Expansion of quiescent lung adenocarcinoma CD8+ T cells by MUC1-8-mer peptide-T2 cell-β2 microglobulin complexes

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Abstract. Adoptive immunotherapy requires the isolation of CD8+ T cells specific for tumor-associated antigens, their expansion *in vitro* and their transfusion to the patient to mediate a therapeutic effect. MUC1 is an important adenocarcinoma antigen immunogenic for T cells. The MUC1-derived SAPDTRPA (MUC1-8-mer) peptide is a potent epitope recognized by CD8+ T cells in murine models. Likewise, the T2 cell line has been used as an antigen-presenting cell to activate CD8+ T cells, but so far MUC1 has not been assessed in this context. We evaluated whether the MUC1-8-mer peptide can be presented by T2 cells to expand CD25+CD8+ T cells isolated from HLA-A2+ lung adenocarcinoma patients with stage III or IV tumors. The results showed that MUC1-8-mer peptide-loaded T2 cells activated CD8+ T cells from cancer HLA-A2+ patients when anti-CD2, anti-CD28 antibodies and IL-2 were added. The percentage of CD25+CD8+ T cells was 3-fold higher than those in the non-stimulated cells (P=0.018). HLA-A2+ patient cells showed a significant difference (2.3-fold higher) in activation status than HLA-A2+ healthy control cells (P=0.04). Moreover, 77.6% of MUC1-8-mer peptide-specific CD8+ T cells proliferated following a second stimulation with MUC1-8-mer peptide-loaded T2 cells after 10 days of cell culture. There were significant differences in the percentage of basal CD25+CD8+ T cells in relation to the cancer stage; this difference disappeared after MUC1-8-mer peptide stimulation. In conclusion, expansion of CD25+CD8+ T cells by MUC1-8 peptide-loaded T2 cells plus costimulatory signals via CD2, CD28 and IL-2 can be useful in adoptive immunotherapy.

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Introduction

Lung adenocarcinoma is the main cause of cancer-related mortality worldwide (1,2). The immune response against tumors requires activation of tumor-specific CD8+ T cells to generate effector CD8+ cytotoxic T lymphocytes (CTLs) with the ability to kill tumor cells (3). The antitumor CD8+ T cells are activated by signals resulting from T cell receptor (TCR) recognition of specific tumor-derived peptide antigens presented by major histocompatibility complex class I (MHC-I) molecules on dendritic cells (DCs) (3,4). The CTL response to cancer is subordinated to the immunosuppressor environment that takes place during the progression of tumors (4). One treatment strategy is adoptive immunotherapy that involves the transfusion of autologous CD8+ CTLs to remove the malignant cells (5). Efforts to activate and expand tumor-specific CTLs *in vitro* have been focused on in the search for immunogenic tumor-associated antigens (TAAs) as well as appropriate tumor antigen-presenting cells (APCs) (5,6). The most significant antigen expressed in the vast majority of adenocarcinomas is a hypoglycosylated isoform from human mucin 1 (MUC1) protein, which exhibits immunogenic peptide sequences (7,8). Among MUC1-derived peptides, the H-2k^b-restricted MUC1-SAPDTRPA (MUC1-8-mer) peptide has proven to be the most immunogenic epitope for murine T cell activation (9,10). MHC-binding epitope prediction analysis showed that the MUC1-8-mer peptide is also restricted to HLA-A2 molecules (11). The T2 cell line expresses HLA-A2 molecules; therefore it has been used as an APC to activate distinctive TAA-specific CD8+ T cells from healthy volunteers (12). Additionally, T2 cells have been used to activate cancer-patient CD8+ T cells specific for TAA-derived peptides, but not MUC1-derived peptides (13). Our aim was to evaluate i) whether T2 cells can present the MUC1-8-mer peptide, and ii) to determine whether MUC1-8-loaded T2 cells activate

and expand $CD8^+$ T cells isolated from lung adenocarcinoma $HLA\text{-}A2^+$ patients.

Materials and methods

Lung adenocarcinoma patients. Nine adult patients with a diagnosis of non-small cell lung cancer established by clinical history, physical examination, chest X-rays, and histopathology were included. The patients were hospitalized at the Oncology Unit at the Instituto Nacional de Enfermedades Respiratorias 'Ismael Cosío Villegas' in Mexico City. The patient recruitment criteria included patients with a diagnosis of lung adenocarcinoma who had not undergone any previous cancer-associated surgery or medical treatment. Patients were classified as stage III and IV according to the standard criteria of the Tumor, Node and Metastasis (TNM) system (14). A peripheral blood sample was obtained from each patient before the start of anticancer chemotherapy or radiotherapy. Ten age-matched and clinically healthy volunteers with no history of cancer were included as controls. The Science and Bioethics Committee of our Institution in accordance with the Declaration of Helsinki approved the study, and patients and healthy volunteers provided informed consent for blood sampling after written information was provided.

