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Generation of Antimelanoma Cytotoxic T Lymphocytes from Healthy Donors after Presentation of Melanoma-associated Antigen-derived Epitopes by Dendritic Cells *in Vitro*¹

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ABSTRACT

MHC class I-restricted CTLs specific for antigens expressed by malignant cells are an important component of immune responses against human cancer. Recently, in melanoma a number of melanocyte differentiation antigens have been identified as potential tumor rejection antigens. In the present study, we show that by applying peptide-loaded dendritic cells, induced by granulocyte-macrophage colony-stimulating factor and interleukin 4 from peripheral blood monocytes of healthy donors, we were able to elicit melanoma-associated antigen-specific CTL *in vitro*. We demonstrate the induction of CTLs directed against HLA-A2.1 presented epitopes derived from tyrosinase, gp100, and Melan A/MART-1. Apart from lysis of peptide-loaded target cells, these CTLs displayed reactivity with HLA-A2.1⁺ melanoma tumor cell lines and cultured normal melanocytes endogenously expressing the target antigen. These data indicate that these CTLs recognize naturally processed and presented epitopes and that precursor CTLs against melanocyte differentiation antigens are present in healthy individuals. The ability to generate tumor-specific CTLs *in vitro*, using granulocyte-macrophage colony-stimulating factor/interleukin 4-induced dendritic cells, illustrates the potential use of this type of antigen-presenting cells for vaccination protocols in human cancer.

INTRODUCTION

CTLs that recognize melanoma tumor cells in a T-cell receptor-mediated and MHC-restricted manner have been isolated from tumor-bearing patients (reviewed in Ref. 1). Using a genetic approach, Boon and colleagues (2-4) have succeeded in identifying a number of antigens recognized by antimelanoma CTLs. Apart from the tumor-specific MAGE-1 and -3 antigens (2, 4), they also identified the melanocyte lineage-specific tyrosinase protein as a target for melanoma-specific CTLs (3). Similarly, we identified the gp100 melanocyte-specific protein as a target antigen for melanoma tumor-infiltrating lymphocytes (5). Recently, two other melanocyte differentiation antigens, Melan A/MART-1 and gp75, were identified as target antigens for antimelanoma CTLs (6-8).

Cox *et al.* (9) reported that a naturally processed gp100 peptide (amino acids 280-288), identified by tandem mass spectroscopy, could direct target cell lysis by HLA-A2.1-restricted antimelanoma CTL lines (9). We demonstrated that the gp100-reactive TIL 1200 line is reactive with two other HLA-A2.1-presented gp100-derived epitopes (amino acids 154-162 and 457-466; Refs. 10 and 11). Recently, two novel epitopes derived from gp100 were shown to direct target cell lysis by HLA-A2.1-restricted tumor-infiltrating lymphocytes (12). Furthermore, a number of peptides derived from the other melanocyte differentiation antigens, tyrosinase and Melan

A/MART-1, have been identified as epitopes for HLA-A2.1-restricted antimelanoma CTLs (13, 14).

The array of epitopes derived from antigens commonly expressed in melanoma tumors now provide a rationale for initiating multitarget tumor vaccination studies. However, successful activation of CD8⁺ cytolytic effector lymphocytes with antitumor reactivity is critically dependent on adequate presentation of the epitopes involved. DCs,³ expressing high levels of MHC class I and II and costimulatory molecules, have been shown to be intimately involved in the generation of tumor-specific cellular immunity (15). Recently, a method has been developed to generate DCs from peripheral blood by culturing progenitor cells in the presence of GM-CSF and IL-4 (16). Thus, obtained DCs proved to be very potent stimulator cells as was demonstrated by their capacity to induce allogenic and autologous mixed lymphocyte reactions (16). Furthermore, these cells are extremely well capable of processing and presenting antigen (17). Taken together, these GM-CSF/IL-4-induced DCs are attractive candidates for the induction of antitumor immune responses.

In the present study, we investigated whether, within the T-cell repertoire of healthy donors, precursor T lymphocytes are present with the capability of recognizing HLA-A2.1-presented peptides derived from the melanocyte differentiation antigens gp100, tyrosinase, and Melan A/MART-1. We demonstrate the *in vitro* induction of tumor-reactive CTLs using DCs generated from peripheral blood monocytes after culture in the presence of GM-CSF and IL-4, as APCs. These results illustrate the potential use of this type of APC for vaccination protocols of human cancer.

MATERIALS AND METHODS

Cell Culture. HLA-A2.1⁺ melanoma lines BLM and BLM gp100 H2.3 were cultured as described previously (5). Melanoma lines Mel 397 (HLA-A2.1⁻) and Mel 624 (HLA-A2.1⁺) were a kind gift from Dr. Y. Kawakami (National Cancer Institute, NIH, Bethesda, MD) and were grown as was reported previously (18). BLM and Mel 624 cells were screened for tyrosinase and Melan A/MART-1 mRNA expression by PCR using oligonucleotides 5'-TTGGCAGATTGTCTGTAGCC-3'/5'-AGGCATTGTGCATGCTGCTT-3' and 5'-AGACGAAATGGATACAGAGC-3'/5'-CATGATTAGTACTGCTAGCG-3', as primer combinations respectively (the tyrosinase-specific PCR primer combination was a kind gift from Dr. N. Brouwenstein, University Hospital Leiden, Department of Clinical Oncology, Leiden, the Netherlands). COS-7 cells were grown in DMEM (GIBCO, Paisley, Scotland, United Kingdom) supplemented with 7.5% FCS (BioWhittaker, Verviers, Belgium). TAP-deficient (T × B) cell hybrid T2 cells (19) were maintained in Iscove's medium (GIBCO) + 7.5% FCS. WEHI 164 clone 13 cells (20) were kindly provided by Dr. P. Coulie (Ludwig Institute for Cancer Research, Brussels, Belgium) and were grown in RPMI 1640 supplemented with 7.5% FCS, L-glutamine (216 mg/liter), L-asparagine (36 mg/liter), and L-arginine (116 mg/liter). Isolation of normal melanocytes from foreskin was performed according to the method of Eisinger and Marko (21), with modifications as

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³ The abbreviations used are: DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor; APC, antigen-presenting cell.

described previously (22). Melanocytes from early passages were used in TNF release assays.

