Tumor-Specific CD8+ T Cell Reactivity in the Sentinel Lymph Node of GM-CSF–Treated Stage I Melanoma Patients is Associated with High Myeloid Dendritic Cell Content

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Abstract

Purpose: Impaired immune functions in the sentinel lymph node (SLN) may facilitate early metastatic events during melanoma development. Local potentiation of tumor-specific T cell reactivity may be a valuable adjuvant treatment option.

Experimental Design: We examined the effect of locally administered granulocyte/macrophage-colony stimulating factor (GM-CSF) on the frequency of tumor-specific CD8+ T cells in the SLN and blood of patients with stage I melanoma. Twelve patients were randomly assigned to preoperative local administration of either recombinant human GM-CSF or NaCl 0.9%. CD8+ T cells from SLN and peripheral blood were tested for reactivity in an IFNγ ELISPOT assay against the full-length MART-1 antigen and a number of HLA-A1, HLA-A2, and HLA-A3–restricted epitopes derived from a range of melanoma-associated antigens.

Results: Melanoma-specific CD8+ T cell response rates in the SLN were one of six for the control group and four of six for the GM-CSF-administered group. Only one patient had detectable tumor-specific CD8+ T cells in the blood, but at lower frequencies than in the SLN. All patients with detectable tumor-specific CD8+ T cells had a percentage of CD1a+ SLN-dendritic cells (DC) above the median (i.e., 0.33%). This association between above median CD1a+ SLN-DC frequencies and tumor antigen–specific CD8+ T cell reactivity was significant in a two-sided Fisher's exact test ($P = 0.015$).

Conclusions: Locally primed antitumor T cell responses in the SLN are detectable as early as stage I of melanoma development and may be enhanced by GM-CSF-induced increases in SLN-DC frequencies.

Melanoma is the most immunogenic tumor identified to date, and as such, it is a prime candidate for the implementation of novel immunotherapeutic approaches. *In vivo* primed T cells reactive to a wide range of melanoma-specific tumor-associated antigens are detectable in tumors, in tumor-draining lymph nodes, and in the blood of patients with melanoma, and most importantly, their frequency can be increased by tumor-associated antigen–specific vaccination (1–8). T cell infiltration of melanoma tumor fields has been identified as an important prognostic factor (9). Indeed, we recently found that in patients with late stage melanoma, tumor-specific tumor-infiltrating CD8+ T cells may afford a survival advantage, whereas circulating tumor-specific CD8+ T cells do not (7, 10). These findings offer a clear indication of the ability of effector T cells to control melanoma development locally.

For the generation of an effective antimelanoma T cell response, high numbers of sufficiently activated myeloid dendritic cells (MDC) are essential (11). MDC take up antigens from the tumor tissue environment, and after sufficient activation, transport these to draining lymph nodes for presentation and activation of specific T cells. Unfortunately, immune effector functions in tumor-conditioned microenvironments are often disturbed, resulting in tolerance rather than immune activation. Specifically, MDC differentiation and activation can both be frustrated by melanoma-derived suppressive factors (e.g., interleukin-10 and gangliosides; refs. 12–15). The first lymph node to directly drain the primary tumor, the so-called sentinel lymph node (SLN), is the preferred site of early metastasis (16–18) and takes the brunt of the tumor-induced immunosuppression (15). In early stages of melanoma development, SLNs show signs of profound MDC suppression, both in terms of numbers and of phenotypic activation (19, 20). This will likely cripple specific T cell

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Table 1. Patient and SLN characteristics

	GM-CSF	Control	*P*
Sex (M/F)	4/2	3/3	0.575*
Age (mean ± SD)	56 ± 11	57 ± 15	0.747*
Breslow thickness (mm, mean ± SD)	1.04 ± 0.33	0.84 ± 0.27	0.262*
Positive SLN	0	0	1.000*
Time from primary excision to SLN procedure (day, mean ± SD)	49 ± 32	66 ± 50	0.470*
HLA-A1	2 of 6	1 of 6	1.000†
HLA-A2	3 of 6	1 of 6	0.545†
HLA-A3	0 of 6	3 of 6	0.182†

*Two-sample Mann-Whitney *U* test.
†Two-tailed Fisher's exact test.

activation, increasing the chances of tumor immune escape and metastatic spread (20, 21).

We recently reported on the dendritic cell (DC)-modulatory effects of intradermal injections of granulocyte/macrophage colony-stimulating factor (GM-CSF) around the excision site of stage I melanoma tumors, resulting in significantly increased numbers and activation state of MDC in the paracortical T cell areas of the SLN (22). In view of the critical role of MDC in the initiation of T cell–mediated immunity, we hypothesized that potentiated MDC functions in the GM-CSF-administered group might be reflected in a higher number of tumor-specific CD8+ T cells in the SLN. This hypothesis is indeed supported by our finding that local priming of melanoma-specific CD8+ T cells, as early as stage I of melanoma development, is associated with a high MDC content of the SLN, as observed in patients receiving locally administered GM-CSF. We conclude that local GM-CSF administration may offer a valuable adjuvant therapy option for patients with early-stage melanoma, aimed at the control of early metastatic events.

