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This information is current as of March 8, 2021.

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*J Immunol* 1999; 163:507-513; ;  
<http://www.jimmunol.org/content/163/1/507>

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# Efficient Transfer of a Tumor Antigen-Reactive TCR to Human Peripheral Blood Lymphocytes Confers Anti-Tumor Reactivity

Timothy M. Clay, Mary C. Custer, Jessica Sachs, Patrick Hwu, Steven A. Rosenberg, and Michael I. Nishimura<sup>1</sup>

The tumor-associated Ag MART-1 is expressed by most human melanomas. The genes encoding an  $\alpha\beta$  TCR from a MART-1-specific, HLA-A2-restricted, human T cell clone have been efficiently transferred and expressed in human PBL. These retrovirally transduced PBL cultures were MART-1 peptide reactive, and most cultures recognized HLA-A2<sup>+</sup> melanoma lines. Limiting dilution clones were generated from three bulk transduced PBL cultures to investigate the function of individual clones within the transduced cultures. Twenty-nine of 29 CD8<sup>+</sup> clones specifically secreted IFN- $\gamma$  in response to T2 cells pulsed with MART-1<sub>(27-35)</sub> peptide, and 23 of 29 specifically secreted IFN- $\gamma$  in response to HLA-A2<sup>+</sup> melanoma lines. Additionally, 23 of 29 CD8<sup>+</sup> clones lysed T2 cells pulsed with the MART-1<sub>(27-35)</sub> peptide and 15 of 29 lysed the HLA-A2<sup>+</sup> melanoma line 888. CD4<sup>+</sup> clones specifically secreted IFN- $\gamma$  in response to T2 cells pulsed with the MART-1<sub>(27-35)</sub> peptide. TCR gene transfer to patient PBL can produce CTL with anti-tumor reactivity in vitro and could potentially offer a treatment for patients with metastatic melanoma. This approach could also be applied to the treatment of other tumors and viral infections. Additionally, TCR gene transfer offers unique opportunities to study the fate of adoptively transferred T cells in vivo. *The Journal of Immunology*, 1999, 163: 507-513.

The isolation of tumor-reactive CTL from tumor lesions and the peripheral blood of cancer patients has enabled the identification of tumor-associated Ags (TAA)<sup>2</sup> in patients with melanoma, head and neck cancer, and renal cancer (1-5). In mice, the transfer of tumor-infiltrating lymphocyte (TIL) clones or TAA-reactive CTL clones generated in vitro by either autologous tumor stimulation or TAA-peptide stimulation has resulted in tumor regression (6-10). These experiments demonstrated that TAA can be relevant targets for cancer therapy and that adoptive transfer of T cells can treat established murine tumors. In human clinical trials, TIL expanded ex vivo and then adoptively transferred to melanoma patients with IL-2 resulted in objective responses in 34% of melanoma patients (11). Thus, cellular immunotherapy can be an effective treatment for patients with melanoma.

Adoptive transfer of TIL requires that T cells be isolated and expanded from individual patients. However, not all patients have accessible tumor lesions of sufficient size (>3 cm) to provide an adequate number of T cells for expansion. In addition, tumor-specific TIL can only be obtained from 50% of TIL cultures. Consequently, TIL therapy is a viable treatment option for only 35% of melanoma patients. An alternative approach would be to stimulate

and expand PBL in vitro with allogeneic human melanoma lines. These mixed lymphocyte tumor cell cultures can generate tumor-reactive CTL, but therapeutic cell numbers are difficult to attain (12, 13). Another approach is to vaccinate patients with a TAA-peptide and isolate TAA reactive CTL from PBMC by in vitro stimulation with TAA peptides (M. Dudley, personal communication). All these approaches require TIL or CTL clones to be generated and expanded for every patient and therefore may be impractical for treating a large number of patients.

We are developing alternative strategies to target TAAs by genetically modifying a patients' PBLs to produce anti-tumor reactivity. Our intention is to redirect the specificity of autologous peripheral blood T cells by the transfer of TCR genes from TAA-specific T cell clones into patient PBLs. We have previously described the cloning of the TCR genes from a tumor-reactive CTL clone (clone 5) derived from a TIL culture from patient 501. The clone 5 TCR is HLA-A2 restricted and is specific for the m9-27 peptide epitope of the melanoma-associated Ag MART-1, which is expressed by most melanoma tumors (14, 15). Transfection of the  $\alpha$  and  $\beta$  TCR genes from clone 5 into Jurkat cells resulted in the expression of a functional TCR on the cell surface (16). These experiments demonstrated that TCR gene transfer can result in the reconstitution of a functional TCR in transfected cells.

Retroviral vectors have been successfully used to transduce T cells (17, 18). A retrovirus that encodes the clone 5 TCR  $\alpha$ - and  $\beta$ -chains has now been constructed, and human PBL transduced with the clone 5 TCR genes recognized melanoma cells in vitro. To examine the reactivity of these gene-modified T cells, T cell clones were generated from transduced PBL cultures. CD4<sup>+</sup> and CD8<sup>+</sup> T cell clones were isolated that specifically secreted cytokine in response to tumor targets and/or T2 cells pulsed with m9-27 peptide. In addition, 23 of 29 CD8<sup>+</sup> clones lysed T2 cells pulsed with the m9-27 peptide, and 15 of 29 CD8<sup>+</sup> clones lysed an HLA-A2<sup>+</sup> melanoma line. These results demonstrate that TCR gene transfer to patient PBL is feasible and can result in CTL with

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Received for publication February 1, 1999. Accepted for publication April 14, 1999.

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<sup>2</sup> Abbreviations used in this paper: TAA, tumor associated Ag; CDR3, third complementarity determining region; cPCR, competitive PCR; TCRBV, TCR V $\beta$ ; TIL, tumor infiltrating lymphocyte.

anti-tumor reactivity *in vitro*. This strategy offers a potential therapy for metastatic melanoma.

## Materials and Methods

### Retroviral vector and supernatant production

A modified SAMEN' backbone was used (Fig. 1) (19). The full-length clone 5  $\alpha$ -chain cDNA was derived from pTA-V $\alpha$ 1 plasmid as a *Xho*I/*Sal*I fragment and ligated into the polylinker of the SAMEN' vector producing the V $\alpha$ 1-SAMEN' construct. The SR $\alpha$  promoter and full-length clone 5  $\beta$ -chain cDNA was isolated from the pcDL-V $\beta$ 7 plasmid and inserted into a *Sal*I/*Bgl*II site engineered downstream of the neomycin resistance gene in the V $\alpha$ 1-SAMEN' construct producing the A7 virus construct. The PG13 retrovirus producer cell line was transfected with the A7 virus construct, and high titer clones were isolated (20). A7/PG13 clone 6 was grown to 80% confluence in DMEM medium (Biofluids, Rockville, MD) supplemented with 10% FBS (Life Technologies, Grand Island, NY) and penicillin (100 U/ml)-streptomycin (100  $\mu$ g/ml)-glutamine (2.92 mg/ml) (Life Technologies). Then, 18 h before supernatant was to be harvested for PBL transductions, the media was exchanged with fresh media.

