Vaccine 29 (2011) 4676–4686

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

ImMucin: A novel therapeutic vaccine with promiscuous MHC binding for the treatment of MUC1-expressing tumors

Riva Kovjazin[a,1], Ilan Volovitz[b,1], Yulia Kundel[c], Eli Rosenbaum[c], Gal Medalia[c], Galit Horn[e], Nechama I. Smorodinsky[e], Baruch Brenner[c,d], Lior Carmon[a,*]

[a] Vaxil BioTherapeutics Ltd., 13 Einstein Street, Weizmann Science Park, Nes-Ziona 74036, Israel
[b] Department of Neurosurgery, the Tel Aviv Sourasky Medical Center (TASMC), Tel Aviv, Israel
[c] Davidoff Cancer Center, Beilinson Campus, Rabin Medical Center, Petach Tikva, Israel
[d] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[e] Department of Cell Research and Immunology, The Alec and Myra Marmot Hybridoma Unit, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 19 December 2010
Received in revised form 21 February 2011
Accepted 27 April 2011
Available online 12 May 2011

Keywords:
MUC1
Cancer vaccine
MHC class I
MHC class II
Signal peptide

ABSTRACT

An optimal cancer vaccine should be able to induce highly potent, long-lasting, tumor-specific responses in the majority of the cancer patient population. One approach for achieving this is to use synthetic peptide vaccines derived from widely expressed tumor-associated antigens, that promiscuously bind multiple MHC class I and class II alleles. MUC1-SP-L (ImMucin, VXL100) is a 21mer peptide encoding the complete signal peptide domain of MUC1, a tumor-associated antigen expressed by over 90% of solid and non-solid tumors. MUC1-SP-L was predicted in silico to bind various MHC class I and MHC class II alleles, covering the majority of the Caucasian population. PBLs obtained from 13 naïve donors all proliferated, with a Stimulation Index (SI ≥ 2), to the MUC1-SP-L peptide, producing mixed $CD4^+$ and $CD8^+$ responses. Similar results were manifested by MUC1-SP-L in PBLs derived from 9 of 10 cancer patients with MUC1 positive tumors. $CD4^+$ and $CD8^+$ T cell populations exhibited CD45RO memory markers and secreted IFN-gamma and IL-2 following stimulation with MUC1-SP-L. These T cells also exhibited proliferation to the MUC1-SP-L inner 9mer epitopes and cytotoxicity against tumor cell lines expressing MUC1 and a concordant MHC class I allele. Cytotoxicity to MUC1-expressing human and murine tumors was shown also in T cells obtained from HLA-A2 transgenic mice and BALB/c syngeneic mice immunized with the MUC1-SP-L and GM-CSF. In an immunotherapy model, BALB/c mice inoculated with metastatic MUC1 transfected murine DA3 mammary tumor cells, exhibited significantly prolonged survival following vaccination with MUC1-SP-L. Our results indicate superior immunological and anti-tumor properties of MUC1-SP-L compared to previously published MUC1-derived epitopes.

1. Introduction

Anti-tumor vaccination using tumor-associated antigens (TAAs) is considered a safe and specific methodology for controlling metastases in cancer patients [1–3]. The rationale behind this approach is that cancer cells that express TAAs in association with their MHC molecules can be subject to recognition and destruction by tumor-specific T cells. However, since these vaccines are often derived from less-immunogenic self-TAAs and are used to treat sick individuals, frequently with compromised immune systems, they should be able to induce a strong and preferably a broad immune response [4].

One approach used to improve a specific immune response was to construct a multi-epitope vaccine that makes use of a mixture of specific MHC class I epitopes derived from different TAAs [5,6]. However, even the enhanced cytotoxic T lymphocytes (CTL) antigenic repertoire in such vaccines could not compensate for the lack of pan-HLA response [7], since they usually comprised of a single MHC class I-restricted epitope. Moreover, these vaccines also lacked MHC class II restricted T helper epitopes. In a few cases, the lack of MHC class II epitopes in such vaccines was shown to induce immunological tolerance to the immunizing antigens [8], rather than long lasting immunity mediated via $CD8^+$ T cell activation. In the past, the limited number of known TAA-derived MHC class II epitopes has led to the use of non-specific 'universal' MHC class II-restricted epitopes [9]. In one report, the use of a pan-HLA class II epitope peptide (PADRE) increased the response against the helper

Abbreviations: CTL, cytotoxic T lymphocytes; DC, dendritic cells; mAb, monoclonal antibody; SI, stimulation index; SP, signal peptide; TAA, tumor-associated antigen; TRA, tandem repeat array; VCs, vaccine candidates.

* Corresponding author at: Vaxil BioTherapeutics Ltd., 3 Hana Robina St., Tel Aviv 69372, Israel. Tel.: +972 8 9396948; fax: +972 8 9396996.
E-mail address: lior.carmon@vaxilbio.com (L. Carmon).

[1] R.K. and I.V. contributed equally to this work.

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.04.103

epitope, but the elevation in $CD8^+$ T cell effectors specific to the MHC class I-restricted epitope was limited [10].

Another approach to improving a specific immune response was to use longer peptides with promiscuous MHC binding properties. There is increasing evidence that vaccines combining MHC class I and class II epitopes, potentiate better anti-tumor effector function and long-term immunity [4,11]. In that setting, antigen specific $CD4^+$ T cells can activate dendritic cells (DC), principally through the interaction of CD40–CD40L. The "conditioned" DC can in turn activate tumor specific $CD8^+$ T cells and cross-present tumor epitopes to them at the tumor site or at remote locations [11–15]. Recent studies, primarily on the Her-2/Neu [16], RAS [17], and NY-ESO-1 [18] TAAs and the human papilloma virus 16 derived E6, E7 [19] antigens, showed that strong, long-term cellular immunity can be induced via longer peptides that encode on a single sequence a combination of one, or less frequently two, $CD4^+$ and/or $CD8^+$ epitopes derived from the same antigen. We recently showed that entire SP domains, usually comprised of 15–40mer long peptides, are inherently immunogenic, owing to their high hydrophobicity, and to their unique sequence motifs (20). This profile makes them a preferred T cell vaccine candidates (VCs) with antigen specific $CD4^+$ and $CD8^+$ epitopes [20]. SPs are usually found in the N-terminus of proteins and share an organelle-related common motif. Nevertheless, different SPs of various antigens exhibit high sequence variability with no particular sequence identity while conforming to the motif needed to maintain their functionality; thereby, they can serve as an ideal VC [20–23].

Following this rationale, our goal in this study was to select and develop a peptide vaccine from the MUC1 TAA that harbors on a single sequence multiple epitopes with more than 50% HLA binding coverage for the Caucasian population, for both $CD4^+$ and $CD8^+$ T cells.

MUC1 is a high-molecular-weight glycoprotein that is overexpressed in a broad range of solid and non-solid tumors [24,25]. The association of MUC1 with cancer progression has been well documented for the past three decades [26–28]. However, the importance of gene amplification as a mechanism leading to increased MUC1 expression in cancer has not been well characterized. Recent studies described the role of MUC1 gene amplification and protein expression in cancer development [29]. Results indicate that MUC1 copy number increases from normal tissue to primary invasive breast carcinomas in correlation with MUC1 protein expression [29]. These results suggest that MUC1 is a preferred TAA for anti-tumor vaccination. Initially, most anti-MUC1 vaccines were directed against the highly immunogenic, extracellular tandem repeat array (TRA), which is under-glycosylated during malignancy, and thereby was considered to expose new TAA epitopes [30]. Although immunity to TRA epitopes was shown to induce antibodies and MHC restricted CTLs [30], studies reported inconclusive results regarding its efficacy in anti-tumor vaccinations [30–33]. Likewise, there is no consensus concerning the correlation between the changes in exposure of sugar moieties and the improved recognition and function of CTLs [34]. More recently, MHC class I epitopes surrounding the TRA and within the SP of MUC1 have also been identified [35]. Experiments with the SP-derived 9mer HLA-A2.1 restricted epitopes MUC1D6 [36] and M1.2 [37] supported the notion that MUC1-SP-derived epitopes associated with MHC molecules are highly expressed on the surface of cancer cells. These epitopes also manifested anti-MUC1 and anti-tumor immunity in mice, as well as safety results in cancer patients [34,36–38].

In the present study, we isolated MUC1-SP-L (ImMucin, VXL100), a 21mer peptide vaccine encoding the entire SP domain of the MUC1 TAA. MUC1-SP-L was selected due to its in silico based promiscuous binding properties to both MHC class I and class II alleles. MUC1-SP-L encodes a number of known MHC class I, 9mer epitopes such as the MUC1D6 [36] (MUC1-SP-S1), M1.2 [37] (MUC1-SP-S2) and several novel epitopes such as MUC1-SP-S4 and MUC1-SP-S5. Our results with MUC1-SP-L showed broad HLA binding and robust antigen specific activation of human $CD4^+$ and $CD8^+$ T cells obtained from healthy volunteers and cancer patients bearing various MUC1 positive tumors. Moreover, MUC1-SP-L induced MUC1-specific CTL response in both syngeneic BALB/c mice and transgenic HLA-A2.1 (HHD-2) mice. The cellular response to MUC1-SP-L in mice was superior to that of the known TRA epitope BLP-25 (MUC1-TRA-L) [39,40]. The use of vaccines such as MUC1-SP-L that induce a strong and a broad T cell response could potentially be translated to better outcomes when using immunotherapy to treat cancer.

2. Materials and methods

2.1. Mice

The derivation of HLA-A2.1/D^b-β2 single-chain, transgenic, *H-2D$^{b-/-}$xβ2M$^{-/-}$* double knockout mice (named HHD-2 mice) has been previously described [14]. Eight- to 12-week-old HHD-2 mice were bred at the Weizmann Institute of Science breeding facility.

Six- to 8-week-old BALB/c mice were bred at the Tel Aviv University breeding facility. All experiments were conducted according to Weizmann Institute of Science and Tel Aviv University institutional rules and regulations.

