The features of circulating and tumor-infiltrating γδ T cells in melanoma patients display critical perturbations with prognostic impact on clinical outcome

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ABSTRACT

γδ T cells hold a pivotal role in tumor immunosurveillance through their prompt activation and cytokine secretion, their ability to kill tumor cells in an Human Leukocyte Antigen (HLA)-unrestricted manner, and their combination of features of both innate and adaptive immunity. These unique properties and functional plasticity render them very attractive both as targets and vectors for cancer immunotherapy. Yet, these potent and fascinating antitumor effectors have not been extensively explored in melanoma. We provided here a detailed investigation of the phenotypic and functional properties of circulating and tumor-infiltrating γδT cells in melanoma patients, and their impact on clinical evolution. High proportions of circulating- and tumor-infiltrating γδT and δ2\textsuperscript{+} subset were associated with better clinical outcome. Notably, melanoma drastically impaired the ability of γδT cells to exhibit activation molecules, secrete cytokines, and display cytotoxicity toward melanoma in response to stimulation with phosphoantigens. It drove them toward regulatory and Th17 profiles associated with poor clinical outcomes. Our study highlights that melanoma hijacked γδT cells to escape from immune control, and revealed that circulating and tumor-infiltrating γδT cell features are promising potential biomarkers of clinical evolution. Such understanding of the physiopathology of γδT cells may help designing new therapeutic approaches exploiting the antitumor potential of γδT cells while counteracting their skewing by tumors to improve patient outcomes.

Introduction

Despite recent improvements in melanoma treatment using targeted therapies or immunomodulatory strategies, the long-term control of the tumor in a majority of patients still remains a challenge. A better understanding of the tumor immune escape mechanisms is crucial to design new therapeutic strategies and potentiate existing immunomodulatory therapies to achieve immune control of the tumor and a better clinical success.

Tumor microenvironment comprises many immune cell types, including γδT cells that could participate in the pathophysiology of the disease, but are still not fully explored despite their fascinating properties. Among cytotoxic effectors, γδ T cells represent 1–10\% of all T cells, but display potent properties and unique contributions to many types of immune responses.\textsuperscript{1,2} γδT cells combine features of both innate and adaptive immunity, display antigen-presenting capacities, and exhibit regulatory and cytotoxic functions in an non-MHC restricted manner.\textsuperscript{3} Different γδT-cell subsets are defined based on particular Vγ or Vδ chains and harbor a particular localization. Vδ1\textsuperscript{+} and Vδ2\textsuperscript{+} cells are predominant subsets, respectively mostly localized in mucosal tissues/ epithelium for Vδ1\textsuperscript{+} cells, and peripheral blood and lymphoid tissues for Vδ2\textsuperscript{+} cells. Vδ1\textsuperscript{+} cells are associated with viral infections, whereas Vδ2\textsuperscript{+} cells are mostly involved in immunity against bacteria and tumors. Vδ1\textsuperscript{+} cells recognize stress-induced ligands such as EPCR, MICA, and MICB, whereas Vδ2\textsuperscript{+} cells recognize phosphoantigens (pAg) derived from the mevalonate or isoprenoid pathways (such as isopentenyl-pyrrophosphate (IPP) from malignant cells or hydroxymethylbutenyl-pyrophosphate (HMB-PP) from bacteria), alkylamines, and synthetic aminobiphosphonates independently of MHC molecules. Upon activation, γδT cells exhibit cytotoxic activity and secrete cytokines, in particular IFNγ and TNFa which allow them to modulate other cells from acquired immunity, especially dendritic cells and classical αβ T cells.

γδT cells are crucial effectors in tumor immunosurveillance\textsuperscript{4,5} due to their rapid activation, their capacity to recognize tumor-
associated ligands neglected by conventional αβT cells in an MHC-unrestricted manner, their ability to kill tumor cells, and their potential to interact with and regulate many other cells leading to coordinated antitumor responses. However, γδT cells can be subverted by tumors to exhibit tumor-promoting functions through polarization toward Th2, Th17, or regulatory profile, recruitment of immunosuppressive myeloid cells, inhibition of antitumor immune responses, all converging to favor tumor progression.  

All these unique and crucial properties together with their functional plasticity render γδT cells attractive both as targets or vectors for cancer immunotherapy. Indeed, anticancer therapies based on the exploitation of the power of γδT cells are emerging. Indirect stimulation of Vδ2+ cells through administration of aminobisphosphonates (zoledronate, pamidronate) can lead to objective tumor responses in prostate cancer and melanoma. The adoptive transfer of ex-vivo expanded γδT cells is feasible in patients and can inhibit melanoma tumor progression in SCID mice, and infusion of preactivated Vδ2+ cells has shown antitumor effects in renal cell carcinoma. γδT cells may also contribute to the efficacy of chemotherapies. Comprehensive and detailed overviews of all clinical studies performed with γδ T cell-based immunotherapies in cancer specifying clinical outcomes have recently been reviewed by others. The in vivo expansion of γδ T cells (with synthetic phosphoantigens or aminobisphosphonates) can lead up to 25% of partial remission (PR) and 42% of stable disease (SD), the adoptive transfer of γδ T cells up to 66% of SD, and the combination of both up to 9% complete remission (CR) and 45% of SD. These promising results on γδT-cell-based immunotherapies sustain further investigations of these cells.

