Characterization of novel breast carcinoma–associated BA46-derived peptides in HLA-A2.1/D^b-β2m transgenic mice

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The human milk fat globule membrane protein BA46 (lactadherin) is highly overexpressed in human breast tumors, making it a potential target for tumor immunotherapy. We have identified BA46-derived peptides that contain the motif recognized by the MHC class I molecule HLA-A2.1 and that are processed and presented by human breast carcinoma cells. In mice lacking normal class I molecules but expressing an HLA-A2.1/D^b-β2 microglobulin single chain (HHD mice), three peptides elicited specific CTL activity. Two of these peptides also stimulated cytotoxic activity in peripheral blood lymphocytes from HLA-A2.1–positive breast carcinoma patients. Adoptive transfer of HHD-derived bulk CTLs to nude mice bearing human breast carcinoma transplants reduced tumor growth. These peptides therefore represent naturally processed BA46-derived CTL epitopes that can be used in peptide-based antitumor vaccines.

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Introduction

The search for novel human tumor-associated antigen (TAA) peptides using $CD8^+$ CTL lines resulted mainly in the identification of melanoma differentiation antigens due to the relatively high immunogenicity of melanomas (1, 2). In contrast, identification of carcinoma-associated antigens encountered technical difficulties. First, contrary to melanomas, it is difficult to establish carcinoma-associated CTL lines in vitro from patients' peripheral blood lymphocytes (PBLs). Second, CTL lines derived from carcinoma patients frequently represent a repertoire of anergized T cells. Finally, in vitro propagation of CTL lines has the potential of enhancing sporadic clones surviving culture condition rather than clones with effective antitumor specificities. Alternative strategies that bypass some of these difficulties involve the use of HLA transgenic mice. The HLA-A2.1 allele is commonly chosen in these mice due to its high prevalence among the Caucasian population. In the last few years, a number of studies have compared the CTL repertoire of defined peptides restricted by HLA-A2.1 in human PBLs from HLA-A2.1–positive patients with CTLs induced in HLA-A2.1 transgenic mice. Good concordance and an overlapping repertoire were found between the endogenous HLA-A2.1 PBL and the murine transgenic HLA-A2.1 CTL repertoires, confirming the potential use of such transgenic mice in the identification of human CTL epitopes (3, 4). However, since vaccination of HLA transgenic mice with multiepitope proteins induced dominant murine H-2–restricted response (5), a combination of classical HLA transgenesis and selective destruction of murine H-2 is needed to obtain exclusive HLA-restricted responses. Recently, this combination was achieved in D^bxβ2 microglobulin (β2M) null mice transgenic for modified HLA-A2.1-β2 microglobulin single chain (HHD) mice (6). In vivo experiments showed that HHD mice, unlike the classical HLA transgenic mice, exhibit HLA-A2.1–restricted responses to multiepitope proteins such as intact influenza virus. In addition, HHD mice selected the same immunodominant CTL epitopes recognized by PBL in influenza-infected HLA-A2.1 individuals (6). Hence, these mice are a useful tool for the identification and characterization of potential tumor-derived, HLA-A2.1–restricted CTL epitopes.

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Nonstandard abbreviations used: tumor-associated antigen (TAA); peripheral blood lymphocytes (PBLs); β2 microglobulin (β2M); nuclear factor 1 (NF-1); trifluoroacetic acid (TFA); effector-to-target (E/T).

The largest group of potential TAA peptides are those derived from normal self proteins (7). These antigens are either tumor specific in their patterns of expression, such as MAGE-1, BAGE, GAGE, and LAGE, or tumor selective by virtue of overexpression, such as HER-2/neu and carcino-embryonic antigen. In the same context, human milk fat globule membrane proteins have been the focus of extensive research, which brought about the isolation of a variety of new breast TAAs (8). The most well-studied TAA within the HFMG-derived antigens is the MUC1 glycoprotein (9). We and others have demonstrated the possibility of inducing MHC-restricted and tumor-specific CTL reactivity to MUC1 in HLA transgenic mice (10) as well as naive (11) and cancer patient–derived PBLs (12).

Another potential human milk fat globule–associated TAA is the 46-kDa glycoprotein named BA46 or lactadherin. BA46 cDNA contains an open reading frame of 387 amino acids with a putative signal peptide, an EGF-like domain with an Arg-Gly-Asp (RGD) cell adhesion sequence, and factor V/VIII C1/C2-like domains (13). BA46 was found to be expressed in a number of breast and ovarian carcinomas (14). In addition, BA46 antigens are released from the tumor from immune complexes (15) and were detected in sera of patients with breast cancer but not in those of either healthy females or from patients harboring tumors of other histological origin (8). Moreover, it has been shown that BA46 can serve as a target for passive immunotherapy; human breast tumors transplanted in nude mice could be eradicated by a radiolabeled anti-BA46 mAb (MC3), which binds to the RGD domain (16). Despite the apparent immunotherapeutic potential of BA46, no CTL reactivity to BA46 epitopes, nor any other cellular antitumor activity, has been reported thus far.

In the current study, we used the HHD mouse system to identify novel breast-associated and HLA-A2.1–restricted anti-BA46 CTL epitopes. We show that antipeptide CTLs lyse BA46-expressing tumor cells in vitro and retard tumor development in vivo. In addition, cytotoxic selectivity patterns were observed in human PBLs from breast cancer patients but not in healthy individuals. These results imply a potential role for BA46-derived peptides as inducers of HLA-restricted CTL responses against human breast carcinomas.

Methods

Mice. The derivation of HLA-A2.1/D^b-β2 monochain, transgenic, *H-2D$^{b-/-}$xβ2M$^{-/-}$* double knockout mice (named HHD mice) was described previously (6). *CD1$^{nude/nude}$* (*CD1$^{nu/nu}$*) 8- to 12-week-old mice were bred in the Weizmann Institute of Science breeding facility.