2.2. MHC binding predictions

Binding predictions were performed for HLA class I (HLA-A, B, C) and HLA class II (HLA-DRB1) alleles that are most frequent worldwide. However, in order to have a defined population, in this study we focused only on the Caucasian population. The binding strength of 9mers to the class I alleles was predicted using BIMAS, http://www.bimas.cit.nih.gov/molbio/hla_bind/ [41]. The prediction of HLA class II peptide binding was done using Propred, http://www.imtech.res.in/raghava/propred/ [42] and Immune Epitope, www.immuneepitope.org [43]. We defined different binding strengths for class I as Strong = peptide score of >100, Medium = 10–100, Weak = 5–10.

Binding strength for DR HLA class II binding was defined in Propred as Strong = top 1% of binders, Medium = 1–2% of binders, Weak = 2–3% of binders. In Immune Epitope, for HLA-DRB1-0901, Strong = IC50 of 0.01–9.9 nM, Medium = 10–99.9 nM, and Weak = 100–10,000 nM.

2.3. Tumor cells

MDA-MB-231 human breast cancer and U266 human multiple myeloma are cell lines positive for both HLA class A2.1 and MUC1. MDA-MB-468 is a human breast cancer line, which is negative for HLA-A2.1, but positive for MUC1 expression. The K-562 human myalo-leukemia cell line and the human MOLT-4 T cell leukemia cell line express neither HLA-A2.1 nor MUC1. The 721.221 B-lymphoblastoid cell line is a TAP-2-deficient lymphoma clone of human origin which is negative for MUC1 expression. The 721.221-A2.1 cell line is the wild type 721.221 line stably transfected with HLA-A2.1 [44]. All cell lines were maintained in RPMI-1640 (Biological Industries, Beit Haemek, Israel) medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and 50 μg/ml gentamycin (Biological Industries, Beit Haemek, Israel) (termed here, complete medium). DA-3 is a metastatic cell line selected from the DMBA-induced mammary tumor in BALB/c mice [45]. DA-3TM are hMUC1 transected DA-3 cells [45].

2.4. Peptide synthesis

MUC1-SP-L was chemically synthesized (EMC Microcollections, Tubingen, Germany) and other peptides were chemically synthesized (GL Biochem, Shanghai, China) by fully automated, solid-phase, peptide synthesis using Fluorenylmethyloxycarbonyl (Fmoc)/tBu-strategy and Rink-amide-polystyrene resin. The purity and identity of the peptide was determined by HPLC-Mass Spectra analysis and was >95% for all peptides.

2.5. Proliferation via peptide-pulsed DC

DCs were enriched from 50 ml buffy-coat samples obtained from naïve donors. Isolated PBMC were cultured at the concentration of 2.5×10^6/ml in complete RPMI for 4 h at 37 °C in tissue culture 150 mm × 25 mm dishes (CellStar, Greiner, Frickenhausen, Germany). Adherent cells were collected and re-cultured in serum-free DCCM-1 medium (Biological Industries, Beit Haemek, Israel) supplemented with human IL-4 (1000 IU/ml) and GM-CSF (80 ng/ml) for 7 days at 37 °C. Next, floating cells were collected and loaded with 50 μg/ml of specified peptide for 18 h at 37 °C. DC-loaded cells were then utilized for the different immunological assays. For T cell proliferation, 2×10^5 PBLs and 25×10^3 peptide-pulsed DC were placed in an U-shaped 96-well plate (Nunc, Roskild, Denmark) with RPMI complete medium supplemented with 5% human AB serum (Sigma Israel, Rehovot, Israel). The following two controls were used in every experiment: 25×10^3 unloaded DC and peptide loaded DC without PBL. Plates were cultured for 5 days followed by additional 18 h with 0.5 μCi/well of ^{3}[H] thymidine (Amersham, Little Chalfont, Buckinghamshire, UK). Cells were then harvested using unifilter 96-well plated (PerkinElmer, Waltham, MA, USA) and the radioactive counts (cpm) were determined by a β-counter (Packard Matrix 96 direct beta-counter, Downers Grove, IL, USA).

2.6. Proliferation of cancer-patient derived PBMC

PBMC were separated from whole blood samples obtained from cancer patients having MUC1 positive tumors. Patients had no prior immunosuppressive therapy and did not receive any blood products within 6 weeks prior to inclusion. Briefly, PBMC were separated in complete RPMI medium and cultured for 4 h, at 37 °C at the concentration of 5.0×10^6/ml and a final volume of 100 μl/well in 96-well flat bottom plates (Costar, Corning, New York, USA), while the remaining PBMC were stored at −80 °C. After 4 h, the non-adherent cells (PBL) were discarded and different peptides at the final concentration of 50 μg/ml and a final volume of 100 μl were suspended in serum free DCCM-1 medium (Biological Industries, Beit Haemek, Israel) and added to the adherent cells for an incubation of 18 h at 37 °C. The next day, the DCCM-1 medium was removed and the plates were rinsed with PBS. The frozen PBMC were then thawed and resuspended at a concentration of 2×10^6 cell/ml and dispensed at a final volume of 200 μl per well in complete RPMI medium supplemented with 5% Hu AB sera (Sigma Israel, Rehovot, Israel). The plates were then cultured for 5 days at 37 °C followed by ^{3}[H] thymidine labeling and analyzed as described.

2.7. ELISA for detecting sera levels of MUC1 and anti-MUC1 antibodies

MUC1 antigen levels were evaluated in ELISA plates (F96 Maxisorp, Nunc, Roskild, Denmark) using a commercial anti-MUC1 monoclonal antibody (mAb) (clone M4H2), ELISA kit (HyTest, Turku, Finland) according to manufacturer's protocol. Clone M4H2, was raised against a TRA peptide and recognizes MUC1's core antigen. MUC1 levels were evaluated using 7 serial dilutions of 100 μl of the patients' sera starting at 1:5. For a MUC1 positive control, we used 6 dilutions (starting at 1:5) of supernatant collected from the DA-3TM [45] cell line. The ELISA plates were developed with TMB/E solution (CHEMICON, Millipore, Billerica, MA, USA). The reaction was terminated by adding 50 μl/well of 10% sulfuric acid. Results were measured at 450 nm.

For evaluating the sera level of anti-MUC1 antibodies, ELISA plates (F96 Maxisorp, Nunc, Roskild, Denmark) were coated with 50 μl of MUC1-TRA-L peptide at 5 μg/ml in carbonate buffer and incubated overnight at 4 °C. Plates were then blocked with 200 μl of PBS with 0.5% gelatin for 2 h at 25 °C. Evaluated sera samples were then diluted 1:100 in PBS with 0.5% gelatin and incubated for 2 h at 25 °C. Next, 50 μl/well of the appropriate secondary anti-IgG antibody HRP-conjugated (CHEMICON, Millipore, Billerica, MA, USA) was added at a final dilution 1:10,000 in a blocking buffer and incubated for 1 h at 25 °C. Plates were then developed as described above. For a positive standard, we used 6 double dilutions starting from 10 μg/ml of the anti-TRA mAb H23 [46]. H23 mAb was raised against the human breast cancer cell line T47D [46] and recognizes the epitope APDTRP on the non-glycosylated from of MUC1. In this assay, naïve sera for MUC1 are $x \leq 200$ μg/ml, based on a mean value determined from 10 healthy individuals.

2.8. Development of MUC1-SP-L-induced T cell lines

Thawed PBLs underwent initial stimulation for 7 days with MUC1-SP-L-pulsed autologous DC at a ratio of 20:1 in complete RPMI medium with 50 IU/ml of human recombinant IL-7 (PeproTech Asia, Rehovot, Israel). Next, PBLs underwent a second stimulation for 5 days with adherent autologous MUC1-SP-L-pulsed PBMC. At the end of the fifth day, the medium was replaced with fresh medium containing 1 μg/ml of MUC1-SP-L and 50 IU/ml of human recombinant IL-2 (PeproTech Asia, Rehovot, Israel) and the cells were re-stimulated for an additional 48 h at 37 °C.

2.9. Cytokine secretion assay from T cells stimulated with MUC1-SP-L and other peptides

Cytokine levels were evaluated in ELISA plates (F96 Maxisorp, Nunc, Roskild, Denmark) using a commercial anti-human TNF-alpha, IL-2, IFN-gamma mAb, ELISA kit (ELISAMAX, Biolegend, Cambridge, UK) according to manufacturer's protocol. Cytokine levels were evaluated by sampling 100 μl of the cell line's growth medium. Results were measured at 450 nm.

2.10. In vitro cytotoxicity assay

Target cells were labeled with ^{35}S methionine (Amersham, Little Chalfont, Buckinghamshire, UK) for 18 h, washed and mixed for 5 h with differing amounts of MUC1-SP-L-specific enriched T cell lines in U-shape microtiter plates (Unifilter, Nunc, Roskild, Denmark). A total of 50 μl of each supernatant was mixed with 150 μl scintillation liquid Microscint^{TM} 40 (PerkinElmer, Waltham, MA, USA) and measured in a Matrix 96 direct beta-counter β counter (Packard Instruments, Meriden, CT, USA). 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 of 10% Triton X-100.

For cytotoxic experiments, two HHD-2 or BALB/c mice per group were sacrificed 10 days after the third vaccination. Their splenocytes were stimulated in vitro with 100 μg/ml MUC1-SP-L

in DCCM-1 for 2 h at 37 °C, 5% CO_2 and then re-stimulated for four additional days in RPMI-complete medium.

2.11. Phenotype analysis of MUC1-SP-L-induced T cell lines

MUC1-SP-L-induced T cell lines were suspended at a final concentration of 2×10^7 cells/ml, in a blocking solution of PBS, containing 3% FCS and 0.1% sodium azide; 50 μl of the cells (1×10^6 cells) were transferred into 5 ml FACS tubes (BD Falcon™, Franklin Lakes, NJ, USA). FITC-conjugated, anti-CD4, anti-CD8 and anti-CD45RO (ebiosciences, San-Diego, CA, USA) were then added for 30 min on ice in the dark. Following this incubation, cells were washed with 2 ml of the blocking buffer and re-suspended in 0.5 ml of PBS. Samples were cytometrically analyzed on LSR II FACS (BD Biosciences, San Jose, CA, USA).