γδT cells have been identified within tumor-infiltrating lymphocytes (TIL) in multiple cancers but their clinical relevance remained mostly unknown. Regarding melanoma, it has been shown that γδT cells infiltrate primary tumors, but little information regarding their features and clinical significance has been reported. Lower proportions of circulating γδT cells especially Vδ2+ cells and higher proportions of Vδ1+ cells have been reported in melanoma patients. Lower frequencies of Vδ1+ and higher frequencies of Vδ2+ cells in blood were associated with better overall survival, and decreased frequency of circulating Vδ2+ cells was observed in patients who progress to advanced stage, sustaining their contribution in the control of melanoma. Few studies reported functional impairments of peripheral γδT cells in melanoma patients compared to healthy controls, but with controversial results, including either similar or lower proliferative capacity, impaired IFNγ/TNFα secretion upon IPP stimulation, and increased, similar or lower cytotoxicity. A skewed differentiation of circulating γδT cells has been pointed out in melanoma patients, with an increased in γδT cells displaying effector or terminally differentiated phenotype after tumor removal.  

The extensive phenotypic and functional features of γδT cells in melanoma patients have not been explored, especially within tumor microenvironment. Previous investigations were limited to analysis of proportions, differentiation stage and functionality, and restricted to peripheral γδT cells. Yet, a better understanding of these effectors with a promising potential in the context of melanoma and in relation with disease outcome could allow their exploitation for cancer immunotherapy. Here we investigated the detailed phenotypic and functional characterization of γδT cells in blood and tumor of melanoma patients in comparison with healthy controls, and assessed their clinical relevance. Our study highlights crucial γδT-cell features and promising potential biomarkers of clinical evolution in melanoma. Such understanding may help harnessing the power of γδT cells against cancer and allow improving cancer immunotherapies and patient outcomes.

**Results**

**High proportions of circulating- and tumor-infiltrating γδT and δ2+ subset are associated with better clinical outcome**

We first evaluated the proportion of γδT cells as well as δ2+ and δ6- subsets in blood and tumor samples of melanoma patients compared to blood and tonsils of healthy donors (HD) ([Figure 1(a)]). As most of tumor samples from patients were metastatic lymph nodes (LN), we used tonsils from healthy donors as control for lymph node tissues of patients, which were the closest available control tissue. Whereas proportions of circulating γδT cells were similar between HD (mean = 2.1%, range 0.4–7.17%) and patients (mean = 2.62%, range 0.51–11.63%), we observed that γδT accumulated within melanoma tumors (mean = 1.6%, range 0.18–13.09%) compared to control tonsils (mean = 0.29%, range 0.15–0.51%) ([Figure 1(b), Supplementary Figure 1A]). Interestingly, for the case of primary tumor (patient #62), the percentage of γδ T cells was the highest of the cohort (13.09% within CD45+). In addition, the Tδ2+δ6- ratio may be altered in melanoma patients, with a tendency to increase in both blood and tumor compared to their respective control, even though not significantly ([Figure 1(c)]). Moreover, when classified according to disease stage, patients with advanced stage III–IV melanoma displayed a lower frequency of circulating γδT cells ([Figure 1(d)]) and a lower Tδ2+δ6- ratio in blood ([Supplementary Figure 1B]) compared to patients at early stage I–II, suggesting a preferential recruitment of γδT cells to the tumor site. Strikingly we found that higher proportion of circulating Tδ2+ subset was significantly associated with longer time to progress (PFS) ([Figure 1e, f, Supplementary Table 3], and higher proportion of tumor-infiltrating γδT cells ([Figure 1g]) predicted better clinical outcome as it was linked with longer PFS and longer overall survival (OS) ([Supplementary Table 4]). Altogether these data enlightened that γδT cells infiltrated melanoma tumors and are associated with better clinical outcome.

**High proportions of γδT cells displaying a naive differentiation stage or a regulatory phenotype are associated with a poor clinical outcome**

