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ORIGINAL ARTICLE

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Vaccination of Stage IV patients with allogeneic IL-4- or IL-2-gene-transduced melanoma cells generates functional antibodies against vaccinating and autologous melanoma cells

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Abstract The antibody (Ab) response to allogeneic Me14932 and autologous melanoma cells was analyzed in 13 Stage IV (AJCC) melanoma patients immunized with Me14932 cells transduced with the IL-4 (Me14932/IL-4) ($n=10$) or IL-2 (Me14932/IL-2) ($n=3$) gene. No Ab response was observed before the 4th vaccination. Among 8 patients that received four vaccinations, 3/5 patients vaccinated with Me14932/IL-4 cells developed Ab (IgG and/or IgM) to Me14932 ($n=3$) and to autologous ($n=2$) melanoma cells, and 2/3 patients vaccinated with Me14932/IL-2 cells developed Ab (IgG) to Me14932, but not to autologous melanoma cells. Further, among these 5 responding patients, circulating Ab against the HLA-A3 allele, expressed only on vaccinating cells, were identified in the immune sera of 4 patients immunized with Me14932/IL-4 ($n=2$) or Me14932/IL-2 ($n=2$) cells. These sera mediated antibody-dependent cell cytotoxicity (ADCC) of Me14932 cells, and a direct correlation ($r=0.85$; $P=0.03$) between intensity of staining (IgG) and extent of lysis was found. Immune serum of one of these patients also induced ADCC of autologous melanoma cells, and serum from another patient mediated complement cytotoxicity of Me14932, but not of autologous melanoma cells. Thus, Abs against vaccinating and autologous melanoma cells were generated in 62% of patients after four vaccinations with cytokine-transduced melanoma cells. These findings demonstrate that the identification and titration of alloreactive Ab helps to monitor the extent of immunization against cellular vaccines, while the induction of Ab reactive to antigens shared between vaccinating and autologous melanoma cells may contribute to their therapeutic efficacy.

Keywords Antibodies · Cellular vaccines · Immunotherapy · Melanoma

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Introduction

The availability of efficient viral and non-viral gene-delivery systems has recently allowed the ex vivo construction of gene-modified autologous and allogeneic tumor cell vaccines carrying different ''therapeutic'' genes [16].

Recently, several shared immunogenic melanoma-associated antigens (MAA), specifically recognized by cytotoxic T lymphocytes (CTL) or by antibodies (Abs), have been identified [for review see:15, 19]. Furthermore, utilizing several in vivo experimental models, it has been demonstrated that melanoma cells transduced or transfected with gene(s) encoding different cytokines enhance their immunogenicity to the host, mostly by activating $CD8^+$ T cells, natural killer cells, and macrophages [9, 12, 17, 21]. Altogether, this evidence strongly supports the idea that immunization of melanoma patients with allogeneic whole-tumor cells engineered with co-stimulatory molecule(s) and/or cytokine genes, might evoke a melanoma-specific systemic immunity.

Therapeutic vaccines based on genetically modified allogeneic tumor cells are also advantageous, since they reduce time and costs necessary to construct autologous

tumor cell vaccines, and allow treatment of patients in early stages of disease or with minimal tumor burden [18]. Additional support to an allogeneic cellular vaccine approach is provided by the evidence that the host's antigen-presenting cells (APC), rather than the vaccinating cells themselves, could prime $CD4^+$ and $CD8^+$ T cells to generate systemic anti-tumor immunity [10].

Our preliminary results from a Phase I-II clinical trial with IL-4- or IL-2-transduced vaccinating allogeneic Me14932 melanoma cells showed limited clinical responses in a minority of treated Stage IV (AJCC) melanoma patients [5, 6]. Nevertheless, these studies also indicated that, in selected patients, repeated vaccinations with gene-modified tumor cells potentiated or induced a CTL response against Melan-A/MART-1, tyrosinase and/or gp100 [3, 4].

