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An approach to the identification of T cell epitopes in the genomic era: application to *Francisella tularensis*

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Abstract

The identification and characterization of epitopes is essential for modern immunologic studies. Here we describe a novel methodology we have developed to identify T cell epitopes exploiting the phenomenon of cross presentation. Particulate antigens, in the form of beads, are very effective in delivering exogenous antigen to both the class I and class II pathways. We will review our efforts to screen entire genomes of pathogens for T cell epitopes taking advantage of the advances in genomics using *Francisella tularensis* as a model. By automating aspects of this technology we will be able to functionally screen the entire genome of *F. tularensis* for T cell epitopes. This technology should be applicable not only to *F. tularensis*, but also to many other pathogens as well.

Keywords

T cells; epitopes; *Francisella tularensis*; immune response

Introduction

The immune response against a pathogen is extremely complex. However, in broad terms, the immune response can be broken down into two key arms: the initial, rapid, non-specific actions of innate immunity; and the delayed, pathogen-specific effectors of the adaptive response involving cellular and antibody-mediated immunity. A key mediator of adaptive cellular immunity, T cells, are crucial effectors against disease causing organisms. Both T cells and antibodies recognize small portions of molecules called epitopes. However, unlike antibodies, T cells must recognize these epitopes in the context of major histocompatibility complex (MHC) molecules which bind these short peptide fragments. These peptide fragments are generated in antigen presenting cells (APCs) by a complex process of proteolytic degradation of antigens. While there are a number of unresolved questions remaining, the general outline of antigen processing is now well-established and several excellent reviews are available [1-3]. Briefly, it was initially thought that two main pathways mediate processing of peptides for MHC presentation. Endogenously produced proteins (such as host cell proteins, bacterial proteins produced by intracellular bacteria, or viral proteins produced by host cells) are degraded and then presented complexed with MHC

class I to activate CD8 T cells. Exogenous proteins derived from outside the APC, such as endocytosed material from extracellular bacteria, are presented in the context of MHC class II molecules to activate CD4 T cells. However, it was realized this distinction was overly simplified, and separation of these pathways was not absolute. Professional APCs, such as dendritic cells and macrophages, have the exquisite capability to shuttle proteins between these two presentation pathways [4-10]. Cross-presentation or cross-priming allows for the display of exogenous proteins in the context of MHC class I. It has been speculated that cross-presentation may have arisen to allow the presentation of viral proteins derived from viruses that do not efficiently infect APCs, and may also increase the 'epitopeome' displayed, adding to the robustness of the immune response [9,11]. Conversely, the phenomenon known as autophagy, a process involving the catabolic degradation of cellular components, provides access of endogenous proteins to the exogenous pathway and presentation of antigens in the context of MHC class II molecules [12]. Much remains to be learned about how these processes are controlled. It is of considerable interest to investigate mechanisms to increase the amount of cross-talk between pathways with the possibility of augmenting an immune response or exploiting this phenomenon to analyze the targets of the immune response as we describe below. Here we will review how we have taken advantage of breakthroughs in understanding of antigen processing to develop a method to identify T cell antigens, and how we are applying these strategies in combination with the advances in genomics to the pathogen *Francisella tularensis*.

Development of the T cell antigen discovery (T-CAD) assay

Studies from a number of labs, including our own, have shown that soluble antigen conjugated to beads (referred to as particulate antigen) results in increased delivery of exogenous antigen to the class I pathway [4-6,8,13,14]. Adsorption to beads has been shown to increase the efficiency of cross-presentation, allowing effective antigen presentation at concentrations 1000-10,000 fold lower than when the protein was soluble [5,8,9,15]. It has also been shown that the bead formulation and bead diameter affect the efficiency of cross-presentation, with a bead diameter of 1-5µm being the optimal size for phagocytic uptake [4,9,13], which may be a mechanism augmenting cross-presentation [16]. Perhaps surprisingly, it has also been shown that CD4⁺ T cell hybridoma activation was greater with APCs that have phagocytosed particulate antigen when compared to soluble antigen [8,13]. Taken together, these findings suggest that beads appear to increase the efficiency of antigen processing and presentation in both class I and class II pathways.

