Generation and Purification of CD8$^+$ Melan-A-specific Cytotoxic T Lymphocytes for Adoptive Transfer in Tumor Immunotherapy[1]

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ABSTRACT

Tumor antigens that might serve as potential targets for adoptive T-cell therapy have been defined in different tumor entities, especially in malignant melanoma. To generate conditions to induce primary T-cell responses against different HLA-A*0201-restricted melanoma peptides and to allow further expansion of peptide-specific T cells for adoptive transfer, CD8$^+$-purified T cells from healthy donors were stimulated with Melan-A-pulsed autologous dendritic cells. Dendritic cells were generated *in vitro* from monocytes with granulocyte macrophage colony-stimulating factor, interleukin-4, and transforming growth factor-β1. After 3–4 weekly stimulation cycles with Melan-A-pulsed DCs, we were able to induce a strong peptide-specific CTL response *in vitro*. MHC-peptide tetramer staining revealed a frequency of up to 3.5% CD8$^+$/Melan-A$^+$ T cells. Additional antigen-independent expansion with anti-CD3/anti-CD28 monoclonal antibodies together with interleukin-2 gave rise to 600-fold expansion of CD8$^+$ CTLs that maintained Melan-A specificity and were able to efficiently lyse Melan-A-expressing melanoma cells. To enrich antigen-specific T cells *in vitro*, we used a recently established technology for analysis and sorting of live cells according to secreted cytokines. In the present study, we demonstrated that Melan-A-specific T cells can be purified by magnetic separation according to secreted IFN-γ. These cells revealed a very potent monospecific CTL response, even at low E:T ratios, against Melan-A-pulsed and Melan-A-expressing target cells. Altogether, our study demonstrated that we have developed an efficient method for generating large numbers of peptide-specific T cells *in vitro* that may be used for adoptive T-cell transfer in tumor immunotherapy.

INTRODUCTION

The cloning and characterization of tumor-associated antigens and tumor-associated antigen-derived peptides recognized by human CTLs in a MHC class I-restricted fashion has opened new possibilities for immunotherapeutic approaches to the treatment of human cancers, particularly malignant melanoma (1). These strategies include either vaccination with antigenic peptides, plasmid DNA, and recombinant viruses encoding tumor-associated antigens (2–4), or the adoptive transfer of *in vitro*-expanded tumor-specific CTLs. Encouraging results have been obtained in animal models using the latter approach (5, 6). However, attempts to generate and expand human tumor-specific T cells *in vitro* for an adoptive transfer in tumor immunotherapy have been conducted with only limited success. This difficulty may have resulted, in part, from the very low frequency of tumor-specific CTL precursors in patients' blood, which may be due to the fact that tumor cells are not potent professional APCs.[3] We therefore searched for *in vitro* conditions that would facilitate the generation and expansion of antigen-specific CTLs for adoptive transfer.

It has been shown that APCs play a dominant role in induction of tumor-specific CTLs *in vivo* and *in vitro* (7, 8). The availability of large numbers of DCs, generated from hematopoietic progenitor cells or monocytes *in vitro*, has profoundly changed preclinical research as well as the clinical evaluation of these cells (9–11). When pulsed with the relevant peptide, DCs are attractive for *in vitro* induction and activation of antigen-specific tumor-reactive CTLs (12).

It has been shown recently that melanoma-reactive CTL lines and clones can be isolated and purified using peptide-MHC tetramers (13–15). Because cytokines are the major parameter of specific T-cell effector function, Manz *et al.* (16) have developed a technology that allows the analysis and separation of activated T cells according to secreted cytokines. An affinity matrix for the secreted cytokine is generated by attaching a specific antibody to the cell surface. Subsequently, the cells are allowed to secrete the cytokine under defined conditions. Cells can then be stained for the secreted molecule, which is now bound to the affinity matrix with specific fluorochrome-labeled detection antibodies (16). This method permits an assessment of the total number of antigen-specific T cells that can be used for

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[3] The abbreviations used are: APC, antigen-presenting cell; DC, dendritic cell; FluM1, influenza matrix protein FluM1$_{58-66}$ peptide GILGFVFTL; mAb, monoclonal antibody; TCGF, T-cell growth factor; PBMC, peripheral blood mononuclear cell; BD, Becton Dickinson; PE, phycoerythrin; IL, interleukin.

purification approaches. In the present study, we used this technique to analyze and purify Melan-A-specific CTLs, generated *in vitro* from CD8-purified T cells after stimulation with Melan-A-pulsed autologous DCs, according to secreted IFN-γ. Purified IFN-γ-secreting T cells showed Melan-A-specific cytotoxic activity even at very low E:T ratios. Our results demonstrate an alternative strategy for generating and purifying antigen-specific CTL from peripheral blood lymphocytes, which can be used for adoptive transfer in tumor immunotherapy.

MATERIALS AND METHODS

Peptides. Each of the peptides used in this study was prepared by Eurogentec (Tilman, Belgium): Melan-A_{26-35}, EAAGIGILTV (17, 18); tyrosinase$_{369-377}$, YMDGTMSQV (19); and FluM1, GILGFVFTL (20). For tetramer refolding and tetramer analysis, a modified Melan-A_{26-35} peptide, ELAGIGILTV, was used. This modified Melan-A peptide (alanine to leucine at position 2) previously has been shown to increase the binding affinity for HLA-A*201 without affecting CTL recognition (21). The identity of each peptide was confirmed by mass spectral analysis. The peptides were >98% pure as assessed by high-pressure liquid chromatography analysis.