Based on the evidence above, and on the notion that cellular and humoral arms of the immune system work in concert, in this study we focused on the analysis of the antibody response against allogeneic Me14932 and autologous melanoma cells in patients immunized with IL-4- or IL-2-transduced Me14932 cells.

Material and methods

Patients and treatment schedule

From January 1994 to July 1996, twenty patients affected by Stage IV (AJCC) melanoma, refusing or refractory to all available therapies, were enrolled in a Phase I-II clinical trial of vaccination with IL-4 ($n=12$) or IL-2 ($n=8$) gene-modified Me14932 melanoma cells. Patients were injected subcutaneously on days 0, 13, 26 and 55 with 5×10^7 ($n=16$) or 1.5×10^8 ($n=4$) irradiated cells (Table 1) resuspended in 1 ml isotonic sterile solution, in the area of draining unaffected lymph nodes. If no disease progression was evident, patients received additional immunizations at monthly intervals. Thirteen patients that received at least 3 doses of vaccine and underwent complete HLA class I typing were investigated in this study (Table 1).

The clinical protocol was reviewed and approved by the Ethical and Scientific Committee of the two participating centers (Istituto Nazionale Tumori of Milan and Centro di Riferimento Oncologico of Aviano) in January 1994. All patients gave written, informed consent before treatment.

Vaccinating cells

The IL-4- or IL-2-transduced melanoma cell lines Me14932/IL-4 and Me14932/IL-2, respectively, and parental Me14932 melanoma cells (A2,3/B7,w50/Cw6,w7/DR2,7) were obtained and characterized as previously described [2, 14]. Me14932/IL-4 and Me14932/IL-2 melanoma cells were amplified in vitro until a number of cells sufficient for all the vaccines was generated; then the cells were washed, irradiated with 200 Gy, aliquoted and frozen at –80 °C until use [5]. Release of IL-4 or IL-2 was 1848 pg/ml and 2344 pg/ml (per 10^5 cells) from Me14932/IL-4 and Me14932/IL-2, respectively.

Cells

Me14932 melanoma cells, autologous melanoma cells obtained from investigated patients as described previously [1], human colorectal carcinoma cells Colo 201 (HLA-A3-negative), lymphoblastoid cells C1R (HLA-A3-negative) and C1R-A3 (that express a transfected genomic clone of HLA-A3) [8] were grown in RPMI-1640 medium (Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Flow) and 2 mM L-glutamine (complete medium) (Flow). C1R-A3 cells were maintained in complete medium supplemented with 0.8 mg/ml hygromycin.

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation (400 *g* for 30 min).

Serological assay, sera and conventional antisera

Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of goat anti-human IgG, dichlorotriazynylaminofluorescein (DTAF)-conjugated $F(ab')_2$ fragments of goat anti-human IgM, and ChromePure mouse Ig were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

Indirect immunofluorescence (IIF) was performed as described previously [13], with minor modifications. Briefly, 50 µl of a cell suspension (2×10^6 cells/ml) were incubated with 50 µl of serum from patients or healthy donors for 30 min at 4 °C. Then, cells were washed three times, and incubated for an additional 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of goat anti-human IgG or with DTAF-conjugated $F(ab')_2$ fragments of goat anti-human IgM. Negative controls were performed by incubating cells with isotype-matched control mouse Ig. After three washings, cells were analyzed with a FACScan flow cytometer. Results are expressed as values of median fluorescence intensity.

Sera collected from patients one week before the first vaccination and one week after each immunization, and normal human

