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Using a Novel Antigen Discovery System

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13. ABSTRACT (Maximum 200 Words)

The goal of this project is to devise a new method for antigen discovery using dendritic cells as antigen-presenting cells to prime autologous naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy donors against breast tumor proteins and peptides. To identify HLA Class I-restricted tumor antigens, we extracted peptides from tumor HLA Class I molecules, fractionated them by RP-HPLC, and loaded them onto DCs to prime naïve CD8<sup>+</sup> T cells *in vitro*. Our results show that we were able to prime naïve CD8<sup>+</sup> T cells to several peptide fractions and generate specificity to the tumor. Electrospray ionization mass spectrometry of the immunostimulatory peptide fractions identified a panel of peptides that could bind MHC-Class I molecules. To identify HLA Class II-restricted tumor antigens, we fractionated tumor protein extracts using RP-HPLC, and loaded them onto DCs to prime naïve CD4<sup>+</sup> T cells *in vitro*. Our results show that we were able to prime naïve CD4<sup>+</sup> T cells to several protein fractions and generate specificity to the tumor. In summary, results stemming from this project show that we have created a novel method that identified a panel of potentially new MHC Class I- and Class II-restricted tumor antigens.

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## INTRODUCTION

The prospect of successful immunotherapy against breast cancer relies in part on the discovery of tumor-specific antigens and their ability to stimulate immune responses in the host. Various approaches have been developed to search for new tumor-specific antigens, i.e. the genetic approach (1, 2), the peptide elution approach (3, 4), the reverse immunology approach (5, 6), and SEREX (7). However, all these approaches depended on the availability of T cell lines or clones from cancer patients that could be used to screen new tumor targets. A panel of melanoma tumor antigens (8) have been identified using these approaches, but very few have been found in tumors of other types, such as breast tumors. Studies have also shown that T cells from cancer patients may be defective (9), thus providing another caveat in using these T cells effectively as reagents to identify new tumor antigens. The goal of this project is to alleviate these concerns and create a new tumor antigen discovery system to identify new breast tumor-specific antigens. We hypothesize that by using dendritic cells from healthy donors to prime naïve T cells to breast tumor proteins and peptides *in vitro*, we could circumvent the problem of tumor-induced immunosuppression *in vivo* and identify new breast tumor-specific antigens.

## BODY

### I. Technical Objectives

The project has followed the original technical objectives, with emphasis placed on Technical Objectives 1 & 2:

1. To use human dendritic cells to prime *in vitro* naïve, autologous CD8<sup>+</sup> T cells to peptides eluted from HLA Class I molecules of the tumor, MS
2. To use human dendritic cells to prime naïve, autologous CD4<sup>+</sup> T cells to fractionated tumor cell lysates of the tumor, MS
3. To clone the genes pertinent to the identified immunogenic tumor peptides and/or proteins

### II. Studies & Results

#### Identification of HLA Class I-restricted tumor antigens (Relevant to Technical Objectives 1 & 3)

To focus on peptides eluted from HLA-A2.1, the most common allele from the Caucasian population, we transfected the MS tumor with the HLA-A2.1 allele (henceforth denoted as MS-A2), as described in the 1998 DOD Annual Report. To address **Task 1 of the *Statement of Work***, we generated large numbers of MS-A2 tumor cells in culture, purified the HLA Class I molecules by immunoaffinity chromatography, acid-extracted the HLA bound peptides, and fractionated them by RP-HPLC. Our results showed that we were able to purify HLA Class I molecules and acid-extract the peptides with reasonable success (10). Electrospray ionization tandem mass spectrometry analysis of these HPLC fractions showed a panel of HLA Class I-bound peptides (Table 1), demonstrating our success in extracting peptides bound to tumor HLA Class I molecules (10,11). Details of these methods have been described in the 1998 DOD

Annual Report as well as a manuscript that is in press for the journal *Clinical Cancer Research* (See Appendix).

To address **Task 2 of the Statement of Work**, we loaded dendritic cells from an HLA-A2.1<sup>+</sup> healthy donor with individual HPLC peptide fractions to prime naïve, autologous CD8<sup>+</sup> T cells. Our results showed that we were able to prime CD8<sup>+</sup> T cells to these peptide fractions and generate specificity to the breast tumor from which the peptides were derived (Figure 1, ref. 11). Interestingly, one of the primed CD8<sup>+</sup> T cell cultures recognized both an HLA-A2.1<sup>+</sup> breast tumor, MS-A2, and lung tumor, 201T-A2, suggesting the presence of shared tumor antigens between these tumors (Figure 2, ref. 11). Most importantly, we were able to reproducibly prime naïve CD8<sup>+</sup> T cells to peptides collected from different HPLC runs (Figure 3, ref. 11) demonstrating our success in priming CD8<sup>+</sup> T cells to eluted peptides and in generating tumor-specific T cell lines. From this part of the work, we have identified 12 HPLC fractions that contain potential breast tumor antigen candidates (Table 1, ref. 11).

To determine the biochemical content and identity of the immunostimulatory HPLC fractions, we analyzed the peptide fractions by electrospray ionization mass spectrometry as proposed for **Task 7 of the Statement of Work**. Our results showed that we have identified a panel of peptides that conform to mass-to-charge ratios of peptides that bind to HLA Class I molecules (Table 1, ref. 11). Our initial sequencing efforts by tandem mass spectrometry yielded a 7 amino acid peptide that bound to HLA-A3 (12), but subsequent experiments showed that it did not confer immunostimulatory capacity. In collaboration with Dr. Donald Hunt from the University of Virginia, we have sequenced peptides from another fraction and found 6 interesting tumor peptides that all bound to HLA-A2.1 with various affinities (Table 2), all having the same sequence except at the C-terminus.

**Table 2. HLA Binding Specificity of Peptides from Fraction #50**

	HLA-A2 <sup>a</sup>	HLA-A3 <sup>b</sup>
<b>Peptide #1</b>	++ <sup>c</sup>	-
<b>Peptide #2</b>	+	-
<b>Peptide #3</b>	+	-
<b>Peptide #4</b>	++	-
<b>Peptide #5</b>	++	-
<b>Peptide #6</b>	+++	-

<sup>a</sup> T2 cells were loaded with the indicated peptides for 18 hours at 27<sup>o</sup>C, and stained for HLA-A2.1 expression using the MA2.1 antibody.

<sup>b</sup> Endogenous peptides of C1R-A3 cells were first eluted from surface HLA-A3 molecules using a citrate-phosphate buffer and the cells were loaded with the indicated peptides at room temperature for 3 hours. HLA-A3 expression was detected using the GAP-A3 antibody.

<sup>c</sup> Relative binding affinity compared to cells without peptide.

Sequence homology search using the NCBI Blast Database showed that these peptides had high homology to one of the cyclin proteins previously reported to be overexpressed in a variety of epithelial cancers, including breast cancer. To confirm if these peptides were immunostimulatory, we synthesized the peptides and tried to use the original CD8<sup>+</sup> T cells



primed with the peptide fractions to see if they responded to the synthetic peptides as well. However, the cells seemed to be on their way to senescence (they have been in culture for 6-7 weeks), because attempts to expand them with either the original tumor or suboptimal anti-CD3 stimulation was unsuccessful. We decided to prime naïve T cells to DCs loaded with the synthetic peptides, and after several rounds of stimulation using autologous macrophages loaded with the synthetic peptides, we tested them using autologous DCs loaded with the peptides in an IFN- $\gamma$  ELISPOT assay. Our results showed that 2 out of 6 peptides were immunostimulatory (Figure 7). We are now currently in the process of writing a paper covering this aspect of our work. We also plan to sequence the remaining immunostimulatory peptide fractions to find additional breast tumor-specific antigens.