Monoclonal antibodies and reagents. Peridinin chlorophyll protein complex-cyanine 5.5 (PerCP-Cy5.5)-labeled anti-human CD3 (clone SK7) monoclonal antibody (mAb), phycoerythrin (PE)-labeled anti-human CD4 (clone OKT4) mAb, fluorescein isothiocyanate (FITC)-labeled anti-human CD8 (clone SK1) and anti-HLA-A2 (clone BB7.2) mAbs, and PerCP-Cy5.5-, PE-, FITC-labeled isotype control (clone MOPC-21) mAbs, and human recombinant IL-2 were purchased from BioLegend, Inc. (San Diego, CA, USA). PE-labeled anti-human CD25 (clone M-A251) mAb and 7-amino-actinomycin-D (7-AAD) were acquired from BD Biosciences (San Jose, CA, USA). Alexa Fluor 594-labeled goat anti-IgG mouse antibody was obtained from Molecular Probes-Life Technologies (Eugene, OR, USA). Human β_2 microglobulin (β_2m) and mouse anti-CA 27-29 (clone M4021209, specific for SAPDTRPA) mAb were obtained from Fitzgerald Industries International (Acton, MA, USA). Blood DNA isolation and Fastype HLA-DNA SSP Typing system kits were provided by Bio-Synthesis Inc. (Lewisville, TX, USA). Lymphoprep™ (Ficoll 1.077 density) was from Axis-Shield PoC As (Oslo, Norway). $CD8^+$ T cell negative isolation kit in a magnetic antibody cell sorting (MACS) system containing biotin-labeled antibodies to human CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR-γδ, CD235a (glycophorin A) and magnetic microbeads coated with mouse Abs against biotin and human CD14; as well as mAbs to human CD2, CD3 and CD28 from the T cell activation/expansion kit were from Miltenyi Biotec (Bergisch Gladbach, Germany). Fetal bovine serum (FBS; Performance Plus), penicillin, streptomycin, L-glutamine and recombinant *Taq* DNA polymerase were purchased from Gibco-Life Technologies (Rockville, MD, USA). Carboxyfluorescein succimidyl ester (CFSE) was from Invitrogen (Camarillo, CA, USA). Vectashield mounting medium with DAPI was from Vector Laboratories, Inc. (Burlingame, CA, USA). RPMI-1640 culture medium, bovine serum albumin fraction V (BSA), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide, agarose, ethidium bromide, trypan blue dyes, and salt reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Peptides. The SAPDTRPA-human mucin 1 (MUC1-8-mer peptide), GILGFVFTL-influenza A virus matrix protein-$1_{58\text{-}66}$ (IVMP1-9-mer peptide), and SIINFEKL-chicken ovalbumin$_{257\text{-}264}$ (OVA-8-mer peptide) (15-17) were synthesized by the Instituto de Biotecnología at the Universidad Nacional Autónoma de México in Cuernavaca (Morelos, Mexico) on a 430A multiple peptide synthesizer (Applied Biosystems, San Diego, CA, USA) according to commercially available manufacturer's protocols. The affinity of these peptides for the HLA-A2 molecule was confirmed by NetMHC 3.4 Server software (18). The purity of the peptides was >95%, and their molecular weights were assessed by high performance liquid chromatography and confirmed by mass spectrometry. The peptides were dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and stored at -70˚C until required.

Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from 20 ml heparinized whole blood by Ficoll density gradient centrifugation for 30 min at 360 g and 10˚C (19). After centrifugation, the interface cells were collected, washed twice in RPMI-1640 medium, and counted in a Neubauer chamber to assess cell viability via the trypan blue dye exclusion test.

The human T2 cell line was obtained from the American Type Culture Collection (ATCC, CRL-1992™; Manassas, VA, USA). T2 cells express an HLA-A2 molecule that lacks TAP function, so it can easily be loaded with exogenous peptides for $CD8^+$ T cell recognition (20). The T2 cell line was cultured in RPMI-1640 medium supplemented with 20% heat-inactivated FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine (20% FBS supplemented-RPMI medium) at 37˚C in a humidified atmosphere containing 5% CO_2.

Purification of $CD8^+$ T cells. Cytotoxic $CD8^+$ T cells were isolated from PBMCs by a negative magnetic selection kit (Miltenyi Biotec). Briefly, PBMCs ($1x10^7$ cells) were suspended in 40 µl phosphate-buffered saline (PBS: 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) supplemented with 0.5% BSA and 2 mM EDTA and incubated with biotin-antibodies to human leukocyte phenotype molecules for 10 min at 4˚C, followed by a second incubation with magnetic microbeads coated with mouse Abs against biotin and human CD14 for an additional 15 min at 4˚C. The purity percentage for magnetically unlabeled $CD8^+$ T cells was always >95%, as determined by flow cytometry via incubation with PE-Cy5.5-anti-human CD3, PE-anti-CD4, and FITC-anti-CD8 mAbs for 30 min at 4˚C. The magnetically labeled $CD8^-$ T cells were used to identify HLA-A2 alleles.

DNA typing for the HLA-A2 allele. Genomic DNA was extracted from $CD8^-$ T cells by a blood DNA isolation kit (Bio-Synthesis) according to the manufacturer's instructions. The total DNA concentration was quantified by spectrophotometry at 260 and 280 nm using an ASP-2680 spectrophotometer (ACTGene Inc., Piscataway, NJ, USA). The DNA was suspended in 100 µl of the elution buffer and

stored at -20˚C until use. Molecular typing was performed with the polymerase chain reaction (PCR) sequence-specific primer (SSP) technique using a Fastype HLA-DNA SSP Typing system kit (Bio-Synthesis). For HLA-A typing, 24 primer pairs were used at a low-resolution modality. Briefly, PCR amplifications were carried out on 1.8 μg of genomic DNA in a 24-μl reaction volume containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3 and 1.5 mM $MgCl_2$; 60 μM of each dNTP (21). Samples were subjected to 20 cycles at 94˚C for 20 sec for denaturing, 20 cycles at 61˚C for 50 sec for annealing, and 20 cycles at 72˚C for 30 sec for extension using an automated thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, Foster City, CA, USA). An additional hold of 94˚C for 20 sec and then 10 cycles at 65˚C for 1 min were added to the denaturing step before the first cycle. After the last cycle, the extension step received an additional 5 min at 72˚C. The amplifications were achieved using recombinant *Taq* DNA polymerase. The integrity of amplified PCR SSP products was assessed by submarine 2% agarose gel electrophoresis and staining with 0.01 mg/ml ethidium bromide for 40 min. Each DNA sample was then visualized in a dual intensity ultraviolet (UV) light transilluminator (UVP Inc., Upland, CA, USA) and analyzed with the Kodak EDAS-290 gel documentation system (Kodak, Rochester, NY, USA) according to the electrophoretic migration of the DNA sample compared with the internal control primer pair specific for the human G3PDH gene (Bio-Synthesis). The results were interpreted following the instructions on the typing sheets from the procedure guide.

