The Melanoma Inhibitor of Apoptosis Protein: A Target for Spontaneous Cytotoxic T Cell Responses

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The identification of tumor antigens which expression is essential for the survival of tumor cells is a new avenue to prevent antigen loss variants emerging due to immunoselection, particularly during immune therapy. The melanoma inhibitor of apoptosis protein, ML-IAP (also named livin) counteracts apoptosis induced by death receptors, hypooxgenic conditions, or chemotherapeutic agents. Thus, elevated expression of ML-IAP renders melanoma cells resistant to apoptotic stimuli and thereby potentially contributes to the oncogenic phenotype. Here, we demonstrate that T cells in a large proportion of melanoma patients infiltrating the tumor or circulating in the peripheral blood specifically recognize ML-IAP-derived peptides. Interestingly, the responses against the peptide epitope ML-IAP$_{280–289}$ were not restricted to melanoma patients but present among peripheral blood T cells in a few healthy controls. *In situ* peptide/HLA-A2 multimer staining, however, confirmed the infiltration of ML-IAP-reactive cells into the tumor microenvironment. Moreover, ML-IAP-reactive T cells isolated by magnetic beads coated with peptide/HLA-A2 complexes were cytotoxic against HLA-matched melanoma cells. In conclusion, out data strongly indicate ML-IAP as a suitable target for immunologic intervention.

Key words: antigens/cytotoxic T lymphocytes/epitopes/human/peptides/tumor immunity.
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It is well established that peptide epitopes derived from human tumor-associated antigens (TAA) can be recognized by cytotoxic T lymphocytes (CTL) in the context of major histocompatibility complex (MHC) molecules (Van den Eynde and Boon, 1997) and that most—if not all—tumors express such antigens. Consequently, several strategies target these TAA in order to generate effective anti-tumor CTL responses in patients (Heslop and Rooney, 1997; Rosenberg *et al*, 1998; Thurner *et al*, 1999). Notably, powerful CTL responses against these antigens have indeed been induced by vaccination and some patients experienced a complete remission of their disease (Rosenberg, 1996; Marchand *et al*, 1999). Immunoselection of antigen loss variants, however, may be an important obstacle for the curative potential of most of the known CTL epitopes in clinical oncology as most characterized peptides are derived from proteins, which are not essential for the survival of the tumor cell. Thus, tumor cells lacking the expression of the targeted antigen escape immune surveillance (Becker *et al*, 1993; Cormier *et al*, 1998). CTL epitopes derived from proteins, which are either linked to the neoplastic transformation such as mutated tumor suppressor genes or are essential for the survival of tumor cells should not be inflicted by this form of immunoselection. We recently identified spontaneous T cell responses against survivin in patients suffering from melanoma, leukemia, or breast cancer (Andersen *et al*, 2001a,b). Survivin is a member of the inhibitor of apoptosis protein (IAP) family, and it is becoming evident that IAP expression enhances the survival of cancer cells and facilitates their escape from immune surveillance and cytotoxic therapies (Jaattela, 1999; Zaffaroni *et al*, 2002). In addition to survivin, a number of different IAP have been described. Their different expression patterns suggest an organ-specific role in promoting cell survival during development and tissue homeostasis. Whereas X-IAP, C-IAP1, and C-IAP2 are relatively ubiquitously expressed, survivin is expressed only in fetal and tumor tissues. Similarly, ML-IAP has a rather selective expression pattern, as it is predominantly detected in melanoma and a limited number of other tissues (Vucic *et al*, 2000; Kasof and Gomes, 2001). ML-IAP and survivin seems to be the only IAP expressed in melanoma. Whereas ML-IAP can be detected in the majority of melanoma cell lines, it is not present in normal melanocytes and high levels of ML-IAP are associated with resistance to drug-induced apoptosis of melanoma cells (Vucic *et al*, 2000; Chen *et al*, 2003). Thus, ML-IAP is a critical cellular factor as increased expression levels confer resistance to apoptotic stimuli, thereby contributing to the pathogenesis and progression of melanoma. These characteristics suggest ML-IAP as a suitable target for immunotherapy against cancer. In that regard, Schmollinger *et al* (2003), recently anecdotally reported that a melanoma patient vaccinated with irradiated autologous melanoma cells engineered to

Abbreviations: CLL, chronic lymphatic leukemia; CTL, cytotoxic T lymphocytes; ML-IAP, melanoma inhibitor of apoptosis protein; TAA, tumor-associated antigens; PBL, peripheral blood lymphocytes; TAP, transporter associated with antigen processing; TIL, tumor-infiltrating lymphocytes.

