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A NOVEL DNA METHYLTRANSFERASE I–DERIVED PEPTIDE ELUTED FROM SOLUBLE HLA-A*0201 INDUCES PEPTIDE-SPECIFIC, TUMOR-DIRECTED CYTOTOXIC T CELLS

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MHC peptides derived from tumor-associated antigens (TAAs) can serve as the basis for the development of immunotherapeutics to treat human malignancies. Previously, we identified novel HLA-A*0201 (HLA-A2)–restricted peptides recovered from soluble HLA molecules secreted by human tumor cell lines, transfected with truncated genes of *HLA-A2* and *HLA-B7*. Here, 4 candidate peptides eluted from soluble HLA-A2 were selected on the basis of their precursor proteins being TAAs. Peptide p1028 (GLIEKNIEL), derived from DNA methyltransferase I (DNMT-1), which is overexpressed in various human tumors, showed the highest affinity to HLA-A2 and was relatively abundant in the sMHC/peptide complexes of all transfected breast, ovarian and prostate cancer cell lines. Peptide p1028-specific CTLs were generated *in vitro* and shown to efficiently lyse not only target cells pulsed with the peptide but also HLA-A2-positive breast cancer cell lines MDA-231 and MCF-7. The peptide induced IFN-γ production in CTLs, which were selectively stained by a p1028 tetramer. Since DNMT-1 is a widely expressed tumor-associated enzyme, the novel DNMT-1-derived, HLA-A2-restricted peptide GLIEKNIEL identified here may provide a suitable candidate for a therapeutic cancer vaccine.

Key words: *soluble HLA; DNMT-1; cancer vaccine; tumor antigen; immunotherapy*

The preferred approach for the elimination of tumor cells is to elicit a specific antitumor CTL response that will select tumor cells presenting the appropriate T-cell epitopes.[1–4] Human clinical trials for a variety of malignant diseases showed that T-cell therapy was effective and curative for some individuals.[5,6] A number of TAAs have been identified, mainly melanoma antigens, such as Melan-A and MART-1. Studies were initiated to vaccinate tumor patients against these antigens, and their ability to induce antitumor T-cell immunity has been shown in clinical studies.[7] Many of the recently identified antigens are nonmutated differentiation antigens overexpressed in tumors, *e.g.,* a peptide derived from protein tyrosinase that was shown to successfully induce the attack and lysis of melanoma cells from cancer patients.[8] Several strategies have evolved to identify novel TAAs, *e.g.,* cloning of epitopes recognized by CTLs[9] and SEREX technology,[10] where a patient's antibodies are used to identify tumor antigens from a cDNA expression library made from mRNA of tumor specimens.

Conventionally, endogenous peptides have been eluted from affinity-purified HLA, such as HLA-A*0201, followed by analysis and sequencing using HPLC combined with tandem mass spectrometry.[11,12] HLA class I molecules are integral membrane glycoproteins; however, the presence of a soluble form of these molecules was demonstrated decades ago.[13,14] Similar to membrane-bound MHC, sMHC class I molecules bind peptides which are 8–10 amino acids long, with a few exceptions having a length of 6, 11 and 12 amino acids.[15]

Purification of MHC molecules is complicated due to their unavoidable contamination with cell debris and detergents after cell lysis. We recently identified HLA-restricted, tumor-specific antigens presented by the sMHC class I molecule by transfecting human tumor cell lines with truncated genes of HLA-A2. After purification of the secreted sHLA by affinity chromatography, peptides were eluted and sequenced.[15] Novel peptides unique to the sMHC variants were identified and screened for tumor-relevant precursor proteins. Detection of these peptides on sMHC indicates that these peptides are properly processed and displayed on transfected sMHC molecules similarly to those displayed on membrane-bound MHC molecules. Peptides recovered from sHLA molecules form similar patterns to those of membrane-bound HLA and include known MHC peptides.[15] Therefore, the repertoire of MHC peptides recovered from sMHC molecules is a good representation of the naturally processed and displayed peptides of transfected cells. Peptides detected this way should be presented at many copy numbers per cell and are likely to be bound with relatively high affinity to HLA.

Use of sHLA as a source of peptides enabled us to identify large numbers of MHC peptides presented by human cancer cells and to screen these for peptides possibly derived from TAAs as candidates for tumor vaccination.[15] We selected 4 peptides from the pool of sMHC-derived peptides identified on the basis of their relevance to tumors and demonstrate the immunogenicity of a DNMT-1-derived peptide that efficiently induces peptide-specific cytotoxic T cells.

MATERIAL AND METHODS

Cell culture

Breast cancer cell lines MCF-7 and MDA-231 and the TAP transporter-deficient lymphoblastic T2 cell line were maintained at 37°C in a 5% CO_2 incubator in RPMI plus HEPES (GIBCO, Grand Island, NY) supplemented with 100 U/ml streptomycin/penicillin (GIBCO). All cell lines were purchased from the ATCC (Rockville, MD).

