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Development of an MHC class I L^d-restricted PSA peptide-loaded tetramer for detection of PSA-specific $CD8^+$ T cells in the mouse

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Abstract

Objectives—We set out to develop a prostate specific antigen (PSA) peptide-loaded tetramer for enumeration of PSA-specific $CD8^+$ T cells in the Balb/c mouse model.

Methods—A candidate MHC class I PSA peptide ($HPQKVTKFML_{188–197}$) was selected based on its ability to restimulate PSA-specific $CD8^+$ T cells to secrete IFN-γ in our assays. Next, H-$2L^d$-restricted peptide-loaded and fluorescently labeled tetramers were produced in conjunction with the NIH Tetramer Core Facility. This tetramer was then tested for staining specificity and optimized for detection of PSA-specific $CD8^+$ T cells induced by our PSA-encoding adenovirus tumor vaccine.

Results—The MHC class I PSA peptide demonstrated successful restimulation of $CD8^+$ T cells isolated from mice previously vaccinated with a PSA-encoding adenovirus tumor vaccine, with no restimulation observed in control vaccinated mice. The peptide-loaded H-$2L^d$ tetramer exhibited the desired binding specificity and allowed for detection and frequency determination of PSA-specific $CD8^+$ T cells by flow cytometry.

Conclusions—We have successfully designed and validated a PSA peptide tetramer for use in the Balb/c mouse model that can be used to test PSA-based prostate cancer vaccines. Until now, PSA-specific $CD8^+$ T cells in the mouse have only been detectable via cytotoxic T lymphocyte (CTL) assays or intracellular cytokine staining, which primarily assess Ag-specific functional activity, not their absolute number. This research tool provides laboratories the ability to directly quantitate $CD8^+$ T cells elicited by PSA-specific immunotherapies and cancer vaccines that are tested in mouse models.

Keywords

Prostate specific antigen; MHC Tetramer; Intracellular cytokine staining; Mouse

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1. Introduction

Induction of tumor antigen-targeting cytotoxic $CD8^+$ T cells is a primary goal of cancer vaccines and immunotherapy given their key role in protective anti-tumor responses (1–7). This has been demonstrated in many mouse tumor models, including colon cancer (3, 8), melanoma (1, 9, 10), and thymoma (6). Consistent with this is the demonstration that this cell population is essential for tumor protection conferred by adenoviral tumor vaccines (11–17). Testing tumor vaccines in mice that use ovalbumin (OVA) as the model tumor antigen is common since OVA-specific $CD8^+$ T cell function and number can be easily detected by cytotoxic T lymphocytes assays (CTL), intracellular cytokine staining (ICS) after antigen restimulation, and OVA peptide (SIINFEKL)-loaded MHC class I tetramer staining. However, when examining $CD8^+$ T cell responses to biologically relevant tumor antigens, e.g. prostate specific antigen (PSA), murine research reagents are not as readily available. Specifically, the lack of a PSA peptide-loaded murine tetramer for detection of PSA-specific $CD8^+$ T cells in the mouse represents a significant gap in the ability to correlate function, i.e. CTL activity and tumor protection, with the total frequency of tumor antigen-specific cytotoxic cells elicited by tumor vaccines. Given the interest in developing vaccines and immunotherapies that target prostate cancer (18), we felt that such a reagent would contribute significantly to this area of research.

Tetrameric MHC molecules complexed with antigenic peptides have a strong track record for enumeration of Ag-specific T cells (19). Production of tetramers for flow cytometry requires two elements: 1) a known MHC class I peptide with confirmed ability to stimulate $CD8^+$ T cells specific for the antigen of interest and 2) identification of the MHC class I allele that binds and presents the peptide of interest. We utilized a candidate PSA peptide that is recognized by PSA-specific $CD8^+$ T cells in the mouse (20, 21). Furthermore, it has been proposed and demonstrated that this peptide binds to the murine MHC class I allele, $H\text{-}2L^d$ (20, 22), which is expressed specifically by our Balb/c mouse model strain (23).

In this study, we designed and validated a PSA peptide-loaded MHC class I tetramer for detection of PSA-specific murine $CD8^+$ T cells in the mouse model. The availability of this reagent provides an important research tool for those laboratories that utilize mouse models for initial prostate cancer vaccine and immunotherapy testing and development.