Materials and Methods

Patients and GM-CSF administration. Twelve patients with stage I melanoma according to criteria of the American Joint Committee on Cancer, scheduled to undergo a SLN procedure were assigned randomly to preoperative local administration of either recombinant human GM-CSF or NaCl 0.9% (22). All patients qualified for a SLN procedure, with Breslow thickness ≥1 mm or with Breslow thickness ≤1 mm, but with Clark level ≥IV, regression, ulceration, or other high-risk factors (23). Patients who had undergone previous immunotherapy or chemotherapy were excluded, as well as patients using immunosuppressive medication, or suffering from any autoimmune disorder. The study was approved by the medical ethical committee of the VU University Medical Center and written informed consent was obtained from each patient before treatment. Patient characteristics are listed in Table 1. Both patient groups received daily intradermal injections, with either 3 μg per kg body weight rhGM-CSF (Leucomax; Schering Plough, Maarssen, the Netherlands), dissolved in 1.0 mL NaCl 0.9%, or 1.0 mL plain NaCl 0.9%. These injections were given directly adjacent to the scar of the primary melanoma excision, from day −3 until day 0 (just prior to surgery).

Triple-technique SLN procedure and isolation of viable SLN cells. On day 0, patients underwent a triple-technique SLN procedure as described previously (24, 25). In short, the day before surgery, patients underwent a dynamic and static lymphoscintigraphy to determine the lymphatic drainage pattern. Just prior to surgery, a blue inert dye was injected adjacent to the site of the primary melanoma excision. During surgery, guided by the blue staining of the draining tissues, the radioactivity and the preoperatively made lymphoscintigraphy, SLNs were removed. Immediately after removal, the SLN was collected in sterile ice-cold complete medium, comprised of Iscove's modified Dulbecco's medium supplemented with 25 mmol/L HEPES buffer (Bio Whittaker, Verviers, Belgium), 50 IU/mL penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L β-mercaptoethanol, supplemented with 10% FCS. Before routine histopathologic examination of the SLN, viable cells were isolated using a previously described cytologic scraping method (26). In short, after measuring the size of the SLN, it was bisected crosswise and the cutting surface of the SLN was scraped 10 times with a surgical blade (size no. 22; Swann Morton, Ltd., England). SLN cells were rinsed from the blade with Iscove's modified Dulbecco's medium containing 0.1% DNase I, 0.14% collagenase A (Boehringer, Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently in PBS with 5 mmol/L EDTA for 10 minutes on ice. Finally, the SLN cells were washed twice in complete medium, counted, and further processed. After isolation of viable SLN cells, the bisected SLN was examined by the pathologist according to routine diagnostic procedures (27).

Isolation of peripheral blood mononuclear cells and flow cytometry. On day 0, before surgery, 40 to 50 mL of blood was drawn from each patient. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). Cells were washed twice with sterile PBS with 0.1% bovine serum albumin. To determine the patients' HLA-A1, HLA-A2, or HLA-A3 status, PBMC were stained with monoclonal antibodies, BB7.2 and MA 2.1 (HLA-A2), GAP A3 (HLA-A3), (American Tissue Culture Collection, Rockville, MD), and A1/A36 (HLA-A1, One Lambda, Canoga Park, CA), whereas CD4/CD8 status was checked with directly labeled anti-CD4 and anti-CD8 (BD, San Jose, CA). Freshly isolated SLN cells were directly stained with PE-labeled CD1a (PharMingen, San Diego, CA) and FITC-labeled CD83 (Immunotech, Marseilles, France) antibodies and analyzed by flow cytometry at 100,000 events per measurement, as previously described (26).

T cell expansion. To obtain sufficient cells for functional analysis, and for the sake of uniformity, T cells from both the SLN and PBMC were expanded as described previously (26). Cells were incubated for 1 hour on ice with 2 μg anti-CD3 and 0.4 μg anti-CD28 per 1×10^6 cells (kindly provided by Dr. René van Lier, CLB, Amsterdam, the Netherlands) in 100 to 200 μL of complete medium with 5% FCS. After incubation and washing, the cells were placed on 24-well plates, coated with affinity-purified goat anti-mouse immunoglobulin (1:100; Dako, Glostrup, Denmark) in complete medium with 10% human pooled serum (CLB) at a concentration of 1×10^6 cells/mL/well for 1 hour at

4°C. The cells were cultured for 48 hours in a humidified 5% CO_2 incubator at 37°C. After 48 hours, the cells were resuspended and the contents of each well was divided over four new uncoated wells at a volume of 250 μL per well. To each new well, 750 μL of fresh culture medium was added and rhIL-2 (CLB) to a final concentration of 10 IU/mL. The cells were cultured for another 5 days, after which they were harvested and counted. This expansion cycle was repeated at least once more or until sufficient numbers were obtained: T cells from four patients in the control group and five patients in the GM-CSF-administered group underwent two expansion cycles, one patient each from the control and the GM-CSF-administered group underwent three expansion cycles, whereas one patient in the control group underwent four expansion cycles. Finally, the expanded T cells were harvested, counted, frozen, and stored for functional analysis at a later date.

