Identification and Characterization of ErbB-3-Binding Protein-1 as a Target for Immunotherapy[1]

Saskia J. A. M. Santegoets,* Marco W. J. Schreurs,* Anneke W. Reurs,* Jelle J. Lindenberg,[†] Esther W. M. Kueter,* Alfons J. M. van den Eertwegh,[†] Erik Hooijberg,* Ricardo J. Brandwijk,[‡] Simon E. Hufton,[2‡] Hennie R. Hoogenboom,[2‡] Rik J. Scheper,[3]* Veerle A. Somers,[4,5‡] and Tanja D. de Gruijl[5†]

Based on immune reactivity in response to a whole-cell colon tumor vaccine and using serological identification of Ags by recombinant cDNA expression cloning, we here describe the molecular and functional identification of a novel human tumor Ag. By screening a cDNA expression library derived from the coloncarcinoma cell line HT-29 with pooled colorectal cancer patients' sera, 26 clones reactive with IgG Abs could be identified. Characterization of these cDNA clones by sequence analysis and alignment, and detailed serological analysis revealed cancer-related immunoreactivity for the ErbB-3-binding protein-1 (Ebp1). Immunohistochemical staining of colorectal tumors and neighboring normal colon tissue indicated the observed cancer-related immunogenicity of Ebp1 to be related to overexpression. Via reverse immunology, five potential HLA-A2-restricted T cell epitopes were identified, of which two ($Ebp1_{45-54}$ and $Ebp1_{59-67}$) bound HLA-A2 with intermediate and high affinity, respectively. Analysis of their immunogenicity in vitro indicated that only the high-affinity $Ebp1_{59}$ epitope gave rise to CD8$^+$ T cells capable of recognizing both exogenously loaded Ebp1 peptide and endogenously expressed Ebp1 on target cells. In addition, in vivo CD8$^+$ T cell responsiveness against the $Ebp1_{59}$ epitope could be detected in two of nine and three of six cancer patients PBMC and tumor draining lymph nodes, respectively, but not in nine of nine healthy donors tested. These data confirm that Ebp1 is an immunogenic protein, capable of eliciting CD8-mediated responses in vivo and in vitro, providing a rationale for further exploration of Ebp1 as a possible target for anticancer immunotherapy. *The Journal of Immunology,* 2007, 179: 2005–2012.

A requisite for successful application of immunotherapeutic approaches like dendritic cell (DC)[6] vaccinations, adoptive transfer of Ag-specific CTL, or Ab infusions for the treatment of cancer, is the recognition of tumor-associated Ags (TAA) by the immune system. The identification of TAA that distinguish normal cells from cancer cells is an important challenge in the field of tumor immunology. Attempts to identify TAA have relied on both T cell- and B cell-mediated responses because immune recognition is a concerted action between the cellular and humoral immune response. The analysis of the T cell repertoire of cancer patients by making use of tumor cell variants and cytotoxic T cell clones and the screening of tumor-derived genomic and cDNA expression libraries has led to the identification of several tumor Ags like MAGE-1, BAGE, GAGE, MART-1/melan-A, and tyrosinase (1–4). However, for many tumors other than melanoma, these approaches have been less successful, particularly for tumors from epithelial origins such as colorectal cancer (CRC). In 1995, Sahin and coworkers (5–7) introduced an alternative strategy to identify immunogenic tumor proteins, without the need to establish tumor cell lines or clone and expand tumor-reactive T cells. This approach, called serological identification of Ags by cDNA expression cloning (SEREX), allows for the direct molecular identification of novel tumor Ags that elicit IgG Ab responses in cancer patients. The screening of prokaryotically expressed cDNA libraries derived from tumor specimens with patient serum, has led to the identification of several novel tumor Ags like NY-ESO-1 (5) and SSX2/HOM-MEL-40 (6). Moreover, Ags such as MAGE-1 and tyrosinase, which had already been defined by their T cell reactivity (5), could also be identified by SEREX methodology, demonstrating the validity of this technology (see also www2.licr.org/CancerImmunomeDB).

Because immunotherapeutically relevant CRC-related Ags are still scarce, we set out to screen a cDNA expression library, derived from the colon carcinoma cell line HT-29, with sera from CRC patients to identify novel Ags that are new potential candidates for immunotherapeutic application. Sequence analysis, sequence alignment in GenBank basic local alignment search tool homology search, and detailed serological analysis of cDNA clones against which seroreactivity was observed, revealed cancer-related immunoreactivity for the ErbB-3-binding protein-1 (Ebp1).

To determine T cell immunogenicity of Ebp1, the reverse immunology approach was undertaken. Using this approach, we were able to identify an HLA-A2-restricted Ebp1-derived CTL epitope, capable of eliciting CD8$^+$ T cell-mediated responses both in vivo

and in vitro, indicative of a possible usefulness of this tumor Ag in antitumor immunotherapy.

Materials and Methods

Cell lines

The HLA-A2$^+$, TAP-deficient cell line T2 was cultured in IMDM (BioWhittaker) supplemented with 10% FCS (Perbio), 100 IE/ml sodium penicillin (Yamanouchi Pharma), 100 μg/ml streptomycin sulfate (Radiumfarma-Fisiopharma), 2.0 mM L-glutamine (Invitrogen Life Technologies), and 0.01 mM 2-ME (Merck) (complete medium). The breast cancer (BrCa) cell line MCF-7 (HLA-A2$^+$), coloncarcinoma cell line HT-29 (HLA-A2$^-$), and the prostate cancer cell line PC-3 (HLA-A2$^-$) (all from American Type Culture Collection) were all cultured in DMEM (BioWhittaker) complete medium.

Serum samples

After informed consent, sera were obtained from CRC patients and healthy individuals. These included serum samples obtained from 10 CRC patients who had undergone active specific immunotherapy (ASI) with autologous tumor cell preparations (8), 10 nonimmunized CRC patients, and 10 healthy individuals.

Sera used for immunoscreening were preabsorbed by repeated passage through columns of Sepharose 6 MB coupled to lysates of *Escherichia coli* Y1090 and phage λ gt11 to remove Abs reactive with Ags related to the phage-host infection. Subsequent to these absorption steps, 1/10 serum dilutions were prepared in 5% nonfat dried milk/TBS and stored at −20°C.

cDNA expression library

A cDNA library, constructed from the colon carcinoma cell line HT-29 and unidirectionally cloned into the UniZAP XR vector (2×10^6 primary recombinants) was obtained commercially (Stratagene) and packaged into phage particles (5, 9, 10).

Immunoscreening of cDNA expression library

Immunoscreening for the detection of reactive clones was performed with serum obtained from colorectal carcinoma patients as described (5). Briefly, *E. coli* transformed with recombinant λ UniZAP phages were plated onto NZY agar plates. Expression of recombinant proteins was induced with isopropyl β-D-thiogalactoside, and plaques were subsequently blotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk/TBS and incubated for 16 h with a 1/100 dilution CRC patient sera. For initial screening, sera from either 10 immunized CRC patients or 10 nonimmunized CRC patients were pooled. Serum Abs binding to recombinant proteins expressed in lytic phages were detected by incubating with a 1/1000 dilution of HRP-conjugated rabbit-anti-human IgG and visualized by staining with ECL (Amersham Biosciences).

