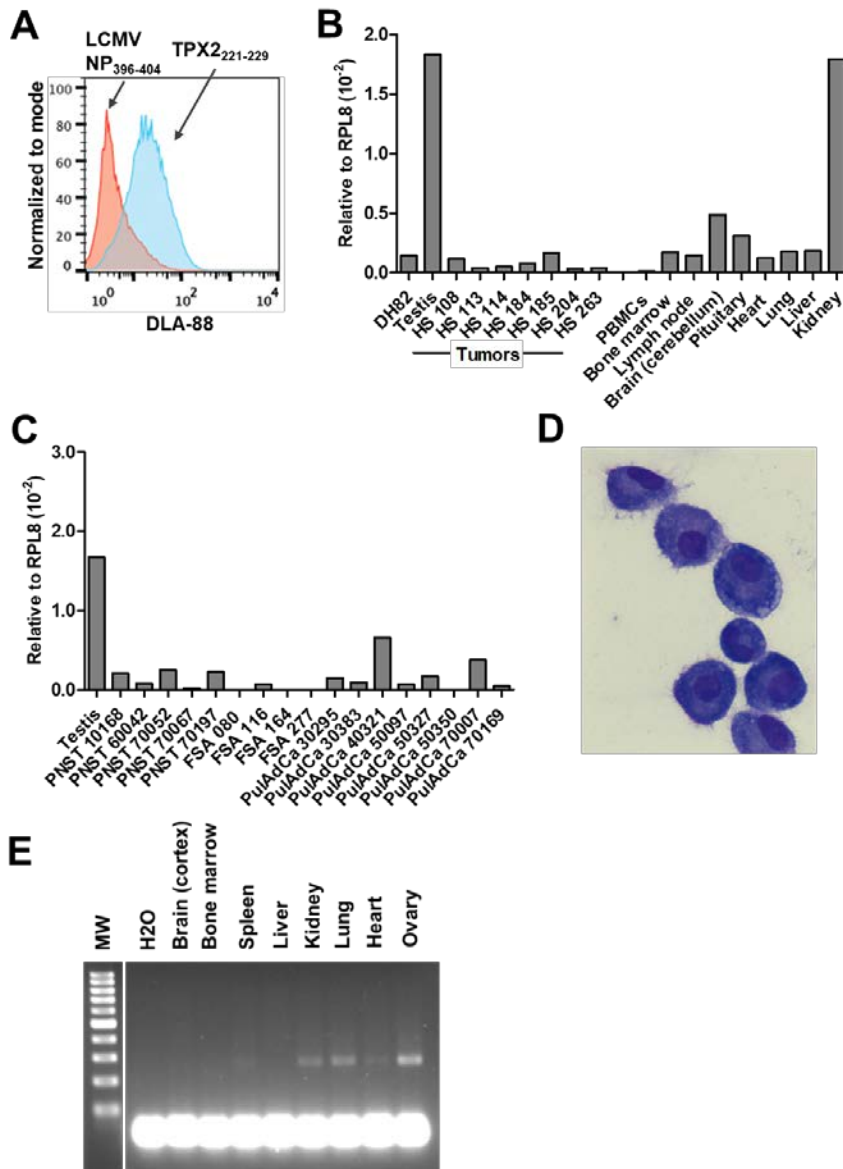


Figure 3.4. Expression of NT5C1B message in the indicated canine tumors and tissues, as determined by qPCR (A-C) or endpoint PCR analyses (D-F). A, NT5C1B is not expressed in HS biopsy specimens. B, NT5C1B is weakly expressed in a single PTCL lymph node specimen; ND, not done. C, NT5C1B is not expressed in normal canine tissues other than testis. Note the discontinuous Y-axes and scale in the graphs in A-C; data represents multiple experiments. D, Gel electrophoresis showing an NT5C1B amplicon in DH82 cells (primer pair 3; mid-range ladder, range 300 – 5000 bp). E, Gel electrophoresis showing NT5C1B amplicons in PTCL lymph node specimens 3, 5, 7, 8, 9, 11, 12 and 13 (primer pair 4; perfect DNA 100 bp ladder, range: 100 – 1000 bp). F, Gel electrophoresis showing induction of NT5C1B message in OSW cells, but not in normal PBMC controls, by 72-hour incubation with 15 μ M 5-aza-dC (primer pair 4; perfect DNA 100 bp ladder, range: 100 – 1000 bp). NT5C1B induction in OSW by 5-aza-dC was confirmed in a second, independent experiment (not shown). The reaction productions from NT5C1B primer pairs 3 and 4 were sequenced to verify amplification of the correct target.



Supplemental Figure S3.1. A, Flow cytometric histogram showing the binding of a peptide derived from TPX2 (TPX2₂₂₁₋₂₂₉; KMQQEVVEM) to DLA-88*508:01. The median fluorescence intensities (MFI) of BARC3 cells at 37°C was 3.68 when either pulsed with NP₃₉₆₋₄₀₄ or not pulsed, and 19.6 when pulsed with TPX2₂₂₁₋₂₂₉. The positive control peptide *K-11* (RFLDKDGFIDK) yielded an MFI of 22.5 (not shown). Data represents three independent experiments. B, Relative expression of AKR1E2 mRNA in the DH82 line, selected HS tumors and normal tissues (from dog #1). C, Relative expression of SPECC1 mRNA in soft tissue sarcomas and pulmonary adenocarcinoma samples. D, Photomicrograph of DCs (day 7) derived from adherent PBMCs using recombinant canine GM-CSF and IL-4 (Wright-Giemsa stain of air-dried spread, x100). Cultured cells were ~50% CD11c⁺ by flow cytometry (not shown). E, Gel electrophoresis showing NT5C1B amplicons in some somatic tissues and the ovary of a healthy dog (#2). Perfect DNA 100 bp ladder, range: 100 – 1000 bp. cDNA integrity was confirmed by obtaining bright bands of the correct length in all samples after PCR for RPL8 (not shown).

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CHAPTER 4: IDENTIFYING THE DLA-88*034:01 PEPTIDE BINDING MOTIF

The prevalent Boxer MHC class Ia allotype Dog Leukocyte Antigen (DLA)-88*034:01 preferentially binds nonamer peptides with a defined motif

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Abstract

Development of effective immunotherapy for chemoresistant malignancies can be advanced by studies in spontaneous cancer models, such as the dog. A crucial first step, T-cell epitope discovery, can be assisted by determination of binding motifs of common Dog Leukocyte Antigen (DLA) class Ia allotypes. Boxers are popular, inbred dogs with increased risks of relevant target cancers and restricted MHC diversity. We sought to identify the motif of DLA-88*034:01, a breed-dominant allotype, to assist peptide prediction from tumor antigens. Mass spectrometry of eluted peptides showed a preference for nonamers with conserved residue preferences: basic at position (P)1; hydrophobic at P2; acidic at P4; H at P6; and F at P9. This data should expedite finding epitopes restricted by this DLA-88 allotype.

Methods and Results

Short peptides loaded and retained in the binding groove formed by the twin α -helices and β -pleated sheet floor of MHC class Ia molecules become available for T-cell receptor (TCR) recognition at the cell surface, thereby directing cognate cytotoxic T lymphocyte (CTL) responses against mutant self- and pathogen-derived antigens. To provide adequate immunity, a

class I molecule must be able to bind a large variety of peptides, within the length constraints imposed by hydrogen bonding of the N- and C-termini at each end of the groove. While class I-eluted ligands show diverse amino acid usage at many positions, at a few specific sites, only invariant or closely related residues are observed. These anchors interact with polymorphic amino acids within the MHC binding pockets to confer high-affinity peptide binding.

Collectively, the preferences for certain anchor residues, as well as peptide length, constitute the motif, which varies with the particular structure and chemistry of the pockets, and is thus allotype-specific.^{1,2} Empty MHC class I molecules initially bind peptides in a much less size- and residue-restricted manner, but canonical peptides dissociate more slowly than those lacking anchors and are competitively retained.³ The motif of a given allotype is discernable by consensus sequence analysis of large numbers of peptides eluted from the binding groove. Defining this motif can be a useful first step in identifying CTL epitopes restricted by that class I molecule, primarily by excluding the vast number of potential peptides within a given antigen that are unlikely to bind. The much smaller pool of predicted binders, which contains a mixture of true and false positives, represents a manageable number of peptides to be tested in CTL-target recognition assays, the final step in epitope confirmation, conserving limiting T-cell resources.⁴

Epitope prediction is a powerful tool for cancer immunotherapy. For example, prediction of mutant peptide binding to autologous HLA molecules led to expedient production of private vaccines containing a limited number of peptides ($n=13-20$) for patients with advanced-stage cutaneous melanoma, inducing T-cell responses against neoantigens.⁵ Such small-scale, phase I studies are encouraging, but far greater investigation will be necessary to determine the optimal means to identify, prime, deliver and sustain neoepitope-reactive T cells capable of mediating

robust and durable anti-tumor responses. Modeling immunotherapies in spontaneous canine cancers could provide critical insights, and propel development beyond what has been furnished by murine studies; however, peptide epitopes from tumor-specific antigens (TSAs) have yet to be reported in dogs. To advance the discovery process, we sought to characterize the binding preferences of the DLA-88*034:01 allele, carried by 82% of Boxers.⁶ Boxers are popular dogs (10th in the latest American Kennel Club ranking), but despite the large population size, are fairly inbred^{7,8}, with restricted MHC class I and II diversity.^{6,9-11} Additionally, the breed has a high incidence of glioma and T-cell lymphoma¹²⁻¹⁴, which are both chemoresistant, deadly tumors in humans for which effective immunotherapies are desperately needed. Canine models of these tumors appear valid (reviewed in Bentley et al.,¹⁵ Richards et al.¹⁶ and Ito et al.¹⁷). Given the constellation of advantageous features of Boxers (large, available population; high incidence of relevant cancers; highly shared DLA alleles), it is not surprising that the breed is becoming an increasing focus of comparative immuno-oncology. We hypothesized that DLA-88*034:01 presents diverse self-peptides with conserved residue preferences at one or more position(s) that could be inferred by pattern analysis. Generating a large dataset of eluted peptides and defining the binding motif of this dominant allele should assist *in silico* determination of candidate CTL epitopes from TSAs in the Boxer breed.

