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Retrovirally Transduced Human Dendritic Cells Can Generate T Cells Recognizing Multiple MHC Class I and Class II Epitopes from the Melanoma Antigen Glycoprotein 100

Réjean Lapointe, Richard E. Royal, Mark E. Reeves, Ivy Altomare, Paul F. Robbins, and Patrick Hwu[1]

Involvement of tumor-Ag specific $CD4^+$ and $CD8^+$ T cells could be critical in the generation of an effective immunotherapy for cancer. In an attempt to optimize the T cell response against defined tumor Ags, we previously developed a method allowing transgene expression in human dendritic cells (DCs) using retroviral vectors. One advantage of using gene-modified DCs is the potential ability to generate $CD8^+$ T cells against multiple class I-restricted epitopes within the Ag, thereby eliciting a broad antitumor immune response. To test this, we generated tumor-reactive $CD8^+$ T cells with DCs transduced with the melanoma Ag gp100, for which a number of HLA-A2-restricted epitopes have been described. Using gp100-transduced DCs, we were indeed able to raise T cells recognizing three distinct HLA-A2 epitopes within the Ag, $gp100_{154-162}$, $gp100_{209-217}$, and $gp100_{280-288}$. We next tested the ability of transduced DCs to raise class II-restricted $CD4^+$ T cells. Interestingly, stimulation with gp100-transduced DCs resulted in the generation of $CD4^+$ T cells specific for a novel HLA-DRβ1*0701-restricted epitope of gp100. The minimal determinant of this epitope was defined as $gp100_{174-190}$ (TGRAMLGTHTMEVTVYH). These observations suggest that retrovirally transduced DCs have the capacity to present multiple MHC class I- and class II-restricted peptides derived from a tumor Ag, thereby eliciting a robust immune response against that Ag. *The Journal of Immunology*, **2001, 167: 4758–4764.**

Proteins preferentially expressed in tumors can be used as targets in T cell-mediated cancer immunotherapy (reviewed in Refs. 1–3). These include tissue differentiation proteins such as gp100 and Melan-A/MART-1, which are proteins expressed in both melanoma and melanocytes (4); tumor/testis Ags such as NY-ESO-1 (2); and mutated Ags such as Ras and p53 (5). Cancer vaccine trials using Ag-specific immunization have resulted in increased levels of circulating T cells capable of recognizing tumors (6). However, clinical responses have been limited (6, 7), and improved methods of immunization are needed.

Among the cells of the immune system, dendritic cells (DCs)[2] have a central role in primary activation of quiescent B and T lymphocytes (8) and may be useful for the generation of T cell-mediated immune responses against tumor Ags (9). Several approaches have been described that use tumor/DC fusions (10) or DCs pulsed with preparations derived from whole tumors such as total RNA (11) or apoptotic bodies (12–14). Although these strategies may induce immune responses against multiple relevant tumor Ags, the use of complex mixtures of proteins may result in competition and attenuation of the most potent immune interactions (15).

DCs have been manipulated to present tumor Ags by pulsing with MHC-restricted peptides (16, 17), but this approach limits the response to identified Ag epitopes and requires that patients express the appropriate HLA alleles. In addition, some epitopes presented by MHC molecules are modified post-transcriptionally (18, 19). Expressing whole tumor Ags in DCs may allow the presentation of multiple epitopes via MHC class I and possibly MHC class II molecules as well as undefined and post-transcriptionally modified epitopes.

Several strategies have been used to transfer genes into DCs, such as recombinant adenoviruses (20), recombinant poxviruses (21), or recombinant retroviruses (22). Because of the potential of viral Ags, often coexpressed in many vector systems, to be immunodominant over tumor Ags, we have developed methods to transduce DCs with recombinant retroviral vectors that solely express the gene of interest (22). Previously, we reported that retrovirally transduced DCs have the capacity to efficiently generate $CD8^+$ T cells reactive against a single HLA-A2 epitope of MART-1 (22). In addition, retrovirally transduced DCs were effective in generating class I-restricted T cells and treating established lung metastases in an experimental murine model system (23). However, it is unclear whether retrovirally transduced DCs can present multiple MHC class I epitopes derived from a tumor Ag.

Besides the generation of class I-restricted T cells, retrovirally transduced DCs may be capable of presenting Ags in the context of class II. Recent observations suggest that the stimulation of both $CD4^+$ and $CD8^+$ T cells against tumor Ags can lead to an effective anti-tumor response (24, 25). Consequently, it is of particular importance to determine whether gene-modified DCs can generate T cells reactive against both MHC class I- and class II-restricted epitopes.

In the present study DCs were retrovirally transduced with the melanoma differentiation Ag gp100 and used to stimulate T cells. The T cells generated were analyzed for the capacity to recognize multiple MHC class I and class II epitopes of gp100. The gp100-transduced DCs were able to generate T cells recognizing three distinct HLA-A2-restricted epitopes of the tumor Ag. Interestingly,

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[2] Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; SCF, stem cell factor; TIL, tumor-infiltrating lymphocytes; VSV, vesicular stomatitis virus.