Tumor cells. MDA-MB-157 is a human breast cancer cell line negative for expression of HLA class I molecules (17). The MDA-MB-157-HHD clone is a HHD transfectant of MDA-MB-157 cells. MDA-MB-231 is a human breast cancer cell line positive for expression of HLA-A2 class I molecules (18). RMA-S is a TAP-2–deficient lymphoma clone of C57B1/6 origin. The RMA-S-HHD clone is a HHD transfectant of RMA-S cells. The RMA-S-HHD-B7.1 clone is an HHD transfectant expressing the murine B7.1 costimulatory molecule. T2 is a TAP-2–deficient lymphoblastoid line of HLA-A2.1 genotype. MDA-MB-157 and MDA-MB-231 cells were maintained in DMEM containing 10% FCS, 1 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, and 1% nonessential amino acids. MDA-MB-157-HHD transfectants were maintained in the same medium supplemented with 500 μg/ml of geneticin (Life Technologies Inc., Paisley, United Kingdom). RMA-S and T2 cells were maintained in RPMI-1640 containing 10% FCS and combined antibiotics.

Plasmids. For pMP6A-B7-1, expression of B7-1 is driven by the enhancer/promoter of the human cytomegalovirus. Intron A of the immediate early gene IE1, which contains the binding site of nuclear factor 1 (NF-1), promotes high expression. The plasmid was a kind gift from Philip Mohan (Applied Immune Science Inc., Santa Clara, California, USA).

pBR32HHD contains the promotor, leader sequence, and the α1 and α2 genomic domains of HLA-A2.1 fused to the genomic α3, transmembrane, and the cytoplasmic domains of the murine H-$2D^b$. The cDNA of β2 microglobulin was fused through a linker to the 5′ of exon 2 (α1 domain) to yield a single chain of HLA-A2.1/D^b-β2 microglobulin product (6).

PCR amplification. Isolation of total RNA from a human tumor cell line as well as patient-derived malignant and normal tissues was performed by Tri-Reagent (Molecular Research Center, Cincinnati, Ohio, USA), according to the manufacturer's instructions. Reverse transcription was on 5 μg of total RNA with on oligo-dt primer. cDNA corresponding to 500 ng of total RNA was PCR amplified (PTC-100; MJ Research Inc. Watertown, Massachusetts, USA) for 35 cycles as follows: 1 minute 92°C, 1 minute 56°C, and 1 minute 72°C. Amplification was followed by a 10-minute incubation at 72°C. We used the following primers for amplification: primer 1, human BA46 gene position 95-121 sense strand, 5′-CCCCGCTGATCACCGCCCCCCGCCTGC-3′; primer 2, human BA46 gene position 1220–1248 antisense strand, 5′-AGACCTCGAGGTGGCAGGTGGCCACTAAC-3′.

As a control, PCR amplification was performed on an irrelevant tumor cell line. In all reactions 10 μl of the 100-μl reaction mixture was subjected to electrophoresis on 1% agarose gels.

Peptide synthesis. Peptides were synthesized on an Abimed AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany), employing the α-*N*-fluorenyl-methoxy-carbonyl (Fmoc strategy following the commercially available manufacturer's protocols). Peptide chain assembly was conducted on a 2-chlorotrityl chloride resin (Novabiochem, Laufelfingen, Switzerland). Crude peptides were purified to homogeneity by reversed-phase HPLC on a semipreparative silica C-8 column (250 × 100 mm, Lichnonorb RP-8; Merck, Darmstadt, Germany).

Elution was accomplished by a linear gradient established between 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in 70% acetonitrile in water (vol/vol). Composition of the products was determined by amino acid analysis (automatic amino acid analyzer; Dionex Corp., Sunnyvale, California, USA) after extraction of acid hydrolysis. Molecular weight was ascertained by mass spectrometry (VG Tofspec, Laser Desorption Mass Spectrometry; Fisons PLC, Manchester, United Kingdom).

Preparation of tumor extract peptides. Total acid-extracted peptides of breast tumor or of normal adjacent tissues were prepared from a pool of five to six postsurgical breast cancer specimens. Non-necrotic (1–2 cm) tumor masses were homogenized in PBS-0.5% Nonidet P-40–10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptine, 8 μg/ml aprotonine, and 0.5 mM PMSF and homogenized using a glass-Teflon homogenizer. Following further stirring for 30 minutes at 4°C, the homogenates were titrated with 10% TFA to a final concentration of 0.1% TFA and stirred for another 30 minutes at 4°C. After ultracentrifugation for 30 minutes at 130,000 *g*, the supernatants were applied to Sephadex G25 columns, and fractions were monitored at OD 230 nm. Peptide fractions below 10 kDa were pooled, lyophilized, and further fractionated by Centripep 3 centrifugation (Amicon, Beverly, Massachusetts, USA). Lyophilized samples were dissolved in sterile double-distilled water, freed from TFA by repeated lyophilization, and relative concentration was monitored by determination of OD at 230 nm. The yields from both tumor and normal tissue were 130–160 OD at 230 nm/g tissue. Following lyophilization, the peptide pool was dissolved in opti-MEM (Life Technologies Inc.) at 30–50 OD at 230 nm/ml for further use.

Peptide loading for FACS analysis. Peptide loading of RMA-S-HHD transfectants was performed as follows. After the cells were washed three times in PBS, the surface expression of HHD monochain was stabilized by a 4-hour culture at 26°C. Synthetic peptides or peptide extracts were added to 5×10^5 cells in 50 μl of Opti-MEM (Life Technologies Inc.) to a concentration of 1–100 μM and/or 0.25–1.0 OD at 230 nm, respectively. The cells were incubated for 2–3 hours at 37°C prior to FACS analysis (Becton Dickinson, Canberra, Australia).

Measurement of peptide binding by stabilization of cell surface MHC. Peptides binding to HHD single chain were measured by stabilization of HHD on RMA-S HHD transfectants, using an indirect FACS assay as follows: 5×10^5 peptide-loaded TAP-2–deficient RMA-S-HHD cells (see peptide loading) were incubated with anti-HLA mAb for 30 minutes at 4°C. After the cells were washed with PBS-0.5% BSA plus 0.1% sodium azide, the second Ab, goat anti-mouse FITC (The Jackson Laboratories, Bar Harbor, Maine, USA), was applied for 30 minutes at 4°C. Following washing, the amount of bound Ab's was detected by FACS scan. Mouse mAb's B-9-12, w6/32 (anti–HLA A,B,C), 28-14-8 (anti–H-2D^b α3 domain), and BB7.2 (anti–HLA-A2.1) were used for analysis.

