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In Vitro and In Vivo Induction of a Th Cell Response Toward Peptides of the Melanoma-Associated Glycoprotein 100 Protein Selected by the TEPITOPE Program[1]

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The melanoma-associated Ag glycoprotein 100 was analyzed by the T cell epitope prediction software TEPITOPE. Seven HLA-DR promiscuous peptides predicted with a stringent threshold were used to load dendritic cells (DC), and induction of a proliferative response was monitored. PBMC of all nine donors including two patients with malignant melanoma responded to at least one of the peptides. The proliferative response was defined as a Th response by the selective expansion of $CD4^+$ cells, up-regulation of CD25 and CD40L, and IL-2 and IFN-γ expression. Peptide-loaded DC also initiated a T helper response in vivo (i.e., tumor growth in the SCID mouse was significantly retarded by the transfer of PBMC together with peptide-loaded DC). Because the use of the TEPITOPE program allows for a prediction of T cell epitopes; because the predicted peptides can be rapidly confirmed by inducing a Th response in the individual patient; and because application of peptide-loaded DC suffices for the in vivo activation of helper cells, vaccination with MHC class II-binding peptides of tumor-associated Ags becomes a feasible and likely powerful tool in the immunotherapy of cancer. *The Journal of Immunology,* **2000, 165: 4731–4741.**

Immunotherapy of cancer has gained in credibility by the definition of tumor-associated Ags and elaboration of their immunogenicity (1, 2). So far, the knowledge of immunogenic tumor-associated Ags has been used in therapeutic protocols mainly by the adoptive transfer of in vitro activated and expanded tumor-specific CTL (3) and, more recently, by vaccination with APCs loaded with MHC class I-restricted peptides (2, 4).

Human tumor-associated Ags have been the first described and until now are the best explored in the melanoma system (5). One of them, glycoprotein 100 (gp100),[4] which belongs to the family of melanocyte differentiation Ags (6), has become of major interest because gp100-specific CTL can frequently be detected in PBMC (4) of patients with malignant melanoma (7). Even PBMC of healthy persons contain gp100-specific CTL precursors (8, 9). The major MHC class I-restricted gp100-derived peptides have been identified, the most immunogenic one being presented by the HLA-A2.1 haplotype (10–15). The frequency of gp100-specific CTL precursors is relatively high (16) and probably based on an appropriate fit of the presented peptide into the TCR binding domain; high levels of cytotoxicity were observed even at low effector to target cell ratios (17). Accordingly, the transfer of gp100-specific CTL in combination with IL-2 revealed good responses in the majority of patients with malignant melanoma (18).

From the clinical, but also from the immunological point of view, vaccination protocols would provide an optimal therapeutic strategy because of the easy handling and the opportunity of an activation of the immune system to end up in immune memory (19–22). In fact, recent reports on vaccination with DNA (23–26) as well as with peptide-loaded dendritic cells (DC; Refs. 9 and 27–37) provided convincing results to support these contentions.

So far, peptide vaccination has mainly been based on MHC class I-restricted peptides. This restriction has historical reasons. First, methods of in vitro generation of DC for presentation of MHC class I- as well as MHC class II-restricted peptides became only recently available (38, 39). Immunogenicity of tumor-associated Ags has so far been evaluated via the induction of and recognition by CTL (40, 41). Second, much progress has been achieved in the prediction of peptide structures fitting into the groove of MHC class I Ags (42, 43). Third, most of the immunogenic tumor-associated Ags are differentiation Ags (44, 45) and it has been supposed that induction of tolerance may be more stringent at the Th cell than at the CTL level (46, 47). Nonetheless, initiation of an immune response naturally proceeds via the activation of Th cells, which are supposed to provide the second signal for the activation of CTL precursors (48), and it can be surmised that vaccination with tumor-derived peptides presented by MHC class II molecules should be advantageous (49–52). The hypothesis has been supported by the fact that vaccination with MHC class II and B7.1 cDNA-transfected tumor cells was highly efficient even in models of metastasizing tumors (53–55). Furthermore, we recently demonstrated that the gp100 protein will be processed such that protein-loaded DC induce both in vitro and in vivo activation and expansion of Th cells (56).

Encouraged by this finding as well as by the identification of a MHC class II-restricted peptide derived from gp100 (57) and the

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[4] Abbreviations used in this paper: gp100, glycoprotein 100; BLM-gp100, BLM cells transfected with gp100 cDNA; DC, dendritic cells; LNC, lymph node cells; TIL, tumor-infiltrating leukocytes; LAK, lymphokine-activated killer.

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recently developed T cell epitope prediction program TEPITOPE (58–61), we analyzed the capability of HLA-DR promiscuous gp100 peptides predicted by TEPITOPE to induce a Th response. DC from nine donors exhibiting distinct HLA-DR haplotypes were loaded with gp100-derived peptides which had been selected by the TEPITOPE program. PBMC of all nine donors mounted an efficient Th cell response toward at least one of seven gp100-derived peptides and no response was observed toward peptides that were supposed not to bind to a given HLA-DR haplotype. Thus, the combined approach of epitope prediction using TEPITOPE and rapid PBMC proliferative response assays could greatly facilitate the selection of MHC class II-restricted peptides suitable for vaccination.

Materials and Methods

Animals and tumors

SCID (H-2^d) mice were bred at the animal facilities of the German Cancer Research Center (Heidelberg, Germany). Animals were housed under specific pathogen-free conditions. They were fed sterilized food and water ad libitum. Mice were used for experiments at the age of 10 wk.

The human melanoma lines BLM (62) and the BLM line transfected with gp100 cDNA (BLM-gp100) (63) were maintained in Dulbecco's minimal essential medium containing 5% FCS. The lines were HLA-A2.1$^+$. The erythroleukemia line K562 was used as a target for lymphokine-activated killer (LAK) activity. The melanoma lines 530C1 (HLA-A2.1$^-$, gp100$^+$; Ref. 62) and FM3 (HLA-A2.1$^+$, gp100$^+$; Ref. 64) were used for cold target inhibition. Both lines were maintained in RPMI 1640 supplemented with 10% FCS.

Collection of peripheral blood and HLA-DR typing

Heparinized blood was collected from seven healthy volunteers (four male, three female, 27–55 years) and two patients with malignant melanoma (one male, 52 years, one female, 46 years). Both patients were at stage IV according to the International Union Against Cancer classification. PBMC were isolated from heparinized blood (30 ml from healthy donors and 10 ml from melanoma patients) by Ficoll gradient centrifugation. HLA-DR typing was preformed by the PCR-SSP method (65).

Monoclonal mAbs

The following hybridomas were used: OKT4 (anti-human CD4; American Type Culture Collection (ATCC), Manassas, VA), OKT8 (anti-human CD8; ATCC), W6/32 (anti-human MHC class I; ATCC), 9.3F10 (anti-human MHC class II; ATCC), HNK1 (anti-human NK; ATCC), 63D3 (anti-human monocytes; ATCC), 15E8 (anti-human CD28; kindly provided by P. H. Krammer, German Cancer Research Center, Heidelberg, Germany), Ox8 (anti-rat CD8; European Cell Culture Collection, Porton Down, U.K.), and K9-18 (anti-mouse H-2D^d; Ref. 66). Culture supernatants were purified by passage over protein G-Sepharose 4B. The eluted fractions were dialyzed against PBS, concentrated to 1 mg/ml, and filter sterilized. Anti-human CD25, CD40, CD40L, CD80, and CD86, as well as anti-human IL-2, IL-4, IL-10, IFN-γ, TNF-α and anti-mouse IL-2, IFN-γ, TNF-α, and FITC or PE-labeled secondary Abs were obtained commercially (PharMingen, Hamburg, Germany).