Peptide loading on the T2 cell line. The HLA-A2-binding ability of the MUC1-8-mer peptide was assessed by HLA-A2 membrane stabilization on the T2 cell line according to a previously described method (13). In brief, T2 cells ($2x10^5$) were placed in flat-bottomed, 96-well cell culture plates (Thermo Scientific Nunc, Roskilde, Denmark) in 10% FBS supplemented-RPMI medium and incubated with both MUC1-8-mer peptide (100 μg/ml) and human β_2m (20 μg/ml) for 24 h at 37˚C in 5% CO_2 atmosphere. The optimal dose of both β_2m and the MUC1-8 peptide was previously obtained from different concentrations tested. The T2 cells incubated either with the HLA-A2 restricted IVMP1-9-mer or the OVA-8-mer peptides were used as positive controls (16,17), whereas cells cultured in the absence of the MUC1-8-mer peptide were considered to be the negative control. After incubation, T2 cells were washed twice with PBS containing 0.2% BSA and sodium azide (PBS-BSA buffer) and stained with FITC-anti-HLA-A2 mAb for 30 min at 4˚C. Finally, the cells were analyzed by flow cytometry where the HLA-A2 expression on T2 cells was evaluated as the mean fluorescence intensity (MFI), which was calculated by obtaining the MFI difference between cells incubated in the presence or absence of the MUC1-8-mer peptide.

Flow cytometry. Cells were acquired using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo software version 8.7.7. (Tree Star Inc., Ashland, OR, USA). To analyze the immunofluorescence staining of cell-surface molecules, 25,000 events were counted in linear mode for both forward scatter (FSC) and side scatter (SSC). Cells were then gated by their physical properties (FSC and SSC) and analyzed with log amplification for immunofluorescence. Data are presented as histograms or two-dimensional dot-plots. Fluorescent staining-labeled isotype-matched control mAbs were used to assess background staining.

Confocal microscopy. T2 cells ($2x10^5$) were cultured in 8-well microchamber glass slides (BD Falcon™, Bedford, MA, USA) in 10% FBS supplemented-RPMI medium and incubated with MUC1-8 peptide plus β_2m for 24 h under the conditions as described above. After the culture, the cells were washed twice with PBS containing 1% BSA and 0.2% sodium azide and incubated with Alexa Fluor 594-labeled goat anti-mouse IgG mAb for 30 min after incubation with the anti-CA 27-29 mAb for 2 h at 4˚C. A second staining was performed with FITC-labeled anti-HLA-A2 mAb for 30 min at 4˚C. Cells incubated with the FITC-isotype control and the Alexa Fluor 594 secondary antibody, were used as controls. For colocalization analysis, the cells were fixed in 1% p-formaldehyde, and the slides were mounted in Vectashield with DAPI diluted 1:3 in PBS. Fluorescence images were acquired with an LSM-510 Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany) using a 63X objective lens. All images were captured under the same exposure, magnification and intensification. The digital images were processed by ImageJ software (Wayne Rasband, NIH, Bethesda, MA, USA) and analyzed using Mander's coefficient (22). Finally, the images were clarified by Adobe Photoshop CS2 software (Adobe Systems, Agrate Bianza, Italy).

In vitro activation and expansion of $CD8^+$ T cells by MUC1-8-mer peptide-loaded T2 cells. MUC1-8-mer peptide-loaded T2 cells were used as APCs to activate freshly obtained $CD8^+$ T cells from lung adenocarcinoma patients or healthy controls, using a modified method described by Jaramillo *et al* (13). In brief, T2 cells ($2x10^5$) were cultured with the MUC1-8-mer peptide plus β_2m for 24 h, as described above. Cells were then harvested, washed in the culture medium, and fixed in 1% p-formaldehyde for 30 min at 4˚C. After washing, fixed T2 cells were cultured with $CD8^+$ T cells ($4x10^5$) in 96-well plates in 10% FBS-supplemented RPMI medium in the presence of 2 μl of bead particle-coupled anti-CD2 and anti-CD28 mAbs from a T cell activation/expansion system (Miltenyi Biotec) for 6 days at 37˚C in a 5% CO_2 atmosphere. Human recombinant IL-2 (20 U/ml) was added after 3 days of cell culture, when half of the culture medium supernatant was removed and the well was replenished with fresh 10% FBS-supplemented RPMI medium. $CD8^+$ T cells cultured with MUC1-8-mer peptide loaded-T2 cells, and $CD8^+$ T cells with anti-CD2 and anti-CD28 mAbs were used as the specific-antigen $CD8^+$ T cell stimulation control. $CD8^+$ T cells only activated with mAbs to CD3, CD2, and CD28 were used as the unspecific-antigen $CD8^+$ T cell stimulation positive control. At the end of the culture, $CD8^+$ T cells were harvested, washed in a PBS-BSA buffer, and stained with FITC-anti-CD8 and PE-anti-CD25 mAbs for 30 min at 4˚C. To exclude dying cells before acquisition in a flow cytometer, cells were additionally incubated in a 7-AAD staining solution for 15 min at 4˚C, washed in PBS-BSA buffer and analyzed by flow cytometry.

Table I. General data of the non-small cell lung adenocarcinoma patients and healthy controls.

