Jos G. A. Houbiers●◇,
Hans W. Nijman●△▼,
Sjoerd H. van der Burg◇,
Jan Wouter Drijfhout●,
Peter Kenemans△,
Cornelis J. H. van de Velde◇,
Anneke Brand●,
Frank Momburg◆,
W. Martin Kast●■ and
Cornelis J. M. Melief●

Department of Immunohaematology and Blood Bank●, Department of Surgery◇, University Hospital, Leiden, Department of Obstetrics & Gynaecology△, Free University Hospital, Amsterdam and Tumor Immunology Program◆, German Cancer Research Center, Heidelberg

In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53*

The central role of the p53 tumor suppressor gene product in oncogenesis is gradually being clarified. Point mutations in the p53 tumor suppressor gene are common in most human cancers and are often associated with p53 protein overexpression. Overexpressed wild-type or mutant determinants of the p53 protein thus represent an attractive target for immunotherapy of cancer directed against a structure involved in malignant transformation. An important step towards this goal is identification of epitopes of p53 that can be recognized by human cytotoxic T lymphocytes. We identified peptides of (mutant) p53 capable of binding to HLA-A2.1 in an *in vitro* assay. These HLA-A2.1-binding peptides were utilized for *in vitro* induction of primary cytotoxic T lymphocyte responses using a human processing-defective cell line (174CEM.T2) as antigen-presenting cell. These cells display "empty" HLA class I surface molecules, that can efficiently be loaded with a single peptide. We obtained $CD8^+$ cytotoxic T lymphocyte clones capable of specifically lysing target cells loaded with wild-type or tumor-specific mutant p53 peptides. This strategy allows the *in vitro* initiation of human cytotoxicT lymphocyte responses against target molecules of choice.

1 Introduction

The promise of T cell therapy of human cancer is looming large, but has not been fulfilled [1, 2]. Recently murine models demonstrated the potential of therapy with *ex vivo* cultured cytotoxic T lymphocytes [3, 4]. Human tumor-specific CTL have been cloned out of peripheral blood or tumor-infiltrating lymphocytes from patients with melanoma [5] and renal cell carcinoma [6]. In some cases the molecular identity of the tumor-associated antigens of these CTL has been elucidated ([17], Th. Boon, personal communication). Instead of natural immunity or immunization with tumor cells or protein, a reverse strategy aimed at generating T cell immunity against a precisely defined epitope in a tumor-associated target protein of choice seems preferable [2]. This strategy offers the advantage of targeting on molecules intrinsically associated with the growth deregulation characteristics of tumor cells. Moreover this approach allows the arousal of a repertoire of T cells against cryptic epitopes that is not normally evoked by immunization with whole antigen [8]. T cell repertoires against subdominant autologous epitopes usually are not subject to clonal deletion [8, 9].

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■ Fellow of the Royal Netherlands Academy of Arts and Sciences (KNAW).
▼ Fellow of the Netherlands Organization for Scientific Research (NWO).

Correspondence: Cornells J. M. Melief, Department of Immunohaematology and Blood Bank, University Hospital Leiden, Building 1, E3-Q, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands (Fax: 31 71 216751)

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CTL recognize peptide fragments of cellular proteins bound in the antigen-presenting groove of MHC class I molecules [10]. *In vitro* peptide induction of primary CTL responses has been achieved with synthetic peptide-loaded murine dendritic cells [11, 12] or mouse processing-defective RMA-S cells [13] as APC. To induce human CTL responses we utilized peptide-loaded human processing-defective ceils, 174CEM.T2 (T2) [14], with properties comparable to those of RMA-S cells. Our strategy of eliciting primary CTL responses *in vitro* includes the following steps: (1) selection of amino acid sequences in target proteins of choice that match peptide motifs for binding to HLA class I [15, 16], using a computerized scoring system, (2) testing of actual binding to HLA class I of these peptides [15, 17], (3) *in vitro* induction of CTL responses against binding peptides, (4) investigation of antigen processing of the chosen target protein using the generated CTL clones and (5) adoptive transfer of the tumor specific CTL [4].

Only few potential target antigens for T cell therapy of human cancer have been identified. Among these is the MAGE family of proteins expressed in melanoma and other tumors [18]. The p53 tumor suppressor gene product might represent another attractive target for CTL therapy. The central role of p53 in oncogenesis is gradually being clarified [19]. In the majority of human cancers the function of p53 is severely impaired by diverse mechanisms [20]. Point mutations in the p53 gene are often associated with p53 overexpression caused by the decreased breakdown of the tetrameric form with mutant components [21]. This may lead to abnormal presentation of p53 to the immune system as evident from the occurrence of p53-specific autoantibodies in patients bearing tumors with p53 overexpression [22]. We therefore concentrated on *in vitro* induction of CTL clones against both (overexpressed) wild-type p53 and (tumor-specific) mutant p53.

