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TITLE: Identification and Characterization of Ovarian Carcinoma Peptide Epitopes
Recognized by Cytotoxic T Lymphocytes

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14. ABSTRACT The purpose of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. The scope of this work involves (1) identifying the peptide antigens recognized by ovarian reactive cytotoxic T lymphocytes (CTL); and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL. Eleven ovarian cancer cell lines were characterized for the expression of class I and II MHC expression, 15 tumor antigens, and immunosuppressive cytokines. This is significant because it will facilitate these and other studies. CTL lines were established from seven patients, but none of them appear to recognize a shared antigen and none recognize any of the predicted antigens from FBP, Her-2/neu, or mesothelin. This is significant because it indicates that ovarian cancer cells may not express shared antigens that can be targeted in a vaccine. The FBP gene was cloned and the DNA demethylating agent 5-aza-2'-deoxycytidine was shown to upregulate cancer testis antigen expression and class I MHC expression on ovarian cancer cells. These results are significant because the ability to upregulate antigens on ovarian cancer cells may make them more amenable to immunotherapeutic intervention.					
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INTRODUCTION

The *subject* of this research is the identification of ovarian cancer antigens that are recognized by cytotoxic T lymphocytes (CTL). The *purpose* of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. Specifically, we are attempting to identify peptide antigens that associate with class I major histocompatibility complex (MHC) coded molecules, and which are capable of stimulating an ovarian cancer cell reactive CTL response. The *scope* of this work involves (1) identifying the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL.

Note: This award has been given a one year no-costs extension. Therefore, this report is being filed as Annual Report rather than a Final Report.

BODY

1. Identify the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification (Months 1-36)

1.1. Establish a panel of ovarian cancer cell tumor lines. (Months 1-30)

Ten previously established ovarian cancer lines including CAOV-3 (1), CAOV-4 (2), COV413 (3), ES-2 (4), OV-90 (5), OVCAR-3 (6), SK-OV-3 (7), SW626 (8), TOV-21G (5), and TOV-112D (5) and one newly established ovarian cancer line (TTB-6) were used in this study. When establishing new ovarian cancer cell lines it is important to determine that the line is of epithelial origin and not fibroblast origin, as the latter cell type can readily become established in a culture initially containing both cell types as is usually the case with patient samples. One characteristic of epithelial cells that can be used to distinguish them from other cell types is the expression of cytokeratins (9). The mAb CAM5.2 (10) which recognizes cytokeratins 7 and 8 (Ck 7/8) (11), and the mAb NCL-5D3 (12) which recognizes cytokeratin 8, and to a lesser extent cytokeratins 18 and 19 (Ck 8/18/19), have been shown to recognize ovarian cancer cells (5, 10, 12, 13). In contrast, mAb AS02 recognizes CD90 on the surface of fibroblasts (14).

To confirm that TTB-6 was epithelial in origin, each of the lines was tested in flow cytometry for reactivity with mAb NCL-5D3 (anti-Ck 8/18) as an epithelial marker and mAb AS02 (anti-CD90) as a fibroblast marker. OVCAR-3, a well-studied ovarian cancer line was positive for Ck 8/18 expression and negative for CD90 expression, while CCD39SK, a skin fibroblast line obtained from the ATCC, demonstrated the opposite pattern of expression (Fig. 1). TTB-6 was positive for Ck 8/18 expression and negative for CD90 expression (Fig. 1), thus confirming the epithelial origin of the cell line. With the exception of ES-2 and TOV-112D, the remaining ovarian cancer lines were also Ck 8/18 positive and CD90 negative (data not shown). ES-2 did not express Ck 8/18, and greater than 90% of the cells were negative for CD90 (Fig. 1). TOV-112D did not express Ck 8/18, but did express CD90 (the small, CD90 negative population was present in two of five experiments).

In contrast to the other nine ovarian cancer cell lines tested here, both ES-2 and TOV-112D were not recognized by mAb NCL-5D3 (Ck 8/18/19 specific) (Fig. 1). TOV-112D was previously shown to be recognized by mAb CAM5.2 (Ck 7/8 specific) (5), which in combination with the present results, indicates the line expresses Ck 7, but not Ck 8/18/19. ES-2 was previously shown to be recognized by mAb OV-TL 12/30 (Ck 7 specific) but not by an antibody specific for Ck 8 (13), which in combination with the results presented here, indicates that the line expresses Ck 7 and not Ck 8/18/19. These results indicate that not all ovarian cancer cell lines are uniform in their expression of particular cytokeratins, and that multiple antibodies may be needed to accurately determine if a particular cell line expresses one or more cytokeratins.

The recognition of CD90 on TOV-112D by mAb AS02 suggests that the line is a fibroblast (Fig. 1), however, several lines of evidence argue against this interpretation. First, it has previously been demonstrated that mAb CAM5.2 binds to TOV-112D (5). As indicated above, this result in combination with our own indicates that the cells express CK 7 associated with epithelial cells and not fibroblast. Second, the line expresses eight tumor antigens (see below), the expression of which is associated with tumor cells and not fibroblasts. Third, even if the small, CD90 negative population in TOV-112D represented epithelial cells and the large, CD90 positive population in TOV-112D represented fibroblasts, this could not be reconciled with the flow cytometry data. In these experiments, the entire TOV-112D population of cells is uniformly Her2/neu positive and MAGE-A1 positive, and the positive populations are clearly separated from the negative control. Thus, expression of Her2/neu and MAGE-A1 cannot be accounted for by a small, sub-population of cells, but rather reflects expression by all the cells in the population. Fourth, the small CD90 negative population was randomly observed in only two of five experiments, thus arguing against this population accounting for the expression of the tumor antigens. Taken as a whole, these results argue that TOV-112D is of epithelial origin, despite the fact that it expresses CD90. As mAb AS02 has been used in conjunction with magnetic beads to deplete cell cultures of fibroblasts (15), caution must be used to first ensure that the epithelial cell population does not also co-express CD90. It is difficult to accurately estimate how frequently ovarian cancer cell lines might express CD90 as the 95% confidence interval for the frequency based on a measurement of one positive line among eleven lines is 0.2 to 41.3%.

In order for the ovarian cancer lines to be useful in studies designed to determine the specificity of tumor reactive CTL it is necessary to know which class I MHC molecules the lines express. This question was addressed by a two-fold approach. First, the class I MHC genotype of each of the ovarian cancer lines was determined by PCR analysis (Table 1). The results of the PCR typing indicate that a minimum of two of the lines (COV413 and SW626) and perhaps an additional two lines (CAOV-3 and CAOV-4) are either homozygous for expression of the HLA-A, -B, and -C alleles, or that they have undergone the deletion of a complete haplotype on one copy of chromosome 6. As the loss of class I and class II MHC expression through chromosomal deletions is a relatively frequent event in cancer cells (16), the loss of a haplotype is a likely explanation for this observation. Homozygous expression cannot be excluded, however, as typing of normal cells from the corresponding patients would be required and such material is not available.

Second, because tumor cells frequently lose the expression of MHC molecules through a variety of mechanisms (16), we also sought to determine if class I MHC molecules could be detected on the surface of the cell lines. mAb W6/32, specific for an epitope present on all class I MHC molecules was used for the analysis (Fig. 2). Each of the lines was positive for class I MHC expression, albeit at levels that are low to moderate in comparison to the B-LCL, JY, which expresses high levels of class I MHC molecules. This information is particularly informative when choosing ovarian cancer lines for use as stimulators or targets when stimulating or assessing the specificity of ovarian cancer-specific CTL.

In the same analysis we also sought to determine if ovarian cancer cells express class II MHC molecules as determined by their ability to bind the class II MHC-specific mAb, L243 (Fig. 2). Most lines do not express class II MHC molecules, although low expression was detected on CAOV-3, CAOV-4, OVCAR-3, SW626, and a subpopulation of ES-2. This indicates that these cell lines may have the ability to stimulate class II MHC restricted responses.

mAbs specific for some of the more prevalent class I MHC molecules in the population are available and were used to assess the expression of individual class I MHC molecules on the ovarian cancer lines (Table 2). Based on the genotype of the cells, each of the HLA-A2, -A3, -

A68, -A69, and -B7 molecules were generally expressed at low to moderate levels in comparison to expression on C1R-A2, C1R-A3, C1R-B7, and JY. Expression of HLA-A2 and/or HLA-B7 on OV-90 and OVCAR-3 was particularly low, while expression of HLA-A3 and HLA-B7 on SW626 was quite high.

To be of value in defining the antigens recognized by ovarian cancer-specific CTL it is also important to have a panel of tumor cell lines that have been characterized for antigen expression. The eleven ovarian cancer cell lines studied here were tested for the expression of twelve cancer/testis antigens (CTA), Her-2/neu, and FBP. PCR was used to determine the mRNA expression levels of these fourteen tumor antigens (Table 3). Each ovarian cancer line had a unique pattern of tumor antigen expression, and expressed between six and twelve of the tested antigens (Table 4). The expression of the individual antigens among the cancer lines ranged from two to eleven positive lines for each of the antigens. The variability in expression occurred within the cancer/testis antigens, while Her-2/neu and FBP were found to be expressed in each line tested.

The availability of antibodies to some of the tested antigens allowed for the further assessment of the antigens at the protein level (Fig. 3). Her-2/neu was clearly over-expressed in SK-OV-3 (100.2-fold over background), and is present at 3.0- to 7.4-fold over background in all the remaining lines with the exception of ES-2. These results are consistent with a previous report demonstrating that SK-OV-3, TOV-21G, and TOV-112D express Her-2/neu as demonstrated by immunohistochemistry (5). Likewise, FBP was clearly over-expressed at the protein level in CAOV-3 (14.1-fold), OV90 (17.5-fold), SW626 (10.1-fold), and TTB-6 (9.2-fold), and to a lesser extent in CAOV-4 (4.9-fold), OVCAR-3 (3.2-fold), and SK-OV-3 (4.0-fold). As with Her-2/neu, a positive PCR at 30 and 40 cycles was poorly predictive of total protein. These results indicate that caution must be used when assessing antigen expression solely on the basis of the strength of the PCR signal. The lack of a strong correlation between PCR reactivity and antibody reactivity could be due to the fact that relatively high mRNA expression saturates the PCR signal even at 30 cycles of amplification, gene-specific mutations preclude the ability of the proteins to be expressed, or that additional factors regulate protein expression.

The results show that the anti-MAGE-A1 antibody bound to three lines (COV413, TOV-21G, and TOV-112D) that were PCR negative for the MAGE-A1 gene (Table 4, Fig. 3). The most likely explanation for binding to MAGE-A1 negative cell lines is cross-reactive binding on other MAGE-A proteins as has been reported for other MAGE-specific antibodies including 57B and 6C1 (17). An analysis of the results does not readily indicate another MAGE-A gene product that might be recognized. As we have used PCR to only test for the seven most prevalent of the eleven expressed MAGE-A genes, the possibility remains that additional, less prevalent MAGE-A genes are expressed in the cell lines and recognized by the antibody. It is also possible that only a small fraction of a line expresses a particular gene when that line is found to be positive by PCR, and that the antibody binding results are an accurate assessment of protein expression for those lines.

As CEA expression can be detected by immunohistochemistry in a low percentage of ovarian cancer samples (18), each of the ovarian cancer cell lines was also tested for CEA expression. As determined by flow cytometry, only OV-90 expressed CEA. Interestingly, SW626 did not express CEA. Although SW626 was originally reported to be an ovarian cancer cell line (8), a more recent report provides evidence that it may actually be of colonic origin (19). The expression of FBP and the lack of expression of CEA is, however, consistent with the line being of ovarian origin.

Both primary and cultured ovarian cancer cells have been shown to express TGF- β (20-23). TGF- β inhibits the *in vitro* generation of CTL (24-26) and blocks *in vivo* tumor

immunosurveillance (27). To the extent that cell lines in our ovarian cancer cell line panel will be used in an attempt to stimulate ovarian-specific CTL, it is important to know whether or not any of the ovarian cancer cell lines in our panel express suppressive cytokines.

The production of TGF- β 1, TGF- β 2, TGF- β 3, and IL-10 was measured in supernatants collected from the ovarian cancer lines following 48 h of growth (Fig. 4). TGF- β exists in two forms, active and latent. Active TGF- β can be measured directly by ELISA, while the latent form must first be activated, and was done here by acid treatment. Total TGF- β is thus a measure of both pre-existing, active TGF- β , and newly activated TGF- β derived from the latent form of the protein.

Only low amounts of active TGF- β 1 were measured in CAOV-3, COV413, and ES-2. Following acid activation, the total TGF- β 1 measured from these same three cell lines was elevated above that endogenously present in FBS. The remaining ovarian cancer lines either did not produce, or only produced negligible amounts of TGF- β 1. Active TGF- β 2 was measured in all of the supernatants obtained from the ovarian cancer cell lines and ranged from about 15-100 pg/ml above that found in FBS. Substantial amounts of total TGF- β 2 (>1,500 pg/ml) were found in COV413, OVCAR-3, SW626, and TOV-112D-derived supernatants, while lesser amounts (>300 pg/ml) were found in ES-2, OV-90, and SK-OV-3. Active and total TGF- β 3 was either absent or present in only small amounts (<25 pg/ml). These concentrations may be biologically significant as TGF- β 1 and - β 2 at concentrations greater than about 500 ng/ml have been shown to suppress the *in vitro* generation of CTL (24-26, 28).

IL-10 is infrequently expressed in ovarian cancer cell lines (23, 29, 30), is present in the ascites of patients with ovarian cancer (30, 31), and is associated with the suppression of T cell responses (32). Only one ovarian cancer cell line (SW626) of the eleven tested here expressed appreciable amounts of IL-10, an amount that was previously shown to be biologically significant in blocking anti-CD3-induced T cell proliferation (33).

The ovarian cancer cell lines characterized here all express class I MHC molecules and a variety of tumor antigens. Some, but not all of the lines, also express immunosuppressive cytokines. This comprehensive analysis will serve to increase the utility of these cell lines in the characterization of antigens recognized by ovarian cancer-specific CTL. This work has now been published (see Appendix #1 and Appendix #4).

1.2. Establish class I MHC-restricted, ovarian cancer cell reactive CTL lines. (Months 1-30)

The goal of this sub-aim is to generate CTL lines which recognize ovarian cancer cells. Patient derived ascites has been used as the source of tumor associated lymphocytes (TAL) and autologous tumor cells. The lymphocytes are generally stimulated four times with autologous tumor before testing in ^{51}Cr -release assays for their ability to kill autologous tumor, class I MHC-matched tumor, and peptide-pulsed, B-LCL. The following is a summary of our attempts to generate such lines:

TAL546 (HLA-A3, -B7). This CTL previously showed some reactivity to the ovarian cancer cell line ES-2. In an attempt to stimulate the population of lymphocytes with this specificity the TAL were subsequently stimulated twice with ES-2 and then tested for their ability to kill ES-2 and additional HLA-A3 $^{+}$ ovarian cancer cell lines (Table 5). The data indicate that stimulation with ES-2 tumor cells has not preferentially stimulated an ES-2-specific response, nor a response that recognizes any other HLA-A3 expressing tumor.

TAL572 (HLA-A2). This CTL initially showed reactivity to autologous tumor and possibly the allogeneic ovarian cancer tumor line SKOV3-A2. Antibody blocking experiments with antibodies directed towards class I MHC molecules were used to determine if an HLA-A2-restricted antigen was being recognized. This experiments demonstrated little to no blocking (data not shown)

suggesting that the response is not HLA-A2 restricted. Cold target inhibition experiments were also performed with both “hot” TAL572 and SKOV3-A2, but reproducible data demonstrating that a shared, HLA-A2-restricted antigen was being recognized could not be obtained (data not shown).

TAL11770 (HLA-A2). TAL11770 initially showed weak reactivity on autologous tumor, however, with repeated stimulations this reactivity was lost and no reactivity was seen on HLA-A2 matched ovarian tumors (Table 6). These results indicate TAL11770 does not have any tumor reactivity.

TAL0319 (HLA-A2). The TAL0319 CTL demonstrated non-specific killing against both HLA-A2 positive and negative ovarian cancer lines, while at the same time losing reactivity against the autologous tumor (Table 7). The line is thus not amenable to antigen identification.

TAL0406 (HLA-A1, -A3). The TAL0406 CTL demonstrated non-specific killing against both HLA-A3 positive and negative ovarian cancer lines, while only demonstrating weak killing against autologous tumor cells (Table 8). These characteristics mean that the line cannot be used for antigen identification.

TAL0511 (HLA-A2, -A3). Following repeated stimulation with autologous tumor cells, the TAL0511 CTL did not recognize either HLA-matched allogeneic ovarian cancer cell lines or the autologous tumor (Table 9). The lack of killing of any tumor target precludes the ability to use these TAL for antigen identification.

TAL567 (HLA-A2). TAL567 CTL recognize the autologous tumor from the sample used to derive the CTL line (TPF567) and they also recognize autologous tumor derived from a second ascites sample from the same patient (TPF568) (Table 10). Unfortunately, the CTL do not recognize any of the HLA-A2 matched ovarian cancer cell lines, thus indicating that the CTL either recognize an antigen unique to the autologous tumor, or at most they recognize an antigen that is not prevalently shared among different HLA-A2 expressing tumors. It has also not been possible to establish a tumor line from either TPF567 or TPF568. Taken together, these results indicate that these CTL are not good candidates for antigen identification.

Taken as a whole, the inability to derive CTL lines that recognize shared ovarian cancer antigens has been problematic from the point of view of identifying new antigens. The inability to derive such lines may, however, indicate that shared antigens are a rarity in ovarian cancer. We will continue with efforts to derive such lines which will either allow us to proceed with antigen identification, or alternatively, may indicate that shared antigens are rare in ovarian cancer. While this latter finding would be a disappointment, it would also indicate that further efforts should not be made to identify new antigens.

1.3. Identify the peptide antigens recognized by the CTL established in 1.2. Each identification project will last an average of 4-6 months, and a given antigen identification project is expected to yield from one to several new peptide antigens. (Months 6-36)

The antigens recognized by cancer-reactive CTL have been broadly categorized as either being unique or shared antigens. Unique antigens are expressed only on the tumor of a particular patient and are not found on tumors obtained from other patients. Conversely, shared antigens are found on the tumors obtained from multiple patients. From the perspective of developing a vaccine for the treatment of ovarian cancer, the identification of shared antigens is the goal of this sub-aim. As indicated in 1.2 above, we have not yet been able to identify ovarian reactive CTL that recognize a shared antigen. Efforts are continuing to identify such CTL, and if they are discovered, work under this sub-aim will begin.

2.0 Identify peptide antigens within the Her-2/neu, folate binding protein, and TAG proteins that give rise to ovarian reactive CTL (Months 1-36)

2.1. Predict the Her-2/neu, folate binding protein, and TAG-derived peptides that conform to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs. Have synthesized the HLA-A1 and -A3 peptides that conform to the rules in specific aim #2. The remaining peptides will be synthesized as needed for step 2.4 below. (Months 1-2)

This aim has been completed. Details were given in the 2nd year annual report. The subset of peptides investigated below are listed in Table 11. As indicated in the 2nd year annual report, we also predicted antigenic peptides from mesothelin and had them synthesized as well as this could be done without incurring further costs.

2.2. PCR isolate the cDNA for Her-2/neu and folate binding proteins, and clone the cDNA for Her-2/neu, folate binding protein, and TAG, into the plasmid pcDNA3.1. Plasmids will be transfected as needed. (Months 1-3)

The folate binding protein (FBP) gene was PCR amplified from the TOV-21G ovarian cancer cell line using a 5'-primer with an incorporated *KpnI* restriction enzyme site and a 3'-primer with an incorporated *EcoRI* site. Both the amplification product and the pcDNA3.1 vector were double-digested with *KpnI* and *EcoRI*. The FBP gene was then directionally ligated into the vector. Following transformation into TOP10 chemically competent *E. coli* and selection of ampicillin-resistant bacterial colonies, plasmid preparations were made from individual colonies. Forward and reverse sequencing was then done to confirm the sequence of the inserted FBP gene. Sequence comparisons were made against the published sequence for FBP (Entrez accession number X62753). One clone was obtained that was free of PCR cloning artifacts and corresponded to the published sequence. The final plasmid is capable of expressing FBP in mammalian cells under the control of the CMV promoter, and the incorporation of the neomycin resistance gene allows for the G418 selection of cells that have taken up the plasmid (Fig. 5).

The cloning, sequencing, transfection, and selection of multiple genes in multiple cell lines is a resource intensive endeavor. We have recently become aware of methodology that might allow us to bypass these procedures and yet express the antigens of interest. The procedure entails the treatment of cancer cell lines with a DNA demethylating agent, a procedure that has been shown to upregulate a variety of CTA (34, 35) and melanoma differentiation antigens (36). Towards this end, we have treated ovarian cancer cell lines with the DNA demethylating drug, 5-aza-2'-deoxycytidine (DAC). In initial experiments the cells were treated with 1 μ M DAC for 3 days, but these conditions of treatment did not reproducibly enhance expression of a variety of CTA (data not shown). Likewise, increasing the dose to 5 μ M also did not reproducibly enhance CTA expression (data not shown). Reasoning that the ovarian cancer cell lines are generally slow growing in comparison to most melanoma cell lines, we extended the incubation period with DAC to 5 and 7 days as demethylation takes place during DNA replication (Table 12). As demonstrated with the OVCAR3 and SKOV3 ovarian cancer cell lines, this treatment could induce the expression of MAGE-A1, MAGE-A3, MAGE-A12, and NY-ESO-1. That this treatment is not universal in its effects was demonstrated by the fact that MAGE-A1 is upregulated in OVCAR3 but not in SKOV3. Likewise, many of the CTA such as MAGE-A2, MAGE-A4, and MAGE-A6 are unaffected by the DAC treatment.