Our lab has developed a novel antigen presentation assay, called the T cell antigen discovery assay (T-CAD), where beads play a critical role in the discovery of T cell stimulating antigens and the subsequent mapping of peptide determinants [17]. We exploited the remarkable efficiency of particulate antigen delivery to develop a system that could be used to identify T cell epitopes. In designing this system, several considerations were taken into account. First, since T cells recognize short peptides complexed with MHC molecules, and thus are not dependent on the native conformation of the protein, we reasoned that bacterially expressed recombinant proteins could be used as a source of antigen. By coupling the antigen to beads, one can isolate protein under strong denaturing conditions necessary for solubilizing the protein antigen from inclusion bodies, which can be coupled directly to

the beads used in presentation assays. An illustration of one form of the T-CAD assay is shown in Figure 1. In the T-CAD assay, the protein of interest is expressed in *Escherichia coli* using recombinant DNA techniques. Here recombinant proteins were engineered using the pQE-40 expression vector from Qiagen to contain N-terminal 6xHis tags so that the antigen could be easily purified from bacterial lysates. The pQE40 vector system expresses the gene of interest as a fusion with murine dihydrofolate reductase (DHFR) protein, which adds stability to the fusion protein and helps protect it from degradation. Proteins are either directly adsorbed to paramagnetic Ni-NTA beads or recombinant protein is first purified using Ni-NTA columns and subsequently conjugated to M-280 tosylactivated paramagnetic Dynabeads. This coupling to beads also allows for purification from denaturing urea buffer and resuspension in PBS or media, which avoids many issues of solubility that plagues other bacterial expression systems. The protein conjugated to beads is then delivered to APCs for uptake, processing, and presentation to hybridomas. A key component of the T-CAD assay is the *lacZ*⁺ hybridoma, allowing for easy and direct analysis of T cell activation by enzymatic analysis using colorimetric substrates. To create the hybridomas, T cells from an immunized animal are fused to the BWZ.36 partner, which contains the beta-galactosidase gene under the control of the IL-2 promoter NF-AT regulatory elements [18]. During T cell activation, IL-2 production is rapidly upregulated, a process that involves regulatory factors binding the NF-AT region and induction of IL-2 synthesis. The coupling of IL-2 promoter elements to *lacZ* allows for assessment of hybrid activation by chromogenic beta-galactosidase substrates such as CPRG or X-gal.

An additional feature of the T-CAD assay is that one can also use it further fine map the epitope using deletion constructs (Figure 2A). In the example shown, nested C terminal truncation constructs are generated by PCR techniques, transformed, and expressed in *E. coli*, and purified proteins are characterized by SDS polyacrylamide gel electrophoresis. Epitope mapping is performed by coupling proteins expressed from the deletion constructs to tosylactivated beads for administration to APCs and subsequent presentation to antigen specific hybridomas. An example T-CAD assay for two hybridomas mapping to different epitopes within the same protein can be seen in Figure 2B. The location of an epitope can be mapped by a loss of hybridoma reactivity, identified by a reduction in the number of activated cells, suggesting that the epitope is no longer present within the protein. Once an epitope region is found, peptide prediction algorithms and over-lapping peptides can be used to further refine the epitope. Unlike some other methods the T-CAD assay [17] makes no *a priori* assumptions about the nature of the epitope or the T cell repertoire and is immediately validated since it uses a functional T cell to identify the epitope. We have used the T-CAD assay to identify class I and class II epitopes in the Prostate Specific Antigen (PSA) [17]. This system can also be applied not just to selected antigens but also to complex antigenic challenges such as pathogens. Below we will describe the application of this technique to the intracellular pathogen *F. tularensis*.

Using the T-CAD system to identify antigens in *F. tularensis*

F. tularensis is a Gram negative intracellular pathogen that has a broad host tropism and is the causative agent of tularemia [19]. Infection and disease severity is dependent upon bacterial strain, the size and route of inoculum, and can ultimately result in sepsis and

systemic dissemination within the host [19-22]. Due to an extremely low infectious dose, a high morbidity/mortality rate, the possibility for generating antibiotic resistance, and the ability to aerosolize the organism allowing widespread dissemination, the Centers for Disease Control has placed *F. tularensis* on the category A select list of potential biological weapons [21,23,24]. An experimental vaccine for tularemia, the attenuated live vaccine strain (LVS) developed in the 1940s [25], has been indispensable for examination of a number of aspects of infection and biology of the lifecycle of *F. tularensis* [26]. However, due to the significant side effects and only partial protection provided, the LVS strain has failed to be approved by the FDA [27-29]. Previous studies have reported a critical role of cellular immunity in the resolution and protection against infection [30-34]. There have only been a limited number of reports describing T cell immunostimulatory antigens [35-39] and which antigens are protective remain very poorly understood. The lack of defined molecular epitopes has greatly hampered the study pathogen-host interactions.

Genomics and antigen discovery

Large-scale sequencing efforts have been applied to many microorganisms and recently the sequence of several strains of *F. tularensis* have been completed. These studies have been useful for a number of studies of the organism including analysis of the immune response. Using 2-D gel immunoblot analyses, coupled with the sequence data and sensitive biochemical approaches, it has been possible to identify several antigens recognized serologically [40-42]. In another approach, investigators have used an *in vitro* transcription and translation system for protein production employing a transcriptionally active PCR product to generate recombinant protein in a cell-free reaction. Using this approach, they have been able to identify a panel of antigens recognized by antibodies [40]. Here we have taken a genomic approach that uses a library of arrayed bacterial clones of *F. tularensis* to identify T cell epitopes using expression screening.

