The shared tumor associated antigen cytochrome P450 1B1 is recognized by

specific cytotoxic T cells


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Footnotes

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4 The abbreviations used in this paper are: CYP1B1, cytochrome P450 1B1; MHC, major histocompatibility complex; HLA, human leukocyte antigen; FL, follicular lymphoma; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APC, antigen-presenting cell; hTERT, human telomerase reverse transcriptase; TCR, T cell receptor; CTL, cytotoxic T lymphocyte; PAH, polycyclic aromatic hydrocarbon; DMBA, dimethylbenzanthracene
Abstract

Cytochrome P450 1B1 (CYP1B1\textsuperscript{4}), a drug metabolizing extrahepatic enzyme, was recently shown to be overexpressed in multiple types of cancer. Such tumor-associated genes may be useful targets for anti-cancer therapy, particularly cancer immunotherapeutics. We identified HLA-A*0201 binding peptides and a naturally processed and presented T cell epitope capable of inducing CYP1B1 specific CTL in HLA-A2 transgenic mice. Furthermore, induction of CYP1B1 specific T cells was demonstrated in healthy donors and cancer patients. These T cells efficiently lysed target cells pulsed with the cognate peptide. More important, HLA-A2 matched tumor cell lines and primary malignant cells were also recognized by CYP1B1 specific CTL. These findings form the basis of a phase I clinical trial exploring a DNA-based vector encoding CYP1B1 for widely applicable cancer immunotherapy conducted at Dana-Farber Cancer Institute.
Introduction

Cytochrome P450 1B1 (CYP1B1) is an extrahepatic cytochrome P450 enzyme that has been associated with the activation of environmental carcinogens. Expression of CYP1B1 is upregulated early during malignant transformation. Furthermore, CYP1B1 was reported to be overexpressed in most human malignancies with minimal expression on critical normal tissues. Such shared tumor-associated antigens may be useful targets for the development of widely applicable cancer immunotherapeutics. The expression of the antigen in some rare normal tissues does not necessarily exclude such genes from serving as immunological targets as has been shown for other tumor antigens such as MUC-1, survivin, telomerase, ras, or p53. We were therefore interested, whether a gene such as CYP1B1 expressed early during the carcinogenic process might serve as a target for CTLs.

In the present report, we characterize the CTL response to HLA-A*0201 restricted epitopes derived from CYP1B1. One CYP1B1-derived epitope was isolated and identified from HLA-A*0201 expressed in several tissue specific tumor cells. Additional epitopes were predicted. The immunogenicity of CYP1B1 was demonstrated in HLA-A2 transgenic mice. Functional CYP1B1-specific T cells capable of lysing tumor cells were established from healthy individuals and cancer patients. Preliminary results from a clinical trial targeting CYP1B1 as a tumor antigen suggest that this antigen might be an attractive candidate to be integrated in widely applicable cancer immunotherapeutics.
Material and Methods

Healthy volunteer and patient samples

Following informed consent and approval by the Dana-Farber Cancer Institute's Review Board, peripheral blood from healthy donors and cancer patients (multiple myeloma, n=6; follicular lymphoma, n=1; prostate cancer, n=1) was obtained by leukapheresis or phlebotomy. Primary FL, ALL, and AML samples and malignant tissue blocks were obtained from discarded specimens. Normal tissue specimens were obtained from the tissue library at IMPATH Biopharmaceutical Services (New York, NY).

Cell lines

The cell lines K029 (melanoma) and 36M (ovarian carcinoma) were kind gifts of Drs. Dranoff and Cannistra (Harvard Medical School, Boston). T2, COS, U266, HS-Sultan, IM-9, SK-MEL-2, SK-OV-3, JY, KATO III, and EL-4 cell lines were obtained from ATCC (Manassas, VA). EL-4 cells were transfected with the HLA-A2/Kb cDNA inserted into the pSV2neo vector.

