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ORIGINAL ARTICLE

HAGE, a cancer/testis antigen with potential for melanoma immunotherapy: identification of several MHC class I/II HAGE-derived immunogenic peptides

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Abstract There remains a need to identify novel epitopes of potential tumour target antigens for use in immunotherapy of cancer. Here, several melanoma tissues and cell lines but not normal tissues were found to overexpress the cancer-testis antigen HAGE at the mRNA and protein level. We identified a HAGE-derived 15-mer peptide containing a shorter predicted MHC class I-binding sequence within a class II-binding sequence. However, only the longer peptide was found to be both endogenously processed and immunogenic for T cells in transgenic mice in vivo, as well as for human T cells in vitro. A different class I-binding peptide, not contained within a longer class II sequence, was subsequently found to be both immunogenic and endogenously processed in transgenic mice, as was a second class II epitope. These novel HAGE-derived epitopes may contribute to the range of immunotherapeutic targets for use in cancer vaccination programs.

Keywords Immunotherapy · Melanoma · HAGE

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Introduction

Since van der Bruggen et al. [19] first identified specific human tumour antigens of the MAGE family, numerous potential immunotherapeutic targets have been discovered, often belonging to the so-called cancer-testis (CT) gene family [18]. Although CT genes are naturally expressed in some normal tissues including testis and placenta, these tissues lack MHC expression and cannot present self antigen to T lymphocytes to elicit an immune response or induce tolerance. However, because these genes are expressed in certain tumours, they can become targets for host immunity and immunotherapy, providing tolerance induction can be avoided. Their exploitation in experimental cancer treatment commonly involves therapeutic vaccination with synthetic peptides together with an appropriate adjuvant. The advantages of using peptides as defined specific immunogens include ease of manufacture and modification to increase affinity or otherwise improve potency and their use for monitoring patient immune responses ex vivo. Peptides containing HLA class II as well as class I-binding motifs may represent optimal immunogens because they target both helper and cytotoxic cells and may also be less prone

to inducing tolerance by inappropriate presentation on non-professional APC. Because of the high frequency of HLA-A*0201 and HLA-DR4 in Caucasians, antigen discovery programs often focus first on identifying peptides which are immunogenic on this background.

In the present study, we have sought to define novel target epitopes within the HAGE molecule. HAGE is a CT antigen, first identified in a human sarcoma [9]. The gene encodes a protein of 648 amino acids, a member of the DEAD-box family of ATP-dependent RNA-helicases. These family members are mainly characterised by the presence of eight conserved motifs including an RNA interaction domain and an ATP hydrolysis motif containing the core amino acid sequence D-E-A-D (Asp-Glu-Ala-Asp). RNA helicases are involved in various steps of RNA metabolism including transcription, pre-mRNA splicing, ribosome biogenesis, cytoplasmic transport, translation, initiation/elongation and mRNA decay [3]. Involvement of these RNA helicases in cancer development has already been reported. Indeed, DDX5 (p68), which shows 55% homology with HAGE and is implicated in growth regulation by acting as a transcriptional co-activator of oestrogen-receptor α ([20], can be associated with abnormal cell proliferation once phosphorylated [21]. These previous findings emphasize the eventual involvement of HAGE in some cancers, suggesting an advantage of targeting this product for immunotherapy, namely that tumours may be unable to escape an adaptive immune response by downregulating the target molecule. HAGE over-expression was reported in different types of cancer (e.g. breast, lung, prostate or colon) and at various frequencies (5, 32, 22 or 31%, respectively). It was also found to be present in 17% of melanoma [18]. Although a number of CT antigens have already been identified in solid tumours such as these and used extensively in clinical trials, there is still a requirement to identify attractive novel targets for immunotherapy in this group of malignancies [14].

Here, we have assessed the mRNA level of HAGE in a panel of melanoma cell lines and found that the cell line ESTDAB-27 expressed the highest amount of HAGE mRNA whereas ESTDAB-07 expression was negative. These results were later found to correlate at the protein level using immunofluorescence microscopy. Using a combination of in silico prediction of binding sequences and proteasome digestion, we first identified a suitable sequence of 15 amino-acid with the potential of stimulating both a class I and class II responses. However, although the MHC class I peptide identified within the class II sequence was found to be immunogenic in HLA-A2 HHDII mice in vivo and in Peripheral blood mononuclear cell (PBMC) from healthy donors in vitro, it was not found to be naturally processed by tumour cells. Nonetheless, the longer HAGE-derived class II peptide (HAGE 506-II) was immunogenic and found to be endogenously processed using standard proliferation assays and cytokine secretion analysis after peptide/DNA immunisation in transgenic mice. Multimer staining and IFNγ ELISpot analysis on PBMC from healthy donors confirmed those results. Moreover, several other peptides were similarly tested using our transgenic mice. One class I peptide and one more class II peptide were found to be endogenously processed and are now currently being investigated for their ability to stimulate T cells using PBMC from healthy donors.

HAGE may thus prove to be a valuable source of tumour-associated derived peptides against which immune responses can be generated. We therefore strongly believe that the combined use of computer-assisted epitope prediction and transgenic mouse screening represent powerful complementary tools for the identification of endogenously processed immunogenic tumour-associated peptides. Tumour mouse models are also currently being generated in order to assess vaccination strategies against HAGE-expressing tumours.

Materials and methods

Peptide prediction and synthesis

The sequence of the putative HAGE protein was taken from the ExPASy database (Acc. No. Q9NXZ2) and screened for predicted HLA-binding peptides using the SYFPEITHI (http://www.syfpeithi.de) and BIMAS (http://www.bimas.cit.nih.gov/) algorithms. Potential processing by the proteasome was screened in silico using the PAProC algorithm (http://www.paproc.de). The selected peptides were then synthesised by Alta Bioscience, Birmingham, UK, using standard solid-phase synthesis, and were deemed >95% pure by HPLC and MS analysis. All peptides were dissolved in 100% DMSO at a concentration of 10 mg/ml and stored at −20°C.

Animals and cell lines

Mouse class I (H-2) knockout HLA-A2.1 transgenic C57BL/6 mice (HHDII) were used, as described by Firat et al. [6]. Mouse class II (I-A^b) knockout HLA-DR4 transgenic C57BL/6 mice were purchased from Taconic. Colonies were bred at Nottingham Trent University animal house in accordance with the UK Home Office Codes of Practice for the housing and care of animals. RMAS-A2 cells, which are transgenic for the HLA-A2.1/Kb class I molecule present in the HHDII transgenic mice, were cultured with RPMI1640 supplemented with 10% FCS and 5 mM L-glutamine (BioWhittaker).