We further examined the detailed properties of circulating and tumor-infiltrating γδT cells. We assessed their differentiation
stage based on CD45RA and CD27 expression, allowing to distinguish naïve (CD45RA+CD27+) and central memory (CD45RA-CD27-) phenotypes that are not yet differentiated, from effector memory (CD45RA-CD27-) and terminally differentiated (CD45RA+CD27-) phenotypes that display effector functions (Supplementary Figure 2A). Even though no significant difference was observed between controls and patients for both blood and lymph node tissue, γδT cells infiltrating tonsils and tumors have a tendency to accumulate in a central memory (CM) stage (CD45RA-CD27+) (mean = 62.5% and 59.98% respectively) with a reduced terminally differentiated effector memory phenotype (EMRA) mean = 3.46% and 6.6% respectively) compared to blood (Figure 2a). Notably, a high proportion of circulating γδT cells in a naïve stage (ranging from 2.9% to 51.6% in patients) was associated with an early relapse (Figure 2b), suggesting that the lack of differentiated γδT cells is predictive of a poorer outcome. Interestingly, the proportion of δ2+ cells in the EMRA stage at the tumor site was negatively linked with PFS and OS parameters (Supplementary Table 6). Besides, when evaluating the regulatory phenotype of γδT cells...
using CD25 marker and FoxP3 transcription factor, we observed an accumulation of CD25hiFoxP3+ γδT cells specifically in tumor from melanoma patients (mean = 5.66%, range 0–23.93%) compared to control tonsils or blood (Figure 2c, Supplementary Figure 2B). Notably, a high proportion of tumor-infiltrating regulatory γδT cells was associated with a shorter PFS and OS (Figure 2d, Supplementary Tables 4 and 6). When separating patients according to the stage of disease at sampling time, we also observed a tendency toward an increase in the proportions of circulating regulatory γδT cells in advanced compared to early stage (Supplementary Figure 1C). Altogether these observations suggest that γδT cells are progressively skewed in both blood and tumor of melanoma patients toward an undifferentiated and regulatory phenotype associated with a poorer clinical outcome.

γδT cells displayed a more activated phenotype in melanoma patients compared to healthy donors

We next investigated basal activation status of γδT cells by analyzing markers of both APC (CD40, CD86) and T cells (CD25, CD69). We observed that tumor-infiltrating γδT cells displayed higher levels of all activation markers compared to circulating γδT cells of patients or HD, but only...
CD40 was significantly higher in tumors (mean = 49.85%, range 14.75–73.23%) compared to tonsils (mean = 35.59%, range 24.1–61.9%) (Figure 3a). Interestingly, high proportions of CD86-expressing tumor-infiltrating γδT cells were associated with shorter survival, whereas high proportions of CD69-expressing tumor-infiltrating γδT cells were linked to better outcome (Figure 3b, Supplementary Tables 4 and 6). These results indicate that γδT cells display a higher basal activation status in melanoma microenvironment, with APC/T-cell characteristics of γδT cells being differentially linked with clinical outcome.

**Circulating and tumor-infiltrating γδT cells from melanoma patients displayed an altered expression of NCR, KIR, and immune checkpoints, with nkp44, PD1, and 41BBL being negative prognosis factors of clinical evolution**

To further gain insight into the mechanism of modulation of γδT cells by melanoma, we examined their expression of NCR, KIR as well as immune checkpoints (ICP) (Supplementary Figure 3), as these molecules will determine the fate of γδT cells and orientate their potentialities to interact with target cells and subsequently modulate other immune cells. We observed that circulating γδT cells from melanoma patients exhibited higher levels of NKG2A (mean = 10.89%, range 3.12–31.42%), NKG2D (mean = 75.79%, range 50.15–96.2%) and NKp46 (mean = 3.8%, range 0.31–14.98%) compared to HD, and tumor-infiltrating γδT cells expressed more NKG2A (mean = 18.56%, range 2.01–36.74%), NKp30 (mean = 40.29%, range 11.88–74.39%), and NKp44 (mean = 20.55%, range 1.5–58.08%) compared to tonsils and blood of HD (Figure 4a, b, Supplementary Figure 3A and 3B). The expression of inhibitory NKG2A and NKp30 receptors on tumor-infiltrating γδT cells tended to positively correlate between them (Supplementary Figure 4A). Notably, NKp44 tended to be more expressed by circulating γδT cells in advanced stage patients compared to early stages (Supplementary Figure 4B), and high proportion of NKp44 + circulating γδT cells was highly predictive of shorter PFS and OS (Figure 4c, Supplementary Tables 3 and 5). Moreover, when separating patients according to short or long progression-free survival either from diagnosis or sampling time, higher expression of NKp30 and NKp44 by

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**Figure 3.** γδT cells displayed a more activated phenotype in melanoma patients compared to healthy donors. Basal activation status of γδT cells was evaluated by flow cytometry in blood and tumor samples from melanoma patients, and blood and tonsils samples from healthy donors. (a) Comparative proportions of Tyδ cells expressing CD40, CD86, CD25, and CD69 (within γδT cells). P values calculated using the Mann-Whitney test. (b) Comparative OS of patients with low or high CD86+ (left panel) or CD69+ (right panel) tumor-infiltrating γδT cells from sampling and diagnosis time respectively. Groups were separated using the median percentage of CD86+ (13.24%) and CD69+ (37.2%) tumor-infiltrating γδT cells (n = 10–11 patients/group). Comparison using Log-rank test.
circulating γδT cells identified patients with a shorter PFS (Supplementary Figure 4C). Interestingly, NKG2D expression appeared to be a good prognosis factor of clinical evolution (Supplementary Tables 3–6).