Cells. Tumor cell lines and T2 cells were maintained in RPMI 1640 supplemented with 200 mM L-glutamine, 50 μM β-mercaptoethanol, 100 mM sodium pyruvate, MEM vitamins, 40 μg/ml streptomycin, 40 units/ml penicillin (standard medium M′), and 10% FCS (PAN Systems GmbH, Aidenbach, Germany). Melanoma cell lines MeE384, MeT413, and MeI493 were established from surgically excised melanoma metastasis. Expression of HLA-A2 and Melan-A was assessed by FACS analysis using an anti-HLA-A2-specific mAb (BB7.2; ATCC, Rockville, MD) and by reverse transcription-PCR, respectively, as described previously (22): MeE384 is HLA-$A2^-$, Melan-A^+; MeT413 is HLA-$A2^+$, Melan-A^+; MeI493 is HLA-$A2^+$, Melan-A^+. T2 cells are HLA-A*0201 human lymphoid cells that are defective in antigen processing but effectively present exogenously supplied peptides (23).

Preparation of TCGF. The preparation of TCGF was described previously (24). TCGF was produced by stimulating 2.5×10^6/ml PBMCs for 2 h with 5 μg/ml phytohemagglutinin (Murex, Dartford, England), 5 ng/ml phorbol myristate acetate (Sigma Chemical Co, St. Louis, MO), and 5,000 rad-irradiated EBV-transformed B cells. The cells were then washed to remove the mitogens and resuspended in RPMI 1640 supplemented with 2.5% human AB serum. After 40 h of incubation, supernatants were harvested, passed through 0.2 μm filters, and stored at −70°C.

mAbs and Flow Cytometry. Surface marker analysis of *in vitro*-cultured cells was performed using a FACScalibur (BD, Mountain View, CA) and the CellQuest software. We used the following mAbs conjugated to FITC or PE for direct fluorescence: anti-CD1a (Coulter Immunology, Hialeah, FL), anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD45RA, anti-CD45RO, anti-CD56, anti-CD80, anti-CD83, and anti-CD86 (all from BD).

Generation of Peptide-specific T-Cell Lines. This study was approved by the institutional ethics committee. All donors and patients gave written informed consent before enrolling in the study. HLA typing was carried out at the Freiburg University HLA Typing Laboratory. $CD8^+$ T lymphocytes were enriched from PBMCs by depletion of $CD4^+$, $CD11b^+$, $CD16^+$, $CD19^+$, $CD20^+$, and $CD56^+$ cells with magnetic cell sorting using a midiMACS device (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The resulting population consisted of >90% $CD8^+$ T cells and was used as the responder cell population. DCs were generated from monocyte-enriched cell fractions as described previously (10). Briefly, monocytes were isolated from PBMCs by plastic adherence. Adherent cells were cultured with standard medium (M′) plus 2% autologous serum, supplemented with 1000 units/ml human granulocyte-macrophage colony-stimulating factor (Essex Pharma, Novartis Pharma, Basel, Switzerland), 100 units/ml IL-4 (CellGenix GmbH, Freiburg, Germany) and 150 units/ml transforming growth factor-β1 (CellGenix). After 5–7 days of culture 100 ng/ml lipopolysaccharide (from *Escherichia coli*, serotype 0111:B4; Sigma) was added for 24 h. Cells displayed typical surface markers of DCs ($CD1a^+$, $CD14^-$, $CD80^+$, $CD83^+$, and $CD86^+$). The monocyte-derived DCs were then harvested and pulsed for 2 h at 37°C with 30 μg/ml of the appropriate peptide and human β_2-microglobulin (10 μg/ml) in serum-free M′-medium. Peptide-pulsed DCs were washed twice and resuspended in M′ medium, and 10^4 responder cells/well and 5×10^3 peptide-pulsed autologous DCs/well were cocultured in 96-well round-bottomed plates in 200 μl M′ medium/well supplemented with 5% autologous plasma and 3% TCGF. Medium and TCGF were replenished twice a week. On day 7, T cells were harvested, counted, and replated at 10^4 T cells/well together with 5×10^3 peptide-pulsed autologous DCs/well in complete medium supplemented with 3% TCGF. Subsequent restimulations with peptide-pulsed DCs were performed once a week; a total of three to four stimulation cycles were conducted before functional analysis. Responder cells were tested for their specificity in a conventional chromium release assay.

Expansion of Antigen-specific CTLs. For expansion of antigen-specific CTLs, T cells were transferred to 25-cm^2 flasks coated with anti-CD3/anti-CD28 mAbs. Briefly, 25-cm^2 flasks (Falcon, Heidelberg, Germany) were coated with anti-human CD3 mAb (OKT3; Ortho Pharmaceutical Corp., Raritan, NJ) and anti-human CD28 mAb (L293; BD) at a concentration of 1 μg/ml in PBS-100 mM HEPES buffer (pH 9) After incubation overnight at 4°C, coated flasks were washed twice with PBS. $CD8^+$ T cells were placed on the precoated and washed flasks at 5×10^5 cells/ml in 10 ml of M′ medium supplemented with 2% autologous serum and 100 IU IL-2/ml (EuroCetus, Amsterdam, the Netherlands). Cells were cultured for 2 weeks; culture medium and IL-2 (100 IU/ml) were changed twice a week.