### Retroviral transduction of PBL

PBL were transduced using an adaption of the method described by Bunnell et al. (21). Briefly, human PBMC were isolated from buffy coats from normal donors at the National Institutes of Health Clinical Center by centrifugation through lymphocyte separation medium (Organon Teknika, Durham, NC). PBMC were cultured at  $1 \times 10^6$  cells/ml for 72 h in AIM-V serum-free medium (Life Technologies) supplemented with 600 IU/ml IL-2 (Cetus, Emeryville, CA) and 10 ng/ml anti-CD3 mAb (OKT3; Ortho Biotech, Raritan, NJ). On day 3, cells were harvested and resuspended at  $1 \times 10^6$  cells/ml in 24-well plates in retroviral supernatant containing 8  $\mu$ g/ml polybrene and 600 IU/ml IL-2. Plates were centrifuged at  $1000 \times g$  at 32°C for 90 min and then incubated overnight at 37°C in a humidified, 5% CO<sub>2</sub> incubator. This transduction procedure was repeated for a further 2 days. On day 6, the cells were harvested and resuspended at  $1 \times 10^6$  cells/ml in AIM-V supplemented with 600 IU/ml IL-2. On day 8, cell concentration was adjusted to  $1 \times 10^6$  cells/ml in AIM-V supplemented with 600 IU/ml IL-2 and 0.5 mg/ml geneticin (Life Technologies). PBL were selected in geneticin for 5 days, and the percent of live cells was monitored daily and their concentration was adjusted to  $10^6$  cells/ml. Following selection, on day 13, PBL were resuspended in AIM-V supplemented with 600 IU/ml IL-2. On day 15, PBL were tested in cytokine release assays. Bulk PBL cultures were cloned on day 16–18.

### Limiting dilution cloning of transduced PBL cultures

Transduced PBL were plated at 10, 1, and 0.3 cells per well in 96-well microtiter plates in 0.2 ml per well. Cells were expanded using anti-CD3 stimulation following the method of Walter et al. (22). Briefly, PBL were plated in RPMI 1640 (Biofluids) containing 11% heat-inactivated human pooled AB serum (Pel Freez, Brown Deer, WI), penicillin (100 U/ml)-streptomycin (100  $\mu$ g/ml)-glutamine (2.92 mg/ml) (Life Technologies), 25 mM HEPES, 25  $\mu$ M 2-ME, with  $2.5 \times 10^5$  irradiated allogeneic PBMC (100 Gy) per ml,  $5 \times 10^4$  irradiated allogeneic EBV-B cells (100 Gy) per ml, and 30 ng/ml anti-CD3 mAb (OKT3; Ortho Biotech). The following day, 120 IU/ml IL-2 was added. On day 5, the medium was exchanged and fresh medium containing 120 IU/ml IL-2, without OKT3, was added. On day 8, fresh IL-2 was added to yield a final concentration of 120 IU/ml. Cells were tested for reactivity on day 12 in cytokine release assays and peptide-specific/tumor-specific cloids were restimulated as described

above. To expand the reactive cloids, the culture volume was increased and the numbers of PBMC and EBV-B were adjusted accordingly.

### Tumor cell lines

HLA-A2-positive and -negative human melanoma cell lines expressing MART-1 and gp100 were established in the Surgery Branch from resected tumor lesions as previously described (23). 397 Mel (HLA-A2<sup>-</sup>), 397-A2 (HLA-A2<sup>-</sup> line transfected to express HLA-A2), 624–28 (HLA-A2<sup>-</sup>), 624–38 (HLA-A2<sup>+</sup>), 888 Mel (HLA-A2<sup>-</sup>), 888-A2 (HLA-A2<sup>-</sup> line transfected to express HLA-A2), 1011 Mel (HLA-A2<sup>-</sup>), 1088 Mel (HLA-A2<sup>+</sup>), and 1300 Mel (HLA-A2<sup>+</sup>) were maintained in complete medium consisting of RPMI 1640 medium supplemented with heat-inactivated 10% FBS (Biofluids), penicillin (100 U/ml)-streptomycin (100  $\mu$ g/ml)-L-glutamine (2.92 mg/ml) (Life Technologies).

### Bulk TIL cultures

TIL cultures were grown as previously described (24). Briefly, tumor samples were enzymatically digested to yield a single-cell suspension, and the cells were grown in AIM-V medium (Life Technologies) supplemented with 10% heat-inactivated pooled human AB serum (Sigma, St. Louis, MO) and 6000 IU/ml IL-2 (Cetus).

### Peptides

Peptides were synthesized on a model 422 peptide synthesizer (Gilson, Worthington, OH) using solid phase methods, as previously (25). The sequences of the peptides used in this study are as follows: gp100 g9-209 (ITDQVPFVS) (26); MART-1 m9-27 (AAGIGILTV) (27); tyrosinase 369-D (YMDGTMSQV) (28).

### Assessment of culture reactivity by cytokine release

PBL cultures and cloids were tested for reactivity in cytokine release assays using commercially available ELISA kits (IL-2 and GM-CSF, R&D Systems, Minneapolis, MN; IFN- $\gamma$ , IL-4, IL-10, and TNF- $\alpha$ , Endogen, Cambridge, MA). Cytokine release was measured with T2 cells either alone or pulsed with peptide (2  $\mu$ g/ml, or as described in figure legends) in complete medium for 2–3 h at 37°C. Cells were also tested for reactivity with MART-1- and gp100-expressing melanoma lines. For “screening” growth-positive cloids for further expansion, cloids were not counted, a quarter of the well volume was taken for the assays against 10,000 888 A2<sup>+</sup> and 888 A2<sup>-</sup> melanoma cells and/or 10,000 T2 cells pulsed with m9-27 peptide or an irrelevant peptide, in a 0.2-ml culture volume. For other assays, 100,000 responder cells and 100,000 stimulator cells were used in a 1.0-ml culture volume. Stimulator cells and responder cells were cocultured for 24 h. Cytokine secretion was measured in culture supernatants.

