

RESEARCH ARTICLE

# Skewed Differentiation of Circulating V $\gamma$ 9V $\delta$ 2 T Lymphocytes in Melanoma and Impact on Clinical Outcome

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## Abstract

### Objective

The aim of this study was to evaluate over time circulating  $\gamma\delta$  T lymphocytes in melanoma patients in terms of frequency, effector functions, and relationship with clinical stage and evolution, by comparing preoperative values to those obtained at a mean follow-up of 36 months or in the event of recurrence or disease progression, and to those of healthy controls. Also, we correlated the presence of tumor-infiltrating  $\gamma\delta$  T lymphocytes with clinical evolution of melanoma.

### Results

Mean frequencies of circulating  $\gamma\delta$  T cells before and after melanoma removal were very similar and comparable to healthy subjects, but patients who progressed to stage III or IV showed a significantly decreased frequency of circulating V $\gamma$ 9V $\delta$ 2 T cells. The distribution of V $\gamma$ 9V $\delta$ 2 memory and effector subsets was similar in healthy subjects and melanoma patients at diagnosis, but circulating  $\gamma\delta$  T cells of patients after melanoma removal had a skewed terminally-differentiated effector memory phenotype. Highly suggestive of progressive differentiation toward a cytotoxic phenotype, V $\gamma$ 9V $\delta$ 2 T cells from patients at follow up had increased cytotoxic potential and limited cytokine production capability, while the opposite pattern was detected in V $\gamma$ 9V $\delta$ 2 T cells from patients before melanoma removal.

### Conclusions

Follow-up data also showed that tumor infiltrating  $\gamma\delta$  T cells were significantly associated with lower mortality and relapse rates, suggesting that they may serve as a prognostic biomarker, for human melanoma.

## Introduction

Malignant melanoma accounts for only 3% of cutaneous tumors, but shows high tendency to metastasize and is responsible for as much as 65% of death for skin cancers [1]. While the standard treatment for localized melanoma is surgical resection, the treatment of metastatic melanoma is still challenging and carries a significant treatment-related morbidity; advanced disease is still associated with a poor prognosis and further research is needed to provide more effective and tolerable therapeutic protocols for stage III and IV melanoma [2–4].

In the last 40 years, increasing interest has developed toward host cellular immune response and its possible therapeutic implication in the adjuvant therapy and advanced disease settings. Spontaneous regression of disease has been reported in patients with melanoma, suggesting a role for host immunity [5], which is indirectly supported by the evidence for lymphocyte infiltration of primary melanomas associated with tumor regression [6,7].

Tumor-infiltrating lymphocytes (TILs) have been shown to play an important role in the anti-tumor surveillance and have been documented in a wide variety of solid tumors including melanoma, breast, renal cell, prostate and colon cancers [8–10]. A potential prognostic and predictive significance has been suggested [11] and numerous clinical trials have focused on their possible use in adoptive immunotherapy [11–15]. CD4<sup>+</sup> and CD8<sup>+</sup> T cells are often reported as the predominant subset of lymphocytes within TILs [16–18] and currently represent a common target for adoptive immunotherapy [19,20]. However, increasing evidence exists for a role of  $\gamma\delta$  T lymphocytes in the anti-tumor surveillance in the periphery [21], which is supported by their localization within epithelia.  $\gamma\delta$  T lymphocytes can be distinguished into two subsets: those expressing the V $\delta$ 1 chain are a minor population in the peripheral blood but predominate in mucosal tissues [22], while the subset expressing the V $\delta$ 2 chain paired to the V $\gamma$ 9 chain (here and after referred to as V $\gamma$ 9V $\delta$ 2 T cells) predominates in peripheral blood and lymphoid tissues in human adults [22]. V $\gamma$ 9V $\delta$ 2 T cells recognize non-peptidic antigens (phosphoantigens, PAg) by a MHC-unrestricted mechanism [23,24] and typically perform anti-tumor immune responses (cytotoxicity, production of IFN- $\gamma$  and TNF- $\alpha$ , and dendritic cell maturation) [25,26]. Thus, there is a substantial interest in  $\gamma\delta$  T cells in the context of immunotherapeutic strategies and several pilot studies have described a partial success of  $\gamma\delta$  T cell-based immunotherapy in different types of cancer after the application of aminobisphosphonates (n-BP) or PAg plus IL-2 *in vivo* or after repetitive transfer of *in vitro* expanded V $\gamma$ 9V $\delta$ 2 T cells [27,28].

In a recent study, we have demonstrated that  $\gamma\delta$  T lymphocytes are well represented amongst TILs in cutaneous melanomas, produce the pro-inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  and exert a strong cytotoxic activity against melanoma cells *in vitro* [29]. Strongly suggestive of their anti-tumor role, percentages of V $\gamma$ 9V $\delta$ 2 T cells, but not total  $\gamma\delta$  or V $\delta$ 1 T cells, correlate with early stage of melanoma and absence of metastasis.

