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ORIGINAL ARTICLE

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In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line

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Abstract The adoptive transfer of in vitro-induced and expanded tumor-specific cytotoxic T lymphocytes (CTL) presents a promising immunotherapeutic approach for the treatment of cancer. The in vitro induction of tumor-reactive CTL requires repeated stimulation of CTL precursors with dendritic cells (DC). To circumvent problems like scarcity of blood DC precursors and donor variability, it would be attractive to use DC from a non-autologous, unlimited source. DCs derived from the human acute myeloid leukemia (AML) cell line MUTZ-3 are attractive candidates since these DCs closely resemble monocyte-derived DC (MoDC) in terms of phenotype and T cell stimulatory capacity. Here we demonstrate that functional CTL clones could be generated against multiple tumor-associated antigens, i.e., human telomerase reverse transcriptase (hTERT), ErbB3-binding protein-1 (Ebp1), carcinoembryonic antigen (CEA) and Her-2/*neu*, by stimulating CD8β^+ CTL precursors with peptide-loaded allogeneic, HLA-A2-matched MUTZ-3-derived DC. A consistent induction capacity, as determined by MHC tetramer-binding, was found in multiple donors and comparable to autologous peptide-loaded MoDC. Functional characterization at the clonal level revealed the priming of CTL that recognized endogenously processed epitopes on tumor cell lines in an HLA-A2-restricted fashion. Our data indicate that MUTZ-3-derived DC can be used as stimulator cells for in vitro priming and expansion of functional TAA-specific effector CTL. MUTZ-3-derived DCs thus represent a ready and standardized source of allogeneic DC to generate CTL for therapeutic adoptive transfer strategies.

Keywords Human dendritic cell line · Tumor-associated antigens · Immunization · Immunotherapy · Adoptive T cell transfer

Abbreviations APC: Antigen-presenting cell · CEA: Carcinoembryonic antigen · CTL: Cytotoxic T lymphocytes · DC: Dendritic cell · GFP: Green fluorescent protein · hTERT: Human telomerase reverse transcriptase · MoDC: Monocyte-derived dendritic cell · ΔNGFR: Truncated form of nerve growth factor receptor · PBMC: Peripheral blood mononuclear cell · PHA: Phytohemagglutin · TIL: Tumor-infiltrating lymphocyte · Tm: Tetramer

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Introduction

The identification and characterization of tumor-associated antigens (TAA) and TAA-derived peptides recognized by cytotoxic T lymphocyte (CTL) have opened new possibilities for immunotherapeutic approaches to treat human cancers. Different strategies can be used, including vaccination with antigenic peptides [26], autologous tumor cells [37], autologous dendritic cells (DC) [21] or the adoptive transfer of in vitro-generated and expanded tumor-specific CTL [7, 16, 25, 41]. The

latter approach seems very promising since adoptive therapy strategies are able to circumvent tolerogenic mechanisms that influence the magnitude and avidity of the anti-tumor response by enabling the selection and activation of highly reactive anti-tumor T cell populations ex vivo. Recent clinical successes have been achieved with adoptive transfer of tumor-reactive CTL in combination with nonmyeloablative chemotherapy in metastatic melanoma patients [7].

DCs, which are professional antigen-presenting cells (APCs), have been shown to be very potent in inducing specific CTL both in vitro and in vivo [9, 15, 23, 32, 33]. DCs loaded with antigenic preparations like synthetic peptides, recombinant proteins or tumor lysates are able to successfully induce specific CTL in vitro from low-frequency healthy donor-derived CTL precursors [9, 23, 30, 33]. To induce robust antigen-specific CTL responses in vitro, CTL precursors need to be stimulated with antigen-loaded DC repeatedly. Due to scarcity of blood DC precursors and donor variability in terms of DC differentiation, generating and expanding tumor-specific CTL remains difficult and labor-intensive. To circumvent these problems, it would be preferable to use DC cultured from an unlimited and readily available source.

We recently showed that DC could be cultured from the MUTZ-3 cell line, a $CD34^+$ human acute myeloid leukemia cell line [19]. Upon stimulation with GM-CSF, IL-4 and TNF-α, MUTZ-3 precursor cells acquire a DC morphology and phenotype consistent with conventional myeloid DC, expressing high levels of MHC, co-stimulatory and adhesion molecules. Furthermore, they are capable of antigen processing and presentation via MHC class I and II, resulting in stimulation of specific $CD8^+$ and $CD4^+$ T cells. Given the fact that the MUTZ-3 cell line expresses the highly prevalent HLA class I antigens HLA-A2, HLA-A3, HLA-B44 and HLA-B56 covering 70% of the Caucasian population, DC generated from this cell line might serve as universal stimulators for the generation of effector CTL in vitro.

In vitro priming of T cells for the generation of a tumor-specific response is dependent on the characterization of epitopes that are able to elicit an efficient immune response. In this study, we selected immunogenic HLA-A2-binding epitopes derived from the classical colorectal carcinoma antigen, CEA [34], the pan-carcinoma antigen, hTERT [38], the adenocarcinoma antigen Her-2/*neu* and the newly identified colorectal carcinoma antigen, Ebp1, which was recently identified by us as an immunogenic protein, capable of eliciting CD8-mediated responses both in vivo and in vitro (Santegoets et al. submitted). Generating tumor-reactive CTL for these adenocarcinoma-associated antigens is technically challenging since these TAAs are also self-proteins, with lower precursor frequencies and preferential outgrowth of low avidity CTL.

Here, we examined the capacity of MUTZ-3-derived DC (hereafter referred to as MUTZ-3 DC) to induce functional tumor-specific CTL from HLA-A2-matched $CD8\beta^+$ CTL precursors and compared it to the priming capacity of commonly used autologous MoDC. We were able to demonstrate that functional hTERT-, Ebp1-, CEA- and Her-2/*neu*-specific CTL clones could be generated by stimulating $CD8\beta^+$ CTL precursors with peptide-loaded allogeneic, HLA-A2-matched MUTZ-3 DC and that the efficiency of this induction was comparable to autologous peptide-loaded MoDC. Our data indicate that MUTZ-3 DCs represent a ready and standardized source of allogeneic DC that can be used as HLA-matched stimulator cells for in vitro priming and expansion of tumor-specific CTL for therapeutic adoptive transfer strategies.

