Reduced Number and Impaired Function of Circulating γδ T Cells in Patients with Cutaneous Primary Melanoma

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We studied the peripheral representation, *in vitro* expansion, cytokine production, and cytotoxicity of γδ T lymphocytes from 23 patients with cutaneous primary melanoma and 28 healthy subjects. We demonstrated that the absolute number and the percentage of circulating $\gamma\delta^+$ T cells were significantly reduced in melanoma patients in comparison with healthy subjects. The decrease was due to a reduction of Vδ2 T cells, whereas the number of Vδ1 T cells was not affected. As a consequence, the Vδ2/Vδ1 ratio was inverted in melanoma patients. A lower percentage of $\gamma\delta^+$ T cells producing tumor necrosis factor-α or interferon-γ was found in melanoma patients. After a 10 d *in vitro* culture, both the percentage and the expansion index of γδ T cells, and in particular of Vδ2 subset, were significantly reduced in melanoma patients in comparison with healthy subjects. The cytotoxicity of sorted γδ T cells against tumor cell lines and the percentage of γδ T cells producing perforins were preserved in melanoma patients. The numerical and functional impairment of γδ T cells could contribute to the inadequate immune response found in melanoma patients and offers the potentiality for the planning of new approaches of immune therapy of malignant melanoma. ***Key words: fluorescence-activated cell sorter/human/melanoma/tumor immunity/γδ T cells J Invest Dermatol 120:829–834, 2003***

Various clinical and experimental observations point to the existence of an immunologic host defense in cutaneous malignant melanoma. $CD3^+$ T cell receptor (TCR) α/β-expressing lymphocytes are considered the prevailing lymphocyte subset in primary as well as secondary malignant melanoma (Strohal *et al*, 1994). Immunomodulatory therapies with cytokines or adoptive transfer of T cells have accomplished complete or partial tumor regression in melanoma patients. Nevertheless, the immune response is in most cases inadequate to control tumor growth as tumor progression often occurs (Lee *et al*, 1999; Dudley *et al*, 2001). Hence, the coexistence of a cellular immune response in melanoma lesions (Kammala *et al*, 1999), demonstrated by the presence of clonally expanded T cells, and the inability of melanoma-specific killer T cells to arrest tumor growth, remains a major paradox of tumor immunology (Thor Straten *et al*, 1999; Nielsen *et al*, 2000).

T lymphocytes bearing the γδ TCR represent a minor population of human peripheral lymphocytes (1–10%) the majority of them expressing the Vδ9/Vδ2 TCR and the $CD3^+CD4^-CD8^-$ phenotype (Groh *et al*, 1989; Poccia *et al*, 1998). The second most frequent subset of peripheral blood γδ T cells expresses Vδ1 in association with various Vγ elements. The ability of γδ T cells to respond to nonprocessed and nonpeptidic phosphoantigens in a major histocompatibility complex (MHC)-unrestricted manner are important features distinguishing them from αβ T cells (Brenner *et al*, 1987; Bukowski *et al*, 1995). γδ T cells are activated by mycobacterial antigens, such as isopentenyl pyrophosphate (IPP) and related phenyl pyrophosphate derivatives (Costant *et al*, 1994; Tanaka *et al*, 1995; Burk *et al*, 1995), stress-associated heat shock proteins (Fisch *et al*, 1999), as well as by several cytokines, such as interleukin (IL)-2 (Kjeldsen-Kragh *et al*, 1993), IL-12 (Fujimiya *et al*, 1997), or tumor necrosis factor (TNF)-α (Ueta *et al*, 1996; Lahn *et al*, 1998). Other signals, such as MHC class I-related chains A and B, are able to engage the activating receptor, NKG2D, present on Vδ2 T cells, substantially enhancing the TCR-dependent Vδ2 T cell response to nonpeptide antigens (Das *et al*, 2001; Wu *et al*, 2002). Although little is known about the physiologic significance of γδ T cells, their marked reactivity toward mycobacterial and parasitic antigens, as well as tumor cells, suggests that γδ T cells play a part in the anti-infectious and anti-tumoral immune surveillance (Poccia *et al*, 1998; Zheng *et al*, 2001). γδ T cells may contribute to the immune defense against cancer, having at least two important functions, i.e., reactivity to tumor cells, and regulatory interactions with αβ T cells (Kabelitz *et al*, 1999). γδ T cells strongly react against certain lymphoma cells, such as Daudi cells, suggesting a cross-reactivity between microbial and tumor-associated antigens (Kunzmann *et al*, 2000). Furthermore, γδ T cells have been identified among tumor-infiltrating lymphocytes in various cancer types (Kabelitz *et al*, 2000). Once activated, γδ T cells produce high levels of cytokines and, mainly, IFN-γ and TNF-α (Poccia *et al*, 1997). Mainly because of their cytokine production, γδ T cells have been proposed to be involved in co-ordinating the interplay between innate and acquired immunity and, in particular, to guide the establishment of acquired immunity contributing to select appropriate antigens and the strategies for their elimination, and to the definition of αβ T cell responses

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toward T helper (Th) 1 or Th2 phenotype (Fearon and Locksley, 1996; Mak and Ferrick, 1998). The strict relationship between γδ and αβ T cells is bidirectional, as demonstrated by the fact that γδ T cell function may depend on αβ T cells (Vila *et al*, 1995; Burns *et al*, 1996).