Culture and adenovirus infection of monocyte-derived dendritic cells. Monocyte-derived dendritic cells (MoDC) were generated according to previously described methods (28). Plastic-adherent monocytes from 3 to 5 × 10^6 PBMC per mL CM were cultured for 7 days in complete medium with 10% FCS in the presence of 100 ng/mL GM-CSF (Schering-Plough) and 1,000 IU/mL interleukin-4 (CLB). The DC phenotype (CD1a+/CD14−) was confirmed by flow cytometry as previously described (29). For each patient, autologous MoDC were infected with E1-deleted adenoviral (type 5) vectors encoding the melanoma-associated Mart-1 protein (Ad-Mart-1) or (as a negative control) green fluorescent protein (Ad-GFP; both kindly provided by Dr. D.T. Curiel, University of Alabama, Birmingham, AL). MoDC were infected at a multiplicity of infection of 100, in a CD40-targeted fashion, using a bispecific antibody conjugate, as previously described by Tillman et al. (29). The day following infection, MoDC were washed and further used in a CD8+ T cell activation assay.

Peptide loading of MoDC and T2 stimulator cells. A panel of HLA-A1, HLA-A2, or HLA-A3–binding peptides, derived from various melanoma-associated tumor antigens and containing previously described CD8+ T cell epitopes (Table 2), was used for CD8+ T cell reactivity testing. Peptide-loaded T2 cells were employed as stimulator cells in ELISPOT readouts for HLA-A2+ patients. Additional inclusion of patients with HLA-A1+ and HLA-A3+ necessitated the use of immature autologous MoDC for this purpose as HLA-A1- or HLA-A3–transduced T2 cell lines were not available to us at that time. T2 cells or MoDC were loaded overnight in serum-free medium with β2-microglobulin (5 μg/mL; Sigma, St. Louis, MO) and melanoma-associated or control peptides (50 μg/mL) at 37°C in a humidified 5% CO_2 incubator. For A2- and A3-binding melanoma CD8+ T cell peptides, A2- and A3-binding control peptides were used, derived from the human papillomavirus type-16 E7 and Bcr-abl protein sequences, respectively. As no appropriate control peptide was available for A1-binding melanoma peptides, unloaded MoDC were used as negative control stimulators. After overnight pulsing, stimulator cells were washed, counted, and used for CD8+ T cell activation testing.

Melanoma-specific* ex vivo *CD8+ T cell activation and IFNγ ELISPOT analysis. To functionally test the expanded CD8+ T cells for melanoma-specific reactivity, an IFNγ ELISPOT assay was done (30, 31). As effector cells, anti-CD3/anti-CD28-stimulated and expanded CD8+ T cells from the SLN and PBMC were used. CD8+ T cells were isolated from the expanded T cell population using the untouched CD8+ mini-MACS selection kit, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation, CD8+ T cells were washed and resuspended in complete medium with 10% FCS and added directly to the transduced or peptide-loaded MoDC or T2 stimulator cells in anti-IFNγ precoated Multiscreen 96-well filtration plates (Millipore, Molsheim, France). Plates were seeded with 10,000 stimulator (S)-cells (MoDC or T2 cells) per well and 100,000 or 50,000 effector (E) CD8+ T cells, resulting in E/S ratios of 10:1 and 5:1, respectively. Assays with adenoviral-transduced MoDC were tested in quadruplicate. All other assays were tested in quadruplicate where possible, but at least in duplicate at each of the E/S ratios. After overnight incubation (18 hours), the cells were flicked off and an ELISPOT assay was done as previously described (32), using a commercially available anti-IFNγ monoclonal antibody pair (Mabtech, Nacka, Sweden). After development of the plates, spots were counted by an automated ELISPOT reader (AID Diagnostika, Strassberg, Germany). CD8+ T cell ELISPOT activity was expressed either as the number of spots per well or as the number of specific effector CD8+ T cells per 1 × 10^6 CD8+ T cells (obtained by subtracting the frequencies of spot-forming CD8+ T cells in control conditions from the spot-forming CD8+ T cell frequencies in the test conditions, based on results from the highest E/S ratio, 10:1). ELISPOT CD8+ T cell responses were considered positive when (*a*) the number of spots in the test condition were significantly higher than the number of spots in the control condition in an unpaired two-sided Student's *t* test ($P < 0.05$), comparing values obtained from both of the tested E/S ratios and recalculating the number of spots per 100,000 CD8+ T cells, (*b*) the mean number of spots for both E/S ratios of the test condition exceeded the number of spots for the corresponding E/S ratios of the control condition by at least 2-fold, and (*c*) the absolute difference in number of spots between the test and control condition in the highest E/S ratio (10:1) was at least five.