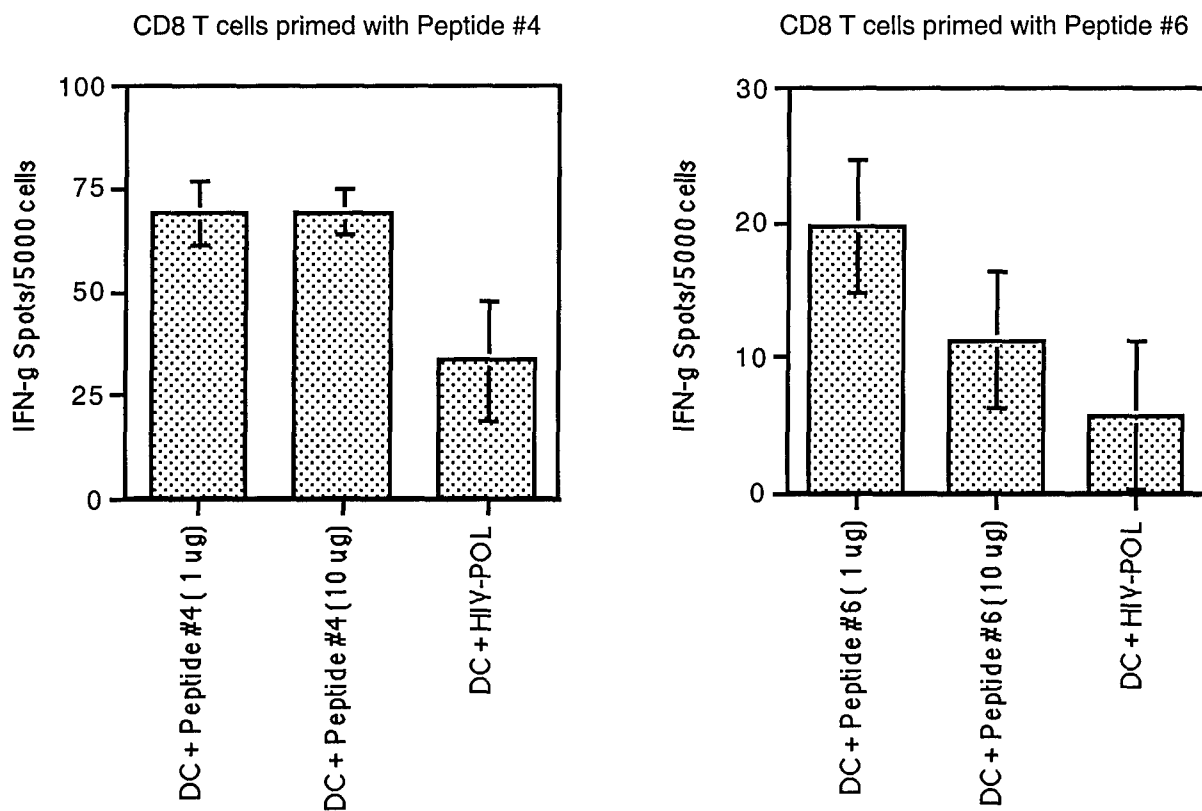


Figure 7. Two peptides identified from our approach contained immunostimulatory activity. DCs loaded with synthetic peptides were tested against autologous CD8 T cells primed to indicated peptides using an IFN- $\gamma$  ELISPOT assay.

As mentioned previously (12), we decided last year to focus our attention on the antigen discovery process and place a hold on **Task 3** of this project, which proposes to look at secondary T cell responses from cancer patients to the eluted peptides of the tumor. Recently, however, with the discovery of the tumor peptides described above, we have started to evaluate T cell responses of breast cancer patients to these peptides, thus starting **Task 3** of this project. This will be done using the IFN- $\gamma$  ELISPOT assay, as well as HLA-A2.1 Class I-tetramers of

these peptides. The results from these ongoing experiments should show conclusively that we have identified a new breast tumor-specific antigen.

#### Identification of HLA Class II-restricted tumor antigens (Relevant to Technical Objectives 2 &3)

To address **Task 4** of the *Statement of Work*, we generated detergent tumor lysate, which we fractionated by RP-HPLC and loaded onto DCs to prime naïve autologous CD4<sup>+</sup> T cells *in vitro*. Details of this work have been described in the DOD '98 Report and will be published in the same manuscript mentioned above. Our results showed that we have been able to obtain the same HPLC profile over different runs (Figure 5B & 5C, ref. 10), allowing us to obtain a constant source of antigen for our studies. We were also able to prime naïve CD4<sup>+</sup> T cells to these protein fractions and generate specificity to the tumor, both in apoptotic form (Figure 4) and in tumor lysate form (Figure 5, ref. 11). From this part of the work, we have identified 14 HPLC protein fractions that contain potential breast tumor antigens (Figure 4, ref. 11). We have also subfractionated an immunostimulatory protein fraction from another run, and was able to narrow our search to two predominant proteins of 17 kD and 19 kD (Figure 6, ref. 11). We are currently in the progress of sequencing these two bands, as well as analyzing the remainder 13 positive protein fractions. However, this work has not progressed further due to our exciting results with the HLA Class I-restricted tumor antigens which have become the focus of most of our effort so that we can bring the work to a favorable completion and publication.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Acid-extraction of peptides bound to tumor HLA Class I molecules
- Reversed-phase HPLC separation of tumor peptides
- Priming of naïve CD8<sup>+</sup> T cells using autologous dendritic cells loaded with fractionated tumor peptides
- Generation of 12 primed-CD8<sup>+</sup> T cell cultures capable of recognizing the original tumor
- Identification of a panel of immunostimulatory peptides by electrospray ionization mass spectrometry analysis
- Confirmation of immunostimulatory capacity in some of the identified peptides
- Reversed-phase HPLC separation of tumor protein extracts
- Priming naïve CD4<sup>+</sup> T cells using autologous dendritic cells loaded with fractionated tumor proteins
- Generation of 14 primed-CD4<sup>+</sup> T cell cultures capable of recognizing the original tumor
- Identification of two immunostimulatory proteins of 17 kD & 19 kD

#### **REPORTABLE OUTCOMES:**

##### Manuscripts:

- Kao, H., Amoscato, A.A., Ciborowski, P., and Finn, O.J. A New Strategy for Tumor Antigen Discovery Based on In Vitro Priming of Naive T Cells with Dendritic Cells. *Clin. Canc. Res.* in press.

##### Oral Presentations:

- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"  
Keystone Symposia, Cellular Immunity and Immunotherapy of Cancer, Santa Fe, New Mexico, January 23<sup>rd</sup>, 2000
- "Identification of MHC Class I-restricted Tumor Peptides Using a Dendritic Cell- Based Antigen Discovery System"  
Pittsburgh Cancer Institute Noon Seminar, January 6th, 2000
- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"  
Fourth Annual University of Pittsburgh Biomedical Student Research Symposium, November 19th, 1999
- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"  
International Conference on Immunology, Shanghai, China, October 26th, 1999
- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"  
Department of Molecular Genetics & Biochemistry Retreat, September 17th, 1999
- "DC-based Tumor Antigen Discovery System: Work in Progress"  
Pittsburgh Cancer Institute Noon Seminar, September 10th, 1998

Poster Presentations:

- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"  
H. Kao, J. Marto, A. A. Amoscato, P. Ciborowski, J. Shabanowitz, D. Hunt, O. J. Finn  
DOD Era of Hope Meeting, Atlanta, Georgia, June 9-11, 2000
- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Tumor Proteins and Peptides using a Dendritic Cell-based Antigen Discovery System"  
Henry Kao, Andrew A. Amoscato, and Olivera J. Finn  
Keystone Symposia, Cellular Immunity and Immunotherapy of Cancer, Santa Fe, New Mexico, January 21-27<sup>th</sup>, 2000
- "Priming CD4<sup>+</sup> and CD8<sup>+</sup> T cells against Epithelial Proteins and Peptides using a Dendritic Cell-based Tumor Antigen Discovery System"  
Henry Kao, Andrew A. Amoscato, and Olivera J. Finn  
Experimental Biology 1999, Washington, D.C. April 17-21, 1999

## CONCLUSIONS

For immunotherapies against breast cancer to be successful, we must identify breast tumor-specific antigens that could effectively stimulate the immune response of the host. We have devised a novel method for tumor antigen discovery that utilizes the *in vitro* priming of naive T cells with dendritic cells loaded with breast tumor proteins and peptides. This approach has advantages over previous approaches in that it seeks to utilize anti-tumor responses generated from healthy, immunocompetent individuals instead of cancer patients. Furthermore, our approach eliminates the use of tumor cells as stimulators and T cells from cancer patients, since studies have shown that either cell may be inadequate or defective to stimulate an effective

immune response. We believe that by using elements that are "healthy", we can be more effective in identifying new tumor antigens.

For the identification of HLA Class I-restricted tumor antigens, we loaded dendritic cells with peptides eluted from tumor HLA Class I molecules and used it to prime naive CD8<sup>+</sup> T cells from a healthy donor. Our results showed that we have been successful in priming T cells to tumor peptides and in generating specificity to the tumor from which the peptides were derived. Mass spectrometry analysis of the immunostimulatory peptides yielded a panel of peptides that could bind HLA Class I molecules, and so far, we have confirmed 2 peptides that bind HLA-A2.1 and contain immunostimulatory capacity. Further work is being done to sequence the remainder 10 peptide fractions to define other new breast tumor-specific antigens.

