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SHORT REPORT

Identification of a new HLA-A*0201-restricted cryptic epitope from CYP1B1

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Cytochrome P450 1B1 (CYP1B1) was recently shown to be a candidate tumor antigen broadly expressed in solid and hematologic malignancies. Nevertheless, use of such self-antigens as targets for immune intervention can be limited because of loss of high-avidity T cells during negative selection in the thymus. Recent data suggest that targeting of cryptic epitopes may represent a way to circumvent such self-tolerance and induce efficient antitumor CTL responses. Here, we present the identification and characterization of a novel, cryptic HLA-A*0201-binding peptide from CYP1B1. The nanomer CYP246 was identified by epitope deduction using algorithms to predict HLA-A*0201-binding peptides. CYP246 is characterized by strong initial HLA-A*0201 binding but a short MHC/peptide binding half-life. Expansion of high-avidity CTL was readily possible using autologous CD40-activated B cells from normal donors and cancer patients as antigen-presenting cells, suggesting that an intact T-cell repertoire can be expanded for this epitope. Lysis of CYP1B1-expressing, HLA-A*0201$^+$ tumor cell lines and primary tumor cells confirmed that sufficient levels of CYP246 are presented by tumor cells for effector CTL killing. These findings indicate that CYP246 is a candidate cryptic epitope for immune interventions in which tumor CYP1B1 is targeted.

Key words: CYP1B1; epitope; cryptic; tumor antigen; immunotherapy

Cytochrome P450 1B1 (CYP1B1) was recently described as an antigen that is widely expressed in human malignancies and can be recognized by cytotoxic T cells (CTL).[1] Due to its broad expression in almost all tumor tissues tested,[2] CYP1B1 represents an important candidate tumor antigen. Consequently, a CYP1B1-based vaccine trial has been initiated.[3] Preliminary results from this clinical trial suggest broad induction of immunity against CYP1B1 after vaccination. Thus far 2 high-affinity HLA-A*0201 epitopes have been identified (CYP239 and CYP190) and shown to induce functional tumor-specific CTL.[1] Targeting of self-proteins as tumor antigens is complicated by the fact that these proteins are frequently expressed in normal tissue including the thymus. Induction of anergy in and/or deletion of T cells expressing high-avidity receptors for candidate tumor/self-antigens such as tyrosinase and p53 have been demonstrated.[4,5] However, irrevocable self-tolerance is more likely to occur with dominant as opposed to cryptic epitopes.[6,7]

Most recently Gross *et al*. suggested that vaccination using heteroclytic variants of low-affinity epitopes would lead to induction of strong antitumor immunity.[8] One approach to targeting cryptic epitopes is the substitution of anchor residues for MHC binding (so-called heteroclytic epitopes) to induce and expand a T-cell repertoire demonstrating strong cross-reactivity with the native epitope.[9] Alternatively, epitopes with high affinity for MHC molecules but short complex stability may be used as immunogens since such epitopes are less prone to tolerize T cells in the thymus[10] at low antigen concentrations but retain the ability to prime T cells when the epitope is supplied at significantly higher concentrations on the surface of a potent APC.

We previously introduced CD40-activated B cells (CD40-B) as potent antigen-presenting cells that prime MHC class I- and II-restricted T-cell responses *in vitro*.[11,12] Using CD40-B cells, we established an efficient CTL induction protocol with which we can expand T-cell populations specific for epitopes of low immunogenicity. Here we present the identification of a novel, cryptic HLA-A*0201-binding epitope from CYP1B1, termed CYP246, using epitope deduction. Furthermore, we demonstrate that peptide-loaded CD40-B efficiently induce autologous, peptide-specific CTL capable of lysing tumor cell lines and primary tumors expressing both CYP1B1 and the appropriate MHC class I allele.

Material and methods

Donor and patient samples

Peripheral blood from healthy volunteers and multiple myeloma patients was obtained by phlebotomy after informed consent and approval by our institute's Review Board. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll density centrifugation. Primary lymphoma samples were obtained from discarded clinical specimen. Samples were disrupted mechanically and single-cell suspensions were prepared by Ficoll density centrifugation.