Table 2. Peptides used for CD8+ T cell IFNγ ELISPOT analysis

Binding to	CD8+ T cell epitope and tumor antigen origin
HLA-A1	Tyrosinase 145-156
	Tyrosinase 243-251
HLA-A2	Mart-1 26-35
	Tyrosinase 369-377
	gp-100 154-162
	Mage-A3
	NY-ESO 157-165
HLA-A3	Mage-A1 96-104
	gp-100 17-25

HLA-A2-tetramer binding analysis. In all HLA-A2+ patients ($n = 4$), expanded T cells were stained with HLA-A2/peptide tetramers, comprising the same HLA-A2-binding melanoma-associated peptides that were used in the ELISPOT analysis (Table 2). APC-conjugated HLA-A*0201 tetramers were generated as described previously (7, 33, 34). Expanded T cells from PBMC and SLN (consisting of only T cells, as checked by flow cytometric analysis with a cocktail of lineage-specific monoclonal antibodies against CD14, CD15, CD16, CD56, and CD19; BD Biosciences, San Jose, CA) were stained with the APC-labeled tetramers (40 μg/mL) by incubation for 15 minutes in CM at 37°C in a humidified 5% CO_2 atmosphere, after which, the cells were washed and kept at 4°C. The cells were subsequently analyzed by flow cytometry, double-staining (after tetramer binding) for CD8 (PE-labeled, BD Biosciences) and gating out dead cells by propidium iodide uptake. The total number of analyzed T cells was at least 1,000,000 events per measurement, as a detection limit of 0.01% of CD8+ T cells was assumed.

Statistical analysis. Differences in patient characteristics between test groups were analyzed using the two-sample Mann-Whitney *U* test and in HLA status and T cell response rates using Fisher's exact test. Differences were considered significant at $P < 0.05$.

Results

Patients. There were no significant differences in patient characteristics between the GM-CSF and the control group (Table 1). Intradermal administration of GM-CSF was well tolerated and none of the SLN showed metastatic tumor cells

on routine histopathologic testing. HLA typing was done using flow cytometry, and in both treatment groups, two patients were negative for either HLA-A1, HLA-A2, or HLA-A3, which made them ineligible for IFNγ ELISPOT testing and HLA-tetramer staining for melanoma-specific epitopes restricted by these HLA types.

CD8+ T cell reactivity in the SLN and peripheral blood. T cell responses against Mart-1 and the tested melanoma peptides (Table 2) were evaluated by IFNγ ELISPOT assay. So as not to interfere with routine diagnostic procedures and to ensure sufficient numbers of T cells for functional analyses, T cells were nonspecifically expanded by anti-CD3 and anti-CD28 stimulation, after which untouched CD8+ T cells were isolated by negative selection, using a magnetic bead cocktail, and tested overnight (26). The number of required expansion rounds for sufficient yields of CD8+ T cells was not significantly different between the control and the GM-CSF-administrated group. Also, the mean preexpansion and postexpansion CD4/CD8 ratios were equivalent for the control group (preexpansion CD4/CD8 ratio, 7.33 ± 3.94; postexpansion CD4/CD8 ratio, 17.0 ± 14.3) and the GM-CSF-administered group (preexpansion CD4/CD8 ratio, 8.03 ± 4.67; postexpansion CD4/CD8 ratio, 16.2 ± 14.5). CD8+ T cell responses against Mart-1 were evaluated independently of HLA status for all patients, using autologous MoDC infected with an adenoviral vector encoding full-length Mart-1. Two GM-CSF-administered patients, but none of the 0.9% NaCl–administered patients, showed a positive Mart-1-specific SLN CD8+ T cell response (Fig. 1). This was not significant in a two-sided Fisher's exact test ($P = 0.227$).

CD8+ T cell reactivity against defined melanoma epitopes was tested in HLA-A1, HLA-A2, or HLA-A3-positive patients and the obtained response rates in the SLN were one of four for the control group and three of four for the GM-CSF-administered group. Results for the positively responding patients, including the calculated specific CD8+ T cell frequencies, are shown in Fig. 2A. One GM-CSF-administered patient (patient 8) showed a T cell response against both full-length Mart-1 and melanoma peptides (including the HLA-A2-restricted peptide Mart-1 26-35; Fig. 2A). Overall SLN CD8+ T cell response rates, to either full-length Mart-1 or the melanoma-associated peptides were one of six for the control group and four of six for the GM-CSF-administered group (Table 3A). Higher melanoma-specific CD8+ T cell reactivity was consistently found in the SLN than in peripheral blood (PB). Only one patient (patient 6) showed any specific CD8+ T cell reactivity in the peripheral blood (Fig. 2A).

For all HLA-A2-positive patients ($n = 4$), HLA-A2-tetramer binding analysis was also done. Cells were analyzed by flow cytometry after double staining for CD8 and HLA-A2 tetramers [showcased in Fig. 2B for patient 9 (a negative responder in the ELISPOT assay) and patient 12 (a positive responder)]. A detection limit of 0.01% was assumed and HLA-A2 tetramer+ populations exceeding this percentage of the total tested CD8+ T cell population were considered to signal positive reactivity against a given epitope. Even though the detected frequencies were not always equivalent, melanoma-specific CD8+ T cell reactivity, as determined by IFNγ ELISPOT assay, could generally be confirmed for all three positively responding HLA-A2+ patients by HLA-A2-tetramer binding analysis (Fig. 2C). Of the 17 peptide tests carried out between the four HLA-A2+ donors, CD8+ T cell reactivity was found both with ELISPOT and by tetramer-binding in seven tests, no reactivity was found with either method in eight tests, whereas ELISPOT reactivity was found which could not be confirmed by tetramer binding in two instances (overall concordance between tests, 88%).

