

# Identification of a new hTERT-derived HLA-A\*0201 restricted, naturally processed CTL epitope

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**Abstract** By the use of a neural network capable of performing quantitative predictions of peptides binding to HLA-A\*0201 molecules, we identified a number of non-amer peptides derived from the catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT). Five nonimmunogenic peptides with measured binding affinities for HLA-A\*0201 ranging from 155 to 1,298 nM were modified at the P1, P2 and P9 positions, respectively, to achieve stronger HLA-A\*0201 binding. One peptide, mp30–38 (mp30), with an L to V substitution at position 9 was subsequently found to be immunogenic in mp30 immunized HLA-A\*0201/H2K<sup>b</sup> or HHD transgenic mice.

The T cell reactivity obtained was directed against both the mp30 and against the unmodified p30. Anti-mp30 specific T cells generated in HLA-A\*0201 transgenic mice were dependent on TCR-CD8/MHC-I  $\alpha 3$  binding and therefore not capable of recognizing mp30-pulsed human HLA-A\*0201<sup>+</sup> cells or murine HLA-A\*0201 transfectants. In order to show reactivity against naturally processed peptide in human tumor cells, an hTERT positive HLA-A\*0201 negative colon carcinoma cell line (CCL220) was transfected with an HLA-A\*0201/H2K<sup>b</sup> cDNA construct and used as target in ELISPOT and cytotoxicity assays. The data show that T cells from mp30 immunized HHD transgenic mice react specifically against the CCL220 transfectant indicating that p30 is naturally processed. In conclusion, we have identified a new CTL HLA-A\*0201 restricted hTERT epitope, which is now, included in an ongoing phase 2 vaccine trial of patients with disseminated cancer.

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## Introduction

Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase and its expression is rate-limiting for telomerase activity, which maintains the protective structure, the telomeres, at the ends of eukaryotic chromosomes [11]. In the majority of human somatic cells, the expression of hTERT is suppressed and the repetitive sequences in the ends of chromosomes are shortened at cell divisions, ultimately resulting in apoptosis [10]. Telomerase expression has been directly linked to tumor development, and inhibition of telomerase in human tumour cells may therefore arrest growth [9, 12]. Thus, the vast majority

of human cancers expresses hTERT and this molecule might therefore qualify as an universal tumor-antigen [7], and as a target for an effective cancer vaccine therapy.

Previously, MHC class I binding peptides derived from hTERT have been shown to elicit CD8<sup>+</sup> T cell responses in vivo capable of recognizing hTERT expressing tumour cells [3, 13, 15, 16, 19, 25–28].

Like most tumour-associated antigens (TAA), hTERT is a self-antigen and central tolerance towards hTERT-derived peptides with high affinity for the MHC molecules is therefore to be expected. However, hTERT-derived peptides with intermediate binding affinities for MHC class I molecules might be insufficiently presented to trigger naive CTLs, but presented at levels capable of stimulating already activated CTLs. In the present work we have used a neural network capable of performing quantitative predictions of peptides binding to HLA-A\*0201 molecules, and identified a number of nonamer hTERT-derived peptides with intermediate binding affinities. These peptides were then modified in order to improve their binding affinity for HLA-A\*0201 above the threshold necessary for activation of naive T cells. This strategy has been used previously by us and others in studies including p53- and hTERT-derived peptides with low or intermediate affinity for the HLA-A\*0201 molecule [8, 13, 18, 19, 24]. However, the position of the residues to be modified and substituted differed between the studies. In the present study modifications were performed in the two primary anchor position residues (P2, P9).

## Materials and methods

### HLA-A\*0201 transgenic mice

C57-A2K<sup>b</sup> transgenic mice, which express a chimeric heavy chain of the MHC-I molecule (HLA-A\*0201  $\alpha$ 1 and  $\alpha$ 2 and H-2K<sup>b</sup>  $\alpha$ 3, transmembrane and intracytoplasmic domains), were kindly provided by Dr. N. Holmes, University of Cambridge, Cambridge, UK. The mice were backcrossed with C57Bl/6Bom (Bomholtgård, Ry, Denmark). HHD transgenic mice, which express the chimeric MHC-I heavy chain (HLA-A\*0201  $\alpha$ 1 and  $\alpha$ 2 and H-2D<sup>b</sup>  $\alpha$ 3, transmembrane and intracytoplasmic domains) with human  $\beta$ 2-microglobulin ( $\beta$ 2 m) covalently linked to the NH<sub>2</sub> terminus of the heavy chain by a 15-amino acid long peptide were kindly provided by F. A. Lemonnier. HHD mice are H-2D<sup>b</sup><sup>-/-</sup> and mouse beta2 m<sup>-/-</sup> double knockout mice.

