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The canine MHC class Ia allele DLA-88*508:01 presents diverse self-and canine distemper virus-origin peptides of varying length that have a conserved binding motif

Peter Ross^{a,1}, Paige S. Nemec^a, Alexander Kapatos^a, Keith R. Miller^c, Jennifer C. Holmes^a, Steven E. Suter^a, Adam S. Buntzman^b, Erik J. Soderblom^e, Edward J. Collins^{c,d,2}, and Paul R. Hess^{a,*}

^aDepartment of Clinical Sciences, North Carolina State University College of Veterinary Medicine, Raleigh, NC, USA 27607

^bDepartment of Immunobiology, University of Arizona, Tucson, AZ, USA 85724

^cDepartment of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, NC, USA 27599

^dDepartment of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA 27599

^eProteomics Core Facility, Institute for Genome Science and Policy, Duke University School of Medicine, Durham, NC 27710

Abstract

Ideally, CD8⁺ T-cell responses against virally infected or malignant cells are defined at the level of the specific peptide and restricting MHC class I element, a determination not yet made in the dog. To advance the discovery of canine CTL epitopes, we sought to determine whether a putative classical MHC class Ia gene, Dog Leukocyte Antigen (DLA)-88, presents peptides from a viral pathogen, canine distemper virus (CDV). To investigate this possibility, DLA-88*508:01, an allele prevalent in Golden Retrievers, was expressed as a FLAG-tagged construct in canine histiocytic cells to allow affinity purification of peptide-DLA-88 complexes and subsequent elution of bound peptides. Pattern analysis of self peptide sequences, which were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS), permitted binding preferences to be

*Corresponding author: Dr. Paul R. Hess, Department of Clinical Sciences, North Carolina State University College of Veterinary Medicine, Box 8401, 1060 William Moore Drive, Raleigh, NC 27607; Telephone: 919-513-6183; Fax: 919-513-6464; paul_hess@ncsu.edu.

¹BD Technologies, Research Triangle Park, NC USA 27709

²Pfizer, Inc. Sanford, NC USA 27330.

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inferred. DLA-88*508:01 binds peptides that are 9-to-12 amino acids in length, with a modest preference for 9- and 11-mers. Hydrophobic residues are favored at positions 2 and 3, as are K, R or F residues at the C-terminus. Testing motif-matched and -unmatched synthetic peptides via peptide-MHC surface stabilization assay using a DLA-88*508:01-transfected, TAP-deficient RMA-S line supported these conclusions. With CDV infection, 22 viral peptides ranging from 9-to-12 residues in length were identified in DLA-88*508:01 eluates by LC-MS/MS. Combined motif analysis and surface stabilization assay data suggested that 11 of these 22 peptides, derived from CDV fusion, hemagglutinin, large polymerase, matrix, nucleocapsid, phosphoprotein, and V proteins, were processed and presented, and thus, potential targets of anti-viral CTL in DLA-88*508:01-bearing dogs. The presentation of diverse self and viral peptides indicates that DLA-88 is a classical MHC class Ia gene.

Keywords

Canine Distemper Virus; CD8⁺ T Lymphocytes; MHC; Peptides/Epitopes; Retrovirus

1. Introduction

CD8⁺ CTL are important effectors in the clearance of virus-infected cells. Studying these responses across unrelated individuals is feasible because of the frequent sharing of certain alleles within the large pool of highly polymorphic classical MHC class Ia genes, and for a prevalent allele, by the predictable focus of T-cell reactivity against a surprisingly small set of peptides within a given antigen. The predominance of these epitopes largely reflects the fact that few peptides are capable of binding a particular class I molecule with sufficient affinity to form stable peptide-MHC (pMHC) complexes at the cell surface that can be recognized by the corresponding TCR. Once identified, these peptide epitopes have proved essential in developing the understanding, in fine detail, of TCR signaling; T-cell selection, homeostasis, differentiation and plasticity; the functional heterogeneity and competition within responding CTL populations; and the kinetics of T-cell expansion and contraction during primary and secondary responses. These peptides are also used to generate pMHC class I multimers, which allow identification and quantification of cognate T cells, independent of their functional status. This tool has been critical in finding “exhausted” anti-viral CTL populations that are invisible in assays where detection is based on T-cell reactivity (Welsh, 2001; Zajac et al., 1998). Finally, peptide vaccines containing established HLA class Ia-restricted viral epitopes can be administered therapeutically to stimulate CD8⁺ T cells and induce meaningful clinical responses, including reductions in viral load (Klade et al., 2012) and complete tumor regressions (Kenter et al., 2009; Welters et al., 2010).

While the dog can be a useful model of immunity to viruses and cancer (da Fontoura Budaszewski and von Messling, 2016; Park et al., 2016), the list of documented CTL in this species is comparatively short, and the methods for characterizing these responses is not well-established (Lebrech et al., 2012). CD8⁺ T-cell activity against inactivated rabies virus vaccine (Hartley and Tarleton, 2015), canine distemper virus (CDV) (Hirama et al., 2003), *Leishmania infantum* (Pinelli et al., 1994; Pinelli et al., 1995), autologous tumor cells (Dow et al., 1998; Mitchell et al., 2012), minor histocompatibility antigens (MiHA) (Georges et

al., 2003; Weber et al., 2003) and shared tumor-associated antigens (Peruzzi et al., 2010) have been reported, without identification of specific epitopes. Recently, Bund *et al.* demonstrated that CTL could be primed in female Beagles against three 9-mer peptides derived from the Y-encoded MiHAg UTY and known to bind HLA-A2; however, the Dog Leukocyte Antigen (DLA) class I molecule restricting these responses was not identified (Bund et al., 2013).

To advance the study of epitope-level CTL in dogs, we sought to determine whether DLA-88 is capable of presenting diverse self- and virus-origin peptides, using a prevalent allele (DLA-88*508:01) as a representative gene product (Ross et al., 2012a). The DLA-88 gene is polymorphic (Graumann et al., 1998; Ross et al., 2012a) and presumably encodes a classical MHC class Ia molecule that restricts CTL activity. Canine distemper virus was used as a model organism, since most dogs are vaccinated with live virus, and as mentioned, T-cell cytotoxicity against infected cells and hemagglutinin (H) protein have been reported (Hirama et al., 2003; Shek et al., 1980). Further, in humans, the importance of CTL in immunity to the related morbillivirus, measles virus (MV), is well-established (Jaye et al., 1998; van Binnendijk et al., 1990; van Els and Nanan, 2002). Our hypothesis was that peptides derived from CDV proteins are presented by DLA-88*508:01. If correct, these peptides should be identifiable by mass spectrometry, and ultimately, could prove useful in defining epitope-specific, anti-CDV CTL responses shared by dogs carrying this allele.