Peptide-binding assay

Each peptide was tested for binding to T2 cells in an HLA-A2 stabilization assay.[16] Briefly, TAP-deficient T2 hybridoma cells

Abbreviations: APC, antigen-presenting cell; ConA, concanavalin A; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DNMT, DNA methyltransferase; ECL, enhanced chemiluminescence; FMOC, 9-fluorenylmethoxycarbonyl; GM-CSF, granuloctye/macrophage colony-stimulating factor; LMP, low molecular weight protein; MAb, monoclonal antibody; NK, natural killer; PBL, peripheral blood lymphocyte; SEREX, serologic identification of antigens by recombinant expression screening; sMHC, soluble major histocompatibility complex; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; TcR, T-cell receptor; TFA, trifluoroacetic acid.

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were incubated with 25 μg/ml peptide and 15 μg/ml β_2-microglobulin (Research Diagnostics, Flanders, NJ) at 37°C for 24 hr. Expression of HLA-A2 was assayed by flow cytometry after staining cells with the anti-HLA-A2 antibody PA2.1 (ATCC) and FITC-conjugated F(ab′)$_2$ goat antimouse Ig (BD Pharmingen, San Diego, CA).

Peptides were synthesized by Dr. M. Fridkin (Weizmann Institute, Israel) using FMOC chemistry and purified by reversed-phase HPLC on a Vydac C18 column (Vydac, Hesperia, CA) in 0.1 TFA and an acetonitrile gradient.[15]

*Preparation of HLA-A*0201-positive PBMCs and DCs*

PBMCs from healthy donors were purified by centrifugation in Ficoll-Hypaque (Sigma, St. Louis, MO) from leukapheresis products obtained from healthy volunteers. For *in vitro* generation of peptide-specific CTLs, we generated autologous DCs as APCs. DCs were generated from fresh PBMCs in 2 steps, as previously described.[17] Briefly, monocyte-enriched cell fractions were prepared by 2 hr plastic adherence of 6.67×10^6 PBMCs/ml and 0.2 ml/cm^2 and cultured for 7 days in X-VIVO-15 medium (BioWhittaker, Walkersville, MD) supplemented with 1% autologous plasma, 800 U/ml GM-CSF and 500 U/ml IL-4. On day 7, the nonadherent cell population was harvested; resuspended in fresh culture medium containing 800 U/ml GM-CSF, 500 U/ml IL-4, 1,000 U/ml IL-6, 10 ng/ml IL-1 and 10 ng/ml TNF-α; and transferred to fresh tissue culture flasks (all cytokines purchased from Preprotech, Rocky Hill, NJ). On day 10, nonadherent cells were harvested and analyzed by flow cytometry.

Flow cytometry

For flow cytometry, 2 to 5×10^5 mature and immature DCs were incubated for 30 min in the dark at 4°C with FITC-labeled anti-CD80, -CD83, -CD86 and -CD11c and PE-labeled anti-HLA-DR antibodies (all antibodies purchased from BD Pharmingen). Stained cells were detected with a FACScan (Becton Dickinson, Mountain View, CA) and analyzed with WinMDI software (Joseph Trotter, URL: http://facs.scripps.edu).

Purification of CD8$^+$ T cells

CD8$^+$ T cells were enriched using MACS Micro beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's directions. MAbs for CD4 and CD8 (BD Pharmingen) were used to monitor cell purity by flow cytometry.

Induction of CTLs

DCs were pulsed for 4 hr at 37°C with 50 μg/ml peptide p1028 in the presence of 3 μg/ml β_2-microglobulin (Research Diagnostics) and inactivated by irradiation before use. CD8$^+$ T lymphocytes were cocultured at 2×10^6 cells/well with 6.7×10^5 peptide-pulsed DCs/well in 24-well plates in 2 ml RPMI-1640 supplemented with 10% autologous plasma, 25 mM HEPES, 0.05 mg/ml gentamicin, 20 U/ml IL-2 and 5 U/ml IL-4 (Preprotech). On day 12 and weekly thereafter, CTLs were restimulated with peptide-pulsed DCs as described above.

Western blotting

Tumor cell lines T2, MCF-7, MDA-231 and PC-3, human PBLs as well as ConA blasts from 4 healthy blood donors were lysed in NP-40 lysis buffer containing 1% NP-40, 0.15 M NaCl and 1 M TRIS (pH 7.4). Protein concentration was measured by spectrophotometry. Equal protein amounts of each lysate were denatured in reducing sample buffer, electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose for 4 hr at 0.8 amp.

The transfer membrane was blocked with 5 % (w/v) BSA (Sigma-Aldrich, St. Louis, MO) in PBS and incubated with a rabbit antihuman DNMT-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was incubated with a goat anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed using ECL Western blot detection reagents (Amersham, Piscataway, NJ).