### Predictions of hTERT peptide binding to HLA-A\*0201

The complete hTERT protein sequence was obtained from NP\_937983 and virtually digested into all possible nonamer

peptides. Artificial neural networks (ANN) were used to predict some of the most important events of antigen processing and presentation: proteasome digestion (NetChop 1.0) and HLA class I binding (NetMHC 1.0, current versions are available at the immunological section of <http://www.cbs.dtu.dk/services/>). Briefly, ANN are trained, using an existing set of data representing the event in question, to capture the general features of that event thereby enabling the ANN to predict the outcome for any nonamer [5, 14].

### HLA-A\*0201-binding assays

Peptides were synthesized using standard Fmoc chemistry at Schafer-N (<http://www.schafer-n.com>). Purity was ascertained by reverse-phase HPLC and identity was confirmed by mass spectroscopy analysis. Peptide-binding affinities to purified HLA-A\*0201 molecules were measured using an in vitro biochemical binding assay [6]. Briefly, the concentration of test peptide needed to effect 50% inhibition (IC<sub>50</sub>) of the binding of a radio labelled tracer peptide was measured by spun column gel filtration.

### Stable transfection of CCL220 with HLA-A\*0201/H2-K<sup>b</sup> cDNA

Human colon carcinoma cells (CCL-220, HLA-A\*0201<sup>-</sup>) were transfected by Lipofectamine 2000 (Invitrogen Corporation, CA, USA) with a plasmid vector encoding the chimeric heavy chain of the MHC class I molecule (A2K<sup>b</sup>) and the neomycin resistance gene. Briefly, 7.5  $\mu$ l Lipofectamine 2000 was diluted in 50  $\mu$ l RPMI-1640 (serum, and antibiotics free) and incubated for 5 min at room temperature (RT) before being mixed with 50  $\mu$ l RPMI-1640 containing 3  $\mu$ g cDNA. After incubating for 20 min at RT, 100  $\mu$ l of the cDNA–Lipofectamine 2000 complexes was added directly to  $0.5 \times 10^6$  cells in 400  $\mu$ l RPMI-1640. Cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 6 h, then additional 500  $\mu$ l RPMI-1640 containing 20% FCS was added. The next day, transfected cells were passaged with complete culture medium supplemented with G418 (Sigma, St Louis, USA) at 800  $\mu$ g/ml. The transfected cells were verified for HLA-A\*0201 expression with flow cytometry by using the PA2.1 (anti-HLA-A\*0201) unlabelled monoclonal antibody (HB-117, ATCC, Rockville, MD, USA) and subsequently FITC-conjugated rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark). Approximately 85% of the CCL220 cells were stably HLA-A\*0201 positive in this culture system.

### Cell lines and anti-CD8 antibody

CCL220, CCL220-A2K<sup>b</sup>, EL4-A2K<sup>b</sup> and EL4-A2 (a kind gift from Dr. Linda A. Sherman) were cultured in complete

culture medium RPMI-1640 (Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin, and  $2 \times 10^{-5}$  M mercaptoethanol. CCL220-A2K<sup>b</sup>, EL4-A2 and EL4-A2K<sup>b</sup> were also cultured in the presence of G418 (800 µg/ml). Ascites generated by hybridoma YTS 169.4 (a kind gift from Dr. J. Reimann) containing 1 mg anti-CD8 mAb/ml were used.

#### Generation of mature dendritic cells from bone marrow cells

Bone marrow cells from transgenic mice were kept over night in culture medium (RPMI 1640 with 10% FCS, penicillin, streptomycin, 2-mercaptoethanol) and the non-adhering cells were isolated by plastic adherence and cultured with GM-CSF, 10 ng/ml, and IL-4, 20 ng/ml (PreproTech Inc., Rocky Hill, NJ, USA). On day 3, fresh culture medium with GM-CSF and IL-4 were supplied. On day 6, the cells were harvested and cultured over night with GM-CSF, IL-4 and LPS, 1 µg/ml. On day 7, the mature dendritic cells were harvested and used for vaccination.

#### Generation of CTLs in transgenic mice

HHD transgenic mice, 8–12 weeks old, were injected subcutaneously in the flank with  $3 \times 10^6$  syngenic mature dendritic cells pulsed with the relevant A2-binding hTERT-peptide and a T-helper peptide PADRE (Pan DR reactive epitope), which binds multiple HLA-DR alleles and H-2 I-A<sup>b/d</sup> or I-E<sup>b/d</sup> antigens [2]. On day 11 after vaccination, the mice were sacrificed and the splenocytes were recovered and re-stimulated with the vaccination-peptide. Briefly, splenocytes ( $100 \times 10^6$  ml) were loaded with the A2-binding vaccination-peptide, 100 µg/ml, for 2 h at 37°C and 5% CO<sub>2</sub>. On day 1 after re-stimulation, IL-2 (Roche, Indianapolis, USA), 50 IU/ml, was added. After 5 days, the CTLs were harvested and depleted for NK and B cells using anti CD49bDX5 antibodies and anti CD19 antibodies (MACS, Milteneyi Biotec, Gladbach, Germany), respectively. Cells were depleted on a column, MACS LS, according to manufacturer's protocol. The non-depleted cells were then used directly for <sup>51</sup>Cr-release assay or kept for another 4 days under resting conditions with IL-2, and then used for ELISPOT assay. C57-A2K<sup>b</sup> transgenic mice were immunized and splenocytes restimulated as above and unfractionated splenocytes were used directly for the ELISPOT assay.