In this study, we have investigated the diagnostic and prognostic potential of a V $\gamma$ 9V $\delta$ 2 T-cell-based blood analysis on the grounds, that it may provide a useful biomarker of the patient's anti-tumor immune response. To this aim, we have evaluated circulating V $\gamma$ 9V $\delta$ 2 T lymphocytes in terms of frequency, effector functions and relationship with clinical stage and evolution, in subjects before and after removal of melanoma, and compared them with healthy controls.

## Materials and Methods

### Characteristics of the study cohort and study design

In this study, we enrolled patients from the same cohort of patients of our previous study on cutaneous melanoma and  $\gamma\delta$  T lymphocytes [29]. The cohort consisted of 74 patients with

histologically confirmed diagnosis of cutaneous melanoma, treated between March 2007 and March 2010 at the Plastic Surgery Unit of the University of Palermo. Forty-two patients were males and thirty-two were females; median age at surgery was 60 years (range 26–90). Sixty-nine patients (93%) presented with primary cutaneous melanoma and 5 patients with cutaneous or subcutaneous metastases (7%). All patients were staged according to the new American Joint Committee on Cancer staging system for cutaneous melanoma [30]. Patients were followed in a multidisciplinary melanoma clinic at the University of Palermo, and disease related mortality and relapse rates were recorded. Data were complemented with those obtained at a mean follow-up of 36 months. A blood sample was obtained from 38 out of 74 patients at the time of diagnosis and a second blood sample was obtained from 32 out of these 38 patients at the follow-up, or in the event of recurrence or disease progression. The clinicopathologic characteristics of the study cohort are given in Table 1. Blood samples from 45 age- and sex-matched healthy subjects in good and stable clinical condition, with laboratory parameters in the physiologic range, were used as a control.

### Ethics Statement

The study was approved by the Ethical Committee of the University Hospital, Palermo, where the patients were recruited. The study was performed in accordance to the principles of the

**Table 1. Clinicopathologic characteristics of the study cohort.**

Characteristic	Patients N (%)
<b>Sex</b>	
Male	20 (62.5)
Female	12 (37.5)
<b>Tumor type</b>	
Primary melanoma	31 (96.9)
Cutaneous/subcutaneous metastasis	1 (3.1)
<b>Melanoma subtype</b>	
Superficial spreading	16 (50)
Nodular	11 (34.4)
Acrallentiginous	2 (6.3)
Spitzoid	3 (9.4)
<b>Localization</b>	
Head and neck	3 (9.4)
Trunk	15 (46.9)
Upper limb	3 (9.4)
Lower limb	11 (34.4)
<b>T Stage</b>	
Tis	3 (9.4)
T1	12 (37.5)
T2	9 (28.2)
T3	3 (9.4)
T4	5 (15.6)
<b>TNM Stage</b>	
0	3 (9.4)
I	14 (43.8)
II	11 (34.4)
III	3 (9.4)
IV	1 (3.1)

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Helsinki declaration and those of the “Good Clinical Practices”, and all individuals gave written informed consent to participate.

## Isolation of PBMC and tumor-infiltrating immune cells, and FACS analysis

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Tissue specimens were obtained from 74 different patients undergoing standard-of-care surgical procedures for cutaneous melanoma, at the time of primary surgery. There were no restrictions (e.g. stage, etc) on tissues included for this study other than confirmation of melanoma by pathology review of H&E slides from the specimen taken for research. Tissue was obtained fresh and immediately transported to the laboratory in sterile saline for processing. Tissue was first minced into small pieces followed by digestion with collagenase type IV and DNAase (Sigma, St. Louis, MO) for 1 hr at 37°C. After digestion, the cells extracted were washed twice in RPMI 1640 medium (Gibco, Grand Island, NY, USA) and tumor-infiltrating mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation. Both PBMC and tumor-infiltrating cells were stained for live/dead discrimination using Invitrogen LIVE/DEAD fixable violet dead cell stain kit (Invitrogen, Carlsbad, CA). Fc receptor blocking was performed with human immunoglobulin (Sigma, 3  $\mu\text{g/ml}$  final concentration) followed by surface staining with different fluorochrome-conjugated antibodies to study the composition of the different subpopulations.

The fluorochrome-conjugated monoclonal antibodies (mAbs) used to characterized the entire population were the following: anti-CD45, anti-CD3, anti-CD14, anti-CD19, anti-pan  $\gamma\delta$  TCR, anti-V $\delta$ 1, anti-V $\delta$ 2, anti-CD27 and anti-CD45RA, all purchased from BD Biosciences (Mountain View, CA). Expression of surface markers was determined by flow cytometry on a FACSCanto II Flow Cytometer with the use of the FlowJo software (BD Biosciences). The cells were first gated for singlets (FSC-H vs FSC-A), then for lymphomonocytes (SSC-A vs FSC-A) and then for T lymphocytes (CD45 vs CD3). The T lymphocyte gate was further analyzed for uptake of the LIVE/DEAD stain to determine live *versus* dead cells and their expression of CD14 and CD19, taking only the live, healthy T cell population (L/D<sup>-low</sup>, CD14<sup>-</sup>, CD19<sup>-</sup>). Pan- $\gamma\delta$  TCR, V $\delta$ 1, V $\delta$ 2, CD27 and CD45RA cell surface expression was then determined from this gated population. For every sample at least 100.000 viable lymphomonocytes were acquired. Negative control (background) values were not subtracted, as the median backgrounds for isotype-matched mAbs was 0.0028% (range, 0.000%-0.0063%). Samples were considered positive if the number of cells was equal to or greater than 0.01% and at least 10 clustered events were apparent. This empiric cut-off value was predicted to be > 90% different from background, at an  $\alpha$  of 0.05 [29,31].