Vaccination. Mice were immunized intraperitoneally three times at 7-day intervals with 2×10^6 irradiated (50 Gy) tumor cells, or with irradiated peptide-loaded RMA-S-HHD-B7.1 transfectants. Peptide loading of RMA-S-HHD-B7.1 cells was performed as follows: the cells were washed three times in PBS, then cell surface expression of HHD monochain was stabilized by a 4-hour culture at 26°C. Synthetic peptides or peptide extracts were added to 10×10^6 cells in 1 ml of Opti-MEM (Life Technologies Inc.) medium to a concentration of 100 μM or 1 OD at 230 nm, respectively. The cells were incubated overnight at 26°C and for an additional 3 hours at 37°C. Peptide-loaded RMA-S-HHD-B7.1 cells were irradiated (50 Gy), washed, resuspended in PBS, and injected intraperitoneally to HHD mice. In mixed synthetic vaccines, RMA-S-HHD-B7.1 cells were loaded separately with each peptide and pooled before vaccination.

In vitro cytotoxicity assay in HHD mice. Mice were immunized intraperitoneally three times at 7-day intervals with 2×10^6 irradiated (50 Gy) tumor cells or with peptide-loaded RMA-S-HHD-B7.1 transfectants. Spleens were removed on day 10 after the last immunization, and splenocytes were restimulated in vitro either with irradiated tumor cells (for mice immunized with tumor cells), or with one-third of the lymphocytes pulsed with 100 μM synthetic peptides, or 1 OD 230 nm patient-derived extract in Opti-MEM (Life Technologies Inc.) medium for 2 hours at 37°C, 5% CO_2. Restimulated lymphocytes were maintained in RPMI-HEPES medium containing 10% FCS, 1 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4, 5×10^{-5} M β-mercaptoethanol, and 1% nonessential amino acids for 5 days. Viable lymphocytes (effector cells) were separated by lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) centrifugation, resuspended in RPMI-HEPES medium, and admixed at different ratios with 5×10^3 ^{35}S methione-labeled, peptide-loaded, RMA-S-HHD cells. CTL assays were performed in U-shaped microtiter wells at 37°C, 5% CO_2, for 5 hours. Cultures were terminated by centrifugation at 100 *g* for 10 minutes at 4°C. A total of 100 μl of the supernatants was mixed with scintillation fluid and measured in a β counter (Becton Dickinson). Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release) × 100. Spontaneous release was determined by incubation of 100-μl labeled target cells with 100 μl of medium. Maximal release was determined by lysis of target cells in 100 μl 0.1 M NaOH.

Adoptive transfer of lymphocytes from HHD mice to CD1$^{nu/nu}$ mice bearing human breast carcinoma. Mice were immunized intraperitoneally with BA46-derived peptides and individually loaded on RMA-S-HHD-B7.1 cells, as described previously. Spleens were isolated from the HHD mice and splenocytes were separated and restimulated with the peptides as described in in vitro cytotoxicity assays. *CD1$^{nu/nu}$* mice were inoculated

with 2.5×10^6 MDA-MB-231 cells per animal into the fat pad region, and 3 days later were injected intravenously with 15×10^6 cells per animal of the HHD-derived restimulated CTLs. The adoptive transfer was followed by intraperitoneal injection of IL-2 once a day for 7 days at a concentration of 1,000 U per animal. Control animals received CTLs induced against unloaded RMA-S-HHD-B7.1 and CTLs induced against an HLA-A2 TAX-binding peptide. IL-2 was administered as above.

Patient selection for human cytotoxicity assay. Patients with localized breast cancer were recruited for HLA typing preceding their selection for the in vitro human cytotoxicity assay. Inclusion criteria included patients who were at least 30 days after their surgical procedure and who did not receive adjuvant chemotherapy. Exclusion criteria included concurrent immunosuppressive treatment or a medical history of an immune-compromised condition. HLA typing of the patient's blood samples were conducted using a routine serological method and were performed in the HLA typing unit of the Rabin Medical Center (Petach Tiqva, Israel).

In vitro cytotoxicity assays of human PBLs. Forty milliliters of blood from HLA-A2.1–positive patients with localized breast cancer was collected using heparinized syringes diluted 1:1 with PBS without calcium and magnesium and separated on Ficoll Hypaque gradients for 30 minutes at 250 *g* at 18°C. Total lymphocytes of each patient were pulsed with 100 μM of various synthetic peptides (one-sixth of the PBL per peptide) for 2 hours at 37°C. Lymphocytes were pooled and divided to 75-cm Falcon bottles at 1 to 2×10^6/ml in RPMI, 10% human serum (Israeli Blood Center, Tel-Aviv, Israel), 1 mM glutamine, 1 mM sodium pyruvate, combined antibiotics, and 1% nonessential amino acids.

On the third day lymphocytes were resensitized with additional 10 μM of synthetic peptide mixture. On the fifth day cells were collected and resuspended (2×10^6/ml) in fresh medium (no peptides) supplemented with 10 U/ml recombinant IL-2. The medium was replaced between days 6 and 11 once the color changed to orange. On day 12, in vitro cytotoxicity assays were performed as described above.

Results

Screening for BA46-derived HLA-A2.1–restricted peptides. The BA46 amino acid sequence was screened for potential HLA-A2.1–restricted peptides and scored according to their ability to stabilize MHC, using the program for independent binding of individual peptide side chains (19). The best HLA-A2.1–binding peptides were selected for synthesis, yet extremely hydrophobic insoluble peptides were excluded. Table 1 summarizes the BA46-derived peptide positions and binding scores. The selected peptides are deduced from the BA46 protein domains as follows: peptide BA46-9 is derived from the cell adhesion (RGD) domain, and peptides BA46-1, BA46-5, and BA46-7 are derived from the C1 and C2 human coagulation factor V and VIII homology domains.

Measurement of BA46 peptide binding by stabilization of cell surface MHC. HLA-A.2.1–binding affinity of BA46-derived peptides was evaluated by FACS analysis. The selected peptides were loaded at 1–100 μM concentration on the murine TAP-2–deficient RMA-S-HHD transfectants, and MHC stabilization was measured (Figure 1). MHC binding at 100 μM was high for all BA46 peptides; however, when peptides were loaded at 1 μM concentration, MHC binding was best by peptides BA46-5, BA46-6, BA46-7, and BA46-9.