Selection of gp100 peptides

The TEPITOPE software was used to predict potential HLA-DR-binding peptides with promiscuous binding characteristics as described elsewhere (58, 59). The prediction threshold was set at 2% and peptides were picked that were predicted to bind to at least four of the following seven HLA-DR molecules DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, and DRB1*1501, which are among the most frequent alleles. Eight peptides, each consisting of 13 aa have been synthesized by the Central Unit of Peptide Synthesis (German Cancer Research Center, Heidelberg, Germany). The peptides covered aa$_{231–243}$ (KHFLRNQPLTFAL), aa$_{290–302}$ (QVVLQAAIPLTSC), aa$_{407–419}$ (SIVVLSGTTAAQV), aa$_{466–478}$ (RLVKRQVPLDCVL), aa$_{552–564}$ (QLVLHQILKGGSG), aa$_{599–611}$ (LIVGILL VLMAVV), aa$_{604–616}$ (LLVLMAVVLASLI), and aa$_{636–648}$ (SHWLRLPR IFCSC). In some experiments, a longer peptide consisting of 15 aa, aa$_{230–244}$ (NKHFLRNQPLTFALQ) has been used (i.e., the peptide aa$_{231–243}$ has been extended one amino acid at each end, the binding core of the peptide being FLRNQPLTF). For comparison, DC also were loaded with the gp100 protein, the preparation of which has recently been described in detail (56).

Generation of DC

DC were generated according to a slight modification (67) of the protocol described by Xu et al. (38). Briefly, DC were generated in 24-well plates, seeding 1×10^6 PBMC/well. After 24 h of culture, nonadherent cells were removed and plastic-adherent cells were cultured in IMEM/10% autologous serum supplemented with 150 U GM-CSF, 50 U IL-4, and 50 U IFN-γ. Mature DC were verified by FACS analysis (MHC class II$^+$, CD40$^+$, CD80$^+$, CD86$^+$, CD14$^-$) and microscopy (veiled cells). DC were loaded for 1.5 h at day 10 of culture with 10 μg peptide, if not indicated otherwise. Cultures were washed to remove unbound peptides and autologous PBMC were added.

Proliferation assay

Autologous PBMC (1×10^6/ml) were cultured for 3 days on peptide-loaded DC, adding 10 μCi/ml [^{3}H]thymidine during the last 6–8 h. Cells were harvested and the incorporation of [^{3}H]thymidine was determined in a beta counter. In most instances, PBMC were added to the peptide-loaded DC within the 24-well plates. In some experiments, peptide-loaded DC were transferred to 96-well microtiter plates before the addition of PBMC. Because experiments run parallel in 24- and 96-well plates revealed comparable results, the difference in setting will not be explicitly mentioned in the individual experiments.

Cytotoxicity assay

Activity of LAK was evaluated in freshly harvested tumor infiltrating leukocytes (TIL) and draining lymph node cells (LNC). For the in vitro restimulation of CTL, draining LNC and TIL were cultured with irradiated (30,000 rad) BLM-gp100 cells for 8 days. The ratio of lymphocytes to irradiated tumor cells was in the range of 20–10:1. After 8 days of culture, blasts were separated from dead cells by Ficoll-Hypaque gradient centrifugation and used for the evaluation of cytotoxic activity. For the determination of CTL and LAK activity, target cells were labeled with ^{51}Cr, washed, and 10^4 cells were seeded in 96-well round-bottom plates together with the stimulated lymphocytes at a ratio of E:T equal to 50–3.1:1. When determining CTL activity, control cultures additionally contained cold target cells (K562 for experimental subtraction of LAK activity or either the

Table I. *Proliferative response of PBMC from healthy volunteers toward gp100 peptide-loaded autologous DC*

HLA-DR Haplotypes	Proliferation Index[a] (potential binding[b])													
	aa$_{231–243}$		aa$_{407–419}$		aa$_{466–478}$		aa$_{552–564}$		aa$_{599–611}$		aa$_{604–616}$		aa$_{636–648}$	
DRB1*1201/1501 (BW)	–	(–/–)	–	(–/+)	–	(–/–)	–	(–/+)	++	(–/+)	++	(–/+)	–	(–/+)
DRB1*0301/0801 (PE)	+++	(+/–)	+	(+/–)	±	(+/+)	±	(+/–)	+	(+/+)	+	(+/+)	++	(+/+)
DRB1*1501/1501 (OC)	–	(–/–)	+++	(+/+)	–	(–/–)	–	(+/+)	–	(+/+)	–	(+/+)	–	(+/+)
DRB1*0401/1501 (MH)	–	(+/–)	–	(+/+)	±	(+/–)	–	(+/+)	–	(+/+)	–	(+/+)	–	(–/+)
DRB1*0101/1101 (MZ)	+++	(+/+)	–	(+/–)	–	(+/+)	–	(–/+)	–	(+/+)	–	(+/+)	±	(+/+)
DRB1*0801/1501 (MS)	–	(–/–)	+	(–/+)	–	(+/–)	±	(–/+)	–	(+/+)	±	(+/+)	–	(+/+)
DRB1*0408/0701 (BC)	+	(–/±)	–	(–/±)	–	(–/±)	–	(–/–)	–	(–/+)	–	(–/+)	–	(–/–)

[a] The proliferation index (proliferation in the presence of peptide-loaded DC:proliferation in the presence of unloaded DC) was classified as follows: +++, > 5-fold; ++, > 4- to 5-fold; +, > 3- to 4-fold; ±, > 2- to 3-fold. All experiments were repeated at least three times. The presented values are derived from the mean increase in three to seven independently performed experiments.

[b] Potential binding was classified as +, stringent threshold of 2%, and ±, less stringent threshold of 3%.