## Flow cytometry-based $\gamma\delta$ T cell functional assays

PBMC were resuspended at a concentration of  $5 \times 10^6/\text{ml}$  in complete RPMI medium in the presence of Zoledronate (Novartis Pharma, Basel, Switzerland, 1  $\mu\text{M}$ ) or with PMA (BD Biosciences, 20 ng/ml) and ionomycin (BD Biosciences, 1  $\mu\text{M}$ ). Anti-CD107a/b mAb (BD Biosciences) was directly added to the culture at this time point [26]. Two hours after stimulation, Monensin (Sigma, 2  $\mu\text{M}$ ) was added and the cells were incubated overnight at 37°C in 5% CO<sub>2</sub>. Then PBMC were stained with anti-TCR V $\delta$ 2 and anti-CD3 mAbs for 15 min at 4°C. Samples were fixed, permeabilized according to manufacturer's instructions and incubated with anti-IFN- $\gamma$  (25723.11, BD Biosciences, Mountain View, CA), anti-perforin ( $\delta\text{G9}$ , Vinci-Biochem, Firenze, Italy) or anti-Granzyme B (GB11, BD Biosciences) mAbs or isotype-matched control

mAbs, for 30 min at 4°C. After washing, the expression of surface CD107a/b and intracellular IFN- $\gamma$ , perforin or Granzyme B was analyzed upon gating on the V $\delta$ 2<sup>+</sup> T cell population.

## Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software. The frequencies of circulating  $\gamma\delta$  T lymphocytes (preoperative values, follow-up values, healthy controls) were compared using nonparametric tests (Wilcoxon test, Mann Whitney test), based on the type of data and the sample size. Mortality and relapse rates in patients with and without tumor-infiltrating V $\delta$ 2 T lymphocytes at first diagnosis were compared using Chi squared test with Yates' correction. Differences between groups with a p value  $\leq 0.05$  were regarded as significant.

## Results

### Ex vivo analysis of $\gamma\delta$ T lymphocytes after melanoma removal

PBMC from 32 subjects were collected before surgical removal of a primary cutaneous melanoma and at a median follow-up of 36 months and were analyzed for the percentage of  $\gamma\delta$  T cells. The results were compared with those in a group of 45 sex- and age-matched healthy subjects (HS) used as control. As shown in Fig 1, the percentages of total  $\gamma\delta$  T cells (A) and their V $\delta$ 1 (B) and V $\gamma$ 9V $\delta$ 2 (C) subsets were similar in melanoma patients before and after tumor removal and comparable to frequencies detected in HS, with no statistically significant differences between the three tested groups.

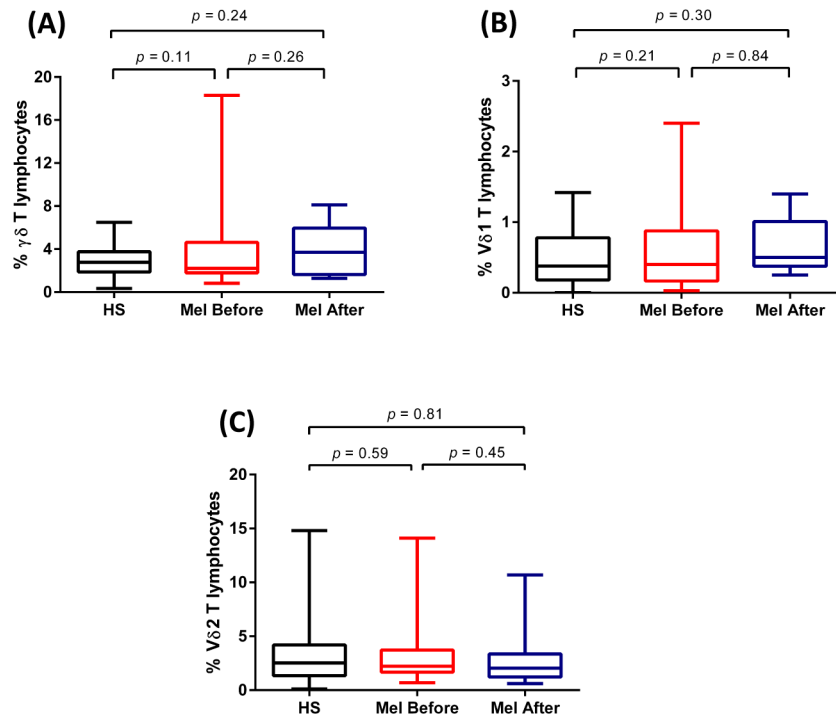
### Phenotypic and functional $\gamma\delta$ T cell responses after melanoma removal