2.12. Vaccination

Eight HHD-2 or BALB/c mice were subcutaneously immunized 3 times at 7-day intervals with 100 μg/mouse of the MUC1-SP-L peptide or the MUC1-TRA-L, in the case of BALB/c mice. Mice injected with peptides and mice in control group (vehicle-GM-CSF) concurrently received intraperitoneally 100 ng of murine GM-CSF (PeproTech Asia, Rehovot, Israel). MUC1-SP-L stock of 10 mg/ml was prepared in DMSO and the final concentration of 100 μg/mouse was reached using PBS. Murine GM-CSF (PeproTech Asia, Rehovot, Israel) was suspended in the PBS buffer.

2.13. Immunotherapy model

Female BALB/c mice, 6–8 weeks old, were inoculated with 1.5×10^5/100 μl DA-3TM at day 0. Seven mice per group were immunized as described above. Briefly, mice were immunized 3 times at weekly intervals with MUC1-SP-L peptide plus GM-CSF, MUC1-TRA-L plus GM-CSF, GM-CSF or PBS starting at day 7 or day 10 post-tumor inoculations. Mice survival was monitored daily.

2.14. Statistics

Results were analyzed with 2-tailed Student's *t*-test or Fisher's exact test. The minimal level of significance was set in a two-tailed *t*-test for $P < 0.05$ and in Fisher's exact for $P < 0.02$.

3. Results

3.1. MUC1-SP-L, a 21mer MUC1-derived peptide has promiscuous HLA-binding

For the selection of appropriate VCs, we used a number of in silico prediction methods as described in Section 2. Using these tools, we searched the SP and TRA domains of MUC1 for sequences that could bind efficiently to a defined list of abundant MHC class I and MHC class II alleles resulting in at least 50% population coverage for both MHC class I and Class II. The outcome of our search was the selection for further development of the 21mer MUC1-SP-L, which spans the entire SP of MUC1. MUC1-SP-L was predicted to bind to MHC class I epitopes in at least 83% of the Caucasian population and MHC class II epitopes in at least 52% of the Caucasian population.

MUC1-SP-L and four of its different inner 9mer epitopes were synthesized for further evaluation. Two were previously identified as the HLA-A2.1 CTL epitopes MUC1-SP-S1 [36] and MUC1-SP-S2 [37,38] and the other two, MUC1-SP-S4 and MUC1-SP-S5, are novel epitopes. For MUC1 TRA domain-derived epitopes, we used the previously identified HLA A2.1-restricted 9mer epitopes M1.1 (MUC1-TRA-S1) [37] and the 25mer peptide vaccine BLP25 [39,40]. (MUC1-TRA-L). As a non-MUC1-SP-derived control, we used a known MHC class I epitope (TRY-SP-S1) derived from the human tyrosinase TAA [47] (Table 1).

3.2. Naïve PBLs proliferate to MUC1-SP-L

To explore the immunogenicity and activation profile of MUC1 epitopes in a diversified HLA setting of an unprimed immunocompetent immune system, we conducted a set of proliferation assays for MUC1 epitopes (Table 1). To ensure efficient presentation, the peptides were pulsed on autologous-DC and cultured with PBLs isolated from the same naïve donors. In all the proliferation experiments, a stimulation index (SI) score of ≥2 was considered to be specific activation, a criterion used in several other reports [48,49]. Fig. 1A, shows that MUC1-SP-L manifested the broadest specific activation (SI ≥ 2) in all 13 samples examined (100%). MUC1-SP-L's inner 9mer epitopes, MUC1-SP-S1, MUC1-SP-S2 and MUC1-SP-S4 induced specific activation in 7/13 donors (53.8%) while MUC1-SP-S5 showed specific activation in 7/12 evaluated donors (58.3%). The MUC1-TRA-S1 epitope manifested specific activation only in 5/13 donors (38%), which was lower than any other SP-derived epitope. The fraction of responders from any class I peptide was lower compared to MUC1-SP-L ($P < 0.02$, Fisher's exact test). Moreover, MUC1-SP-L absolute SI was the highest in 8/13 donors (61%) and the stimulation to MUC1 was statistically higher ($P < 0.01$, *t*-test) compared to the stimulation of the 9mer epitopes MUC1-SP-S1, MUC1-SP-S2, MUC1-SP-S5 or the TRA 9mer epitope MUC1-TRA-S1, excluding its inner epitope MUC1-SP-S4 ($P = 0.082$).

We next evaluated the immunogenicity and specific activation of the MUC1 longer peptides MUC1-SP-L and MUC1-TRA-L, which can potentially bind multiple MHC class I and II molecules. Fig. 1B shows that MUC1-SP-L manifested specific activation in 6/6 (100%) evaluated donors, while MUC1-TRA-L manifested specific activation in only 1/6 (16.6%) of these donors ($P < 0.02$, Fisher's exact). Although we did not perform a full HLA typing analysis of the analyzed donors, there is a good reason to assume that MUC1-SP-L superior activation profile vs. MUC1-TRA-L correlates with its wider HLA in silico predicted binding (Table 1). We tested this assumption in one of the six evaluated donors, having the following HLA typing: A0205, A26.1, B3801, B4101, CW0701, CW1203, DRB1 1302, DRB1 1305. MUC1-TRA-L was not predicted to bind any of the class I or II alleles of this donor and indeed did not manifest specific activation. In contrast, MUC1-SP-L was predicted to bind the class I A0205 and the class II DRB 1302 and indeed the donor PBL responded positively to MUC1-SP-L. This result is of further interest as the donor's class I allele A0205, was not used for our binding prediction due to its low frequency (~1% of the Caucasian population).

3.3. PBLs from cancer patients having MUC1-positive tumors proliferate to MUC1-SP-L

Next, we evaluated in a proliferation assays, the activation profile of the MUC1 epitopes (Table 1) in PBMC obtained from cancer patients bearing MUC1-positive colorectal, lung, prostate and testicular tumors (Fig. 2). To check MUC1 expression, we evaluated the sera levels of both MUC1 antigen and anti-MUC1 IgG antibodies. It was previously shown that both could indicate MUC1 expression in tumor cells [50–52].

The clinical characteristics and the MUC1/anti-MUC1 antibody sera levels of the patients enrolled in these experiments are presented in Table 2. In this set of experiments, the MUC1 peptides were presented directly via PBMC and not via DC to the PBLs of the cognate cancer patient. This is based on the assumption that immune cells in these patients have already been primed by the MUC1 antigen, as was shown in the case of the CA125 TAA [53]. The highest specific activation in 90% (9/10) of evaluated patients was

Table 1
MHC class I (HLA-A, B, C) and class II (HLA-DRB1) binding prediction of epitopes used in this study.

VXL ID[a]	Published ID	Sequence	Target	Class I alleles predicted binding	Class II alleles predicted binding
MUC1-SP-S1	MUC1D6 [36]	LLLTVLTVV	MUC1 (SP) 13–21	A0201 (S)	
MUC1-SP-S2	MUC1C6 [36] M1.2 [37]	LLLLTVLTV	MUC1 (SP) 12–20	A0201 (S)	
MUC1-SP-S4	–	TQSPFFLLL	MUC1 (SP) 5–13	A0201 (M), A24 (W), Cw0602 (W)	
MUC1-SP-S5	–	SPFFLLLLL	MUC1 (SP) 7–15	B7 (M), B51.1 (S), Cw4.1 (S), 6.2 (M)	
MUC1-TRA-S1	M1.1 [37]	STAPPVHNV	MUC1 (TRA) 950–958[b]	B5801 (W)	
TYR-SP-S1	NO [47]	MLLAVLYCL	Tyrosinase (SP) 1–9	A0201 (S), A24 (W), A3 (W), Cw4.1(W) H-2Kd (S)	
MUC1-SP-L	ImMucin VXL100	MTPGTQSPFFLLLLLTVLTVV	MUC1 (SP) 1–21	A24 (W), A0201(S), B7 (M), B35.1 (M), B51.1 (S), B58.1 (M), Cw7.2 (W), Cw4.1 (S), Cw6.2 (M) H-2Kd (S)	DRB10401 (S), 1101 (S), 13.2 (S), 0301 (M), 1501 (M), 0701 (W) Murine-I-A^d (S), I-A^b (S)
MUC1-TRA-L	BLP25 [39,40]	STAPPAHGVTSAPDTRPAPGSTAPP	MUC1 (TRA) 130–154	A68.1 (S), B5101 (W) H-2Kd	Murine-I-A^d (S)

[a] VXL ID nomenclature: TYR, tyrosinase; SP, signal peptide; TRA, tandem repeat array; S, short; L, long.
[b] MUC1-TRA-S1 epitope also appears with one amino acid mismatch in position 130–138 on the MUC1 protein.