Participant	Gender/age (years)	HLA-A2	Stage/classification	Histopathology for MUC1
HC1	M/38	-	-	-
HC2	M/59	-	-	-
HC3	F/34	-	-	-
HC4	F/62	-	-	-
HC5	M/59	-	-	-
HC6	F/62	+	-	-
HC7	F/59	+	-	-
HC8	M/64	+	-	-
HC9	M/51	+	-	-
HC10	M/63	+	-	-
P1	F/74	-	T4N3M3	+
P2	M/53	-	T4N3M0	+
P3	F/62	+	T4N3M0	+
P4	F/74	+	T4N3M1	+
P5	M/53	+	T4N2M0	+
P6	M/71	+	T4N3M2	+
P7	M/51	+	T3N2M0	+
P8	F/69	+	T3N2M0	+
P9	F/66	+	T3N3M1	+

HC, healthy control; P, patient; M, male; F, female; TNM, tumor node metastasis; + abnormal overexpression of MUC1 in the histopathological study.

Table II. HLA-A2 peptide binding predictions of the NetMHC 3.4 Server software.

Peptide name	Sequence	Logscore	Affinity (nM)	Binding level
MUC1-8-mer	SAPDTRPA	0.073	22,690	Medium
OVA-8	SIINFEKL	0.206	5,377	Medium
IVMPI-9	GILGFVFTL	0.769	12	Strong binder

Binding scores were estimated using NetMHC 3.4 software (www.cbs.dtu.dk/services/NetMHC/): Strong binder threshold, 50 nM; weak binder threshold, 500 nM (18).

For clonal expansion detection, the $CD8^+$ T cells suspended in RPMI-1640 medium were stained with 15 μl of 0.5 mM CFSE (prepared from a 5-mM stock solution dissolved in dimethyl sulfoxide) for 15 min at 37˚C in darkness (23). After incubation, the cells were washed twice in 10 ml of 10% FBS-supplemented RPMI medium and the cell viability was evaluated by trypan blue dye exclusion test. CFSE-labeled $CD8^+$ T cells ($4x10^5$) were then cultured with fixed MUC1-8-mer peptide-loaded T2 cells ($2x10^5$) in the presence of costimulatory antibodies plus IL-2 (added every third day) for 10 days. An additional stimulation of $CD8^+$ T cells was carried out through adding fixed MUC1-8-mer peptide-loaded T2 cells ($2x10^5$) on day 7 of culture after removing half of the culture medium supernatant and replenishing the well with fresh 10% FBS-supplemented RPMI medium.

Statistical analysis. The entire analysis was performed by STATA™ 10 software using a Shapiro-Wilk test to identify population distributions. Because the variables were asymmetrically distributed, a Wilcoxon rank-sum (Mann-Whitney) test was carried out for their comparison. Values are shown as median (md) and interquartile range (iqr). Some data showed symmetry; thus, they were analyzed using the Student's t-test, and the values are displayed as mean ± standard deviation. Differences between groups were considered statistically significant at $P<0.05$.

Results

Patient characteristics. The mean patient group age was 63.67±10.7 years (range, 51-74 years); among these, 4 were male (44%) and 5 were female (56%); 7 out of 9 patients were $HLA\text{-}A2^+$. Four of the patients had metastasis; 6 of the 9 total patients presented with tumor stage IV, whereas the remaining 3 had tumor stage III (Table I). The control group mean age was 59.1±9.3 years (range, 34-64 years); 6 were male and 4 female.

MUC1-8-mer peptide plus β_2m increases the expression of HLA-A2 molecules on the T2 cell surface. The HLA-A2-specific affinity of the MUC1-8-mer peptide to T2 cells was predicted by NetMHC 3.4 software (18), and compared to the affinity

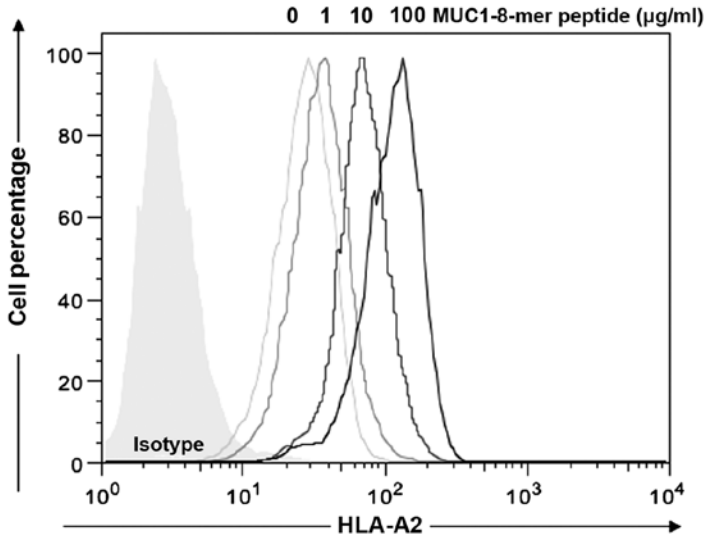

Figure 1. HLA-A2 expression on the T2 cell surface is dose-dependent on the MUC1-8 peptide concentration. T2 cells were incubated with MUC1-8-mer peptide concentrations ranging from 0 to 100 μg/ml in the presence of β_2m (20 μg) for 24 h at 37˚C in a 5% CO_2 atmosphere. After peptide loading, the T2 cells were harvested, washed, and stained with FITC-labeled anti-HLA-A2 mAb for flow cytometry. A representative histogram was constructed where the x-axis denotes the HLA-A2 fluorescence intensity of the T2 cells, and the y-axis indicates the cell percentage.