For the identification of HLA Class II-restricted tumor antigens, we loaded dendritic cells with fractionated tumor lysate and used it to prime naive CD4<sup>+</sup> T cells. Our results showed that we have been successful in priming T cells to tumor proteins and generate specificity to the original tumor. Further biochemical analysis of one of the immunostimulatory protein fractions narrowed our search to 2 proteins of 17 & 19 kD. Further work is being done to analyze the remainder 13 protein fractions and define new breast tumor-specific antigens.

In summary, our results show that our approach has been successful in identifying a panel of HLA Class I- and Class II-restricted breast tumor-specific antigens. We are excited at the prospect of this discovery, and currently, we are working to further characterize and define these immunostimulatory tumor peptides and proteins. While the goal of this project was to search for new breast tumor-specific antigens, the universality of our approach allows it to be used to search for new tumor antigens in other types of tumors as well.

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12. Kao, H. 1999. Department of Defense Annual Report DAMD17-97-1-7057.

#### APPENDICES

- Manuscript in press for *Clinical Cancer Research*.

**A New Strategy for Tumor Antigen Discovery Based on *In Vitro* Priming of Naïve T Cells  
With Dendritic Cells<sup>1</sup>**

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Key Words: *in vitro* priming, CD4, CD8

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## ABSTRACT

We describe a method for discovery of new tumor antigens that utilizes dendritic cells as antigen-presenting cells to prime autologous naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy donors against tumor proteins and peptides. For the identification of HLA Class I-restricted tumor antigens, peptides were extracted from tumor HLA Class I molecules, fractionated by RP-HPLC, and loaded onto *in vitro* generated DCs to prime naïve CD8<sup>+</sup> T cells. Our results show that we were able to prime naïve CD8<sup>+</sup> T cells *in vitro* to several peptide fractions and generate specificity for the tumor. Electrospray ionization mass spectrometry was used to confirm that these fractions contained peptides derived from MHC-Class I molecules and the primed CD8<sup>+</sup> T cells were used to further analyze the immunostimulatory peptide fractions. For the identification of HLA Class II-restricted tumor antigens, we fractionated tumor protein extracts using RP-HPLC, and loaded individual fractions onto DCs to prime naïve CD4<sup>+</sup> T cells. Our results show that we were also able to prime naïve CD4<sup>+</sup> T cells to several protein fractions and generate specificity for the tumor. These results illustrate the potential of this method to identify new immunostimulatory MHC Class I- and Class II-restricted tumor antigens.

## INTRODUCTION

The prospect of successful immunotherapy against tumors relies in part on the discovery of tumor-specific antigens that are able to stimulate effective immune responses in the host. Several approaches have been developed over the years for the identification of tumor antigens. The "genetic approach" utilizes tumor cDNA libraries transfected into target cells expressing appropriate HLA molecules (1, 2). The "peptide elution approach" utilizes peptides eluted from tumor HLA and loaded on target cells bearing the same HLA molecules (3, 4). The "reverse immunology approach", utilizes peptide sequences derived from already known oncogenes or other putative tumor associated genes that contain desired HLA anchor motifs (5, 6). All these approaches depend on the availability of tumor-specific T cell lines or clones derived from cancer patients and used to recognize the new targets. Most recently, the SEREX approach has been used where tumor cDNA expression libraries are screened with sera from cancer patients (7). These approaches have led to the identification of a panel of tumor antigens, primarily in melanomas (8-15). Very few tumor-specific antigens have been described in epithelial tumors, the best known being the HER-2/neu derived peptides (16), and the core peptides of the MUC-1 tandem repeat (17). In addition to the overall number of tumor antigens being small, there is a concern that they were all identified using an immune response from individuals with cancer that has clearly not been effective as a tumor rejection response. Therefore, we devised and tested a new antigen discovery system that seeks to determine not what a cancer patient recognizes on his/her tumor, but rather, what a healthy immune system recognizes as foreign among numerous peptides and proteins derived from tumor cells. Our approach is based on *in vitro* cultured dendritic cells that are used to prime autologous healthy naïve T cells.



Dendritic cells have been shown to be the most potent APCs in the immune system, expressing high levels of MHC molecules, costimulatory molecules and adhesion molecules essential for T cell activation (18). Furthermore, dendritic cells are capable of inducing primary T cell responses *in vitro* to both viruses and synthetic peptides (19-22), whereas other APCs<sup>3</sup> can only stimulate previously sensitized T cells (23). DCs have been successfully used to prime naïve T cells *in vitro* against several known tumor antigens (24-30).

Studies have shown marked alterations in the signal-transduction molecules in the T cells of human cancer patients, such as alterations in expression of p56<sup>lck</sup> tyrosine kinase, TCR- $\zeta$  chain (31), and NF- $\kappa$ B p65 transcription factors (32). Therefore, in our approach we have used T cells from healthy donors. The results here illustrate the utility of this new approach for priming naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells against proteins and peptides isolated from a breast epithelial tumor cell line and presented by DCs grown *in vitro*, and generation of specificity for the tumor. This has led to the isolation of candidate HLA Class I- and HLA Class II-restricted epithelial tumor antigens.

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<sup>3</sup> The abbreviations used are: APC, antigen presenting cell; DC, dendritic cell; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; PBMC, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocytes

## MATERIALS AND METHODS

**Cell lines.** MS (A3, B7, B7, C7, C7; DR15, DQ6 homozygous) is a breast epithelial adenocarcinoma cell line derived from the metastasis of a breast cancer patient. This cell line does not express either MUC-1 or HER-2/neu (data not shown), the two major epithelial tumor antigens. MS-A2 is the same cell line that we stably transfected with the HLA-A2.1 plasmid (33) using the calcium phosphate precipitation method (Stratagene, La Jolla, CA). The B lymphoma cell line Raji (A3, B15, C7 homozygous; DR3, DR10, DQ1, DQ2) was purchased from the American Type Culture Collection (Manassas, VA). The chronic myelogenous leukemia cell line K562 was also purchased from ATCC. The melanoma cell line Mel 624 (A2, A3, B7 homozygous) was a kind gift from Dr. Walter Storkus, University of Pittsburgh. The lung tumor cell line, 201T (A10, A29, B15, B44), which we also transfected with the HLA-A2.1 plasmid (designated 201T-A2), was a kind gift from Dr. Jill Siegfried, University of Pittsburgh. Naïve CD4<sup>+</sup>, CD8<sup>+</sup> T cells, dendritic cells, and macrophages were derived from a leukaphoresis product of a healthy platelet donor (A2, A29, B7, B44, C7, C7; DR15, DR7, DQ6, DQ2) obtained through the Central Blood Bank of Pittsburgh (Pittsburgh, PA).

**Antibodies.** Mouse anti-human HLA-DR (L243), CD3 (Leu-4), CD4 (Leu-3a), CD8 (Leu-2a), and CD56 (Leu-19) were purchased from Becton Dickinson (San Jose, CA). Mouse anti-human CD45RO (UCHL1) and CD20 were purchased from DAKO (Carpinteria, CA). Goat anti-mouse IgG antibodies were obtained from Zymed Laboratories, Inc. (South San Francisco, CA). W6/32, a mouse anti-human MHC Class I antibody, was produced by the W/632 hybridoma obtained from ATCC (Manassas, VA) and purified via a Protein A-Sepharose column (Sigma, St. Louis, MO) in the laboratory.