#### ELISPOT assay

The generated CTLs were assayed in 20-h ELISPOT cultures (96-well nitrocellulose microtitre plates, Millipore, Billerica, MA, USA) for IFN-γ production. Briefly microtitre plates were coated overnight with 50 µl per well

of anti-IFN-γ monoclonal antibody (BD Pharmingen, San Diego, CA, USA) at 10 µg/ml in carbonate buffer, pH 9.6. Effector:stimulator cell ratio, 3:1, was added in at least triplicates with titration of cells ranging from 300,000:100,000 down to 18,750:6,250. The plates were incubated in 37°C, 5% CO<sub>2</sub> for 20 h. The plates were processed using biotinylated anti-IFN-γ (5 µg/ml, biotin rat antimouse IFN-γ BD Pharmingen, San Diego, CA, USA), streptavidin-conjugated horseradish peroxidase (1:1,000, DAKOCytomation, Glostrup, Denmark) and Alkaline Phosphate Conjugate Substrate Kit (BIORAD, Hercules, CA, USA). Spots were quantified using the ELISPOT reader, ELR02 (Autoimmun Diagnostika GmbH, Strassberg, Germany). Peptide pulsed (40 µg/ml for 2 h, 37°C, 5% CO<sub>2</sub>) or unpulsed EL4-A2 or EL4-A2K<sup>b</sup> transfectants, CCL220-A2K<sup>b</sup> transfectants or wildtype CCL220 cells were used as stimulator cells in the assay.

In the case of human blood cells, ELISPOT analysis was performed after one week in vitro culture of separated mononuclear cells with peptide (5 µg) and IL-2 (40 IU/ml). We cultured  $3 \times 10^5$  to  $3 \times 10^4$  cells per well, depending on the availability of patient material, together with hTERTmp30 peptide (5 µg/ml) and TAP deficient HLA-A\*0201<sup>+</sup> 10<sup>4</sup> T2 cells. After an overnight incubation, IFN-γ spots were developed. The number of spots was determined by a digitalized ELISPOT counter (Immunospot, CTL Inc., CA, USA).

#### Cytotoxic assay

Target cells, CCL220 and CCL220-A2K<sup>b</sup>, were either left unpulsed or pulsed with the vaccination-peptide or the wildtype peptide in a final concentration of 40 µg/ml for 2 h at 37°C in culture medium. Target cells were labelled (30 µCi Na<sub>24</sub><sup>51</sup>CrO per  $1 \times 10^6$  cells), then washed four times and resuspended to  $3 \times 10^4$  ml. Target cells (100 µl) were then mixed with effector cells at effector:target ratios (E:T) of 100:1, 50:1, 25:1 and 12.5:1. After incubation for 4 h at 37°C in 5% CO<sub>2</sub>, <sup>51</sup>Cr-release was measured in 50 µl supernatant using Lumaplates (Perkin-Elmer, Boston, USA) and a microplate scintillation counter. Spontaneous and total <sup>51</sup>Cr-releases were measured by adding culture medium or a detergent (Triton X-100) 2%, respectively. The percentage of specific lysis was calculated as  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})$ .

#### Western blot

For detection of hTERT in human CCL220 tumor cells, cell lysates of  $5 \times 10^6$  cells were subjected to electrophoresis on 10% SDS polyacrylamide gels. The separated proteins were electrotransferred to nitrocellulose membranes. The

membranes were treated with hTERT-specific rabbit polyclonal antibody H-231 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by swine anti-rabbit IgG horseradish peroxidase (DAKOcytomation, Glostrup, Denmark). The HeLa nuclear extract (sc-2120, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a positive control for hTERT expression. The ECL Plus kit (Amersham Biosciences, Piscataway, NJ, USA) was used to detect antigen/antibody complexes and chemiluminescent signals were detected using X-ray film. The western blot positive HeLa extract control showed one band at 74–76 kDa and the same band was found in the cell-lysate of CCL220.