The genome sequence of *F. tularensis* SchuS4 was used to generate a genome-wide gene collection for *F. tularensis* [43,44]. The *F. tularensis* library has approximately 1800 open reading frames (ORFs) from *F. tularensis* SchuS4. This library was made using the Gateway cloning system, in which donor clones containing the ORFs in entry vectors can be shifted into a variety of destination vectors. The entry vectors are designed to be transcriptionally silent, but can be shifted to other expression vectors that can be expressed in bacteria or eukaryotic cells. As we will describe below we are using this arrayed library of the individual proteins from *F. tularensis* in combination with the T-CAD system to identify T cell epitopes.

Expression of *F. tularensis* antigens

During the process of high-throughput protein production it is crucial to have multiple validation steps that can be assayed in the process. The first step of shifting the library was amplification of the clone sets in preparation for shuttling from the Gateway entry vector to a particular destination expression vector using an enzyme-mediated recombination reaction. This recombination reaction uses the site-specific properties of bacteriophage lambda and *att* sites within the entry and destination vectors to provide high efficiency, directional recombination to an assortment of destination expression vectors. Before shifting clone sets

into an expression vector, we found it important to analyze the donor clones by agarose gel electrophoresis, since initial experiments revealed that some of the donor clones either had, or were in the process, of undergoing deletion events. In most cases we were able to rescue the correct clone by subcloning and validation by sequencing. Upon verification of entry vector DNA, we investigated several different destination vectors available for protein expression. Ultimately, we chose to move forward with the pBAD-Dest49 Gateway vector, in part because of the tight regulation of protein expression. In this vector, protein production is tightly controlled by the arabinose promoter, which is repressed by glucose and can be induced by arabinose. The pBAD-Dest49 vector expresses the gene of interest fused in frame with thioredoxin to help stabilize the proteins and contains V5 and 6xHis tags that are useful for expression analysis and purification.

After shifting the library into a destination vector, the next phase was high-throughput protein production and purification. An illustration of the methodology employed is shown in Figure 3. Starting from freezer stocks, *F. tularensis* library bacterial pBAD-Dest49 clones were grown in 96 deep well plates in 2ml volumes supplemented with glucose to repress recombinant protein production, for 18-20 hours to reach stationary phase, and then an aliquot is used to inoculate 5ml glucose-free cultures in four 24 well block plates allowing maintenance of the 96 well array format (Figure 3a-c). When these cultures are growing exponentially (OD600 between 0.4-0.6), protein expression is induced with arabinose and allowed to grow for 4 hours before harvest. The four 24 well block plates are pelleted and proteins purified using the Qiagen Biorobot 3000, which employs an automated high-throughput Ni-NTA column purification of 6xHis tagged proteins and elution into 96 deep well plates, maintaining the arrayed format (Figure 3d-f). Upon completion of protein purification, a purified protein array sampling of the *F. tularensis* proteome is available for downstream analyses (Figure 3g).

To assay for protein production, we developed an immunodot blot assay where an aliquot of each purified protein is blotted onto nylon membrane using a 96 well dot blot apparatus, again retaining the 96 well format. The membrane is then probed with anti-V5 antibody followed by a secondary antibody conjugated to HRP allowing for detection and approximate quantification of V5-tagged proteins (Figure 4a). Subsets of the purified proteins can be further validated by molecular weight analysis by separation on SDS-PAGE and immunoblotting techniques probing with anti-V5 or anti-thioredoxin antibodies (Figure 4b). By analyzing every protein from the library by dot blot analysis it is possible to determine if there are any holes in the library. In some cases it may be possible to achieve better protein expression by altering the growth and induction procedures.

Analysis of the *F. tularensis* proteins for T cell epitopes

Once a plate had been purified and protein production validated by immunodot blot (Figure 4a), an aliquot of each protein is individually conjugated to tosylactivated beads and washed by magnetic separation before addition to APCs. T cell hybridoma reactivity was then screened in a T-CAD assay as described earlier, all done while maintaining a 96 well format (Figure 4c). Hybridoma activation was assessed by CPRG or X-gal assay and T cell reactive proteins directly identified without any of the difficulties associated with deconvoluting

pooled proteins or peptides (Figure 4d). Upon identification of immunoreactive antigens, deletion constructs once again become a valuable tool for mapping both T cell epitopes, utilizing the T-CAD assay (Figure 2). Thus in principle, it is possible to scan the entire genome for T cell epitopes using the T-CAD assay.