**Isolation of tumor HLA Class I-bound peptides.** Isolation of HLA Class I-associated peptides was similar to previously described methods (3, 34). MS-A2 tumor cells were grown in 10-chamber cell factories (Nalge Nunc, Naperville, IL), and expanded weekly until  $>1.5 \times 10^{10}$  cells were obtained. The cells were washed three times in ice-cold PBS, pelleted and stored at  $-80^{\circ}\text{C}$  for later use. Detergent lysis buffer (1% CHAPS) and a cocktail of protease inhibitors (2 mM PMSF, 100  $\mu\text{M}$  Iodoacetamide, 5  $\mu\text{g}/\text{ml}$  Aprotinin, 10  $\mu\text{g}/\text{ml}$  Leupeptin, 10  $\mu\text{g}/\text{ml}$  Pepstatin A, 3  $\text{ng}/\text{ml}$  EDTA, and 0.04% sodium azide) (Sigma, St. Louis, MO) were used to solubilize the cells at  $4^{\circ}\text{C}$  for 1 hr. The cell lysate was spun at  $100,000 \times g$  for 1 hour to remove insoluble proteins, and the supernatant was filtered through a  $0.22 \mu\text{m}$  filter (Millipore, Bedford, MA) to further remove debris from the suspension. The supernatant was then passed through a Protein A-Sepharose anti-class I (W6/32) column (BioRad, Hercules, CA) overnight. The column was then washed 30 times sequentially with low salt (150 mM NaCl, 20 mM Tris pH 8.0) buffer, high salt (1 M NaCl, 20 mM Tris pH 8.0) buffer, and Tris buffer (20 mM Tris pH 8.0). Class I molecules were then eluted from the column using 0.2N acetic acid, and peptides were extracted from the Class I molecules by boiling in 10% acetic acid for 5 minutes. The released peptides were further purified using 5 kD cut-off microconcentrators (Amicon, Bedford, MA), vacuum centrifuged to reduce the volume, and frozen at  $-80^{\circ}\text{C}$ .

**High Performance Liquid Chromatography.** 1) Fractionation of peptide extracts. The peptide extracts were fractionated by reverse phase HPLC on a Rainin HPLC separation system (Varian, Woburn, MA). The peptide extracts were concentrated to 150  $\mu\text{l}$  via vacuum centrifugation, and injected into a Brownlee Aquapore (Applied Systems Inc., San Jose, CA) C18 column (column dimensions: 2.1mm x 3 cm, pore size: 300  $\text{\AA}$ , particle size: 7  $\mu\text{m}$ ) on the HPLC. The peptides

were eluted with a 65 minute trifluoroacetic acid/acetonitrile gradient [v/v 0-15% for 5 minutes, 15-60% for 50 minutes, and 60-100% for 10 minutes solvent B (60% acetonitrile in 0.085% TFA) in solvent A (De-ionized water in 0.1% TFA)] with a flow rate of 200  $\mu$ l/min. Two hundred microliter fractions were collected at one minute intervals, concentrated via vacuum centrifugation to 40  $\mu$ l, and divided into 4 aliquots, 3 for the use in T cell stimulation and 1 for mass spectrometry analysis.

2) Fractionation of protein extracts.  $>1 \times 10^9$  MS tumor cells were lysed in detergent buffer, spun at 100,000 x g, and then filtered using a 0.22  $\mu$ m filter as above. The supernatant was dialyzed overnight in Tris Buffered Saline pH 7.2 (TBS) (Sigma) to remove detergent. The protein extract was concentrated by vacuum centrifugation, and one-tenth of the extract ( $\sim 1 \times 10^8$  cell equivalents) was fractionated by reverse-phase HPLC using a Phenomenex Jupiter C4 column (column dimensions: 4.6 mm x 150 mm, pore size: 300  $\text{\AA}$ , particle size: 7  $\mu$ m) (Torrence, CA). The proteins were eluted with a 60 minute TFA/acetonitrile gradient [v/v 10-80% solvent B (100% acetonitrile in 0.1 TFA) in solvent A (De-ionized water in 0.1% TFA)] at a flow rate of 500  $\mu$ l/min. Five hundred microliter fractions were collected at one minute intervals, concentrated by vacuum centrifugation to 100  $\mu$ l, and divided into 4 aliquots, 3 for the use in T cell stimulation, and 1 for protein content analysis.

3. Sub-Fractionation of protein fractions. 25% of a specific protein fraction obtained from the primary fractionation was further sub-fractionated by reverse-phase HPLC using a Phenomenex Jupiter C4 column (column dimensions: 4.6 mm x 150 mm, pore size: 300  $\text{\AA}$ , particle size: 7  $\mu$ m) (Torrence, CA). The proteins were eluted with a shallow 10 minute TFA/acetonitrile gradient [v/v 55-62% solvent B (100% acetonitrile in 0.1 TFA) in solvent A (De-ionized water in 0.1% TFA)] at a flow rate of 500  $\mu$ l/min, and fractions were collected at one minute intervals.

The sub-fractions were then further concentrated by vacuum centrifugation, with 33% of the material loaded onto a 15% SDS-PAGE gel and visualized using a silver stain analysis kit (BioRad), and 33% was loaded onto macrophages and used in a proliferation assay (see below). All solvents were HPLC grade and were obtained from VWR Scientific Products (West Chester, PA).

**Generation of DCs *in vitro*.** DCs were cultured *in vitro* for 7 days as described previously (28). PBMCs from a healthy donor were isolated after centrifugation over Lymphocyte Separation Medium™ (Organon Teknika, Durham, NC) and washed extensively with PBS to eliminate residual platelets. The cells were plated in a T-75 flask for two hours in serum-free AIM-V (Life Technologies, Grand Island, NY) medium, after which the non-adherent cells were removed and used as the source of naïve T cells (see below). The adherent cells were treated with 1000 U/ml GM-CSF and 26 ng/ml IL-4 (Schering Plough, Kenilworth, NJ) for 7 days in serum-free AIM-V (Life Technologies) medium supplemented with 2 mM L-glutamine and penicillin/streptomycin. DCs were fed with additional media and cytokines on Day 4 of culture, and purified on Day 7 by negative selection of contaminating T, B, and NK cells. The cells were stained with anti-CD3, -CD19, and -CD56 antibodies for 45 minutes in cold PBS and washed in PBS supplemented with 5% human AB serum (Gemini Products, Calabasas, CA). Magnetic DYNAL™ beads (Lake Success, NY) coated with goat anti-mouse IgG were then added to the cells for 45 minutes, and the contaminating cells were removed by magnetic separation. Flow cytometry analysis of the remaining cells showed they were high HLA-DR<sup>+</sup> and B7-2<sup>+</sup>.

**Purification of naïve CD8<sup>+</sup> and CD4<sup>+</sup> T cells.** Non-adherent cells obtained after plastic adherence for DC isolation was used as the source of naïve CD8<sup>+</sup> or naïve CD4<sup>+</sup> T cells. To purify naïve CD8<sup>+</sup> T cells, the cells were stained with anti-CD4, -CD20, CD56, and CD45RO antibodies for 45 minutes in cold PBS and washed in PBS with 5% HAB serum. Four 100-mm petri dishes (Nunc LabTek, Naperville, IL) were precoated with 10 µg/ml goat anti-mouse IgG antibodies (Zymed) in 0.05 M Tris, pH 9.5 at room temperature for 1 hr and washed with PBS. Cells were added to each plate and incubated at 4<sup>0</sup>C for 1 hr. The non-adherent cells collected were the CD45RA<sup>+</sup>CD8<sup>+</sup> T cells. The same procedure was used for purifying naïve CD4<sup>+</sup> T cells, except that anti-CD8 antibodies are used instead. All T cell cultures were grown in RPMI medium (ICN, Costa Mesa, CA) supplemented with 10% human AB sera (Gemini Products), L-glutamine, and penicillin/streptomycin (Life Technologies).

**Priming naïve CD8<sup>+</sup> T cells to tumor peptides.** To prime naïve CD8<sup>+</sup> T cells, 2 x 10<sup>4</sup> dendritic cells were incubated for 2-4 hours first with 25% of each peptide containing RP-HPLC fraction (10 µl), and then overnight in the presence of 1000 U/ml TNF-α (Genzyme, Cambridge, MA) in 96-well U-bottom plates (Falcon, Franklin lakes, NJ). 2 x 10<sup>5</sup> autologous naïve CD8<sup>+</sup> T cells were added the next day to the DCs in the presence of 2 ng/ml IL-1β (R & D Systems, Minneapolis, MN), 20 U/ml IL-2 (DuPont, Wilmington, DE), and 26 ng/ml IL-4 (Schering Plough). The CD8<sup>+</sup> T cell cultures were fed every 3-4 days with 10 U/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. In addition, 10 ng/ml IL-7 (Pharmingen, San Diego, CA) was included in the cytokine mixture after the 2<sup>nd</sup> restimulation. The CD8<sup>+</sup> T cell cultures were restimulated every 7-10 days using autologous macrophages (obtained by plastic adherence) loaded with the individual peptide fractions, until the 3<sup>rd</sup> restimulation, where autologous

macrophages loaded with irradiated (12, 000 Rads) MS-A2 tumors (macrophages : tumor = 1 : 5) were used as stimulators (T cells : loaded macrophages = 10 : 1).