#### Clinical vaccination trial

Two HLA-A2 positive patients with progressive metastatic renal cell carcinoma were enrolled in a clinical phase I/II study testing the efficacy and toxicity of dendritic cell (DC) based vaccination (ClinicalTrials.gov Identifier: [NCT00197860](#)). Briefly, autologous DCs were pulsed with HLA-A2 matched hTERT mp30 peptide. On the day of vaccination,  $1 \times 10^7$  peptide pulsed DCs were injected. Vaccination no. 1–4 were given weekly and no. 5–10 were given biweekly. A leukapheresis sample collected prior to treatment (week-2) and blood samples drawn at successive time points during treatment were analyzed (manuscript in preparation). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation (Lymphoprep, Nycomed Diagnostika, Oslo, Norway) and cryo-preserved.

## Results

#### Selection of new HLA-A\*0201-binding hTERT-derived epitopes

Four peptides were chosen with predicted HLA-A\*02 binding affinities from 15 to 382 nM and with at least one

suboptimal anchor residue for HLA-A\*0201 binding that theoretically, by substitution of an optimal amino acid, might improve the binding (Table 1).

Table 1 also includes the peptides, p540–548 [15, 27, 28] and p572–580 [13, 19], since the immunogenicity of p540–548 and a p572–580 analogue peptide have been demonstrated previously. Table 1 shows also the biochemically measured peptide binding affinities for HLA-A\*0201. In order to improve the HLA-A\*0201 binding affinity of these peptides, they were subjected to aminoacid substitutions as indicated in the table. However, after modification only minor changes in the affinity were observed: the affinities of three peptides were increased, in one peptide, there was hardly any change and in another, the affinity decreased after modification. The discrepancies between the predicted and the measured binding affinities are within the ranges normally observed in this system [5].

#### Immunogenicity of analogue hTERT peptides and cross-reactivity with the wild type epitope

Immunogenicity was assessed by immunizing C57-A2K<sup>b</sup> transgenic mice. Each peptide was pulsed on dendritic cells and subsequently injected into transgenic mice and IFN- $\gamma$  ELISPOT was used as readout for immunogenicity. Except for the known immunogenic peptide p540–548 [15, 27, 28], none of the hTERT-derived wild type peptides in Table 1 were capable of eliciting IFN- $\gamma$  spots (data not shown).

Next, the aminoacid modified peptides depicted in Table 1 were tested for immunogenicity by injection of peptide pulsed dendritic cells into individual groups of three transgenic mice. As shown in Fig. 1, the mp30–38 peptide (mp30) was found to be immunogenic in vivo. The data also show that T cells against mp30 crossreact with wildtype p30. Like the wildtype counterpart, p540–548 [15, 27, 28], the analogue mp540–548 epitope was shown to be immunogenic, and the peptide specific T cells crossreacted with

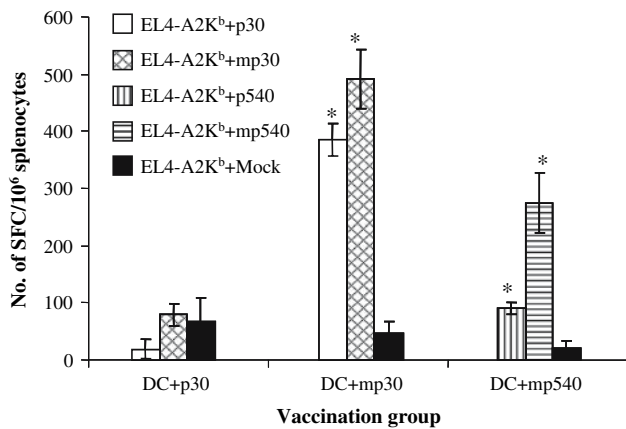
**Table 1** HLA-A\*0201 binding affinity (IC<sub>50</sub> in nM) of six wild type and five amino acid modified peptides of hTERT

Position	Wild type peptide			Modified peptide		
	Sequence	pIC <sub>50</sub>	mIC <sub>50</sub>	Modification	Sequence	mIC <sub>50</sub>
p30–38	RLGPQGWRL	15	620	p 38 L–V	RLGPQGWRV	423
p122–30	YLPNTVTDA	382	155	p130 A–V	YLPNTVTDV	160
p359–67	RLVETIFLG	144	900	ND	–	–
p540–48 <sup>a</sup>	ILAKFLHWL	38	340	p548 L–V	ILAKFLHWV	525
p572–80 <sup>b</sup>	RLFFYRKSV	ND	1,298	p572 R–Y	YLFFYRKSV	206
p1017–25	LQLPFHQQV	172	576	p1018 Q–L	LLLPFHQQV	340

pIC<sub>50</sub> predicted IC<sub>50</sub>, mIC<sub>50</sub> measured IC<sub>50</sub>, ND not done

<sup>a</sup> p540–48 [15, 27, 28]

<sup>b</sup> p572–80 [13, 19]



