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13. ABSTRACT ***(Maximum 200 Words):*** Recent clinical successes have fostered renewed optimism among tumor immunologists. We exploited recent advances to identify immunogenic peptides from "self" proteins and to present those peptides to generate breast tumor-specific $CD8^+$ killer T cell (CTL) *in vitro*. Two target proteins, the aryl hydrocarbon receptor/transcription factor (AhR) and the AhR-regulated cytochrome P450 CYP1B1 enzyme, were selected for these studies because of their high level expression in breast tumors and their role in environmental chemical carcinogenesis. Three computer-based algorithms identified AhR- and CYP1B1 peptides predicted to bind a common human HLA class I allele, HLA-A*0201. HLA stabilization experiments confirmed high affinity binding of several AhR and CYP1B1 peptides to HLA-A*0201. Presentation of these peptides on activated B cells to autologous human $CD8^+$ T cells *in vitro* induced CTL which lysed tumors expressing AhR or CYP1B1 in an antigen- and HLA-class I-specific manner. CTL frequency analysis with fluorescent peptide/HLA-A*0201 tetramers indicated that 1%-4% of the $CD8^+$ T cell population generated *in vitro* was responsible for the killing activity. Enrichment of the peptide/HLA-A*0201-specific T cells significantly enriched the killing activity. The data demonstrate the feasibility of developing breast cancer vaccines with a platform that combines bioinformatics and advanced strategies for tumor antigen presentation.

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Introduction:

Immunotherapy has long been viewed as an important approach to cancer treatment. While recent clinical successes have been encouraging, several technical and theoretical limitations remain. To circumvent some of these limitations, our efforts have been directed toward *in vitro* induction of human tumor-specific cytotoxic T cells (CTL) for adoptive T cell transfer followed by tumor-specific vaccination. We have shown that adoptive transfer of CTL induced with CD40 ligand (CD40L)-activated whole leukemic cells is a practical, safe, and potentially effective approach to human cancer treatment. However, a similar whole cell immunogen approach to breast cancer is problematic since breast epithelial cells cannot be made immunogenic without extensive modification by gene transfection. Consequently, we have used peptides derived from breast tumor-associated proteins to generate tumor-specific CTL. These studies represent an extension of our previous work in which it was demonstrated that breast tumors express extremely high levels of the aryl hydrocarbon (dioxin) receptor/transcription factor (AhR) and AhR-regulated CYP1B1 (cytochrome P450-1B1). The AhR contributes to dysregulated cell growth and both proteins play a role in environmental chemical-induced cancer. Immunogenic human AhR and CYP1B1 peptides, predicted with complementary peptide-MHC class I binding algorithms, were loaded onto CD40L-activated B cells for stimulation of autologous $CD8^+$ T cells. The resulting T cell lines specifically killed cognate peptide-loaded target cells and a variety of AhR^+ and $CYP1B1^+$ tumor lines in an MHC-restricted fashion. Given these results, we hypothesized that breast cancer eventually can be treated by induction of CTL specific for a spectrum of peptides derived from AhR-related, tumor-associated proteins.

Body:

Please refer to the attached manuscript with bibliography for a more detailed description of experiments carried out with CYP1B1 peptides under the auspices of the DOD Concept Award.

CYP1B1 protein is highly expressed in malignant but not normal cells

CYP1B1 has been implicated in carcinogenesis by environmental carcinogens, as well as endogenous estrogen-related carcinogenesis in human breast and uterine tumors. Immunohistochemistry of tissue sections for CYP1B1 protein has previously shown that >95% of human tumors stained positive. Within a series of human breast and ovarian carcinomas, the expression of CYP1B1 was limited to the malignant cells and was not detectable in stromal cells or infiltrating lymphocytes in the same specimen.

We extended the analyses of CYP1B1 protein expression through *in situ* and *in vitro* studies. Breast, ovarian, and colon carcinoma (Attached manuscript, Fig. 1 a) demonstrated high to very high CYP1B1 staining in neoplastic cells while stromal compartments and surrounding normal tissue were negative. Significant CYP1B1 protein over-expression was observed in all cancer cell lines and primary tumor specimens as compared to normal adjacent tissue (Attached manuscript, Fig. 1b and c and data not shown). These data support the contention that CYP1B1 is a nearly universal tumor antigen with limited expression in normal cells. Considering that CYP1B1 is not expressed in the most essential normal tissues and that homogenous high intensity staining of all tumor samples significantly exceeded expression in normal tissue samples, we proceeded to evaluate CYP1B1 as a candidate universal tumor antigen.

Identification of immunogenic peptides from CYP1B1

Three computational epitope prediction algorithms (BIMAS, SYFPEITHI and LPpep) were applied to CYP1B1 with respect to HLA-A*0201 restriction to determine a candidate list of possible high affinity binding peptides. Of the ten most likely candidates, the CYP239 epitope consistently showed the highest binding affinity in a cellular binding assay (Attached manuscript, Table 1). Therefore, CYP239 was chosen for further analysis.

CYP239- reactive T cells exist in the repertoire of healthy volunteers and cancer patients

In an autologous system, we expanded peptide-specific T cells through multiple rounds of stimulation with CYP239 peptide presented on antigen presenting cells (APC). In over 70% of the healthy individuals

tested, CYP239-specific $CD8^+$ T cells were generated that specifically lysed peptide-pulsed T2 (Attached manuscript, Fig. 2a and 2b) or autologous CD40-activated B cells (Figure I). CYP239-specific T cells were successfully expanded *in vitro* from 8/9 patients. T cell lines were peptide-specific since target cells loaded with irrelevant peptides were not lysed (Attached manuscript, Fig. 2a). HLA-restriction was demonstrated by the lack of lysis of HLA-A2 mismatched target cells (data not shown). Peptide/HLA-A2 tetramers containing CYP239 were synthesized to analyze the frequency of peptide-specific CTL in the expanded T cell lines. Between 0.5 and 3.0% of all $CD8^+$ T cells expanded with the CYP239 peptide stained with the CYP239 tetramer but not with a control tetramer (Attached manuscript, Fig. 2d).

Figure I: CYP239-specific $CD8^+$ T Cells Selectively Kill Cognate Peptide-Pulsed Target Cells

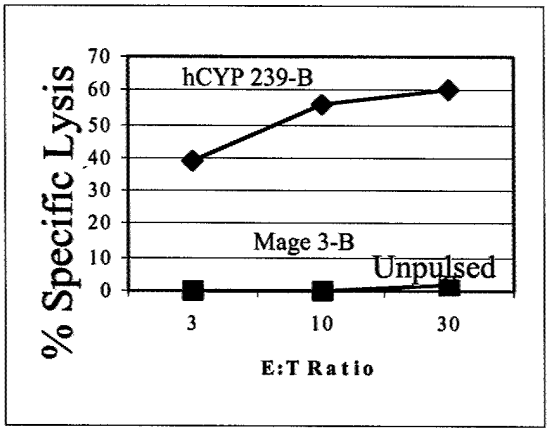


Figure II: CYP 239-specific $CD8^+$ T Cells Selectively Kill $AhR^+/CYP1B1^+$, $HLA\text{-}A2^+$ Human Tumors

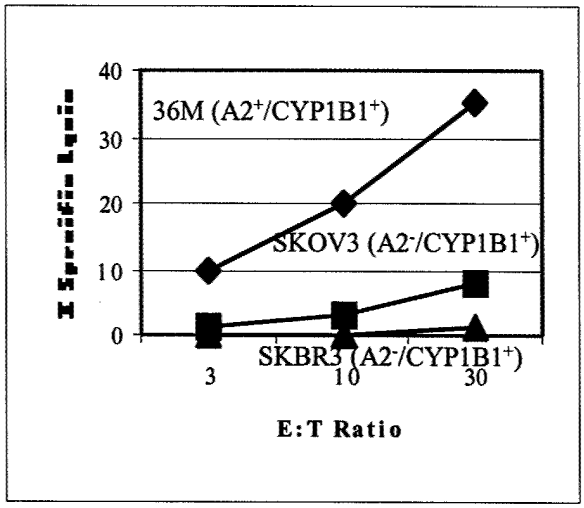
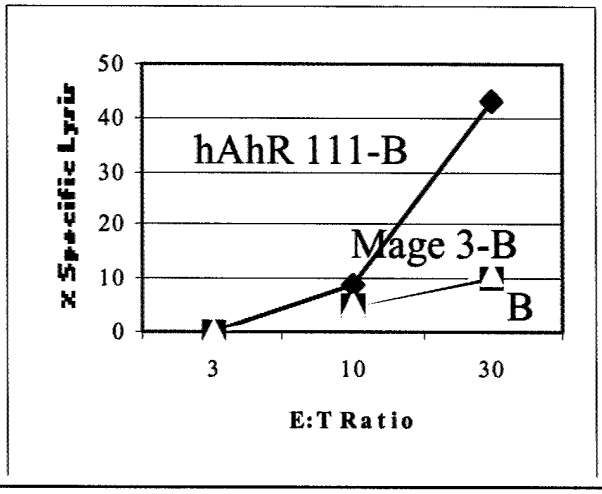