The expression of immune checkpoints by γδT cells in patients was highly heterogeneous, especially for tumor-infiltrating γδ T cells. We observed higher proportions of circulating γδT cells expressing 4-1BB (mean = 9.81%, range 0.79–43.33%), TIM3 (mean = 2.44%, range 0.42–16.49%), LAG3 (mean = 3.39%, range 0–13.76%), and ICOSL (mean = 8.7%, range 1.97–35.71%) in melanoma patients compared to HD, whereas only proportions of TIM3+ (mean = 11.12%, range 1.54–31.36%) and LAG3+ (mean = 15.9%, range 0.71–50%) tumor-infiltrating γδT cells were higher compared to tonsils (Figure 5(a), Supplementary Figure 3C and 3D). The other immune checkpoints analyzed were similarly expressed between the groups (Supplementary Figure 5A). Interestingly, on tumor-infiltrating γδT cells, TIM3 and LAG3 expressions highly positively correlated between them, as well as PD1 and TIM3 or LAG3 (Supplementary Figure 5B). When assessing the clinical relevance of the immune checkpoint expression on γδT cells, we found that PD1 on circulating γδT cells and 4-1BB, TIM3 and LAG3 on tumor-infiltrating γδT cells

Figure 4. Circulating and tumor-infiltrating γδT cells from melanoma patients displayed an altered expression of NKR and KIR, with NKp44 being a negative prognosis factor of clinical evolution.

The expression of KIR and NKR by γδT cells was evaluated by flow cytometry in blood and tumor samples from melanoma patients (black symbols), and blood and tonsils samples from healthy donors (white symbols). (a) Comparative proportions of γδT cells expressing NKG2A and NKG2D. (b) Comparative proportions of γδT cells expressing NKP30, NKp44, and NKp46. P values calculated using the Mann-Whitney test. (c) Comparative PFS (upper panels) and OS (lower panels) from diagnosis and sampling time of patients with low or high NKp44+ circulating γδT cells. Groups were separated using the median percentage of NKp44+ circulating γδT cells 6.3% \( n = 12–14 \) patients/group. Comparison using Log-rank test.
were associated with earlier relapse and shorter overall survival (Figure 5(b,c), Supplementary Tables 3–6), even if these molecules were similarly expressed on the corresponding γδ T cell melanoma patients compared to controls. Altogether these data enlightened that γδ T cells are skewed in their expression of NKR, KIR, and immune checkpoints in melanoma microenvironment, and we identified modulations both in blood and tumor as critical negative prognosis factors of clinical evolution.

The heatmap representation of all phenotypic features of γδ T cells highlighted differences between the groups analyzed (Supplementary Figure 6A), and multidimensional analysis revealed clear specific features for each group (Supplementary Figure 6B). Notably, principal component analysis of circulating and tumor-infiltrating γδ T cells of melanoma patients also allowed segregating patients with short and long PFS (Supplementary Figure 6C and 6D).

Figure 5. Circulating and tumor-infiltrating γδ T cells from melanoma patients displayed an altered expression of immune checkpoints, with PD1 and 41BB associated with a poor clinical outcome. The expression of immune checkpoints by γδ T cells was evaluated by flow cytometry in blood and tumor samples from melanoma patients (black symbols), and blood and tonsils samples from healthy donors (white symbols). (a) Comparative proportions of γδ T cells expressing 4-1BB, 4-1BBL, PD1, TIM3, LAG3, and ICOSL. P values calculated using the Mann-Whitney test. (B,C) Comparative PFS and OS from diagnosis time of patients with (b) low or high PD1+ circulating γδ T cells (n = 10–12 patients/group) and (c) low or high 4-1BBL+ tumor-infiltrating γδ T cells (n = 11–13 patients/group). Groups were separated using the median percentage of PD1+ circulating γδ T cells 21.5% and 4-1BBL+ tumor-infiltrating γδ T cells 38.1%. Comparison using Log-rank test.
Circulating and tumor-infiltrating γδT cells from melanoma patients have an impaired ability to respond to specific and global stimulation

We further investigated whether melanoma could impact the ability of γδT cells to respond to stimulation. To this end, we purified γδT cells from blood samples of HD or blood and tumor samples of melanoma patients and stimulated them with phosphoantigens (IPP, HMB-PP) that specifically activate Tδ2+ cells and PMA/ionomycin that globally stimulate all γδT cells. As expected, for the HD group, expression of CD69 and CD25 were drastically increased on Tδ2+ cells upon triggering with phosphoantigens and PMA/ionomycin, whereas Tδ2- cells responded only to PMA/ionomycin stimulation (Supplementary Figures 7 and 8, white symbols). We observed that circulating- and tumor-infiltrating γδT cells from melanoma patients have an impaired ability to upregulate CD69 and CD25 in response to phosphoantigens and PMA/ionomycin compared to HD (Figure 6(a)), and this failure affected both Tδ2+ and Tδ2- cells (Supplementary Figures 7 and 8). In addition, stimulated γδT cells from HD
secreted high levels of IFNγ and TNFα, which were dramatically altered with circulating- and tumor-infiltrating γδT cells from melanoma patients (Figure 6(b)). Stimulated γδT cells secreted also IL17-A, which level was not significantly different between HD and patients (Figure 6(c)). However, γδT cells from both blood and tumor of melanoma patients secreted IL17-A already at baseline in absence of external stimulation, and notably, basal IL17-A levels from tumor-derived γδT cells were linked with worse clinical outcome (Figure 6(d), Supplementary Table 7). The other cytokine analyzed (IL4, IL10, and TGFβ) were found not to be differentially secreted between melanoma patients and controls (Supplementary Figure 9), but high IL10 levels upon stimulation were associated with shorter PFS and OS (Supplementary Table 7). Therefore, melanoma drastically impaired the ability of γδT cells to exhibit activation molecules and secrete cytokines in response to stimulation, and drove them toward a Th17 profile associated with a poor clinical outcome.