To test our hypothesis, we sought to sequence peptides eluted from the binding groove of affinity-isolated DLA-88*034:01 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The full-length coding sequence of DLA-88*034:01 was obtained by reverse transcription (RT)-PCR of archived RNA from a previously-genotyped Boxer.⁶ To permit affinity purification of the allele, we employed a C-terminal epitope tag approach, as previously described.^{18,19} Accordingly, the DLA-88*034:01 amplicon was ligated into a modified

pcDNA3.1 vector containing the FLAG epitope coding sequence downstream of a *NotI* site. The sequence translation is shown in Supplemental Figure 1. The canine histiocytic cell line DH82, obtained from the ATCC (atcc.org; CRL-10389) and grown in Dulbecco's Modified Eagle's Medium containing 15% FBS, penicillin (100 IU ml⁻¹) and streptomycin (100 µg ml⁻¹), was transfected with the pcDNA3.1-DLA-88*034:01-FLAG construct using Lipofectamine 3000 (Thermo Fisher Scientific; thermofisher.com), following the manufacturer's protocol. After 48 h, transfectants were placed under G418 selection at 800 µg ml⁻¹. Drug-resistant colonies were retrieved by ring-cloning and screened for the presence of intracellular FLAG by flow cytometry, using a FITC-conjugated, anti-FLAG murine monoclonal antibody (mAb; Sigma-Aldrich, sigmaaldrich.com). Clone PN62 was selected based on a strongly positive FLAG signal (not shown). Expression of DLA-88*034:01 at the PN62 cell surface was verified by surface biotinylation assay (Supplemental Figure 2).

Lysates were prepared from pooled PN62 cell pellets (~5 x 10⁸ cells total) that had been harvested weekly. After centrifugation to remove large contaminants, peptide-DLA-88*034:01 complexes were immunoprecipitated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich). Bound DLA-88*034:01-FLAG was eluted, and pooled protein samples were acidified, boiled, and filtered by size exclusion (3 kD) centrifugation. The peptide sample was further cleaned up and concentrated by hydrophilic-lipophilic-balanced (HLB) reversed phase solid phase extraction (SPE; Oasis HLB cartridge; Waters, waters.com). Eluted samples were analyzed by LC-MS/MS, using a nanoACQUITY UPLC system (Waters) coupled to a Thermo Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Mass spectra data were searched against a supplemented *Canis lupus familiaris* protein database using Mascot 2.5.1 (Matrix Science;

matrixscience.com). Peptide and protein identifications were validated using Scaffold 4.8.4 (Proteome Software; proteomesoftware.com).

The length distribution of unique peptides identified by LS-MS/MS is shown in Supplemental Figure 3A. Nonamer peptides (blue bar) are dominant. We obtained 265 unique nonamers from 207 parent proteins (listed in Supplemental Table 1, Experiment 1). These diverse proteins originated from all subcellular locations, including the plasma membrane, cytosol, cytoskeleton, Golgi apparatus, endoplasmic reticulum, mitochondrion, nucleus and other internal membrane-bound organelles (not shown). Upon inspection of the 9-mer peptide list, strong preferences at several positions were immediately evident, as visualized by sequence logo representation (Supplemental Figure 3B). An F occupies the C-terminal position in slightly more than 50% of peptides (Table 1). When amino acids were grouped according to chemical characteristics of their side chains (hydrophobic [A, V, I, L, F, W, M and P], charged-acidic [D, E], charged-basic [K, R], polar [S, T, C, N, Q, Y and H], or left unassigned [G]), it can be seen that 83, 50 and 71% of P2, P3 and C-terminal residues were hydrophobic, respectively (Table 1). By classical definition, the P2 hydrophobic residue constitutes an anchor (frequency >80%), while the P3 and P9 residues are considered auxiliary anchors (frequency 50-80%).²⁰ In HLA class Ia molecules, the P2 and P9 positions are the most common residues recognized as anchors.

Because amino acids naturally occur at varying frequencies in the mammalian proteome, however, this characterization is simplistic and potentially misleading. Therefore, individual and group amino acid preferences were also analyzed at each position as the fold-change in occurrence relative to their expected frequency.²¹ As with the preliminary assessment, altered frequencies of individual peptides and groups were evident at multiple positions in nonamers (Table 2). At the N-terminus, basic residues are favored and acidic are disfavored. Charged

amino acids occur rarely at P2, while most hydrophobic residues, with the exception of F, are highly favored. Some polar-group residues are also observed less frequently than expected at this position. Acidic amino acids at P4 are preferred by DLA-88*034:01. Not surprisingly, with the exception of H at P6, there were no biased frequencies from P5 through P8, since residues at these positions generally interact with the TCR. As before, F strongly dominates the C-terminus, while acidic, polar (except Y), and A, G, and P residues are disfavored. A few self-peptides that conformed strongly to these collective preferences, including KITDIHIKY (VPS13C₃₁₉₋₃₂₇), RLRDGHFKY (TADA1₁₉₈₋₂₀₆), and KIYDDHRKF (TENM4₁₉₄₆₋₁₉₅₄), were identifiable in the eluted peptide pool.

While a preference for nonamers was observed, it seems likely that DLA-88*034:01 can also accommodate longer-length peptides. When 10- and 11-mer peptides were analyzed for frequency of residue usage across positions, the same motif was apparent (Supplemental Figure 3C), suggesting that they had been eluted from the binding groove. Interestingly, the frequency of P at P5 is increased in 11-mer peptides, possibly reflecting the need for reduced flexibility in the backbone of these longer peptides in order to hold anchor residues in their binding pockets.²² Peptides that were 8 (Supplemental Figure 3C) and 12 ($n=35$; not shown) amino acids in length do not match the motif and are thought to be unlikely binders.

To obtain additional support for these conclusions, a second DLA-88*034:01-FLAG immunoprecipitation, peptide elution and LC-MS/MS analysis were performed. A comparable distribution of peptide lengths was observed (Supplemental Figure 3D; nonamers indicated by blue bar). We obtained 86 unique nonamers from 74 parent proteins (Supplemental Table 1, Experiment 2). Fifty of these nonamers (58%) had also been recovered in the first LC-MS/MS analysis as well (indicated by boldface type), including the peptide in this second pool that most

closely represented the motif, KVADPVVTF (EFTUD2₆₅₄₋₆₆₂). When the second nonamer list was analyzed for altered frequencies of amino acid occurrence at each position, as individuals or groups, very similar preferences were seen (Supplemental Figure 3E; Supplemental Table 2, compare to Table 2). The only notable difference between the two analyses is that the frequency of basic residues at P9 was lower in the second experiment, and there was increased usage of M at this position. A summary of the binding preferences of DLA-88*034:01, based on the combined datasets, is shown in Table 3. This work brings the total number of DLA-88 allotypes for which a binding motif has been deduced from eluted peptide sequences to three (DLA-88*501:01; *508:01 and *034:01).^{19,23} Of course, binding assays with synthetic peptides that have various combinations of favored and disfavored residues, as well as A substitutions at these positions, will be needed to verify the conclusions of this study.

In summary, the canine MHC class I allele DLA-88*034:01 presents diverse self-peptides, and appears to preferentially bind nonamers with the motif, Basic-I/L/V-X-Acidic-X-H-X-X-F, based on two independent analyses of eluted peptides. This data supports our hypothesis, and should allow more accurate prediction of TSA-derived peptides recognized by CTL in a majority of Boxers, and foster the development of glioma and lymphoma immunotherapy models in this breed.

Table 4.1. The frequency of amino acid occurrence (as individual or chemical group) by position (P) in nonamer peptides eluted from DLA-88*034:01 (1st experiment).

| Residue or group | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|------------------|-------------------|------|------|------|------|------------------|------|------|------|
| A | 10.9 ^a | 5.3 | 7.5 | 5.7 | 3.8 | 6.0 | 6.4 | 8.7 | 1.1 |
| V | 9.4 | 20.8 | 9.8 | 2.3 | 5.3 | 7.9 | 10.2 | 5.7 | 3.8 |
| I | 6.4 | 29.1 | 12.8 | 1.5 | 6.4 | 7.2 | 6.0 | 2.3 | 4.5 |
| L | 3.8 | 23.8 | 7.9 | 0.8 | 3.0 | 4.2 | 9.1 | 4.9 | 1.5 |
| F | 1.1 | 0 | 6.4 | 0.4 | 1.9 | 3.0 | 1.5 | 0 | 54.3 |
| W | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| M | 0.4 | 2.3 | 2.6 | 0 | 1.1 | 1.5 | 2.3 | 0.8 | 5.3 |
| P | 1.5 | 1.9 | 3.0 | 7.2 | 12.1 | 10.6 | 9.8 | 1.1 | 0 |
| D | 0.4 | 1.1 | 4.5 | 18.1 | 1.9 | 4.2 | 1.5 | 3.0 | 0.4 |
| E | 0.8 | 1.5 | 1.9 | 19.6 | 5.7 | 4.5 | 6.0 | 6.0 | 0 |
| K | 28.7 | 0.4 | 6.8 | 7.2 | 17.4 | 12.8 | 3.0 | 16.6 | 8.7 |
| R | 11.3 | 0.8 | 7.9 | 3.0 | 2.6 | 1.1 | 4.2 | 11.7 | 9.1 |
| S | 9.4 | 1.5 | 6.0 | 8.7 | 3.4 | 4.5 | 8.3 | 9.8 | 0 |
| T | 3.8 | 4.5 | 4.2 | 3.8 | 5.7 | 4.9 | 9.1 | 6.0 | 0.8 |
| C | 0 | 0 | 0 | 0 | 0 | 0.4 ^b | 0 | 0 | 0 |
| N | 1.5 | 0.4 | 6.0 | 4.9 | 3.4 | 5.7 | 6.0 | 3.4 | 0 |
| Q | 2.6 | 3.8 | 1.9 | 4.9 | 5.3 | 4.5 | 6.8 | 9.4 | 0 |
| Y | 2.6 | 0.4 | 5.3 | 1.9 | 1.5 | 1.5 | 3.0 | 0.8 | 9.4 |
| H | 0.4 | 0 | 4.2 | 1.5 | 5.3 | 7.5 | 1.5 | 3.0 | 0 |
| G | 4.5 | 2.3 | 0.8 | 8.3 | 14.0 | 7.5 | 4.9 | 6.4 | 0.8 |
| PHOB | 33.6 | 83.0 | 50.2 | 17.7 | 33.6 | 40.4 | 45.3 | 23.4 | 70.6 |
| ACIDIC | 1.1 | 2.6 | 6.4 | 37.7 | 7.5 | 8.7 | 7.5 | 9.1 | 0.4 |
| BASIC | 40 | 1.1 | 14.7 | 10.2 | 20 | 14.0 | 7.2 | 28.3 | 17.7 |
| POLAR | 20.4 | 10.6 | 27.5 | 25.7 | 24.5 | 29.1 | 34.7 | 32.5 | 10.2 |

PHOB: hydrophobic amino acid group.