Sequence analysis of identified Ags

Immunoreactive cDNA clones were isolated and subcloned and subsequently converted to pBluescriptSK phagemids (Stratagene) by in vivo excision and plasmid DNA was isolated using the Qiagen plasmid mini kit system (Qiagen). cDNA inserts were sequenced at CPRO Wageningen using ABI Prism (PerkinElmer) automated sequencers. Homology search was performed with basic local alignment search tool software of the National Center for Biotechnology Information (NCBI).

Immunohistochemistry

Tumor samples were fixed in neutral buffered formaldehyde and processed to paraffin according to standard procedures. Sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. Next, endogenous peroxidase activity was blocked using 0.3% (v/v) H_2O_2 in methanol for 30 min. After Ag retrieval in 10 mM citric acid (pH 6.0) for 10 min, the slides were first incubated with 2% normal swine serum (DakoCytomation) in PBS/1% BSA for 10 min. Subsequently, tissue sections were incubated overnight at 4°C with either 1/200 dilution (0.5%) of normal rabbit serum (DakoCytomation) or 1/200 dilution (0.76 mg/ml) of anti-Ebp1 polyclonal Ab (Upstate Biotechnology), followed by incubation with biotinylated swine anti-rabbit Igs (1/100) and streptavidin-HRP (1/500). Bound peroxidase was visualized with 0.4 mg/ml 3-amino-9-ethylcarbazole and 0.02% (v/v) H_2O_2 in 0.1 M NaAc (pH 5.0), nuclei were counterstained with hematoxylin, and the tissue preparations were mounted with Kaiser's glycerol gelatin (Merck).

Immunoblot analysis

Cells were lysed in PBS containing 1 mM EDTA, 1 mM PMSF, and 1% Nonidet P-40 for 2 min at 4°C, followed by an ultrasound boost. Cell fragments were removed by centrifugation at 14,000 rpm and supernatants were frozen at −20°C. Protein concentration was determined with a Bio-Rad protein assay. Cell lysates (20 μg) or control HeLa nuclear extract (10 μg) were fractionated on an 8% polyacrylamide slab gel and transferred onto a nitrocellulose membrane by electroblotting. After blotting, the filters were blocked for 3 h in block buffer containing PBS, 1% (w/v) BSA, 1% (w/v) milk powder, and 0.05% (v/v) Tween 20, followed by an overnight incubation with 1 μg/ml anti-Ebp1 polyclonal Ab (Upstate Biotechnology) in block buffer at 4°C. Immunoreactivity was visualized with peroxidase-conjugated swine anti-rabbit Igs (DakoCytomation) in block buffer, followed by staining with ECL.

Synthetic peptides and HLA-A2 binding assays

Peptides derived from Ebp1 protein that contain potential HLA-A2-binding motifs were selected using the SYFPEITHI database (11). Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II; MultiSyntech) using Fmoc-chemistry (Leiden University Medical Center, Leiden, The Netherlands). Peptides were >90% pure as analyzed by reversed-phase HPLC, dissolved in DMSO (Merck), and stored at −20°C.

Peptide binding to HLA-A2 was determined by an immunofluorescence-based T2 peptide binding assay and JY competition assay as described (12–14). Briefly, a T2 peptide binding assay was performed as follows: T2 cells were incubated with two-step dilutions (in a range of 100–6.25 μg/ml) of Ebp1-derived peptides for 14 h at 37°C in serum-free IMDM in the presence of 5 μg/ml β_2-microglobulin (Sigma-Aldrich). Stabilization of HLA-A2 molecules on T2 cells was determined by flow cytometry using the anti-HLA-A2 mAb BB7.2 (American Type Culture Collection). Background mean fluorescence values were measured by incubating T2 cells with DMSO at equal dilutions. The fluorescence index was expressed as follows: [experimental mean fluorescence/background mean fluorescence] − 1. The binding affinity of the peptides was also determined by JY competition assay (14). In brief, after elution of naturally HLA-A2-bound peptides by mild acid treatment, JY cells were incubated with different concentrations of the test (competitor) peptide in the presence of fluorescein-labeled reference peptide (FLPSDC[fl]FPSV) for 24 h at 4°C. Next, fluorescence intensity was measured by flow-cytometric analysis. Binding capacity of competitor peptides was determined as the concentration of peptide required to inhibit 50% of reference peptide binding. Peptides were considered to be high-affinity binders when $IC_{50} < 5$ μM, intermediate-affinity binders when 5 μM $< IC_{50} < 15$ μM, and low-affinity binders when $IC_{50} > 15$ μM.

Abs, tetramers, and flow cytometry

PE- or FITC-labeled Abs directed against human CD8α, CD107a, CD80, CD86 (BD Biosciences), and CD83 (Immunotech) were used for flow cytometric analysis. PE- and/or allophycocyanin-labeled HLA-A2 tetramers (Tm) with the Ebp1$_{45-54}$, and Ebp1$_{59-67}$ epitopes were prepared as described previously (15). Ab and/or tetramer staining was performed in PBS supplemented with 0.1% BSA and 0.02% natrium-azide for 30 min at 4°C and 15 min at 37°C, respectively. Stained cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. To exclude dead cells in flow cytometric tetramer analysis, 0.5 μg/ml propidium iodide (ICN Biomedicals) was used.

In vitro generation of DC

Monocyte-derived DC (MoDC) and MUTZ-3-derived DC were generated as described (16, 17).

Primary CTL induction in vitro

Ag-specific CD8$^+$ T cells were generated by repeated stimulation with peptide-loaded autologous HLA-A2$^+$ MoDC or allogeneic HLA-A2-matched MUTZ-3 DC as described (18, 19). One day before each restimulation, a sample was taken and analyzed for the presence of Ebp1-specific CD8$^+$ T cells by IFN-γ ELISPOT analysis or by flow cytometry using PE- and allophyco-cyanin-labeled Ebp1$_{59}$ tetramers. Ag-specific CD8$^+$ T cell lines were derived from 1000 cell/well cultures. For this purpose, CD8$^+$ T cells derived from the original bulk cultures were weekly stimulated with irradiated feeder mix consisting of allogeneic PBMC and JY cells in Yssel's medium supplemented with 100 ng/ml PHA (Murex Biotech) and 20 U/ml IL-2.

Chromium release assay

Cytotoxic activity of generated CD8$^+$ T cell lines was determined by standard chromium release assay as described (18).

