In Vitro Generation of Cytolytic T Cells Against Human Melanoma Cells Overexpressing HDM2

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Abstract. *Background: Previous experiments have shown that tumour-associated antigens can be exploited for a successful anti-tumour immunisation. Previous reports demonstrated that oncoprotein MDM2 (HDM2) contains two highly conserved MHC class I binding motifs, $MDM2_{100}$ and $MDM2_{441}$, and that dendritic cells (DC) presenting $MDM2_{100}$ stimulate an effective CTL reaction against melanoma cells. Materials and Methods: In this study, we investigated the CTL-inducing capacity of autologous human dendritic cells pulsed with fragment $HDM2_{441}$. Results: In vitro $HDM2_{441}$-primed T lymphocytes revealed a strong proliferation activity, released Th-1-associated cytokines, and possessed an effective anti-tumour activity causing apoptosis in $HDM2_{441}$-overexpressing melanoma cells. Cytotoxic assay demonstrated that, in parallel to melanoma cells, up to 65% of primed T cells also underwent apoptosis. Conclusion: These data suggest that $HDM2_{441}$ may be exploited for broad-spectrum DC-based trials against metastatic melanomas overexpressing HDM2, and point out that the efficacy of such immunotherapeutical approaches may be limited via T cell apoptosis.*

Malignant melanoma is the prototype of an extremely aggressive tumour, against which many immunotherapeutical approaches have been designed. These include administration of pro-inflammatory cytokines (1, 2), transfection of melanoma cells with genes encoding co-stimulatory molecules (3), usage of a superagonist variant of the melanoma-associated peptide MART1/Melan A (4), vaccination with hybridomas of melanoma cells and autologous DC (5) and application of dendritic cells (DC) presenting tumour antigens to cytotoxic T lymphocytes (CTL) (6-11). Nestle *et al.* translated, for the first time, the DC approach into a successful clinical therapy in which *ex vivo* generated DC were loaded with a cocktail of melanoma-associated peptides (tyrosinase, MART1/Melan A, gp100, MAGE-1 and MAGE-3), prior to injection into the healthy lymph nodes of patients suffering from metastatic melanoma (12). For a broad-spectrum DC-based immunotherapy of metastatic malignant melanoma, however, it is necessary to identify further tumour-associated antigens that are expressed at high levels in the majority of melanomas, but not in normal cells.

The Murine double minute-2 (MDM2) gene encoding a 90-kDa zinc finger oncoprotein is a critical, negative regulator of the p53 pathway, the overexpression of which plays a crucial role in tumorigenesis and progression of different malignancies (13). Of special note for a DC-based immunotherapy are data showing that the MDM2 protein contains two highly conserved MHC class I binding motifs, $MDM2_{100}$ and $MDM2_{441}$ (14). Sadovnikova and Stauss demonstrated that antigen-presenting cells loaded with $MDM2_{100}$ induce CTL that are capable of killing murine melanoma cells, effectively (15). Based on these data we hypothesised that HDM2, the human homologue of MDM2, might be exploited for a DC-based immunotherapy of malignant melanomas in humans. This hypothesis became more attractive since, recently, Polsky *et al.* have shown that HDM2 is strongly overexpressed in invasive and metastatic malignant melanomas, but not in nevi (16).

In the present work we show that *in vitro* human DC loaded with $HDM2_{441}$, the human homologue of $MDM2_{441}$, stimulate an effective CTL reaction, which leads to apoptosis of human melanoma cells overexpressing $HDM2_{441}$.

Materials and Methods

Generation of two melanoma cell lines. Two human melanoma cell lines (KaII and LIR) were established from surgically removed lymph nodes of patients with metastatic melanoma. The lymph nodes were spliced for microscopic examination and tumour cell preparation. Immediately after diagnosis on frozen sections, fresh

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tissue probes were passed repeatedly through stainless-steel sieves with mesh sizes of 80 μm (Sigma, Deisenhofen, Germany). Isolated cells were cultured in RPMI 1640 medium containing 10% human serum. They were then characterised by applying RT-PCR, immunocytochemistry, immunofluorescence and confocal laser-scanning microscopy.

Preparation and characterisation of fibroblasts. Cell preparation from surgically removed tissues has been described in detail previously (5). Briefly, healthy connective tissue probes (n=3) were passed repeatedly through stainless-steel sieves with mesh sizes of 380 μm and 230 μm, respectively. Isolated cells were cultured for one week in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% autologous serum. They were then characterised by applying immunofluorescence for the human fibroblast antigen (clone 5B5, Dako, Hamburg, Germany), as described previously (5).

Analysis of HDM2 mRNA. Total RNA was isolated from cell lines with the RNeasy Total RNA Kit® (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After isolation RNA was eluted from the affinity columns in a final volume of 20 ml DEPC-H20. Then 2 ml isolated total RNA was applied to RT-PCR with oligonucleotide primers yielding a 1,536 bp cDNA corresponding to the full-size HDM2 mRNA as previously described by Sigalas *et al.* (17). Primers were produced by MWG Biotech (Munich, Germany). Reverse transcription and amplification of the HDM2 mRNA was performed with a OneStep RT-PCR Kit® (QIAGEN) on a DNA thermal cycler UNOII (Biometra, Goettingen, Germany) using thin-walled reaction tubes (Perkin Elmer, Weiterstadt, Germany)(for primer design, PCR conditions and PCR mixture see Sigalas *et al.* (17)). PCR products were separated on a 3% (w/v) agarose gel containing 0.5 mg ethidium bromide per ml. Results were documented by means of a CCD camera (Biometra).

The HDM2 PCR fragments were purified from agarose gel using QIAEX II Gel Extraction Kit® (QIAGEN). Two hundred ng of isolated cDNA was labelled with the PRISM-Ready Reaction Dye Deoxy-TM Terminator Cycle Sequencing Kit® according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany). Automatic sequencing was performed on an ABI 310 DNA analyser (Applied Biosystems). Oligonucleotides, previously used for amplification of fragments, served as sequencing primers.

Immunocytochemistry. Melanoma cells were transferred onto glass slides, fixed in ice-cold acetone for 5 min and washed three times with PBS. The cells were then incubated at 4˚C overnight with the primary monoclonal anti-HDM2 antibody (mAb) SMP 14 (working dilution, 1:50; Santa Cruz Biotechnology, Heidelberg, Germany) which recognises the epitope corresponding to amino acids 154-167 of HDM2. After washing with PBS, a rabbit anti-mouse antibody (DAKO, Hamburg, Germany) was added to the samples at a working dilution of 1:20. After 1h cells were washed three times with PBS, then APAAP complex (DAKO) diluted 1:20 in human serum was pipetted onto the tumour cells. Having incubated the cells for 1h at room temperature (RT), staining was performed with substrate solution prepared according to the published protocol (18).