DAC treatment has also been shown to upregulate the expression of class I MHC molecules on melanomas (37, 38). As with CTA expression, we also show that this is true in the ovarian cancer cell lines OVCAR3 and SKOV3 (Fig. 6). mAb W6/32, which recognizes all class I MHC molecules, detected an upregulation of class I MHC protein expression at both 5 and 7 days of treatment (Fig. 6C, F). HLA-A2 (Fig. 6A, D), HLA-B7 (Fig. 6B), and HLA-A3 (Fig. 6E) were also shown to be individually upregulated. These findings are currently being extended to the remaining ovarian cancer cell lines in our panel.

While these findings are significant from a practical point of view – in many cases they allow the

use of a target cell because the treatment with DAC upregulates a tumor antigen or class I MHC molecule that is ordinarily not expressed, they are also significant from a clinical point of view. That is, the ability to treat a patient with DAC and upregulate the expression of known antigens and class I MHC molecules may make tumors amenable to immunotherapeutic vaccines that would have otherwise had no effect.

2.3. Stimulate TIL/TAL samples from HLA-A1⁺ and HLA-A3⁺ ovarian patients with ovarian peptides predicted to bind to the respective class I MHC molecules. Test the specificity of the ensuing cultures for reactivity with peptide-pulsed target cells and with ovarian cancer cells expressing the appropriate class I MHC molecule and cognate protein. Confirm peptide identity with SRM mass spectrometry. (Months 4-12)

As indicated in section 1.2 above, we have been establishing ovarian cancer reactive CTL lines by stimulating the TAL obtained from ovarian cancer patients with autologous tumor. The CTL that develop in response to autologous tumor are specific for those peptides which are naturally processed and presented by the tumor. By using these CTL lines to screen for reactivity against synthetic peptides, one eliminates the possibility of identifying a peptide that is immunogenic, but not naturally processed and presented.

TAL0406 CTL were used to test HLA-A1-restricted peptides including one from FBP, three from Her-2/neu, and two from mesothelin (Table 13). Because of the high background killing on C1R-A1 without peptide, the analysis is best restricted to the results obtained at an E:T of 10:1. There is no evidence, however, that any of the tested peptides were recognized as none of the peptide-pulsed targets demonstrates a significant level of killing above that of the unpulsed targets.

TAL0406 and TAL0511 CTL were used to test HLA-A3-restricted peptides including four from FBP, two from Her-2/neu, and two from mesothelin (Tables 14, 15). TAL0406 also had high background killing of C1R-A3 without peptide, but this issue was not seen with TAL0511. The results demonstrate that none of the peptides are reproducibly recognized by the CTL.

Efforts will continue over the course of the next year to determine if any of the peptides are antigenic. This will be done both by testing the reactivity of CTL stimulated with autologous tumor, as well as by stimulating CTL with the peptides and then looking for reactivity against peptide-pulsed cells as well as tumor.

2.4. Repeat 2.3 for peptides associated with HLA-A2, B7, and B8. The order in which this is done will be dictated by order in which patient material becomes sufficiently available to conduct the experiments. (Months 13-36)

TAL0319, TAL0511, and TAL567 CTL were used to test HLA-A2 restricted peptides including four from FBP and three from mesothelin (Tables 16, 17, 18). The TAL0319 had high background reactivity on the unpulsed C1R-A2, but the this activity was not seen with the TAL0511 and TAL567 CTL. The results demonstrate that none of the peptides are reproducibly recognized by the CTL.

Efforts will continue in this area over the course of the next year as described in 2.4 above.

2.5 Determine the ability of CTL generated in 1.2 above to recognize target cells transfected or infected with the gene of interest and identify the peptide antigen. Confirm with SRM mass spectrometry. (Months 6-36)

As indicated in 1.2 above, we have not yet obtained CTL that can be used for this specific aim. Efforts are continuing, however, to derive such CTL.

Table 1. Class I MHC Geneotype of Ovarian Cancer Lines

Tumor Line	Pathology	Molecular Class I MHC Typing		
		HLA-A	HLA-B	HLA-C
CAOV-3	Adenocarcinoma	6901	(B49) or (4704, 4901)	07
CAOV-4	Adenocarcinoma	02	(15) or (15, 46)	03
COV413	Advanced ovarian cancer	02	07	07
ES-2	Clear cell carcinoma	03, 68	14 (65), 41	07, 08
OV-90	Adenocarcinoma	02	(4902, 58) or (50, 58)	06, 07
OVCAR-3	Adenocarcinoma	02, 29	07, 5805	07
SK-OV-3	Adenocarcinoma	03, 68	18, 35	04, 05
SW626	Adenocarcinoma	03	07	07
TOV-21G	Clear cell carcinoma	11, 26	(15, 40) or (40, 95)	02, 04
TOV-112D	Endometrioid carcinoma	03	14, 41	07, 08
TTB-6	Adenocarcinoma	(02, 68) or (02)	4037, 44	02, 07

Table 2. Expression of Class I MHC Proteins on Ovarian Cancer Lines^a

mAb	Tumor Cell Lines											Controls		
	CAOV-3 (A69) ^b	CAOV-4 (A2)	COV413 (A2, B7)	ES-2 (A3, 68)	OV-90 (A2)	OVCAR-3 (A2, B7)	SK-OV-3 (A3, 68)	SW626 (A3, B7)	TOV-21G	TOV-112D (A3)	TTB-6 (A2, 68)			
Exp. 1												C1R-A2 (A2)	C1R-A3 (A3)	C1R-B7 (B7)
BB7.2 (A2, 69) ^c	89.8^d			1.1	13.2	8.6	2.3		2.0	1.0		247.8	1.1	1.0
CR11-351 (A2,68, 69)	62.6			12.5	13.6	8.7	22.8		1.0	3.4		301.7	11.4	1.0
GAP-A3 (A3)	3.0			33.8	3.0	1.5	42.3		1.0	26.0		2.0	105.7	1.0
ME1-1.2 (B7, 27)	2.7			1.9	1.7	14.8	1.9		1.0	1.8		2.4	1.1	199.8
W6/32 (All class I)	205.8			56.8	31.1	36.7	75.6		55.3	36.6		287.0	129.8	238.1
2nd Only	2.0			1.0	1.2	1.3	1.5		1.0	1.0		1.0	1.0	1.0
Exp. 2												C1R-A2 (A2)	C1R-A3 (A3)	C1R-B7 (B7)
BB7.2 (A2, 69)		190.1				25.5		9.4			47.7	937.3	3.5	3.2
CR11-351 (A2,68, 69)		226.7				38.1		64.4			92.0	978.8	33.9	2.1
GAP-A3 (A3)		7.8				4.2		572.9			4.0	4.2	470.8	3.3
ME1-1.2 (B7, 27)		27.8				30.8		452.6			4.8	7.9	2.7	565.4
W6/32 (All class I)		502.9				88.5		1063.7			208.6	1107.0	668.0	1214.7
2nd Only		7.5				4.5		8.8			3.6	2.4	2.4	2.1
Exp. 3												JY (A2, B7)		
BB7.2 (A2, 69)			105.8	1.6	20.8		2.2			1.5		259.5		
GAP-A3 (A3)			7.9	56.7	3.9		69.1			34.0		1.3		
ME1-1.2 (B7, 27)			69.2	6.8	2.0		2.0			1.4		313.5		
W6/32 (All class I)			217.9	116.3	49.2		181.0			47.2		809.3		
2nd Only			7.0	1.2	1.6		1.7			1.2		1.0		

^aFlow cytometry with antibodies directed against class I MHC molecules was performed as indicated in the Materials and Methods section.

^bThe class I MHC molecules potentially expressed by the cell lines (see Table 2 for the complete molecular typing) and for which mAbs are available to confirm protein expression.

^cThe specificity of the mAb is given in the parentheses.

^dMedian fluorescence activity. Bolded values correspond to reactions expected to be positive if the corresponding class I MHC gene in the line is expressed. Data are representative of a minimum of two independent experiments.

Table 3. Tumor Antigen Primer Pairs Used for PCR

Gene	Annealing temp (°C)	Product size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	60	598	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC
FBP	60	633	AGC CAG GCC CCG AGG ACA AGT	TGA GCA GCC ACA GCA GCA TTA GG
Her-2/neu	60	907	GCA CGG GCC CCA AGC ACT CTG ACT	ACT CGG CAT TCC TCC ACG CAC TCC
MAGE-A1	65	421	CGG CCG AAG GAA CCT GAC CCA G	GCT GGA ACC CTC ACT GGG TTG CC
MAGE-A2	68	317	AAG TAG GAC CCG AGG CAC TG	GAA GAG GAA GAA GCG GTC TG
MAGE-A3	66	725	TGG AGG ACC AGA GGC CCC C	GGA CGA TTA TCA GGA GGC CTG
MAGE-A4	68	446	GAG CAG ACA GGC CAA CCG	AAG GAC TCT GCG TCA GGC
MAGE-A6	69	727	TGG AGG ACC AGA GGC CCC C	CAG GAT GAT TAT CAG GAA GCC TGT
MAGE-A10	65	485	CAC AGA GCA GCA CTG AAG GAG	CTG GGT AAA GAC TCA CTG TCT GG
MAGE-A12	56	392	GGT GGA AGT GGT CCG CAT CG	GCC CTC CAC TGA TCT TTA GCA A
NY-ESO-1	66	458	GCG GCT TCA GGG CTG AAT GGA TG	AAG CCG TCC TCC TCC AGC GAC A
TAG-1	62	672	AGG AAG GGG CTC CCA CAG TGC	CCC AGG TTA GAA CGG TCA GCA GAA
TAG-2a	62	528	AGC GGC GGG CTG AAG GA	GAG GGT AGG GTG GTC ATT GTG TCA
TAG-2b	62	401	AGC GGC GGG CTG AAG GAC TC	CAG CAC AAC AGG AAC ATT CAG TGG
TAB-2c	62	536	AGC GGC GGG CTG AAG GA	GGG GGA TTT TAT TGC GGT GAA AGT

Table 4. Expression of Tumor Antigen Genes in Ovarian Cancer Cell Lines^a

Antigen	Tumor Cell Lines											Antigen
	CAOV-3	CAOV-4	COV413	ES-2	OV-90	OVCAR-3	SK-OV-3	SW626	TOV-21G	TOV-112D	TTB-6	Positive Cell Lines
MAGE-A1	-	-	-	+++	+++	-	+++	-	-	-	-	3
MAGE-A2	+	++	+	+	+	+	+	+	++	+	+	11
MAGE-A3	-	-	-	++++	++++	-	++	+	-	+	-	5
MAGE-A4	-	-	++	++	++	-	-	-	-	+	-	4
MAGE-A6	-	+	-	+++	+++	-	++	+	+	+	+	8
MAGE-A10	-	-	-	++	++	+++	-	-	-	-	-	3
MAGE-A12	+++	+++	+++	++++	++++	+++	+++	+++	+++	+++	+++	11
NY-ESO-1	++	-	++	++	+++	++	++	++	-	++	++	9
TAG-1	++	+	-	+	++	++++	+	++	+	-	+	9
TAG-2a	+	-	+	-	++	++++	-	++	-	-	++++	6
TAG-2b	-	-	-	-	-	++++	-	-	-	-	+++	2
TAG-2c	-	-	-	-	-	++++	-	+	-	-	++++	3
Her-2/neu	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	11
FBP	++++	++++	+++	+	++++	++++	++++	++++	++++	+++	++++	11
# of Expressed Antigens	7	6	7	11	12	10	9	10	6	8	10	

^aGene expression was determined by PCR. Data represent the average expression levels obtained using a minimum of two replicate experiments at each of 30 and 40 cycles of analysis. Data are reported as: (++++) easily visible at 30 cycles; (+++) weakly visible at 30 cycles, easily visible at 40 cycles; (++) not visible at 30 cycles, easily visible at 40 cycles; (+) not visible at 30 cycles, weakly visible at 40 cycles; and (-) not visible at 30 or 40 cycles.

Table 5. TAL546 Specificity^a

E:T	Target Cells (Class I MHC)					
	ES-2 HLA-A3	SKOV3.A2 HLA-A2, A3	TOV112D HLA-A3	SKOV3 HLA-A3	OV90 HLA-A2	TTB6 HLA-A2
60	0.4 ^b	5.6	-7.8	2.7	2.1	1.6
30	0.0	1.6	-7.8	-1.2	0.4	2.1
15	0.7	1.3	-7.8	-0.5	-1.3	2.0
7.5	-0.3	-0.5	-7.8	-1.8	-0.7	0.7

^aTAL546 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 6. TAL11770 Specificity^a

E:T	Target Cells (Class I MHC)								
	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR3 HLA-A2	SK-OV-3.A2 HLA-A2	TTB-6 HLA-A2	OAT 11770 HLA-A2	TOV-112D HLA-A3	ES-2 HLA-A3
80	9.1 ^b	11.3	12.1	6.6	11.7	9.1	10.2	44.6	12.7
40	4.7	9.9	3.4	2.6	10.6	0.7	8.2	35.5	5.1
20	5.7	6.7	2.2	2.0	6.4	0.4	9.4	9.3	4.9
10	3.3	3.5	-0.4	2.4	3.8	-0.2	5.8	6.0	0.1

^aTAL11770 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 7. TAL0319 Specificity^a

E:T	Target Cells (Class I MHC)							
	CAOV-4 HLA-A2	COV413 HLA-A2	OV-90 HLA-A2	OVCAR-3 HLA-A2	SKOV3.A2 HLA-A2	TTB6 HLA-A2	OAT0319 HLA-A2	SKOV3 HLA-A3
80	66.0 ^b	89.4	58.1	60.6	72.6	62.9	8.8	49.7
20	53.6	66.2	42.9	48.0	63.7	45.3	3.7	26.5

^aTAL0319 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 8. TAL0406 Specificity^a

E:T	Target Cells (Class I MHC)					
	ES-2 HLA-A3	SKOV3 HLA-A3	SW626 HLA-A3	TOV-112D HLA-A3	OAT0406 HLA-A3	OV-90 HLA-A2
80	87.7 ^b	42.9	75.0	88.2	19.8	51.1
20	63.0	29.3	58.4	75.6	6.4	38.7

^aTAL0406 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 9. TAL0511 Specificity^a

E:T	Target Cells (Class I MHC)												
	CAOV-4 HLA-A2	COV413 HLA-A2	ES-2 HLA-A3	OV-90 HLA-A2	OVCAR-3 HLA-A2	SK-OV-3 HLA-A3	SK-OV-3.A2 HLA-A2, A3	SW626 HLA-A3	TOV-112D HLA-A3	TTB6 HLA-A2	OAT0319 HLA-A2	OAT0511 HLA-A2, A3	TOV-21G A2 ⁺ , A3 ⁺
40	3.9 ^b	10.2	5.0	3.4	0.8	2.9	3.1	1.4	6.9	4.0	13.7	18.1	32.2
20	2.0	5.3	2.7	3.2	1.7	2.0	1.6	-0.2	4.8	3.0	6.5	13.1	22.9
10	1.0	3.5	0.8	3.2	1.1	-0.3	0.2	-2.4	3.1	2.2	1.1	7.8	11.1
5	0.0	1.1	-0.3	0.6	-0.5	-0.7	-1.4	-2.1	-1.5	1.1	-0.1	2.5	1.7

^aTAL0511 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 10. TAL567 Specificity^a

E:T	Target Cells (Class I MHC)								
	CAOV4 HLA-A2	COV413 HLA-A2	OV90 HLA-A2	OVCAR3 HLA-A2	SKOV3.A2 HLA-A2,3	TTB6 HLA-A2	TPF567 HLA-A2	TPF568 HLA-A2	SKOV3 HLA-A3
80	5.5 ^b	0.9	3.5	2.5	10.5	1.5	53.1	54.4	0.8
40	3.9	0.7	1.0	1.0	8.6	0.9	45.5	43.0	0.6
20	2.1	-0.5	1.6	1.4	5.6	0.2	33.0	29.7	-0.2
10	0.3	0.1	1.0	-0.3	1.0	1.8	20.1	17.5	-0.3

^aTAL567 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 11. Peptides Synthesized for Testing^a

Source Protein	Tested Peptides Segregated by Class I MHC Binding Molecule		
	HLA-A1	HLA-A2	HLA-A3
FBP	P NEEVARFY	L LLVWVAVV F LLSLALML L LSLALMLL S LALMLLWL	L LNVSMAK R VLNVPLSK Y LYRFNWNH A VVGEAQTR
HER-2/neu (HER)	H LDMLRHLY L LDIDETAY V SEFSRMAR		I LIKRRQK I LKETELRK
Mesothelin (Mes)	E IDESLIFY T LDTLTAFY	F LLFSLGWV S LLFLLFSL V LPLTVAEV	E LAVALAQK A LQGGPPY

^aPeptides tested in Tables 13-18. Bolded amino acids are the code used for each peptide in subsequent tables.

Table 12. Effect of DAC Treatment on the Expression of Tumor Antigen Genes in Ovarian Cancer Cell Lines^a

	OVCAR3						SKOV3					
	3 Days		5 Days		7 Days		3 Days		5 Days		7 Days	
	DAC-	DAC+	DAC-	DAC+	DAC-	DAC+	DAC-	DAC+	DAC-	DAC+	DAC-	DAC+
Cells plated (x10 ⁶)	2.0	2.0	1.0	1.0	1.0	1.0	2.0	2.0	1.0	1.0	1.0	1.0
Cells harvested (x10 ⁶)	5.7	3.6	4.9	2.3	7.2	1.8	6.6	6.2	7.1	4.7	4.8	2.9
MAGE-A1	0	2	0	4	0	4	0	2	0	0	0	0
MAGE-A2	0	0	0	0	0	0	0	0	0	0	0	0
MAGE-A3	0	2	0	3	0	3	0	0	0	0	0	2
MAGE-A4	0	0	0	0	0	0	0	0	0	0	0	0
MAGE-A6	0	0	0	0	0	0	0	0	0	0	0	0
MAGE-A10	4	4	4	4	4	4	0	0	0	0	0	1
MAGE-A12	2	2	1	3	1	3	2	2	2	3	2	3
NY-ESO-1	0	2	0	4	0	4	0	2	0	3	0	4
TAG-1	4	2	4	2	4	2	0	0	0	0	0	0
TAG-2a	2	1	2	1	2	1	0	0	0	0	0	0
TAG-2b	2	1	2	1	2	1	0	0	0	0	0	0
TAG-2c	1	1	1	1	1	1	0	0	0	0	0	0

^aIntensity of PCR bands was scored from “0” (no band observed) to “4” (very intense band).

Table 13. TAL0406 Reactivity Against HLA-A1-Restricted Peptides^a

Peptide: Source: Status: E:T	Target Cells								
	C1R-A1 + PNEE FBP	C1R-A1 + HLDM HER	C1R-A1 + LLDI HER	C1R-A1 + VSEF HER	C1R-A1 + EIDE Mes	C1R-A1 + TLDT Mes	C1R-A1 + EADP MAGE-1 Known	C1R-A1	K562
40	54.3 ^b	50.7	59.4	53.1	61.8	57.9	57.4	55.6	78.2
10	30.7	33.9	33.5	30.9	25.1	29.1	32.5	32.2	53.3

^aTAL567 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

Table 14. TAL0406 Reactivity Against HLA-A3-Restricted Peptides^a

Peptide: Source: Status: E:T	Target Cells											
	C1R-A3 + LLNV FBP	C1R-A3 + RVLN FBP	C1R-A3 + YLYR FBP	C1R-A3 + AVVG FBP	C1R-A3 + ILIK HER	C1R-A3 + ILKE HER	C1R-A3 + ELAV Mes	C1R-A3 + ALQG Mes	C1R-A3 + SLF MAGE-A1 Known	C1R-A3 + VLR HER Known	C1R-A3	K562
40	43.0 ^b	49.2	45.1	51.6	53.8	46.8	59.2	43.5	43.0	49.7	51.3	78.2
10	28.3	27.3	23.1	23.1	26.3	28.2	36.2	17.4	22.6	27.9	23.1	53.3

^aTAL0406 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.^b% Specific ⁵¹Cr-release.**Table 15.** TAL0511 Reactivity Against HLA-A3-Restricted Peptides^a

Peptide: Source: Status: E:T	Target Cells											
	C1R-A3 + LLNV FBP	C1R-A3 + RVLN FBP	C1R-A3 + YLYR FBP	C1R-A3 + AVVG FBP	C1R-A3 + ILIK HER	C1R-A3 + ILKE HER	C1R-A3 + ELAV Mes	C1R-A3 + ALQG Mes	C1R-A3 + SLF MAGE-A1 Known	C1R-A3 + VLR HER Known	C1R-A3	K562
40	14.4 ^b	15.3	16.6	18.0	16.6	15.3	17.8	16.3	12.4	14.7	13.0	12.6
20	12.9	10.0	12.2	10.2	9.0	10.5	10.8	9.4	8.4	9.5	9.4	9.2
10	6.0	6.4	8.3	7.4	6.4	6.7	8.6	6.7	6.1	6.0	5.6	6.4
5	3.6	4.8	3.8	6.5	4.5	4.3	4.6	3.4	0.9	1.4	3.1	2.7

^aTAL0511 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.^b% Specific ⁵¹Cr-release.

Table 16. TAL0319 Reactivity Against HLA-A2-Restricted Peptides^a

Peptide: Source: Status: E:T	Target Cells											
	C1R-A2 + LLL V FBP	C1R-A2 + FLL S FBP	C1R-A2 + LLS L FBP	C1R-A2 + SLA L FBP	C1R-A2 + FLL F Mes	C1R-A2 + SLL F Mes	C1R-A2 + VLPL Mes	C1R-A2 + SLG TAG Known	C1R-A2 + KIF HER Known	C1R-A2 + EIW FBP Known	C1R-A2	K562
40	78.6 ^b	77.7	82.7	73.9	78.5	77.0	83.1	82.6	84.6	86.3	89.0	66.7
10	67.7	74.2	64.7	64.7	70.9	68.8	72.4	77.7	58.5	64.5	60.8	62.0

^aTAL0319 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.^b% Specific ⁵¹Cr-release.**Table 17.** TAL0511 Reactivity Against HLA-A2-Restricted Peptides^a

Peptide: Source: Status: E:T	Target Cells											
	C1R-A2 + LLL V FBP	C1R-A2 + FLL S FBP	C1R-A2 + LLS L FBP	C1R-A2 + SLA L FBP	C1R-A2 + FLL F Mes	C1R-A2 + SLL F Mes	C1R-A2 + VLPL Mes	C1R-A2 + SLG TAG Known	C1R-A2 + KIF HER Known	C1R-A2 + EIW FBP Known	C1R-A2	K562
40	25.0 ^b	33.9	31.5	38.1	27.5	29.6	32.8	32.2	31.1	31.1	31.2	12.6
20	20.0	21.8	22.2	23.5	19.9	18.6	24.1	23.4	24.3	23.2	21.5	9.2
10	16.3	16.7	16.9	17.6	16.4	15.1	15.6	17.3	17.6	16.1	14.2	6.4
5	12.1	10.1	14.3	11.2	7.5	10.1	12.1	11.2	14.3	10.9	8.1	2.7

^aTAL0511 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.^b% Specific ⁵¹Cr-release.