Immunohistochemistry of CYP1B1

Sections of paraffin embedded tumor specimens were prepared for immunostaining following standard procedures. Slides were then incubated for 1 hour with 150-200 µl polyclonal rabbit anti-CYP1B1 (generous gift of Dr. Marcus, UNM, Albuquerque, NM) or non-specific polyclonal rabbit Ig. Slides were rinsed and treated with Link, Label, and Substrate (Biogenix StrAviGen Multilink kit, Biogenex) and counter-stained with haematoxylin. Analysis of normal tissue sections was performed by IMPATH
Biopharmaceutical Services (New York, NY) on frozen tissue sections. In brief, OCT compound (Miles Laboratories, Inc., Naperville, IL) embedded tissues were cut and fixed in acetone. After blocking by hydrogen peroxide, slides were incubated with the monoclonal mouse anti-human CYP1B1 5D3 antibody (10 µg/ml) or a murine IgG1k control antibody (DAKO Corporation, Carpinteria, CA) for 30 minutes. Tissues were counterstained with hematoxylin (AMTS Inc., Lodi, CA). Breast cancer specimens were employed as positive controls, and normal liver was included as negative control. Samples 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 protein was isolated following standard protocols for preparation of the microsomal protein fraction by differential speed centrifugation. Recombinant human CYP1B1 (0.015 pmol, Gentest Woburn, Massachusetts) was used as a control. Membranes were probed with purified monoclonal mouse-anti human CYP1B1 clone 5D3 (14) and secondary goat-anti mouse-HRP (SC Biotechnologies, Santa Cruz, CA). Bands were visualized by enhanced chemiluminescent detection (Amersham Pharmacia, Piscataway, NJ).
Peptides and peptide prediction

Peptides were purchased from Sigma Genosys Biotechnologies (The Woodlands, TX), Harvard Medical School Biopolymers Laboratory (Boston, MA), New England Peptides (Fitchburg, MA) or Multiple Peptide Systems (San Diego, CA): CYP77 (LARRYGDV), CYP190 (FLDPRPLTV), CYP239 (SLVDVMPWL), HTLV-TAX11 (LLFGYPVYV), EBV-BMLF1 280 (GLCTLVAML), the idioype derived peptide (AHTKDGFNF), MAGE-3 F271 (FLWGPRALV), and HBV core F18 (FLPSDYFPSV). Binding of peptides to HLA-A*0201 was predicted using three algorithms: “BIMAS”,15 “LP pep” (kindly provided by Z. Weng, Boston University) and “SYFPEITHI”.16 The peptides were ranked for each algorithm and sorted by a cumulative score.

HLA-A*0201 peptide binding and complex stability assay

Following previously described methods, peptide binding was assayed using T2 cells.10 For complex stability T2 cells were washed three times in serum-free IMDM after peptide incubation, 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-A*0201 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-A*0201 complexes on the surface was calculated using linear regression analysis (y=yo+a*e^{-b*x}; SigmaPlot). Peptides were also tested for their capacity to bind
recombinant HLA-A*0201 molecules in vitro as previously described. The HBV core 18-27 peptide was used as the radiolabeled probe.

**HLA-A*0201 isolation and peptide repertoire analysis**

An automated HPLC based immunoaffinity chromatography system was used to rapidly purify the HLA molecules. The intact peptide repertoire was isolated by acid extraction and separated by reverse phase HPLC. Peptide sequencing was accomplished by automated microcapillary LC/MS/MS analysis utilizing ion trap technology as previously described. Briefly, aliquots (0.5-5 µL) of each peptide-containing fraction were concentrated using a microtrap (peptide captrap; Michrom BioResources, CA, USA) in place of the sample loop in the autoinjector and analyzed by either of two ion trap systems (LCQ classic or LCQ Deca; Thermo-Finnigan, San Jose, CA, USA) equipped with automated data dependent selection of precursor ions for subsequent MS/MS analysis. Following acquisition of an MS/MS spectrum the precursor ion was dynamically written to an exclusion list where it resided for 30s before a MS/MS spectrum for this precursor ion could be collected for a second time. The complete data set of MS/MS spectra was searched against a protein database that contained homologues of the CYP1B1 protein. A synthetic homologue of CYP190 was analyzed by LC/MS/MS to confirm the detection of naturally processed CYP1B1 peptides.

**Generation of CTL**

CTL were generated as previously described. CTL cultures always contained >90% CD3+CD8+, <5% CD4+ and <5% CD56+. 