We previously reported on a scoring system predicting peptide binding to HLA-A2.1 and an *in vitro* peptide

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binding assay [15]. We now demonstrate that *in vitro* induction of primary CTL responses is feasible and that CTL against autologous and mutant epitopes of p53 can be raised.

2 Materials and methods

2.1 Peptide scoring system

We recently published a computer scoring system for predicting peptide binding to HLA molecules [15]. Briefly, for peptide binding to HLA-A2.1 the scores are based on the 9-amino acid HLA-A2.1-restricted peptide motifs [23, 24]. These motifs designate leucine, isoleucine or methionine at position 2 and an aliphatic residue (valine, leucine, isoleucine or alanine) at the C terminal end (position 9) as anchor residues in addition to various "strong" and "weak" motif residues at other positions. Peptide binding studies, performed since our recent report [15], with a variety of unselected overlapping 9-, 10- and 11-mer peptides from several proteins showed that at the C terminal end cysteine and threonine can also be designated as anchor residues [unpublished observations, J.G.A.H., H.W.N., W.M.K., M.C.W. Feltkamp (Leiden) and C.J.M.M.]. Our scoring system assigns 6. 4 or 2 points for an anchor, a strong or a weak residue match, respectively. The scores of all 9 amino acids are multiplied to reach the final peptide score. Peptides lacking anchor residues at position 2 and the C-terminal end were discarded. The scoring for 10- and 11-mer peptides was performed similarly, but multiple anchors at positions 9, 10 or 11 within one peptide were scored only once. A correlation can be expected between high score and binding to HLA-A2.1, which is exemplified by the observation that all HLA-A2.1-restricted CTL epitopes [25, 26] analyzed by us score over 71 points.

2.2 Cell lines and cytotoxicity assay

The 174CEM.T2 (T2, a gift from R Cresswell, Dept. of Immunology, Yale University, New Haven, CT) cell line showing no HLA-B5 and low HLA-A2.1 surface expression [14] was cultured in Iscove's modified Dulbecco's medium (IMDM) with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml kanamycin and 10 % FCS.

The T2/TAP1+2 cell line is the T2 cell line transfected with the genes encoding for the subunits TAP1 and TAP2 [27] of a putative peptide transporter. T2/TAP1+2 cells have restored HLA class I surface expression and show HLA class I-restricted antigen presentation [28, 29]. The T2/TAP1 cell line was transfected with only the gene for the TAP1 subunit of the TAP heterodimer and showed properties comparable to those of T2 [28].

The cytotoxicity of CTL against sensitized target cells was tested in a standard 4-h ^{51}Cr-release assay. The peptide was loaded onto the target cells during 10 min before the assay and was present in the medium during the assay. All HLA-A2.1 positive cell lines were effectively lysed by an HLA-A2.1 alloreactive CTL clone [30].

2.3 Peptide binding assay

The peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Abimed AMS 422) using Fmoc-chemistry and HPLC checked for contaminants. Peptides were dissolved in PBS, pH corrected, brought to a peptide concentration of 4 mg/ml and stored at −20 °C.

The T2 *in vitro* peptide binding assay was published recently [15]. Briefly, T2 cells were washed twice; 80000 T2 cells in 40 ul serum-free IMDM medium and 10 ul synthetic peptide (final peptide concentration in well: 50 μg/ml) were incubated overnight. After incubation the T2 cells were washed and stained successively with the HLA-A2 specific mouse monoclonal antibody BB7.2 and FITC-labeled F(ab')2 fragments of polyclonal goat anti-mouse IgG. Fluorescence of viableT2 cells was measured at 488 nm on a FACScan flow cytometer. The fluorescence ratio was calculated by the formula: mean fluorescence experimental sample/mean fluorescence background. A fluorescence ratio higher than 1.2 correlated with an experimental sample mean fluorescence higher than background mean fluorescence plus 2 standard deviations. Binding of the synthetic peptides was tested in at least three separate assays and was highly reproducible.