Table 18. TAL567 Reactivity Against HLA-A2-Restricted Peptides ^a						
Peptide: Source: Status: E:T	Target Cells					
	C1R-A2 + FLLF Mes	C1R-A2 + SLLF Mes	C1R-A2 + VLPL Mes	C1R-A2 + KIF HER Known	C1R-A2	K562
40	3.7 ^b	4.1	6.0	6.3	4.7	6.9
20	3.6	2.5	3.3	4.3	6.5	4.5
10	1.8	-1.3	2.1	2.3	4.8	2.3
5	-0.7	-1.4	1.2	-0.3	2.5	1.2

^aTAL567 CTL were tested in a ⁵¹Cr-release assay against the indicated targets.

^b% Specific ⁵¹Cr-release.

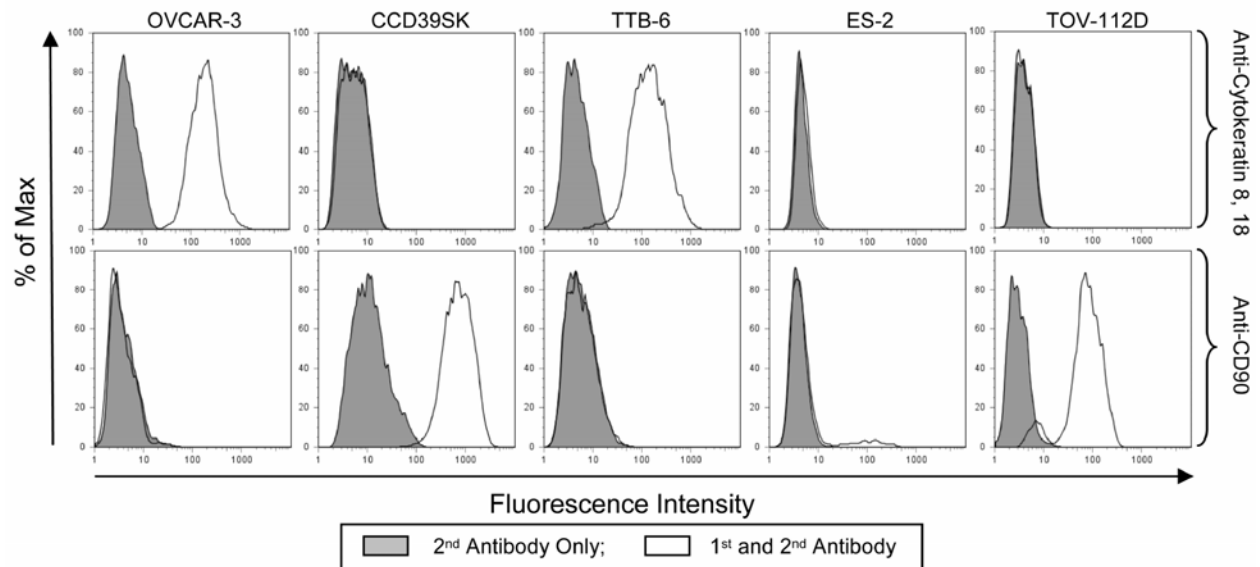


Fig. 1 Expression of epithelial and fibroblast markers by ovarian cancer lines. mAb NCL-5D3 (anti-cytokeratin 8, 18) was used to identify epithelial cells and AS02 (anti-CD90) was used to identify fibroblasts by flow cytometry. Background staining was determined in the absence of added first antibody. OVCAR-3, ES-2, and TOV-112D are previously identified ovarian cancer cell lines, CCD39SK is a skin fibroblast line, and TTB-6 is being tested to determine if it is of epithelial origin. The data are representative of a minimum of two independent experiments for each cell line.

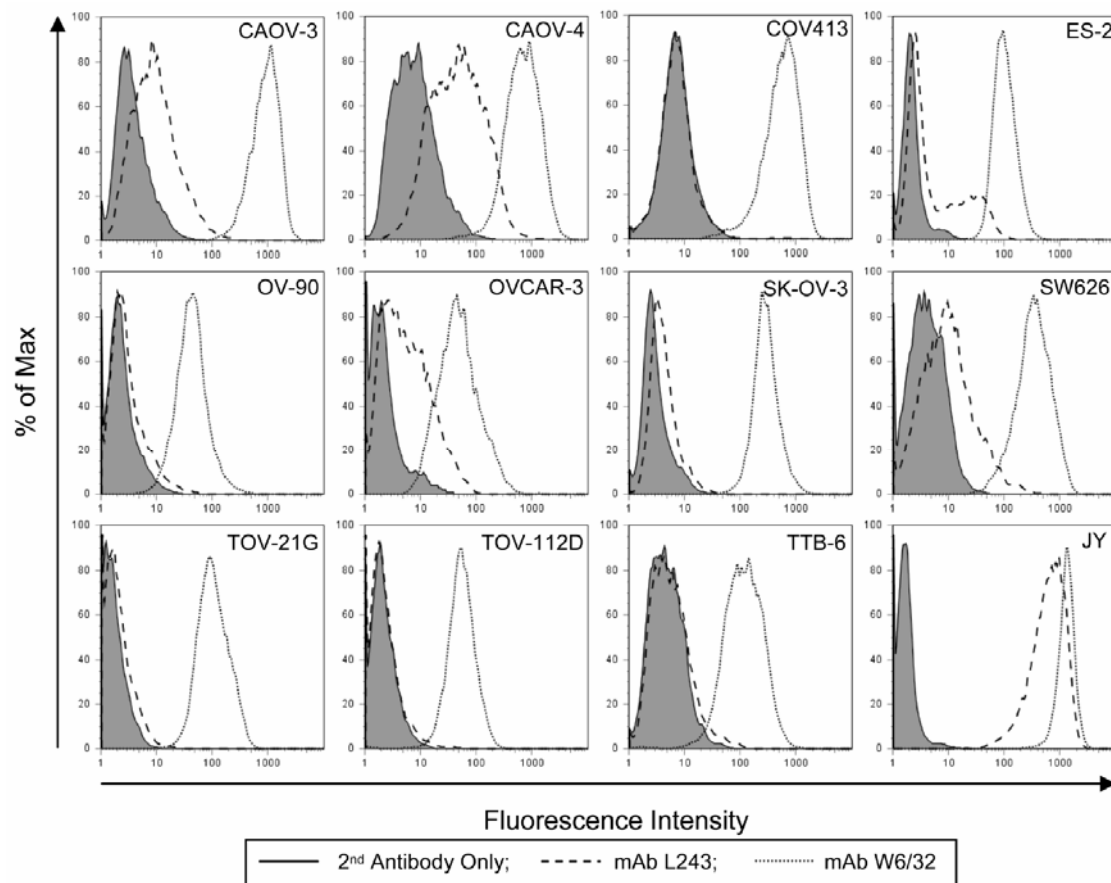


Fig. 2 Class I and class II MHC protein expression as determined by flow cytometry. Class I MHC proteins were detected using mAb W6/32 and class II MHC proteins were detected using mAb L243. Background staining was determined in the absence of added first antibody. JY is a B-LCL which was used as a positive binding control for mAbs W6/32 and L243. The data are representative of a minimum of two independent experiments for each cell line.

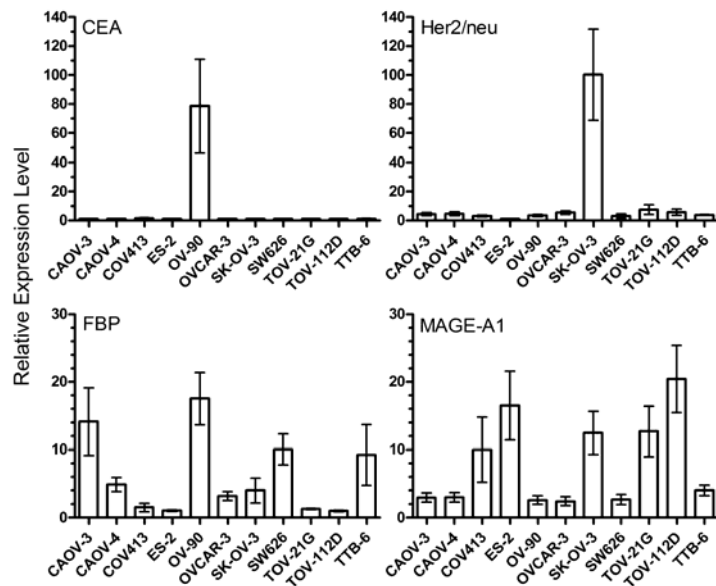


Fig. 3. Tumor antigen expression as determined by flow cytometry. mAb COL-1 (anti-CEA), mAb TA-1 (anti-Her-2/neu), and mAb Mov18/ZEL (anti-FBP) were used to stain unfixed cells, while mAb 3F257 (anti-MAGE-A1) was used to stain cells that had been fixed and permeabilized. Background staining was determined in the absence of added first antibody. Data are presented as the ratio of fluorescence activity obtained in the presence of both first and second antibody to that obtained in the presence of second antibody only, and are the average ($\pm$ SD) of three independent experiments for each cell line.

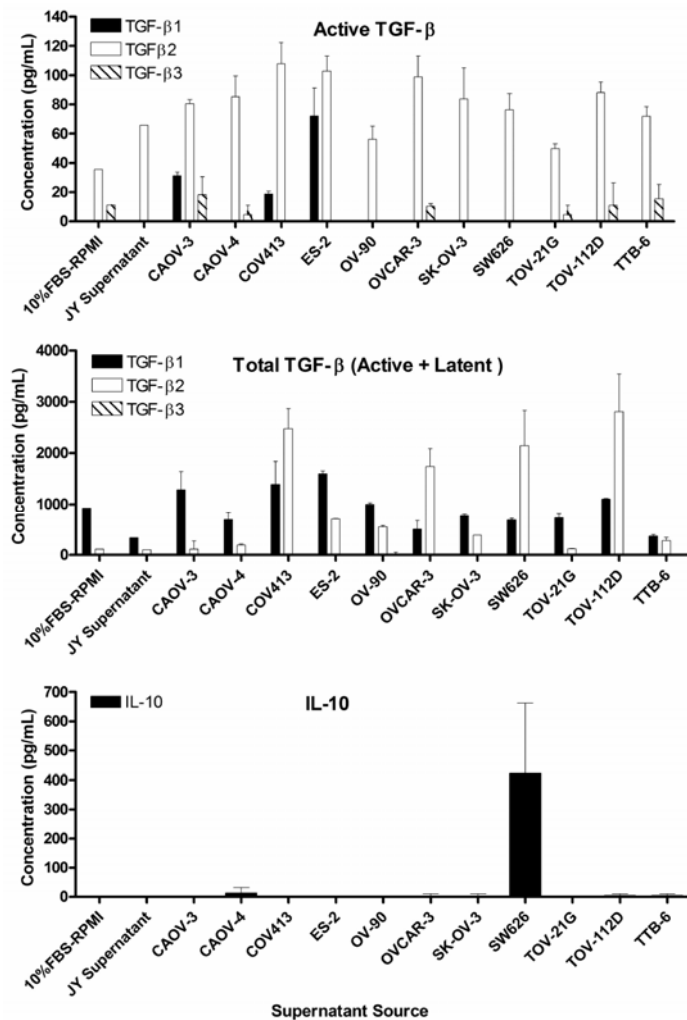


Fig. 4. TGF-β and IL-10 expression as determined by ELISA. Supernatants from ovarian cancer cell lines were obtained 48 h after 3×10^6 cells were added to T75 flasks in 8 ml of RPMI-10FBS. Total TGF-β was determined by acid-activation of the latent form of the cytokine. Cytokine concentrations were determined using antibody pairs for TGF-β1, TGF-β2, TGF-β3, and IL-10. Data are presented as the average ($\pm$ SD) of two independent experiments.

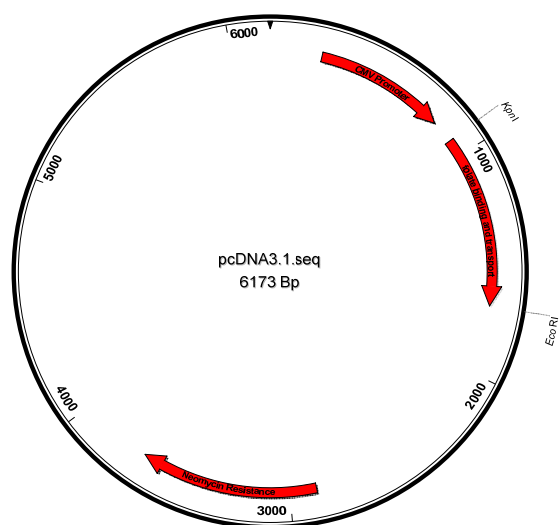


Fig. 5. FBP gene construct in pcDNA3.1. The FBP gene was cloned as a *KpnI*/*EcoRI* fragment.

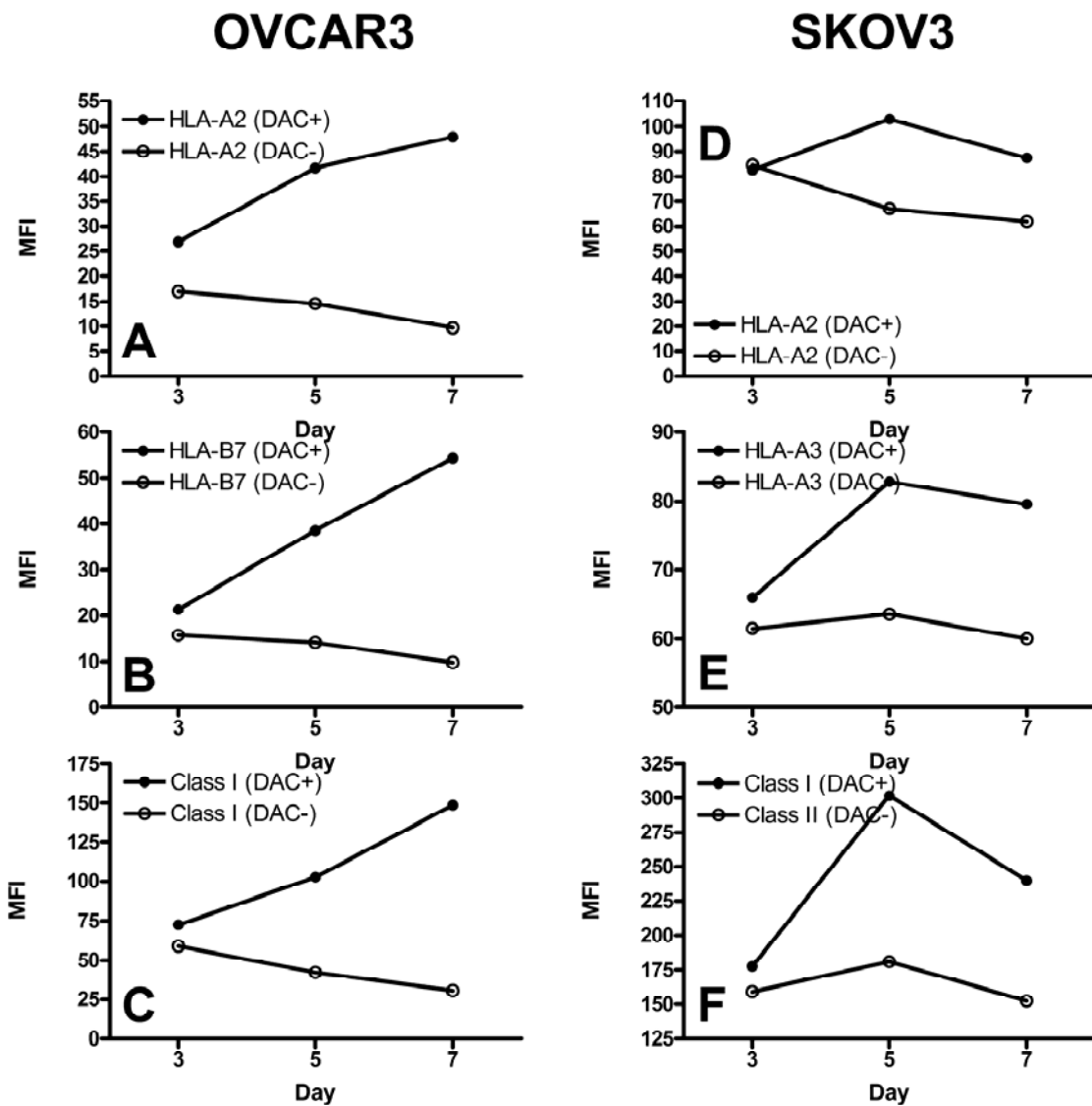


Fig 6. Effect of 1 μ M DAC treatment on the expression of class I MHC molecules on the ovarian cancer cell lines OVCAR3 and SKOV3. The cells were all plated at the same time and DAC added in a staggered fashion such that all of the cells could be harvested at the same time. Class I MHC expression was determined by flow cytometry using the following antibodies: mAb CR11-351 (HLA-A2); mAb ME1-1.2 (HLA-B7); mAb GAP-A3 (HLA-A3), and mAb W6/32 (all class I MHC molecules).

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that a newly established cell line (TTB-6) derived from an ovarian cancer patient is of epithelial origin and is an ovarian cancer line
- Demonstrated that the pattern of expression of cytokeratins differs among ovarian cancer cell lines and that no single pattern uniquely identifies ovarian cancer cells
- Demonstrated that CD90, a marker associated with fibroblasts, is sometimes expressed on ovarian cancer cells
- Established the class I MHC genotype of eleven ovarian cancer cell lines
- Established the class I MHC phenotype (overall class I MHC expression, HLA-A2, HLA-A3, and HLA-B7) and class II MHC phenotype (overall expression) of eleven ovarian cancer cell lines
- Characterized the eleven ovarian cancer cell lines for the expression of the FBP, Her-2/neu, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, TAG-2c, and CEA antigens
- Demonstrated that there is not always a linear relationship between mRNA and protein expression for Her-2/neu, FBP, and MAGE-A1 – in the latter case this may be do to cross reactivity of the anti-MAGE-A1 antibody on other MAGE family members
- Demonstrated that TGF- β 1 and TGF- β 2 are variously produced by the ovarian cancer lines, but that TGF- β 3 is only minimally produced
- Demonstrated that IL-10 was only produced by the ovarian cancer cell line SW626
- Established CTL lines from seven ovarian cancer patients, however, none of these appeared to recognize shared antigens and thus were not useful antigen identification
- Cloned the FBP gene
- Demonstrated that DAC is capable of upregulating the expression of CTA and class I MHC molecule expression in ovarian cancer cell lines
- Demonstrated that none of the generated CTL recognize any of the predicted peptide antigens from FBP, Her-2/neu, or mesothelin

REPORTABLE OUTCOMES

Publications

Carr, T.M., Adair, S.J., Fink, M.J., and Hogan, K.T. (2008) Immunological profiling of a panel of ovarian cancer cell lines. *Cancer Immunol. Immunotherapy*. 57:31-42.

Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2007) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 -restricted epitopes. *J. Immunotherapy*. In press.

Abstracts

Adair, S.J., Carr, T.M., and Hogan, K.T. (2005) Identification of cytotoxic T lymphocyte epitopes derived from the cancer/testis antigen, TAG. *iSBTc Annual Meeting. J. Immunotherapy* 28:639.

Hogan, K.T., Carr, T.M., Adair, S.J., and Fink, M.J. (2007) Immunological characterization of eleven ovarian cancer cell lines. *iSBTc Annual Meeting. J. Immunotherapy* 30:888.

Patent Application

Hogan, K.T. TAG-derived epitopes and uses thereof. US Provisional Patent Application Serial No. 60/856,510 filed on November 3, 2006.

Ovarian Cancer Cell Lines Established

OAT11770 was established.

CONCLUSION

The ability to identify ovarian cancer antigens for the development of a therapeutic vaccine is critically dependent upon the availability of well-characterized ovarian cancer cell lines, which until now, have not been available. These lines are used both to stimulate CTL and to act as targets when testing the specificity of existing CTL. The characterization of the eleven ovarian cancer cell lines in this study fills the previously existing void and will now allow us to continue with the establishment and characterization of ovarian reactive CTL.

The cloning of the FBP gene provides a molecular “reagent” that can be used for the future dissection of any FBP reactive CTL that are generated. Having the gene in hand is important as it can be transfected into appropriate cell lines to determine the specificity of the CTL.

The upregulation of CTA and class I MHC molecule expression by the DNA demethylating agent DAC has important research and clinical consequences. From a research perspective, the ability to upregulate these molecules greatly enhances the utility of the existing ovarian cancer cell lines. Cell lines which do not express a particular antigen can sometimes be made to express that antigen, and cell lines that express too little class I MHC molecules on their surface to be recognized by CTL may have that deficit erased following DAC treatment. From a clinical perspective, these results indicate that combination therapy involving a cancer vaccine and a DNA demethylating agent may have synergistic effects when the vaccine targets antigens that can be upregulated by a DNA demethylating agent.

The inability to generate CTL that recognize a shared antigen or any of the predicted antigens is problematic from a research perspective in which the desire is to identify additional antigens for inclusion in a therapeutic vaccine for the treatment of ovarian cancer. This may in fact represent a paucity of such antigens and will become more clear as we generate additional ovarian cancer reactive CTL. From a clinical standpoint this would be an important finding, if it stands up, as it would indicate that development of such a vaccine is not technically feasible because of the lack of common antigens.