2. Materials and methods

2.1 Culture of cell lines

DH82, a histiocytic sarcoma cell line derived from the bone marrow of a Golden Retriever (Wellman et al., 1988), was obtained from the ATCC (CRL-10389), and maintained in DMEM medium containing 15% FBS and penicillin/streptomycin (D-15 medium). BARC3 cells were cultured as previously described in RPMI-1604 medium supplemented with 10% FBS and 2mM L-glutamine (R-10), and containing 200 µg/ml G418 (Ross et al., 2012b).

2.2 Generation of a stable canine cell line expressing a FLAG-labeled DLA-88*508:01 molecule

The coding sequence of DLA-88*508:01, from the start codon through Q350 (GenBank, JQ733514), was determined by PCR amplification with DLA88-F and DLA88-R primers (Supplemental Table 1), using DH82cDNA as template. DLA-88-specific antibodies are not available for immunoprecipitation. Thus, for affinity isolation of recombinant pMHC complexes, the amplicon was ligated into a modified pcDNA3 vector containing the FLAG epitope coding sequence (Supplemental Figure 1, underlined in red), which had been placed directly downstream of the *NotI* restriction enzyme site in the multiple cloning region (gift of Jeff Yoder, NCSU). DH82 cells transfected with this plasmid using Lipofectamine 2000 (Invitrogen) were selected with G418 (400 µg/ml), cloned by limiting dilution, and subsequently screened for intracellular FLAG expression by flow cytometry (FACSCalibur; BD Biosciences), using the anti-FLAG Ab M2 (Sigma) after permeabilization (Cytofix/Cytoperm; BD Biosciences). Clone 9-15, which was strongly and uniformly positive for FLAG, was used throughout the study. To verify surface expression of recombinant

DLA-88*508:01, cell surface proteins of 9-15 cells were isolated with the Pierce Cell Surface Protein Isolation Kit (ThermoScientific) and analyzed by Western blotting, using M2 and HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) Abs and chemiluminescent detection (SuperSignal West Dura Chemiluminescent Substrate, ThermoScientific).

2.3 Infection of 9-15 cells with CDV

The production of an enhanced Green Fluorescent Protein (eGFP)-expressing CDV clone (Onderstepoort vaccine strain) has been described previously (Suter et al., 2005). Infection of 9-15 cells was initiated by adding CDV-eGFP stock (tissue culture supernatant stored at -80°C) to a subconfluent flask. By fluorescence microscopy, $\sim 10\%$ and $\sim 100\%$ were eGFP+ by 4 and 7 days post-inoculation, respectively. Subsequently, infected cell cultures were continuously maintained by transferring $\sim 10\%$ of cells from a uniformly eGFP+ flask into a new flask seeded with uninfected 9-15 cells at 40–60% confluency; the remaining $\sim 90\%$ of infected cells were pelleted and stored for immunoprecipitation. Newly-infected cultures expanded at rates comparable to their uninfected counterparts and typically became completely eGFP+ by 2–3 days post-inoculation, at which point they were again harvested, to insure maximum cell viability and expression of viral proteins.

2.4 Isolation of DLA-88*508:01-bound peptides

Peptides were isolated using an established protocol (Sherman et al., 2008), with minor modifications. Briefly, 9-15 cells were grown in D-15 medium containing 400 $\mu\text{g/ml}$ G418, and harvested twice weekly for storage as cell pellets at -80°C . Continued FLAG expression in cultures was verified by flow cytometry performed on a biweekly basis. Once $3 - 8 \times 10^9$ 9-15 cells had been 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, and 1% protease inhibitor cocktail [Sigma]), incubated for 1 hour at 4°C , and pooled. To remove large contaminants, the lysate was centrifuged ($3220 \times g$ for 5 min at 4°C), and then clarified further by ultracentrifugation ($100,000 \times g$ for 1 hour at 4°C) and passage through a 0.22 μm filter (Millipore). Following pre-clearing by 1-hour incubation with Mouse IgG-Agarose, DLA-88*508:01 complexes were immunoprecipitated overnight at 4°C using Anti-FLAG M2 Affinity Gel (both Sigma). After extensive washing, bound peptides were eluted with 0.2 N acetic acid, boiled for 5 min, filtered through a 3kD centrifugal filtration device (Amicon Ultra-4 3K; Millipore), purified over Hydrophilic-Lipophilic-Balanced reversed-phase cartridges (Waters Corp.), eluted with increasing concentrations of methanol in water, and concentrated to $\sim 100 \mu\text{l}$ final sample volume with a SpeedVac vacuum centrifuge (ThermoScientific).

2.5 Sequence determination of eluted peptides by nano-flow Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Following buffer exchange into 50 mM ammonium bicarbonate, pH 8.0, peptides were applied to a detergent removal spin column (Norgen Biotek), and eluted peptide quantities were estimated using a BCA assay. Samples were brought to dryness by vacuum centrifugation, resuspended in 10 μl 2% acetonitrile, 0.1% formic acid, and analyzed by LC-MS/MS using a NanoAcquity UPLC (Waters) equipped with a 1.7 μm BEH C₁₈ 75 $\mu\text{m} \times 2.5$

cm column coupled through an electrospray interface to a Synapt G2 HDMS QToF mass spectrometer (Waters), operating in a data-dependent mode of acquisition. Mass spectra were processed with Mascot Distiller (Matrix Science) and submitted to Mascot searches against an NCBI_mammal database appended with reverse entries of each protein. Data were searched at 5 ppm precursor and 0.03 Da product ion mass tolerances, with no enzyme rules selected. Searched spectra were imported into Scaffold v3.0 (Proteome Software) and scoring thresholds were set to yield a peptide false discovery rate of 1.4% (implemented by the PeptideProphet algorithm) based on decoy database searches (Keller et al., 2002). Raw data from the first analysis are available for public download here: https://discovery.genome.duke.edu/express/resources/3441/Ross_April2013_MHC_Peptides.sf3

2.6 Estimation of peptide binding to DLA-88*508:01

The canine peptides *K-9* (KLFSGELTK; SC5D₂₈₉₋₂₉₇) and *K-11* (RFLDKDGFIDK; CYP1B1₄₄₄₋₄₅₄) and their glycine (G)-substituted variants, as well as multiple peptides derived from CDV gene products, were synthesized by Peptide 2.0. Stock solutions were prepared in DMSO and frozen for later use. The pMHC stabilization assay was performed as previously described (Ross et al., 2012b). BARC3 cells (10⁵ per 100 µL) were cultured in R-10 in 96-well, round-bottomed plates overnight at 27°C. At that time, or 16 hours later, cells were incubated with synthetic peptides (100 – 200 µM final concentration) or an equivalent volume of DMSO. Cells were then cultured for an additional 5 hours at 37°C, washed with FACS buffer (PBS containing 2% FBS, 2 mM EDTA and 0.02% sodium azide), stained with a mouse anti-MHC class I mAb (H58A; Monoclonal Antibody Center – Washington State University), washed again, secondarily stained with Alexa Flour 647-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), washed additionally, and analyzed by flow cytometry (FACScan, BD Biosciences; Cytoflex, Beckman Coulter). For both Abs, staining was performed at a 1:200 dilution in FACS buffer at 4°C for 25 min. Peptide-independent controls were generated by staining BARC3 cells cultured solely at 27°C with both 1° and 2° Abs (positive control) and by staining BARC3 cells pulsed with DMSO and cultured at 37°C with only the 2° Ab (negative control). Additional controls were established by incubating cells with motif-matched and -unmatched peptides, followed by staining with 1° and 2° Abs.