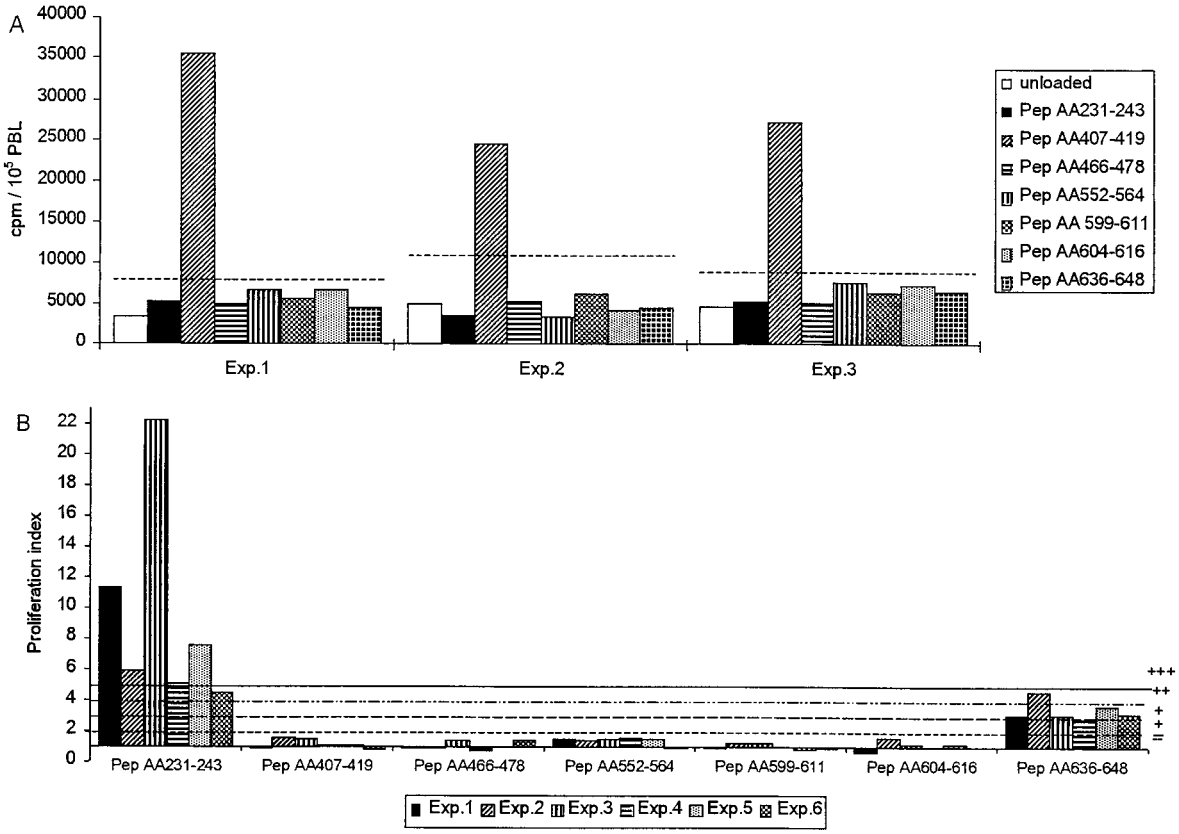

FIGURE 1. Proliferative responses of PBMC from healthy donors toward gp100-derived peptides. PBMC of donor OC (*A*) and MZ (*B*) were seeded on autologous DC (PBMC:DC = 50:1) which had been loaded with 10 μg gp100-derived peptides. Control cultures contained PBMC and unloaded DC. [^{3}H]Thymidine incorporation was determined after 3 days of culture. *A*, [^{3}H]Thymidine uptake as observed in three independently performed experiments. *B*, Proliferation indices (mean cpm/1 $\times$ 10^5 PBMC cultured on loaded DC:mean cpm/1 $\times$ 10^5 PBMC cultured on unloaded DC) from six independently performed experiments are shown. Proliferative responses were classified as $\pm$ (>2–3, dotted line), + (>3–4, dashed line), ++ (>4–5, dotted/dashed line), >5 (+++, solid line)

melanoma cell line 530C1, which is HLA-A2.1$^-$ and gp100$^+$ or the melanoma cell line FM3, which is HLA-A2.1$^+$ and gp100$^+$ for restriction analysis). The ratio of cold target to target was 10:1. Plates were incubated for 6 h at 37°C. After centrifugation, aliquots were removed and released radioactivity was determined in a gamma counter. Cytotoxicity was calculated as follows: % cytotoxicity = 100 $\times$ ((counts in test well − counts in control well)/(maximal releasable counts − counts in control well)). The spontaneous release was in the range of 7–12%. SDs of triplicates were in the range of 3–5%.

Flow cytometry

FACS analysis of DC and PBMC was performed according to routine procedures using 1–5 $\times$ 10^5 cells. For the analysis of cytokine expression, cells have been permeabilized before staining. When analyzing the phenotype of human lymphocytes after transfer into SCID mice, murine lymphocytes have been depleted by two rounds of panning on anti-mouse H-2D^d-coated plates, collecting the nonadherent fraction. Panning was performed according to the method described by Wysocki and Sato (68).

In vivo protocols

SCID mice were conditioned by irradiation (300 rad) and were treated with anti-asialoGM1 (10 μl injected i.p. according to the manufacturer's suggestion) to restrain inherent NK cell activity (69). The animals received one day later an i.v. injection of 1 $\times$ 10^5 peptide-loaded DC together with 1 $\times$ 10^7 freshly harvested autologous PBMC. Spleen cells were harvested 5 days thereafter. Human cells were selected as described above by panning. Survival, expansion, and gain of functional activity in vivo were evaluated by flow cytometry and proliferative activity. To control the efficacy of vaccination with peptide-loaded DC, SCID mice received 1 $\times$ 10^5 peptide-loaded DC together with 1 $\times$ 10^6 autologous PBMC at day 0. BLM-gp100 (5 $\times$ 10^4) were injected s.c. 5 days later. The injections of DC and PBMC were repeated in 10-day intervals. Tumor growth and survival time as well as in vivo activation of lymphocytes and cytotoxic activity in the draining node and within the tumor were monitored.

Statistics

Significance of differences was evaluated by the two-tailed Student *t* test.

Results

Induction of a proliferative response to gp100-derived peptides

Eight synthetic peptides corresponding to sequence segments predicted by TEPITOPE (58, 59) were synthesized. Peripheral blood-derived DC of seven healthy volunteers were generated as described in *Materials and Methods* and were loaded after 10 days of culture with the above-mentioned eight peptides. Unbound peptides were removed by washing after 1.5 h and autologous PBMC were added. After 48 h of culture, [^{3}H]thymidine was added for the last 6–8 h to determine the proliferative activity. Table I summarizes the reactivities obtained in three to eight independent settings and indicates (in parentheses), whether the individual peptides are supposed to bind or not to bind to the given HLA-DR haplotype. None of the donors responded to the peptide covering $aa_{290-302}$ (data not shown). However, because this peptide was very poorly water soluble, the failure of obtaining a response toward this peptide should be taken with caution. With five peptides, at least one of the donors displayed good reactivity. The observed proliferative reactivity profiles correlated with the prediction of peptide binding to a given HLA-DR haplotype. Yet none of the donors responded to all the predicted peptides, and different donors sharing HLA-DR

Table II. *Dose dependence of Th cell activation by gp100 peptide-loaded DC*

Dose of Peptide (μg/ml)	Proliferation Index with Peptide[a]		
	$aa_{231–243}$	$aa_{407–419}$	$aa_{636–648}$
Expt. 1			
1	1.28	1.09	0.98
5	3.88	1.00	2.43
10	5.49	1.21	3.23
25	5.76	1.20	3.12
50	5.73	1.24	3.25
Expt. 2		$aa_{231–243}$	$aa_{230–244}$
0.78		1.58	3.35
1.56		2.11	4.21
3.13		3.43	8.41
6.25		4.73	10.55
12.50		7.28	11.08
25.00		7.50	12.26
50.00		7.69	15.77

[a] PBMC of donor MZ (HLA-DRB1*0101/1101) were seeded on autologous DC (PBMC:DC = 50:1) that had been loaded with 1–50 μg/ml gp100-derived peptides. Control cultures contained PBMC and unloaded DC. [^{3}H]Thymidine incorporation was determined after 2 days of culture. Proliferation indices (mean cpm/1 $\times$ 10^5 PBMC cultured on loaded DC:mean cpm/1 $\times$ 10^5 PBMC cultured on unloaded DC) are shown.

haplotypes did not essentially respond toward the same peptides. However, as evident by the presentation of mean proliferation indices in Table I and demonstrated for donors OC and MZ (Fig. 1), reactivity profiles of individual donors were stable. Using 10 μg/ml 13-mer peptides, peak responses were observed. When loading with 0.1–1 μg/ml 13-mer peptides, hardly any proliferative response was observed. Yet, as has been described before (70), the efficacy of response induction could be improved by using 15-mer peptides, where a high proliferation index was seen already when loading DC with 1 μg/ml peptide (Table II).