Table I. *Genes identified after immunoscreening of CRC cell line HT-29 cDNA expression library with either nonimmunized or immunized CRC patient sera (SEREX)[a]*

Clone No.	Homology	NCBI/GenBank No.	Serum
HT-1[b]	ErbB-3 binding protein Ebp1	AAD00646	Immunized[c]
HT-6[b]	26S proteasome subunit p40.5	AAD43442	Immunized[c]
HT-11[b]	TATA box binding prot-assoc factor TAF2H	AAM14627	Immunized[c]
HT-16[b]	Unknown		Immunized[c]
HT-17[b]	Unknown		Immunized[c]
HT-18[b]	Unknown		Immunized[c]
HT-23[b]	EST	AA151686	Immunized[c]
HT-25[b]	Human keratin 18	AAA59461	Immunized[c]
HT-27[b]	Growth factor receptor-bound protein	AAC72075	Immunized[c]
HT-28[b]	Human elongation factor 1-α	P68104	Immunized[c]
HT-32	BAC clone CTA-276O3 from 7q22-q31.1	AC004668	Immunized[c]
HT-33[b]	Transmembrane 4 superfamily member 4	AAH01386	Immunized[c]
HT-7	Smad6	AAC82331	Nonimmunized[d]
HT-8	Ribosomal protein S18	CAB56794	Nonimmunized[d]
HT-9	Chlordecone reductase homolog	AAD14013	Nonimmunized[d]
HT-10	EST	AA402539	Nonimmunized[d]
HT-12	EST	AI810918	Nonimmunized[d]
HT-14	DNA-binding protein TAXREB107	BAA04491	Nonimmunized[d]
HT-15[b]	Euk transl init fact 3, subunit 4 mRNA	O75821	Nonimmunized[d]
HT-20	Ribosomal protein L23	BAB79465	Nonimmunized[d]
HT-21	EST	AU081097	Nonimmunized[d]
HT-24	GATA-binding protein GATA2	AAH15613	Nonimmunized[d]
HT-26	Spermidine synthase	NP003123	Nonimmunized[d]
HT-29	DKFZp761M2324	AAH25305	Nonimmunized[d]
HT-30	Ribosomal protein 5S	NP00100	Nonimmunized[d]
HT-31	LDL receptor gene	AAD14091	Nonimmunized[d]

[a] Abbreviations: EST, Expressed sequence tag; LDL, low density lipoprotein.
[b] Analyzed for reactivity against individual sera from healthy individuals and CRC patients.
[c] Ag identified by screening with sera obtained from CRC patients who had undergone ASI with autologous tumor cell preparations (8).
[d] Ag identified by screening with sera obtained from nonimmunized CRC patients.

CD107a membrane expression

Cytotoxic activity of CD8$^+$ T cells was also determined by a sensitive, flow cytometric degranulation assay, in which the potential for granule-dependent perforin/granzyme-mediated target cell killing was measured by the detection of cumulative expression of granular membrane protein CD107a on the cell surface of responding Ag-specific T cells (20, 21). For this purpose, Ebp1$_{59}$-specific CD8$^+$ T cells were stimulated with various target cells for 5 h at 37°C in a 1:1 ratio in the presence of anti-CD107a-PE (BD Biosciences) and 4 μM monensin (Sigma-Aldrich). Following stimulation, cells were washed, stained with allophycocyanin-labeled tetramer and FITC-labeled anti-CD8 mAb, respectively, and analyzed by flow cytometry.

Intracellular IFN-γ staining

To determine the capacity of the CD8$^+$ T cell lines to produce IFN-γ upon recognition of a specific target, intracellular IFN-γ staining was performed. CD8$^+$ T cells were cultured with target cells at an E:T ratio of 2:1 in the presence of 0.5 μl of GolgiPlug (BD Biosciences) After 5 h, cells were harvested and stained with tetramer and anti-CD8 mAb. After fixation and permeabilization by using a fixation/permeabilization kit (Cytofix/Cytoperm; BD Biosciences), cells were labeled with anti-IFN-γ Ab (BD Biosciences) and analyzed on a FACSCalibur.

Blood and lymph node samples

Heparinized blood samples were obtained from HLA-A2$^+$ CRC and BrCa patients and PBMC were isolated by centrifugation on Ficoll-Hypaque gradient (Pharmacia) and subsequently cryopreserved in liquid nitrogen until analysis. Tumor-draining lymph node (TDLN) material was obtained from stage III BrCa patients undergoing surgical resection of the tumor. Pathological evaluation revealed lymph nodes to be tumor positive. These materials were obtained after informed consent in accordance with guidelines of the local ethical committee.

Detection of Ebp1-specific CD8$^+$ T cells in cancer patients

The presence of Ebp1-specific CD8$^+$ T cells in either peripheral blood or tumor-involved lymph nodes of HLA-A2$^+$ CRC and BrCa patients was analyzed by IFN-γ ELISPOT assay as described (22, 23). In brief, microbead-isolated untouched CD8$^+$ T cells, obtained from lymph node dis-

sociates upon anti-CD3/anti-CD28-mediated expansion, as described (24), were stimulated overnight in an ELISPOT plate with T2 cells loaded with control peptide or the relevant peptide, while PBMC were directly incubated with 50 μg/ml peptide in the presence of β_2-microglobulin. Each sample was tested in duplicate or triplicate, and the response was expressed as the number of spots per 100,000 effector cells. A response was considered to be specific when the mean number of spots was at least 10 per 100,000 cells and when the p value of the relevant peptide vs the control peptide was <0.05 in a one-sided t test.

Statistical analysis

Frequencies of reactive sera between the test groups were compared using a two-sided Fisher's Exact test. Differences were considered significant when $p < 0.05$.

Results

SEREX analysis of the HT-29 cDNA library

A cDNA expression library of 2×10^6 primary clones was obtained from the CRC cell line HT-29 and subsequently immunoscreened with pooled sera obtained from either nonimmunized CRC patients or CRC patients that had been immunized with autologous whole-cell tumor vaccines in a 1/100 dilution. Of a total of 6.8×10^5 plaques tested, 85 were found to be reactive with serum IgG Abs. After secondary and tertiary screening, 26 positive clones were identified. These clones were selected for further analysis. After purification, excision, and isolation, the cDNA inserts were analyzed by restriction enzyme analysis. Subsequent DNA sequencing led to the identification of 19 genes with homology to known gene products and 7 genes without homology to any functionally annotated sequence in public databases (Table I).

Table II. *Analysis of Ebp1 for immunoreactivity with multiple sera using SEREX*

Reactivity for Ebp1 in SEREX	Sera		
	Number of immunized CRC[a]	Number of nonimmunized CRC[b]	Number of healthy controls[c]
− (negative)	1	0	9
+ (positive)	6	8	1
++ (high)	2	2	0
+++ (very high)	1	0	0
Total positive	9/10[d]	10/10[e]	1/10

[a] Sera from ASI-treated CRC patients.
[b] Sera from nonimmunized CRC patients.
[c] Sera from healthy controls.
[d] Vs healthy control sera, $p = 0.001$, two-sided Fisher's Exact test.
[e] Vs healthy control sera, $p = 0.0001$, two-sided Fisher's Exact test.

Detailed serological analysis of SEREX-defined Ags

To determine whether the obtained cDNA clones showed cancer-related immunogenicity, sera from 10 CRC patients who had been vaccinated with autologous tumor cells, sera from 10 nonimmunized CRC patients, and sera from 10 healthy controls were tested for their reactivity against the selected Ags (as indicated in Table I). Of the 12 Ags screened, 11 reacted with sera from both healthy individuals and CRC patients and showed no clear tumor-related immunogenicity (data not shown). Ebp1 was defined as having a cancer-related serological profile because this clone reacted preferentially with sera from CRC patients compared with sera from healthy controls (Table II). Of note, Ebp1 reactivity was found at high frequencies both in vaccinated and in nonvaccinated CRC patients, indicating its natural immunogenicity and apparently high prevalence in patients with colon cancer. Moreover, comparing sera from vaccinated and nonvaccinated patients revealed that no differences were observed in the strength of the Ebp1 Ab response, further emphasizing the natural immunogenicity of Ebp1.