### Assessment of reactivity by lysis assay

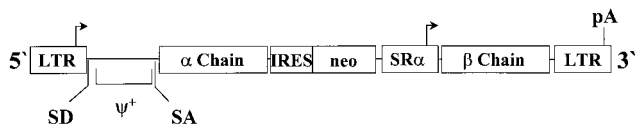
PBL cloids were tested for their ability to lyse HLA-A2<sup>+</sup>, MART-1<sup>+</sup> targets in <sup>51</sup>Cr-release assays. Target cells were T2 cells pulsed with either the MART-1 m9-27 peptide (2  $\mu$ g/ml) or an irrelevant peptide (either gp100 g9-209 or tyrosinase 369-D), and melanoma cell lines 888 HLA-A2<sup>-</sup> and 888 HLA-A2<sup>+</sup>, as previously described (29). Briefly, targets were labeled for 90 min with 200  $\mu$ Ci <sup>51</sup>Cr (10–35 mCi/ml, Amersham, Arlington Heights, IL) per  $1 \times 10^7$  cells, washed three times with HBSS and then plated in triplicate with responders at the following E:T ratios: 80:1, 20:1, 5:1, and 1.25:1, or as described in figure legends. Plates were incubated for 4 h at 37°C and then supernatants were harvested. <sup>51</sup>Cr release was measured in supernatants on a Wallac 1470 automatic  $\gamma$  counter (Wallac, Gaithersburg, MD), and percent specific lysis was calculated.

### Cell surface immunofluorescence

Bulk PBL cultures and cloids were stained with the following Abs: FITC isotype control, PE isotype control, human CD8 FITC, and human CD4 PE (Becton Dickinson, San Jose, CA). Cell surface immunofluorescence was measured on a FACScan flow cytometer (Becton Dickinson).

### Transduction efficiency measured by competitive PCR (cPCR) analysis

A genomic DNA competitor for cPCR analysis of transduction efficiency was generated from the clone 5 TCR  $\beta$ -chain cDNA by PCR (M. I. Nishimura, manuscript in preparation). The competitor is identical in sequence to the clone 5  $\beta$ -chain but contains a 50-bp deletion in the 5' end of the constant region, to allow discrimination of the competitor and clone 5



**FIGURE 1.** Structure of the A7 retrovirus. A modified SAMEN' pg1 Moloney murine leukemia virus backbone. Expression of the TCR  $\alpha$ -chain is driven by the promoter in 5' long terminal repeat (LTR), which also drives expression of the neomycin phosphotransferase gene (neo) via an internal ribosomal entry site (IRES).  $\beta$ -chain expression is driven by the hybrid HTLV-I/SV40 SR $\alpha$  promoter. SD, splice donor site; SA, splice acceptor site; pA, polyadenylation signal;  $\Psi^+$ , packaging signal. Arrows denote transcription start sites.

Table I. MART-1 Ag recognition by TIL 5 TCR transduced bulk PBL cultures

Stimulators	HLA-A2	Responders (pg/ml IFN- $\gamma$ released)					
		Medium	65 SHAM <sup>a</sup>	65 TRAN <sup>a</sup>	1235 TIL <sup>b</sup>	1520 TIL <sup>b</sup>	
Medium	—	0.0	60.7	16.1	63.1	29.5	
T2 alone	+	0.7	62.9	65.8	51.8	140.9	
T2 + g9-209	+	0.4	70.1	80.9	45.4	<b>12875.0<sup>c</sup></b>	
T2 + m9-27	+	0.6	54.6	<b>980.0</b>	<b>8082.5</b>	158.1	
397 MEL	—	0.0	67.4	71.1	42.0	21.6	
397 A2 MEL	+	1.6	74.8	129.8	<b>2210.0</b>	<b>1717.5</b>	
624-28 MEL	—	0.3	58.2	131.1	36.6	15.6	
624-38 MEL	+	12.6	59.0	205.0	<b>5612.5</b>	<b>3515.0</b>	
1300 MEL	+	0.4	50.2	243.6	<b>11902.5</b>	<b>13627.5</b>	

Stimulators	HLA-A2	Medium	66 SHAM	66 TRAN	67 SHAM	67 TRAN	1235 TIL	1520 TIL
Medium	—	1.5	8.2	13.2	8.3	14.7	39.5	72.5
T2 alone	+	1.6	15.7	15.7	15.5	16.5	16.4	109.6
T2 + g9-209	+	0.4	13.3	15.2	16.7	12.2	32.6	>2000
T2 + m9-27	+	1.6	15.3	<b>506.5</b>	13.2	<b>214.3</b>	>2000	119.3
397 MEL	—	1.1	15.3	56.8	19.5	46.1	34.8	19.9
397 A2 MEL	+	1.5	11.7	74.6	13.8	51.0	>2000	>2000
1011 MEL	—	1.1	11.7	17.7	9.9	15.0	34.9	170.8
888 A2 MEL	+	1.2	39.3	<b>603.1</b>	31.4	<b>473.9</b>	>2000	>2000
1558 MEL	+	0.8	91.9	<b>292.0</b>	57.6	<b>174.0</b>	<b>226.5</b>	<b>252.0</b>
1300 MEL	+	0.0	56.8	<b>249.1</b>	51.0	<b>163.1</b>	>2000	>2000
SK23 MEL	+	1.6	20.9	<b>281.4</b>	22.1	<b>177.9</b>	>2000	>2000

<sup>a</sup> Abbreviations: SHAM, untransduced; TRAN, transduced.

<sup>b</sup> 1235 TIL is a MART-1-reactive culture that recognizes the m9-27 peptide. 1520 TIL is a gp100-reactive culture and recognizes the g9-209 peptide.

<sup>c</sup> Bold results represent specific IFN- $\gamma$  release that is >50 pg/ml and at least 2-fold over background secretion. Values marked >2000 were in excess of the maximum standard used in that assay. Results presented are representative of multiple experiments.