Verification of the activation of gp100-specific Th cells by peptide-loaded autologous DC

To see whether the selected peptides actually represent naturally processed and presented epitopes of the gp100 protein, DC of donor MZ were loaded with the peptide $aa_{231–243}$, which initiated a proliferative response; with the peptide $aa_{407–419}$, which did not induce a proliferative response; or with the gp100 protein. T cells were collected after 48 h and rested for 3 days. Thereafter, they were challenged in a criss-cross fashion with the peptides/gp100 protein used for priming (Table III). Only T cells primed with gp100 protein or peptide $aa_{231–243}$ displayed a significantly higher proliferation index upon challenge with the corresponding peptide/protein. Furthermore, T cells primed with gp100 protein readily responded toward peptide $aa_{231–243}$. The same observation accounted for T cells primed with peptide $aa_{231–243}$ when challenged with the gp100 protein. By these features of mutual protein vs peptide priming it becomes very likely that peptide $aa_{231–243}$ represents a naturally processed and presented epitope.

Table III. *Activation of gp100-specific Th cells by peptide-loaded autologous DC: specificity of response*

Peptide	Proliferation Index[a]				
	Primary response	Response after challenge with			
		None	$aa_{407–419}$	$aa_{231–243}$	gp100
None	1.00	1.00	0.86	3.58	4.69
$aa_{407–419}$	1.01	1.13	2.27	4.67	4.91
$aa_{231–243}$	3.34	1.00	2.42	15.01	14.54
Gp100	6.80	1.15	2.01	23.48	30.52

[a] PBMC of donor MZ were primed and restimulated with peptide-/gp100-loaded DC as described in *Materials and Methods.* The proliferation index is shown (reference values of 5 $\times$ 10^5 PBL/well cultured on unloaded DC were 24,264 and 20,237 cpm in the primary response and after "restimulation," respectively).

To differentiate between the induction of a Th response rather than of a MHC class I-restricted CTL response, PBMC were analyzed after 3 days of coculture with autologous peptide-loaded DC for the relative expansion of $CD4^+$ vs $CD8^+$ cells as well as for up-regulation of CD25, CD40L, and CD28 (Table IV). A selective expansion of $CD4^+$ cells has only been observed in PBMC responding to a presented peptide. The relative expansion of $CD4^+$ cells corresponded to the proliferation index (i.e., the highest percentage of $CD4^+$ cells was recovered from cultures exhibiting strong proliferative activity). In addition to a selective expansion of $CD4^+$ cells, expression of CD25 and CD40L was consistently found to be up-regulated in responding cultures. Up-regulation of CD28 was seen only occasionally.

The MHC class II-restricted Th response was also confirmed by the pattern of cytokine production (Table V). Only PBMC cultured in the presence of autologous DC loaded with a peptide, which initiated a proliferative response, contained an increased percentage of cells expressing IL-2 and IFN-γ. In contrast, expression of IL-4 was unaltered, indicating that a Th-1-type response has been initiated. As demonstrated with PBMC from donor MZ (Fig. 2), the majority of cytokine expressing cells were $CD4^+$.

Unimpaired response of PBMC from patients with metastatic malignant melanoma to gp100 peptides

We next asked whether PBMC of patients with a growing melanoma may show a similar response profile. Because it is unlikely

Table IV. *Activation of gp100-specific Th cells by peptide-loaded autologous DC: in vitro expansion and activation of CD4-positive cells in response to DC loaded with gp100-derived peptides*

DC and PBMC Donor	Peptide	Proliferation Index	% Stained PBMC[a]				
			CD4	CD8	CD25	CD28	CD40L
OC (DRB1*1501/1501)	None		16.9	25.4	22.7	50.4	30.4
	Pep $aa_{407–419}$	7.43	22.5*	22.8	43.0*	49.6	44.6*
	Pep $aa_{636–648}$	1.12	18.9	25.8	23.8	50.9	29.5
PE (DRB1*0301/0801)	None		14.1	35.7	30.3	49.3	29.6
	Pep $aa_{231–243}$	5.94	26.2*	31.2	46.2*	50.6	40.9*
	Pep $aa_{552–564}$	1.52	13.4	36.9	31.1	47.7	30.6
MZ (DRB1*0101/1101)	None		12.3	39.5	12.3	30.3	28.9
	Pep $aa_{231–243}$	11.31	31.9*	37.8	30.1*	45.4*	45.2*
	Pep $aa_{407–419}$	1.11	10.9	40.2	12.2	31.9	31.1

[a] Mean values of three experiments are shown. *, Significance of differences ($p < 0.05$).

Table V. *Cytokine expression in response to DC loaded with gp100-derived peptides*

DC and PBMC Donor	Peptide	Proliferative Response[a]	% Cytokine Expressing Cells[b]		
			IL-2	IFN-γ	IL-4
OC (DRB1*1501/1501)	None		7.5	7.0	2.1
	Pep $aa_{407–419}$	+++	14.2*	19.4*	3.9
	Pep $aa_{636–648}$	−	5.0	4.1	2.2
PE (DRB1*0301/0801)	None		21.1	9.9	7.4
	Pep $aa_{231–243}$	+++	44.1*	29.2*	6.9
	Pep $aa_{552–564}$	−	16.7	9.7	5.8
MZ (DRB1*0101/1101)	None		6.9	7.3	3.6
	Pep $aa_{231–243}$	+++	38.1*	36.3*	9.8*
	Pep $aa_{407–419}$	−	4.6	9.1	2.1
MS (DRB1*0801/1501)	None		16.1	10.4	5.7
	Pep $aa_{407–419}$	+	30.5*	21.4*	3.1
	Pep $aa_{552–564}$	±	22.8*	18.6*	5.6

[a] Proliferation indices were classified as described in Table I.
[b] Mean values of three experiments are shown.
*, Significance of differences ($p < 0.05$).

that in the melanoma-bearing patient gp100 peptides will be presented by professional APCs, the possibility had to be taken into account that a state of tolerance or anergy had been created. Alternatively, PBMC of patients with malignant melanoma could have been sensitized toward gp100 peptides such that they would mount a memory response upon in vitro (re)stimulation. To obtain a hint as to a natural in vivo sensitization or, more importantly, induction of tolerance or anergy, PBMC of two patients with metastatic recurrence of a $gp100^+$ malignant melanoma were tested for reactivity toward the panel of peptides shown above.

The patients' PBMC responded to at least one of the gp100 peptides, in correlation with the predicted HLA-DR binding specificity (Fig. 3). The proliferation indices were in the same range as those observed with PBMC of healthy volunteers. The fact that PBMC of patients with a recurrent malignant melanoma responded toward selected peptides of gp100 does not exclude, but strongly argues against induction of tolerance or anergy by the growing tumor.

In vivo activation of Th cells by peptide-loaded autologous DC

We next explored whether by the transfer of loaded DC a Th response would be induced in vivo. SCID mice were conditioned by irradiation and anti-asialoGM1 treatment. One day later they received a single i.v. injection of 1×10^6 unloaded or loaded DC and 1×10^7 freshly harvested autologous PBMC. Mice were sacrificed after 5 days and the spleens were excised. The number of human lymphocytes, their phenotype, and their state of activity were analyzed in vitro (Table VI). From animals that had received loaded DC, roughly 40% of the injected cells were recovered as compared with <20% from animals injected only with PBMC or with PBMC plus unloaded DC (data not shown). Furthermore, the percentage of $CD4^+$ and of $CD25^+$ cells was significantly increased in mice receiving loaded as compared with unloaded DC. Notably (Table VI), only the lymphocytes recovered from mice injected with loaded DC exerted a profound proliferative activity when challenged in vitro with DC presenting the same peptide. Finally, and in line with the in vitro finding (Table VI), a high percentage of these $CD4^+$ cells produced IL-2 and IFN-γ. $CD4^+$ cells recovered from mice receiving no or unloaded DC did not produce cytokines at a measurable level.