^aExpressed as a percentage; group totals do not add up to 100% because G is omitted. In the group analysis, anchor residues (>80%) are marked in darker gray, and auxiliary anchors (50-80%) in light gray.

^bOnly one peptide was recovered with a C at P6.

Table 4.2. Fold-change in occurrence of individual amino acids or chemical group by position (P) in nonamer peptides eluted from DLA-88*034:01 (1st experiment).

| Residue or group | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|------------------|------------------|-----|-----|-----|-----|------------------|-----|-----|------|
| A | 1.3 ^a | 0.6 | 0.9 | 0.7 | 0.5 | 0.7 | 0.8 | 1.1 | 0.1 |
| V | 1.4 | 3.0 | 1.4 | 0.3 | 0.8 | 1.2 | 1.5 | 0.8 | 0.5 |
| I | 1.1 | 4.9 | 2.2 | 0.3 | 1.1 | 1.2 | 1.0 | 0.4 | 0.8 |
| L | 0.4 | 2.5 | 0.8 | 0.1 | 0.3 | 0.4 | 0.9 | 0.5 | 0.2 |
| F | 0.3 | 0 | 1.7 | 0.1 | 0.5 | 0.8 | 0.4 | 0 | 14.1 |
| W | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| M | 0.2 | 0.9 | 1.1 | 0 | 0.5 | 0.6 | 0.9 | 0.3 | 2.2 |
| P | 0.3 | 0.4 | 0.6 | 1.5 | 2.6 | 2.2 | 2.1 | 0.2 | 0 |
| D | 0.1 | 0.2 | 0.8 | 3.3 | 0.3 | 0.8 | 0.3 | 0.6 | 0.1 |
| E | 0.1 | 0.2 | 0.3 | 2.9 | 0.8 | 0.7 | 0.9 | 0.9 | 0 |
| K | 4.9 | 0.1 | 1.2 | 1.2 | 3.0 | 2.2 | 0.5 | 2.8 | 1.5 |
| R | 2.0 | 0.1 | 1.4 | 0.5 | 0.5 | 0.2 | 0.8 | 2.1 | 1.6 |
| S | 1.4 | 0.2 | 0.9 | 1.3 | 0.5 | 0.7 | 1.3 | 1.5 | 0 |
| T | 0.7 | 0.8 | 0.8 | 0.7 | 1.1 | 0.9 | 1.7 | 1.1 | 0.1 |
| C | 0 | 0 | 0 | 0 | 0 | 0.3 ^b | 0 | 0 | 0 |
| N | 0.4 | 0.1 | 1.5 | 1.2 | 0.8 | 1.4 | 1.5 | 0.8 | 0 |
| Q | 0.7 | 1.0 | 0.5 | 1.2 | 1.3 | 1.2 | 1.7 | 2.4 | 0 |
| Y | 0.9 | 0.1 | 1.8 | 0.6 | 0.5 | 0.5 | 1.0 | 0.3 | 3.2 |
| H | 0.2 | 0 | 1.8 | 0.7 | 2.3 | 3.3 | 0.7 | 1.3 | 0 |
| G | 0.6 | 0.3 | 0.1 | 1.2 | 2.0 | 1.1 | 0.7 | 0.9 | 0.1 |
| PHOB | 0.8 | 1.9 | 1.2 | 0.4 | 0.8 | 0.9 | 1.1 | 0.5 | 1.6 |
| ACIDIC | 0.1 | 0.2 | 0.5 | 3.1 | 0.6 | 0.7 | 0.6 | 0.7 | 0 |
| BASIC | 3.5 | 0.1 | 1.3 | 0.9 | 1.8 | 1.2 | 0.6 | 2.5 | 1.6 |
| POLAR | 0.8 | 0.4 | 1.0 | 1.0 | 0.9 | 1.1 | 1.3 | 1.2 | 0.4 |

PHOB: hydrophobic amino acid group.

^aExpressed as fold-change relative to the naturally-expected frequency in mammalian proteins. Highly disfavored residues or groups (≤ 0.1) are marked in light gray, while highly favored (>3.0) are marked in darker gray.

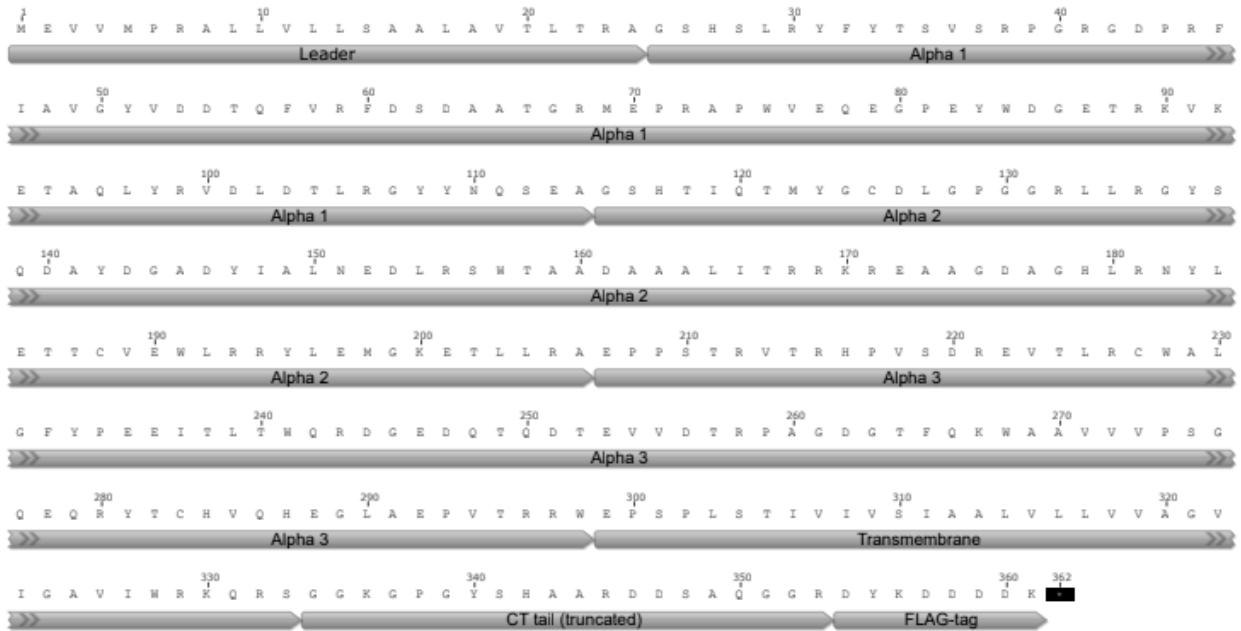
^bOnly one peptide was recovered with a C at P6.

Table 4.3. Individual and group amino acid preferences of DLA-88*034:01 by position (P).

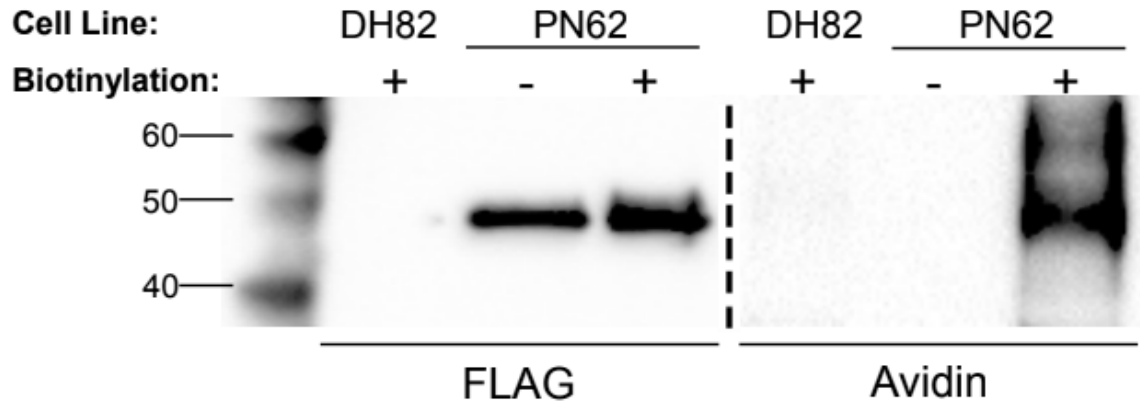
| Preference | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 |
|---------------------------------------|--------|---------------------------------|----|---------|----|----|----|----|--|
| Highly favorable | Basic | I, V | | Acidic | | H | | | F>>Y |
| Highly unfavorable^a | Acidic | Acidic; Basic; F, H, N, Y | G | F, L, M | | | | F | Acidic; Polar except Y; A, G, P |

^aC and W were only observed at one position in one peptide each.