Immunofluorescence and confocal laser-scanning microscopy. Melanoma cells were mounted onto slides by cytocentrifugation. After fixation in cold acetone for 5 min, the samples were incubated with the polyclonal Ab against the melanoma marker S100 (working dilution 1:50, DAKO), or with the anti-HDM2 mAb SMP 14 (working dilution 1:50). The samples were then incubated for 60 min with a fluorescein isothiocyanate (FITC)- conjugated rabbit-anti-mouse or with a FITC-conjugated goat-anti-rabbit secondary antibody (working dilution 1:50, DAKO) before being mounted with Fluorescent Mounting Medium (DAKO). Analysis of stained cells was performed by fluorescence microscopy (Zeiss, Jena, Germany). Double fluorescence labelling of melanoma cells was performed using serum $HDM2_{441}$ and MHC class I mAb. The serum $HDM2_{441}$ was derived from rabbits immunised with a synthesised peptide corresponding to amino acid 441-452 of HDM2 . The peptide $HDM2_{441}$ was synthesised on an ABi433A peptide synthesiser (Applied Biosystems, Foster City, CA, USA) using 2-(1H-9Azabenzotnazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluoro-phosphate as activation reagent and standard 9-Fluorenylmethoxy-carbonyl/test-Butyl protection chemistry. Conditional double coupling and conductivity monitoring with monitor-previous-peak-algorithm were applied (for work up, purification and analysis as well as immunisation see (19)). The mAb against human MHC class I (clone W6/32) was obtained from Sigma. Cells were fixed in cold formalin (4%) for 10 sec or were non-fixed. Then they were washed three times in PBS and incubated with rabbit serum (DAKO). After 10 min, the cells were incubated for 3 h with the primary anti-human MHC class I mAb (working dilution 1:50). After washing, a secondary rabbit phycoerythrin (PE)-conjugated anti-mouse antibody (working dilution 1:10, DAKO) was pipetted onto the slides. After 2 h, samples were washed and incubated with mouse IgG (working dilution 1:20, DAKO) for 20 min. Then the serum $HDM2_{441}$ diluted 1:10 in PBS was added to the samples. After 3 h, the secondary FITC-conjugated swine anti-rabbit antibody (DAKO) was applied to the samples at a working dilution of 1:50. After 2 h, slides were washed three times with PBS on a rocking platform, mounted with Fluorescent Mounting Medium (DAKO) and analysed on a LEICA TCS confocal laser scanning system (Bensheim, Germany). Cells with co-fluorescence were further tested by the x-y axis scanning modus. The specificity of the staining reactions was proved by blocking experiments. Positive staining of HDM2 was completely abolished by pre-incubation of the HDM2 serum with a 500-fold molar excess of HDM_{441} peptide for 1 h at room temperature, and positive staining of MHC I molecules by pre-incubation of melanoma cells with the blocking anti-MHC class I mAb G46-2.6 (Pharmingen, Hamburg, Germany) for 2 h at room temperature.

Epitope prediction analysis. The protein sequence of $HDM2_{441}$ was analysed for its potential binding to MHC class I molecules. The epitope prediction analysis was performed using the "SYFPEITHI" data base (http://syfpeithi.bmi-heidelberg.com). The lowest score used for binding capacity was set to 16. The following MHC class I molecules were found to be relevant for the $HDM2_{441}$ binding: HLA-A*03, HLA-B*08, HLA-B*2709 and HLA-B*5101. In this database peptides binding to HLA-C cannot be predicted.

Isolation of blood T lymphocytes and monocytes and generation of DC. Leukapheresis was obtained from venous blood of healthy volunteers (22 to 35 years old). PBMC were isolated and typed for HLA class I and II alleles (Department for Transfusion Medicine, University Hospitals Goettingen, Germany). Leukapheresis from HLA-A*03-, HLA-B*08- or HLA-B*5101-positive volunteers were centrifugated on a Ficoll-Hypaque discontinuous gradient. After isolation of PBMC, lymphocytes were separated by "rosetting"

using sheep erythrocytes and monocytes by glass adherence, as described previously (7). Flow cytometry revealed that 93±4 of lymphocytes express CD3. $5x10^7$ adherent monocytes were cultured in RPMI 1640 supplemented with 2.5% heat-inactivated autologous serum in flat-bottom plates (Hereaus, Hanau, Germany). To generate dendritic cells, the adherent PBMC fractions were cultured in RPMI 1640 medium supplemented with GM-CSF (300 U/ml), IL-4 (300 U/ml) (R&D Systems, Wiesbaden, Germany) and 2.5% autologous serum as described (28). DC expressed HLA-DR, CD40, CD54, CD86, but did not express CD83 as described previously (5). On day 6, DC were incubated with a different concentration of HDM_{441} peptide and recombinant human CD40 ligand (working protocol: 100ng/ml; Alexis, San Diego, CA, USA) for a further 24 h. On day 7, DC were yielded and characterised by flow cytometry.

Flow cytometry. $2x10^6$ cells were transferred to 96-well, round-bottom microtiter plates (Nunc, Wiesbaden, Germany), which had been pre-coated with blocking buffer (10% heat-inactivated rabbit serum and 0.1% NaN_3 in PBS). For membrane staining cells were incubated for 30 min with PE-conjugated primary antibodies. The mAb against HLA-DR (clone L243) was obtained from Leinco Technologies (St. Louis, MO, USA), the mAb against CD80 (clone BB1) from Pharmingen and the mAbs against CD3 (clone B-B11), CD14 (clone RMO52), CD83 (clone HB15A) and CD86 (clone Bu63) from Immunotech (Marseille, France). For the staining of the intracellular interleukin 12 (IL-12), a complete flow cytometry kit was obtained from R&D Systems and applied as described by the manufacturer. After washing with PBS, the cells were fixed with 2% paraformaldehyde, and subjected to flow cytometric analysis using FACStar^{plus} (Becton Dickinson, San Jose, CA, USA).

Autologous $HDM2_{441}$-supplemented mixed leukocyte culture (MLC). $1x10^6$ DC were pulsed with a synthesised $HDM2_{441-452}$ peptide for 2 h (working concentrations: 10, 100, or 1000 $HDM2_{441-452}$ ng/ml) before being incubated with $1x10^7$ autologous T lymphocytes. After 6 days, co-cultured lymphocytes were analysed for their proliferation, Th1/Th2 differentiation and cytotoxic activity.

Proliferation assay. To determine the proliferation activity of lymphocytes, 0.2 μCi [^{3}H]thymidine was added to each well of the autologous $HDM2_{441}$-supplemented MLC on day 5. After 24 h, cells were harvested by an automated Inotech cell harvester (Dunn, Ansbach, Germany) and [^{3}H]thymidine incorporation was measured by a Matrix 96 direct β counter (Hewlett-Packard, Meriden, CT, USA). Mixed cultures composed of T cells and non-pulsed autologous DC and cultures consisting solely of peripheral blood T cells served as controls. Results were given in counts per minute (cpm).

In vitro cytokine release assay. To determine the Th1/Th2 differentiation of lymphocytes, supernatants (500 μl) of day 6 autologous MLC supplemented with $HDM2_{441}$ were harvested and assessed by commercial ELISA for the production of IFN-γ, IL-5, IL-10 and IL-12 (p70) (R&D, Systems). The lower limit of sensitivity for each assay was: IFN-γ, <3 pg/ml; IL-5, <3 pg/ml; IL-10, <0.5 pg/ml; IL-12, <5 pg/ml. For controls supernatants removed from autologous T cell/non-pulsed DC mixed cultures were subjected to *in vitro* cytokine release assays. Further controls were supernatants from cultures consisting solely of peripheral blood T cells.