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Cytotoxicity assay

CTL lines were used after at least four antigenic stimulations in standard $^{51}$Cr release assays as previously described. 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 aa170-213, EGFP linked to huCYP1B1 aa205-352, or EGFP alone. COS cells were sorted for EGFP expression prior use. Alternatively, a recombinant vaccinia virus containing full length human CYP1B1 cDNA was generated and used to infect HLA-A*0201 $^+$ monocyte-derived and matured DC for 16-18 hours (multiplicity of infection, MOI, 10).

Human IFN-γ ELISPOT assay

ELISPOT analysis for IFN-γ secretion using human PBMC was carried out as previously described. Peptides from HTLV-1 and EBV BMLF-1 were used as negative and positive controls, respectively.

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

Female HLA-A2/K$^b$ transgenic mice were obtained from The Scripps Research Institute (La Jolla CA). v/huCYP1B1d3 consists of a 1.6kb full-length human CYP1B1 cDNA coding sequence with introduced nucleotide changes that produce 3 single amino acid substitutions (W57C, G61E, and G365W) in the huCYP1B1 coding sequence, to inhibit the enzymatic activity of the CYP1B1 protein (data not shown) cloned into CMV promoter-based mammalian expression vector. Plasmid DNA was made with plasmid purification kits (QIAGEN, Chatsworth, CA) and encapsulated in PLG microparticles.
and injected intramuscular (total dose of 100 µg DNA) at two-week intervals 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 (R&D Systems). Pooled spleen cells from two mice per treatment group were enriched for CD8⁺ T cells and plated at 1x10⁵ cells/well. T cells were stimulated with 1x10⁵ EL4-A2Kᵇ cells/well pulsed with 10 µg/ml peptide or infected with recombinant vaccinia virus prior to plating (MOI, 10). Plates were incubated for 24 hours, developed, and analyzed by automated image analysis (Zellnet Consulting, Inc., New York, NY).
Results

CYP1B1 protein is highly expressed in malignant but not normal cells

Among the proteins associated with early events of malignant transformation induced by chemical carcinogens, cytochrome P450 1B1 protein has been reported to be overexpressed in the majority of cancers tested. Expression of mRNA for CYP1B1 has been reported for some normal tissues and a single report has also suggested protein expression in several human tissues. We have extended these findings and demonstrate homogenous and significant protein overexpression in multiple randomly selected tumor specimens tested (Fig. 1a-c). A comprehensive screen of 32 different normal tissues (three specimen for each) derived from autopsy material from otherwise healthy patients who died of trauma (Fig. 1d) was also conducted. Breast, ovarian, and colon carcinoma (Fig. 1a) 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 9 of 9 cancer cell lines of various histologies, as well as in primary tumor specimens compared to normal adjacent tissue (Fig. 1b and c and data not shown). Among all normal tissues tested Fallopian tube showed the highest level with an apical cellular distribution. Breast, uterine, and ureter specimen showed intermediate to high levels of staining (+) with <20% of tissue-specific cells displaying high intensity. Weak to intermediate staining (+) was detected in two of three skin samples with <20% of cells displaying intermediate intensity. In addition, variable and weak staining (+/-) was detected in 10-25% of cells of prostate (2 of 3 samples), 20% of pancreas (2/3), 50% of pituitary (1/3), 40% of colon (1/3), 10% of
bladder (1/3), 20% of small intestine (1/3), and 10% of thymus (1/3) tissue-specific cells.

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 (30 µg 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, normal and malignant breast tissue from two separate individuals. As a positive control, 0.015 pmol of recombinant human (rh) CYP1B1 was loaded. (c) Summary of CYP1B1 expression in malignant tissue. Tissues were analyzed by immunohistochemistry (IHC) (†) or Western blot (‡). Analysis was performed on primary tumor tissue except for cell line samples marked with an asterisk. +++ intensity resembles high expression in all tumor cells (IHC) 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. Symbols: +++ high expression, >20% of cells with high expression in all three samples; ++ intermediate expression and <20% cells with high expression in at least one sample; + low expression and <20% of cells with intermediate expression in at least one sample; +/- weak expression in at least one sample; - no expression in any sample tested.

Taken together, CYP1B1 is expressed in some normal tissues but strongly overexpressed in all malignancies tested to date.