Supplemental Information



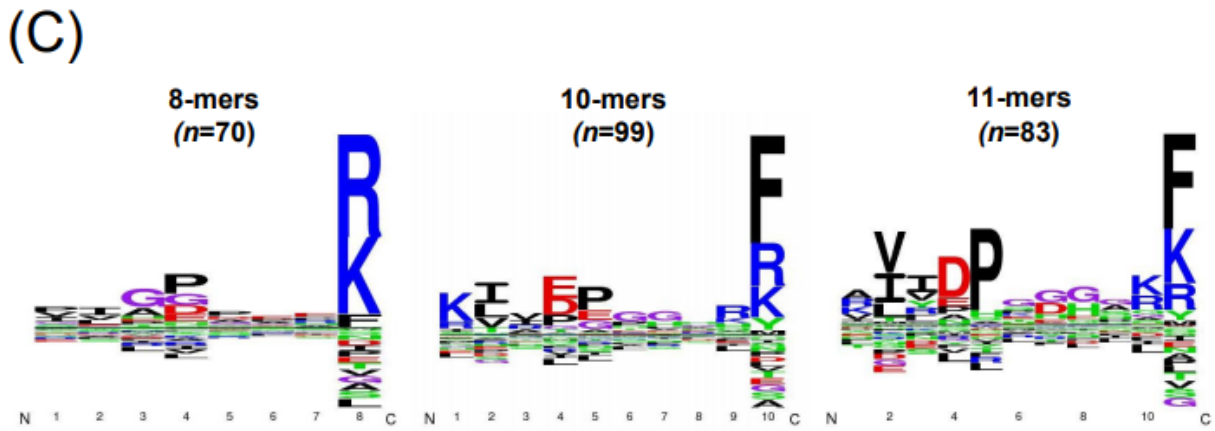
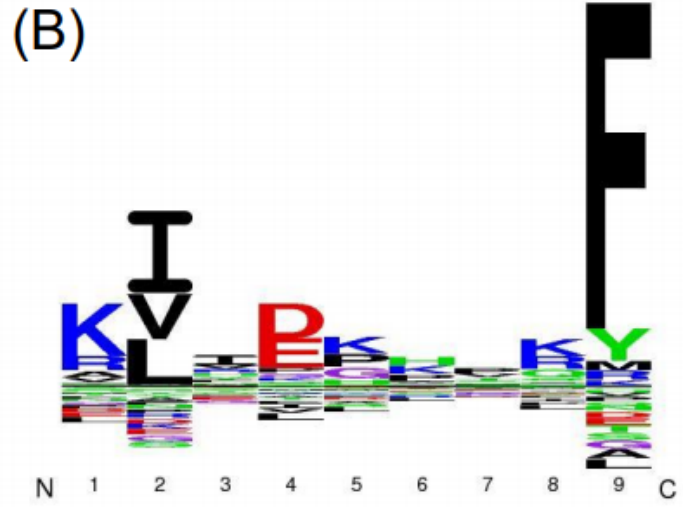
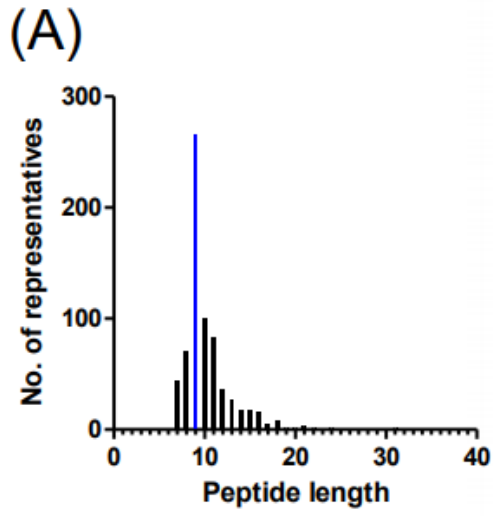
Supplemental Figure S4.1. The putative translation of the DLA-88*034:01-FLAG construct used in this study (GenBank no. MG904874). DLA-88*034:01 was amplified from cDNA (2 μ l) prepared from peripheral blood mononuclear cell RNA isolated from a Boxer. The 50 μ l PCR was performed with a Qiagen HotStar HiFidelity Polymerase kit (qiagen.com) using 1 μ M primers. DLA88-F (5'-GGAATTCGGAGATGGAGGTGGTGA-3') adds an EcoRI site upstream of the initial ATG (underlined), and DLA99-R (5'-ATTAAGCGGCCCGCCCTGGGCACTGTCATCGCGT-3'), adds an Not1 site immediately after the Q350 codon (underlined) in-frame to the vector FLAG sequence, as previously described.¹ The gel-purified ~1300 bp amplicon was digested with EcoRI and Not1 and ligated into pcDNA3.1. The resulting construct was transformed into and propagated in NEB 5-alpha competent *E. coli* (New England BioLabs; neb.com). Cloned plasmids were verified by Sanger sequencing (Eurofins; eurofinsgenomics.com). The MHC class Ia functional domains and FLAG epitope are annotated below the sequence

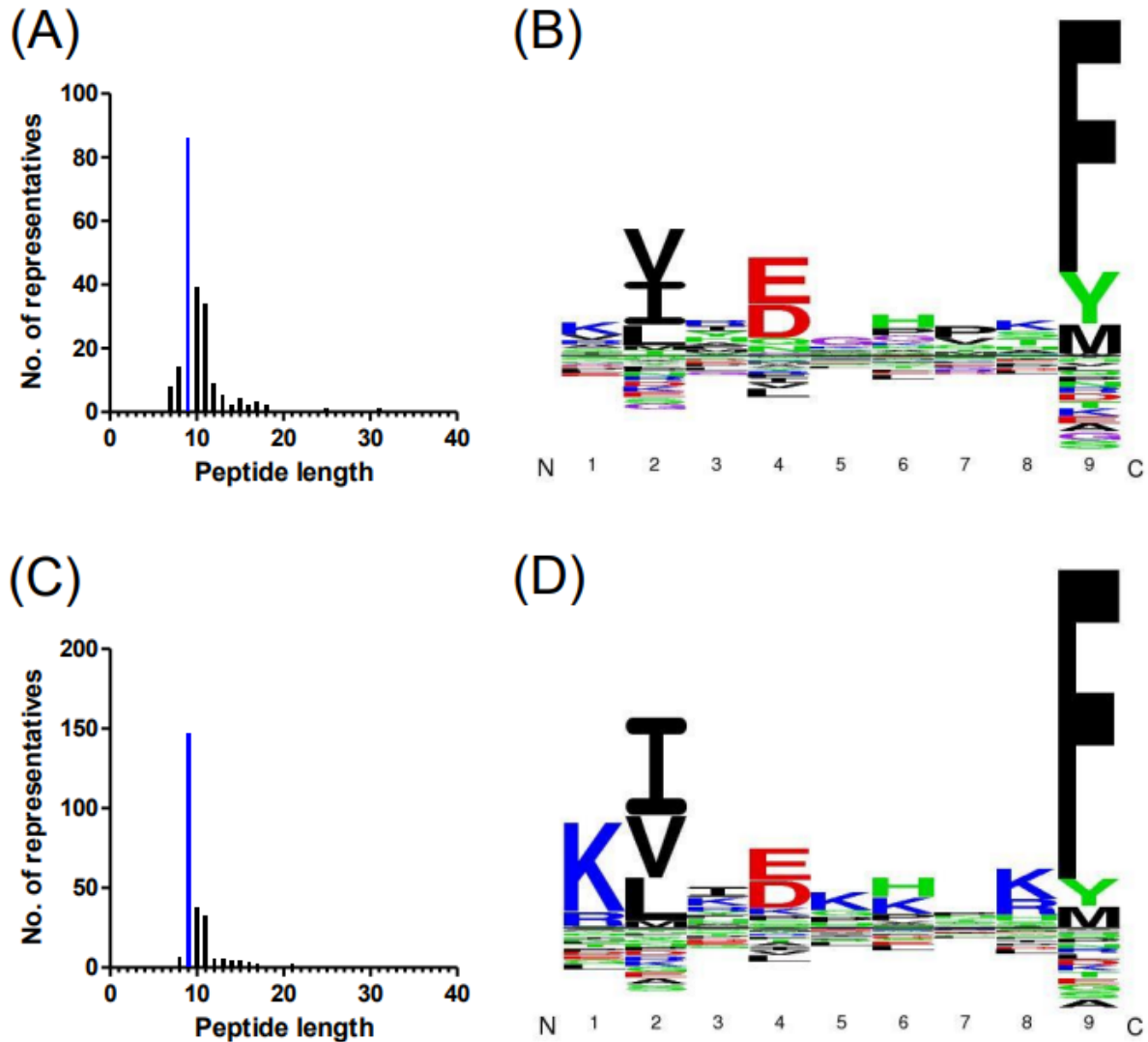


Supplemental Figure S4.2. DLA-88*034:01 is expressed at the cell surface of the PN62 clone. Intact PN62 and DH82 cells grown side-by-side were biotinylated using a Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific). After lysis, FLAG-tagged proteins were isolated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich). Western blotting was performed to verify FLAG capture. Protein samples were denatured at 95C for 4 min, and separated by SDS-PAGE, using a NuPAGE 4-12% Bis-Tris Gel running at 150 V for 45 min with 1X MOPS SDS running buffer in an XCell SureLock Mini-Cell Electrophoresis System (all Thermo Fisher Scientific). The gel was then transferred to a 0.45 μ M nitrocellulose membrane (Bio-Rad; www.bio-rad.com) using an XCell II Blot module, running at 25 V for 90 min in transfer buffer (1xTris-glycine, 20% methanol, 0.1% SDS). The nitrocellulose was blocked in PBS-0.1% TWEEN 20 (PBS-T20) with 10% non-fat dry milk (NFDM) on a shaker at RT for 30 min. The membrane was halved to assay for FLAG and biotin in parallel. The FLAG blot was incubated with a primary rabbit anti-FLAG polyclonal antibody (GenScript; genscript.com) at 1:1,000 in PBS-T20 with 5% NFDM on a shaker at RT for 1 h. After triple washing, the blot was incubated with a secondary horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H+L) antibody (GeneseeScientific; geneseesci.com) at 1:5,000 at RT for 1 h. The biotin blot was incubated with a Pierce High Sensitivity NeutrAvidin HRP Conjugate (Thermo Fisher Scientific) at 1:10,000 in PBS-T20 with 5% NFDM on a shaker at RT for 1 h. After repeated washing, blots were visualized with chemiluminescent detection (SuperSignal West Dura Extended Duration Chemiluminescent Substrate; Thermo Fisher Scientific) on a ChemiDoc XRS+ System (Bio-Rad). The left gel shows that a FLAG-tagged protein at the correct molecular weight (~45 kD; Thermo Super Signal Molecular Weight Protein Ladder is the reference) was isolated from PN62 cells. The right panel shows an avidin signal in biotinylated PN62 cells, indicating that this protein was present at the cell surface during labeling. The same results were observed in two experiments.