To provide a semi-quantitative comparison of binding strength between CDV peptides, a binding score was derived from the mean fluorescence intensity (MFI) of stained BARC3 cells, according to the formula: $(MFI_X - MFI_{no\ pep@37C}) / (MFI_{no\ pep@27C} - MFI_{no\ pep@37C})$, where X is the experimental variable. Surface MHC class I staining therefore was arbitrarily defined as 0 at 37°C and 1 at 27°C. Negligible binding was defined as the mean binding score ($n=4$) of the motif-unmatched lymphocytic choriomeningitis virus (LCMV) peptide NP₃₉₆₋₂₀₄ (FQPQNGQFI; GenScript) plus 2 standard deviations (Ross et al., 2012b).

2.7 Statistical Analysis

The length variability of eluted peptides was compared between DLA-88*508:01 and the murine MHC class I molecule H2-D^b (Delgado et al., 2009). Using the peptide set discovered for each allele by LC-MS/MS, variability estimates were computed by the

equation: $(\text{individual peptide lengths} - \text{median length})^2$ and compared by Wilcoxon rank sum test, as implemented in the R software package (<http://www.R-project.org>).

3. Results

Affinity isolation of MHC class Ia complexes and LC-MS/MS of eluted peptides is an expedient means of discovery of potential CTL epitopes derived from viral antigens. However, not all peptides identified in such experiments originate from the binding groove, as similarly-sized fragments are also generated in lysates by *in vitro* proteolysis. In order to improve the confidence that any recovered CDV sequences represented actual processed and presented peptides, we first analyzed self peptides from uninfected canine cells in order to deduce the binding preferences of the DLA-88*508:01 allele. The ability of peptides that matched or contradicted the putative motif to stabilize DLA-88 complexes both *in vitro* and at the cell surface was then tested. These tools were used to evaluate presumptive DLA-88*508:01 binders from CDV, and produce a list of likely viral epitopes recognized by CTL restricted by this canine class I allele.

3.1 Recombinant DLA-88 is expressed at the surface of a canine cell line

A stably-transfected cell line (9-15) that expresses DLA-88*508:01-FLAG was generated by dilution cloning. To verify that DLA-88*508:01 was present at the cell surface, we biotinylated intact 9-15 cells, and then isolated the labeled proteins from cell lysates with avidin-conjugated agarose. When immunoblotted for the FLAG epitope, a band of the appropriate molecular weight (~44 kDa) was detected in the bound fraction (Fig. 1A, left), confirming cell-surface expression. This effect was not simply due to non-specific binding of lysate proteins to the avidin gel, as no FLAG signal was observed in the eluate when the biotinylation step was omitted (Fig. 1A, right).

3.2 Determination of the peptide-binding motif of DLA-88*508:01

Classical MHC class Ia alleles differ in their peptide-binding preferences (Buxton et al., 1992; Guo et al., 1992; Jardetzky et al., 1991; Silver et al., 1992). To identify the preferences of DLA-88*508:01, we sought to survey the sequences of naturally processed and loaded self peptides and infer conserved residue types at each position. Bound peptides were acid-eluted from DLA-88*508:01 complexes that had been FLAG affinity-purified from 9-15 lysates and were analyzed by LC-MS/MS to determine individual sequences. The size distribution of the 49 peptides recovered in our first experiment is shown in Fig. 1B. Forty-three (88%) were 9-to-12 amino acids in length, which we considered likely binders. Of these, four were potential contaminants and excluded from analysis. Three (GLAEKFLKG; SLVKYISK; KSRHPGRIP) did not align with predicted proteins from either dog or cow (FBS), and the fourth (PQIVKWDRDN) was from the C-terminus of canine beta-2-microglobulin precursor. The remaining 39 peptides, and the 36 unique proteins from which they presumably originated, are listed in Table 1.

To identify potential residue preferences of DLA-88*508:01 at each position from the 39 peptides, independent of their length, 3, 2 or 1 “gap” positions (designated position 5', position 5'', and position 7') were added to 9-, 10- and 11-mers, respectively, to permit all

sequences to be aligned at the amino (N) and carboxy (C-) termini (Table 1). Peptide and MHC heavy chain binding interactions are generally conserved at C-terminus sites (Collins et al., 1995; Fremont et al., 1992). For this analysis, amino acids were divided into four groups, based upon side-chain chemical characteristics: hydrophobic (A, V, I, L, F, W, M, and P), charged-acidic (D and E), charged-basic (K and R), and polar (S, T, C, N, Q, Y, and H), with glycine (G) unassigned. When inspected for conservation (~60% or greater occurrence) of amino acid group by position, pattern analysis of the aligned sequences revealed that hydrophobic (Phob) residues were preferred at position 2, position 3 and position 5, while charged-basic (K/R) residues were preferred at position 9 (Supplemental Fig. 2A).

Due to the small number of peptide sequences recovered from this initial LC-MS/MS run, defining conserved residues solely by frequency analysis conceivably could identify dominant amino acid groups that do not reflect actual MHC binding preferences. To minimize this hazard, we also evaluated potential binding interactions between amino acids at the identified positions and the heavy chain by creating *in silico* molecular models of the canine pMHC complex. Because DLA-88*508:01 had not been solved at the time of this analysis, modeling was based upon the most homologous human class I allele with a solved crystal structure, HLA-A*68:01 (Guo et al., 1992), using two peptides from our dataset that represented the most commonly observed lengths: the 9-mer peptide *K-9* and the 11-mer peptide *K-11*. This analysis supported the deduced preferences at all positions except for position 5 (see Supplemental Results; Supplemental Fig. 2A and 2B). Based on combined pattern and modeling analyses, we concluded that the likely binding motif of DLA-88*508:01 is X-Phob-Phob-X₍₅₋₈₎-K/R, which was further supported by *in silico* analysis using NetMHCpan (Supplemental Results; Supplemental Figure 2D), and has subsequently been confirmed in a crystallographic study (Xiao et al., 2016), based on the peptides *K-9* and *K-11*, and predicted CDV H peptides (Ross, 2012).

3.3. Confirmation of the peptide-binding motif DLA-88*508:01

To determine whether the eluted self peptides identified by LC-MS/MS that matched the proposed motif were actually capable of binding DLA-88*508:01, pMHC complexes were prepared with peptides *K-9* or *K-11*, or as a control, an H2-K^d-restricted CTL epitope from influenza hemagglutinin (HA) that lacked the preferred residues (having a Y at position 2, S at position 3, and L at position 9) (Kuwano et al., 1989). Circular dichroism (CD) spectrometry was performed to assess the thermal stability of the purified complexes, as the change in CD signal intensity measured as a function of temperature correlates with the free energy of peptide binding to the MHC (Batalia et al., 2000; Fahnestock et al., 1992). The CD data (Supplemental Results; Supplemental Figure 2E) demonstrate that both 9- and 11-mer peptides that match the proposed motif bind DLA-88*508:01.