2. Materials & Methods

2.1. Mice and cell lines

Inbred 6–8 week old male Balb/c were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in filtered cages. The murine B cell lymphoma, A20 ($H\text{-}2^d$ background), was previously transfected with the human PSA gene, resulting in the PSA-expressing cell line designated A4, as previously described (14). These cells were maintained in RPMI-1640 (GIBCO, Invitrogen, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA), 1mM sodium pyruvate (GIBCO, Invitrogen, CA), 10 mM HEPES (GIBCO, Invitrogen, CA), 0.05 mM 2-mercaptoethanol, and 50 μg/mL gentimicin sulfate (Mediatech, Inc., VA).

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2.2. Viral vaccines and vaccination

Replication deficient adenoviruses encoding human PSA (Ad5-PSA) or LacZ (Ad5-LacZ) were obtained from the University of Iowa Gene Transfer Vector Core, as previously described (12, 15). Mice received subcutaneously delivered viral vaccine at a fixed dose of 10^8 pfu.

2.3. PSA peptide, tetramer and antibodies

The MHC class I human PSA peptide ($HPQKVTKFML_{188–197}$) was selected because it is an MHC class I-restricted T cell epitope capable of restimulating murine $CD8^+$ T cells *in vitro* (20, 21). The peptide used in these studies had greater than 98% purity (New England Peptide^{TM}, Gardner, MA). H-$2L^d$ class I tetramers were produced according to a previously established protocol (24). First, expression of the murine MHC class I heavy chain (H-$2L^d$) and human light chain β2-microglobulin (β2m) from bacterial expression vectors was carried out in *E. coli*. Human β2m has been shown to have a greater affinity for murine MHC class I heavy chain, and therefore is preferred over murine β2m for tetramer production. Upon isolation, these soluble proteins were incubated with the PSA peptide ($HPQKVTKFML_{188–197}$) to allow refolding of the MHC- peptide complex. Refolded monomeric complexes were then concentrated, biotinylated and multimerized into fluorescently labeled tetramers with streptavidin (NIH Tetramer Core Facility, Atlanta, GA; Fig. 1). MAbs specific for CD8 (FITC), CD3ε (PE-Cy5), and IFN-γ (PE) were all purchased from eBioscience (San Diego, CA).

2.4. Detection of Ag-specific IFN-γ producing cells by intracellular cytokine staining

Fourteen days after viral vaccination, splenocytes were prepared and restimulated with 5 μg of PSA peptide (described in section 2.3) or with PSA-expressing A4 cells (described in section 2.1) at a 10:1 ratio. These cells served as a positive PSA stimulation control, as previously described (14). All restimulations were done in the presence of Golgistop^{TM} (BD Cytofix/Cytoperm^{TM} Fixation/Permeabilization Kit, BD Biosciences, San Diego, CA). After 4hrs, cells were treated with Fc block (eBioscience, San Diego, CA) and then stained for CD8 and CD3. Following surface marker staining, cells were fixed, permeabilized and stained for IFN-γ, according to manufacturer's protocol (BD Cytofix/Cytoperm^{TM} Fixation/ Permeabilization Kit, BD Biosciences, San Diego, CA). Samples were acquired on a FACScan flow cytometer (Becton Dickinson, NJ) and analyzed with FlowJo software (TreeStar, OR).

2.5. Enumeration of Ag-specific $CD8^+$ T cells by PSA-tetramer staining

Fourteen days after vaccination with either Ad5-PSA or Ad5-LacZ, splenocytes were isolated and the L^d-PSA tetramer (see section 2.3) was tested for its ability to detect PSA-specific $CD8^+$ T cells. The tetramer was titrated to determine its optimal working dilution and to confirm its specificity. Surface molecules were stained for CD8 and CD3. Samples were acquired and analyzed as described in section 2.4.

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2.6 Statistical analysis

Two way ANOVA was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3 Results

3.1. PSA peptide can restimulate CD8$^+$ T cells in vitro after Ad5-PSA vaccination

Our first goal was to confirm that the proposed MHC class I PSA peptide, HPQKVTKFML$_{188–197}$ (20, 21, 25), was capable of restimulating murine CD8$^+$ T cells obtained from mice previously vaccinated with our Ad5-PSA viral vaccine. Fourteen days after viral vaccination with Ad5-LacZ or Ad5-PSA, splenocytes were restimulated *in vitro* with either PSA-expressing A4 cells or PSA peptide followed by intracellular detection of IFN-γ. We observed that the PSA peptide was specifically stimulatory to only the splenocytes from Ad5-PSA vaccinated mice, as measured by detection of IFN-γ-producing CD8$^+$ T cells by flow cytometry (Fig. 2A). The difference in levels of stimulation observed between Ad5-LacZ and Ad5-PSA vaccinated mice was statistically significant when the cells were restimulated with either PSA-expressing A4 cells (positive control) or the PSA peptide, demonstrating that successful stimulation occurred *in vitro* when the peptide was used (Fig. 2B). These results demonstrated to us that this peptide was a good candidate for PSA tetramer development.