Supplemental Figure S4.3. Length and sequence characteristics of peptides eluted from DLA-88*034:01, as determined by LC-MS/MS. The pcDNA3.1-DLA-88*034:01-FLAG plasmid was transfected into DH82 cells using Lipofectamine 3000 (Thermo Fisher Scientific; thermofisher.com), following the manufacturer's protocol. After 48 h, transfectants were placed under G418 selection at 800 µg ml⁻¹. Drug-resistant colonies were retrieved by ring-cloning and screened for the presence of intracellular FLAG by flow cytometry, using a FITC-conjugated, anti-FLAG murine monoclonal antibody (mAb; Sigma-Aldrich, sigmaaldrich.com). Cell pellets from weekly harvests of the PN62 clone, cultured continuously with G418 (200 µg ml⁻¹) and validated monthly for FLAG expression, were stored at -80C. When ~5 x 10⁸ cells were collected, pellets were thawed in lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% NP-40, DNase [20 µl]) and 1% protease inhibitor cocktail [Sigma-Aldrich]), incubated on a rocker at 4C for 1 h, and combined. Large contaminants were removed by centrifugation (3,220 x g at 4C for 5 min) and lysates were further clarified by additional centrifugation (15,996 x g at 4C for 1 h). Peptide-DLA-88*034:01 complexes were immunoprecipitated with Anti-FLAG M2 Affinity Gel (Sigma-Aldrich) and 1% protease inhibitor cocktail on a rocker at 4C overnight. The beads were then poured into a polypropylene column fitted with a 30 µm pore size polyethylene frit and extensively washed. DLA-88*034:01-FLAG was eluted with 0.1 M Tris, pH 12.0, 0.5 M NaCl buffer, in 500 µl aliquots. Western blotting of samples taken at each step of the immunoprecipitation showed that FLAG in the lysates was efficiently captured by the M2-agarose beads, and subsequently detached completely by alkaline elution (not shown). Glacial acetic acid was added to the pooled protein-containing samples to a 10% final concentration (pH 2.0). After boiling for 5 min, samples were filtered through a 3 kD centrifugal filtration device (Amicon Ultra-4 3K; Millipore Sigma; emdmillipore.com), and further purified and concentrated by hydrophilic-lipophilic-balanced (HLB) reversed phase solid phase extraction (SPE; Oasis HLB cartridge; Waters, waters.com).. After washing with 2% formic acid (FA), peptides were eluted with 25% methanol/0.1% FA and 50% methanol/0.1% FA and lyophilized. A portion of the resuspended sample was then injected into a nanoACQUITY UPLC system equipped with a 1.7 µm BEH C18 75 µm x 25 cm column coupled through an electrospray interface to a Thermo Q Exactive HF-X mass spectrometer for 60-min gradient data-dependent acquisition. Tandem MS data were analyzed using the search engine Mascot 2.5.1 (Matrix Science; matrixscience.com), set with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 5.0 PPM and no enzyme rules selected, against a modified *Canis lupus familiaris* protein database containing an additional 106 custom entries representing endogenous and exogenous retroviral proteins (total proteins: 29,713), appended with reverse entries of each protein. Searched spectra were imported into Scaffold 4.8.4 (Proteome Software; proteomesoftware.com) for validation of peptide and protein identifications. Peptide identifications were accepted at >56% probability to achieve a <1.0% false discovery rate (FDR) by the Peptide Prophet algorithm, based on decoy database searches. 2 Protein identifications were accepted at >97% probability to achieve a <5.0% FDR by the Protein Prophet algorithm, with a 1 peptide minimum. 3 Prior to motif analysis, internal quality control and obvious contaminating peptides (e.g., derived from human keratin or porcine trypsin) were discarded. When necessary, peptide and protein identities were verified by searching the dog (taxid: 9615) and mammal (taxid: 40674) databases, using the NLM NCBI BLASTP program available at the URL: <https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>. (A) In the first experiment, a total of 696 unique peptides, representing 335 different proteins, were recovered. For convenience, the blue bar highlights nonamers. (B) Graphical display of amino acid

preferences of DLA-88*034:01, based on the relative residue frequencies at each position of eluted nonamers (n=265). (C) Sequence logo representations of residue usage by position for 8-, 10- and 11-mer peptides recovered in the first experiment. In (B) and (C), the P-weighted Kullback-Leibler-type sequence logos were generated by Seq2Logo 2.0 (<http://www.cbs.dtu.dk/biotools/Seq2Logo/>), using Hobohm1 clustering, with a specificity threshold of 0.63.4 In these representations, amino acids were assigned the following color codes: hydrophobic (A, V, I, L, F, W, M, and P), black; charged-acidic (D and E), red; charged-basic (K and R), blue; polar (S, T, N, Q, Y and H), green; and unassigned (G), purple. On the X-axis, amino-acid positions are indicated by numbers; N and C represent the amino- and carboxy-termini, respectively.





Supplemental Figure S4.4. Length and sequence characteristics of peptides eluted from DLA-88*034:01, as determined in two additional independent experiments. (A) In a second experiment, a total of 210 unique peptides, representing 135 different proteins, were recovered. The blue bar again highlights nonamers. (B) Sequence logo representation of amino acid preferences of DLA-88*034:01 based on the nonamers (n=86) identified in the second experiment. (C) Length distribution of the 248 peptides recovered in a third experiment. One hundred forty-seven nonamers, derived from 144 parent proteins, are highlighted by the blue bar. (D) Sequence logo representation of amino acid preferences of DLA-88*034:01 based on the nonamers identified in the third experiment. In (B) and (D), sequence logos were generated and labelled in the identical manner as described in Supplemental Figure 3.

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CHAPTER 5: THE DLA-88*034:01 PRESENTS CDV PEPTIDES WITH A DEFINED BINDING MOTIF

Abstract

DLA-88*034:01 is a highly prevalent class I allele in Boxers—a popular dog breed that is 4x more likely than mixed breeds to develop lymphoma—and therefore can be useful in cancer immunotherapy development. Our lab has previously eluted DLA-88*034:01 self-peptides and established a tentative binding motif for this classical class I canine allele. When studying anti-tumor CTL responses, a positive control against a known CTL epitope is needed. In humans, there are known CTL epitopes from ubiquitous viruses such as CMV, EBV and the flu virus, but this is not the case for the dog; no CD8⁺ T-cell-restricted minimal epitope has been described in canines. However, CTL against the canine distemper virus (CDV) protein, hemagglutinin (H), has been reported. Moreover, dogs are often vaccinated multiple times with live CDV, so CTL should be detectable *ex vivo*. We have previously reported CDV peptides restricted to DLA-88*508:01. Using our BARC3 cell line, an RMA-S derivative expressing DLA-88*034:01, we were able to confirm the class I binding motif and binding of CDV peptides. Here, we generated a BARC3 equivalent, BB13, that instead, expresses DLA-88*034:01 and was used to confirm the predicted class I allele motif and verified binding of CDV peptides. The immunogenicity of these peptides will determine their value as positive controls in identifying a tumor epitope CTL in the dog. This information is critical for tracking cancer vaccine efficacy.

Introduction

Spontaneous cancers in dogs parallels human disease in regards to incidence, clinical presentation, molecular profiles and resistance to therapy. These similarities make the canine a great model for examining CTL-based immunotherapies in cancer. However, this approach is

currently complicated by the fact that minimal epitopes have yet to be discovered in the dog, and canine class I major histocompatibility complexes (MHC I) that restrict these responses are undefined. We and others have provided indirect evidence that dog leukocyte antigen (DLA)-88 is a classical class I locus and most likely regulates CTL responses¹⁻⁴. We have also defined DLA-88*034:01 as a highly prevalent allele in the Boxer, a popular breed with increased risk of chemoresistant cancer⁵.

To study anti-tumor CTL in Boxers, however, we will need a positive control. In human studies, it is common to use peptides from ubiquitous viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV) and influenza. An analogous approach in the dog is challenging because a highly frequent viral infection is not common in dogs. However, canine distemper virus (CDV) is probably the most reasonable target because most domestic dogs are repetitively vaccinated with live CDV as puppies and throughout their lifetime. Furthermore, CTL against the hemagglutinin (H) protein has been demonstrated and CDV has only 6 genes, increasing the likelihood of identifying a CDV minimal epitope that dictates a CTL⁶. For these reasons, we sought to determine which CDV peptides are processed and presented by DLA-88*034:01, as a first step in defining CTL in the Boxer. These peptides are necessary for identifying an epitope-level class I allele-restricted CTL against CDV, which can be used as a positive control to find epitope-specific tumor CTL for cancer immunotherapy development.

Materials and Methods

Cell culture and RMA-S cell transfection/cloning

The murine lymphoma line, RMA-S, was cultured with RPMI-1640 supplemented with 10% FBS, penicillin (100 IU mL⁻¹)/streptomycin (100 µg mL⁻¹) (P/S) and 2 mM L-glutamine (R-10). The DLA-88*034:01-FLAG expressing cell line, PN62, was cultured in DMEM

supplemented with 15% FBS, penicillin (100 IU mL⁻¹)/streptomycin (100 µg mL⁻¹) (P/S) and G418 (0.2 mg/ml).

A modified pcDNA3 expression plasmid encoding DLA-88*034:01 heavy chain with a FLAG epitope-tag at the carboxyl terminus and geneticin (G418) antibiotic resistance gene, was transfected into RMA-S cells using Lipofectamine 3000 (Invitrogen). Briefly, RMA-S cells (1.25x10⁶ cells) were plated in a 24-well flat bottom tissue culture plate (Corning). The DLA-88*034:01 containing plasmid (1 µg) was diluted in P3000 (2 µl) and 50 µl Opti-MEM Media (Gibco, Grand Island, NY). The Lipofectamine 3000 reagent (2.5 µl) was diluted in 25 µl Opti-MEM Medium. The DNA and Lipofectamine (DNA/Lipo) were combined and incubated for 30 min. at room temperature in the dark. The DNA/Lipo was added dropwise to the RMA-S cells (in Opti-mem for at least 30 minutes prior) and incubated for 6 hrs. After 6 hrs. antibiotic free R-10 (400 µl) was added to the cells and incubated overnight. After 48 hours, the cells were placed under selection with R-10 selection media (R-10 + G418 antibiotic (0.8 mg/ml)) for 7 d, then 0.2 mg/ml thereafter). Clones were isolated by limiting dilution and screened for vector expression after fixation and permeabilization (Cytotfix/Cytoperm; BD Biosciences) and intracellular staining with anti-FLAG mab M2 (Sigma-Aldrich) by flow cytometry. Clone number 13 (BB13) was used throughout the study and continuously maintained under G418 selection (0.2 mg/ml).