In addition, peptide *K-9* stabilized DLA-88*508:01 on the surface of TAP-deficient murine cells (RMA-S) stably expressing the canine allele (BARC3 cells) (Ross et al., 2012b), consistent with peptide-MHC binding (Fig. 2A). To further assess the contribution of preferred residues at position 2, position 3 and the C-terminus, altered peptide ligands with various glycine (G) substitutions were synthesized and tested via surface stabilization assay.

The replacement with a G residue of a strongly hydrophobic residue at either position 2 or position 3, or the charged-basic K at the C-terminus, abolished binding, while the collective presence of the native peptide *K-9* residues L, F and K at anchor positions was sufficient to confer DLA-88*508:01 binding (Fig. 2C), supporting the motif. An M substitution of L at position 2 enhanced binding but in itself was insufficient to overcome the negative effects of G substitution at either position 3 or position 9 (Fig. 2D). Peptide *K-11* also binds DLA-88*508:01 (Fig. 2E), with greater affinity than peptide *K-9*, as we have previously reported (Ross et al., 2012b). As might be expected, peptides created by substituting like-group amino acids at position 2 (Fig. 2F), position 3 (Fig. 2G) or the C-terminus (Fig. 2H), which maintained the motif, had no detrimental effects. The consequences of losing preferred residues at anchor positions with the longer peptide *K-11* was similar to peptide *K-9*, but less absolute; a G at position 2 or the C-terminus either reduced or abolished binding, respectively, while substitution at position 3 caused no change (Fig. 2I). Surprisingly, unlike peptide *K-9*, the native F, L and K anchors of peptide *K-11* in themselves were insufficient for binding (Fig. 2J), possibly due to negative effects of G residues at non-anchor positions (Hudrisier et al., 1996), although other 11-mer peptides with minimal motif elements (GMFGGGGGGGK [Fig. 2J, rightmost plot]; GFIGGGGGGGK [not shown]) did stabilize DLA-88*508:01. Overall, the data from these cell-based assays are consistent with the preferences deduced from our initial LC-MS/MS analysis. DLA-88*508:01 binds both 9- and 11-mers with hydrophobic residues at positions 2 and 3 and charged-basic residues at the C-terminus. The preference at position 3 appears less stringent than for the other two anchor residues.

3.4. DLA-88*508:01 presents diverse self- and viral-origin peptides

The defining functional characteristic of classical MHC class I molecules is the display of peptides derived from a wide variety of intracellular proteins, allowing effective CD8⁺ T-cell immunosurveillance. To investigate whether diverse peptides from an intracellular pathogen were also displayed by DLA-88*508:01, class I-eluted peptides from CDV-eGFP-infected 9-15 cells (Supplemental Figure 3A) were again sequenced by LC-MS/MS. A larger number of peptides were recovered (Supplemental Figure 3B, upper graph), representing more than 120 canine proteins; 64% were 9- to 12-mers (gray bars). Fifteen of the 49 peptides found in the first analysis were recovered in this second run; of those, the 12 matching the preferred length are listed in Supplemental Table 2. Prior to evaluation for the DLA-88*508:01 binding motif, the new longer list of self peptides was extensively curated. Excluded from analysis were cell lysate and immunoprecipitation contaminants that have been reported repeatedly (Mellacheruvu et al., 2013; Rees et al., 2011; Trinkle-Mulcahy et al., 2008), as well as peptides that were nested within multiple fragments of slightly longer lengths, which could have been generated by *in vitro* proteolysis. Alignment of the remaining peptides was again performed (Fig. 3), showing the same residue preferences at positions 2 and 3, and the C-terminus, as in our first experiment (Supplemental Fig. 2A). The ability of DLA-88*508:01 to accommodate peptides ranging from 9 to 12 amino acids in length was also confirmed. When the first and second curated self peptide datasets were merged, a preference for 9- to 11-mers was evident (Fig. 4A). Similar length distributions were observed when the two peptide lists were combined without prior editing for contaminants (Fig. 4B), and when unedited LC-MS/MS data from a third experiment investigating

idiotype antigen presentation (Kapatos & Hess, unpublished) were added (Fig. 4C). Of course, these distributions are rough estimates of the binding length preferences of DLA-88*508:01, since MS analyses were not performed using quantitative methods (Ong and Mann, 2005), and the relative frequencies assume that every unique species in a dataset is present as a single peptide.

The negative-stranded RNA genome of CDV encodes six genes: fusion (F); hemagglutinin (H); large polymerase (L); matrix (M); nucleocapsid (N); and phosphoprotein (P). Two accessory proteins involved in evading innate host defenses, V and C, are also encoded by the P gene (Otsuki et al., 2013a; Otsuki et al., 2013b). Thirty-nine unique peptides representing all six viral gene products (and V) were identified (Supplemental Fig. 3B, lower panel, and 3C). Of those, 22 (56%) were 9- to 12-mers. While any of these peptides potentially had been eluted from the binding groove of DLA-88*508:01, some appeared suspect and were thought to have been produced *in vitro*. Specifically, some peptides had few or no favored residues at anchor positions, were contained within (“nested in”) multiple peptides ranging from 13 to 20 amino acids in length that had also been recovered, and were located at the C-terminus of the protein (Table 2). To evaluate this possibility further, we synthesized the 22 candidates and tested them for binding to DLA-88*508:01 in a semi-quantitative manner. Representative data from these experiments (H candidate peptides are shown as examples) can be seen in Supplemental Fig. 3D. Peptides that bound the allele, were not nested in larger peptides, and had one or more anchor residues were considered genuine ($n=11$). Conversely, those peptides considered suspect by the criteria described above and without measurable binding were designated as artifactual ($n=7$). For example, all F-origin peptides were judged to be artifactual. The status of the remaining four peptides was considered uncertain. Finally, eight peptides also originated from eGFP: one 9-mer, three 10-mers, and four that were 13 residues in length.

4. Discussion

Identifying viral peptides presented by classical MHC class I molecules is necessary to define CD8+ T-cell responses at the epitope level. Quantitative estimates from vaccinia virus suggest these peptides are rare, on the order of ~1 in 250, reflecting in part the low probability of any given peptide being processed by the proteasome (~15%) and binding to the restricting class Ia element with high affinity (~2.5%) (Assarsson et al., 2007). Mass spectrometric analysis of peptides eluted from affinity-purified MHC class Ia complexes is a useful means of discovering processed/presented peptides. For example, 70 HLA A, B and C-bound peptides from MV were recently found by this approach (Schellens et al., 2015). Ultimately, the winnowed list of candidate epitopes can then be used to screen T-cell lines or clones, or to generate pMHC class I tetramers, to find CTL. Our results support the designation of DLA-88 as a MHC class Ia gene in dogs. DLA-88 is expressed at the cell surface. The peptides eluted from DLA-88*508:01 appear to be diverse, while most (but not all) non-classical MHC class Ib loci bind a very restricted repertoire of peptides (Apostolopoulos et al., 2008). In canine cells infected with CDV, DLA-88 presents a variety of viral-origin peptides.