Immunisation procedure

HHDII mice were immunised by an injection at the base of the tail with the peptides (100 μg of class I peptide and 140 μg Hep B as a Class II helper peptide) emulsified in Incomplete Freunds Adjuvant (IFA) (Sigma-Aldrich) at a ratio of 1:1. HLA-DR4-transgenic mice were immunised twice, with a 1 week interval, with 100 μg of class II peptide emulsified in IFA at a ratio of 1:1. All vaccines were delivered as a 100 μl intradermal bolus inoculum at the base of the tail. For testing endogenous processing, a pcDNA3 expression vector encoding HAGE (a kind gift from Professor T. Boon, Ludwig Institute, Brussels) was coated with gold and used to immunise transgenic mice using gene gun technology. Each HHD or C57BL/6-DR4 mouse was immunised with one bullet containing 1 μg of DNA using a Helios gene gun (BioRad). Three rounds of immunisation with the same bullets were undertaken at 7-day intervals.

In vitro generation of CTLs and Cr^{51} cytotoxicity assay from HLA-A2 transgenic mice

Spleens were harvested from naïve animals 2–3 days before spleens of immunised animals were taken. Splenocytes were isolated as previously described [15]. They were washed and placed into a T75 flask at 1.5×10^6 cells/ml with 25 μg/ml of lipopolysaccharide (LPS), 7 μg/ml of dextran sulphate and 15 μg/ml Vitamin E (Sigma-Aldrich) before incubation at 37°C, 5% CO_2. On the day spleens were harvested from immunised animals, these cultured cells were harvested and irradiated with 5,000 rads for 8 min using a caesium gamma source. They were then washed and suspended at a concentration of 20×10^6 cells/ml with 10 μg/ml of peptide for 60 min, at 37°C, 5% CO_2. Cell suspensions were divided into two aliquots, and set up with either the peptide used in immunisation or with a control immunogenic peptide. Following incubation, cells were washed and plated at 5×10^5 cells/well with splenocytes from immunised animals.

Spleens from immunised animals were harvested 7 days after immunisation. Splenocytes were flushed from the spleen as described above, washed and resuspended at a concentration of 2.5×10^6 cells/well with peptide-pulsed splenocytes in a 24-well plate giving a final volume of 1 ml/well. These were cultured for 5–6 days at 37°C, 5% CO_2. To measure cytokine production, culture supernatants were collected on day 3 and 5, and stored at −20°C until analysed by ELISA. On day 5 of in vitro restimulation, splenocytes were harvested, counted and used as effector cells. A standard 4-h chromium release assay was performed to determine percentages of specific cytotoxicity using peptide-pulsed RMAS-A2 cells labelled with chromium-51 (Cr^{51}) (Amersham Biosciences) as targets.

BM-DC preparation for antigen presentation

BM-DC were generated as previously described [15]. On day 7 of culture, they were replated in 24-well plates at a density of 5×10^5 cells/well/ml in the presence of 1 μg/ml of LPS to induce maturation, and pulsed with 10 μg/ml of peptide. On day 8, mature BM-DC were further pulsed with 10 μg/ml of peptide for 4–6 h at 37°C. Cells were washed twice and used as antigen presenting cells (APC) at a concentration of 5×10^3 cells per well in proliferation assays and at 5×10^4 cells/well in 48-well plates for cytokine measurements.

In vitro peptide restimulation of splenocytes

Spleens from immunised animals were collected and processed as described above. Splenocytes were plated in 24-well plates with 10 μg/ml of peptide at a density of 3.5×10^6 per ml for C57BL/6-DR4. After a week in incubation, 60 IU/ml of IL-2 (Biosource) was added to the cells. For cytokine measurements, CD8-depleted splenocytes were co-cultured in 48-well plates at 5×10^5 cells/well with 5×10^4 BM-DC/well. Supernatants were collected after 72 h of culture. Anti-HLA-DR antibody L243 (purified from culture supernatant, ATCC: HB55) and its isotype-matched antibody (mouse IgG2a, azide-free, BD Biosciences) were added at a final concentration of 2 μg/ml.

Murine proliferation assays

Splenocytes from peptide-immunised mice were depleted of CD8+ T cells after 6 days of in vitro culture using mouse CD8 dynabeads (Dynal) according to the manufacturer's protocol. Preparations were shown to be typically 98%-free of CD8+ T cells by flow cytometry. CD8-depleted splenocytes were used as responder cells in proliferation assays performed at a density of 5×10^4 cells/well. Cells were co-cultured with 5×10^3 BM-DC either pulsed with the specific or an immunogenic HLA-DR-restricted control peptide in quadruplicates in round-bottom 96-well plates. Blocking of the response with 2 μg/ml of the anti-HLA-DR antibody L243 was systematically performed. An isotype-matched control antibody for L243 clone (mouse IgG2a) was used at the same concentration. Cultures were incubated for approximately 60 h at 37°C, and [^{3}H]-thymidine was added at 37 kBq/well for the last 18 h. Plates were harvested onto 96 Uni/Filter plates (Packard Instrument), scintillation liquid (Microscint 0, Packard) was added and the plates were counted in a Top-Count counter (Packard). Results are expressed as counts per minute (cpm) as means

of quadruplicates. Statistical analysis was performed using unpaired Student's *t*-test.

ELISA

Samples of supernatant were collected on day 2 and 5 of the murine cell cultures and stored at −20°C until required. Cytokines analysis using ELISA kits (R&D) was carried out according to the manufacturer's protocols.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated as previously described. PBMC and cell lines were typed for HLA class I and II alleles. Standard molecular methods were used to establish the HLA-A and B types, as well as HLA-DR and DQ.