Figure III: Human AhR 111-specific $CD8^+$ T Cells Selectively Kill Cognate Peptide-Pulsed Target Cells



Recognition of endogenously processed CYP239 peptide

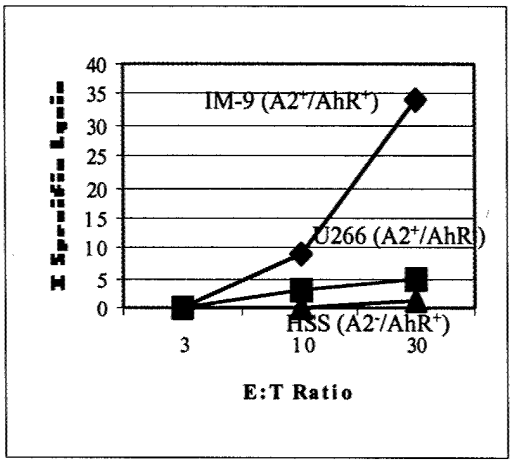
To determine if tumor cells process and express CYP239 peptide in association with MHC class I and in a form recognizable by CYP239-specific CTL, CTL were induced *in vitro* and tested for their ability to lyse human tumors. As exemplified in Fig. 3c of the attached manuscript, a variety of tumor cells including multiple myeloma (U266), ovarian carcinoma (36M), melanoma (K029), and EBV-transformed lymphoid cell lines (IM-9) were lysed by CYP1B1-specific CTL. HLA-A2 negative tumor cell lines were not killed (Figure II). These data demonstrate the feasibility of targeting the CYP1B1 protein for immunotherapy.

Identification of Immunogenic peptides from AhR

As for CYP1B1 peptides, 3 algorithms were used to predict human AhR peptides likely to bind HLA-A*0201. One peptide, AhR 111, was used to induce peptide-specific human CTL as described for the CYP 239 peptide. Significant CTL activity was observed when AhR 111-educated T cells were tested for their ability to lyse cognate (i.e. AhR 111) peptide-pulsed B cells (Figure III) or untreated tumor cell lines (Figure

IV) in an HLA-A2-restricted fashion. These data demonstrate the feasibility of targeting the human AhR for immunotherapy.

Figure IV: Human AhR111-specific $CD8^+$ T Cells Selectively Kill $AhR^+/CYP1B1^+$, $HLA\text{-}A2^+$ Human Tumors



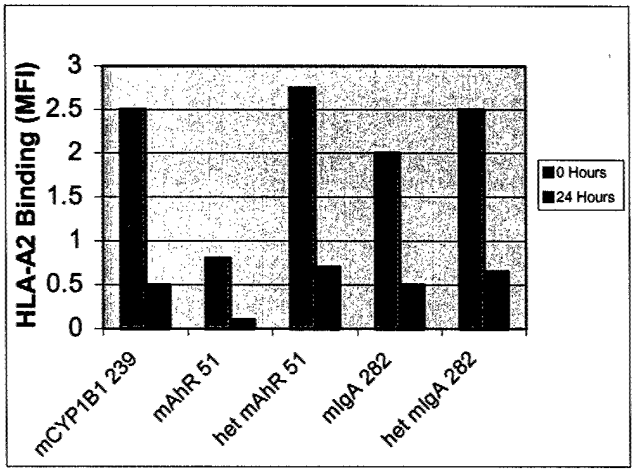
Identification of murine AhR and CYP1B1 peptides for development of a mouse model for breast cancer immunotherapy

Despite the production of human CYP1B1 and AhR-specific CTL, the above studies cannot address the potential for pathologic autoimmune responses or treatment efficacy *in vivo*. To this end we have begun to develop an animal model for AhR and CYP1B1-specific immunotherapy by attempting to identify peptides from murine AhR and CYP1B1 predicted to bind human HLA-A*0201 expressed in HLA-A2 transgenic mice. Indeed, several candidate peptides were identified (Table I). Furthermore, potentially immunogenic peptides from another endogenous protein, IgA, have similarly been postulated. (IgA-derived peptides were evaluated to provide the opportunity to demonstrate breaking of tolerance to an abundant "self" protein). In addition, we predicted the sequence of "heteroclitic" peptides which would be expected to bind HLA-A2 with a higher affinity than their native counterparts. All murine peptides were synthesized and tested for HLA-A2 binding in the T2 cell binding assay. The murine AhR 51 and CYP239 peptides were shown to bind HLA-A2 as predicted (Figure V).

Table I: Potentially Immunogenic Peptides Derived from Murine AhR, CYP1B1, and IgA

	Y	
	Y Y	
	Y Y	

Figure V: Binding of Predicted Mouse Peptides to HLA-A2 (T2 Binding Assay)



Key Research Accomplishments:

1. Demonstration of high level expression of AhR and CYP1B1 in rodent and human breast carcinomas
2. Identification of human AhR- and CYP-1B1-derived, HLA-A2 binding peptides
3. Standardization of a system for expanding normal B cells as antigen-presenting cells *in vitro*
4. Confirmation of the ability to induce peptide-specific CTL responses *in vitro*
5. Demonstration of the ability of $CD8^+$ T cells from cancer patients to recognize and respond to AhR and CYP1B1-derived peptides.
6. Establishment of tetramer technology for identifying AhR and CYP1B1 peptide/HLA-A2-specific CTL

7. Identification of corresponding murine tumor peptides

Reportable outcomes:

Presentations and Publications

Submission of manuscript entitled "Human Cytochrome P450 1B1, A Potential Universal Tumor Antigen" by Drs. Maecker, Sherr, Vonderheide, von Bergwelt-Baildon, Hirano, Anderson, Xia, Butler, Wucherpfennig, O'Hara, Cole, Ramstedt, Tomlinson, Chicz, Nadler, and Schultze.

Symposium presentation at ERA of Hope meeting, Orlando, FLA ("The aryl hydrocarbon (dioxin) receptor and CYP1B1 as targets for breast cancer immunotherapy" by Drs. Maecker, Flies, Anderson, Schultze, and Sherr)

Funding resulting from Concept Award:

NHLBI PO1 program project, Project 4 ("A novel strategy for AL amyloid immunotherapy") $200,000 direct costs/year x 5 years

Conclusions:

The studies supported by the DOD Concept Award demonstrate for the first time that proteins related to cellular responses to environmental chemicals, i.e. the AhR and CYP1B1, are highly expressed in mammary tumors. As a consequence, they can be considered viable targets for cancer immunotherapy. Our ability to break immune tolerance to peptides derived from these "self" antigens, demonstrates the feasibility of inducing effective, tumor-specific killer T cell responses to human cancers. Furthermore, the technology employed to generate these immune responses is adaptable for generating animal models of tumor vaccination and are readily scalable for human trials.

References:

See accompanying manuscript.

Appendices:

Manuscript: "Human Cytochrome P450 1B1, A Potential Universal Tumor Antigen" by Drs. Maecker, Sherr, Vonderheide, von Bergwelt-Baildon, Hirano, Anderson, Xia, Butler, Wucherpfennig, O'Hara, Cole, Ramstedt, Tomlinson, Chicz, Nadler, and Schultze

Human Cytochrome P450 1B1, A "Credentialled" Universal Tumor Antigen

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Running Title: The widely expressed tumor antigen CYP1B1

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Keywords: Tumor antigen - Cytochrome P450 - Tumor - CTL

Abstract

Although many patients demonstrate immune recognition of tumor antigens (TA), the role of TA in the prevention, rejection, and treatment of established human cancers remains questionable. Failure of the tumor bearing host to mount a clinically significant immune response may be due in part to the status of the host's immune system or, alternatively, to the nature of target rejection TA. We proposed that a "credentialled TA" must be over-expressed on most, if not all, cancers with limited expression on normal essential tissues; be strongly and homogeneous expressed on all tumor cells; be central to the neoplastic process; and be processed and presented to a responsive T cell repertoire in the cancer bearing patient. Here, we present evidence that cytochrome P450 1B1 (CYP1B1), an enzyme involved in activation of environmental carcinogens, meets these criteria of credentialling. CYP1B1 is over-expressed on all human tumors examined with limited expression on normal essential tissues. CYP1B1 displays strong and homogeneous expression on all cells within a tumor. CYP1B1 specific peptides are highly expressed in the groove of HLA-A2 positive tumor cells. Although neither normals nor tumor-bearing patients have detectable CYP1B1 responsive T cells in their peripheral blood, CYP1B1-specific T cells capable of lysing tumor cells have been generated from most normals and cancer patients demonstrating that CYP1B1 is both processed and presented to a responsive T cell repertoire. Autoimmunity was not observed in animal models following immunization with CYP1B1 peptides. Thus, we propose that CYP1B1 is the first credentialled universal tumor antigen. Further validation of this contention can only be addressed through clinical experimentation.