HLA-A2.1⁺ Lymphocytes. Healthy Caucasian volunteers were phenotyped HLA-A2.1⁺ by flow cytometry using monoclonal antibodies BB7.2 (23) and MA2.1 (24). After obtaining signed consent, the donors underwent leukapheresis, and PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. The cells were cryopreserved in aliquots of 4×10^7 PBMCs.

Peptides. For induction of CTLs and chromium release assays, peptides were synthesized with a free COOH-terminal either by f-moc peptide chemistry using an ABIMED Multiple Synthesizer or by t-boc chemistry on a Biosearch SAM2 peptide synthesizer. All peptides were >90% pure as indicated by analytical HPLC. Peptides were dissolved in DMSO and stored at -20°C .

In Vitro CTL Induction. Using thawed PBMCs, DCs were generated as described by Romani *et al.* (16). PBMCs were resuspended at 4×10^6 cells/ml in Iscove's medium containing 5% FCS and were distributed in 6-well tissue culture plates (Costar, Badhoevedorp, the Netherlands) at 3 ml/well. After 2 h of incubation at 37°C , the nonadherent cells were washed away, and 3 ml Iscove's medium containing 5% FCS supplemented with 800 units/ml GM-CSF (Schering-Plough, Amstelveen, the Netherlands) and 500 units/ml IL-4 (Schering-Plough) were added to the wells. After 5 days of incubation, IL-1 α (Hoffman LaRoche Inc., Nutley, NJ) and IFN- γ (Boehringer Ingelheim, Alkmaar, the Netherlands) were added to the medium at final concentrations of 50 LAF units/ml and 150 units/ml, respectively, and the cells were transferred to an incubator at 30°C . One day later, the APCs were loaded with peptide according to the procedure described by Celis *et al.* (25), with slight modifications. The DCs were collected and resuspended at 5×10^6 cells/ml in Iscove's medium without serum containing 40 $\mu\text{g/ml}$ peptide and 3 $\mu\text{g/ml}$ human $\beta 2$ -microglobulin (Sigma, St. Louis, MO) and were incubated at 30°C for 4 h. Subsequently, the peptide-loaded APCs were irradiated at 5000 rad, centrifuged, and resuspended in Iscove's medium containing 5% pooled human AB⁺ serum. Autologous CD8⁺-enriched responder T-lymphocytes were prepared by adhering thawed PBMCs for 2 h and by subsequent depletion of the nonadherent fraction of CD4⁺ T cells using the anti-CD4 monoclonal antibody RIV-7 (26) and sheep antimouse IgG-coated magnetic beads (Dynal, Oslo, Norway). The resulting population consisted of approximately 60% CD8⁺ T cells and 10% remaining CD4⁺ T cells. At the onset of stimulation 2×10^5 peptide-loaded DCs and 2×10^6 responder cells/well were cocultured in a 24-well tissue culture plate (Costar) in 2 ml Iscove's medium containing 5% human serum and 5 ng/ml IL-7 (Genzyme, Cambridge, MA).

On day 10 and weekly thereafter, the responder populations were restimulated. Irradiated PBMCs (4×10^6 /well) were incubated for 2 h in a 24-well plate, nonadherent cells were removed, and 0.5 ml serum free Iscove's medium was added per well containing 20 $\mu\text{g/ml}$ peptide and 2 $\mu\text{g/ml}$ $\beta 2$ -microglobulin. After 2 h of incubation, the peptide was removed and 10^6 responder cells were added to the wells in 1 ml medium. One day later, 1 ml medium was added containing IL-2 (Perkin Elmer/Cetus Corp., Emeryville, CA) and IL-7 at final concentrations of 10 units/ml and 5 ng/ml, respectively. Responder populations were tested for their specificity after at least four rounds of restimulation.

Transfection of COS-7 Cells. Transfection was performed using the DEAE-dextran-chloroquine method (27). Briefly, 9×10^5 semiconfluent COS-7 cells were transfected with 5 μg pBJ1HLA-A2.1neo (11) alone or in combination with 5 μg pBJ1gp100neo (5). After 48 h of transfection, COS-7 cells were used as stimulator cells in TNF release experiments.

Chromium Release Assay. The melanoma tumor cells used as targets were incubated for 48 h with 50 units/ml IFN- γ . Chromium release assays were performed as described previously (5). Briefly, 10^6 target cells were incubated with 100 μCi Na₂⁵¹CrO₄ (Amersham, Bucks, United Kingdom) for 1 h. Various amounts of effector cells were then added to 2×10^3 target cells in triplicate wells of U-bottomed microtiter plates (Costar) in a final volume of 150 μl . In peptide recognition assays, T2 target cells were preincubated with various concentrations of peptide for 90 min at 37°C in a volume of 100 μl prior to the addition of effector cells. After 5 h of incubation, a portion of the supernatant was harvested, and its radioactive content was measured.

TNF Release Assay. CTL responder cells (3×10^3) were incubated with either 3×10^4 melanoma tumor cells, 4×10^4 cultured normal melanocytes, or 2×10^4 transiently transfected COS-7 cells in 100 μl Iscove's medium containing 5% human serum and 5 ng/ml IL-7. After 24 h, 50 μl of the

supernatant were harvested, and its TNF content was measured by testing its cytolytic effect on WEHI 164 clone 13 cells (20) using a colorimetric assay, as described previously (28).