Human DC generation and in vitro sensitisation

Monocyte-derived DC were generated as previously described [7], except DC were matured with 1 μg/ml soluble rCD40L (PeproTech) and 10 ng/TNFα (BD Biosciences). Mature DC were pulsed with 10 μg/ml of peptide for 4 h at 37°C, followed by a wash step. 1×10^5 DC were co-cultured with 2×10^6 autologous PBMC in 2 ml serum-free ex vivo 15 medium (BioWhittaker), in 24-well cluster plates (Greiner), for 1 week. On day 7, frozen autologous DC were thawed and pulsed (as previous) for 4 h, followed by washing. 1×10^5 DC were added per well in 1 ml of fresh medium. Following 24 h co-culture, 60 IU/ml of IL-2 (Chiron GmbH) was added per ml medium. Medium was exchanged and fresh IL-2 added every third day. On day 14, cells were restimulated with 5×10^5 peptide-pulsed (as previous), irradiated (30 Gy) autologous PBMC and IL-2 added. T cell lines were tested for their specificity on day 21. T cell lines were maintained by restimulation with autologous PBMC every 7–10 days. For the generation of the HAGE-specific CD8+ T cell clones, CD8 T cells were first isolated from whole PBMC using CD8 positive selection with MACS magnetic beads (Miltenyi Biotec) and 2×10^6 CD8+ T cells were co-cultured with 2×10^5 peptide-pulsed monocyte-derived mature DC. Restimulation was carried out as in the same way. Peptide specific CD3+ CD8+ cells, as determined by the CD107a assay (below) were single-cell sorted into 96-well cell culture plates using a BD FACSAria (BD Biosciences) at the Institute for Biomedical Engineering, ETH & Uni Zurich. 2×10^5 allogeneic pooled-feeder cells, 150 IU/ml IL-2 (R&D systems) and 1 μg/ml PHA (HA16, Sigma) were added and eventual expanding colonies picked and maintained in 150IU/ml IL-2 containing medium.

IFNγ ELISpot assay

The test was performed using MAHA S4510 filter plates (Millipore GmbH) and human IFNγ development module (R&D systems) according to the manufacturer's protocol. The number of IFNγ spot-forming cells was measured by an automated ELISpot reader (Aelvis GmbH) and analysed using AELVISV3.3 software. Spots measured in the presence of APC pulsed with the control peptide were considered as non-specific background (negative control). Samples were considered to contain peptide-specific T cells when the mean number of spots in test triplicates exceeded control triplicates at least twofold. Statistical analysis was performed using unpaired Student's *t*-test.

RT-PCR and real time PCR

RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc.). Reverse transcription using the SuperScript II reverse transcriptase kit (Gibco-BRL) was performed with a mixture of oligo-dT and random hexamers according to the manufacturer's protocol. For conventional RT-PCR amplification was carried out using primers specific for HAGE cDNA according to GenBank accession number NM_018665 (HAGE-897 5′-CCTTTCAATGTTATCCTGAG-3′, HAGE-1331 5′-TTATATTCTTCAGATTGACG-3′; product size 435). The PCR amplification was carried out for 32 cycles of 94, 60 and 72°C, using a hot start procedure. Real-time PCR was carried out using primers specific for HAGE cDNA according to GenBank accession number NM_018665 (HAGE-496 5′-GGAGATCGGCCATTGATAGA-3′, HAGE-716 5′-GGATTGGGGATAGGTCGTTT-3′; product size 221 bp), for 45 cycles of 95, 66 and 72°C, using a Sybr Green Supermix (BioRad) and a hot start procedure.

Immunofluorescence

To date, HAGE has been described as a putative protein due to the fact that this antigen has only been detected at the message level. As no commercial antibodies are available for the HAGE gene product, an anti-serum, against HAGE peptides, was generated in rabbits. ESTDAB-07 and -27 cells were plated in an eight-chamber slide (BD Biosciences) at a concentration of 1×10^4 cells/well. After overnight incubation at 37°C, 5% CO_2, cells were fixed and permeabilised at room temperature using a solution of 1% (w/v) paraformaldehyde and 70% (v/v) ethanol, respectively. Cells were washed and then stained on ice with 10 mg/ml HAGE-specific rabbit polyclonal anti-serum and 10 μg/ml fluorescein-conjugated mouse anti-rabbit Ig (BD Biosciences). After multiple washes in PBS (0.1% (w/v) BSA, 0.02% (w/v) NaN3), the slide was left to dry at room

temperature and prepared with fluorescent mounting media (DakoCytomation). Cells were observed under confocal microscopy using a Leica TCS-NT microscope and captured using Leica TCS-NT software.

Results

Analysis of HAGE expression

Taking advantage of the availability of the unique ESTDAB collection of >120 immunologically characterised melanoma cell lines (www.ebi.ac.uk/ipd/estdab/index.html); we were able to analyse 72 different melanomas for the expression of HAGE using RT-PCR. We found that 23 of 72 melanoma lines (32%) (Fig. 1a shows the expression of some of these as examples) expressed HAGE mRNA, where beta-actin was used as an internal control (data not shown). Quantitative real-time PCR was used to confirm the expression of HAGE in ESTDAB-17, -27, -34 and -95, and absence of expression in ESTDAB-07 (as an example of negative HAGE expression)(Fig. 1b). HAGE was also shown to be expressed in five out of ten primary melanomas. Moreover, expression of HAGE in two of these five samples was found to be almost two times the expression of HAGE in testis (Fig. 1c). A polyclonal antiserum raised against HAGE-specific polypeptides was used on fixed and permeabilised ESTDAB-07, -17, -27, -34 and -95 cells (Fig. 2b–f, respectively). Secondary antibody tagged with fluorescein highlights the expression and localisation of HAGE within the cells. High levels of protein could be detected in ESTDAB-17, -27, -34 and -95 (Fig. 2c–f, respectively) but none in ESTDAB-07 cells (Fig. 2b) and none in the secondary alone (Fig. 2a). It can also be noticed that the expression of HAGE was shown to be cytoplasmic. These data along with the fact that no real time RT-PCR HAGE expression could be detected in normal tissues (data not shown) demonstrate that HAGE is a suitable target for immunotherapy and that the search for immunogenic peptides is a valid follow on step.