The role of γδ T cells in the early anti-tumor defense against melanoma has been suggested by the evidence that γδ T cells may constitute up to 25% of lymphocyte infiltrate in cutaneous primary melanomas, whereas they are not present in metastatic melanoma (Bachelez *et al*, 1992). These cells exert potent cytotoxic activity against autologous tumor cells (Bachelez *et al*, 1992; Nanno *et al*, 1992). In another study, the survival of patients with necrotizing choroidal melanomas was increased with evidence of Vγ1 and Vδ1 TCR^+ cells (Bialasiewicz *et al*, 1999).

On the basis of the pivotal role that γδ T cells may have in the immune response against melanoma acting directly on tumor cells and, secondarily, by modulating the phenotype of T cell responses, we evaluated the peripheral representation and the *in vitro* expansion, cytokine production, and cytotoxicity of γδ T cells from melanoma patients, comparing the results with those obtained in healthy controls. This study demonstrated for the first time an alteration of circulating γδ T cells in melanoma patients, with a significant decrease of the number of γδ T cells in the peripheral blood, an altered pattern of cytokine production, and an impaired *in vitro* expansion of these cells. The knowledge about the deterioration of γδ T cells could account for the melanoma-related alterations of T cell-mediated adoptive responses and may be helpful for the planning of new approaches of immune therapy in melanoma patients.

MATERIALS AND METHODS

Cell preparation and stimulation Human peripheral blood was obtained from 23 melanoma patients (mean age ± SD 59.6 ± 16.1 y; median: 64.5 y; range 32–80) and 28 healthy subjects (mean age ± SD 57.5 ± 18.9 y; median: 67.0 y; range 32–79). Healthy subjects were volunteers in good and stable clinical condition, and had laboratory parameters in the physiologic range. Melanoma patients have been admitted to the Dermatology Unit of the I.N.R.C.A. Hospital of Ancona (Italy). Melanoma patients were in good health other than for the existence of melanoma as checked on the basis of clinical and laboratory parameters. Melanoma patients and healthy subjects were equally distributed according to sex and the percentage of male and female inside each group was about the same. The investigations were performed after approval by a local institutional review board. A written informed consent was obtained from each subject. Diagnosis of melanoma was histologically confirmed. All patients brought cutaneous primary nonmetastatic melanoma and were staged according to the new American Joint Committee on Cancer staging system for cutaneous melanoma (Balch *et al*, 2000). In detail, the number of patients and the relative tumor thickness was as follows: ⩽1.0 mm (n = 15), 1.01–2.0 mm (n = 4), 2.01–4.0 mm (n = 2), and ⩽4.0 mm (n = 2). A blood drawing was taken before the surgical excision.

Peripheral blood mononuclear cells (PBMC) were fractionated on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and separated by density gradient centrifugation ($400 \times g$, 30 min). Cells from the interface of the gradients were washed twice with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (Gibco, Life Technologies, Gaithersburg, MD) and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U per ml) and streptomycin (100 μg per ml) (all from Life Technologies, complete medium) at a concentration of 1×10^6 per ml. The viability was always greater than 98% as determined by trypan blue exclusion. Lymphocytes from both melanoma patients and healthy donors were tested fresh. In each experiment a similar number of melanoma patients and healthy donors was examined. Each donor was tested once and all the tests were carried out with a single blood sample. Mononuclear cells were cultured in the complete medium supplemented with 100 U per ml of IL-2 (Chiron Milano, Italia). Phosphoantigen-specific stimulation of γδ T cells was performed using the nonpeptidic antigen isopentenylpyrophosphate (30 μg per ml, IPP; Sigma, St Louis, MO). After 1 wk of culture, the volume corresponding to half the culture medium was replaced by fresh medium. On day 10 of culture, viable cells were counted and used for fluorescence-activated cell sorter analysis and cytotoxicity. The expansion of γδ T cells was followed by cytometric analysis through double staining of stimulated cells with anti-CD3 [phycoerythrin (PE)] and anti-pan γδ, anti-Vδ1, or anti-Vδ2 T cells [fluorescein isothiocyanate (FITC)] monoclonal antibody (MoAb). The absolute number of γδ T cells in each culture was calculated as follows: (percentage of γδ T cells among total cells) × (total cell count)/100. The γδ T cell expansion index was then calculated by dividing the absolute number of γδ T cells in stimulated cultures by the absolute number of γδ T cells before culture (Poccia *et al*, 1997).

MoAb and fluorescence-activated cell sorter analysis The PE-conjugated MoAb anti-CD3 was purchased from EuroClone (Devon, Wetherby, West York, UK). The FITC-conjugated anti-pan-TCR γδ, anti-TCR Vδ1, and anti-TCR Vδ2 were purchased from Endogen (Boston, MA). IgG1 (Becton Dickinson Milano, Italy) was used as isotype control.

PBMC (1×10^6) were labeled with 10 μl of anti-CD3 or anti-TCR Vδ1 MoAb or 5 μl of anti-pan-TCR γδ or anti-TCR Vδ2 MoAb in a final volume of 150 μl of RPMI 1640 with 10% fetal bovine serum for 30 min in ice. At the end of the incubation, cells were washed in PBS, resuspended in Isoton II (Coulter, Euro Diagnostics, Hialeah, FL) and immediately analyzed with a Coulter XL flow cytometer.