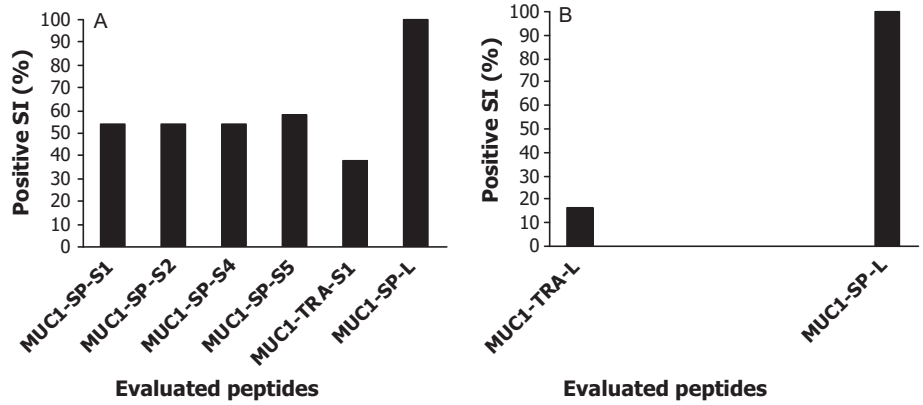

Fig. 1. Proliferation in naïve individuals. (A) PBLs from 12 or 13 naïve donors or (B) six donors were individually stimulated with MUC1-SP-L, MUC1-TRA-L or other 9mer peptides on cognate dendritic cells as described in Section 2. Results are presented as the fraction of donors exhibiting proliferation to each peptide. Responders were considered as such if their SI ≥ 2. SI = Stimulation Index.

directed to MUC1-SP-L, as compared to any other 9mer epitopes, which manifested positive proliferation in 20–50% of the patients (Fig. 2A). The fraction of responders to MUC1-SP-S1, MUC1-SP-S2 and MUC1-SP-S4 peptides was significantly lower compared to those of MUC1-SP-L ($P < 0.02$ to MUC1-SP-S1, and MUC1-SP-S2 vs. MUC1-SP-L, and $P < 0.05$ to MUC1-SP-S4 vs. MUC1-SP-L, Fisher exact; Fig. 2B).

When comparing the two MUC1-longer peptide vaccines, MUC1-SP-L manifested specific (SI ≥ 2) activation in 5/6 (83.3%) evaluated patients, while MUC1-TRA-L did not manifest any specific activation in all six evaluated patients (Fig. 2C, $P < 0.02$, Fisher exact). MUC1-SP-L also manifested stronger absolute SI levels comparing to MUC1-TRA-L (Fig. 2D). The results here indicate the immunodominant properties of MUC1-SP-L compared to any other

Table 2
Characteristics of cancer patients.

Patient no.	Age	Sex M/F	Indication	Stage	Interval from surgery[a]	Macroscopic disease present	MUC1 antigen[b] (titer)	Anti-MUC1 antibodies[b] (μg/ml)
001	50	M	Colorectal	III	34	No	0	294
002	59	M	Colon	III	29	No	1:16	382
003	66	F	Colorectal	II	No surgery	Yes	1:16	266
004	52	F	Colorectal	II	No surgery	Yes	1:4	260
005	60	F	Lung	I	32	No	0	816
006	57	M	Colon	II	31	No	1:4	756
007	77	M	Prostate	I	No surgery	Yes	1:64	758
008	72	M	Prostate	II	No surgery	Yes	1:32	267
009	54	M	Prostate	II	No surgery	Yes	1:4	571
010	71	M	Colon	IV	18	Yes	0	290

[a] Interval from surgery for primary tumor to blood draws (days).
[b] Sera levels of MUC1 antigen and anti-MUC1 antibodies were measured by ELISA assay as described in Section 2. Positive MUC1 level is titer of $x \geq 1{:}4$, positive anti-MUC1 IgG antibodies are $x > 200$ μg/ml. Results represent one out of two similar repeated experiments.

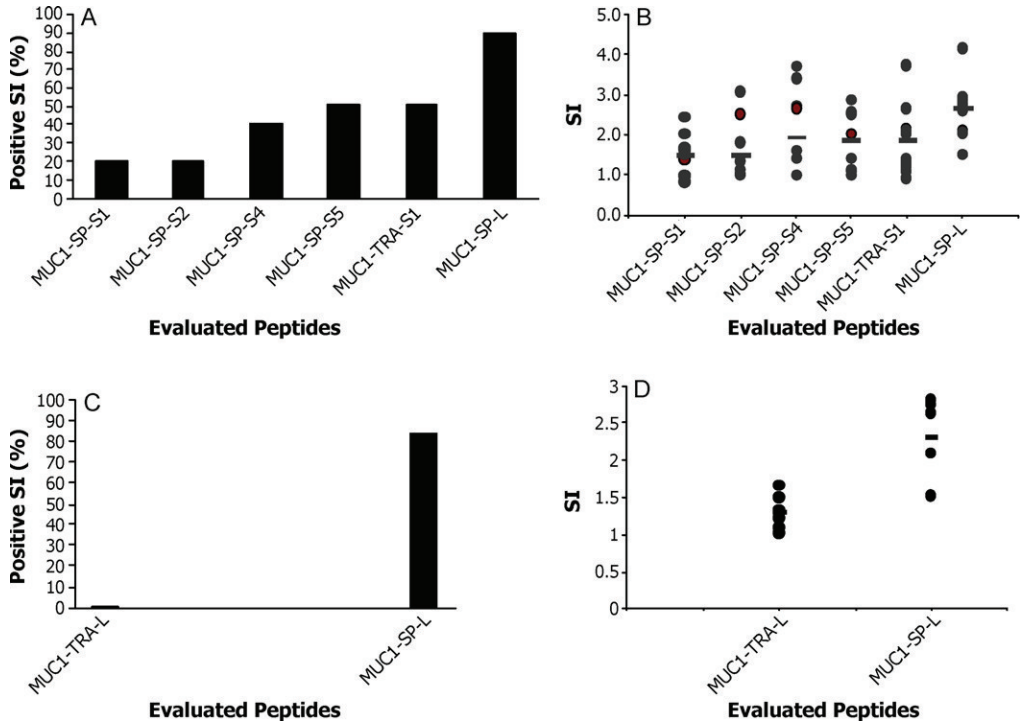

Fig. 2. Proliferation in cancer patients. PBMCs from 10 (A and B) or 6 (C and D) MUC1 positive cancer patients were individually stimulated as described in Section 2 by MUC1-SP-L or 9mer peptide-loaded PBMC (A and B), and MUC1-SP-L or MUC1-TRA-L peptide-loaded PBMC (C and D). Positive responders (A and C) were considered as such if their SI > 2. Individual SI are depicted in (B and D). SI = Stimulation Index.

evaluated MUC1 epitope in a heterogeneous population with diversified HLA settings. The results also suggest a wide response profile for MUC1-SP-L in patients with MUC1-positive cancers. Combining the responder fractions of the healthy donors (13/13) and the cancer patients (9/10), the confidence interval for the fraction of responders in this mixed population was 90–100% (confidence level of 95%).

3.4. Establishment and characterization of MUC1-SP-L-induced T cell lines

To test which T cell populations ($CD4^+$ or $CD8^+$) were activated and could change their memory or functional phenotype following activation with MUC1-SP-L, naïve PBLs were consecutively stimulated with MUC1-SP-L-loaded cognate DC. The memory characteristics $CD45RO^+$ of the $CD4^+$ or $CD8^+$ cells were evaluated by FACS analysis (Fig. 3A). The results indicate that following even one stimulation, MUC1-SP-L could induce the maturation of naïve PBLs into $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ T cells. Additional stimulations increased the absolute number of $CD45RO^+$ cells, although their percentages in the total population decreased. The total number of cells exhibiting memory markers doubled compared to the initial levels recorded in both populations; from 43% to 85% in the $CD45RO^+CD4^+$ cells and from 15% to 36% in the $CD45RO^+CD8^+$ cells.

One of the parameters of functionally activated T cells is the secretion of key cytokines such as TNF-alpha, IL-2, and IFN-gamma in response to antigen stimulation. The levels of cytokine secretion depends on the type of cell, e.g., $CD4^+$ vs. $CD8^+$, on the stage of activation, in particular early vs. late stage activation, and on the type of memory induced [54]. We therefore analyzed the levels of cytokine secretion by the stimulated cells during MUC1-SP-L line development (Fig. 3B). We observed a peak of TNF-alpha after the first round of stimulation with MUC1-SP-L, which decreased after the second stimulation, and completely disappeared after the third stimula-

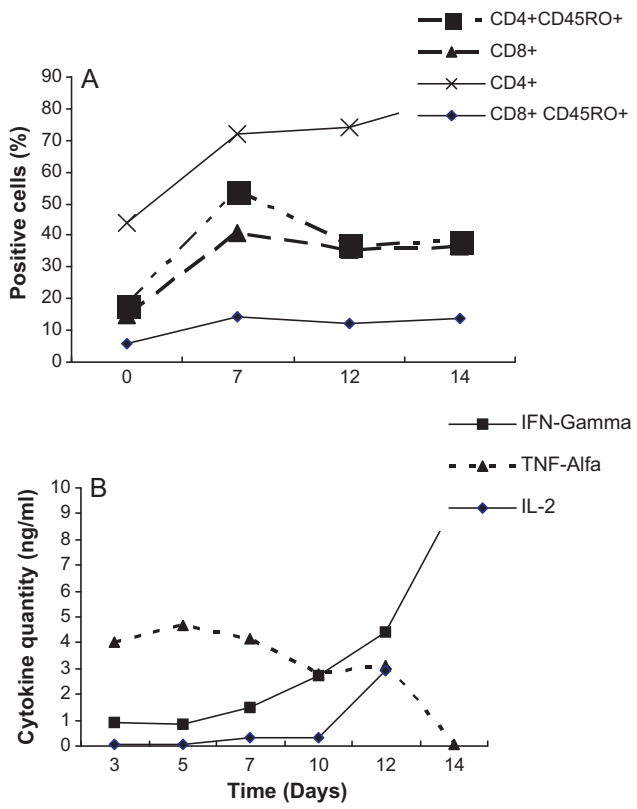

Fig. 3. (A) Characterization of MUC1-SP-L-raised T cell lines. Samples of cells and growth media were collected following each T cell stimulation (on days 7, 12, 14), and evaluated in FACS for CD4, CD8, and CD45RO memory phenotypes. (B) ELISA measured secreted cytokines. Results shown are from one of the two similar experiments averaging results from 4 donors.