Chromium Release Assay. The cytotoxic activity of T-cell lines was measured by a conventional 4-h ^{51}Cr release assay using triplicate cultures in V-bottomed plates. Target cells analyzed included HLA-$A2^+$, Melan-A^+, or HLA-$A2^-$, Melan-A^+ melanoma cell lines, peptide-pulsed or nonpulsed T2 cells, and the natural killer target K562. For the peptide recognition assay, T2 target cells were preincubated with 30 μg peptide /ml overnight at 37°C in serum-free M′ medium. E:T ratios were 25:1, 5:1, and 1:1 on 2000 target cells/well. Triplicate wells were averaged and the percentage of specific cytotoxicity was calculated as [(sample − spontaneous release)/

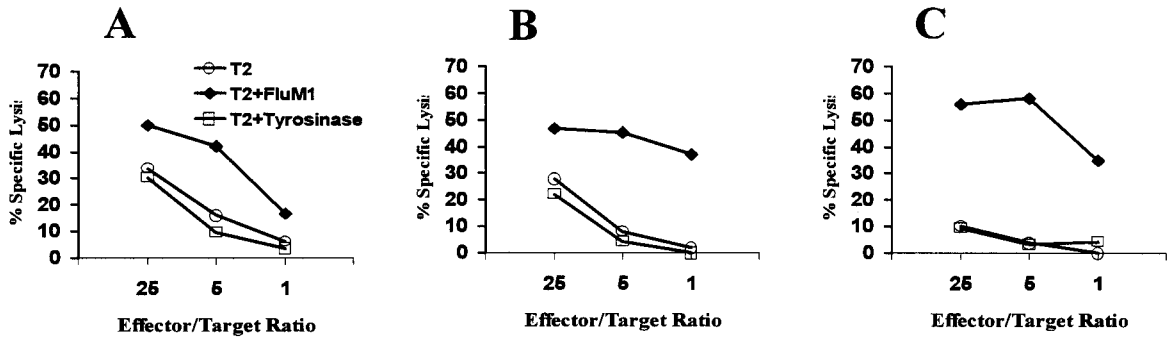

Fig. 1 Repeated stimulation with peptide-pulsed DCs is optimal for induction of antigen-specific T cells. CD8-purified T cells from healthy donors were stimulated once a week with FluM1-pulsed autologous DCs. A total of one (day 0; *A*), two (days 0 and 7; *B*), or three (days 0, 7, and 14; *C*) stimulations with peptide-pulsed DCs were carried out. Cytotoxic activity against T2 cells was measured on day 7 after stimulation in a 4-h ^{51}Cr release assay in the absence (○) or presence of the relevant (*FluM1*; ♦) or irrelevant (*tyrosinase*; □) peptide. The cytotoxic activity of one representative CTL line is shown. Values represent triplicates at E:T ratios of 25:1, 5:1, and 1:1.

(maximum release − spontaneous release)]. For spontaneous release, targets were plated without T cells in M′ medium plus 5% autologous plasma. For maximum release, targets were plated with 5% NP40 (IGEPAL; Sigma) detergent.

HLA-Peptide Tetrameric Complexes and Flow Cytometry. Tetrameric peptide-HLA-A*0201 complexes were synthesized as described previously (25, 26). In brief, purified HLA heavy chain and β2-microglobulin were synthesized using a prokaryotic expression system (pET; Novagen, Milwaukee, WI). The heavy chain was modified by deletion of the transmembrane/cytosolic tail and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The expressed heavy chain and β2-microglobulin were solubilized and refolded together *in vitro* in the presence of a modified Melan-A peptide 26-35 ELAGIGILTV. The 45-kDa refolded product was isolated using fast protein liquid chromatography and biotinylated by BirA (Avidity, Denver, CO) in the presence of biotin (Sigma), ATP (Sigma), and Mg^{2+} (Sigma). The biotinylated product was separated from free biotin by gel filtration and ion exchange using fast protein liquid chromatography. Streptavidin-PE conjugate (Sigma) was added in a 1:4 molar ratio, and the tetrameric product was concentrated to 1 mg/ml. Analysis of cells for the expression of cell surface markers was performed using FACScan (BD) and CellQuest software (BD). Frozen CTLs were thawed and cultured in RPMI 1640 supplemented with 5% human serum. After 24 h of incubation, cell viability was assessed. For tetramer staining, 10^6 PBMCs or *in vitro*-cultured T cells were centrifuged at 300 × *g* for 5 min and resuspended in 50 μl of cold PBS. Tetramer and anti-CD8-Tricolor (Caltag Laboratories, Burlingame, CA) were added, and the cells incubated for another 30 min. The samples were washed twice with PBS before formaldehyde fixation. Double-color analysis was performed with tetramer-PE and anti-CD8-Tricolor.

Detection and Purification of Antigen-specific T Cells by Staining for Secreted IFN-γ with the Affinity Matrix Technology. Antigen-specific CTLs were detected and purified from T-cell lines using the IFN-γ secretion assay (Miltenyi Biotec GmbH) according to the manufacturer's instructions. This method has been described by Manz *et al.* (16) and Assenmacher *et al.* (27). Briefly, an antibody matrix is attached to the cell surface. Cells, stimulated with the specific antigen, secrete cytokines for a defined period of time, allowing the secreted cytokine to bind to the affinity matrix. The bound cytokine is then stained as an artificial surface molecule. To induce IFN-γ secretion in peptide-specific CTLs, T-cell lines were stimulated *in vitro* for 6 h with T2 cells pulsed with the appropriate peptide (30 μg/ml). Cells were then labeled with an affinity matrix for the secreted IFN-γ (catch reagent). After 45 min of incubation at 37°C, the secreted cytokine, bound to the catch reagent, was stained with a PE-conjugated IFN-γ-specific antibody (detection reagent). IFN-γ^+ T cells were then isolated by magnetic cell sorting using anti-PE MicroBeads.