Ebp1 expression in normal and malignant colon

To determine the mechanism behind the observed colon cancer-related immunogenicity as defined by IgG seroreactivity profiling, immunohistochemical analysis of colorectal tumors and neighboring healthy tissue as an internal control was performed. Staining of paraffin-embedded tissue sections with anti-Ebp1 polyclonal Ab revealed that Ebp1 is expressed in the cytoplasm and that Ebp1 is overexpressed in colorectal tumor fields compared with neighboring normal colon tissue in eight of eight samples tested (Fig. 1).

Selection of HLA-A2-resticted peptides derived from Ebp1

It is generally accepted that immune recognition of tumor Ags is a concerted action between the cellular and humoral immune response. The development of high-titer Abs requires $CD4^+$ T cell help, and the identification of potential tumor Ags using SEREX methodology requires high IgG Ab levels. To determine whether Ebp1 can serve as a target for cell-mediated immunotherapy and is able to trigger $CD8^+$ T cell responses in vitro and in vivo, putative Ebp1-specific, HLA-A2-restricted epitopes were selected and analyzed for their immunogenicity. By screening the Ebp1 amino acid sequence using computer algorithms, five potential HLA-A2-binding sequences with high predicted HLA-A2-binding scores were selected and checked for their actual capacity to bind to HLA-A2 by T2-binding assay and JY competition assay. From the five peptides analyzed, only Ebp1$_{45}$ and Ebp1$_{59}$ were able to stabilize HLA-A2 expression, indicating affinity for HLA-A2 (Table
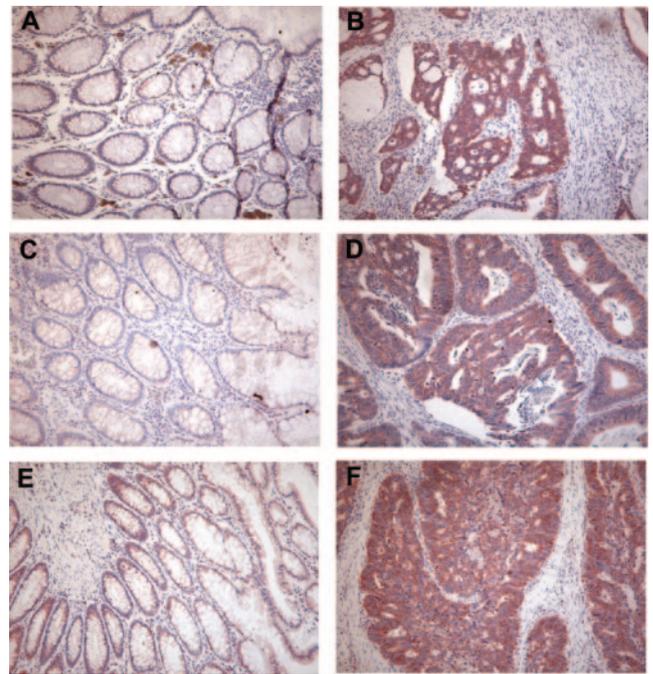

FIGURE 1. Ebp1 staining of colorectal tumors and accessory healthy tissue reveals strong overexpression in tumor cells. Formalin-fixed, paraffin-embedded tissue preparations of tumor samples were stained with anti-Ebp1 polyclonal Ab. *A–F*, Ebp1 expression is shown in colorectal tumor fields (*B*, *D*, and *F*) and accessory healthy tissue (*A*, *C*, and *E*). Data shown are three representative patients of eight patients analyzed. Colorectal tumor fields and adjoining healthy tissue shown are from the same slides.

III). For these two peptides, binding affinity was confirmed by JY competition assay. Consequently, Ebp1$_{45-54}$ and Ebp1$_{59-67}$ peptides, with an intermediate and high HLA-A2 binding affinity, respectively, were selected to further study their immunogenicity in vitro and in vivo.

Induction of human $CD8^+$ T cell responses against Ebp1 in vitro

To investigate the immunogenicity of the two selected HLA-A2-binding Ebp1-derived epitopes, the ability of the peptides to induce a $CD8^+$ T cell response in vitro was determined. For this purpose, $CD8\beta^+$ CTL precursors isolated from PBMC of HLA-A2$^+$

Table III. *Affinity for HLA-A2 of Ebp1-derived peptides[a]*

Epitope	Sequence[b]	Rammensee Score[c]	T2 Binding Capacity[d]	JY Competition Assay IC$_{50}$ (μM)[e]
1–9	MIMEETGKI	23	−	n.t.
44–52	YILKEGDLV	22	−	n.t.
45–54	ILKEGDLVKI	29	+	12.5
50–58	DLVKIDLGV	21	−	n.t.
59–67	HVDGFIANV	23	+	2