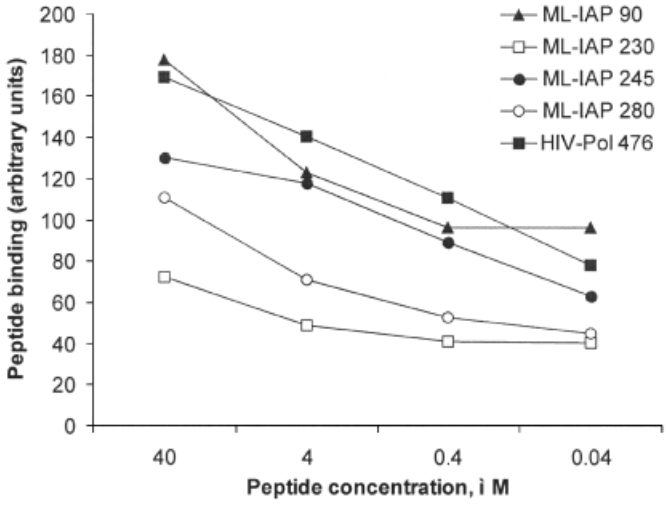

Figure 1
Identification of HLA-A2-binding peptides from ML-IAP. Class I MHC heavy chain bands were quantified on a PhosphorImager. The mount of stabilized HLA-A2 heavy chain is directly related to the binding affinity of the added peptide. The binding of the HLA-A2-restricted-positive control peptide HIV Pol_{476} (*black square*) was compared with the peptides ML-IAP_{90} (*black triangle*), ML-IAP_{230} (*white square*), ML-IAP_{245} (*black circle*), and ML-IAP_{280} (*white circle*).

Table I. Peptides examined in this study

Protein[a]	Sequence	C_{50} (μM)[b]
HIV-1 pol_{476}	ILKEPVHGV	0.2
ML-IAP_{245}	RLQEERTCKV	1
ML-IAP_{280}	QLCPICRAPV	20
ML-IAP_{90}	RLASFYDWPL	0.2
ML-IAP_{154}	LLRSKGRDFV	10
ML-IAP_{230}	VLEPPGARDV	>100
ML-IAP_{98}	PLTAEVPPEL	>100
ML-IAP_{261}	SIVFVPCGHL	Not binding
ML-IAP_{34}	SLGSPVLGL	1
ML-IAP_{54}	QILGQLRPL	1
ML-IAP_{99}	LTAEVPPEL	0.9
ML-IAP_{83}	GMGSEELRL	30
ML-IAP_{200}	ELPTPRREV	Not binding

[a]The value range listed in subscript indicates the position of the first amino acid in the sequence.
[b]The C_{50} value is the concentration of the peptide required for half maximal binding to HLA-A2.

secrete granulocyte-macrophage colony-stimulating factor, developed CTL reactivity against two ML-IAP-derived peptides. In this study, we searched for and detected spontaneous T cell reactivity in tumor-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) against ML-IAP-derived peptides in a larger series of melanoma patients by ELISPOT assay. Furthermore, we took advantage of the recently established techniques allowing *in situ* detection as well as *ex vivo* isolation of antigen-reactive T cells to analyze the functional capacity of ML-IAP-specific CTL.

Results

Binding of ML-IAP-derived peptides to HLA-A2 The amino acid sequence of the ML-IAP protein was screened for the most probable HLA-A2 nona-mer and deca-mer peptide epitopes, using the main HLA-A2-specific anchor residues (Andersen *et al*, 2000). Twelve ML-IAP deduced peptides were synthesized and examined for binding to HLA-A2 by comparison with the HLA-A2 high-affinity positive control epitope from HIV-1 $pol_{476–484}$ (ILKEPVHGV) by the assembly assay. The assembly assay is based on stabilization of the class I molecule after loading of different concentrations of peptide to the TAP-deficient cell line T2. Subsequently, correctly folded stable MHC heavy chains are immunoprecipitated using conformation-dependent antibodies. The extent of stabilization of class I MHC molecules is directly related to the binding affinity of the added peptide as exemplified in Fig 1. The peptide concentration required for half maximal recovery of class I MHC molecules (C_{50} value) were 0.2 μM for the HIV-1 $pol_{476–484}$ (Table I). Five ML-IAP peptides bound with similar high affinity as the positive control; ML-IAP_{245}, ML-IAP_{90}, ML-IAP_{34}, ML-IAP_{54}, and ML-IAP_{99} (C_{50} = 1, 0.2, 1, 1, and 0.9 μM, respectively) (Table I). The peptides ML-IAP_{280}, ML-IAP_{83}, and ML-IAP_{154} bound only with intermediate affinity (C_{50} = 20, 30, and 10 μM, respectively), and ML-IAP_{230} and ML-IAP_{98} bound only weakly to HLA-A2 (C_{50} > 100 μM). Two of the peptides examined (ML-IAP_{261}, ML-IAP_{200}) did not bind to HLA-A2 at all (Table I).

CTL responses against ML-IAP-derived peptides in melanoma patients Using the ELISPOT interferon-γ secretion assay, we examined for the presence of specific T cell responses against the ML-IAP deduced, HLA-A2 binding peptides in peripheral blood T cells and TIL from melanoma patients. This method has previously been shown to be highly effective to identify tumor-specific CTL in cancer patients (Herr *et al*, 1999; Andersen *et al*, 2001a; Scheibenbogen *et al*, 2002).

The strongest and most frequent CTL responses were detected against the intermediate HLA-A2 binding peptide ML-IAP_{280} and responses against this peptide were actually present in both TIL and PBL. Figure 2(*A*) exemplifies such strong spontaneous responses; each spot represents a peptide-reactive, interferon-γ producing cell. Additionally, we examined PBL from 45 patients and TIL from 16 patients for responses against ML-IAP_{280}, identifying spontaneous responses in PBL from 14 patients (≈ 35%), and in TIL from six patients (≈ 43%) (Fig 2*B*).