RESULTS

Repetitive Stimulation with Peptide-pulsed DCs Is Optimal for Induction of Antigen-specific T Cells. We analyzed how many stimulation cycles with peptide-pulsed DCs would be necessary to generate optimal culture conditions for *in vitro* induction of antigen-specific T cells. Because the frequency of influenza-specific T lymphocytes in peripheral blood is much higher than the frequency of melanoma antigen-specific CTLs, we used FluM1 for stimulation. CD8-purified T cells from healthy donors were stimulated once a week with peptide-pulsed DCs; culture medium was changed twice a week. A total of one (day 0), two (days 0 and 7), or three (days 0, 7, and 14) stimulations with peptide-pulsed DCs were carried out. It became evident, as shown in Fig. 1, that three stimulation cycles with antigen-pulsed DCs provided the most efficient conditions for the generation of antigen-specific T cells. T cells exhibited a high cytotoxic activity against FluM1 peptide-pulsed T2 cells but did not lyse unlabeled T2 cells or T2 cells labeled with an irrelevant peptide (see Fig. 1*C*). In contrast, T cells stimulated for only 1 or 2 weeks with peptide-pulsed DCs revealed a nonspecific cytotoxic potential against unlabeled T2 cells (Fig. 1, *A* and *B*).

The same culture conditions were used for induction of Melan-A-specific CTLs. CD8-purified T cells were stimulated once a week using Melan-A_{26-35} decapeptide-pulsed DCs.

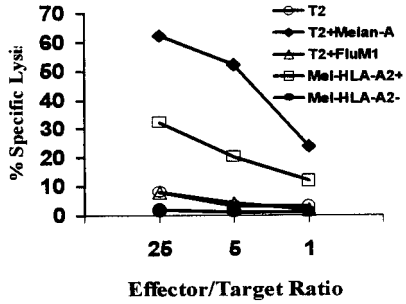

Fig. 2 Induction of Melan-A-specifc CTLs from PBMCs *in vitro* using DCs pulsed with Melan-A peptide. CD8-purified T cells from healthy donors were stimulated once a week with Melan-A-pulsed DCs. Seven days after the fourth stimulation (day 28), T cells were tested for their cytotoxic activity in a 4-h ^{51}Cr release assay against Melan-A-expressing HLA-A2$^+$ (□) or HLA-A2$^-$ (●) melanoma cells or against T2 cells in the absence (○) or presence of the relevant (*Melan-A*; ♦) or irrelevant (*FluM1*; △) peptide. Values represent triplicates at E:T ratios of 25:1, 5:1, and 1:1. The results shown are representative of one experiment.

Table 1 Induction and expansion of Melan-A-specific CTLs in 10 healthy donors

	Lysis of T2 cells[a]	
Donor	+Melan-A	+FluM1
1	65.8	6.4
2	22.3	6.7
3	46.1	22.9
4	60.0	8.5
5	15.0	8.3
6	40.6	10.7
7	50.1	1.8
8	36.7	6.0
9	52.0	4.3
10	54.5	3.8

[a] CD8-purified T cells, stimulated weekly with Melan-A-pulsed autologous DCs for 4 weeks, were tested for cytotoxic activity in a 4-h ^{51}Cr release assay at an E:T ratio of 25:1 against Melan-A or FluM1 peptide-pulsed T2 cells. Results expressed as the percentage of lysis.

Seven days after the fourth stimulation (day 28), CD8$^+$ T cells were tested for cytotoxic activity against different targets. As shown in Fig. 2, CD8$^+$ T cells demonstrated Melan-A peptide specificity by lysis of Melan-A-pulsed T2 cells but not by lysis of FluM1 peptide-pulsed or nonpulsed T2 cells. Melan-A-specific T cell lines also showed specific killing of the HLA-A2$^+$, Melan-A-expressing melanoma cells with no reactivity against HLA-A2$^-$ melanoma cells, indicating that Melan-A-specific CTLs recognize the endogenously processed and presented Melan-A antigen. Peptide titration revealed that a half-maximal lysis for Melan-A-specific CTLs was obtained at a peptide concentration of ~50 ng/ml (data not shown).

Induction and Expansion of Melan-A-specific CTLs from PBMCs *in Vitro* Using DCs Pulsed with Melan-A Peptide. CD8-purified T cells from 10 healthy donors were stimulated with Melan-A$_{26-35}$ decapeptide-pulsed autologous DCs once a week. A total of three to five stimulation cycles were conducted. On day 7 after each stimulation cycle, the number of CD8$^+$ T cells was analyzed. During the first 2 weeks of stimulation, the total number of CD8$^+$ T cells increased only moderately, whereas a significant increase was noted after the third and fourth round of stimulation. After 4 weeks of culture, we could obtain as many as 4×10^7 CD8$^+$ T cells from an initial number of 10^6 cells, corresponding to a mean expansion of 40-fold (data not shown). As shown in Table 1, functional analysis of CD8$^+$ T cells after 4 weeks of antigen-specific stimulation revealed a mean specific lysis of 38.9% (range, 15–65.8%) against Melan-A-pulsed T2 cells, whereas the mean cytotoxic activity against an irrelevant peptide was only 7.9% (range, 1.8–22.9%). T cells derived from PBMCs of melanoma patients showed similar results (data not shown).

The phenotype of the cultures was measured by flow cytometry (data not shown). At the beginning of the *in vitro* stimulation, the mean percentage of CD3$^+$/CD8$^+$ T cells after purification was 91%. By the end of the 4-week culture period with Melan-A-pulsed autologous DCs, all cultures contained a mean of 96% CD3$^+$/CD8$^+$ T cells. Melan-A-specific CD8$^+$ T cells showed the classical phenotype for CD8$^+$ effectors: CD4$^-$, CD16$^-$, CD56$^-$, CD45RO$^+$, CD45RA$^-$.

