Deciphering human γδ T cell response in cancer: lessons from tumor-infiltrating γδ T cells

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ABSTRACT

The finding that γδ T cells are present amongst tumor-infiltrating lymphocytes in humans suggest they participate in tumor immune surveillance, but their relevance is unclear because the relative frequencies of tumor-infiltrating γδ T cells correlate with tumor remission or progression, or do not correlate at all with prognosis. This is suggestive of the fact that tumor-infiltrating γδ T cells may play substantially different effector or regulatory functions, and correlation with patient's prognosis relies on distinct γδ T cell subsets in the context of the tumor. There is interest to exploit γδ T cells in tumor immunotherapy, but to make this approach successfull there is urgent need to fully understand the biological functions of γδ T cells and of how they can be manipulated *in vivo* and *ex vivo* to safely provide benefit to the host.

This review focuses on our previous and ongoing studies of tumor-infiltrating γδ T lymphocytes in different types of human cancer. Moreover, we discuss the interaction of tumor-infiltrating γδ T cells with other cells and molecules present in the tumor microenvironment, and their clinical relevance on the ground, that better knowledge in this field can be used further for better immunotherapeutic intervention in cancer.

Running title: Tumor-infiltrating γδ T lymphocytes.

Key words: γδ T lymphocytes, colon cancer, tumor-infiltrating lymphocytes; tumor microenvironment; clinical correlation.

1. GENERAL INTRODUCTION ON ANTIGEN RECOGNITION BY γδ T CELLS.

Human $\gamma\delta$ T lymphocytes are typically classified based on the Vδ chain used to make their T cell receptor (TCR). The prevalent (>70%) $\gamma\delta$ T lymphocytes in human blood and secondary lymphoid organs of adult individuals express a TCR eterodimer composed of the Vγ9 and Vδ2 chains [1]. These cells are commonly named as Vγ9Vδ2 T lymphocytes, and recognize non proteic pyrophosphate metabolites called phosphoantigens (PAgs), which derive either from the mevalonic acid (MVA) pathway [2] or the microbial non-mevalonate Rohmer cycle ((E)-4-Hydroxy-3-methylbut-2-enyl pyrophosphate, HMB-PP) [3].
Butyrophilin-3A1 (CD277) is necessary but not sufficient for PAg recognition by Vγ9Vδ2 T cells [4-6]. Another BTN molecule, BTN2A1, has been recently identified as a key ligand that associates on the cell surface with BTN3A1 independent of PAg and binds to the Vγ9Vδ2 TCR. In particular, the surface of the BTN2A1 IgV domain directly binds to germline-encoded Vγ9 regions of the Vγ9Vδ2 TCR, while somatically recombined CDR3 regions implicated in PAg recognition are uninvolved. It is then likely that binding of a second ligand, possibly BTN3A1, to a separate TCR Vδ2 domain is also required [7,8].
In agreement with the very restricted antigen recognition pattern, the Vγ9Vδ2 TCR repertoire is invariant or semi-invariant, suggestive of an innate-like modality of PAg recognition [9,10]. However, there is evidence [11] that in addition to the PAg-reactive Vγ9$^+$ T lymphocytes, the Vδ2 population also includes another subset of Vγ9$^-$ T lymphocytes with a diverse repertoire and with adaptive-like characteristics, as indicated by the finding that this population selectively clonally expands during acute cytomegalovirus (CMV) infection.

$\gamma\delta$ T lymphocytes expressing the Vδ1 or Vδ3 TCRs, are less represented than Vγ9Vδ2 cells in adult blood but predominate in tissues [1], and have quite different antigen reactivity. Vδ1 T lymphocytes physiologically reside in the skin, lung, intestine and colon epithelia where they represent the major γδ T cell subset and recognize stress antigens overexpressed upon CMV-infecion and tumor transformation [12-15]. Vδ3 cells are even rarer that Vδ1 T cells in blood but abound in the liver and gut and are expanded in patients with CMV infection or B cell leukemia. Few studies have provided evidence for MHC class I and CD1d restricted Vδ1 T cells [16] and a Vγ8Vδ3 T lymphocyte population recognize Annexin A2 overexpressed by stressed and tumor cells [17].