RESULTS

Induction of CTLs Recognizing Melanocyte Differentiation Antigen-derived Epitopes. To assess whether within the T-cell repertoire of healthy donors precursor T lymphocytes are present with the capability of recognizing melanocyte differentiation antigen-derived epitopes, we initiated *in vitro* cultures of peptide-loaded stimulator cells along with autologous responder T lymphocytes. As stimulator cells we used highly potent DCs. HLA-A2.1⁺ DCs were generated using GM-CSF and IL-4 according to the procedure described by Romani *et al.* (16). These cells were loaded with 40 $\mu\text{g/ml}$ peptide, irradiated, and added to CD8-enriched T cells. The CTL cultures were initiated in the presence of IL-7. As peptides we used HLA-A2.1-presented epitopes derived from the melanocyte differentiation antigens gp100 [amino acids 280–288, YLEPGPVTA (9)], tyrosinase [amino acids 369–377, YMNGTMSQV (13)], and Melan A/MART-1 [amino acids 27–35, AAGIGILTV (14)].

At day 10, the responder populations were restimulated with irradiated adherent blood mononuclear cells pulsed with the appropriate peptide. After 24 h, IL-7 and IL-2 were added to the cultures. Prior to the second restimulation the responder populations were depleted for contaminating CD4⁺ T lymphocytes (30–50%). The resulting cultures consisted of >90% CD3⁺/CD8⁺ T lymphocytes (data not shown).

After two more rounds of restimulation, responder cells were tested for cytotoxic activity (Fig. 1). All three bulk CTL populations efficiently lysed HLA-A2.1⁺ T2 target cells incubated with the relevant peptides, whereas only low background lysis was observed in the presence of an irrelevant peptide. CTLs directed against the gp100 280–288 epitope were particularly efficient in lysing target cells loaded with the YLEPGPVTA peptide (Fig. 2A). Peptide titration showed that half-maximal lysis for this CTL culture was obtained at a gp100 280–288 peptide concentration of approximately 500 pM (Fig. 2B). These results demonstrate that peptide-specific CTL bulk cultures could be obtained with the described induction protocol.

Peptide-induced CTLs Display Reactivity with Melanocyte Differentiation Antigen-expressing Cells. To address the question whether the CTL responder populations could also recognize endogenously processed and presented antigen, we performed chromium

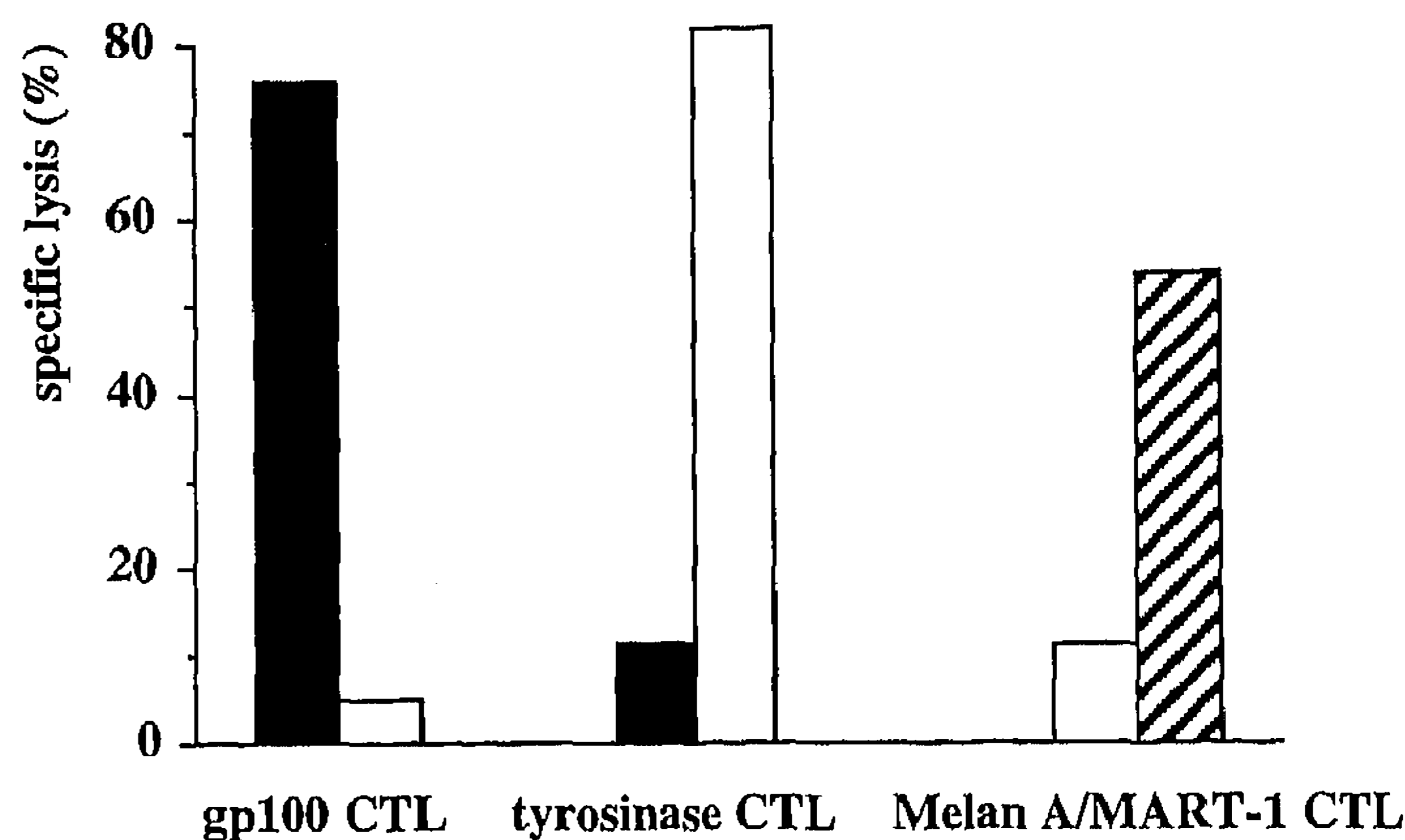


Fig. 1. Peptide-specific reactivity of *in vitro*-induced melanocyte differentiation antigen-specific CTL cultures. Chromium-labeled HLA-A2.1⁺ T2 target cells were preincubated for 90 min with 1 μM of the indicated peptides. The different CTL cultures were added at an E:T ratio of 40:1. After 5 h of incubation, chromium release was measured. ■, T2 + YLEPGPVTA (gp100 280–288); □, T2 + YMNGTMSQV (tyrosinase 369–377); ▨, T2 + AAGIGILTV (Melan A/MART-1 27–35).

