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ORIGINAL ARTICLE

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A TGFβRII frameshift-mutation-derived CTL epitope recognised by HLA-A2-restricted CD8$^+$ T cells

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Abstract Microsatellite instability (MSI) is recognised as genome-wide alterations in repetitive DNA sequences caused by defects in the DNA mismatch repair machinery. Such mutation patterns have been found in almost all analysed malignancies from patients with hereditary non-polyposis colorectal cancer, and in approximately 15% of sporadic colorectal cancers. In cancers with the MSI phenotype, microsatellite-like sequences in coding regions of various cancer-related genes, including transforming growth factor β receptor type II (TGFβRII), are targets for mutations. The TGFβRII gene harbours a 10-bp polyadenine tract, and mutations within this region are found in 90% of colorectal cancers with MSI. The frameshift mutations result in new amino acid sequences in the C-terminal part of the proteins, prematurely terminating where a novel stop codon appears. In this study we have defined a new cytotoxic T lymphocyte (CTL) epitope (RLSSCVPVA), carrying a good HLA-A*0201 binding motif, and resulting from the most common frameshift mutation in TGFβRII. A CTL line and several CTL clones were generated from an HLA-A2$^+$ normal donor by repeated stimulation of T cells with dendritic cells pulsed with the peptide. One of the CTL clones was able to kill an HLA-A2$^+$ colon cancer cell line harbouring mutant TGFβRII. This epitope may be a valuable component in cancer vaccines directed at MSI-positive carcinomas.

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Introduction

Transforming growth factor-β (TGFβ) is a potent inhibitor of epithelial cell growth, and loss of this negative regulation is thought to contribute to tumour development [17]. Cancers, including colon cancer cell lines, demonstrate resistance to the growth inhibitory effect of TGFβ [6, 21]. The TGFβ growth inhibitor signal is transduced by a complex composed of type I (RI) and type II (RII) receptors [16]. If one of the receptors is absent or inactivated the cells lose their responsiveness to TGFβ. In colorectal cancer cell lines, mutation of TGFβRII is one reason for the loss of TGFβ responsiveness [15]. Inactivating TGFβRII mutations have been found in about 90% of all colon cancers that show microsatellite instability (MSI) due to defects in DNA mismatch repair [15, 18]. These inactivating TGFβRII mutations occur as 1- or 2-base deletions or insertions in a 10-bp microsatellite-like polyadenine tract in the 5' coding half of the gene. Since the mutations occur in the coding region of TGFβRII, they result in a shift in the reading frame (frameshift) and give rise to synthesis of new truncated versions of TGFβRII. These proteins contain amino acid sequences that are foreign to the body and are potential cancer vaccine candidates provided they will bind to HLA molecules and be presented at the cell surface to T lymphocytes.

If novel peptides generated by frameshift mutations in MSI$^+$ tumours can be recognised by the T cell immune system, they might lead to a strong selection for general escape from immune elimination. This could account for the suggestive indication that β_2-microglobulin (β_2-m) mutations leading to loss of HLA class I have been found more frequently in MSI$^+$ tumours than in MSI$^-$ tumours [2, 3]. It has been indicated that patients with MSI$^+$ colorectal cancers (CRCs) show better survival rates than cases carrying MSI$^-$ tumours [26].

Many studies have also shown that MSI^+ CRCs are characterised by a pronounced infiltration of lymphocytes and the majority of these lymphocytes are activated cytotoxic T cells [5, 10, 12]. Moreover, MSI^+ CRCs also showed a significantly higher percentage of tumour cells undergoing apoptotic cell death [5]. One attractive hypothesis to explain these phenomena is that the MSI^+ phenotype increases the production of abnormal peptides able to elicit cytotoxic immune responses against tumour cells.

Previously, frameshift mutations in the adenomatosis polyposis coli gene have been reported to induce cytotoxic T cell responses in mice [24]. The mutations arose by short deletions or insertions that altered the reading frame, and several of these reading frames contained regions of sequences that were likely to bind to HLA class I molecules. Although frameshift mutations occur more frequently in adenomatosis polyposis coli than in MSI^+ tumours, they are spread all over the gene, rather than confined to a repeat sequence defined hotspot, such as is the case for TGFβRII and other genes [7].

$CD4^+$ T cell responses against several TGFβRII frameshift-derived peptides have recently been described both in cancer patients with HNPCC and sporadic MSI^+ colon cancer (Sæterdal et al., in press). These results indicated that one component of the prominent T lymphocyte infiltration observed in MSI^+ CRCs may indeed be directed against frameshift mutations generated by the lack of efficient DNA mismatch repair. Novel CTL epitopes generated by frameshift mutations in MSI^+ CRCs that may lead to $CD8^+$ T cell response have not yet been characterised.