Cytotoxicity assay

Lysis of target cells by CTLs recognizing peptide p1028 was tested in a standard 4 hr ^{51}Cr-release assay. Briefly, 1.25×10^5 target cells (T2 cells pulsed with 25 μg/ml peptide or unpulsed, MCF-7, MDA-231, K562, DC) were labeled for 1 hr at 37°C with 180 μCi of $Na2^{51}CrO_4$ (Perkin-Elmer, Boston, MA), washed 3 times, resuspended in RPMI/10% FCS and plated in conical 96-well plates. To block HLA-A2, MCF-7 cells were incubated before labeling with $Na2^{51}CrO_4$ for 30 min with anti-HLA-A2 antibody PA2.1 (ATCC) and washed subsequently. CTLs were added to target cells at the indicated E:T ratios to a total volume of 200 μl/well. After 4 hr at 37°C, supernatants were harvested using harvesting frames and detected in a gamma counter. Percent specific lysis was calculated as *(cpm experimental counts – cpm media control)/(cpm Triton X-100 – cpm media control) × 100%.*

Spontaneous release was determined from wells to which 100 μl of medium were added instead of effector cells. Total releasable radioactivity was obtained after treating target cells with 1% Triton X-100.

Intracellular IFN-γ assay

The specificity of CTLs for p1028 was further tested by measuring intracellular IFN-γ release. CTLs (1×10^5) were coincubated with 1×10^3 peptide-pulsed T2 cells in 96-well plates in a volume of 100 μl/well. Control wells contained CTLs alone or in the presence of unloaded T2 cells. Intracellular IFN-γ was measured after 24 hr incubation at 37°C. Cells were stained with anti-CD8-FITC for 30 min at 4°C. After permeabilization, following the manufacturer's protocol, they were incubated with PE-conjugated IFN-γ antibody (antibodies and Cytofix/Cytoperm Kit from BD Pharmingen). Cell fluorescence was analyzed by flow cytometry.

Tetramer staining

To further determine the specificity of CTLs, tetramers were generated. They were produced by ProImmune (Oxford, UK). CTLs (2 to 5×10^5) were washed with PBS and resuspended in 50 μl PBS/1% BSA. Cells were incubated for 15 min on ice with 1 μg/ml FITC-labeled tetramer, while PE-conjugated anti-CD8 antibody (BD Pharmingen) was added for 20 min. After washing, stained cells were detected with a FACScan.

TABLE I – sHLA-A2-ELUTED PEPTIDES

Peptide	Sequence	Precursor protein	Score MHC database[19]	NCBI accession number
p981	SLIGHLQTL	Protein tyrosine phosphatase	32	U16996
p1028	GLIEKNIEL	DNA methyl transferase I	28	X63692
p1258	FLFDGSPTYVL	Fatty acid synthase	N/A	U29344
p1145	FLFDGSPTYV	Fatty acid synthase	23	U29344

Soluble HLA-A2-eluted peptides were sequenced, synthesized and precursor proteins identified.[15] Theoretical binding affinity for peptides was calculated in the database for MHC ligands and peptide motifs by Rammensee.[19]

RESULTS

Selection of sHLA-A2-derived peptides

Four peptides eluted from sHLA-A2 were selected on the basis of their relative abundance and tumor-associated precursor proteins. These peptides were synthesized and retested to ascertain their binding affinity to HLA-A2 on human cell lines. The sMHC-derived peptides characterized here are 8–11 amino acids long (Table I). Binding affinities of these peptides to HLA-A2 were evaluated using the TAP-deficient human T2 cell line. The low level of peptides in TAP-mutant T2 cells allows most MHC class I molecules to remain empty and to associate with exogenously added high-affinity peptides. Upon exposure to exogenous HLA-A2-binding peptides, enhanced HLA-A2 expression can be observed and detected by flow cytometry. The percentage of HLA-A2 reconstitution after peptide pulsing with the different peptides is shown in Figure 1*a.* Peptide p1028 (GLIEKNIEL) has the highest binding affinity to HLA-A*0201 of the 4 peptides. It has the typical length of MHC class I ligands (9 residues) and contains the characteristic anchor residue leucine at positions 2 and 9.[18]

The relatively high affinity of p1028 for HLA-A2 is consistent with the theoretical score in the database for MHC ligands and peptide motifs SYFPEITHI,[19] where this epitope is scored with 28 out of a maximal score for HLA-A2 peptides of 36. For instance, the well-known epitope GILGFVFTL derived from the influenza A matrix protein scores 30. Peptides p1028 and p1258 have already been described as high-affinity binders,[15] which is confirmed here for the human T2 cell line. Previously, the murine TAP-deficient HLA-A2.1/Db-β2m-transfected RMA-S-HHD cell line was used to determine binding affinities. We identified peptide p1028 as the highest-affinity binder compared to peptides p981, p1145 and p1258. In these experiments, maximal HLA-A2 expression was achieved with a peptide concentration of 25 μg/ml after peptide pulsing for 24 hr. We therefore focused on p1028 for the generation of peptide-specific T cells.