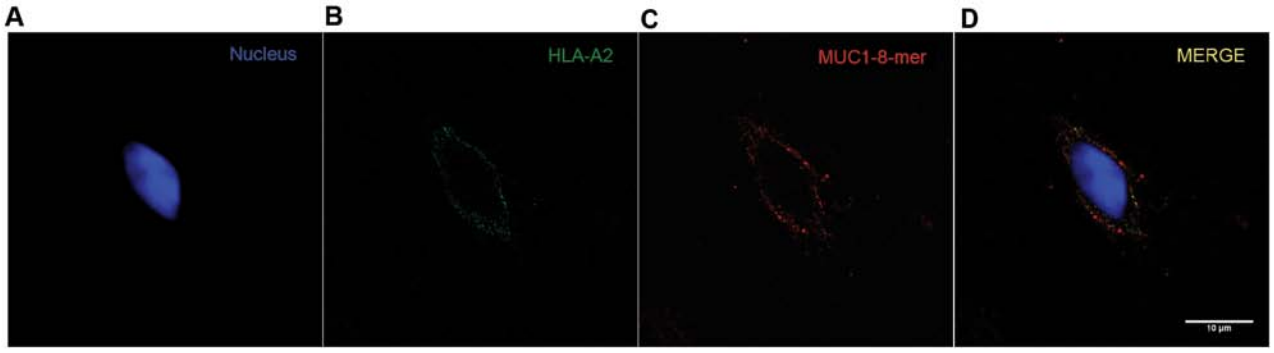

Figure 2. Colocalization of the HLA-A2 molecule with the MUC1-8-mer peptide on the T2 cell surface. T2 cells were loaded with the MUC1-8-mer peptide (100 μg) in the presence of β_2m (20 μg) for 24 h at 37˚C in a 5% CO_2 atmosphere. The T2 cells were then harvested, washed, stained with FITC-labeled anti-HLA-A2 and Alexa Fluor 594-labeled goat anti-mouse IgG mAb after anti-CA 27-29 mAb (specific for SAPDTRPA), and prepared for confocal microscopy. (A) Image from one cell with DAPI nuclear staining (blue), (B-D) Same images as (A) where the HLA-A2 molecule is stained green (B), the MUC1-8-mer peptide is stained red (C), and the image merged by triple immunofluorescence (D), revealing colocalization (yellow) between HLA-A2 molecule and MUC1-8-mer peptide. Images were visualized using an LSM-510 Zeiss confocal microscope. Data shown are representative of two individual experiments. Scale bar, 10 μm.

of the well-recognized HLA-A2-specific IVMP1-9-mer and OVA-8-mer epitopes (Table II). T2 cells pulsed with the MUC1-8-mer peptide in the presence of β_2m showed an increase in HLA-A2 molecule expression in a dose-dependent manner (0, 1, 10 and 100 μg); the basal value was 31.6±12.7; for 1 μg/ml peptide the value increased to 40.3±18.9; for 10 μg/ml, 77.1±32.3 and for 100 μg/ml, 123±52, respectively (Fig. 1). Concentrations >100 μg/ml of the MUC1-8-mer peptide did not increase HLA-A2 expression on the T2 cells. Therefore MUC1-8-mer peptide at 100 μg/ml was considered as the optimal concentration for expression of HLA-A2 molecules and this was the concentration used in all the subsequent experiments.

MUC1-8-mer peptide and the HLA-A2 molecule colocalize on the T2 cell surface. To confirm the assembly of the MUC1-8-mer peptide-HLA-A2 complex onto the T2 cell surface, an anti-CA 27-29 mAb (antibody specific for the SAPDTRPA peptide sequence) was used together with the anti-HLA-A2 mAb. The analysis of the overlap of fluorescent emissions from the green channel (HLA-A2) and the red channel (MUC1-8-mer peptide) on the T2 cell membrane showed significant values for the Mander's coefficient, which were from 0.04 to 1 (22). As shown in Fig. 2, one T2 cell is shown separately in the green and red channels; in the third image, both fluorescence emissions are merged (yellow color) and distributed along the cell membrane, indicating that the MUC1-8-mer peptide is found in the same place as the HLA-A2 molecule (22).

MUC1-8-mer peptide-loaded T2 cells plus costimulatory antibodies induce activation and expansion of CD8$^+$ T cells from HLA-A2 patients. The percentage of CD25$^+$CD8$^+$ T cells after stimulated with the T2 cell-MUC-1-8-mer complex and