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$CD4^+$ T cells specific for a new HLA-DRβ1*0701 epitope of gp100 were generated. The ability of retrovirally transduced DCs to generate T cells reactive against multiple MHC class I and class II epitopes of a tumor Ag may be significant for in vitro analysis of tumor Ags and, more importantly, for cancer patient immunotherapy.

Materials and Methods

Media and cell culture

Complete medium consisted of IMDM (Biofluids, Rockville, MD and Life Technologies, Gaithersburg, MD) supplemented with 10% human AB serum (male, heat inactivated; Gemini Bio-Products, Calabasas, CA), 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Biofluids), and 50 μg/ml gentamicin (Life Technologies). DC medium consisted of complete medium supplemented with 100 ng/ml GM-CSF, 100 ng/ml TNF-α (both obtained from PeproTech, Rocky Hill, NJ), and 40 ng/ml stem cell factor (SCF; R&D Systems, Minneapolis, MN). T cell medium consisted of complete medium supplemented with 60–300 IU/ml rIL-2 (Chiron, Emeryville, CA). T cell clones and tumor-infiltrating lymphocytes (TIL) 1520 were cultured in AIM-V medium (Life Technologies) supplemented with 5% human serum, 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamicin. rIL-2 at a concentration of 6000 IU/ml was added for TIL 1520 and at 300 IU/ml for the T cell clones.

Melanoma cell lines from patient A as well as cell lines 624.38 mel and 586 mel were developed at the Surgery Branch (National Cancer Institute, National Institutes of Health, Bethesda, MD) (26), and SK23 mel and MDA231 (breast cancer) were obtained from American Type Culture Collection (Manassas, VA). Because the melanoma line from patient A failed to express gp100, this line was transduced using a vesicular stomatitis virus (VSV)-pseudotyped retroviral vector expressing either gp100 or green fluorescent protein as described above. EBV-immortalized B cells were generated as previously described (26). Transduced cells were cultured in RPMI 1640-based medium containing 500 μg/ml geneticin sulfate (Life Technologies). All tumor and EBV-B cell lines were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS (Biofluids and Life Technologies) and antibiotics. T2 cells (American Type Culture Collection) are a TAP-deficient, HLA-A2-positive cell line and were grown in RPMI 1640 supplemented with 10% human AB serum (heat-inactivated) and antibiotics.

$CD34^+$ hemopoietic progenitor cells were obtained from HLA-A2-positive patients undergoing treatment for melanoma as part of an institutional review board-approved protocol (Surgery Branch, National Cancer Institute). $CD34^+$ cells were mobilized in peripheral blood by five daily s.c. injections of 10 μg/kg G-CSF (Neupogen; Amgen, Thousand Oaks, CA), followed by lymphocytopheresis to obtain PBMCs on day 6. $CD34^+$ cells were selected by an immunoaffinity column (CellPro, Bothell, WA) and cryopreserved. A single leukapheresis typically yielded 2–5 $\times$ 10^8 $CD34^+$ cells.

Retroviral vectors

The PG13-based gp100 retroviral packaging cell line was generated by inserting the complete gp100 cDNA into the retroviral vector SAMEN (27) as described previously (22). Producer cell medium consisted of DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS and antibiotics.

The gp100-VSV-pseudotyped retroviral system was prepared by first inserting the complete gp100 sequence in the pCLNC retroviral plasmid (28). The pCLNC-gp100 and pMDG-VSV plasmids were cotransfected in 293-gag-pol packaging cells using Lipofectamine Plus (Life Technologies). The 293-gag-pol packaging cells, provided by I. Verma (The Salk Institute, La Jolla, CA), were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. Medium was changed 16 and 48 h after transfection. Culture supernatants were harvested on days 3 and 4 after transfection of the 293-gag-pol cells. Producer cells were removed from retroviral supernatant by filtration with a 0.2-μm filter (Nalgene, Rochester, NY). Supernatants were immediately frozen at −70°C for future use.

Transduction of $CD34^+$-derived DCs

$CD34^+$ cells were differentiated to DCs according to the protocol previously described (22). Briefly, $CD34^+$ cells were thawed, washed in complete medium, and plated at 5 $\times$ 10^6 cells/well in a six-well plate or at 5 $\times$ 10^5 cells/well in a 24-well plate in complete DC medium containing TNF-α, SCF, and GM-CSF. Cultured cells were harvested on days 5 and 10, centrifuged, and resuspended in complete DC medium with cytokines. Cells were used on day 14, and DC phenotype was confirmed by morphological and FACS analyses (22).

For transduction with the PG13 system, $CD34^+$ cells were cocultured with irradiated PG13-SAM-gp100-EN producer cells (31 Gy; 7 $\times$ 10^5 cells/well in a six-well plate) in complete DC medium with cytokines containing 8 μg/ml polybrene (Aldrich, Milwaukee, WI). After 30 h DCs were replated on fresh irradiated producer cells in DC medium including cytokines without polybrene for 24 h. On day 3, transduced DCs were resuspended in fresh complete DC medium in a six-well plate, and differentiation was completed as described above.