[a] Abbreviation: n.t., Not tested.
[b] Single-letter abbreviations for amino acids.
[c] Determined at http://sypheiti.bmiheidelberg.com/Scripts/MHCServer.dll/EpPredict.html.
[d] As determined by HLA-A2 stabilization at 50 μM peptide. Peptide scored positive when the fluorescence intensity that is expressed as follows: [(experimental mean fluorescence/background mean fluorescence) − 1] ≥ 1 in two independent experiments.
[e] HLA-A2 binding affinity was determined in a JY competition assay. Increasing amounts of competitor (test) peptide was used to inhibit HLA-A2 binding of a FITC-labeled reference peptide to determine the concentration of peptide required to inhibit 50% of reference peptide binding (IC$_{50}$).
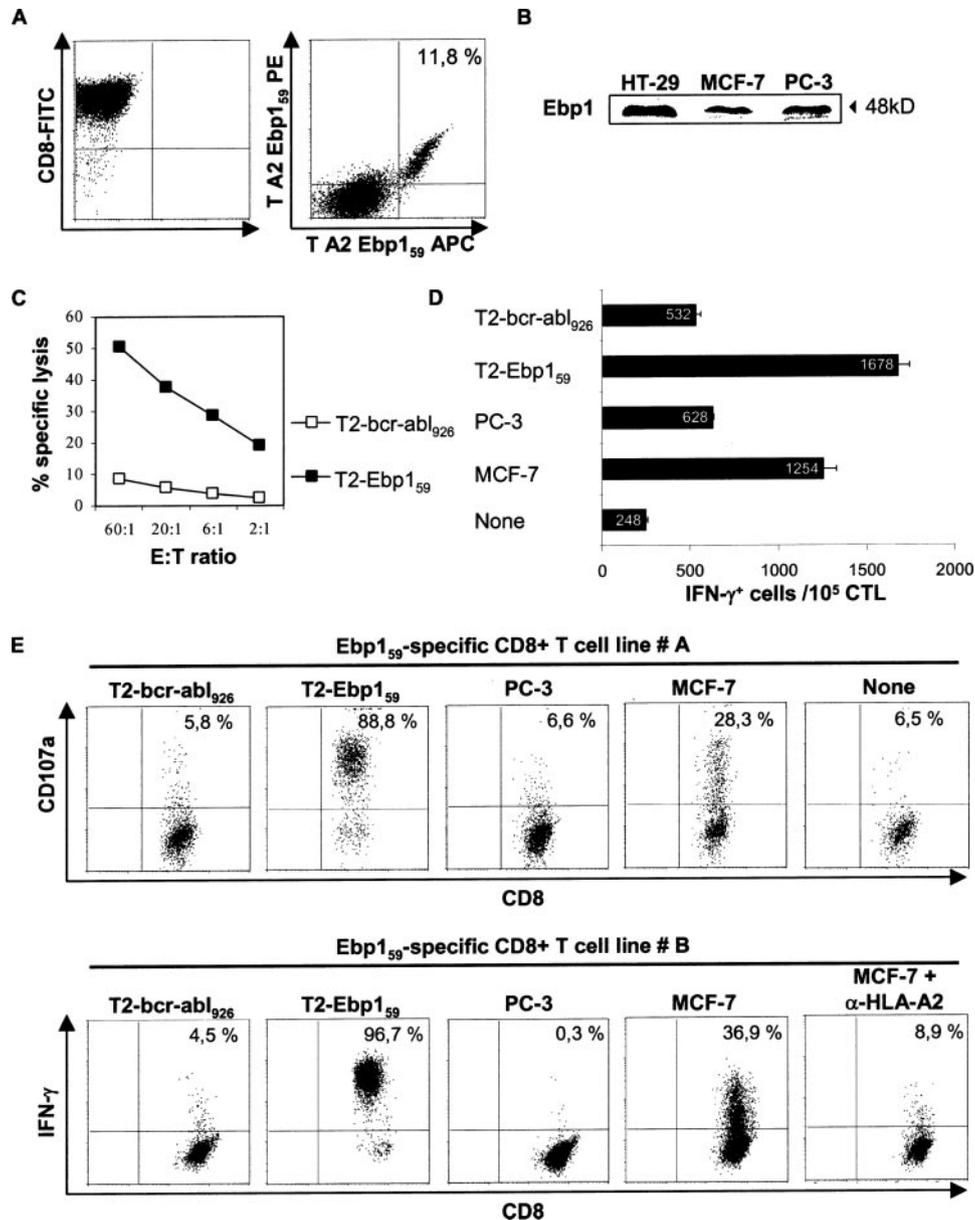
FIGURE 2. Immunogenicity of the Ebp1$_{59}$ epitope in vitro. Ebp1$_{59}$-specific CD8$^+$ T cell lines, obtained from CD8β^+ CTL precursors from two healthy donors after repeated in vitro stimulations with peptide-loaded MoDC of MUTZ-3 DC, were analyzed for their Ebp1$_{59}$ specificity by flow cytometric HLA-A2$^+$ Tm binding analysis and functionally by chromium release, IFN-γ ELISPOT, and CD107a flow cytometric degranulation assay. A, Flow cytometric analysis of an Ebp1$_{59}$-specific CD8$^+$ T cell line A upon staining with FITC-labeled CD8 and PE/allophycocyanin-labeled tetramer staining. Staining with both PE- and allophycocyanin-labeled Tm was performed to exclude false positive background staining by single Tm. Propidium iodide-negative, viable cells are shown. In all subsequent functional tests, the following target cells were used as follows: HLA-A2$^+$ T2 cells loaded with the Ebp1$_{59}$ peptide or the HLA-A2-restricted control peptide bcr-abl$_{926}$ and the Ebp1-expressing tumor cell lines MCF-7 (HLA-A2$^+$) and PC-3 (HLA-A2$^-$). B, Ebp1 expression in the tumor target cell lines MCF-7 and PC-3 was confirmed by Western blot analysis. HT29 was included and shown as a positive control. However, due to ubiquitous but low expression of Ebp1 in normal healthy tissues, a negative control sample was not included. C, Ebp1$_{59}$ peptide-specific cytolytic activity of CD8$^+$ T cell line A was determined against peptide-loaded T2 cells in a chromium release assay. D and E, Specific recognition of Ebp1$_{59}$, both exogenously loaded onto T2 cells and endogenously expressed and processed in MCF-7 for HLA-A2-associated presentation, was shown by IFN-γ ELISPOT assay for CD8$^+$ T cell line A (results expressed as the number of IFN-γ spots per 100,000 total CD8$^+$ effector cells) (D) and by CD107a flow cytometric degranulation assay (i.e., CD8$^+$ T cell line A; top) and intracellular IFN-γ staining (i.e., CD8$^+$ T cell line B; bottom) of two individual Ebp1$_{59}$-specific T cell lines (E). The events shown were gated for live tetramer-Ebp1$_{59}$$^+$CD8$^+$ T cells, and the percentages of CD8$^+$CD107a$^+$ T cells of CD8$^+$IFN-γ^+ T cells are shown in the upper right quadrants.

healthy donors were weekly stimulated with mature, peptide-pulsed DC. From the second round of stimulation, CD8$^+$ T cell cultures were analyzed for the expansion of Ebp1-specific CD8$^+$ T cells through IFN-γ ELISPOT or flow cytometric HLA-tetramer analysis. In four of five healthy donors tested, Ebp1$_{59}$-specific CD8$^+$ T cells could be detected after three in vitro stimulation rounds, whereas Ebp1$_{45}$-specific CD8$^+$ T cells could not be detected in four of the four donors tested (data not shown). In con-

clusion, the Ebp1$_{59}$ CTL epitope with high HLA-A2-binding affinity was shown to be immunogenic in vitro in multiple donors.