In this study, we first defined the self peptide repertoire of DLA-8*508:01. MHC class Ia ligands are derived from intracellular polypeptides. We assigned a putative precursor protein for all 39 canine sequences in Table 1, based upon findings in other species that most antigenic peptides derive from translations of the standard reading frame (van Endert, 2011). The canine proteome is poorly characterized, and accordingly, most candidate proteins were predicted from the annotated genome. The locations of the self peptides within their parent proteins appeared widely distributed; only two (19, 31) originated from the C-terminus. Virtually all proteins were represented by a single peptide, consistent with other studies (Hickman et al., 2004; Hoof et al., 2012); however, elongation factor 1- α 1 was a precursor for three overlapping peptides (26, 27 and 37). Interestingly, in the repertoire of self peptides presented by HLA-B*18:01, four peptides were generated from the same gene product (Hickman et al., 2004), perhaps reflecting its cytosolic abundance, a factor predictive for increased presentation (Hoof et al., 2012). The AHNAK gene product also yielded two peptides (22, 23), presumably reflecting the fact that each sequence occurs multiple times in the parent protein (SMPDFDLHLK, 3; SMPDVDLHLK, 4). By inference from analysis of their human and murine orthologs, slightly more than half of the canine source proteins are present in the cytoplasm (Table 1, bolded), which might be expected, given the importance of proteasomal degradation to the generation of peptide ligands; however, other cellular compartments are also represented, including the nucleus, plasma and ER membranes, and mitochondria. This distribution is consistent with the ligand survey reported for HLA-B*18:01, where source proteins were found in virtually all compartments: 53% were cytoplasmic, 43% were nuclear and 18% bound to membranes (Hickman et al., 2004). Presumably, such widespread intracellular sampling is important for maintaining robust immunosurveillance. How peptides originating from non-cytosolic source proteins can access the transporters associated with antigen processing (TAP) for delivery into the endoplasmic reticulum and loading onto MHC class Ia molecules is not entirely clear. Nuclear proteins can be degraded by proteasomes resident in that compartment, with the subsequent diffusion of peptides through nuclear pores to reach TAP (Reits et al., 2003). Alternatively, peptide ligands could be derived from the substantial fraction (~30%) of all proteins that are destroyed by cytosolic proteasomes shortly after synthesis (Schubert et al., 2000), before transport to their final cellular location. Such rapidly degraded polypeptides (RDPs) are thought to represent either defective ribosomal products (DRiPs) (Yewdell, 2011), or occur as the result of protein generation by ribosomes unassociated with heat-shock protein chaperones (Eisenlohr et al., 2007). Another possible source of class Ia peptides from non-cytosolic proteins may be so-called “pioneer translation products” – polypeptides produced during an early round of translation that serves as quality control for mRNA transcript integrity (Apcher et al., 2011). From a functional perspective, class Ia-bound self peptides in humans have been shown to be preferentially derived from RNA- and nucleic acid-binding proteins, chaperone and heat shock protein molecules, and ATP-dependent helicase activity proteins, and here, representatives from all these same categories can be found among the DLA-88*508:01 ligands (Table 1, peptides 2, 3, 6, 9, 10, 14, 16, 24, 26/27/37, 29, 32, 36 and 39). Another potential source of self peptides that are not completely represented in the current canine genomic dataset (derived from an individual dog) are endogenous retroviruses (ERVs) (Jo et al., 2012), which appear to be broadly transcribed in normal canine cells (Tarlinton et al., 2013), and translated under specific

conditions (i.e., during placentation) (Cornelis et al., 2012). While there is little information on potential canine ERV (CfERV) protein sequences, we extended the search of our mass spectra data against the CfERV reverse transcriptase sequences provided by Jo et. al (Jo et al., 2012) (94 γ and 10 β variants), but identified no peptides originating from these elements. A more thorough evaluation for CfERV peptides in the future seems warranted. In humans, CTL have been demonstrated against ERV-derived peptides, supporting their presentation by HLA class I molecules (Mullins and Linnebacher, 2012; Schiavetti et al., 2002; Tu et al., 2017).

Our frequency analysis suggests that DLA-88*508:01 strongly prefers to bind peptides with hydrophobic amino acids at position 2. In particular, M, F and V are favored, occurring 31, 22 and 26 times in 138 peptides, respectively. These frequencies represent approximate fold-increases of ~9.3, 4.1 and 2.8 over their naturally-occurring distribution in proteins (Bairoch, 2013; McCaldon and Argos, 1988). In addition to residue specificity, class Ia alleles have strong biases for binding peptides of particular lengths. For example, 96.2% of the peptides that bind H2-K^b are 8-mers, and 89.9, 80 and 100% of peptides that bind H2-D^b, HLA-B7 and HLA-A2, respectively, are 9-mers (Delgado et al., 2009; Huczko et al., 1993; Sudo et al., 1995). In contrast, species of four different lengths constituted the spectra of peptides eluted from DLA-88*508:01, with 9-mer (39%) and 11-mer (34%) peptides observed most frequently (Figure 4A). The variability in the distribution of lengths of peptides bound by the canine allele was significantly different ($p < 2.2e-16$) from that of peptides eluted from H2-D^b (Delgado et al., 2009). This wide range in peptide lengths is not unprecedented, however, and is similar to what has been observed for the homologous human MHC class I allele, HLA-A*68:01, which binds peptides ranging in length from 9 – 11 amino acids (Guo et al., 1992).

The binding motifs of DLA-88*508:01 and HLA-A*68:01 are also similar, except for the preference of the human allele at position 2 for a T residue (Guo et al., 1992). This amino acid is permitted by DLA-88*508:01 as well, as 8 of the 19 peptides (of 138 total) that lack a hydrophobic residue at position 2 possess a T. This finding serves as a reminder that class I binding motifs reflect a preference, not a strict requirement, for particular anchors, and non-canonical peptides can be identified (Lazoura et al., 2009). Similar to HLA-A*68:01, many of the peptides bound by DLA-88*508:01 have a K or R at the C-terminus, with fold-enrichments of roughly 8.6 and 2.2, respectively, compared to their normal usage in proteins. It is important to note that peptides containing basic residues anywhere within the linear sequence ionize more efficiently during positive mode electrospray ionization. This phenomenon does not, however, preclude the ability of the LC-MS/MS system to efficiently identify other non-R- or K-containing peptides, as all peptides contain an N-terminal primary amine that can sequester protons, and the qualitative dynamic range of the platform is in excess of three orders of magnitude. These factors argue that the preference of the canine allele for K or R at this last residue is genuine, rather than an artifact of the discovery process. Twenty-three of the 138 peptides ended with F (~4.3-fold enrichment). Support for F at this position has been shown in another study (Xiao et al., 2016). Overall, K, R or F were found at the C-terminus in 79% of eluted peptides, and collectively, these residues appear to be important determinants of binding to DLA-88*508:01 at this position.