For transduction with the VSV-pseudotyped retroviruses, retroviral supernatant was added to cultured $CD34^+$ cells on days 2 and 3 at a ratio of 1:1 with culture medium. GM-CSF, SCF, TNF-α, and polybrene were added, and cells were spun in the plate at 1000 $\times$ g for 1 h. On day 4 transduced DCs were resuspended in fresh complete DC medium in a six-well plate, and differentiation was completed as described above.

Mixed leukocyte reaction

MLR was performed as described previously (22) with minor modifications. Briefly, 1 $\times$ 10^5 allogeneic enriched T cells (human T cell immunoaffinity columns; R&D Systems) were cocultured with increasing numbers of irradiated (1500 rad) DCs in 96-well, flat-bottom tissue culture plates for 6 days. T cell proliferation was monitored by [^{3}H]thymidine (DuPont-New England Nuclear, Boston, MA) incorporation for the last 16 h. Results were corrected for [^{3}H]thymidine incorporation by irradiated DCs and T cells alone.

Stimulation of autologous T lymphocytes with transduced DCs

Autologous human PBMC were obtained from leukapheresis of patients at the Surgery Branch (National Cancer Institute) and were cryopreserved for use in experiments. T lymphocytes were isolated from PBMC using a human T cell immunoaffinity column (R&D Systems). Purified T cells (2 $\times$ 10^6) were cocultured with irradiated gp100-transduced DCs (2 $\times$ 10^5, 15 Gy) in a 24-well plate in 2 ml complete medium without rIL-2. rIL-2 (60–300 U/ml) was added on day 2, and the cells were diluted with fresh complete medium and rIL-2 to keep the cell concentration at 1–2 $\times$ 10^6 cell/ml. T cells from patient A were obtained before any nonsurgical treatment of melanoma and were stimulated once with gp100-transduced DCs. T lymphocytes from patient B were obtained 8 mo after immunization with $gp100_{280-288}$ peptide in IFA (29). In some cultures a combination of soluble trimeric recombinant CD40 ligand (CD40L; Immunex, Seattle, WA) and LPS prepared from *Salmonella typhimurium* (Sigma, St. Louis, MO) was added to the T cell stimulation with the gp100-transduced DCs. CD40L, and LPS can increase the capacity of DCs to stimulate the generation of Ag-specific T cells (30).

Where indicated, the bulk T cell cultures were cloned by limiting dilution at 1, 2, or 10 cells/well in 96-well, round-bottom plates (31). Cloning was performed in complete medium using AIM-V medium supplemented with 5% human AB serum, 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml gentamicin in the presence of 5 $\times$ 10^4 irradiated PBMC prepared from three different donors (from normal volunteers at the Clinical Center, National Institutes of Health), 30 ng/ml anti-CD3 (OKT3; Ortho-Biotech, Raritan, NJ), and 300 U/ml rIL-2. Relevant clones were expanded in T-25 tissue culture flasks at 1–5 $\times$ 10^5 cells/flask with 2.5 $\times$ 10^7 irradiated PBMC feeders prepared from three different donors (Blood Bank, Clinical Center, National Institutes of Health) and 30 ng/ml anti-CD3 in 25 ml of AIM-V complete medium. On day 2, 300 U/ml rIL-2 was added, and on day 5, 20 ml of medium was replaced with fresh AIM-V complete medium containing 300 U/ml rIL-2. On day 8, cells were counted, and the cell concentration was maintained at 1–2 $\times$ 10^6 cells/ml in AIM-V complete medium with 300 U/ml rIL-2.

T cell assays

T cells were analyzed for their capacity to recognize tumors expressing gp100 and HLA-A2 or T2 cells pulsed with gp100-derived HLA-A2 binding peptides $gp100_{154-162}$ (KTWGQYWQV), $gp100_{209-217}$ (ITDQVPFSV), $gp100_{280-288}$ (YLEPGPVTA) (32), or an irrelevant HLA-A2-restricted peptide derived from the influenza M-1 protein. T2 cells were pulsed with peptides at 1 μg/ml for 3 h at room temperature and washed twice with PBS. Peptide-pulsed T2 or tumor cells (1 $\times$ 10^5) were incubated with 1 $\times$ 10^5 specific T cells in a 250-μl volume in 96-well, flat-bottom plates. Supernatants were harvested after 24 h, and human IFN-γ was assayed by ELISA using a commercially available kit (R&D Systems).