Intracellular detection of IFN-γ, TNF-α, and perforin Mononuclear cells were stimulated with IPP and IL-2 for 18 h, and GolgiPlug (a protein transport inhibitor containing brefeldin A; PharMingen, Milton Keynes, U.K.) was added during the last 12 h of culture to block intracellular transport processes and allow cytokine accumulation. Stimulated cells (1×10^6) were stained with the anti-pan-TCR γδ MoAb for 30 min at 4°C. Fixation–permeabilization of cells was performed in PBS/2% paraformaldehyde for 15 min at 4°C, followed by incubation for 30 min at room temperature in the dark with PE-conjugated anti-human IFN-γ MoAb or anti-human TNF-α MoAb diluted in PBS, 1% bovine serum albumin, and 0.05% saponin. Cells were finally washed twice in PBS, 1% bovine serum albumin, and 0.01% saponin and analyzed on a XL flow cytometer (Coulter). Perforins were measured after 10 d of incubation using an anti-human perforin-PE (clone δG9, BD PharMingen) at the same conditions above reported. The controls for unspecific staining included incubation with isotype-matched MoAb (PharMingen).

Isolation of γδ T lymphocytes and cytotoxic assay γδ T lymphocytes, *in vitro* expanded with IPP and IL-2 for 10 d, were isolated through cytofluorimetric cell sorting (Vantage, Becton Dickinson). The purity of γδ T cells, assessed by cytofluorimetric analysis, was greater than 95%.

Cytotoxic assay was performed by a fluorimetric method as recently reported (Provinciali *et al*, 1992). The natural killer resistant cell line Daudi and the natural killer cell sensitive K562 cell line were used as target cells. Daudi is a human lymphoblastoid B cell line derived from a Burkitt lymphoma, which constitutively expresses antigens recognized by Vδ9 Vδ2 T cells. K562 is a human myeloid cell line derived from chronic myelogenous leukemia. The fluorescence was read with a 1420 VICTOR^2 multilabel counter (Wallac, Turku, Finland). The percentage of specific lysis was calculated as follows:

$$\%\,\text{specific lysis} = [(F_{med} - F_{exp})/F_{med}] \times 100$$

where F represent the fluorescence of the solubilized cells after the supernatant has been removed; med = F from target incubated in medium alone; exp = F from target incubated with effector cells.

Lytic units ($LU_{20}/10^7$ cells) were calculated by using a computational method (Bryant *et al*, 1992). One LU corresponded to the number of effector cells required to produce 20% of specific lysis.

Statistical analysis Statistical analysis was performed using parametric (Student's t test) or nonparametric (Mann–Whitney rank sum test) tests on the basis of the distribution of the data. Difference between means were considered significant at $p < 0.05$. Correlation between γδ T cell number or expansion and tumor thickness was compared by simple linear regression analysis. The statistical analysis was performed with SigmaStat software version 1.03 (Jandel Scientific, Erkrath, Germany).

RESULTS

Ex vivo* analysis of γδ T lymphocytes** Peripheral blood lymphocytes from 23 melanoma patients and 28 healthy subjects were analyzed for the percentage and the absolute number of γδ T cells through double staining with anti-CD3 and anti-γδ MoAb. As shown in **Table I** and **Fig 1**(A***), the absolute number of γδ T cells was significantly reduced in melanoma patients in comparison with healthy subjects (66.1 ± 33.8 *vs* 92.9 ± 32.6;

Table I. Absolute number of lymphocytes, γδ T cells, Vδ1 T cells, and Vδ2 T cells, in healthy subjects and melanoma patients

	Absolute no. (n × mm^3)				
Donors	Lymphocytes	γδ T Cells	Vδ1 T Cells	Vδ2 T Cells	Vδ2/Vδ1 ratio
Healthy subjects	2159 ± 118[a]	92.9 ± 32.6	38.5 ± 13.1	68.2 ± 35.8	1.8
Melanoma patients	2268 ± 539	66.1 ± 33.8*	33.1 ± 19.4	31.7 ± 13.7*	0.9

[a]Data are expressed as mean ± SD.
*$p < 0.01$ vs healthy subjects.

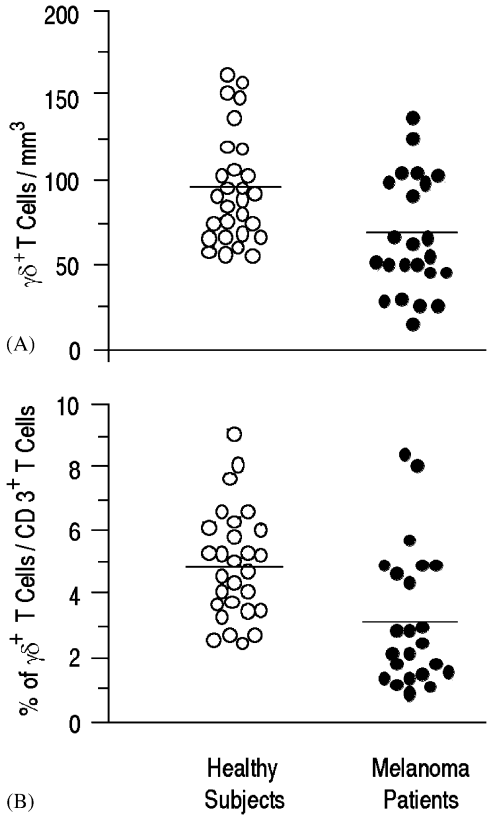

Figure 1. Absolute number and percentage of γδ T cells in the peripheral blood of melanoma patients and healthy donors. Freshly isolated PBMC from patients with cutaneous primary melanomas (n = 23) or healthy subjects (n = 28) were double stained with MoAb anti-pan γδ (FITC) and anti-CD3 (PE) and analyzed by flow cytometry. (*A*) The absolute number of γδ T cells, and (*B*) the percentage of γδ T cells among total CD3$^+$ T cells, were both significantly reduced in melanoma patients in comparison with healthy subjects ($p < 0.01$).