Table 1 Characteristics of vaccinated Stage IV (AJCC) patients

Patient	Vaccinations (*n*)	Vaccine	Dose	HLA		
				-A	-B	-C
#1	3	Me14932/IL-4	5×10^7	2,30	35,62	w3,w4
#2	3	Me14932/IL-4	5×10^7	1,2	8,13	–, –
#3	3	Me14932/IL-4	5×10^7	2,32	51,38	–, –
#4	3	Me14932/IL-4	5×10^7	2,32	49,38	–, –
#5	3	Me14932/IL-4	5×10^7	1,2	8, –	w4,w8
#6	6	Me14932/IL-4	5×10^7	2,10	5,49	w6, –
#7	4	Me14932/IL-4	5×10^7	2,19	5, –	w1, –
#8	4	Me14932/IL-4	5×10^7	2,11	5,18	w1, –
#9	4	Me14932/IL-4	5×10^7	2, –	5,16	–, –
#10	4	Me14932/IL-4	5×10^7	1,2	5,8	–, –
#11	4	Me14932/IL-2	5×10^7	1,2	5,17	w2, –
#12	4	Me14932/IL-2	1.5×10^8	2,10	15,16	w1, –
#13	6	Me14932/IL-2	5×10^7	2,30	55,37	w1,w6

sera (NHS) from healthy lab volunteers, were aliquoted and stored at –80 °C until use.

HLA typing and detection of alloreactive Ab

Serological typing for HLA-A, -B, and -C alleles and detection of alloreactive Ab were performed by the standard two-stage National Institutes of Health complement-dependent microlymphocytotoxicity assay utilizing the Lymphotype HLA-ABC 144-1 and -2 Italia trays from Biotest (Milan, Italy) and the Lambda Cell Trays kit (One Lambda, Calif.), respectively.

Cytotoxicity assays

Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays were performed in triplicate in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.) as previously described [7]. Briefly, for ADCC assay, ^{51}Cr-labeled target cells (1×10^4 cells/well) were incubated with 20 µl of heat-inactivated sera at 37 °C. After a 20-min incubation, 100 µl of PBMC from healthy donors or from patients were added to each well, at the effector/target (E/T) ratios of 100:1, 50:1, 25:1, 12.5:1. After a 4-h incubation, supernatant (0.1 ml) was harvested from each well and counted in a gamma counter. The percentage of cytotoxicity was determined as described [7]. For CDC assay, ^{51}Cr-labeled target cells (3×10^4 cells/well) were incubated with 20 µl of heat-inactivated sera at 37 °C. After a 20-min incubation, 100 µl of normal human serum (NHS) was added to each well in a final volume of 200 µl. After a 1-h incubation, supernatant (0.1 ml) was harvested from each well and counted in a gamma counter. The percentage of cytotoxicity was determined as described [7].

Statistical analysis

Correlation analysis with a value of $r > 0.7$, giving a value of $P < 0.05$, obtained by using the least square linear regression, was considered statistically significant.

Results

Vaccination-induced Ab response to Me14932 and autologous melanoma cells

To analyze the Ab response (IgG and/or IgM) elicited by repeated immunizations with IL-4- or IL-2-transduced allogeneic Me14932 melanoma cells against Me14932 and autologous melanoma cells, pre-immune and immune sera from 13 patients were tested by IIF, followed by flow cytometry.

Pre-immune and control sera from healthy donors did not bind to Me14932 and/or autologous melanoma cells; furthermore, no Ab response was observed in the 5 patients that received a maximum of 3 vaccinations (data not shown). In contrast, among the 8 patients that received at least 4 doses of vaccine, 3 (#6, #7, #9) out of the 5 patients vaccinated with Me14932/IL-4 cells and 2 (#12, #13) out of the 3 patients vaccinated with Me14932/IL-2 cells, developed an IgG response against Me14932 melanoma cells after the 4th immunization (Table 2). Among these 5 responding patients, a concomitant IgM response against Me14932 cells was detected exclusively in 2 (#6, #7) out of the 3 patients immunized with Me14932/IL-4 cells (Table 2).