The gp100 peptide library consisted of 68 peptides of 17–21 residues overlapping by 10–12 aa. The peptides were synthesized by solid phase

F-moc methodology as previously described (33). A control peptide derived from the Ig κ-chain known to bind to HLA-DRβ1*0701 was also prepared (34). Recombinant gp100 protein was made and purified as previously described (35). Recombinant NY-ESO-1 protein is another tumor Ag (2) used as a negative control and was made and purified as previously described (36). EBV-B cells (1×10^5) were pulsed with gp100-purified protein or peptides for 18 h in 96-well, flat-bottom plates, and T cells were added directly to the pulsed B cells for a 24-h recognition assay. In some experiments EBV-B cells (5×10^5) were pulsed in 48-well plates for 18 h, and cells were washed twice with PBS. T cells were then cocultured with the pulsed EBV-B cells in 96-well, flat-bottom plates for 24 h. Supernatants were harvested, and human IFN-γ was assayed by ELISA using a commercially available kit (R&D Systems).

Results

Generation of retrovirally transduced, CD34$^+$-derived DCs

DCs derived from CD34$^+$ hematopoietic stem cells were generated according to the procedure previously described (22). Because the CD34$^+$ cells actively proliferate upon differentiation into DCs, retroviral vectors can be used to introduce genes in the genome. gp100 was used as a model melanoma Ag and was cloned into retroviral vectors. T cells recognizing an HLA-A2-restricted epitope of gp100 were used with transduced DCs to monitor the expression and presentation of gp100 through MHC class I. As shown in Fig. 1*A*, two T cell lines specific to an HLA-A2-restricted epitope of $gp100_{209-217}$ recognized gp100-transduced DCs, but not DCs transduced with the control retroviral vector. Similar results were obtained using different CD34 donors.

Because DCs are potent T cell activators, an MLR was next performed to determine whether their capacity to stimulate T cells was affected by retroviral transduction. As shown in Fig. 1*B*, gp100 retrovirally transduced DCs equally stimulated the proliferation of allogeneic T cells compared with DCs transduced with control vectors or untransduced DCs.

Because DCs were successfully transduced with gp100 without affecting their capacity to stimulate T cells, gp100-transduced DCs were next used in T cell sensitization studies.

Generation of T cells recognizing multiple HLA-A2-restricted epitopes of gp100

To determine whether gp100-transduced DCs could stimulate the generation of specific CD8$^+$ T cells recognizing multiple epitopes within gp100, resting T cells from melanoma patients were cultured with autologous gp100-transduced DCs. After one or two T cell stimulations using transduced DCs, two of four patients tested had T cells reactive against HLA-A2$^+$/gp100$^+$ tumor cells (Table I). T cell cultures derived from patients A and B were reactive against two HLA-A2$^+$/gp100$^+$ tumor cell lines (624.38 mel and SK23 mel). These T cells failed to react with HLA-A2$^-$/gp100$^+$ or HLA-A2$^+$/gp100$^-$ tumor cells (586 mel and MDA231, respectively), confirming the HLA-A2 and gp100 specificity. Importantly, these cells recognized all three defined HLA-A2-specific, gp100-restricted epitopes tested using peptides $gp100_{154-162}$, $gp100_{209-217}$, and $gp100_{280-288}$ when pulsed on T2 cells. Peptide recognition using T cells from both patients were specific for gp100, because they failed to react against an unrelated peptide from the influenza M-1 protein. These results demonstrate that gp100-transduced DCs can stimulate autologous T cells reactive against multiple gp100-restricted HLA-A2 defined epitopes.

*Generation of T cells recognizing a new HLA-DRβ1*0701 epitope of gp100*

In addition to presentation of epitopes by MHC class I, transduced DCs may present peptides in the context of MHC class II molecules. T cell stimulations were next performed to determine whether gp100-transduced DCs could generate Ag-specific CD4$^+$ T cells recognizing gp100 peptides presented by MHC class II. T cells from patient A were stimulated twice with DCs retrovirally transduced with gp100. Stimulated T cells were analyzed for their capacity to recognize a gp100-expressing autologous melanoma cell line in an MHC class II-restricted fashion. The cultured T cells were reactive against the autologous tumor line expressing