$\beta$ -chain PCR products on agarose gels. DNA was extracted from transduced PBL cultures before and after selection in G418, as previously described (30). Briefly,  $1 \times 10^6$  cells were suspended in  $1 \times$  PCR buffer containing 0.1 mg/ml proteinase K (Life Technologies) and 0.5% Tween 20 (J. T. Baker, Phillipsburg, NJ) in a 200- $\mu$ l volume and were incubated at 56°C for 45 min followed by 70°C for 15 min. Then, 1  $\mu$ l of DNA was amplified in two series of cPCR reactions in a 50- $\mu$ l volume containing  $1 \times$  PCR buffer (Life Technologies), 2.5 U DNA *Taq* polymerase (Life Technologies), 200  $\mu$ M dNTP (Life Technologies), and 400 nM of both forward and reverse primers. Series 1 used a complementarity-determining region-3 (CDR-3)-specific forward primer (TIL 5b VDJ; 5'-GATCTCCTGAGTTGGGATGA-3') and a constant region specific reverse primer (C $\beta$ con I1R, 5' CCACCTTGCCACTCTGGC-3'). Series 2 used a constant region forward primer (C $\beta$ F' 5'-GTTCCCACCCGAGGTCGC-3') and the same constant region reverse primer (C $\beta$ con I1R). The amount of sample DNA was held constant, and the competitor was serially diluted in each series of PCR reactions. PCR was performed in a Perkin-Elmer 9600 DNA thermocycler (Perkin-Elmer, Foster City, CA) and consisted of 35 cycles of 92°C denaturation for 30 s, 60°C annealing for 30 s, and 72°C extension for 1 min. Products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining. Gels were documented using a Stratagene Eagle-eye (Stratagene, La Jolla, CA), and the amount of PCR product in template and competitor bands was measured using ImageQuANT software (Molecular Dynamics, Sunnyvale, CA). Regression analyses were performed with Excel software (Microsoft, Redwood, WA).

#### TCR V $\beta$ (BV) subfamily analysis of clonoids

The official nomenclature proposed by the International Union of Immunological Societies (IUIS) subcommittee on nomenclature has been used throughout this manuscript (31). Designation of TCR  $\beta$  gene segments was according to Arden et al. (32). Total cellular RNA was isolated using Trizol reagent, as per manufacturers instructions (Life Technologies). cDNA was synthesized using oligo(dT) (12–18) and Superscript Preamplification System reagents (Life Technologies). Oligonucleotide sequences of the BV subfamily-specific PCR primers and PCR methodology were as previously described (M. D. McKee et al., manuscript in preparation). Briefly, 1  $\mu$ l of cDNA was amplified in a 50- $\mu$ l reaction volume containing 2.5 U *Taq* DNA polymerase (Life Technologies),  $1 \times$  PCR buffer, 200  $\mu$ M dNTP (Life Technologies), and 400 nM of both forward and reverse primer. Amplifications were performed with a Perkin-Elmer 9600 DNA thermocycler (Perkin-Elmer) under the following conditions: 35 cycles of 92°C dena-

uration for 30 s, 60°C annealing for 30 s, and 72°C extension for 1 min. PCR products were resolved on 2% agarose gels and visualized using ethidium bromide staining.

## Results

The A7 retrovirus encodes the full-length clone 5 TCR  $\alpha$ - and  $\beta$ -chain cDNAs and the neomycin resistance gene as a selectable marker (Fig. 1). PBL from three normal donors were stimulated with anti-CD3 Ab and IL-2 and were transduced with the A7 virus (PBL 65 was transduced separately from PBL 66 and 67). The percentage of transduced cells was measured before and after selection with G418 by cPCR. Twenty-two to 38% of the unselected cells were transduced, and, following G418 selection, 60–92% of the cells were transduced (data not shown). Transduced and selected PBL cultures were 80–95% CD8<sup>+</sup> T cells by immunofluorescence analysis (data not shown). Cytokine release assays with bulk PBL cultures demonstrated that transduction with the clone 5 TCR genes conferred Ag-specific reactivity to the cultures (Table I). In two separate transduction experiments, we successfully transferred a functional MART-1-reactive TCR to normal PBL-derived T cells as demonstrated by their specific recognition of T2 cells pulsed with the m9-27 peptide (Table I). In addition, the PBL-66 and PBL-67 cells recovered from the second transduction showed specific IFN- $\gamma$  secretion in response to HLA-A2<sup>+</sup> melanoma lines expressing MART-1 (Table I). These results demonstrate that peripheral blood T cells from normal donors can be genetically modified to react with a human melanoma Ag.

Clone 5 TCR-transduced PBL cultures were cloned in limiting dilution to examine the characteristics of individual clones in the bulk PBL populations. Clones were screened for Ag-specific reactivity in cytokine release assays. Forty-three of 200 (21.5%) clones from PBL-65, 34 of 91 (37.4%) clones from PBL-66, and 47 of 104 (45.2%) clones from PBL-67 specifically secreted IFN- $\gamma$  in response to T2 cells pulsed with m9-27 peptide. Approximately



Table II. Characteristics of clones derived from bulk transduced PBL cultures

Culture	Phenotype <sup>a</sup>	Total No. of Clones	IFN- $\gamma$ Release vs. T2 + m9-27 <sup>b</sup>	IFN- $\gamma$ Release vs. Tumor <sup>c</sup>	Specific Lysis of T2 + m9-27 <sup>d</sup>	Specific Lysis of 888-A2 <sup>e</sup>
PBL-65	CD4	4	4	0	0	0
	CD8	8	8	1	2	2
	CD4/CD8	1	1	1	1	0
PBL-66	CD4	0	—	—	—	—
	CD8	9	9	9	9	8
PBL-67	CD4	0	—	—	—	—
	CD8	12	12	8	12	5

<sup>a</sup> Cell-surface immunofluorescence analysis. CD4/CD8: clod containing both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

<sup>b</sup> Specific IFN- $\gamma$  release measured in culture supernatants from clones cocultured with m9-27 peptide-pulsed T2 cells.

<sup>c</sup> Specific IFN- $\gamma$  release measured in culture supernatants from clones cocultured with HLA-A2<sup>+</sup> melanoma cell lines.

<sup>d,e</sup> Specific lysis measured in 4-h <sup>51</sup>Cr release assays at an E:T ratio of 80:1. Clones that were considered to be specific exhibited at least 15% lysis over background (T2 + m9-27, range 18–100%, mean 63.5%; 888-A2<sup>+</sup> MEL, range 15–85%, mean 42.1%). In all experiments, background lysis of T2 + g9-209 or 888 MEL was <10%.