Differently that αβ T cells, and with the exception of very rare Vδ3 T cells, γδ T cells lack genetical (i.e. HLA, CD1 or MR1) restriction, and are indipendent on co-stimulatory signals (i.e. CD28) [1]. However, γδ T cells share several features with αβ T cells: in fact, similar to T CD8 αβ subset, they can be distinguished into “naive”, “central memory”, “effector memory” and “terminally differentiated” subsets with different recirculation pattern and effector functions [18]. Moreover, γδ T cells may acquire highly diverse effector functions in the presence of certain cytokine combinations and polarize to Th1, Th2, Th17, follicular T helper, Th9 and T regulatory (Treg) cells [19-29] and this wide flexibility highlights the capacity of γδ T cells to rapidly respond to different antigenic challenges.

2. γδ T CELL FUNCTIONS IN TUMOR IMMUNOLOGY.

The evolutionary divergence between human and mice has been the major obstacle in the scientific research in the field of γδ T lymphocyte. In fact, cells with repertoire and reactivity of human γδ T lymphocytes, particularly Vγ9Vδ2 T lymphocytes, are only found in humans and higher primates, but absent from rodents [1], in which pre-clinical studies are performed. Thus, the lack of a suitable animal model has greatly limited our knowledge of the role of γδ T lymphocytes in immune responses to pathogens.

Nevertheless, robust evidence that γδ T cells have anti-tumor activity has been firmly established by studies of Hayday and coworkers showing that γδ T lymphocytes control cutaneous carcinogenesis in mice [30], and by the finding that human γδ T lymphocytes are able to exert potent HLA-unrestricted cytotoxicity *in vitro* against a variety of tumor cells (reviewed in 31). Moreover, human γδ T lymphocytes expanded *ex vivo* and then adoptively transferred into immunodeficient mice xenografted with tumor cells have demonstrated efficient anti-tumor activity [30].

The most important effector function of γδ T cells which is relevant to tumor immunology is their cytotoxic activity. γδ T cells recognize antigens (stress molecules, PAgs/BTN3A1 complexes, other unknow molecules) broadly expressed on many different tumor cells and in the absence of genetic-restriction. Thus, the capacity to recognize antigens which are common to many different tumors and in the absence genetic restriction, may allows a broader clinical utilization of γδ T cells in the global heterogenic population and across many different tumor types. Upon tumor antigen recognition by the γδ TCR or NKG2D and NCRs receptors [32-35], γδ T lymphocytes establish immunological synapses with target tumor cells and kill them using the same mechanisms as NK and $CD8^+$ cytotoxic T cells, namely granule exocytosis with release of perforin, granzymes and granulysis and activation of death receptors by the respective ligands [36-40]. Moreover, human γδ (both Vδ1 and Vδ2) T lymphocytes express CD16 (the low affinity FcγRIII) and perform antibody-dependent cell-mediated cytotoxicity (ADCC) [41].

In addition to their prominent cytolytic activity, upon antigen stimulation in the presence of polarizing cytokines, human γδ T lymphocytes polarize towards different phenotypic and functional subsets [18-29] and acquire different cytokine secretion patterns [see above], thereby modulating tumor growth by orchestrating downstream immune responses.

Thus, human γδ T lymphocytes help B lymphocytes to produce different antibody classes [26-28], promote 4-1BB-mediated NK cell cytotoxicity [42], induce dendritic cells maturation [43,44] and reprogram their functions to become professional antigen-presenting cells to induce proliferation and expansion of conventional CD8 cytotoxic T lymphocytes [45-47].