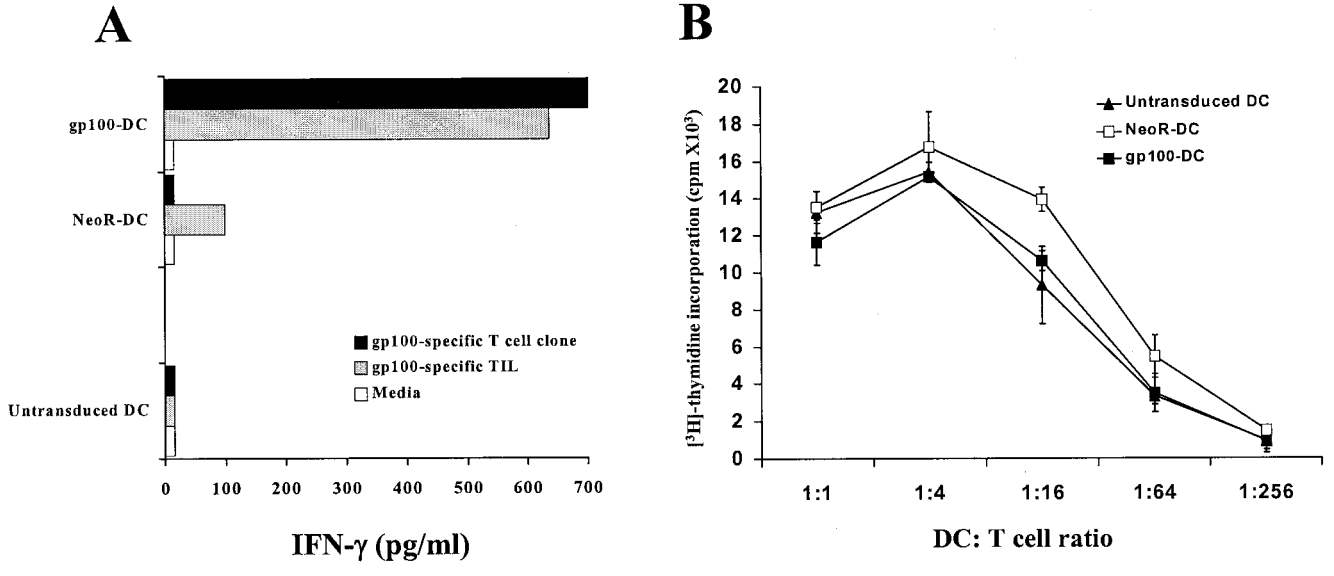

FIGURE 1. Characterization of gp100-transduced DCs for the presentation of an HLA-A2 epitope of gp100 and their capacity to stimulate allogeneic T cells in MLR. DCs were cultured and gp100-transduced as described in *Materials and Methods*. *A*, gp100-transduced (gp100-DC) or control (NeoR-DC) DCs were incubated with TIL or cloned T cells recognizing the HLA-A2-restricted $gp100_{209-217}$ epitope. Results are expressed as picograms per milliliter of IFN-γ released following a 24-h coculture. *B*, Transduced or control DCs were irradiated and incubated with 1×10^5 allogeneic T cells at various cell ratios as indicated. [^{3}H]Thymidine was added for the last 16 h of a 6-day proliferation assay, and incorporation was measured as described in *Materials and Methods*.

Table I. *HLA-A2-reactivity of T lymphocytes generated with gp100-transduced DCs*[a]

	IFN-γ (pg/ml)								
		T2 pulsed with Peptides				Tumors			
	Media	fluM-1	G9-154	G9-209	G9-280	624.38	SK23	586	MDA231
Patient A									
Media	<16	<16	<16	<16	<16	<16	<16	<16	<16
DC-control	33	129	82	115	119	53	100	34	31
DC-gp100	47	102	**1654**	1384	**2170**	**340**	**700**	63	45
Patient B									
DC-control	<16	<16	17	<16	<16	<16	<16	<16	<16
DC-gp100	<16	<16	**464**	**499**	**1629**	**250**	**384**	<16	<16

[a] gp100-transduced DCs were used to stimulate autologous T cells prepared from melanoma patients. Patient A: T cells received one stimulation with DCs, and the recognition assay was performed 14 days later. Patient B: autologous T cells received two stimulations with DCs 14 days apart, and the recognition assay was performed 28 days after the first stimulation. Specificity of stimulated T cells from both patients was analyzed using T2 cells pulsed with the influenza M-1 peptide (fluM1) or with the gp100-specific peptides $gp100_{154-162}$ (G9-154), $gp100_{209-217}$ (G9-209), or $gp100_{280-288}$ (G9-280). Also, HLA-$A2^+$/$gp100^+$ tumor cells (624.38 and SK23) or HLA-$A2^-$/$gp100^+$ (586) or HLA-$A2^+$/$gp100^-$ (MDA231) tumor cells were used as targets. Results are expressed in picograms per milliliter of IFN-γ released following a 24-h coculture. DC-gp100, gp100-transduced DCs. DC-control, DCs transduced with the parental retroviral vector.

HLA-DR and gp100, but not against gp100-negative melanoma cells or autologous cultured B cells (data not shown).

The reactive bulk T cell culture was cloned by limiting dilution, and growing wells were screened using autologous melanoma cells expressing gp100 or green fluorescent protein as a negative control. Forty-six of 167 wells were specifically reactive against the autologous melanoma expressing gp100 and class II. The reactivity of nine representative positive clones is presented in Fig. 2*A* along with four representative negative wells (neg1 to neg4). Eight of the 46 positive clones were expanded and further characterized. All eight clones were $CD4^+$ (data not shown), suggesting recognition of gp100 peptides presented by MHC class II. To further characterize the reactivity, some of the clones were cocultured with autologous B cells pulsed with a bacterially produced gp100 protein. All the $CD4^+$ T cell clones tested secreted IFN-γ when exposed to autologous B cells pulsed with gp100, but not those pulsed with an irrelevant tumor Ag prepared using a similar procedure. The results from two representative clones are presented in Fig. 2*B*. Clones 6 and 8 failed to react against NY-ESO-1, a protein produced similarly to recombinant gp100. To identify the MHC restriction element, Abs known to bind and block presentation by defined MHC molecules were used. Recognition by clones 6 and 8 of target cells pulsed with the gp100 protein was inhibited when using blocking Abs against MHC class II and HLA-DR, but the recognition was unchanged when using a blocking MHC class I Ab. Also, both clones failed to recognize gp100 when pulsed on unmatched HLA-DR B cells. By using HLA-DR-matched B cells, the restriction element was identified as HLA-DRβ1*0701 (patient A is HLA-DRβ1*0701 and *1601; Fig. 3).