Retardation of tumor growth by vaccination with peptide-loaded DC

To explore whether vaccination with peptide-loaded DC has an influence on tumor growth, SCID mice received repeated injections of peptide-loaded DC and autologous PBMC. The donor was

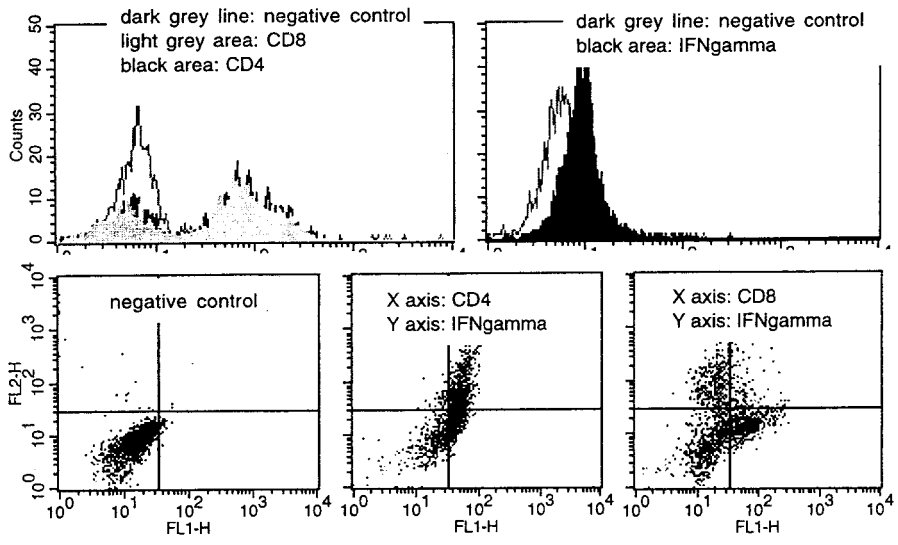

FIGURE 2. Cytokine expression by CD4-positive cells cultured on gp100 peptide-loaded DC. PBMC of donors MZ (HLA-DRB1*0101/1101) were cultured for 3 days in the presence of DC loaded with Pep $aa_{231–243}$ (proliferative response). PBMC were stained with anti-CD4-FITC, anti-$CD8^-$ FITC, or were double stained with anti-CD4-FITC or anti-CD8-FITC and anti-IFN-γ-PE. The majority of IFN-γ^+ cells were stained by anti-CD4.

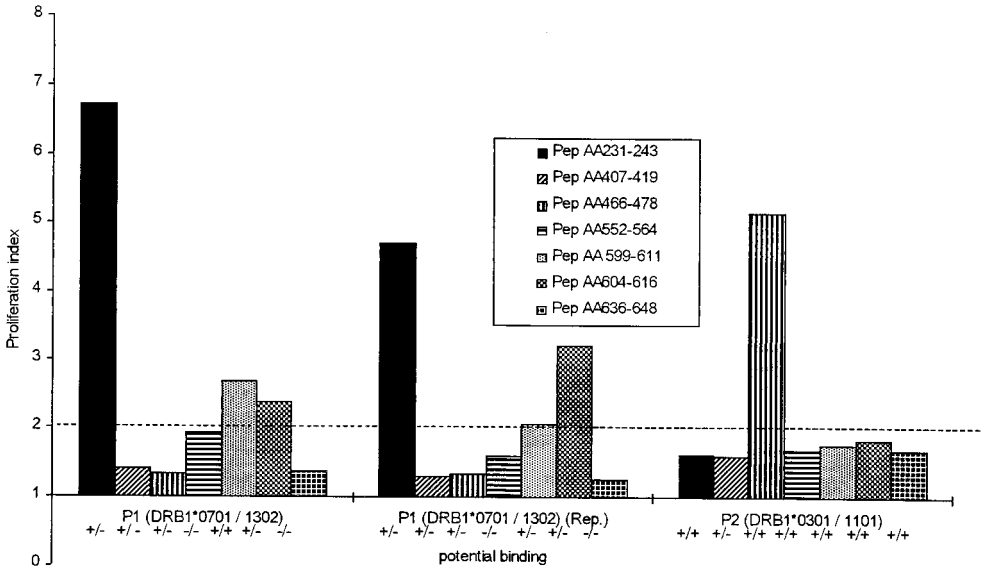

FIGURE 3. Responsiveness of PBMC from patients with malignant melanoma toward gp100-derived peptide-loaded DC. PBMC of two patients with malignant melanoma were seeded on autologous DC loaded with the gp100-derived peptides described above. After 3 days of culture, [^{3}H]thymidine incorporation was determined. Proliferation indices (mean cpm/1 $\times$ 10^5 PBMC cultured on loaded DC:mean cpm/ 1 $\times$ 10^5 PBMC cultured on unloaded DC) are shown. A proliferation index of >2 has been marked by a dotted line.

of the HLA-A2.1 haplotype and had been tested to respond to peptide $aa_{231–243}$ (proliferation index: >10). Five days after the first application of DC plus PBMC, SCID mice received a s.c. injection of 5 $\times$ 10^4 BLM-gp100 cells, which also are of the HLA-A2.1 haplotype (Fig. 4). The injection of DC plus PBMC was repeated six times in intervals of 10 days. Tumor growth was slightly retarded by the application of allogeneic PBMC. This has been observed with BLM as well as with BLM-gp100 cells. BLM-gp100 growth was significantly retarded in mice receiving peptide-loaded DC as compared with PBMC without DC or PBMC with unloaded DC. In fact, rapid tumor growth only started after cessation of DC/PBMC injections. The mean survival time was prolonged by a factor of 2. Because neither the in vivo growth rate of BLM cells nor the survival time of BLM-bearing mice were significantly changed when mice received peptide-loaded DC in addition to the allogeneic PBMC, the effect on BLM-gp100 cells can be considered as gp100 specific.

An in vitro analysis of draining LNC was performed 4 wk after tumor cell application. TIL were collected when the mean tumor diameter reached 0.5 cm (i.e., 4 wk after tumor cell application when mice received either PBMC or PBMC plus unloaded DC and after 6 wk when mice received PBMC plus peptide-loaded DC (Table VII)). An efficient population of draining lymph nodes was seen only in mice receiving peptide-loaded DC. In these mice, also a high number of TIL was recovered. The majority of draining LNC and TIL were of human origin (data not shown) and a higher percentage of $CD4^+$ cells was recovered in mice receiving PBMC plus peptide-loaded DC as compared with mice receiving PBMC or PBMC plus unloaded DC. In the draining lymph node of mice receiving either unloaded or peptide-loaded DC, a high percentage of cells expressed MHC class II molecules and the HNK1.1 marker. In TIL, an increase in MHC class II and HNK1.1 expression was only seen after application of PBMC plus peptide-loaded DC. IL-2, TNF-α and, most impressively, IFN-γ expression was up-regulated in draining LNC and TIL. Cytokine expression of murine cells was only slightly increased (Table VIII).

Draining LNC and TIL from mice receiving PBMC or unloaded DC plus PBMC weakly proliferated in response to peptide-loaded DC and irradiated BLM-gp100 cells (Fig. 5*A*). The proliferative response was significantly increased when mice had received peptide-loaded DC plus PBMC. The fact that particularly TIL from mice receiving peptide-loaded DC did not proliferate in response to irradiated BLM cells supports our interpretation that the peptide-loaded DC induced a gp100-specific response.