**Circulating and tumor-infiltrating γδT cells from melanoma patients have an impaired cytotoxic potential toward melanoma tumor cells**

We then explored how melanoma affects the cytotoxic capacities of γδT cells. We noticed an increased proportion of CD56+ circulating γδT cells in melanoma patients (mean = 43.79%, range 22.4–83.34%) compared to HD (Figure 7(a)), that was associated with better PFS (Figure 7(b)). We then evaluated the cytotoxic potential of γδT cells toward melanoma tumor cells by assessing the degranulation of their intracellular cytotoxic vesicles that are covered with membrane-bound CD107a/b and containing perforin and granzyme B. γδT cells purified from HD and stimulated with phosphoantigens or PMA/ionomycin secreted granzyme B and upregulated CD107a/b at their surface while secreting high levels of perforin when facing their target melanoma tumor cells (Figure 7(c–e), white symbols). Even though circulating- and tumor-infiltrating γδT cells from melanoma patients displayed higher levels of CD107a/b at their surface compared to HD in these conditions (Figure 7(c)), that was negatively link to OS (Supplementary Table 8), their ability to secrete perforin and/or granzyme B was impaired especially within tumor microenvironment (Figure 7(d–e)), revealing a failure in their cytotoxic capacities toward melanoma tumor cells.

**Melanoma tumor cells directly impaired the capacity of γδT cells to mature and exhibit their functionalities in response to stimulation**

We next wondered if melanoma tumor cells could directly impact the features of γδT cells. We submitted γδT cells
purified from HD to melanoma-derived supernatants before assessing their ability to respond to stimulation with phosphoantigens and PMA/ionomycin. We observed that γδT cells preincubated with melanoma-derived supernatants had an impaired capacity to upregulate CD25 and/or CD69 and to secrete IFNγ in response to phosphoantigens and PMA/ionomycin (Supplementary Figure 10A and 10B). Furthermore, they displayed lower level of granzyme B (Supplementary Figure 10C) despite similar CD107a/b surface expression when facing the tumor target cells (Supplementary Figure 10D). Therefore, melanoma tumor cells directly impaired the capacity of γδT cells to exhibit their functionalities in response to stimulation.

**Discussion**

γδT cells are fascinating cells due to their unique properties and functional plasticity that render them very attractive for immunotherapy. Yet, these potent antitumor effectors have not been extensively explored in melanoma, limited to the identification of γδT cells within primary tumors and the report of alterations in frequencies and functions of circulating γδT cells. Little and controversial information is available on their functional features and clinical significance. There is a total lack of information regarding immune checkpoint expression by γδT cells in the context of cancer. We provided here a detailed investigation of....

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**Figure 7.** Circulating and tumor-infiltrating γδT cells from melanoma patients have an impaired cytotoxic potential toward melanoma tumor cells. (a,b) The expression of CD56 by γδT cells was evaluated by flow cytometry in blood and tumor samples from melanoma patients (black symbols), and blood and tonsils samples from healthy donors (white symbols). (a) Comparative proportions of γδT cells expressing CD56. P values calculated using the Mann-Whitney test. (b) Comparative PFS from diagnosis time of patients with low or high CD56+ circulating γδT cells. Groups were separated using the median percentage of CD56+ circulating γδT cells 40.9% (n = 10–11 patients/group). Comparison using Log-rank test. (c–e) γδT cells were purified from blood of healthy donors (white symbols), or blood (gray symbols) and tumor samples (black symbols) from melanoma patients and activated or not with phosphoantigens (IPP, HMB-PP) or PMA/ionomycin for 20 h. Cells were further cocultured with melanoma tumor cells (A375, COLO829) in a 20:1 ratio for 5 h with GolgiSTOP for the last 4 h. (c) Degranulation of γδT cells was assessed by adding CD107a/b Abs during the culture of γδT cells with target cells and revealed by flow cytometry. (d) Perforin production was monitored in the coculture supernatants. (e) Granzyme B secretion was evaluated in the culture supernatants of stimulated γδT cells. P values calculated using the Wilcoxon matched pairs test (straight line) or the Mann-Whitney test (dotted lines).
circulating and tumor-infiltrating γδT cells in term of frequency, phenotype, functional properties, and relationship with clinical stage and clinical evolution in melanoma compared to control. Our study highlights that γδT-cell features are promising potential biomarkers of clinical evolution in melanoma, and brings better understanding of the physiopathology of γδT cells in melanoma (summarized in Figure 8) that may help designing new therapeutic approaches exploiting the potential of γδT cells to improve patient outcomes.