Maximal Expansion of Melan-A-specific CTLs *in Vitro* Using Antigen-independent Stimulation with Anti-CD3 and Anti-CD28 mAbs. Our goal was to define optimal culture conditions for obtaining large numbers of antigen-specific CTLs for an adoptive transfer in melanoma immunotherapy. It has been shown that antigen-specific T cells can be expanded by secondary anti-CD3/anti-CD28 mAb activation (15, 28, 29). After 3–4 weeks of weekly stimulation with peptide-pulsed DCs, Melan-A-specific CD8$^+$ T cells were further expanded with anti-CD3/anti-CD28 mAbs supplemented with IL-2 (100 IU/ml). Culture flasks (25 cm^2) were coated with anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (1 μg/ml). Medium and IL-2 (100 IU/ml) were replenished twice a week. A representative experiment is shown in Fig. 3, demonstrating significant expansion of CD8$^+$ T cells under these culture conditions. After 2 weeks of antigen-independent stimulation, we could obtain as many as 6×10^8 CD8$^+$ T cells from an initial number of 4×10^7 cells, corresponding to a mean expansion of 15-fold. CD8$^+$ T cells were tested before and 2 weeks after antigen-independent stimulation for their cytotoxic activity against HLA-A2$^+$ Melan-A-expressing melanoma cells and Melan-A-pulsed T2 cells. As shown in Fig. 4, expanded CD8$^+$ T cells exhibited the same specific cytotoxic activity against Melan-A-pulsed T2 cells before and after antigen-independent stimulation. In addition, Melan-A-specific T-cell lines exhibited specific killing of HLA-A2$^+$, Melan-A-expressing melanoma cells (Fig. 4). These results demonstrate that antigen-independent stimulation with anti-CD3/anti-CD28 mAbs together with IL-2 provides an efficient strategy for secondary activation and expansion of tumor-specific CD8$^+$ T cells that maintains their antigen-specific cytotoxic activity.

Detection and Purification of *in Vitro*-stimulated Melan-A-specific CTLs according to MHC-Peptide Tetrameric Complexes and IFN-γ Secretion. We next analyzed the frequency of CD8$^+$ T cells specific for the HLA-A*0201-binding melanoma-associated peptide Melan-A ELAGIGILTV, using MHC-peptide tetrameric complexes. T cells from three

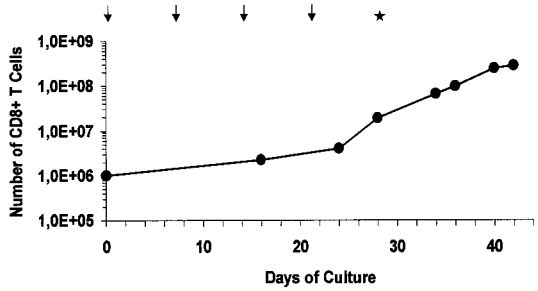

Fig. 3 Maximal expansion of antigen-specific CTLs after sequential stimulation based on initial antigen-specific activation with autologous peptide-pulsed DCs followed by secondary antigen-independent stimulation with anti-CD3/anti-CD28 mAbs. CD8-purified T cells were stimulated once a week with Melan-A-pulsed DCs (*arrows*). Seven days after the fourth stimulation (*day 28*), T cells were further expanded with anti-CD3/anti-CD28 mAbs coated on tissue flasks (⋆). Medium and IL-2 (100 IU/ml) were replenished twice a week. Results of one representative experiment show the cell number of $CD8^+$ T cells at different time points measured by trypan blue exclusion.

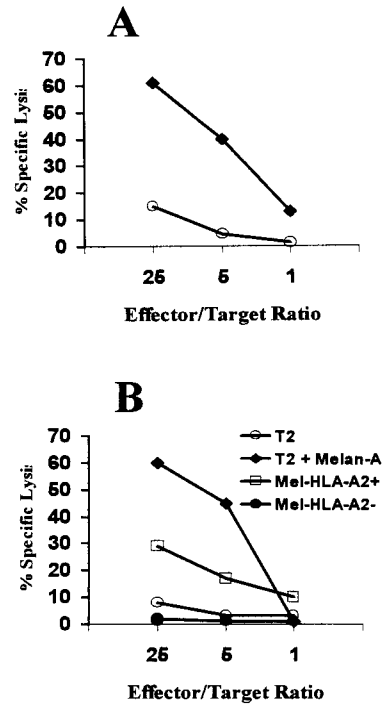

Fig. 4 Maintainence of antigen-specific activity of Melan-A-specific CTLs after antigen-independent stimulation. CD8-purified T cells were stimulated for 4 weeks with Melan-A-pulsed DCs followed by antigen-independent activation with anti-CD3/anti-CD28 mAbs coated on tissue flasks. Cytotoxic activity in a 4-h ^{51}Cr release assay against Melan-A-expressing HLA-$A2^+$ (□) or HLA-$A2^-$ (●) melanoma cells or against T2 cells in the absence (○) or presence of the relevant (*Melan-A*; ♦) or irrelevant (FluM1; not shown) peptide was measured on day 28 of antigen-specific stimulation (*A*) and after 2 additional weeks of antigen-independent stimulation (*B*). Values represent triplicates at E:T ratios of 25:1, 5:1, and 1:1.

selected donors, obtained either directly from PBMCs by Ficoll separation or after 5 weeks of *in vitro* culture, including 3 weeks of antigen-specific and 2 weeks of antigen-independent stimulation, were stained with the PE-conjugated tetrameric complexes and with an anti-CD8 antibody conjugated to Tricolor. The frequency of $CD8^+$/Melan-A^+ cells in unstimulated PBMCs varied between 0.01% and 0.48% (see Fig. 5*A*). After 5 weeks of *in vitro* stimulation, the number of Melan-A-specific $CD8^+$ T cells increased from 0.01% to 0.04–0.07% and from 0.48% to 3.42%, respectively (Fig. 5*B*).