Introduction

Ever increasing numbers of potential human tumor associated antigens (TA)[1-6] have been discovered employing either tumor-specific T cell clones[7] or auto-antibodies[8,9] recognizing the tumor target followed by molecular cloning of the corresponding gene. Alternatively, a number of potential TA have been identified by elution of epitopes from tumor cell HLA[10] or by analysis of sequences corresponding to neoplastic pathogenetic chromosomal translocations[11,12], tumor-associated mutations[13,14], and molecular rearrangements of T and B cell receptors[15-17]. Translational strategies employing these TA to either vaccinate or generate TA-specific T cells for adoptive transfer have shown that the majority of TA evoke detectable cellular and serologic immune responses in most patients. Mixed clinical responses are observed to a variable degree depending on the trial design, and a significantly smaller number of patients experience clinically significant anti-tumor responses[17-19].

Despite these successes, strategies to induce antigen-specific anti-tumor immunity have yet to become the standard of care for human cancer. Several obstacles contribute to these limited successes. First and foremost, is the capacity of most neoplastic cells to mutate genes that are non-essential to the neoplastic process resulting in heterogeneous TA expression. This continuous process results in tumor cells capable of evading TA-specific T cells thereby leading to the outgrowth of immune escape antigen loss-variants[20]. In both vaccine and adoptive TA-specific T cell therapy this obstacle has repeatedly been shown to be operative with either transient, partial, or absent clinical responses[20-22]. Second is the observed failure in the majority of patients of detectable TA-specific T cells to sufficiently expand and differentiate into functional effectors which might generate clinically significant anti-tumor immune responses. Inability of these T cells to evoke and sustain robust anti-tumor immunity even in patients with minimal disease burden suggests that endogenously generated anti-tumor specific T cells are either non-functional or are negatively regulated. Finally, generalized applicability of these strategies has been significantly hampered by restricted expression of many TA to a limited number of diseases and histologic subtypes.

An approach to overcome these obstacles would be to identify TA that are homogenously expressed in the majority of tumors, resistant to the generation of antigen-loss variants, and not subject to ongoing down-regulatory influences interfering with the development of a robust anti-tumor immune response. Such a "credentialled" TA would need to meet the following criteria: (1) over-expression in the great majority of all cancers, (2) stable expression throughout the malignant transformation, (3) function of the gene critical to the neoplastic process, (4) peptide sequences capable of being presented in the context of HLA, (5) a functional and expandable TA-specific T cell repertoire, and (6) absence of any regulatory mechanisms preventing the induction and amplification of immunity to this specific TA. We suggest that two complementary approaches

will have the highest likelihood of identifying optimal candidates. First, candidate genes are identified by systematically analyzing data obtained from cancer genetics, genomics and biology. Once potential candidates are identified that are expressed in the majority of cancers with little or no expression in normal tissues they must be analyzed for their capacity to generate anti-tumor-specific T cell-mediated immunity. The demonstration of T cell function does not definitively prove that the specific antigen is processed and presented. Plasticity of antigen-specific T cells and their capacity to recognize multiple antigens has been demonstrated in many experimental systems[23,24]. Therefore, while 'reverse immunology' is rapid and robust, confirmation that the putative candidate antigen is truly presented by the tumor cell requires biochemical proof. In this approach, peptides are directly eluted from tumor cell HLA molecules followed by subsequent sequencing and identification of the full-length gene[10,25]. Again, it is necessary to demonstrate that T cells exist in the repertoire capable of responding to such biochemically defined epitopes. While this approach provides proof of presentation of TA-derived epitopes on tumor cells, it is limited by the need to analyze significant quantities of both tumor and normal tissues. Therefore, the combination of the above two approaches yields the highest likelihood of defining a universal TA meeting the stringent criteria proposed above.

In the present report we have used the above strategies and identified the cytochrome P450 1B1 (CYP1B1) as a potential universal TA. CYP1B1 is an enzyme expressed early during malignant transformation and mediates the activation of environmental carcinogens. Its nearly uniform expression on human tumors, absence of heterogeneity within a single cancer, minimal expression on critical normal tissues, and the demonstration that antigen-specific T cells can be generated and function in both normal and cancer patients makes this an attractive candidate to "credential as a universal TA."

Results

CYP1B1 protein is highly expressed in malignant but not normal cells

In an attempt to identify genes that are widely expressed in lung, breast, colon, skin and lymphoid tumors, but not in normal tissue, we screened CGAP genomic databases. Using the 'Differential Gene Expression Displayer' on CGAP, which allows comparison of RNA expression between normal and malignant tissues, we identified >700 genes that were expressed in up to three of the above tumor types but not in normal tissue. However, as of September 13, 2001, using these stringent criteria no single gene fulfilled our requirement of being expressed in all five tumor types but not in normal tissue.

In contrast, a keyword search ('lung', 'breast', 'colon', 'skin', 'lymph node', 'cancer', 'protein') of the PubMed database identified three potential widely expressed candidate genes. Two of these genes have been excluded because of excessive normal tissue expression. Therefore, only the cytochrome P450 1B1 (CYP1B1) fulfilled these stringent selection criteria[26]. CYP1B1 has been implicated in carcinogenesis by environmental carcinogens[27,28] as well as endogenous estrogen-related carcinogenesis in human breast and uterine tumors[29]. Immunohistochemistry of tissue sections for CYP1B1 protein has previously shown that >95% of human tumors stained positive but no non-malignant tissues[26]. Within a series of human breast[30] and ovarian[31] carcinomas, the expression of CYP1B1 was limited to the malignant cells and was not detectable in stromal cells or infiltrating lymphocytes in the same specimen.

We extended the analysis of CYP1B1 protein expression through *in situ* and *in vitro* studies. Breast, ovarian, and colon carcinoma (**Fig. 1 a**) demonstrated high to very high CYP1B1 staining in neoplastic cells while stromal compartments and surrounding normal tissue were negative. Significant CYP1B1 protein over-expression was observed in all cancer cell lines and primary tumor specimens compared to normal adjacent tissue (**Fig. 1b and c** and data not shown). To resolve the recent controversy surrounding CYP1B1 expression in normal tissues[26,30-38], 32 different tissues (three specimen each) derived from autopsy material from otherwise healthy patients who died of trauma were screened (**Fig. 1d**). CYP1B1 protein could be detected at high intensity in 80% to 90% of tissue specific cells in fallopian tube showing an apical cellular distribution, and at intermediate intensity in 70% to 100% of all ureter and breast epithelial cells with strong membrane and cytoplasmic staining in scattered cells. Normal tissue from uterus showed very heterogeneous expression with the majority of tissue specific cells (between 20-40%) showing low intensity membrane staining. Some low to intermediate intensity staining was observed in prostate and skin in all three samples (2-30% of tissue specific

cells). Weak staining was observed for pancreas (20% of tissue specific cells in 2 of 3 samples), pituitary (50% in 1 of 3), colon (40% in 1 of 3), bladder (10% in 1 of 3), small intestine (20% in 1 of 3), and thymus (10% in 1 of 3). All other tissues were negative for CYP1B1. Although CYP1B1 did not fulfill our most stringent criteria, the above data supports the contention that CYP1B1 was a nearly universal TA with limited expression in normal cells. Considering that CYP1B1 was not expressed in the most essential normal tissues and that homogenous high intensity staining of all tumor samples significantly exceeded expression in normal tissue samples, we proceeded to evaluate CYP1B1 as a candidate universal TA.

Identification of immunogenic peptides from CYP1B1

Three computational epitope prediction algorithms (BIMAS, SYFPEITHI and LPpep) were applied to CYP1B1 with respect to HLA-A*0201 restriction to determine a candidate list of possible high affinity binding peptides. Of the ten most likely candidates, the CYP239 epitope consistently showed the highest binding affinity (FI) in a cellular binding assay (**Table 1**), while the peptide/HLA-A*0201 complex stability ($t_{1/2}$) was the highest for CYP190. These results were confirmed by affinity measurements to recombinant HLA-A*0201 (IC_{50}) using the established inhibition affinity assay[60] (**Table 1**). Based on these criteria CYP190 and CYP239 were chosen for further analysis.

CYP239 and CYP190 reactive T cells exist in the repertoire of healthy volunteers and cancer patients

The first critical step to credential a candidate TA is the demonstration of peptide-specific T cells in the repertoire. Once identified, such T cells are essential as readout for the analysis of endogenous processing and presentation of antigen-derived peptides. In an autologous system, we expanded peptide-specific T cells through multiple rounds of stimulation with either CYP190 or CYP239 peptide presented on antigen presenting cells (APC). In over 70% of the healthy individuals tested, CYP190 or CYP239-specific $CD8^+$ T cells were generated that specifically lysed peptide-pulsed T2 (**Fig. 2a and 2b**) or autologous CD40-activated B cells (data not shown). Specific T cells were successfully expanded *in vitro* from 2/2 cancer patients against CYP190, and from 8/9 patients against CYP239. T cell lines were peptide-specific since target cells loaded with irrelevant peptides were not lysed **(Fig. 2a)**. HLA-restriction was demonstrated by the lack of lysis of HLA-A2 mismatched target cells (data not shown). Avidity of CTL lines was estimated in peptide titration studies (**Fig. 2c**) indicating that CYP190 and CYP239-specific CTL were of at least intermediate avidity[39]. Peptide/HLA-A2 tetramers containing CYP190 or CYP239 were synthesized to analyze the frequency of peptide-specific CTL in the expanded T cell lines. Between 0.5 and 3.0% of all $CD8^+$ T cells expanded with the CYP239 peptide stained with the CYP239 tetramer but not with a control tetramer (**Fig. 2d**). These results are comparable to previously published data for gp100[40] or proteinase-3[41] specific CTL lines. CYP190-specific CTL could not be detected using the CYP190 tetramer, instead epitope-specific CTL were enumerated by IFN-γ ELISPOT assay (1 to 1.5%, data not shown). A similar dissociation between CTL effector function and TCR-peptide/MHC complex half-life has been recently reported for tyrosinase-specific T cells[42].

