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Short communication

A cell-based MHC stabilization assay for the detection of peptide binding to the canine classical class I molecule, DLA-88

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ABSTRACT

Identifying immunodominant CTL epitopes is essential for studying CD8+ T-cell responses in populations, but remains difficult, as peptides within antigens typically are too numerous for all to be synthesized and screened. Instead, to facilitate discovery, *in silico* scanning of proteins for sequences that match the motif, or binding preferences, of the restricting MHC class I allele – the largest determinant of immunodominance – can be used to predict likely candidates. The high false positive rate with this analysis ideally requires binding confirmation, which is obtained routinely by an assay using cell lines such as RMA-S that have defective transporter associated with antigen processing (TAP) machinery, and consequently, few surface class I molecules. The stabilization and resultant increased life-span of peptide–MHC complexes on the cell surface by the addition of true binders validates their identity. To determine whether a similar assay could be developed for dogs, we transfected a prevalent class I allele, DLA-88*50801, into RMA-S. In the BARC3 clone, the recombinant heavy chain was associated with murine β2-microglobulin, and importantly, could differentiate motif-matched and -mismatched peptides by surface MHC stabilization. This work demonstrates the potential to use RMA-S cells transfected with canine alleles as a tool for CTL epitope discovery in this species.

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1. Introduction

CD8+ T cells chiefly function to eliminate virus-infected and malignant cells, which they detect by specifically recognizing short peptides – their cognate epitopes – bound by MHC class I molecules on the target surface. Remarkably, despite the vast diversity of TCR repertoires and large number of peptides within a given antigen, CTL responses across individuals sharing class I alleles are predictably directed against one or a few common epitopes. For example, of the 550 possible nonamer peptides within lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP), all C57BL/6 mice are dominated by reactivity against a single epitope, NP396 (Yanagi et al., 1992), while in BALB/c mice, the major response is directed against NP118 (Schulz et al., 1991). Multiple mechanisms underlie this immunodominance phenomenon, including antigen density and processing, TCR availability, peptide–MHC class I binding affinity, and competition between T cell specificities (reviewed in Yewdell, 2006). While the study of specific CTL responses is made possible by the restrictions on diversity imposed by immunodominance, identifying epitopes still remains a difficult, resource-intensive task.

Abbreviations: β2M, beta-2-microglobulin; BARC, Bispecies Antigen Recognition Cells; BFA, brefeldin A; DLA, Dog Leukocyte Antigen; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; NP, nucleoprotein; TAP, transporter associated with antigen processing.

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Ultimately, epitopes are confirmed by demonstrating CTL effector activity, such as IFN-γ production or target killing, upon incubation with the peptide and the appropriate class I restriction element. Yet simply testing all possible peptides within an antigen is usually not a feasible strategy, because of the costs of peptide synthesis, and the low frequency of specific memory T cells (10^{-2} to 10^{-4}), which limits the number of peptides that can be evaluated in a single assay. The preferred approach is therefore to generate a smaller list of likely candidates by attempting to predict the effects of immunodominance on peptide selection, usually through a combination of *in silico* analysis and empirical determination. Of course, not all effects are weighted equally: some factors are minimally limiting (TCR availability; transporter associated with antigen processing [TAP] specificity) or difficult to reproducibly measure or estimate (proteasomal cleavage; TCR binding) (Assarsson et al., 2007; Lundegaard et al., 2007), and consequently, are often not included in prediction analysis. Quantitatively, the most important parameter in epitope selection is the affinity for class I molecules, which are relatively poor at binding peptides (Yewdell, 2006). Importantly, in those peptides that do bind, common amino acid groups in certain positions (anchor residues) can be identified. These residues (and peptide length) collectively form a motif, which can be used to scan proteins by computer program to eliminate unlikely binders for that allele (Rammensee et al., 1999). While useful, such predictions typically generate many more false than true positive binders – in one study, of the 1657 predicted peptides from vaccinia virus, only 263 strongly bound HLA-A*0201 (Assarsson et al., 2007) – and therefore, require experimental confirmation. One of the simplest, most cost-effective means of testing peptide binding to class I alleles is the peptide-induced MHC stabilization assay, which uses TAP-deficient cell lines such as T2 (human) or RMA-S (mouse). Without efficient TAP-mediated transport of cytosolic peptides into the ER, assembled class I complexes are structurally unstable, and retained only transiently at the cell surface. However, when RMA-S or T2 are incubated with an exogenous peptide capable of binding class I, surface pMHC complexes are stabilized and easily detected by flow cytometry with an anti-class I mAb.