Table 2 Binding of sera from vaccinated patients to Me14932 and autologous melanoma cells as assessed by IIF analysis. Cells were sequentially incubated with pre-immune (day 0) and immune sera collected at different time intervals and number of vaccinations (*in brackets*) as indicated, and with FITC-conjugated F(ab')$_2$ fragments of goat anti-human IgG or with DTAF-conjugated F(ab')$_2$ fragments of goat anti-human IgM; then cells were analyzed by flow cytometry. Data represent the values of median fluorescence intensity (*NT* not tested)

	Me14932 melanoma cells		Autologous melanoma cells	
	IgG	IgM	IgG	IgM
Me14932/IL-4-vaccinated patients				
#6				
Day 0	4	6	4	10
Day 65 (4th)	83	41	19	183
Day 139 (6th)	112	27	14	106
#7				
Day 0	4	4	6	5
Day 65 (4th)	64	4	6	5
Day 137 (4th)	103	29	18	32
#9				
Day 0	4	5	5	4
Day 85 (4th)	22	7	5	6
Me14932/IL-2-vaccinated patients				
#12				
Day 0	4	4	NT	NT
Day 63 (4th)	53	6	NT	NT
#13				
Day 0	4	4	4	4
Day 63 (4th)	40	3	4	3
Day 100 (5th)	23	4	5	5
Day 154 (6th)	17	6	6	3

Sera from the 5 patients that stained Me14932 cells were also tested for binding to autologous melanoma cells, and to Colo 201 colorectal carcinoma cells utilized as negative control. As reported in Table 2, an IgG and IgM response to autologous melanoma cells was observed in 2 (#6, #7) out of the 3 patients immunized with Me14932/IL-4 cells; in contrast, no Ab response to autologous melanoma cells was found in the 2 patients vaccinated with Me14932/IL-2 cells. Furthermore, investigated sera did not bind (IgG and/or IgM) to Colo 201 colorectal carcinoma cells (data not shown).

Vaccination-induced alloantibodies

Complement-mediated microlymphocytotoxicity assays demonstrated that immune sera from 3 (#6, #7, #12) out of 4 HLA-A3-negative patients that reacted with Me14932 cells, had Ab directed to HLA-A3 allele expressed on vaccinating Me14932 melanoma cells.

To further investigate the specificity of generated anti-HLA-A3 Ab, to quantitate them, and to determine their kinetics of appearance, available sera from patients #7, #12, and #13 were tested for reactivity against C1R-A3$^+$ and C1R lymphoblastoid cells. Flow