Albeit less frequent, spontaneous CTL responses against three additional ML-IAP-derived peptides were detected. PBL from 37 patients and TIL from 20 patients were examined for a response against ML-IAP_{245}, ML-IAP_{90}, and ML-IAP_{230} revealing responses against the ML-IAP_{245} peptide in two of the PBL (≈ 5%), and five of the TIL samples (≈ 25%) (Fig 3), and responses against the ML-IAP_{90} peptide in two PBL (≈ 5%), and seven TIL samples (≈ 35%) (Fig 3). Surprisingly, we also detected a response against the weak HLA-A2 binding peptide ML-IAP_{230} in PBL of two patients (≈ 5%), and TIL of six patients (≈ 30%)

even though this peptide was not able to stabilize the HLA-A2 molecule (Fig 3).

ML-IAP-reactive T cells in healthy individuals PBL from 10 healthy, HLA-A2-positive persons were analyzed to investigate whether a response against ML-IAP$_{245}$, ML-IAP$_{90}$, and ML-IAP$_{230}$ would be present. No responses were observed in any of the controls against any of these peptides. As the spontaneous CTL responses against ML-IAP$_{280}$ were very frequent in melanoma patients, however, we additionally examined 32 healthy individuals for responses against this peptide. Surprisingly, we detected a response in four of these (Fig 4).

Detection of ML-IAP-reactive T cells *in situ* ML-IAP$_{280}$/HLA-A2 and ML-IAP$_{245}$/HLA-A2-specific monomers were multimerized using dextran molecules, which were conjugated with both streptavidin and fluorescein isothiocyanate. Multimerized MHC complexes were used to stain acetone-fixed, frozen material as described previously (Andersen *et al*, 2001b; Schrama *et al*, 2002) and antigen-specific cells were visualized using a confocal laser microscope. Sections of primary melanoma from six patients were analyzed, and ML-IAP$_{280}$- and ML-IAP$_{245}$-reactive CTL could readily be detected *in situ* in the tumor microenvironment in two of the patients (Fig 5).

ML-IAP/HLA-A2-reactive CTL lyze HLA-matched melanoma cells To characterize the functional capacity of ML-IAP-reactive CTL, these cells were isolated by means of magnetic beads coated with HLA-A2/ML-IAP complexes. Recently, we isolated gp100- and Mart-1-specific T cells in a similar manner showing that this method is highly efficient to enrich specifically peptide/MHC-reactive T cells (Schrama *et al*, 2001). ML-IAP$_{245}$-specific cells were directly isolated from PBL (Fig 6*A*). ML-IAP$_{280}$-reactive cells were enriched from TIL of a melanoma infiltrated lymph node after being stimulated once *in vitro* with peptide. These cells