The ability to directly detect and isolate antigen-specific T cells according to secreted cytokines allows their separation from the rest of the bulk cultures at an early phase of specific *in vitro* stimulation. To evaluate the feasibility of this technique, the frequency of $CD8^+$ IFN-γ-secreting T cells after stimulation with Melan-A-pulsed target cells was measured by two-color FACS analysis. Nonstimulated Melan-A-specific CTLs revealed no IFN-γ secretion (data not shown). The frequency of $CD8^+$/IFN-γ^+ cells that had been stimulated with Melan-A-pulsed DCs once a week for a total of 3 weeks and expanded with anti-CD3/anti-CD28 mAbs for another 2 weeks varied between 2.3% and 6.6%. Enrichment of IFN-γ^+ T cells was performed with one cycle of purification using anti-IFN-γ mAb-labeled immunomagnetic beads and subsequent antigen-specific restimulation for 1 week. As shown in Fig. 6, cells could be purified from 3.7% (Fig. 6*A*) to 51.8% (Fig. 6*B*) $CD8^+$/IFN-γ^+ T cells after one round of immunomagnetic separation. The T-cell fraction that did not bind to anti-IFN-γ mAb-labeled beads (negative fraction) and consisted of 1.24% $CD8^+$/IFN-γ^+ cells (Fig. 6*C*). Functional characterization of these three different T-cell lines demonstrated that $CD8^+$/IFN-γ^+-purified T cells exhibited a high level of specific cytotoxicity against Melan-A-pulsed T2 cells and to a lesser extent against HLA-$A2^+$ Melan-A-expressing melanoma cells (Fig. 6*B*). In contrast, the negative fraction displayed lower levels of cytotoxic activity against Melan-A-pulsed T2 cells but higher levels against non-pulsed T2 cells (Fig. 6*C*). These results demonstrate that T cells, purified by magnetic separation according to secreted IFN-γ, exhibit a specific CTL response against Melan-A-pulsed and Melan-A-expressing target cells. In addition, similar to the unsorted T-cell population, purified Melan-A-specific CTLs can be further expanded *in vitro* (data not shown).

DISCUSSION

The adoptive transfer of *in vitro*-induced and expanded tumor-specific CTLs provides a promising approach to the immunotherapy of cancer. Previous attempts to determine the impact of adoptive transfer in tumor immunotherapy have been limited by the difficulty of isolating T cells of known antigen specificity. Melanoma and renal cell carcinoma were the first tumors in humans to be treated with adoptive immunotherapy (30). Infusions of polyclonal T-cell populations isolated from the tumor (tumor-infiltrating lymphocytes) and nonspecifically expanded *in vitro* with high concentrations of IL-2 suggested the therapeutic potential of this approach in cancer patients (30). The recent characterization of melanoma-associated antigens recognized by human CTLs has opened new possibilities for the adoptive transfer of T cells in tumor immunotherapy. Adoptive transfer of tumor-infiltrating lymphocyte populations containing CTL specificities for melanocyte differentiation antigens such