CTL responses induced by BA46-derived peptides in HHD mice. Most BA46-derived peptides bind effectively to HLA-A2.1 on RMA-S-HHD cells. Since no CTL reactivity to BA46 was reported so far, we first tested in vivo the immunogenic profile of BA46-derived peptides. Hence, the lysis pattern of target cells loaded with individual BA46 peptides was examined following immunization with a pool of all BA46-derived synthetic peptides as described in Methods. Anti-BA46 CTLs (Figure 2a) showed significant lysis of BA46-loaded RMAS-HHD target cells. Anti–BA46-6, –BA46-7, and –BA46-9 peptides elicited high specific lysis: 42%, 60%, and 43%, respectively. Lysis of 10–20% were obtained with the other BA46 peptides BA46-1, BA46-2, BA46-5, and BA46-8. Therefore, BA46-6, BA46-7, and BA46-9 seem to harbor better immunogenic properties in HHD mice.

Based on the above results, we next evaluated the efficacy and specificity of the three most immunogenic BA46-derived peptides. HHD mice were immunized either with BA46-6, BA46-7, or BA46-9 individual peptides, a known breast-associated HER2/Neu–derived peptide as a positive control, or with a melanoma-associated tyrosinase-derived peptide as a negative control (Figure 2b). All three BA46-derived peptides show high lysis patterns: BA46-6, 36%; BA46-7, 76%; and BA46-9, 60%. These results were comparable to those obtained by two known HLA-A2–restricted tumor-derived CTL epitopes, the breast-associated HER2/Neu and the melanoma-associated tyrosinase (60% lysis for both). Moreover, the lysis was specific as demonstrated by the limited lysis of targets pulsed with the melanoma-associated tyrosinase

Table 1
Selection of BA46-derived HLA-A2.1–restricted peptides

Peptide[A]	Position No.[B]	Sequence[C]	Score[D]
BA46-1	271	KQGNFNAWV	515.839
BA46-2	131	NLLRRMWVT	195.006
BA46-5	356	NLFETPILA	75.365
BA46-6	194	NLFETPVEA	75.365
BA46-7	97	GLQHWVPEL	49.134
BA46-8	313	VQFVASYKV	44.356
BA46-9	5	RLLAALCGA	42.278

BA46-derived peptides were selected according to the known consensus motifs for peptides bound by HLA-A2.1. [A]Peptides designation. [B]The position of the first and last amino acid in the protein sequence.[C]The amino acid sequence of the peptides.[D]Calculated score that estimates the half time for dissociation of the peptide-HLA complex.

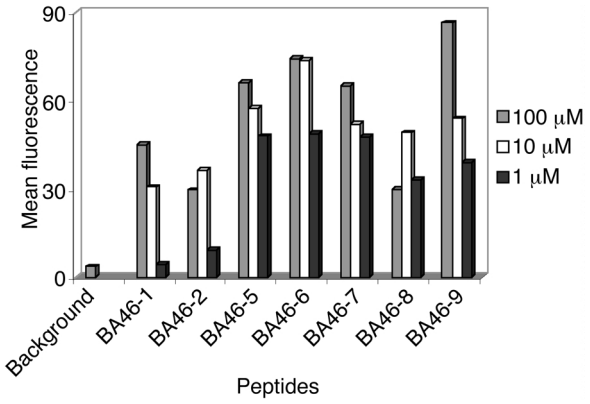

Figure 1
Stabilization of cell surface MHC by BA46-derived peptides. BA46-derived peptides were loaded at various concentrations (1–100 μM) on TAP2-deficient RMA-S-HHD cells as described, and indirect FACS analysis was performed by incubating 5×10^5 loaded cells with anti–HLA-A2.1 MAb BB7.2 for 30 minutes at 4°C. After the cells were washed with PBS-0.5% BSA plus 0.1% sodium azide, the secondary Ab, goat anti-mouse-FITC, was applied for 30 minutes at 4°C. Following another wash, the amounts of bound Ab's were detected by a FACScan. Mean fluorescence at 1–100 μM peptide concentrations is shown. Results are representative of three similar experiments.

peptide (between 5–13%) by CTLs induced against BA46-derived peptides or HER2/Neu-derived peptides.

HLA-A2.1–restricted lysis of breast carcinoma cell lines by CTLs induced against BA46-derived peptides or human breast cancer–derived tumor extract. To determine whether BA46 protein processing results in presentation of BA46-derived peptides by breast carcinoma tumors, we first had to obtain an HHD- and BA46-expressing breast carcinoma cell line. As a model we chose MDA-MB-157-HHD, an HLA-negative breast carcinoma cell line transfected by the HLA-A2.1-β2m single chain. This cell line was shown to express the HHD molecule (10) and was also shown to express BA46, as determined by PCR analysis and DNA sequencing (see the second lane in Figure 5b).

MDA-MB-157-HHD, its parental HLA-A2.1–negative MDA-MB-157, and the HLA-A2.1–positive MDA-MB-231 cell lines were used as targets for CTL analysis using lymphocytes activated against BA46-derived peptides. HHD mice were immunized with RMA-S-HHD-B7.1 cells loaded with BA46-selected peptides, and lysis of the breast carcinoma cell lines was monitored (Figure 3). Activated lymphocytes against BA46-derived peptides BA46-6, BA46-7, and BA46-9, or against the peptide fraction of fresh breast tumor samples show up to 30 times higher lysis of MDA-MB-157-HHD transfectants than of the parental untransfected cells, suggesting MHC-restricted lysis. Similarly, anti-peptide CTLs lysed the native HLA-A2.1–expressing