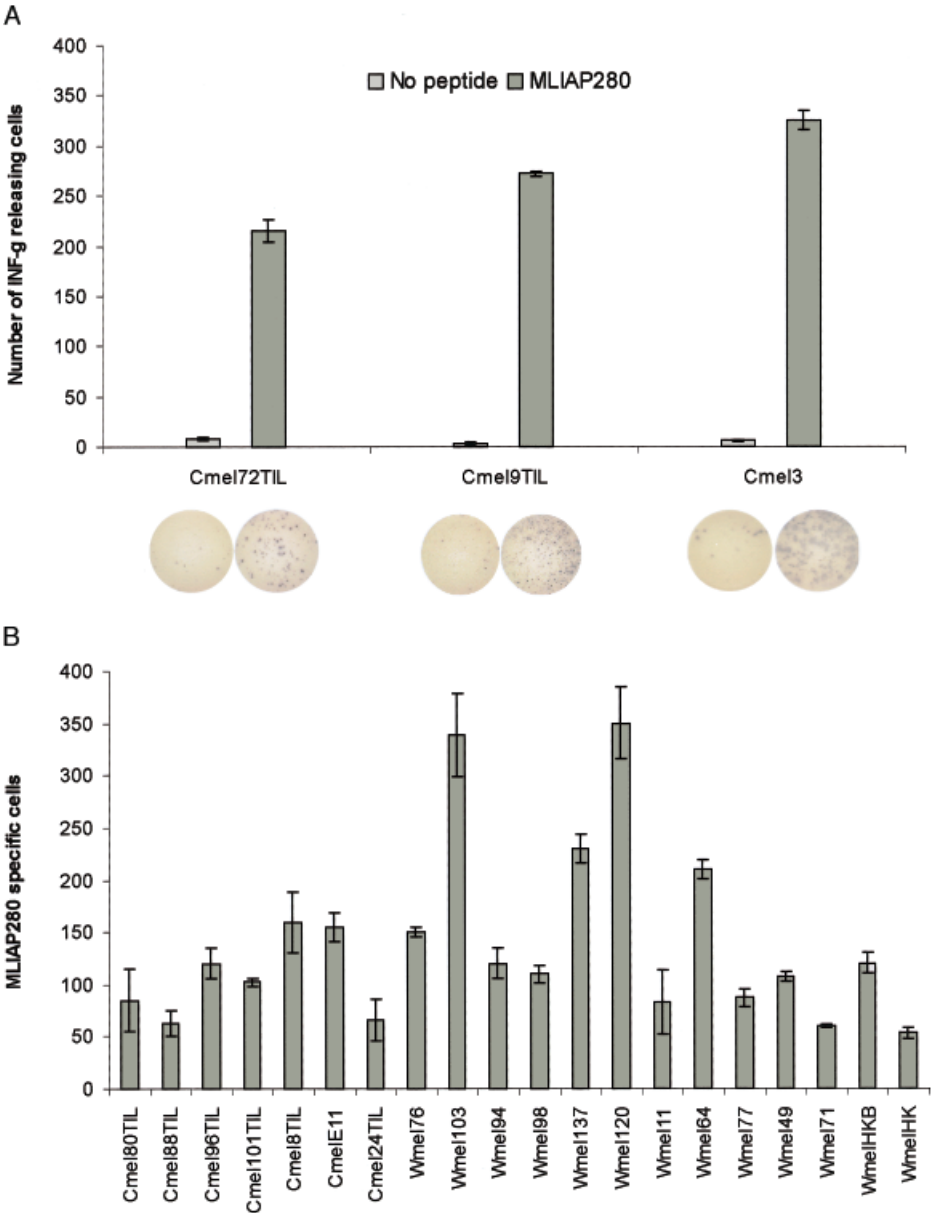

Figure 2
T cell response against the ML-IAP$_{280}$ (QLCPICRAPV) peptide. (*A*) Strong responses was seen in TIL from the melanoma patients Cmel72 and Cmel9 and in PBL from the melanoma patient Cmel3. T lymphocytes were stimulated once with peptide before plated at 3×10^5 cells per well in duplicates either with T2 cells without (*light gray*) or with peptide (*dark gray*). The graph depicts the quantification of interferon-γ producing cells. (*B*) Patients hosting weaker ML-IAP$_{280}$ responses. The average number of ML-IAP$_{280}$-specific cells was calculated after subtraction of spots without added peptide for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC). PBL from 45 melanoma patients and TIL from 16 patients was examined. $p < 0.05$ (evaluated by Student's t test for unpaired samples) calculated as the number of spots in wells with added peptide as compared with the number of spots in wells without peptide.

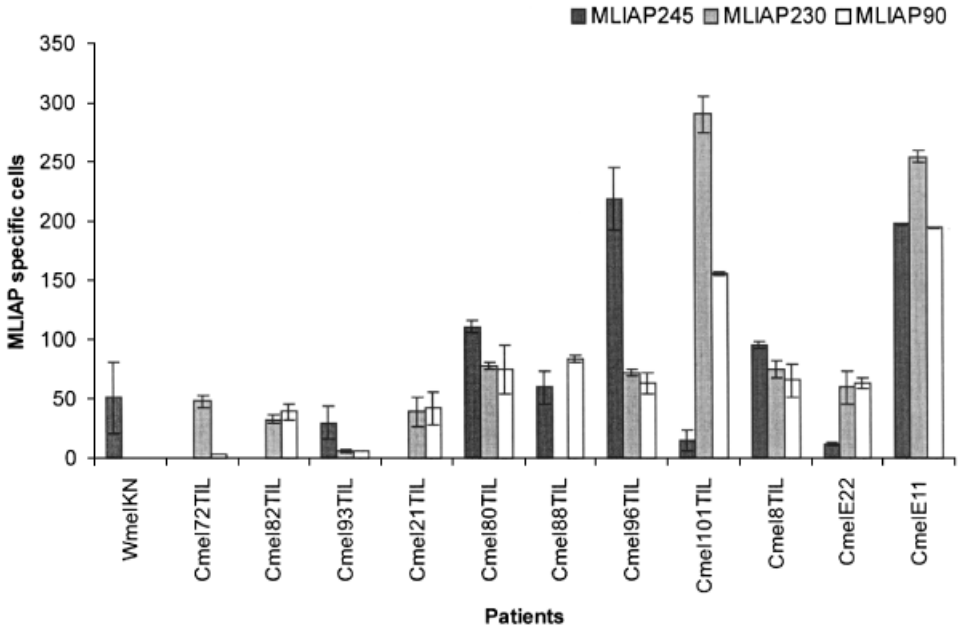

Figure 3
T cell response against the peptides ML-IAP$_{245}$ (RLQEERTCKV), ML-IAP$_{230}$ (VLEPPGARDV), and ML-IAP$_{90}$ (RLASFYDWPL). TIL samples from 20 patients and in PBL from 37 melanoma patients were analyzed. T lymphocytes were stimulated once with peptide before plated at 3×10^5 cells per well in duplicates either without or with peptide. The average number of peptide-specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC). $p < 0.05$ (evaluated by Student's t test for unpaired samples) calculated as the number of spots in wells with added peptide as compared with the number of spots in wells without peptide.