Peptides and peptide prediction

Peptides were purchased from Sigma Genosys Biotechnologies (The Woodlands, TX) and New England Peptides (Fitchburg, MA): CYP246 (WLQYFPNPV), HTLV-TAX11 (LLFGYPVYV) and MAGE-3 271 (FLWGPRALV). Binding of peptides to HLA-A*0201 was predicted using 3 algorithms: ''BIMAS'';[13] ''LPpep'' (kindly provided by Z. Weng, Boston University) and ''SYFPEITHI''.[14] 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.[15] For complex stability T2 cells were washed 3 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 monoclonal antibody BB7.2 (ATCC, Manassas, VA) at 0, 2, 4, 6 and 24 hr after peptide withdrawal. An 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

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TABLE I – PREDICTION OF CYP1B1-DERIVED EPITOPES AND PEPTIDE BINDING TO HLA-A*0201

Peptide	Position	Sequence	BIMAS	LPpep	SYFPEITHI	Cumulative score[1]	Binding affinity[2] (FI)	MHC/Peptide halflife[3] (hr)
CYP25	25–33	LLLSVLATV	1006	3.54	32	8	Could not be synthesized	
CYP88	88–96	RLGSCPIVV	29	4.61	20	55	2.4	2.6
CYP190	190–198	FLDPRPLTV	128	6.52	26	31	3.7	10.0
CYP239	239–247	SLVDVMPWL	1108	2.88	24	13	3.8	3.3
CYP246	**246–254**	**WLQYFPNPV**	**1216**	**6.23**	**21**	**35**	**3.4**	**0.9**
CYP292	292–300	MMDAFILSA	21	3.31	20	51	3.4	3.0
CYP344	344–352	LLFTRYPDV	656	4.69	24	18	3.1	2.7
CYP377	377–385	NLPYVLAFL	270	7.10	25	35	2.6	0.8
CYP380	380–388	YVLAFLYEA	65	1.56	20	42	Insoluble	
CYP486	486–495	ILAHQCDFRA	49	3.87	18	54	3.0	2.9
CYP528	528–536	LLDSAVQNL	33	4.08	26	24	3.4	4.1

[1]Predicted epitopes were ranked according to their score in each individual algorithm. The cumulative score was calculated as the sum of 3 individual ranks (lowest possible 3).–[2]Binding affinity shown as fluorescence index [FI = (mean fluorescence with peptide − mean fluorescence without peptide)/(mean fluorescence without peptide)] in the T2 binding assay. Results are representative of 4 experiments.–[3]Time to half-maximal FI in the peptide/MHC complex stability assay using T2 cells.

using linear regression analysis (y = yo + a*e exp(−b*x); SigmaPlot).

Cell lines

T2, U266, HS-Sultan and IM-9 cell lines were obtained from ATCC.

Generation of CTL lines and cytotoxicity assay

CTL lines from healthy individuals and cancer patients were generated as described and tested for cytotoxicity using standard 4 hr ^{51} chromium-release assay.[1,12]

Tetramer analysis

Tetrameric A2/peptide complexes with peptides from HTLV TAX (negative control) and CYP246 were synthesized essentially as described (kindly provided by Dr. K. Anderson, Dana-Farber Cancer Institute, Boston, MA)[16] and multimerized with streptavidin-Phycoerythin (SA-PE; Molecular Probes, Eugene, OR). Staining of cells with tetramers, monoclonal antibodies and annexin V for exclusion of dead cells as well as flow cytometry was performed as previously described.[17] At least 5,000 $CD8^+$ T cells were acquired and analyzed.

Results

Candidate epitopes from the enzyme CYP1B1 were identified using 3 computational epitope prediction algorithms (BIMAS, SYFPEITHI and LPpep). All candidate epitopes were ranked according to the sum of the individual prediction's rank. Besides the known epitopes CYP239 and CYP190,[1] CYP246 was among the peptides predicted to bind highly to HLA-A*0201 (Table I). Indeed, using a cellular binding assay strong initial binding was demonstrated for CYP246 (FI 3.4, Table I). However, the stability of CYP246/HLA-A*0201 complex was very low as evaluated by the half-life of peptide/MHC complexes after peptide withdrawal (Table I). Of the 7 candidates with sufficient initial binding (FI $\geq$ 3.0), the half-life of the peptide/HLA-A*0201 complex ($t_{1/2}$) was the shortest for CYP246 (0.9 hr), significantly shorter than that of the previously identified epitopes CYP190 and CYP239 (Table I). Based on these results, we tested whether CYP246 might be a cryptic CYP1B1 epitope.