$p < 0.01$). The absolute number of peripheral lymphocytes was similar in the two groups of subjects (**Table I**). As shown in **Fig 1**(***B***), the percentage of CD3$^+$ γδ T cells in peripheral blood was significantly lower in melanoma patients than in healthy donors (mean ± SD, 3.2 ± 2.1 *vs* 4.8 ± 1.7; $p < 0.01$). As shown in **Table I**, the absolute number of Vδ1 T cells did not show significant difference in the two groups of donors (38.5 ± 13.1 and 33.1 ± 19.4, in healthy subjects and melanoma patients, respectively). Differently, the absolute number of Vδ2 T cells was significantly reduced in melanoma patients in comparison with healthy subjects (31.7 ± 13.7 *vs* 68.2 ± 35.8; $p < 0.01$). The Vδ2 and Vδ1 subsets were differently represented in the two groups: in healthy controls the Vδ2 subset was predominant (Vδ2/Vδ1 ratio = 1.8), whereas in melanoma patients the proportion of Vδ2 and Vδ1 subsets was similar (Vδ2/Vδ1 ratio = 0.9) (**Table I**). No significant correlation was found plotting the number of γδ T cells and the tumor thickness of the respective patient.

Cytokine production by γδ T lymphocytes As it has been demonstrated that activated γδ T cells produce IFN-γ and TNF-α, we studied the intracellular production of these lymphokines in 1 d stimulated γδ T cells from healthy subjects and melanoma patients. As shown in **Figs 2 and 3**, the percentage of γδ T cells producing IFN-γ was significantly reduced in melanoma patients in comparison with healthy controls (mean ± SD, 9.3 ± 5.5 *vs* 18.3 ± 9.5; $p < 0.01$). In a similar way, the percentage of γδ T cells producing TNF-α was lower in melanoma patients than in healthy controls (11.5 ± 3.8 *vs* 17.9 ± 7.7 for melanoma patients and healthy subjects, respectively; $p < 0.02$).

Expansion of γδ T lymphocytes The expansion of γδ T cells from healthy controls and melanoma patients was evaluated after 10 d of culture in the presence of IPP and low-dose IL-2. Both the proportion of γδ T cells, evidenced by double staining fluorescence-activated cell sorter analysis, and their relative increase in comparison with the γδ T cell number found on day 0 (expansion index) were evaluated. As shown in **Fig 4**, the proportion of γδ T cells reached on day 10 was significantly lower in melanoma patients than in healthy donors (mean ± SD, 11.9 ± 8.4 *vs* 36.5 ± 17.6; $p < 0.01$). In a similar way, the expansion index of γδ T cells on day 10 *vs* day 0 was significantly reduced in melanoma patients in comparison with healthy controls (4.4 ± 3.8 *vs* 8.6 ± 6.7; $p < 0.02$) (**Fig 4**). As shown in **Table II**, the expansion index of the Vδ2 subset was significantly lower in melanoma patients than in healthy donors (3.3 ± 3.6 *vs* 5.8 ± 4.4; $p < 0.03$).

Cytotoxicity of γδ T lymphocytes In order to evaluate the cytotoxic potential of γδ T cell cultures after *in vitro* expansion, we tested the cell activity of γδ T cells against Daudi and K562 tumor cell lines and their perforin content through flow cytometry. Purified cultures of activated γδ T cells (10 d incubation), obtained through cytofluorimetric cell sorting, were cytotoxic against Daudi tumor cells. A great heterogeneity of cytotoxic activity was found in both groups considered. Mean levels of cytotoxicity were similar between healthy subjects and melanoma patients (438.2 ± 141.7 *vs* 402.5 ± 359.2, data not shown). The cytotoxic activity of γδ T cells was also tested against the K562 tumor cell line, with a similar distribution of cytotoxicity in normal donors and melanoma patients (data not shown).

The percentage of γδ T cells producing perforins among total γδ$^+$ T cells was not significantly different in melanoma patients in comparison with healthy subjects (42.8 ± 37.6 *vs* 51.4 ± 8.8, data not shown).

DISCUSSION

In this study we have demonstrated for the first time the existence of numerical and functional alterations of γδ T cells from patients with cutaneous primary melanomas. The number of circulating γδ T cells, the percentage of cells producing IFN-γ or TNF-α, and their *in vitro* expansion, were all decreased in melanoma patients when compared with healthy age-matched subjects.