In this study, peptides from all CDV gene products, except C, were presented by the class Ia allele, and some or all may represent epitopes targeted by anti-viral CTL responses in dogs carrying DLA-88*508:01. In mice, a majority of class I – restricted CD8+ T cells reacting to processed and presented viral peptides appear to represent so-called subdominant responses; that is, capable of recognizing infected cells but not routinely observed in a natural infection. Typically, only a small percentage (~10%) of CTL are immunodominant (Assarsson et al., 2007; Sercarz et al., 1993). Such strict hierarchical ordering presumably reflects the tight control of pathogen and host factor variability imposed in murine models, and antiviral responses in infected humans appear much more “immunodemocratic” (reviewed in (Yewdell, 2006)). In measles, for example, HLA class I presentation of peptides is broad-based, as studies with the super type HLA-A2 illustrate. Using peptide elution from MV-infected cells, as well as class I-binding prediction and peptide-MHC cell-surface stabilization assays, 12 candidate epitopes restricted by HLA-A2 were reported in early studies, derived from C (C₈₄₋₉₂), F (F₅₇₋₆₅), H (H₂₉₋₃₇; H₃₀₋₃₈; H₂₅₀₋₂₅₉; H₅₁₆₋₅₂₅; H₅₇₆₋₅₈₄), L (L₅₁₉₋₅₂₇), M (M₂₁₁₋₂₁₉), and N (N₂₁₀₋₂₁₈; N₂₂₆₋₂₃₄; N₃₄₀₋₃₄₈) proteins (Nanan et al., 1995; Oh et al., 2006; van Els et al., 2000). Only CTL reactivity against C₈₄₋₉₂, H₃₀₋₃₈ and M₂₁₁₋₂₁₉ was initially demonstrated though, suggesting that responses might be oligospecific (Jaye et al., 2003; van Els and Nanan, 2002). However, a 2007 study showed broad, balanced CD8+ T-cell IFN- γ responses against 19 peptides from C, F, H, L, M, N, and P proteins in 60% of HLA-A2⁺ vaccinees, with no epitope dominating (Ota et al., 2007). Tetramer- or dimer-staining showed that anti-MV CTL in each individual targeted 5 or more different peptides at similar frequencies (Ota et al., 2007). Whether antiviral T-cell responses in dogs tend to be immunodominant (like mice) or immunodemocratic (like humans) is unknown, but our set of 11 CDV candidate epitopes should permit this question to be investigated, at least for one representative DLA-88 allele. Currently, we are assessing CTL responses in DLA-88*508:01⁺ dogs immunized with the Onderstepoort strain of CDV. It seems reasonable to expect CD8+ T-cell reactivity against the processed and presented H peptides, AISDGVY GK and QVIDVLTP LFK. Both bind the DLA-88 allele with moderate-to-high affinity (Table 2; Supplemental Fig. 3D). As previously mentioned, CTL responses were elicited in two dogs by immunization with a recombinant adenovirus expressing CDV H (Yanaka strain) (Hirama et al., 2003). Further, in humans immunized with the measles-mumps-rubella vaccine, CTL against H are most numerically abundant (Ota et al., 2007).

There are likely other CDV peptides presented by DLA-88*508:01 that we did not identify in these experiments. Considering the total number of possible 9-, 10-, 11- and 12-mers in the CDV proteome, our 11 peptides represents a recovery rate of 0.05% (Supplementary Table 3), considerably less than the theoretically expected rate of 0.1–4% (assuming 10–15% processing and 1–2.5% presentation (Assarsson et al., 2007; Cascio et al., 2001)). On the other hand, our results are on par with comparable empirical findings in MV. Schellens *et al.* found 11 HLA-A2-restricted peptides, including 4 established and 7 novel peptides (F₁₄₀₋₁₅₂; F₁₆₅₋₁₇₅; H₄₇₇₋₄₈₅; L₂₆₃₋₂₇₁; L₉₇₁₋₉₈₁; M₂₀₄₋₂₁₂; and M₃₂₃₋₃₃₄), in MV-infected cells by methods similar to those used here (Schellens et al., 2015). Nonetheless, *in vitro* infection of a monotypic cell line cannot be expected to yield the exact same repertoire of class I-bound peptides as during natural infection of diverse cell types, so some potential epitopes may have been missed. Moreover, the expression of morbillivirus proteins changes

over the time course of infection (Herberts et al., 2003). Our data is primarily derived from 9-15s infected for 48–72 hours, although a small percentage (~10% of cells) were infected for longer periods. For example, in an MV-infected B-cell line, MV C₈₄₋₉₂ presentation by HLA-A2 is not detectable until 24 hours post-infection, but is presented at extremely high density at 48 hours, reflecting increased synthesis of the parent protein at that delayed time point (Herberts et al., 2003). Interestingly, we did not see C-origin peptides. It may be that production of CDV C in the DH82 histiocytic cell line is suppressed, or occurred mainly after our harvest point.

5. Conclusions

Our data provides additional evidence that DLA-88 is a classical MHC class I molecule in dogs. The allele DLA-88*508:01 binds peptides of varying length, with binding preferences for hydrophobic amino acids at position 2 (and to a lesser extent at position 3), and a K, R or F residue at the C-terminus. In virus-infected cells, DLA-88*508:01 presents diverse viral peptides for CTL immunosurveillance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

β2M	Beta2-Microglobulin
CDV	Canine Distemper Virus
CD	Circular Dichroism
DLA	Dog Leukocyte Antigen
CfERV	Canine Endogenous Retrovirus
DRiPs	Defective Ribosomal Products
eGFP	Enhanced Green Fluorescent Protein
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
MV	Measles Virus
MiHAg	Minor Histocompatibility Antigen
pMHC	Peptide-Major Histocompatibility Complex

RDPs	Rapidly Degraded Polypeptides
TAP	Transporters Associated with Antigen Processing

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Highlights

- DLA-88*508:01 binds peptides 9-12 amino acids in length, preferring 9- and 11-mers
- Hydrophobic residues at positions 2, 3, and K, R or F at the C-terminus are favored
- DLA-88*508:01 presents 11 different peptides from 6 canine distemper virus proteins
- Infection of DH82 with RD114 leads to class I presentation of gag and env peptides
- Presentation of diverse self and viral peptides supports DLA-88 as a class Ia gene

Fig. 1.