CD1a+ SLN-MDC content in relation to melanoma-specific CD8+ T cell reactivity. Previously, we found a significant increase in the frequency of mature CD1a+ CD83+ SLN-MDC after GM-CSF administration (22). Mean percentages of SLN-MDC were 0.68% in the GM-CSF group and 0.15% in the control group ($P = 0.006$). When patients were classified according to the percentage of CD1a+ SLN-MDC, an association with positive melanoma antigen-specific CD8+ T cell reactivity became apparent (Fig. 3). Melanoma-specific CD8+ T cell reactivity was found in five of six patients with a percentage of SLN-MDC above the median (i.e., 0.33% SLN-MDC—based on all 12 tested patients), whereas no reactivity was detected in any of the patients with a percentage of SLN-MDC below the median (Table 3B). This T cell reactivity was significant in two-sided Fisher's exact test ($P = 0.015$).

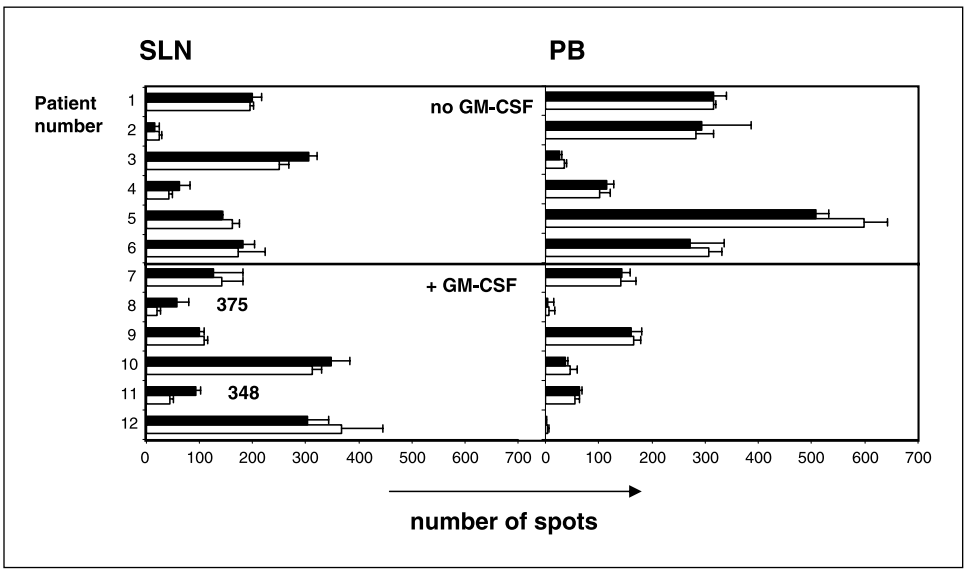

Fig. 1. CD8+ T cell responses against full-length Mart-1 were tested in a 1-day IFNγ ELISPOT analysis in SLN and PB from patients with stage I melanoma receiving intradermally injected NaCl 0.9% (no GM-CSF) or GM-CSF (+GM-CSF). ELISPOT reactivity is expressed as the number of spots per 100,000 CD8+ effector T cells. For each patient, autologous MoDC were infected with an adenoviral type 5 vector encoding melanoma-associated full-length Mart-1 (Ad-Mart-1; *closed columns*) or with an adenovirus encoding green fluorescent protein (Ad-GFP; negative control; *open columns*), and used to stimulate expanded and isolated CD8+ T cells. Two GM-CSF-administered patients (patients 8 and 11) showed a positive Mart-1-specific CD8+ T cell response in the SLN [specific CD8+ T cell frequencies (per 1×10^6 total CD8+ T cells) are listed].

Discussion

Cutaneous melanoma is the most aggressive type of skin cancer, for which complete surgical excision at an early stage remains the only curative treatment option. Adjuvant therapy options are limited and show no survival benefits. Naturally occurring melanoma-specific T cell responses have mostly been described in patients with advanced-stage melanoma, but are already detectable in the early stages of melanoma development (35). This underlines the immunogenicity of this particular tumor type and raises the possibility to boost these early immune responses by novel immunotherapeutic approaches in order to curb early metastatic events. Unfortunately, melanoma-infiltrating T cells and T cells in melanoma SLN have often been described to be in a state of tolerance due to tumor-induced immune suppression (5, 36). This is effected both indirectly at the level of DC and their interactions with T cells, and directly on the T cells themselves (37, 38). As shown in adoptive transfer studies, this state of functional tolerance is reversible *in vitro* and after activation and expansion, tumor-derived T cells can mediate significant tumor regression in patients with refractory melanoma (39–41). DC-based vaccination studies in patients with advanced-stage melanoma have also resulted in long-lasting anti-melanoma T cell responses but generally with limited clinical effects (35, 42). The ability to generate or boost melanoma-specific T cell responses *in vivo* and at earlier, more localized stages of melanoma development,