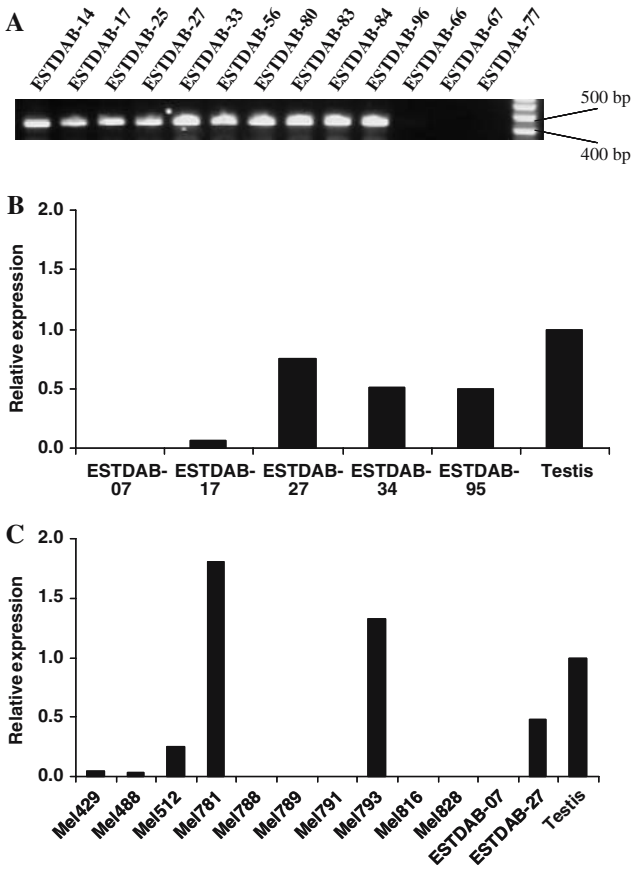

Fig. 1 Analysis of HAGE expression. **a** RT-PCR analysis of the expression of HAGE in ESTDAB melanoma cell lines using water as negative and testis as positive controls. PCR products for 18S ribosome and HAGE should be of 110 and 432 bp, respectively. Relative expression of HAGE obtained by real time RT-PCR analysis of **b** five tumour cell lines and **c** ten primary tumours from melanoma patients. The data are expressed relative to the mRNA level in normal testis, arbitrarily set as 1. The value of each sample was determined in triplicate reactions

Selection of peptides based on multiple-parameter algorithm analysis

The putative protein sequence for the HAGE gene product was screened using the SYFPEITHI algorithm (www.syfpeithi.com) for potential promiscuous class II-binding peptides that also contain HLA-A*0201 binding motifs. Moreover, screening for prediction of potential epitopes was further restricted to those where the class I motif would have the C-terminus of the peptide correctly cleaved by the human 20S proteasome, as predicted using the PaProC (www.paproc.de) algorithm (the human proteasome type III model). The 15-mer HAGE position 506–520 (termed HAGE 506-II throughout) containing the predicted HLA-A2 9-mer, position 506–514 (HAGE 506-I), met these conditions. Accordingly, synthetic peptides corresponding to this sequence as well as those scoring high using the SYFPEITHI algorithm, were obtained (Table 1) and tested for their immunogenicity.

HAGE 506-I peptide is immunogenic but not processed

The immunogenicity of all the class I HAGE derived peptides listed in Table 1, included the short HAGE-derived peptide 506-I, predicted to bind to HLA-A2, were first tested in HHDII transgenic mice. Following peptide immunisation and in vitro irradiated peptide-pulsed LPS blasts restimulation, several peptides were proved to be immunogenic by inducing a peptide-specific cytotoxic response against peptide-pulsed RMAS/A2 and by promoting peptide-specific IFNγ secretion (Fig. 3a, Table 1).

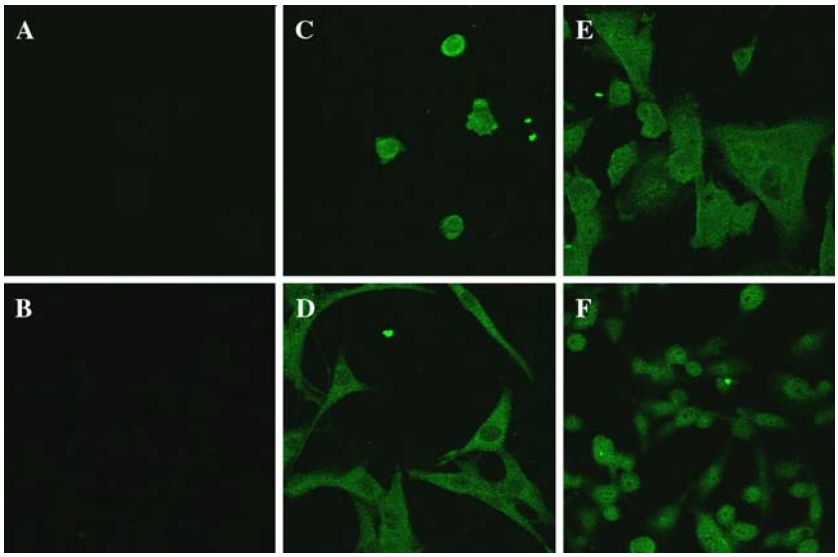

Fig. 2 HAGE protein expression in ESTDAB melanoma cell lines. Cells were grown onto chamber slides until 70% confluent was reached. Cells were then fixed and permeabilised and HAGE protein expression was assessed after a two step antibody staining. Immunofluorescence was observed under a confocal microscope (×40 magnification) in HAGE-positive ESTDAB-17 (**c**), -27 (**d**), -34 (**e**) and -95 (**f**) but not in HAGE-negative ESTDAB-07 (**b**). Cytoplasmic HAGE protein localisation can be observed. No non-specific secondary antibody staining was observed (**a**)

Table 1 Immunogenicity and endogenous process of HAGE-derived class I and class II peptides in HHDII and HLA-DR4 transgenic mice

Name	Sequence	Syfpeithi (A2 or DR4)	Murine immunisation (IFNγ)	
			Peptide	DNA
HAGE 103	IIQEQPESL	24	2/3	0/6
HAGE 126	AVIDNFVKKL	24	1/2	3/6
HAGE 296	YLMPGFIHLV	30	4/4	0/6
HAGE 506-I	DLILGNISV	25	1/2	0/6
HAGE 109	ESLVKIFGSKAMQTK	26	4/4	0/4
HAGE 195	KKNFYKESTATSAMS	28	3/4	0/4
HAGE 338	KYSYKGLRSVCVYGG	28	3/4	2/4
HAGE 506-II	DLILGNISVESLHGD	20	3/4	4/4

In vitro assays using HLA-A*0201-positive PBMC from healthy donors stimulated with autologous peptide-pulsed mature DC did indeed confirmed HAGE 506-I as an immunogenic peptide (Fig. 4). Polyclonal T cell lines derived from 5/5 healthy donor PBMC generated with the HAGE 506-I peptide showed peptide-specific IFNγ release and T cell degranulation (Data not shown).