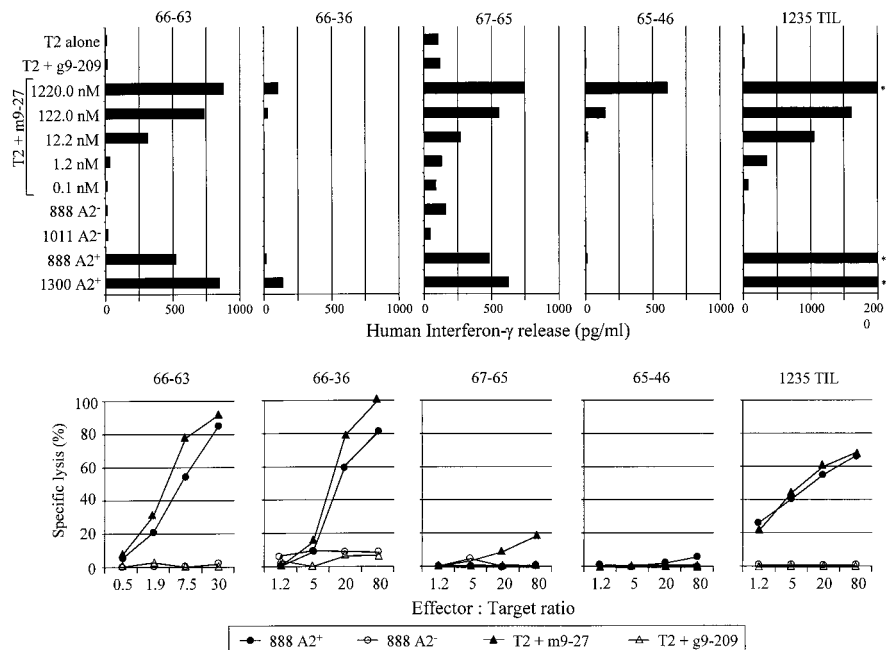
half of the peptide reactive clones, 15 of 34 from PBL-66 and 20 of 47 from PBL-67 (PBL 65 clones were not tested), also specifically released IFN- $\gamma$  when stimulated by 888-A2<sup>+</sup> MEL. Therefore, a high percentage of cells from each of the transduced PBL populations were Ag reactive.

A representative group of reactive clones from each transduction were expanded to further characterize their phenotype and Ag reactivity. These results are summarized in Table II. Immunofluorescence analysis revealed that all of the clones tested from PBL-66 and PBL-67 were CD8<sup>+</sup> (9 of 9 and 12 of 12, respectively), while clones from PBL-65 were CD4<sup>+</sup> (4 of 13), CD8<sup>+</sup> (8 of 13), or a mixture of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1 of 13). In cytokine release assays, each of the 29 CD8<sup>+</sup> clones secreted IFN- $\gamma$  in response to m9-27 peptide-pulsed T2 cells and 18 of 29 CD8<sup>+</sup> clones secreted IFN- $\gamma$  in response to tumor cells (Table II). In <sup>51</sup>Cr release assays, 23 of the 29 CD8<sup>+</sup> clones lysed T2 cells pulsed with m9-27 peptide, and 15 of the 29 CD8<sup>+</sup> clones lysed the HLA-A2<sup>+</sup> melanoma line 888 MEL (Table II). None of the CD4<sup>+</sup> clones lysed m9-27 peptide-pulsed T2 cells or 888-A2 melanoma cells (Table II). The CD4<sup>+</sup> clones could secrete IFN- $\gamma$  when stimulated with T2 cells pulsed with m9-27 peptide but not tumor cells.

Most clones were also tested for their ability to secrete cytokines other than IFN- $\gamma$ . All 24 clones tested (6 from PBL-65, 9 from PBL-66, and 9 from PBL-67) specifically secreted IFN- $\gamma$  and GM-CSF but not TNF- $\alpha$ , IL-2, IL-4, or IL-10 in response to m9-27 peptide-pulsed T2 cells (data not shown).

Individual clones fell into one of four groups based on their CD4 vs CD8 phenotype, their ability to lyse MART-1<sup>+</sup> targets, and their ability to secrete cytokines following Ag stimulation. A representative clone from each group is shown in Fig. 2. The first group of clones (23 of the 34 clones tested), represented by clone 66-63, were CD8<sup>+</sup> T cells, lysed HLA-A2<sup>+</sup> MART-1<sup>+</sup> targets, and secreted IFN- $\gamma$  when stimulated by m9-27 peptide-pulsed T2 cells or HLA-A2<sup>+</sup> melanoma cell lines. The ability of most of these clones to lyse targets and secrete cytokine was generally comparable to the MART-1-reactive TIL 1235. One of the 34 clones tested (clone 66-36) was CD8<sup>+</sup> and lysed HLA-A2<sup>+</sup> MART-1<sup>+</sup> targets but secreted little or no IFN- $\gamma$  when stimulated by m9-27 peptide-pulsed T2 cells or HLA-A2<sup>+</sup> melanoma cell lines. The third group of clones (6 of the 34 clones tested), represented by clone 67-65, were CD8<sup>+</sup> and secreted IFN- $\gamma$  when stimulated by m9-27 peptide-pulsed T2 cells or HLA-A2<sup>+</sup> melanoma

**FIGURE 2.** Cytokine release and lysis assays for representative transduced PBL clones. A, Human IFN- $\gamma$  release measured in culture supernatants from clones incubated with unpulsed T2 cells or T2 cells pulsed with either decreasing concentrations of the m9-27 peptide or the irrelevant g9-209 peptide. Recognition of HLA-A2<sup>+</sup> and HLA-A2<sup>-</sup> melanoma lines was also tested. Specific recognition is defined as >100 pg/ml of IFN- $\gamma$  released and >2-fold above background release by relevant controls. \*, Values were above the maximum standard (2000 pg/ml) used in the assay. B, Specific lysis of T2 cells pulsed with either m9-27 peptide or the irrelevant g9-209 peptide, and of melanoma lines 888 Mel (HLA-A2<sup>-</sup>) and 888-A2<sup>+</sup> (HLA-A2<sup>+</sup>), by transduced clones in 4-h <sup>51</sup>Cr release assays.



cell lines but weakly lysed HLA-A2<sup>+</sup> MART-1<sup>+</sup> targets. The fourth group of clones (4 of the 34 clones tested), represented by clone 65-46, were CD4<sup>+</sup> and secreted IFN- $\gamma$  when stimulated by m9-27 peptide-pulsed T2 cells but did not lyse HLA-A2<sup>+</sup> MART-1<sup>+</sup> targets. To insure that the properties observed in these clones were due to a single clone and not mixed clones, the clonality of a small group of clones was tested by determining their TCRBV usage with a panel of TCRBV subfamily-specific primers. Of the 13 clones tested, 10 expressed only the introduced BV7 gene and one other TCRBV subfamily, indicating that most of the T cell lines were clonal (data not shown). These results indicate that MART-1-reactive clones obtained by transducing normal donor PBL with the TIL 5 TCR were heterogenous with respect to their ability to lyse and specifically secrete cytokines when cultured with MART-1<sup>+</sup> cells.