Cytotoxicity assay. T cells separated from autologous T cell/DC mixed cultures with or without $HDM2_{441}$ peptide, or from control mono-cultures, were co-incubated with allogeneic KaII or LIR cells, or allogeneic fibroblasts (n=3). To prove whether $HDM2_{441}$-primed T cells specifically react against tumour cells delivering $HDM2_{441}$ in the context of MHC class I molecules, in some experiments melanoma cells were pre-incubated for 1 h with an anti-MHC-I blocking antibody (working dilution: 1μg/ml, clone G46-2.6, Pharmingen) before being co-cultured with T cells (n=3), as described previously (5). After 24 h, the mixed population of melanoma cells and T cells were tested for apoptosis by TdT-mediated FITC-dUTP nick end labelling (Tunel). For control, mixed cultures of allogeneic fibroblasts and $HDM2_{441}$-primed T cells were also tested for apoptosis by Tunel (n=3).

Tunel. Cells ($1x10^6$) were incubated for 15 min with 5 ml of 1% paraformaldehyde. After centrifugation (3x5 min, 300 g), cells were re-suspended in 0.5 ml PBS and incubated with 5 ml ice-cold 70% ethanol for 30 min. Then the cell suspension was centrifuged (3x5 min, 300 g). The supernatant was then removed by aspiration and cells were re-suspended in 50.75 μl of the staining solution of the APO-DIRECT™ Kit (Pharmingen). The staining solution contained 10 μl reaction buffer, 0.75 μl TdT enzyme, 8 μl FITC-dUTP and 32 μl H_2O. After incubation for 60 min at 37°C, cells were washed with rinse buffer (2x5 min, 300 g) of the APO-DIRECT™ Kit. For control the Tunel technique was performed omitting the TdT enzyme. To determine whether apoptotic cells were target cells (melanoma cells or fibroblasts) or effector cells (T lymphocytes), the Tunel technique was combined with immune staining for CD3 as described above. Then the cells were subjected to flow cytometry. CD3-, Tunel+ cells were considered as apoptotic target cells and CD3+, Tunel+ ones as apoptotic T lymphocytes. Differences in apoptotic rates were statistically examined by two-tailed *t*-test. Results were considered significant for p values <0.05.

Results

Stimulation of T lymphocytes against $HDM2_{441}$. After stimulation with CD40 ligand and HDM_{441} peptide, DC expressed high levels of HLA-DR, CD83, CD86 and IL-12 (Figure 1). After characterisation DC were co-cultured with autologous T lymphocytes for 6 days (autologous MLC supplemented with $HDM2_{441}$). Then the proliferation rate and Th1-/Th2-differentiation of T cells stimulated in MLC were analysed.

The [^{3}H]thymidine assay showed a very high proliferation activity in T lymphocytes generated in autologous MLC supplemented with 100 or 1000 ng/ml $HDM2_{441}$ peptide. Significantly lower proliferation activity was observed in mixed cultures composed of T cells and autologous DC without $HDM2_{441}$ peptide. No or only weak [^{3}H]thymidine incorporation was measured in cultures consisting of T cells and the synthesised peptide $HDM2_{441}$ or solely of peripheral blood T cells (Figure 2A). Because the highest proliferation activity was observed in MLCs with 1000 ng/ml $HDM2_{441}$, all experiments in the next steps were carried out using this

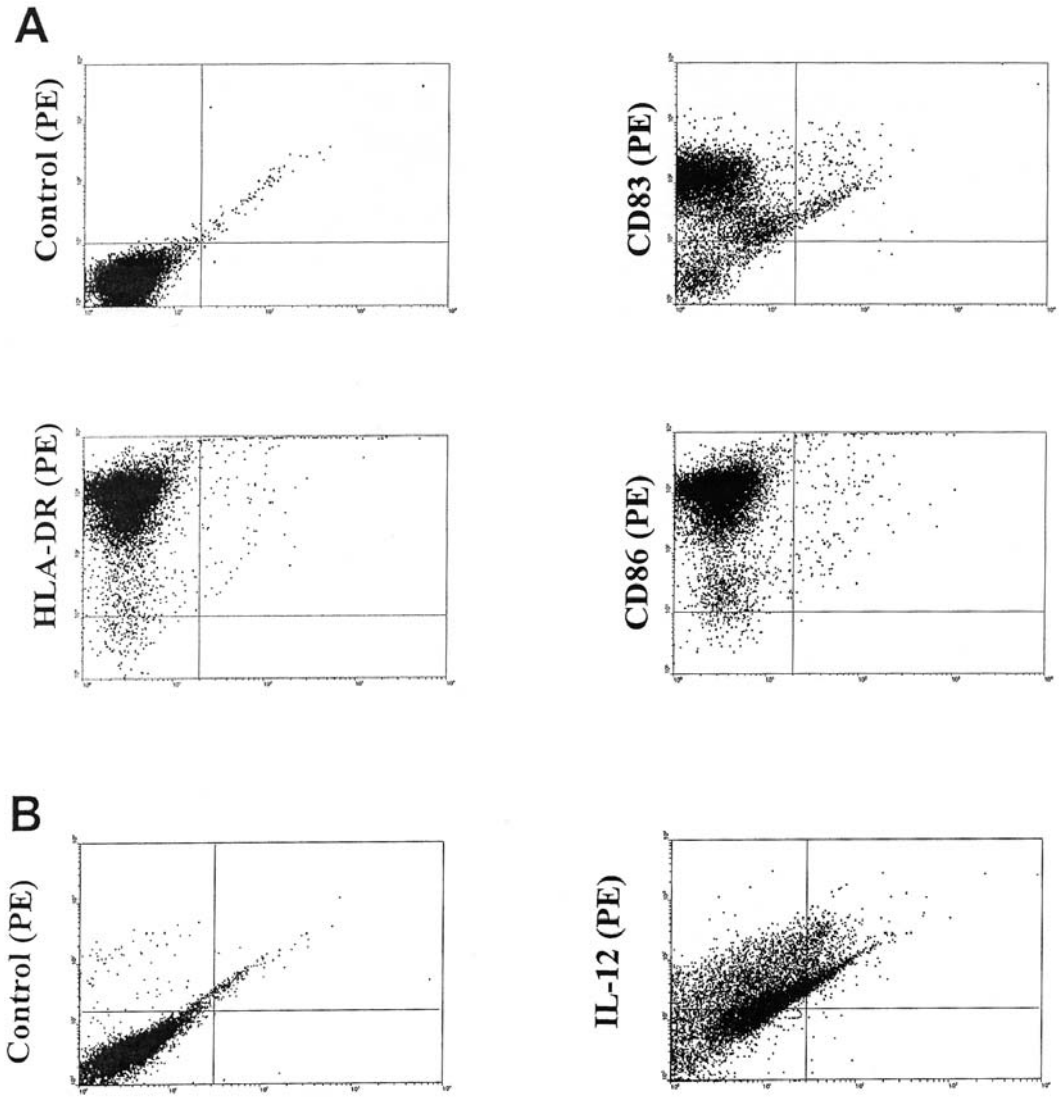

Figure 1. *Characterisation of DC. After stimulation with CD40 ligand and HMD2441 peptide DC were characterised. Flow cytometric analysis of one representative experiment shows that DC express high levels of HLA-DR, CD83, CD86 (A) as well as IL-12 (B).*

optimal antigen concentration. By *in vitro* cytokine release assay significantly higher concentrations of IFN-γ and IL-12 (p70) were noted in supernatants of autologous $HDM2_{441}$-supplemented MLC as compared to controls (Figure 2B). No significant difference was noted among concentrations of IL-5 or IL-10 in supernatants of MLC and controls (Figure 2B). Then the cytotoxic efficacy of $HDM2_{441}$-stimulated T cells against $HDM2_{441}$-positive and -negative melanoma cells was proved in a cytotoxic assay.