**Priming naïve CD4<sup>+</sup> T cells to tumor proteins.** To prime naïve CD4<sup>+</sup> T cells, dendritic cells were loaded with 25% of each protein fraction (~ 25 µl), and treated the same as described for CD8<sup>+</sup> T cell priming above. The CD4<sup>+</sup> T cell cultures were restimulated every 10-14 days using autologous macrophages loaded with the individual protein fractions (T cells: macrophages = 10:1), and fed every 4-5 days with 10 U/ml IL-2 and 13 ng/ml IL-4, depending on growth kinetics. 10 ng/ml IL-7 (Pharmingen) was added to the CD4<sup>+</sup> T cell cultures after the 2<sup>nd</sup> restimulation. For the 3<sup>rd</sup> restimulation, MS tumor was irradiated for 7 minutes (2.18 J/cm<sup>2</sup>) using a Spectra Mini II UV-B irradiator (Daavlin, Bryan, Ohio) and loaded onto macrophages overnight (5:1 = apoptotic tumor : macrophages) that were used as stimulators (T : loaded macrophages = 10:1) the next day.

**Cytotoxicity assays.** 1-2 x 10<sup>6</sup> target cells were labeled with 50 µCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham, Arlington Heights, IL) for 90 minutes at 37<sup>0</sup>C. The labeled cells were then washed three times and plated at 1 x 10<sup>3</sup> cells/well in a 96-well V-bottom plate (Costar, Cambridge, MA) with various numbers of effector T cells. In addition, a 50-fold excess of unlabeled K562 (5 x 10<sup>4</sup>) was added to the wells for 15 minutes prior to the addition of T cells to prevent the detection of lymphokine-activated killer (LAK) activity in the assay. The plates were centrifuged and incubated for 4 hours at 37<sup>0</sup>C. All determinations were done in triplicate. Supernatants were harvested using a Skatron harvesting press (Skatron Instruments, Sterling, VA) and counted on a Cobra II series auto gamma counting system (Packard, Meriden, CT). Maximum release was

obtained by adding 50  $\mu$ l of 1% Triton X-100 to the labeled target cells. Spontaneous release was obtained by incubating the labeled cells in the absence of T cells. Percent specific lysis was calculated from the following formula: % specific lysis = 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release). In blocking experiments, anti-MHC Class I Ab (W6/32) was added to the labeled target cells for 30 minutes prior to the addition of the effector T cells.

**Proliferation Assays.** Autologous macrophages loaded with UV-induced apoptotic MS tumor cells (5:1 = apoptotic tumor : macrophages) were seeded in round-bottom 96-well microplates (Costar, Cambridge, MA) with primed T cell cultures at a T cell : stimulator ratio of 20 : 1. For proliferation assays using tumor lysate, MS tumor cell lysate was generated as described previously, and  $1.75 \times 10^8$  cell equivalents were loaded onto  $2 \times 10^6$  autologous macrophages for 2 hours. T cells were added at a T cell : stimulator ratio of 10 : 1. For the proliferation assay using the sub-fractions of #44, 33% of the sub-fraction was loaded onto  $5 \times 10^4$  macrophages overnight and added to T cells with a T : stimulator ratio of 1:1 the next day. The wells were pulsed with [ $^3$ H]TdR (Amersham, Life Science) for the last 18 hours of the 5-day period, harvested by a Skatron semiautomatic cell harvester (Skatron Instruments), and counted on a Wallac 1205 beta plate scintillation counter (Gaithersburg, MD). The results are expressed as mean values of triplicate determinations.

**Mass spectrometry analysis.** 25% of the RP-HPLC peptide fraction was concentrated by vacuum centrifugation to near dryness and resuspended in 5  $\mu$ l of 0.1M acetic acid. One microliter of this material was loaded onto a microcapillary C18 column (150 mm x 75  $\mu$ m i.d.),



and eluted with a 20 minute linear gradient [v/v 0-80% solvent B (0.1 M acetic acid in 100% acetonitrile) in solvent A (De-ionized water in 0.1 M acetic acid)]. Flow rates for the nanospray probe (186 nl/min) was achieved by coupling the Rainin HPLC system with an Accurate microflow processor (LC Packings, San Francisco, CA) for flow splitting. The nanospray probe was operated at a voltage differential of +3.2 keV. The source temperature was maintained at 30<sup>0</sup>C. Mass spectra were obtained by scanning from 300-1500 every 3 seconds and summing individual spectra on a Fisons Quattro II triple quadrupole mass spectrometer (Micromass Inc., Loughborough, U.K.).

## RESULTS

*Identification of HPLC fractions containing immunogenic tumor peptides.* CD8<sup>+</sup> T cell cultures were primed and restimulated with HPLC fractions as described in the *Materials & Methods*. Due to the low amount of peptide, later restimulations were done using macrophages loaded with irradiated MS-A2 tumor cells. Monitoring the CD8<sup>+</sup> T cell cultures with an inverted microscope over four restimulations clearly showed that while there was T cell proliferation in all wells, several of the CD8<sup>+</sup> T cell cultures were expanding at a much higher rate, suggesting the presence of immunostimulatory peptides in the fractions used for priming in these wells. Most of the unstimulated CD8<sup>+</sup> T cell cultures reached senescence after 8 weeks in culture.

Figure 1 shows the result of one priming experiment in which after the 4<sup>th</sup> restimulation, we were able to test all the T cell cultures for their ability to recognize the original tumor, MS-A2, from which the peptides were derived. Out of the 65 CD8<sup>+</sup> T cell cultures primed on the individual peptide fractions, 12 (Fractions 15, 22, 30, 32, 37, 38, 43, 44, 50, 51, 52, 63) exhibited strong cytotoxicity against the tumor. Since the PBL donor and the tumor were mismatched at the HLA-A3 allele, we also used the Raji tumor cell line, which was matched only at the HLA-A3 allele with the PBL donor, to ensure that the cytotoxic CD8<sup>+</sup> T cell cultures were not alloreactive. None of the cultures that killed MS-A2 tumor cells recognized the Raji targets.

The 12 cytotoxic CD8<sup>+</sup> T cell cultures were also tested for their ability to recognize another epithelial adenocarcinoma, a lung tumor, 201T-A2, to look for shared tumor antigens. As shown in Figure 2, a CD8<sup>+</sup> T cell culture, primed with peptide fraction #32, recognized again the original tumor, and also the lung tumor. Since the lung tumor and the breast tumor shared

only the HLA-A2.1 allele, this suggested that the peptide being recognized was a shared antigen restricted by HLA-A2.1.

To determine the extent of reproducibility of this approach, we repeated the acid extraction, peptide fractionation, and priming procedure. We decided to pool several consecutive HPLC fractions for two reasons: 1) to compensate for small shifts in fraction number between HPLC runs, and 2) to reduce the total number of T cell cultures *in vitro*, making the approach less labor intensive. Naive CD8<sup>+</sup> T cells were primed on pooled fractions that were composed of the immunostimulatory fractions identified earlier as well as flanking fractions. The patterns of T cell stimulation and expansion induced by the pooled fractions were consistent with the patterns induced by the immunostimulatory fractions observed in the previous priming. An example is shown in Figure 3, in which we primed CD8<sup>+</sup> T cell cultures to pooled fractions 41-46 (Figure 3A) and 61-65 (Figure 3B), and generated specificity to the original tumor, MS-A2. This corresponded to immunostimulatory fraction # 44 and fraction # 63 from the previous run, respectively (Figure 1). We also showed that CD8<sup>+</sup> T cells primed on pooled fractions 41-46 were blocked by the anti-MHC Class I antibody, W6/32 (Figure 3A). Furthermore, we showed that the specific antigen was present in the epithelial tumor, and not in the perfectly HLA-matched melanoma, Mel 624 (Figure 3B).

To evaluate the content of these 12 immunostimulatory fractions, we analyzed them by electrospray ionization nanospray mass spectrometry. We obtained a panel of peptide species (Table 1) conforming to mass-to-charge ratios of 700-1300 Daltons, indicative of HLA Class I-binding peptides. These results showed that we were successful in extracting peptides from HLA Class I molecules, and that there were immunostimulatory peptides in the HPLC fractions that were capable of stimulating naïve CD8<sup>+</sup> T cells to proliferate and expand *in vitro*. We are

in the process of sequencing these peptides, and so far, we have identified a series of peptides that belong to a new tumor antigen (Kao et al., manuscript in preparation).