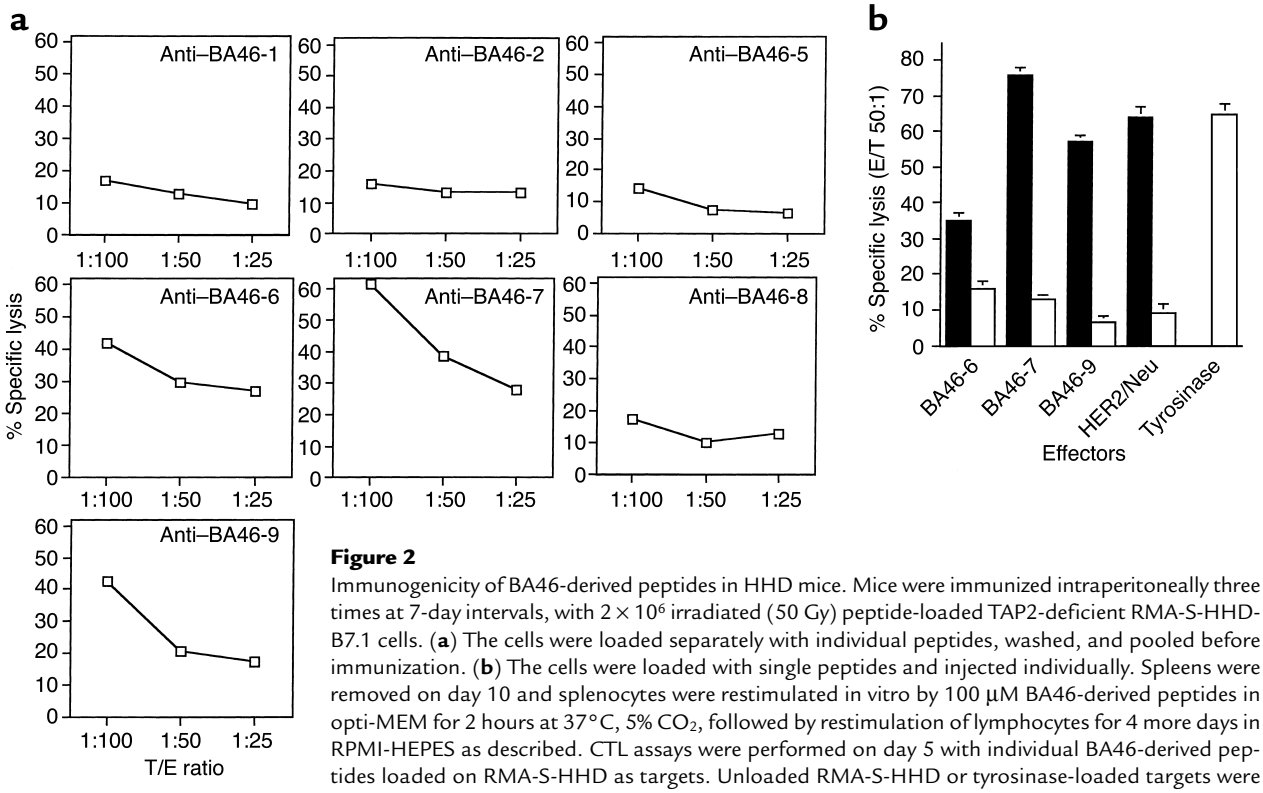

Figure 2
Immunogenicity of BA46-derived peptides in HHD mice. Mice were immunized intraperitoneally three times at 7-day intervals, with 2×10^6 irradiated (50 Gy) peptide-loaded TAP2-deficient RMA-S-HHD-B7.1 cells. (**a**) The cells were loaded separately with individual peptides, washed, and pooled before immunization. (**b**) The cells were loaded with single peptides and injected individually. Spleens were removed on day 10 and splenocytes were restimulated in vitro by 100 μM BA46-derived peptides in opti-MEM for 2 hours at 37°C, 5% CO_2, followed by restimulation of lymphocytes for 4 more days in RPMI-HEPES as described. CTL assays were performed on day 5 with individual BA46-derived peptides loaded on RMA-S-HHD as targets. Unloaded RMA-S-HHD or tyrosinase-loaded targets were used as negative controls. An effector-to-target (E/T) ratio of 50:1 is shown. Black bars represent the targets loaded with BA46 peptides and white bars represent targets loaded with the tyrosinase-specific peptides. Specific lysis of all three peptides, BA46-6, BA46-7, and BA46-9, is statistically significant ($P < 0.001$) compared with lysis of the tyrosinase peptide. Results represent the average of three similar experiments. T/E ratio, tumor/effector ratio.

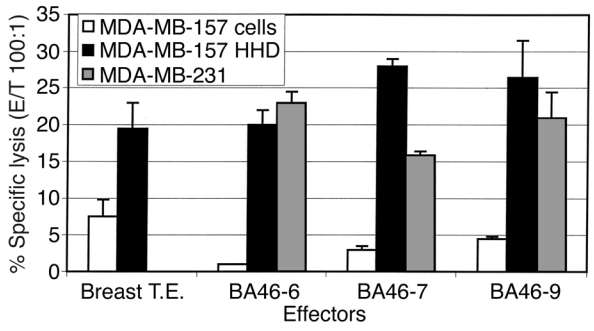

Figure 3
HLA-A2.1–restricted lysis of the breast-carcinoma cell lines. MDA-MB-157-HHD and MDA-MB-231 by CTL against BA46-derived peptides and patient-derived tumor extract (T.E). Mice were immunized as described in Methods with patient-derived tumor extract or with BA46-6, BA46-7, and BA46-9 peptides loaded on RMA-S-HHD-B7.1 transfectants. Lysis of MDA-MB-157(HLA-A2.1–negative), MDA-MB-157-HHD, and MDA-MB-231 (HLA-A2.1–positive) breast carcinoma cells was monitored by CTL assays. Specific lysis of HHD-transfected MDA-MB-157-HHD was significantly higher ($P < 0.0006$) than that of wild-type MDA-157 target cells. The E/T ratio of 100:1 is shown. Results are representative of three similar experiments for MDA-MB-157/HHD and two experiments for MDA-MB-231.

MDA-MB-231 cells. These data show that processing and presentation of BA46-derived peptides takes place in human breast carcinoma cells.

When the same procedure was performed using CTLs induced against breast cancer patient-derived tumor extract peptides, a strong HHD-restricted lysis of MDA-MB-157-HHD cells was observed. This finding implies that an overlapping T cell epitope repertoire exists between the breast tumor cell line and fresh breast tumor extracts.