In the present study we provide evidence showing that frameshift mutations in the TGFβRII gene may lead to new tumour antigens recognised by cytotoxic T cells. CTLs were generated in vitro using a protocol involving dendritic cells (DCs). We were able to obtain a CTL line and CTL clones specifically recognising a 9mer frameshift peptide derived from the deletion of a single A in the 10-bp polyadenine [poly $(A)_{10}$] tract of the TGFβRII gene. The new epitope binds to HLA-A2, an HLA class I allele expressed by nearly 50% of the Caucasian population [9]. We also demonstrate that the CTL clones are able to recognise endogenously processed antigen. Our results indicate that frameshift peptides derived from mutated TGFβRII represent a new group of tumour-specific antigens that may be used in cancer vaccines.

Materials and methods

Cells and media

Buffy coats from normal healthy donors were purchased from the blood bank at Ullevål Hospital, Oslo, Norway. $HLA\text{-}A2^+$ individuals were selected by using 8G12 mAb (anti HLA-A2; G. Gaudernack) coupled to Dynabeads (Dynal, Oslo, Norway). Peripheral blood mononuclear cells (PBMCs) were prepared by centrifuging the buffy coats over a Ficoll-Hypaque gradient (Lymphoprep) (Nycomed, Oslo, Norway). The HLA type of donor B was HLA-A2; B17, 4; C7; DQ1, 3; DR2, 4. Autologous B-lymphoblastoid cell lines (B-LCLs) were generated by Epstein-Barr virus transformation of B cells from the donors. B-LCL 9005 used as APC was from the 11th International Histocompatibility Workshop cell panel. The HLA class I type of 9005 was HLA-A3; B27; C1. Colon cancer cell lines HCT 116, SW 480 and LS174T were obtained from ATCC (Rockville, Md.). The antigen processing defective T2 cell line was used as a target in cytotoxicity assays. The erythroleukemia cell line K562 was used as a NK target. All T cells were grown in X-vivo 15 (BioWhittaker Europe, Verviers, Belgium) supplemented with gentamicin and 1% autologous serum (standard medium) (generation of T cell lines) or 10% heat-inactivated human pool serum (T cell clones and further propagation of T cell lines). Other cell lines were grown in RPMI-1640 (BioWhittaker) supplemented with gentamicin, 500 U/ml IFN-γ (colon cancer cell lines) (Amersham, Aylesbury, UK) and 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Life Technologies). Conditioned medium (CM) was produced by Ig adherent PBMCs, as described by Romani et al. [20].

Peptides

Peptides were ordered from Neosystem S.A. (Strasbourg, France) or provided by Hydro Research Center (Porsgrunn, Norway). The peptides represent frameshift mutations in the TGFβRII gene and their amino acid sequences are listed in Table 1.

Antibodies

In flow cytometry analysis the following antibodies were used: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-CD3, anti-CD8, (Caltag laboratories, Burlingame, Calif.); anti-CD4 (Dako, Denmark); anti-CD83 (Immunotech, Marseilles, France), anti-CD86 (Pharmingen, San Diego, Calif., USA); anti HLA class II (Dako); anti-CD14 (Caltag) and anti-CD19 (Dako). Isotype-matched FITC- and PE-conjugated irrelevant mouse monoclonal antibody (Dako) served as a control. W6/32, anti-HLA class I hybridoma cells (ATCC, Rockville, Md.) were used in blocking experiments.