Analysis of melanoma cell lines for the expression of HDM2 and localisation of $HDM2_{441}$. To design the cytotoxic assay, we first we identified melanoma cell lines overexpressing (target cells) or failing to express $HDM2_{441}$ (control cells). RT-PCR showed that the melanoma cell line KaII contained the full-size transcript of HDM2 mRNA and low amounts of an aberrant HDM2 mRNA splicing transcript (Figure 3). In contrast, the cell line LIR only expressed the splicing transcript (Figure 3). Sequence analysis demonstrated that the aberrant transcript in both cell lines lacked exon 4-11 and parts of exon 12 (Figure 3). However, gene mutations were not detectable in either the full-size or in the aberrant transcripts (data not shown). Using the monoclonal antibody SMP14, we next analysed the expression of the HDM2 protein in KaII and LIR cells. This antibody recognises an epitope which corresponds to amino acids 154-167 of the

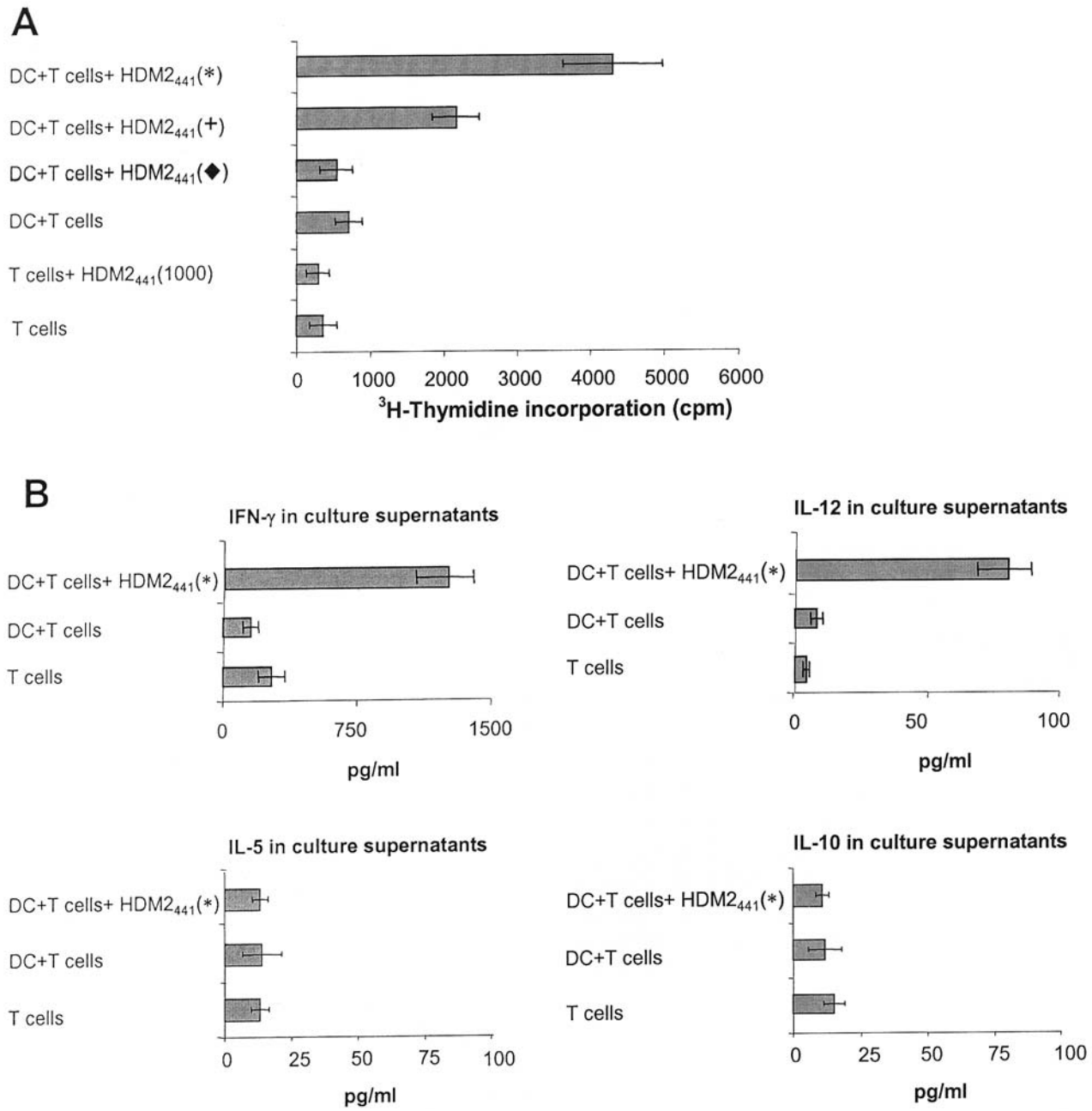

Figure 2. *Proliferation and differentiation of T cells stimulated by $HMD2_{441}$-pulsed DC. T lymphocytes were cultured with autologous DC pulsed with 1000 (*), 100 (+) or 10 (◆) ng/ml $HMD2_{441}$ peptide. Mixed cultures composed of T cells and non-pulsed autologous DC and cultures consisting solely of peripheral blood T cells served as controls. After 6 days, uptake of [^{3}H]thymidine and release of Th1-/Th2-associated cytokines were measured. The results of a representative experiment are expressed as mean + SD of three replicates. The highest proliferation activity of T lymphocytes is observed in mixed cultures consisting of 1000 ng/ml $HMD2_{441}$ peptide (A). Cytokine release assays demonstrate that in mixed cultures with 1000 ng/ml $HMD2_{441}$ peptide (*) the production and secretion of IFN-g and IL-12, but not IL-5 and IL-10, are increased when compared to controls (B).*

HDM2 protein and is encoded by a nucleotide stretch of *HDM2* exon 8. Applying immunocytochemistry, almost all KaII cells revealed moderate to strong nuclear and membranous signals for the HDM2 protein (Figure 4A) whereas only weak background signals were detectable in small numbers of LIR cells (Figure 4B). Then we proved whether HDM2 protein detected on the surface of melanoma cells bear the $HDM2_{441}$ epitope, the human