Human V $\gamma$ 9V $\delta$ 2 T cells include those with naive or central-memory phenotypes ( $T_{\text{naive}}$ , CD45RA<sup>+</sup>CD27<sup>+</sup>;  $T_{\text{CM}}$ , CD45RA<sup>-</sup>CD27<sup>+</sup>) that home to secondary lymphoid organs, but that lack immediate effector function, and those with effector-memory ( $T_{\text{EM}}$ , CD45RA<sup>-</sup>CD27<sup>-</sup>) and terminally-differentiated ( $T_{\text{EMRA}}$ , CD45RA<sup>+</sup>CD27<sup>-</sup>) phenotypes that home to sites of inflammation and that display immediate effector functions as cytokine production and cytotoxic activity [32]. The phenotype and the functional responses of V $\gamma$ 9V $\delta$ 2 T cells were analyzed before and at follow-up after melanoma removal and compared to those observed in healthy subjects.

As shown in Fig 2A, V $\gamma$ 9V $\delta$ 2T cells obtained from PBMC of HS and melanoma patients before tumor removal showed a similar subset distribution with predominance of the  $T_{\text{CM}}$  (76.8% and 69.6%, respectively) and the  $T_{\text{EM}}$  (13% and 21%, respectively) phenotypes, which together accounted for approximately 90% of the whole V $\gamma$ 9V $\delta$ 2 compartment. Conversely,  $T_{\text{Naive}}$  and  $T_{\text{EMRA}}$  subsets were very poorly represented amongst circulating V $\gamma$ 9V $\delta$ 2 T cells of HS and patients before melanoma removal. As shown in Fig 2A, the majority of circulating V $\gamma$ 9V $\delta$ 2 T lymphocytes in patients at follow up after melanoma removal expressed the  $T_{\text{EMRA}}$  phenotype, while the other three subsets were poorly represented.

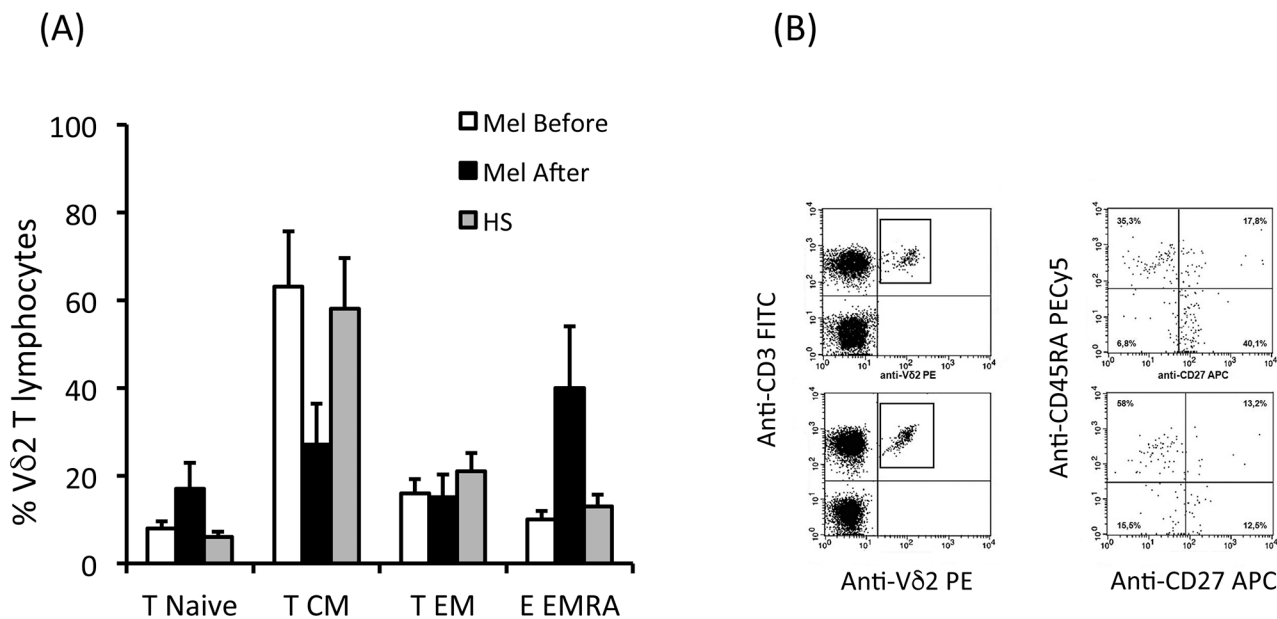
This skewed phenotype of peripheral V $\gamma$ 9V $\delta$ 2 T cells was strikingly depicted by the raw data for patient #7 shown in Fig 2B. Overall, the statistically significant changes in circulating V $\gamma$ 9V $\delta$ 2T cell subset distribution indicate that a long-term effector maturation and mobilization of peripheral blood V $\gamma$ 9V $\delta$ 2 T cells occur in subjects after melanoma removal and evoke previous observations after Zoledronate injection in cancer patients [33,34].