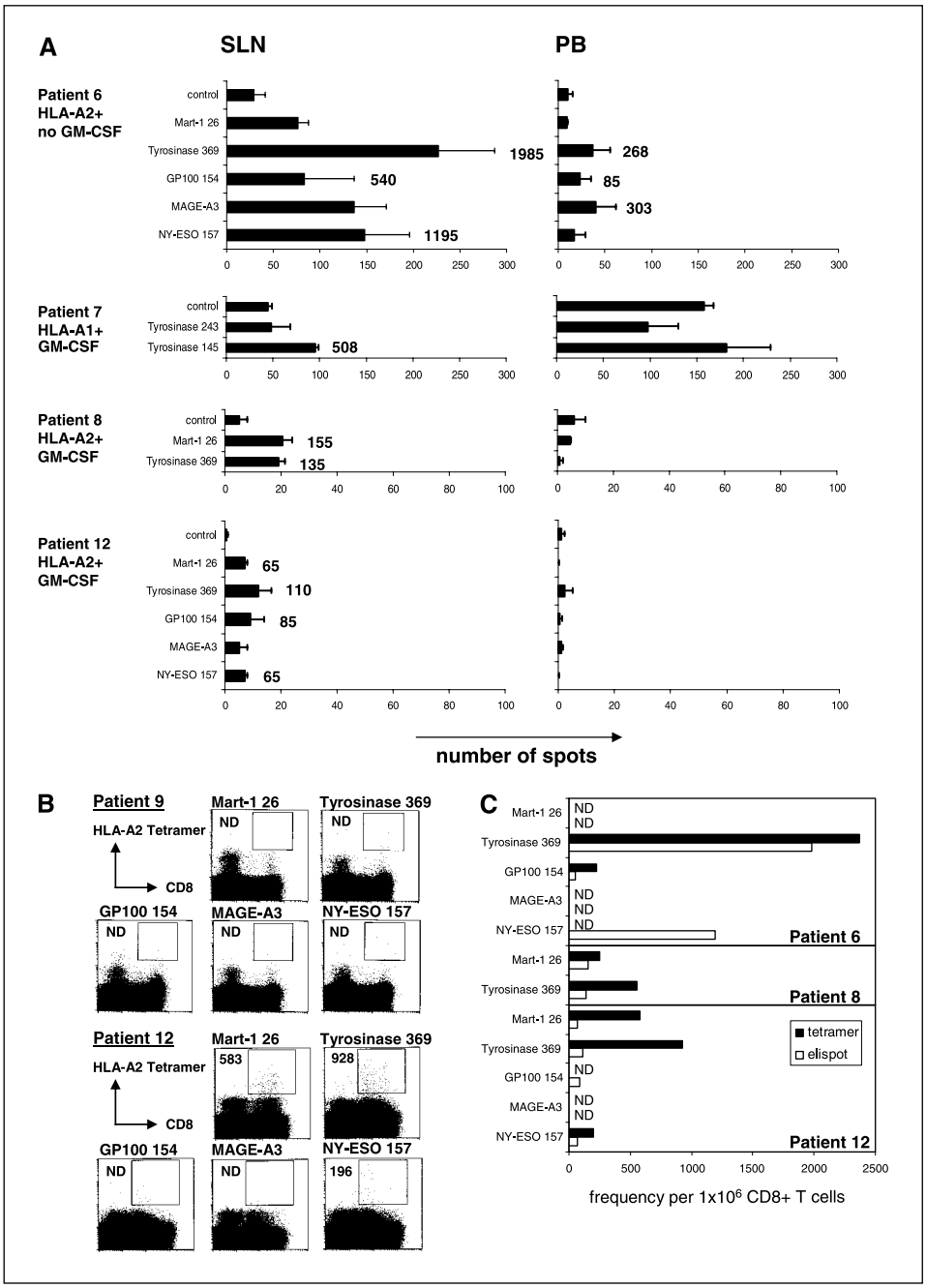

Fig. 2. Melanoma-specific CD8+ T cells from patients with stage I melanoma recognize HLA-A1- and HLA-A2-binding epitopes from multiple melanoma-associated antigens; responses are more prominent in the SLN than in PB. *A,* IFNγ ELISPOT reactivity is expressed as the number of spots per 100,000 CD8+ effector T cells. CD8+ T cell responses against peptides derived from various melanoma-associated antigens were tested in a 1-day ELISPOT analysis in SLN and PB. Melanoma-specific and control peptides were loaded onto autologous MoDC (in case of HLA-A1 or HLA-A3 positivity) or T2 cells (in case of HLA-A2 positivity), which were used to stimulate expanded and isolated CD8+ T cells. Data are only shown from positively responding patients; patient number, treatment allocation, and HLA status are indicated. Positive responses (against the listed epitopes) are denoted by the calculated specific CD8+ T cell frequencies (per 1×10^6 total CD8+ T cells). *B,* in HLA-A2+ patients, expanded T cells from SLN were stained with HLA-A2/peptide tetramers, comprising the same HLA-A2-binding melanoma-associated peptides that were previously tested in the ELISPOT analysis. Cells were analyzed by flow cytometry after double staining for CD8 and HLA-A2 tetramers; results are shown for patient 9, who previously did not respond in the IFNγ ELISPOT assay to any of the indicated epitopes from Mart-1, tyrosinase, GP100, MAGE-A3, and NY-ESO, and for patient 12, who previously responded to the epitopes derived from Mart-1, tyrosinase, GP100, and NY-ESO (*A*). Tetramer-binding CD8+ T cell frequencies (per 1×10^6 total CD8+ T cells; detection limit at 100) are listed (*ND,* not detectable). *C,* for all three positively responding HLA-A2+ patients melanoma-specific CD8+ T cell reactivity determined by IFNγ ELISPOT-assay (*open columns*) could be confirmed by HLA-A2-tetramer binding analysis (*closed columns*), although not for all tested epitopes. Nor were the calculated frequencies of specific CD8+ T cells (per 1×10^6 total CD8+ T cells) always equivalent between both methods (*ND,* not detectable).