We highlighted that despite similar proportions of circulating γδT cells between melanoma patients and HD, their rate decreased in advanced stage melanoma, but δ2+ cells were positively associated with longer PFS, which is concordant with another study revealing longer survival of patients with higher circulating δ2+ cell proportions.24 In previous studies, total circulating γδT cells were found to be either similar23 or reduced22 in melanoma patients compared to HD, whereas lower frequencies of circulating δ2+ cells have been reported both in early22 and late23,26,29 stage melanoma. Interestingly, the characterization of γδT cells in the context of immunomodulatory therapies highlighted that decreasing proportions of circulating Vδ2 cells during ipilimumab treatment was associated with lower clinical benefit.24

We found an increased frequency of γδT cells in tumors compared to control tonsils revealing an active migration of γδT cells toward melanoma, which was associated with better clinical outcome. Within γδT cells, higher proportions of tumor-infiltrating δ2+ cells showed a trend toward longer PFS and OS. This is in line with other studies describing
that γδT cells including δ2+ infiltrate primary melanoma tumor, representing 15–25% of TIL, and their proportion correlated negatively with disease stage and positively with progression-free and overall survival. Furthermore TCRγ-/- mice lacking γδT cells are highly sensitive to tumor development upon exposure to chemical carcinogens or tumor inoculation. All these observations proved that γδT cells participate in the immunosurveillance of melanoma, and their proportion may be a promising candidate biomarker of clinical outcome and/or response to immunotherapies.

The differentiation stage of γδT cells has also been examined, as it brings information regarding their migratory pathway and functional potential. We observed that circulating and tumor-infiltrating γδT cells harbored predominantly a T_{CM}/TEMRA or T_{CM} status respectively, even though no difference was noticed between patients and controls. Notably, we highlighted that higher proportions of circulating naïve γδT cells were associated with early relapse. In line with these data, it has been shown that circulating γδT cells in late stages and tumor-infiltrating γδT cells are skewed toward TEMRA status, and early differentiated Vδ1+ cells in blood were associated with a poorer survival. Melanoma may therefore either block the differentiation of γδT cells keeping them in a non-functional naïve status or favor their differentiation toward an exhausted phenotype by chronically stimulating them with phosphoantigens that accumulate in tumor cells in order to escape from γδT cells’ antitumor action.

γδT cells are known to exert both tumor-preventing and tumor-promoting activity (23). Our work allows depicting protumor activity of γδT cells in the context of melanoma. Indeed, we reported an accumulation of FoxP3+ regulatory γδT cells within melanoma tumors, which was dramatically associated with early relapse and shorter survival. However, their presence was not associated with increased levels of immunoregulatory cytokines TGFβ or IL10. It is known that FoxP3 expression by γδT cells does not necessarily correlate with suppressive functions. The precise characterization of regulatory γδT cells would require investigating their ability to inhibit the proliferation of stimulated CD4+ αβT cells. An indirect regulatory role of γδT cells can be anticipated anyway through their IL17 secretion. Indeed, we found that γδT cells were skewed toward Th17 profile in melanoma, independently of external stimulation, and such polarization was associated with worse clinical outcome. Very interestingly, Th17-skewed γδT cells were reported to favor tumor escape by promoting breast cancer metastasis, supporting accumulation of immunosuppressive myeloid-derived suppressor cells (MDSCs) in colorectal cancer and angiogenesis.
Analysis of γδT cells' functionality in melanoma is controversial, revealing in blood either impaired cytokine secretion, altered cytotoxic activity and poor proliferative capacity, or preserved cytotoxic potential associated with limited cytokine secretion upon stimulation. In tumors one study reported a strong production of pro-inflammatory cytokines (IFNγ and TNFα) together with potent cytotoxic activity. We demonstrated that melanoma severely impaired the ability of γδT cells to exhibit activation molecules and secrete cytokines in response to stimulation, and altered their cytotoxic potential toward melanoma tumor cells. Discrepancies between studies may reflect the nature of the samples (melanoma staging, purified untouched γδT cells or γδT-cell lines generated upon 10days culture with IPP and IL2), the comparison group (HD or not), the type of stimulation performed (phosphoantigens or PMA/ionomycin), the way to measure cytotoxic activity (CD107 exposure by γδT cells, perforin secretion, or annexinV/iodure propidium-based viability of target cells), and the target cell used. Most of the functional evaluations in previous studies were performed on γδT cell lines which may not be representative of the state of the γδT cells in situ. We used γδT cells directly upon their purification which is more likely to represent their status within melanoma microenvironment. Our observation of a higher proportion of cytotoxic CD56+ γδT cells in blood of melanoma patients is in line with a previous study showing elevated numbers of circulating cytotoxic γδT cells defined as being CD28 negative and harboring a high perforin content. It has been shown that CD56-expressing cells display strong effector functions including Th1 cytokine production and cytotoxic capacity, especially CD56+ Vγ9δ2 T cells harbor superior cytotoxic activity compared to their CD56-counterparts.