Materials and methods

Cell lines

The $CD34^+$ human acute myeloid leukemia cell line MUTZ-3 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany) was cultured in MEM-α medium containing ribonucleosides and deoxyribonucleosides (Life Technologies, Paisley, UK) supplemented with 20% Fetal calf serum (Perbio, Helsingborg, Sweden), 100 I.E./ml sodium penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands), 100 µg/ml streptomycin sulfate (Radiumfarma-Fisiopharma, Naples, Italy), 2.0 mM L-glutamine (Invitrogen, Breda, The Netherlands), 0.01 mM 2-mercapoethanol (Merck, Darmstadt, Germany) and 10% 5637-conditioned medium (CM) [13, 19].

The EBV-transformed B cell line JY and the TAP-deficient cell line T2 (both $HLA\text{-}A2^+$) were cultured in IMDM (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 I.E./ml sodium penicillin, 100 µg/ml streptomycin sulphate, 2.0 mM L-glutamine and 0.01 mM 2-mercapoethanol (complete medium). The breast cancer cell lines MCF-7, MDA-MB231 ($HLA\text{-}A2^+$), colon carcinoma cell lines SW620, SW403 ($HLA\text{-}A2^+$), HT-29 ($HLA\text{-}A2^-$), gastric carcinoma cell line KATO-3 ($HLA\text{-}A2^+$), prostate cancer cell line PC-3 ($HLA\text{-}A2^-$) (all from ATCC, Manassas, VA, USA) and melanoma cell line melAKR ($HLA\text{-}A2^+$; Netherlands Cancer Institute, Amsterdam, The Netherlands) were all cultured in DMEM (BioWhittaker) complete medium.

Synthetic peptides

The HLA-A2-restricted peptides $hTERT_{540}$ (ILAKFLHWL) and $hTERT_{988Y}$ (YLQVNSLQTV), CEA_{571} (YLSGANLNL), $Ebp1_{59}$ (HVDGFIANV) and Her-2/neu_{369} (KIFGSLAFL) were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, MultiSyntech, Witten, Germany) using Fmoc-chemistry. Peptides were analyzed by reversed-phase high-performance liquid chromatography (HPLC), dissolved in DMSO (Merck) and stored at −20°C.

In vitro generation of monocyte-derived and MUTZ-3-derived DC

MoDCs were generated as described [2]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood of normal human volunteers by density centrifugation over Lymphoprep (Nycomed AS, Oslo, Norway). PBMCs were allowed for 1–2 h to adhere to the bottom of plastic culture flasks (Nunc, Intermed, Denmark) at 37°C. Non-adherent cells were removed and the adherent cells were cultured for 5–7 days in IMDM complete medium supplemented with 100 ng/ml GM-CSF (Schering-Plough, Madison, NJ, USA) and 1,000 U/ml IL-4 (Strathmann Biotec, Hamburg, Germany).

MUTZ-3 DCs were generated as described [19]. Briefly, MUTZ-3 progenitors were cultured in 12 well tissue culture plates at a concentration of 1×10^5/ml in MEM-α medium without 5637-CM in the presence of 100 ng/ml GM-CSF, 1,000 U/ml IL-4 and 2.5 ng/ml TNF-α (Strathmann Biotec) for 7 days. Every 3 days new cytokines were added. At day 7, maturation of both MoDC and MUTZ-3 DC was induced by adding MCM mimic, a cytokine cocktail consisting of 50 ng/ml TNF-α, 100 ng/ml IL-6, 25 ng/ml IL-1β (Strathmann Biotec) and 1 μg/ml PGE2 (Sigma).

Antibodies, tetramers and flow cytometry

PE- or FITC-labeled Abs directed against human CD34 (Strathmann Biotec), CD40, CD83, CD8β (Immunotech, Marseille, France), CD1a, CD8α, CD14, CD27, CD28, CD45RO, CD45RA, CD80, CD86, CCR7, DC-SIGN, TCR$\alpha\beta$, TCR$\gamma\delta$ (all from BD Biosciences, Mountain view, CA, USA) and APC-labeled-anti-ΔNGFR (Chromoprobe, Aptos, CA, USA) were used for flow cytometric analysis. PE- and/or APC-labeled HLA-A2 tetramers (Tm) presenting the $hTERT_{988Y}$, $hTERT_{540}$, $Ebp1_{59}$, Her-2/neu_{369}, and CEA_{571} epitopes were prepared as described previously [11]. Antibody and/or tetramer staining was performed in PBS supplemented with 0.1% BSA and 0.02% natrium-azide for 30 min at 4°C and 15 min at 37°C, respectively. Stained cells were analyzed on a FACScalibur (BD Biosciences) using Cell Quest software. To exclude dead cells in flow cytometric tetramer analysis, 0.5 μg/ml propidium iodide (ICN Biomedicals, Zoetermeer, The Netherlands) was used. mAb- and tetramer-guided flow sorting was performed on a FACStar^{Plus} (BD Biosciences) using CellQuest software.

Primary CTL induction in vitro

Antigen-specific CTLs were generated as described [30]. Briefly, CD8β^+ CTL precursors were isolated from PBMC of HLA-A2$^+$ healthy donors and a prostate cancer patient by positive selection on an automated magnetic cell sorting (MACS) device (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). For this purpose, total PBMCs were stained with unlabeled anti-CD8β mAb (Immunotech) and microbead-conjugated anti-mouse IgG Abs (Miltenyi Biotec). Mature MoDC and MUTZ-3 DCs, prepared as described above, were loaded with 25 μg/ml peptide in the presence of 3 μg/ml β2-microglobulin (Sigma-Aldrich, St. Louise, MO, USA) for 2–4 h at room temperature and irradiated (40 Gy). 1×10^5 peptide-loaded DCs were cultured for 10 days with 1×10^6 CD8β^+ CTL precursors and 1×10^6 irradiated (80 Gy) CD8β^- autologous PBMC in Yssel's medium [43] supplemented with 1% hAB serum (ICN Biochemicals), 10 ng/ml IL-6 and 10 ng/ml IL-12 in a 24 well tissue-culture plate. At day 1, 10 ng/ml IL-10 (R&D Systems) was added. From day 10, CTL cultures were stimulated every week for 5 weeks with 1×10^5 fresh peptide-loaded DCs in the presence of 5 ng/ml IL-7 (Strathmann Biotec). Two days after each restimulation, 10 U/ml IL-2 (Strathmann Biotec) was added. One day prior to each restimulation, a sample was taken and analyzed by flow cytometry using both PE- and APC-labeled tetramers presenting the relevant epitope. Tetramer-positive CTL were isolated by CD8$^+$/tetramer$^+$ flow sorting and subsequently cloned by limiting dilution [43]. For this purpose, CTLs were weekly stimulated with irradiated feeder-mix consisting of allogeneic PBMC and JY cells in Yssel's medium supplemented with 100 ng/ml phytohemagglutin (PHA; Murex Biotech, Dartford, UK) and 20 U/ml IL-2. To allow extensive characterization of the generated CEA_{571}-specific CTL clones, the clones were rescued from replicative senescence by introducing human telomerase reverse transcriptase (hTERT) as described elsewhere [12]. Briefly CEA_{571}-specific CTLs, stimulated for 48 h with feeder-mix as described above, were transduced with retrovirus encoding LZRS-hTERT-IRES-ΔNGFR, in fibronectin-coated plates (Retronectin, Takara, Japan) in the presence of 100 U/ml IL-2. During transduction, the plates were centrifuged at 2,000×g for 90 min at 23°C and subsequently incubated at 37°C. After 4,5 h, cells were washed and cultured overnight in Yssel's medium containing 20 U/ml IL-2. Next day, retroviral transduction was repeated. After 48 h, transduction efficiency was checked by flow cytometric analysis of nerve growth factor receptor (ΔNGFR)-marker gene expression. Subsequently, ΔNGFR$^+$ CTLs were positively selected by MACS and cloned by limiting dilution as described.