Infection of PN62 cells with eGFP-CDV

A CDV clone (Onderstepoort vaccine strain) expressing enhanced Green Fluorescent Protein (eGFP) has been described previously⁷. Infection of PN62s was initiated by adding CDV-eGFP stock (tissue culture supernatant stored at -80 °C) to a 40% confluent T-182 flask. Cells were ~100% eGFP⁺ by 7 days post-inoculation. Infected PN62 cells were maintained by transferring ~10-20% of eGFP⁺ cells to a new flask seeded with uninfected PN62 cells at 40-

60% confluency while the remaining ~90% of CDV infected cells were pelleted, washed and stored at -80 °C for immunoprecipitation. This cycle of inoculation of uninfected cultured followed by harvesting occurred every 2-3 days ensuring high cell viability and expression of viral proteins.

*Isolation of DLA-88*03:401 bound CDV peptides*

Peptides were isolated as previously described⁸. CDV infection was monitored via fluorescent microscopy where ~100% of PN62 cells were expressing eGFP at time of harvest. Briefly, once a total of 5×10^8 eGFP-CDV infected PN62s were harvested, cell pellets were thawed in lysis buffer (10 M Tris pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% NP-40, DNase (20 μ l) and 1% protease inhibitor cocktail (Halt Protease and Phosphatase Inhibitor, ThermoFischer Scientific) and incubated at 4 °C for 1 h. To remove large contaminants, the lysate was centrifuged (3220 x g for 5 min at 4 °C) and further clarified by ultracentrifugation (100,000 x g for 1 h at 4 °C). DLA-88*034:01 complexes were immunoprecipitated overnight at 4 °C using Anti-FLAG M2 Affinity Gel (Sigma). After extensive washing, bound peptides were eluted with 0.1 M Tris, 0.5 M NaCl elution buffer (pH 12), boiled for 5 minutes and filtered through a 3 kD centrifugal filtration device (Amicon μ ltra-4 3K; Millipore). Eluted peptides were analyzed by LC-MS/MS, using a nanoACQUITY UPLC system (Waters) coupled to a Thermo Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific). Mass spectra data were searched against a supplemented *Canis lupus familiaris* protein database, and peptide and protein identifications were validated using Scaffold 4.8.4 (Proteome Software; proteomesoftware.com).

*MHC class I, DLA-88*508:01, motif and CDV peptide binding assays*

To bring MHC class I molecules to the cell surface, RMA-S and BB13 cells were incubated at 27°C overnight, as previously described⁹. Peptides (synthesized by Peptide 2.0) from DMSO stock solutions (10 mM) or DMSO only were added to overnight cell cultures (10⁵ in 200 µl) at 100 µM in a 96-well flat-bottom cell culture plate (Genesee Scientific), followed by 5 hours of incubations at 37°C. As a negative and positive control for peptide binding, peptide 34-6 (AAAAAAAAAA) and 34-16 (KIYDDHRKF) were added to a well of BB13s in every experiment and some BB13s were kept at 27°C as a positive control for MHC surface expression. Table 1 lists the motif peptides used to test the DLA-88*034:01 binding motif. Table 2 lists the peptides used to test DLA-88*034:01 binding of CDV peptides that were identified from the PN62 CDV+ IP/mass spectrometry analysis.

Flow Cytometry for Peptide Binding Verification

Cells were transferred to a 96-well round-bottom polypropylene plate and washed in FACS buffer (PBS containing 2% FBS, 2 mM EDTA and 0.02% sodium azide) with 5% normal goat serum and primary and secondary staining was performed at 4 °C for 20 minutes. The primary antibody, anti-MHC class I (DG-H58A, Monoclonal Antibody Center, Washington State University, Washington) and secondary antibody, Alexa Fluor 647-conjugated F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch, Pennsylvania) were both diluted at 1:200 in FACS and cells were stained in 100 µl volumes. Background staining was determined by omission of the primary antibody. Cells were analyzed using a Cytoflex flow cytometer (Beckman Coulter) and data was analyzed with the CytExpert Software (Beckman Coulter). The APC channel MFI was used to represent binding strength of each peptide which was determined with the following equation: $(\text{MFI}_{\text{Peptide}} - \text{MFI}_{\text{DMSO}}) / (\text{MFI}_{27^\circ\text{C}} - \text{MFI}_{\text{DMSO}})$.

Results and Discussion

We intended on infecting PN62s with CDV and sequencing the DLA-88*034:01 presented peptides returned from MS. However, not all peptides identified by MS are processed and presented; some are artifactual¹⁰. To have confidence that the CDV peptides truly originate from the binding groove, we accepted those that 1) match the motif and 2) stabilize DLA-88*034:01 on the surface of RMA-S cells. To experimentally verify this motif, we generated the BB13 cell line. These cells functioned as expected and produced positive MHC staining when incubated at 27°C for 24 hours. Furthermore, the highly-motif matched peptide, returned by MS, stabilized MHC complexes at the cell-surface, verifying class I binding. We therefore used the BB13 cell line to confirm CDV peptides from our MS data.

*Production of DLA-88*034:01 expressing murine line*

We have previously developed a DLA-88*508:01 expressing clone, BARC3, that was successfully able to differentiate between motif matched and unmatched peptides¹¹. We used the same approach here to isolate the RMA-S clone, BB13, this time expressing the prevalent Boxer allele, DLA-88*034:01⁵. After antibiotic selection, the highly FLAG-positive clone 13 (BB13) was chosen for subsequent peptide binding experiments (Figure 1).

*BB13 cell line stably expresses DLA-88*034:01 on the cell surface at 27 °C*

When RMA-S cells are cultured at 27 °C, MHC complexes accumulate on the cell-surface⁹. To determine if MHC complexes were being accumulated on the RMA-S derivative, BB13s, cells were placed at 27 °C overnight and stained with a MHC antibody. Indeed, we observed highly express DLA-88*034:01 at the cell surface. BB13 cells that were placed at 37 °C for 5 hours after overnight incubation displayed no MHC expression (Figure 2). This verified

that our FLAG expressing clone expresses the transfected MHC allele at the cell surface and further allow confirmation of the DLA-88*304:01 motif.

Determination of optimal peptide concentration for binding assay

When RMA-S cells are incubated with exogenous peptide able to bind in the MHC class I groove, surface pMHC complexes are stabilized⁹. To exclude the possibility of peptide binding and stabilization of class I complexes simply due to oversaturation of peptide, we first identified the optimal concentration of peptide to use in our binding assays. We analyzed MHC expression on BB13 cells when pulsed with the DLA-88*034:01 self-peptide, 34-6, as well as the motif peptide, 34-7, at concentrations ranging from 0.1 μ M to 100 μ M (Figure 3). We found MHC expression plateaued with addition of peptide at 50 μ M, so this concentration was used in subsequent experiments.

*Confirmation of the DLA-88*034:01 peptide-binding motif*

To verify DLA-88*034:01 class I surface expression, we pulsed BB13 cells with a canine self-peptide with the DLA-88*034:01 binding motif along with a 9-mer peptide consisting of all alanine (A) amino acids (34-16) as a negative control (Figure 4A). We previously identified the canine self-peptide, KIYDDHRKF (teneurin transmembrane protein, TENM4₁₉₄₆₋₁₉₅₄), as conforming strongly to the DLA-88*034:01 binding motif through IP and mass spectrometry experiments.⁸ We hypothesized this motif containing self-peptide would bind strongly to the class I complex, giving high H58A signal and therefore could be used as a positive control for BB13 peptide binding assays. As expected, the self-peptide demonstrated an increase in H58A staining, indicating binding and stabilization of the DLA-88*034:01 complexes at the cell surface. The absence of H58A staining after 34-16 peptide addition demonstrated that the self-peptide binding was specific and the BB13 cells can be used to verify DLA-88*034:01 peptide

binding. Furthermore, peptides consisting of the DLA-88*034:01 motif with alanine substitutions at non-binding/non-motif positions were also tested using the BB13 cell line to verify the binding motif (Table 1, Figure 4B).

Peptide 34-7 represented the ideal DLA-88*034:01 motif with a K, I, D, H and F at positions (P)1, P2, P4, P6, and P9, respectively. Indeed, this peptide demonstrated high H58A expression, indicating these positions are important for binding in the MHC pocket. To further refine the class I binding motif, peptides were synthesized to include a substitution with an A at each of the motif positions. As expected, peptides with motif amino acids at P2 and P9 (34-8, 13 and 14, Table 1)—the two most common positions in HLA class Ia molecules—demonstrated strong MHC stabilization (Figure 5). Loss of MHC stabilization with replacement of the motif residue, isoleucine (I), with an A at anchor P2 indicates that at least two motif residues are needed for sufficient binding (Figure 5). Residues at P3 and P9 are considered auxiliary motifs (frequency 50%-80%) and this is illustrated by peptide 34-9, showing slightly less class I stabilization than peptide 34-14, which increases stabilization with the addition of the motif residue at P2¹². Interestingly, P6, a position associated with the motif, but not typically with strong anchor or auxiliary positions¹², seemed to be important for peptide binding as demonstrated with strong MHC stabilization with 34-11 (Figure 5). This information is valuable for class I peptide binding prediction, an important step identifying antigens to target for vaccine development.

*CDV peptides are presented by DLA-88*034:01*

We have previously identified CDV peptides that are presented by DLA-88*508:01. We sought to determine the CDV peptides presented by DLA-88*034:01. After elution of peptides from CDV-eGFP infected PN62 cells and subsequent sequencing via LC-MS/MS, we obtained

13 peptides from five CDV genes (Fusion (F), Hemagglutinin (H), Large (L), Matrix (M)) and one accessory peptide from V which is encoded by the phosphoprotein (P) gene (Table 2).

Knowing the DLA-88*034:01 motif helped us identify peptides that were most likely artifactual and non-binders (peptides in red, Table 2). This allowed us to refine the list of peptides to test empirically to five peptides (peptides in black, Table 2). Using the previously described MHC stabilization assay with BB13 cells, we verified binding of both H peptides as well as the M and L peptides, however, the V peptide proved to be a non-binder (Figure 5).

Summary

Overall, we accomplished our objectives of 1) developing a DLA-88*034:01 expressing cell line, BB13, that can be used to experimentally verify peptide binding, 2) analyze binding contribution of preferred motif residues and 3) identify CDV peptides restricted to DLA-88*034:01. We have previously confirmed the DLA-88*508:01 peptide binding motif and identified allele-restricted CDV peptides using similar methods¹. For each motif, P2 and P9 anchor positions were identified as important residues for binding and P3 seemed to be less stringent than the former two anchor residues. Surprisingly, P6 was a relevant residue for peptide binding. This position was not identified as part of the DLA-88*508:01 motif, and so was not specifically tested.