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APPENDIX

1. Carr, T.M., Adair, S.J., Fink, M.J., and Hogan, K.T. (2008) Immunological profiling of a panel of ovarian cancer cell lines. *Cancer Immunol. Immunotherapy*. 57:31-42.
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Immunological profiling of a panel of human ovarian cancer cell lines

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Abstract

Purpose The efficient identification of peptide antigens recognized by ovarian cancer-specific cytotoxic T lymphocytes (CTL) requires the use of well-characterized ovarian cancer cell lines. To develop such a panel of cell lines, 11 ovarian cancer cell lines were characterized for the expression of class I and class II major histocompatibility complex (MHC)-encoded molecules, 15 tumor antigens, and immunosuppressive cytokines [transforming growth factor β (TGF- β) and IL-10].

Methods Class I MHC gene expression was determined by polymerase chain reaction (PCR), and class I and class II MHC protein expression was determined by flow cytometry. Tumor antigen expression was determined by a combination of polymerase chain reaction (PCR) and flow cytometry. Cytokine expression was determined by ELISA.

Results Each of the ovarian cancer cell lines expresses cytokeratins, although each cell line does not express the same cytokeratins. One of the lines expresses CD90, which is associated with a fibroblast lineage. Each of the cell lines expresses low to moderate amounts of class I MHC molecules, and several of them express low to moderate amounts of class II MHC molecules. Using a combination of PCR and flow cytometry, it was determined that each cell line expressed between six and thirteen of fifteen antigens tested. Little to no TGF- β 3 was produced by any of the cell lines, TGF- β 1 was produced by three of the cell lines, TGF- β 2 was produced by all of the cell lines, with four of the cell lines producing large amounts of the latent

form of the molecule, and IL-10 was produced by one of the cell lines.

Conclusions Each of the 11 ovarian cancer lines is characterized by a unique expression pattern of epithelial/fibroblast markers, MHC molecules, tumor antigens, and immunosuppressive cytokines. Knowledge of these unique expression patterns will increase the usefulness of these cell lines in identifying the antigens recognized by ovarian cancer-specific CTL.

Keywords Ovarian cancer · Class I MHC molecules · Cancer antigens · Immune suppression · Cytokine

Abbreviations

B-LCL	B-lymphoblastoid cell line
CEA	Carcinoembryonic antigen
CTL	Cytotoxic T lymphocyte
Ck	Cytokeratin
FBP	Folate binding protein
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
TGF- β	Transforming growth factor β

Introduction

Early studies indicated that T cells infiltrate solid, ovarian tumors [24, 27]. Immunohistochemical analysis subsequently showed CD8⁺ T cell infiltrates in biopsy samples [38], and flow cytometry showed that CD3⁺ T cells are the major leukocyte population detected in both tumor-infiltrating lymphocytes and tumor-associated lymphocytes [56].

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An immunohistochemical analysis of advanced-stage ovarian carcinoma specimens indicated that the presence of tumor-infiltrating CD3⁺ T lymphocytes strongly correlates with increased time of survival and increased time to recurrence of stage III and IV patients following surgical resection and chemotherapy [66]. More recently, in ovarian cancer tumors and ascites, it was shown that the presence of CD4⁺25⁺FOXP3⁺ T regulatory cells is correlated with poor survival [11]. Taken together, this evidence suggests that T cell-mediated immunity plays a significant role in the pathology of ovarian cancer, and further suggests that T cell-mediated immunotherapy might be a viable approach to treating the disease.

One form of T cell-mediated immunotherapy that is currently under development involves the immunization of ovarian cancer patients with antigenic peptides that bind to class I MHC molecules and stimulate a tumor reactive CTL response [26, 43, 47]. While a large number of peptide antigens are available for clinical trials in melanoma, comparatively few peptides are available for clinical trials in ovarian cancer [39]. Tumor antigens known to be expressed in ovarian cancer and for which antigenic peptides have been identified include Her-2/neu [14, 16, 46], folate binding protein (FBP) [29, 44, 45], the aminoenhancer of split protein [4], and the cancer/testis antigens NY-ESO-1 [40], LAGE-1 [40], MAGE-A1 [20, 65], and TAG [25].

Although a variety of approaches have been taken to identify peptide antigens that can be used to stimulate tumor reactive CTL [51, 58], all of these approaches ultimately rely on the availability of cell lines that have been characterized with respect to their expression of MHC molecules and tumor antigen proteins. As part of our effort to identify such antigens we have characterized eleven established ovarian cancer cell lines for their expression of class I and class II MHC molecules, and for their expression of the tumor antigens carcinoembryonic antigen (CEA), FBP, Her-2/neu, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, and TAG-2c. As these cell lines may also be used in attempts to stimulate a CTL response, we also determined if they express the immunosuppressive cytokines TGF- β 1, TGF- β 2, TGF- β 3, and IL-10.

Materials and methods

Cell culture medium

RPMI-1640 was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum (RPMI-10FBS).

Cell lines

The ovarian cancer lines CAOV-3 [10], CAOV-4 [28], ES-2 [32], OV-90 [48], OVCAR-3 [23], SK-OV-3 [17], SW626 [18], TOV-21G [48], and TOV-112D [48] were obtained from the ATCC (Manassas, VA). The ovarian cancer cell line TTB-6 was established at the University of Virginia, and the ovarian cancer cell line COV413 [30] was obtained from Dr. Angela Zarling (University of Virginia). The lines were maintained in RPMI-10FBS.

The B-lymphoblastoid cell line (B-LCL) JY (HLA-A2; –B7) was maintained in RPMI-10FBS. Class I MHC transfectants of Hmy2.C1R were maintained in RPMI-10FBS supplemented with 300 μ g/ml G418 or 300 μ g/ml hygromycin and included C1R-A1 (HLA-A1, hygromycin), C1R-A2 (HLA-A2, G418), and C1R-A3 (HLA-A3, G418). The skin fibroblast cell line CCD39SK was maintained in RPMI-10FBS containing 1 mM sodium pyruvate and 0.1 mM nonessential amino acids.

Primary antibodies

Monoclonal antibodies (mAb) BB7.2 (anti-HLA-A2, A69; γ_{2b}) [42], CR11-351 (anti-HLA-A2, A68, A69; γ_1) [52], GAP-A3 (anti-HLA-A3; γ_{2a}) [6], ME1-1.2 (anti-HLA-B7,B27; γ_1) [15], and W6/32 (anti-HLA-A, B, C; γ_{2a}) [41] were produced in our laboratory from the corresponding hybridoma and were used at a concentration of 10 μ g/ml.

mAbs clone 3F257 (anti-MAGE-A1; γ_{2a}) (United States Biological, Swampscott, MA), Mov18/ZEL (anti-FBP; γ_1) (Axxora, San Diego, CA), TA-1 (anti-Her-2/neu; γ_1) (Calbiochem, La Jolla, CA), and COL-1 (anti-CEA; γ_{2a}) (BD Biosciences, San Diego, CA), were used at a concentration of 10 μ g/ml. mAb AS02 (anti-CD90; γ_1) (Calbiochem) was used at a concentration of 5 μ g/ml. mAb NCL-5D3 (anti-Ck 8/18; γ_{2a}) (MP Biomedicals, Solon, OH) was used at a 1:10 dilution.

Flow cytometry

For all of the antibody binding experiments except those using mAbs 3F257 and NCL-5D3, the primary antibodies were added to $2\text{--}5 \times 10^5$ cells and incubated for 30 min on ice. The cells were washed twice, 50 μ l of a 1:50 dilution of sheep anti-mouse IgG-FITC (ICN, Irvine, CA) was then added, and the cells were incubated an additional 30 min on ice. The cells were then washed once, fixed with 2% paraformaldehyde in PBS, and analyzed on a FACSCalibur instrument (BD Biosciences, San Jose, CA).

Determination of 3F257 and NCL-5D3 antibody binding was done on cells that had been fixed and permeabilized according to the Cytotfix/Cytoperm kit instructions (BD

Biosciences). Both the primary and secondary antibodies were diluted in perm/wash buffer.

TGF- β 1, TGF- β 2, TGF- β 3, and IL-10 ELISA

Confluent cells were trypsinized, washed, and seeded at 3×10^6 cells per T75 flask in 8 ml of RPMI-10FBS. Forty-eight hours later, the media was collected, centrifuged, aliquoted, and stored at -80°C .

TGF- β 1, TGF- β 2, TGF- β 3, and IL-10 concentrations were measured using DuoSet ELISA Development kits for each cytokine (R&D Systems, Minneapolis MN). Standard curves for each cytokine ranged from 2,000 to 15.625 pg/ml. Supernatants from each cell line were assayed in duplicate according to the manufacturer's instructions. Concentrations of active and active plus latent TGF- β were measured for all three isoforms. The TGF- β isoforms were activated in accordance with the protocol provided in the TGF- β 1 kit. Following the addition of stop solution, absorbances were measured (450 nm test wavelength, 540 nm reference wavelength) on a DYNEX Technologies MRX II microplate reader (Chantilly, VA).

DNA, RNA, and cDNA preparation

DNA was obtained from 5×10^6 cells using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was prepared from $2-10 \times 10^6$ cells using the RNeasy Mini kit (Qiagen) as per the kit instructions. DNA and RNA were quantified by absorbance at 260 nm. Total RNA was converted to cDNA

by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA).

Class I MHC gene typing

Class I MHC typing was done using the Micro SSP Generic HLA Class I typing tray SSP1L (One Lambda, Inc., Canoga Park, CA).

Polymerase chain reaction

Primer sequences used are shown in Table 1 and are as previously published for GAPDH [33], MAGE-A1 [8], MAGE-A2 [13], MAGE-A3 [12], MAGE-A4 [12], MAGE-A6 [12], MAGE-A10 [12], MAGE-A12 [12], NY-ESO-1 [64], TAG-1 [25], TAG-2a [25], TAG-2b [25], and TAG-2c [25]. Primers for FBP and Her-2/neu were designed using Primer-Select software (DNASTAR, Inc., Madison, WI).

PCR was performed on 250 ng of cDNA using Platinum *Taq* High Fidelity (Invitrogen). The PCR mixes were heated to 94°C for 2 min, 30 and 40 cycles of amplification were performed (1 cycle = 30 s denaturation at 94°C , 30 s annealing at the temperature given in Table 1, 60 s extension at 72°C), and a final extension completed at 72°C for 5 min. The PCR products were visualized on ethidium bromide-stained agarose gels.

Human subjects research approval

This research was approved by the University of Virginia Human Investigation Committee in accordance with an

Table 1 Tumor antigen primer pairs used for PCR

Gene	Annealing temp ($^\circ\text{C}$)	Product size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	60	598	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC
FBP	60	633	AGC CAG GCC CCG AGG ACA AGT	TGA GCA GCC ACA GCA GCA TTA GG
Her-2/neu	60	907	GCA CGG GCC CCA AGC ACT CTG ACT	ACT CGG CAT TCC TCC ACG CAC TCC
MAGE-A1	65	421	CGG CCG AAG GAA CCT GAC CCA G	GCT GGA ACC CTC ACT GGG TTG CC
MAGE-A2	68	317	AAG TAG GAC CCG AGG CAC TG	GAA GAG GAA GAA GCG GTC TG
MAGE-A3	66	725	TGG AGG ACC AGA GGC CCC C	GGA CGA TTA TCA GGA GGC CTG
MAGE-A4	68	446	GAG CAG ACA GGC CAA CCG	AAG GAC TCT GCG TCA GGC
MAGE-A6	69	727	TGG AGG ACC AGA GGC CCC C	CAG GAT GAT TAT CAG GAA GCC TGT
MAGE-A10	65	485	CAC AGA GCA GCA CTG AAG GAG	CTG GGT AAA GAC TCA CTG TCT GG
MAGE-A12	56	392	GGT GGA AGT GGT CCG CAT CG	GCC CTC CAC TGA TCT TTA GCA A
NY-ESO-1	66	458	GCG GCT TCA GGG CTG AAT GGA TG	AAG CCG TCC TCC TCC AGC GAC A
TAG-1	62	672	AGG AAG GGG CTC CCA CAG TGC	CCC AGG TTA GAA CGG TCA GCA GAA
TAG-2a	62	528	AGC GGC GGG CTG AAG GA	GAG GGT AGG GTG GTC ATT GTG TCA
TAG-2b	62	401	AGC GGC GGG CTG AAG GAC TC	CAG CAC AAC AGG AAC ATT CAG TGG
TAB-2c	62	536	AGC GGC GGG CTG AAG GA	GGG GGA TTT TAT TGC GGT GAA AGT

Primer references and cycling conditions are given in the “[Materials and methods](#)”

assurance filed with and approved by the Department of Health and Human Services.

Results

Ovarian cancer lines

Ten previously established ovarian cancer lines including CAOV-3 [10], CAOV-4 [28], COV413 [30], ES-2 [32], OV-90 [48], OVCAR-3 [23], SK-OV-3 [17], SW626 [18], TOV-21G [48], and TOV-112D [48] and one newly established ovarian cancer line (TTB-6) were used in this study. To confirm that TTB-6 was epithelial in origin, each of the lines was tested in flow cytometry for reactivity with mAb NCL-5D3 (anti-Ck 8/18) as an epithelial marker and mAb AS02 (anti-CD90) as a fibroblast marker. OVCAR-3, a well-studied ovarian cancer line was positive for Ck 8/18 expression and negative for CD90 expression, while CCD39SK, a skin fibroblast line obtained from the ATCC, demonstrated the opposite pattern of expression (Fig. 1). TTB-6 was positive for Ck 8/18 expression and negative for CD90 expression (Fig. 1), thus confirming the epithelial origin of the cell line. With the exception of ES-2 and TOV-112D, the remaining ovarian cancer lines were also Ck 8/18 positive and CD90 negative (data not shown). ES-2 did not express Ck 8/18, and greater than 90% of the cells were negative for CD90 (Fig. 1). TOV-112D did not express Ck 8/18, but did express CD90 (the small, CD90 negative population was present in two of five experiments).

Class I and II MHC expression

The class I MHC genotype of each of the ovarian cancer lines was determined by PCR analysis (Table 2). Because tumor cells frequently lose the expression of MHC molecules through a variety of mechanisms [57], we also sought to determine if class I MHC molecules could be detected on the surface of the cell lines. mAb W6/32, specific for an epitope present on all class I MHC molecules was used for the analysis (Fig. 2). Each of the lines was positive for class I MHC expression, albeit at levels that are low to moderate in comparison to the B-LCL, JY, which expresses high levels of class I MHC molecules. In the same analysis we also sought to determine if ovarian cancer cells express class II MHC molecules as determined by their ability to bind the class II MHC-specific mAb, L243 (Fig. 2). Most lines do not express class II MHC molecules, although low expression was detected on CAOV-3, CAOV-4, OVCAR-3, SW626, and a subpopulation of ES-2.

mAbs specific for some of the more prevalent class I MHC molecules in the population are available and were used to assess the expression of individual class I MHC molecules on the ovarian cancer lines (Table 3). Based on the genotype of the cells, each of the HLA-A2, -A3, -A68, -A69, and -B7 molecules were generally expressed at low to moderate levels in comparison to expression on C1R-A2, C1R-A3, C1R-B7, and JY. Expression of HLA-A2 and/or HLA-B7 on OV-90 and OVCAR-3 was particularly low, while expression of HLA-A3 and HLA-B7 on SW626 was quite high.

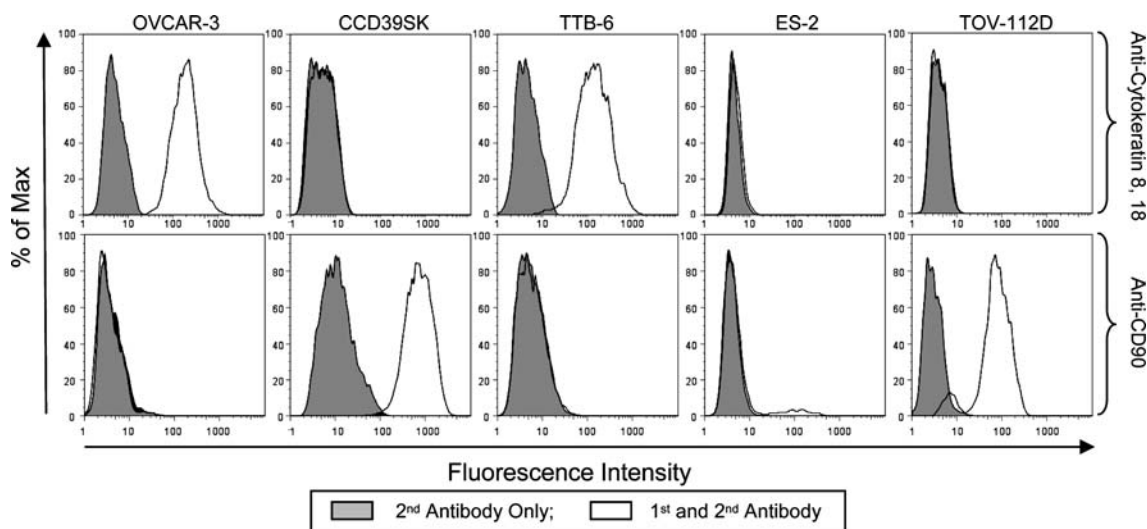
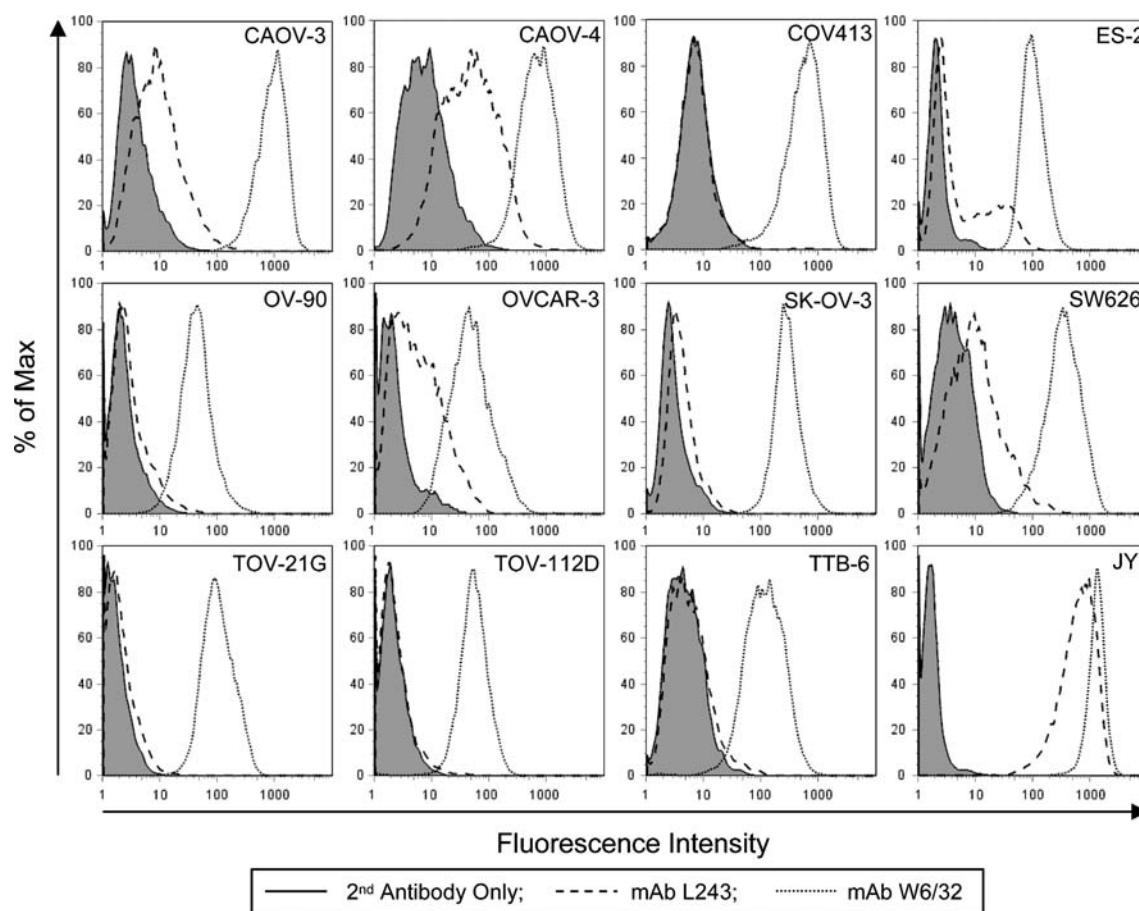


Fig. 1 Expression of epithelial and fibroblast markers by ovarian cancer lines. mAb NCL-5D3 (anti-cytokeratin 8, 18) was used to identify epithelial cells and AS02 (anti-CD90) was used to identify fibroblasts by flow cytometry. Background staining was determined in the absence of added first antibody. OVCAR-3, ES-2, and TOV-112D are

previously identified ovarian cancer cell lines, CCD39SK is a skin fibroblast line, and TTB-6 is being tested to determine if it is of epithelial origin. The data are representative of a minimum of two independent experiments for each cell line

Table 2 Class I MHC genotype of ovarian cancer lines

Tumor Line	Pathology	Molecular class I MHC typing		
		HLA-A	HLA-B	HLA-C
CAOV-3	Adenocarcinoma	6901	(49) or (4704, 4901)	07
CAOV-4	Adenocarcinoma	02	(15) or (15, 46)	03
COV413	Advanced ovarian cancer	02	07	07
ES-2	Clear cell carcinoma	03, 68	14 (65), 41	07, 08
OV-90	Adenocarcinoma	02	(4902, 58) or (50, 58)	06, 07
OVCAR-3	Adenocarcinoma	02, 29	07, 5805	07
SK-OV-3	Adenocarcinoma	03, 68	18, 35	04, 05
SW626	Adenocarcinoma	03	07	07
TOV-21G	Clear cell carcinoma	11, 26	(15, 40) or (40, 95)	02, 04
TOV-112D	Endometrioid carcinoma	03	14, 41	07, 08
TTB-6	Adenocarcinoma	(02, 68) or (02)	4037, 44	02, 07

**Fig. 2** Class I and class II MHC protein expression as determined by flow cytometry. Class I MHC proteins were detected using mAb W6/32 and class II MHC proteins were detected using mAb L243. Background staining was determined in the absence of added first antibody.