Both the percentage and the absolute number of circulating γδ T cells were significantly reduced in patients with cutaneous primary melanoma, thus suggesting a specific deficit for this lymphocyte population. The reduction of γδ T cell number well

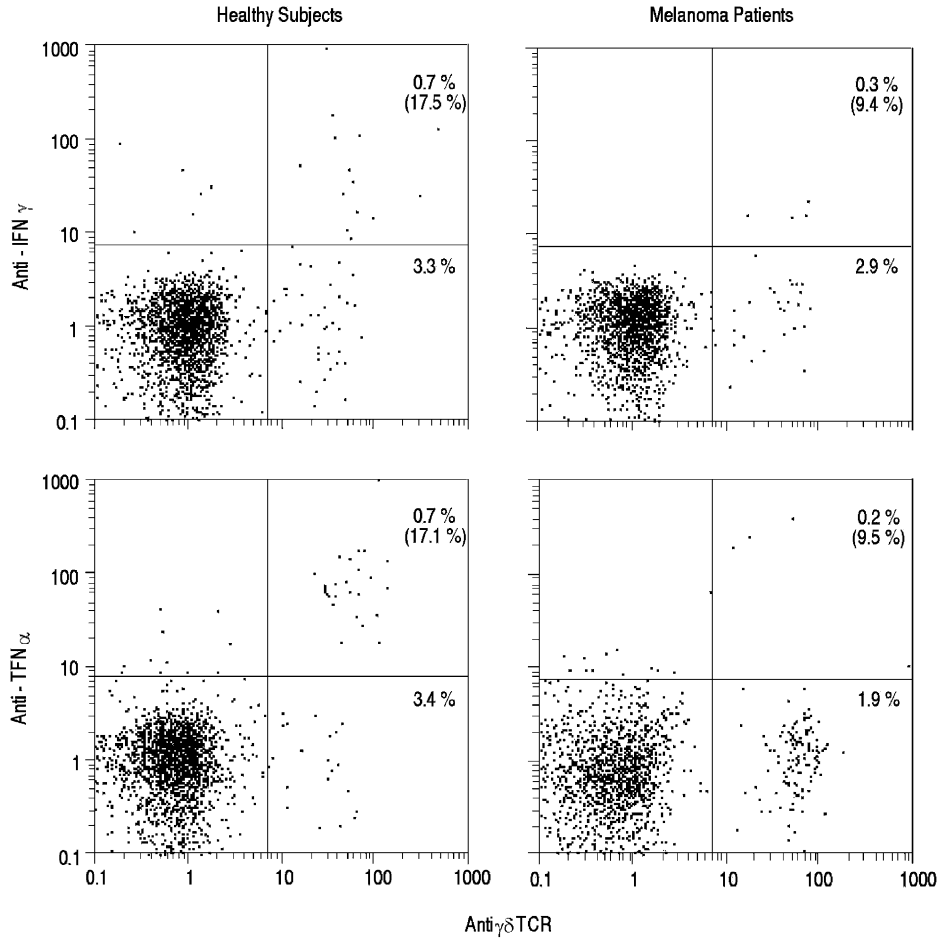

Figure 2. Analysis of cytokine production by γδ T cells in melanoma patients and healthy donors. PBMC from patients with cutaneous primary melanomas or healthy subjects, were stimulated for 18 h in the presence of IPP (30 μg per ml) and IL-2 (100 U per ml). The last 12 h of culture were performed in the presence of GolgiPlug, a protein transport inhibitor containing brefeldin. Single-cell analysis of cytokine synthesis in γδ T cells from a representative healthy subject or melanoma patient was performed following dual staining with cell surface anti-γδ (FITC) MoAb and intracellular anti-IFNγ or anti- TNF-α (PE) MoAb. Numbers in brackets indicate the percentages of γδ cells synthesizing a given cytokine among total γδ T lymphocytes.

correlated with the decrease of the Vδ2 T cell subset, i.e., the most frequent subset of circulating γδ T cells (Groh *et al*, 1989; Poccia *et al*, 1998). The Vδ2 population is involved in the reactivity toward microbial antigens and tumor cell antigens (Poccia *et al*, 1998; Zheng *et al*, 2001). The role of Vδ2 T cells in the immune defense against cancer has been demonstrated on the basis of their reactivity against certain lymphoma cells, such as Daudi cells (Kunzmann *et al*, 2000), and for their presence among tumor infiltrating lymphocytes in various cancer types (Kabelitz *et al*, 2000). On this basis, our data strongly suggest that an impaired γδ T cell potential may contribute to a lower immune defense against melanoma. Both Vδ1 and Vδ2 T cell populations have been reported to contribute to anti-tumor immunity because of their cytotoxic and Th1-type cytokines producing activities (Wu *et al*, 2002). Furthermore, both γδ T cell subsets have been found able to respond to stress-induced expression of the MHC class I-related chains A and B (Wu *et al*, 2002). MHC class I-related chains function as ligands for NKG2D, an activating receptor complex that triggers natural killer cells, costimulates CD8 αβ and Vγ9δ2 γδ T cells, and is required for stimulation of Vδ1 T cells (Groh *et al*, 1999; Das *et al*, 2001; Girardi *et al*, 2001; Wu *et al*, 2002). The role of γδ T cells in the early anti-tumor defense against melanoma has been previously suggested by the evidence that a significant accumulation of Vδ1 T cells was found in three of 11 primary cutaneous melanomas, with proportions of γδ T cells ranging from 15 to 25% of lymphocyte infiltrate, whereas γδ T cells were not present in eight metastatic melanomas (Bachelez *et al*, 1992). In our study, we did not find numerical changes of the Vδ1 T cell population in melanoma patients in comparison with controls, whereas the Vδ2 subset was significantly reduced in melanoma patients. Almost half of our melanoma patients had an absolute number of circulating γδ T cells similar or lower than half of the mean number found in controls (**Fig 1**). Phenotypic analysis of the tumor infiltrating γδ T cells revealed that they use the products of the Vδ9 and Vδ2 genes in a way similar to most of circulating TCR γδ. It is noteworthy that γδ lines or clones developed from lymphocytes infiltrating primary melanoma display a potent autologous MHC-unrestricted tumor cell cytotoxicity (Bachelez *et al*, 1992; Nanno *et al*, 1992). In a more recent study, the presence of γδ T cells well correlated with the survival of patients with necrotizing choroidal melanoma (Bialasiewicz *et al*, 1999). In our study, not only the number but also the function of γδ T cells was altered in melanoma patients. The *in vitro* expansion of γδ T cells, that represent one of the most relevant functional parameters for γδ T cells, was significantly reduced in patients with cutaneous primary melanomas. This would imply that γδ T cells from melanoma patients have a decreased proliferative capacity in comparison with healthy subjects. Under normal conditions, γδ T cells respond to antigen challenge by secreting large quantities of TNF-α and IFN-γ (Poccia *et al*, 1997; Kabelitz *et al*, 2000), which contribute to the activation of both specific and aspecific immune responses. In this study, we show that the percentage of γδ T cells producing either TNF-α or IFN-γ is significantly reduced in melanoma