Intracellular IFN-γ detection

To determine the capacity of CTL clones to produce IFN-γ upon recognition of a specific target, intracellular IFN-γ staining was performed. Target cells used included HLA-A2$^+$/CEA$^+$, HLA-A2$^+$/Ebp1$^+$, HLA-A2$^-$/CEA$^+$ or HLA-A2$^-$/Ebp1$^+$ tumor cell lines and JY cells or T2 cells pulsed with either relevant or irrel-

evant peptide. CTLs were cultured with target cells at an effector:target cell (E:T) ratio of 2:1 in 96-well round-bottom plate. One hour after the start of stimulation, 0.5 µl of GolgiPlug (BD Biosciences) was added to each well. After 6 h, cells were harvested, washed, stained with APC-labeled tetramer and PE-labeled anti-CD8 mAb. After fixation with 4% paraformaldehyde (Merck) and permeabilization with 1×BD Perm/wash solution (BD Biosciences), cells were labeled with FITC-conjugated anti-IFN-γ Ab (BD Biosciences). Stained cells were analyzed on a FACScalibur.

Chromium release assay

Cytotoxic activity of CTL clones was determined by standard chromium release assay as described [30].

CD107a membrane expression

Cytotoxic potential was determined by a flow cytometric degranulation assay as described [3, 27]. In this assay, the potential for granule-dependent perforin/granzyme-mediated target cell killing was determined. As a marker for degranulation, the cumulative exposure of granular membrane protein CD107a (also known as lysosomal-associated membrane protein-1 (LAMP-1)) on the cell surface of a responding antigen-specific T cell was measured by flow cytometry. For this purpose, CEA_{571}- or Her-2/neu_{369}-specific CTLs were stimulated with various target cells for 5 h at 37°C in a 1:1 ratio in the presence of anti-CD107a-PE (BD Biosciences) and 4 µM monensin (Sigma). Following the stimulation, cells were washed, stained with APC-labeled tetramer and FITC-labeled anti-CD8 mAb, respectively, and analyzed on a FACScalibur.

Adenoviral infection

Replication-deficient recombinant type-5 adenoviruses (with deletion of the E1 region), encoding green fluorescent protein (GFP) and CEA were used (kindly provided by Dr. Nikolay Korokhov, Vectorlogics Inc., Birmingham, AL, USA). MelAKR cells were incubated with the Ad5GFP-CEA at a multiplicity of infection (MOIvp) of 1,000, in 100 µl of serum-free DMEM medium, at 37°C for 1 h, after which the cells were washed and cultured overnight in complete DMEM medium with 10% FCS.

Results

Characterization of mature MoDC and MUTZ-3 DC

Like DCs cultured from monocytes (MoDC) or $CD34^+$ bone marrow-derived precursors (CD34-derived DC), DCs cultured from the human cytokine-dependent myeloid cell line MUTZ-3 (MUTZ-3 DC) exhibit true DC morphology and characteristics. After differentiation in the presence of GM-CSF, IL-4 and TNF-α and maturation with a cytokine cocktail containing TNF-α, IL-6, IL-1β and PGE2, MUTZ-3 DC expressed intermediate to high levels of the co-stimulatory molecules CD80, CD86 and CD40, and of DC-specific molecules CD1a, DC-SIGN and CD83 (Fig. 1). Thus, MUTZ-3 DCs phenotypically resemble MoDC (see Fig. 1) and CD34-derived DC, as previously described [19].

Induction of $hTERT_{988Y}$-specific $CD8^+$ T cells in vitro using peptide-pulsed autologous MoDC or allogeneic HLA-A2-matched MUTZ-3 DC

DCs have the unique ability to induce and activate tumor-specific CTLs both in vivo and in vitro. In order to determine whether MUTZ-3 DC can be used to generate primary tumor-specific CTL in vitro and whether their priming efficiency is comparable to that of MoDC, both autologous MoDC and allogeneic MUTZ-3 DC were used as stimulator cells in an in vitro CTL induction

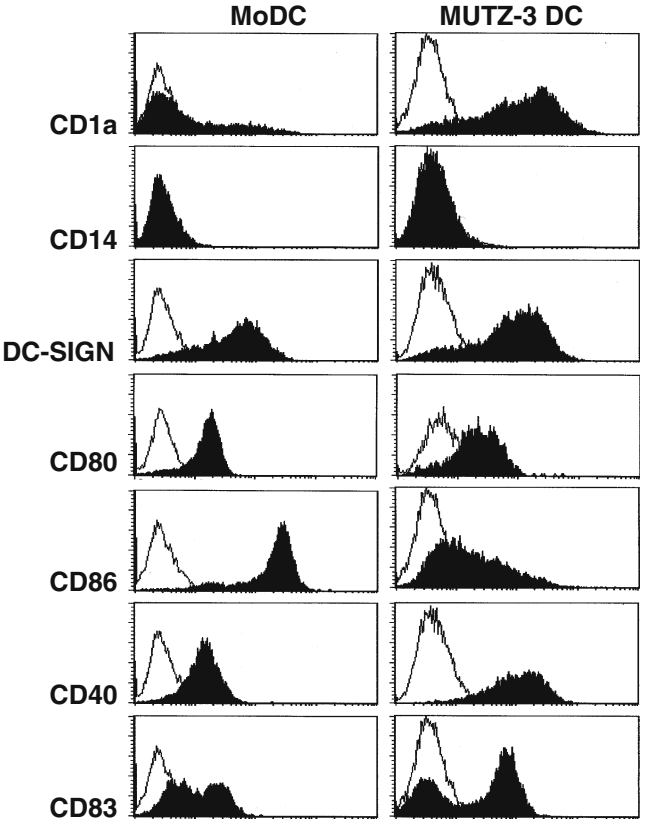

Fig. 1 Phenotypic characterization of mature MoDC and MUTZ-3-derived DC. DCs were stained with FITC and/or PE-conjugated antibodies against CD1a, CD14, CD86, CD40, DC-SIGN and CD83 and analyzed by flow cytometry. *Open histograms* isotype-matched controls; *closed histograms* the marker as indicated to the left