To find the epitope recognized, an overlapping gp100 peptide library consisting of 68 peptides of 17 or 21 residues overlapping by 10–12 aa was prepared. The autologous EBV-B cells were pulsed with the peptide library and used as targets in a T cell recognition assay using three different T cell clones. Two clones failed to recognize any peptides, and one clone specifically recognized peptide $gp100_{170-190}$ (Fig. 4*A*; data from two clones shown). Using a $gp100_{170-190}$-specific T cell clone, a titration experiment using the $gp100_{170-190}$ peptide pulsed on EBV-B cells revealed that recognition was undetectable below 4–10 μM (Fig. 4*B*). Interestingly, the $gp100_{170-190}$-specific T cell clone recognized B cells pulsed with gp100 protein at a concentration as low as 22 nM. The difference between the concentrations of protein and peptide required for recognition may be due to the greater susceptibility of peptide to degradation by serum proteases.

Finally, several peptides were synthesized to define an optimal HLA-DRβ1*0701 epitope. As shown in Table II, no peptides were better recognized at 10 or 1 μM compared with the $gp100_{170-190}$ peptide. Finally, substitutions were made at position 5 of the

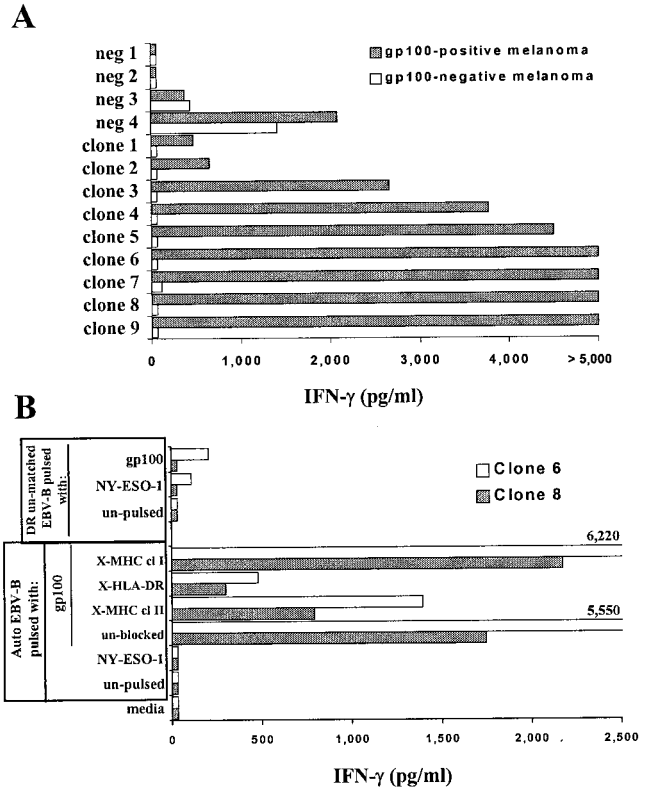

FIGURE 2. Generation of HLA-DR-specific T cells using gp100-transduced DCs. gp100-transduced DCs were used to stimulate autologous T cells in the presence of CD40L and LPS. After two stimulations, the cultured T cells were cloned by limiting dilution, and reactivity was assessed by recognition of autologous melanoma cells expressing gp100. *A*, Reactivity of some representative clones are shown. *B*, Reactivity of clones 6 and 8 against autologous (Auto) or HLA-DR-unmatched EBV-B cells after pulsing with control protein (NY-ESO-1) or gp100-purified protein. Abs blocking MHC class I (X-MHC cl I), MHC class II (X-MHC cl II), or HLA-DR (X-HLA-DR) recognition were added to the assay. Results are expressed as picograms per milliliter of IFN-γ released following a 24-h coculture. neg, Negative.