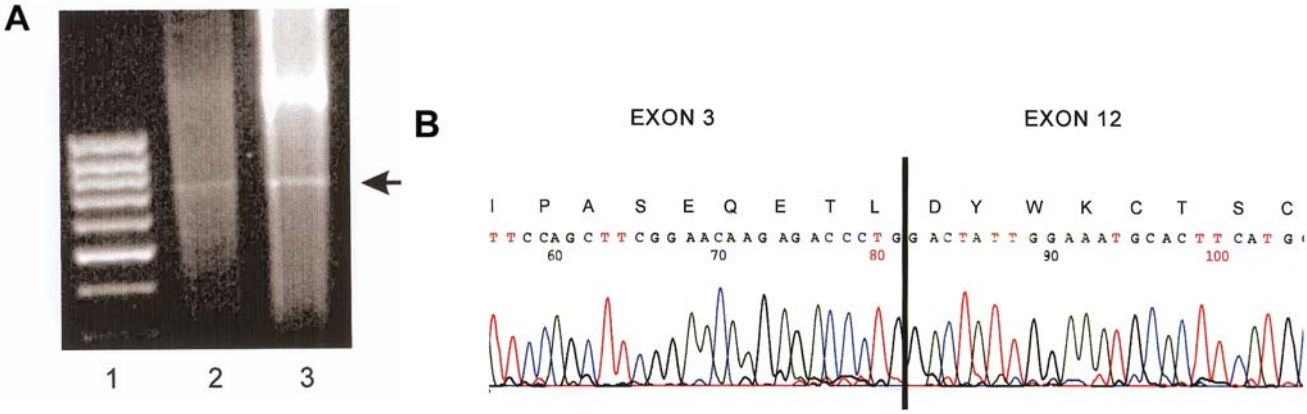

Figure 3. *Analysis of HDM2 mRNA expression by RT-PCR and sequence analysis. Part A: the expression of HDM2 mRNA was studied by RT-PCR. PCR products were separated on a agarose gel. Lane 1 shows molecular weight standard. Lanes 2 and 3 contains HDM2 cDNA of melanoma cell lines LIR and KaII, respectively. Note that only the cell line KaII (lane 3) expresses large amounts of full-length HDM2 mRNA (the diffuse and intense cDNA band above) and both cell lines contain low quantities of a splicing variant (arrow). Part B: the HDM2 PCR fragments were purified from agarose gel before sequence analysis. Results showed that the aberrant transcript in both cell lines lacked exon 4-11 and parts of exon 12. Sequence analysis of the HDM2 splicing variant of the KaII cell line is shown.*

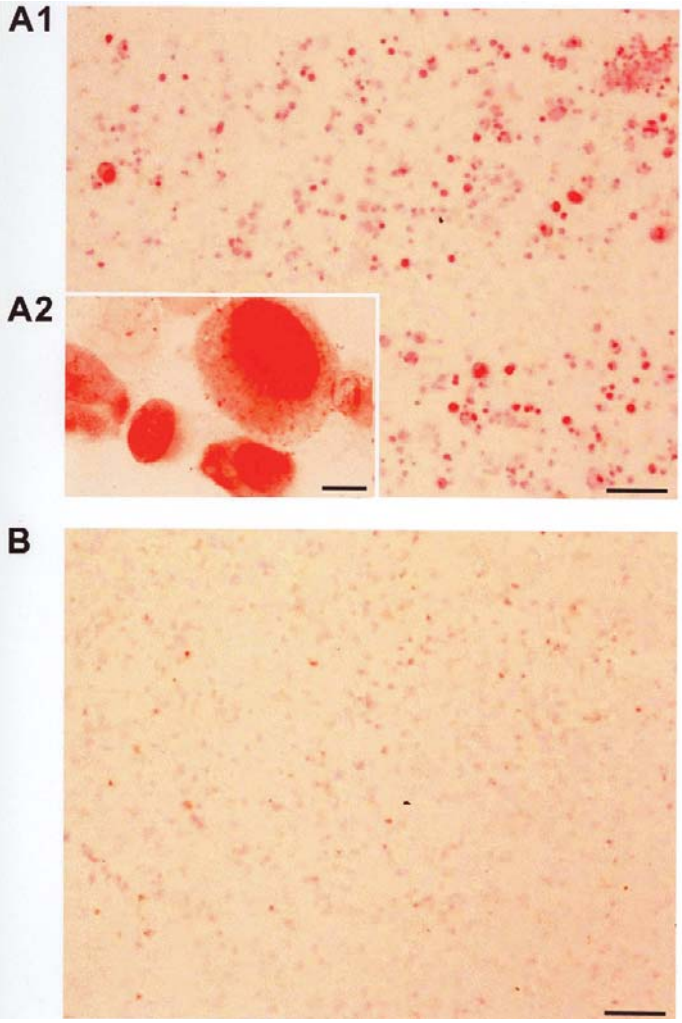

Figure 4. *Analysis of HDM2 protein expression in cytospins obtained from melanoma cell lines. The expression of HDM2 was investigated by the monoclonal Ab SMP14 recognising the epitope corresponding to amino acids 154-167 of the HDM2 protein. Part A shows the overexpression of HDM2 in KaII cells (photograph A1, bar: 25 nm). Note that the majority of KaII cells contains large amounts of HDM2 which is mainly located in the nucleus (photograph A2, bar: 5 nm). Part B demonstrates that the vast majority of LIR cells does not express HDM2. Note that only a few LIR cells show a very low expression of HDM2 (photograph B, bar: 25 nm).*