JY is a B-LCL, which was used as a positive binding control for mAbs W6/32 and L243. The data are representative of a minimum of two independent experiments for each cell line

Tumor antigen expression

PCR was used to determine the mRNA expression levels of fourteen tumor antigens (Table 4). Each ovarian cancer line

had a unique pattern of tumor antigen expression, and expressed between six and twelve of the tested antigens. The expression of the individual antigens among the cancer lines ranged from two to eleven positive lines for each of

Table 3 Expression of class I MHC proteins on ovarian cancer lines

Tumor cell lines													
	CAOV-3 (A69) ^a	CAOV-4 (A2)	COV413 (A2, B7)	ES-2 (A3, 68)	OV-90 (A2)	OVCAR-3 (A2, B7)	SK-OV-3 (A3, 68)	SW626 (A3, B7)	TOV-21G (A3)	TOV-112D (A3)	TTB-6 (A2, 68)	Controls	
												CIR-A2 (A2)	CIR-A3 (A3) CIR-B7 (B7)
Experiment 1													
BB7.2 (A2, 69) ^b	89.8 ^c			1.1	13.2	8.6	2.3		2.0	1.0		247.8	1.1 1.0
CR11-351 (A2,68, 69)	62.6			12.5	13.6	8.7	22.8		1.0	3.4		301.7	11.4 1.0
GAP-A3 (A3)	3.0			33.8	3.0	1.5	42.3		1.0	26.0		2.0	105.7 1.0
ME1-1.2 (B7, 27)	2.7			1.9	1.7	14.8	1.9		1.0	1.8		2.4	1.1 199.8
W6/32 (All class I)	205.8			56.8	31.1	36.7	75.6		55.3	36.6		287.0	129.8 238.1
2nd Only	2.0			1.0	1.2	1.3	1.5		1.0	1.0		1.0	1.0 1.0
Experiment 2													
BB7.2 (A2, 69)		190.1				25.5		9.4			47.7	937.3	3.5 3.2
CR11-351 (A2,68, 69)		226.7				38.1		64.4			92.0	978.8	33.9 2.1
GAP-A3 (A3)		7.8				4.2		572.9			4.0	4.2	470.8 3.3
ME1-1.2 (B7, 27)		27.8				30.8		452.6			4.8	7.9	2.7 565.4
W6/32 (All class I)		502.9				88.5		1063.7			208.6	1107.0	668.0 1214.7
2nd only		7.5				4.5		8.8			3.6	2.4	2.4 2.1
Tumor cell lines													
	CAOV-3 (A69) ^a	CAOV-4 (A2)	COV413 (A2, B7)	ES-2 (A3, 68)	OV-90 (A2)	OVCAR-3 (A2, B7)	SK-OV-3 (A3, 68)	SW626 (A3, B7)	TOV-21G (A3)	TOV-112D (A3)	TTB-6 (A2, 68)	Controls	
												JY (A2, B7)	
Experiment 3													
BB7.2 (A2, 69)			105.8	1.6	20.8		2.2			1.5			259.5
GAP-A3 (A3)			7.9	56.7	3.9		69.1			34.0			1.3
ME1-1.2 (B7, 27)			69.2	6.8	2.0		2.0			1.4			313.5
W6/32 (All class I)			217.9	116.3	49.2		181.0			47.2			809.3
2nd only			7.0	1.2	1.6		1.7			1.2			1.0

Flow cytometry with antibodies directed against class I MHC molecules was performed as indicated in the Materials and Methods section

^a The class I MHC molecules potentially expressed by the cell lines (see Table 2 for the complete molecular typing) and for which mAbs are available to confirm protein expression

^b The specificity of the mAb is given in the parentheses

^c Median fluorescence activity. Bolded values correspond to reactions expected to be positive if the corresponding class I MHC gene in the line is expressed. Data are representative of a minimum of two independent experiments

Table 4 Expression of tumor antigen genes in ovarian cancer cell lines

Antigen	Tumor cell lines											Antigen positive cell lines
	CAOV-3	CAOV-4	COV413	ES-2	OV-90	OVCAR-3	SK-OV-3	SW626	TOV-21G	TOV-112D	TTB-6	
MAGE-A1	–	–	–	+++	+++	–	+++	–	–	–	–	3
MAGE-A2	+	++	+	+	+	+	+	+	++	+	+	11
MAGE-A3	–	–	–	++++	++++	–	++	+	–	+	–	5
MAGE-A4	–	–	++	++	++	–	–	–	–	+	–	4
MAGE-A6	–	+	–	+++	+++	–	++	+	+	+	+	8
MAGE-A10	–	–	–	++	++	+++	–	–	–	–	–	3
MAGE-A12	+++	+++	+++	++++	++++	+++	+++	+++	+++	+++	+++	11
NY-ESO-1	++	–	++	++	+++	++	++	++	–	++	++	9
TAG-1	++	+	–	+	++	++++	+	++	+	–	+	9
TAG-2a	+	–	+	–	++	++++	–	++	–	–	++++	6
TAG-2b	–	–	–	–	–	++++	–	–	–	–	+++	2
TAG-2c	–	–	–	–	–	++++	–	+	–	–	++++	3
Her-2/neu	++++	++++	++++	+	++++	++++	++++	++++	++++	++++	++++	11
FBP	++++	++++	+++	+	++++	++++	++++	++++	++++	+++	++++	11
# of Expressed Antigens	7	6	7	11	12	10	9	10	6	8	10	

Gene expression was determined by PCR as indicated in the “Materials and methods”. Data represent the average expression levels obtained using a minimum of two replicate experiments at each of 30 and 40 cycles of analysis. Data are reported as: (+++++) easily visible at 30 cycles; (++++ weakly visible at 30 cycles, easily visible at 40 cycles; (+++) not visible at 30 cycles, easily visible at 40 cycles; (++) not visible at 30 cycles, weakly visible at 40 cycles; and (–) not visible at 30 or 40 cycles

the antigens. The variability in expression occurred within the cancer/testis antigens, while Her-2/neu and FBP were found to be expressed in each line tested.

Antibodies recognizing CEA, Her-2/neu, FBP, and MAGE-A1 are available, and were used with flow cytometry to assess the expression of the respective molecules at the protein level (Fig. 3). CEA was expressed in OV-90 but not in any other ovarian cancer cell line. Her-2/neu was expressed at high levels in SK-OV-3, but no other ovarian cancer cell lines. FBP was expressed at elevated levels in CAOV-3, OV-90, SW626, and TTB-6, while MAGE-A1 was expressed at elevated levels in COV413, ES-2, SK-OV-3, TOV-21G, and TOV-112D.

TGF- β 1, TGF- β 2, TGF- β 3, and IL-10 expression

The production of TGF- β 1, TGF- β 2, TGF- β 3, and IL-10 was measured in supernatants collected from the ovarian cancer lines following 48 h of growth (Fig. 4). TGF- β exists in two forms, active and latent. Active TGF- β can be measured directly by ELISA, while the latent form must first be activated, and was done here by acid treatment. Total TGF- β is thus a measure of both pre-existing, active TGF- β , and newly activated TGF- β derived from the latent form of the protein.

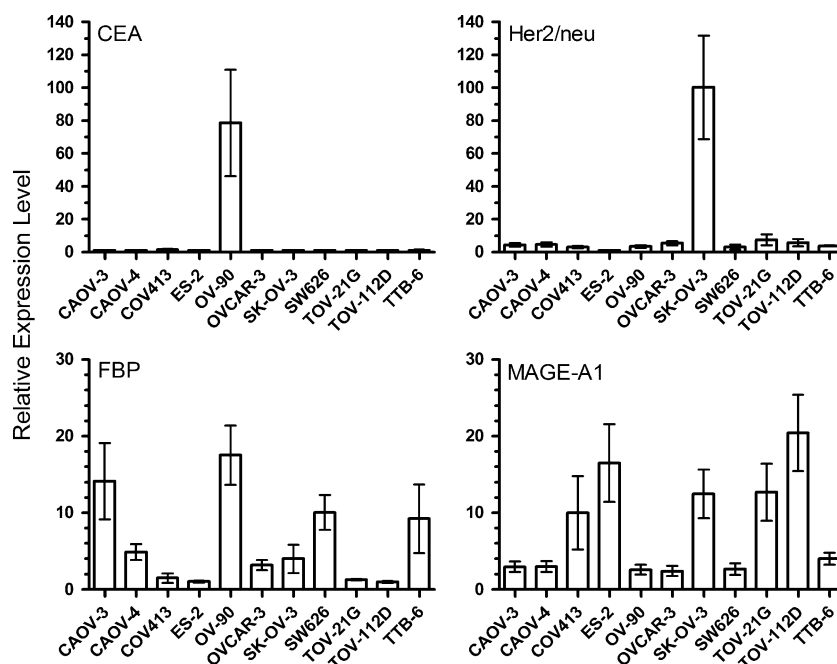
Only low amounts of active TGF- β 1 were measured in CAOV-3, COV413, and ES-2. Following acid activation,

the total TGF- β 1 measured from these same three cell lines was elevated above that endogenously present in FBS. The remaining ovarian cancer lines either did not produce, or only produced negligible amounts of TGF- β 1. Active TGF- β 2 was measured in all of the supernatants obtained from the ovarian cancer cell lines and ranged from about 15 to 100 pg/ml above that found in FBS. Substantial amounts of total TGF- β 2 (>1,500 pg/ml) were found in COV413, OVCAR-3, SW626, and TOV-112D-derived supernatants, while lesser amounts (>300 pg/ml) were found in ES-2, OV-90, and SK-OV-3. Active and total TGF- β 3 was either absent or present in only small amounts (<25 pg/ml). Only SW626 produced significant amounts of IL-10.

Discussion

When establishing new ovarian cancer cell lines it is important to determine that the line is of epithelial origin and not fibroblast origin, as the latter cell type can readily become established in a culture initially containing both cell types as is usually the case with patient samples. One characteristic of epithelial cells that can be used to distinguish them from other cell types is the expression of cytokeratins [35]. The mAb CAM5.2 [34], which recognizes cytokeratins 7 and 8 (Ck 7/8) [59] and the mAb NCL-5D3 [3], which recognizes cytokeratin 8, and to a lesser extent cytokeratins 18

Fig. 3 Tumor antigen expression as determined by flow cytometry. mAb COL-1 (anti-CEA), mAb TA-1 (anti-Her-2/neu), and mAb Mov18/ZEL (anti-FBP) were used to stain unfixed cells, while mAb 3F257 (anti-MAGE-A1) was used to stain cells that had been fixed and permeabilized. Background staining was determined in the absence of added first antibody. Data are presented as the ratio of fluorescence activity obtained in the presence of both first and second antibody to that obtained in the presence of second antibody only, and are the average ($\pm$ SD) of three independent experiments for each cell line



and 19 (Ck 8/18/19), have been shown to recognize ovarian cancer cells [3, 34, 48, 60]. In contrast, mAb AS02 recognizes CD90 on the surface of fibroblasts [53]. The newly described TTB-6 ovarian cancer cell line described here is recognized by mAb NCL-5D3 and not by mAb AS02 (Fig. 1), thus confirming that it is of epithelial origin.

In contrast to the other nine ovarian cancer cell lines tested here, both ES-2 and TOV-112D were not recognized by mAb NCL-5D3 (Ck 8/18/19 specific) (Fig. 1). TOV-112D was previously shown to be recognized by mAb CAM5.2 (Ck 7/8 specific) [48], which in combination with the present results, indicates the line expresses Ck 7, but not Ck 8/18/19. ES-2 was previously shown to be recognized by mAb OV-TL 12/30 (Ck 7 specific) but not by an antibody specific for Ck 8 [60], which in combination with the results presented here, indicates that the line expresses Ck 7 and not Ck 8/18/19. These results indicate that not all ovarian cancer cell lines are uniform in their expression of particular cytokeratins, and that multiple antibodies may be needed to accurately determine if a particular cell line expresses one or more cytokeratins.

The recognition of CD90 on TOV-112D by mAb AS02 suggests that the line is a fibroblast (Fig. 1), however, several lines of evidence argue against this interpretation. First, it has previously been demonstrated that mAb CAM5.2 binds to TOV-112D [48]. As indicated above, this result in combination with our own indicates that the cells express CK 7 associated with epithelial cells and not fibroblast. Second, the line expresses eight tumor antigens (Table 4), the expression of which is associated with tumor cells and not fibroblasts. Third, even if the small, CD90 negative population in TOV-112D represented epithelial cells and

the large, CD90 positive population in TOV-112D represented fibroblasts, this could not be reconciled with the flow cytometry data. In these experiments, the entire TOV-112D population of cells is uniformly Her2/neu positive and MAGE-A1 positive, and the positive populations are clearly separated from the negative control. Thus, expression of Her2/neu and MAGE-A1 cannot be accounted for by a small, sub-population of cells, but rather reflects expression by all the cells in the population. Fourth, the small CD90 negative population was randomly observed in only two of five experiments, thus arguing against this population accounting for the expression of the tumor antigens. Taken as a whole, these results argue that TOV-112D is of epithelial origin, despite the fact that it expresses CD90. As mAb AS02 has been used in conjunction with magnetic beads to deplete cell cultures of fibroblasts [54], caution must be used to first ensure that the epithelial cell population does not also co-express CD90. It is difficult to accurately estimate how frequently ovarian cancer cell lines might express CD90 as the 95% confidence interval for the frequency based on a measurement of one positive line among eleven lines is 0.2–41.3%.

In order for the ovarian cancer lines to be useful in studies designed to determine the specificity of tumor reactive CTL it is necessary to know which class I MHC molecules the lines express. This question was addressed by a two-fold approach: first, by using molecular PCR typing to determine the class I MHC genotype of the cells (Table 2); and second, by assessing the surface expression of select class I MHC molecules for which mAbs are available (Table 3). The results of the PCR typing indicate that a minimum of two of the lines (COV413 and SW626) and

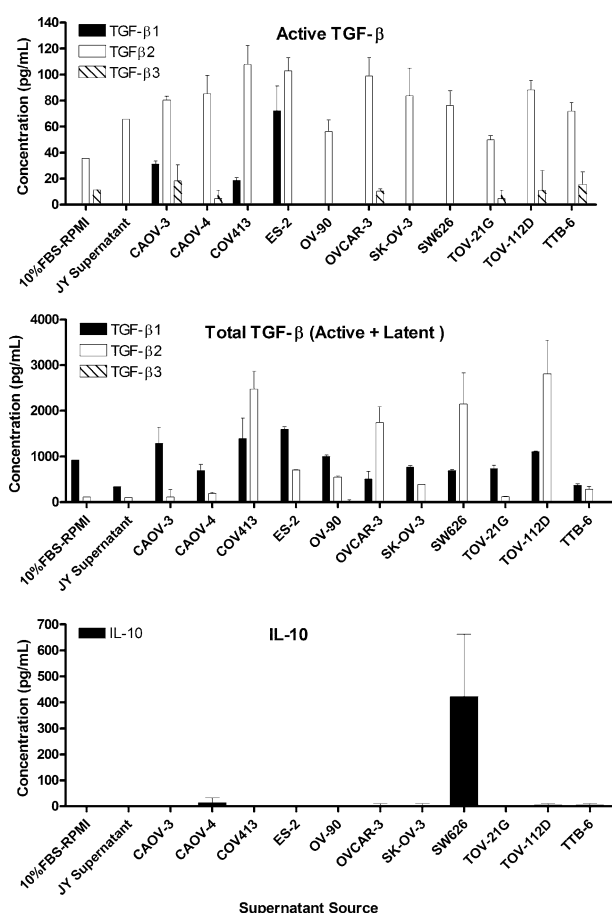


Fig. 4 TGF- β and IL-10 expression as determined by ELISA. Supernatants from ovarian cancer cell lines were obtained 48 h after 3×10^6 cells were added to T75 flasks in 8 ml of RPMI-10FBS. Total TGF- β was determined by acid-activation of the latent form of the cytokine. Cytokine concentrations were determined using antibody pairs for TGF- β 1, TGF- β 2, TGF- β 3, and IL-10. Data are presented as the average ($\pm$ SD) of two independent experiments

perhaps an additional two lines (CAOV-3 and CAOV-4) are either homozygous for expression of the HLA-A, -B, and -C alleles, or that they have undergone the deletion of a complete haplotype on one copy of chromosome 6. As the loss of class I and class II MHC expression through chromosomal deletions is a relatively frequent event in cancer cells [57], the loss of a haplotype is a likely explanation for this observation. Homozygous expression cannot be excluded, however, as typing of normal cells from the corresponding patients would be required and such material is not available.

The use of mAb W6/32, specific for an epitope present on all class I MHC molecules, confirmed that each of the ovarian cancer cell lines in comparison to B-LCL lines, expresses low to moderate levels of class I MHC molecules (Fig. 2; Table 3). The use of mAbs specific for particular class I MHC molecules allowed us to further confirm the expression of individual class I MHC molecules, again at

levels that are low to moderate in comparison to levels found on B-LCL. This information is particularly informative when choosing ovarian cancer lines for use as stimulators or targets when stimulating or assessing the specificity of ovarian cancer-specific CTL. As part of this same analysis, several ovarian cancer cell lines were also shown to express class II MHC molecules, thus indicating that they may have the ability to stimulate class II MHC restricted responses.

To be of value in defining the antigens recognized by ovarian cancer-specific CTL it is also important to have a panel of tumor cell lines that have been characterized for antigen expression. The eleven ovarian cancer cell lines studied here were tested for the expression of twelve cancer/testis antigens, Her-2/neu, and FBP (Table 4; Fig. 3). As determined by PCR, each of the tested antigens was expressed in between three and all eleven of eleven lines tested. When antigen expression is assessed on individual ovarian cancer cell lines, it is seen that each line expresses between six and twelve of the fourteen studied antigens.

The availability of antibodies to some of the tested antigens allowed for the further assessment of the antigens at the protein level (Fig. 3). Her-2/neu was clearly over-expressed in SK-OV-3 (100.2-fold over background), and is present at 3.0- to 7.4-fold over background in all the remaining lines with the exception of ES-2. These results are consistent with a previous report demonstrating that SK-OV-3, TOV-21G, and TOV-112D express Her-2/neu as demonstrated by immunohistochemistry [48]. Likewise, FBP was clearly over-expressed at the protein level in CAOV-3 (14.1-fold), OV90 (17.5-fold), SW626 (10.1-fold), and TTB-6 (9.2-fold), and to a lesser extent in CAOV-4 (4.9-fold), OVCAR-3 (3.2-fold), and SK-OV-3 (4.0-fold). As with Her-2/neu, a positive PCR at 30 and 40 cycles was poorly predictive of total protein. These results indicate that caution must be used when assessing antigen expression solely on the basis of the strength of the PCR signal. The lack of a strong correlation between PCR reactivity and antibody reactivity could be due to the fact that relatively high mRNA expression saturates the PCR signal even at 30 cycles of amplification, gene-specific mutations preclude the ability of the proteins to be expressed, or that additional factors regulate protein expression.

The results show that the anti-MAGE-A1 antibody bound to three lines (COV413, TOV-21G, and TOV-112D) that were PCR negative for the MAGE-A1 gene (Table 4; Fig. 3). The most likely explanation for binding to MAGE-A1 negative cell lines is cross-reactive binding on other MAGE-A proteins as has been reported for other MAGE-specific antibodies including 57B and 6C1 [50]. An analysis of the results does not readily indicate another MAGE-A gene product that might be recognized. As we have used PCR to only test for the seven most prevalent of the eleven

expressed MAGE-A genes, the possibility remains that additional, less prevalent MAGE-A genes are expressed in the cell lines and recognized by the antibody. It is also possible that only a small fraction of a line expresses a particular gene when that line is found to be positive by PCR, and that the antibody binding results are an accurate assessment of protein expression for those lines.

As CEA expression can be detected by immunohistochemistry in a low percentage of ovarian cancer samples [31], each of the ovarian cancer cell lines was also tested for CEA expression. As determined by flow cytometry, only OV-90 expressed CEA. Interestingly, SW626 did not express CEA. Although SW626 was originally reported to be an ovarian cancer cell line [18], a more recent report provides evidence that it may actually be of colonic origin [19]. The expression of FBP and the lack of expression of CEA is, however, consistent with the line being of ovarian origin.

Both primary and cultured ovarian cancer cells have been shown to express TGF- β [5, 21, 37, 62]. TGF- β inhibits the in vitro generation of CTL [36, 49, 63] and blocks in vivo tumor immunosurveillance [61]. To the extent that cell lines in our ovarian cancer cell line panel will be used in an attempt to stimulate ovarian-specific CTL, it is important to know whether or not any of the ovarian cancer cell lines in our panel express suppressive cytokines. Some, but not all of the cell lines, produced TGF- β 1 and/or TGF- β 2, but not TGF- β 3. Four of the lines (COV413, OVCAR-3, SW626, and TOV-112D) produced substantial amounts of the latent form of TGF- β 2 as none of them produced more than 100 pg/ml of active TGF- β 2, and the total amount measured for each was >1,500 pg/ml. These concentrations may be biologically significant as TGF- β 1 and - β 2 at concentrations greater than about 500 ng/ml have been shown to suppress the in vitro generation of CTL [1, 36, 49, 63].

IL-10 is infrequently expressed in ovarian cancer cell lines [7, 22, 62], is present in the ascites of patients with ovarian cancer [22, 55], and is associated with the suppression of T cell responses [2]. Only one ovarian cancer cell line (SW626) of the eleven tested here expressed appreciable amounts of IL-10, an amount that was previously shown to be biologically significant in blocking anti-CD3-induced T cell proliferation [9].