**Fig. 1** Immunogenicity and cross reactivity in the efferent arm of the immune response. Groups of C57-A2K<sup>b</sup> transgenic mice ( $n = 3$ ) were vaccinated with  $3 \times 10^6$  syngenic DCs pulsed with p30, mp30 or mp540 (10  $\mu\text{g/ml}$ ), and PADRE (5  $\mu\text{g/ml}$ ). Ten days after immunization, the mice were sacrificed and the pooled splenocytes (from 3 mice) were pulsed with the peptide used for immunization (100  $\mu\text{g/ml}$ ) and expanded in vitro for 5 days, on day 5, the splenocytes were tested in ELISPOT wells for IFN- $\gamma$  production after exposure to EL4-A2K<sup>b</sup> cells pulsed with wildtype peptide, modified peptide and Mock (control), respectively, \* $P < 0.001$  ( $t$  test)

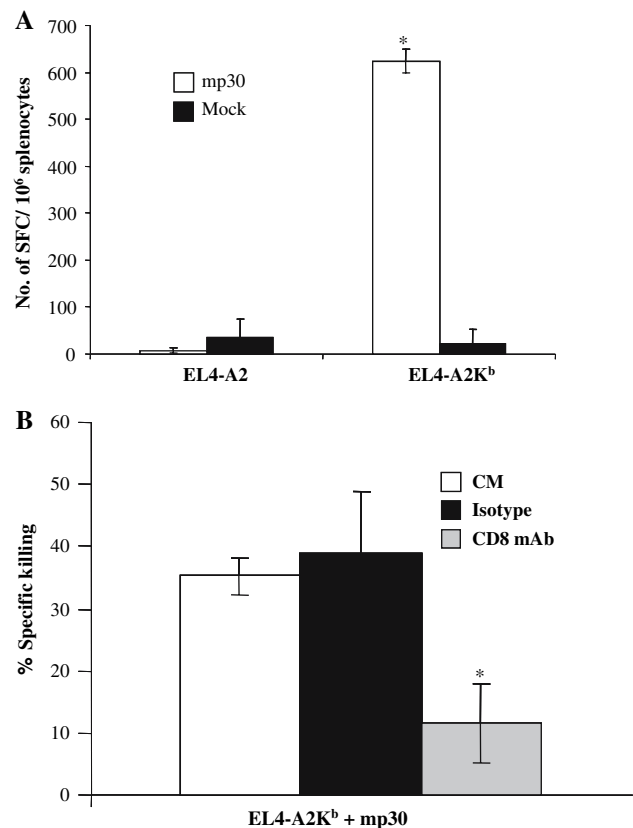
wildtype p540–548. None of the other modified peptides were found to be immunogenic, although, their binding affinity for HLA-A\*0201 were measured to be comparable to the intermediate binding affinity of mp30.

#### CD8 dependence of T cells with specificity for mp30–38

The function of anti-mp30 specific T cells generated in transgenic mice was found to be dependent on their CD8 mediated interaction with MHC-I  $\alpha 3$  domain on target cells (Fig. 2a). Thus, if peptide-pulsed mouse EL4 cells transfected with HLA-A\*0201 were used as stimulator cells in an ELISPOT assay, there was no response. In contrast, peptide-pulsed target EL4 cells transfected with the chimeric A2K<sup>b</sup> cDNA construct provided the necessary contact between TCR/CD8 and the peptide-MHC-I complex to induce IFN- $\gamma$  production by mp30 specific T cells. In addition, when 5  $\mu\text{g}$  anti-CD8 antibody was added to the cytotoxicity assay, the level of CTL mediated killing dropped by 66% (Fig. 2b).

Target cells are more efficiently sensitized for lysis by modified than by non-modified peptide

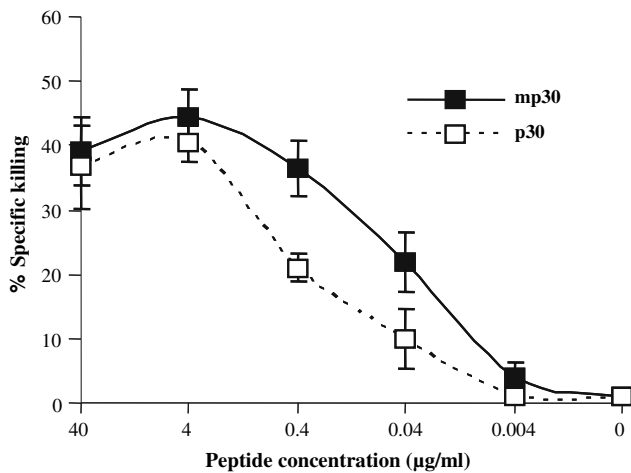
Spleen cell-derived mp30 specific CTLs from immunized mice were studied for their capacity to lyse target cells pulsed with a dose titration of mp30–38 versus p30–38 peptide. As shown in Fig. 3 mp30–38 peptide sensitized target cells show half-maximal lysis at approximately tenfold lower peptide concentrations than p30–38 peptide.