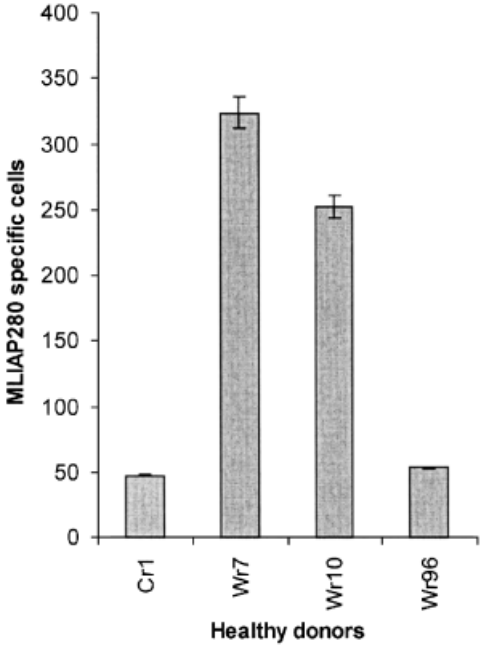

Figure 4
T cell response against the peptide ML-IAP$_{280}$ (QLCPICRAPV) in healthy individuals. PBL from 34 HLA-A2-positive, healthy individuals were analyzed. T lymphocytes were stimulated once with peptide before plated at 3×10^5 cells per well in duplicates either without or with peptide. The average number of peptide-specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC). $p < 0.05$ (evaluated by Student's t test for unpaired samples) calculated as the number of spots in wells with added peptide as compared with the number of spots in wells without peptide.

lyzed T2-cells in a peptide-specific manner (Fig 6*B*). Additionally, we tested the cytotoxicity of the ML-IAP$_{280}$-reactive CTL against the autologous melanoma line FM72, the HLA-A2-matched melanoma cell line FM93 and the HLA mismatched melanoma cell line FM56. This analysis revealed that the ML-IAP$_{280}$-reactive T cells efficiently lyzed both the autologous and the HLA-matched melanoma cell lines. In contrast, no cytotoxicity was observed against the HLA-A2-negative melanoma cell line FM56 or the natural killer target cell K562 (Fig 6*C*).

Discussion

In order to define new targets for specific immunotherapy, we scanned the ML-IAP protein for the presence of HLA-A2 binding motifs and—after successful identification—used these peptides to test for the presence of specific T cell reactivities in melanoma patients ($n = 55$) by ELISPOT assay. Following this strategy, we identified strong CTL responses against the intermediate HLA-A2 binding peptide ML-IAP$_{280}$, as well as intermediate responses to three additional peptide epitopes, i.e., the strong HLA-A2 binding peptides ML-IAP$_{245}$ and ML-IAP$_{90}$ and the very weak binding peptide ML-IAP$_{230}$. Notably, the ML-IAP$_{90}$ was also identified as an epitope in the one melanoma patient examined by Schmollinger *et al* (2003).

Vucic *et al* (2000) demonstrated by data from northern blot analysis that elevated levels of ML-IAP can be detected in melanoma cells, but not in normal tissue, except to some extent in the testis. This were supported by Kasof and Gomes (2001), who were able to detect elevated levels of ML-IAP in a large panel of melanoma cells, whereas they could not detect ML-IAP in healthy melanocytes or any normal tissues except placenta. Responses against one of the identified epitopes, ML-IAP$_{280}$, however, were not only detected in a large proportion of the melanoma patients, but also in a limited number of healthy individuals. The significance of this finding is unclear, as neither of the healthy donors included in the study showed any signs of autoimmunity despite the fact that they hosted a T cell response against ML-IAP. In that regard, it is well established that circulating precursor CTL against melanocyte