2.4 *In vitro* CTL induction protocol

T2 cells - to be used as APC - in a concentration of 2 X 10^6 cells per m! were incubated overnight with 80 μg/ml peptide at 37 °C in serum-free IMDM medium with 2 mM glutamine, antibiotics [100 IU/ml penicillin (Brocases Pharma, Leiderdorp, The Netherlands), 100 μg/ml kanamycin (Sigma, St. Louis, MO)]. After peptide incubation the T2 cells were treated with mitomycin C to prevent proliferation. After triple washing in RPMI (Gibco, Paisley, Scotland) 10^5 peptide-loaded T2 cells in 50 μl standard medium with 80 μg/ml peptide were seeded into each well of a 96-well U-bottom culture plate. Standard medium is RPMI, glutamine (2 mM) and antibiotics. Responder cells were Ficoll (Lymphoprep, Nycomed-pharma, Oslo, Norway) discontinuous gradient separated PBMC from an HLA-A2.1 subtyped healthy individual. Washed PBMC (4 x 10^5) in 50 μl standard medium with 30 % pooled human serum (tested and found negative for suppression activity in mixed lymphocyte cultures) were added to the peptide-loaded T2 cells and cultured at 37 °C in an incubator (5 % CO_2 and 90 % humidity). On day 7 the responding, viable PBMC of all 96 wells were harvested on Ficoll. "Responder cells" were seeded into a 96-well culture plate: 5 x 10^4 responder cells in 50 μl standard medium with 30 % pooled human serum per well. Per well 2 x 10^4 cryopreserved, autologous, irradiated (2500 rad) PBMC and 10^4 autologous, irradiated (5000 rad) B-LCL in 50 μl standard medium with 80 μg/ml peptide were added. On day 14 a similar restimulation was performed. On day 21 viable responder cells were harvested on Ficoll and washed in RPMI. This bulk was cloned by limiting dilution: 10 or less responder cells in 50 μl standard medium with 30 % pooled human serum were seeded per well. As stimulators (APC) and feeders, 2 x 10^4 pooled and irradiated (3000 rad) PBMC from at least three different donors and 10^4 pooled and irradiated (10000 rad) B-LCL from at least two different HLA-A2.1

Table la. Peptides of wild-type p53 that bind to HLA-A2.1

Position[a)]	Score[b)]		Peptides	FR[c)]
21- 31	576		DLWKLLPENNV	1.2~
24- 32	144		KLLPENNVL	1.7
25- 35	1152		LLPENNVLSPL	2.8
65- 74	288		RMPEAAPPVA	1.4
65- 73	288	D6:	RMPEAAPPV	2.2
113-122	2304		FLHSGTAKSV	1.3
129-138	144		ALNKMFCQLA	1.4
129-137	144		ALNKMFCQL	1.3
132-140	384		KMFCQLAKT	1.2~
136-145	288		QLAKTCPVQL	1.3
168-176	1152	B7:	HMTEVVRRC	1.2~
187-197	288		GLAPPQHLIRV	2.6
193-203	72		HLIRVEGNLRV	1.4
256-265	72		TLEDSSGNLL	1.3
263-272	72		NLLGRNSFEV	1.6
264-274	288		LLGRNSFEVRV	1.2~
264-272	288	A8:	LLGRNSFEV	1.9
339-407	288		EMFRELNEA	1.8

Table lb. Peptides of p53 mutants that bind to HLA-A2.1

Position[a)]	Score[b)]		Peptides	FR[c)]
129-137	288		ALNKMLCQL	1.4
129-137	288		ALNKMFYQL	1.2~
132-140	192		NMFCQLAKT	1.5
132-140	384		KLFCQLAKT	1.3
132-140	384		KMFYQLAKT	1.3
168-176	1152	I7:	HMTEVVRHC	1.3
187-197	288		GLAPPQHFIRV	1.2~
264-273	288		LLGRNSFEVCV	1.2~
264-274	288		LLGRNSFEVC	1.2~

a) Position of the peptide in the amino acid sequence of p53.
b) Arbitrary score of our scoring system, based on peptide motifs. Only 9-, 10- or 11-merpeptides scoring over 71 points with two anchor residues have been synthesized; see Sect. 2.1.
c) Fluorescence ratio of at least three separate peptide binding assays; see Sect. 2.3. — A weakly binding peptide.
The underlined letter marks the point mutation. A8, I7: peptides chosen for further studies; D6. B7 control peptides.

subtyped donors resuspended in 50 µl standard medium with 2 % leuco-agglutinin (Pharmacia, Uppsala, Sweden), human recombinant IL-2 (240 IU/ml, Eurocetus, Amsterdam, The Netherlands) and peptide (40 µg/ml) were added per well. Growing clones were expanded.

3 Results

3.1 Scoring of (mutant) peptides of p53 and actual binding of selected peptides

Computerized application of the scoring system to the 393 amino acids of wild-type p53 [31] and the 20 amino acids around each one of 32 published point mutations in colorectal and ovarian cancer (references available) resulted in a minority of p53 peptides scoring over 71 points and having two anchor residues: 41 peptides (14 9-mer, 15 10-mer and 12 11-mer) out of the 1152 possible wild-type peptides and 22 peptides (11 9-mer, 7 10-mer and 4 11-mer) out of the 960 possible mutant peptides.