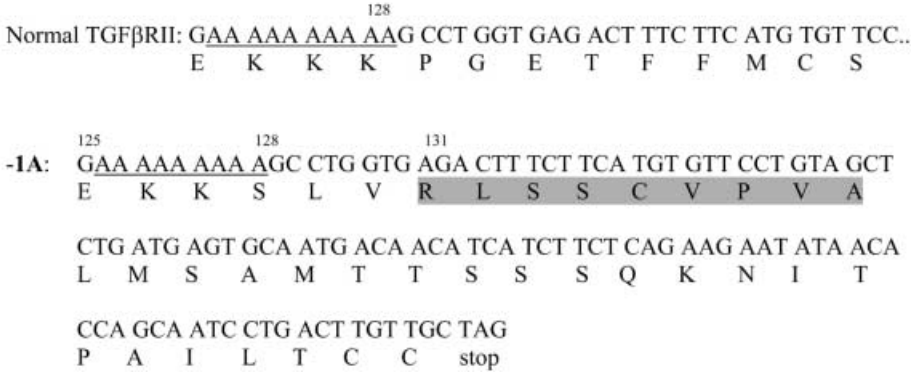
Peptide-specific T cell stimulation

PBMCs from 4 healthy $HLA\text{-}A2^+$ donors (A, B, C and D) were stimulated with peptides using a protocol involving dendritic cells (DCs) (EUCAPS, http://ww.medizin.uni-tuebingen.de/eucaps/home/). Briefly, DCs were generated by plating 3 ml PBMCs (4×10^6 cells/ml) in 6-well tissue culture plates in standard medium. After 1–2 h incubation at 37°C, nonadherent cells were washed away and frozen. Three millilitres of standard medium supplemented with 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Schering-Plough, Berlin, Germany) and 500 U/ml IL-4 (BioSource Int., Nivelles, Belgium) were added to the adherent cells. Cytokines were added every other day. At day six, 10 ng/ml TNF-α (BioSource Int.) was added. After one week, the DCs were collected and pulsed with 20 μM peptides and 3 μg/ml β_2-m (Sigma, Oslo, Norway) at 37°C, as follows: A1, p523; B1, p523; B2, p573; C1, p523; D1, p523; D2, p573; and D3, p581. After 4 h the cells were washed and irradiated (60 Gy) and seeded as 0.3×10^6 cells /well in 24-well culture plates (Costar). Responder T cells were prepared using thawed nonadherent autologous cells. 3×10^6 responding T cells were co-cultured with the peptide-loaded DCs in standard medium. After two days 20 ng/ml IL-7 was added. On day 12 and weekly thereafter, responder populations were restimulated with adherent, irradiated (60 Gy), autologous peptide-pulsed PBMCs (10–20 μM peptide for 2 h at 37°C). On day two after restimulation, 20 U/ml IL-2 (Amersham) was added. The responder cells were separated over lymphoprep before the first

Table 1 Amino acid sequences and HLA-A*0201 binding of peptides derived from mutated TGFβRII

Name	Sequence[b]	Score[a]
p523	[128]SLVRLSSCV	23
p573	[131]RLSSCVPVA	19
p581	[122]IMKEKKKSL	23
p357/39	[139]ALMSAMTTSSSQKNITPAILTCC	-
p538	[128]SLVRLSSCVPVALMSAMTTSSSQ	-

[a]From SYFPEITHI database of MHC ligands and peptide motifs, http://134.2.96.221/.
[b]Position of the start amino acid in the protein is indicated.



restimulation. After the third restimulation, the cultures were tested for peptide specificity in a cytotoxicity assay.

Generation of T-cell clones

T cells from one responding culture stimulated with peptide p573 were cloned by limiting dilution (5, 1, or 0.5 cells per well) onto Terasaki plates (Nunc, Roskilde, Denmark). As feeder cells, 2×10^4 allogeneic, irradiated (30 Gy) PBMCs were used and the cells were propagated with 1 μg/ml phytohaemagglutinin (PHA) (Wellcome, Dartford, England) and 100 U/ml IL-2.

Cytotoxicity assays

^{51}Cr-release cytotoxicity assay was performed by the labelling of 2×10^6 target cells in FCS and ^{51}Cr (7.5 MBq) (Laborel, Oslo, Norway), in a total volume of 0.5 ml for 1 h, with gentle mixing every 15 min. Cells were washed three times in cold RPMI-1640 and seeded at 2×10^3 target cells in 96-well, U-bottomed microtitre plates. Target cells were pulsed with 10 μM (or as indicated) peptide for about 0.5–1.0 h at 37°C. Effector cells (1×10^5 or as indicated) were added and the plate was left for 4 h at 37°C. DCs used as target cells were generated as described above. On day 5, the DCs were pulsed with peptide. Two days later the peptide-pulsed DCs were matured for 48 h with 10 ng/ml TNF-α (BioSource Int., Nivelles, Belgium), 100 U/ml INF-α (Schering-Plough) and 25% CM, before they were labelled with ^{51}Cr. The maximum and spontaneous ^{51}Cr release of target cells was measured after incubation with 5% Triton X-100 (Sigma) or medium, respectively. Supernatants were harvested and radioactivity was measured in a Packard (Meriden, Conn.) Topcount microplate scintillation counter. The percentage of specific chromium release was calculated by the formula: [(experimental release – spontaneous release)/(maximum release – spontaneous release)]×100.