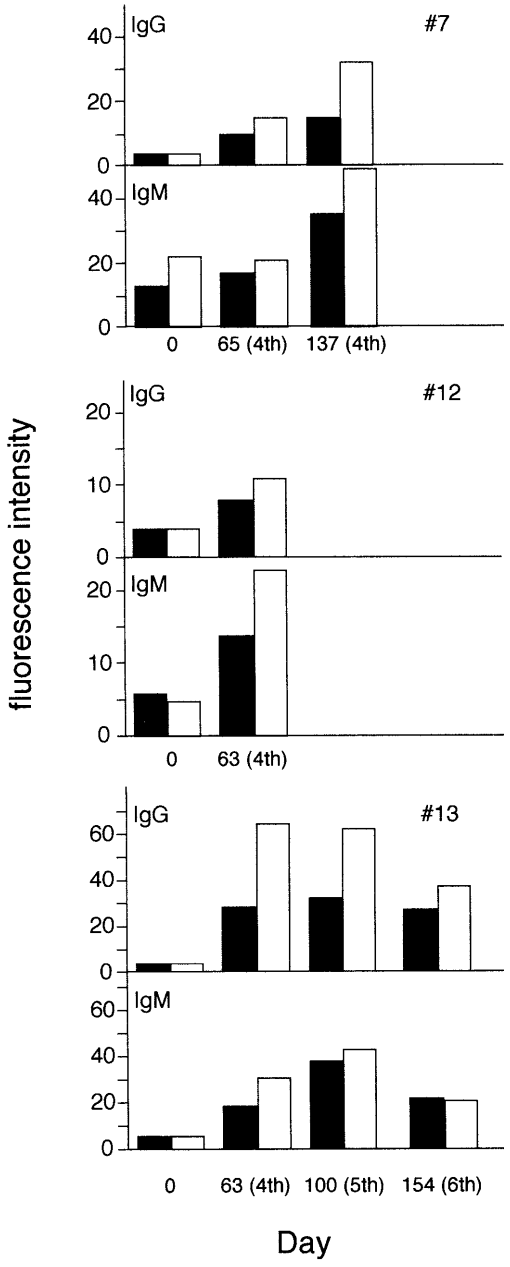

Fig. 1 Binding of sera from patients #7, #12 and #13 on C1R and C1R-A3$^+$ lymphoblastoid cells. C1R (*black bars*) and C1R-A3$^+$ (*white bars*) lymphoblastoid cells were sequentially incubated with pre-immune (day 0) and immune sera collected at different time intervals and number of vaccinations (*in brackets*) as indicated, and with FITC-conjugated F(ab')$_2$ fragments of goat anti-human IgG or with DTAF-conjugated F(ab')$_2$ fragments of goat anti-human IgM. Then, cells were analyzed by flow cytometry. Data represents the values of median fluorescence intensity

cytometry data demonstrated that circulating IgG and IgM, which stained C1R-A3$^+$ cells with a higher intensity than C1R cells, were detectable in all investigated patients after the 4th vaccination. Furthermore, in selected patients (#7, #13) the titer of circulating anti-HLA-A3 Ab increased with additional vaccinations (Fig. 1). Control sera from healthy individuals stained (IgG and/or IgM) neither C1R-A3$^+$ nor C1R cells (data not shown).

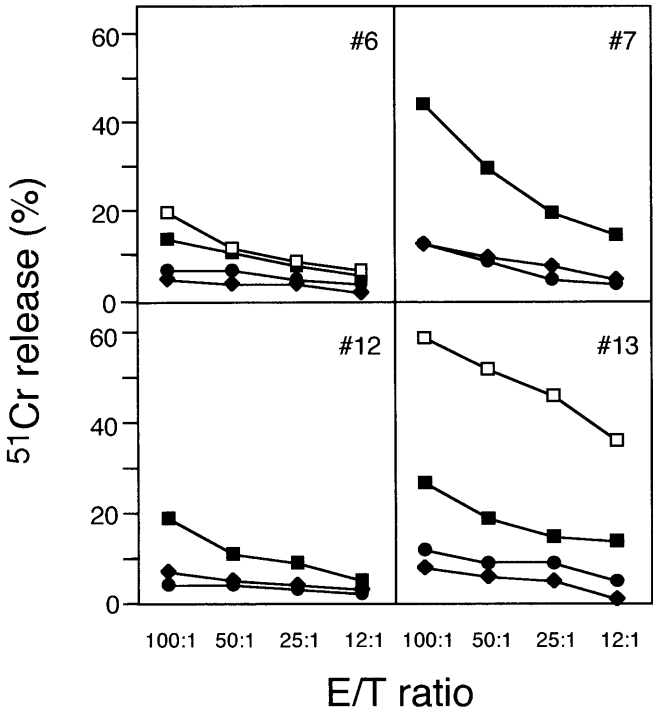

Fig. 2 ADCC of Me14932 melanoma cells by sera of patients #6, #7, #12 and #13. ^{51}Cr-labeled Me14932 melanoma cells were incubated with NHS (◆), pre-immune (●) or immune sera collected after 4 (■) or 6 (□) vaccinations. At the end of a 20-min incubation, cells were added with scalar concentrations of PBMC from a healthy subject to reach the indicated E/T ratios. After a 4-h incubation at 37 °C, supernatant (0.1 ml) was harvested from each well and released radioactivity was counted in a gamma counter. Data represent the percentage of specific ^{51}Cr release

Immune sera mediated ADCC and CDC of Me14932 and autologous melanoma cells

To investigate the functional activity of vaccination-generated Ab, immune sera from the 5 patients that developed circulating Abs against Me14932 cells were evaluated for their ability to mediate ADCC and/or CDC of Me14932 cells and, when available, of autologous melanoma cells.