CTLs induced by BA46-derived peptides lyse more efficiently RMA-S-HHD pulsed with tumor extract than with normal tissue extract. An important criteria for selection of tumor-associated peptide-based vaccines were their expression in patients' tumors as well as the frequency of this expression in comparison with normal tissues. BA46 is a nonmutated protein overexpressed in breast carcinoma tumors. Hence, it is of obvious interest to examine the abundance of BA46 peptides in patient-derived breast tissue extracts in comparison with tumor extract. Moreover, these data might explain the CTL reactivity to BA46. CTLs generated to individual BA46-derived peptides were two- to threefold more active in lysis of targets pulsed with tumor extract versus targets pulsed with equal amounts of normal extracts (Figure 4). BA46-6 and BA46-7, as well as the breast-associated HER2/Neu peptide, showed the highest potency in eliciting breast-associated lysis, 48%, 37%, and 38%, respectively. In addition, CTLs to these peptides manifested the most pronounced differential lysis between targets pulsed with tumor-derived peptides and targets pulsed with normal tissue-derived peptides (Figure 4). Vaccination with breast tumor–extracted peptides showed threefold preferential tumor versus normal tissue recognition, supporting the window of specificity in terms of lysis between normal and tumor tissues. Vaccination with the HLA-A2.1 melanoma-associated peptide tyrosinase did not show tumor-specific recognition and resulted in 10–15% lysis. Overall, the results of this tumor versus the normal tissue extract system suggest that BA46-6– and BA46-7–derived peptides can be regarded as tumor-associated antigen peptides.

Screening of human tumor cell lines for expression of BA46. In an attempt to evaluate the relevance of the results to breast and nonbreast malignancies, we have screened various cell lines and patient-derived tumor samples for the expression of BA46. Therefore, total RNA-derived cDNAs were prepared, and BA46 expression was monitored by PCR (Figure 5). A PCR product of 383 bp, representing BA46, could be amplified in the majority of the cDNA samples tested, including those from the MDA-MB-157, MCF-7, and MDA-MB-231 breast carcinoma cell lines. The identity of the PCR product was verified by sequencing. A 890-bp PCR product identical in size and intensity was obtained in all cDNAs tested, using primers derived from the G3PDH housekeeping gene (not shown). These results indicate that BA46, like MUC1, is expressed in different epithelial tumors and is not necessarily limited to breast and ovarian tumors.

Adoptive transfer of lymphocytes from HHD mice to CD1$^{nu/nu}$ mice bearing human breast carcinoma. In order to analyze whether the immunogenic BA46–derived

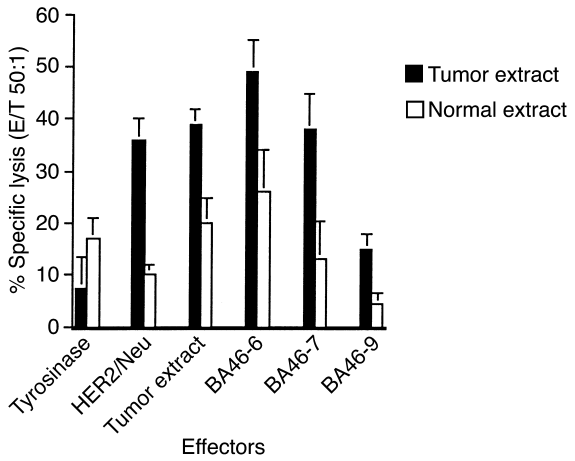

Figure 4
Differential lysis of targets loaded with tumor or normal breast tissue–derived peptides by anti–BA46-induced CTL. CTL assays using anti–BA46-6, BA46-7, and BA46-9 or antitumor extract peptide-activated lymphocytes were performed as described in Methods, using RMA-S-HHD cells loaded with equal amounts (1 OD 230/5 × 10^5 cells/100 μl at the loading step) of tumor- and normal tissue–extracted peptides as targets. Specific lysis on tumor extract by CTLs induced by vaccination with different peptides was significantly higher than lysis of normal extract ($P < 0.01$). Results of a CTL assay using anti-Her2/Neu and tyrosinase-activated lymphocytes are presented as negative and positive controls, respectively. The E/T ratio of 50:1 is shown. Results represent the average of three similar experiments.

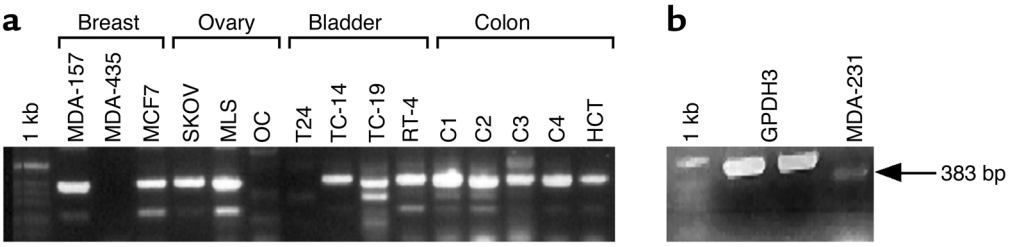

Figure 5
BA46 expression in different cell lines and patient tumor samples detected by RT-PCR. RNA from cell lines and fresh tumor samples was extracted, and cDNA was produced as described in Methods. The cDNA served as a template in PCR amplification with BA-46–specific primers. A 383-specific band was detected by gel electrophoresis. The lanes were as follows. (**a**) 1-kb marker, breast carcinomas: MDA-MB-157, MDA-MB-435, MCF7; ovarian carcinomas: SKOV, MLS, OC; bladder carcinomas: T24, sample 14, sample 19, RT4; colon carcinomas: sample 1, sample 2, sample 3, sample 4, HCT. (**b**) 1-kb marker, GPDH3, GPDH3, breast carcinoma: MDA-MB-231.

peptides are indeed tumor rejection antigen peptides, we created an in vivo model of adoptive transfer to nude mice bearing human tumors. The human breast carcinoma cell lines MDA-MB-231 (HLA-A2 and BA-46 positive), MDA-MB-157-HHD (HHD and BA46 positive), and MCF7 (HLA-A2 and BA46 positive) were tested for progressive tumor growth in HHD and *CD1*$^{nu/nu}$ mice. None of the lines formed tumors in HHD mice. MDA-MB-231 formed tumors in 100% of the *CD1*$^{nu/nu}$ mice, while MCF7 formed tumors in 70% when implanted with 17β-estradiol (0.72 mg/pellets, 60-day release; Innovative Research of America, Sarasota, Florida, USA) subcutaneously. Thus, MDA-MB-231 was chosen as the tumor target.