3.2. PSA-peptide loaded MHC class I tetramer displays titratable specificity

The strategy for tetramer production was to incorporate the peptide into the H-2L^d murine MHC class I molecule, which is expressed in the Balb/c mouse strain. This MHC allele has been previously demonstrated to bind the PSA peptide specifically (20, 22). The PSA tetramer was then tested and optimized for flow cytometric staining. To do this, splenocytes were harvested fourteen days after Ad5-LacZ or Ad5-PSA vaccination of Balb/c mice. These cells were then stained for the T cell markers CD8 and CD3, as well as the PSA tetramer at one of four dilutions (1:100, 1:200, 1:400 or 1:800), followed by flow cytometric acquisition and analysis (Fig. 3A). We observed titratable staining of CD8$^+$ T cells from Ad5-PSA vaccinated mice that was significantly higher than staining of CD8$^+$ T cells from Ad5-LacZ vaccinated mice at all dilutions tested (Fig. 3B).

4. Conclusions

Prostate cancer is the second most common cancer and the second leading cause of cancer-related death in American men (American Cancer Society, 2009). Given the impact of this disease, recent research has focused on the development and testing of tumor-targeted immunotherapies and vaccines. Because of its common overexpression by prostate malignancies, PSA is an attractive target for immune stimulation by such therapies and vaccines. Studies have clearly shown that eliciting PSA-specific CD8$^+$ T cell responses is critical for optimal tumor protection and regression. However in mouse prostate cancer models where we and other labs focus immunological testing, the absence of a PSA tetramer has limited the extent to which this cell population can be studied and quantified. Because of this, we set out to produce such a reagent that would allow for specific identification and

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enumeration of these cells, providing more complete detail about how effective certain prostate cancer vaccines might be.

Here we have described the successful identification of an MHC class I PSA peptide ($HPQKVTKFML_{188–197}$) that is stimulatory for PSA-specific $CD8^+$ T cells in our prophylactic prostate cancer adenovirus vaccine mouse model. A tetramer loaded with this peptide was then produced that could be used for flow cytometric enumeration of PSA-specific $CD8^+$ T cells in the mouse. Once completed, we confirmed and optimized its use for detecting this cell population in the Balb/c mouse after Ad5-PSA vaccination. This novel tetramer is a powerful research tool that will allow for important immunological evaluation of tumor antigen-specific $CD8^+$ T cells in PSA-based tumor models in the mouse.

Acknowledgments

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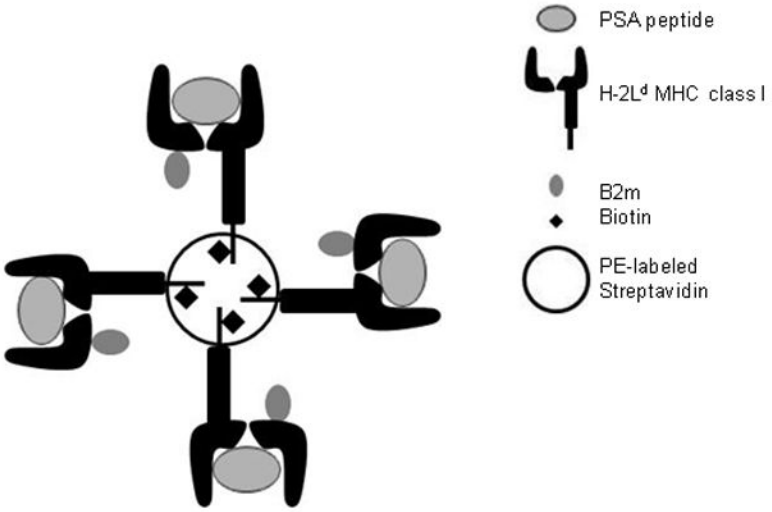

Figure 1.
Schematic representation of the PSA peptide-loaded MHC class I tetramer. The basic construction is detailed in the Materials & Methods.