**Elution of CYP1B1-derived epitopes from tumor cells**

A biochemical approach was undertaken to identify epitopes presented by HLA-A*0201 from several tumor samples including multiple myeloma, gastric carcinoma, colorectal adenocarcinoma, 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.33

**Figure 2**

Comparison of LC/MS/MS spectra for the HLA-A*0201 associated naturally processed and presented CYP190 peptide isolated from the EBV transformed B cell line JY (middle panel) and the myeloma cell line U266 (lower panel) versus the synthetic homologue (upper panel). The primary sequence of CYP190 is depicted in the upper left corner. Retention times were identical (data not shown) and the fragmentation patterns confirm the CYP190 sequence identity. Similar spectra were obtained from gastric carcinoma, several colorectal adenocarcinomas, and EBV transformed B cell lines derived from multiple individuals (data not shown).
A search of MS/MS spectra against a protein database containing only homologues of the CYP1B1 protein revealed one epitope derived from CPY1B1 (referred to as CYP190; FLDPRPLTV; Fig. 2). To confirm the nature of CYP190, a synthetic homologue was characterized with respect to HPLC elution profile and the precursor ion and MS/MS fragmentation pattern. CYP190 was confirmed using the ion trap by specifically targeting and fragmenting all m/z values in the appropriate peptide containing fractions isolated from tumor cells (Fig. 2).

**Prediction of additional CYP1B1-derived epitopes**

Additional epitopes for characterization of CTL responses against CYP1B1 were predicted using three computational algorithms (BIMAS, SYFPEITHI and LPpep). Among the ten most likely candidates, the peptide/HLA-A*0201 complex stability ($t_{1/2}$) was the highest for CYP190, while the predicted CYP239 epitope (SLVDVMPWL)

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<th>$t_{1/2}$ (hrs)</th>
<th>IC$_{50}$ (nmol)</th>
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Table 1
Binding affinity and HLA/peptide complex stability of CYP1B1-derived and control peptides to human HLA-A*0201.

1 Fluorescence index (FI) = mean fluorescence with peptide / mean fluorescence without peptide. The known HLA-A*0201 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 HBV core 18-27 to HLA-A*0201 by 50%.
consistently showed the highest binding affinity (FI) in a cellular binding assay (Table 1). These results were confirmed by affinity measurements to recombinant HLA-A*0201 (IC₅₀) using an inhibition affinity assay¹⁷ (Table 1). Based on these findings the epitopes CYP190 and CYP239 were chosen for immunologic analysis.

**Immunity against CYP1B1 in HLA-A2 transgenic mice**

*In vivo* immunogenicity of CYP1B1 was assessed using HLA-A2/Kᵇ transgenic mice.²⁵ Mice were vaccinated with a plasmid encoding full-length mutated human CYP1B1 (v/huCYP1B1d3) or vector control (v). To target DNA for uptake by antigen presenting cells (APC), the plasmid DNA was encapsulated in biodegradable
microparticles composed of PLG. After three i.m. vaccinations, CD8\(^+\) enriched splenocytes showed specific interferon (IFN)-\(\gamma\) reactivity against EL4-A2/K\(^b\) cells expressing full-length huCYP1B1 (Fig. 3a). Non-immunized animals or animals immunized with a control vector showed no IFN-\(\gamma\) response. We further investigated whether mice immunized with full-length huCYP1B1d3 would show reactivity against the HLA-A*0201 binding epitopes defined above. All animals immunized with v/huCYP1B1d3 regularly showed strong IFN-\(\gamma\) production when EL4-A2/K\(^b\) cells were pulsed with CYP190 (3/3 experiments, Fig. 3b). Weak reactivity against CYP239 was only seen in one experiment, suggesting that CYP190 is the immunodominant epitope, when stimulating with the whole huCYP1B1 cDNA.

Human and mouse CYP1B1 show 75% sequence identity and 81% homology. Nevertheless, CYP190 differs in 4 and CYP239 in 2 amino acids between mouse and human. Therefore we elected to identify an additional human epitope that was 100% identical to the murine sequences. Using MHC binding prediction algorithms (SYFPEITHII, BIMAS) and IFN-\(\gamma\) 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 same vaccination strategy with the full-length v/huCYP1B1d3 construct. Immunity was detected against CYP77 in two of two vaccination experiments (Fig. 3c). 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 all major organs including fallopian tube, mammary gland, and uterus did not reveal any pathological changes associated with autoimmune phenomena in empty vector- or v/huCYP1B1d3-vaccinated mice (data not shown). Taken
together, immunity to CYP1B1 was induced efficiently in HLA-A2/Kb transgenic mice. Despite efficient induction of immunity to the shared self-peptide CYP77 there was no evidence of autoimmune phenomena.