Flow cytometry

Phenotypic analysis of T cells and DCs was determined by flow cytometry. Cells (1×10^5/well) were stained with the appropriate FITC- or PE-conjugated antibody at a final concentration of 5–10 μg/ml for 30 min at 4°C and then washed and analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

Analysis of TCR V-region usage

RNA was extracted using the Purescript Isolation Kit (Gentra Systems, Minn.). Synthesis of cDNA and the quantitation of TCR cDNA in each sample were carried out as described [4]. Subsequently, the cDNA was PCR amplified using a primer panel that amplifies the BV regions 1–24 [22]. Amplifications were carried out in a total volume of 25 μl containing 1×PCR buffer [50 mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol red, 12% sucrose, 0.005% (w/v) BSA (Boehringer-Mannheim, Mannheim, Germany)], 2.5 pmol of each primer, 40 mM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 1.25 U AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, Calif.). Parameters used for amplification were: 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s,

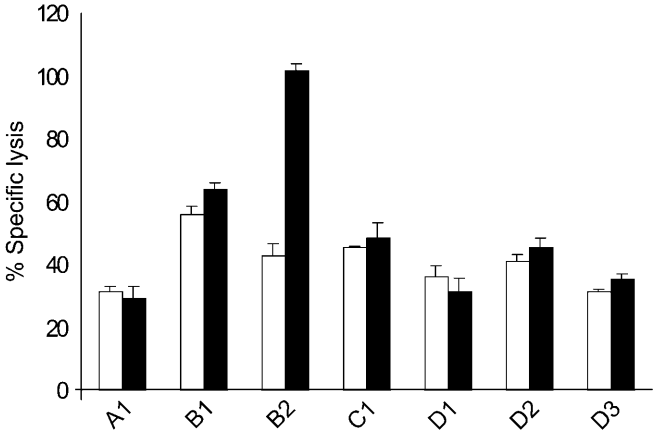


Fig. 1 Cytotoxic activity of seven bulk cultures against T2 pulsed with synthetic peptides. The bulk cultures were induced using peptide-pulsed, autologous DC as APC. Cytotoxic activity of the cultures was measured in a standard ^{51}Cr-release assay against T2 (□) or T2 pulsed with the peptide corresponding to that used to generate the bulk (■)

for 32 (quantitative PCR) or 40 cycles (DGGE). Taq polymerase and dNTPs were added to the reaction tube at an 80°C step between the denaturation and annealing steps of the first cycle (hot start).

The resulting PCR products were analysed by denaturing gradient gel electrophoresis (DGGE) [22, 23]. Thus, 10 µl aliquots were loaded onto a denaturing gradient gel containing 6% polyacrylamide and a gradient of urea and formamide from 20% to 80%. Gels were run at 160 V for 4.5 h in 1×TAE buffer kept at a constant temperature of 58°C. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

Results

Induction of frameshift-peptide-specific CTLs

PBMCs from four HLA-A2$^+$ healthy donors, A, B, C and D, were stimulated weekly by peptide-pulsed APCs in order to generate tumour-antigen-specific T cell lines. The first stimulation was performed with DCs displaying the immature phenotype CD14$^-$, CD19$^-$, CD86$^+$, HLA-class II$^+$ and CD83$^-$ (data not shown). The tumour antigens were 3 different peptides derived from frameshift mutations in the 10-bp poly(A) tract (bp 375–384) of TGFβRII, all selected to be good HLA-A2 binders (Table 1). Seven different cultures were generated, as described in Materials and methods. After the second restimulation, culture B2 showed evidence of specific killing of T2 cells pulsed with p573 (data not shown). For the other cultures mostly unspecific lysis was observed. After a third restimulation, the pattern was even more obvious (Fig. 1), indicating that an immune response against the p573 peptide was taking place during in vitro culturing. The B2 cell line was extremely efficient in killing peptide-loaded target cells, and strong lytic activity was obtained with an effector to target ratio of 6:1 (Fig. 2B). Peptide titration showed that the half-maximal lysis with p573 was approximately 0.01 µM (Fig. 2A). The NK target cell line K562 was not killed, indicating that killing with the B2 cell line was not due to NK like activity (Fig. 2B). Cold target inhibition with K562 used as cold target in the cytotoxicity assay confirmed that the cell line was peptide specific (Fig. 3A). When T2 cells pulsed with p573 were used as cold targets, specific lysis was inhibited by 39% (Fig. 3A). T2 cells pulsed with the frameshift-mutated TGFβRII-derived peptide, p581, originating from an insertion of one A in the poly(A)$_{10}$ tract, resulting in an amino acid sequence that is different from the stimulating peptide, were not killed by the B2 cell line (data not shown).