protocol. To this end, CD8β^+ CTL precursors isolated from PBMC of HLA-A2$^+$ healthy donors were stimulated weekly with either peptide-pulsed autologous MoDC or allogeneic but HLA-A2-matched MUTZ-3 DC in 12 parallel cultures at 1×10^6 CD8β^+ CTL precursors per culture. The epitopes selected were the previously described HLA-A2-restricted hTERT-derived peptides, hTERT$_{540}$ and/or the P1Y heteroclitic variant of hTERT$_{988}$ (hTERT$_{988Y}$), in which aspartic acid (D) at position 988 is substituted for tyrosine (Y) for increased binding affinity for HLA-A2 [29, 38]. From the second round of stimulation onwards, T cell cultures were monitored for the expansion of hTERT-specific CD8$^+$ T cells through HLA-A2-tetramer binding (see Fig. 2). Table 1 shows the capacity of both MoDC and MUTZ-3 DC to induce hTERT-specific CD8$^+$ T cells, as tested in two individual HLA-A2$^+$ healthy donors by tetramer binding. Comparable T cell priming efficiencies were observed against the hTERT$_{988Y}$ epitope with allogeneic HLA-A2-matched MUTZ-3 DC and fully HLA-matched autologous MoDC. Of note, both MoDC and MUTZ-3 DC were equally ineffective in generating hTERT$_{540}$-specific CD8$^+$ T cells (Table 1). The highest achieved percentage of Tm-hTERT$^+_{988Y}$ CD8$^+$ T cells using MoDC and MUTZ-3 DC was comparable, varying between 0.15 and 3.5% for the different inductions, and was reached at various stimulation rounds (see Table 1). No difference was observed in the number of in vitro stimulations (IVS) needed to detect the first hTERT$_{988Y}$-specific CD8$^+$ T cells by tetramer analysis; both MoDC- and MUTZ-3 DC-induced hTERT$_{988Y}$-specific CD8$^+$ T cells could already be detected as early as round two or three of stimulation (Table 1). In conclusion, equivalent T cell priming efficiencies (i.e., induction rates) were observed for autologous MoDC and allogeneic HLA-A2-matched MUTZ-3 DC.

Induction of antigen-specific, HLA-A2-restricted CD8$^+$ T cells directed against different tumor-associated epitopes by allogeneic MUTZ-3 DC in vitro

To further establish the MUTZ-3 DCs' utility as universal HLA-A2$^+$ stimulator cells for in vitro priming and expansion of CTL against different TAA across a range of allogeneic backgrounds, we included the immunodominant HLA-A2-restricted CEA-derived peptide CEA$_{571}$, also known as CAP-1 [34] and the recently identified, HLA-A2-restricted Ebp1-derived peptide Ebp1$_{59}$ (Santegoets et al. submitted). hTERT$_{540}$-specific CD8$^+$ T cells could be generated in 1/12 individual cultures from one donor tested, while hTERT$_{988Y}$-specific CD8$^+$ T cells could be generated in 6/36 individual cultures from two out of three donors tested. Furthermore, detectable Ebp1$_{59}$-specific CD8$^+$ T cells were generated in 9/24 individual cultures from two donors (2/2 donors positive), while CEA$_{571}$-specific CD8$^+$ T cells could be induced in 6/48 individual cultures from four different donors (4/4 donors positive) (Table 2). Frequency of MUTZ-3 DC-induced tetramer-positive T cells was shown to be variable, ranging between 0.15 and 4.12%. These findings are summarized in Table 2. Importantly, tumor peptide-reactive CD8$^+$ T cells could be primed by MUTZ-3 DC in all donors tested. These data demonstrate that MUTZ-3 DC-mediated and HLA-A2-restricted T cell priming is reproducible for different peptides in multiple donors against a variety of allogeneic MHC backgrounds.