Binding-length peptides are eluted from a FLAG-tagged, surface DLA-88 molecule. (A) Recombinant DLA-88*508:01 is expressed on the cell surface. Biotin-labeled and -unlabeled 9-15 cells were lysed and incubated with avidin-conjugated agarose gel. Bound proteins were subsequently eluted with DTT-containing SDS-PAGE buffer. A Western blot was probed with an anti-FLAG Ab (1:1000), followed by HRP-conjugated anti-mouse IgG (1:10,000). Lys, lysate; Unb, unbound fraction; Bound, bound fraction, eluted from avidin-agarose. The data represents multiple independent experiments. (B) The distribution by length of peptides eluted from DLA-88*508:01 and identified by LC-MS/MS.

Fig. 2.

A pMHC surface stabilization assay confirms binding of the *K-9* and *K-11* peptides and the consensus-deduced preferences of DLA-88*508:01 at anchor residues. Axes labels are shown in the upper right panel. The position 2, position 3 and C-terminus are highlighted by larger, bold typeface. For each plot, the peptide tested is shown above in black; for comparison, the native reference sequence is shown below in gray. A dot indicates concordance of the residues; a line indicates a substituted residue from the same amino acid group. In this assay, tested peptides that bind DLA-88*508:01 are right-shifted from the control (gray line: BARC3 cells cultured with DMSO at 37°C and stained with the 2° Ab only). Additional negative controls are BARC3 cells pulsed with 9-mer and 11-mer peptides containing G at every position, and stained with 1° and 2° Abs; neither peptide resulted in an increase in MFI (not shown). The same results were obtained in two independent pMHC stabilization experiments. (A) Peptide *K-9* is a true DLA-88*508:01 binder. The presence of the native amino acids at each of the three anchor positions were necessary (B), since G substitutions at any one abolished binding, and collectively sufficient, since these residues preserved binding when G substitutions were made at all non-anchor positions (C). Those latter G substitutions were not responsible for binding, since a G-substituted peptide with non-preferred residues at position 2, position 3 and position 9 (GNDGGGGGE) did not bind. (D) The substitution of the highly preferred M for L at position 2 in peptide *K-9*

improves binding strength (greater rightward shift), but is not sufficient to overcome the negative effects of preferred residue loss at either the position 3 or C-terminus anchors. (E) Peptide *K-11* is a true DLA-88*508:01 binder. The substitution of amino acids from the same groups as the native peptide *K-11* residues at position 2 (F) (M or L for F), position 3 (G) (F or I for L) or the C-terminus (H) (R for K) was well-tolerated by DLA-88*508:01. (I) Glycine substitution at two anchor residues results in minimal (position 2) or no (C-terminus) binding, while a G residue at position 3 has no effect. Unlike peptide *K-9*, the presence of native peptide *K-11* residues at position 2, position 3 and the C-terminus alone is insufficient to confer binding, although that lack could be overcome by substituting M for F at position 2 (J, rightmost plot), or an I for L at position 3 (not shown). GFIGGGGGGGK bound with less affinity than GMFGGGGGGGK.

Peptides	Position												
	P1	P2	P3	P4	P6	P6'	P6''	P8	P7	P7'	P8	P9	
8-mers (n=38)	Phob	Phob	Polar	D/E	Phob			Phob	Phob		Phob	K/R	
Frequency (%)	50	82	42	34	63			61	42		37	58	
10-mers (n=24)	Phob	Phob	Phob	D/E	Phob		Phob	Phob	Phob		Phob	K/R	
Frequency (%)	38	92	63	38	46		38	50	46		38	58	
11-mers (n=32)	Phob	Phob	D/E	D/E	Phob	D/E	Phob	Phob	Phob		Phob	K/R	
Frequency (%)	44	88	44	44	56	34	47	59	50		53	66	
12-mers (n=6)	Phob	Phob	Phob	n/c	Phob	n/c	Polar	Phob	D/E	Phob	Phob	K/R	
Frequency (%)	60	80	80		80		60	60	40	60	60	80	
All (n=88)	Phob	Phob	Phob	D/E	Phob	n/c	n/c	Phob	Phob	n/c	Phob	K/R	
Frequency (%)	45	88	59	38	59			59	44		44	64	

Fig. 3.

Amino acid conservation by position in 9-, 10- 11- and 12-mer peptides eluted from DLA-88*508:01. Based on side chain chemical properties, amino acids were grouped into four categories (hydrophobic [Phob], charged acidic [D/E], charged basic [K/R], or polar). The N- and C-termini of all sequences were aligned by adding “gap” positions to the shorter peptides, which were placed in a manner that maximized residue conservation at all positions. The numbers below the boxes indicate the frequency at which a member of the predominant group at that position was found across all peptides of a given length; n/c indicates no consensus (i.e., >1 group present at equal frequencies, or no group having a frequency ≥ 25%). Titles that are italicized indicate that the dominant group at a specific position differs from the initial findings shown in Supplementary Fig. 2A. An amino acid group present at a particular position in ≥ 60% of all peptides is highlighted by gray shading.

Fig. 4.

Relative frequencies of 9- to 12-mer peptides compiled from separate LC-MS/MS analyses of eluates from DLA-88*508:01 complexes. (A) Length distributions of curated peptides from uninfected and CDV-GFP-infected 9-15 cells. (B) Length distributions of all peptides (without curation) from the same analyses as in (A). (C) Length distributions of all peptides from (B), as well as from an additional LC-MS/MS analysis of a 9-15 subclone stably transfected with a plasmid encoding canine idiotypic-derived peptides and eGFP.

Table 1

Amino acid sequences of 39 canine self peptides eluted from DLA-88*508:01, and their putative proteins of origin.

Peptide	Position ^d											Predicted protein ^b	NCBI accession no.
	P1	P2	P3	P4	P5	P5'	P5''	P6	P7	P7'	P8	P9	
1	K	T	N	K	F			T	Y		G	F	XP_859260
2	F	F	K	P	H			W	D		E	K	serpin peptidase inhibitor, clade H (HSP 47) member 1 (collagen binding protein 1) CAZ17012 ^e
3	H	V	I	E	T			L	I		G	K	T-complex protein 1 subunit epsilon XP_013965795
4 ^c	K	L	F	S	G			E	L		T	K	lathosterol oxidase XP_853004
5	K	M	N	P	Q			S	A		F	F	non-specific lipid-transfer protein XP_866084
6	K	M	K	E	I			A	E		A	Y	heat shock protein 70 BAB78505 ^e
7	K	M	N	P	L			W	N		T	M	proteasome subunit beta type-4 XP_533057
8	K	I	K	Y	P			E	N		F	F	protein phosphatase type 1 catalytic subunit delta isoform AAM88380 ^e
9	K	L	K	G	V			G	E		S	F	hypoxia up-regulated protein 1 isoform X1 XP_005619743
10	L	M	K	E	I			L	D		K	K	heat shock protein HSP 90-beta XP_532154
11	R	F	F	D	K			V	I		E	K	rab-like protein 3 isoform X1 XP_850572
12	Y	L	V	E	K			P	K		Y	K	actin-related protein 10 XP_851943
13	K	L	G	P	N			D	Q		Y	K	cytochrome c oxidase subunit NDUF44 XP_003639527
14	K	F	K	E	V			G	E		A	F	dnaI homolog subfamily C member 7 isoform X1 XP_537639
15	N	M	I	K	Y			I	K		E	K	inosine-5'-monophosphate dehydrogenase 2 isoform X1 XP_005632624
16	K	F	I	E	I			A	A		R	K	heterogeneous nuclear ribonucleoprotein U isoform X1 XP_005622662
17	K	M	I	P	P			G	I		H	F	protein AAR2 homolog isoform X2 XP_534409
18	H	V	I	S	W			E	D		R	K	transforming growth factor beta type II receptor AAR19223 ^e
19	K	T	F	N	Q			G	K		F	K	elongation factor 1-gamma XP_853577
20	K	L	K	D	W			Q	E		G	Y	long-chain-fatty-acid--CoA ligase 4 isoform X2 XP_863157
21	F	A	I	G	S			Q	T		K	K	PEST proteolytic signal-containing nuclear protein XP_535723
22	S	M	P	D	F			D	L		L	K	neuroblast differentiation-associated protein AHNAK XP_013976488
23	S	M	P	D	V			D	L		L	K	neuroblast differentiation-associated protein AHNAK XP_013976488
24	R	V	N	L	F	T		D	F		K	Y	eukaryotic translation initiation factor 3 subunit B XP_536894