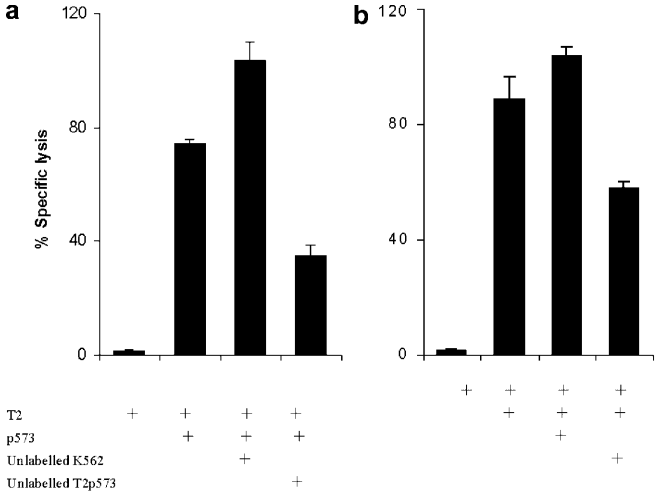


Fig. 3a, b Cold target inhibition assay with the B2 cell line and CTL clone 102. The cytotoxic activity of B2 (**A**) and CTL clone 102 (**B**) was measured against T2 with addition of p573 (1 µM), unlabelled peptide-pulsed T2 or unlabelled K562 as indicated with +. Unlabelled to labelled target ratio was 50:1

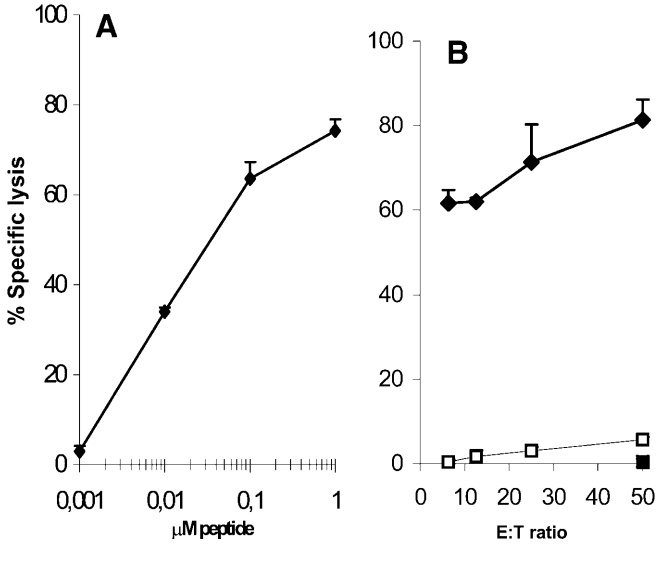


Fig. 2A, B Cytotoxic activity of the B2 culture. **A** Peptide sensitivity was tested using T2 pulsed with various concentrations of peptide p573 at an E:T ratio of 50:1. **B** Effector to target ratio using peptide pulsed (p573, 10 µM) T2 (◆) and K562 (□) as target cells. Unpulsed T2 cells (■) were used as a negative control

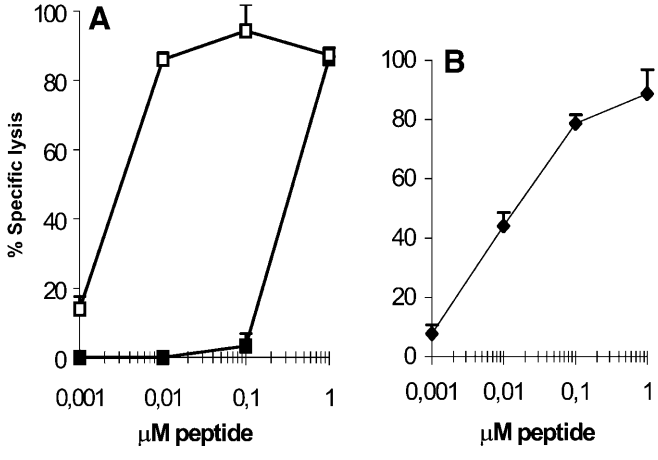


Fig. 4A, B Peptide sensitivity of CTLs. Peptide sensitivity of **A** CTL clones 37 (■), 44 (□) and **B** CTL clone 102 (◆) was tested using T2 pulsed with various concentrations of peptide p573 (E:T ratio was 50:1)

Together these results demonstrate the peptide specificity of the B2 cell line.

Generation and characterisation of CTL clones

To examine the TGFβRII frameshift-derived peptide-specific response in more detail, CTL clones were generated. The B2 cell line was cloned by limiting dilution using allogeneic PBMCs as feeder cells. From the first cloning experiment, 70 CTL clones (out of 150 tested) were obtained that specifically recognised the TGFβRII frameshift-derived peptide p573. These clones were highly cytotoxic, killing 63–100% of peptide-pulsed T2 cells (data not shown). Further characterisation of these clones could not be undertaken due to mycoplasma infection of the cultures. A second cloning attempt from the cell line gave rise to eight p573-specific CTL clones. Of these, CTL clones 37, 44, 54 and 102 were characterised further. Phenotypic analysis demonstrated a $CD3^+$, $CD8^+$ and $CD4^-$ phenotype for all four clones (data not shown). Peptide titration experiments demonstrated wide differences in peptide sensitivity among the clones. CTL clone 44 and 102 both killed T2 pulsed with p573 efficiently at a peptide concentration of 0.01 μM. CTL clone 37 was a low affinity clone, and only able to kill T2 target pulsed with 1 μM p573 (Fig. 4). It is our general experience that the protocol used to generate the CTL culture and clones produces predominantly low affinity clones, but also some high affinity clones worth further study. To further demonstrate the peptide specificity of the clones, we performed a cold target inhibition assay using the CTL clone 102. The results of these experiments were the same as with the starting B2 cell line (Fig. 3B).