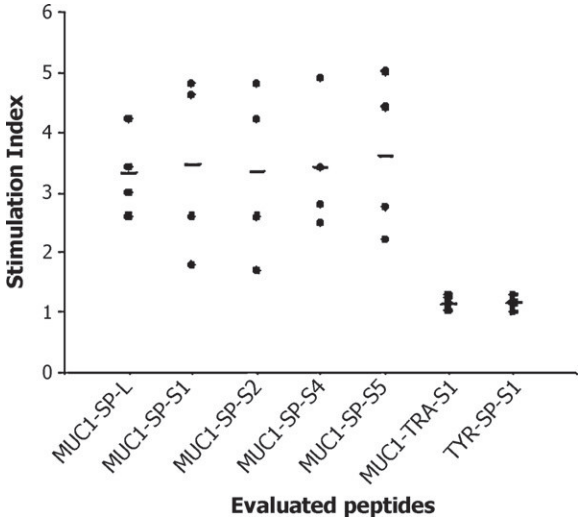

Fig. 4. Cross priming mediated by MUC1-SP-L-induced T cell line as measured by proliferation response. T cell lines that were raised to MUC1-SP-L, were evaluated in proliferation to various MUC1 class I 9mer epitopes, namely MUC1-SP-S1, MUC1-SP-S2, MUC1-SP-S4 and MUC1-SP-S5. Proliferation to MUC1-SP-L, MUC1-SP-S1, 2, 4 and 5 are statistically significant ($P < 0.001$) compared to the proliferation of the MUC1-TRA-S1 or TYR-SP-S1. Results represent one out of two similar experiments using 4 different donors.

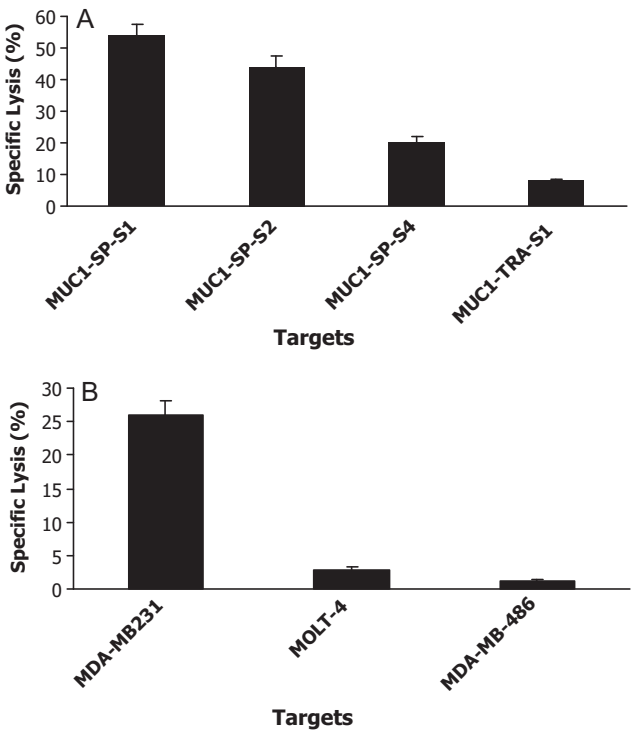

Fig. 5. (A) Cytotoxic properties of MUC1-SP-L-induced T cell lines. Specific lysis of the TAP-deficient 721.221-A2.1 loaded with the MUC1-epitopes or (B) MUC1 and HLA-A2.1 positive tumors MDA-MB-231. Cytotoxicity is greater ($P < 0.002$) for MUC1-SP-S1, MUC1-SP-S2, MUC1-SP-S4 compared with the lysis of MUC1-TRA-S1 and also statistically higher ($P < 0.00002$) for MDA-MB-231 compared with the lysis of the HLA-A2.1-negative cell line MDA-MB-486 or the MUC1-negative cell line MOLT-4. Effector-to-target (E:T) ratio of 100:1 is shown. Standard deviation in this assay is of triplicate well. Results represent one of the two similar experiments using the same T cell-line.

tion. In contrast, the levels of IFN-gamma and IL-2 significantly increased after the second and third stimulations with MUC1-SP-L. These results suggest that MUC1-SP-L could induce Th1 activation in naïve donor T cells.

3.5. MUC1-SP-L-specific T cell lines proliferate to MUC1-SP-L or to the SP inner epitopes

To test whether APC cross-presents MUC1-SP-L to T cells, we used T cell lines that were raised to MUC1-SP-L, to check whether they respond to various MUC1 class I 9mer epitopes, namely MUC1-SP-S1, MUC1-SP-S2, MUC1-SP-S4 and MUC1-SP-S5. Fig. 4 shows that T cell lines raised on MUC1-SP-L proliferated to MUC1-SP-L, but also to its inner epitopes with a mean SI = 4, supporting cross-presentation. The response was highly specific and statistically significant ($P < 0.001$, *t*-test) for proliferation to MUC1-SP-L and its inner epitopes compared to the control tyrosinase SP 9mer epitope TYR-SP-S1 peptide, or to the MUC1 TRA-derived epitope MUC1-REA-S1.

Specific proliferation by MUC1-SP-L-induced T cell lines to MUC1-SP-L, to its inner epitopes MUC1-SP-S1, MUC1-SP-S2, MUC1-SP-S4, and to some extent to MUC1-SP-S5 corresponded with the release of the Th1 cytokines IL-2 and IFN-gamma. In contrast, secretion of the proinflammatory cytokine TNF-alpha by MUC1-SP-L-induced T cell lines was similar when stimulated with most epitopes and did not correspond with MUC1-SP-L activation (data not shown).

3.6. Cytotoxicity responses mediated by MUC1-SP-L-induced T cell lines

To check the cytotoxic potential of the CTLs that developed while raising the mixed CD4/CD8 T cell lines, we conducted a set of cytotoxicity assays against MUC1 positive HLA-A2.1 positive tumor cells or MUC1 peptide-loaded TAP-deficient cells (Fig. 5). MUC1 expression in all evaluated cell lines was confirmed by FACS analysis, using the H23 anti-MUC1 mAb [46] (see Supplementary Fig. S1). To overcome HLA restriction barriers between our T cell lines and MUC1 positive tumor cells, we evaluated MUC1-SP-L derived cell lines for the expression of the MHC class I HLA-A2.1 allele and proceeded with one of the four lines that were found positive (data not shown).

The HLA-A2.1 positive T cell line was tested for its ability to lyse TAP-deficient HLA-A2.1-restricted 721.221-A2.1 transfected B-lymphoblastoid cells loaded with single MUC1-derived HLA-A2.1-restricted peptides. Fig. 5A shows that the MUC1-SP-L-raised CTL line lysed MUC1-SP-L inner peptides, loaded onto 721.221-A2.1 target cells. HLA-A2.1-restricted MUC1-SP-S1, MUC1-SP-S2, and MUC1-SP-S4 peptides elicited high-to-moderate specific lysis of 52%, 40%, and 20% of targets, respectively in 1:100 target-to-effector ratio, supporting antigen processing and presentation of these epitopes in APCs. A significantly lower ($P < 0.0002$, *t*-test) lysis of 8% was recorded to the HLA-A2.1-restricted TRA peptide MUC1-TRA-S1 indicating the specificity of the induced response to epitopes derived from MUC1-SP.

We also evaluated the ability of the MUC1-SP-L-raised HLA-A2.1 positive T cell line to lyse MUC1 expressing tumor cells in a similar cytotoxic assay. The results in Fig. 5B show MUC1-Specific and HLA-A2.1-restricted lysis of the MUC1 positive breast tumor cell line MDA-MB-231 was stronger than the lysis of any irrelevant target ($P < 0.00002$, *t*-test). The breast cell line MDA-MB-468, which expresses MUC1, but not HLA-2.1, was not lysed in this assay. Likewise, no lysis was detected of the acute lymphoblastic leukemia cell line MOLT-4, which does not express MUC1 or HLA-2.1. These results confirm the natural processing and presentation of MUC1-SP-L epitopes on MUC1 expressing tumors.

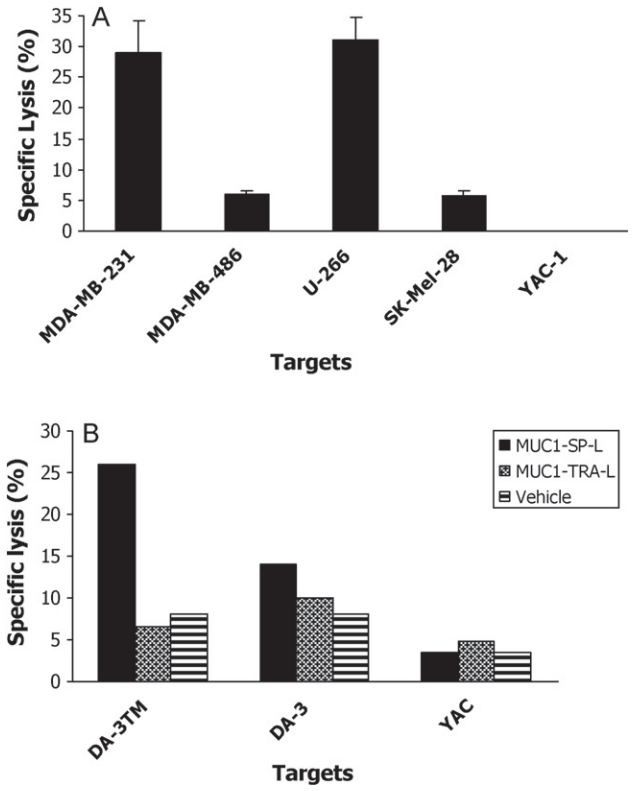

Fig. 6. Immunogenicity of MUC1 long peptides in (A) HLA-A2.1 transgenic HHD-2 mice and (B) BALB/c syngeneic mice. Mice were vaccinated with MUC1-SP-L, MUC1-TRA-L with GM-CSF or GM-CSF alone (Vehicle) as described in Section 2. MUC1-SP-L specific lysis of MUC1 and HLA-A2.1 positive tumors MDA-MB-231 and U266 is greater ($P < 0.001$) compared with lysis of the controls MDA-MB-486, SK-Mel-28 and Yac. Results represent one out of two similar experiments using 2 mice for each immunization group per experiment. An effector-to-target (E:T) ratio of 100:1 is shown.