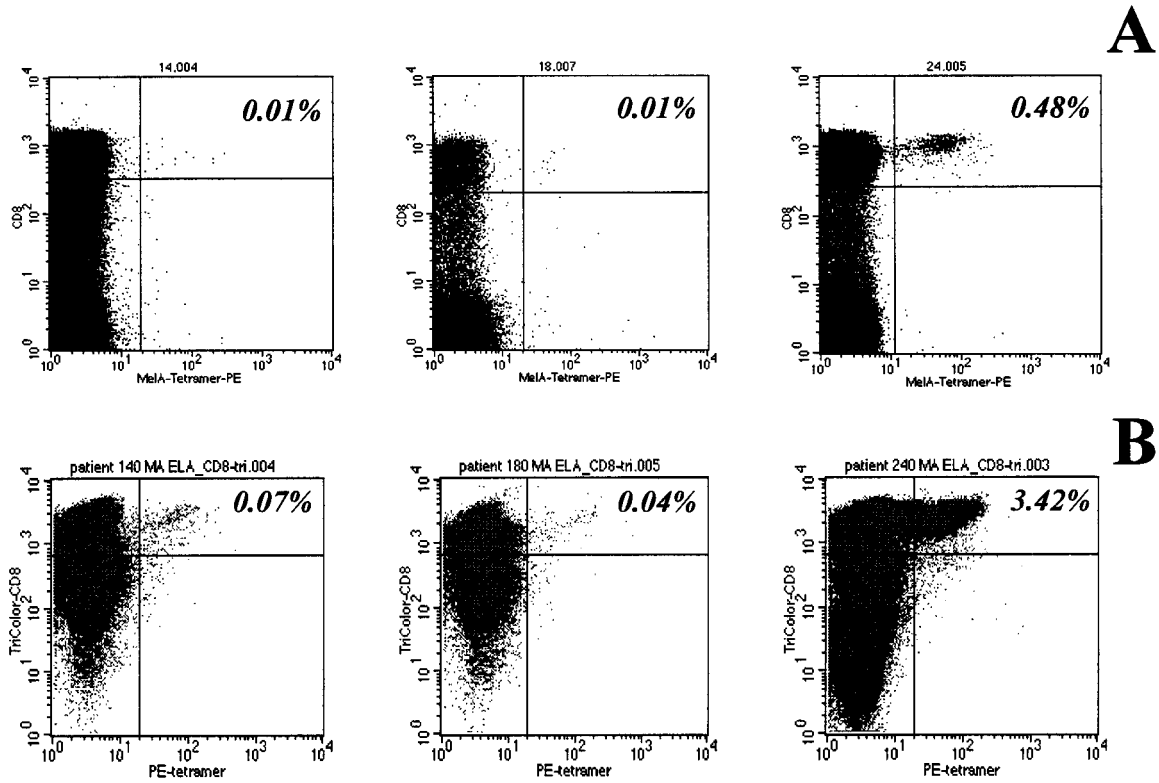

Fig. 5 Frequency of tetramer-reactive Melan-A+/CD8$^+$ T cells from three selected donors before and after peptide-specific *in vitro* stimulation. CD8$^+$ T cells from three healthy donors, obtained either directly from PBMCs by Ficoll separation (*A*) or after 5 weeks of *in vitro* culture including 3 weeks of antigen-specific and 2 weeks of antigen-independent stimulation (*B*), were stained with Tricolor-conjugated anti-CD8 and PE-conjugated Melan-A-ELAGIGILTV HLA-A*0201 tetrameric complexes. Percentages of CD8$^+$/Melan-A$^+$ T cells are shown in the *upper right quadrants* of the dot blots.

as tyrosinase resulted in tumor regression in melanoma patients (31).

The ability to induce tumor-reactive T cells *ex vivo* using peptide-pulsed DCs as APCs suggests an important role for DCs in adoptive T-cell immunotherapies (12). In the present study, we demonstrate that repetitive stimulation with Melan-A-pulsed autologous DCs is an essential component for *in vitro* induction of Melan-A-specific CD8$^+$ CTLs. A requirement for achieving effective adoptive therapy is the ability to obtain large numbers of tumor-specific CTLs from circulating CTL precursors after short-term *in vitro* stimulation. The present study indicates that antigen-specific CTLs can expand up to 600-fold after antigen-specific and subsequent nonspecific stimulation. Our results also demonstrate that CD8$^+$ peptide-specific CTLs, induced by *in vitro* stimulation with antigen-pulsed DCs, can be expanded by coculture with immobilized mAbs against the CD3 and CD28 molecules without losing their specificity. Anti-CD3 plus anti-CD28 mAbs have been used by other groups to expand T-cell lines and T-cell clones (15, 28, 29). Riddell and Greenberg (28) demonstrated long-term culture (3 months) of CMV-specific CD8$^+$ T-cell clones that maintained their antigen-specific activity.

Replicative senescence may have important implications for adoptive immunotherapy with antigen-specific T cells. Our results demonstrate that 4–5 weeks are required for induction and expansion of antigen-specific CTL lines to reach sufficient numbers of CTLs suitable for adoptive transfer. In contrast, isolation and expansion of peptide-specific CTL clones, as described by Yee *et al.* (14), requires more than 2 months of *in vitro* culture.

The induction and expansion of T lymphocytes recognizing Melan-A within healthy donors, as demonstrated by our study and by those of other investigators (12), indicates that the T-cell repertoire is capable of generating efficient immune responses against these differentiation antigens. The frequency of circulating Melan-A-specific CTLs measured by peptide-MHC tetramer staining varied from 0.01% to 0.48% (Fig. 5*A*). These results are comparable to the frequencies of circulating CD8$^+$ T cells detected in melanoma patients (13). One limitation of our approach concerns the fact that induction and expansion of T cells in melanoma patients may fail because of defects in antigen presentation or in functional responsiveness of the T cells. Our strategy, which allows the rapid preparation of antigen-specific T cells from healthy donors, may also be used for the generation of donor lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens after allogeneic bone marrow transplantation (32).

The isolation and purification of Melan-A-specific CTLs according to secreted IFN-γ allowed us to separate them from the rest of the bulk cultures at an early phase of specific *in vitro*