The phenotypic modifications of V $\gamma$ 9V $\delta$ 2 T cells in subjects at follow up after melanoma removal were paralleled by modifications in their functional responses *in vitro*. Analysis performed in 14 patients showed (Fig 3A), that intracellular IFN- $\gamma$  response to Ionomycin/PMA stimulation declined over time after melanoma removal, as compared to the response observed



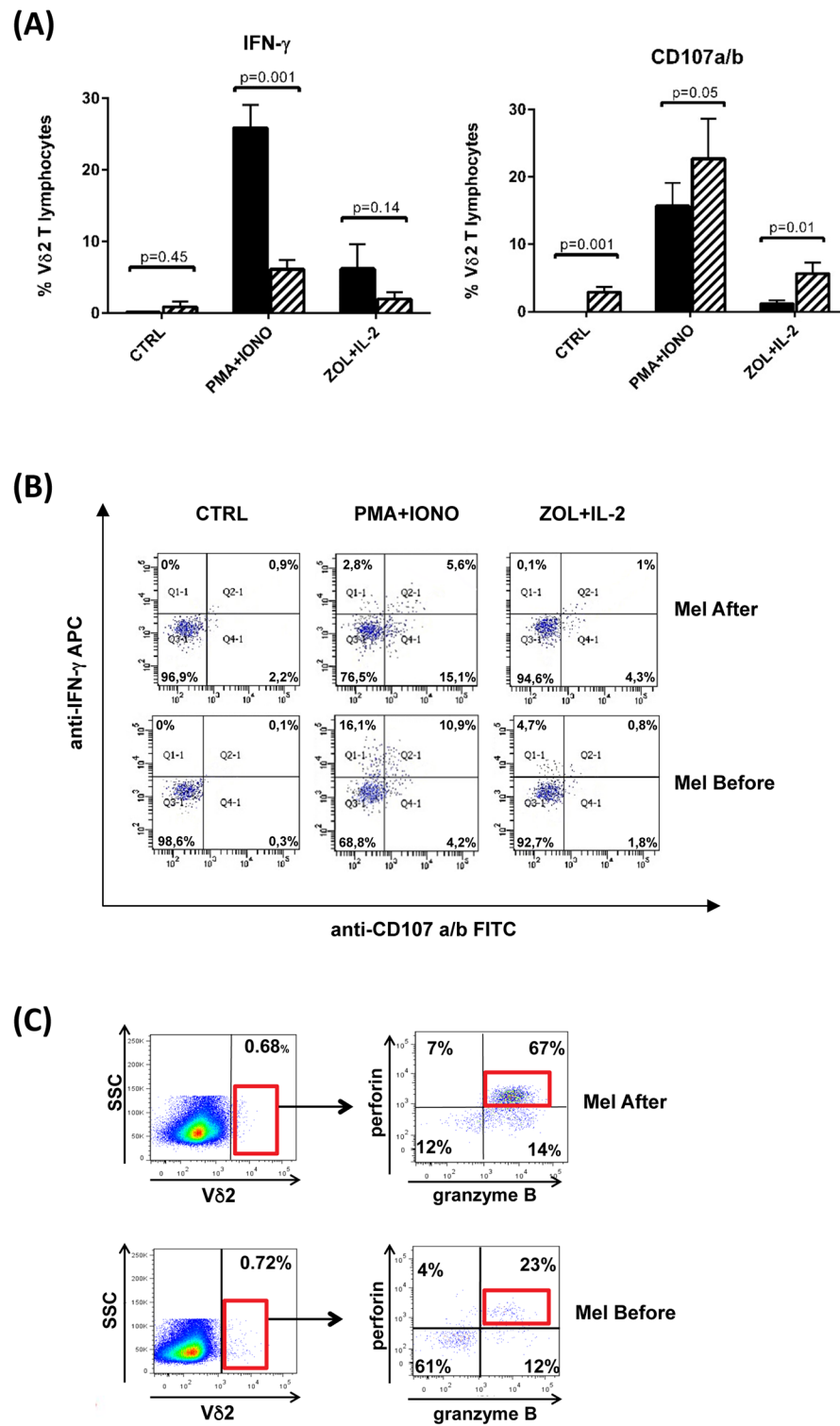
**Fig 1. Percentages of total  $\gamma\delta$  T cells (A) and their V $\delta$ 1 (B) and V $\delta$ 2 (C) subsets in healthy subjects and in melanoma patients before and after tumor removal.** Box plots of percentages of  $\gamma\delta$  T cells subsets in 32 melanoma patients before and after melanoma removal and in 45 healthy subjects. Boxes represent 25th to 75th percentiles; middle bar identifies median; whiskers show minimum and maximum.