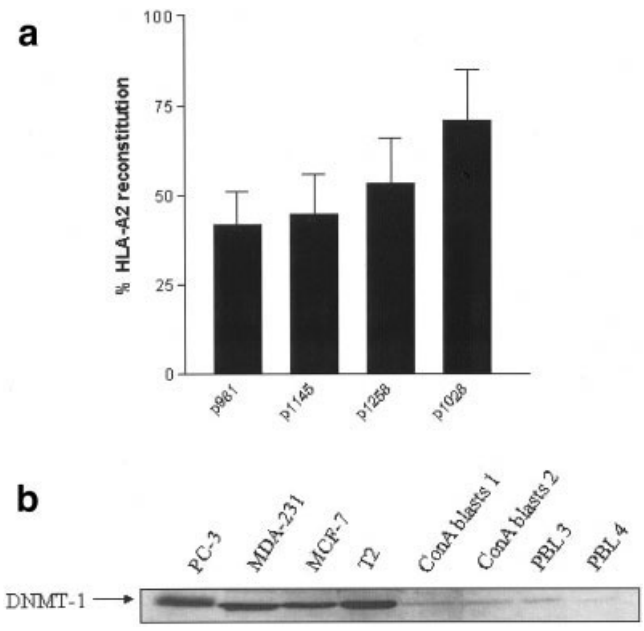

FIGURE 1 – *(a)* Peptide p1028 has strong binding affinity for HLA-A2. TAP-deficient T2 hybridoma cells were incubated with 25 μg/ml sHLA-A2 peptide and 15 μg/ml β2-microglobulin at 37°C for 24 hr to reconstitute HLA-A2 expression on the cell surface. Expression of HLA-A2 was measured by flow cytometry using the anti-HLA-A2 MAb PA2.1. Highest reconstitution of HLA-A2 was achieved with p1028. The percentage of HLA-A2 reconstitution induced by peptide pulsing is indicated on the *y* axis. *(b)* DNMT-1 expression in tumor cell lines compared to activated and naive PBLs. Peptide p1028 is derived from DNMT-1, an enzyme associated with cancer. Cell lysates derived from tumor cell lines T2, MCF-7, MDA-231 and PC-3 as well as PBLs and ConA-activated human T lymphocytes from 4 healthy blood donors were analyzed for DNMT-1 expression by Western blotting using an anti-human DNMT-1 antibody. DNMT-1 was highly expressed in all tumor cell lines, whereas moderate expression was observed for PBLs as well as ConA blasts of all 4 donors.

DNMT-1 is strongly expressed in cancer cell lines MCF-7, MDA-231, PC-3 and T2

The selected peptide, p1028, was derived from DNMT-1, which is a highly abundant protein found in tumors.[20] Elevated levels of DNMT-1 mRNA and DNMT activity have been observed in many cancer cells *in vitro* and tumors *in vivo.*[21,22] However, DNMT-1 mRNA is highly expressed in most fetal as well as adult tissues containing continuously renewing cell populations (*e.g.,* liver),[20] showing a relationship between DNMT mRNA levels and the proliferative state of the tissue. Here, we show high expression of DNMT-1 in several tumor cell lines. We compared DNMT-1 expression in 2 breast cancer cell lines, MCF-7 and MDA-231, the T2 lymphoma cell line and the prostate cancer cell line PC-3 with that in PBLs and activated T lymphocytes from 4 healthy blood donors by Western blotting (Fig. 1*b*). The enzyme is highly expressed in all tumor cell lines used here and was at very low levels in PBLs as well as activated T lymphocytes. The relative overexpression of DNMT-1 in many tumor cell lines in comparison to nonmalignant cells and tissue suggests that p1028 could be a good candidate for tumor immunotherapy.

Generation of DCs and induction of CTLs

DCs were separated from leukapheresis products of healthy HLA-A2-positive volunteer blood donors. Immature DCs were obtained by culturing adherent cells with cytokines, as described above. DCs were phenotyped after 7 days as well as after full maturation, at which point they expressed high levels of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as MHC class II (Fig. 2). In addition, expression of CD11c and CD83 was enhanced. Mature DCs were pulsed with sHLA-A2-derived peptides and used as stimulator cells. Autologous $CD8^+$ T cells were used as responder cells. Peptide p1028-loaded DCs induced clonal expansion of T cells after the first restimulation, whereas no expansion was observed for the lower-affinity peptides. These findings indicate that the exogenously added sHLA-A2-derived p1028 is efficiently presented by DCs, confirming that the peptide is a natural HLA-A2 ligand and able to induce a T-cell response.

Specific CTL cytotoxicity to p1028-pulsed T2 cells

CTLs were phenotyped and subsequently tested for lytic activity in a standard ^{51}Cr-release assay. As expected, >90% of CTLs were $CD8^+$ (Fig. 3*a*). To test whether $CD8^+$ T cells specifically lyse p1028-presenting cells, T2 cells were incubated overnight with 25 μg/ml p1028 or the irrelevant control peptide p1258 and β_2-microglobulin and used as target cells. CTLs lysed p1028-presenting T2 cells but not p1258-loaded or unloaded T2 cells (Fig. 3*b*). These data demonstrate that target cell killing is specifically dependent on p1028.

Peptide p1028-specific CTLs lyse tumor cells

To investigate whether p1028-specific CTLs are able to lyse tumor cells expressing DNMT-1, we used the HLA-A2-expressing breast cancer cell lines MCF-7 and MDA-231 as target cells, pulsed T2 cells as a positive control and DCs as a negative control representing nononcogenic cells. K562 cells were used as target cells to exclude NK cell-like activity. MCF-7, MDA-231 and T2 cells expressed high levels of DNMT-1. If p1028 is potentially presented by membrane-bound MHC molecules, expression of HLA-A2 should make tumor cells susceptible to lysis by p1028-specific CTLs. Furthermore, DNMT-1 was degraded to p1028 by the 2 breast cancer cell lines as it was displayed in the sHLA-A2 molecule of transfected breast cancer cells. As expected, p1028-specific CTLs efficiently lysed T2 cells pulsed with p1028 (Fig. 4).