While the ability to confirm the predictions of binding algorithms is critical for streamlining epitope discovery, there is unfortunately no corresponding cell line for evaluating putative CTL epitopes in dogs. The range of class I molecules of mice and humans that can be tested has been has been expanded beyond the endogenous alleles of RMA-S and T2 by production of transfectants; accordingly, we sought to determine whether peptide binding at the canine classical class I locus, Dog Leukocyte Antigen (DLA)-88 (Graumann et al., 1998), could be evaluated using this same strategy. An RMA-S clone expressing a prevalent allele, DLA-88*50801 (Ross et al., 2012), was therefore generated. Like the parent line, these cells could discriminate motif-matched and -mismatched peptides in a standard stabilization assay. This methodology should constitute a valuable immunologic tool for investigating and defining epitope-specific $CD8^+$ T-cell responses in the dog.

2. Materials and methods

2.1. Cell culture and cloning of DLA-88-transfected RMA-S cells

The murine lymphoma line RMA-S was cultured in RPMI-1640 containing 10% FBS and 2 mM L-glutamine (R-10). A modified pcDNA3 expression plasmid encoding a DLA-88*50801 heavy chain (GenBank, JQ733514) with a FLAG epitope tag at the carboxyl terminus, previously generated in our lab (Ross P and Hess PR, manuscript submitted), was transfected into RMA-S using Lipofectamine 2000 (Invitrogen). Following G418 selection (1 mg/ml for 7 d, then 0.2 mg/ml for 8 d), individual clones were isolated by limiting dilution and screened for vector expression after permeabilization (Cytofix/Cytoperm; BD Biosciences) and intracellular staining with the anti-FLAG mAb M2 (Sigma–Aldrich) by flow cytometry. Clone number 3 (BARC3) was used throughout the study and maintained continuously under G418.

2.2. MHC class I surface stabilization assays

In order to accumulate class I molecules on the cell surface, RMA-S and BARC3 cells were cultured overnight at 27 °C, as previously described (Ljunggren et al., 1990). In some experiments, accumulated surface class I molecules were peptide-loaded by adding K9 (KLFSGELTK), K11 (RFLDKDGFIDK) (both synthesized by Peptide 2.0), NP396 (FQPQNGQFI) or NP366 (ASNENMETM) (both synthesized by GenScript) peptides from DMSO stock solutions to overnight cultures, followed by an additional 5 h of incubation at 37 °C. Peptide loading of RMA-S and BARC3 cells (10^5 in 100 μl) was performed in R-10 or serum-free Opti-MEM I medium (Gibco/Life Technologies) in 96-well flat-bottom cell culture plates. To assess time-dependent stability of pMHC complexes, peptide-loaded BARC3 cells were washed with PBS and cultured with 5 μg/ml brefeldin A (BFA; BioLegend) for various lengths of time prior to collection.

2.3. Flow cytometry and data analysis

For staining, cells were washed in FACS buffer (PBS containing 2% FBS and 0.1% NaN_3) and incubated with the relevant primary or secondary Ab for 15 min at 4 °C in 96-well round-bottom polypropylene plates. The following unconjugated mAbs were used (clone names are listed parenthetically) at pre-determined optimal concentrations: anti-canine MHC class I (H58A, VMRD; 3F10, Ancell), anti-$H2\text{-}D^b$ (28-14-8), anti-$K^{b/d}$ (34-1-2S) (both eBioscience), and anti-murine β2M (S19.8) (BD Pharmingen). In all experiments, Alexa Fluor 647-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) was used as a secondary detecting Ab; background staining was established by omission of the primary mAb. Flow cytometric list mode data was collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). Viable cells were differentiated using forward and side scatter gating. Data were graphed and nonlinear regression analysis was performed using Prism 5 (GraphPad).

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