**CYP190- and CYP239-reactive T cells in healthy volunteers and cancer patients**

After demonstrating immunogenicity of CYP1B1 derived epitopes in HLA-A2 transgenic mice we investigated whether these epitopes would also trigger specific and functional CTL responses in HLA-A*0201 positive healthy donors and cancer patients. Peptide-specific T cells were expanded by weekly stimulations with either CYP190 or CYP239 peptide presented on autologous APC. In over 70% of healthy HLA-A*0201 positive individuals tested, CYP190- and CYP239-specific CD8\(^+\) T cells were generated that specifically lysed peptide-pulsed T2 cells (Fig. 4a and b). Moreover, specific T cells

**Figure 4**

CTL recognizing the CYP190 or CYP239 peptide can be generated from cancer patients and healthy donors. (a) After four \textit{ex vivo} antigenic stimulations CTL raised from healthy HLA-A*0201 individuals against CYP190 or CYP239 peptide specifically lyse T2 cells pulsed with 20 \(\mu\)g/ml of cognate peptide (■), but not unpulsed T2 cells (□), or T2 cells pulsed with an irrelevant peptide (○; 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 two independent experiments.

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<tr>
<td>CYP239</td>
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17
were also successfully expanded \emph{in vitro} from 2/2 cancer patients against CYP190, and from 5/5 patients against CYP239. T cell lines were peptide-specific since target cells loaded with irrelevant peptides were not lysed (Fig. 4a). HLA-restriction was demonstrated by the lack of lysis of HLA-A*0201 mismatched target cells (data not shown). Avidity of CTL lines was estimated in peptide titration studies (Fig. 4c) indicating that CYP190- and CYP239-specific CTL were of intermediate to high avidity.\textsuperscript{34} Epitope-specific CTL were enumerated by interferon (IFN)-\textgamma ELISPOT assay. For both peptides the frequency of epitope-specific T cells ranged between 0.5% and 3% in expanded CTL lines (data not shown). These results are comparable to previously published data for hTERT-,\textsuperscript{24} gp100-,\textsuperscript{35} or proteinase-3.\textsuperscript{36} To assess whether these CYP1B1-derived epitopes are part of a pre-existing anti-tumor immune response, we analyzed T cells derived from peripheral blood of 5 HLA-A*0201 positive healthy donors and 8 HLA-A*0201 positive cancer patients by IFN-\textgamma ELISPOT assay (Table 2).

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\textbf{Table 2}
Detection of INF-\textgamma secreting cells by ELISPOT analysis in the peripheral blood of cancer patients and healthy donors.
\textsuperscript{1} MM, multiple myeloma; FL, follicular lymphoma; CaP, cancer of the prostate; HD, healthy donor.
\textsuperscript{2} all values given in spot-forming cells/100,000 CD8\textsuperscript{+} T cells.
\textsuperscript{3} HTLV-TAX, negative control; EBV-BMLF-1, positive control.
We were unable to detect CYP190- or CYP239-specific CTL above background level in both healthy individuals and cancer patients suggesting that these CYP1B1-derived epitopes are not targeted by the endogenous anti-tumor immune response.

**Recognition of endogenously processed CYP190 and CYP239 epitopes**

Recognition of endogenously processed CYP1B1-derived peptides by human CTL was evaluated using 1) COS cells transfected with HLA-A*0201 and minigene constructs containing the CYP190 (aa173-205) and CYP239 (aa213-352) epitopes, 2) dendritic cells (DC) infected with a vaccinia construct encoding full length CYP1B1 cDNA, and 3) 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.
cells (Fig. 5a). Similarly, DC infected with the vaccinia construct containing CYP1B1, but not the wild type vaccinia virus, were lysed by CYP190- and CYP239-specific CTL (data not shown). As exemplified in Fig. 5b, 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. Lysis of tumor cell lines was equally demonstrated for CTL derived from healthy individuals or cancer patients. HLA-A*0201 negative tumor cell lines were not killed (data not shown). As exemplified in Fig. 3c using follicular lymphoma (FL) cells CYP190- and CYP239-specific CTL demonstrated comparable lysis of HLA-A*0201-matched primary tumor cells. The particular HLA-A*0201 mismatched FL sample used as the control consistently showed a higher background for CYP190-specific CTL. In addition, CYP190- and CYP239-specific CTL displayed specific lysis of HLA-A*0201+ acute leukemia blasts (data not shown).
Discussion