The ovarian cancer cell lines characterized here all express class I MHC molecules and a variety of tumor antigens. Some, but not all of the lines, also express immunosuppressive cytokines. This comprehensive analysis will serve to increase the utility of these cell lines in the characterization of antigens recognized by ovarian cancer-specific CTL.

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The TAG Family of Cancer/Testis Antigens is Widely Expressed in a Variety of Malignancies and Gives Rise to HLA-A2–Restricted Epitopes

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Summary: The TAG-1, TAG-2a, TAG-2b, and TAG-2c cancer/testis genes, known to be expressed in an unusually high percentage of melanoma cell lines, are shown here to be expressed in a variety of tumor lines of diverse histologic type, including cancers of the brain, breast, colon, lung, ovary, pharynx, and tongue. The genes are also expressed in fresh, uncultured melanoma, and ovarian cancer cells. Epitope prediction algorithms were used to identify potential HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 epitopes, and these potential epitopes were tested for their ability to stimulate a peptide-specific cytotoxic T lymphocyte response using lymphocytes from healthy donors. Two HLA-A2–restricted epitopes (SLGWLFLLL and LLLRLECNV) were identified using this approach. Cytotoxic T lymphocytes specific for each of these peptides were capable of recognizing tumor cells expressing both the corresponding class I major histocompatibility complex encoded molecule and the TAG genes. These results indicate that TAG-derived peptides may be good components of a therapeutic vaccine designed to target melanoma and a variety of epithelial cell-derived malignancies.

Key Words: cancer/testis antigen, CTL, epitope, immunotherapy (*J Immunother* 2007;00:000–000)

Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity and cytokine secretion have emerged as major mechanisms by which tumor growth is controlled by the mammalian immune system.¹ Vaccination of mice with immunogenic peptides has been shown to control tumor growth in both therapeutic and prophylactic models of

cancer.^{2,3} Vaccine trials have begun in humans, with most efforts using antigenic peptides known to bind to class I major histocompatibility complex (MHC) molecules, although class II MHC molecule binding peptides are also being tested.^{4–7} Although a substantial number of peptides have been discovered that can be used for the treatment of melanoma, there are relatively fewer peptide antigens that can be used for the treatment of other malignancies.^{8–10} Identification of additional peptide antigens would expand the number of malignancies that are amenable to vaccine-mediated therapy, and the use of a large number of peptides in a vaccine would minimize the impact of antigen loss variants that arise in the presence of immunoselection.^{11–14}

The categories of proteins giving rise to the tumor antigens recognized by CTL include cancer/testis antigens, differentiation antigens, mutated gene products, widely expressed proteins, and viral proteins.^{15–17} Cancer/testis antigens are particularly attractive candidates for use in tumor vaccines, as these antigens are only expressed in the testis and occasionally the placenta, which are both immunologically privileged sites.¹⁸ A consequence of this pattern of expression is that the peripheral CTL are not rendered tolerant to cancer/testis antigens and can thus recognize the antigens when they are expressed on tumor cells. The cancer/testis antigen family now contains a wide variety of proteins, prototypic members of which are exemplified by the MAGE¹⁹ and NY-ESO-1²⁰ antigens. More recently, we have identified multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) of a gene coding for a new cancer/testis antigen.²¹

The TAG-1, TAG-2a, TAG-2b, and TAG-2c genes were previously shown to be expressed in almost 90% of 32 melanoma lines tested.²¹ Unlike most cancer/testis antigens, which are rarely expressed in leukemia or myeloma cells, the TAG genes are also expressed in K562, a myelogenous leukemia, and they are homologous with chronic myelogenous leukemia-derived clones in the human EST database.²¹ Importantly, TAG is naturally immunogenic as the TAG-derived peptide RLSNRLLLR was recognized by HLA-A3–restricted CTL obtained from a melanoma patient.^{21,22} We have expanded on this work in the present study by demonstrating that the TAG gene is expressed in a variety of epithelial cell-derived

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tumors and by identifying additional TAG-derived peptides that elicit tumor-reactive CTL responses.

MATERIALS AND METHODS

Growth Medium

RPMI-1640 supplemented with 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (complete RPMI) served as the base medium. Base medium was supplemented with 10% fetal bovine serum (RPMI-10FBS) or 10% human serum (RPMI-10HS). For the growth of some tumor lines, RPMI-10FBS was additionally supplemented with 1 mM of sodium pyruvate, 5 ng/mL of epidermal growth factor, 10 µg/mL of transferrin, and 7.3 µg/mL of insulin (RPMI-10FBS-PETI).

Cell Lines

The melanoma lines AVL3-Mel, DM6, DM13, DM93, DM281, SK-Mel-5, VMM5A, VMM12, and VMM39 were available from our laboratory. Melanoma lines indicated as TAG⁺ in the cytotoxicity experiments described below were all previously shown to be strongly positive for TAG-1 and TAG-2a mRNA expression as determined by polymerase chain reaction (PCR) amplification.²¹ The brain cancer lines SW-1088, T98G, U-87MG, and U-373MG were obtained from the ATCC (Manassas, VA). The breast cancer lines MCF7, MDA-MB-453, MDA-MB-468, and SK-BR-3 were obtained from the ATCC; the breast cancer lines VAB5-A, BRC-173, and BRC-751 were established at the University of Virginia. The colon cancer lines HT-29, LS174T, and SW480 were obtained from the ATCC; the colon cancer line VCR-8 was established at the University of Virginia. The lung cancer lines Calu-1, SK-LU-1, and SK-MES-1 were obtained from the ATCC; the lung cancer lines TTB-250, VBT-2, VLU-6, VLU-13, VLU-18, and VLU-19 were established at the University of Virginia. The ovarian cancer lines CAOV-3, CAOV-4, ES-2, OV-90, OVCAR3, SK-OV-3, SW626, TOV-21G, and TOV-112D were obtained from the ATCC. The ovarian cancer cell line TTB-6 was established at the University of Virginia, and the ovarian cancer cell line COV413 was obtained from Dr Angela Zarling (University of Virginia). The squamous cell carcinomas of pharyngeal (FaDu), tongue (SCC4), and cervical (SiHa) origins were obtained from the ATCC. The cancer lines were maintained in RPMI-10FBS or RPMI-10FBS-PETI.

The B-lymphoblastoid cell lines (B-LCL) JY, MST, and T2 were maintained in RPMI-10FBS. C1R-A2, C1R-A3, and C1R-B7 were maintained in RPMI-10FBS supplemented with 300 µg/mL G418.

Patient Material

Cryopreserved tumor digest was obtained from the Tissue Procurement Facility at the University of Virginia. The tissue was obtained in an anonymized fashion and in accordance with established Institutional Review Board protocols.

PCR

Total RNA was prepared from 2 to 10 × 10⁶ cells using the RNeasy Mini kit (Qiagen, Valencia, CA) as per the kit instructions. RNA was quantified by absorbance at 260 nm. Total RNA was converted to cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Previously designed primers were used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1361, 1362; 598 bp), TAG-1 (A52, C723; 672 bp), TAG-2a (A73, E600; 528 bp), TAG-2b (A73.92, F473; 401 bp), and TAG-2c (A73, G608; 536 bp).²¹ PCR was performed on 250 ng of cDNA using Platinum Taq High Fidelity (Invitrogen). The PCR mixes were heated to 94°C for 2 minutes, 30 and 40 cycles of amplification were performed, and a final extension completed at 68°C for 5 minutes. When amplifying the TAG genes, the 30 and 40 cycles consisted of 94°C for 30 seconds, 62°C for 30 seconds, and 68°C for 60 seconds. When the GAPDH gene was amplified, the 30 cycles consisted of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 seconds. The PCR products were visualized on ethidium bromide-stained agarose gels.

Epitope Prediction

The TAG-1 and TAG-2 genes can each be potentially translated into 3 different isoforms (TAG-1α, TAG-1β, TAG-1γ, TAG-2α, TAG-2β, and TAG-2γ), with the putative α, β, and γ forms differing in the length at the N-terminal end of the protein. For the purposes of epitope prediction, the largest isoforms of each, TAG-1α and TAG-2α, were used and are referred to herein as TAG-1 and TAG-2.

The SYFPEITHI (www.syfpeithi.de)²³ and Parker (bimas.cit.nih.gov/molbio/hla_bind)²⁴ epitope prediction algorithms were used to identify peptides that have a high predicted binding affinity for HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8. The peptides were synthesized (New England Peptide Inc, Gardner, MA) and resuspended at 2 to 10 µg/mL in 100% dimethyl sulfoxide.

Peripheral Blood Mononuclear Cells

The buffy coat fraction from a unit of blood was obtained as a byproduct of voluntary blood donations by healthy individuals (Virginia Blood Services, Richmond, VA). Peripheral blood mononuclear cells (PBMC) were obtained after centrifugation on Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) and washed twice. Monocytes were enriched from the PBMC by adherence to tissue culture flasks for 2 hours and were subsequently used for the generation of dendritic cells. The plastic nonadherent cells were used as peripheral blood lymphocytes (PBL) and were cryopreserved until use.

Class I MHC Gene Typing

DNA was obtained from 5 × 10⁶ PBMC using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Class I MHC typing was carried out

using the Micro SSP Generic HLA Class I typing tray SSP1L (One Lambda Inc, Canoga Park, CA).

Dendritic Cell Preparation

Dendritic cells were prepared using a modification of a previously published protocol.²⁵ In brief, adherent monocytes were incubated for 6 to 8 days in the presence of 800 U/mL granalocyte macrophage colony-stimulating factor and 500 U/mL interleukin (IL)-4 to produce immature dendritic cells. The nonadherent, immature dendritic cells were then incubated in the presence of 800 U/mL granalocyte macrophage colony-stimulating factor, 500 U/mL IL-4, 10 ng/mL IL-1 β , 1,000 U/mL IL-6, 10 ng/mL tumor necrosis factor- α , and 1 μ g/mL prostaglandin E2. Mature dendritic cells (mDC) were obtained as nonadherent cells after 3 to 4 days of incubation. mDC were incubated with peptide (40 μ g/mL) and β_2 -microglobulin (3 μ g/mL) for 2 hours at room temperature. The peptide-pulsed mDC were irradiated (3500 Rad) and washed once to remove free peptide. The cells were then used immediately for CTL stimulation or were cryopreserved for future stimulations.

Stimulation of Peptide-specific CTL

CTL were stimulated using a modification of the protocol of Lu and Celis.^{26,27} Equal volumes of PBL (2×10^6 cells/mL) were mixed with autologous, peptide-pulsed mDC (1×10^5 cells/mL) to give a responder to stimulator ratio of 20:1 in RPMI-10HS supplemented with 10 ng/mL IL-7. Wells (generally 48) on a 48-well plate were seeded with 0.5 mL of the mixed responder/stimulator cells. One day after the initial priming, IL-10 at a final concentration of 10 ng/mL was added to each well. The cultures were restimulated every 7 days. For secondary stimulations, peptide-pulsed stimulator cells were added to each well in 0.5 mL RPMI-10HS. IL-10 (10 ng/mL) was added 1 day later, and IL-2 at a final concentration of 10 Cetus U/mL was added 2 days later. Two to 3 days later, IL-2 (10 Cetus U/mL) was added again to each culture. Tertiary and later stimulations were performed in a similar fashion except that IL-2 was the only added cytokine. For all stimulations, 2.5×10^4 mDC were used as the stimulator cells in the restimulations until they were depleted, after which 1×10^6 autologous PBL or 2.5×10^5 to 5.0×10^5 B-LCL matched for the class I MHC molecule of interest were used. T-cell cultures showing activity against peptide-pulsed targets in a screening assay were expanded with anti-CD3.²⁸

Cytotoxicity Assays

A standard 4 hours ^{51}Cr -release assay was used and modified as indicated below for the indicated analyses.²⁹ Target cells were labeled with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ for 2 hours and then washed. The ^{51}Cr -labeled cells were then incubated with peptide (10 $\mu\text{g}/\text{mL}$) for 1 hour at room temperature, washed, and added to wells containing CTL on a 96-well plate at 2000 cells/well. Maximal ^{51}Cr -release was determined by incubating labeled target cells in the presence of 3% NP-40, and spontaneous ^{51}Cr -release was

obtained by incubating with assay medium alone. Counts per minute (CPM) present in the collected supernatants were measured using a Wallac WIZARD automatic gamma counter (Perkin Elmer, Downers Grove, IL). The percent specific release was calculated as: % specific ^{51}Cr -release = $100 \times (\text{ER}-\text{SR})/(\text{MR}-\text{SR})$, where ER = CPM experimental release, SR = CPM spontaneous release, and MR = CPM maximal release.

Cytotoxicity Screening Assay

T-cell cultures were tested for cytotoxic activity against peptide-pulsed cells 5 to 6 days after the fourth stimulation. Cells from 4 to 6 randomly selected wells from each 48-well plate to be tested were counted and averaged to obtain a mean effector cell concentration. Average effector-to-target (E:T) ratios of 40:1 and 10:1 were tested in duplicate for each culture. In some experiments, unlabeled K562 cells were added at an unlabeled-to-labeled target ratio of 20:1 to decrease nonspecific killing attributable to natural killer cells.

CTL Specificity Analysis

^{51}Cr -labeled tumor cells and peptide-pulsed B-LCL were used as targets. CTL cultures were counted and resuspended at a concentration giving an initial E:T ratio which generally ranged from 20:1 to 80:1, with subsequent 1:2 dilutions used to give 4 E:T ratios for each CTL being analyzed.

Peptide Titration

Peptides, starting at 10 $\mu\text{g}/\text{mL}$, were diluted through a 10-fold dilution series, after which ^{51}Cr -labeled targets were added for 1 hour at room temperature. The target cells were then resuspended at 20,000 cells/mL and added to the appropriate wells of the 96-well plates in 100 μL aliquots. Effector cells were then added at 100 $\mu\text{L}/\text{well}$ to give the desired E:T ratio.

Cold Target Inhibition

Cold targets, starting at a 60:1 cold-to-hot (C:H) ratio were added in a final volume of 50 μL per well. Cold target cells, when appropriate, were first incubated with peptide (0.1 to 10 $\mu\text{g}/\text{mL}$ depending on avidity) for 1 hour at room temperature. Before plating, peptide-pulsed cold target cells were washed with $10 \times$ volume of RPMI 1640 containing 1% FBS. Effector cells, in a volume of 50 $\mu\text{L}/\text{well}$, were then added. The 96-well plates were then centrifuged at 800 rpm for 2 minutes at room temperature and incubated at 37°C for 1 hour. After the incubation, ^{51}Cr -labeled targets were added in 100 μL aliquots. Each cell line used in the assay was also evaluated in triplicate, without cold targets, at the appropriate E:T ratios.

Human Subjects

All research involving human subjects and human tissues was approved by the University of Virginia Institutional Review Board in accordance with an assurance filed with and approved by the Department of Health and Human Services.

RESULTS

Expression of TAG Genes in Established Tumor Lines

The TAG genes were previously shown to be expressed in a large percentage of melanoma cell lines.²¹ To determine if the TAG genes are expressed in other cancers, a broad survey was taken using established tumor lines of nonmelanocytic origin. Brain tumors, which share an ectodermal origin with melanocytes, expressed the TAG-1, TAG-2a, and TAG-2c genes, but

not the TAG-2b gene (Table 1). Tumor lines of epithelial cell origin including breast, colon, lung, ovarian, pharyngeal, tongue, and cervical origin were also tested for expression of the TAG genes (Table 1). With the exception of the pharyngeal, tongue, and cervical tumor lines for which only 1 cell line each was tested, each of the TAG genes was expressed in at least 1 line of each cancer type. As with melanoma,²¹ TAG-1 was the most frequently expressed TAG gene, whereas TAG-2b was the least frequently expressed TAG gene. By performing

TABLE 1. Expression of TAG Genes in Established Tumor Lines*

Tumor Tissue Origin and Type	TAG-1	TAG-2a	TAG-2b	TAG-2c
Tumors of neural origin				
Brain	75% (19%-99%)	50% (7%-93%)	0% (0%-60%)	50% (7%-93%)
SW-1088	+	+	—	—
T98G	+	+	—	+
U-87MG	+	—	—	+
U-373MG	—	—	—	—
Tumors of epithelial origin				
Breast	71% (29%-96%)	14% (4%-58%)	14% (4%-58%)	14% (4%-58%)
BRC-173	—	—	—	—
BRC-751	+	—	—	—
MCF7	—	—	—	—
MDA-MB-453	+	—	—	—
MDA-MB-468	+++	+++	++	++
SK-BR-3	+	—	—	—
VAB5-A	+	—	—	—
Colon	100% (40%-100%)	50% (7%-93%)	25% (6%-81%)	50% (7%-93%)
HT-29	+	—	—	—
LS174T	+	+	—	+
SW480	+	—	+	—
VCR-8	+++	+	—	+
Lung	67% (30%-93%)	78% (40%-97%)	44 (14%-79%)	44 (14%-79%)
Calu-1	—	+	—	—
SK-LU-1	+	—	—	—
SK-MES-1	++	++	+	+
TTB-250	—	+	—	—
VBT-2	+	+	—	—
VLU-6	—	—	—	—
VLU-13	+++	+++	+++	+++
VLU-18	+++	+++	++	++
VLU-19	++	+	+	+
Ovarian	82% (48%-98%)	55% (23%-83%)	18% (23%-52%)	27% (6%-61%)
CAOV-3	+	+	—	—
CAOV-4	+	—	—	—
COV413	—	+	—	—
ES-2	+	—	—	—
OV-90	+	+	—	—
OVCAR3	+++	+++	++	+++
SK-OV-3	+	—	—	—
SW626	+	+	—	+
TOV-21G	+	—	—	—
TOV-112D	—	—	—	—
TTB-6	+++	+++	++	+++
Pharyngeal	100% (2%-100%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
FaDu	+	—	—	—
Tongue	100% (2%-100%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
SCC4	+	—	—	—
Cervical	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)	0% (0%-98%)
SiHa	—	—	—	—

*PCR was performed as described in the Materials and Methods. The PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: + + +, product was easily visualized after 30 cycles of amplification; + +, product was weakly visible at 30 cycles and easily visible after 40 cycles of amplification; +, product was only visible after 40 cycles of amplification; —, product was not visualized after 40 cycles of amplification. The percentage positive with 95% confidence intervals is given for each tumor type. Each sample exhibited a strong signal when GAPDH was PCR amplified.

the PCR amplification at both 30 and 40 cycles, it was also possible to categorize the expression levels in individual cell lines. The data demonstrate that the TAG genes are strongly expressed in some tumor lines, whereas weakly expressed in others (Table 1). Because a strong positive amplification signal of the GAPDH gene was obtained for each of the cDNA samples (data not shown), the lack of TAG gene amplification for a given sample cannot be attributed to the quality of the cDNA preparation.

Expression of TAG Genes in Uncultured Melanoma and Ovarian Carcinoma Cells

It was previously shown that the TAG genes were expressed in the uncultured melanoma cells from which the VMM18 melanoma line was established and from which the TAG genes were originally cloned and identified, thus demonstrating that the expression of the genes is not an artifact of in vitro cell culture.²¹ That work has been extended here by showing that the TAG genes are expressed in a relatively high frequency of uncultured melanomas and ovarian carcinomas (Table 2). As with the tumor lines, TAG-1 is the most frequently expressed and TAG-2b is the least frequently expressed TAG gene.

Prediction of Class I MHC Binding Peptides From TAG-1 and TAG-2

The SYFPEITHI²³ and Parker et al²⁴ epitope prediction algorithms were used to predict HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 binding peptides from the TAG-1 and TAG-2 proteins (Table 3). The top 5 peptides predicted by each algorithm were reviewed and those peptides not containing canonical

anchor residues were eliminated. The remaining 2 to 5 top ranked peptides were then selected for synthesis and testing. The peptide RLSNRLLLLR was predicted to bind HLA-A3 (ranking in the top 1 to 4 scores for TAG-1 and TAG-2), but was not chosen for study as we previously demonstrated that it is recognized by a tumor-reactive CTL line that naturally developed in a melanoma patient.²¹

Generation of CTL Specific for TAG-derived Peptides

Each peptide listed in Table 4 was synthesized and tested for its ability to prime a peptide-specific CTL response using PBL obtained from 3 to 9 healthy donors. PBL were stimulated with peptide-pulsed, autologous mDC, generally in 48 individual microcultures per donor. After the fourth restimulation, the individual cultures were tested for reactivity with peptide-pulsed target cells in a ⁵¹Cr-release assay. Cultures, with killing that was more than 20% above that found on the target cells not pulsed with peptide, were selected for additional characterization. Initially, individual microcultures were stimulated with only a single peptide, and these cultures identified 2 peptides for further study, including the HLA-A2-restricted peptide SLGWLFLLL and the HLA-B8-restricted peptide LSRLSNRLL (Table 4). Selected cultures reactive with SLGWLFLLL and LSRLSNRLL were expanded with anti-CD3 antibody for further analysis. The reactivities of 6 SLGWLFLLL cultures are shown in Figure 1. The culture reactive with the LSRLSNRLL peptide lost its peptide-specificity after expansion, and although it recognized tumor, it was not studied further.

TABLE 2. Expression of TAG Genes in Uncultured Melanoma and Ovarian Carcinoma Cells

Sample Numbers	Gene Expression Pattern*			
	TAG-1	TAG-2a	TAG-2b	TAG-2c
Melanoma				
204, 415, 7719	++	++	+	++
956	++	++	+	+
2241	++	++	—	++
8062	++	+	—	—
3540	+	+	+	+
1302	+	+	—	—
278, 550, 1435, 2348, 8353	+	—	—	—
123, 243, 482, 509, 2201, 4479, 8326, 8542, 8899	—	—	—	—
Total positive	13/22	8/22	5/22	6/22
% positive	59%	36%	23%	27%
95% confidence interval	36%-79%	17%-59%	8%-45%	11%-50%
Ovarian carcinoma				
6, 519	++	++	+	++
144, 632	+	+	—	—
117, 121, 189, 1006	+	—	—	—
1130	—	+	—	—
29, 94, 125, 136, 185, 212, 227, 258, 546, 567, 572, 834, 1288, 3883	—	—	—	—
Total positive	8/23	5/23	2/23	2/23
% positive	35%	22%	9%	9%
95% confidence interval	16-57%	8-44%	1-28%	1-28%

*PCR was performed as described in the Materials and Methods. Following 30 rounds of amplification the PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: (++) product was easily visualized; (+) product could be visualized, but the band was very light; (—) product was not visible. Each sample exhibited a strong signal when GAPDH was PCR amplified.