Although CDV peptides were recovered in both motifs, mass spectrometry analysis revealed a larger number of CDV peptides for DLA-88*508:01 (22) than for DLA-88*034:01 (13), but both retrieved peptides from all CDV 6 genes. This output is similar to the gene/peptide profile for HLA genes from cells infected with measles virus (MV)¹³--the human CDV equivalent. As previously mentioned, many peptides returned from mass spectrometry are artifactual and require experimental confirmation. Subjecting the CDV peptides to binding

assays indeed narrowed down the list for both motifs. The percentage of peptides determined, “genuine”, was also lower in DLA-88*034:01 (~31%) than DLA-88*058:01 (50%). However, this is not necessarily translate to lack of success, because the four binders are subject for CTL testing to determine immunogenicity, our ultimate goal. Human studies have previously found that of the MV peptides restricted by HLA-A2, 25% demonstrated CTL activity¹⁴⁻¹⁷. If this is true, at least one of the four CDV class I-restricted peptides should produce an immunogenic response. Furthermore, two of the four genuine peptides are products of the H gene, the only CDV protein in which an epitope CTL has been defined⁶. In conclusion, four CDV-DLA-88*034:01-restricted peptides identified in this study seem to be genuinely processed and presented by class I molecules and their immunogenic potential will be determined in subsequent CTL assays.

Conclusion

Our data has verified that DLA-88*034:01 has a defined binding-motif and presents CDV peptides. Also, we have developed a cell line, BB13, that successfully distinguishes between DLA-88*034:01-restricted peptide binders and non-binders. This information will allow us to test the true CDV peptide binders for immunogenicity and is the first step in obtaining a positive-control viral pMHC tetramer for identifying tumor epitope CTL and development of a canine cancer vaccine.

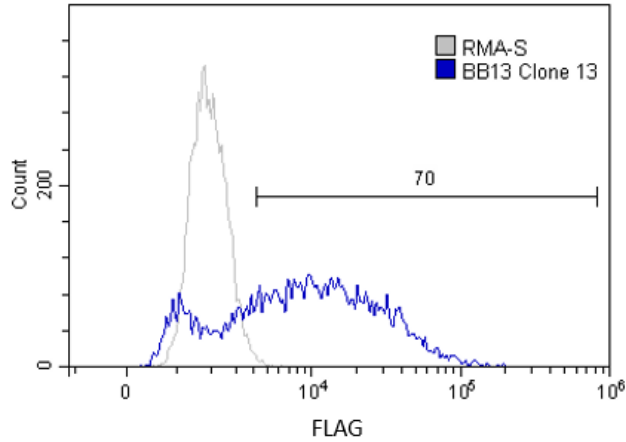


Figure 5.1. DLA-88*34:01 expressing RMA-S clone. Expression of DLA-88*034:01 on the RMA-S cell surface was verified by intracellular FLAG staining and subsequent analysis via flow cytometry. RMA-S cells without MHC transfection are shown as a negative control.

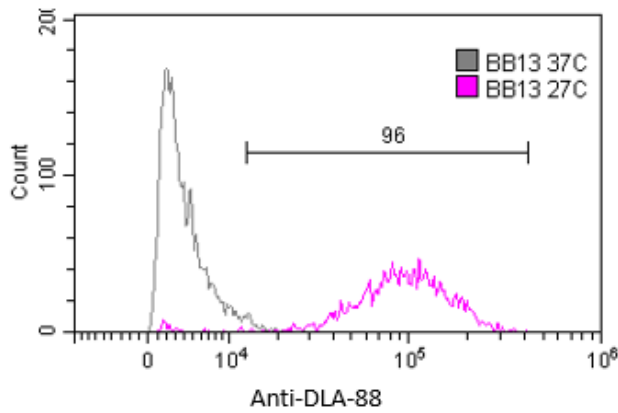


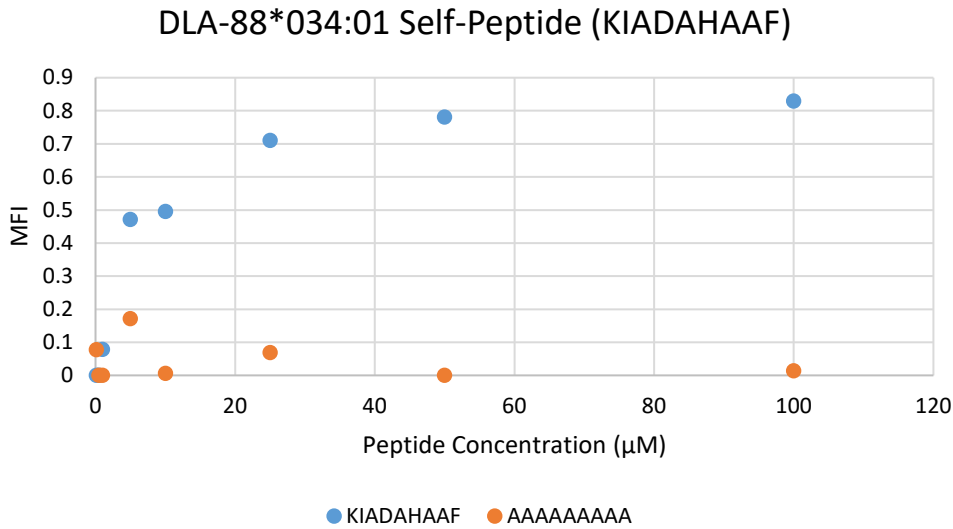
Figure 5.2. MHC class I is expressed on the cell surface of BB13 cells at 37 °C but not at 27 °C. BB13 cells were cultured at 37 °C and then transferred to 27 °C overnight, or kept at 37 °C followed by H58A staining for detection of MHC class I molecules at the cell surface.

Table 5.1. DLA-88*034:01 Motif Peptides.

| Peptide ID Name | Peptide Sequence | Motif Positions |
|------------------------|-------------------------|------------------------|
| 34-16 | AAAAAAAAA | No motif |
| 34-6* | KIYDDHRKF | P1, P2, P4, P6, P9 |
| 34-7 | KIADAHAAF | P1, P2, P4, P6, P9 |
| 34-8 | AIAAAAAAF | P2, P9 |
| 34-9 | AAADAAAAF | P4, P9 |
| 34-10 | KAAAAAAAF | P1, P9 |
| 34-11 | AAAAAHAAF | P6, P9 |
| 34-12 | AAAAAAAF | P9 |
| 34-13 | KIAAAAAAF | P1, P2, P9 |
| 34-14 | AIADAAAAF | P2, P4, P9 |
| 34-15 | KAADAAAAF | P1, P4, P9 |

*Self-Peptide (TENM4₁₉₄₆₋₁₉₅₄) detected by mass spectrometry from IP of PN62 cells

A)



B)

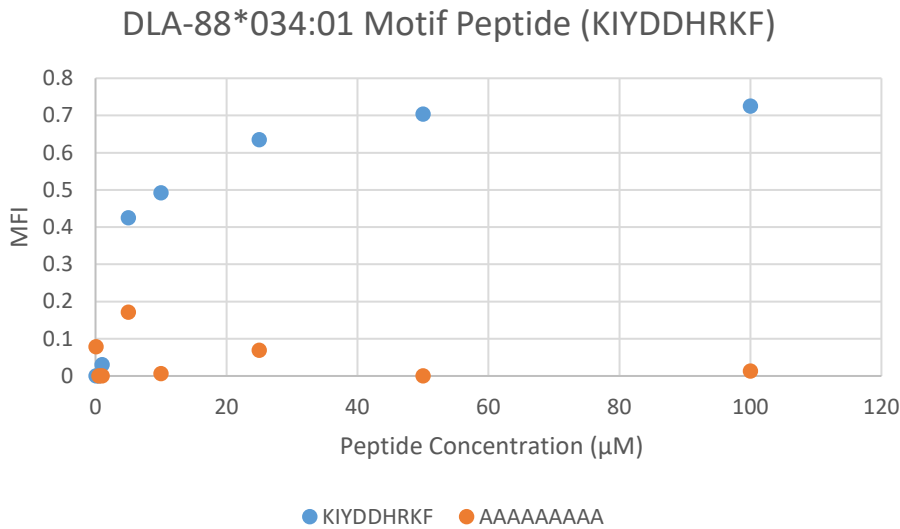


Figure 5.3. DLA-88*034:01 motif and self-peptide binding is concentration dependent. Self-peptide, TENM4₁₉₄₆₋₁₉₅₄ (KIADAHAAF), (A), motif peptide, 34-7 (KIYDDHRKF), (B) or negative control peptide, 34-16 (AAAAAAAAAA) (A and B) was added to BB13 cells that had been cultured overnight at 27°C and then placed at 37°C for 5 hours followed by staining with H58A and flow cytometry analysis. MHC expression plateaued at 50 µM and therefore was the concentration chosen for subsequent experiments.