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**Fig 2. Phenotype of circulating V $\gamma$ 9V $\delta$ 2 T cells before and after melanoma removal.** PBMC were stained with anti-CD3, anti-V $\delta$ 2, anti-CD45RA and CD27 mAbs. Percentages of T<sub>naive</sub> (CD45RA<sup>+</sup>CD27<sup>+</sup>), T<sub>CM</sub> (CD45RA<sup>+</sup>CD27<sup>+</sup>), T<sub>EM</sub> (CD45RA<sup>+</sup>CD27<sup>+</sup>) and T<sub>EMRA</sub> (CD45RA<sup>+</sup>CD27<sup>+</sup>) cells were determined by FACS analysis. (A) Shows cumulative data for V $\delta$ 2 T cells and (B) shows representative flow cytometry panels from patient # 7 upon gating on CD3<sup>+</sup>V $\delta$ 2<sup>+</sup> T cells and staining with CD27 and CD45RA.

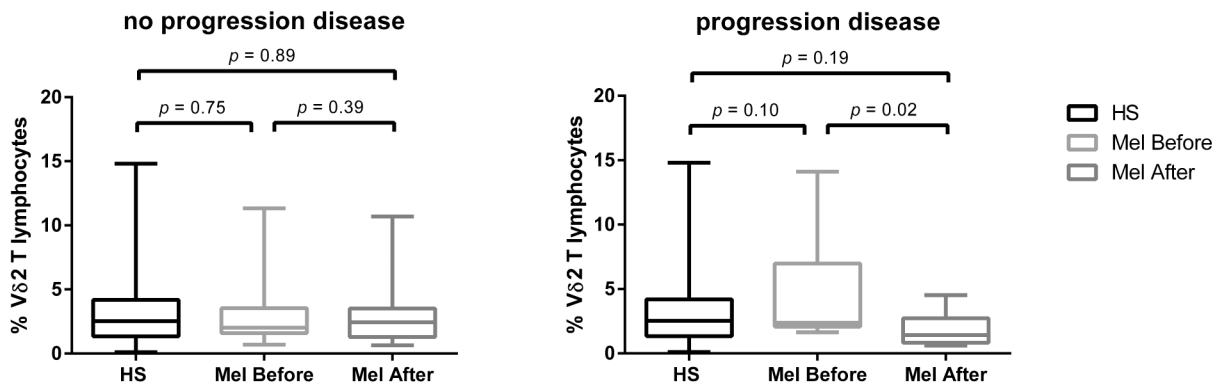
doi:10.1371/journal.pone.0149570.g002



**Fig 3. Functional responses of circulating V $\gamma$ 9V $\delta$ 2 T cells in patients before and after melanoma removal.** (A) Cumulative data and of intracellular IFN- $\gamma$  production and CD107a mobilization assays upon *in vitro* stimulation with Zoledronate or Ionomycin/PMA. (B) and (C) show raw data of intracellular IFN- $\gamma$  production and CD107a mobilization (B) and intracellular perforin and granzyme B expression by V $\gamma$ 9V $\delta$ 2 of patient #21.

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**Fig 4. Percentages of  $V\gamma 9V\delta 2$  T cells in “progressor” and “not progressor” groups of melanoma patients and in healthy subjects.** Boxes represent 25th to 75th percentiles; middle bar identifies median; whiskers show minimum and maximum.

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in the same patients before melanoma removal;  $IFN-\gamma$  response to Zoledronate stimulation also decreased after melanoma removal, but differences did not attain statistical significance. Conversely, the cytotoxic capability of  $V\gamma 9V\delta 2$  T cells (measured by the CD107 mobilization assay after Ionomycin/PMA or Zoledronate stimulation) consistently increased in patients after melanoma removal, and differences to values in patients before melanoma removal attained statistical significance. In accordance with the CD107a mobilization results, intracellular FACS analysis carried out on  $V\gamma 9V\delta 2$  T cells from 4 patients after melanoma removal revealed increased expression of perforin ( $70.3 \pm 8.6\%$  versus  $25.3 \pm 6.1\%$ ) and granzyme B ( $79.5 \pm 10.4\%$  versus  $28.7 \pm 6.7\%$ ). This trend is illustrated by raw data for patient #21 (Fig 3B and 3C).

### Correlation between circulating $V\gamma 9V\delta 2$ T cells and clinical outcomes

Twelve out of the 32 studied patients relapsed and progressed after melanoma removal; six of them died before another blood sample was collected and thus follow-up data are available for only 6 patients, who progressed to stage III (1 patient) or stage IV (5 patients). Progression was invariably associated to substantial declines in  $V\gamma 9V\delta 2$  T cell numbers: as shown in Fig 4, patients who progressed to stage III or IV showed a decreased frequency of circulating  $V\gamma 9V\delta 2$  T cells at 36-months follow-up compared to values at diagnosis (4.55% to 1.81%). Conversely, patients who did not progress during the follow-up period showed a sustained or slightly increased frequency of circulating  $V\gamma 9V\delta 2$  T cells over time (2.94% to 3.15%). The Wilcoxon test for paired data showed a significant difference between preoperative and follow-up values in the “progressor” group of patients ( $p = 0.02$ ), but not in the “non progressor” group ( $p = 0.39$ ). Also, the Mann Whitney test showed a significant difference in the frequency variation over time between the “progressor” and “nonprogressor” groups ( $p = 0.01$ ).