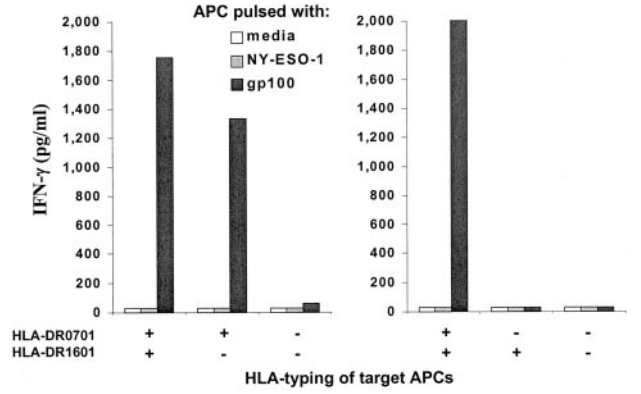

FIGURE 3. Specific recognition by a T cell clone of HLA-DR-matched B cells pulsed with purified gp100. T cells were cocultured with EBV-B cells prepared from donors sharing different HLA-DR alleles after a 16-h pulsing with purified proteins (NY-ESO-1 or gp100). Results are expressed as picograms per milliliter of IFN-γ released following a 24-h coculture.

$gp100_{174-194}$ peptide, presuming that this is position 1 according to the defined motif for HLA-DRβ1*0701 (SYFPEITHI web site, http://syfpeithi.de/). However, none of the peptides was better recognized when pulsed at 10 or 1 μM compared with the wild-type $gp100_{174-194}$ peptide.

The data presented demonstrate that CD34-derived DCs retrovirally transduced with gp100 can generate T cells reactive against both MHC class I and class II gp100 epitopes.

Discussion

DCs are potent APC in the generation of specific T cells and may be critical in the generation of an anti-tumor immune response against a specific Ag. Optimally, the nature of such a response should include the activation of both $CD8^+$ and $CD4^+$ T cells recognizing multiple MHC class I and class II epitopes derived from the tumor Ag. The work presented in this paper suggests that retrovirally transduced DCs expressing a tumor Ag have the capacity to generate $CD8^+$ T cells reactive against multiple MHC class I-restricted epitopes, which could potentially be an advantage for the establishment of a broad immune response against tumors. Interestingly, the same DCs were efficient in the generation of $CD4^+$ T cells recognizing MHC class II epitopes of the Ag, and a new HLA-DRβ1*0701 epitope of gp100 was identified. Because 15–28% of the population express this HLA-DR allele, this gp100 peptide represents a potential candidate for patient immunization. To our knowledge this represents the first example of the generation of $CD4^+$ T cells recognizing a defined HLA class II epitope using tumor Ag-transduced DCs.

The generation of $CD4^+$ T cells using transduced DCs suggests that peptides derived from the endogenously expressed gp100 were presented by MHC class II molecules. Also, tumor cells expressing MHC class II and gp100 were efficiently recognized when using $CD4^+$ T cells specific for an HLA-DRβ1*0701 epitope of gp100. Classically, peptides presented by MHC class I molecules are derived from endogenously expressed proteins, while Ag presentation by MHC class II occurs mainly from exogenous Ags. This study suggests that endogenously expressed gp100 can traffic to the lysosomal/endocytic pathway to be presented by MHC class II. Interestingly, a dileucine-based melanosomal transport signal motif known to target proteins to the melanosome is present in gp100 (37). A similar motif derived from gp75/tyrosinase-related protein-1 has been previously shown to be critical for targeting to the lysosomal/endocytic compartment and for the presentation by MHC class II (38). It is unclear whether all tumor Ags will endogenously traffic to MHC class II, or whether this will require the addition of specific signaling motifs.

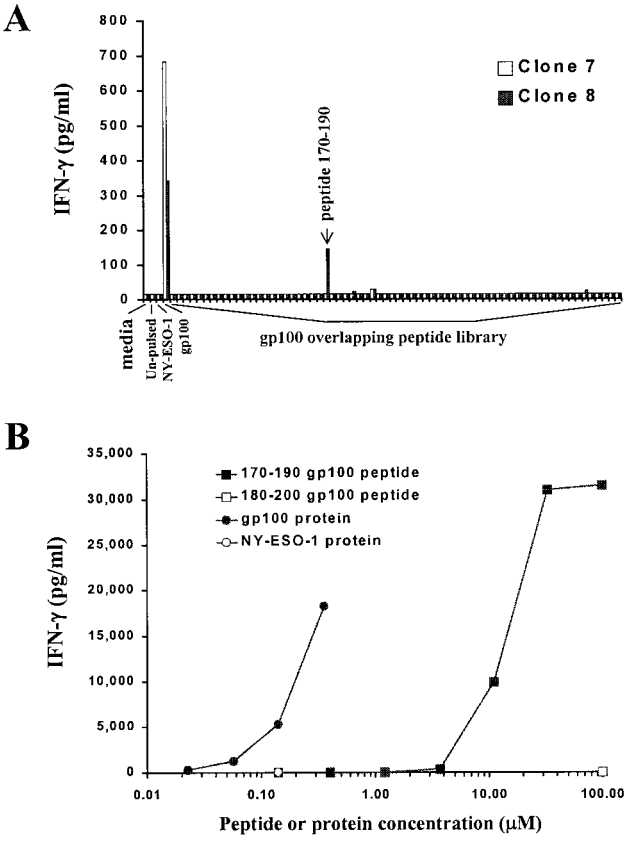

FIGURE 4. Identification of an HLA-DRβ1*0701 gp100-epitope using an overlapping peptide library. *A*, T cell clones 7 and 8 were cocultured with autologous EBV-B cells pulsed with NY-ESO-1 or gp100 proteins (10 μg/ml) or with 68 overlapping peptides derived from gp100 (all at 100 μM). *B*, A $gp100_{170-190}$-specific T cell clone was cocultured with autologous EBV-B cells after pulsing with gp100 protein or the $gp100_{170-190}$ peptide at different concentrations. Results are expressed as picograms per milliliter of IFN-γ released following a 24-h coculture.