These 63 potentially binding peptides were synthesized and tested for actual binding. The peptides that bind to HLA-A2.1 are shown in Table la. Eighteen of the 41 selected peptides (44 %) of wild-type p53 appeared to bind: 7 9-mer. 6 10-mer and 5 11-mer peptides, whereas 9 out of the 22 selected mutant peptides (41 %) of p53 showed binding properties to HLA-A2.1 (Table lb).

3.2 Comparison of different *in vitro* protocols for CTL induction

Several *in vitro* protocols for CTL induction have been compared to find optimal conditions. We used in all protocols PBMC from HLA-A2.1-positive healthy donors as "responders" cells. Protocols with different types of APC (T2, T2/TAP1, T2/TAP1+2 or PBMC) loaded with either 40 µg/ml or 0.4 µg/ml peptide and with responder cells being or not harvested on Ficoil were run in parallel (Table 2). The bulk cultures did not exert any cytotoxicity. During the bulk stimulations the "high peptide" and "Ficoil" protocols resulted in the lowest yields of "responder" cells. The "Ficoil" and "T2" protocols resulted in the lowest cloning efficiency (data not shown). Only T2 cells loaded with a high concentration of peptide combined with harvesting of responder cells on Ficoil generated stable, peptide-specific CTL clones (Table 2). The generated CTL clones are TcRα/β$^+$, CD2$^+$, CD3$^+$, CD8$^+$, CD25$^+$, HLA-DR$^+$, HLA-A2$^+$ and CD16$^-$ (data not shown).

PBMC used as responders cells were depleted of CD4$^+$ cells before start of the CTL induction to test whether CD4$^+$ lymphocytes are essential for this protocol. Induction

Table 2. Comparison of different CTL induction protocols

APC	F[a)]	Pep[b)]	Cln-CTX[c)]
PBMC	–	H	
PBMC	+	H	
PBMC	–	L	
PBMC	+	L	
T2	–	H	
T2	+	H	3
T2	–	L	
T2	+	L	
T2-CD4[–d)]	+	H	2
T2/TAP1+2	+	H	
T2/TAP1	+	H	3

a) Harvesting of responders cells +/– on Ficoil.
b) High (40 µg/ml) or low (0.4 µg/ml) peptide concentration.
c) Number of stable, peptide specific CTL clones generated.
d) The responder PBMC were depleted of CD4$^+$ cells.

without CD4+ responder cells generated stable, peptide-specific CTL. Utilization as APC of T2/TAP1+2 cells, that have restored antigen processing and HLA class I surface expression, failed to generate stable CTL clones, whereas T2/TAP1 cells, that are functionally comparable to T2, could act as useful APC (Table 2).

3.3 CTL clones against wild-type and mutant p53 peptides

Examples of *in vitro* induced CTL clones are 1A5 and 3C5 which recognize p53 {264–272} (A8, Table 1 and Fig. 2). The CTL clones show dose-response relationships in lytic capacity (Fig. 1). To check whether the specificity of CTL was not directed against a contaminant, the A8 peptide was HPLC purified and successfully used to sensitize target cells (Fig. 2). HLA-A2.1 restriction of the CTL clones was confirmed by blocking of T2-directed lysis with the anti-HLA class I mAb W6/32 (data not shown) and by lack of lysis of A8-sensitized HLA-A2.1− EBV-transformed lymphoblastoid B cells (LCL, Fig. 2). Cytotoxicity of the clones was also blocked by incubation of the CTL with an anti-CD8 mAb (data not shown).

The fine specificity of the CTL was studied by alanine and arginine replacements in the synthetic A8 peptide. Residues at positions 2, 3, 9 and to a lesser extent 6. 7 influence peptide binding to HLA-A2.1, whereas replacements at positions 4, 5 and 8 interfere with recognition by the T cell receptor. Substitutions at positions 6 and 7 might also affect CTL recognition (Table 3).

Using the same induction protocol we primed HLA-A2.1-restricted CTL precursors with specificity for an HLA-A2.1 binding peptide of p53 with an arginine to histidine mutation common in colon [32] cancer (peptide I7: p53{168–176,175H} see Table 1b). The CTL clone 10F10, shown in Fig. 2. does not lyse target cells that are sensitized with the wild-type p53{168-176} peptide (B7) and is therefore able to recognize a tumor-specific antigen.