In order to obtain specific CTLs for adoptive transfer to *CD1*$^{nu/nu}$ mice, we vaccinated HHD mice with BA46-derived peptides individually loaded on RMA-S-HHD-B7 cells. *CD1*$^{nu/nu}$ mice were inoculated with the MDA-MB-231 into the fat pad region and 3 days later, HHD-derived, in vitro–restimulated CTLs were injected intravenously. The CTLs induced by any of the BA46-derived peptides in HHD mice were able to suppress MDA-MB-231 growth in *CD1*$^{nu/nu}$ mice (Figure 6). CTLs to peptides BA46-7 and BA46-9 (and less to peptide BA46-6) were potent in decreasing the tumor volumes compared with the control group. Statistical analysis using the alternate Welch *t* test (unpaired, assuming unequal variances) showed one-tailed *P* values of 0.0026–0.0078 for the group receiving anti–BA46-7 relative to the group receiving anti–RMA-S-HHD-B7.1 on the various days after inoculation. *P* values of 0.0191–0.0409 were determined for the group receiving anti–BA46-9 relative to the control, while *P* values of 0.0340, 0.0426, 0.0800, and 0.0885 were calculated on days 29, 32, 36, and 39, respectively, for the group receiving anti–BA46-6 relative to the control. The group receiving anti-TAX showed no significant difference (*P* values 0.0617–0.1933) from the control at any time. At the end of the experiment two of eight animals in the group of BA46-6, four of eight animals in the group of BA46-7, and three of eight animals in the group of BA46-9 were tumor free, compared with the control groups of "no peptide" and TAX, where all the mice developed the tumor mass.

PBLs derived from breast carcinoma patients show epitope-specific and HLA-A2.1–restricted cytotoxic activity upon stimulation with BA46 peptides. To test whether BA46 peptides can induce cytotoxicity in human PBLs, we concentrated on BA46-6, BA46-7, and BA46-9 peptides, which were found to be the most immunogenic peptides in the HHD murine model. The primed bulk lymphocytes were generated as described in Methods. These CTLs were tested for their ability to lyse the TAP-deficient T2 target cells loaded with individual BA46 peptides and the HLA-A2.1–restricted melanoma-associated tyrosinase peptide, as well as the target K562, a human cell line that is very sensitive to NK cells. Results from six individual patients showed specific lysis mediated by two BA46-derived peptides, BA46-6 and BA46-7, in PBLs of patients 1, 2, 3, and 6. PBLs from patient 4 reacted specifically only on targets pulsed with BA46-7, while PBLs from patient 5

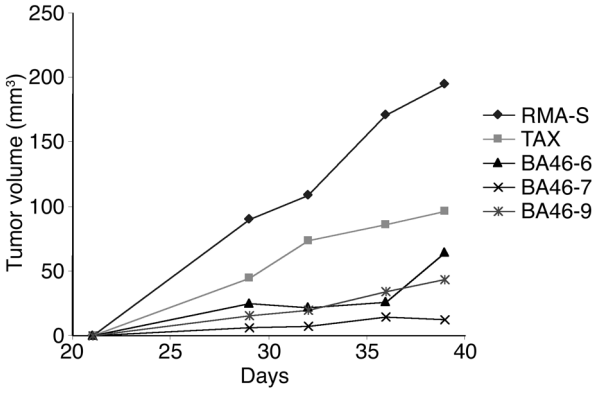

Figure 6
Adoptive transfer of HHD-derived anti–BA46-specific CTLs to MDA-MB-231 tumor-bearing *CD1*$^{nu/nu}$ mice. CTLs specific to BA46 peptides were obtained from HHD mice and adoptively transferred to *CD1*$^{nu/nu}$ MDA-MB-231 tumor-bearing mice (eight mice per group) as described in Methods. Tumor growth was followed for 40 days. CTLs from mice immunized with unloaded RMA-S-HHD-B7.1 (seven mice per group) and RMA-S-HHD-B7.1 loaded with unrelated HIV TAX-derived peptide (four mice per group) are the negative controls in the experiment. The experiment was performed once. For statistical analysis see text.

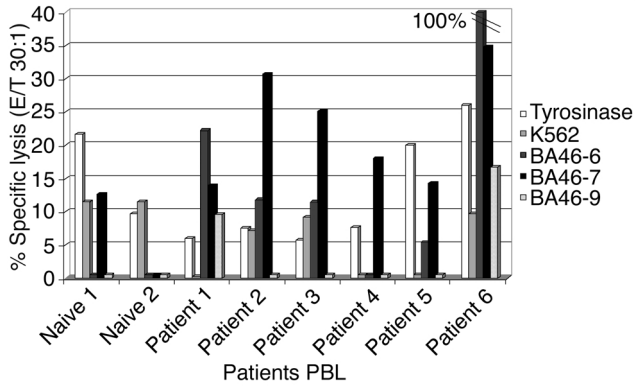

Figure 7
HLA-restricted and epitope specific CTL reactivity in breast cancer patients. T lymphocytes were generated from the peripheral blood of six patients with localized breast cancer, as described in Methods. These CTLs were tested for their ability to lyse Tap-deficient T2 cells loaded with individual BA-46 peptides, the HLA-A2.1–restricted melanoma-associated tyrosinase peptide, and the human NK target cell line K562. Each bar represents the average of triplicates. The experiment was performed once.

showed similar lysis of targets pulsed with the tyrosinase-derived peptides and BA46-7 peptide. PBLs from control volunteers did not show high reactivity against any of the BA46 peptides. PBLs from the breast carcinoma patients did not lyse specifically targets pulsed with BA46-9 as compared with targets pulsed with the tyrosinase peptide that served as background in the assay. The low lysis of K562 indicated that NK activity was not involved (Figure 7). Despite the high variability among individual patients as to the extent to which target cells were lysed, the lysis pattern was relatively reproducible and showed that reactivity against BA46-6 and BA46-7 can be stimulated in PBLs of patients by short incubation with peptides.

Discussion

Tumor cells express defined antigens that can be recognized by tumor-destroying ($CD8^+$) CTLs. However, since most cancer patients do not mount efficient T cell responses against their tumors, effective immunization protocols should be defined to induce cancer cell killing by T cells in patients. One of the obstacles in vaccine development is the fact that for most epithelial tumors, only a few TAAs and their corresponding CTL epitopes have been defined so far (12).