For the first time, we investigated the expression of a large panel of NCR, KIR, and immune checkpoints by γδT cells in the context of melanoma, and highlighted crucial clinical correlations. Circulating- and tumor-infiltrating γδT cells from melanoma patients exhibited higher levels of inhibitory receptors NKG2A and/or Nkp30, but also expressed more of activating NCR Nkp44 and/or Nkp46 compared to control groups. We also surprisingly identified that high proportions of Nkp44+ circulating γδT cells were predictive of poor clinical outcomes. Such modulated NCR and KIR expression will affect their capacity to recognize and kill target tumor cells. Notably, splice variants of human NCR encoding receptors with inhibitory functions have been recently discovered, influencing outcomes in many immunopathological contexts. Such differential splicing arises in some tissue microenvironments where inhibitory NCR are triggered by specific ligands, as this is the case for the inhibitory isoform of Nkp44 that cross-link Proliferating Cell Nuclear Antigen (PCNA) on tumor cells, a ligand expressed by many cancer types. Interestingly, the inhibitory form of Nkp44 has been found on tumor-infiltrating NK cells, and blocking the interaction Nkp44-PCNA results in inhibition of tumor growth including melanoma in mouse models. Hence, NCR can be considered as novel innate immune checkpoints, which, based on our results, offer an exciting potential therapeutic target to manipulate in cancer.

We also observed higher proportions of circulating γδT cells expressing 4-1BB, TIM3, LAG3 and ICOSL, and higher proportions of TIM3+ and LAG3+ tumor-infiltrating γδT cells in melanoma patients compared to control groups. We clearly identified that high proportions of PD1+ and 4-1BBBL+ circulating- or tumor-infiltrating γδT cells were predictive of poor clinical outcomes. Such correlations were known for PD1-expressing classical αβT cells, but have never been reported for γδT cells before. Of note, one study reported that the expression of 41BBBL could predict patient outcome in acute myeloid leukemia. Our work revealed that PD1, 41BB/41BBL, TIM3, and LAG3 are crucial checkpoints on γδT cells allowing immune escape and tumor progression. These observations thus pointed out that γδT cells may be interesting cells to look at in the context of current immunotherapies with immune checkpoint blockers, as they displayed a perturbed immune checkpoint panel expression and could be targeted by these therapies. Our data also validate a combinatorial therapeutic approach as three major inhibitory immune checkpoints that currently undergo clinical developments are expressed by tumor-infiltrating γδT cells.

Such phenotypic and functional alterations of γδT cells driven by melanoma may contribute to an inefficient antitumor immune response, by directly impairing killing of tumor cells and by altering subsequent interactions of γδT cells with other immune effectors. Melanoma disables γδT cells by altering their antitumor potentialities, by actively driving them toward a Th17 profile, or by skewing their immune checkpoint expression. Despite their hijacking by melanoma, positive correlates of some γδT features with clinical outcome revealed them as crucial antitumor effectors, which prompt their use as vectors or targets for cancer immunotherapy. γδT cells recognize stress-induced molecules and endogenous mevalonate metabolites in HLA-unrestricted manners, which accumulate in tumor cells and whose expression can be enhanced by bishophonates such as zoledronate. By inhibiting the farnesyl pyrophosphate synthase, zoledronate blocks the mevalonate pathway, leading to accumulation of phosphoantigens favoring recognition and killing of tumor cells by γδT cells. Such observations may foster the development of new therapeutic approaches using or targeting γδT cells. Successful clinical trials aiming at stimulating γδT cells with zoledronate such as in lymphoid malignancies, prostate cancer, breast cancer, or at adoptively transferring ex-vivo expanded γδT cells proved that γδT cell-based therapeutic approaches are promising to fight tumors and improve patient outcome.

Thus γδT cells have a pivotal role in antitumor immune responses, but impairment of their potentialities may contribute to tumor escape from immunity. Altogether, our study demonstrates that melanoma hijacked γδT cells to escape from immune control. Yet γδT cells have the potential to exhibit potent antitumor activity to successfully drive favorable clinical outcome. A better understanding of the functional plasticity of γδT cells may help designing new therapeutic approaches exploiting the potential of γδT cells while counteracting their skewing by tumor cells.
Patients and methods