Lack of an expanded pool of CYP1B1-specific CTL *in vivo*

Except for one patient with multiple myeloma (MM, 0.12%, **Table 2**) we were unable to detect CYP239-specific CTL above background (cut off 0.09%) in HLA-A*0201 positive cancer patients or healthy individuals. Moreover, the frequency of CYP239-specific T cells did not increase upon a single *ex vivo* stimulation with the cognate peptide (data not shown) again suggesting a very low precursor frequency of CYP1B1-specific T cells. Results obtained by tetramer staining were confirmed by ELISPOT analysis for IFN-γ in a subset of patients and healthy individuals (n=20, data not shown). Similarly, IFN-γ ELISPOT and tetramer analysis failed to detect CYP190-specific T cells in healthy individuals or cancer patients (n=12, data not shown).

Recognition of endogenously processed CYP190 and CYP239

Three independent approaches were applied to evaluate recognition of endogenously processed CYP1B1-derived peptides by human CTL: 1) CTL-mediated lysis of COS cells transfected with HLA-A2*0201 and minigene constructs containing the CYP190 (aa173-205) and CYP239 (aa213-352) epitopes, 2) CTL-mediated cytotoxicity against dendritic cells (DC) infected with a vaccinia construct encoding full length CYP1B1 cDNA, and 3) lysis of HLA-matched tumor cell lines and primary tumors. CYP190 and CYP239-specific CTL showed significant lysis of CYP1B1 minigene-transfected COS cells compared to vector control transfected

COS cells (**Fig. 3a**). CYP239-mediated lysis was further confirmed by lysis of HLA-A*0201 positive DC expressing full length CYP1B1 (**Fig. 3b**). As exemplified in **Fig. 3c** a variety of tumor cells including multiple myeloma (U266), ovarian carcinoma (36M), melanoma (K029), and EBV-transformed lymphoid cell lines (IM-9) were lysed by CYP1B1-specific CTL. HLA-A*0201 positive normal monocytes used as controls were not lysed by CYP239-specific CTL. In 1 of 4 experiments we observed lysis of monocytes by CYP190-specific CTL (up to 22% at E:T = 30:1). Lysis of tumor cell lines was equally demonstrated for CTL derived from healthy individuals or cancer patients. HLA-A2 negative tumor cell lines were not killed (data not shown). Using HLA-A2-matched or mismatched follicular lymphoma (FL), acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL) samples we observed CYP190 and CYP239-specific CTL lysis of primary tumors (**Fig. 3d**). Lysis of FL cells was comparable for CYP190 and CYP239 CTL. The HLA-A2 mismatched FL control sample consistently showed a higher background for CYP190-specific CTL. Lysis of ALL cells was higher for CYP190-specific CTL, while CYP239-specific CTL demonstrated higher lysis of AML cells.

Elution of CYP1B1-derived epitopes from tumor cells

In addition to functional analysis, a bioanalytical approach was undertaken to identify epitopes presented by HLA-A*0201 from tumor cells including multiple myeloma and EBV-transformed B cells. Automated HPLC based immunoaffinity chromatography was followed by HPLC based peptide repertoire fractionation and mass spectrometry. Peptide sequencing was accomplished by automated LC/MS/MS analysis utilizing ion trap technology[43]. A search of the complete data set of MS/MS spectra against a protein database containing only homologues of the CYP1B1 protein revealed the CYP190 epitope (**Fig 4**). In addition, synthetic homologues of CYP1B1-derived peptides were characterized with respect to their HPLC elution profiles and the precursor ion and MS/MS fragmentation patterns. The ion trap was used to specifically target and fragment all m/z values in the appropriate peptide containing fractions isolated from tumor cells. This approach confirmed the identification of the CYP190 peptide as the only biochemically characterized HLA-A*0201 restricted epitope identified in tumor cells (**Fig 4**).

***In vivo* immunity without autoimmunity**

To test whether CYP1B1 is immunogenic in the HLA-A2/K^b transgenic mouse model[44], groups of three mice were vaccinated with a pcDNA3 construct encoding full-length human CYP1B1 (pcDNA3huCYP1B1) or vector control (pcDNA3). After two *i.m.* vaccinations, $CD8^+$ enriched splenocytes showed specific interferon (IFN)-γ reactivity against EL4-A2/K^b cells expressing full-length huCYP1B1 (**Fig. 5a**). Non-immunized animals or animals immunized with a control vector showed no IFN-γ response. Similar data were obtained after three or four vaccinations (data not shown). We further investigated whether mice immunized with full-length huCYP1B1 would show reactivity against the HLA-A2 binding epitopes defined above. All animals immunized with huCYP1B1 regularly showed strong IFN-γ production when EL4-A2/K^b cells were pulsed with CYP190 (3/3 experiments, **Fig. 5b**). Weak reactivity against CYP239 was only seen in one experiment, suggesting that CYP190 is the immunodominant epitope, when stimulating with the whole huCYP1B1 gene.

Induction of immunity to self antigens raises the potential for inducing autoimmunity[4]. Human and mouse CYP1B1 show 73% sequence identity and 79% homology. Nevertheless, CYP190 differs in 4 and CYP239 in 2 amino acids between mouse and human. Therefore we elected to identify a human epitope that was 100% identical to the murine sequences. Using MHC binding prediction algorithms (SYFPEITHII, BIMAS) and IFN-γ ELISPOT screening, we identified a K^b-restricted peptide, CYP77, that is identical in human and mouse. HLA-A2/K^b transgenic mice were immunized using the identical vaccination strategy with the full-length huCYP1B1. Immunity was detected against CYP77 in two of two vaccination experiments (**Fig. 5c**). The frequency of CTL reacting against the self-epitope CYP77 in these experiments reached 50% of the frequency of CYP190-specific CTL. Complete histological examination of mice vaccinated with huCYP1B1 did not reveal any pathological changes associated with autoimmunity. Taken together, immunity to CYP1B1 was induced efficiently in HLA-A2/K^b transgenic mice confirming epitopes identified by epitope prediction and *in vitro* studies. Despite efficient induction of immunity to the self-peptide CYP77 there was no evidence of autoimmunity.

Discussion

Increasing evidence demonstrates that treatment and prevention of human disease must be directed at unique targets involved in the pathogenesis and pathophysiology of that disease. Antigen-specific immunotherapy is the ultimate targeted therapy, however, to date this modality has yet to become standard of care for any neoplasm. Although numerous candidate TA have been identified and evaluated clinically, presently it is unknown whether the paucity of clinically significant immune responses observed following vaccination is due to fallacies in the concept, selection of the TA, status of patient, and/or the therapeutic approach. To address the issue of the selection of the TA, we now propose the concept of credentialling. A "credentialled TA" must be (1) over-expressed in the overwhelming majority of human cancers, (2) stably and non-heterogeneously expressed throughout tumor cell ontogeny, (3) critical to the neoplastic process, (4) presented in the context of HLA, and (5) recognized by a functional and expandable TA-specific T cell repertoire. We propose that selection of a TA based upon these criteria will improve the potential for it to be a clinically significant tumor rejection antigen.

Here, we present CYP1B1 as the first "credentialled human TA." It is the most broadly expressed human TA and, to date, we and others have yet to find a single tumor cell type that is CYP1B1 negative. Homogeneous over-expression of CYP1B1 protein has been observed in all tumors tested. In contrast, no major essential target organ expresses significant levels of CYP1B1 protein and with the exception of the fallopian tube, breast, and ureter, its limited expression on normal tissues is both low level and heterogeneous. Molecular and functional analysis demonstrates that CYP1B1 protein is processed and presented by tumor cells and serves as a target for CYP1B1-specific cytolytic T cells. Screening of peripheral blood from both normals and cancer patients using ELISPOT and tetramer analysis demonstrates that CYP1B1-specific T-cells are below the level of detection. However, patients are not tolerant to CYP1B1 since CYP1B1-specific T cells have been generated from most normal donors and cancer patients tested demonstrating an intact and expandable CYP1B1-specificT cell repertoire. Immunization of HLA-A2 transgenic mice with huCYP1B1 DNA demonstrates the induction of immunity against a human and murine CYP1B1 shared epitope without evidence of autoimmunity. Considering the above findings and its role as a carcinogen-activating enzyme during the early events of malignant transformation and/or progression, we propose that CYP1B1 is an ideal candidate to be validated in human clinical trials.