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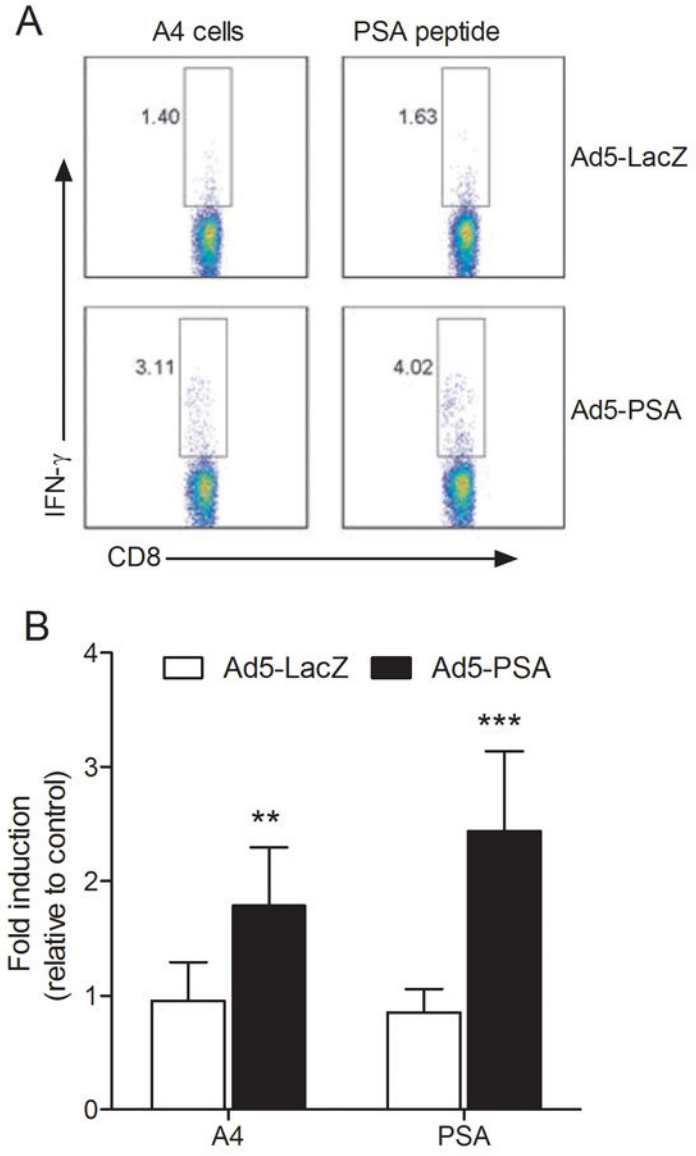

Figure 2.

MHC class I PSA peptide (HPQKVTKFML$_{188–197}$) can stimulate IFN-γ production by CD8$^+$ T cells from mice previously vaccinated with Ad5-PSA. Splenocytes were harvested 14 days after vaccination of mice with either Ad5-LacZ or Ad5-PSA and restimulated *in vitro* with either PSA-expressing A4 cells or PSA peptide, followed by intracellular cytokine staining (ICS) for IFN-γ. *A*, Representative flow cytometric plots of CD8$^+$IFN-γ$^+$ T cells. *B*, Statistical analysis of ICS data from 5 mice/vaccination group; frequencies of CD8$^+$IFN-γ$^+$ T cells determined by flow cytometry after A4- or PSA-restimulation were normalized to those observed after incubation in media alone (negative control). **, p<0.01; ***, p<0.001.

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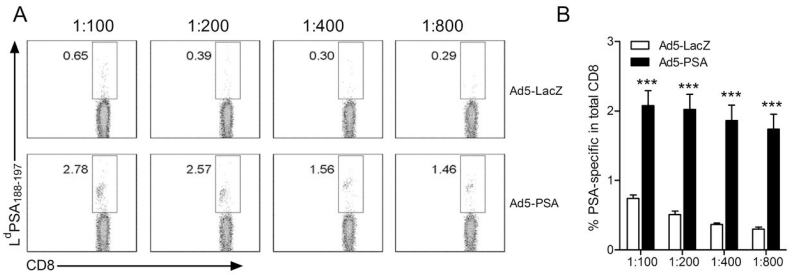

Figure 3.

Optimization and demonstration of specificity of PSA peptide-loaded MHC class I tetramer. Splenocytes were harvested 14 days after vaccination of mice with either Ad5-LacZ or Ad5-PSA and stained with H-2L^{d}-PSA$_{188–197}$ tetramer at four dilutions. *A*, Representative flow cytometric plots of CD8^{+}PSA tetramer^{+} T cells. *B*, Statistical analysis of CD8^{+}PSA tetramer^{+} (PSA-specific) T cell frequencies observed at each dilution of PSA tetramer. Data is from 5 mice/vaccination group. ***, p<0.001.