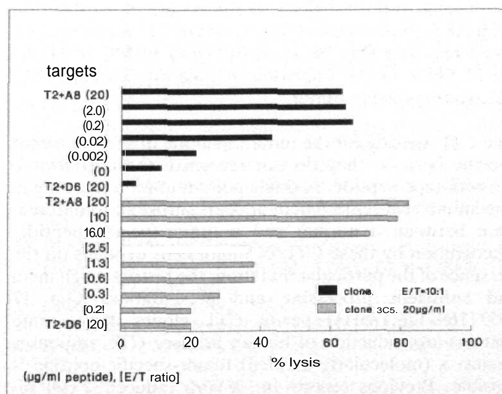

Figure 1. Dose-response relationship of cytotoxicity of two CTL clones that were induced *in vitro* against peptide A8 (p53{264–272} see Table 1a) in a standard 4-h ^{51}Cr-release assay. Target cells: T2 cells (T2) loaded with 20 ug/ml down to 2 ng/ml A8 peptide, with 20 ng/ml of the HLA-A2.1 binding p53{65–73} peptide (D6) or with PBS alone (0). The effector-to-target ratio [E/T] was 10:1 and the peptide concentration (µg/ml) was 20 µg/ml except where indicated otherwise.

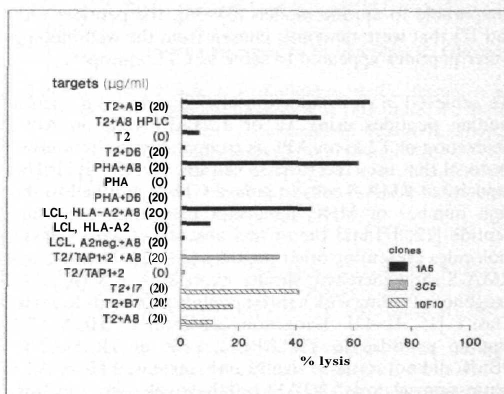

Figure 2. Standard 4-h ^{51}Cr-release cytotoxicity assay of two CTL clones specific for peptide A8 (p53{264–272}) and one CTL clone specific for peptide I7 (p53{168–176,175H}) with several types of target cells. T2 cells (T2), antigen presentation restored T2 cells (T2/TAP1+2), PHA blasts (PHA) or B-LCL (LCL) with HLA-A2.1+ or HLA-A2.1− (A2neg.) phenotype. The effector-to-target ratio was 10:1 and the peptide concentration 20 µg/ml, except for "T2+A8 HPLC" were the T2 target cells were sensitized with HPLC-purified A8 peptide. CTL against the mutant p53 peptide I7 do not lyse peptide B7 (wild-type p53 {168–176})-sensitized target cells.

Table 3. Amino acid replacements **in** p53 {264–272}[a]

Alanine replacement									
L	L	G	R	N	S	F	E	V	
+	−	±	+	+	+	+	+	−	binding
+	±	+	−	−	±	±	−	−	CTL lysis
Arginine replacement									
L	L	G	R	N	S	F	E	V	
+	±	-	+	+	−	-	±	−	binding
+	−	−	+	−	−	−	−	−	CTL lysis

a) Amino acid replacement study. Every single amino acid in the A8 peptide was replaced sequentially by alanine or arginine. Of all 17 different peptides the binding to HLA-A2.1 in the T2 peptide binding assay was assessed. T2 cells were peptide loaded (20 ug/ml) and used as target cells in a standard ^{51}Cr-release cytotoxicity assay to study recognition by the A8-specific CTL clone 1D5 in an effector-to-target ratio of 10:1. Symbols: + for high, ± for moderate and — for no binding or cytotoxicity. Moderate binding is a fluorescence ratio below 1.5 and moderate cytotoxicity is lysis between 20% and 60% of the maximum lysis.

4 Discussion

The pre-selection of peptides with the computerized scoring system is useful [15, 33], since out of 2112 possible peptides of (wild-type and mutant) p53, 63 peptides were selected, over 40% of which showed actual binding to HLA-A2.1. Ten- and 11-mer peptides were shown again to be among HLA-A2.1 binding peptides [33]. Furthermore, these first two steps of our reverse strategy resulted in precise prediction of HLA-A2.1-restricted epitopes since, comparable to murine models [34-36], the peptides (A8 and I7) that were randomly chosen from the well-binding 9-mer peptides appeared to serve as CTL epitopes.