**Fig. 2** CD8-dependence of the generated CTLs. C57-A2K<sup>b</sup> transgenic mice (3 mice) were immunized with  $3 \times 10^6$  syngenic DCs pulsed with mp30 (10  $\mu\text{g/ml}$ ) and PADRE (5  $\mu\text{g/ml}$ ). Ten days after immunization, the mice were sacrificed and the pooled splenocytes (from 3 mice) were pulsed with mp30 (100  $\mu\text{g/ml}$ ) and expanded in vitro for 5 days. Day 5, splenocytes (300,000/well) were tested in ELISPOT wells for IFN- $\gamma$  production when stimulated with either EL4-A2 cells (100,000) pulsed with mp30 or an HLA-A\*0201-binding irrelevant peptide (Mock) or EL4-A2K<sup>b</sup> cells (100,000) pulsed with mp30 or an HLA-A\*0201-binding irrelevant peptide (a). In some cases, splenocytes were tested in the 4 h cytotoxicity assay (b). Target cells were mp30-pulsed EL4-A2K<sup>b</sup> and incubated with effector cells at ratio of 1:50 in the presence of anti-CD8 antibody or isotype control. \* $P < 0.001$  ( $t$  test)

#### Natural processing and presentation of the p30

CCL220 cells express telomerase as judged from western blot experiments (data not shown). HLA-A\*0201 negative CCL220 cells were transfected with the chimeric HLA-A\*0201/K<sup>b</sup> cDNA construct. Transfected and nontransfected cells were used as targets for CTL immunity generated in HHD transgenic mice immunized with dendritic cells pulsed with mp30. Data in Fig. 4 show that only transfected CCL220 cells are recognized by anti-mp30–38 T cells both in the ELISPOT assay (Fig. 4a) and in the cytotoxicity assay (Fig. 4b). Further, the data in Fig. 4b illustrate that pulsing of the transfected target cells with wildtype p30 does not significantly increase their sensitivity to killing, suggesting



**Fig. 3** Target cells are more efficiently sensitized for lysis by modified than by non-modified peptide. Spleen cells derived from the HHD transgenic mice immunized with mp30 pulsed DCs were studied for their capacity to lyse EL4-A2K<sup>b</sup> cells pulsed with a dose titration of mp30–38 versus p30–38 peptide. The effector cells and target cells pulsed with different concentration of peptides were cultured at ratio of 50:1 for 4 h. Filled squares are EL4-A2K<sup>b</sup> pulsed with mp30, open squares are EL4-A2K<sup>b</sup> pulsed with p30. Error bars represent standard deviations

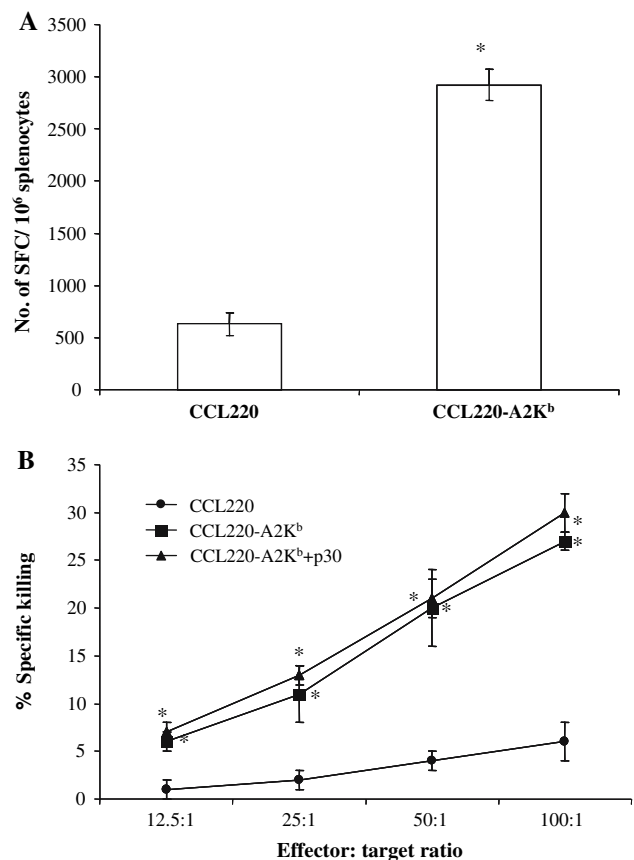
that the p30 epitope from the point of view of immunogenicity is optimally processed and presented by the CCL220 cells.