Functional analysis of in vitro-primed Ebp1$_{59}$-specific CD8$^+$ T cells

To further analyze the functional activity of the Ebp1$_{59}$-specific CD8$^+$ T cells and to address whether the Ebp1$_{59}$ epitope is naturally processed by cells expressing the Ebp1 endogenously,
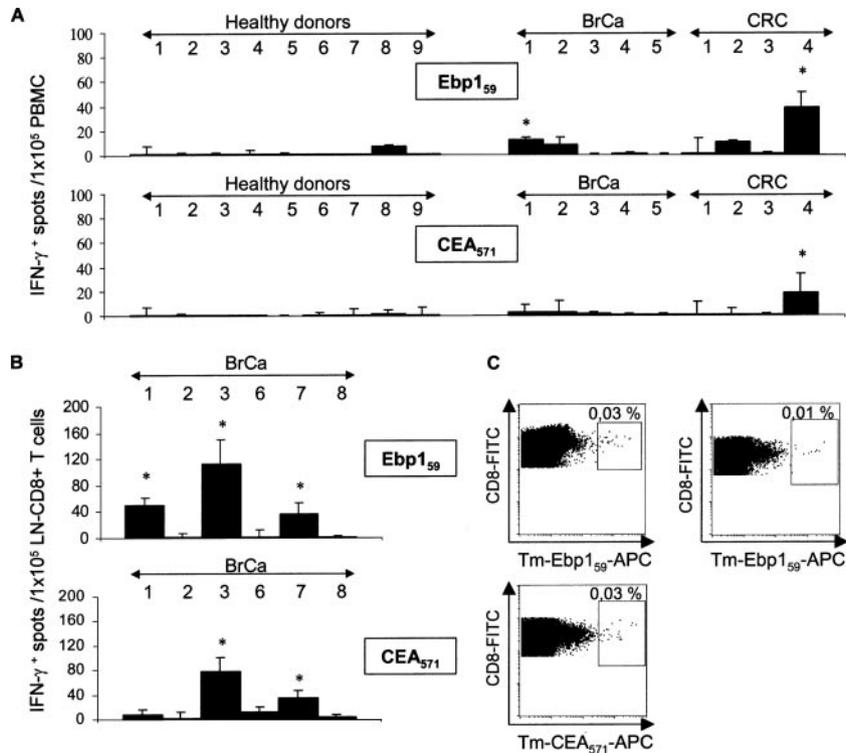
FIGURE 3. Detection of Ebp1-specific $CD8^+$ T cells in HLA-A2$^+$ CRC and BrCa patients. The presence of Ebp1-specific $CD8^+$ T cells in HLA-A2$^+$ BrCa (BrCa 1–5) and CRC (CRC 1–4) patients was analyzed by IFN-γ ELISPOT. *A* and *B*, Data shown from PBMC from nine healthy donors, five BrCa and four CRC patients (*A*) and $CD8^+$ T cells, which were obtained by lymph node dissociation and anti-CD3/anti-CD28-mediated expansion from six BrCa patients (BrCa 1–3 and 6–8) for the Ebp1$_{59}$ epitope (*B*, *top panel*) and CEA$_{571}$ epitope (*B*, *lower panel*). PBMC and $CD8^+$ T cells were stimulated overnight with either peptide alone or peptide-loaded T2 cells. The response is expressed as the number of spots minus the number of background spots per 100,000 effector cells. A response was considered to be specific, when the mean number of spots was at least 10 per 100,000 cells and when the *p* value of the relevant peptide vs the control peptide was <0.05 in a one-sided *t* test. Specific response is indicated by an asterisk (*) with given *p* value. Note: For BrCa patients 1–3, both PBMC (*A*) and TDLN-derived $CD8^+$ T cells (*B*) have been analyzed (with corresponding numbering), whereas BrCa PBMC samples 4 and 5 and TDLN samples 6–8 were all obtained from different patients. *C*, TDLN-derived $CD8^+$ T cells were also analyzed for the presence of Ebp1$_{59}$ and CEA$_{571}$-specific T cells by HLA-A2-guided tetramer analysis. The events shown were gated for live cells and propidium iodide-negative population. The percentage of $CD8^+$tetramer$^+$ cells are depicted in the *upper right corner*.

Ebp1$_{59}$-specific $CD8^+$ T cell lines (from 1000 cell/well bulk cultures) were expanded from two individual donors and tested for Ebp1 specificity by tetramer analysis, standard chromium release assay, IFN-γ ELISPOT, and CD107a flow cytometric degranulation assay. Tetramer analysis revealed TCR specificity for the Ebp1$_{59}$ epitope in the tested $CD8^+$ T cell lines at 11.8% (i.e., T cell line A; Fig. 2*A*) and 50% (i.e., T cell line B; data not shown). Functional analysis by chromium release assay revealed that the obtained $CD8^+$ T cell lines were able to specifically lyse Ebp1$_{59}$-loaded target cells, as shown for T cell line A in Fig. 2*C*. In addition, IFN-γ ELISPOT further showed that the Ebp1$_{59}$-specific $CD8^+$ T cells were able to produce IFN-γ upon recognition of HLA-A2$^+$ target cells, exogenously loaded with Ebp1$_{59}$ peptide, but not when loaded with an irrelevant peptide as shown for T cell line A in Fig. 2*D*. To assess whether the Ebp1$_{59}$-specific $CD8^+$ T cells were capable of recognizing endogenously processed and presented Ebp1, their capacity to recognize Ebp1$^+$HLA-A2$^+$ MCF-7 tumor cells was determined by IFN-γ ELISPOT, CD107a flow cytometric degranulation assay, and intracellular IFN-γ staining. Target cells used were the BrCa cell line MCF-7 and prostate cancer cell line PC-3, in both of which Ebp1 expression was confirmed by Western blot analysis (Fig. 2*B*). The recognition of the Ebp1$^+$HLA-A2$^+$ cell line MCF-7, but not the Ebp1$^+$HLA-A2$^-$ cell line PC-3, demonstrated the correct endogenous processing and expression of the Ebp1$_{59}$ epitope in HLA-A2$^+$ tumor cells, as well as the ability of the generated $CD8^+$ T cells to react to tumor

cells expressing endogenous Ebp1 in an HLA-A2-restricted manner (i.e., $CD8^+$ T cell line A in Fig. 2, *D* and *E*, *top panel*, and $CD8^+$ T cell line B in Fig. 2*E*, *lower panel*). Importantly, CD107a mobilization of the Ebp1$_{59}$ tetramer-negative $CD8^+$ T cells was determined to be at background level (data not shown). In addition, incubating the tumor cells with an anti-HLA-A2 Ab inhibited $CD8^+$ T cell reactivity almost completely, confirming that the recognition was indeed HLA-A2 restricted and not mediated by NK cells (shown for $CD8^+$ T cell line B in Fig. 2*E*). In conclusion, the Ebp1$_{59}$ epitope is an immunogenic, naturally processed epitope capable of inducing specific and functional effector $CD8^+$ T cells in vitro from healthy donor-derived $CD8\beta^+$ CTL precursors.

Detection of Ebp1$_{59}$-specific $CD8^+$ T cells in BrCa and CRC patients

To explore the in vivo immunogenicity of Ebp1, the presence of Ebp1-specific $CD8^+$ T cell responses in cancer patients was tested. Because Ebp1 has previously also been described to be expressed in BrCa cells (25), BrCa were also analyzed. To this end, PBMC and/or TDLN-derived $CD8^+$ T cells obtained from HLA-A2$^+$ patients were analyzed for the presence of $CD8^+$ T cells directed against the HLA-A2-restricted Ebp1$_{59}$ epitope and the immunodominant epitope derived from the well-known colon and breast carcinoma-associated Ag CEA, CEA$_{571}$. First, in an ex vivo, overnight IFN-γ ELISPOT assay, $CD8^+$ T cell responses could be detected against the Ebp1$_{59}$ epitope in the PBMC of one of five

BrCa patients and one of four CRC patients tested, whereas no $CD8^+$ T cell responses could be detected against the CEA_{571} epitope (Fig. 3A). Importantly, no $Ebp1_{59}$- and CEA_{571}-specific $CD8^+$ T cells could be detected in PBMC from nine $HLA-A2^+$ healthy donors (Fig. 3A), indicating Ebp1's tumor-associated specificity.

To determine whether local priming of $Ebp1_{59}$-specific $CD8^+$ T cells might occur in TDLN, we also analyzed TDLN material from six BrCa patients for the presence of $Ebp1_{59}$ and CEA_{571}-specific $CD8^+$ T cells. As shown in Fig. 3B, IFN-γ ELISPOT analysis revealed that $Ebp1_{59}$- and CEA_{571}-specific $CD8^+$ T cells could be detected in TDLN of three of six and two of six BrCa patients, respectively. However, detection of these $Ebp1_{59}$- and CEA_{571}-specific $CD8^+$ T cells could not be confirmed by HLA-A2-guided tetramer analysis. Although IFN-γ ELISPOT revealed the presence of $Ebp1_{59}$ and CEA_{571}-specific $CD8^+$ T cells in TDLN, the percentages were generally low, ranging between 0.03 and 0.1% (i.e., 34–100 spots per 100,000 cells). Because tetramer analysis is relatively insensitive with a detection limit of 0.05–0.1%, these low frequencies are hardly detectable. Indeed, as also shown in Fig. 3C, the percentage of tetramer-positive cells ranged between 0.01 and 0.03% (Fig. 3C). Of note, comparing TDLN and peripheral blood samples revealed that, in BrCa patient 1, $Ebp1_{59}$-specific $CD8^+$ T cells could be detected in both lymph node and blood, whereas in BrCa patient 3, $Ebp1_{59}$-specific $CD8^+$ T cells could only be detected in the TDLN. In summary, the detection of $Ebp1_{59}$-specific $CD8^+$ T cells in cancer patients' PBMC and TDLN indicates the tumor-related immunogenic potential of Ebp1.