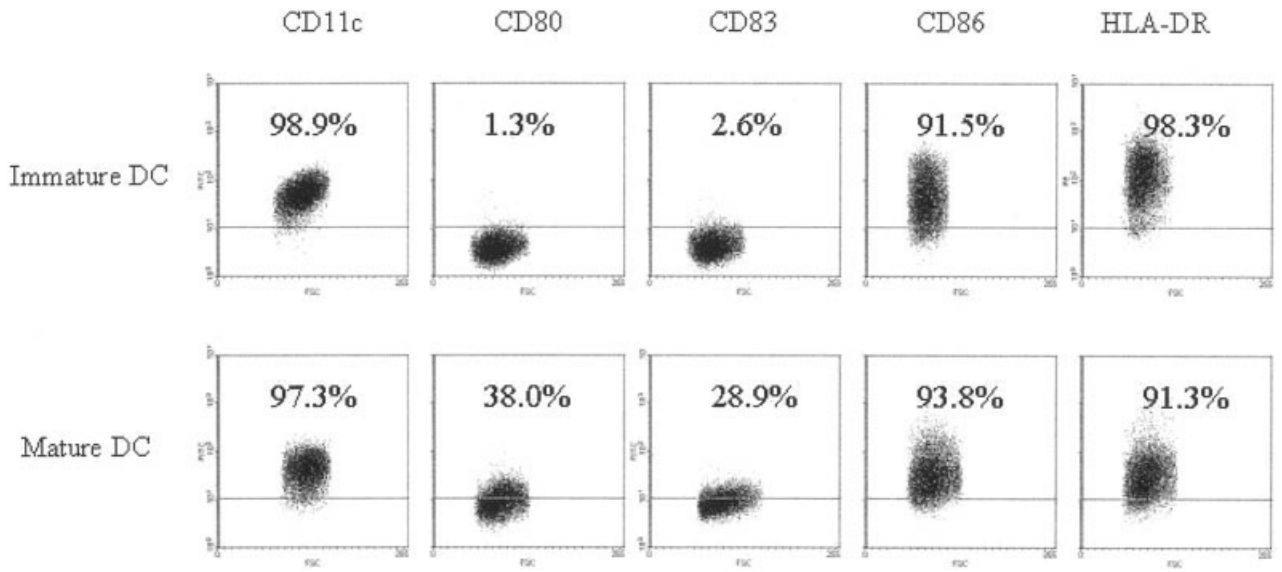

FIGURE 2 – Phenotyping of immature and mature DCs. Adherent PBMCs were cocultured in the presence of GM-CSF and IL-4. At day 7, nonadherent cells were harvested and cultured for 3 days with additional cytokines (TNF-α/IL-1/IL-6). Mature and immature DCs were stained with MAbs for 30 min at 4°C and analyzed by flow cytometry. Characteristically, mature DCs showed enhanced CD80 and CD83 expression.

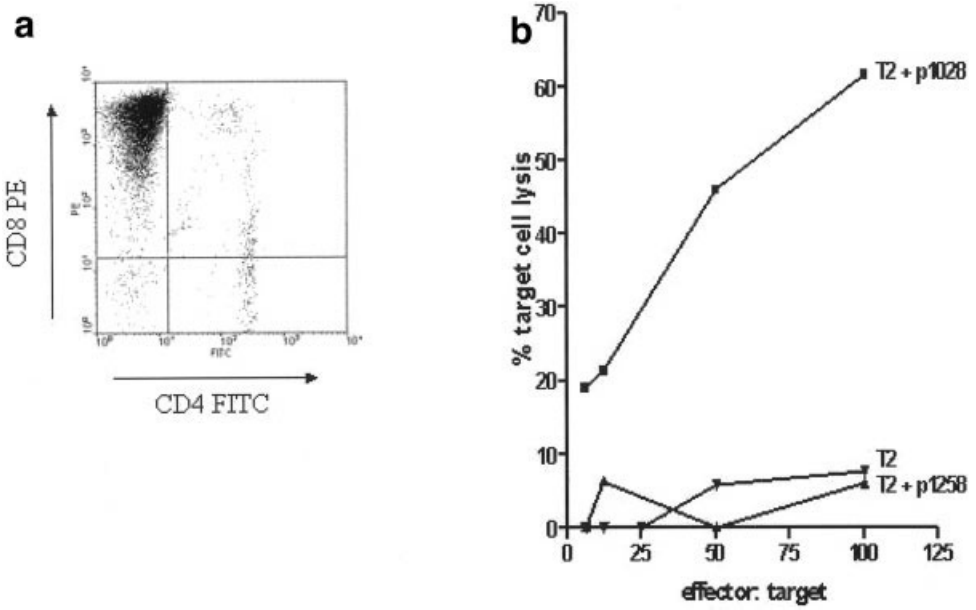

FIGURE 3 – Effector CTLs specifically recognize p1028-loaded T2 cells. CTLs were phenotyped before testing their cytotoxicity. The CTL culture contained >90% $CD8^+$ T cells, as analyzed by flow cytometry *(a)*. Peptide-pulsed (25 μg/ml) and unpulsed HLA-A2-positive T2 cells were labeled with ^{51}Cr and used as target cells in a 4 hr ^{51}Cr-release assay. CTLs specifically lysed p1028-pulsed T2 cells, while no significant lysis was detected in unpulsed T2 cells or T2 cells pulsed with p1258, an irrelevant sHLA-A2-derived control peptide.