## Discussion

PBL from normal blood donors were successfully transduced with the genes encoding a MART-1-specific TCR, producing TAA-specific PBL cultures that had MART-1-specific anti-tumor reactivity. This is the first report describing the genetic modification of human PBL with TAA-specific TCR genes. PBL from all three normal donors were successfully transduced to express a MART-1-reactive TCR, demonstrating that we could reliably redirect the specificity of naive T cells. Clones isolated from these transduced PBL cultures secreted cytokines following Ag stimulation and lysed HLA-A2<sup>+</sup> MART-1<sup>+</sup> targets. Many of these clones were as effective as TIL 1235 cells in lysing m9-27 peptide-pulsed T2 cells or HLA-A2<sup>+</sup> melanoma cells and secreting IFN- $\gamma$  when stimulated by very low concentrations of m9-27 peptide pulsed onto T2 cells. These gene-modified T cells capable of recognizing melanoma Ags thus provide an alternative approach for treating patients with metastatic melanoma.

Suprisingly, CD4<sup>+</sup> clones were also isolated from a transduced PBL culture that recognized T2 cells pulsed with the m9-27 peptide in cytokine release assays. Recognition occurred in the absence of CD4 coreceptor interaction with the presenting MHC molecule, because CD4 cannot bind to HLA-A2.1 (33). The interaction between a TCR and the peptide-MHC complex is thought to be a low-affinity interaction (34). CD4 and CD8 facilitate the interaction between a T cell and an APC by binding to class II or class I MHC molecules, respectively. This is thought to occur before the TCR/peptide-MHC interaction, increasing the overall avidity of the TCR/peptide-MHC complex (35–37). In addition to their roles as adhesion molecules, there is a close association between the cytoplasmic tail of CD4 with p56<sup>lck</sup> suggesting that CD4 may also have a role in signal transduction during T cell activation (38, 39). The cytoplasmic tail of the CD8  $\alpha$ -chain also associates with p56<sup>lck</sup>, and a role in signal transduction has also been suggested for CD8 (40, 41). The recognition of m9-27 peptide-pulsed T2 cells by transduced CD4<sup>+</sup> clones demonstrated that the clone 5 cells had a TCR with sufficient affinity to transduce an activation signal without the interaction of CD8 coreceptors with HLA-A2. However, the clone 5 TCR, when expressed in Jurkat cells or normal CD4<sup>+</sup> T cells, was unable to recognize melanoma cells. This suggests that the high levels of m9-27 peptide present on T2 cells can overcome the low-affinity of a TCR leading to T cell activation. We and others have isolated CD8-independent T cell clones that recognized human melanoma cells in a class I-restricted manner (M.I.N., unpublished observations) (42–44). The TCR genes from these CD8-independent T cell clones likely encode high-affinity TCR, which would be better candidates for gene transfer.

A potential advantage of transferring a specifically reactive bulk transduced PBL culture to cancer patients is that CD8<sup>+</sup> and CD4<sup>+</sup> clones expressing different levels of the transferred TCR can have different functional characteristics. The CD4<sup>+</sup> clones might augment the activity of CD8<sup>+</sup> clones by providing stimulatory cytokines such as IL-2 at the tumor site. However, the relative frequency of lytic CD8<sup>+</sup> clones and reactive CD4<sup>+</sup> clones in the transduced bulk PBL cultures will be variable and their reactivity may be low. An alternate treatment strategy would be to isolate T cell clones with defined reactivities from these bulk transduced PBL cultures. Tumor-reactive clones from the transduced bulk cultures can be expanded to numbers suitable for adoptive transfer (10<sup>10</sup> cells) using the procedures described (22). Clones or pools of these clones could be adoptively transferred into melanoma patients. The transfer of tumor-reactive CD4<sup>+</sup> clones with tumor-reactive CD8<sup>+</sup> clones may be more effective than transferring the CD8<sup>+</sup> clones alone.

The HLA-A2-restricted TCR used in this study could enable us to treat ~50% of all melanoma patients because ~50% of all melanoma patients express HLA-A2 (45). However, there are no HLA class II alleles expressed by >34% of melanoma patients (46). Furthermore, most melanoma-reactive CD4<sup>+</sup> T cells recognize only their autologous tumors rather than shared Ags. The capacity to produce HLA-A2-restricted CD4<sup>+</sup> T cells that recognize shared melanoma Ags could enable us to provide T cell help to more patients than we could treat with MHC class II-restricted cells.

Because of the unique CDR3 sequences of the clone 5 TCR  $\alpha$ - and  $\beta$ -chains, cPCR can be used to follow adoptively transferred PBL clones to examine their persistence in the peripheral blood of patients (47). Biopsies or fine needle aspirates of accessible tumor lesions could also allow the trafficking of TCR gene-modified T cells to tumors to be assessed. Because individual transduced PBL clones express a second unique TCR, the CDR3 region of this TCR will allow individual transduced clones in a pool of adoptively transferred clones to be monitored. These studies could provide new insights into the fate of adoptively transferred T cells. Previous trafficking studies of adoptively transferred CTL have relied upon the transfer of radiolabeled cells and therefore were imprecise (48).

A number of other potential applications of TCR gene transfer to PBL are under investigation. T cell clones that are specific for a number of different TAAs have been isolated and a panel of retroviral vectors encoding the TAA-specific TCR from these clones is being assembled. We envision using multiple viruses to transduce separate PBL cultures from a melanoma patient to generate cultures with anti-tumor reactivity against different TAA. Adoptive transfer of CTL pools recognizing multiple TAA could circumvent in vivo immunoselection of tumor cells lacking expression of a single target TAA, which has been reported to be a mechanism of tumor escape from cellular immunotherapies (49). It is conceivable that a single PBL culture could be sequentially transduced with two or more TCR to generate clones expressing multiple tumor-Ag-specific TCR. We are also investigating the transfer of TAA-specific TCR into T cell clones that already have an existing anti-tumor reactivity. We can routinely generate MART-1 m9-27-reactive CTL by multiple rounds of in vitro peptide stimulation of melanoma patient PBMC in the majority of patients tested (50, 51). Bispecific clones could also be generated by transducing T cell clones that target the tumor vasculature, such as clones specific for the vascular endothelium growth factor receptor. These studies could provide unique opportunities to study T cell biology. And finally, the transfer of TCR genes to human PBL might also be used to identify new TAA. Where a CTL clone with a unique reactivity has been isolated but cannot be expanded

to sufficiently high numbers to permit the screening of a cDNA library, transfer of the clone TCR to PBL could provide an inexhaustible supply of T cells for library screening.