In order to assess whether any of these peptides were endogenously processed and presented in vivo, gene gun immunisation of the HHDII mice using DNA-coated gold particles coding for the full cDNA of HAGE and in vitro irradiated peptide-pulsed LPS blasts restimulation were performed. The same experiment was conducted using DNA coding for a mutant p53 protein (273His) as a positive control, because this peptide is known to be naturally processed [11, 12]. Results presented in Fig. 3 confirm that the p53 DNA immunisation resulted in processing and presentation of the p53 epitope, as shown by the specific killing of peptide-pulsed RMAS/A2. No killing could be observed after DNA vaccination with HAGE against HAGE 506-I peptide, suggesting that this peptide is not naturally endogenously processed, at least not in mice. On the other hand peptide specific killing was observed against HAGE 126 peptide suggesting that this peptide is naturally endogenously processed (Fig. 3b, Table 1).

It was however necessary to evaluate whether the HAGE 506-I peptide was produced by human melanoma cells. For that purpose, HAGE 506-I-specific CD8+ T cell clones were co-cultured with HLA-A*0201-positive melanoma cell lines, ESTDAB-07 and -27. No specific recognition of ESTDAB-27 was observed, although this cell line was killed when pulsed with the class I peptide by the clone (Fig. 4). Incubation of the tumour cell lines with 100 IU/ml IFNγ for >5 days in order to induce the immunoproteasome also failed to result in any specific recognition (data not shown). Therefore, these data demonstrate that although the 506-I HAGE-derived peptide is immunogenic it is not endogenously processed for presentation to CD8+ T cells. Further work is being carried out in order to find out whether the same is true for peptide HAGE 126.

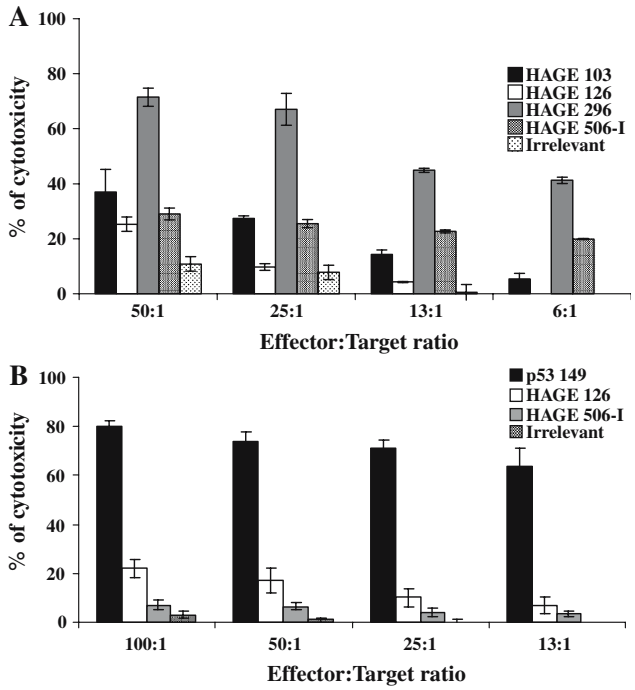

Fig. 3 Immunogenicity and endogenous processing of class I HAGE derived peptides after peptides or DNA immunisation of HHDII mice and in vitro re-stimulation. Cytolytic properties of CTLs generated after immunisation of HHDII mice with class I HAGE derived peptides in combination with the HepB class II peptide in IFA are shown in panel **a**. Endogenous processing of any of these peptides was assessed after immunisation of HHDII with full length cDNA of either p53 as a control or HAGE, and one in vitro re-stimulation with p53 149 peptide or HAGE 506-I peptide, respectively, panel **b**. Target cells used were T2 pulsed with either p53 149, HAGE 506 class I (specific) or PAP135 (non-specific). The results presented here are the average of cytotoxicity obtained from six different mice for each peptide

HAGE 506-II is both immunogenic and endogenously processed

HLA-DR4 transgenic mice were immunised twice, at 1 week intervals, with selected class II HAGE peptides (Table 1) emulsified in IFA as per written in Sect. 2.3. Thereafter, splenocytes were stimulated once in vitro with the same peptide and the specific proliferation of CD8-depleted T cells was measured 7 days later. BM-DC pulsed with either specific HAGE peptides or control MHC class II-restricted influenza HA (flu) peptides were incubated with these CD4+ T cells for 3 days. These T cells were found to specifically proliferate when co-cultured with BM-DC pulsed with HAGE class II peptides but not with flu (irrelevant) peptide (Fig. 5a). The proliferation was shown to be DR restricted since no proliferation was observed when the L243 antibody was added to the culture. Moreover, peptide-specific IFNγ but not IL-5 secretion was observed. Table 1 summarises the results obtained.

Results found with the HAGE 506-II peptide also correlated with those found in in vitro assays following priming of PBMC from healthy donors with peptide-pulsed mature DC and two rounds of in vitro stimulation; T cell lines were assessed for peptide specificity by assaying IFNγ and GM-CSF secretion in culture supernatants taken 24 h following co-culture of stimulated T-cells with autologous PBMC pulsed with either the HAGE 506-II peptide or a control, DR4-binding peptide. This peptide was found to be immunogenic in 5/5 healthy donors as assessed by the specific IFNγ production (Table 2).

The natural processing and presentation of the HAGE class II peptides was then investigated by immunising HLA transgenic mice with HAGE cDNA using the gene gun methodology. After three rounds of DNA immunisation, spleens of immunised animals were harvested and splenocytes were stimulated in vitro with class II peptides. After a week of stimulation and a week of rest in the presence of murine IL-2, proliferation assays were performed with peptide-pulsed BM-DC. HAGE class II peptides were able to induce specific CD4+ T cell proliferation in all six mice tested (Fig. 5b). Peptide-specific IFNγ but not IL-5 production was observed, suggesting a Th1-type response (Fig. 5b, Table 1). Moreover, after three rounds of DNA immunisation with the empty expression vector and in vitro re-stimulation of splenocytes of immunised animals with class II peptides, no proliferation was observed in both mice tested (data not shown). These results indicate that unlike the class I epitope it contains, HAGE 506-II epitope as well as the HAGE 338 are naturally processed and presented, at least in mice.