Interestingly, CTLs were also able to lyse the 2 breast cancer cell lines, though at a lower percentage than pulsed T2 cells. As control for primary human cells, we used mature DCs as target cells. DCs were not lysed, confirming the poor physiologic expression of p1028 in nononcogenic primary cells, also confirmed by Western blotting in Figure 1*a*. There was no significant NK-like activity of the generated CTLs as K562 cells were not susceptible to lysis.

p1028 CTLs are HLA-A2-restricted

To test whether the tumor cell lysis by p1028 CTLs is HLA-A2-restricted, we used prostate cancer cell line PC-3 (HLA-A2-negative) and breast cancer cell line MCF-7 (HLA-A2-positive) (Fig. 5*a*) as targets in a cytotoxicity assay. In addition, we blocked HLA-A2 on A2-positive breast cancer cell line MCF-7 using the anti-HLA-A2 MAb PA2.1. p1028 CTLs specifically lysed the A2-expressing tumor cell line MCF-7, whereas the A2-negative cell line PC-3 as well as the A2-blocked MCF-7 cells were not susceptible to CTL killing (Fig. 5*b*), proving that HLA-A2 was a requirement for CTL recognition of target cells.

Characterization of CTLs by intracellular IFN-γ production and tetramer staining

To further characterize the p1028-generated CTLs, we tested their ability to produce IFN-γ upon interaction with p1028-pulsed T2 cells. IFN-γ production was measured intracellularly after 24 hr coincubation with peptide-loaded or native T2 cells (Fig. 6*a*). Clearly, p1028-pulsed T2 cells induced significant IFN-γ production compared to controls.

To confirm the peptide-dependent cytotoxicity of p1028-specific CTL, we stained CTLs with newly generated MHC–peptide tetramers. Tetramers expressing either peptide p1028 or the irrelevant

peptide p898 were commercially acquired. Peptide p898 is derived from an IFN-γ-inducible protein, which was also recovered from sHLA-A2 molecules,[11,15] and thus has the same characteristics as p1028 regarding MHC restriction. CTLs were costained with anti-CD8 and tetramer p1028 or p898, respectively. The p1028 tetramer stained 74% of CTLs, whereas the control tetramer failed to show any significant binding (Fig. 6*b*). These results confirm that the generated CTLs are highly specific for p1028. Thus, we describe a novel HLA-A*0201-eluted peptide that induces CTLs *in vitro,* which lyse tumor cells. This peptide provides a novel candidate tumor vaccine.

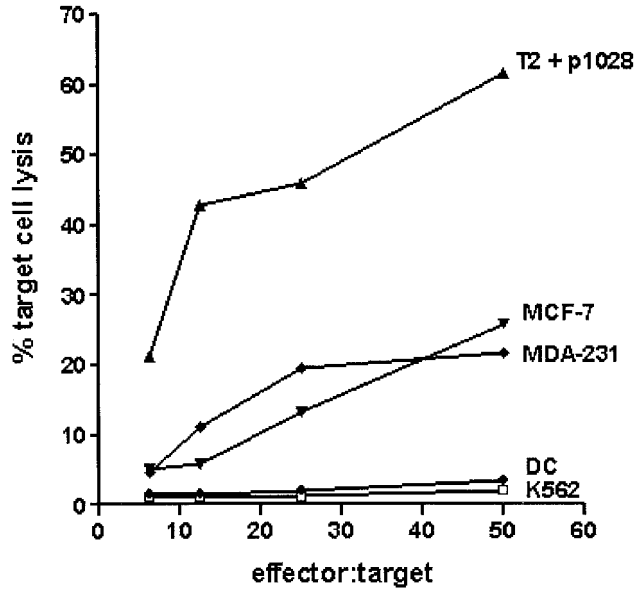

FIGURE 4 – Breast cancer cell lines MDA-231 and MCF-7 are susceptible to lysis by p1028-specific CTLs. MCF-7, MDA-231, K562 and p1028-pulsed T2 cells as well as DCs were used as target cells in a 4 hr ^{51}Cr-release assay. Peptide-pulsed T2 cells were highly susceptible to CTL killing. Both breast cancer cell lines, MCF-7 and MDA-231, were also lysed by p1028-specific CTLs. K562 target cells were not susceptible to cell lysis. No lytic activity was detectable against unpulsed DCs used as target cells, indicating that the tumor cells, but not primary human cells, display peptide p1028 on their surface MHC.