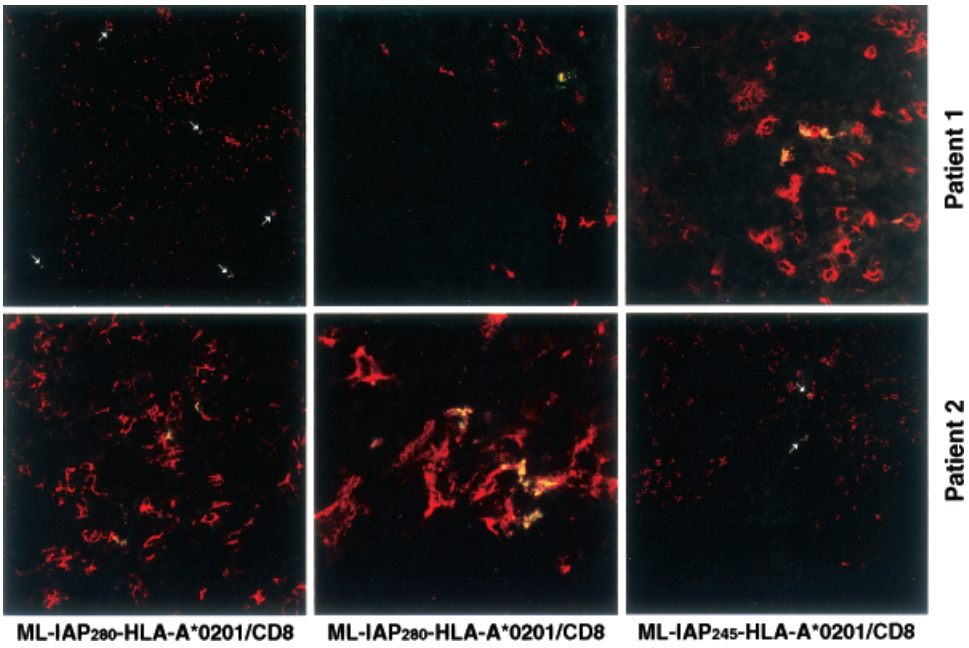

Figure 5
***In situ* detection of ML-IAP-reactive CTL**. Confocal laser scanning microscopy was used to detect CTL reacting with a Cy3-conjugated anti-CD8 antibody (red channel) and a fluorescein isothiocyanate-conjugated multimeric HLA-A2/ML-IAP$_{280}$ construct (green channel) (first and second columns) or with a fluorescein isothiocyanate conjugated multimeric HLA-A2/ML-IAP$_{245}$ construct (last column) in primary tumors from two HLA-A2-positive melanoma patients.

differentiation antigens, such as MART-1/Melan-A, gp100, and tyrosinase can be detected not only in melanoma patients but also in some normal donors (Jäger *et al*, 1996; D'Souza *et al*, 1998; Griffioen *et al*, 2001). Interestingly, major differences in the differentiation status of circulating CTLp to the immunodominant Melan-A$_{26-35}$ epitope of melanoma patients and healthy individuals have been reported. In contrast to the latter, patients with metastatic melanoma Melan-A-specific CTLp are characterized by a memory phenotype (D'Souza *et al*, 1998).

It should be noted that we were able to detect ML-IAP-reactive CTL not only in circulation but also in the inflammatory infiltrate of the tumor. There is a consensus that effective tumor immunity requires proper priming of the T cell. It is equally important, however, that the T cells acquire the ability to home to the site of action. The combined detection of ML-IAP-specific T cells in the blood and in the tumor lesions indicates that these cells are capable of circulating and homing to the tumor site. This is a significant finding, as several clinical reports have suggested a functional dissociation between local and systemic anti-melanoma T cell responses. Thus, the presence of TAA-specific T cells in circulation may not lead to clinically relevant responses (Lee *et al*, 1998; Rosenberg *et al*, 1998; thor Straten *et al*, 1999).

The ELISPOT methodology represents a strong tool to monitor peptide-specific T cell responses. Although it has been shown that ELISPOT reactivity in most cases correlates with the capacity to lyze the target cell, the formal prove for this notion can only be obtained directly. We provided such evidence by isolation of ML-IAP-reactive T cells, which possess the functional capacity of peptide-specific target cell lysis. Moreover, ML-IAP-reactive T cells killed not only the autologous tumor cell line, but also a HLA-matched melanoma cell line. This finding further suggests that melanoma cells indeed process and present the ML-IAP peptide.

Two of the epitopes identified (ML-IAP$_{280}$ and ML-IAP$_{230}$) only weakly bound to HLA-A2. In that respect, it is worth noting that many factors may influence the CTL response against any given peptide. These include expression level of the relevant source protein, processing, TAP transport, peptide affinity to the class I MHC molecule, surface expression of the class I MHC, T cell receptor repertoire, etc. (Yewdell and Bennink, 1999). Thus, peptide binding to class I is only one in a number of factors that determine the immunogenicity of a given peptide. Additionally, in contrast to foreign peptides, self peptides expressed on the cell surface at high density due to high MHC-binding affinity, seem rather to induce tolerance as reactive T cells are eliminated or inactivated (Moudgil and Sercarz, 1994). Thus, many prominent epitopes for CTL responses to self proteins are subdominant or cryptic. This notion is substantiated by the observation that many epitopes of melanoma antigens, which are nonmutated self proteins, such as gp100 and MART-1, have relatively low binding affinities to class I MHC (Parkhurst *et al*, 1996). As the efficacy of tumor immunotherapy largely depends on the avidity of recruited CTL, rather than the affinity of the epitope to the MHC molecule (Zeh *et al*, 1999), low-affinity epitopes from oncogenic nonmutated self proteins are suitable targets, provided that they are presented by tumor cells efficiently enough to be recognized by CTL. For ML-IAP$_{280}$ we directly demonstrated this by direct cytotoxicity assays and *in situ* multimer staining.

The attractiveness of using IAP such as ML-IAP and/or survivin for vaccination purposes is based on the fact that downregulation or loss of expression of these proteins as some form of immune escape would impair sustained tumor growth. For therapeutic strategies, targeting of antigens that plays an insignificant role in relation to tumor cell growth and survival, the selection of antigen deficient tumors is a well-recognized limitation (Jäger *et al*, 1997; Thurner *et al*, 1999; Yee *et al*, 2000). As elevated expression of ML-IAP in