Since T2 only expresses HLA-A2 and the peptides used for stimulation are selected to be good HLA-A2 binders, all evidence indicates that the CTL clones really are HLA-A2 restricted. To confirm the HLA class I restriction we accordingly performed blocking experiments with the HLA-class I antibody W6/32. In these experiments, which were repeated several times, no blocking was observed (data not shown). To provide further evidence for the HLA-A2 restriction, we compared killing of different peptide-pulsed targets by the CTL clone 44. As shown in Fig. 5, p573-pulsed B-LCL 9005, expressing a set of HLA-class I molecules different from that of donor B, were not killed above background killing, while the autologous B-LCL and T2 targets pulsed with p573 were efficiently killed.

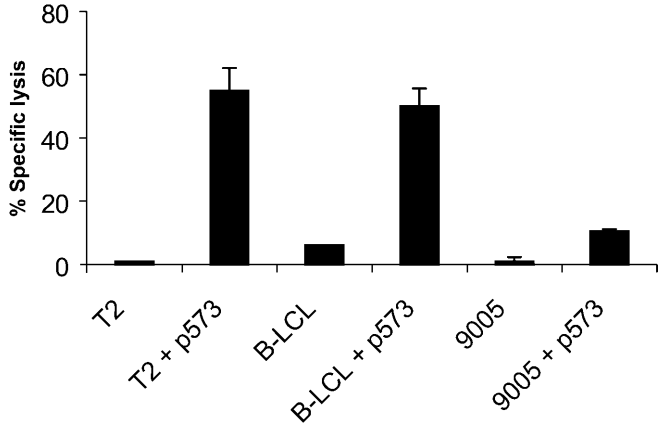


Fig. 5 Cytotoxicity of CTL clone 44 against B-LCLs. T2 cells, autologous B-LCLs and B-LCL 9005 were pulsed with p573 (10 μM) and used as targets (E:T ratio was 10:1). The HLA class I molecules of 9005 is different from donor B's

Analysis of the TCR BV usage of the CTL clones

Since recognition and clonal expansion of T cells specific for TGFβRII frameshift-mutation-derived tumour antigens may occur spontaneously in vivo during tumour development (Sæterdal et al., in press), information on the TCR variable region expressed by T cells recognising these new epitopes may be of importance to monitor T cell immunity in the patients. We therefore analysed the expression of TCR BV regions of the CTL clones. CTL clones 37, 54 and 102 express TCR VB 2, however, clone 37 differs from clone 102 and 54 at the DNA level (data not shown). CTL 44 expresses TCR VB 13. The diverse usage of TCR VB genes of this small number of clones indicates that the T cell repertoire against the p573 peptide epitope may be very broad.

Cytotoxic activity against endogenously processed antigen

To determine whether the CTLs generated were able to kill endogenously processed TGFβRII peptide, the bulk culture and the CTL clones were tested for cytotoxic activity against various colon cancer cell lines. The cell

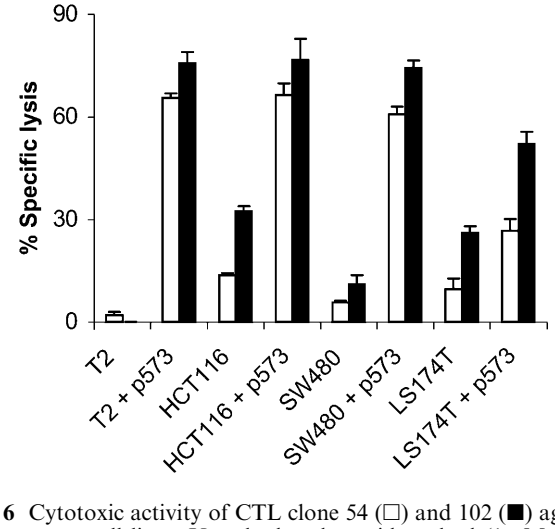


Fig. 6 Cytotoxic activity of CTL clone 54 (□) and 102 (■) against colon cancer cell lines. Unpulsed and peptide-pulsed (1 μM p573) T2 and the colon cancer cell lines HCT116, SW480 and LS174T were used as targets (E:T ratio of clone 54 was 25:1 and of clone 102, 50:1)