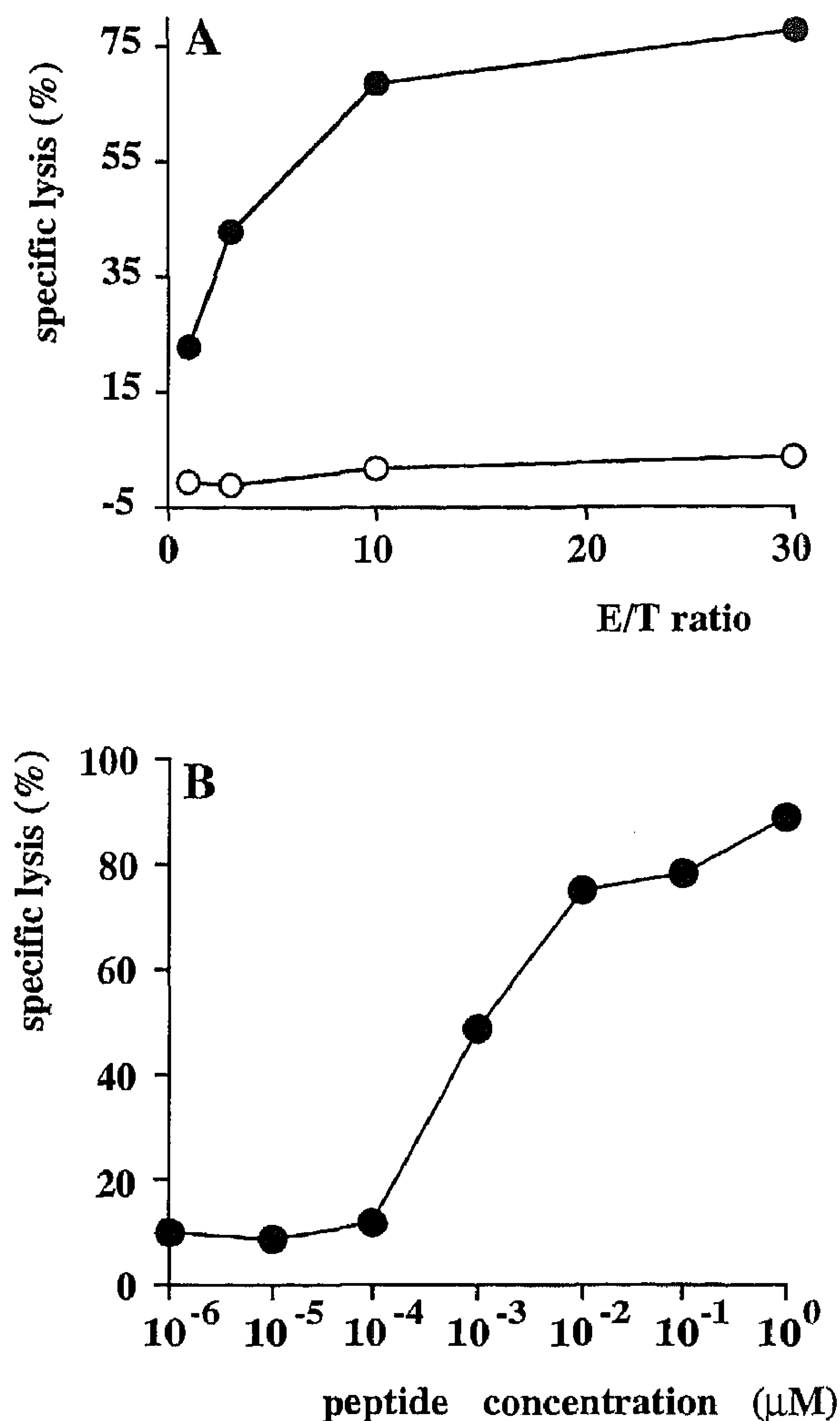


Fig. 2. Bulk anti-gp100 CTLs efficiently recognize T2 cells loaded with peptide YLEPGPVTA. *A*, chromium-labeled T2 target cells were preincubated with 1 μ M peptide. The anti-gp100 280-288 CTL culture was then added at different E:T ratios. After 5 h of incubation, chromium release was measured. Peptides: ●, gp100 280-288 YLEPGPVTA; ○, tyrosinase 369-377 YMNGTMSQV. *B*, chromium-labeled T2 target cells were preincubated with various concentrations of gp100 280-288 YLEPGPVTA peptide. The anti-gp100 280-288 CTL culture was then added at an E:T ratio of 15:1. After 5 h of incubation, chromium release was measured.

release experiments now using HLA-A2.1⁺ melanoma cell lines BLM and Mel 624 as targets. BLM cells have lost expression of the gp100 antigen, both at the protein and at the mRNA level (29). Furthermore, they lack expression of both tyrosinase and Melan A/MART-1 as was assessed by PCR (data not shown). In contrast, Mel 624 cells express all three antigens. As shown in Fig. 3A, all peptide-induced CTL cultures lysed the antigen-expressing Mel 624 cells, whereas no lysis was observed against antigen-negative BLM cells. These data show that the three different CTL cultures induced using peptide-loaded DCs, all recognized native epitopes derived from gp100, tyrosinase, and Melan A/MART-1 endogenously processed and presented by HLA-A2.1⁺ Mel 624 cells. The finding that gp100-expressing BLM transfectants were lysed by the anti-gp100 280-288 CTL culture confirmed this notion (Fig. 3B), and demonstrated that transfected BLM cells are able to process and present antigenic peptides.