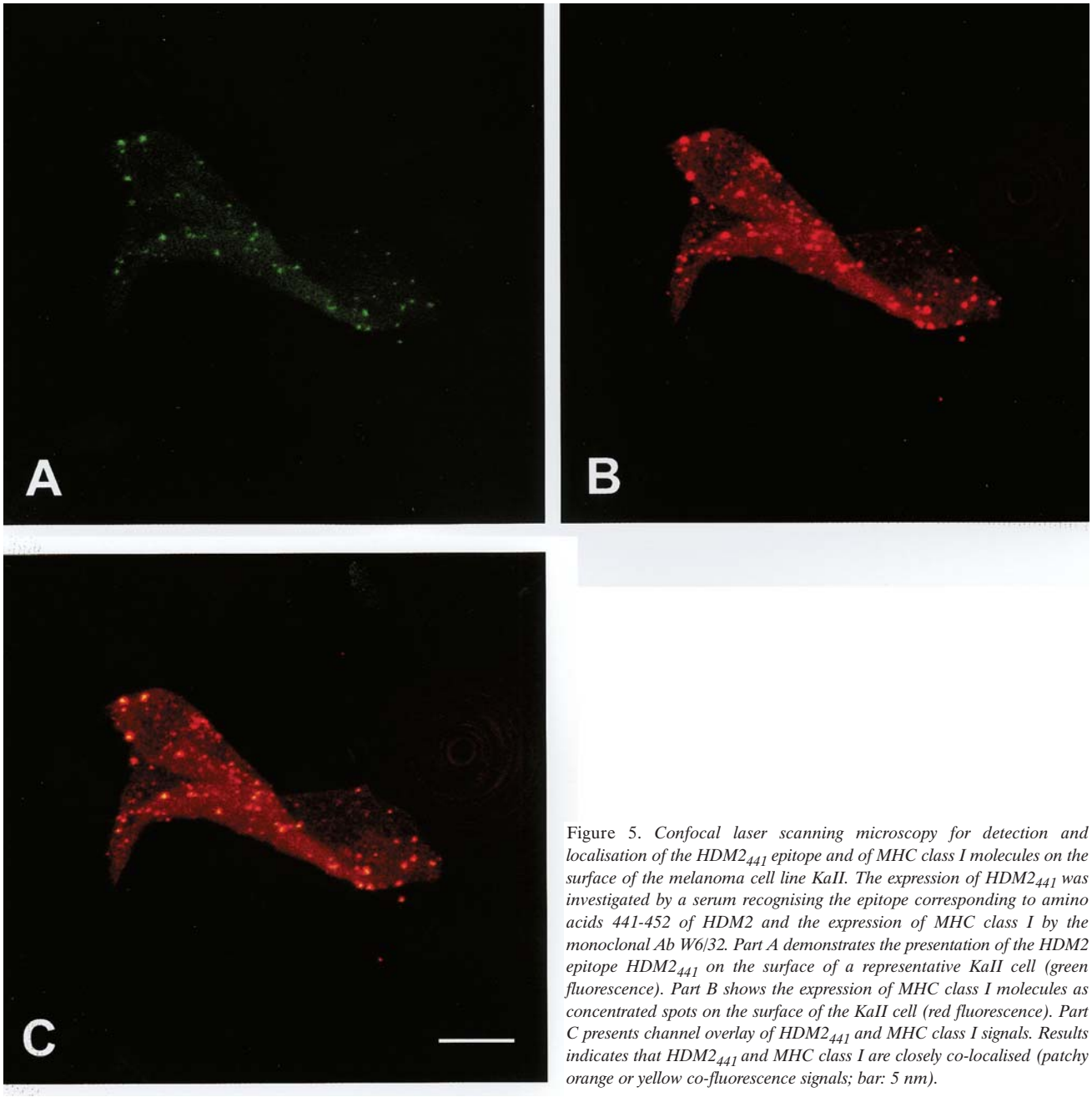

Figure 5. *Confocal laser scanning microscopy for detection and localisation of the $HDM2_{441}$ epitope and of MHC class I molecules on the surface of the melanoma cell line KaII. The expression of $HDM2_{441}$ was investigated by a serum recognising the epitope corresponding to amino acids 441-452 of HDM2 and the expression of MHC class I by the monoclonal Ab W6/32. Part A demonstrates the presentation of the HDM2 epitope $HDM2_{441}$ on the surface of a representative KaII cell (green fluorescence). Part B shows the expression of MHC class I molecules as concentrated spots on the surface of the KaII cell (red fluorescence). Part C presents channel overlay of $HDM2_{441}$ and MHC class I signals. Results indicates that $HDM2_{441}$ and MHC class I are closely co-localised (patchy orange or yellow co-fluorescence signals; bar: 5 nm).*

homologue of the MHC class I binding motif MDM2441. Results indicated $HDM2_{441}$ signals in KaII (Figure 5) but not in LIR cells (data not shown). Double fluorescence labelling and confocal laser-scanning microscopy demonstrated that $HDM2_{441}$ and MHC class I molecules were strictly co-localised on the cell surface of KaII cells (Figure 5). After characterisation, KaII and LIR cells were subjected to a cytotoxic assay.

Cytotoxic effect of $HDM2_{441}$-specific T cells. In the cytotoxic assay T cells were co-incubated with KaII or LIR cells. After 24 h, the mixed population of melanoma cells and T cells were tested for apoptosis by Tunel. Results indicated that T cells derived from MLC supplemented with 1000 ng/ml $HDM2_{441}$ possessed anti-tumour effects resulting in apoptosis of 61±9% of KaII and 36±7% of LIR cells, whereas control (unprimed) T cells separated from DC/T cell mixed cultures led to death

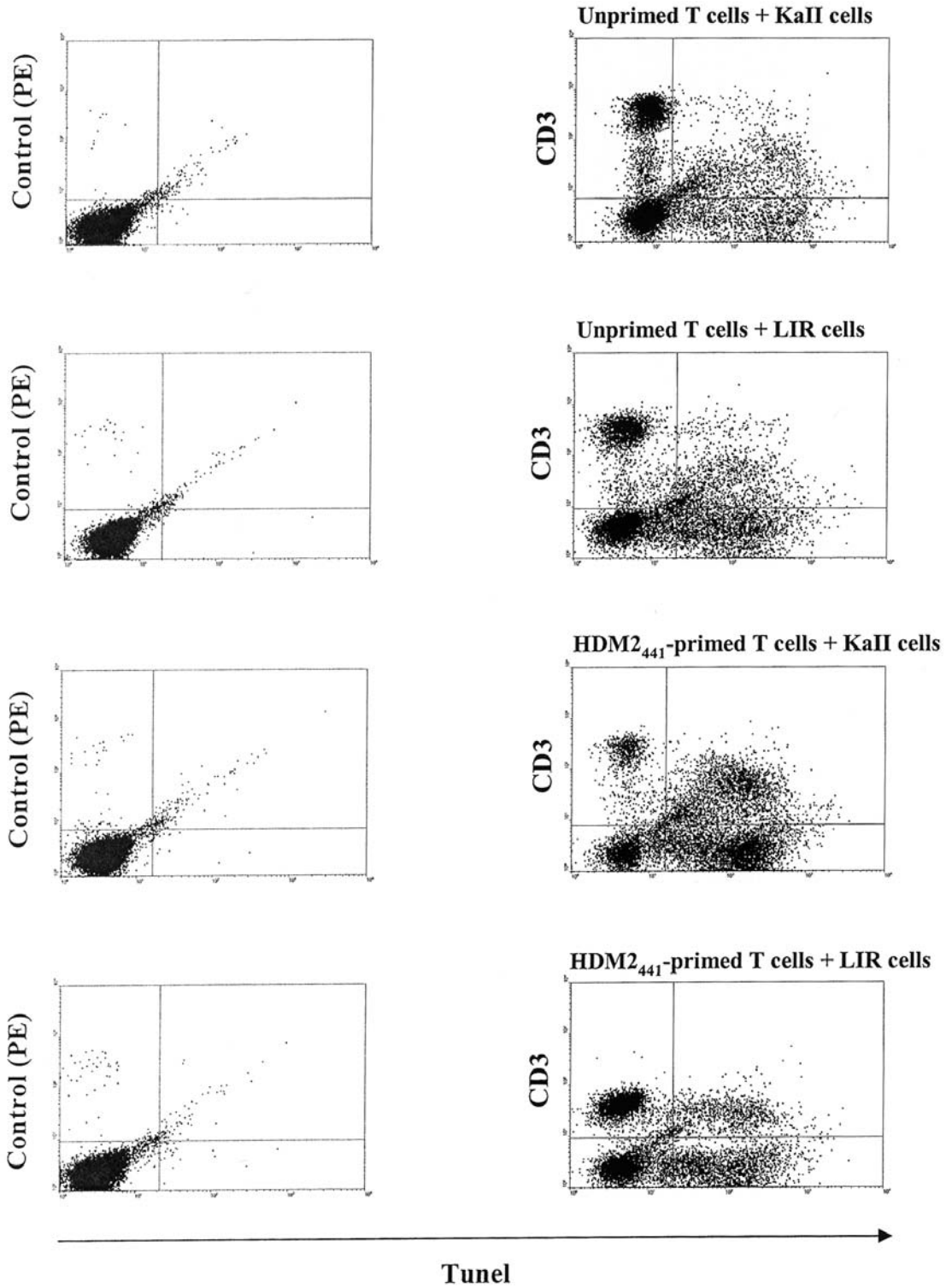

Figure 6. *Characterisation of $HDM2_{441}$-primed T-lymphocyte. T cells were cultured with autologous DC in the absence or presence of a synthesised $HDM2_{441}$ peptide. After 6 days unprimed and $HDM2_{441}$-primed T cells were co-incubated with $HDM2_{441}$-positive KaII or with $HDM2_{441}$-negative LIR melanoma cells. Twenty-four hours later, the mixed population of melanoma cells and T cells were tested for apoptosis by Tunel followed by flow cytometry. Results of a representative experiment is shown. CD3-negative, Tunel-positive cells represent apoptotic melanoma cells (lower right fields) and CD3-positive, Tunel-positive ones represent apoptotic T cells (upper right fields). For control corresponding mixed populations of tumour cells and T cells were double-stained with PE-labelled irrelevant IgG and TdT-omitted Tunel. Controls are depicted on the left and corresponding experiments on the right side.*