There are several recently reported TA that are also excellent candidates to be fully "credentialled." The human catalytic domain of telomerase (hTERT) is one such antigen[45]. Approximately 85% of human tumors demonstrate telomerase activity and the only normal cells that express hTERT are dividing cells. Like CYP1B1, hTERT specific T cells cannot be detected in normals or tumor bearing patients but can be generated in normals and cancer patients upon antigen specific immune stimulation[46]. However, we and others have yet to be able to elute and identify hTERT peptides from the groove of HLA-$A2^+$ tumor cells. Thus, hTERT does not yet fulfill our criteria for credentialling. Recently, MDM2 has been proposed as a universal human TA[47]. Unlike CYP1B1 and hTERT, MDM2 expression on normal and neoplastic cells has not yet been fully evaluated. Moreover, the heterogeneity within tumor is unknown. Like CYP1B1, MDM2- derived peptides have been eluted from tumor cell HLA-A2. To date, all T cells from normals appear to be tolerant to MDM2. For MDM2 to be credentialled, functional autologous T cells directed against an alternative MDM2 derived epitope must be identified. Cyclin B1 has been recently reported as a shared epithelial tumor associated antigen recognized by T cells[48]. This antigen was identified by elution of peptides from the groove of an HLA-$A2^+$ adenocarcinoma of the breast. Cyclin B1 peptides are reported to be expressed on many adenocarcinomas but their characterization on normal and neoplastic cells still requires further validation. $CD8^+$ T cells could be generated from normal donors which could lyse peptide-pulsed antigen presenting cells but not tumor cells. Therefore, like hTERT and MDM2, cyclin B1 does not yet fulfill the criteria for credentialling. This is not a critique of the validity and importance of any of these or other known antigens, but rather an attempt to set the bar for TA credentialling with the objective of improving the potential of that antigen to be successful in the clinic.

In addition to fulfilling the criteria for being credentialled, CYP1B1 displays certain unique properties that may enhance its potential performance as a clinical TA target. Longitudinal studies in animal models have established stable over-expression of CYP1B1 throughout the malignant transformation[35]. CYP1B1 has been

implicated in carcinogenesis by environmental carcinogens such as dioxins[49] and polycyclic aromatic hydrocarbons (PAH)[50]. PAH are metabolized by CYP1B1 to highly active epoxides thereby causing DNA adduct formation[51,52], an early step in tumor development. CYP1B1 has also been linked to endogenous estrogen-related carcinogenesis in human breast, uterine, and other tumors[29,53]. CYP1B1 catalyzes the 4-hydroxylation of 17β-estradiol, and the product (4-hydroxyestradiol) and its metabolites have been implicated in direct and indirect free radical-mediated DNA damage[54]. Further support for the pivotal role of CYP1B1 in carcinogenesis is derived from studies of CYP1B1$^{-/-}$ mice[55]. Challenge of these mice with the prototypic PAH 7,12-dimethyl-benz[a]anthracene, DMBA, leads to a significantly reduced incidence of lymphoma and skin tumors as compared with wildtype mice. Likewise, the expression of CYP1B1 on normal tissues appears unique. Although not expressed on critical end organs that would obviate clinical applicability if autoimmunity were induced, the localization of cells in the ureter, fallopian tube, and breast deserve comment. One explanation for this finding might be related to the observation that CYP1B1 has been linked to endogenous estrogen-related carcinogenesis as well as the finding that estrogen metabolites may be involved in direct and indirect free radical-mediated DNA damage in these tissues.

By definition, our concept of TA credentialling requires merging of several divergent approaches and technologies. Specifically, epitope deduction based on prediction algorithms and T cell epitope screening must be complemented by direct biochemical elution of peptides from the groove of HLA to insure the identification of all potential antigens and their epitopes. For example, CYP239 was predicted *in silico* and was shown through T cell screening to be an immunogenic epitope. However, CYP239 was not detected biochemically. Ongoing studies will address whether this discrepancy can be explained by the density and biochemical characteristics of the epitope expressed by tumor cells, the efficacy of antigen processing of the specific epitope[56], the avidity of the epitope specific T cells, or the plasticity of the T cell receptor thus allowing cross reactivity[23,24]. Likewise, prediction of the functionality of the antigen-specific T cell repertoire must be integrated with the concept of T cell tolerance. Here, the epitope density on the target cell plays an important role. While too low copy number might prevent the induction of immunity, too high copy number might be responsible for the deletion of high avidity T cells. These events result in a functionally tolerant T cell repertoire incapable of recognizing endogenously processed and presented antigen on tumor cells[47,48]. Alternatively, the induction of high avidity T cells specific for epitopes expressed at low density on tumor cells might potentially cause autoimmunity[4]. Finally, the observation that T cell function and T cell avidity do not linearly correlate adds another dimension of complexity to the credentialling process[42]. In this context the identification of fully functional CYP190-specific $CD8^+$ T cells that did not bind CYP190-peptide/MHC tetramers was less surprising[42]. While we have functionally excluded the induction of low avidity T cells[57], the lack of tetramer binding might be a consequence of enhanced T cell receptor down-regulation secondary to enhanced antigen exposure[58]. Since CYP190 demonstrates an extremely long half-life when bound to HLA-A2, our culture conditions might not be optimized for such peptides[59] even when reduced amounts of peptide were used for stimulation (data not shown). We are further addressing this issue by using antigen-presenting cells endogenously processing the CYP190 epitope after infection of a minigene containing the CYP190 epitope. All these issues appear to be confounding when credentialling a TA and must be considered prior to proceeding to clinical evaluation.

So why is the concept of credentialling essential? The answer lies in the commitment to find TA that will induce a clinically significant immune response for the majority of patients with cancer. Therefore, we believe that our proposed stringent criteria provide the greatest hope for success. Even if an optimal credentialled TA can be identified, there are still several obstacles that must be overcome. It is unlikely that a clinically significant immune response will be observed in toxicity trials due to immunodeficiency or advanced disease. No pre-clinical model supports success of this concept for cancer or even pathogens. Success will require the optimal credentialled TA administered to the optimal patient (normal T cell repertoire to the TA and minimal tumor burden). Whether this will be sufficient or additional enhancements to accelerate T cell expansion or improve antigen presentation are necessary awaits clinical evaluation.

Material and Methods

Donor and Patient Samples

For CTL generation, peripheral blood from healthy blood donors and cancer patients (multiple myeloma, stage I-III, n=3; follicular lymphoma, stage IV, n=1; prostate cancer, stage IV, n=1) was obtained by leukapheresis and peripheral blood mononuclear cells (PBMC) were purified by Ficoll-density centrifugation[60]. For *ex vivo* analysis of CYP1B1-specific T cells, peripheral blood from healthy individuals (n=11) and cancer patients (see table 2) was drawn by phlebotomy, followed by isolation of $CD8^+$ T cells by RosetteSep® technology (StemCell Technologies, Vancouver, Canada). Primary NHL, ALL, and AML samples as well as normal and malignant tissue blocks were obtained from discarded specimens. Leukapheresis products, phlebotomy samples, and tumor tissue were obtained following informed consent and approval by our Institute's Review Board.

In silico analysis of differentially expressed genes

The publicly available cDNA libraries provided by CGAP were regularly screened for differentially expressed genes primarily using the Differential Gene Expression Displayer using all EST, CGAP, MGC, ORESTES, and SAGE Libraries. Up to 6126 genes in 1228 libraries derived from tumor tissue (900129 total sequences) and 1902 libraries derived from normal tissue (2093234 total sequences) were included in this analysis. Libraries derived from malignant tissue were compared either globally to all libraries derived from normal tissue or only to libraries of the normal counterpart. Widely expressed candidate genes for further analysis were defined by expression in libraries derived from breast, lung, colon, skin and lymphatic tissue without expression in normal tissue. Furthermore, PubMed was regularly searched for candidate genes using the following keywords: 'cancer', 'lung', 'breast', 'colon', 'skin', 'lymph node', and 'protein'.

Cell Lines

The melanoma cell line K029 was a kind gift of Dr. G. Dranoff (Dana-Farber Cancer Institute, Boston). The 36M ovarian carcinoma cell line was a kind gift of Dr. S. Cannistra (Beth Israel Deaconess Hospital, Boston). The TAP-deficient T2 cell line; the multiple myeloma cell lines U266 and HS-Sultan; the EBV-transformed B lymphoblastoid cell line IM-9; the melanoma cell line SK-MEL-2; the ovarian carcinoma cell line SK-OV-3; and COS cells were obtained from ATCC (Manassas, VA). The murine thymoma cell line EL4 was obtained from ATCC and was transfected with the HLA-A2/Kb cDNA inserted into the pSV2neo vector.