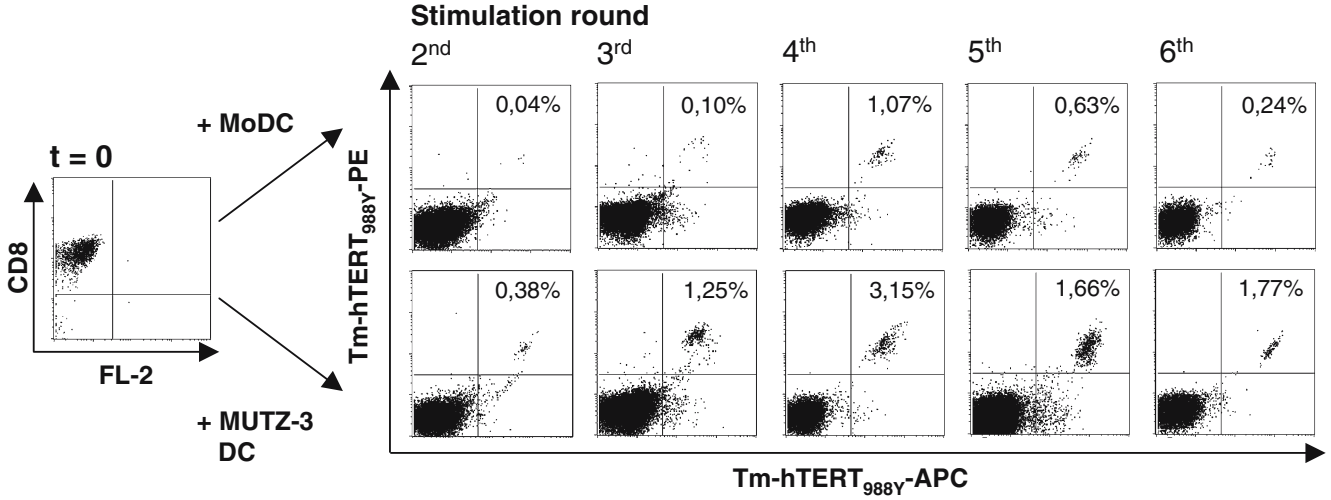

Fig. 2 Flow cytometric HLA-A2$^+$ tetramer (Tm)-binding analysis of CD8β^+ CTL precursors stimulated with mature hTERT$_{988Y}$-loaded MoDC or MUTZ-3 DC. From day 0 ($t=0$) magnetic bead-isolated CD8$^+$ CTL precursors were stimulated repeatedly with either peptide-pulsed autologous MoDC or HLA-A2-matched MUTZ-3 DC. Flow cytometric PE-/APC-labeled tetramer analysis on CD8β^+ CTL precursors was performed on day 7 after second, third, fourth, fifth and sixth stimulation cycles. Staining with both PE- and APC-labeled Tm was performed to exclude false positive staining by single Tm. Results for live, propidium iodide-negative cells are shown. Percentages Tm-hTERT$_{988Y}$-PE$^+$/Tm-hTERT$_{988Y}$-APC$^+$ CD8$^+$ T cells are shown in the *upper right quadrants*

Table 1 MoDC- versus MUTZ-3 DC-mediated induction of hTERT-specific $CD8^+$ T cells

Donor	Antigen	Epitope	MoDC			MUTZ-3 DC		
			Induction efficiency[a]	Earliest Tm+ frequency (%) (number of IVS)[b]	Max. Tm^+ T cell frequency (%) (number of IVS)[c]	Induction efficiency[a]	Earliest Tm+ frequency (%) (number of IVS)[b]	Max. Tm^+ T cell frequency (%) (number of IVS)[c]
1	hTERT	988Y	2/12	0.13 (3) 1.30 (3)	0.80 (4) 3.50 (4)	2/12	0.26 (2) 0.10 (3)	0.40 (3) 2.10 (6)
		540	0/12	NA	NA	0/12	NA	NA
2	hTERT	988Y	5/12	0.17 (2) 0.29 (2) 0.10 (3) 0.18 (1) 0.10 (3)	0.25 (4) 0.29 (2) 0.15 (4) 0.48 (2) 1.07 (4)	3/12	0.22 (3) 0.10 (2) 0.38 (2)	0.72 (4) 1.50 (5) 3.15 (4)

[a]Determined by flow cytometric HLA-$A2^+$ tetramer analysis and expressed as proportion of positively responding bulk cultures. For each peptide, 12 parallel $CD8\beta^+$ T cell cultures were started. Cultures were scored positive when percentage of tetramer-(Tm) positive cells $>0.1\%$ of live cells

[b]Earliest frequencies listed are first observed tetramer-positive cells for each individual culture detected after indicated number of in vitro stimulations (IVS) in parentheses

[c]Maximum frequencies listed are highest achieved percentage of tetramer-positive cells for each individual culture detected after indicated number of in vitro stimulations (IVS) in parentheses

NA not applicable

Isolation, expansion and characterization of functional $CD8^+$ T clones

To further characterize the MUTZ-3 DC-generated $CD8^+$ T cells in terms of phenotype and cytotoxic potential, MUTZ-3 DC-induced CEA_{571}- and $Ebp1_{59}$-specific $CD8^+$ T cells were isolated and cloned. Tetramer$^+$$CD8^+$ T cells in the priming cultures tended to reach a maximum percentage after varying numbers of restimulation cycles, after which their numbers waned (see Fig. 2). This overgrowth by non-specific clones most likely signals a decreased proliferative potential of the antigen-specific effector T cells. To optimize the chances of successful cloning and expansion, specific tetramer$^+$ T cells were therefore isolated before this point was reached. CEA_{571}- and $Ebp1_{59}$-specific $CD8^+$ T cells were isolated by CD8/tetramer-guided flow sorting from individual cultures containing 0.17% and 0.44% Tm^+ T cells, respectively (not shown). After cloning by limiting dilution and further expansion, CEA_{571}- and $Ebp1_{59}$-specific T cell cultures of up to 95 and 70% pure, respectively, could be isolated and expanded (Figs. 3a, 4a).

Table 2 MUTZ-3 DC-mediated induction of CEA-, hTERT- and Ebp1-specific $CD8^+$ T cells

Donor	Antigen	MUTZ-3 DC	
		Induction efficiency[a]	Max. Tm^+ T cell frequency (%) (number of IVS)[b]
3	$hTERT_{988Y}$	2/12	0.41 (4) 2.89 (5)
	$hTERT_{540}$	1/12	0.25 (5)
4	$hTERT_{988Y}$	4/12	0.24 (3) 0.56 (3) 0.44 (3) 4.12 (5)
	CEA_{571}	1/12	0.15 (7)
	$Ebp1_{59}$	5/12	1.82 (3) 0.54 (3) 0.79 (6) 0.61 (3) 0.12 (3)
5	$hTERT_{988Y}$	0/12	NA
	CEA_{571}	1/12	0.78 (7)
6	CEA_{571}	2/12	0.55 (2) 0.57 (5)
	$Ebp1_{59}$	4/12	0.28 (5) 0.89 (5) 1.49 (5) 2.66 (5)
7	CEA_{571}	2/12	0.26 (7) 1.36 (7)

[a]Determined by flow cytometric HLA-$A2^+$ tetramer analysis and expressed as proportion of positively responding bulk cultures. For each peptide, 12 parallel $CD8\beta^+$ T cell cultures were started. Cultures were scored positive when percentage of tetramer-positive cells $>0.1\%$ of live cells

[b]Maximum frequencies listed are highest achieved percentage of tetramer-positive cells for each individual culture detected after indicated number of in vitro stimulations (IVS) in parentheses

NA not applicable

After isolation and expansion, CEA_{571}- and $Ebp1_{59}$-specific T cell clones were used for phenotypic and functional analysis. Flow cytometric analysis revealed that the generated $CD8^+$ T cells could be defined as effector memory T cells, according to the $CD8^+CD27^-CD28^-CD45RA^+$ $CD45RO^{+/-}CCR7^-$ $TCR\alpha\beta^+$ phenotype (Table 3) [10, 28].