*Identification of HPLC fractions containing immunogenic tumor proteins.* We have devised and tested a similar strategy to identify HLA Class II-restricted tumor antigens by analyzing immunostimulatory properties of fractionated tumor proteins. CD4<sup>+</sup> T cell cultures were primed and restimulated as described in the *Materials & Methods*. By the 3<sup>rd</sup> restimulation, macrophages loaded with apoptotic MS tumor cells were used to stimulate the CD4<sup>+</sup> T cell cultures. Similar to the CD8<sup>+</sup> T cell cultures, observation of the CD4<sup>+</sup> T cell cultures with an inverted microscope over five restimulations showed that not all the CD4<sup>+</sup> T cell cultures were growing equally well, suggesting that the CD4<sup>+</sup> T cells were responding to immunostimulatory proteins present in some of these fractions, and not in others. Most of the unstimulated CD4<sup>+</sup> T cell cultures reached senescence after 10 weeks in culture.

Figure 4 shows the results of one priming experiment in which after the 2<sup>nd</sup> restimulation, we tested all the T cell cultures for their ability to recognize the original tumor, MS, from which the proteins were obtained. Autologous macrophages were loaded with apoptotic tumor and used in a 5-day proliferation assay as stimulators of the primed CD4<sup>+</sup> T cell cultures. Out of the 52 CD4<sup>+</sup> T cell cultures primed on the individual protein fractions, 14 (Fractions 5, 10, 11, 12, 13, 22, 28, 35, 37, 38, 39, 40, 46, 51) proliferated in response to macrophages loaded with apoptotic tumor. We also tested the CD4<sup>+</sup> T cell cultures for cytotoxicity against the original tumor via a CTL assay. None of the T cells tested killed the original tumor (data not shown). Some of the positive CD4<sup>+</sup> T cell cultures were also tested for their ability to recognize autologous macrophages loaded with tumor lysate. As shown in

Figure 5, CD4<sup>+</sup> T cell cultures primed with protein fractions #5-6 and #15 were able to proliferate to macrophages loaded with tumor lysate, consistent with results shown in Figure 4.

To determine the content of the immunostimulatory protein fractions, we evaluated the protein fractions using SDS-PAGE and silver stain analysis. Because of a large number of proteins in each fraction, the fractions of interest were further subfractionated by RP-HPLC. An example is shown in Figure 6 where we analyzed the immunostimulatory protein fraction # 44 from the second HPLC run that corresponded to fraction #46 from the first run (Figure 4), due to slight variations in HPLC fraction number between runs. From fraction #44 we derived 10 sub-fractions, and analyzed them for protein content (Figure 6A) and immunostimulatory capacity (Figure 6B). We detected immunostimulatory capacity in 4 of the 10 subfractions (#44.1, #44.2, #44.4, and #44.7). Silver stain analysis detected two prominent bands, one at 17 kD, and the other at 19 kD in fraction 44.4 (Figure 6A). We are in the process of sequencing these bands, as well as determining the immunostimulatory proteins in the other HPLC fractions.

## DISCUSSION

We have devised and tested a new approach to tumor antigen discovery that utilizes dendritic cells as antigen-presenting cells to prime naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells against tumor

proteins and peptides. This approach has advantages over previous approaches in that it seeks to explore anti-tumor responses that can be generated in healthy, immunocompetent individuals, to tumor antigens presented by professional APCs. This new approach eliminates the use of T cells from cancer patients and the use of tumor cells as APCs. Studies have shown that T cells in cancer patients may be defective, and tumor cells are poor APCs (35, 36). In addition, by using T cells from healthy donors, we are relying on a T cell repertoire that has not been affected by the presence of the tumor.

We have used this approach in search of new epithelial tumor antigens which have been limiting to date mainly due to the difficulty in generating tumor cell lines and consequently tumor-reactive T cell lines (8). For the identification of HLA-Class I-restricted tumor antigens, we showed that we were able to prime naive CD8<sup>+</sup> T cells to tumor peptides and generate specificity to the original tumor from which the peptides were derived. In some cases, the primed CD8<sup>+</sup> T cells also recognized another tumor of the same tissue type, suggesting the existence of shared tumor antigens. We also showed that we could reproduce the whole procedure, demonstrating that there were indeed tumor peptides present in similar fractions that conferred immunostimulatory capacity.

To determine the composition of immunostimulatory peptide fractions, we used electrospray ionization mass spectrometry to look for masses that would conform to what is expected of HLA Class I-associated peptides which have mass-to-charge ratios between 700-1300 Daltons. The advantage of using the mass spectrometer to identify HLA Class I-associated peptides have been extensively documented (37). It offers high sensitivity for detection of small amounts of peptides. We were able to identify a panel of peptides that fit the criteria of HLA Class I-binding peptides, suggesting that these immunostimulatory fractions contained peptides

that could be MHC Class I-restricted candidate tumor antigens. The identification and sequencing of these naturally processed peptides is currently ongoing. Fraction #50 (Figure 1) has already yielded a new tumor antigen (Kao et al., manuscript in preparation) not found by other approaches to date.

For the identification of HLA Class II-restricted tumor antigens, we demonstrated that we were able to prime naive CD4<sup>+</sup> T cells to fractionated tumor proteins and generate specificity against macrophages fed the original tumor, both in the apoptotic form and the tumor lysate form. Not surprisingly, SDS-PAGE and silver stain analysis of each of the positive protein fractions yielded an array of protein bands (data not shown), requiring further sub-fractionation and confirmation of immunostimulatory capacity. Sub-fractionation of one of these protein fractions yielded two predominant proteins of 17 kD and 19 kD, narrowing our search to two specific tumor antigen candidates. Investigations are currently underway in our lab to further sub-fractionate and sequence other positive protein fractions identified by *in vitro* priming of CD4<sup>+</sup> T cells.

Although we expect that this new approach will be capable of identifying numerous new tumor antigens, it is not without its disadvantages. As all the other previously used methods, it is very labor intensive. Furthermore, it is very dependent on the ability to grow and expand tumor peptide or tumor protein-specific T cells. In our experience, we have been able to generate only 3-5 x10<sup>6</sup> CD8<sup>+</sup> and CD4<sup>+</sup> T cells per HPLC fraction, which severely limited the number of cytotoxicity and proliferation assays that could be done to evaluate their specificity. This problem is becoming less critical, however, with the advent of new functional assays that use very few T cells, such as ELISPOT (38). The technical complexities of using the mass spectrometer to detect and sequence peptides from these fractions could also be a daunting task,

although we were moderately successful in identifying a panel of peptides at the 100 femtomole level.

In summary, by devising a system that relies on healthy donors, we have utilized the full immune potential of the donor's T cells and DCs as reagents to screen for new tumor antigens, and we have detected a number of candidate tumor antigens that are being further characterized. While we focused our search on epithelial tumor antigens, the strength of our approach is its universal applicability to all tumors, including those that do not readily grow *in vitro*. While we have utilized a tumor cell line as a source of peptides and proteins, they can just as easily be derived from pieces of tumor removed at the time of surgery. And lastly, in this approach the peptides and proteins that are found to be immunostimulatory are already known to be naturally processed and presented, an important factor in the design of cancer vaccines.

#### **ACKNOWLEDGMENTS**

We would like to thank Robert A. Henderson and Elizabeth M. Hiltbold for their help at the beginning of this project. We would also like to thank Joseph Ahearn for sharing his flow cytometer and UV-irradiator.