Immunohistochemistry of CYP1B1

5 μ sections of paraffin embedded specimens were mounted on barrier slides and baked for 1 hour at 60°C. Slides were then deparaffinizied, hydrated and dried. After blocking of endogenous peroxidase activity by 3% hydrogen peroxide and heat induced antigen retrieval slides were incubated for 1 hour with 150-200 μl polyclonal rabbit anti-CYP1B1 (generously provided by Dr. Craig Marcus, U. New Mexico, Albuquerque, NM)[26] or, as a negative control, non-specific polyclonal rabbit Ig. Slides were rinsed and sequentially treated with Link, Label, and Substrate as per the manufacturers instructions (Biogenix StrAviGen Multilink kit, Biogenex). Samples were counter-stained with haematoxylin and then dehydrated to xylene.

Immunohistochemical staining of normal tissue sections was performed by IMPATH Biopharmaceutical Services (New York, NY) on snap frozen tissue sections. Three specimens per tissue type, isolated from individual donors, were selected at random from the IMPATH Tissue Library. In brief, OCT compound (Miles Laboratories, Inc., Naperville, IL) embedded tissues were cut at 5 μm, and fixed in acetone . After blocking of endogenous peroxidase activity by hydrogen peroxide, slides were incubated with either the monoclonal mouse anti-human CYP1B1 5D3 antibody[30] (10 μg/ml) or a murine IgG_{1k} negative control antibody (DAKO Corporation, Carpinteria, CA) for 30 minutes at room temperature. Staining was detected using the Envision+™ Kit (DAKO Corporation) according to the manufacturer's recommendations. Tissues were counterstained with Mayer's hematoxylin solution (American Master Tech. Scientific Inc., Lodi, CA). Frozen breast cancer specimens were employed as a positive tissue control, and normal liver was included as a negative tissue

control. Slides were evaluated by pathologists, and the staining intensity of test slides was judged relative to the intensity of a control slide containing an adjacent section stained with an irrelevant negative control. In keeping with standard pathology practice, staining intensity was reported at the highest level of intensity observed in all tissue elements.

Western blot analysis

CYP1B1 expression was determined in microsomal cell fractions. Microsomal protein was isolated by differential speed centrifugation. Tissue or cells were homogenized (10mM Tris-HCl, pH7.4/0.25M sucrose/15% glycerol), and high-density particles were pelleted by centrifugation for 20 min at 15,000g. The supernatant was collected and centrifuged for 1 hour at 180,000g. The pellet was washed once and resuspended in 100mM Tris-HCl, pH7.4/1mM EDTA/15% glycerol. 20-30μg of microsomal protein was separated by SDS-PAGE and transferred to a PVDF membrane. Recombinant human CYP1B1 (0.015 pmol, Gentest) was used as a positive control. Membranes were probed with purified monoclonal mouse-anti human CYP1B1 clone 5D3 and secondary goat-anti mouse-HRP (Santa Cruz Biotechnologies, Santa Cruz, CA). Bands were visualized by enhanced chemiluminescent detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Peptides

The CYP1B1-peptides CYP190 (FLDPRPLTV) and CYP239 (SLVDVMPWL), the HTLV-TAX11 (LLFGYPVYV), the peptide AHTKDGFNF derived from a clonal idiotype sequence, and the peptide F271 (FLWGPRALV) derived from MAGE-3 were purchased from Sigma Genosys Biotechnologies (The Woodlands, TX), from Harvard Medical School Biopolymers Laboratory (Boston, MA), or from New England Peptides (Fitchburg, MA) . The peptide CYP77 (LARRYGDV) is shared between human and mouse CYP1B1 and was obtained from Multiple Peptide Systems (San Diego, CA).

Peptide Prediction

Binding of peptides to HLA-A*0201 was predicted using three independent algorithms to increase specificity: a matrix algorithm available at the BioInformatics & Molecular Analysis Section (BIMAS) on the NIH) web site[61]; a linear programming algorithm (LPpep) at Boston University (Z. Weng kindly provided access[62]); and a two-dimensional matrix algorithm (SYFPEITHI)[63]. The peptides were initially ranked individually for each algorithm and subsequently sorted by a cumulative ranking score.

HLA-A*0201 peptide binding and complex stability assay

TAP-deficient T2 cells were pulsed with 40 μg/ml of peptide and 3 μg/ml of β2-microglobulin (Sigma, St. Louis, MO) for 18 hr in serum-free IMDM (Life Technologies, Rockville, MD) at 37°C. Cells were washed three times in serum-free IMDM, and aliquots of cells were replated and incubated at 37°C in the absence of exogenous peptide. HLA-A*0201 expression was measured by flow cytometry using FITC-conjugated mAb BB7.2 (ATCC) at 0, 2, 4, 6, and 24 hours after peptide withdrawal. Increase of HLA-A2 expression on T2 cells reflects stabilization of MHC complexes by the addition of exogenous peptides and was quantified using the fluorescence index (FI = ($MFI_{peptide\ pulsed\ T2}$ / $MFI_{unpulsed\ T2}$) - 1). The half-life of HLA-A2 complexes on the surface was calculated using linear regression analysis (y=yo+ a*e exp(-b*x); SigmaPlot). Peptides were tested for their capacity to bind recombinant HLA-A*0201 molecules *in vitro* as previously described[64]. The HTLV tax 11-19 peptide was used as the radiolabeled probe.

HLA-A2 isolation and peptide repertoire analysis:

HLA-A2 peptide complexes were purified by immunoaffinity chromatography using a BB7.2 immobilized Protein A support. An automated HPLC based immunoaffinity chromatography system was used to rapidly purify the HLA molecules and better capture low affinity peptides[61]. This system allows peptide mass spectrometry analyses to proceed within 18-36 hours after cell or tissue harvest. The intact peptide repertoire was isolated by acid extraction and separated by reverse phase HPLC. Peptide sequencing was accomplished by automated LC/MS/MS analysis[61] utilizing ion trap technology operated in two distinct modes. First, peptide-

containing fractions were analyzed by automated data dependent selection of precursor ions for subsequent MS/MS analysis. The complete data set of MS/MS spectra was searched against a protein database that contained only homologues of the CYP1B1 protein. Synthetic homologues of both CYP190 and CYP239 were analyzed by LC/MS/MS to confirm the detection of naturally processed CYP1B1 T-cell epitopes.

Generation of CTL

CTL were generated as previously described[45,60]. Assessment of cytotoxic effector function and tetramer analysis were performed with CTL cultures always >90% $CD3^+CD8^+$, <5% $CD4^+$ and <5% $CD56^+$.

Cytotoxicity Assay

To assess cytolytic function CTL lines were used after at least four antigenic stimulations in standard ^{51}Cr release assays as previously described[45]. Percent specific lysis was calculated from cpm of (experimental result - spontaneous release)/(maximum release - spontaneous release) x100%. Monocytes as targets were isolated from PBMC by RosetteSep® following the manufacturer's recommendations. For testing endogenous processing of CYP1B1-derived peptides COS cells stably expressing HLA-A*0201 were transfected with mini-gene constructs encoding either EGFP linked to huCYP1B1 aa 170-213, EGFP linked to huCYP1B1 aa 205-352, or EGFP alone. Target cells were sorted for EGFP expression and used in cytotoxicity experiments. Alternatively, a recombinant vaccinia virus containing full length human CYP1B1 cDNA[49] (kindly provided by Dr. T. Sutter, University of Memphis, TN) was generated by established techniques[65] and used to infect $HLA\text{-}A2^+$ monocyte-derived GM-CSF and IL-4 induced DC that were matured with TNFα (Endogen, Woburn, MA) and recombinant CD40L (kindly provided by Immunex, Seattle, WA)[66] for 16-18 hours (multiplicity of infection, MOI, 10). DC were then used as targets in ^{51}Cr release assays.

Tetramer analysis

Tetrameric HLA-A2/peptide complexes with CYP239, CYP190, and TAX11, an immunogenic peptide derived from HTLV-1, were synthesized essentially as described[67]. Biotinylation was confirmed by gel shift assay with unlabelled streptavidin (Molecular Probes, Eugene, OR). Biotinylated monomers were purified by size exclusion chromatography, and conjugated to Phycoerythrin (PE; Molecular Probes). For staining of CTL lines and patient $CD8^+$ T cells, cells were incubated with the tetramer for 30 minutes at room temperature; after 20 minutes CD8-PC5 (Beckman Coulter, Fullerton, CA) was added. Non-vital cells were excluded by Annexin V-FITC staining according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN). To amplify the frequency of tetramer positive cells in patient samples $CD8^+$ were stimulated with cognate peptide (1μg/ml), cytokines, and autologous irradiated PBMC as described[68] and re-evaluated by tetramer after 10 days.

Human IFN-γ ELISPOT assay

PBMC at $1x10^5$ cells/well were added to ImmunoSpot plates (Cellular Technology Ltd., Cleveland, OH) precoated with 10 μg/ml of anti-IFN-γ mAb (Mabtech, Nacka, Sweden) in the presence or absence of peptide (10μg/ml) and incubated for 20 hours at 37°C. After washing, wells were incubated with 1 μg/ml biotin-conjugated anti-IFN-γ mAb (Mabtech) followed by streptavidin-alkaline phosphatase (Mabtech). Spots were developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium color development substrate (Promega, San Luis Obispo, CA). Spots were analyzed using an ImmunoSpot analyzer and software (Cellular Technology Ltd.).