In order to find out whether the HAGE 506-II peptide was also presented by HAGE-positive human tumour cells, HAGE 506-II-specific T cell lines were generated from healthy donor PBMC and assessed for their recognition of naturally processed peptides. Figure 6a shows a CD4+ T cell line which specifically recognises autologous APC pulsed with the HAGE 506-II peptide. This T cell line responded to the HLA-DR4+, HAGE-positive melanoma cell line ESTDAB-27 but significantly less to the HLA-DR4+, HAGE-negative melanoma cell line ESTDAB-07 ($P < 0.01$).

Both cell lines were shown to express significant-levels of surface HLA-DR as part of their characterisation by the ESTDAB project (http://www.ebi.ac.uk/ipd/estdab/index.html). Further in vitro data consistent with the natural processing of the HAGE 506-II epitope are shown in Fig. 6b, depicting a HAGE 506-II-specific CD4+ T cell line responding to autologous DC pre-cultured with cell lysates from the HAGE-positive cell line ESTDAB-27 but not from the HAGE-negative cell line ESTDAB-07 ($P < 0.01$).

Using HAGE 506-II specific pentamers in combination with a CD4 antibody, we were able to show that the T cells from this T cell line were all CD4 positive and 8.3% of them were specific for the HAGE 506-II peptide (data not

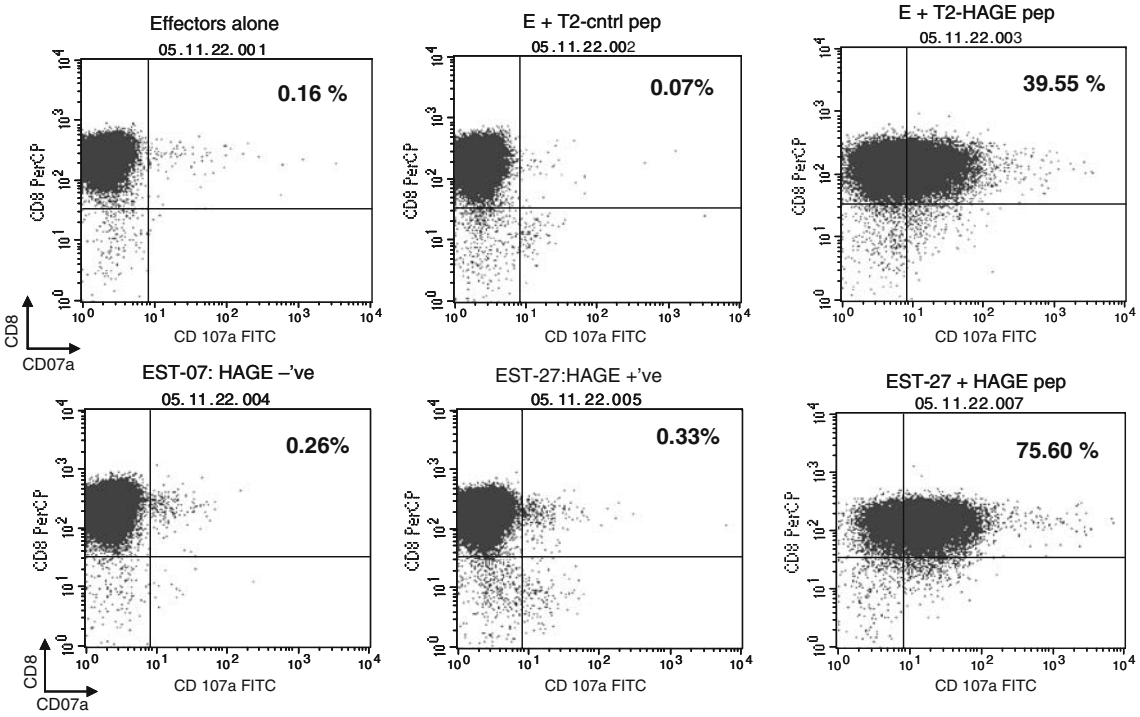

Fig. 4 HAGE 506-I-specific T cell clone co-cultured with HAGE-expressing melanoma cell line. A HAGE 506-I-specific CD8 T cell clone was challenged with either T2 cells pulsed with the HAGE 506-I or control peptide, or HAGE expressing (ESTDAB-27) or non-expressing (ESTDAB-07) HLA-A*0201-positive melanoma cell lines followed by CD107a measurement as a marker of cytolytic activity. HAGE expressing ESTDAB-27 was pulsed with the HAGE 506-I peptide as a positive control

shown); strengthening the conclusion that the proliferation observed with the lysate experiments was due to the true processing of the HAGE 506-II peptide. Taken together, the mouse and human data support the contention that the HAGE 506-II epitope is naturally processed and presented in the context of HLA-DR molecules.

Discussion

Since the cloning of MAGE-1 [19], the first gene reported to encode a tumour antigen recognised by T cells, molecular identification and characterisation of novel tumour antigens has rapidly progressed [8, 10]. There are a number of different approaches that can be applied to identify new class I/II-restricted target antigens for immunotherapy. In this paper, the application of the reverse immunology approach to identify potential T cell epitopes from the product of the cancer-testis gene HAGE is described. Since its discovery in a human sarcoma [9], HAGE expression has been reported in more than 50% of myelogenous leukaemias [1], and in some cases of benign and malignant neoplasms of the salivary glands [13]. HAGE is also reported to be frequently expressed in brain tumours, and colon and non-small-cell lung carcinomas [18]. Here, the expression of this cancer-testis gene was analysed by conventional and real time RT-PCR in a wide range of melanoma cell lines as well as primary melanoma and normal tissues. It was found that HAGE was strongly over-expressed in 32% (23/72) of a large number of melanoma cell lines tested and in 20% (2/10) of primary melanoma tissues which is in line with previously described by Scanlan et al. [18], and at very low level in normal tissues (data not shown), which was then found to be negative using real time RT-PCR. HAGE protein was then assessed in the ESTDAB-27 and ESTDAB-07 cell lines and found to be highly expressed in the ESTDAB-27 but not in the ESTDAB-07 as anticipated. Moreover its cellular location appears to be mainly cytoplasmic. Together, the over-expression of HAGE in melanoma cell lines can validate the use of this putative cancer-testis antigen as a target for immunotherapy against melanoma and justify the search for relevant CTL and T-helper epitopes using reverse immunology.