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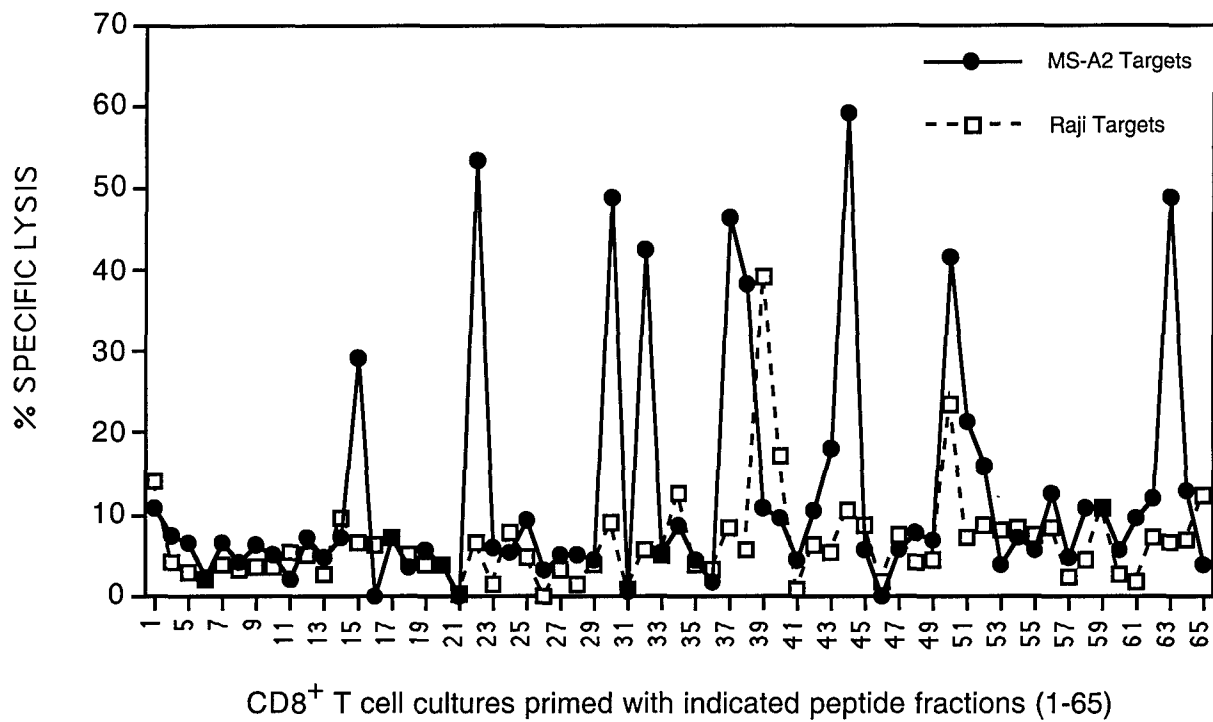


Figure 1

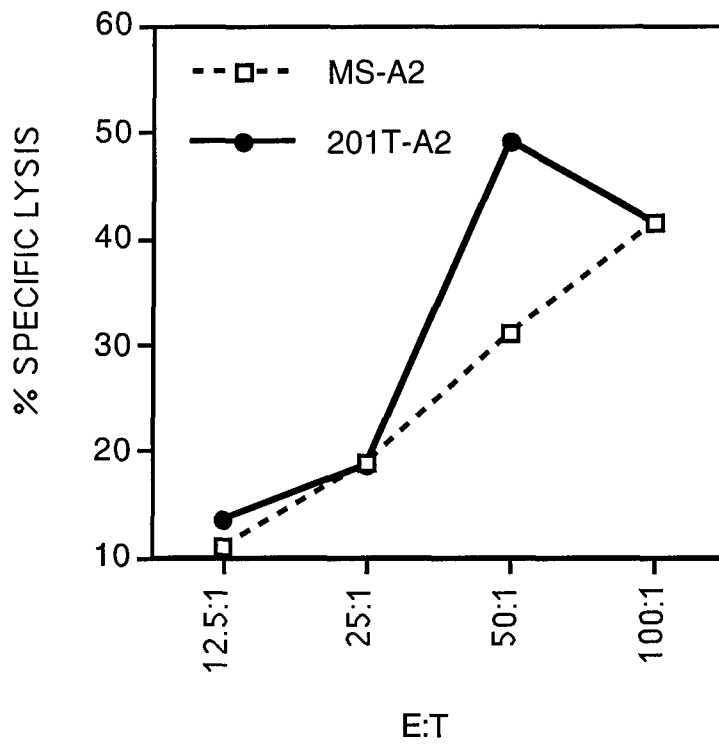


Figure 2

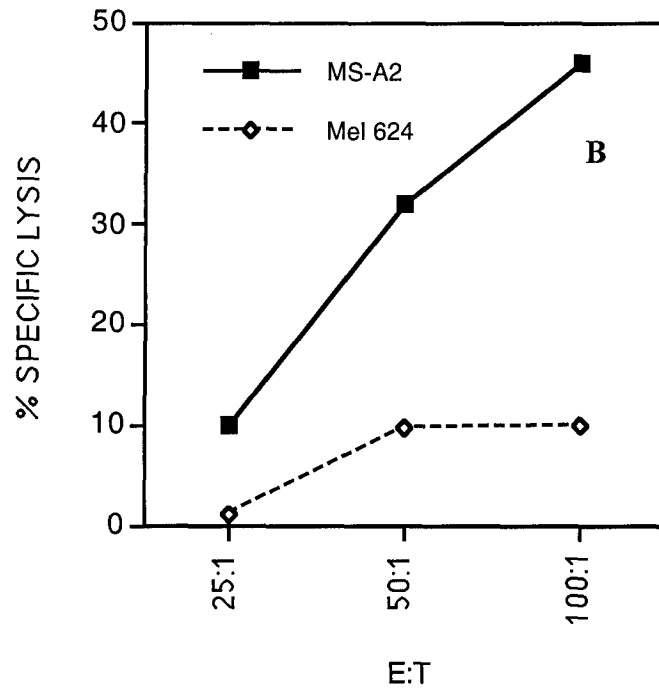
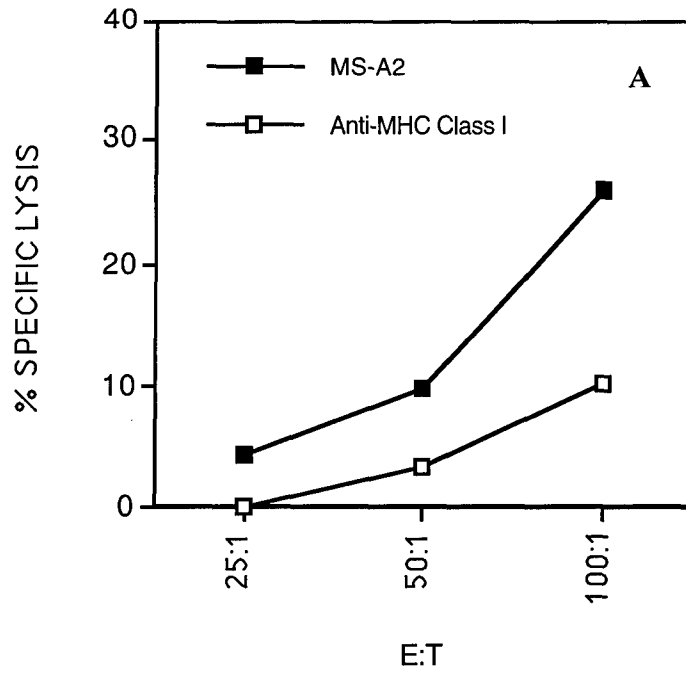


Figure 3

Table 1: Mass spectrometry analyses of immunostimulatory peptide fractions<sup>a</sup>.

Fraction #	m/z <sup>b</sup>	Fraction #	m/z
15	851.7, 879.8 <sup>c</sup>	44	615.2, 1229.5, 942.3, 921.4, 1061.5
22	921.3, 1061.4	50	728.1, 949.2, 1256.5
30	717	51	949
32	717	52	949, 885.9
37	None	63	805.6
38	921.3		
43	949.2, 816		

<sup>a</sup>HPLC peptide fractions that tested positive in the CTL assay were analyzed by nanospray microcapillary HPLC mass spectrometry.

<sup>b</sup> Mass-to-charge ratio.

<sup>c</sup> Peptide mass to charge ratios (m/z) conforming to peptides that bind HLA Class I molecules (700-1300 Daltons) were considered candidates for tumor antigens.