CTL induction in HLA-A2/K^b transgenic mice

HLA-A2/Kb transgenic mice used in this study were obtained from The Scripps Research Institute and have been described elsewhere[44]. Female mice 6-8 weeks of age were used in all experiments. The plasmid pcDNA3hu1B1 consists of a 1.6kb full-length human CYP1B1 cDNA coding sequence cloned into the unique EcoRV site of pcDNA3 mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Plasmid DNA for injection was made with endotoxin-free plasmid purification kits according to the manufacturer's instructions

(QIAGEN Inc., Chatsworth, CA.). A 25µl volume was injected into the tibialis anterior muscle of each leg for a total dose of 100 µg of DNA. Animals were immunized at two-week intervals (three mice per group) and assayed 9-12 days after last immunization.

Mouse IFN-γ ELISPOT Assay

Murine $CD8^+$ T cell responses to CYP1B1 were analyzed by IFN-γ ELISPOT assay using a commercial IFN-γ ELISPOT assay kit according to the manufacturer's recommendations (R&D Systems). As effector cells, pooled spleen cells from two mice per treatment group were enriched for $CD8^+$ T-cells and plated in duplicate at $1x10^5$ cells/well. T cells were stimulated with $1x10^5$ EL4-$A2K^b$ cells/well pulsed with 10µg/ml peptide or infected with recombinant vaccinia virus for 16-18 hours prior to plating (MOI, 10). PHA (10µg/ml; Sigma-Aldrich, St. Louis, MO) was used as a positive control, and $CD8^+$ T-cells in medium alone as a negative control. Plates were incubated for 24 hours, developed, and analyzed by automated image analysis (Zellnet Consulting, Inc., New York, NY). Antigen-specific T-cell frequencies are reported as spot forming cells/$1x10^6$ $CD8^+$ T-cells and have been subtracted for background spontaneous IFN-γ secretion.

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Legends

Table 1

Binding affinity and HLA/peptide complex stability of CYP1B1-derived and control peptides to human HLA-A*0201.

Notes

1 Fluorescence index (FI) = mean fluorescence with peptide / mean fluorescence without peptide. The known HLA-A2 binding peptide from MAGE 3 and a non-binding peptide from an idiotype sequence were used as positive and negative controls. One representative of 5 experiments is shown.
2 Time to half-maximal FI after withdrawal of peptide was calculated using linear regression analysis.
3 Peptide concentration necessary to inhibit binding of a labeled reference peptide (TAX) to HLA-A*0201 by 50%.

Table 2

Prevalence of CYP239 tetramer binding cells in cancer patients and healthy controls

Notes

MM multiple myeloma; CLL chronic lymphocytic leukemia; DLCL diffuse large B cell lymphoma; FL follicular lymphoma; ND normal donor

* cut off was calculated from frequencies in HLA-A2 negative individuals as previously described[69]: mean + 3x standard deviation

Figure 1

Expression of CYP1B1 protein in normal and malignant tissue. (a) Expression of CYP1B1 in human tumors detected by immunohistochemistry using a polyclonal antibody. All tissues were also stained with an Ig control (upper left panel and data not shown. (b) Western blot analysis of microsomal fractions (30μg per sample) from normal and malignant tissue using the monoclonal CYP1B1 antibody (similar results were obtained using the polyclonal antibody). Normal and malignant lung tissue were from the same individual. As a positive control, 0.015pmol of recombinant human (rh) CYP1B1 was loaded. (c) Summary of CYP1B1 expression in malignant tissue. Tissues were analyzed by IHC ([1]) or Western blot ([2]). Analysis was performed on primary tumor tissue except for cell line samples marked with an asterisk. High expression (+++) in all tumor cells (IHC) resp. strong expression by Western blot as exemplified in a and b. (d) Tissue sections from three individuals were evaluated for CYP1B1 expression by immunohistochemistry; a breast carcinoma was used as positive control always showing high expression. Intermediate expression in the majority of cells (++) in all three individuals; low expression in the majority of cells (+) in all three individuals; low expression in the minority of cells (-/+) in all three individuals; low expression in the minority of cells in one or two individuals ([-/+]); no expression in any individual (-).

Figure 2

CTL recognizing the CYP190 or CYP239 peptide can be generated from cancer patients and healthy donors. (a) After four *ex vivo* antigenic stimulations CTL raised from healthy HLA-A2$^+$ individuals against CYP190 or CYP239 peptide specifically lyse T2 cells pulsed with 20 μg/ml of cognate peptide (■), but not unpulsed T2 cells (□), or T2 cells pulsed with an irrelevant peptide (O; F271 from MAGE-3). (b) Efficiency for the induction of peptide-specific CTL from healthy donors and cancer patients. (c) Cytotoxicity of CYP190-specific (●) and CYP239-specific (◆) CTL against T2 cells pulsed with increasing concentration of cognate peptide (effector:target ratio = 10:1). Dashed lines reflect the peptide concentration at which half-maximal lysis was achieved. Results are representative of 2 independent experiments. For CYP239 similar results were obtained when bulk CTL were sorted for tetramer-positive cells prior to lysis assay (data not shown) (d) One representative tetramer analysis CYP239-specific CTL after 4 weeks in culture. The A2/TAX tetramer served as a negative control. Non-vital cells were excluded by annexin V staining. Percent tetramer$^+$ per CD8$^+$ T cells is shown.

Figure 3

CYP1B1-derived peptides are endogenously processed and presented by tumor cells.

(a) Specific lysis of COS/A2 cells transfected with a construct encoding EGFP + CYP1B1 amino acids 170-213 by CYP190-specific CTL (left panel, ◆) or 205-352 by CYP239-specific CTL (right panel, ◆) but not control EGFP-transfected COS cells (◇). (b) Specific lysis of HLA-A2$^+$ DC infected with a vaccinia construct encoding full length CYP1B1 cDNA (◆) or vector control only (◇) by CYP239-specific CTL. (c) Lysis of HLA-A2$^+$ tumor cell lines U266 (multiple myeloma), 36M (ovarian carcinoma), K029 (melanoma), and IM-9 (EBV lymphoblastoid B cell line) by CYP190-specific and CYP239-specific CTL. Lysis of HLA-A2- control cell lines was consistently less than 10% at effector:target ratio = 30:1 (data not shown). The NK cell target K562 was not lysed (data not shown). In 1 of 4 experiments HLA-A2$^+$ monocytes were lysed by CYP190-specific CTL (22% at 30:1). Results are representative for at least 2 and up to 15 experiments. In some experiments lysis of HLA-A2$^+$ tumor cell lines exceeded 80% specific lysis although lysis of irrelevant targets

was also increased in these experiments. Expression of CYP1B1 in target cells was confirmed by Western blot analysis (**Fig. 1** and data not shown).s shown below the table. (d) Lysis of primary tumor cells by CYP190-specific and CYP239-specific CTL. Solid symbols represent HLA-A2 positive samples, open symbols HLA-A2 negative. Targets were primary follicular lymphoma cells (FL), acute lymphoblastic leukemia cells (ALL), and acute myeloid leukemia cells (AML). Experiments with ALL cells were performed once, all others represent at least two independent experiments. Expression of CYP1B1 by tumor cells was confirmed by Western blotting.

Figure 4

Comparison of LC/MS/MS spectra for the HLA-A2 associated naturally processed and presented CYP190 peptide purified from the EBV transformed B cell line JY and the myeloma cell line U266 versus the synthetic homologue. Retention times were identical (data not shown) and the fragmentation patterns confirm the CYP190 sequence identity. **Figure 5**

Ex vivo IFN-γ ELISPOT analysis of T cell reactivity in HLA-A2/K^b mice vaccinated with a huCYP1B1 encoding DNA construct. (a) HLA-A2/K^b mice were primed and boosted once with pcDNA3huCYP1B1, pcDNA3 control, or not vaccinated. 12 days after boost $CD8^+$ enriched spleen cells were tested for reactivity against EL4-A2/K^b cells infected with vaccinia encoding huCYP1B1 (vac-huCYP1B1), vaccinia wild-type (vac-wt), or a non-infected control. Results are shown as IFN-γ spot-forming cells (SFC)/10^6 $CD8^+$ T cells. (b) The same spleen cells from vaccinated and control mice were used to measure IFN-γ release in response to EL4-A2/K^b cells untreated or pulsed with the CYP190 peptide. (c) IFN-γ ELISPOT analysis of spleen cells from mice primed and boosted with pcDNA3huCYP1B1. EL4-A2/K^b cells pulsed with the HLA-A2 binding human epitope CYP190 or the K^b binding shared mouse/human epitope CYP77 were used as stimulators. In all assays PHA was used as a positive control (data not shown), and IFN-γ background secretion was determined without stimulation. **References**