Functional activity of the generated T cell clones was determined by IFN-γ production assay, CD107a flow cytometric degranulation assay and standard chromium release assay. IFN-γ production assays showed that the obtained CEA_{571}-specific $CD8^+$ T cell clones were able to recognize the exogenously loaded CEA_{571} epitope, but not its endogenously expressed counterpart in the $HLA\text{-}A2^+/CEA^+$ gastric carcinoma cell line KATO-3 (Fig. 3b), nor in the breast or coloncarcinoma lines MCF-7, SW-620 or SW-403 (data not shown). Similarly, in a chromium release assay, CEA_{571}-specific $CD8^+$ T cells were able to specifically lyse peptide-loaded JY target cells, but not the $HLA\text{-}A2^+/CEA^+$ KATO-3 cells unless exogenously loaded with CEA_{571} peptide, excluding intrinsic apoptosis resistance or defective HLA expression as possible causes of the observed lack of CTL-induced lysis (data not shown). Functional avidity analysis, based on cytolysis of JY targets loaded with titered peptide, revealed the CEA_{571}-specific $CD8^+$ T cell clones to exhibit low avidity antigen recognition (half maximum lysis at 500 nM, data not shown). We also assessed tumor cell recognition in the high-sensitivity CD107a degranulation assay [3, 27]. Again, only exogenously loaded and not endogenously expressed CEA_{571} epitope on target cells was recognized by the CEA_{571}-specific CTL clones, unless over-expression of CEA was induced by high-efficiency adenoviral transduction (Fig. 3c). Transduction efficiency of the $HLA\text{-}A2^+/CEA^-$ melanoma cell line MelAKR was 100% as assessed by GFP transgene expression upon infection with an adenovirus encoding both CEA and GFP (data not shown). These data indicate that despite low avidity antigen recognition, CEA_{571}-specific CTL generated by MUTZ-3 DC are able to detect endogenously processed and presented CEA_{571} peptide.

Functionality of the $Ebp1_{59}$-specific $CD8^+$ T cell line was determined by IFN-γ production. As shown in Fig. 4b, the $Ebp1_{59}$-specific CTL line was able to recognize both exogenously loaded target cells and $HLA\text{-}A2^+$ tumor cells expressing endogenous Ebp1. The observation that the recognition of $HLA\text{-}A2^+/Ebp1^+$ tumor cells could be blocked almost completely by incubating the target cells with an anti-HLA-A2 antibody, confirmed that the recognition of these tumor cells is HLA-A2 restricted and not mediated by NK cells.

To demonstrate the feasibility of generating high avidity cytolytic T cell clones from cancer patients, we

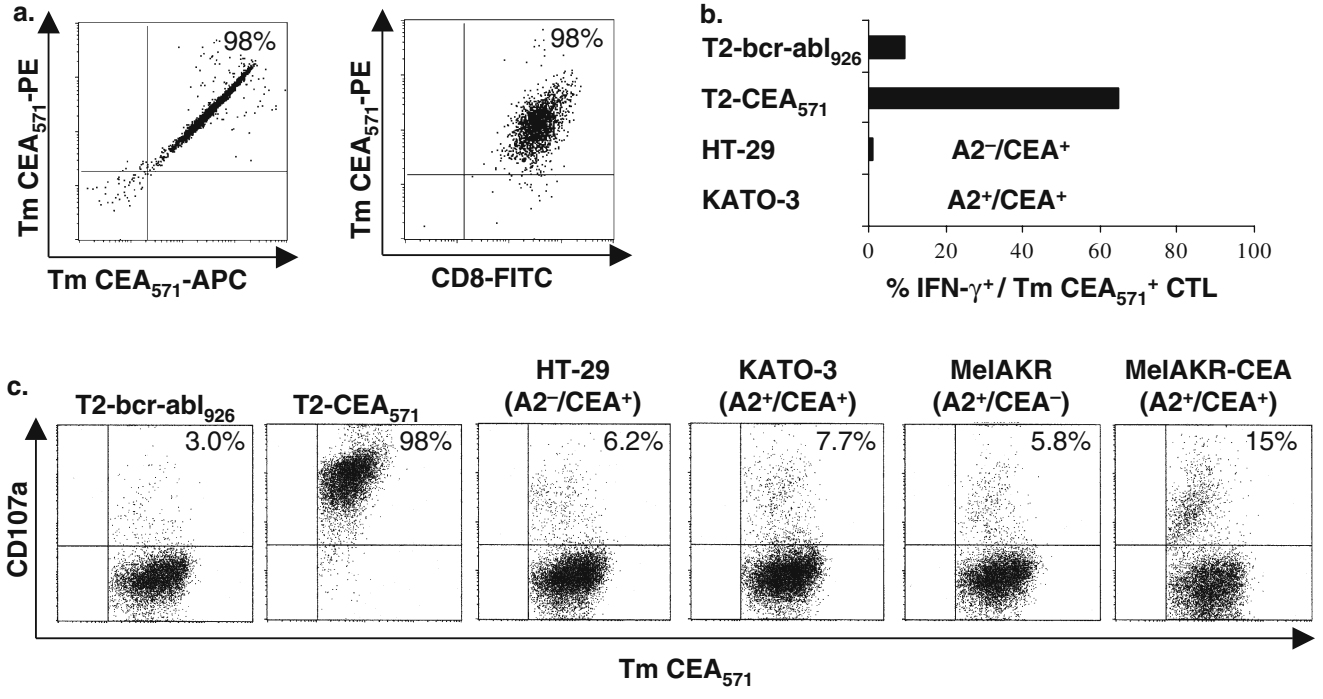

Fig. 3 Flow cytometric tetramer-binding analysis and functional analysis of CEA_{571}-specific CTL clones. CEA_{571}-specific $CD8^+$ T cells were generated by repeated stimulation of $CD8\beta^+$ CTL precursors with peptide-pulsed MUTZ-3 DC. **a** Flow cytometric PE-/APC-labeled tetramer (Tm)-binding analysis and PE-labeled Tm/FITC-CD8 staining of CEA_{571}-specific $CD8^+$ T cells after Tm CEA_{571}/CD8-guided FACSsort and limiting dilution cloning. Lytic activity of CEA_{571}-specific $CD8^+$ T cell clones were analyzed by **b** intracellular IFN-γ staining and **c** flow cytometric degranulation assay. Data shown are representative of two separately tested clones. Target cells used were $HLA\text{-}A2^+$ JY or T2 cells loaded with the CEA_{571} peptide or control HLA-A2-restricted peptides $Flu\text{-}M1_{58}$ or $Bcr\text{-}abl_{926}$, CEA expressing cell lines KATO-3 and the HT-29, wild-type $CEA^-/HLA\text{-}A2^+$ MelAKR and AdGFP-CEA-transduced MelAKR. Percentage IFN-γ^+ cells and $CD107a^+$ cells shown were gated for live tetramer-$CEA^+_{571}/CD8^+$ T cells