Immunogenicity to CYP246 was assessed in an optimized *in vitro* T-cell screening system. CYP246-specific CTL were generated from the peripheral blood of healthy HLA-A*0201$^+$ volunteers by priming highly purified $CD3^+$ $CD8^+$ $CD16^-$ $CD56^-$ T cells and restimulating weekly for 3–4 weeks with peptide-pulsed autologous CD40-activated B cells. These CTL efficiently lysed T2 cells pulsed with CYP246 peptide but did not kill unpulsed T2 cells or T2 cells loaded with an irrelevant peptide (Fig. 1*a*).

To determine if CYP246 is presented by tumor cells, HLA-A*0201$^+$ and HLA-A*0201$^-$ cell lines were tested for susceptibility to lysis by CYP246-specific CTL. Multiple myeloma cells were chosen as representative targets because CYP1B1 is strongly expressed in all myeloma cell lines tested so far.[1] In 4 independent experiments, HLA-A*0201$^+$ IM-9 and U266 were efficiently lysed by CYP246-specific CTL, whereas the HLA-A*0201$^-$ line, HS-Sultan, was killed only at background level (Fig. 1*b*). Addition of nonlabelled CYP246-loaded T2 cells at a ratio cold:hot targets of 15:1 to lysis experiments resulted in 50% reduced lysis of IM-9 targets (data not shown). Similarly, CYP246-specific CTL were able to kill primary lymphoma cells from 2 HLA-A*0201$^+$ patients but failed to lyse lymphoma cells from a patient not expressing the HLA-A*0201 allele (data not shown).

We next evaluated whether CYP246-specific CTL could be generated from HLA-A*0201$^+$ untreated multiple myeloma patients. $CD8^+$ T cells from 2 patients were stimulated for 5 weeks with CYP246-loaded CD40-B cells. The resulting CTL efficiently lysed CYP246 peptide-pulsed T2 cells (data not shown). Furthermore, they efficiently killed CYP1B1-expressing myeloma cell lines U266 and IM-9 in an HLA-A*0201-restricted fashion (Fig. 1*c*).

MHC/peptide-specific T-cell frequency analyses using HLA-A2/CYP246 tetramers revealed a relatively low frequency of CYP246-tetramer binding cells among T-cell lines (*e.g.,* 0.35%, Fig. 1*d*) despite exhibiting a high level of cytolytic activity. Such highly efficient low-frequency CTL have been observed by other investigators.[18,19] Initial analysis of patients with multiple myeloma did not reveal a pre-expanded pool of CYP246-tetramer binding cells (data not shown). Collectively, these data indicate the induction of efficient, autologous peptide-specific, MHC-restricted CTL with the cryptic CYP246 peptide from very low precursor frequencies.

Discussion

Broadly expressed tumor antigens identified by comparative expression studies and epitope deduction may serve as target structures for widely applicable tumor immunotherapy. Here, we introduce a novel HLA-A*0201-binding peptide derived from cytochrome P450 1B1, an enzyme overexpressed in most human tumors.[2] Among the epitopes identified from CPY1B1 thus far, CYP246 is unique, owing to its MHC binding properties. Despite strong initial binding, the MHC-CYP246 peptide complex is relatively unstable with a binding half-life approximately 3 and 10 times shorter than that of other CYP1B1 epitopes (CYP239 and CYP190, respectively). This was consistent with the observation that, in contrast to the previously identified epitope CYP190,[1] CYP246 was not detected by mass spectrometry among peptides