Melanoma patients and healthy donor (HD)’ samples

Blood and tumor (primary tumor, cutaneous metastases and lymph node metastases) samples not needed for pathological investigations were obtained from 46 and 44 melanoma patients respectively, stage I–IV. Patients were staged according to the American Joint Committee on Cancer (AJCC) staging system for melanoma 2009 (V7). Clinical features are shown in Supplementary Tables S1 and S2. Progression free survival (PFS) and overall survival (OS) were calculated both from diagnosis and sampling time. Patients who underwent more than one relapse between diagnosis date and date of death/last news were removed when analyzing survival based on PFS calculated from diagnosis time (n = 3 patients) because PFS from diagnosis time could relate to the first or second relapse, and the PFS calculated from sampling time will be higher than the PFS calculated from diagnosis time. Blood samples were also obtained from 25 healthy volunteers (HD), and lymph nodes tissues (tonsils) obtained from 9 volunteers who underwent tonsillectomy due to repeating angina. PBMCs were purified by Ficoll-Hypaque density-gradient centrifugation (Eurobio). Tumor samples were mechanically dilacerated and digested with 2 mg/ml collagenase-D (Roche) 20U/ml DNase (Sigma). The resulting tumor-infiltrating cell suspensions were filtered and washed. Small whole tumor fragments (10 mm3) were incubated 24 h in complete RPMI1640 10% FCS (Invitrogen) to generate tumor supernatants. The study was conducted in accordance with the Declaration of Helsinki. All procedures were approved by the Ethics committee of Grenoble University Hospital and the French Blood Agency and declared under the reference #DC-2008–787. All participants signed informed consent forms.

Tumor cell lines

Human melanoma lines COLO829 and A375 were purchased from ATCC (LGC-Standards). Cultures were performed in RPMI1640-Glutamax (Invitrogen) supplemented with 1% nonessential amino-acids, 1 mM sodium pyruvate (Sigma), 100 µg/ml gentamycin, and 10% FCS (Invitrogen).

Phenotypic analyses

Phenotypic analyses were performed on both cutaneous tumors (primary tumor and metastases) and lymph node metastases. Cell suspensions were stained with anti-human antibodies in PBS 2% FCS. Total γδT cells were identified as CD45+ CD3+ panTCRγδ+, and further divided into δ2+ and δ2– subsets using the corresponding antibodies (Abs). The features of γδT cells were analyzed using anti-human antibodies and their isotype-matched controls: the basal activation status was determined using anti-CD40 (Beckman), -CD86, -CD69, -CD25 (BD) Abs; immune checkpoints were analyzed using anti-OX40, -OX40L, -ICOS, -41BB, -41BBL, -PD1, -PD1L, -PD2 (BD), -ICOSL, -TIM3, -CTLA4, -LAG3 (eBiosciences) Abs; activating and inhibitory NKR were depicted using anti-NKG2A, -NKG2C (BioTechne), NKG2D (BD), NKP30, NKP44, NKP46 (Beckman) Abs; differentiation stage was assessed by labelling with anti-CD27 and -CD45RA Abs (BD); regulatory γδT cells were evaluated using FoxP3 intranuclear labelling (eBiosciences) and cytotoxic γδT cells using CD56 labelling (BD). Suspensions were subjected to flow cytometry analysis using a FACS CantoII and DIVA software (BD). To ensure quality control during the study, we performed a systematic standardization of the fluorescence intensities using cytometer setup and tracking beads (CST) (BD).

Functional assays

Functional studies were performed only from lymph node metastases. To perform functional assays, γδT cells were purified using EasySep human γδT-cell enrichment kit (StemCell) according to manufacturer’s instructions. The purity obtained was routinely above 95%. In some experiments, purified γδT cells from HD were pre-incubated with supernatants derived from melanoma tumors (50% in volume) for 24 h before performing functional assays.

Response to stimulation

Purified γδT cells were resuspended at 1.106/ml in complete RPMI1640 10% FCS and cultured for 24 h alone or with phosphoantigens, isopentyl pyrophosphate (IPP, 80 µM) (Sigma) or 1-hydroxy-2-methyl-2-butenyl 4-pyrophosphate (HMB-PP, 200 nM) (Sigma) together with IL2 (0.1 UI/ml) (Peprotech), or PMA (20 ng/ml) and ionomycin (1 µg/ml) (Sigma). The activation phenotype of γδT cells was analyzed using anti-CD40 (Beckman), -CD69, -CD25 (BD) antibodies. Human soluble IL4, IL10, IL17-A, IFNγ, TNFα, TGFβ, and granzyme B production were measured in culture supernatants by a Cytometric Bead Array assay (CBA, BD).

Cytotoxic activity

γδT cells cytotoxic activity was evaluated by a CD107 degranulation assay and perforin measurement upon coculture with target cells. Upon stimulation of purified γδT cells as previously described, the cells were washed and cocultured with melanoma tumor cells (A375, COLO829) in a 20:1 ratio for 5 h. Anti-human CD107a/b Abs (BD) were added at the start of the coculture together with GolgiSTOP (BD) for the last 4h. The cells were then labeled with CD45, CD3, panTCRγδ Abs (BD) before flow cytometry analysis. Perforin production was evaluated in the coculture supernatants using Human PRF1 ELISA kit (AbCam).

Statistical analysis

The statistical analyses were performed by Prism software using the Mann-Whitney test, the nonparametric U test, the Wilcoxon matched t-test, the one-way ANOVA, the Kruskal-Wallis nonparametric test, the Spearman correlation and the Log-rank test. Cox regressions, principal components analyses (PCA) and heatmap were done in Rstudio using the packages FactoMineR and p heatmap.
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Disclosure of Potential Conflicts of Interest

The authors report no conflict of interest.

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