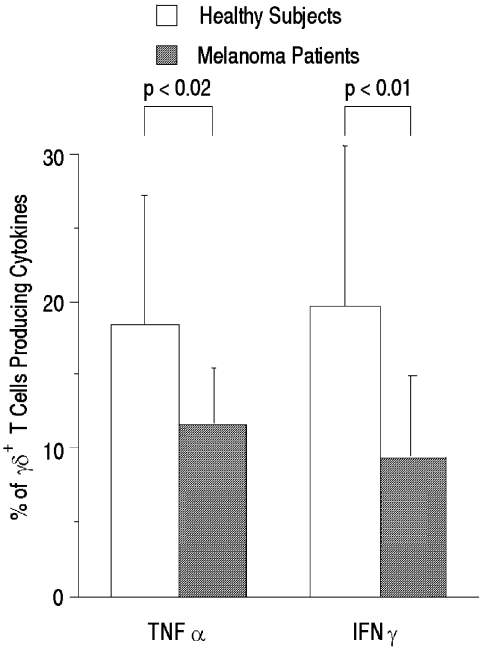

Figure 3. Percentage of γδ T cells producing cytokines in melanoma patients and healthy donors. $\gamma\delta^+$ T cells from patients with cutaneous primary melanomas or healthy subjects were activated as reported in **Fig 2** and analyzed for cytokine production after dual staining with cell surface anti-pan γδ (FITC) MoAb and intracellular anti-IFN-γ or anti-TNF-α (PE) MoAb. The mean percentage of γδ T cells producing either IFN-γ or TNF-α was significantly lower in melanoma patients in comparison with healthy subjects ($p < 0.01$ and $p < 0.02$ for IFN-γ or TNF-α, respectively).

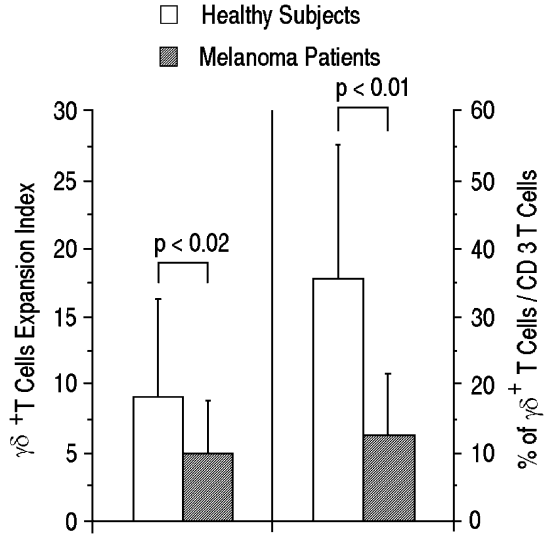

Figure 4. Evaluation of γδ T cell expansion in melanoma patients and healthy donors. PBMC from patients with cutaneous primary melanomas or healthy subjects were stimulated for 10 d in the presence of IPP (30 μg per ml) and IL-2 (100 U per ml). The expansion index (left panel) and the percentage (right panel) of *in vitro* expanded $\gamma\delta^+$ T cells among total $CD3^+$ T cells were significantly lower in melanoma patients in comparison with healthy subjects ($p < 0.02$ and $p < 0.01$).

patients. Instead, the lytic activity of γδ T cells, evidenced by their cytotoxicity toward Daudi and K562 tumor cells and their production of perforins, seems to be well preserved in melanoma patients.

On the basis of the data reported in this paper, we suggest that the impairment of γδ T cell number and function may contribute

Table II. Expansion index of γδT cells and Vδ2 T cells in healthy subjects and melanoma patients.