### Correlation of circulating and intratumoral $V\gamma 9V\delta 2$ T cells with clinicopathological parameters of melanoma

To investigate further the clinical significance of  $V\gamma 9V\delta 2$  T cells in melanoma, the percentages of circulating and tumor-infiltrating  $V\gamma 9V\delta 2$  T cells at the time of diagnosis were analyzed relative to clinicopathological factors of melanoma patients, including cancer-specific survival rates, relapse-free survival (RFS) and overall survival (OS). We observed no statistically significant associations between the percentage of circulating  $V\gamma 9V\delta 2$  T cells before melanoma removal and stage, RFS and OS (data not shown). We then determined the impact of tumor-



**Table 2. Correlations between tumor-infiltrating V $\gamma$ 9V $\delta$ 2 T cells and clinicopathologic characteristics in melanoma patients.**

Parameter	V $\gamma$ 9V $\delta$ 2 T cells amongst TILs		p value
	+ (n = 46)	- (n = 28)	
<b>Stage</b>			
0-I-II	68%	32%	0.035
III-IV	29%	71%	0.025
<b>Mortality rate</b>	0%	21.4%	0.005
<b>Relapse rate</b>	13%	35.7%	0.045

Mortality and relapse rate among 74 patients with cutaneous melanoma (median 36 months follow-up).

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infiltrating V $\gamma$ 9V $\delta$ 2 T cells on survival rates in a cohort of 74 previously studies melanoma patients [29] to determine the prognostic significance of intratumoral V $\gamma$ 9V $\delta$ 2T cells. Previously, we reported that the presence of V $\gamma$ 9V $\delta$ 2 T cells among tumor-infiltrating lymphocytes was positively correlated with earlier tumor stages (0, I and II). As shown in Table 2, the presence of V $\gamma$ 9V $\delta$ 2 T cells among tumor-infiltrating lymphocytes was positively correlated not only with stage, but also with RFS and OS of melanoma patients: at a median follow-up of 36 months, the relapse rate was 13.0% when V $\gamma$ 9V $\delta$ 2 T cells were present, and 35.7% in the absence of V $\gamma$ 9V $\delta$ 2 T cells (Table 2). Similarly, the mortality rate was 21.4% in the absence of V $\gamma$ 9V $\delta$ 2 T cell infiltration, but 0% when V $\gamma$ 9V $\delta$ 2 T cells were present. The Chi squared test with Yates' correction showed a significant difference between the “ $\gamma\delta$  positive” and “ $\gamma\delta$  negative” groups in both the mortality rate (p = 0.005) and the disease relapse rate (p = 0.045).

## Discussion

$\gamma\delta$  T cells possess a combination of innate and adaptive immune cell qualities rendering them attractive for immunotherapy [21]. Two recent surveys [27,28] have reviewed the available published studies on the *in vivo* activation and adoptive transfer of *ex vivo*- expanded  $\gamma\delta$  T cells in cancer patients, providing evidence that V $\gamma$ 9V $\delta$ 2 T cell-based immunotherapy improves overall survival and, in view of its low toxicity grade, provides a proof of principle for its utilization as adjuvant to conventional therapies for resistant/refractory patients care. Although  $\gamma\delta$  T cell-based immunotherapy has delivered promising results, several factor influence its success, amongst which is the finding of a low number and/or functionally unresponsive  $\gamma\delta$  T cells in patients with several types of tumors ([21,35]). For instance, Provinciali *et al.* [36,37] found that the number of circulating  $\gamma\delta$  T cells and their V $\gamma$ 9V $\delta$ 2 subset was reduced in patients with melanoma and was not recovered after melanoma removal, as compared to the levels found in controls. However, V $\gamma$ 9V $\delta$ 2 T cells had increased proliferative and cytokine-producing capability after melanoma removal, in comparison with the same subjects before surgical intervention or with control donors. Conversely, Petrini and coworkers [38] have found similar percentages of circulating  $\gamma\delta$  T cells in healthy control subjects and patients with melanoma, which had normal proliferative capacity but impaired cytotoxic activity, and Campillo *et al.* found increased frequencies of  $\gamma\delta$  T cells in patients with melanoma, which had high perforin content [39]