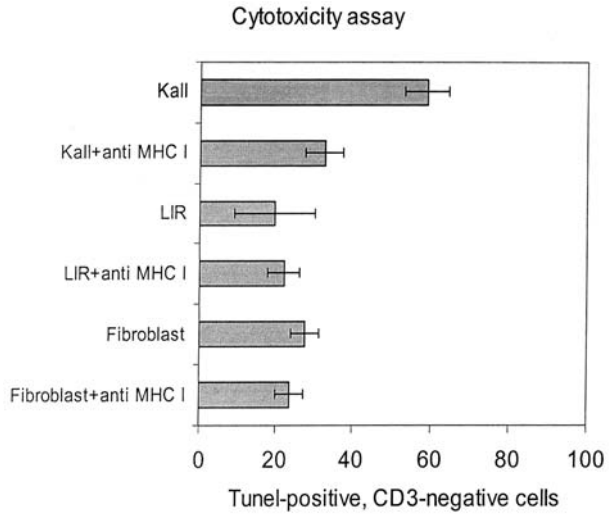

Figure 7. *Blocking of MHC class I on melanoma cells. To prove whether $HDM2_{441}$-primed T cells specifically react against tumour cells delivering $HDM2_{441}$ in the context of MHC class I molecules, KaII cells, LIR cells and allogeneic fibroblasts were pre-incubated for 1 hour with an anti-MHC-I blocking antibody before being co-cultured with $HDM2_{441}$-primed T cells (n=3). Twenty-four hours later, the mixed population of melanoma cells and T cells were tested for apoptosis by Tunel followed by flow cytometry. Results of a representative experiment is shown. CD3-negative, Tunel-positive cells were considered as apoptotic melanoma cells or apoptotic fibroblasts.*

of only17±6% KaII, or 21±5% LIR cells (Figure 6). Results also demonstrated that, following co-incubation with tumour cells, not only CD3-negative melanoma cells but also CD3-positive lymphocytes underwent apoptosis. Co-incubation with KaII cells led to apoptosis in 54±11% of $HDM2_{441}$-primed and in 11±3% of unprimed T cells, whereas co-incubation with LIR cells caused apoptosis in 15±5% of $HDM2_{441}$-primed and in 12±3% of unprimed T cells (Figure 6). To determine whether primed T cells recognise and eliminate melanoma cells presenting $HDM2_{441}$ *via* MHC class I molecules, KaII were pre-treated with a blocking anti-MHC I antibody. As shown in Figure 7, following class I blockade on KaII cells, the cytotoxic efficiency of $HDM2_{441}$-primed T cells was reduced significantly when compared to the control (36±3% versus 59±8%, $p<0.05$) (Figure 7).

Discussion

Identifying antigenic targets on human tumour cells has become an exciting field of immunotherapeutical research. Of special interest are antigens overexpressed in tumour cells, but not in normal ones. Studies on mouse tumour models demonstrated that the oncoprotein MDM2 contains two highly conserved MHC class I binding motifs, $MDM2_{100}$ and MDM_{2441} (14), the presentation of which by DC stimulates CTL against murine melanoma cells (15). Considering recent data that HDM2 is strongly overexpressed in human malignant melanomas but not in nevi (16), we questioned whether $HDM2_{441}$ might be exploited as a tumour antigen, against which a DC-based CTL reaction could be induced.

At first monocyte-derived DC were generated by virtue of IL-4 and GM-CSF as described by others (20). After loading with $HDM2_{441}$ peptide and stimulation with CD40 ligand, DC expressed not only high levels of HLA-DR, CD86 and IL-12, but also became strongly positive for CD83 (21). Functional tests showed that $HDM2_{441}$–primed DC effectively induced a T cell proliferative response, when compared to controls. Evidence from cytokine release assays indicated that in cultures with $HDM2_{441}$–primed, proliferating lymphocytes the production and secretion of Th-1-(IFN-γ and IL-12) but not Th-2-associated cytokines (IL-5 and IL-10) were increased (22). Based on these data, we speculated that peptide–pulsed DC present $HDM2_{441}$ and provide co-stimulatory signals (*e.g.* CD86) and pro-inflammatory cytokines (*e.g.* IL-12) needed for sensitisation and Th-1 differentiation of T cells against $HDM2_{441}$, as described for $MDM2_{100}$ (15).

Using a cytotoxic assay, we next proved the CTL activity of $HDM2_{441}$-primed T cells against melanoma cells as target. To design this assay we first characterised human melanoma cell lines for the expression of HDM2 and $HDM2_{441}$. RT-PCR demonstrated that KaII cells contained the full-size transcript of HDM2 mRNA and low amounts of an aberrant HDM2 mRNA splicing transcript, whereas LIR cells only expressed the splicing transcript. Sequence analysis showed that the aberrant transcript in both cell lines lacked exon 4-11 and parts of exon 12 corresponding to the HDM2 mRNA variant type b (17). Immunocytochemistry confirmed the RT-PCR data and illustrated that KaII but not LIR cells overexpressed HDM2 and $HDM2_{441}$. Since no mutation was detected in HDM2 transcripts, it could be ascertained that accumulation of HDM2 and $HDM2_{441}$ in KaII cells was not caused by abnormalities in the HDM2 protein as formerly found in different cancers (23, 24). This finding was important because it excluded the possibility that point mutations alter $HDM2_{441}$ structure with the consequence that $HDM2_{441}$-positive melanoma cells remain unrecognisable for CTL. Of importance was also the evidence of confocal scanning of double fluorescence-labelled KaII cells, that $HDM2_{441}$ and MHC class I molecules were strictly co-localised on the surface of KaII cells, because it supports the hypothesis that overexpressed $HDM2_{441}$ in KaII cells is presented in the context of MHC I molecules, as previously reported for MDM_{2441} (14). After characterisation melanoma cells were incubated with MLC-derived T cells. When co-cultured with $HDM2_{441}$-sensitised T cells 61±9% of KaII cells underwent apoptosis in comparison with 36±7% of LIR cells ($p<0.05$).

Thus, these data suggested that $HDM2_{441}$-sensitised T cells preferentially eliminated $HDM2_{441}$-overexpressing tumour cells and not $HDM2_{441}$-negative ones. Considering the result that the $HDM2_{441}$ epitope and MHC I molecules were co-localised on the surface of KaII cells, we hypothesised that primed T cells recognised KaII cells *via* $HDM2_{441}$ presented in the context of MHC I molecules. Indeed, this interpretation could be supported by the evidence that the cytotoxicity of primed T cells was significantly reduced, when MHC class I molecules were blocked on melanoma cells. Nevertheless $HDM2_{441}$-sensitised T cells seem not only to kill the target cells through HDM2 recognition but also *via* HDM2-independent mechanisms, because the degree of apoptosis of LIR cells was higher in cytotoxic experiments with allogeneic $HDM2_{441}$-sensitised T cells in comparison with controls ($p<0.05$).

Surprisingly, the results demonstrated that, besides melanoma cells, different numbers of lymphocytes also underwent apoptosis following co-culturing with target cells. Interestingly the highest apoptotic rate was observed among $HDM24_{41}$-primed T cells co-cultured with KaII cells. In this regard, one may speculate that products of tumour cells such as Fas ligand may lead to apoptosis of activated T cells through engagement of Fas, which is known to be up-regulated on activated T cells in comparison to naive ones (25). Although we did not prove whether KaII cells express Fas ligand or other cytotoxic ligands, the importance of this mechanism must be considered with caution because such ligands have been detected on only a few spontaneous tumours and, when overexpressed by gene transfection, it is not always protective for the tumour (26-29). A more attractive hypothesis may be the occurrence of activation-induced cell death (AICD) in T cells. When the T cell receptor of antigen-primed lymphocytes (*e.g.* $HDM2_{441}$-specific T cells) is engaged by antigens delivered by non-professional antigen-presenting cells (*e.g.* $HDM2_{441}$ delivery by melanoma cells), T cells are re-activated followed by apoptosis (AICD) through death factors such as tumour necrosis factor and/or CD95 ligand, engaging corresponding receptors on the same or a neighbouring cell (30, 31). This phenomenon may limit the expansion of an anti-tumour immune response by eliminating CTL.