Table 3. CD8+ Tcell reactivity rates in SLN and PB of the patients tested for stage I melanoma

(A) NaCl 0.9% or GM-CSF administration

	Control		GM-CSF	
	SLN	PB	SLN	PB
Ad-Mart-1	0 of 6	0 of 6	2 of 6	0 of 6
CD8+ Tcell peptides	1 of 4	1 of 4	3 of 4	0 of 4
Overall CD8+ Tcell reactivity	1 of 6	1 of 6	4 of 6	0 of 6

(B) CD1a+ SLN-DC content

	‹0.33% SLN-DC*		›0.33% SLN-DC	
	SLN	PB	SLN	PB
Ad-Mart-1	0 of 6	0 of 6	2 of 6	0 of 6
CD8+ Tcell peptides	0 of 3	0 of 3	4 of 5	1 of 5
Overall CD8+ Tcell reactivity	0 of 6†	0 of 6	5 of 6	1 of 6

*SLN-DC rate: percentage of CD1a+ cells of the total SLN cell population; overall median SLN-DC rate = 0.33%.
†Significantly lower than the corresponding SLN CD8+ Tcell reactivity rate in the "›0.33% SLN-DC" group (5 of 6) in a two-sided Fisher's exact test (P = 0.015).

might have clinical benefit through the clearance of early occurring micrometastases.

Lymphatic mapping and selective SLN excision in patients with melanoma is a minimally invasive procedure, which allows for the identification of patients at-risk for lymph node metastasis who should undergo a full therapeutic tumor-draining lymph node dissection. Recent studies have confirmed the SLN procedure to be safe and offers a possible survival benefit (23, 24, 43). Routine application of this procedure in patients with early stage melanoma presents a unique translational setting to study adjuvant therapies *in vivo* aimed at the potentiation of immune reactivity within the SLN. We therefore set up a small-scale clinical trial to investigate the effects of GM-CSF, injected around the scar of the previously removed primary melanoma, on the SLN immune status.

We hypothesized that a superior activation state of MDC in the melanoma SLN would be effected through the local administration of GM-CSF, and that such a microenvironment would be more conducive to the generation of T cell–mediated antitumor immunity (44, 45). We previously reported a significant increase in the number, maturation, and activation state of CD1a+ SLN-MDC after local GM-CSF treatment (22). We also found a significant increase in the number of T cells bound to the SLN-DC after GM-CSF administration and concluded that this might well be a reflection of protracted periods of binding between DC and T cells upon specific antigen recognition. In keeping with this, we now report that melanoma-specific CD8+ T cell response rates were higher in the GM-CSF-administered patients and correlated with CD1a+ SLN-MDC content. Moreover, a more robust melanoma-specific CD8+ T cell reactivity was observed in the SLN of the stage I melanoma patients compared with the peripheral blood.

To investigate melanoma-specific CD8+ T cell response rates in the SLN, viable T cells were obtained by scraping the cutting surface of bisected SLN. Previous validation of this technique showed that the viabilities and phenotypic characteristics of SLN cells obtained by scraping were entirely comparable to SLN cells obtained by dissociation of the total lymph node (26, 46). T cell functionality was also comparable between both methods, with equal T cell expansion factors and similar frequencies of CD8+ T cells specifically recognizing the M1 matrix protein of influenza haemophilus or the tumor antigen Her-2/neu (26).

To facilitate standardized functional testing of tumor-specific CD8+ T cells from all SLN samples, polyclonal T cell expansion was required. The employed polyclonal expansion method was previously shown to efficiently induce the proliferation of tumor-specific T cells from tumor-draining lymph nodes, while maintaining specificity at the clonal level, even after 3 months of culture (47, 48).

The number of HLA-A2-positive patients was more prevalent in the GM-CSF cohort, with three HLA-A2-positive patients in the GM-CSF- and only one in the saline-administered patient group. As this was a relatively small study, it was important to, as much as possible, avoid any bias in the T cell readouts that might result from this imbalance in HLA-A2 status between the two test groups. We therefore also included HLA-A1 and HLA-A3 binding melanoma antigen-derived epitopes in our panel to enable us to also test HLA-A1+ and HLA-A3+ patients. This resulted in a more balanced distribution with four evaluable patients in both test groups. Although more evaluable peptides were available for HLA-A2 (Table 1), the number of peptides tested per patient was not solely dictated by HLA status but by the number of CD8+ T cells that were isolated upon T cell expansion as well. Overall, the total number of ELISPOT assays per group was balanced with 16 peptides tested in four GM-CSF-administered patients and 13 peptides tested in four saline-administered patients. Of note, in a separate study carried out