In addition, we assayed TNF release by the anti-gp100 CTLs to demonstrate engagement of gp100-specific T-cell receptors. Fig. 4 clearly demonstrates that gp100 280-288-specific CTLs are only triggered to secrete TNF by HLA-A2.1⁺ melanoma tumor cells that express the gp100 antigen. These results confirm the data we obtained with the chromium release assays. To demonstrate that the observed

TNF production is HLA-A2.1 restricted and gp100 specific, we co-transfected COS-7 cells with the HLA-A2.1 and gp100-encoding cDNAs and used these cells to stimulate the anti-gp100 CTLs (Fig. 5). These double transfectants stimulated the bulk anti-gp100 CTLs to produce a significant level of TNF, while COS-7 cells transfected without DNA or with the HLA-A2.1-encoding plasmid alone failed to stimulate the CTLs. Therefore, we conclude that the anti-gp100 CTLs recognize the gp100 280-288 epitope endogenously presented in HLA-A2.1.

Since gp100 is expressed in all cells of the melanocytic lineage, one would predict that normal melanocytes are also recognized by the gp100 280-288-specific CTLs, as was previously shown for patient-derived antimelanoma CTLs (5, 30). To investigate this, short-term cultures of HLA-A2.1⁺ and HLA-A2.1⁻ normal melanocytes were

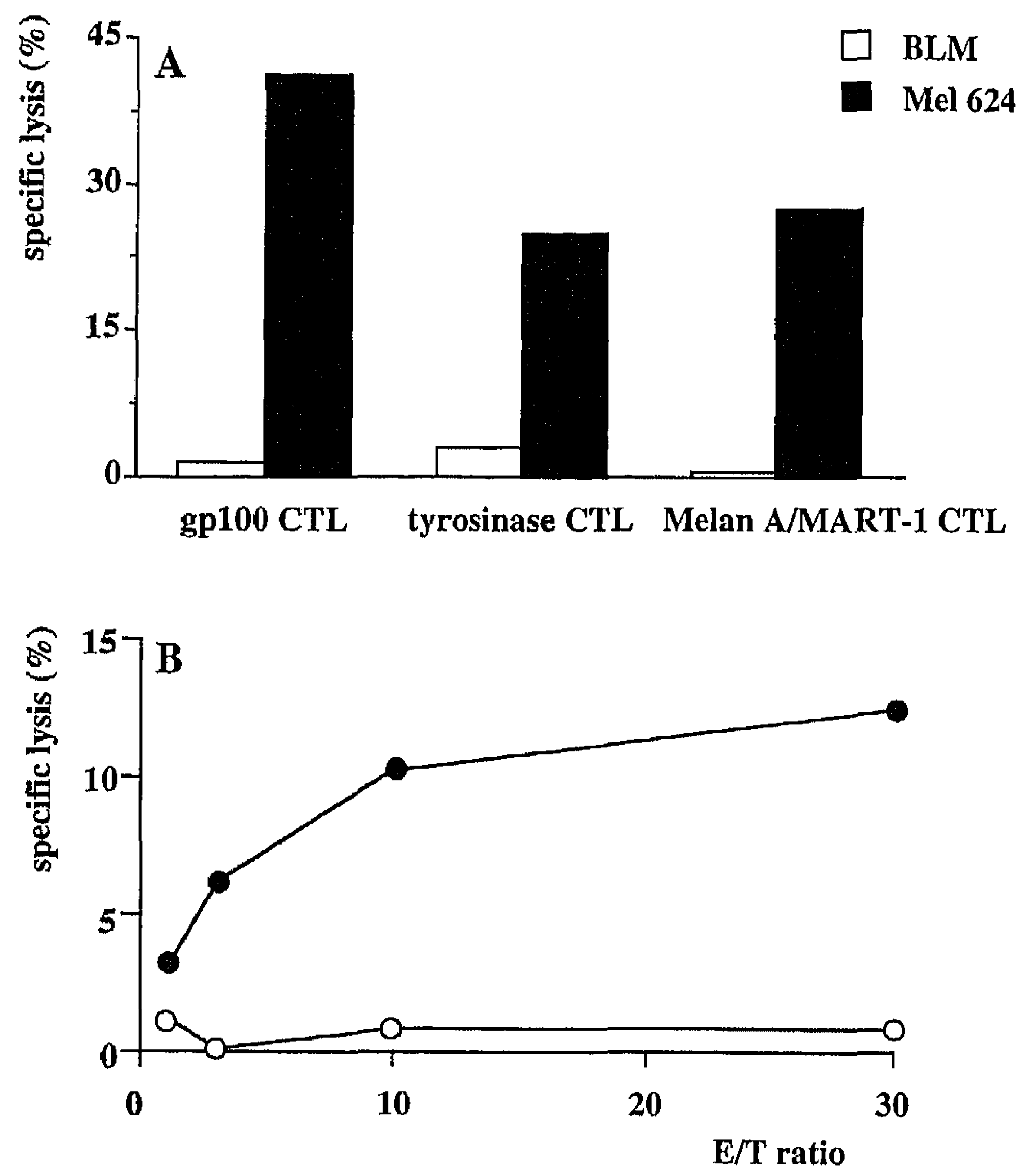


Fig. 3. Peptide-induced melanocyte differentiation antigen-specific CTL cultures specifically lyse antigen-positive melanoma cells. *A*, chromium-labeled HLA-A2.1⁺ BLM and Mel 624 melanoma cells were used as target cells. BLM cells lack expression of gp100, tyrosinase, and Melan A/MART-1. The different CTL cultures were added at an E:T ratio of 40:1. After 5 h of incubation, chromium release was measured. *B*, the anti-gp100 CTL culture was added at different E:T ratios to chromium-labeled BLM (○) or BLM gp100 H2.3 (●) target cells. After 5 h of incubation, chromium release was measured.