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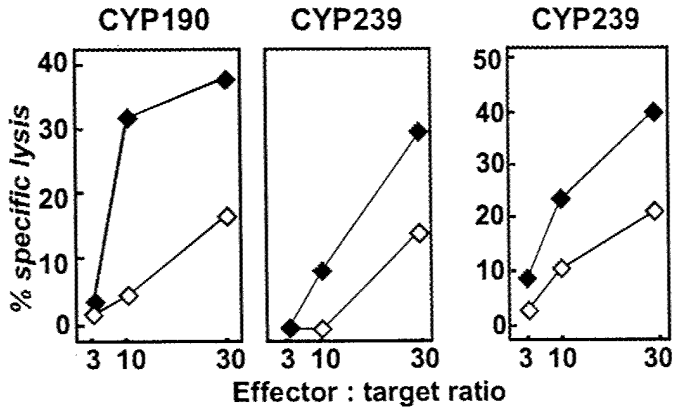
a

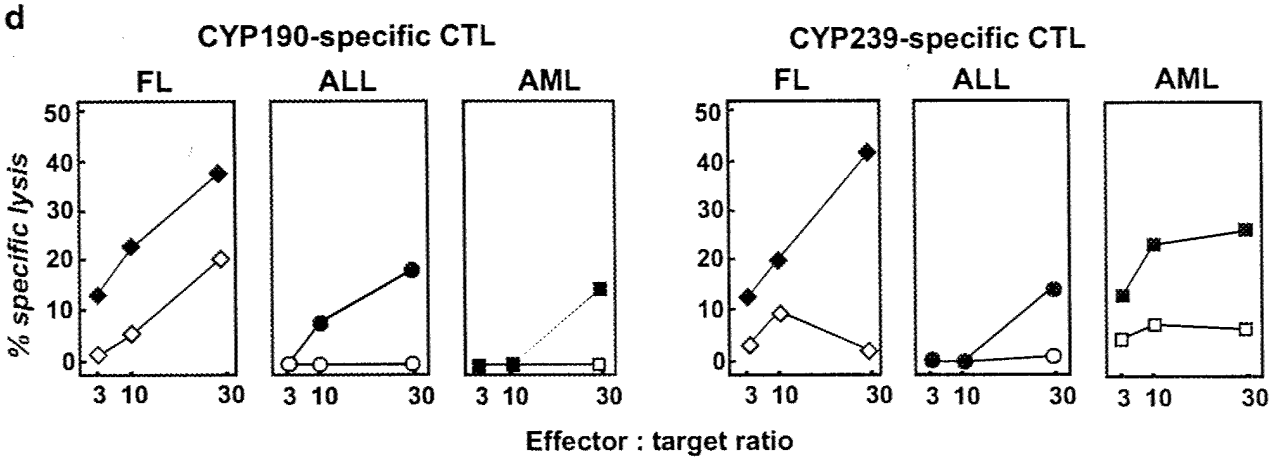


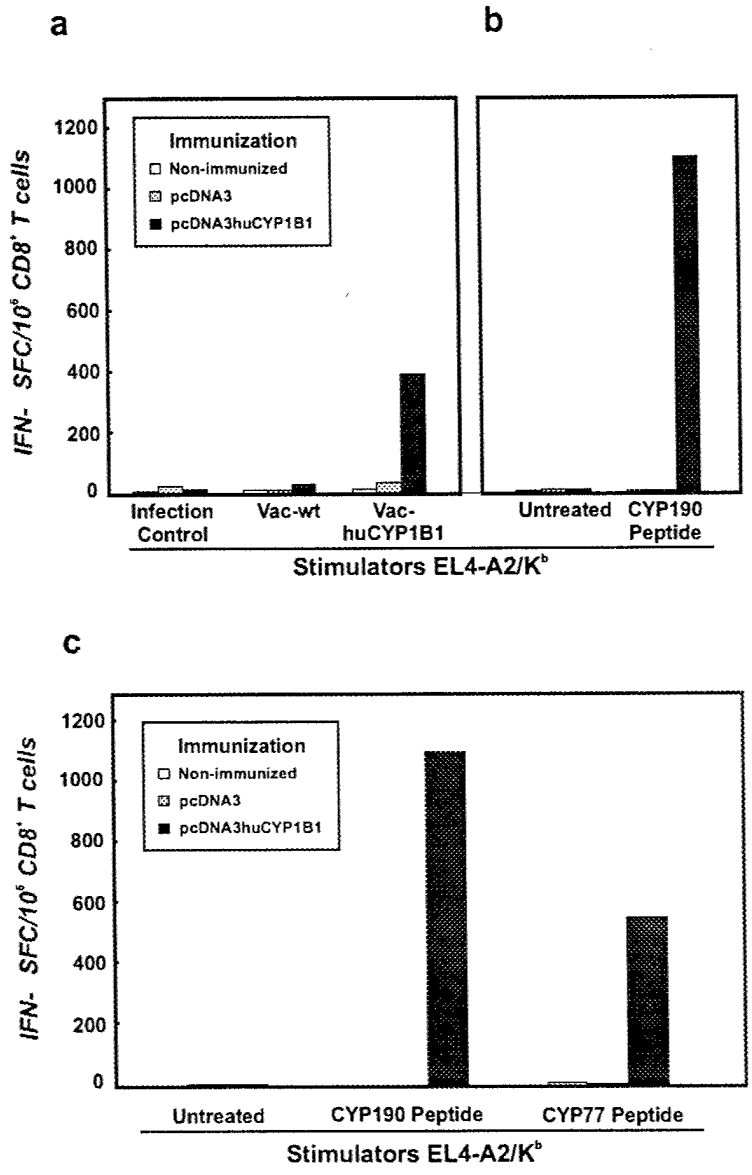
b

c

	CYP190			CYP239		
E:T ratio:	30	10	3	30	10	3
U266	31	14	6	38	23	10
36M	14	4	0	35	20	10
K029	30	14	7	45	18	2
IM-9	44	29	12	36	16	4
mono A2+	2	0	0	0	0	0

d




a
b
Immunization
Non-immunized
pcDNA3
pcDNA3huCYP1B1
IFN- SFC/10^6 CD8+ T cells
1200
1000
800
600
400
200
0
Infection Control
Vac-wt
Vac-huCYP1B1
Untreated
CYP190 Peptide
Stimulators EL4-A2/K^b
c
Immunization
Non-immunized
pcDNA3
pcDNA3huCYP1B1
IFN- SFC/10^6 CD8+ T cells
1200
1000
800
600
400
200
0
Untreated
CYP190 Peptide
CYP77 Peptide
Stimulators EL4-A2/K^b

Peptide	Protein	Position	Sequence	T2 assay		
				max (FI)[1]	$t_{1/2}$ (hrs)[2]	IC_{50} (nmol)[3]
CYP190	CYP1B1	190	FLDPRPLTV	3.7	10.1	67
CYP239	CYP1B1	239	SLVDVMPWL	3.9	3.3	63
controls						
positive	MAGE-3	271	FLWGPRALV	3.2	3.4	nd
negative	Idiotype	98	AHTKDGFNF	0	nd	nd

Peptide	Protein	Position	Sequence	T2 assay		
				max (FI)[1]	$t_{1/2}$ (hrs)[2]	IC_{50} (nmol)[3]
CYP190	CYP1B1	190	FLDPRPLTV	3.7	10.1	67
CYP239	CYP1B1	239	SLVDVMPWL	3.9	3.3	63
controls						
positive	MAGE-3	271	FLWGPRALV	3.2	3.4	nd
negative	Idiotype	98	AHTKDGFNF	0	nd	nd

HLA-A2 negative patients		HLA-A2 positive patients		HLA-A2 positive healthy donors	
diagnosis	**CYP239-tetramer$^+$/CD8$^+$**	**diagnosis**	**CYP239-tetramer$^+$/CD8$^+$**	**diagnosis**	**CYP239-tetramer$^+$/CD8$^+$**
MM	0.01%	breast cancer	0.02%	ND	0.00%
MM	0.02%	prostate cancer	0.00%	ND	0.03%
MM	0.02%	CLL	0.08%	ND	0.05%
MM	0.05%	DLCL	0.02%	ND	0.05%
MM	0.07%	FL	0.04%	ND	0.08%
MM	0.06%	**MM**	**0.12%**	ND	0.00%
MM	0.07%	MM	0.04%	ND	0.01%
MM	0.02%	MM	0.01%	ND	0.01%
MM	0.01%	MM	0.08%	ND	0.07%
MM	0.02%	MM	0.04%	ND	0.00%
MM	0.00%	MM	0.05%	ND	0.05%
MM	0.02%	MM	0.01%	ND	0.04%
MM	0.00%	MM	0.02%		
MM	0.03%	MM	0.04%		
MM	0.01%	MM	0.00%		
MM	0.02%	MM	0.02%		
MM	0.02%	MM	0.01%		
MM	0.00%	MM	0.02%		
MM	0.06%	MM	0.03%		
MM	0.06%	MM	0.05%		
		MM	0.00%		
		MM	0.01%		
		MM	0.01%		
		MM	0.02%		
		MM	0.05%		
		MM	0.02%		
average	**0.03%**		**0.03%**		**0.03%**
standard deviation	**0.02%**		**0.03%**		**0.03%**
cut off*	**0.09%**				