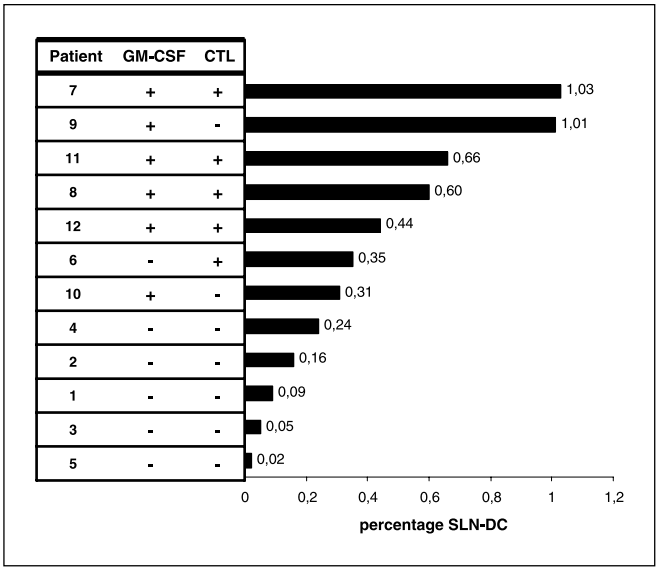

Fig. 3. High SLN-MDC frequencies correlate to melanoma-specific CD8+ Tcell reactivity. The GM-CSF (+) treated and NaCl 0.9% (−) treated patients are indicated (patient numbers listed) in relation to CD8+ Tcell reactivity (+, positive ELISPOT response against at least one of the tested tumor antigen epitopes) and the frequency (% of total SLN leukocytes) of CD1a+ SLN-MDC.

subsequent to this trial, we have tested six HLA-A2+ stage I/II melanoma patients that were similarly administered saline, and none of these displayed any reactivity against the same panel of tested peptides used in this study. This supports our assumption that the under-representation of HLA-A2+ patients in the saline control group of this study is not related to the lack of detection of tumor-specific CTL reactivity in this group. Moreover, as a measure of CTL reactivity independent of HLA status, we also included full-length MART-1 in our assays.

The higher melanoma-specific CD8+ T cell reactivity rate found in the SLN compared with the peripheral blood is consistent with the literature (1, 49), and is indicative of local priming. No external source of antigen was provided in the currently applied intradermal GM-CSF administration scheme. This suggests that the detected CD8+ T cells were primed against endogenous tumor-derived antigen sources, despite the fact that at the time of GM-CSF administration, no discernable tumor load remained in these patients. Interestingly in this context, recent studies from the Boon-Coulie laboratory showed that even in the case of melanoma antigen-specific vaccination, reinvigorated preexistent antitumor CTLs, rather than newly primed antivaccine CTLs, were likely responsible for the subsequent antitumor effects (8, 50). By way of explanation, they suggested that a temporary break of tumor-induced immunosuppression because of vaccination (possibly due to immunoactivating signals from the newly primed antivaccine CTL) might reactivate previously primed antitumor CTL and set into motion an antitumor response. A similar effect may be achieved by breaking immunosuppression with DC-activating agents such as GM-CSF. Our observation of an association between GM-CSF administration, high MDC frequencies, and increased antimelanoma CD8+ T cell frequencies certainly seems in line with this notion. The fact that we did not add an external tumor antigen source for new T cells to be primed against, suggests that either sufficient tumor-derived antigen traces lingered in the SLN (possibly in the context of residing macrophages or DC) to stimulate the melanoma antigen-reactive CD8+ T cells or that previous activation of antitumor CD8+ T cells had already induced a shift in the T cell repertoire resulting in increased antitumor T cell frequencies in the SLN, which may have been further reinforced by the general immunostimulation provided by GM-CSF. Importantly, no relationship between T cell reactivity rates and the length of interval between primary melanoma excision and the SLN procedure was found: in three of six patients with a below median interval and in two of six patients with an above median interval, melanoma-specific CD8+ T cells were detected. In line with this, the interval length between the responding and nonresponding patients did not differ significantly either (64 ± 46 versus 49 ± 35 days, respectively; $P = 0.371$ in the Mann-Whitney U test). Thus, the difference in T cell reactivity rates observed between the two test groups was not attributable to a possible difference in the retention of melanoma-derived antigens due to testing at varying time points subsequent to the removal of the primary tumor (i.e., the melanoma antigen source).

Although this is a small study and these data await confirmation in a larger trial, they are nevertheless important in that they show that (*a*) antitumor CD8+ T cells can already be primed *in vivo* in the earliest stages of melanoma development at a relatively low Breslow thickness and that (*b*) these CD8+ T cell responses seem to be enhanced by immunostimulatory agents such as GM-CSF. In future studies, we aim to also include patients with higher Breslow thickness, who are at greater risk for SLN metastasis. SLN metastases in these patients will increase the risk of immune suppression and further metastatic spread, but will also provide a ready source of tumor antigens for immune priming or boosting. Local GM-CSF administration in these patients will not only allow the further study of such processes as reversal of immune suppression and tumor-specific T cell priming, but also of any clinical benefit in terms of (disease-free) survival.

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