The *F. tularensis* library is also useful for selection of antigens for conventional immunization and epitope discovery. The library can also be used to select antigens, chosen because of their role in virulence, for use in immunization generating an immune response directed at a single antigen. This immunization technique could prove useful for identifying epitopes that might prove protective but would have otherwise been masked by dominant antigens in a response against a whole pathogen. As alluded to previously, it is possible to use protein arrays to identify antigens recognized serologically [40,45-47]. While such studies identify only those antibodies which are capable of recognizing antigens adsorbed to a solid support, it nevertheless is a powerful technique. It is possible, perhaps likely, that antigens recognized by antibodies contain T cell epitopes, due to the nature of B – T cell interaction and antibody production. Intriguingly, serological analysis of recombinant cDNA expression libraries (SEREX) from cancer patients has proven to be a useful tool for identifying immunogenic tumor antigens and have revealed that cytotoxic T lymphocyte (CTL) immunity is often elicited against SEREX-defined antigens [48-50]. This raises the possibility that serologically defined antigens may also be enriched for both CD4 and/or CD8 T cell epitopes. Using the approaches outlined here, we can test this hypothesis by re-arraying a subset of the library which are reactive with antisera from infected or immunized animals to screen for reactivity for T cells. Work done by others using 2D electrophoresis techniques and the *in vitro* transcription/translation system has elucidated a panel of B cell reactive antigens in *Francisella* [40-42]. Based on their findings we have selected approximately 40 antigens for analysis in our functional screenings, hypothesizing that they might be enriched for T cell reactive antigens. Ongoing work is aimed at examining whether such antigens are enriched for T cell epitopes.

Concluding remarks

In conclusion, we have taken advantage of paramagnetic beads to augment an antigen presentation assay designed for antigen and epitope discovery (T-CAD). We have further developed the T-CAD assay into a powerful, fully comprehensive, and flexible high-throughput assay for identifying both T cell immunoreactive antigens that is compatible with any pathogen. The comprehensive analysis of T cell epitopes of organisms will prove extremely useful in understanding host-pathogen interactions as well as have important implications for vaccine development.

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Figure 1. Schematic of the T-CAD assay

6xHis tagged proteins are purified from bacterial lysates using the Qiagen Biorobot 3000. Purified proteins are subsequently removed from Ni-NTA elution buffer and washed using magnetic separation with M-280 tosylactivated paramagnetic beads. The protein-coated beads are then administered to syngeneic splenocytes (APCs) for uptake, processing, and presentation of protein to hybridomas. After overnight incubation, a beta-galactosidase assay is done to identify and enumerate activated (blue) cells by the presence of a colorimetric precipitate.

Figure 2. Generation of DHFR fusion protein deletion constructs for epitope identification (A) Schematic example of full length protein of interest (a) and eight carboxy-terminal deletion constructs (b-h) used in the pQE40 bacterial expression vector. The black box indicates 6xHis tag, the gray box indicates murine DHFR protein, and open box denotes portions of protein of interest fused in frame with DHFR. (B) Representative reactivity screen against a panel of deletion proteins. Two hybridomas were incubated with splenocytes, tosylactivated beads adsorbed with various DHFR deletion proteins (a-h) or DHFR-OVA (negative control). After overnight incubation, a beta-galactosidase assay was done to identify activated cells. A loss in reactivity by a hybridoma, indicated by a decrease in activated cells, suggests that the epitope recognized is no longer present in the deletion construct to be presented. Representative data for reactivity patterns from two different hypothetical hybridomas that recognize two different epitopes are shown.

Figure 3. Illustration of the methodology employed for high-throughput protein purification

a) Freezer stocks of bacterial pBAD-Dest49 clones are used to seed; *b)* 96 deep well plates in 2mL volumes supplemented with glucose and grown for 18-20 hours to reach stationary phase. Aliquots from (*b*) were used to inoculate; *c)* 5mL glucose-free cultures in four 24 well block plates. Protein expression was induced with arabinose when the cultures reached an OD600 between 0.4-0.6 and allowed to grow for 4 hours before harvest. *d)* Upon harvest, cultures are pelleted and lysed in the 24 well blocks and; *e)* placed upon the Qiagen Biorobot 3000 for high-throughput Ni-NTA purification and *f)* elution of purified proteins into a 96 well plate. *g)* The purified protein sampling, once again in a 96 well arrayed format, was now available for downstream analyses.

Figure 4. Downstream analyses utilizing high throughput purified proteins

(a) An immunodot blot probed with anti-V5 antibody to ascertain protein expression. (b) A western blot with purified protein samples probed with anti-V5 antibody to ascertain molecular weight. (c) A T-CAD assay where 96 purified proteins are individually conjugated to beads, screened for reactivity against NFAT-*lacZ*⁺ hybridomas, which can be identified by CPRG or X-gal (represented in d) (d) Representative positive well from an X-gal assay where an antigen from the throughput production process has been identified as T cell reactive.