Collectively these data suggest that $HDM2_{441}$-pulsed DC may induce a CTL reaction against melanoma cells overexpressing the oncoprotein HDM2 and therefore may be applied within the scope of a broad-spectrum DC-based immunotherapy of metastatic malignant melanomas. The data also show that, in the course of melanoma cell/lymphocyte interaction, not only tumour cells but also T cells undergo apoptosis and suggest that the T cell apoptosis may be one of the mechanisms limiting the efficacy of the DC-mediated CTL reactions against malignant melanomas.

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References

1 Lejeune FJ, Ruegg C and Leinard D: Clinical application of TNF-alpha in cancer. Curr Opin Immunol *10*: 573-580, 1998.
2 Margolin KA, Liu PY, Unger JM *et al*: Phase II trial of biochemotherapy with interferon alpha, decarbazine, cisplatin and tamoxifen in metastatic melanoma: a Southwest Oncology Group trial. J Cancer Res Clin Oncol *125*: 292-296, 1999.
3 Yang S, Darrow TL and Seigler HF: Generation of primary tumor-specific cytotoxic T lymphocytes from autologous and human lymphocyte antigen class I-matched allogeneic peripheral blood lymphocytes by B7 gene-modified melanoma cells. Cancer Res *57*: 1561-1568, 1997.
4 Rivoltini L, Squarcina P, Loftus DJ *et al*: A superagonist variant of peptide MART1/Melan A27-35 elicits anti-melanoma CD8+ T cells with enhanced functional characteristics: Implication for more effective immunotherapy. Cancer Res *59*: 301-306, 1999.
5 Soruri A, Fayyazi A, Neumann C *et al*: *Ex vivo* generation of human anti-melanoma autologous cytolytic T cells by dendritic cell/melanoma cell hybridomas. Cancer Immunol Immunother *50*: 307-314, 2001.
6 Schuler-Thurner B, Dieckmann D, Keikavoussi P *et al*: Mage-3 and influenza-matrix peptide-specific cytotoxic T cells are inducible in terminal stage HLA-A2.1+ melanoma patients by mature monocyte-derived dendritic cells. J Immunol *165*: 3492-3496, 2000.
7 Soruri A, Fayyazi A, Gieseler R *et al*: Specific autologous anti-melanoma T cell response *in vitro* using monocyte-derived dendritic cells. Immunobiology *198*: 527-538, 1998.
8 Thurner B, Haendle I, Roder C *et al*: Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med *190*: 1669-1678, 1999.
9 Henry F, Boisteau O, Bretaudeau L *et al*: Antigen-presenting cells that phagocytose apoptotic tumor-derived cells are potent tumor vaccines. Cancer Res *59*: 3329-3332, 1999.
10 Kaplan JM, Yu Q, Piraino ST *et al*: Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. J Immunol *163*: 699-707, 1999.
11 Banchereau J, Palucka AK, Dhodapkar M *et al*: Immune and clinical responses in patients with metastatic melanoma to CD34+ progenitor-derived dendritic cell vaccine. Cancer Res *61*: 6451-6458, 2001.
12 Nestle FO, Alijagic S, Gilliet M *et al*: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med *4*: 328-332, 1998.
13 Piette J, Neel H and Marechal V: Mdm2: keeping p53 under control. Oncogene *15*: 1001-1010, 1997.
14 Dahl AM, Beverley PC and Stauss HJ: A synthetic peptide derived from the tumor-associated protein mdm2 can stimulate autoreactive, high avidity cytotoxic T lymphocytes that recognize naturally processed protein. J Immunol *157*: 239-246, 1996.

15 Sadovnikova E and Stauss HJ: Peptide-specific cytotoxic T lymphocytes restricted by nonself major histocompatibility complex class I molecules: reagents for tumor immunotherapy. Proc Natl Acad Sci USA *93*: 13114-13118, 1996.
16 Polsky D, Bastian BC, Hazan C *et al*: HDM2 protein overexpression, but not gene amplification, is related to tumorigenesis of cutaneous melanoma. Cancer Res *61*: 7642-7646, 2001.
17 Sigalas I, Calvert AH, Anderson JJ, Neal DE and Lunec J: Alternatively spliced mdm2 transcripts with loss of p53 binding domain sequences: transforming ability and frequent detection in human cancer. Nat Med *2*: 912-917, 1996.
18 Cordell LJ, Falini B, Erber WN *et al*: Immunoenzymatic labeling of monoclonal antibodies using immune complex of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem *3*: 219-229, 1984.
19 Schlott T, Scharf J, Soruri A *et al*: Fragments of human oncoprotein MDM2 reveal variable distribution within and on cultivated human hepatoma cells. Biochem Biophys Res Com *283*: 956-963, 2001.
20 Sallusto F and Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med *179*: 1109-1118, 1994.
21 Zhou LJ and Tedder TF: CD14 blood monocytes can differentiate into functionally mature CD83 dendritic cells. Proc Natl Acad Sci USA *93*: 2588-2592, 1996.
22 Mosmann TR and Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today *17*: 138-146, 1996.
23 Schlott T, Reimer S, Jahns A *et al*: Point mutations and nucleotide insertions in the MDM2 zinc finger structure of human tumours. J Pathol *182*: 54-61, 1997.
24 Schlott T, Ahrens K, Ruschenburg I, Reimer S, Hartmann H and Droese M: Different gene expression of MDM2, GAGE-1,-2 and FHIT in hepatocellular carcinoma and focal nodular hyperplasia. Br J Cancer *80*: 73-78, 1999.
25 Hahne M, Rimoldi D, Schroter M *et al*: Melanoma cell expression of Fas (Apo-1/CD95) ligand: implication for tumor immune escape. Science *274*: 1363-6, 1996.
26 Chappell DB, Zaks TZ, Rosenberg SA and Restifo NP: Human melanoma cells do not express Fas (Apo-1/CD95) ligand. Cancer Res *59*: 59-62, 1999.
27 Real LM, Jimenez P, Kirkin A *et al*: Multiple mechanisms of immune evasion can coexist in melanoma tumor cell lines derived from the same patient. Cancer Immunol Immunother *49*: 621-628, 2001.
28 Shimizu M, Fontana A, Takeda Y, Yagita H, Yoshimoto T and Matsuzawa A: Induction of antitumor immunity with Fas/APO-1 ligand (CD95L)-transfected neuroblastoma neuro-2a cells. J Immunol *162*: 7350-7357, 1999.
29 Behrens CK, Igney FH, Arnold B, Moller P and Krammer PH: CD95 ligand-expressing tumors are rejected in anti-tumor TCR transgenic perforin knockout mice. J Immunol *166*: 3240-3247, 2001.
30 Abbas AK: Die and let live: eliminating dangerous lymphocytes: Cell *84*: 655-657, 1996.
31 Janssen O, Sanzenbacher R and Kabelitz D: Regulation of activation-induced cell death of mature T-lymphocyte populations. Cell Tissue Res *301*: 85-99, 2000.

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