We achieved *in vitro* induction of CTL responses against binding peptides using T2 or T2/TAP1 cells as APC. Utilization of T2 as an APC is comparable to the murine protocol that uses the RMA-S cell line as APC [13]. The capacity of RMA-S cells to induce CTL is ascribed to the high number of MHC molecules presenting the same peptide [12, 37] and the virtual absence of MHC class I molecules presenting other peptides [38–40]. Both T2 and RMA-S show increased surface expression of MHC after exogenous loading with a single peptide that binds to MHC Class I [15, 41–43]. Exogenous addition of HLA-A2.1 binding peptides to T2/TAP1+2 cells or HLA-A2.1$^+$ PBMC did not result in significantly increased HLA-A2.1 expression, whereas T2/TAP1 cells have cell surface expression comparable to that of T2 (data not shown). We conclude that under the circumstances of our *human* protocol a high number of relevant peptide/MHC complexes on the cell surface is important for *in vitro* CTL response induction. This is comparable to the murine model of De Bruijn et al. [12]. This induction of CTL precursor cells does not seem to be dependent on help from CD4$^+$ ceils, since CD4-depleted PBMC responders resulted in CTL clones as well (Table 2). An influence of irradiated CD4$^+$ cells, present among the feeder cells during the re-stimulations (2nd and 3rd week) can not be excluded. De Bruijn et al. showed that in the mouse primary CTL response induction by peptide-loaded dendritic cells or RMA-S cells is CD4$^+$ T cell independent [12].

Our strategy may be helpful to select by in vitro methodology peptides (epitopes) that are suitable for peptide-based vaccines for human cancer. In a mouse model, a peptide-based vaccine, selected on the basis of MHC class I binding capacity, was shown to prevent outgrowth of tumors (Feltkamp, M.C.W. et al., manuscript submitted).

The alanine and arginine replacement study defined the binding requirements of A8 to HLA-A2.1 and the fine specificity of the A8 specific clones. The importance of positions 2, 3. 9 and to a lesser extent 6, 7 for peptide binding is reflected in the published peptide motifs for HLA-A2.1 [23, 24, 26] and other replacement studies [33]. The amino acid side chains at positions 4, 5 and 8 (and possibly 6, 7) seem essential for TcR recognition which largely confirms previous reports on HLA-A2.1 restricted CTL [25, 26]. Our *in vitro* human CTL induction protocol might be a useful tool in studies on the precise composition of the TcR, *e.g.*, Vα, Vβ usage [44] and their precise sequences, of human class I-restricted CTL. From PBMC of a single donor several CTL clones could be generated against peptides that only differ in one amino acid.

Our A8 (p53 wild-type)-specific CTL clones supplement observations of T cell responses against autologous structures [22, 45]. Nonresponsiveness for the self antigen p53 *in vivo* can result from several mechanisms: (1) clonal deletion of p53 reactive T cells, (2) anergy or suppression of established CTL, (3) localization of the self antigen or (4) absent or insufficient antigen processing. It has already been stated that tolerance only exists towards dominant epitopes. whereas the repertoire against "cryptic" self epitopes is largely intact [8, 46].

The A8-specific CTL disprove clonal deletion (1) while a possible anergic state of the CTL (2) might have been overcome in the induction protocol [47], although the CTL most probably result from a primary induction by our *in vitro* protocol. In our study we even have observed that induction of primary CTL responses against the wild-type p53 (self) peptide was easier, more efficient, than induction against the mutant p53 (foreign) peptide. P53 is present in every cell (3) at very low concentration [19], but the clones specific for peptide A8 are not capable of lysing T2/TAP1+2 cells, PHA-blasts and B-LCL without peptide loading. Absence or insufficiency of processing and presentation (4) of the CTL epitope (A8) is a possible explanation for the lack of lysis of non sensitized "normal" cells. A murine model showed that autoreactive (cytochrome c) T cells escape tolerance when the self-peptide lacks processing [48]. Alternatively, the epitope can be presented at levels insufficient for recognition [9, 49, 50] by the A8-specific CTL clones. The A8-specific CTL clones could be blocked with an anti-CD8 mAb, which has been associated with "low-affinity" T cell receptor [37, 51, 52].

In the case of tumor cells with overexpression of p53, specifically under conditions of abnormal routing of p53 through the cytoplasm [53], the processing might be qualitively and/or quantitively different. IgG autoantibodies against overexpressed p53 in breast cancer patients have been reported [22] indicating activation of CD4$^+$ T helper lymphocyte responses. Since we can exclude clonal deletion of all A8 peptide specific CTL, a primary or even secondary T cell response may be inducible from PBMC of HLA-A2.1$^+$ (40% of the Caucasoid population) patients with p53 overexpressing tumor.

The CTL specific for the mutant peptide of p53 are tumor specific because they do not recognize the corresponding wild-type peptide. A single point mutation resulting in one amino acid replacement appears sufficient to differentiate between a normal and a tumor-specific peptide. Recognition by these CTL of tumor cells depends on the presence of the particular mutation, the restriction element and sufficient processing and presentation. Our I7 (p53{168–176,175H})-specific CTL clones demonstrate that *in vitro* induction of human primary CTL responses against a (molecularly defined) tumor-specific epitope is feasible. Previous reports on *in vitro* induced T cell responses concerned non cytotoxic, CD4$^+$, HLA class II-restricted T cells that proliferate upon stimulation with peptides of point-mutated ras protein [54, 55].