3.7. Cytotoxicity responses induced by MUC1-SP-L in two mouse strains

To test MUC1-SP-L immunogenic potential in vivo, we immunized HLA-A2.1 transgenic mice (HHD-2) and BALB/c syngeneic mice with MUC1-SP-L. Murine GM-CSF was admixed with MUC1-SP-L, as this combination previusely exhibited high-quality adjuvant properties when used with other TAA peptide vaccines [55,56]. Fig. 6 shows that splenocytes from HHD-2 mice immunized with MUC1-SP-L lysed both the MDA-MB-231 (29% lysis) breast cell line and the U266 multiple myeloma cell lines (33% lysis), both expressing MUC1 and HLA-A2.1 (Fig. 6A). This lysis was statistically greater ($P < 0.0007$, t-test) compared to the lysis of the control MUC1 positive HLA-A2.1 negative cell line MDA-MB-468 and MUC1, HLA-A2.1 negative cell line SK-Mel-28 (6–8% lysis). No lysis was recorded to the Yac-1 cell line; ruling out the involvement of NKs. Likewise, MUC1-SP-L immunized BALB/c mice produced splenocytes with the greatest lysis of DA-3TM (26%), the murine mammary tumor cell line transfected with the human MUC1 gene. The lysis was probably HLA-A2.1 restricted in HHD-2 mice and K^d-restricted in BALB/c mice, as MHC class I epitopes were predicted in silico for MUC1-SP-L in these mice (Table 1). Moreover, in both strains we could predict MHC class II epitopes, I-A^b for HHD-2 mice and I-A^d for BALB/c mice (Table 1), which plausibly assisted in immune priming. Less lysis, at the range of 6–8%, was manifested by MUC1 TRA epitope MUC1-TRA-L, in-spite of the existence of class I epitopes on this peptide (Table 1). Less lysis at the range of 8–14% was also observed against the wild type DA-3 line. The lysis of the wild type DA-3 can be explained by MUC1-SP-L shared epitopes between the human and murine MUC1. Horn et al. showed that murine MUC1 was expressed on wild type DA-3 cells [57]. No lysis was recorded against the Yac-1 cell line, ruling out the involvement of NKs in these mice. These results indicated that MUC1-SP-L also has strong immunogenic properties when injected in vivo to mice with different MHC backgrounds.

3.8. Anti-tumor responses induced by MUC1-SP-L in BALB/c mice tumor model

To check the therapeutic potential of MUC1-SP-L, BALB/c mice were inoculated with DA-3TM syngeneic tumor cells. Vaccination with MUC1-SP-L or MUC-TRA-L plus GM-CSF was performed as described in Section 2 beginning 7 days prior (protection mode) or 10 days after (therapy mode) tumor inoculation (Fig. 7). Interestingly, while both peptides exhibited similar effects ($P = 0.29$, t-test) on the survival of mice (~60% on day 120) in protection mode (Fig. 7A and C), in the therapy mode, the activity of MUC1-SP-L far surpassed that of MUC-TRA-L (85% vs. 0% survival, respectively on day 120). The survival of MUC1-SP-L immunized mice on day 120 was statistically significant ($P < 0.01$, t-test) in comparison with the survival of MUC-TRA-L immunized mice. Immunization with GM-CSF also had a significant effect on mice survival (42% vs. 0% on day 120) compared to MUC1-TRA-L. This plausibly suppressive effect by MUC1's TRA has been previously shown by Agrawal et al. These results suggest an advantage for the MUC1-SP domain in a therapeutic setting.

4. Discussion

Many single epitopes of MHC class I-restricted TAA peptides showed promising preclinical results both in vitro and in vivo [58–60]. Yet, thus far, these vaccines have demonstrated limited efficacy when tested in clinical trials [61]. There is increasing evidence that vaccines combining TAA specific MHC class I and II epitopes may potentiate better anti-tumor effector functions and long-term immunity [4,13,62]. To date, very few peptides with promiscuous binding of both MHC class I and II alleles have been identified. Previous reports of using longer peptides, encoding multiple epitopes used either of two strategies. The first strategy uses peptides which promiscuously bind several MHC class II alleles [63,64]. Fern and Good reported two malaria-derived peptides with broad activation of naïve PBLs [64]. These malaria peptides mediated positive (SI > 2) proliferation by $CD4^+$ and $CD8^-$ T cells. The second strategy used peptides that combine a single class I and a single class II epitope [16–18]. Gjertsen et al. reported a response of both $CD4^+$ and $CD8^+$ T cells to a RAS-derived peptide [17]. A similar approach was also shown using an HER-2/neu-derived peptide [16]. Likewise, Odunsi et al. presented an NY-ESO-1-derived 14mer peptide with binding properties for two class I alleles (HLA-A2.1, A24) and two class II alleles (HLA-DPB1*0401 and HLA-DPB1*0402) [18]. Vaccination with this peptide produced prolonged immunity of up to 12 months in ovarian cancer patients. Nevertheless, although a combination of both MHC class I and II epitopes led to a strong immune response, none of these vaccines had a wide MHC coverage for both MHC class I and MHC class II.

While past research sought the best epitopes based on their specific antigenicity, we recently presented a third alternative in which we used specific defined domains like SP, that inherently contain significantly larger amount of epitopes than other protein domains [20]. The improved binding of this domain relies on its hydrophobic nature and on its specific sequence [20]. Support for our approach was published by Jiang et al., who showed that vaccination of mice with an 18mer SP derived from the bacterial antigen Ag2/PRA, either as a gene or synthetic peptide, could induce pro-

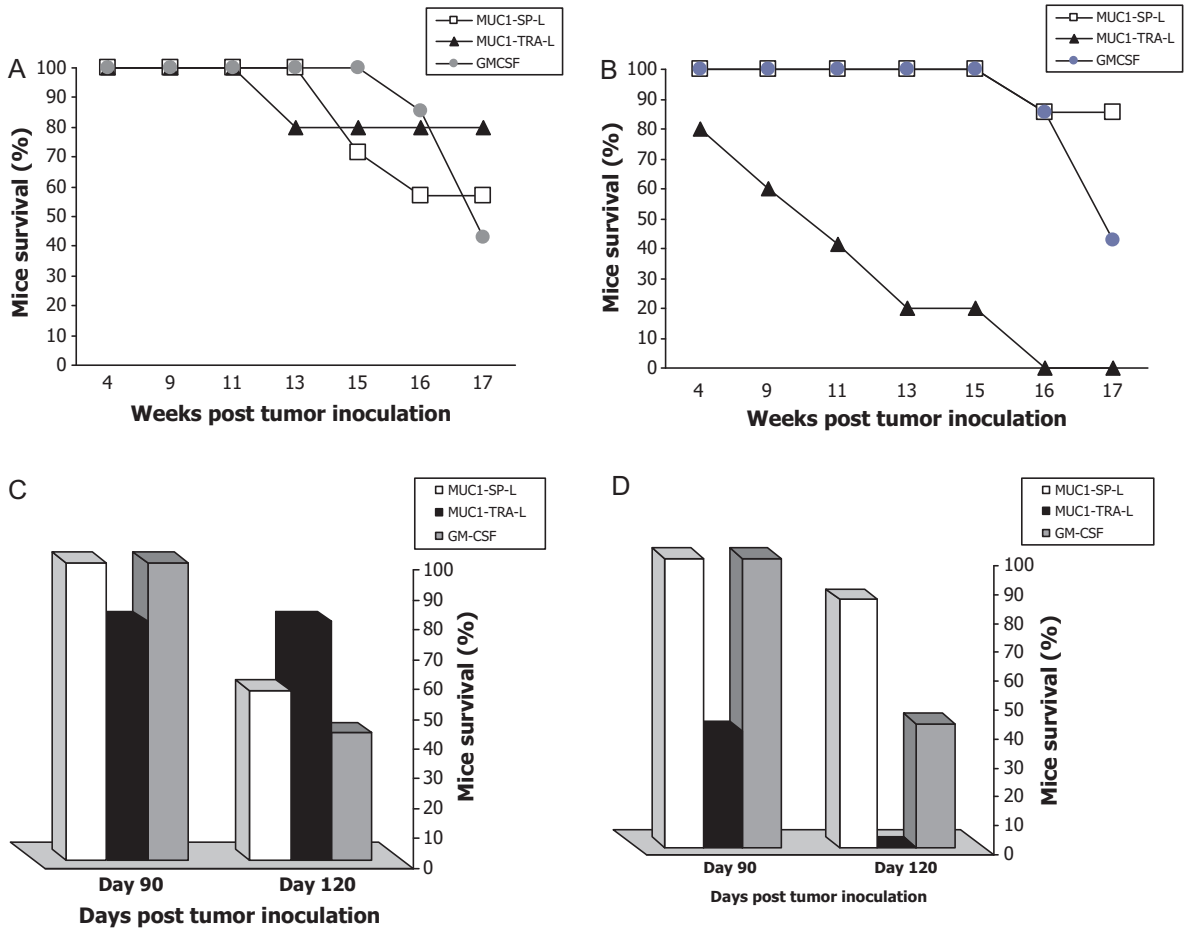

Fig. 7. Anti-tumor properties of MUC1 long peptides in BALB/c syngeneic mice. Mice were vaccinated with MUC1-SP-L or MUC1-TRA-L plus GM-CSF, as described in Section 2. (A and C) DA-3TM tumor cell inoculation was performed 7 days before or (B and D) 10 days after vaccine regimen initiation. Survival of MUC1-SP-L tumor bearer mice was significantly higher ($P < 0.01$) compared with the survival of MUC1-TRA-L immunized mice (B and D).

tective immunity against the Coccidiomycosis microbial pathogen. The protective response was superior to that of the mature protein without the SP. Moreover, the protection was highly specific, as frame-shift mutation in Ag2/PRA SP abolished the specific activity [65]. In a separate study, McMurry et al. identified a MTb originated promiscuous SP derived epitope, which induced IFN-gamma secretion in a large percentage of PBMC from MTb immune subjects [66].