Peptides are convenient, easy to synthesise for clinical use, easy to modify and to administer to patients. Disis et al. [4] described that the administration of peptides derived from a self tumour protein rather than the whole protein was able to induce antibody production and T cell activation. Moreover, peptides can easily be given together with adjuvants and cytokines or dendritic cells using wild-type sequences or sequences optimised to increase their binding affinity to MHC molecules. Using ex vivo monitoring based on tetramer technology, it was shown that T cell responses

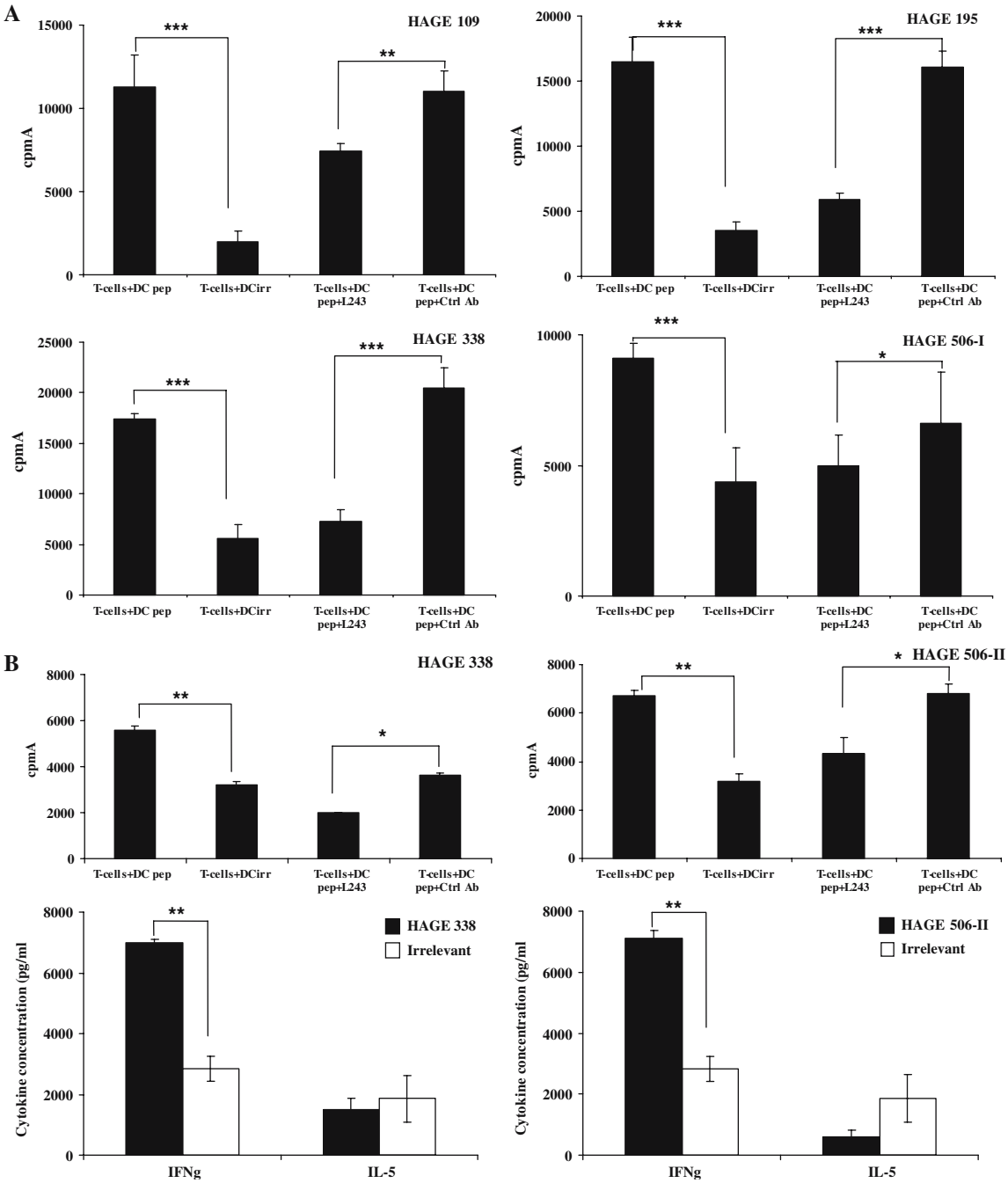

Fig. 5 Immunogenicity and endogenous processing of HAGE 506 class II peptide in DR4-transgenic mice. CD4+ T cells from three groups of two DR4 mice immunised with class II HAGE derived peptides (**a**) or full length HAGE cDNA (**b**) were stimulated in vitro with HAGE derived class II peptides, and then co-cultured with peptide-pulsed BM-DC. Peptide specificity is shown by means of a ^{3}H-thymidine incorporation proliferation assay. $^{**}P < 0.01$, unpaired Student's *t*-test

to peptide vaccines can be followed and that in most cases the clinical outcome correlates with an immunological response [11]. One of the major advantages of identifying immunogenic tumour-associated peptides is the subsequent identification of T cell receptors specific for these peptides. Once identified, vectors encoding these TCR can be engineered and used to transfect lymphocytes from peripheral blood for transfer to patients. A recent study conducted by Morgan et al. [12] indicated the presence of circulating transduced lymphocytes 1 year post-transfusion and metastatic regression in two of 17 treated patients. Numerous studies have demonstrated the feasibility of cancer immunotherapy

Table 2 Immunogenicity of HAGE 506 class II restricted peptide in healthy donors

Name	Cytokine secretion	Human in vitro stimulation
HAGE 506-I	IFNγ	5/5
HAGE 506-II	IFNγ/GM-CSF	5/5