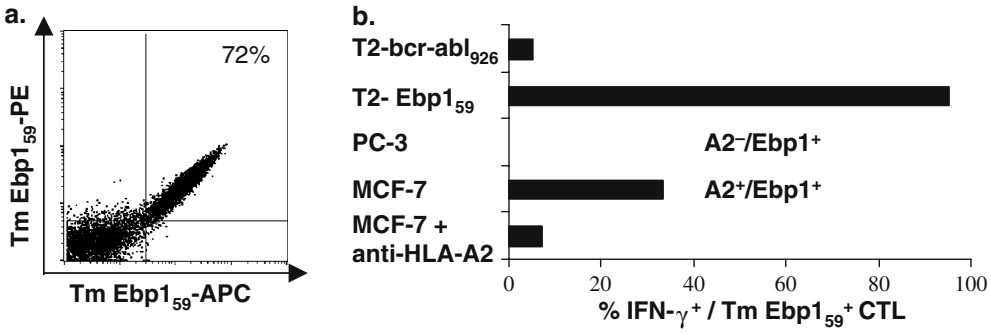

Fig. 4 Flow cytometric tetramer-binding analysis and functional analysis of an Ebp1$_{59}$-specific CTL clone. Ebp1$_{59}$-specific CD8$^+$ T cells were generated by repeated stimulation of CD8β^+ CTL precursors with peptide-pulsed MUTZ-3 DC. **a** Flow cytometric PE-/APC-labeled tetramer (Tm)-binding analysis of an Ebp1$_{59}$-specific CD8$^+$ T cell line after Tm Ebp1$_{59}$/CD8-guided FACSsort and limiting dilution cloning. **b** Functional activity of the Ebp1$_{59}$-specific CD8$^+$ T cell line was determined by intracellular IFN-γ staining. Target cells used were HLA-A2$^+$ T2 cells loaded with the Ebp1$_{59}$ peptide or control HLA-A2-restricted peptide bcr-abl$_{926}$, Ebp1$^+$/HLA-A2$^-$ cell line PC-3 and Ebp1$^+$/HLA-A2$^+$ cell line MCF-7, with or without anti-HLA-A2 neutralizing antibody BB7.2. Percentage IFN-γ^+ cells shown was gated for live tetramer-Ebp1$_{59}^+$/CD8$^+$ T cells

stimulated CD8β^+ CTL precursors obtained from a prostate cancer patient with MUTZ-3 DC loaded with low peptide concentrations (1 μg/ml for the first stimulation and 10 ng/ml thereafter). In doing so, we were able to generate high avidity Her-2/*neu*$_{369}$-specific CTL (up to 95% pure, Fig. 5a), with half maximal CD107a translocation at 1 pM peptide as analyzed by avidity analysis on HLA-A2$^+$ JY targets cells loaded with tenfold dilutions of the Her-2/*neu*$_{369}$ peptide (data not shown). This CTL clone was indeed able to specifically recognize and lyse the HLA-A2$^+$/Her-2/*neu*$^+$ tumor cell line SW620 (Fig. 5b). Importantly, the recognition of this tumor cell line was as effective as the recognition of the exogenously loaded JY target cells. In conclusion, employing allogeneic HLA-A2-matched MUTZ-3 DC, tumor peptide-specific CTL clones with endogenously expressed epitope and tumor recognition capabilities could be generated.

Table 3 Phenotype of CEA$_{571}$-specific CD8$^+$ T cells

Antigen	Expression*	
	Clone 5#1	Clone 6#1
CD8α	99.1	98.4
CD8β	99.4	97.9
CD27	0	0
CD28	1.0	0.7
CD45RO	13.2	43.8
CD45RA	97	96.4
TCRαβ[a]	61.6	57.9
TCRγδ	0	0
CCR7	0	0

*Indicated as percentage positive cells, determined by flow cytometry

[a]TCR expression varies with time after stimulation and increases to 100% ≥7 days after stimulation

Discussion

The adoptive transfer of in vitro-induced and expanded tumor-specific CTL presents a promising immunotherapeutic approach for the treatment of cancer. Recently, objective clinical responses were observed in refractory metastatic melanoma patients receiving in vitro-generated and expanded tumor-reactive T cells and IL-2 infusions, either with or without immunodepleting chemotherapy [7, 41]. Tumor-infiltrating lymphocytes (TILs) are considered to be an appropriate source of tumor-reactive T cells, as they recognize a variety of TAA. However, since TILs are often not available due to lack of accessible tumors, it would be useful to generate tumor-reactive CTL from peripheral blood-derived CTL precursors. Due to low precursor frequencies in the blood, repeated IVS with APC is needed to generate sufficient numbers of tumor-reactive CTL. DCs are preferably used for these stimulations since they have been shown to be very potent inducers of specific CTL both in vitro and in vivo [9, 23, 33]. In this study, we report on the applicability of MUTZ-3 DC as stimulator cells for the generation and expansion of TAA-specific effector CTL for adoptive transfer in a clinical setting.

It is well known that the mature MoDCs are capable of inducing functional tumor-specific CTL in vitro [9, 30, 33]. Here we show that allogeneic HLA-A2-matched MUTZ-3 DC can similarly induce tumor antigen-specific CD8$^+$ T cells. Importantly, T cell priming efficiencies were shown to be equivalent in terms of induction rate for allogeneic MUTZ-3 DC and autologous MoDC. From previous studies in our lab employing the same priming method with autologous MoDC [30, 31], we know that tumor antigen-specific CD8+ T cells with similar functional avidity (i.e., low to intermediate) were induced as described here for MUTZ-3 DC. Our observation that both MoDC and MUTZ-3 DC induce tumor antigen-specific CD8$^+$ T