The current study presents MUC1-SP-L, a 21mer peptide, which is the entire SP domain of the MUC1 TAA. The peptide employs both strategies, a long peptide harboring many overlapping epitopes with a predicted binding to a wide range of MHC class I and II alleles. This strategy allows the use of synthetic peptide vaccines with wide population coverage and a potentially more robust response. Several studies presented strong correlations between high affinity binding of peptides to MHC class I [67] and MHC class II molecules [68] as predicted by in silico algorithms, to the immunogenicity of these peptides. Our results with the 21mer peptide MUC1-SP-L corroborate these findings. We showed in human PBL and in murine splenocytes, a strong, wide response to MUC1-SP-L compared to any other MUC1 evaluated epitopes. MUC1-SP-L was the only peptide that induced proliferation in all PBLs from 13 naïve donors examined, compared to a 16.6–58.3% response rate induced by any other MUC1-derived epitope (Fig. 1A and B). Since the 13 independent, responding donors represent a statistically large variety of HLA alleles, the response to MUC1-SP-L is likely to be mediated via multiple different MHC-epitope pairs (Fig. 1A). This result was also strengthened by the ability of MUC1-SP-L to bind rare alleles such as HLA-A0205, expressed in one of the 13 donors, an allele not included in our prediction strategy. A similarly wide activation profile for MUC1-SP-L was also observed in PBLs derived from cancer patients bearing MUC1-positive tumors. Response to MUC1-SP-L in cancer patients, induced directly via PBMC without autologous DC, was stronger than all other MUC1 defined peptides in terms of the fraction of positive responders ($SI \geq 2$), as well as in absolute SI values (Fig. 2). MUC1-SP-L induced proliferation in 90% of the samples examined, compared to a 20–50% response rate induced by its inner epitopes, and 0% binding of response rate by MUC1's TRA peptide MUC-TRA-L (Fig. 2A and B).

T cell lines induced via repeated stimulation with MUC1-SP-L showed specific responses to the inner 9mer epitopes MUC-SP-S1, MUC-SP-S2, MUC-SP-S4, and MUC-SP-S5, but not to the MUC1 TRA-derived epitope MUC-TRA-S1 or the TYR-SP-S1 epitope derived from the SP domain of tyrosinase (Fig. 4). These results corroborate the MUC1-SP-L cross presentation. $CD4^+$ and $CD8^+$ T cells activated by MUC1-SP-L exhibited a Th1 Cytokine release profile (Fig. 3). Moreover, MUC1-SP-L-induced T cells exhibited lysis of target cells loaded with the 9mer HLA-A2.1 epitopes MUC1-SP-S1, MUC1-SP-S2, and MUC1-SP-S4 and the lysis of tumor cells expressing HLA-A2.1 and MUC1 (Fig. 4). Lysis of these targets was either observed using MUC1-SP-L-raised human T cell lines (Fig. 5) or by splenocytes derived from MUC1-SP-L immunized HHD-2 and BALB/c mice (Fig. 6). The immunogenicity of MUC1-SP-L was demonstrated in the process of generating MUC1-SP-L-induced T cell lines through the increase of the detected percentages of $CD4^+CD45R0^+$, and $CD8^+CD45R0^+$ positive cells secreting high lev-

els of IL-2 and IFN-gamma (Fig. 3). The polyfunctional memory T cells generated following vaccination has been shown to correlate with an effective clinical response to pathogens such as to Leishmania major [69].

Proliferation to self peptides, in general, and specifically to TAA derived peptides is usually limited to indirect presentation, as they originate from self proteins and therefore are considered poor immunogens [70]. Likewise, historically, peptides injected without adjuvant were shown to be minimally immunogenic and usually required co-administration with immunologic adjuvants to induce detectable T cell responses [70]. Our in vitro and in vivo results with MUC1-SP-L were somewhat different from these observations. MUC1-SP-L was able to induce lymphocyte proliferation in vitro by direct activation using PBMC. Moreover, immunization of HLA-A2.1 transgenic mice and BALB/C syngeneic mice with MUC1-SP-L and murine GM-CSF induced a strong anti-MUC1 response without the use of any adjuvant. CTL response by MUC1-SP-L alone could even be generated in BALB/c mice using a longer vaccination regimen (data not shown). We can ascribe this strong immune response observed with MUC1-SP-L to the following underlying reasons: first, MUC1-SP-L, as a SP, contains lipophilic sequences which are known to be more immunogenic; past studies demonstrated augmented immune responses to MHC class I epitopes from MART-1 [71] or from ovalbumin [72] antigens via linking these epitopes to a SP sequence derived from the E3/19 protein of adenovirus type 2. In the case of ovalbumin, the immune response produced by the SP linked epitopes was comparable to that achieved via incomplete Freund's adjuvant [72]. Second, MUC1-SP-L also has MHC promiscuous binding to mice of the H-2d and H-2b haplotypes (Table 1) [20]. Third, SP sequences, like MUC1-SP-L, could also induce a preferred immunity via TAP-independent presentation [23,73,74]. This unique property is due to the signal peptidases found on the lumenal side of the ER membrane. A previous report by Dorfel et al. showed that TAP inhibition could only affect the presentation of the MUC1 TRA epitope MUC1-TRA-S1, but not of MUC1-SP-derived epitope MUC1-SP-S2 [73].

Although MUC1-SP-L was selected upon it's promiscuous binding to many human HLA alleles, we also tested efficacy on inbred mice expressing a limited number of MHC class I and II molecules. Nonetheless, in spite of these limitations, in vivo results of MUC1-SP-L in BALB/c were encouraging. Interestingly, MUC1-SP-L immunized tumor bearer mice manifested extended survival, especially in a therapeutic setting, which was significantly better compared to the TRA peptide MUC1-TRA-L. Although, a similar phenomena was previously described using an anti-PD1 agonist antibody [75], additional studies need to be performed in order to further explain these results. Since MUC1-TRA-L did not produce highly cytotoxic T lymphocytes in our assays, we speculate that manifested anti-tumor properties were related to the generation of anti-MUC1 antibodies.

The results presented here suggest a simple, straightforward strategy for immunotherapy of MUC1-expressing tumors. The MUC1-SP-L, composed of the entire SP domain of MUC1, is an attractive target for immunotherapy; since it is a safe, synthetic peptide, it stimulates responses in a broad portion of the population, it activates both the cytotoxic and the T helper arms, and it probably functions in a TAP-dependent and independent manner. Moreover, it leads to potent protective immunity, as shown in a metastatic murine mode. The safety and efficacy profile of MUC1-SP-L is currently being evaluated in cancer patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.103.

References

[1] Kedar E, Klein E. Cancer immunotherapy: are the results discouraging? Can they be improved? Adv Cancer Res 1992;59:245–322.
[2] Parmiani G, Castelli C, Dalerba P, Mortarini R, Rivoltini L, Marincola FM, et al. Cancer immunotherapy with peptide-based vaccines: what have we achieved? Where are we going? J Natl Cancer Inst 2002;94:805–18.
[3] Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415–32.
[4] Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 2008;8:351–60.
[5] Kawashima I, Hudson SJ, Tsai V, Southwood S, Takesako K, Appella E, et al. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum Immunol 1998;59:1–14.
[6] Fikes JD, Sette A. Design of multi-epitope, analogue-based cancer vaccines. Expert Opin Biol Ther 2003;3:985–93.
[7] Barve M, Bender J, Senzer N, Cunningham C, Greco FA, McCune D, et al. Induction of immune responses and clinical efficacy in a phase II trial of IDM-2101, a 10-epitope cytotoxic T-lymphocyte vaccine, in metastatic non-small-cell lung cancer. J Clin Oncol 2008;26:4418–25.
[8] Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced tumor growth through specific T cell tolerance induction. Proc Natl Acad Sci U S A 1996;93:7855–60.
[9] Diethelm-Okita BM, Okita DK, Banaszak L, Conti-Fine BM. Universal epitopes for human CD4+ cells on tetanus and diphtheria toxins. J Infect Dis 2000;181:1001–9.
[10] Weber JS, Hua FL, Spears L, Marty V, Kuniyoshi C, Celis E. A phase I trial of an HLA-A1 restricted MAGE-3 epitope peptide with incomplete Freund's adjuvant in patients with resected high-risk melanoma. J Immunother 1999;22:431–40.
[11] Ossendorp F, Mengede E, Camps M, Filius R, Melief CJ. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. J Exp Med 1998;187:693–702.
[12] Porgador A, Snyder D, Gilboa E. Induction of antitumor immunity using bone marrow-generated dendritic cells. J Immunol 1996;156:2918–26.
[13] van Mierlo GJ, Boonman ZF, Dumortier HM, den Boer AT, Fransen MF, Nouta J, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol 2004;173:6753–9.
[14] Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 1997;185:2043–51.
[15] Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. Nature 1998;393:480–3.
[16] Knutson KL, Schiffman K, Disis ML. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T cell immunity in cancer patients. J Clin Invest 2001;107:477–84.
[17] Gjertsen MK, Bjorheim J, Saeterdal I, Myklebust J, Gaudernack G. Cytotoxic CD4+ and CD8+ T lymphocytes, generated by mutant p21-ras (12Val) peptide vaccination of a patient, recognize 12Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. Int J Cancer 1997;72:784–90.
[18] Odunsi K, Qian F, Matsuzaki J, Mhawech-Fauceglia P, Andrews C, Hoffman EW, et al. Vaccination with an NY-ESO-1 peptide of HLA class I/II specificities induces integrated humoral and T cell responses in ovarian cancer. Proc Natl Acad Sci U S A 2007;104:12837–42.
[19] Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, et al. Induction of tumor-specific CD4+ and CD8+ T cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008;14:178–87.
[20] Kovjazin R, Volovitz I, Daon Y, Vider-Shalit T, Carmon L, Louzoun Y. Signal peptides and trans-membrane regions are broadly immunogenic and have high CD^{8+} T cell epitope densities: implications for vaccine development. Mol Immunol 2011;48:1009–18.
[21] Lyko F, Martoglio B, Jungnickel B, Rapoport TA, Dobberstein B. Signal sequence processing in rough microsomes. J Biol Chem 1995;270:19873–8.
[22] Martoglio B. Intramembrane proteolysis and post-targeting functions of signal peptides. Biochem Soc Trans 2003;31:1243–7.
[23] Martoglio B, Dobberstein B. Signal sequences: more than just greasy peptides. Trends Cell Biol 1998;8:410–5.
[24] Graham RA, Burchell JM, Taylor-Papadimitriou J. The polymorphic epithelial mucin: potential as an immunogen for a cancer vaccine. Cancer Immunol Immunother 1996;42:71–80.
[25] Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, et al. Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res 1993;53:641–51.
[26] Hareuveni M, Tsarfaty I, Zaretsky J, Kotkes P, Horev J, Zrihan S, et al. A transcribed gene, containing a variable number of tandem repeats, codes for a human epithelial tumor antigen. cDNA cloning, expression of the transfected gene and over-expression in breast cancer tissue. Eur J Biochem 1990;189:475–86.
[27] McGuckin MA, Walsh MD, Hohn BG, Ward BG, Wright RG. Prognostic significance of MUC1 epithelial mucin expression in breast cancer. Hum Pathol 1995;26:432–9.