In conclusion, selection of HLA class I-restricted epitopes from a target molecule of choice is possible and *in vitro* peptide-mediated induction of human CTL clones is feasible. These or other CTL clones directed against the

above-mentioned peptides may be capable of selectively lysing tumor cells with the relevant mutation of p53 or overexpression of p53. Such CTL are potentially useful for cellular immunotherapy of cancer. The same strategy can be applied to other molecularly defined HLA-binding epitopes in target proteins of choice.

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5 References

1 Rosenberg, S. A., *Cancer Res.* 1991. *51 (18suppl):* 5074s.
2 Melief, C. J. M., *Adv. Cancer Res.* 1992. *58:* 143.
3 Greenberg, P. D., *Adv. Immunol.* 1991. *49:* 281.
4 Kast, W. M., Offringa, R., Peters. P. J., Voordouw, A. C, Meloen, R. H., Eb, A. J. van de and Melief, C. J. M., *Cell* 1989. *59:* 603.
5 Cerottini, J. C., FJeidner. V. von and Boon, T.. *Ann. Oncol.* 1992. *3:* 11.
6 Koo, A. S., Tso, C. L., Shimabukouro, T., Peyret. C, deKernion. J. B. and Belldegrun, A., *J. Immunother.* 1991. *10:* 347.
7 Boon, T., *Adv. Cancer Res.* 1992. *58:* 177.
8 Gammon, G., Sercarz, E. E. and Benichou, G., *Immunol. Today* 1991. *12:* 193.
9 Ohnó, S., *Immunogenetics* 1992. *36:* 22.
10 Townsend, A. and Bodmer, H., *Annu. Rev. Immunol.* 1989. *7:* 601.
11 Macatonia, S. E., Taylor, P. M., Knight. S. C. and Askonas. B. A., *J. Exp. Med.* 1989. *169:* 1255.
12 Bruijn, M. L. H., de, Nieland, J, D., Schumacher. T. N. M., Ploegh, H. L., Kast. W. M. and Melief, C. J. M.. *Eur. J. Immunol.* 1992. *22:* 3013.
13 Bruijn, M. L. H., de, Schumacher, T. N. M., Nieland, J. D., Ploegh. H. L., Kast, W. M. and Melief, C. J. M., *Eur. J. Immunol.* 1991. *21:* 2963.
14 Salter, R. D. and Cresswell, P., *EMBO J.* 1986, 5: 943.
15 Nijman, H. W., Houbiers, J. G. A., Vierboom, M. P M.. Burg, S. H.. van der. Drijfhout, J. W, D'Amaro, J., Velde, C. J. H. van de. Kenemans. P., Melief. C. J. M. and Kast, W. M., *Eur. J. Immunol.* 1993. *23:* 1215.
16 Hill, A. V S., Elvin, J., Willis. A. C, Aidoo, M., Allsopp, C E. M., Gotch, F. M., Gao, X. M., Takiguchi, M., Greenwood, B. M., Townsend. A. R. M., McMichael, A. J. and Whittle, H. C., *Nature* 1992. *360:* 434.
17 Stuber, G., Modrow. S., Hoglund, P. Franksson, L., Elvin, *L,* Wolf, H., Karre, K. and Klein, G., *Eur. J. Immunol.* 1992. *22:* 2697.
18 Bruggen, P. van der, Traversari. C., Chomez, P., Lurquin, C., Plaen, E. de, Eynde, B. van den, Knuth, A. and Boon. Th.. *Science* 1991. *254:* 1643.
19 Lane, D. P, *Nature* 1992. *358:* 15.
20 Vogelstein, B. and Kinzier, K. W., *Cell* 1992. *70:* 523.
21 Levine, A. J., Momand, J. and Finlay, C. A., *Nature* 1991. *351:* 453.
22 Crawford. L. V., Pim, D. C. and Bulbrook. R. D., *Int. J. Cancer* 1982. *30:* 403.
23 Falk. K., Rötzschke, O., Stevanovic, S., Jung, G. and Rammensee, H.-G., *Nature* 1991. *351:* 290.
24 Hunt, D. F., Henderson, R. A., Shabanowitz, J., Sakhuchi, K., Michel, H., Sevilir, N., Cox, A. L., Appella, E. and Engelhard, V. H., Science 1992. 255; 1261.
25 Gotch, F. M., McMichael, A. J. and Rothbard, J., *J. Exp. Med.* 1988. *168:* 2045.
26 Morrison, J., Elvin, J., Latron, F., Gotch, E, Moots, R., Strominger, J. L. and McMichael, A. J., *Eur. J. Immunol.* 1992. *22:* 903.