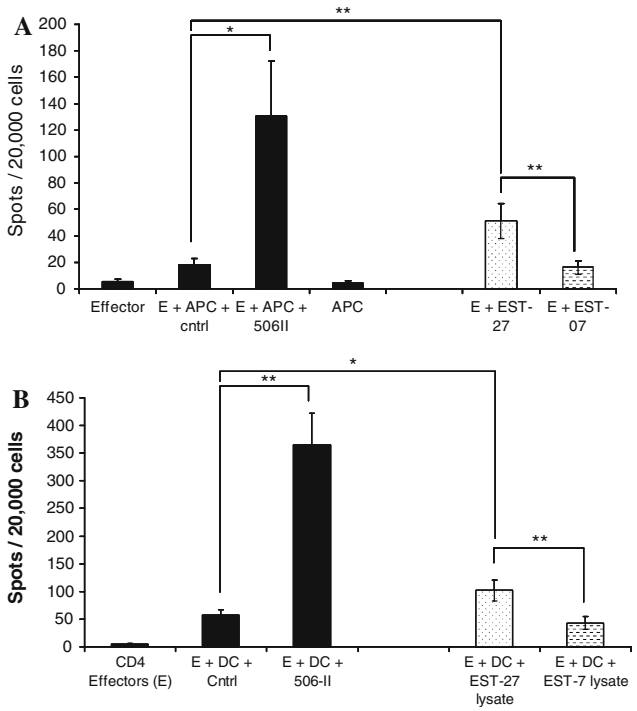

Fig. 6 Endogenous processing of HAGE 506-II peptide by a HAGE-expressing melanoma cell line or by autologous DC co-cultured with a cell lysate from a HAGE expressing cell line. Panel **a** shows the results of a HAGE 506-II-specific T-cell line challenged with either peptide-pulsed autologous PBMC or HLA-DR4+ melanoma cell-lines that were HAGE positive or negative. IFN-γ secretion by the T-cell line was monitored by ELISpot; for which 1×10^4 T cells (Effector *E*) were co-cultured for 40 h in an anti-IFN-γ-coated plate with either PBMC pulsed with HAGE 506-II or a control DR4 binding peptide or the melanoma cell lines (EST-07/27). The number of spots represents means of triplicate wells. Panel **b** shows the results of a HAGE 506-II specific T-cell line challenged with autologous DC pulsed with relevant or control (*Ctrl*) peptides or autologous immature monocyte-derived DC loaded with tumour cell lysate and matured overnight with TNFα and CD40L. The numbers of spots indicated are means of triplicate wells with the standard deviations indicated. $^*P < 0.05$, $^{**}P < 0.01$, unpaired Student's *t*-test

using tumour antigens recognised by CD8+ T cells [16, 22]. However, the responses observed were unfortunately mostly weak and transient. CD4+ T cells play a central role in initiating and maintaining anti-tumour therapy. Thus, optimal immunisation will require the recruitment of both CD4+ and CD8+ T cells, in order to generate a long-lasting anti-tumour immune response [2].

To this end, HAGE 506-I predicted to bind strongly to HLA-A*0201 and HAGE 506-II for HLA-DRβ1*0401, containing the former putative class I epitope, were selected using the evidence-based computer algorithm SYFPEITHI. CD8+ T cell cytotoxicity to HAGE 506-I peptide, as well as IFNγ production, were demonstrated in both transgenic mice and human in vitro work. However, attempts to demonstrate that this peptide was naturally processed by antigen-presenting cells and tumour cells were unsuccessful. Lack of lysis of HAGE-positive targets could have been due to lack of expression of the peptide at the tumour cell surface, or possibly because the T cells clones antigen receptors were of too low avidity. To address the latter possibility, peptide-titration experiments on peptide-pulsed T2 cells were performed. This indicated that the clones had a moderate-to-high avidity, with an IC_{50} of approximately 10^{-10} M (data not shown), thus rendering it less likely that the lack of recognition was the result of low-avidity T receptors. These results make it unlikely that the HAGE 506-I epitope is endogenously processed and presented in the context of HLA-A2 molecules. This class I peptide was initially predicted by the Paproc I program to be correctly cleaved by the proteasome, but this was no longer the case with the updated version, Paproc II, as with the different proteasomal cleavage prediction algorithm, FragPredict. However, these algorithms are still based on limited sets of training data and comparison of such techniques by Saxova et al. [17] demonstrated that the accuracy of these algorithms was in the range of only 40–80%.

In contrast to these results, peptide-specific CD4+ T cell proliferation and IFNγ secretion was demonstrated in both transgenic mice and in in vitro human work in protocols designed to reveal endogenous processing. Following immunisation of HLA-DR4-positive mice with DNA encoding the HAGE protein, peptide-specific CD4+ T cell proliferation was observed with the HAGE 506-II peptide. This was blocked by anti-HLA-DR antibody. Moreover, mice immunised with the empty expression vector did not show any proliferative response when splenocytes were re-stimulated with the same peptides (data not shown). Taking together, these data show that the proliferation observed was peptide-specific, HLA-DR-restricted. The in vitro generation of CD4+ T cell lines from human PBMC showed specific HLA class II-restricted recognition of HLA-DR matched tumour cell lines, as well as DC pulsed with HAGE-positive cell lysates. These results are therefore in agreement with those obtained from DNA immunisation of transgenic mice, suggesting that the HAGE protein is produced and cleaved to give rise to the 506-II in ways that are similar in mice HLA-DR4 and human derived DC. This processing of endogenous proteins and presentation of tumour-derived class II peptides is still not fully understood. However, Dissanayake et al. [5] recently reported that the generation of tumour-derived MHC class II-restricted epitopes were independent of the proteasome and

the TAP complexes. They suggested that MHC class II-restricted endogenously synthesised epitopes might overlap with the classical endosomal pathway for presentation of exogenously synthesised molecules, providing an explanation of how peptides derived from intra-cellular proteins such as HAGE can be found at the surface presented by MHC class II molecules to CD4+ T cells. Thereafter several other peptides chosen for their SYFPEITHI binding score to HLA-A2 or HLA-DR4, were synthesised and tested in our transgenic mice for their immunogenicity as well as their endogenous processing using the gene gun technology. One class I and one additional class II HAGE-derived peptide were found to be both immunogenic and processed in our mouse model. These peptides are now investigated for their ability to generate in vitro CTL or T-helper cells capable of specifically responding to target cells expressing the appropriate HLA molecules as well as HAGE. Together, the data presented here indicate that HAGE represents a valid candidate target for certain melanomas, and could potentially be included future immunotherapeutic vaccine design.

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