In this paper, we have evaluated the frequencies and functional properties of circulating V $\gamma$ 9V $\delta$ 2 T lymphocytes in subjects before and after removal of melanoma, in relationship with clinical stage and evolution, and compared them with healthy controls. Data here reported clearly show that percentages of total  $\gamma\delta$  T cells and their V $\delta$ 1 and V $\gamma$ 9V $\delta$ 2 subsets did not

significantly differ in melanoma patients before and after tumor removal and in healthy control subjects. We do not have any explanation for the difference between our results and the previously reported  $\gamma\delta$  T cell studies in patients with melanoma. One possibility might be that changes in V $\gamma$ 9V $\delta$ 2 T cell numbers and functions might be age- and sex-related and not a consequence of tumor growth. Accordingly, V $\gamma$ 9V $\delta$ 2 T cells change characteristically with age, gradually decreasing beyond 30 years of age and drop more strikingly in men than in women [40]. Moreover, this loss is accompanied by a substantial depletion of V $\gamma$ 9V $\delta$ 2 effector T cells, which is mirrored by a reduced IFN- $\gamma$  secretion [40]. Accordingly, the absolute number of V $\gamma$ 9V $\delta$ 2 T cells in a cohort of breast cancer patients receiving chemotherapy did not differ from age-matched breast cancer patients without treatment [41] and the decrease in absolute numbers of V $\gamma$ 9V $\delta$ 2 T cells in a cohort of 41 patients with pancreatic ductal adenocarcinoma did not correlate with cancer stage/progression, but rather with patient age [42].

While we did not find any statistically significant difference in the distribution of V $\gamma$ 9V $\delta$ 2 memory and effector subsets between healthy subjects and melanoma patients, lymphocytes expressing the T<sub>EMRA</sub> phenotype were the dominant V $\gamma$ 9V $\delta$ 2 T cell subset in patients at follow-up after melanoma removal. Highly suggestive of progressive differentiation toward a cytotoxic phenotype, V $\gamma$ 9V $\delta$ 2 T cells from patients at follow-up had increased cytotoxic potential and limited cytokine production capability. Whether or not this predominant V $\gamma$ 9V $\delta$ 2 T<sub>EMRA</sub> phenotypic and functional status is associated with clinically relevant melanoma features is actually unknown. Intuitively, the accumulation of antigen-experienced V $\gamma$ 9V $\delta$ 2 T<sub>EMRA</sub> cells with a powerful cytotoxic potential could be considered beneficial in terms of disease control. However, recent studies in patients with chronic lymphatic leukemia have demonstrated that elevated numbers of circulating V $\gamma$ 9V $\delta$ 2 T cells with dominant T<sub>EM</sub> and T<sub>EMRA</sub> phenotypes are a negative prognosticator [43], which is reminiscent of data previously reported in conventional CD4<sup>+</sup> and CD8<sup>+</sup> cells [44,45]; in these patients, T<sub>EMRA</sub> CD8<sup>+</sup> cells displayed the phenotypic hallmarks of functional exhaustion, further supporting the concept that long-lasting tumor-induced chronic activation may lead to the undesired accumulation of cells unable to exert effective antitumor activity. Indeed, several *in vitro* and *in vivo* data indicate that chronic V $\gamma$ 9V $\delta$ 2 T cell activation may induce functional exhaustion or anergy. Anergy has been reported in HIV-infected individuals [46], whereas functional exhaustion has been described in a preclinical nonhuman primate model after repeated stimulations with PAg and low-dose IL-2 [47] and has been associated with a differentiation shift, which is well in line with the accumulation of V $\gamma$ 9V $\delta$ 2 T<sub>EMRA</sub> cells.

In our study, we also evaluated the impact of baseline and follow-up V $\gamma$ 9V $\delta$ 2 T cell counts on clinicopathological parameters of melanoma, to investigate whether these counts could be used as an alternative to predict outcome. While baseline percentages of circulating V $\gamma$ 9V $\delta$ 2 T cells were not significantly correlated to any of the well established prognostic factors for melanoma (stage, RFS and OS), progression was invariably associated to substantial declines in V $\gamma$ 9V $\delta$ 2 T cell numbers, reminiscent of our previous observations after Zoledronate and IL-2 injection in cancer patients.

We further determined prognostic significance of tumor-infiltrating V $\gamma$ 9V $\delta$ 2 T cells for the prediction of cancer development during the follow-up period in a cohort of 74 previously studied melanoma patients, and we show here that the presence of V $\gamma$ 9V $\delta$ 2 T cells among tumor-infiltrating lymphocytes was positively correlated with RFS and OS of melanoma patients.

In this regard, our study, albeit small, emphasizes strongly a correlation of peripheral blood V $\gamma$ 9V $\delta$ 2 T cells with arrested disease progression, and this evokes a comparable correlation of V $\gamma$ 9V $\delta$ 2 T cell numbers (and most likely activities) with arrested disease progression in other scenarios of advanced malignancy [33,48]. We note, however, that the declining health of

patients could not be attributed to poor functional conversion of V $\gamma$ 9V $\delta$ 2 T cells (which was equivalent in all patients, data not shown). Therefore, the critical difference seems to be a failure to sustain robust V $\gamma$ 9V $\delta$ 2 T cell numbers, implying that high response frequencies compose a key property of lymphoid stress-surveillance [8]. Hence, any future treatment optimizations should respect the need to achieve sufficiently large numbers of relevant V $\gamma$ 9V $\delta$ 2 T cells.

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## Author Contributions

Conceived and designed the experiments: FT SB SM AC FD FM. Performed the experiments: SB FT SM. Analyzed the data: FT SB AA SM FD. Contributed reagents/materials/analysis tools: FT,SB SM AA. Wrote the paper: FT SB SM AC FD FM.

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