CTL activity was evaluated after in vitro restimulation with irradiated BLM-gp100 cells. Both draining LNC and TIL from mice that had received peptide-loaded DC, exhibited a significantly higher cytotoxic activity as compared with draining LNC and TIL from mice treated with PBMC or PBMC plus unloaded DC. The response was at least partly gp100 specific, because BLM cells not expressing gp100 were lysed at a significantly lower degree. Furthermore, the response was predominantly mediated by $CD8^+$

Table VI. *In vivo induction of a Th cell response by peptide-loaded DC: expansion of CD4-positive cells, cytokine expression by human PBMC, and proliferative response of human PBMC after recovery in the SCID mouse*

PBMC[a]	DC[a]	% Human PBMC in SCID SC	% Stained Spleen Cells			Ratio CD4:CD8	% Cytokine Expressing $CD4^+$ Cells[c]			CPM/10^5 Cells Recovered from the Spleen of SCID Mice and Cultured on[d]		Proliferation Index
			CD4	CD8	CD25		IL-2	IFN-γ	IL-4	Unloaded DC	Loaded DC	
+	None	49.2 ± 4.2	18.5 ± 2.9	30.7 ± 1.5	10.6 ± 2.3	0.60:1	9.8 ± 2.6	2.9 ± 0.8	4.2 ± 1.3	285 ± 21	599 ± 53	2.1
+	Unloaded	44.9 ± 2.9	15.5 ± 2.1	29.4 ± 2.7	8.9 ± 0.7	0.53:1	9.9 ± 2.5	3.9 ± 1.8	2.6 ± 1.2	307 ± 29	706 ± 68	2.3
+	Loaded	61.7 ± 2.2*	37.7 ± 5.5*	23.9 ± 3.5	21.7 ± 2.3*	1.58:1	27.7 ± 5.7*	16.4 ± 2.6*	5.8 ± 1.8	343 ± 48	5351 ± 283	15.6

[a] PBMC and DC were derived from donor MZ (DRB1*0101/1101). Where indicated, DC were loaded with 10 μg/ml of Pep $aa_{231–243}$. DC (1 $\times$ 10^6) and 1 $\times$ 10^7 freshly harvested PBMC were injected i.v. into SCID mice. Spleen cells were collected 5 days thereafter.

[b] The percentage of human cells was evaluated by staining with W6/32.

[c] $CD4^+$ cells were separated by panning. The CD4-enriched population was permeabilized and stained with biotinylated anti-IL-2, IFN-γ and IL-4 plus streptavidin-PE.

[d] Unseparated SC were cultured for 3 days in the presence or absence of unloaded or loaded DC. [^{3}H]Thymidine was added during the last 16 h of culture.

*, Significance of differences ($p < 0.05$).

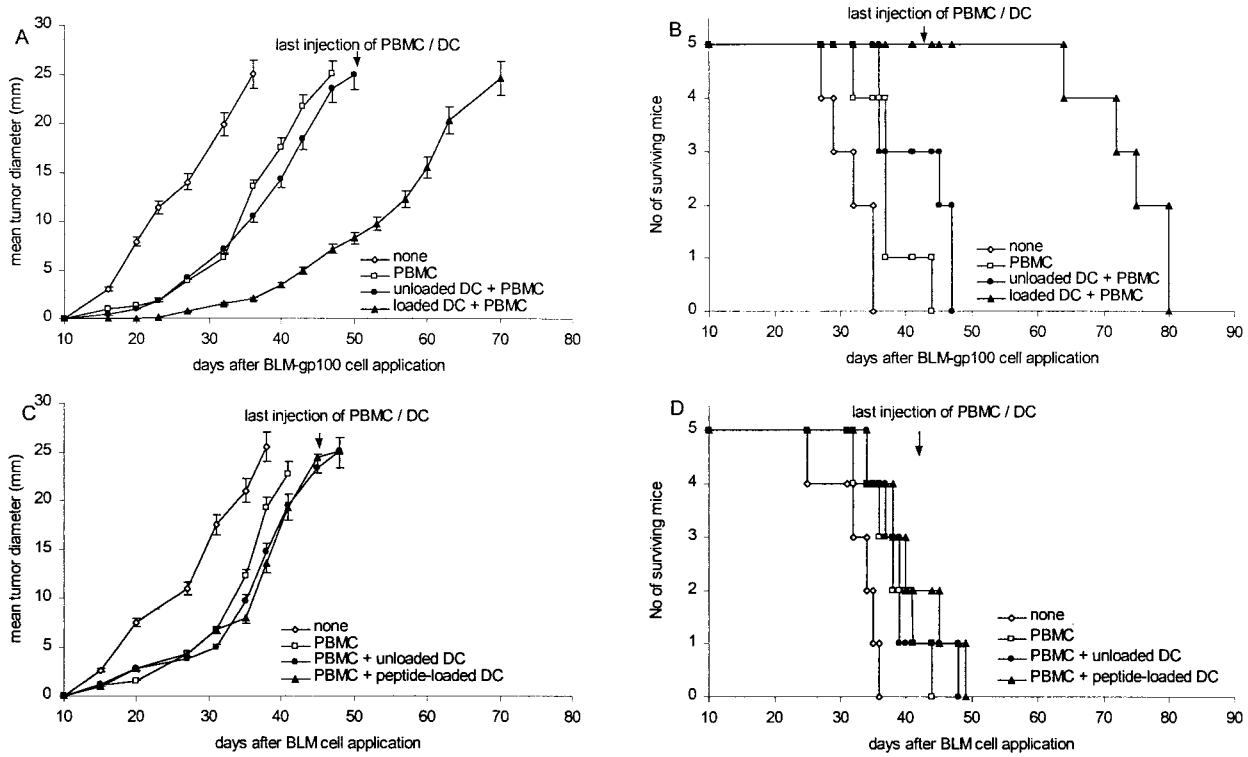

FIGURE 4. Retardation of tumor growth by vaccination with gp100 peptide-loaded DC. SCID mice conditioned by irradiation with 3 Gy and anti-asialoGM1 treatment, received six injections of 1×10^5 gp100 peptide ($aa_{231-243}$)-loaded DC and 1×10^6 autologous PBMC. The DC/PBMC were of the HLA-A2.1 haplotype, the proliferation index in response to DC loaded with peptide $aa_{231-243}$ was 10.8. Mice received a s.c. injection of 5×10^4 BLM or BLM-gp100 (HLA-A2.1$^+$) 5 days after the first injection of DC plus PBMC. Tumor growth (*A* and *C*) and survival time (*B* and *D*) were monitored.

cells, because the cytotoxic activity was only slightly reduced by cold target inhibition with a gp100$^+$ melanoma line of a different HLA-A haplotype, but was strongly inhibited in the presence of a gp100$^+$, HLA-A2.1$^+$ cold target melanoma line (Fig. 5, *C* and *D*). An estimate on LAK activity was obtained by testing cytotoxic activity of freshly harvested TIL and draining LNC for K562 cells (Fig. 5*B*). Although draining LNC from mice treated with either unloaded or peptide-loaded DC exhibited high LAK activity, a recruitment of LAK into the tumor was only observed in mice treated with peptide-loaded DC.