lines used were HCT116, SW480 and LS174T. The HLA-A2$^+$ MSI$^+$ cell line HCT 116 has a single A deletion in the poly(A)$_{10}$ tract of TGFβRII [8] This deletion gives rise to the frameshift peptide p573, used to generate CTLs in this study. SW480 and LS174T were used as control cells. The HLA-A2$^+$ cell line SW480 is MSI$^-$ and has a wild-type TGFβRII [25]. Like HCT 116, LS174T also harbours a single A deletion in the TGFβRII gene [25], but despite the presence of the HLA-A2 gene in its genomic DNA it lacks measurable expression of HLA-A2 [4]. The CTL clones did not kill SW480 above background killing, CTL clone 102 was able to specifically lyse HCT116 (Fig. 6) and, to a lesser degree, also LS174T. For the CTL clone 54, the same specific pattern of lysis was observed, but killing was less efficient. With addition of exogenous p573 peptide, all cell lines were killed to a higher degree by both the clones, indicating that addition of exogenous peptide resulted in expression of sufficient amount of HLA-A2/p573. Interestingly, the low level HLA-A2-expressing cell line LS174, like T2, expressed sufficient amounts of HLA-A2 to be efficiently killed following peptide pulsing. To further provide evidence for processing of the TGFβRII frameshift-derived CTL epitope, we used another high affinity clone, CTL clone 44, as effector cells and tested peptide-pulsed HLA-A2$^+$ DCs as targets. Mature DCs were loaded with p573; immature DCs were loaded with a 23-amino-acid (aa)-long peptide, p538, containing an embedded p573 sequence (Table 1). Another 23 aa-long TGFβRII frameshift peptide only sharing one aa with p573 was used as control. The DC pulsed with the long peptides were matured to allow optimal presentation before we performed the cytotoxicity assay. In Fig. 7, we demonstrate that the CTL clone

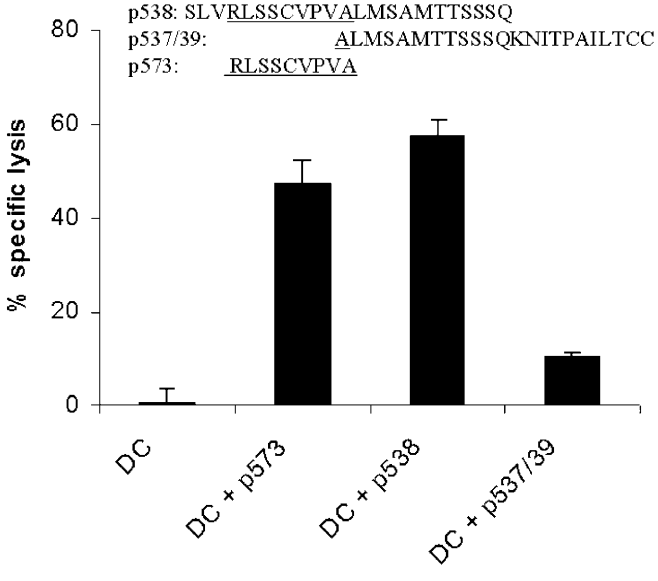


Fig. 7 Cytotoxic activity of CTL clone 44 against peptide-pulsed DCs. DCs were pulsed with p573 (10 μM), p538 or p537/39 (25 μM) and used as targets (E:T ratio was 12:1). The amino acid sequences of the peptides are given and the p573 epitope is underlined

lysed DCs pulsed with p573 and p538, whereas unpulsed DCs and DCs pulsed with p537/39 were not lysed. Killing was not due to NK activity, since the NK target K562 was not lysed (data not shown). To exclude that killing of p538-pulsed DCs was due to contamination of p573 in the p538 peptide, processing defective T2 cells were loaded with p538 and used as target cells in a cytotoxicity assay. However, these cells were not killed by clone 44 (data not shown). This experiment indicates that also TGFβRII frameshift antigens provided by an exogenous route are processed to yield the p573 CTL epitope.

Discussion

Cytotoxic T cells, specific for aberrant gene products of cancer cells, are of potential importance in cancer immunotherapy. Tumour-antigen-specific CTLs constitute the basis for development of immunotherapy strategies and/or adoptive cellular therapy approaches. Generation of CTLs recognising tumour-specific antigens, such as TGFβRII frameshift-derived peptides, and the definition of key elements, such as the antigen-presenting molecules, are considered to be useful tools in the development of immunotherapy. Here we describe several HLA-A2-restricted CD8$^+$ T cell clones generated from a normal donor that recognise a nonamer peptide derived from a frameshift (single A deletion) mutation in the poly(A)$_{10}$ tract of the TGFβRII gene. The CTLs are highly peptide-specific, able to efficiently lyse T2 pulsed target cells at low effector to target ratios (6:1) and at low peptide concentrations (0.01 μM). Importantly, these CTLs also lyse tumour targets expressing the relevant HLA molecule and the corresponding mutation. These results indicate that peptides derived from frameshift mutations in TGFβRII are immunogenic in man and provide evidence for the possible usefulness of such peptides in cancer vaccines.