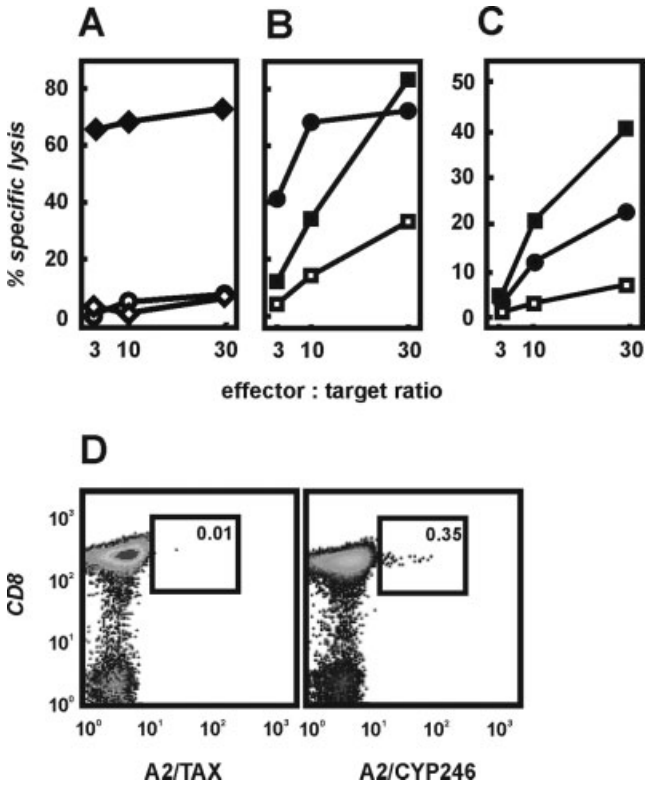

Figure 1 – Identification and characterization of a new HLA-A*0201-binding epitope derived from CYP1B1. CYP246-peptide (WLQYFPNPV) loaded onto autologous CD40-B cells was used to generate peptide-specific cytotoxic T-cell lines by 4–5 weekly stimulations. CTL lines routinely were >95% $CD8^+$ and <2% $CD56^+$. Cytotoxicity was measured by standard 4 hr ^{51}chromium-release assay. *(a)* CYP-246-specific CTL from a healthy HLA-A*0201^+ donor efficiently lyse T2 cells pulsed with CYP246 peptide (solid diamonds) but fail to lyse either unpulsed T2 cells (open diamonds) or T2 cells loaded with an irrelevant HLA-A*0201-binding peptide derived from MAGE-3 (open circles). *(b)* Cytotoxicity of CYP246-specific CTL against multiple myeloma cell lines. HLA-A*0201^+ cell lines IM-9 (solid squares) and U266 (solid circles) are killed by CYP246-specific CTL, whereas cytotoxicity against HLA-A*0201^- line HS-Sultan (open squares) is only at background level. All cell lines strongly express CYP1B1 as confirmed by Western blot analysis.[1] *(c)* Similar to CTL generated from healthy individuals, CYP246-specific CTL generated by repetitive stimulations from a patient with stage III multiple myeloma efficiently lyse both HLA-A*0201^+ MM cell lines IM-9 (solid squares) and U266 (solid circles), whereas HS-Sultan (open squares) is spared from cell lysis. *(d)* Tetramer analysis of this T-cell line shows a small population of cells (0.35% of total $CD8^+$ T cells) binding to the A2/CYP246-tetramer. A tetramer loaded with an immunogenic epitope derived from the HTLV TAX protein was used a negative control.

eluted from HLA-A*0201 molecules from CYP1B1$^+$ tumor cells (data not shown). Nevertheless, we could expand CYP246-specific $CD8^+$ T-cell populations as postulated from both healthy individuals and myeloma patients using CD40-activated B cells as antigen-presenting cells. These CTL were highly lytic against CYP246 peptide-pulsed targets as well as against primary tumor cells, although limited in number as demonstrated by MHC/peptide tetramer analysis.

Interestingly, despite the inability to detect CYP246 peptide eluted from HLA-A*0201 tumor cells and despite the relative instability of the complex it forms with HLA-A*0201, presentation of this epitope by tumor cell lines and primary tumor tissue was sufficient for lysis by CYP246-specific CTL. For such epitopes with limited MHC-peptide stability, killing with antigen-specific T cells may represent a more sensitive method to detect the epitopes than peptide elution techniques.

Of note, CYP246 is located in the same region and overlaps with the previously identified epitope CYP239.[1] However, a third immunogenic epitope, CYP190, maps to a different region, and other potential candidate epitopes have been identified throughout the CYP1B1 molecule (Table I). Extensive search of published data did not reveal whether there is any functional significance to this observation.

As expected, multiple myeloma patients did not appear to expand a preexisting population of T cells specific for this low-affinity epitope (data not shown). These findings indicate that CYP246 is a promising target complementary to the previously identified HLA-A*0201-binding CYP1B1 epitopes, which, unlike other tumor peptide targets, does not induce peptide-specific T-cell tolerance. Furthermore, highly active CTL can be induced and expanded from $CD8^+$ T-cell populations from cancer patients. Evaluation of CTL responses against cryptic epitopes such as CYP246 represents a valuable extension to analysis of ongoing clinical trials utilizing vaccination approaches against the whole CYP1B1 protein. It will be important to determine with tetramer and functional CTL assays if T cells specific for this ''cryptic'' and likely subdominant epitope are produced in patients immunized with whole CYP1B1 protein, with the various forms of the CYP1B1 gene or with the CYP246 peptide itself.

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