	Expansion Index[a]	
Donors	γδT cells	Vδ2 T cells
Healthy subjects	8.6 ± 6.7[b]	5.8 ± 4.4
Melanoma patients	4.4 ± 3.8*	3.3 ± 3.6**

[a]The expansion index was calculated by dividing the absolute number of γδ T cells in stimulated cultures by the absolute number of γδ T cells before culture.
[b]Data are expressed as mean ± SD.
*$p < 0.02$ and **$p < 0.03$ vs healthy subjects.

to the inefficacious immune defense against melanoma with at least two different mechanisms. One of these is based on the lower γδ T cell potential, determined by the reduced number and expansion of γδ T cells, and particularly of Vδ2 subset, found in melanoma patients. In this case, the impairment of γδ T cells may represent a new mechanism by which melanoma tumor cells escape an efficacious immune response. The second mechanism is based on the regulatory interactions between γδ T cells and αβ T cells. Several studies have demonstrated that the type and the functioning of the specific lymphocyte response is dependent on signals provided by the innate recognition system (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Indeed, even if the recognition of foreign or nonself proteins is controlled by the rearranging genes that encode specific receptors, the functional outcome of most responses to pathogens and the initiation of the response itself is determined by the type of innate immune response they elicit (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Recent evidence has suggested that one of the crucial players involved in the regulation of innate and acquired immunity is the γδ T cell population (Mak and Ferrick, 1998). There is substantial cross-talk between γδ and αβ T cells. It has been demonstrated that the proliferative response of human peripheral blood γδ T cells towards microbial antigens or Daudi tumor cells, depends on helper signals provided by $CD4^+$ αβ T cells (Burns *et al*, 1996a,b). Moreover, some γδ T cell function may depend on αβ T cells, as, for example, the γδ T cell proliferation in response to activated $CD4^+$ αβ T cells (Burns *et al*, 1996a,b), and the antigen presentation by $CD4^+$ αβ T cells to γδ T cells (Vila *et al*, 1995; Collins *et al*, 1998). On the other hand, substantial evidence has suggested that γδ T cells regulate certain αβ T cell-mediated immune responses, pointing the definition of αβ T cell responses toward a Th1 or Th2 phenotype (Fearon and Locksley, 1996; Mak and Ferrick, 1998; Kabelitz *et al*, 2000). This fact is particularly related by the cytokines secreted by γδ T cells that, in turn, are able to mediate both innate and acquired immunity (Ferrick *et al*, 1995). We propose that, in analogy with what has been recently demonstrated by us in aged people and centenarians (Argentati *et al*, 2002), the numerical and functional impairment of γδ T cells demonstrated in this study could determine a derangement in the establishment of acquired immunity, with consequent difficulties in selecting appropriate antigens and the strategies for their elimination. Further studies will be performed to investigate the specific causes involved in the impairment of γδ T cells in primary cutaneous melanoma patients. At present, we may only suggest that an impairment of γδ T cells may determine a lower immune defense against melanoma which, in turn, may predispose to melanoma. The healthy and stable conditions of melanoma patients examined in this study make improbable that the γδ T cell defect is secondary to the malignancy, even if this possibility may not be definitively excluded. Finally, the fact that approximately 10% of patients with melanoma have family histories of the disease, suggesting a genetic susceptibility (Platz *et al*, 1997), raises the question on whether the differences we have found might be attributable to the genetic background of the melanoma patients. We do not think this is the case, as melanoma patients and controls enrolled in our study were identified in the

same geographical area (Ancona area in Italy), and melanoma patients did not have family histories of melanoma.

In conclusion, the demonstration of a numerical and functional derangement of γδ T cells, which we have found in patients with cutaneous primary melanomas, make them a potentially useful tool for the planning of new approaches of immune therapy in malignant melanoma.

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REFERENCES

Argentati K, Re F, Donnini A, *et al*: Numerical and functional alterations of circulating γδ T lymphocytes in aged people and centenarians. *J Leukocyte Biol* 72:65–71, 2002

Bachelez H, Flageul B, Deos L, Boumsell L, Bensussan A: TCR gamma delta bearing T lymphocytes infiltrating human primary cutaneous melanomas. *J Invest Dermatol* 98:369–374, 1992

Balch CM, Buzaid AC, Atkins MB, *et al*: A new American Joint Committee on Cancer staging system for cutaneous melanoma. *Cancer* 88:1484–1491, 2000

Bialasiewicz AA, Ma JX, Richard G: Alpha/beta- and gamma/delta TCR(+) lymphocyte infiltration in necrotising choroidal melanomas. *Br J Ophthalmol* 83:1069–1073, 1999

Brenner MB, McLean J, Scheft H, *et al*: Two forms of the T-cell receptor gamma protein found on peripheral blood cytotoxic T lymphocytes. *Nature* 325: 689–694, 1987

Bryant J, Day R, Whiteside T, Heberman RB: Calculation of lytic units for the expression of cell-mediated cytotoxicity. *J Immunol Methods* 146:91–103, 1992

Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB, Band H: Vγ2Vδ2 TCR–dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J Immunol* 154:998–1006, 1995

Burk MR, De Mori L, Libero G: Human Vδ9-Vδ2 cells are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur J Immunol* 25:2052–2058, 1995

Burns J, Bartholomew B, Lobo S: Induction of Vγ2Vδ2 T cell proliferation by activated antigen-specific $CD4^+$ T cells and IL-2. *Clin Immunol Immunopathol* 80:38–43, 1996a

Burns J, Lobo S, Bartholomew B: Requirement of $CD4^+$ T cells in the γδ proliferative response to Daudi Burkitt's lymphoma. *Cell Immunol* 174:19–23, 1996b

Collins RA, Werling D, Duggan SE, Bland AP, Parsons KR, Howard CJ: γδ T cells present antigen to $CD4^+$ αβ T cells. *J Leukoc Biol* 63:707–712, 1998

Costant P, Davodeau F, Peyrat MA, Pouquet Y, Puzo G, Bonneville M, Fournier JJ: Stimulation of γδ T cells by nonpeptidic mycobacterial ligands. *Science* 264:267–270, 1994