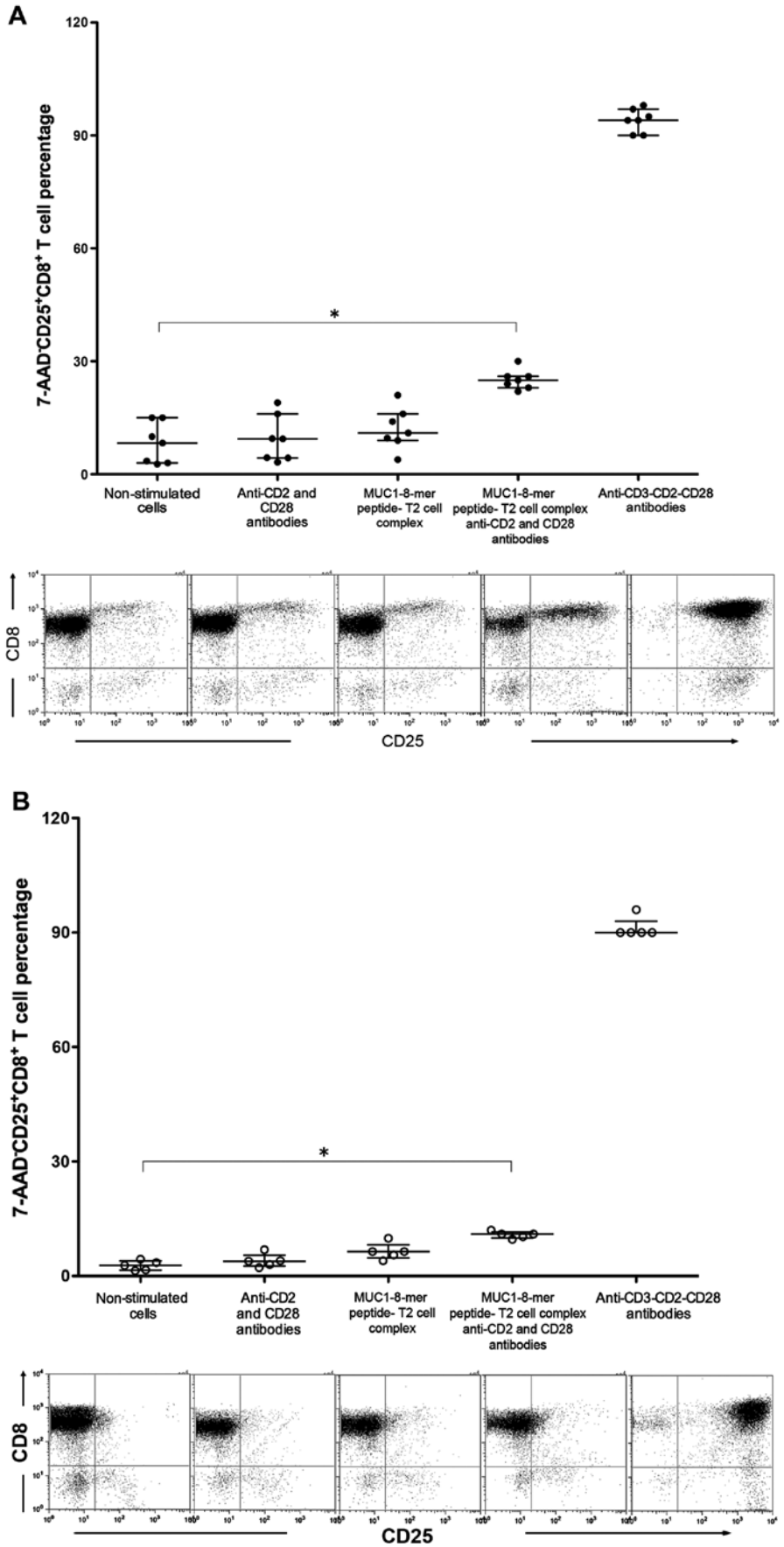

Figure 3. MUC1-8-mer peptide-T2 cell complex induces CD25 expression on $CD8^+$ T cells from HLA-$A2^+$ lung adenocarcinoma patients and healthy individuals. Purified $CD8^+$ T cells from patients (A) or healthy individuals (B) were cultured under the different indicated conditions over 6 days. Cells were harvested, stained with FITC-labeled anti-CD8 and PE-labeled anti-CD25 mAbs, and analyzed using flow cytometry (images are shown in panels below each condition). An additional incubation with 7-aminoactinomycin-D (7-AAD) was performed to exclude any dying cells. Each solid circle denotes a sample from one patient, whereas an open circle represents a sample from one healthy control. $^*P<0.05$.

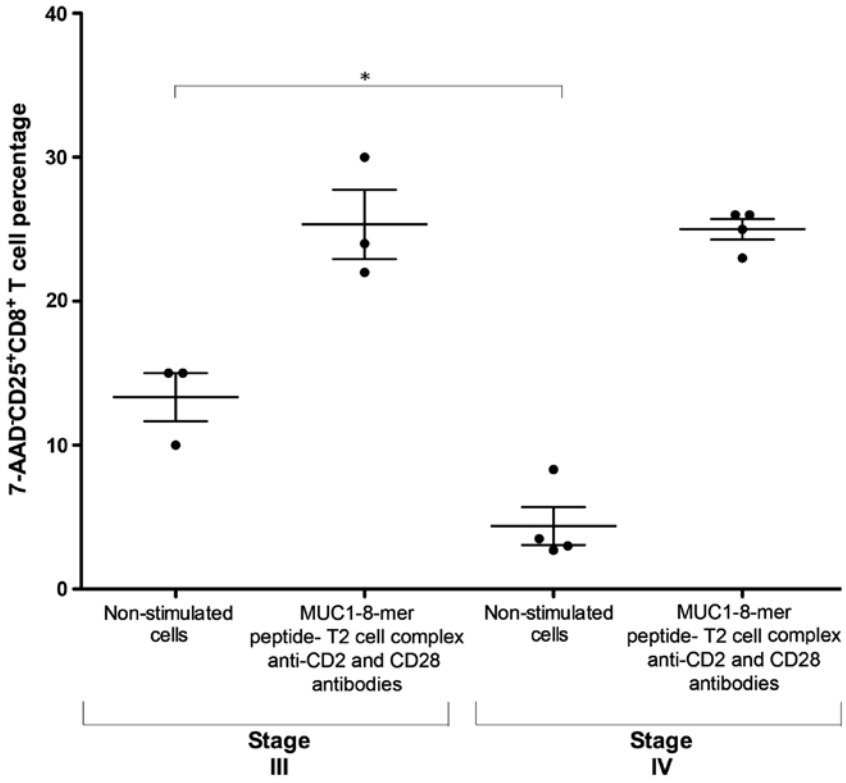

Figure 4. Similar increase in $CD25^+CD8^+$ T cells from HLA-$A2^+$ patients with stage III or IV tumor disease progression after stimulation by the MUC1-8-mer peptide-T2 complex and costimulatory antibodies. Purified $CD8^+$ T cells from patients with stage III or IV were cultured with MUC1-8-mer peptide loaded-T2 cells and costimulatory antibodies to CD28 and CD2 plus IL-2 over 6 days. The cells were harvested, washed and stained with FITC-labeled anti-CD8 and PE-labeled anti-CD25 mAbs, and analyzed using flow cytometry. An additional incubation with 7-aminoactinomycin-D (7-AAD) was performed to exclude dying cells. Each circle denotes a sample from one patient. *P<0.03.

anti-CD2 and CD28 was 4.2-fold higher in the HLA-$A2^+$ patients than those from the HLA-$A2^-$ patients (md 25%, iqr 22-30 vs. md 6%, iqr 5.3-6.7).