Discussion

Vaccination against tumor-associated Ags is supposed to provide a powerful therapeutic approach (20–22) and DC have been proven to be the most efficient cell type for Ag presentation (39, 71–75). Although vaccination with whole tumor extracts is a possible way to go (76–78), the likelihood of providing sufficient amounts of tumor-associated Ags will be low. Pulsing DC with defined tumor-associated Ags or with immunogenic peptides could significantly increase the chance to initiate lymphocyte activation. Furthermore, induction of an immune response naturally proceeds via activation of Th cells (48), which support among others cytotoxic T cells and monocytes, which both have been demonstrated to be of importance in tumor defense (52, 79–82). However, knowledge of peptides derived from tumor-associated Ags and presented by MHC class II molecules has been sparse and only recently a new epitope prediction software, TEPITOPE (58, 60, 61), for the prediction of HLA-DR binding sequences has become available. Because gp100 is known to induce a significant cytotoxic response in many patients with malignant melanoma (9–17), we considered this molecule as a suitable target to explore the likelihood of inducing a Th

Table VII. *In vivo induction of an anti-tumor response by gp100 peptide-loaded DC: in vivo expansion of human leukocytes and infiltration into the tumor*

PBMC[a]	DC[a]	No. ($\times 10^6$) of Draining LNC	% Stained Draining LNC[b]				No. ($\times 10^6$) of TIL	% Stained TIL$^+$				
			CD4	CD8	MHC II	NK		CD4	CD8	MHC II	NK	Mϕ
+	None	1.2	7.8	14.1	11.2	10.5	8.5	4.5	14.7	1.0	6.2	2.3
+	Unloaded	2.0	13.1*	33.4*	25.0*	39.9*	11.3	6.0	11.8	4.3*	4.7	2.5
+	Loaded	5.2	28.3*	21.1*	30.3*	36.0*	22.5	20.6*	29.6*	16.5*	29.1*	8.9*

[a] PBMC and DC were derived from an HLA-A2.1$^+$ donor. Where indicated, DC were loaded with 10 μg/ml of Pep $aa_{231-243}$. DC (1×10^6) and 1×10^7 PBMC were injected i.v. into SCID mice in intervals of 10 days. Five days after the first injection, mice received a s.c. injection of 5×10^4 BLM-gp100. Draining lymph node cells were harvested after 4 wk and TIL were harvested when the mean tumor diameter reached 0.5 cm.

[b] The percentage of stained cells was derived from the total population of draining LNC or TIL (i.e., without preselecting human leukocytes). Values represent the mean of five animals. SD, not presented for clarity of presentation, was in the range of 3–10%.

*, Significance of differences ($p < 0.05$).

Table VIII. *In vivo induction of an anti-tumor response by gp100 peptide-loaded DC: cytokine expression of human PBMC and murine leukocytes in draining LNC and TIL*

		% Stained Draining LNC[b]						% Stained TIL[b]					
PBMC[a]	DC[a]	hIL-2	hIFN-γ	hTNFα	mIL-2	mIFN-γ	mTNFα	hIL-2	hIFN-γ	hTNFα	mIL-2	mIFN-γ	mTNFα
+	None	5.0	1.9	5.3	3.6	10.8	5.9	10.8	6.3	2.3	2.2	20.4	6.8
+	Unloaded	10.8*	2.8	8.6*	nt	nt	nt	9.3	7.1	5.9*	nt	nt	nt
+	Loaded	27.1*	24.2*	15.6*	4.2	25.9*	9.8*	29.2*	38.1*	19.3*	2.9	21.3	7.0

[a] PBMC and DC were derived from an HLA-A2.1$^+$ donor. Where indicated, DC were loaded with 10 μg/ml of Pep $aa_{231–243}$. DC (1×10^6) and 1×10^7 PBMC were injected i.v. into SCID mice in intervals of 10 days. Five days after the first injection, mice received a s.c. injection of 5×10^4 BLM-gp100. Draining lymph node cells were harvested after 4 wk and TIL were harvested when the mean tumor diameter reached 0.5 cm.

[b] The percentage of stained cells was derived from the total population of draining LNC or TIL (i.e., without preselecting human leukocytes). Values represent the mean of five animals. SD, not presented for clarity of presentation, was in the range of 3–10%.

*, Significance of differences ($p < 0.05$).

cell response by peptides predicted to potentially bind to a HLA-DR haplotype.

Gp100 HLA-DR promiscuous binding peptides were predicted by the TEPITOPE software package using a stringent threshold (58, 60, 61). The observed response correlated with the predicted HLA-DR binding specificity. Yet none of the donors reacted with all potentially binding peptides. There are two possible explanations which are not mutually exclusive. The TEPITOPE prediction software is a useful tool for the identification of candidate T cell epitopes in any protein Ag, allowing a considerable reduction of the number of peptides to be assayed in vitro. A recent report using TEPITOPE to identify candidate T cell epitopes in the tumor Ag MAGE-3 shows that PBMC of a healthy donor responded strongly to one of five peptides (59). In search for DR4-restricted MART-1 epitopes, three of six predicted peptides were actually binding (82). We find these reports in line with our proliferative response results (one to two of seven peptides). Alternatively, a state of partial tolerance cannot be excluded. Like many tumor-associated Ags, gp100 is a differentiation Ag (6). Although it has been shown repeatedly that CTL precursors with specificity for weakly abundant self Ags are not necessarily deleted, it has been expected that tolerance at the level of Th cells may be more stringent, such that the presence of CTL precursors could not become harmful because of the missing help for maturation and activation (46). Thus, the