Human clinical trials with adoptive transfer of TCR gene-modified PBL clones can evaluate the effectiveness of these approaches for the treatment of patients with metastatic melanoma. The success of retroviral transduction in three PBL tested in this study demonstrates that this technique would provide a source of anti-tumor CTL for immunotherapy protocols. As CTL that recognize TAA in other tumor histologies are identified, the TCR genes from these T cells could potentially be used for the therapy of other cancers. These approaches could also be applied to the treatment of viral infections.

## Acknowledgments

We thank Arnold Mixon for cell surface immunofluorescence analysis and the staff of the National Institutes of Health Clinical Center Department of Transfusion Medicine for supplying normal donor PBMC samples.

## References

- Robbins, P. F., and Y. Kawakami. 1996. Human tumor antigens recognized by T cells. *Curr. Opin. Immunol.* 8:628.
- Boon, T., and P. van der Bruggen. 1996. Human tumor antigens recognized by T lymphocytes. *J. Exp. Med.* 183:725.
- Mandruzzato, S., F. Brasseur, G. Andry, T. Boon, and P. van der Bruggen. 1997. A CASP-8 mutation recognized by cytotoxic T lymphocytes on a human head and neck carcinoma. *J. Exp. Med.* 186:785.
- Brandle, D., F. Brasseur, P. Weynants, T. Boon, and B. Van den Eynde. 1996. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma. *J. Exp. Med.* 183:2501.
- Gaugler, B., N. Brouwenstijn, V. Vantomme, J. P. Szikora, C. W. Van der Spek, J. J. Patard, T. Boon, P. Schrier, and B. J. Van den Eynde. 1996. A new gene coding for an antigen recognized by autologous cytolytic T lymphocytes on a human renal carcinoma. *Immunogenetics* 44:323.
- Kast, W. M., R. Offringa, P. J. Peters, A. C. Voordouw, R. H. Meloan, A. J. van der Eb, and C. J. Melief. 1989. Eradication of adenovirus E1-induced tumors by E1A-specific cytotoxic T lymphocytes. *Cell* 59:603.
- Rodolfo, M., C. Bassi, C. Salvi, and G. Parmiani. 1991. Therapeutic use of a long-term T cell line recognizing a common tumor-associated antigen: the pattern of in vitro reactivity predicts the in vivo effect on different tumors. *Cancer Immunol. Immunother.* 34:53.
- Feltkamp, M. C., G. R. Vreugdenhil, M. P. M. Vierboom, E. Ras, S. H. van der Burg, J. ter Schegget, C. J. M. Melief, and W. M. Kast. 1995. Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. *Eur. J. Immunol.* 25:2638.
- Burger, U. L., M. P. Chang, M. Nagoshi, P. S. Goedegebuure, and T. J. Eberlain. 1996. Improved in vivo efficiency of tumor-infiltrating lymphocytes after restimulation with irradiated tumor cells in vitro. *Ann. Surg. Oncol.* 3:580.
- Shilyansky, J., J. C. Yang, M. C. Custer, P. Spiess, A. Mixon, D. J. Cole, J. J. Mule, S. A. Rosenberg, and M. I. Nishimura. 1997. Identification of a T-cell receptor from a therapeutic murine T-cell clone. *J. Immunol.* 20:247.
- Rosenberg, S., J. Yannelli, J. Yang, S. Topalian, D. Schwartzentruber, J. Weber, D. Parkinson, C. Seipp, J. Einhorn, and D. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* 86:1159.
- Crowley, N. J., C. L. Slingluff, Jr., T. L. Darrow, and H. F. Seigler. 1990. Generation of human autologous melanoma-specific cytotoxic T-cells using HLA-A2-matched allogeneic melanomas. *Cancer Res.* 50:492.
- Stevens, E. J., L. Jacknin, P. F. Robbins, Y. Kawakami, M. El Gamil, S. A. Rosenberg, and J. R. Yannelli. 1995. Generation of tumor-specific CTLs from melanoma patients by using peripheral blood stimulated with allogeneic melanoma tumor cell lines: fine specificity and MART-1 melanoma antigen recognition. *J. Immunol.* 154:762.
- Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki, and S. A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA* 91:3515.
- Marincola, F. M., Y. M. Hijazi, P. Fetsch, M. L. Salgaller, L. Rivoltini, J. Cormier, T. B. Simonis, P. H. Duray, M. Herlyn, Y. Kawakami, and S. A. Rosenberg. 1996. Analysis of expression of the melanoma-associated antigens MART-1 and gp100 in metastatic melanoma cell lines and in situ lesions. *J. Immunother.* 19:192.
- Cole, D. J., D. P. Weil, J. Shilyansky, M. Custer, Y. Kawakami, S. A. Rosenberg, and M. I. Nishimura. 1995. Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen. *Cancer Res.* 55:748.
- Rosenberg, S. A., O. Aebersold, K. Cornetta, A. Kasid, R. A. Morgan, R. Moen, E. M. Karson, M. T. Lotze, J. C. Yang, S. L. Topalian, M. J. Merino, K. Culver, A. D. Miller, R. M. Blaese, and W. F. Anderson. 1990. Gene transfer into hu-
- mans: immunotherapy of patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med.* 323:570.
- Lam, J. S., M. E. Reeves, R. Cowherd, S. A. Rosenberg, and P. Hwu. 1996. Improved gene transfer into human lymphocytes using retroviruses with the gibbon ape leukemia virus envelope. *Hum. Gene Ther.* 7:1415.
- Treisman, J., P. Hwu, S. Minamoto, G. E. Shafer, R. Cowherd, R. A. Morgan, and S. A. Rosenberg. 1995. Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. *Blood* 85:139.
- Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden. 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* 65:2220.
- Bunnell, B. A., L. M. Muul, R. E. Donahue, R. M. Blaese, and R. A. Morgan. 1995. High-efficiency retroviral-mediated gene transfer into human and nonhuman primate peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* 92:7739.
- Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med.* 333:1038.
- Topalian, S. L., D. Solomon, and S. A. Rosenberg. 1989. Tumor specific cytotoxicity by lymphocytes infiltrating human melanomas. *J. Immunol.* 142:3714.
- Rosenberg, S. A., B. Packard, P. Aebersold, D. Solomon, S. Topalian, S. Toy, P. Simon, M. Lotze, J. Yang, C. Seipp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J. Wei, and D. White. 1988. Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: preliminary report. *N. Engl. J. Med.* 319:1676.
- Parkhurst, M. R., M. L. Salgaller, S. Southwood, P. F. Robbins, A. Sette, S. A. Rosenberg, and Y. Kawakami. 1996. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A\*0201-binding residues. *J. Immunol.* 157:2539.
- Kawakami, Y., S. Eliyahu, C. Jennings, K. Sakaguchi, X-Q. Kang, S. Southwood, P. F. Robbins, A. Sette, E. Appella, and S. A. Rosenberg. 1995. Recognition of multiple epitopes in the human melanoma antigen gp100 associated with in vivo tumor regression. *J. Immunol.* 154:3961.
- Kawakami, Y., S. Eliyahu, K. Sakaguchi, P. F. Robbins, L. Rivoltini, J. R. Yannelli, E. Appella, and S. A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *J. Exp. Med.* 180:347.
- Mosse, C. A., L. Meadows, C. J. Luckey, D. J. Kittleson, E. L. Huczko, C. L. Slingluff, Jr., J. Shabanowitz, D. F. Hunt, and V. H. Engelhard. 1998. The class I antigen-processing pathway for the membrane protein tyrosinase involves translation in the endoplasmic reticulum and processing in the cytosol. *J. Exp. Med.* 187:37.
- Kawakami, Y., S. A. Rosenberg, and M. T. Lotze. 1988. Interleukin 4 promotes the growth of tumor-infiltrating lymphocytes cytotoxic for human autologous melanoma. *J. Exp. Med.* 168:2183.
- Kawasaki, E. S. 1990. Sample preparation from blood, cells and other fluids. In *PCR Protocols*. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA, pp. 146-152.
- WHO-IUIS nomenclature sub-committee on TCR designation. 1995. Nomenclature for T-cell receptor (TCR) gene segments of the immune system. *Immunogenetics* 42:451.
- Arden, B., S. P. Clark, D. Kabelitz, and T. W. Mak. 1995. Human T-cell receptor variable gene segment families. *Immunogenetics* 42:455.
- Parnes, J. R. 1989. Molecular biology and function of CD4 and CD8. *Adv. Immunol.* 44:265.
- Matsui, K., P. A. Reay, H. Schild, D. S. Fazekas, and M. M. Davis. 1991. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* 254:1788.
- Doyle, C., and J. L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* 330:256.
- Norment, A. M., R. D. Salter, P. Parham, V. H. Engelhard, and D. R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class II molecules. *Nature* 336:79.
- Salter, R. D., R. J. Benjamin, P. K. Wesley, S. E. Buxton, T. P. Garrett, C. Clayberger, A. M. Krensky, A. M. Norment, and D. R. Littman. 1990. A binding site for the T-cell co-receptor CD8 on the  $\alpha 3$  domain of HLA-A2. *Nature* 345:41.
- Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85:5190.
- Glaichenhaus, N., N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of p56<sup>lck</sup> with CD4 in antigen-specific signal transduction in T cells. *Cell* 64:511.
- Dembic, Z., W. Haas, R. Zamoyska, J. Parnes, M. Steinmetz, and H. von Boehmer. 1987. Transfection of the CD8 gene enhances T-cell recognition. *Nature* 326:510.
- Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56<sup>lck</sup>. *Cell* 55:301.
- Hayashi, Y., D. S. Hoon, M. S. Park, P. I. Terasaki, L. J. Foshag, and D. L. Morton. 1992. Induction of CD4<sup>+</sup> cytotoxic T cells by sensitization with allogeneic melanomas bearing shared or cross-reactive HLA-A. *Cell. Immunol.* 139:411.
- LeMay, L. G., J. Kan-Mitchell, P. Goedegebuure, W. Harel, and M. S. Mitchell. 1993. Detection of melanoma-reactive CD4<sup>+</sup> HLA-class I-restricted cytotoxic T