[28] Engelmann K, Shen H, Finn OJ. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. Cancer Res 2008;68:2419–26.
[29] Lacunza E, Baudis M, Colussi AG, Segal-Eiras A, Croce MV, Abba MC. MUC1 oncogene amplification correlates with protein overexpression in invasive breast carcinoma cells. Cancer Genet Cytogenet 2010;201:102–10.
[30] Magarian-Blander J, Domenech N, Finn OJ. Specific and effective T cell recognition of cells transfected with a truncated human mucin cDNA. Ann N Y Acad Sci 1993;690:231–43.
[31] Agrawal B, Krantz MJ, Reddish MA, Longenecker BM. Cancer-associated MUC1 mucin inhibits human T cell proliferation, which is reversible by IL-2. Nat Med 1998;4:43–9.
[32] Fung PY, Longenecker BM. Specific immunosuppressive activity of epiglycanin, a mucin-like glycoprotein secreted by a murine mammary adenocarcinoma (TA3-HA). Cancer Res 1991;51:1170–6.
[33] van de Wiel-van Kemenade E, Ligtenberg MJ, de Boer AJ, Buijs F, Vos HL, Melief CJ, et al. Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction. J Immunol 1993;151:767–76.
[34] Stepensky D, Tzehoval E, Vadai E, Eisenbach L. O-glycosylated versus non-glycosylated MUC1-derived peptides as potential targets for cytotoxic immunotherapy of carcinoma. Clin Exp Immunol 2006;143:139–49.
[35] Correa I, Plunkett T, Coleman J, Galani E, Windmill E, Burchell JM, et al. Responses of human T cells to peptides flanking the tandem repeat and overlapping the signal sequence of MUC1. Int J Cancer 2005;115:760–8.
[36] Carmon L, El-Shami KM, Paz A, Pascolo S, Tzehoval E, Tirosh B, et al. Novel breast-tumor-associated MUC1-derived peptides: characterization in Db−/− x beta2 microglobulin (beta2m) null mice transgenic for a chimeric HLA-A2.1/Db-beta2 microglobulin single chain. Int J Cancer 2000;85:391–7.
[37] Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL, Stevanovic S, et al. Identification of HLA-A2-restricted T cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. Blood 1999;93:4309–17.
[38] Brossart P, Wirths S, Stuhler G, Reichardt VL, Kanz L, Brugger W. Induction of cytotoxic T-lymphocyte responses in vivo after vaccinations with peptide-pulsed dendritic cells. Blood 2000;96:3102–8.
[39] Palmer M, Parker J, Modi S, Butts C, Smylie M, Meikle A, et al. Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer. Clin Lung Cancer 2001;3:49–57 [discussion 58].
[40] Sangha R, North S. L-BLP25: a MUC1-targeted peptide vaccine therapy in prostate cancer. Expert Opin Biol Ther 2007;7:1723–30.
[41] Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163–75.
[42] Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. Bioinformatics 2001;17:1236–7.
[43] Vita R, Zarebski L, Greenbaum JA, Emami H, Hoof I, Salimi N, et al. The immune epitope database 2.0. Nucleic Acids Res 2010;38:D854–862.
[44] Shimizu Y, DeMars R. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. J Immunol 1989;142:3320–8.
[45] Herbert LM, Grosso JF, Dorsey Jr M, Fu T, Keydar I, Cejas MA, et al. A unique mucin immunoenhancing peptide with antitumor properties. Cancer Res 2004;64:8077–84.
[46] Keydar I, Chou CS, Hareuveni M, Tsarfaty I, Sahar E, Selzer G, et al. Production and characterization of monoclonal antibodies identifying breast tumor-associated antigens. Proc Natl Acad Sci U S A 1989;86:1362–6.
[47] Wolfel T, Van Pel A, Brichard V, Schneider J, Seliger B, Meyer zum Buschenfelde KH, et al. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. Eur J Immunol 1994;24:759–64.
[48] Smith SH, Brown MH, Rowe D, Callard RE, Beverley PC. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. Immunology 1986;58:63–70.
[49] Akbar AN, Terry L, Timms A, Beverley PC, Janossy G. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. J Immunol 1988;140:2171–8.
[50] Croce MV, Isla-Larrain MT, Demichelis SO, Gori JR, Price MR, Segal-Eiras A. Tissue and serum MUC1 mucin detection in breast cancer patients. Breast Cancer Res Treat 2003;81:195–207.
[51] MacLean GD, Reddish MA, Koganty RR, Longenecker BM. Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic STn vaccine. J Immunother Emphasis Tumor Immunol 1996;19:59–68.
[52] Reddish MA, MacLean GD, Poppema S, Berg A, Longenecker BM. Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and CD69+ lymphocytes correlate with effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. Cancer Immunol Immunother 1996;42:303–9.
[53] Schultes BC, Whiteside TL. Monitoring of immune responses to CA125 with an IFN-gamma ELISPOT assay. J Immunol Methods 2003;279:1–15.
[54] Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol 2003;4:835–42.
[55] Holmes JP, Benavides LC, Gates JD, Carmichael MG, Hueman MT, Mittendorf EA, et al. Results of the first phase I clinical trial of the novel II-key hybrid preventive HER-2/neu peptide (AE37) vaccine. J Clin Oncol 2008;26:3426–33.
[56] Schaed SG, Klimek VM, Panageas KS, Musselli CM, Butterworth L, Hwu WJ, et al. T cell responses against tyrosinase 368–376(370D) peptide in HLA*A0201+ melanoma patients: randomized trial comparing incomplete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, and QS-21 as immunological adjuvants. Clin Cancer Res 2002;8:967–72.
[57] Horn G, Gaziel A, Wreschner DH, Smorodinsky NI, Ehrlich M. ERK and PI3K regulate different aspects of the epithelial to mesenchymal transition of mammary tumor cells induced by truncated MUC1. Exp Cell Res 2009;315:1490–504.
[58] Finn OJ. Cancer vaccines: between the idea and the reality. Nat Rev Immunol 2003;3:630–41.
[59] Carmon L, Bobilev-Priel I, Brenner B, Bobilev D, Paz A, Bar-Haim E, et al. Characterization of novel breast carcinoma-associated BA46-derived peptides in HLA-A2.1/D(b)-beta2m transgenic mice. J Clin Invest 2002;110:453–62.
[60] Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. Annu Rev Immunol 1989;7:601–24.
[61] Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909–15.
[62] Surman DR, Dudley ME, Overwijk WW, Restifo NP. Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. J Immunol 2000;164:562–5.
[63] Barker CJ, Beagley KW, Hafner LM, Timms P. In silico identification and in vivo analysis of a novel T cell antigen from Chlamydia, NrdB. Vaccine 2008;26:1285–96.
[64] Fern J, Good MF. Promiscuous malaria peptide epitope stimulates CD45Ra T cells from peripheral blood of nonexposed donors. J Immunol 1992;148:907–13.
[65] Jiang C, Magee DM, Ivey FD, Cox RA. Role of signal sequence in vaccine-induced protection against experimental coccidioidomycosis. Infect Immun 2002;70:3539–45.
[66] McMurry J, Sbai H, Gennaro ML, Carter EJ, Martin W, De Groot AS. Analyzing Mycobacterium tuberculosis proteomes for candidate vaccine epitopes. Tuberculosis (Edinb) 2005;85:95–105.
[67] Tian S, Maile R, Collins EJ, Frelinger JA. CD8+ T cell activation is governed by TCR-peptide/MHC affinity, not dissociation rate. J Immunol 2007;179:2952–60.
[68] Southwood S, Sidney J, Kondo A, del Guercio MF, Appella E, Hoffman S, et al. Several common HLA-DR types share largely overlapping peptide binding repertoires. J Immunol 1998;160:3363–73.
[69] Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 2007;13:843–50.
[70] Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 1999;80:219–30.
[71] Minev BR, Chavez FL, Dudouet BM, Mitchell MS. Synthetic insertion signal sequences enhance MHC class I presentation of a peptide from the melanoma antigen MART-1. Eur J Immunol 2000;30:2115–24.
[72] Minev BR, McFarland BJ, Spiess PJ, Rosenberg SA, Restifo NP. Insertion signal sequence fused to minimal peptides elicits specific CD8+ T cell responses and prolongs survival of thymoma-bearing mice. Cancer Res 1994;54:4155–61.
[73] Dorfel D, Appel S, Grunebach F, Weck MM, Muller MR, Heine A, et al. Processing and presentation of HLA class I and II epitopes by dendritic cells after transfection with in vitro-transcribed MUC1 RNA. Blood 2005;105:3199–205.
[74] Weinzierl AO, Rudolf D, Hillen N, Tenzer S, van Endert P, Schild H, et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. Eur J Immunol 2008;38:1503–10.
[75] Hardy B, Yampolski I, Kovjazin R, Galli M, Novogrodsky A. A monoclonal antibody against a human B lymphoblastoid cell line induces tumor regression in mice. Cancer Res 1994;54:5793–6.