In the HLA-$A2^+$ patients, the percentage of $CD25^+CD8^+$ T cells stimulated with anti-CD2 and anti-CD28 or the MUC1-8-mer-T2 cell complex was identical to that observed in the non-stimulated $CD8^+$ T cells; notably when the $CD8^+$ T cells were stimulated with the MUC-1-8-mer T2 cell complex and anti CD2-CD28 mAbs, there was a 3-fold increase compared to the non-stimulated cells (md 25.0%, iqr 22-30 vs. md 8.3%, iqr 3.7-15, P=0.018) (Fig. 3A). Similarly, the proportions of CD25-expressing $CD8^+$ T cells from HLA-$A2^+$ healthy controls showed an identical behavior but with lower values (md 11%, iqr 9.6-12 stimulated cells vs. md 2.8%, iqr 1.4-4.4 non-stimulated cells, P=0.018) (Fig. 3B). Non-stimulated $CD8^+$ T cells from HLA-$A2^+$ patients showed that there was dispersion in the percentages of $CD25^+CD8^+$ T cells (Fig. 3A), therefore samples from cancer patients were divided according to their TNM classification (14). Fig. 4 shows that non-stimulated cells from patients with stage III cancer exhibited a higher proportion of CD25 expression than non-stimulated cells from patients with stage IV (md 15%, iqr 10-15 vs. md 3.2%, iqr 2.7-8.3, P=0.03). Notably, the proportion of $CD25^+CD8^+$ T cells was similar in both patient groups after stimulation with the MUC1-8-mer peptide-T2 cell complex in the presence of anti-CD2 and anti-CD28 mAbs plus IL-2 (md 24%, iqr 22-30 vs. md 25.5%, iqr 23-26, respectively). In contrast, the polyclonal activation of $CD8^+$ T cells from healthy controls and patients with anti-CD3, anti-CD28 and anti-CD2 showed similar activation values, but the expression intensity of $CD25^+$ cells was lower in the cancer patients (Fig. 3).

To evaluate the proliferative response of antigen-specific cells, restimulation of $CD8^+$ T cells with the MUC1-8-mer peptide-T2 complex was performed after 7 days of culture. Three days later, the cells were fixed and clonal expansion was determined by CFSE treatment. Fig. 5A shows the proliferative response of CFSE-labeled $CD8^+$ T cells specific to the MUC1-8-mer peptide. This cell population had a 77.6% increase after the restimulation in comparison to the 26.7% observed in CFSE-labeled $CD8^+$ T cells activated with the MUC1-8-mer peptide-T2 complex plus anti-CD2 and anti-CD28 antibodies (Fig. 5B).

Discussion

Conventional treatments against lung cancer are unsatisfactory in most cases, thus a more effective therapy for the removal of tumor cells is urgent (24,25). Adoptive T cell therapy has shown promising results as a treatment strategy for cancer patients (5). However, the challenge that faces this type of therapy is the *ex vivo* activation and expansion of CTLs from a limited number of peripheral blood mononuclear cells from patients with advanced cancer (26). The identification of immunogenic TAA epitopes and selection of appropriated APCs are crucial to activate tumor-specific CTLs (6,27). Our results confirmed the binding of the MUC1-8-mer peptide into