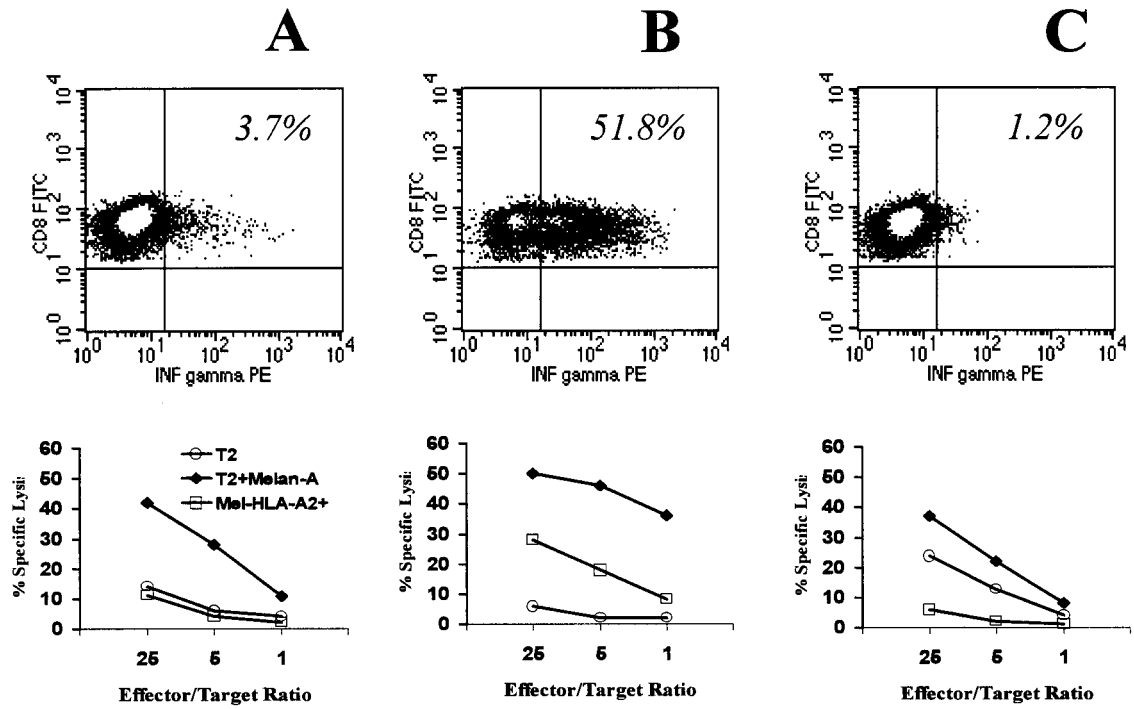

Fig. 6 Detection and purification of *in vitro*-stimulated Melan-A-specific CTLs according to IFN-γ production. CD8-purified T cells that had been stimulated with Melan-A-pulsed DCs once a week for a total of 3 weeks and expanded with anti-CD3/anti-CD28 mAbs for another 2 weeks were restimulated for 6 h with Melan-A-pulsed T2 cells. Cells were then labeled with the affinity matrix specific for IFN-γ, incubated for 45 min at 37°C, washed, and stained for captured IFN-γ and CD8 (*A, top panel*). Enrichment of IFN-γ T cells was performed with an anti-IFN-γ mAb using immunomagnetic beads. Immune fluorescence analysis demonstrated that T cells could be purified from 3.7% (before purification; *A, top panel*) to 51.8% (after purification; *B, top panel*) $CD8^+$/IFN-γ^+ T cells. The T-cell fraction that did not bind to anti-IFN-γ mAb-labeled beads (negative fraction; *C, top panel*) consisted of 1.2% $CD8^+$/IFN-γ^+ T cells. *A–C, bottom panels*, cytotoxic activities of the three different T-cell fractions were measured with a 4 h ^{51}Cr release assay against Melan-A-expressing HLA-$A2^+$ melanoma cells (□) or against T2 cells in the absence (○) or presence (♦) of the Melan-A peptide. Values represent triplicates at E:T ratios of 25:1, 5:1, and 1:1.

stimulation. These findings confirm and extend observations of other investigators who succeeded in generating and purifying peptide-specific CTLs (13, 14). Those investigators have recently demonstrated that Melan-A-specific CTLs can be generated *in vitro* from the patient's peripheral blood lymphocytes by stimulation with autologous peptide-pulsed APCs and then isolated using peptide-MHC tetramers. Dunbar *et al.* (15) have obtained rapid cloning of melanoma-specific CTLs from different tissues by tetramer-guided sorting. CTLs were not restimulated with peptides, but with solid-phase mitogenic signals. These results demonstrate that peptide-specific CTLs can be purified before further expansion. A novel approach used in our study consists of cytokine-guided purification of a specific lymphocyte subpopulation. It has been demonstrated by Manz *et al.* (16) and Assenmacher *et al.* (27) that T cells activated with an antigen of interest can be analyzed and sorted according to expression of cytokines like IFN-γ or IL-2, using the cellular affinity matrix technology. It has been demonstrated that expression of surface IFN-γ is precisely linked to secretion of IFN-γ (27). Our results demonstrate that surface IFN-γ^+ cells purified from polyclonal Melan-A-specific $CD8^+$ T cells exhibit a higher cytotoxic activity against Melan-A-pulsed and Melan-A-expressing target cells than the IFN-γ-negative fraction or unseparated T cells. Whether this may be due to up-regulation of MHC molecules on target cells induced by IFN-γ is not yet clear. An additional advantage of this strategy is its applicability to other tumors, such as renal cell carcinoma, where tumor antigens are mostly unknown. There are some limitations to the adoptive transfer of antigen-specific CTLs. One important limiting factor of this method is the lack of $CD4^+$ T-cell help that may be necessary for an efficient antitumor immune response induced by $CD8^+$ T cells (33). The addition of cytokines such as IL-2 and IL-12, however, might be suitable to overcome this problem (34). An alternative strategy implies the co-administration of melanoma-antigen-specific $CD4^+$ T-cell lines (35, 36). Again, the novel strategy of cytokine-guided isolation and purification of specific $CD4^+$ helper T-cell populations would be helpful for adoptive transfer regimens. A second limitation of our strategy relates to the generation of monospecific CTLs. Tumor escape mechanisms include immunoselection of antigen-loss variants. Thus, a therapeutic strategy should optimally be based on adoptive transfer of CTL lines specific for a broader range of antigens.

In summary, we have shown that a sequential approach based on initial antigen-specific activation with autologous peptide-pulsed DCs, followed by secondary antigen-independent stimulation with anti-CD3/anti-CD28 mAbs, represents an efficient tool for generation and expansion of antigen-specific CTLs

that can be used for adoptive transfer in tumor immunotherapy. In addition, cytokine-driven purification is a new method for selection of antigen-specific CTLs after a short period of specific *in vitro* stimulation. Further studies are needed to compare the efficiency of this approach with other techniques such as tetramer-guided selection (13–15) or T-cell receptor β-chain variable region-driven selection (37).

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