TABLE 3. Test Peptides From TAG Used for In Vitro CTL Priming

Class I MHC Binding Protein*	Peptide Sequence†	Residue Numbers‡	Presence in TAG-1 or TAG-2	Parker Score§	SYFPEITHI Score
HLA-A1	ESERGLPAS	32-40	1, 2	0.27	16
	NLEPLVSRD	64-72	1, 2	0.90	16
	SRDPPASAS	70-78	1, 2	0.25	17
HLA-A2	TL [~] SRLSNRL	41-49	1, 2	21.4	22
	LLRLECNV	49-57	1, 2	487.5	25
	SLGWLFLLL	78-86	1	40.6	24
HLA-A3	FLLLNSTT	83-91	1	126.8	20
	GLPASTLSR	36-44	1, 2	24.0	21
	LLLNSTTK	84-92	1	30.0	28
HLA-B7	LPAQEGAPT	1-9	1, 2	2.0	20
	VQRRAEGLL	10-18	1, 2	40.0	12
	LPASTLSRL	37-45	1, 2	80.0	21
HLA-B8	LSRLSNRLL	42-50	1, 2	40.0	12
	DPPASASLF	72-80	2	0.4	11
	TVQRRAEGL	9-17	1, 2	4.0	18
	VQRRAEGLL	10-18	1, 2	1.2	17
	LSRLSNRLL	42-50	1, 2	4.0	18

*Antigenic peptides from TAG-1 and TAG-2 were predicted based on the predicted ability of the peptides to bind to the indicated class I MHC molecule.

†Underscored residues correspond to canonical amino acid residues typically found at that position of a peptide binding to the respective class I MHC molecule.

‡Position of the peptide within the linear sequence of TAG-1 and TAG-2.

§Score obtained from predictive algorithm of Parker et al.²⁴

||Score obtained from the predictive algorithm of Rammensee et al.²³

Screening individual test peptides for their ability to stimulate a CTL response is an inherently time and resource intensive endeavor. To determine the feasibility of screening multiple peptides simultaneously, PBL of 1 of the donors reactive against the SLGWLFLLL peptide in association with HLA-A2 was also stimulated with a mix of 4 peptides, including SLGWLFLLL. Because of limitations on the number of responder cells in each microculture, the initial screening was performed against targets pulsed with all 4 peptides, and this yielded positive cultures (Table 4). Cultures with cytotoxic reactivity were then expanded with anti-CD3 and tested against targets individually pulsed with each of the peptides used in the stimulation. Not only was a response against

SLGWLFLLL in the mix detected, but a response was also measured against LLLRLECNV in association with HLA-A2, thus validating that multiple peptides can be tested simultaneously for their ability to induce a CTL response (Table 4; Figs. 2A, C). A similar peptide mix lacking the SLGWLFLLL peptide was also used to identify another LLLRLECNV reactive culture in an additional donor (Table 4). Likewise, a mix of HLA-B7 peptides led to the identification of LPAQEGAPT as a candidate epitope (Table 4; Figs. 2B, D). Although LPAQEGAPT-reactive CTL were shown to recognize tumor (data not shown), their specificity could not be confirmed in cold target inhibition experiments and results with this peptide are not discussed further.

TABLE 4. Summary of CTL Reactivity Against Stimulating Peptides

Class I MHC Binding Protein		No. Positive Cultures Obtained/No. Cultures Initiated†		Specificity of Obtained CTL‡
HLA-A1	SRDPPASAS	0/6	NT	
HLA-A1	ESERGLPAS NLEPLVSRD	0/6	NT	
HLA-A2	SLGWLFLLL	7/9	SLGWLFLLL	
HLA-A2	SLGWLFLLL TLSRLSNRL LLLRLECNV FLLLNSTT	1/1	SLGWLFLLL LLLRLECNV	
HLA-A2	TLSRLSNRL LLLRLECNV FLLLNSTT	1/5	LLLRLECNV	
HLA-A3	GLPASTLSR	0/6	NT	
HLA-A3	LLLNSTTK	0/6	NT	
HLA-B7	LPASTLSRL	0/6	NT	
HLA-B7	LPAQEGAPT VQRRAEGLL LSRLSNRLL DPPASASLF	2/3	LPAQEGAPT	
HLA-B8	LSRLSNRLL	1/9	LSRLSNRLL	
HLA-B8	TVQRRAEGL VQRRAEGLL	0/6	NT	

*Peptide priming of CTL was performed as indicated in the Materials and Methods, and was carried out with either a single peptide or a pool of 2 to 4 peptides as indicated.

†A culture was considered positive if the responder cells in at least 1 well from a given donor recognized target cells pulsed with the corresponding peptide(s) in both initial screening assays.

‡Initial screening for positive cultures used target cells pulsed with a pool of peptides when a peptide pool was used for the stimulations. Once a CTL line was established, it was tested against target cells pulsed with the individual peptides.

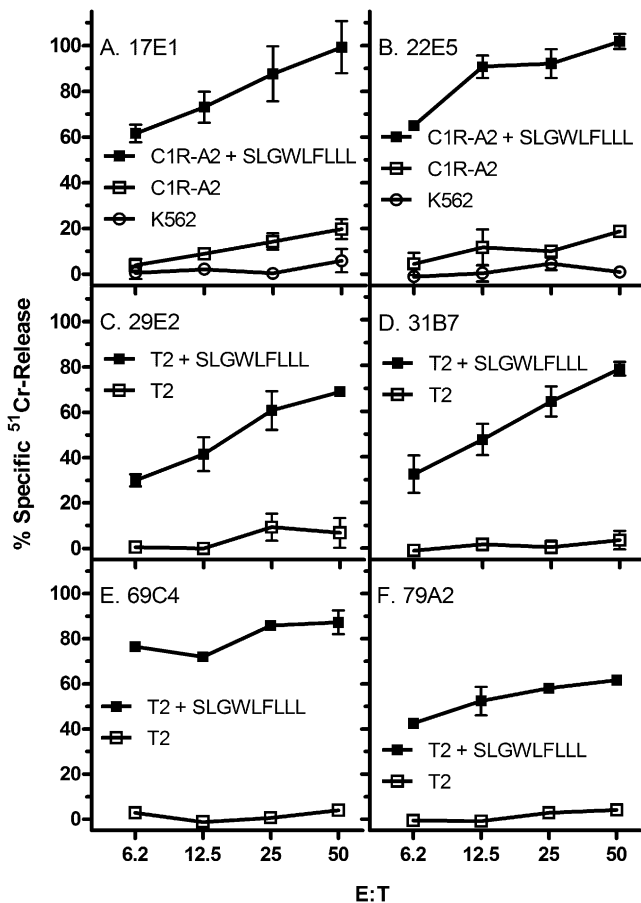


FIGURE 1. Peptide reactivity of anti-CD3 expanded, SLGWLFLL (SLG) peptide-stimulated microcultures. Expanded microcultures were tested in a ⁵¹Cr-release assay against the indicated targets. Six independent cultures are shown, each derived from a separate donor. C1R-A2 and T2 are HLA-A2⁺ targets.

MHC Restriction of the SLGWLFLL and LLLRLECNV Peptides

To confirm that the SLGWLFLL and LLLRLECNV peptides are presented by HLA-A2, the respective CTL cultures were tested against a panel of target cells that were either matched or unmatched for the class I MHC molecule of interest. Included among these targets were C1R-A2 and C1R-B7 that are class I MHC gene transfectants (HLA-A*0201 and HLA-B*0702, respectively) of the class I MHC null cell line, Hmy2.C1R. These transfectants can be used to unambiguously determine the class I MHC restriction of a CTL line or clone. The target cells were incubated in the presence of the test peptide and then tested for their susceptibility to lysis by the peptide-specific CTL.

CTL lines 69C4 and 22E5, specific for the SLGWLFLL peptide, recognized the HLA-A2 positive cell lines T2 and C1R-A2 when pulsed with peptide, but did not recognize the HLA-A2 negative MST and C1R-

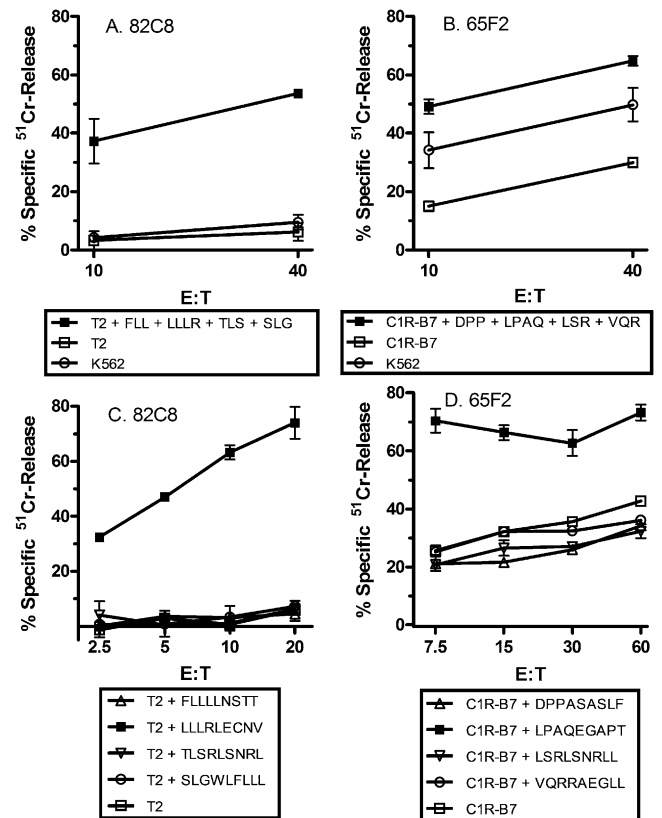


FIGURE 2. Peptide specificity of peptide mix-stimulated microcultures. Microcultures 82C8 (A) and 65F2 (B) were tested in a ⁵¹Cr-release assay against targets, expressing either a mix of peptides predicted to associate with HLA-A2 (A) or HLA-B7 (B). After expansion with anti-CD3, 82C8 (C) and 65F2 (D) were tested against the individual peptides, which comprised the original mix. T2 is an HLA-A2⁺ target; C1R-B7 is an HLA-B7⁺ target. Peptides used were FLLLNSTT (FLL), LLLRLECNV (LLLR), TLSRLSNRL (TLS), SLGWLFLL (SLG), DPPASALF (DPP), LPAQEGAPT (LPAQ), LSRLSNRL (LSR), and VQRRAEGLL (VQR).

B7 lines when pulsed with peptide (Fig. 3A, B). The same results were obtained with the CTL line 82C8, specific for the LLLRLECNV peptide (Fig. 3C). These results demonstrate that both the SLGWLFLL and LLLRLECNV peptides are presented by HLA-A2.

Peptide Dose-response of Peptide-specific CTL

The relative affinity of the peptide-specific CTL lines was determined by testing the ability of the CTL to recognize target cells incubated with 10-fold dilutions of peptide, beginning at 10 µg/mL (~10 µM). CTL lines recognizing the SLGWLFLL peptide showed a broad range of peptide concentrations over which half-maximal killing was achieved, with most having half-maximal activity between 0.1 and 10 nM (Fig. 4A). CTL lines recognizing the LLLRLECNV peptide had half maximal activity between 1 and 20 nM (Fig. 4B).

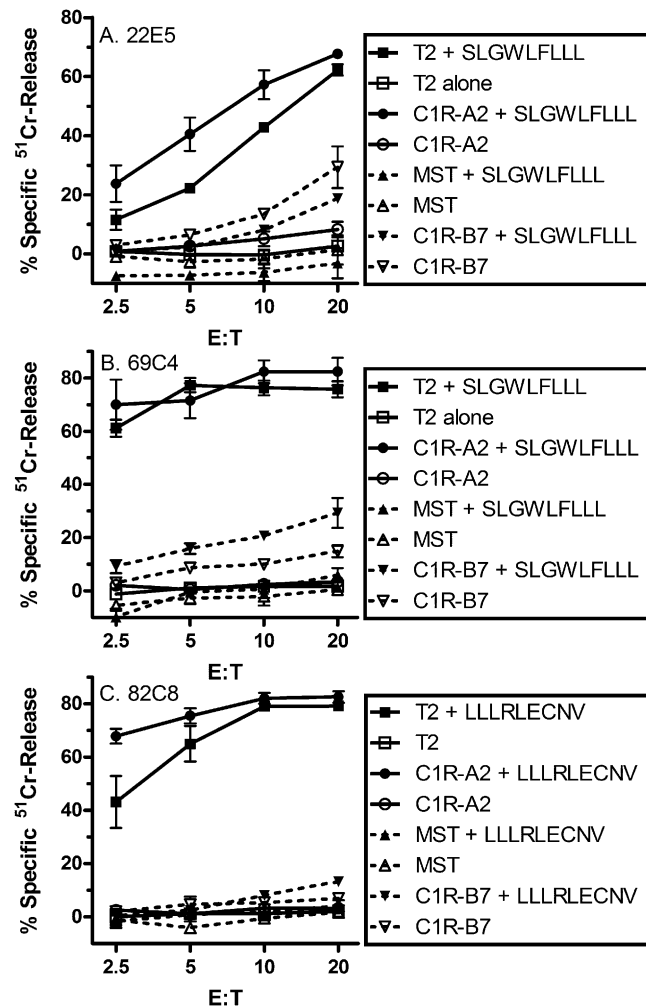


FIGURE 3. MHC restriction of the SLGWLFLLL (SLG) and LLRLECNCV (LLR) peptides. CTL lines 22E5 (A), 69C4 (B), and 82C8 (C) were tested in a ^{51}Cr -release assay for their ability to kill the indicated targets. Solid symbols indicate that the targets cells were pulsed with 2.5 $\mu\text{g}/\text{mL}$ of the indicated peptide as described in the Materials and Methods. Open symbols indicate that the targets were not pulsed with peptide; solid lines, indicate that the targets are HLA-A2 $^{+}$; dashed lines indicate that the targets are HLA-A2 $^{-}$.

Recognition of Tumors by Peptide-specific CTL

To determine if the peptide-specific CTL also recognize tumor cells, the CTL were tested for their ability to recognize tumors expressing both the appropriate class I MHC molecule and the TAG gene. CTL lines 22E5 and 69C4 (SLGWLFLLL specific) recognized some, but not all, tumors expressing both HLA-A2 and the TAG genes; however, tumors expressing only HLA-A2 or the tumor antigen alone were not recognized (Figs. 5A, B, and data not shown). Likewise, CTL line 82C8 (LLRLECNCV specific) recognized some, but not all tumor lines expressing HLA-A2 and the TAG genes; however, tumors expressing either the HLA-A2 or the

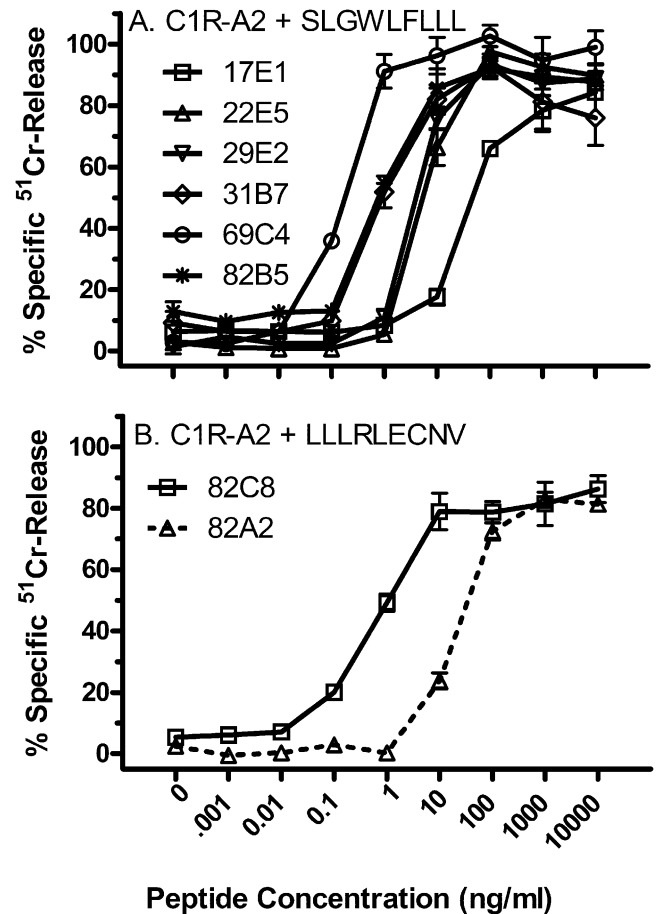


FIGURE 4. Peptide dose-response of peptide-specific CTL. The indicated target cells were preincubated with peptide at the indicated concentrations for 1 hour at 37°C and then used as targets in a standard ^{51}Cr -release assay with the indicated CTL lines. CTL were used at an E:T of 5:1.

tumor antigen alone were not recognized (Fig. 5C and data not shown).

The tumor reactivity of the CTL lines was further confirmed in cold target inhibition experiments. The recognition of ^{51}Cr -labeled DM13 tumor cells (HLA-A2 $^{+}$, TAG $^{+}$) by the CTL line 69C4 (HLA-A2-restricted, SLGWLFLLL-specific) was inhibited by unlabeled SLGWLFLLL-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Fig. 6A). Similarly, the recognition of ^{51}Cr -labeled DM6 tumor cells (HLA-A2 $^{+}$, TAG $^{+}$) by the CTL line 82C8 (HLA-A2-restricted, LLRLECNCV-specific) was inhibited by unlabeled LLRLECNCV-pulsed T2 cells, but not by T2 cells alone or T2 cells pulsed with the irrelevant GILGFVFTL peptide (Fig. 6B). These results confirm that the TAG-derived SLGWLFLLL and LLRLECNCV peptides are naturally processed and presented by HLA-A2 on tumor cells.

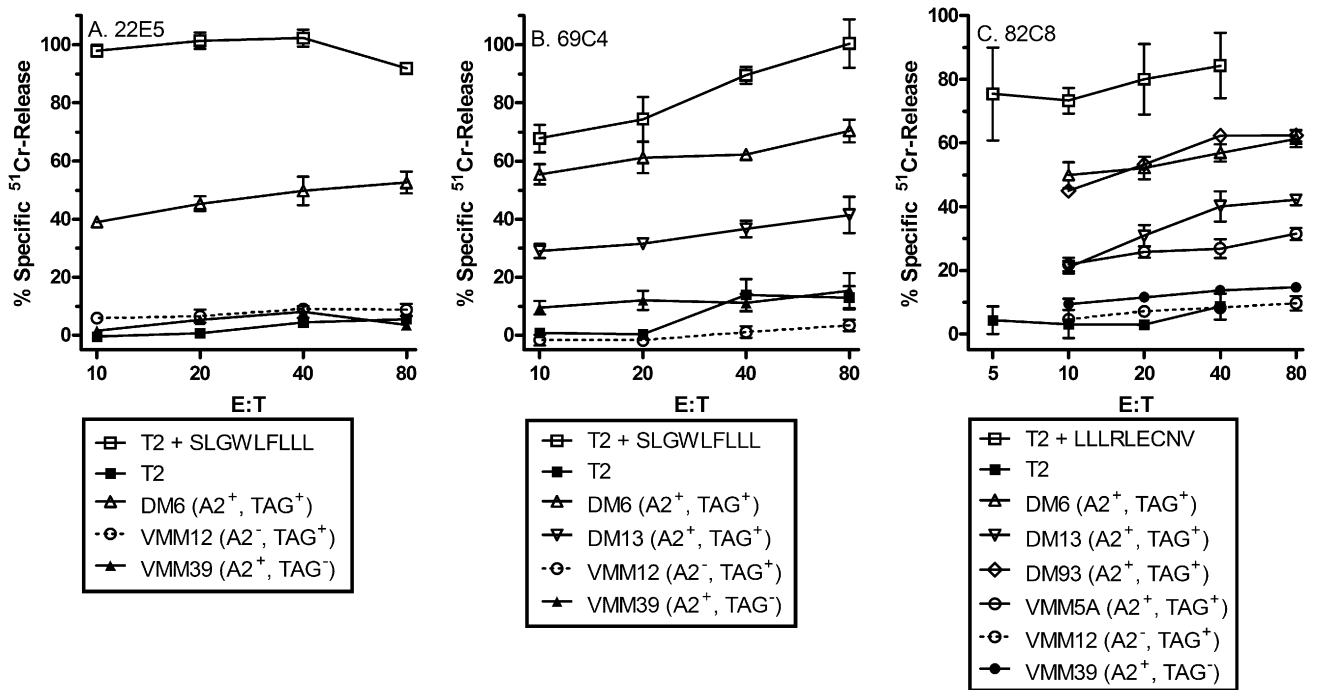


FIGURE 5. Recognition of tumor lines by peptide-specific CTL. CTL lines 22E5 (A), 69C4 (B), and 82C8 (C) were tested in a standard ^{51}Cr -release assay against the indicated targets. Open symbols indicate that the target was incubated with the indicated TAG peptide or expresses the TAG gene; closed symbols indicate that the target was neither incubated with the cognate TAG peptide nor expressed the TAG gene. Solid lines indicate that the target cells are matched with the CTL for expression of HLA-A2; dashed lines indicate that the target cells do not share HLA-A2 in common with the CTL.