Peptide	Position ^a											Predicted protein ^b	NCBI accession no.
	P1	P2	P3	P4	P5	P5'	P5''	P6	P7	P7'	P8	P9	
25	R	V	V	D	F	D	E	T	W		N	K	XP_535140
26	A	V	G	V	I	K	A	V	D		K	K	NP_001184045 ^e
27	V	A	V	G	V	I	K	A	V		D	K	NP_001184045 ^e
28	A	F	F	G	P	E	G	F	H		E	K	XP_005636350
29	K	A	I	K	E	M	G	F	T		N	M	XP_533327
30	S	F	M	G	P	K	G	S	P		S	R	XP_005631564
31	P	K	F	E	V	L	D	K	P		Q	S	XP_013965323
32	K	T	K	E	F	T	G	I	D		N	L	XP_005627692
33 ^d	R	F	L	D	K	D	G	F	I		D	K	NP_001153156.1 ^e
34	T	F	F	P	F	S	D	G	D		K	K	XP_546246
35	A	T	K	E	S	D	G	F	H		R	F	XP_005634602
36	I	M	G	P	N	Y	T	P	G		K	K	XP_533949
37	V	A	V	G	V	I	K	A	V	D	K	K	NP_001184045 ^e
38	F	F	F	D	L	N	E	K	Q	G	R	K	XP_534991
39	S	M	A	E	F	L	K	V	K	G	E	K	NP_001273908 ^e

^a Amino acid side chain color code: black, hydrophobic; dark gray, charged basic; light gray, charged acidic; white, polar or gap position.

^b Proteins indicated with bold face are putatively expressed in the cytosol, based on a comparison to the orthologous mammalian proteins (UniProtKB).

^c K-9;

^d K-11

^e Proteins deduced from cDNA sequences; the remainder were predicted from the canine genomic sequence (NW_003726047) by automated analysis.

Table 2

CDV-origin peptides (9- to 12-mers) eluted from DLA-88*508:01 isolated from CDV-eGFP-infected 9-15 cells

CDV protein	Peptide sequence ^a	Positional designation	Length	# of + anchors	Nested in peptides >12 residues in length ^b	C-terminal peptide?	Mean net binding ^c	Assessment
Fusion (F)	LTGTSKSYVR	F ₆₅₁₋₆₆₀	10	1	y - 13, 14, 15, 16, 17, 18	y	-0.009	artifactual
	PAFKPDLTGTS	F ₆₄₅₋₆₅₅	11	2	y - 13, 14, 15, 16, 17, 18	y	-0.015	artifactual
	LTGTSKSYVRS	F ₆₅₁₋₆₆₂	12	0	y - 13, 14, 15, 16, 17, 18	y	-0.005	artifactual
Hemagglutinin (H)	PAFKPDLTGTSK	F ₆₄₅₋₆₅₆	12	3	y - 13, 14, 15, 16, 17, 18	y	-0.001	artifactual
	AISDGVYGR	H ₂₂₂₋₂₃₀	9	2	n	n	0.291	genuine
	QVIDVLTPLFK	H ₈₇₋₉₇	11	3	n	n	0.674	genuine
Large polymerase (L)	ISNGIGKYFK	L ₅₆₄₋₅₇₃	10	1	n	n	0.254	genuine
	KAIEKGFDRY	L ₈₇₇₋₈₈₆	10	2	n	n	0.139	genuine
	SLHGPPFAK	L ₂₀₀₃₋₂₀₁₂	10	2	n	n	0.769	genuine
Matrix (M)	FMVHIGNFSRK	M ₂₁₇₋₂₂₇	11	3	n	n	0.910	genuine
	GRLLPQVRVID	M ₂₉₋₃₉	11	1	n	n	0.012	uncertain
	RVIDPGLGDRK	M ₃₆₋₄₆	11	3	n	n	0.870	genuine
Nucleocapsid (N)	RHDDPDVSIK	N ₉₀₋₁₀₀	11	3	n	n	0.649	genuine
	SSPVYNDRELL	N ₅₁₂₋₅₂₂	11	0	y - 16, 20	y	-0.013	artifactual
	VANQQPPTNK	N ₄₂₇₋₄₃₇	11	2	n	n	0.296	genuine
	SSPVYNDRELLN	N ₅₁₂₋₅₂₃	12	0	y - 16, 20	y	0.002	artifactual
	YMVILENSVQNK	N ₃₁₂₋₃₂₃	12	3	n	n	0.752	genuine
Phosphoprotein (P)	FPHNPEGKTRD	P ₂₁₂₋₂₂₂	11	0	y - 16, 17, 23	n	-0.005	artifactual
V protein (V)	YATPSQDIK	V ₂₉₁₋₂₉₉	9	2	y - 16, 17, 18	y	0.133	uncertain
	LQFPHNPEGK ^d	V ₂₁₀₋₂₁₉	10	2	y - 16, 17, 23	n	0.997	uncertain
	LVVPAGAVSNR	V ₁₂₉₋₁₃₉	11	3	n	n	0.385	genuine
	RVWYATPSQDIK ^e	V ₃₈₈₋₂₉₉	12	3	y - 16, 17, 18	y	0.571	uncertain

^aResidues at anchor positions consistent with the motif are underlined

^by (yes) indicates that the 9- to 12-mer sequence was also observed within one or more longer peptides of the lengths specified; n (no)

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Mean values from pMHC stabilization assays ($n = 2$), calculated as described in the Materials and Methods. Negligible binding was defined as 0.011 , computed as the mean score $+ 2$ standard deviations for NP396-404 ($n=4$). $K-11$ ($n=4$) bound with a mean score of 0.616 ± 0.178

d_p Peptide also found in Phosphoprotein

e_g Sequence is RVWYATPSQDLK in GenBank, ACD92993.1