Discussion

The identification of Ags that distinguish normal cells from cancer cells is an important challenge in the field of tumor immunology and immunotherapy. In this study, we report on the identification and characterization of a novel tumor Ag by analyzing the humoral immune response of CRC patients using SEREX methodology. By screening a colon carcinoma cell line-derived cDNA expression library with sera obtained from CRC patients, we were able to identify several Ags of which the ErbB-3-binding protein Ebp1 showed cancer-related specificity. Undertaking the reverse immunology approach, we were able to identify an Ebp1-derived CTL epitope, which is endogenously processed and presented by tumor cells in the context of HLA-A2.

Ebp1 was first described by Yoo et al. (26) as a modulator of the ErbB-3 signal transduction pathway, mediating the biological effects of heregulin. In recent years, it has become clear that Ebp1 is involved in multiple signal transduction pathways and plays a role in cellular proliferation and differentiation processes (25–30). The involvement of Ebp1 in these cell cycle processes and the observation that, in 20.9% of all CRC patients, the *Ebp1* gene carries a coding region frameshift mutation caused by microsatellite instability, point to a possible role of (mutated) Ebp1 in human carcinogenesis.

An important aspect in defining a novel tumor Ag is determining the mechanism by which this potential tumor Ag can elicit an immune response in the autologous host. As described previously (26, 31), Ebp1 is expressed in a wide range of tumor cell lines but also in various normal human tissues, i.e., lung, pancreas, prostate, kidney, placenta, liver, and breast. This ubiquitous expression pattern and the observed increase in Ebp1 expression in colorectal tumor fields compared with neighboring normal colon tissue, as observed by immunohistochemical analysis in all colorectal tumors tested, indicate that overexpression is the most likely mechanism of immunogenicity observed in CRC for the novel Ag Ebp1. However, screening of a larger group of CRCs is needed to deter-

mine the actual prevalence of overexpression of Ebp1 in CRC. Yet, the observation that 19 of 20 CRC patients (either immunized or nonimmunized) mounted a serological immune response against Ebp1, suggests that Ebp1 is prevalent in CRC. Whether Ebp1 is overexpressed in other epithelial tumors, such as BrCa, remains to be determined. Even though overexpression in BrCa tissue could not be confirmed by immunohistochemistry in our hands (data not shown), the detection of Ebp1-specific $CD8^+$ T cell reactivity in BrCa patients' PBMC and lymph node demonstrates the absence of tolerance and the in vivo immunogenicity of Ebp-1. This was further supported by the observation that the precursor frequency of Ebp1-specific $CD8^+$ T cells is relatively high, given our ability to generate $Ebp1_{59}$-specific $CD8^+$ T cells in 9 of 24 cultures from two different healthy donors ($1 \times 10E6$ $CD8^+$ T cells/culture; data not shown). In addition, the observed lack of functional $Ebp1_{59}$-specific T cell responses in peripheral blood of healthy donors provides evidence for Ebp1's tumor-related immunogenicity.

The presence of $Ebp1_{59}$-specific $CD8^+$ T cells in the blood of cancer patients suggested the possibility of augmentation of such responses by vaccination. However, we were unable to detect any $Ebp1_{59}$-specific $CD8^+$ T cell responses in three HLA-A2-positive ASI patients who were available for testing. Yet, the observation that Ebp1-specific $CD8^+$ T cells had been generated in vivo in BrCa and CRC patients is indicative of a possible application of Ebp1 as a target for anticancer immunotherapy. Because Ebp1 is expressed at low level by normal healthy tissues, such induction of a potent immune response to this self-Ag might lead to the induction of autoimmunity, as also described for other self-Ags (32, 33). Whether autoimmunity will be induced when Ebp1 will be used as a target for anticancer immunotherapy is difficult to predict. Phase I clinical trials, in which tumor cells are targeted through self-proteins, like p53 and CEA, indicate that vaccination with self-protein pulsed DC can be safe and without severe toxicity (34–36). However, in terms of CTL responses, it is not known generally what level of self-Ag expression/presentation is required to render cells sensitive for immune recognition. Therefore, further in vivo research in animal models is necessary. The fact that human Ebp1 is highly homologous to murine Ebp1 with comparable expression patterns (31, 37) indicates the potential value of murine models to study this for Ebp1.

In conclusion, by screening a cDNA expression library from a CRC cell line with patients' sera, we were able to isolate and characterize Ebp1 as a novel immunogenic tumor Ag, capable of eliciting $CD8^+$ T cell-mediated responses both in vivo and in vitro, confirming its possible usefulness in anticancer immunotherapeutic therapies.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki, and S. A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA* 91: 3515–3519.
2. van der, B. P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, E. B. Van den, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254: 1643–1647.
3. Coulie, P. G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J. P. Szikora, et al. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180: 35–42.

4. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 178: 489–495.

5. Sahin, U., O. Tureci, H. Schmitt, B. Cochlovius, T. Johannes, R. Schmits, F. Stenner, G. Luo, I. Schobert, and M. Pfreundschuh. 1995. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc. Natl. Acad. Sci. USA* 92: 11810–11813.

6. Tureci, O., U. Sahin, I. Schobert, M. Koslowski, H. Scmitt, H. J. Schild, F. Stenner, G. Seitz, H. G. Rammensee, and M. Pfreundschuh. 1996. The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res.* 56: 4766–4772.

7. Chen, Y. T., M. J. Scanlan, U. Sahin, O. Tureci, A. O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh, and L. J. Old. 1997. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. USA* 94: 1914–1918.

8. Vermorken, J. B., A. M. Claessen, H. van Tinteren, H. E. Gall, R. Ezinga, S. Meijer, R. J. Scheper, C. J. Meijer, E. Bloemena, J. H. Ransom, et al. 1999. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. *Lancet* 353: 345–350.

9. Hufton, S. E., P. T. Moerkerk, E. V. Meulemans, A. de Bruine, J. W. Arends, and H. R. Hoogenboom. 1999. Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. *J. Immunol. Methods* 231: 39–51.

10. Somers, V. A., R. J. Brandwijk, B. Joosten, P. T. Moerkerk, J. W. Arends, P. Menheere, W. O. Pieterse, A. Claessen, R. J. Scheper, H. R. Hoogenboom, and S. E. Hufton. 2002. A panel of candidate tumor antigens in colorectal cancer revealed by the serological selection of a phage displayed cDNA expression library. *J. Immunol.* 169: 2772–2780.

11. Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50: 213–219.

12. Nijman, H. W., J. G. Houbiers, M. P. Vierboom, S. H. van der Burg, J. W. Drijfhout, P. D'Amaro, P. Kenemans, C. J. Melief, and W. M. Kast. 1993. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. *Eur. J. Immunol.* 23: 1215–1219.