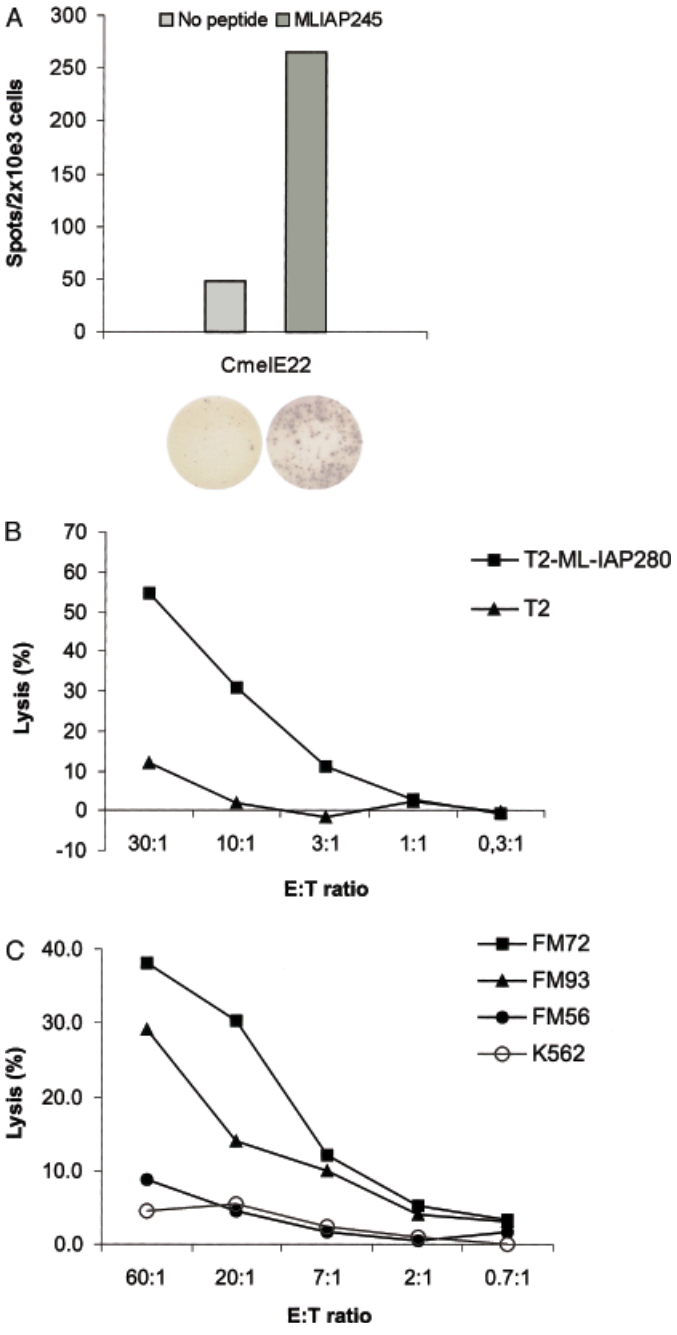

Figure 6
Cytolytic capacity of ML-IAP-specific CTL. (*A*) ML-IAP$_{245}$-reactive CTL were isolated from PBL from the melanoma patients CmelE22 using peptide-coated magnetic beads before being plated at 2×10^3 cells per well in duplicates either with T2 cells without or pulsed with ML-IAP$_{245}$. (*B*) ML-IAP$_{280}$-reactive CTL were isolated from a melanoma-infiltrated lymph node from patient Cmel72 using peptide-coated magnetic beads. These cells was analyzed for specific lysis of T2 cells with (*square*) or without (*triangle*) ML-IAP$_{280}$ peptide. (*C*) Lysis by ML-IAP$_{280}$-isolated T cells of the autologous melanoma cell line FM72 (*square*), the HLA-A2-positive melanoma cell line FM93 (*triangle*), the HLA-A2 negative cell line FM56 (*black circle*) and the natural killer target cell line K562 (*white circle*).

cells is correlated with drug resistance (Vucic *et al*, 2000; Ashhab *et al*, 2001), the combination of a ML-IAP-based immunotherapy with cytotoxic chemotherapy might be an effective way to treat cancer.

In conclusion, we demonstrate that IAP represents a novel group of proteins of tumor antigens in cancer patients recognized by cytotoxic T cells. Thus, proteins that confer the oncogenic phenotype of tumor cells may serve as targets for immune responses. Our data thereby stress the importance of combining the conceptual understanding of cellular immunity with the phenotypic characteristics of cancer cells.

Materials and Methods

Patients and normal control PBL or T lymphocytes from tumor infiltrated lymph nodes were obtained from HLA-A2 positive melanoma patients or healthy individuals. Informed consent was otained from the patients prior to any of these measures.

Peptides All peptides were purchased from KJ Ross-Petersen APS (Holte, Denmark) and provided at more than 80% purity as verified by high-performance liquid chromatography and mass spectrometry analysis. All peptides used are listed in Table I.

Assembly assay for peptide binding to class I MHC molecules Assembly assays for binding of the synthetic peptides to class I MHC molecules metabolically labeled with [^{35}S]-methionine were carried out as described (Andersen *et al*, 1999a,b). The assembly assay is based on stabilization of the class I molecule after loading of peptide to the peptide transporter deficient cell line T2. Subsequently, correctly folded stable MHC heavy chains are immunoprecipitated using conformation-dependent antibodies. After IEF electrophoresis, gels were exposed to PhosphorImager screens, and peptide binding was quantitated using the Image-quant PhosphorImager program (Molecular Dynamics, Sunnyvale, California).