27 DeMars, R. and Spies, T., *Trends Cell Kol.* 1992. *2:* 81.
28 Momburg, F., Ortiz-Navarrete, V., Neefjes, J., Houlmy, E., Wal. Y van de, Spits, H., Powis, S. J., Butcher, G. W., Howard, J. C, Walden, P and Hämmerling, G. J., *Nature* 1992. 360: 174.
29 Arnold, D., Driscoll. J., Androlewicz, M., Hughes, E., Cresswell, P. and Spies, T., *Nature* 1992. *360:* 171.
30 Horai. S., Poel, J. van de and Goulmy, E.. *Immunogenetics* 1992. *16:* 135.
31 Soussi, T., Caron de Fronmental, C and May. P. Oncogene 1990. 5: 945.
32 Nigro. J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, R, Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. and Vogelstein. B., *Nature* 1989. *342:* 705.
33 Parker, K. C, Bednarek, M. A., Hull, L. K., Utz, U., Cunningham, B., Zweerink, H, J., Biddison. W. E. and Coligan. J. E., *J. Immunol* 1992. *149:* 3580.
34 Pamer, E. G., Harty, J. T, and Bevan, M. J., *Nature* 1991. *353:* 852.
35 Rötzschke. O., Falk, K., Stevanovic. S., Jung, G., Walden, P. and Rammensee, H.-G., *Eur. J. Immunol.* 1991. *21:* 2891.
36 Zhou. X., Abel Motal. U. M., Berg. L. and Jondal. M., *Eur. J. Immunol.* 1992. *22:* 3085.
37 Alexander, M. A., Damico, C A., Wieties. K. M., Hansen. T. H. and Connolly, J. M., *J. Exp. Med.* 1991. *173:* 849.
38 Townsend, A. R. M., Öhlén. C., Bastin, F., Ljunggren, H. G., Foster. L. and Karre, K., *Nature* 1989. *340:* 443.
39 Sijts, E. J. A. M., Bruijn, M. L. H. de, Nieland, J. D., Kast. W. M. and Melief, C. J. M., *Eur. J. Immunol.* 1992. 22: 1639.
40 Stauss, H. J., Davies, H., Sadovnikova, E., Chain, B., Horowitz, N. and Sinclair, C, *Proc. Natl. Acad. Sci. USA* 1992. *89:* 7871.
41 Cerundolo. B., Alexander, J., Anderson, K., Lamb. C, Cresswell. P., McMichael. A., Gotch, F. and Townsend, A., *Nature* 1990. *345:* 449.
42 Ljunggren, H.-G., Stam. N. J., Öhlén. C, Neefjes, J. J., Hoglund. P., Heemles, M.-T., Bastin, J., Schumacher, T. N. M., Townsend, A. R. M. and Karre, K., *Nature* 1990. *346:* 476.
43 Schumacher, T. N. M.. De Bruijn, M. L. H.. Vernie, L. N., Kast. W. M., Melief, C. J. M., Neefjes. J. J. and Pioegh. H. L., *Nature* 1991. *350:* 703.
44 Nanda. N. K., Arzoo, K. K. and Sarcarz. E. E., *J. Exp. Med.* 1992. *176:* 297.
45 Wraith, D. C. Smilek. D. E., Mitchel, D. J., Steinman. L. and McDevitt, H. O., *Int. Rev. Immunol.* 1990. *6:* 37.
46 Ohno, S., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 4643.
47 Grossman. Z. and Paul, W. E., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 10365.
48 Mamuia, M. J., *J. Exp. Med.* 1993. *177:* 567.
49 Christinck, E. R., Luscher, M. A., Barber, B. H. and Williams. D. B., *Nature* 1991. 352: 67.
50 Schild. H., Rötzschke. O., Kalbacher, H. and Rammensee, H.-G., *Science* 1990. 257: 1587.
51 Maryanski, J. L., Pala, P., Cerottini, J.-C. and MacDonald, H. R., *Eur. J. Immunol.* 1988. *18:* 1863.
52 Moretta, A., Pantaleo. G., Mingari, M. C, Moretta, L. and Cerottini, J., *J. Exp. Med.* 1984. 759: 921.
53 Moll, U. M.. Riou, G. and Levine. A. J., *Proc. Natl. Acad. Sci. USA* 1992. *89:* 7262.
54 Jung. S. and Schluesener. H. J., *J. Exp. Med.* 1991. *173:* 273.
55 Gedda-Dahl III, T., Eriksen, J. A., Thorsby, E. and Gaudernack, G., *Hum. Immunol.* 1992. 33: 266.