In the current study, we evaluated the potential use of novel BA46-derived peptides as inducers of antitumoral immunity. To this end, we used HHD mice whose class I–restricted T cell repertoire is selected on a single class I allele, the HLA-A2.1. Using this mouse system, we and others have identified previously HLA-A2.1–restricted epitopes from the TAAs MAGE-A1, HER-2/neu, and MUC1 in epithelial tumors (10, 20, 21). Most known TAAs were categorized in five groups: group 1, viral proteins in virally associated tumors such as human papillomavirus and Epstein-Barr virus; group 2, tumor-specific mutations such as caspase-8 and β-catenin or generalized mutations such as P53 and Ras; group 3, tissue-differentiation antigens such as MART-1, tyrosinase, and gp100; group 4, proteins sharing expression in tumor and testis such as MAGE-1, MAGE-3, BAGE, SART3, and NY-ESO-1; and group 5, proteins overexpressed in tumors compared with normal tissue such as nonmutated P53, telomerase catalytic subunit, survivin, prostate-specific antigen, prostrate-specific membrane antigen, FGF-5, and MUC1. BA46 appears to belong to group 5 of tumor antigens, although it was earlier reported as expressed in lactating breast and in breast and ovarian tumors only (8, 14). However, we show that BA46 expression is not limited to breast and ovarian carcinomas, but expression is detected in transitional cell carcinomas of bladder and colon cancers as well, similar to the epithelial TAA MUC1.

BA46 was shown to be involved in adhesion to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and to endothelial cells. Little is known about the physiological function of lactadherin; in the rat it was identified as an *O*-acetylganglioside synthase (22). In milk it was also shown to be an antiviral protein, inhibiting infection by rotavirus (23). Recently, a 50-amino fragment of human BA46, medin, was characterized as an amyloid deposit constituent found around small vessels entering the aortic wall (24). Interestingly, the close mouse homologue MFG-E8 was recently described as a macrophage-derived protein that binds to apoptotic cells and targets them to phagocytes for engulfment (25). Overexpression of BA46 suggests a potential role of BA46 as a target for immunotherapy in various human cancers, possibly other than breast and ovarian carcinomas. We may also speculate that BA46 might be involved in the malignant process. Couto et al. (26) suggested that the RGD-dependent cell-binding activity of BA46 with membrane integrins modulates cell polarity, position, growth, and differentiation. Giuffrida et al. (27) proposed that defective release of the EGF domain is possibly related to the malignant process. Any of these mechanisms can be in concordance with a potential basic role of BA46 in the malignant process.

Seven HLA-A2.1–restricted peptides derived from the BA46 protein sequence were selected according to their calculated MHC-binding affinities. This approach was based on previous reports demonstrating a positive correlation between binding affinity and immunogenicity of potential CTL epitopes (19, 28). Although all seven peptides could bind to MHC, there was little correlation between predicted and measured complex stabilization. Peptide immunogenicity, in vivo, was evaluated by CTL analysis using a pool of peptides in the immunization phase and individual peptides in the recognition and lysis phase. Three peptides, BA46-6, BA46-7, and BA46-9, all high binders, were immunogenic in HHD mice, yet another high-binding peptide, BA46-5, was nonimmunogenic. Sequence alignment between BA46 and the murine homologue MFG-E8 shows 76% identity between cDNAs and 57% identity (68%

positives) in protein sequences. All seven HLA-A2.1–binding BA46 peptides have close homologues in the murine MFG-E8 that preserves the anchor motives in position 2 and 9. The immunogenic peptides BA46-7 and BA46-9 homologues show only conservative amino acids changes (GLQHWVPEL→GLQRWGPEL and RLLAALCGA→RVLAALCGM, respectively), while some of the nonimmunogenic peptides show larger variation, indicating that immunogenicity is not directed against the differences between human and mouse sequences. Lymphocytes activated against each of the three immunogenic peptides preferentially lysed the breast carcinoma cell lines MDA-MB-157-HHD and MDA-MB-231, confirming that BA46-derived peptides are indeed processed and presented by the native HLA-A2.1 and by the single-chain HHD molecule on breast carcinoma cell lines and rules out the possibility of false-positive lysis due to exogenous pulsing of target cells. Moreover, CTLs induced against peptide extracts of fresh human breast tumors show HLA-A2.1–restricted lysis of the MDA-MB-157-HHD cell line. While the identity of the cross-reactive peptides in the tumor extract and the cell line are unknown, CTLs induced against at least two BA46 peptides, BA46-6 and BA46-7, as well as those induced against the HER2/Neu peptide, contained subpopulations that recognized and lysed targets presenting tumor extract peptides, suggesting the antigenicity of these CTL epitopes. The largest group of potential TAA peptides is that derived from proteins that are overexpressed in tumors compared with the corresponding normal tissues; therefore, a crucial parameter for selection of TAA peptide vaccines is their frequency and magnitude of expression in tumors compared with normal tissues. Our data showed at least 2.5-fold higher CTL reactivity to tumor peptides versus normal tissue peptides was generated by BA46-6 and BA46-7 peptides. The same preferential recognition of tumor peptides but not of normal peptides was reproduced upon vaccination with breast tumor extract peptides. BA46-6 and BA46-7 were also efficient in inducing human CTLs in blood lymphocytes from breast carcinoma patients but not those derived from healthy individuals. These data support the hypothesis that the peripheral blood of patients, but not of healthy individuals, contains primed CTLs capable of recognizing TAA-derived peptides after short peptide stimulation, while extended protocols, usually using dendritic cells as antigen-presenting cells, are necessary to prime CTLs in PBMCs from healthy donors (29). A small number of human CTL epitopes has been tested for induction of tumor rejection in animal models. Notably, MUC1 peptide vaccines were shown to protect transgenic A2/Kb mice from B16-A2-MUC1 tumors in active vaccination protocols, and adoptive transfer of a MUC1-specific CTL line isolated from MUC1 transgenic mice could prevent formation of MUC1-expressing tumors (30, 31). Here we show that anti-BA46 peptide CTLs adoptively transferred to *CD1*$^{nu/nu}$ mice orthotopically injected with human MDA-MB-231 breast carcinoma cells significantly reduced tumor growth after a single lymphocyte transfer.

In summary, in this article we have shown, we believe for the first time, that a CD8-mediated CTL immune response to BA46 can be induced in vivo. The CTL reactivity to BA46 peptides is HLA-A2.1 restricted as well as tumor specific. Our results suggest a potential role for BA46-6 and BA46-7 as tumor-associated antigen peptides in breast carcinoma and possibly in other tumors, too. Further study of the antigenicity of these peptides in various human cancer cell lines, as well as the nature of the BA46 protein itself, is warranted for designing proper immunotherapeutic protocols.

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