#### Response against mp30–38 in vaccinated patients

Two patients with metastatic renal carcinoma were enrolled into a vaccination program (see “Materials and methods”). PBMC were obtained prior to and at various intervals after initiation of the mp30/DC vaccine injections. The ELISPOT data in Fig. 5 demonstrate that none of the patients had detectable levels of reactivity prior to vaccination, but both patients showed a vaccine-dose-related increase in anti-mp30 reactivity with a maximum after six vaccinations.

#### Discussion

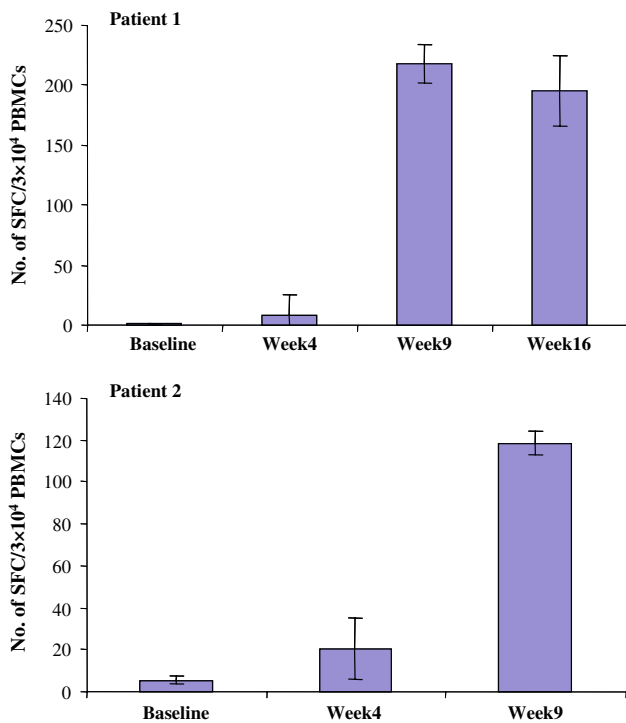
In the present study, we focused on hTERT peptides with a relatively low affinity for the HLA-A\*0201 molecule. Consequently, T cell immunity against these peptides could be expected not to be intrathymically deleted but preserved and available for tumour rejection responses. Aminoacid substitutions were performed primarily at one of the peptide anchor positions in order to improve binding to HLA-A\*0201 and immunogenicity of the modified peptides. Only one of these modified peptides (mp30–38), with a selected residue modification in the P9 anchor position, was found to have an improved binding affinity for the HLA-A\*0201 molecule and by immunization capable of inducing peptide



**Fig. 4** T cells specific for mp30 epitope release IFN- $\gamma$  after exposure to CCL220-A2K<sup>b</sup> transfectants and kill CCL220-A2K<sup>b</sup> transfectants. HHD transgenic mice ( $n = 4$ ) were vaccinated with  $3 \times 10^6$  syngenic DCs pulsed with mp30–38 (10  $\mu$ g/ml) and PADRE (5  $\mu$ g/ml). Eleven days after immunization, the mice were sacrificed and the pooled splenocytes (from 4 mice) were pulsed with the vaccination peptide (100  $\mu$ g/ml) and expanded in vitro for 5 days. On day 5, the splenocytes were harvested and left under resting conditions (IL-2 was added) for another 4 days. After resting, the splenocytes were tested in ELISPOT wells for IFN- $\gamma$  production in response to CCL220-A2K<sup>b</sup> and CCL220, respectively (a). For the cytotoxicity assay (b), the splenocytes were tested on day 5. Target cells were CCL220-A2K<sup>b</sup>, p30 pulsed CCL220-A2K<sup>b</sup> and CCL-220, respectively. \* $P < 0.001$  ( $t$  test)

specific T cells. Thus, dendritic cells pulsed with mp30 peptide and injected into HLA-A\*0201 transgenic mice induced a specific HLA-A\*0201 restricted and CD8 dependent response. We also demonstrated that the generated CTLs cross reacted with the p30 wild type peptide counterpart and showed that mp30–38 peptide sensitized target cells show half-maximal lysis at approximately tenfold lower peptide concentrations than p30–38 peptide. Thus it appears that the modified p30–38 peptide is improved not only as an immunogen, but also as an antigen.