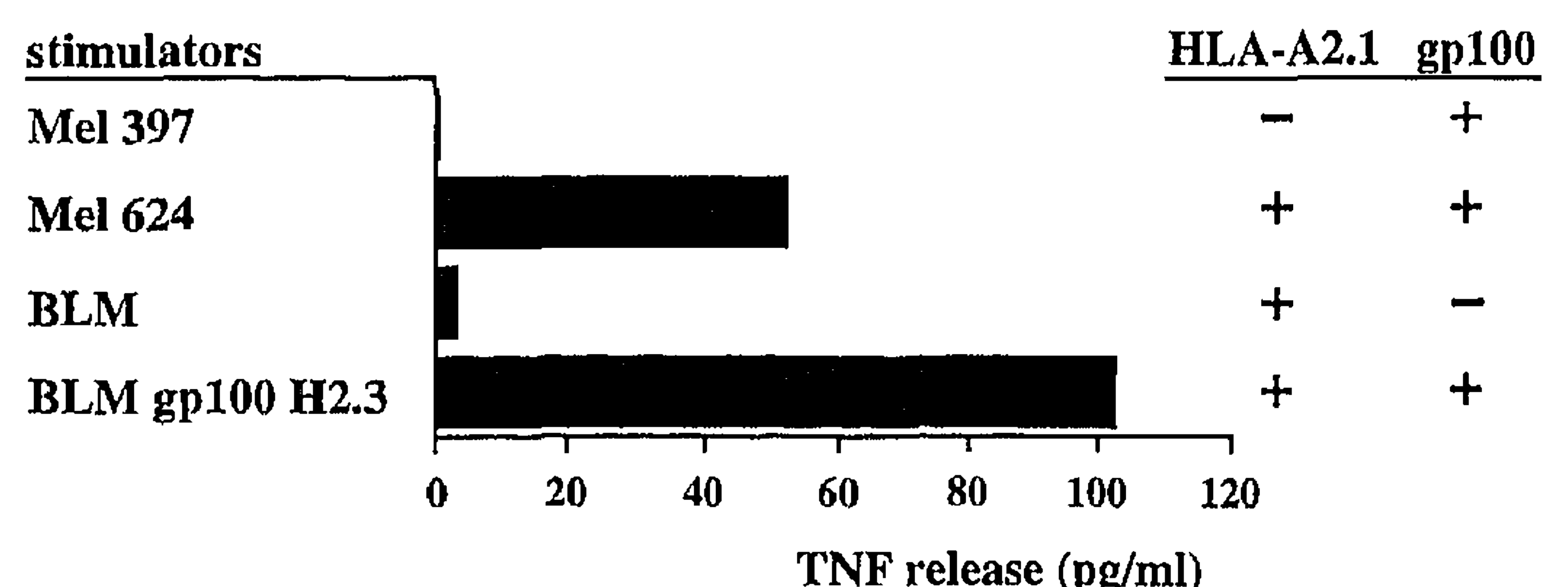


Fig. 4. Recognition of HLA-A2.1⁺ gp100⁺ melanoma cells by anti-gp100 280-288 CTLs. The anti-gp100 CTL culture was cocultured in combination with different melanoma tumor cells. After 24 h of incubation, a portion of the supernatant was harvested, and its TNF content was measured in a bioassay using WEHI 164 clone 13 cells.