It is not clear why gp100-specific $CD8^+$ T cells were raised in only two of four patients tested. This may be due to patient-to-patient variability in the number of pre-existing T cells capable of recognizing tumors. In addition, in this study, only one or two in vitro T cell stimulations with DCs were used. Further rounds of stimulation may have resulted in the detection of specific T cells in additional patients.

The generation of both $CD4^+$ and $CD8^+$ T cells using transduced DCs could be important for an optimal anti-tumor immune response. For anti-viral responses and in autoimmunity, $CD4^+$ T cells appear to help control and sustain $CD8^+$ T cell activity (39–41). Recent observations suggest that $CD4^+$ T cells help to enhance the anti-tumor activity of $CD8^+$ T cells (24, 25). However, the precise mechanism of this help by $CD4^+$ T cells is not well defined. Possible mechanisms may include the activation of APC through the expression of CD40L (42) or the secretion of cytokines such as IL-2. In addition, activated $CD4^+$ T cells may have indirect effector functions, such as the recruitment of eosinophils and macrophages due to the secretion of both Th1- and Th2-type cytokines (25).

Therefore, the ability of transduced DCs to stimulate both $CD4^+$ and $CD8^+$ T cells could be exploited in tumor immunology in vitro for Ag characterization and in vivo for patient immunization. For example, in vitro strategies are needed to validate the immunologic

Table II. *Optimization of the DRβ1*0701 epitope of gp100*[a]

gp100		IFN-γ (pg/ml) 10 μM	1 μM
170–190	L SIGTGRAMLG THTMEVTVYH	21,000	944
168–188	SGL SIGTGRAMLG THTMEVTV	1,655	64
172–192	IGTGRAMLG THTMEVTVYH RR	17,050	257
174–194	TGRAMLG THTMEVTVYH RRGS	17,140	388
176–196	RAMLG THTMEVTVYH RRGSRS	912	<8
178–198	MLG THTMEVTVYH RRGSRSYV	52	<8
174–190	TGRAMLG THTMEVTVYH	14,010	186
174–191	TGRAMLG THTMEVTVYH R	11,100	146
174–192	TGRAMLG THTMEVTVYH RR	11,550	87
174–193	TGRAMLG THTMEVTVYH RRG	17,910	273
173–193	GTGRAMLG THTMEVTVYH RRG	22,910	502
173–190	GTGRAMLG THTMEVTVYH	18,770	456
172–190	IGTGRAMLG THTMEVTVYH	17,590	469
171–190	SIGTGRAMLG THTMEVTVYH	20,710	899
171–191	SIGTGRAMLG THTMEVTVYH R	12,210	185
174–194*5F	TGRA**F**LG THTMEVTVYH RRGS	19,390	516
174–194*5L	TGRA**L**LG THTMEVTVYH RRGS	23,140	559
174–194*5Y	TGRA**Y**LG THTMEVTVYH RRGS	11,640	191
	Ig κ-chain 188–201	<8	
	unpulsed	<8	
	NY-ESO-1 (142 nM)	<8	
	gp100 (142 nM)	13,730	

[a] Autologous EBV-B cells were pulsed with the different peptides or proteins at indicated concentrations for 18 h. Cells were washed and cocultured with a $gp100_{170-190}$-specific T cell clone. Results are expressed in picograms per milliliter of IFN-γ released following a 24-h coculture.

relevance of candidate tumor Ags that are overexpressed in tumors compared with normal tissues. Retrovirally transduced DCs could be used to characterize the potential immunogenicity of candidate tumor Ags and may allow the identification of both MHC class I- and class II-restricted epitopes. Moreover, effective methods are needed to evaluate the efficiency of patient immunization following vaccination with defined tumor Ags. Retrovirally transduced DCs could be used for the in vitro analysis of peripheral blood precursor frequency of tumor-specific T cells before and after patient vaccination. Finally, because retrovirally transduced DCs are efficient at generating both $CD4^+$ and $CD8^+$ T cells in vitro, they may also have applications in direct patient immunization. Such an immunization strategy would require the mobilization of $CD34^+$ hematopoietic stem cells from cancer patients, followed by retroviral transduction with the tumor Ag and in vitro differentiation to DCs. This approach would be feasible, because $CD34^+$ cell mobilization, retroviral transduction, and treatment with cultured cells are all techniques that have been used clinically. Optimal DC maturational signals as well as the most effective schedule and route of immunization remain to be defined.

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