The results reported in Fig. 2 show that immune sera from patients #6, #7, #12 and #13 induced ADCC of Me14932 cells and to a different extent. Interestingly, linear regression analysis identified a direct correlation between intensity of staining (IgG) and extent of lysis of Me14932 cells ($r = 0.85$, $r^2 = 0.72$, $P = 0.03$). In addition, immune serum of patient #7, that showed IgG and IgM binding to autologous melanoma cells, induced ADCC also of autologous neoplastic cells (Fig. 3). Comparable ADCC results were obtained using allogeneic (Fig. 2 and Fig. 3) or autologous (data not shown) PBMC as effector cells.

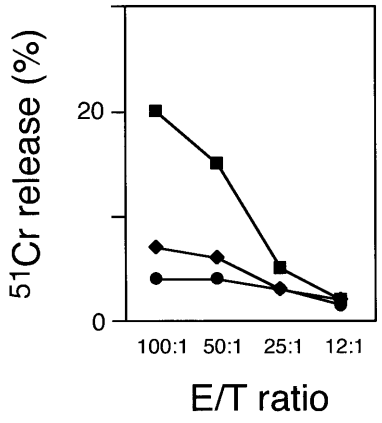

Fig. 3 ADCC of autologous melanoma cells by sera of patient #7. ^{51}Cr-labeled melanoma cells were incubated with NHS (◆), pre-immune (●) or immune sera collected after 4 (■) vaccinations. At the end of a 20-min incubation, cells were added with scalar concentrations of PBMC from a healthy subject to reach the indicated E/T ratios. After a 4-h incubation at 37 °C, supernatant (0.1 ml) was harvested from each well and released radioactivity was counted in a gamma counter. Data represent the percentage of specific ^{51}Cr release

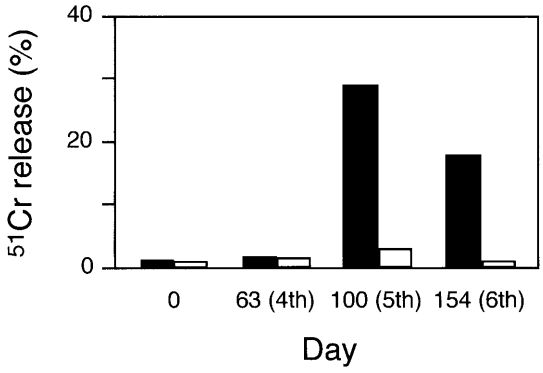

Fig. 4 CDC of Me14932 melanoma cells by sera of patient #13. ^{51}Cr-labeled Me14932 (*black bars*) and autologous (*white bars*) melanoma cells were incubated with pre-immune (day 0) or immune sera collected at different time intervals and number of vaccinations (*in brackets*) as indicated. At the end of a 20-min incubation, cells were added with NHS as source of C. After a 1-h incubation at 37 °C, supernatant (0.1 ml) was harvested from each well and released radioactivity was counted in a gamma counter. Data represent the percentage of specific ^{51}Cr release

Among investigated sera, serum collected after the 5th immunization from patient #13 induced CDC of Me14932 melanoma cells, but not of autologous melanoma cells (Fig. 4).

Immune sera of patients #6, #7, #12 and #13 did not induce ADCC or CDC of Colo 201 colorectal carcinoma cells (data not shown). Additionally, sera from healthy donors did not mediate ADCC or CDC of Me14932 melanoma cells, and of melanoma cells from patient #7.

Discussion

The results of our study demonstrate that vaccination with allogeneic melanoma cells transduced with a IL-4 or IL-2 gene generated circulating Abs directed against vaccinating and autologous melanoma cells in 5 out of 8 (62%) patients after the 4th vaccination. This finding clearly indicates that repeated immunizations with genetically engineered Me14932 cells, in addition to inducing or amplifying a TAA-specific CTL response [3, 4], efficiently induced a humoral immune response in the majority of treated patients. Additionally, the finding that selected patients also developed Ab directed to autologous melanoma cells, suggests that this vaccination approach could break immune tolerance, priming a humoral immune response against the autologous tumor.