DISCUSSION

Peptides derived from otherwise physiologic proteins, overexpressed in various tumors, are targets for immunologic response; *e.g.,* a peptide derived from protein tyrosinase successfully induced the attack and lysis of melanoma cells from cancer patients.[8,9] Identification of peptide p1028[15] revealed a previously unknown immunogenic, HLA-A2-restricted peptide derived from DNMT-1, using a novel method of identifying peptides eluted from sMHC. It has been eluted from the sMHC of ovarian, prostate and breast cancer cell lines.

Here, we selected the most abundant peptides recovered from the sMHC/peptide complex, which were derived from TAAs, and evaluated their binding affinity to HLA-A2. The peptides presented by an HLA class I molecule are usually 8–10 amino acids long with 2 or 3 primary anchor residues.[18] As shown here, the length of peptides eluted from recombinant sHLA-A2 does not appear to differ from that of peptides displayed on membrane-bound MHC class I molecules.[15] However, high-sensitivity analysis of MHC peptides based on recovery from sHLA followed by capillary HPLC and tandem mass spectrometry enabled the identification of a few "outlier" peptides, such as p1258, with a length of 11 amino acids.

The peptide further characterized in our study, p1028 (GLIEKNIEL), displays the characteristic anchor residues for HLA-A2, with L at the second and ninth positions. A high binding predictive value was calculated in the database for MHC ligands[19] for p1028, which was confirmed by our binding affinity studies on T2 cells. We observed slightly different results for peptide affinities using either the murine HLA-A2-expressing RMA-S-HHD cell line or the T2 cell line. This might be due to the higher basal expression of self-peptides presented by T2 cells at 37°C compared to RMA-S-HHD cells at 26°C, therefore creating a higher threshold for exogenously added peptides to bind to the surface MHC. Also, our results indicate a relatively low binding affinity for p981, which was sustained by the lack of ability to induce expansion of T cells in response to p981.

In contrast, stimulation of $CD8^+$ T cells with p1028 promoted the expansion of specific CTLs. However, such a profound T-cell response was observed for only 1 of 4 healthy individuals. These CTLs were capable of killing p1028-loaded T2 cells but not T2 cells loaded with an irrelevant sHLA-A2-derived control peptide (p1258), showing p1028-dependent cytotoxicity. CTLs also showed lytic activity against the breast cancer cell lines MDA-231 and MCF-7, whereas DCs and K562 cells remained unaffected. The lower cytotoxicity against those tumor cell lines compared to loaded T2 cells is likely due to lower expression of the naturally

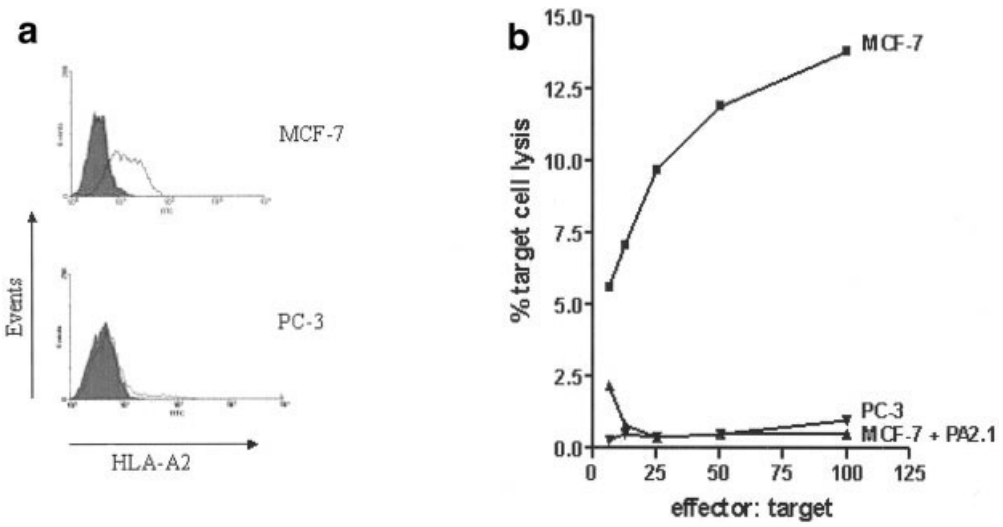

FIGURE 5 – HLA-A2 restriction of p1028 CTLs. To determine HLA-A2 expression in MCF-7 and PC-3, cell lines were stained with PA2.1 MAb (anti-HLA-A2). Breast cancer cell line MCF-7, but not prostate cancer cell line PC-3, expressed HLA-A2 on the cell surface, as analyzed by flow cytometry *(a).* Both cell lines were used as targets for p1028 CTLs in a ^{51}Cr-release assay. The HLA-A2-negative, DNMT-1-expressing PC-3 cells were not susceptible to lysis by p1028-specific CTLs, whereas the HLA-A2-expressing MCF-7 cells were susceptible. Anti-HLA-A2 antibody inhibited lysis of MCF-7. Both results prove HLA-A2 restriction of the generated CTLs.