The presence of multiple TCRs capable of binding a given peptide/HLA class I complex in a normal T cell repertoire provides an optimal framework for effective control of an eventual neoplastic outgrowth. In this study we have identified the usage of TCR BV 13 and two forms of TCR VB 2 in CTLs recognising the TGFβRII frameshift epitope p573/RLSSCVPVA. The presence of TILs in colorectal cancer with high-level DNA microsatellite instability (MSI-H) is well known and the majority of these cells are found to be CD8$^+$ [5, 10, 12]. Their function is still unknown and so far no information is available regarding the TCR usage and clonal composition of these T cells. However, since CTL clones from a normal donor express different TCRs capable of recognising the p573 epitope, it is possible that TILs from MSI$^+$ cancer patients comprise T cells specific for frameshift-derived peptides using different TCRs.

One of the final goals for immunotherapy is to induce tumour-antigen-specific T cells capable of both recogn-

ising and killing tumour cells in vivo. These results confirm the feasibility of using DC-based protocols to elicit frameshift mutated TGFβRII-specific CTL responses in vitro and may also indicate that such responses could be obtained in cancer patients. However, some of the CTL clones generated using this protocol did not seem to recognise endogenously processed antigen, either due to the use of low affinity TCR, or indicating that the p573 epitope is not optimally processed and expressed in vivo. Selection of the p573 epitope described in our study was based on HLA-A2 binding prediction, but a recent study by Kessler et al. [13] reported a more precise epitope prediction method. In their study, CTLs were stimulated in vitro using predicted epitopes generated from the tumour-associated antigen PRAME. To predict the epitopes, they developed an improved multistep epitope prediction procedure, in which in vitro proteasome-mediated digestion of candidate epitopes is incorporated. Using this approach, they showed that CTL clones with high sensitivity for a high-affinity-binding PRAME peptide, which were not produced in vitro by proteasome-mediated digestion, were unable to lyse PRAME and HLA-A*0201-expressing tumour cell lines. CTL clones recognising epitopes based on the improved epitope prediction procedure did recognise and lyse tumour cells. In future characterisation of p573 and other CTL epitopes derived from frameshift-mutated TGFβRII, the improved multistep epitope prediction procedure should be taken into account.

Crucial for the initiation of effective immune responses, both in vitro and in vivo, is the presentation of antigenic peptides in an immunogenic context. DCs are APCs specialised to regulate T cell immunity [1]. We tested a protocol involving DCs to elicit in vitro immune responses in four different donors, using three peptides all derived from frameshift mutations in TGFβRII. In only one of the donors, induction of CTLs against one of the peptides, epitope p573, was successful. One explanation for this rather low success rate can be provided by a recent study, which reported that only mature DCs, not immature DCs, could expand and differentiate CTL precursors into cytotoxic effector cells [14]. Flow cytometric analysis of the DCs used in our work revealed a $CD83^-$ phenotype after maturation with TNF-α, indicating that the DCs used as APCs were not fully mature.

TGFβRII mutations are found in 90% of all MSI^+ colorectal cancers [18] and may therefore be a good target gene for immunotherapy. Here, we have reported the identification of CTL epitope RLSSCVPVA, derived from the most common TGFβRII mutation. In a parallel study, a CD4 epitope recognised by T cells derived from both a normal donor and MSI^+ cancer patients has been identified (Sæterdal et al., in press). Together, our results indicate that frameshift peptides from TGFβRII represent a new group of tumour-specific antigens that may be used in cancer vaccines targeting spontaneous and inherited forms of MSI^+ cancers. By immunisation with a combination of MHC class I and II epitopes a broader and more effective immune response may be generated. Other aspects supporting an immunisation with distinct frameshift-derived peptides representing several T cell epitopes are the findings that MSI^+ patients often harbour mutations in more than one gene carrying nucleotide repeat sequences (recent studies by Thorstensen et al., submitted), and the risk for tumour escape by immune selection after immunisation in vivo [11, 19]. It is therefore tempting to design a vaccine based on a combination of peptides generated from frameshift mutation in candidate genes, such as TGFβRII, Bax and Caspase-5.

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Note added in proof. Recently, Linnebacher et al. (2001, Int. J. Cancer 93: 6) described results very similar to ours regarding the RLSSCVPVA peptide.

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