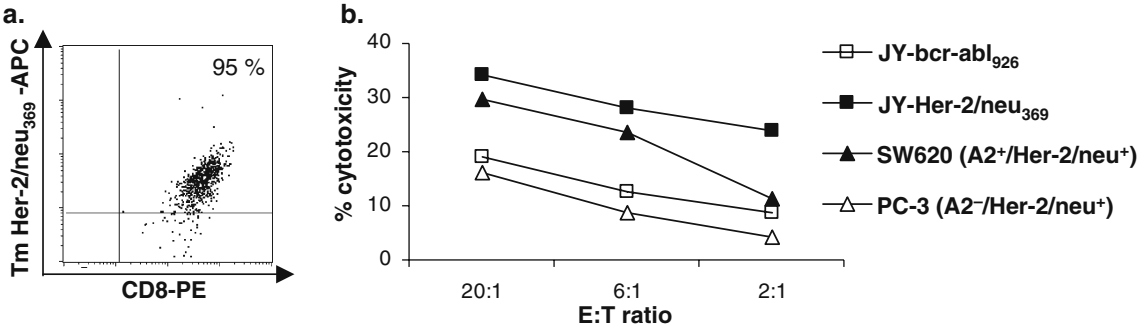

Fig. 5 Flow cytometric tetramer-binding analysis and functional analysis of a prostate cancer patient-derived Her-2/neu_{369}-specific CTL clone. Her-2/neu_{369}-specific CD8$^+$ T cells were generated by repeated stimulation of CD8β^+ CTL precursors with peptide-pulsed MUTZ-3 DC. **a** Flow cytometric APC-labeled Tm/PE-labeled CD8 staining of an Her-2/neu_{369}-specific CD8$^+$ T cell line after Tm Her-2/neu_{369}/CD8-guided FACSsort and limiting dilution cloning. Results for live, propidium iodide-negative cells are shown. Percentage Tm Her-2/neu_{369}-APC$^+$/CD8-PE$^+$ T cells is shown in the upper right quadrants. **b** Functional activity of the Her-2/neu_{369}-specific CD8$^+$ T cell clone was determined by chromium release assay. Target cells used were HLA-A2$^+$ JY cells loaded with the Her-2/neu_{369} peptide or control HLA-A2-restricted peptide bcr-abl$_{926}$, the Her-2/neu^+/HLA-A2$^-$ cell line PC-3 and the Her-2/neu^+/HLA-A2$^+$ cell line SW620

cells with comparable efficiency and avidity indicates that there is no interference of allogeneic CTL responses against mismatched HLA antigens with the CTL response against the selected antigen. This is in line with findings from other groups, observing no or low-level allogeneic CTL response when priming with either allogeneic melanoma cell lines or peptide-pulsed allogeneic DC [5, 24]. Overall, these results indicate that allogeneic MUTZ-3 DC can be used to induce TAA-specific CD8$^+$ T cells from low frequency CTL precursors directed against various TAAs. Importantly, these tumor-specific CD8$^+$ T cells were generated in all donors tested, independent of HLA background.

A requirement for achieving effective adoptive T cell therapy is the ability to generate tumor-reactive CTLs capable of recognizing tumor cells expressing the antigen of interest. Functional characterization of MUTZ-3 DC-induced and expanded Ebp1$_{59}$- and CEA$_{571}$-specific CD8$^+$ T cells revealed that the Ebp1$_{59}$-specific CD8$^+$ T cells were able to recognize both exogenously loaded target cells and HLA-A2$^+$ tumor cells expressing endogenous Ebp1, whereas the primed and expanded CEA$_{571}$-specific CD8$^+$ T cells were only able to recognize target cells exogenously loaded with CEA$_{571}$ peptide. The fact that we generated CEA$_{571}$-specific CD8$^+$ T cells that are of low avidity may account for the observation that endogenously processed and presented CEA$_{571}$ epitope could only be detected on tumor cells transduced with adenoviral vectors to induce high levels of the CEA protein, but not on wild-type tumor cells expressing physiological levels of CEA.

The selection of low avidity CEA$_{571}$-specific CD8$^+$ T cells in the current study can be explained by the possible absence and/or unresponsiveness of high and intermediate avidity CEA-specific CTL. This is most likely due to negative selection in the thymus and/or the induction of peripheral tolerance [14, 36]. Furthermore, as reported previously [1, 4, 39] and currently appreciated as a well-known phenomenon, the repetitive stimulation of antigen-specific CTL with APC expressing high densities of peptide/MHC complexes favors low avidity CTL outgrowth. Consequently, the induction of low avidity CEA$_{571}$-specific CD8$^+$ T cells in this study is most likely due to the selected antigen and the priming method rather than to the use of MUTZ-3 DC. Indeed, we were able to select tumor-reactive CTL directed against Her-2/*neu* and Ebp1, another self-protein which is known to be involved in multiple signal transduction pathways and cellular proliferation and differentiation processes [40, 42, Santegoets et al. submitted].

In addition, from previous studies it has become clear that generating tumor-reactive CEA$_{571}$-specific CTL from normal individuals remains difficult. Most CEA$_{571}$-specific CTL reported originate from CEA vaccinated cancer patients, in which CEA-specific T cell responses may have been potentiated and possible tolerance may have been broken [34, 35, 45]. Moreover, most of those tumor-reactive CEA$_{571}$-specific CTL reported previously were not CD8$^+$ CTL clones as we describe here, but CD8 and CD4 containing bulk T cell lines. Nevertheless, it has been shown possible to generate CEA$_{571}$-specific tumor-reactive CTL in vitro from normal individuals [17].

Although it is well appreciated that high avidity CTLs show superior anti-tumor activity in vivo [1, 6, 44], it has been demonstrated that low avidity CTLs can also mediate tumor rejection in vivo [18, 20]. For future clinical application, it would be preferable to induce tumor-reactive CTL with a broad range of avidities. We are therefore currently optimizing our CTL induction protocol in order to generate higher avidity tumor-specific CTL displaying anti-tumor activity at lower TAA-expression levels. Employing MUTZ-3 DC loaded with lower concentrations of Her-2/*neu*-derived HLA-A2-binding peptides, we are indeed able to generate higher avidity effector CTL from cancer patient-derived CTL

precursors with the ability to recognize and kill tumor cells. In addition, for future clinical application we are developing methods to generate MUTZ-3 DC under serum-free GMP conditions [8, 22].

In summary, our data clearly show that MUTZ-3 DC can be used to induce tumor-reactive CTL clones in vitro. MUTZ-3 DCs thus represent a ready and standardized source of allogeneic HLA-matched DC capable of generating and expanding functional TAA-specific effector CTL for therapeutic adoptive transfer strategies. Moreover, the capacity of MUTZ-3 DC to induce antigen-specific CTL against a variety of HLA backgrounds also suggests its possible utility as DC vaccine in in vivo cancer immunotherapy.

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