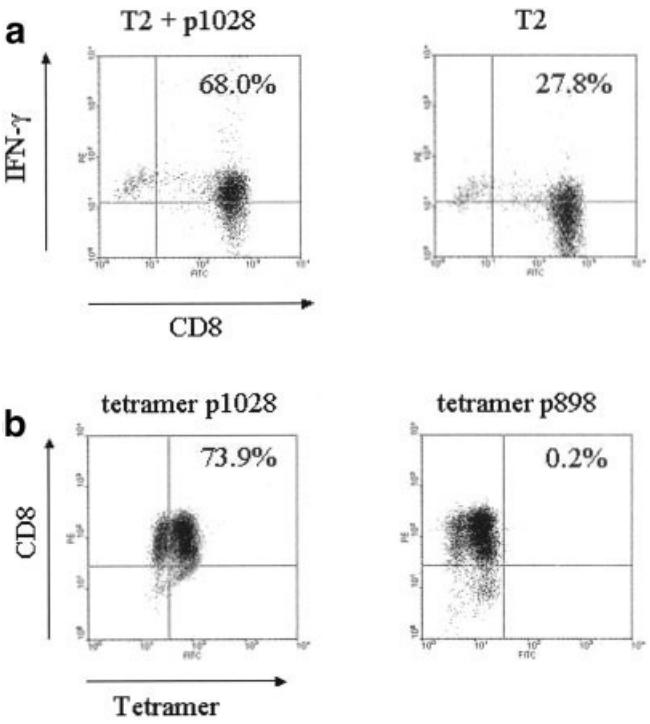

FIGURE 6 – Tetramer binding and intracellular IFN-γ production of p1028-specific CTLs. p1028-specific CTLs express intracellular IFN-γ to p1028-loaded T2 cells. IFN-γ was measured after 24 hr incubation with either pulsed or unpulsed T2 cells. Cells were stained with anti-CD8, permeabilized and incubated with a PE-conjugated IFN-γ antibody. Data were analyzed by flow cytometry. *(a)* 68% of the CTL specifically produced IFN-γ to p1028-loaded T2 cells, compared to only 28% of those responding to unloaded T2 cells. *(b)* CTLs were co-stained with FITC-conjugated A2.1/p1028 or control A2.1/p898 tetramers and anti-CD8-PE. The p1028 tetramer stains approximately 74% of CTLs, while the irrelevant tetramer showed no significant binding, indicating that the generated CTLs are highly specific for peptide p1028.

processed peptide p1028 on membrane-bound MHC molecules on those cell lines relative to T2 cells loaded with a high concentration of synthetic peptide. Natural levels of p1028 in these cells are, however, sufficient for recognition by p1028-specific CTLs. Although high levels of DNMT-1 are expressed in the T2 cell line, unloaded T2 cells are not susceptible to CTL lysis, probably due to the TAP transporter defect, which impedes the natural presentation of endogenous peptides. p1028 CTLs were not able to lyse HLA-A2-negative, DNMT-1-expressing PC-3 cells or MCF-7 cells incubated with HLA-A2 blocking antibody. These experiments prove the HLA-A2 restriction of the generated CTLs. Peptide-dependent cytotoxicity was confirmed by staining CTLs with p1028.

Many tumors overexpress physiologic proteins, like DNMT-1, thereby presenting a different set of self-peptides associated with MHC class I molecules relative to normal cells. DNMT-1 is highly elevated in many cancer cells but is also elevated in tissues containing continuously renewing cell populations, such as fetal tissue.[20] The relative overexpression of a particular cancer-associated protein allows triggering of tumor-specific CTLs against self-antigens but may lead to autoimmunity induction and therefore raises the question of how to increase the specificity to tumor cells. One approach to increase tumor specificity is to use CTL clones equipped with an NK cell inhibitory receptor, taking advantage of the usually low expression of MHC class I on tumor cells.[23] Another limitation to generating tumor-specific T cells against self-peptides for use in adoptive immunotherapy is that self-peptide-specific CTLs undergo negative selection and peripheral tolerance, which leads to their elimination. Since tolerance to self-antigens is self-MHC-restricted, one could generate allorestricted CTLs against the peptide of interest. These would be self-peptides but bound by non-self MHC molecules on allorestricted CTLs, thereby avoiding the MHC-restricted tolerance to self-antigens.[24] The peptide identified here is derived from DNMT-1, which is overexpressed in various tumors and accordingly expressed in normal tissues,[20] as shown by comparison of breast cancer, prostate cancer and lymphoma cell lines with naive and activated lymphocytes. In malignancy, methylation patterns change, which can lead to genetic instability and repression of tumor-suppressor genes.[25] DNMT-1 has been identified as a target for cancer therapy. Pharmacologic DNA methyltransferase inhibitors exerted antitumor effects in *in vitro* and *in vivo* laboratory models, as reviewed by Goffin and Eisenhauer.[26]

Put together, the overexpression of DNMT-1 in various tumors and the finding of a novel immunogenic peptide that can elicit an immune response against tumor cells suggests that this epitope may indeed be suitable for T cell–mediated immunotherapy. We believe that the strategy of recovering peptides from sHLA antigens will provide more attractive candidates for vaccination and adoptive T-cell immunotherapeutic approaches against tumors by avoiding contamination of MHC molecules with cell debris and detergents usually encountered after cell lysis.

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