In order to include the mp30 peptide epitope, in a vaccine for immunotherapy, it is necessary to prove that the wild type p30–38 is processed and presented at the surface of human telomerase expressing HLA-A\*0201 positive



**Fig. 5** Changes in the frequency of hTERTmp30 specific T cells in 7 days cultures of PBMCs from two renal cell carcinoma patients vaccinated for 6 (Patient 2) or 10 (Patient 1) times (week 1–16) with mp30 loaded autologous dendritic cells. Vaccination-induced T cell responses against mp30 were measured by ELISPOT analysis and data show the mean number of peptide specific IFN- $\gamma$  spots in response to mp30 by  $3 \times 10^4$  in vitro stimulated PBMCs. Non-specific IFN- $\gamma$  spots are subtracted. Bars, range of duplicates

tumor cells. For this purpose, we used a HLA-A\*0201 negative, but HLA-A\*0201/K<sup>b</sup> transfected, telomerase positive colon carcinoma cell line, CCL-220, as target cells for mp30–38 immune murine T cells, and we showed reactivity in this system against the naturally processed peptide p30–38. The non-transfected, HLA-A\*0201 negative cell line was used as a convenient genetically identical background control. Thus, we propose that CTL precursors for p30–38 peptide can be triggered in cancer patients by a vaccine including the mp30 peptide. In support of this notion, an immune responses against mp30 was recorded in PBMC of two HLA-A\*0201<sup>+</sup> renal cell carcinoma patients after vaccination with autologous dendritic cells pulsed with the mp30 peptide (Fig. 5), strongly supporting that the human TCR repertoire is capable of recognizing and responding to this peptide in vivo.

Previously, the immunogenic HLA-A\*0201-restricted hTERT epitope, p540–48, included as an immunogenic control in the present work, has been extensively studied and used in vaccines for cancer patients [4, 15, 23, 26–28]. This epitope might be poorly processed as judged from our own predictions, since the C-flush value [14] is as low as 0.04 (data not included). The C-flush value is the probability

of processing of an epitope ( $0 \leq \text{C-flush value} \leq 1$ ). In comparison, the predicted C-flush value of the p30 peptide is 1. The fact that three to four in vitro restimulations are needed to induce p540-specific CTLs from CD8-enriched PBMCs in healthy as well as in cancer patients [28] raises some doubt about the efficiency of presentation of the p540 epitope. In addition, Ayyoub et al. [4] incubated a long hTERT precursor peptide (amino acids 534–554) with proteasomes and analyzed the products by mass spectrometry and cytotoxic assays. It showed that p540 is inefficiently processed. Finally, CTLs from HLA-A\*0201<sup>+</sup> melanoma patients only show cytotoxicity against A2<sup>+</sup> target cells in the presence of exogenously added p540–48 peptide but no direct cytotoxicity against HLA-A\*0201<sup>+</sup> hTERT<sup>+</sup> melanoma or colon carcinoma cells [4]. The clinical outcome from a phase I trial [26] in which patients were vaccinated with DCs pulsed with p540 is hard to judge because of the small number of patients included and the absence of a patient control group. The results from other vaccination studies with p540 are not encouraging [17].

A number of other immunogenic HLA-A\*0201-restricted epitopes have been identified using HLA-A\*0201 transgenic mice as well as by in vitro immunization of donor PBMCs. Minev et al. [15] used transgenic mice and generated a peptide specific response against p865. They also showed that patient CTLs specific for p865 lysed a variety of telomerase expressing, HLA-A\*0201 positive cell lines of breast, colon, lung and melanoma origin. Another two A2-restricted hTERT-derived epitopes, p572 [13, 19] and p988 [19], were identified coincidentally. In contrast to p540 and p865, p572 and p988 show relative low binding affinity for HLA-A\*0201 and therefore (as for p30) likely spared from deletion due to self-tolerance. Consequently, analogue peptides of p572 and p988 (the affinity for HLA-A\*0201 was increased by aminoacid substitution in P1) were identified to be immunogenic in HLA-A\*0201 transgenic mice, and the wildtype peptide counterparts were shown to be endogenously processed and presented by HLA-A\*0201 positive tumour cells and recognized by CTLs raised by the analogue peptide. In the present study, a substitution in the anchor P9 in the p30 epitope was introduced, since the binding to HLA-A\*0201 is affected positively by this change without interfering with TCR binding. This is not the case as with substitution in P1 in which position there is a risk for TCR binding interference.

In addition to the above mentioned A2-restricted epitopes, one HLA-A3-restricted [25], two HLA-A24-restricted [3], three HLA-B0702-restricted [1] and finally, one HLA-A1 restricted [20] hTERT-derived epitope have been identified to meet the needs of candidate epitopes for a cancer vaccine.

From an immunotherapeutic point of view, it has been speculated that an effective tumor vaccine may require the

induction of both CTL as well as T-helper cell responses against hTERT [29]. Two hTERT MHC class II-restricted epitopes have been identified capable of inducing CD4<sup>+</sup> T cell responses in the context of several commonly found HLA-DR alleles [21, 22].

The result of the present study may extend the number of hTERT epitopes that can potentially be targeted by CTLs in an hTERT-based vaccine. A vaccine that include both common MHC class II and class I allele-restricted hTERT epitopes may have greater success than vaccines comprising only one or a few allele-restricted epitopes.

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