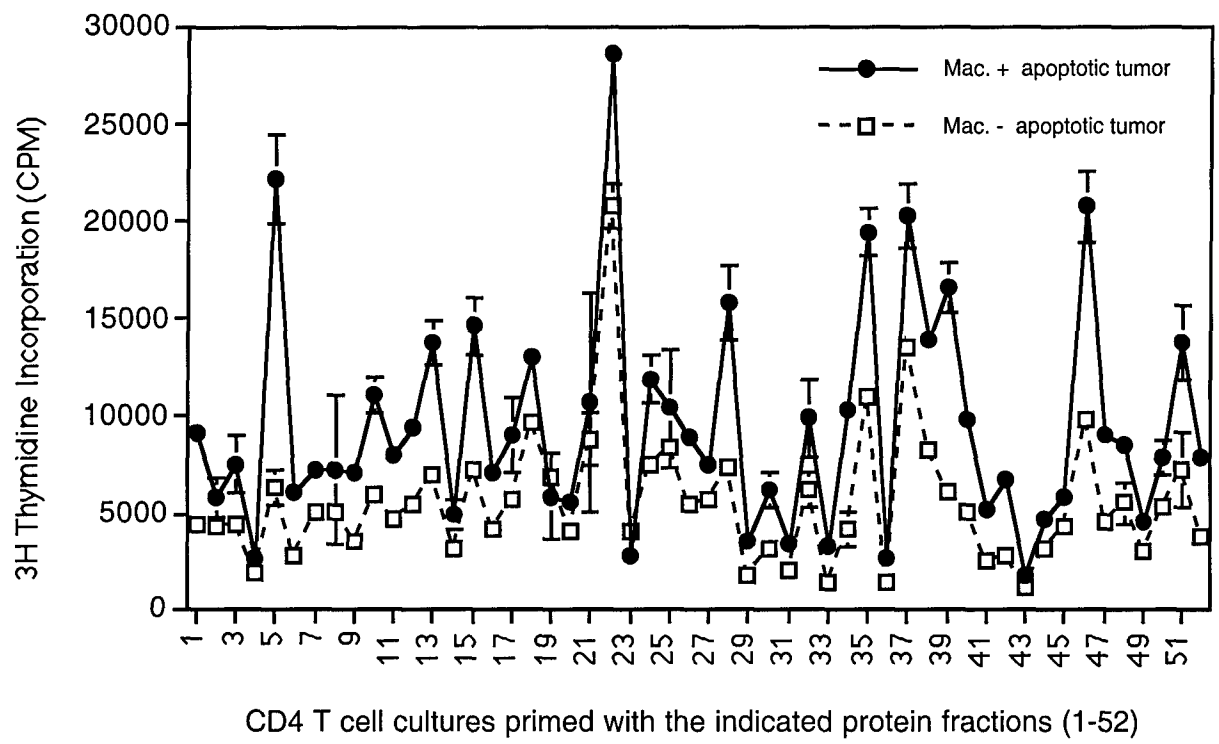


Figure 4

Figure 5

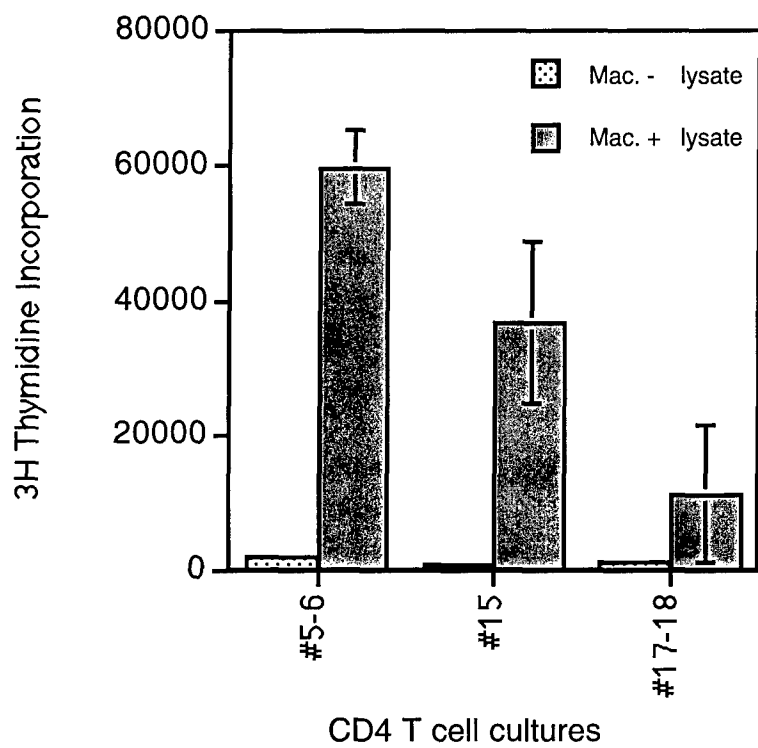
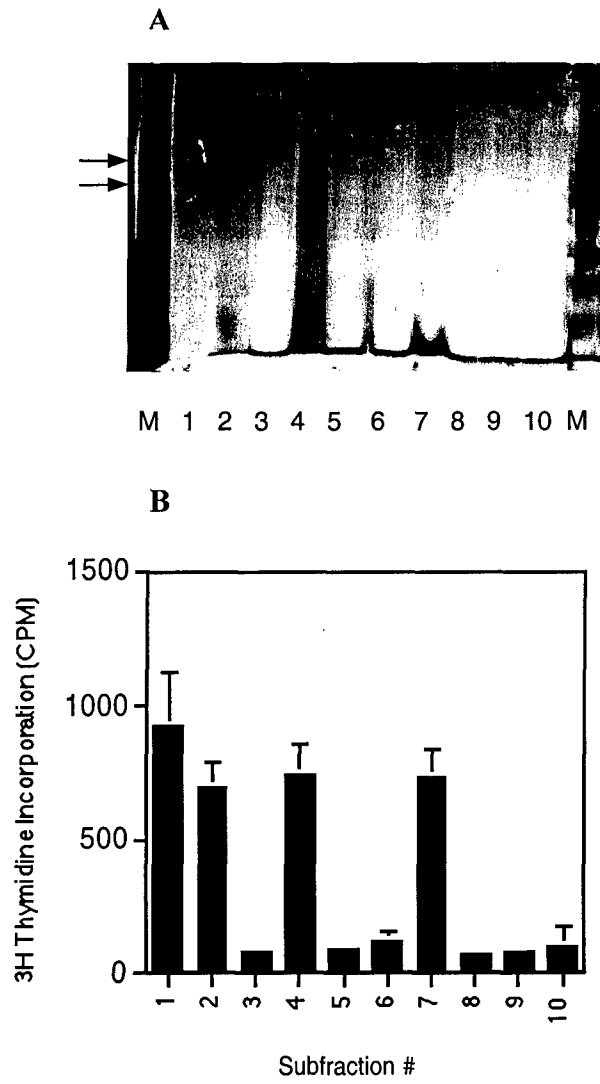


Figure 6





## FIGURE LEGENDS

Figure 1: Identification of 12 primed CD8<sup>+</sup> T cell cultures that recognized the original tumor, MS-A2, from which the tumor peptides were derived. The primed T cells were tested after the 4<sup>th</sup> restimulation. E : T ratio was at 100:1. The Raji cell line (A3, B15, C7) was a control for alloreactivity.

Figure 2: CD8<sup>+</sup> T cells primed with peptide fraction # 32 from MS-A2 tumor recognized a shared tumor antigen on a lung tumor cell line, 201T-A2. CD8<sup>+</sup> T cells generated from priming to eluted peptides from fraction # 32 were used in a CTL assay after the 5<sup>th</sup> restimulation.

Figure 3: CD8<sup>+</sup> T cells primed with pooled peptide fractions recognized the original tumor and were HLA Class I-restricted. A) CD8<sup>+</sup> T cells primed with peptide fractions #41-46 recognized the original tumor, MS-A2 and were blocked by the anti-MHC Class I antibody, W6/32. B) CD8<sup>+</sup> T cells primed with peptide fractions #61-65 recognized the original tumor (MS-A2), and not an HLA-matched tumor, Mel 624.

Figure 4: Identification of 12 primed CD4<sup>+</sup> T cell cultures that recognized the original tumor, MS, from which the proteins were obtained. The primed CD4<sup>+</sup> T cells were tested in a proliferation assay using macrophages loaded with UV-B induced apoptotic tumor (20 : 1 = T : macrophages) after the 2<sup>nd</sup> restimulation.

Figure 5: CD4<sup>+</sup> T cell cultures primed with protein fractions recognize autologous macrophages loaded with tumor lysate. 2 x 10<sup>6</sup> autologous macrophages were loaded with ~ 1.75 x 10<sup>8</sup> cell

equivalents of tumor lysate for 2 hours and used as stimulators of primed CD4<sup>+</sup> T cell cultures in a proliferation assay. T cell cultures were pooled as indicated. T cells were added at a T cell : macrophage ratio of 10 : 1.

Figure 6: SDS PAGE and functional analysis of subfractions # 44.1 - # 44.10. Fraction #44 was subfractionated as described in *Materials & Methods*, and 10 subfractions were collected. A) 33% of each HPLC sub-fraction (# 44.1-# 44.10) was resolved on a 15% SDS-PAGE gel and silver stained. B) 33% of each sub-fraction was loaded onto  $5 \times 10^4$  macrophages overnight and added to T cells primed to Fraction # 44 with a T : stimulator ratio of 1 : 1 for a 5-day proliferation assay. M, molecular weight markers.

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