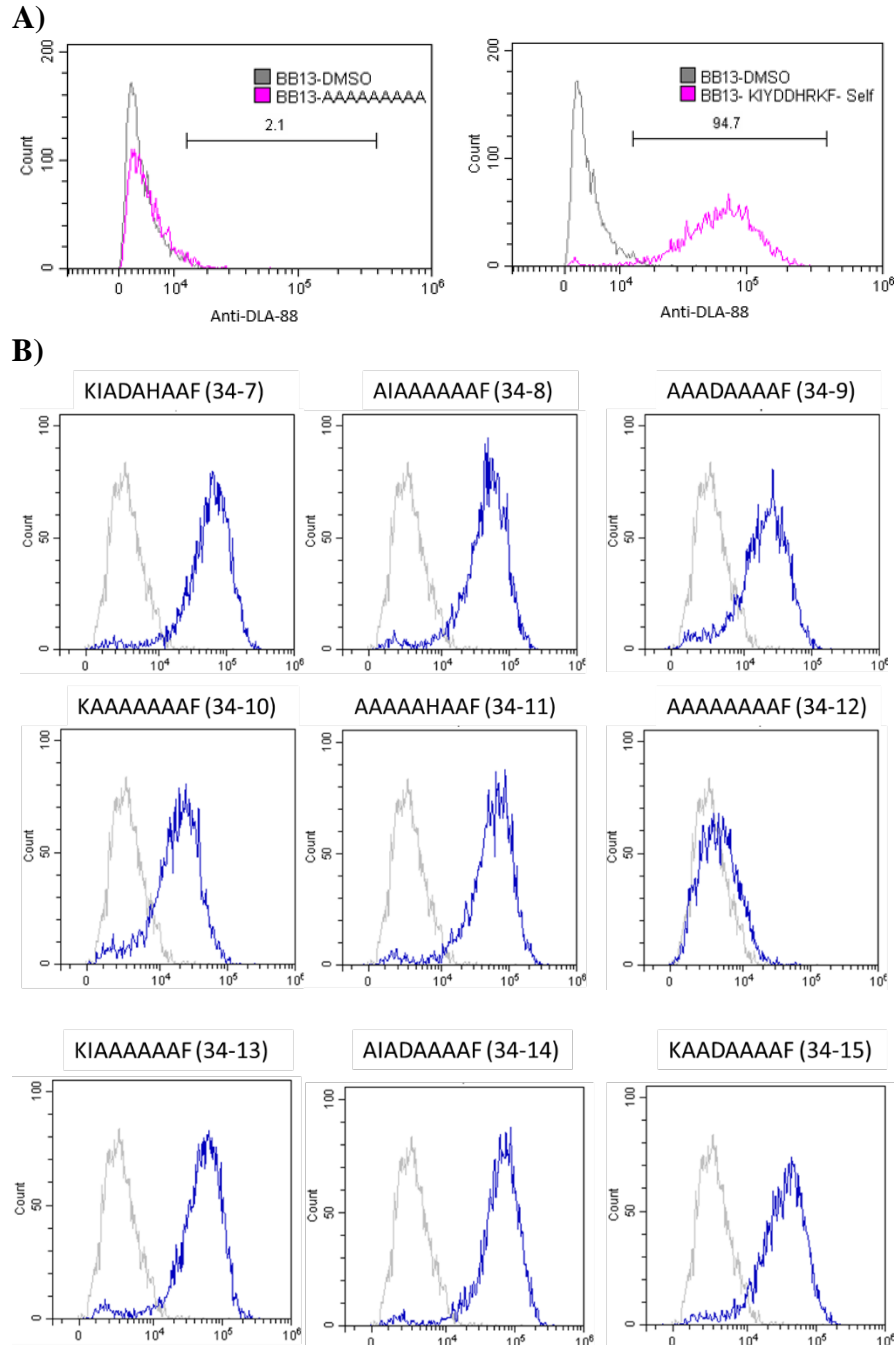


Figure 5.4. BB13 cells verify DLA-88*034:01-restricted peptide binding and confirm the predicted DLA-88*034:01 binding motif. A) Self-peptide, TENM4₁₉₄₆₋₁₉₅₄ (KIADAHAAF), motif peptide, 34-7 (KIYDDHRKF), negative control peptide, 34-16 (AAAAAAAAA) (pink) or DMSO alone (gray) was added to BB13 cells that had been cultured overnight at 27°C and then placed at 37°C for 5 hours followed by staining with H58A and flow cytometry analysis. B) Motif peptides (summarized in Table 1) were also tested for binding to experimentally determine the DLA-88*034:01 binding motif. Representative histograms from three separate experiments. All peptides were added at a concentration of 50 μ M.

Table 5.2. DLA-88*034:01 CDV Peptides Returned from MS.

| Gene | Peptide |
|------|---|
| F | PAFKPDLTGTSK, PAFKPDLTGTSKSY, LTGTSKSYVRSL |
| H | ATTSVGKVF, YVYDPIRTI |
| L | KLAKGRPIY |
| M | KVLTSGSVF |
| N | SSPVYNDRELLN, SPVYNDRELLN |
| V | YATPSQDLK, GYSFGLKPDRAAD, FPHNPEGKTRD, PEMQTRVW |

Peptides in red don't possess DLA-88*034:01 binding motif and most likely artifactual peptides

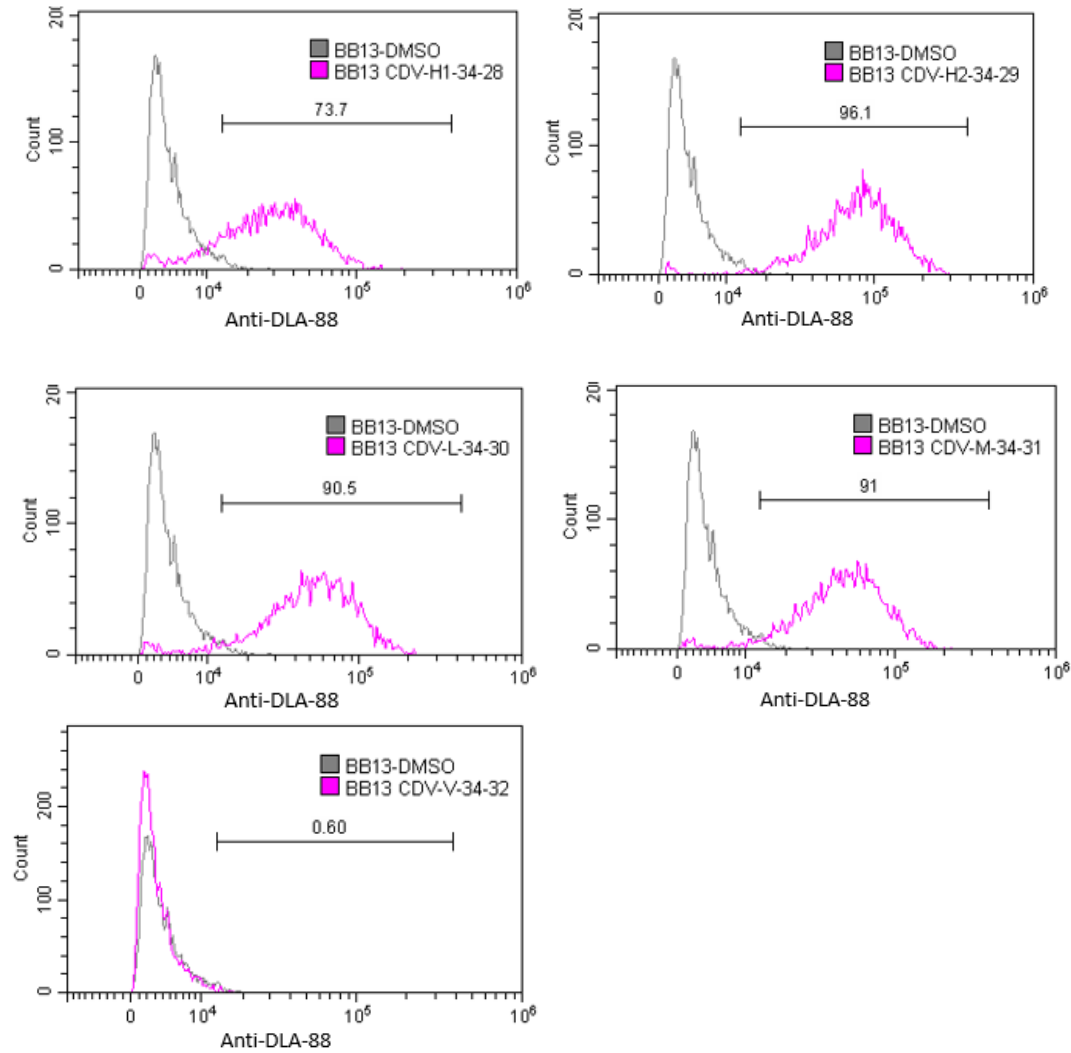


Figure 5.5. CDV peptides are presented by DLA-88*034:01. Five CDV peptides that matched the DLA-88*034:01 motif (peptides in black, Table 2) or DMSO only were added to the MHC stabilization assay (50 μ M) as previously described, stained with H58A and analyzed via flow cytometry. Histograms show the percentage of H58A positive staining for each peptide (pink) and DMSO (gray).

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CHAPTER 6: FINAL CONCLUSIONS AND FUTURE DIRECTION

The work performed in this dissertation provides a stepping stone for immunologic research in the dog to move forward. We have successfully defined the peptide binding motif in the highly prevalent Boxer allele, DLA-88*034:01, making it the third canine allele with a known binding motif. This information will allow further investigation of the pMHC and T cell interaction in dogs with this allele. Moreover, the methods described to obtain this data can be applied to other prevalent DLA alleles. We also specify methods for analyzing tumor associated antigens in the dog in which we found two genes, NT5C1B and CCTA-4, that are attractive cancer vaccine target candidates. At the transcript level, CCTA-4 demonstrated overexpression in canine histiocytic sarcoma and peripheral T-cell lymphoma, with only minimal expression in lung and gall bladder. However, when we probed these tissues at the protein level with a CCTA-4 antibody, we did not find expression.

This data allowed us to receive funding for a phase 1 clinical trial to test a peptide vaccine against CCTA-4 in dogs with lymphoma. The peptide vaccine will consist of overlapping long peptides from CCTA-4, which assist in activating CD4 T helper cells, and an adjuvant to augment the immune response. An adjuvant is included in vaccines to augment the immune response by initiating innate immunity and potentially stimulating dendritic cells (DC) so they can more effectively activate tumor specific T-cells produce a long-lasting adaptive response. Adjuvants stimulate pattern recognition receptors (PRRs), such as, various toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)- like receptors, nucleotide-binding oligomerization domain NOD-like receptors (NLRs) and C-type lectin receptors (CLRs)^{1,2}. Four common adjuvants, Poly I:C (TLR-3; RIG-1), monophosphoryl lipid A (MPLA) (TLR-4), Imiquimod (TLR-7) and CpG ODN 2006 (TLR-9), have been tested in the dog with two, MPLA

and Imiquimod, having FDA approval. Our next objective is to identify a strong adjuvant candidate from a pool of commercially available PRR ligands by screening DC activation in-vivo. The prevailing adjuvant will then be used in our peptide vaccine study against the CCTA-4 antigen. The first step is to confirm the safety of this vaccine so a phase 2 trial, analyzing efficacy, can be implemented. If successful, this vaccine will be added to the options of cancer therapy in a pool of dogs with chemoresistant cancers. It will also progress canine cancer immunotherapy development and potentially gain insight to human cancer immunotherapies as well.

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