Here, we propose CYP1B1 as a shared tumor-associated antigen expressed in almost all human malignancies tested so far. Biochemical analyses revealed expression of at least one CYP1B1-derived epitope (CYP190) on HLA-A*0201 molecules derived from tumor cells. Further epitopes are most likely to be presented as demonstrated for the CYP239 epitope. However, at least in HLA-A*0201 transgenic animals, CYP190 seems to be the immunodominant epitope. Importantly, immunity to epitopes from murine CYP1B1 could also be induced in vivo. Although not directly detectable in peripheral blood of healthy donors and cancer patients, fully functional CYP1B1-specific T cells were generated from most healthy volunteers and all cancer patients tested demonstrating an intact and expandable T cell repertoire for CYP1B1. Considering the above findings and its role as a carcinogen-activating enzyme during the early events of malignant transformation and/or progression, we have initiated clinical trials targeting CYP1B1.

CYP1B1 displays certain unique properties. Longitudinal studies in animal models have established stable over-expression of CYP1B1 throughout the malignant transformation. CYP1B1 has been implicated in carcinogenesis by environmental carcinogens such as dioxins and polycyclic aromatic hydrocarbons (PAH). PAH are metabolized by CYP1B1 to highly active epoxides thereby causing DNA adduct formation, an early step in tumor development. CYP1B1 has also been linked to endogenous estrogen-related carcinogenesis in human breast, uterine, and other tumors. 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. Further support for the role of CYP1B1 in
carcinogenesis is derived from studies of CYP1B1\(^{-}\) mice.\(^{41}\) 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 wild type mice. Likewise, the expression of CYP1B1 on normal tissues appears unique. The pattern of expression might be related to the linkage of CYP1B1 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. The expression of CYP1B1\(^{+}\) cells in the fallopian tube, breast, and uterus is of some concern when targeting this self antigen in immunotherapy strategies. The detection of whole protein by immunohistochemistry or Western blotting in normal tissue is an important aspect during the characterization of novel tumor antigens. However, it needs to be taken into account that the use of different antibodies and techniques\(^{32}\) will lead to somewhat different results. For tumor antigen discovery it therefore can only function as a screening tool followed by detailed immunological analysis as demonstrated here. Moreover, protein expression does not necessarily reflect antigen processing of the immunologically relevant epitopes in tissues tested positive for protein expression. There might be significant differences between various cell types in processing and presenting immunogenic epitopes and it might be possible that some normal tissues only poorly present these epitopes. This is best addressed in vivo in animal models or early clinical trials. Indeed, preliminary results from a phase I clinical trial conducted at Dana-Farber Cancer Institute targeting CYP1B1 with the same DNA construct described in this study did not reveal any autoimmunity against CYP1B1 despite the fact that anti-CYP1B1
specific immunity was induced in basically all patients vaccinated\textsuperscript{42} (John Gribben, Dana-Farber Cancer Institute, personal communication).

Success of cancer immunotherapeutics might require a combination of tumor antigens administered to cancer patients capable of responding to antigen challenge. Optimally, such patients would have both a normal T cell repertoire and minimal tumor burden. CYP1B1 might therefore be integrated into a cancer immunotherapeutic together with other widely expressed tumor antigens including but not limited to NY-ESO1,\textsuperscript{43} hTERT,\textsuperscript{10} MDM-2,\textsuperscript{44} Cyclin B1,\textsuperscript{45} and survivin.\textsuperscript{7} The clinical benefit of combining these widely expressed tumor-associated antigens are now under consideration.
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References


The shared tumor-associated antigen cytochrome P450 1B1 is recognized by specific cytotoxic T cells