Das H, Groh V, Kuijl C, Sugita M, Morita CT, Spies T, Bukowski JF: MICA engagement by human Vγ2Vδ2 T cells enhances their antigen-dependent effector function. *Immunity* 15:83–93, 2001

Dudley ME, Wunderlich J, Nishimura MI, *et al*: Adoptive transfer of cloned melanoma-reactive T lymphocytes for the treatment of patients with metastatic melanoma. *J Immunother* 24:363–373, 2001

Fearon DT, Locksley RM: The instructive role of innate immunity in the acquired immune response. *Science* 272:50–54, 1996

Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H: Differential production of interferon-γ and interleukin-4 in response to Th- and Th-stimulating pathogens by γδ T cells in vivo. *Nature* 373:255–257, 1995

Fisch P, Malkovsky M, Kovats S, *et al*: Recognition by human Vδ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science* 250:1269–1273, 1999

Fujimiya Y, Suzuki Y, Katakura R, Miyagi T, Yamaguchi T, Yoshimoto T, Elbina T: In vitro interleukin 12 activation of peripheral blood CD3+CD56+ and CD3+CD56− gammadelta T cells from glioblastoma patients. *Clin Cancer Res* 3:633–643, 1997

Girardi M, Oppenheim DE, Steele CR, *et al*: Regulation of cutaneous malignancy by γδ T cells. *Science* 294:607–609, 2001

Groh V, Porcelli S, Fabbi M, *et al*: Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. *J Exp Med* 169:1277–1294, 1989

Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T: Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICAA and MICB. *Proc Natl Acad Sci USA* 96:6879–6884, 1999

Kabelitz D, Wesch D, Hinz T: γδ T cells, their T cell receptor usage and role in human diseases. *Springer Semin Immunopathol* 21:55–75, 1999

Kabelitz D, Glatzel A, Wesch D: Antigen recognition by human γδ T lymphocytes. *Int Arch Allergy Immunol* 122:1–7, 2000

Kammula US, Lee KU, Riker AI, Wang E, Ohnmacht GA, Rosenberg SA, Marincola FM: Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J Immunol* 163:6867–6875, 1999

Kjeldsen-Kragh J, Quayle AJ, Skalhegg BS, Sioud M, Forre O: Selective activation of resting gamma delta T lymphocytes by interleukin-2. *Eur J Immunol* 23: 2092–2099, 1993

Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M: Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood* 96:384–392–99, 2000

Lahn M, Kalataradi H, Mittelstadt P, *et al*: Early preferential stimulation of γδ T cells by TNF-α. *J Immunol* 160:5221–5230, 1998

Lee KH, Wang E, Nielsen MB, *et al*: Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J Immunol* 163:6292–6300, 1999

Mak TW, Ferrick DA: The γδ T-cell bridge: Linking innate and acquired immunity. *Nature Med* 4:764–765, 1998

Medzhitov R, Janeway CA Jr: Innate immunity: Impact on the adaptive immune response. *Curr Opin Immunol* 9:4–9, 1997

Nanno M, Seki H, Mathioudakis G, *et al*: Gamma/delta T cell antigen receptors expressed on tumor-infiltrating lymphocytes from patients with solid tumors. *Eur J Immunol* 22:679–687, 1992

Nielsen MB, Monsurro V, Migueles SA, *et al*: Status of activation of circulating vaccine-elicited $CD8^+$ T cells. *J Immunol* 165:2287–2296, 2000

Platz A, Hansson J, Mansson-Brahme E, *et al*: Screening of germline mutations in the CDKH2A and CDKNZB genes in Swedish families with hereditary cutaneous melanoma. *J Nat'l Cancer Inst* 89:676–678, 1997

Poccia F, Cipriani B, Vendetti S, *et al*: CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive Vδ9Vδ2 T lymphocytes. *J Immunol* 159:6009–6017, 1997

Poccia F, Gougeon ML, Bonneville M: Innate T cell immunity to nonpeptidic antigens. *Immunol Today* 19:253–256, 1998

Provinciali M, Di Stefano G, Fabris N: Optimization of cytotoxic assay by target cell retention of the fluorescent dye carboxyfluorescein diacetate (CFDA) and comparison with conventional ^{51}CR release assay. *J Immunol Methods* 155:19–24, 1992

Strohal R, Marberger K, Pehamberger H, Stingl G: Immunohistological analysis of anti-melanoma host responses. *Arch Dermatol Res* 287:28–35, 1994

Tanaka I, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR: Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature* 375:155–158, 1995

Thor Straten P, Becker JC, Guldberg P, Zeuthen J: In situ T cells in melanoma. *Cancer Immunol Immunother* 48:386–395, 1999

Ueta C, Kawasumi H, Fujiwara H, *et al*: Interleukin-12 activates human gamma delta T cells. synergistic effect of tumor necrosis factor-alpha. *Eur J Immunol* 26:3066–3073, 1996

Vila LM, Haftel HM, Park H-S, Lin M-S, Romzek NC, Hanash SM, Holoshitz J: Expansion of mycobacterium-reactive γδ T cells by a subset of memory helper T cells. *Infect Immun* 63:1211–1216, 1995

Wu J, Groh V, Spies T: T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial γδ T cells. *J Immunol* 169:1236–1240, 2002

Zheng BJ, Chan KW, Im S, *et al*: Anti-tumor effects of human peripheral gammadelta T cells in a mouse tumor model. *Int J Cancer* 92:421–425, 2001