13. Kessler, J. H., B. Mommaas, T. Mutis, I. Huijbers, D. Vissers, W. E. Benckhuijsen, G. M. Schreuder, R. Offringa, E. Goulmy, C. J. Melief, et al. 2003. Competition-based cellular peptide binding assays for 13 prevalent HLA class I alleles using fluorescein-labeled synthetic peptides. *Hum. Immunol.* 64: 245–255.

14. van der Burg, S. H., E. Ras, J. W. Drijfhout, W. E. Benckhuijsen, A. J. Bremers, C. J. Melief, and W. M. Kast. 1995. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells: identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. *Hum. Immunol.* 44: 189–198.

15. Heemskerk, M. H., E. Hooijberg, J. J. Ruizendaal, M. M. van der Weide, E. Kueter, A. Q. Bakker, T. N. Schumacher, and H. Spits. 1999. Enrichment of an antigen-specific T cell response by retrovirally transduced human dendritic cells. *Cell. Immunol.* 195: 10–17.

16. Masterson, A. J., C. C. Sombroek, T. D. De Gruijl, Y. M. Graus, H. J. van der Vliet, S. M. Lougheed, A. J. van den Eertwegh, H. M. Pinedo, and R. J. Scheper. 2002. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. *Blood* 100: 701–703.

17. Bender, A., M. Sapp, G. Schuler, R. M. Steinman, and N. Bhardwaj. 1996. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* 196: 121–135.

18. Schreurs, M. W., K. B. Scholten, E. W. Kueter, J. J. Ruizendaal, C. J. Meijer, and E. Hooijberg. 2003. In vitro generation and life span extension of human papillomavirus type 16-specific, healthy donor-derived CTL clones. *J. Immunol.* 171: 2912–2921.

19. Santegoets, S. J., M. W. Schreurs, A. J. Masterson, Y. P. Liu, S. Goletz, H. Baumeister, E. W. Kueter, S. M. Lougheed, A. J. van den Eertwegh, R. J. Scheper, et al. 2006. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. *Cancer Immunol. Immunother.* 55: 1480–1490.

20. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* 281: 65–78.

21. Rubio, V., T. B. Stuge, N. Singh, M. R. Betts, J. S. Weber, M. Roederer, and P. P. Lee. 2003. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat. Med.* 9: 1377–1382.

22. Banchereau, J., A. K. Palucka, M. Dhodapkar, S. Burkeholder, N. Taquet, A. Rolland, S. Taquet, S. Coquery, K. M. Wittkowski, N. Bhardwaj, et al. 2001. Immune and clinical responses in patients with metastatic melanoma to CD34+ progenitor-derived dendritic cell vaccine. *Cancer Res.* 61: 6451–6458.

23. Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael. 1997. Rapid effector function in CD8+ memory T cells. *J. Exp. Med.* 186: 859–865.

24. Vuylsteke, R. J., P. A. van Leeuwen, S. Meijer, P. G. Wijnands, M. G. Status Muller, D. H. Busch, R. J. Scheper, and T. D. de Gruijl. 2002. Sampling tumor-draining lymph nodes for phenotypic and functional analysis of dendritic cells and T cells. *Am. J. Pathol.* 161: 19–26.

25. Xia, X., A. Cheng, T. Lessor, Y. Zhang, and A. W. Hamburger. 2001. Ebp1, an ErbB-3 binding protein, interacts with Rb and affects Rb transcriptional regulation. *J. Cell. Physiol.* 187: 209–217.

26. Yoo, J. Y., X. W. Wang, A. K. Rishi, T. Lessor, X. M. Xia, T. A. Gustafson, and A. W. Hamburger. 2000. Interaction of the PA2G4 (EBP1) protein with ErbB-3 and regulation of this binding by heregulin. *Br. J. Cancer* 82: 683–690.

27. Lessor, T. J., J. Y. Yoo, X. Xia, N. Woodford, and A. W. Hamburger. 2000. Ectopic expression of the ErbB-3 binding protein ebp1 inhibits growth and induces differentiation of human breast cancer cell lines. *J. Cell. Physiol.* 183: 321–329.

28. Zhang, Y., N. Woodford, X. Xia, and A. W. Hamburger. 2003. Repression of E2F1-mediated transcription by the ErbB3 binding protein Ebp1 involves histone deacetylases. *Nucleic Acids Res.* 31: 2168–2177.

29. Zhang, Y., J. D. Fondell, Q. Wang, X. Xia, A. Cheng, M. L. Lu, and A. W. Hamburger. 2002. Repression of androgen receptor mediated transcription by the ErbB-3 binding protein, Ebp1. *Oncogene* 21: 5609–5618.

30. Squatrito, M., M. Mancino, M. Donzelli, L. B. Areces, and G. F. Draetta. 2004. EBP1 is a nucleolar growth-regulating protein that is part of pre-ribosomal ribonucleoprotein complexes. *Oncogene* 23: 4454–4465.

31. Xia, X., T. J. Lessor, Y. Zhang, N. Woodford, and A. W. Hamburger. 2001. Analysis of the expression pattern of Ebp1, an ErbB-3-binding protein. *Biochem. Biophys. Res. Commun.* 289: 240–244.

32. Overwijk, W. W., D. S. Lee, D. R. Surman, K. R. Irvine, C. E. Touloukian, C. C. Chan, M. W. Carroll, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1999. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4+ T lymphocytes. *Proc. Natl. Acad. Sci. USA* 96: 2982–2987.

33. Ludewig, B., A. F. Ochsenbein, B. Odermatt, D. Paulin, H. Hengartner, and R. M. Zinkernagel. 2000. Immunotherapy with dendritic cells directed against tumor antigens shared with normal host cells results in severe autoimmune disease. *J. Exp. Med.* 191: 795–804.

34. Babatz, J., C. Rollig, B. Lobel, G. Folprecht, M. Haack, H. Gunther, C. H. Kohne, G. Ehninger, M. Schmitz, and M. Bornhauser. 2006. Induction of cellular immune responses against carcinoembryonic antigen in patients with metastatic tumors after vaccination with altered peptide ligand-loaded dendritic cells. *Cancer Immunol. Immunother.* 55: 268–276.

35. Lesterhuis, W. J., I. J. De Vries, D. H. Schuurhuis, A. C. Boullart, J. F. Jacobs, A. J. de Boer, N. M. Scharenborg, H. M. Brouwer, M. W. van de Rakt, C. G. Figdor, et al. 2006. Vaccination of colorectal cancer patients with CEA-loaded dendritic cells: antigen-specific T cell responses in DTH skin tests. *Ann. Oncol.* 17: 974–980.

36. Svane, I. M., A. E. Pedersen, H. E. Johnsen, D. Nielsen, C. Kamby, E. Gaarsdal, K. Nikolajsen, S. Buus, and M. H. Claesson. 2004. Vaccination with p53-peptide-pulsed dendritic cells, of patients with advanced breast cancer: report from a phase I study. *Cancer Immunol. Immunother.* 53: 633–641.

37. Radomski, N., and E. Jost. 1995. Molecular cloning of a murine cDNA encoding a novel protein, p38–2G4, which varies with the cell cycle. *Exp. Cell Res.* 220: 434–445.