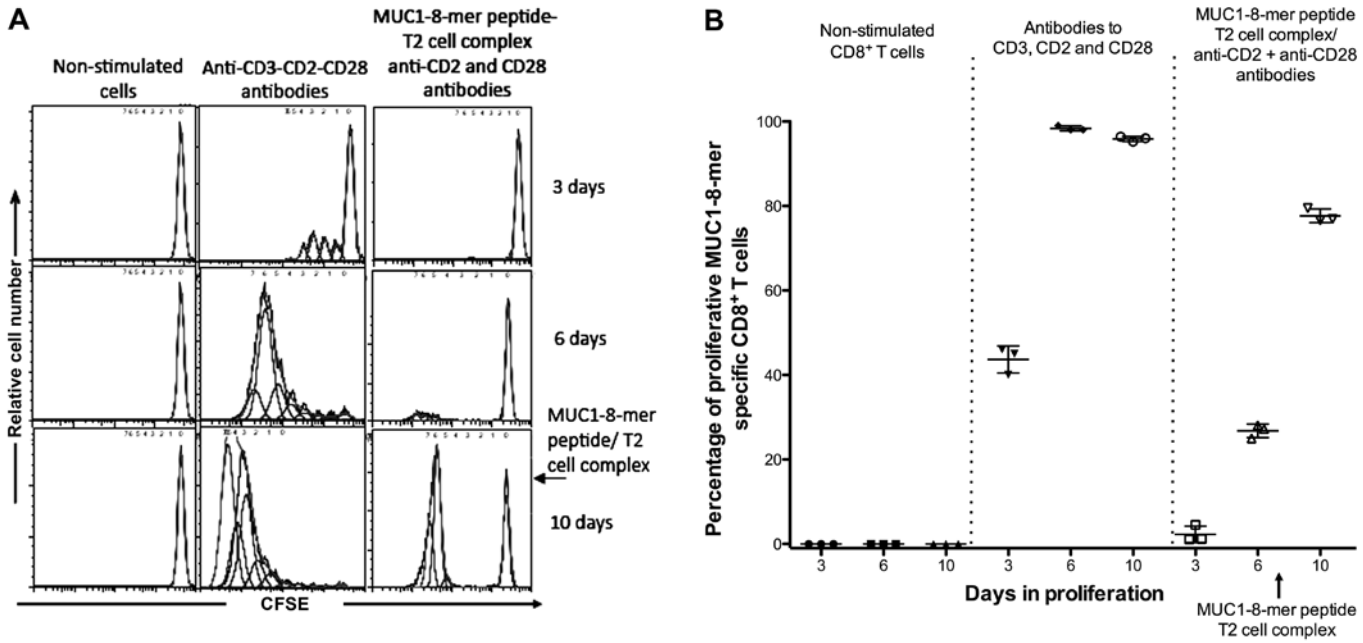

Figure 5. Expansion of antigen-specific CD8+ T cells after restimulation by the MUC1-8-mer peptide-T2 complex. CFSE-labeled CD8+ T cells from HLA-A2+ patients were cultured for 10 days under the indicated times and conditions. (A) Histograms show the CFSE intensity of the non-divided cell population in the absence of stimulus (left panels), and cells that have divided numerous times based on sequential fluorescence intensity (middle panels), as well as antigen-specific cells restimulated with the MUC1-8-mer peptide-T2 complex (right panels). (B) Graphic image showing the CD8+ T cell proliferation percentage on different days of culture under the conditions indicated.

β_2m established T2 cells according to MHC-binding epitope predicting analysis (11). Type 1 mucin (MUC1) is an important TAA in adenocarcinomas; diverse MUC1-derived peptides have been used to induce a CD8+ CTL response in patients with adenocarcinomas (6). Among MUC1-derived peptides, the MUC1-8-mer peptide SAPDTRPA has been shown to be an immunogenic epitope that activates murine T cells (10). The advantage of T2 cells is that they lack TAP function and consequently they have empty and unstable HLA-A2 molecules (20). This characteristic gives them a potential advantage as APC peptides can be loaded exogenously onto the HLA-A2 molecules to activate TAA-specific CTLs (12,13). The use of autologous monocyte-derived DCs as APCs would be ideal to generate efficient costimulatory and antigen-specific signals for activation of autologous T cells (11). However, the number of functional monocyte-derived DCs from cancer patients is low in long lasting cultures due to their limited replicative potential (5).

Our results indicated that the MUC1-8-mer peptide-T2 complex only induced activation of purified CD8+ T cells when anti-CD28 and anti-CD2 antibodies plus IL-2 were added to the cell culture. The additional costimulation generated by beads coupled with anti-CD2 and anti-CD28 antibodies and recombinant IL-2 allowed clonal expansion of MUC1-8 peptide-specific CD8+ T cells isolated from HLA-A2 adenocarcinoma patients. The antibodies to costimulatory molecules induce formation of actin filaments between the beads and CD8+ T cell surface mimicking the immunological synapsis between APCs and T cells (28,29). Our results also showed that the activation of MUC1-8 peptide-specific CD8+ T cells was similar to those from different activation systems using other MUC1-derived epitopes (30-33). This finding confirms reports in which CD8+ T cells from HLA-A2+ healthy donors recognize distinct MUC1-derived peptides (11,31,33). Mamaglobulin A-derived peptides loaded into T2 cells stimulated with soluble anti-CD28 antibodies plus recombinant human IL-2 have been used to activate HLA-A2+ CD8+ T cells in breast cancer patients (13). However, in this system, expansion of CTLs required necessarily, several weekly restimulations to maintain the activity against diverse cell lines (13). Our results were similar but opposed to Jaramillo *et al* (13), as we evaluated the percentage of proliferative cells.

Notably, we observed the preexistence of CD8+ T cells reactive to the MUC1-8 peptide in HLA-A2+ patients as well as in HLA-A2+ healthy controls; the latter could be because the MUC1-8 peptide has been shown to be highly immunogenic in a murine model (10), thus suggesting that there is a basal T cell population recognizing MUC1 in healthy individuals. The proportion of CD8+ T cells from HLA-A2+ healthy controls activated by MUC1-8 peptide-loaded T2 cells was significantly lower than those from HLA-A2+ cancer patients after 6 days of culture. This suggests that MUC1-recognizing T cells in healthy individuals are capable of continuously limiting the development of a tumor whereas in adenocarcinoma patients this ability has been lost probably as a results of the immunosuppressive environment that has been well established (34-36).

Our results also showed that the proportions of CD25+CD8+ non-stimulated T cells isolated from stage III and IV patients were significantly different, but that after stimulation with the MUC1-8-mer peptide T2 complex in the presence of anti-CD2 and CD28 antibodies the same amount of CD25+CD8+ T cells was induced. This confirms that the potential to respond to APCs is maintained in both stages, but the amount

of CD25 expression in the stage IV cells was diminished, as possibly the cells keep their ability to respond to an external antigen-presenting system (37). The range of possible explanations include different cytokine environment (34-36); rescue of the cell signaling mechanism by the direct and continuous contact with the anti-CD28 antibody that surpasses the inhibitory signal mediated by CTLA-4 as observed in $CD4^+$ T cells (38); lack of contact with Treg cells as the experiments were performed *in vitro*, or tumor progression (35,36,39). Ongoing experiments are being performed in our laboratory to try to determine a possible mechanism.

Taken together, we modified an *in vitro* system that uses MUC1-8 peptide-pulsed T2 cells and improved it through the stimulation of $CD8^+$ T cell costimulatory molecules, such as CD2 and CD28. Under these conditions, we found that preexistent MUC1-specific $CD8^+$ T cells from HLA-$A2^+$ lung adenocarcinoma patients and HLA-$A2^+$ healthy controls were efficiently activated. The specificity of this system allowed us to distinguish between cancer patients and healthy individuals. Furthermore, clonal expansion of MUC1-specific $CD8^+$ T cells from cancer patients occurred independently of tumor disease progression. This activation system could be an innovative tool to induce and expand tumor-specific CTLs from HLA-$A2^+$ patients with adenocarcinoma.

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