Antigen stimulation of PBL To extend the sensitivity of the ELISPOT assay, PBL were stimulated once *in vitro* prior to analysis (McCutcheon *et al*, 1997; Pass *et al*, 1998). At day 0, PBL or crushed lymph nodes were thawed and plated in 2 mL per well at a concentration of 2×10^6 cells in 24-well plates (Nunc, Denmark) in X-vivo medium (BioWhittaker, Walkersville, Maryland), 5% heat-inactivated human serum, and 2 mM of L-glutamine in the presence of 10 μM of peptide. Two days later 20 IU per mL recombinant interleukin-2 (Chiron, Ratingen, Germany) was added to the cultures. The cultured cells were tested for reactivity in the ELISPOT on day 12.

ELISPOT assay The ELISPOT assay was used to quantify peptide epitope-specific interferon-γ releasing effector cells as described previously (Berke *et al*, 2000). Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45, Millipore, Hedehusene, Denmark) were coated with anti-interferon-γ antibody (1-D1K, Mabtech, Nacka, Sweden). The wells were washed, blocked by X-vivo medium, and cells added in duplicates at different cell concentrations. Peptides were then added to each well and the plates were incubated overnight. The following day, media was discarded and the wells were washed prior to addition of biotinylated secondary antibody (7-B6-1-Biotin, Mabtech). The plates were incubated for 2 h, washed, and avidin–enzyme conjugate (AP-Avidin, Calbiochem, Life Technologies, Roskilde, Denmark) was added to each well. Plates were incubated at room temperature for 1 h and the enzyme substrate NBT/BCIP (Gibco, Life Technologies, Roskilde, Denmark) was added to each well and incubated at room temperature for 5 to 10 min. The reaction was terminated by washing with tap-water upon the emergency of dark purple spots. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC, Cleveland, Ohio) and the peptide-specific CTL frequency could be calculated from the numbers of spot-forming cells. All assays were performed in duplicates for each peptide antigen.

Immunohistochemistry stainings Multimerized peptide/HLA complexes were used to identify antigen-specific T cells *in situ* in tumor lesions of cancer patients, as previously described (Anderson *et al*, 2001b). Biotinylated ML-IAP_{280}/HLA-A2 and ML-IAP_{245}/HLA-A2 monomer was supplied by Proimmune Ltd (Oxford, UK). The biotinylated monomers were multimerized with streptavidin–fluorescein isothiocyanate-conjugated dextran molecules (kindly provided by L. Winther, DAKO, Glostrup, Denmark) to generate multivalent HLA-dextran compounds for immunohistochemistry. Tissue sections were dried overnight and subsequently fixed in cold acetone for 5 min. All incubation steps were performed in the dark at room temperature: (1) 45 min of the primary antibody (1:100 diluted); (2) Cy3-conjugated goat anti-mouse (1:500 diluted; code 115-165-100; Jackson ImmunoResearch, obtained from Dianova, Hamburg, Germany) for 45 min; and, finally, (3) the multimers for 75 min. Between each step, the slides were washed two times for 10 min in phosphate-buffered saline/bovine serum albumin 0.1%. The slides were mounted in Vectashield and kept in the refrigerator until observed under the confocal microscope (TCS 4D, Leica, Mannheim, Germany).

Isolation of peptide-specific T cells Antigen-specific cells were isolated by means of ML-IAP_{280}/HLA-A2- and ML-IAP_{245}/HLA-A2-coated magnetic beads as previously described (Anderson *et al*, 2001b). Biotinylated monomers (ProImmune, Oxford, UK) were coupled to streptavadin coated magnetic beads (Dynabeads M-280, Dynal A/S, Oslo, Norway) by incubating 2.5 μg monomers with 5×10^6 beads in 40 μL phosphate-buffered saline, for 20 min at room temperature. The magnetic complexes were washed three times in phosphate-buffered saline in a magnetic field (Dynal A/S) and subsequently mixed with PBL, at a ratio of 1:10 in phosphate-buffered saline with 5% bovine serum albumin, and rotated very gently for 1 h. Antigen-specific $CD8^+$ T cells associating with the magnetic complexes were gently washed three times. Isolated cells were resuspended numerous times in X-vivo with 5% human serum, and incubated for 2 h, before the magnetic beads were released and removed from the cell suspension. The isolated cells were cultured in a 96-well plate in X-vivo with 5% 5% human serum. One day after isolation 20 units interleukin-2 per mL was added, and on day 5 the capacity of these cells to kill target cells was tested either by ELISPOT or in standard ^{51}Cr release assays.

Cytotoxicity assay Conventional [^{51}Cr]-release assays for CTL-mediated cytotoxicity was carried out as described elsewhere (Andersen *et al*, 1999a). Target cells were T2 cells with or without the relevant peptide, autologous melanoma cell line FM72, the HLA-A2-positive melanoma cell line FM93 (Kirkin *et al*, 1995), and the HLA-A2 negative melanoma cell line FM56 (Kirkin *et al*, 1995). All cancer cell lines expressed ML-IAP as examined by reverse transcription–polymerase chain reaction (data not shown).

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