Supporting recent in vivo evidence in the murine model demonstrating that IL-4 plays a role in the development of CTL-mediated tumor immunity [20], we found that local release of IL-4 from engineered vaccinating Me14932 cells generated a systemic T-cell-mediated immunity in selected vaccinated patients [4]. Similar results were observed in patients vaccinated with IL-2-releasing Me14932 cells [3]. Our present data show a stronger Ab (IgG and IgM) response to Me14932 cells in patients vaccinated with IL-4-releasing Me14932 cells, compared to patients vaccinated with IL-2-releasing Me14932 cells. Furthermore, an Ab response to autologous melanoma cells was identified only in patients vaccinated with IL-4-releasing melanoma cells. These findings are consistent with the role of IL-4 in supporting humoral immunity [11], and suggest that vaccination with IL-4- rather than with IL-2-transduced allogeneic melanoma vaccines is more effective in potentiating the immunogenicity of vaccinating cells.

Vaccination-induced circulating Abs were, at least partially, directed against the HLA-A3 allele expressed on vaccinating cells, but not on autologous melanoma cells. This finding, together with the demonstration that the titer of anti-HLA-A3 Ab persisted, or even increased, with additional vaccinations, suggests that the identification and titration of alloreactive Ab may represent a useful strategy for monitoring the extent of immunization against allogeneic cellular vaccines in the course of treatment. Furthermore, immune sera from selected patients reacted weakly with C1R cells (Fig. 1), indicating that Ab reactive to cell surface antigens shared between vaccinating and C1R cells were also generated.

Additionally, the finding that immune sera mediated ADCC of Me14932 cells demonstrates that repeated immunizations with IL-4- or IL-2-transduced allogeneic melanoma cells generates a functional humoral response to vaccinating cells. This experimental evidence is further supported by the identified correlation between the intensity of IgG binding and the extent of lysis of Me14932 cells by immune sera. These results, together with the observation that selected immune sera also mediated CDC of Me14932 cells, indicate that a vaccination-induced host humoral immune response might significantly contribute to effectively reject vaccinating

cells in vivo. This assumption is supported by the evidence that repeated administration of vaccinating cells rapidly evoked a local skin reaction that was characterized by erythema, swelling and induration, and by the absence of detectable vaccinating cells seven days after their administration (data not shown).

Vaccination of patient #7 with Me149323/IL-4 cells generated a strong anti-Melan-A/MART-$1_{27–35}$ peptide CTL response [4], together with functional Ab to autologous tumor cells (Table 2, Fig. 3). Noteworthy, Melan-A/MART-$1_{27–35}$ peptide was found to be expressed on the cell surface of melanoma cells of this patient, but not of vaccinating cells that expressed Melan-A/MART-$1_{27–35}$ protein only at the cytoplasmic level [4]. The identification of an anti-Melan-A/MART-$1_{27–35}$ peptide CTL response in this patient led us to speculate that cross-priming through the host's APC had occurred following physical and/or cell-mediated destruction of vaccinating cells at injection sites [4]. However, our present identification in patient #7 of treatment-generated functional Abs directed against both vaccinating and autologous melanoma cells, strongly suggests that these Abs could have significantly contributed to the cross-priming against Melan-A/MART-$1_{27–35}$ peptide through its release by ADCC-destroyed vaccinating and/or autologous melanoma cells.

Taken together, the results of this study demonstrate that repeated immunizations with IL-4- or IL-2-transduced allogeneic melanoma cells elicit functional Abs that, in addition to mismatched HLA class I antigens expressed on vaccinating cells, recognize antigens expressed on autologous melanoma cells. Thus, vaccination-generated Abs may contribute to the in vivo immunogenicity of selected tumor antigens and may contribute to the therapeutic efficacy of cellular vaccines mainly designed to induce/up-regulate a tumor-specific CTL response.

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