DISCUSSION

Cancer/testis antigen expression has most often been studied at the mRNA level by PCR analysis.^{30,31} In melanoma, mRNA expression for different cancer/testis antigens ranges from about 4% to 90%.^{21,30} TAG (~90%) and MAGE-3 (~50% to 90%) are the most frequently expressed cancer/testis antigens, whereas SAGE is infrequently expressed (4%). Many cancer/testis antigens including BAGE, CT7, GAGE, MAGE-1, NY-ESO-1, and SSX-2 are expressed in about 25% to

75% of the melanoma samples tested. In a study of 8 cancer/testis antigens in 47 different melanoma tumors, 91% expressed at least 1 of the antigens, and 13% expressed all of the antigens.³⁰ The present work extends our previous findings with TAG in melanoma by demonstrating that the family of genes is expressed in 23% to 59% of 22 fresh melanomas tested (Table 2). Although this level of expression is somewhat less than that found in cultured melanoma cell lines, it is still a relatively high frequency of expression in comparison with other cancer/testis antigens.

As cancer/testis antigens are expressed in a wide variety of cancers of diverse histologic types,¹⁸ it was also of interest to determine if tumors of epithelial origin expressed the TAG genes. Established tumor lines including breast, colon, lung, ovarian, pharyngeal, and tongue all expressed TAG, with TAG-1 being the gene most often expressed and at the highest levels (Table 1). A similar finding was also observed with brain tumors which, like melanoma cells, are of an ectodermal lineage (Table 1). As is the case with uncultured melanoma cells, the TAG genes can also be detected in uncultured ovarian tumor cells (Table 2). The expression of the TAG genes in a relatively high frequency of tumors of different tissue origins indicates that TAG-derived antigens would be useful components of vaccines targeting a variety of malignancies.

A frequent goal when immunizing with tumor vaccines is to elicit a tumor-specific CTL response. A

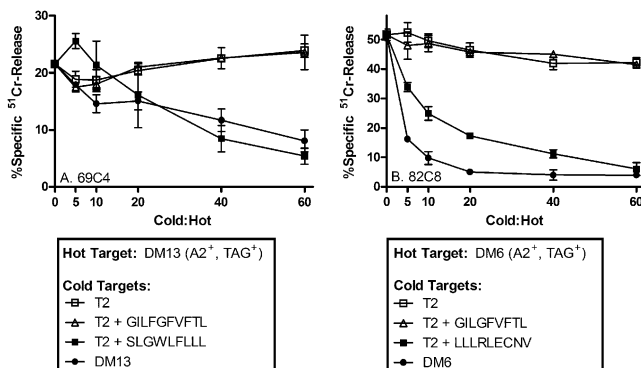


FIGURE 6. Cold target inhibition analysis of peptide-specific CTL. CTL were preincubated for 1 hour at 37°C with the indicated ratio of cold targets, after which the hot targets were added and incubation continued for an additional 4 hours. CTL lines 69C4 and 82C8 were used at an E:T of 40:1.

common approach to designing such vaccines is to include 1 or more short antigenic peptides (most often 9 amino acids in length) capable of binding to class I MHC molecules. Class I MHC molecules that are prevalent in the population are most often chosen, because it maximizes the utility of the vaccine at the population level. For example, by concentrating on the 3 most prevalent class I MHC molecules in the white population (ie, HLA-A1 = 28.1%, A2 = 49.1%, and A3 = 25.0%),³² coverage for approximately 82% of the population can be obtained. By targeting additional prevalent class I MHC molecules (HLA-B7 = 22.9%, B8 = 17.9%), it is possible to approach coverage of the entire population. In the present study, we used 2 predictive approaches to identify candidate TAG derived peptides that could be presented by HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8.^{23,24}

A challenge presented by using the predictive approach to antigen identification is that different predictive algorithms result in different peptide rankings. By combining the results of 2 or more predictive algorithms, it is possible to minimize this limitation by focusing on the top-ranked peptides from different algorithms, an approach that has been shown to work with both prostate specific membrane antigen²⁶ and carcinoembryonic antigen.²⁷ We have applied that approach to the cancer/testis antigen TAG and have attempted to identify new peptide antigens presented in association with HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 (Table 3). Using this approach, we tested a total of 15 peptides that were predicted to bind to 1 or more of these class I MHC molecules. Of these peptides, 4 were shown to elicit a response. Of these, LSRLSNRLL was initially shown to be recognized, but as the CTL line eventually lost recognition of the peptide, it was not studied further. CTL that recognized LPAQEGAPT in association with HLA-B7 were also generated, but although the CTL could recognize tumor expressing HLA-B7 and the TAG genes, the specificity of the CTL could not be confirmed in a cold target inhibition experiment.

Two antigenic peptides restricted by HLA-A2 were identified. The SLGWLFLLL peptide was recognized by CTL derived from 7 different individuals. This peptide could sensitize targets for lysis with half-maximal killing occurring at 0.1 to 10 nM, which suggests that the CTL have a high affinity for the peptide/HLA-A2 complex and makes it likely that the complex could be recognized on the surface of a tumor cell, with such recognition occurring on DM6 and DM13 melanomas. Not all HLA-A2⁺, TAG⁺ tumors were recognized by the CTL, however, indicating that either the peptide/MHC complex is present at very low levels on the surface of some tumor cells or that not all tumors positive for TAG expression by PCR express the TAG protein. To begin to address this question, we have recently produced recombinant TAG-1 and TAG-2 proteins for the purpose of generating TAG-specific antisera.

The LLLRLECNV peptide stimulated an HLA-A2-restricted response from 2 of 6 different donors. The peptide sensitized targets for lysis at 1 to 20 nM, suggesting that these CTL had receptors of a slightly lower affinity than those used to recognize the SLGWLFLLL peptide. In contrast to CTL recognizing the SLGWLFLLL peptide, CTL recognizing the LLLRLECNV peptide recognized multiple tumors expressing both HLA-A2 and TAG. Taken together with the results obtained with the SLGWLFLLL peptide-specific CTL, this suggests that the TAG protein is expressed in many tumors positive for TAG expression by PCR and that the LLLRLECNV epitope is selectively expressed over the SLGWLFLLL epitope.

It is common to use peptide binding experiments as an intermediate step between the predictive step and the CTL elicitation step, with only peptides demonstrating high affinity binding being selected for further study. The experiments of Lu and Celis^{26,27} have demonstrated, however, that it is possible to dispense with peptide binding experiments when using a predictive approach to peptide epitope identification. Because binding experiments can be both time-consuming and expensive to perform, their elimination can greatly streamline antigen identification. The results obtained here confirm that peptide antigens can be successfully identified in the absence of performing preliminary binding experiments. Importantly, our results also demonstrate that the antigen identification process can be further consolidated and made more efficient by simultaneously testing multiple peptides. Although there is a theoretical concern that competition among multiple peptides for binding to a limited number of class I MHC molecules might preclude the identification of an antigenic peptide in a peptide mix, we have successfully used the approach here to identify 3 different peptides when 3 or 4 peptides are included in the mix. Because the in vitro stimulations are resource intensive experiments to perform, the ability to simultaneously screen up to 4 peptides will greatly enhance the utility of the predictive approach to peptide epitope antigen identification.

In combination with a previously identified HLA-A3-restricted TAG epitope,²¹ a total of 3 TAG-derived epitopes have now been identified from TAG, including 2 HLA-A2-restricted epitopes and 1 HLA-A3-restricted epitope. Because the TAG family of genes is expressed in a high percentage of melanomas and in a variety of tumors of epithelial origin, these epitopes are ideal candidates for inclusion in a vaccine for the therapeutic treatment of a variety of malignancies, for the ex vivo stimulation of T cells for use in adoptive therapy, and as reagents for studying the T-cell-mediated immune response to tumors.

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Among the clinicopathologic variables that contribute to the stratification of neuroblastoma patients as high risk for adverse outcome is amplification of the N-myc oncogene within the tumor. Further, a transgenic mouse with tissue-specific overexpression of the N-myc oncogene has been generated (Weiss WA, et al. *EMBO J* 16: 2985, 1997), and develops spontaneous neuroblastoma tumors. We have now established a panel of 10 novel transplantable neuroblastoma cell lines derived from spontaneous tumors that arise in these N-myc transgenic mice. We have conditioned these lines for transplantation via serial orthotopic passage in the adrenal gland, and in some instances subcutaneously and in vitro. We have constructed tissue arrays containing both transplantable cell lines and spontaneous tumors that arise in N-myc transgenic mice. By staining with H+E, these tumors are noted to represent relatively typical small round blue cell tumors with predominantly immature and some differentiated elements. The tumors that form after implantation of these cell lines are highly vascular as evidenced by H+E staining and CD31 immunohistochemistry. Consistent with human neuroblastoma, these tumors demonstrate positive immunohistochemical staining for neurofilament, tyrosine hydroxylase, synaptophysin and chromogranin A, and overproduce large amounts of catecholamines (DOPA, dopamine and norepinephrine). Electron micrographs demonstrate that these tumor cell lines form relatively undifferentiated tumors in vivo. We have now profiled the patterns of gene expression within both spontaneous tumors that arise in these mice as well as the transplantable cell lines using a 19,942 gene cDNA microarray. Analysis of gene expression patterns using hierarchical clustering and principal component analysis demonstrates close correspondence between passaged cell lines compared to spontaneous tumors that arise in N-myc transgenic mice. Comparison of these tumor cell lines with a panel of human neuroblastoma tumors demonstrates a set of over 376 genes that are overexpressed in common by these cell lines and human neuroblastoma tumors, including prosurvival factors such as survivin, and a wide range of cell-cycle genes among others. Further, a set of 88 genes was found to be underexpressed in common by these murine cell lines and human neuroblastoma tumors. In vivo, these cell lines form aggressive tumors that lead to mortality in mice both after subcutaneous implantation of $1.0\text{--}2.5 \times 10^6$ cells, but also after orthotopic implantation of 10-fold fewer cells in the adrenal gland. Collectively, these studies provide important new high throughput tools for investigation of the microenvironment of murine neuroblastoma tumors, and novel model systems for the preclinical discovery of new agents for the treatment of neuroblastoma in children.

TAG, A Cancer/Testis Antigen, Is Widely Expressed in Human Cancers of Diverse Histological Type

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Although immunization of melanoma patients with tumor antigens is beginning to show promising results, it is likely that truly effective therapy will require vaccination with multiple peptides derived from different proteins and with peptides that target multiple class I MHC molecules. Cancer/testis antigens, which are expressed in a variety of tumors, but not in normal tissue except the testis and placenta, are particularly promising tumor vaccine candidates. Using melanoma-reactive cytotoxic T lymphocytes, we have recently identified a new cancer/testis antigen termed TAG which gives rise to an HLA-A3-restricted epitope (RLSNRLLLR). TAG was cloned and identified using 5' and 3' RACE, and was localized to chromosome 5. The gene coding for TAG has multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) and is expressed in 84–98% of the melanoma lines tested. The TAG protein is coded for in an open reading frame that is initiated by one of three nonstandard initiation codons, and the nucleotide sequence coding for the RLSNRLLLR peptide crosses an exon-exon boundary. The TAG gene has homology to two chronic myelogenous leukemia-derived clones and a hepatocellular carcinoma clone in the human expressed sequence tags (EST) database. TAG is not expressed in B- or hybrid T-B-lymphoblastoid cell lines, although all four isoforms are expressed in K562, a myelogenous leukemia cell line. One or more of the TAG genes has also been shown to be expressed by PCR in four of nine (44%) lung cancer cell lines, one of eight (13%) breast cancer cell lines, one of three (33%) ovarian cancer lines, seven of seventeen (41%) ovarian cancer clinical isolates, two of five (40%) colon cancer cell lines, zero of four brain cancer cell lines (0%), and three of nine (33%) gastric cancer cell lines. To characterize TAG expression at the protein

level, the TAG-1 and TAG-2a genes have been cloned into the pET100/D vector for expression as His-tagged fusion proteins. Experiments are presently in progress to determine optimal conditions for the expression and purification of the fusion proteins. The purified proteins will then be used for the production of TAG-specific antisera, and the antisera will be used to assess TAG protein expression in tumor lines expressing the TAG genes. The characterization of TAG expression at both the mRNA and protein levels in different tumors will allow us to determine the extent to which TAG will serve as a useful antigenic target. (Supported by NIH/NCI CA90815)

Identification of Cytotoxic T Lymphocyte Epitopes Derived From the Cancer/Testis Antigen, TAG

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Cancer/testis antigens are excellent candidates for inclusion in cancer vaccines as they are naturally immunogenic, are expressed in a high percentage of tumors of diverse histological origin, and their expression in normal tissue is limited to the testis and placenta. We have recently identified a new cancer/testis antigen termed TAG which is expressed as multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) at the mRNA level. TAG is known to be immunogenic as it gives rise to an HLA-A3-restricted epitope (RLSNRLLLR) that is recognized by cytotoxic T lymphocytes (CTL) that naturally occur in a melanoma patient. To determine if TAG gives rise to additional epitopes recognized by CTL we have used two predictive algorithms (SYFPEITHI [www.syfpeithi.de] and Parker [bimas.cit.nih.gov/molbio/hla_bind]) to identify those peptides that have a high predicted binding affinity for HLA-A1, -A2, -A3, -B7, -B8, and -B44. The top three to five predicted binders for each algorithm/HLA combination have been synthesized and are being tested for their ability to elicit tumor reactive CTL from healthy blood donors. Donor lymphocytes are initially stimulated with peptide-pulsed, autologous, mature dendritic cells (mDC), and are then restimulated thereafter on a weekly basis with peptide-pulsed stimulator cells (autologous mDC, autologous peripheral blood mononuclear cells, or class I MHC-matched allogeneic B-LCL). Following a total of four stimulations, the cultures are tested for reactivity against peptide-pulsed, class I MHC-matched target cells. Using this protocol, CTL responses have developed in response to SLGWLFLL peptide in association with HLA-A2 and LRSNRLL peptide in association with HLA-B8, but not to SRDPPASAS peptide in association with HLA-A1, LLLLNSTTK peptide in association with HLA-A3, or LPASTLSRL peptide in association with HLA-B7. SLGWLFLL reactive CTL also lyse the melanoma line DM6 (HLA-A2⁺, TAG⁺) indicating that the SLGWLFLL peptide is naturally processed. Experiments are in progress to further characterize the CTL response to SLGWLFLL and LRSNRLL, and to determine the immunogenicity of additional TAG-derived peptides. (Supported by DOD W81XWH-05-1-0012 and NIH/NCI CA90815)

The Anti-Tumor Effect of Apo2L/TRAIL on Patient Pancreatic Adenocarcinomas Grown as Xenografts in SCID Mice

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Considerable effort has been devoted to identifying molecules that can induce death of tumor cells while sparing normal cells. In this regard, Apo2L/TRAIL shows considerable promise for cancer therapy since this member of the tumor necrosis factor family induces apoptosis in a large number of human tumor cell lines, both in vitro and in vivo, while normal cells are not similarly susceptible. Furthermore, in many cells, when Apo2L/TRAIL is combined with chemotherapy, the effect is synergistic. We are investigating the Apo2L/TRAIL sensitivity of patient-derived tumors using a patient tumor xenograft/ SCID mouse model. We have found that pancreatic tumors grown as xenografts exhibit a range of sensitivity to Apo2L/TRAIL. While both sensitive and resistant tumors have been identified, other tumors show a heterogeneity of response. Changes in apoptotic signaling molecules in a sensitive tumor were analyzed by Western blot following Apo2L/TRAIL

proinflammatory cytokines such as IL-1 β , IL-2, or IL-6 in CM of PBLs have no role in induction of TGF- β 1. A role of TNF- α evaluated in TGF- β 1 expression. Exogenous recombinant TNF- α (5 ng/mL) induced TGF- β 1 expression in cancer cell lines, and when it is combined with IFN- γ , the effect is synergistically enhanced. Moreover, exogenous IFN- γ and TNF- α -induced endogenous TNF- α mRNA level in tumor cells, this autocrine loop also may have a role.

Our data demonstrate that tumor-derived TGF- β 1 is induced by proinflammatory cytokines IFN- γ and TNF- α . These findings suggest that some immune cells can induce TGF- β 1 overexpression in tumor cells, leading to immune evasion.

Adoptive Immunotherapy of Malignancies With Gene Modified, EBV-specific, Cytotoxic T-Cell Lines

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Immunotherapy has long been suggested as an option for the treatment of cancer. The clinical and commercial successes of monoclonal antibodies means that there are now convincing data to show significant tumor responses in a substantial number of treated patients. Monoclonal antibodies, however, represent only one arm of the immune system. The effectiveness of donor lymphocyte infusion for treating relapse after stem cell transplantation has encouraged further investigation aimed at exploiting T-cell immunity against cancer. A cellular immune response may have superior ability to kill malignant cells and improved biodistribution (transit through multiple tissue planes), as well as increased long-term persistence. Moreover, improvements in cellular biology and our ability to manipulate gene expression in cells of the immune system have facilitated and enhanced efforts aimed at developing T-cell therapies for malignancy.

This presentation will describe how adoptively transferred T cells can be used in the therapy of both hematological and epithelial malignancy. Although many challenges remain, significant response rates (including > 50% sustained CR in relapsed and primary resistant EBV+ lymphoma and nasopharyngeal carcinoma) are now obtained, even in patients with relapsed or primary resistant disease. These CTL can be further modified with chimeric antigen receptors (CARs) to provide them with dual specificities, for EBV-infected and for tumor targets. In preclinical studies such CTL have superior persistence and effector function against malignant cells than CAR expressing primary T cells, apparently because of the costimulation they receive when their native receptor is engaged by EBV-expressing target cells. Our current clinical study in patients with neuroblastoma, confirms the *in vivo* superiority of CAR-CTL over CAR-T cells and indicates a means by which T-cell therapy for cancer can be improved.

5-AZA-2'-deoxycytidine Treatment Increases Expression of Tumor Associated Antigens in Human Melanoma

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We previously established that coordinated loss of several melanocytic antigens in human melanomas is generally attributable to down-regulation of antigen gene expression rather than irreversible deletion or mutation. More importantly, we have shown that distinct agents can enhance antigen expression in melanomas. Our previous publications have shown that either inhibitors of MAPK signaling pathways or treatment with the cytokine interferon-beta (IFN- β) can up-regulate tumor antigen expression in melanoma cell lines, leading to increased killing by cytotoxic T cells specific for Melan-A/MART-1. We have now identified 5-aza-2'-deoxycytidine as an additional agent that can increase antigen expression in melanomas, including both differentiation antigens such as Melan-A/MART-1 and gp100, as well as the cancer-testis antigen, MAGE-A1. 5-aza-2'-deoxycytidine (5-aza) is a nucleoside analog with DNA methyl-transferase inhibitor activity. 5-aza treatment of 3 different antigen-negative tumor cell lines for 4 to 7 days augmented both protein and mRNA expression of several antigens in all of the cells tested to date. Of note, antigen increases were more pronounced in the antigen-negative tumor cells than in several antigen-positive tumor cells. The antigen-

enhancing effects of the 5-aza could be attributed to the master melanocytic regulator MITF-M, which was shown to increase upon treatment. This may explain why we failed to see as large an effect of 5-aza on melanoma cell lines with high constitutive levels of MITF-M and melanoma-specific gene expression. Demethylation of the promoters or induction of DNA damage stress pathways could also be involved. Combination of 5-aza and IFN- β treatment of cells with low levels of melanoma antigen expression (antigen-negative cell lines) caused melanoma differentiation antigens to increase to levels comparable to that seen in constitutively antigen-positive cell lines. While many immunotherapeutic investigations have focused on improving the effector limb of the antitumor response, few studies have addressed the loss of tumor-associated antigen expression, associated with immune escape by tumors. Heterogeneous expression of melanocytic antigens occurs frequently in melanomas and represents a potent barrier to immunotherapy. Treatment of melanomas with 5-aza, particularly in combination with such agents as IFN- β , may assist the immune system's ability to kill tumor cells because of the increased levels of target antigens.

Immunological Characterization of Eleven Human Ovarian Cancer Cell Lines

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The study of the cellular immune response to ovarian cancer requires the use of well-characterized tumor cell lines. To develop such a panel of cell lines, 11 ovarian cancer cell lines were characterized for the expression of epithelial cell markers, class I and class II major histocompatibility complex (MHC)-encoded molecules, 15 tumor antigens (MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, NY-ESO-1, TAG-1, TAG-2a, TAG-2b, TAG-2c, Her-2/neu, folate-binding protein, and carcinoembryonic antigen), and immunosuppressive cytokines [transforming growth factor β (TGF- β) and IL-10]. Each of the ovarian cancer cell lines expresses cytokeratins, although each cell line does not express the same cytokeratins. One of the lines expresses CD90 which is associated with a fibroblast lineage. Each of the cell lines expresses low to moderate amounts of class I MHC molecules, and several of them express low to moderate amounts of class II MHC molecules. Treatment of the cell lines with 1 μ M 5'-aza-2'-deoxycytidine (DAC) for 72 hours, a treatment that can up-regulate the expression of class I and II MHC molecules in other malignancies, did not reproducibly up-regulate the expression of these molecules on the 11 ovarian cancer cell lines. Using a combination of PCR and flow cytometry, it was determined that each cell line expressed between 6 and 13 of 15 antigens tested. Although *in vitro* treatment of other malignancies with DAC has been shown to up-regulate the expression of a variety of cancer/testis antigens, only the expression of NY-ESO-1 was reproducibly up-regulated in a majority of the cell lines tested. TGF- β 1 was produced by 3 of the cell lines, TGF- β 2 was produced by all of the cell lines, with 4 of the cell lines producing large amounts of the latent form of the molecule, and little to no TGF- β 3 was produced by any of the cell lines. SW626 was the only cell line that produced IL-10. These results demonstrate that each of the 11 ovarian cancer lines is characterized by a unique expression pattern of epithelial/fibroblast markers, MHC molecules, tumor antigens, and immunosuppressive cytokines. The results further demonstrate that unlike other malignancies, treatment of ovarian cancer cells with DAC may not lead to an increase in class I MHC expression, class II MHC expression, and the expression of many cancer/testis antigens. This knowledge increases the utility of these cell lines for use in antigen identification experiments and suggests that therapeutic treatment of ovarian cancer patients with DAC may not be useful in enhancing the expression of antigens targeted in vaccines designed to stimulate a cytotoxic T lymphocyte response.

Development of an Immunocompetent Mouse Model of Ovarian Cancer

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