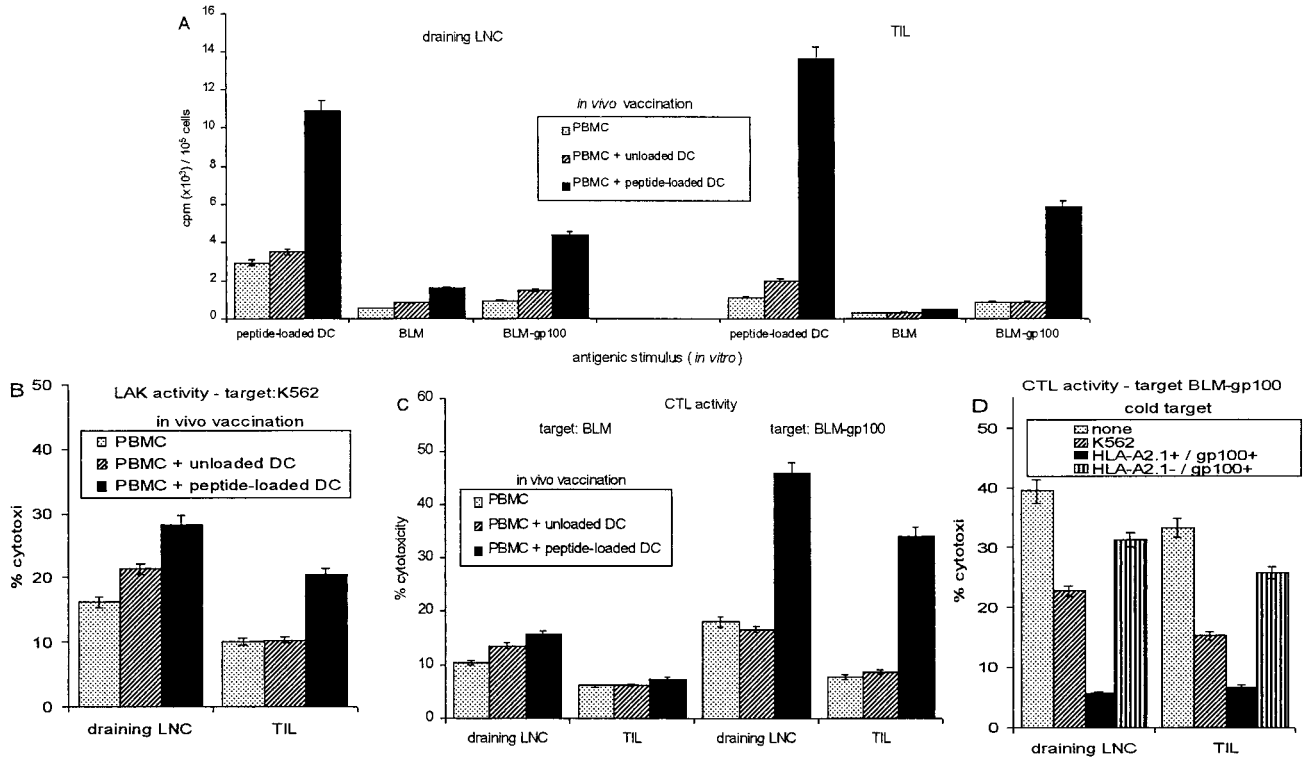

FIGURE 5. Proliferative and cytotoxic activity of draining LNC and TIL of tumor-bearing mice vaccinated with gp100 peptide-loaded DC. SCID mice were treated as described above. Draining lymph nodes were harvested after 4 wk. TIL were harvested after 4 wk (mice receiving PBMC or PBMC plus unloaded DC) or after 6 wk (mice receiving PBMC plus peptide-loaded DC) when the tumor reached a mean diameter of 0.5 cm. *A*, Proliferation was evaluated in response to peptide ($aa_{231–243}$)-loaded DC (1×10^4/well), irradiated BLM cells (1×10^4/well), or irradiated BLM-gp100 cells (1×10^4/well). The mean cpm ± SD at 10^5 cells/well is shown. *B*, LAK activity was evaluated using freshly harvested draining LNC or TIL and K562 cells as target. The percentage cytotoxicity at a ratio of E:T = 50:1 is shown. *C*, CTL activity was evaluated after restimulation of draining LNC and TIL with irradiated BLM-gp100 cells for 7 days. The percentage cytotoxicity toward BLM-gp100 cells and for comparison toward BLM cells at a ratio of E:T = 25:1 is shown. *D*, The HLA-A2.1 restriction of the CTL response shown in *C* was controlled by adding the following cold target: K562 cells, 530C1 (HLA-A2.1$^-$, gp100$^+$), or FM3 (HLA-A2.1$^+$, gp100$^+$). Cold targets were added at a ratio of cold target:target equal to 10:1. The E:T ratio was 25:1.

observation that individuals sharing HLA-DR alleles did react with different peptides could well be due to tolerance toward selected epitopes. In addition, it has been reported particularly for gp100 that even at the CTL level a state of anergy toward dominant epitopes may be observed, while responsiveness toward subdominant entities remained unaltered (11). Finally, the reactivity of PBMC from patients with gp100$^+$ malignant melanoma was neither enhanced nor reduced as compared with healthy donors. There are two mutually not exclusive explanations. The most likely explanation for not being confronted with a state of tolerance/anergy may rely on the low amount of presented peptide available in the in vivo situation. This would be in line with our observation that a large amount of peptide was required to induce an efficient Th response. Alternatively, we actually may have measured a memory response because gp100 is a differentiation Ag to which some immune response can be preexistent (83). This could explain why healthy donors and melanoma patients responded equally well. In this case too, one could argue that only low affinity T cells, which escaped tolerance induction, may have been selected. Even if this holds true, it would appear promising for therapeutic interventions, because it implies that a therapeutically efficient response can even be induced by low affinity T cells. Finally, it has been described that externally loaded peptides may differ slightly from naturally processed peptides, which could result in a reduced responsiveness of T cells toward the latter (84). Although we cannot exclude the possibility of induction of a suboptimal response, we clearly could demonstrate that T cells primed with peptide-loaded DC recognize naturally processed gp100 and vice versa. Similar findings have recently been reported by Touloukian et al. (84). In an elegant study, the authors showed that HLA-DR transgenic mice responded to the gp100 protein and that response was specific for a computer-predicted epitope. In the reverse setting, human PBMC primed with peptide-loaded DC recognized and lysed melanoma cells (84).

Taken the facts that the observed response correlated with the peptide binding specificity and the donor haplotype, that reactivity as well as nonreactivity of an individual donor's PBMC were stable attributes, and that PBMC of none of the donors responded to all peptides, both the use of a prediction program like TEPITOPE and a procedure to evaluate the reactivity profile in PBMC of individual patients appear to be necessary, but probably also are sufficient for setting up an "individual-specific" vaccination protocol. A HLA-DR binding assay (85) as recently reported for MART-1 by Zarour et al. (86) could provide a valuable additional means of reassuring binding.

Activation of Th cells by pulsing DC with peptides predicted to bind to a given HLA-DR haplotype has been suggested by the rapid induction of a proliferative response, the preferential expansion of CD4$^+$ cells in vitro as well as in vivo, the up-regulation of the costimulatory molecule CD40L, and the strong expression of the high avidity α-chain of the IL-2R on CD4$^+$ cells. The high percentage of CD4$^+$ cells which produced IL-2 and IFN-γ provided additional evidence for an efficient activation of gp100 peptide-specific Th cells. A significant increase in the frequency of IFN-γ-secreting cells has also been described for a MART-1 peptide presented by HLA-DR4 (86). Furthermore, vaccination with DC which had been loaded with a gp100 peptide sufficed for the activation and expansion of peptide-specific Th in vivo. Most convincingly, growth of an HLA-A-matched malignant melanoma was significantly retarded by repeated application of peptide-loaded DC plus autologous PBMC.

The efficacy of peptide-loaded DC in tumor rejection/growth retardation preferentially would have been tested with a gp100-positive melanoma and autologous PBMC. Such a system not being available, we used the HLA-A2.1$^+$ melanoma BLM-gp100 and DC/PBMC of a HLA-A2.1$^+$ healthy donor, the PBMC strongly responding to the gp100 peptide aa$_{231–243}$. By repeated application of peptide-loaded DC and PBMC, tumor growth could be significantly retarded. This accounted selectively for BLM-gp100 and not for BLM cells, pointing toward induction of a gp100-specific response on top of the allogeneic reaction. Furthermore, an efficient recruitment of PBMC into the tumor was only observed in mice receiving peptide-loaded DC. TILs selectively proliferated in response to the peptide used for DC loading and displayed high levels of CTL and LAK activity. We interpret these findings in the sense that only in the presence of peptide-loaded DC gp100-specific Th cells became selectively activated and homed into the tumor. Apparently the gp100-specific Th cells did not only recruit CTL, but also NK cells. A recent report by Hung et al. (87) describes a broad role of CD4$^+$ cells in orchestrating the host response including the activation of Th1 and Th2 cells, which activate eosinophils and macrophages. After vaccination with peptide-loaded DC we did not observe activation of Th2 cells (i.e., expression of IL-4 and IL-10 in TIL was low (data not shown)). Yet, the analysis of TIL confirmed that elements of the nonadaptive immune defense (i.e., monocytes and NK cells) were recruited only by DC loaded with an MHC class II-restricted peptide and only in this setting NK cells recovered from the tumor-displayed high cytotoxic activity. Furthermore, we observed a strong gp100-specific, HLA-A2.1 restricted (i.e., CD8-mediated) CTL response, which implies that activation of gp100 peptide-specific Th sufficed for activation of CD8$^+$ cells and their recruitment into the tumor. Whether CD4$^+$ T cells also contributed to tumor cell lysis cannot be elaborated on in our experimental setting. Finally, we want to mention that we consider it of utmost importance to see whether, by vaccination with DC that have been pulsed with MHC class II-binding peptides, a memory T cell response can be induced. Because this is not possible in the humanized SCID mouse due to the appearance of GvH reactions, we are currently exploring this question in the murine B16 melanoma model.

Taken together, there are four important messages. First, TEPITOPE proved a useful prediction tool to identify candidate T cell epitopes from protein Ags of interest and minimize the subsequent laboratory work. Second, PBMC of healthy donors, but also of patients with malignant melanoma contain Th cells specific for gp100-derived peptides. Third, the screening for reactivity of an individual patient's PBMC toward a given peptide can be performed in a short time. Fourth, vaccination with DC loaded with an MHC class II-binding peptide suffices for the induction of a potent Th cell response in vivo with recruitment of monocytes, NK and CD8$^+$ CTL into the tumor.

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