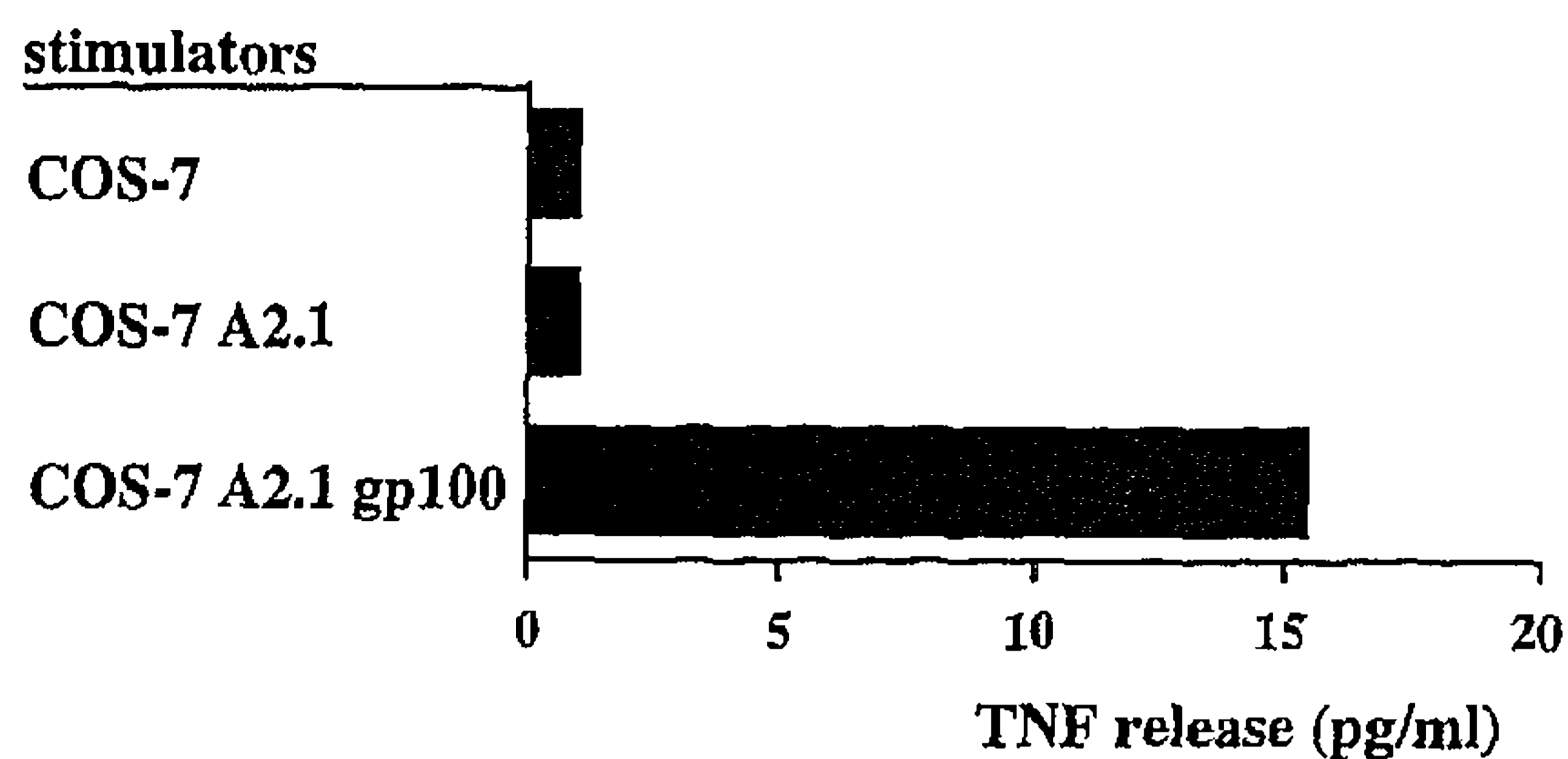


Fig. 5. Recognition of COS-7 cells transfected with HLA-A2.1 and gp100 cDNA by anti-gp100 CTLs. Bulk anti-gp100 CTLs were added to the transfectants, and after 24 h of incubation, a portion of the supernatant was harvested, and its TNF content was measured in a bioassay using WEHI 164 clone 13 cells. As controls, COS-7 cells transfected without DNA or with HLA-A2.1 cDNA alone were tested.

tested for recognition by the anti-gp100 bulk CTL culture. Melanocytes used in this study all expressed the gp100 antigen to a similar extent (data not shown). As shown in Fig. 6, HLA-A2.1⁺ melanocytes efficiently stimulated the anti-gp100 CTL, whereas HLA-A2.1⁻ melanocytes failed to do so. Together, these results demonstrate that peptide-induced *in vitro* bulk CTL cultures recognize epitopes endogenously processed and presented by both antigen-positive HLA-A2.1⁺ melanoma tumor cells and transfectants as well as by short-term cultured normal melanocytes.

DISCUSSION

In the present report, we demonstrate that HLA-A2.1-restricted CD8⁺ CTLs specific for three distinct melanocyte differentiation antigens can be induced from peripheral blood lymphocytes of healthy individuals. These CTLs were generated *in vitro* using peptide-pulsed dendritic cells induced from adherent PBMCs using GM-CSF and IL-4. The obtained bulk CTL populations directed against gp100 (YLEPGPVTA), tyrosinase (YMNGTMSQV), and Melan A/MART-1 (AAGIGILTV) displayed reactivity with HLA-A2.1⁺ melanoma tumor cells expressing these three antigens. These findings confirm and extend observations of other investigators who succeeded in generating peptide-specific CTLs capable of recognizing endogenously processed antigen using autologous B lymphoblast cells as stimulator cells (25, 31, 32). The amount of peptide needed for target cell recognition by the obtained CTLs in these studies was approximately 1 nM (31, 32). Using DCs as stimulator cells, we show that gp100 280–288-specific CTLs can be induced that require a comparable amount of peptide.

The presence of CTLs in the T-cell repertoire of healthy donors capable of recognizing melanocyte differentiation antigens indicates that deletion of potential auto-antigen-reactive CTL precursors has not occurred. It may be that these precursor T lymphocytes have not yet encountered their antigen and exist in the periphery as ignorant T lymphocytes (33). In this case, the *in vitro* activation of melanocyte differentiation antigen-reactive CTLs reflects the induction of a primary immune response. Alternatively, such lymphocytes may have seen melanocyte-derived epitopes *in vivo*, resulting in an elevated precursor frequency. In this light the described *in vitro* CTL induction can be regarded as a secondary response. *In vivo* priming of melanocyte antigen-specific CTLs could be one of the causes of melanocyte destruction as is seen in part of the vitiligo patients. The elevated precursor frequency of melanocyte antigen-specific CTLs might explain the results of Rivoltini *et al.* (34), who were able to generate donor-derived Melan A/MART-1-specific CTLs by just pulsing PBMCs with peptide without applying specialized APCs. Therefore, it remains to be established whether the induced CTL reactivities de-

scribed here are primary immune responses or reflect weak secondary responses.

So far four melanocyte differentiation antigens have been identified as target antigens for antimelanoma CTLs. Adoptive transfer of tumor-infiltrating lymphocyte populations containing CTL specificities for gp100, tyrosinase, and gp75 resulted in objective tumor regression in melanoma patients (8, 10, 35). This suggests that patients may benefit from an immune response against these antigens. Apart from occasionally occurring vitiligo, no toxic side effects against normal tissues expressing these antigens, like retina and skin, were observed in these patients. The occurrence of T lymphocytes recognizing melanocyte differentiation antigens within healthy donors, as demonstrated by our study and by those of others (32, 34), indicates that the T-cell repertoire is capable of generating responses against these differentiation antigens. Moreover, with these results the two basic requirements for vaccination, well-characterized target antigens and the presence of precursor T lymphocytes capable of recognizing the target antigens, have been fulfilled. Therefore, melanocyte differentiation antigens are attractive candidates for active immunotherapy in melanoma.