- cell clones with long-term assay and pretreatment of targets with interferon- $\gamma$ . *Cancer Immunol. Immunother.* 37:187.
44. Darrow, T. L., Z. Abdel-Wahab, M. A. Quinn-Allen, and H. F. Seigler. 1996. Recognition and lysis of human melanoma by a CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>-</sup> T-cell clone restricted by HLA-A2. *Cell. Immunol.* 172:52.
  45. Player, M. A., K. C. Barracchini, T. B. Simonis, L. Rivoltini, F. Arienti, C. Castelli, A. Mazzocchi, F. Belli, G. Parmiani, and F. M. Marincola. 1996. Differences in frequency distribution of HLA-A2 subtypes between North American and Italian white melanoma patients: relevance for epitope specific vaccination protocols. *J. Immunother. Emphasis. Tumor Immunol.* 19:357.
  46. Marincola, F. M., P. Shamamian, L. Rivoltini, M. Salgaller, J. Cormier, N. P. Restifo, T. B. Simonis, D. Venzon, D. E. White, and D. R. Parkinson. 1996. HLA associations in the antitumor response against malignant melanoma. *J. Immunother.* 18:242.
  47. McKee, M. D., T. M. Clay, S. A. Rosenberg, and M. I. Nishimura. 1999. Quantitation of T-cell receptor frequencies by competitive PCR: generation and evaluation of novel TCR subfamily and clone specific competitors. *J. Immunother.* 22:93.
  48. Pockaj, B. A., R. M. Sherry, J. P. Wei, J. R. Yannelli, C. S. Carter, S. F. Leitman, J. A. Carasquillo, S. M. Steinberg, S. A. Rosenberg, and J. C. Yang. 1994. Localization of <sup>111</sup>Indium-labelled tumor infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy. *Cancer* 73:1731.
  49. Jager, E., M. Ringhoffer, J. Karbach, M. Arand, F. Oesch, and A. Knuth. 1996. Inverse relationship of melanocyte differentiation antigen expression in melanoma tissues and CD8<sup>+</sup> cytotoxic-T-cell responses: evidence for immunoselection of antigen-loss variants in vivo. *Int. J. Cancer* 66:470.
  50. Rivoltini, L., Y. Kawakami, K. Sakaguchi, S. Southwood, A. Sette, P. F. Robbins, F. M. Marincola, M. L. Salgaller, J. R. Yannelli, E. Appella, and S. A. Rosenberg. 1995. Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. *J. Immunol.* 154:2257.
  51. Marincola, F. M., L. Rivoltini, M. L. Salgaller, M. Player, and S. A. Rosenberg. 1996. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of in vivo priming by tumor cells. *J. Immunother.* 19:266.