DCs have been shown to play a critical role in the initiation of cellular immune responses (15). Hence, it is tempting to speculate that administration of DCs may be effective in generating cellular tumor immunity *in vivo*. Thus, the laborious approach of adoptive transfer of tumor-specific CTLs might be circumvented. So far, *in vitro* CTL induction protocols using APCs and peptide were only successful when using autologous B lymphoblasts or DCs as APCs. Regarding the *in vivo* induction of tumor-reactive CTL using peptide-pulsed APCs, usage of autologous DCs may be preferable to usage of autologous B lymphoblasts because, in addition to their antigen-presenting capacity, DCs may be better equipped to migrate throughout the body and home to the lymph nodes. Efficacy of peptide-pulsed DCs as APCs for the induction of antigen-specific CTLs *in vivo* was demonstrated in a murine setting using marrow-derived, *in vitro*-generated DCs (36). Until recently, the use of DCs as immunotherapeutic agents has been hampered by their low frequency, causing great difficulty to obtain sufficient amounts of pure DCs. However, with this recently developed method, it has become possible to generate sizable numbers of pure DCs by culturing progenitor cells in the presence of GM-CSF and IL-4 (16). We showed that using such DCs, CTL responses could be elicited *in vitro* directed against melanocyte differentiation antigen-derived epitopes. The next step would be to generate large amounts of DCs *in vitro*, expose them to antigen, and reinfuse them into patients. Concomitant administration of cytokines like GM-CSF and IL-2 may further support *in vivo* induction of tumor immunity. Although this promising immunotherapeutic concept may at the moment be only applicable using broadly expressed

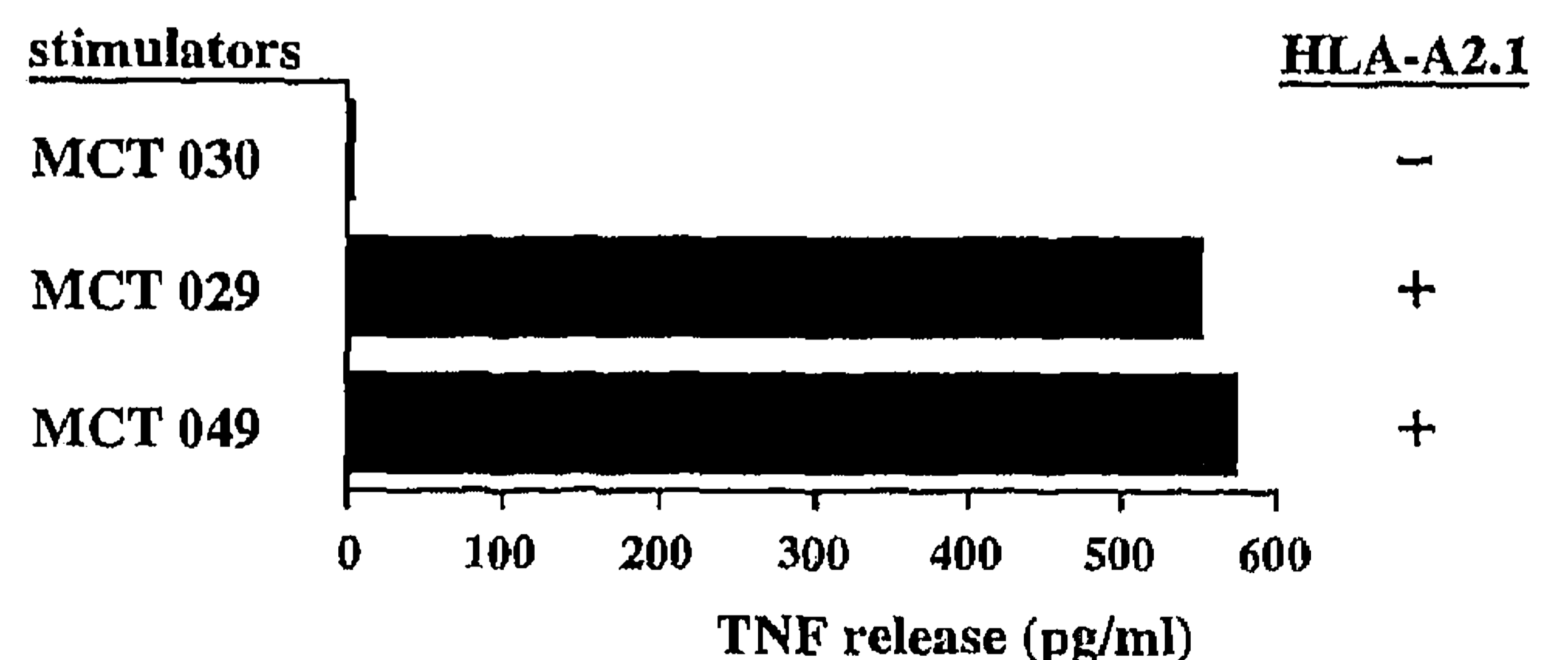


Fig. 6. Recognition of HLA-A2.1⁺-cultured normal melanocytes by anti-gp100 CTLs. Bulk anti-gp100 CTLs were added to the transfectants, and after 24 h of incubation, a portion of the supernatant was harvested, and its TNF content was measured in a bioassay using WEHI 164 clone 13 cells. Melanocytes from early passages were used, and HLA-A2.1 expression was assessed by flow cytometry using anti-HLA-A2 monoclonal antibodies MA2.1 and BB7.2.

antigens in melanoma, a similar approach may be applicable to other solid tumors.

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