3. TUMOR-INFILTRATING γδ T CELLS: HOW CAN WE DETECT THEM?

The identification of tumor-infiltrating γδ T lymphocytes in humans is a major issue for establishing their prognostic value and for cancer immunotherapy, as these pleiomorphic cells which are located in most healthy organs are often misidentified when not unidentified at all [48,49]. Typically, detection and characterization of γδ T lymphocytes in healthy or tumor tissues relies on a combination of cell surface markers labeled by immunostaining and visualized by immunohistochemistry. This technique requires access to fresh tumor samples and furthermore, immunohistochemical spotting of γδ T cells remains largely hampered by the paucity of anti-TCR reagents compatible with staining of formalin-fixed paraffin embedded samples. There is only one anti-pan γδ TCR mAb on the market which works well on paraffin embedded tissues and which has been made commercially available only very recently [50], but this mAb does not distinguish between Vδ1, Vδ2 and Vδ3 subsets of γδ T cells. Alternatively, or in addition, γδ T lymphocytes in tissues may be visualized by flow cytometry [48,49]. However, this is a quite demanding process which requires digestion of tissue samples large enough to obtain a sufficient number of viable cells for subsequent phenotypic and functional analysis. Nevertheless, both these techniques have been used to identify the frequency, phenotypes and functions by γδ T lymphocytes obtained from different types of human cancer tissues.

However, given the recent developments in γδ T cell-based therapies of cancer, it is important to know not only the rate of tumor-infiltrating γδ lymphocytes from any tumor biopsy, but also their subset distribution, maturation/activation/exhaustion states and functional profiles likewise, but this has remained unclear so far. The large number of transcriptomic datasets obtained using microarrays or mRNA sequencing (RNAseq) of bulk tumor biopsies, and currently available from public databases (GEO database, The Cancer Genome Atlas, etc.) represented an unvaluable resource and unique opportunity to address these issues by datamining approaches. One such method, called deconvolution, consists in algorithmic learning transcriptomic profiles of several specific cell types taken individually, followed by algorithmic deduction of their respective rates from the bulk transcriptome of a complex cell mixture. Hence theoretically, deconvolution can determine the cell composition from any bulk tumor biopsy, provided the cell type components have previously been learned [51]. Accordingly Gentles et al. deconvoluted 19,000 tumors using their algorithm CIBERSORT to decipher the composition of TILs and found that γδ TILs were the most significant favorable cancer-wide prognostic populations [52]. In a subsequent deconvolution of 15,000 biopsies from 50 solid and hematologic malignanices however, we showed that due to insufficiently learnt γδ T cell transcriptomes (*n*= 2 low resolution microarrays), CIBERSORT massively misidentifes both γδ, CD8, and NK cell types [53]. These three closely related cell types have highly multicolinear transcriptomes [54], which require much larger learnings from many more γδ T cell transcriptomes to return reliable results. In addition, determining their TCRV subsets and further intrinsic hallmarks such as stage of maturation or exhaustion are out of reach for the same reasons. Hence current published deconvolution studies might provide us with misleading conclusions about γδ T cell infiltrating human cancers.

The recent developments in single cell RNA sequencing are complementing and updating immunochemistry and flow cytometry methodologies, and now provide us with the finest level of resolution for the gene expression patterns of large sets of cells, unveiling much finer aspects of cells and allowing their classifications from human tissues.

Nevertheless, for the same above reasons, plus impossibility to determine TCRVγ and δ gene usage from short reads of 3' mRNA ends, the identification of γδ T lymphocytes in scRNA-seq data remained elusive, so γδ T remain barely identified in most scRNAseq studies of PBMC or tissues. By providing the first scRNA-seq dataset of cell-sorted TCRVδ1 and TCRVδ2γδ T lymphocytes purified from PBMC of healthy individuals with known CMV serology, we defined a gene signature identifying human γδ T cells in scRNAseq datasets [55] (Figure 1). By this approach, scarce γδ TILs of either TCRVδ1 and TCRVδ2 subtypes could be detected in a small series of published cancer scRNASeq datasets, with cell counts suggesting a decorrelation of αβ TILs and γδ TILs across human cancers. These advances paved the way to further metaanalyses of γδ TILs aiming at reliably determining their presence, TCR usage, maturation/differentiation, and activation/exhaustion status in the tumor microenvironment of a large spectrum of human cancers.

4. γδ T LYMPHOCYTES IN CHRONIC INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER

Patients with inflammatory bowel disease (IBD) have an elevated risk to develop colorectal cancer (CRC), that is associated with the duration and extent of chronic inflammation [56,57]. The role of γδ T cells in the pathogenesis of IBD is still poorly known, because of the conflicting findings of studies showing either increased or decreased frequencies of γδT cells in IBD patients, as compared to control subjects. Moreover, most studies have looked at circulating γδ T cells, which do not necessarily mirror the situation in gut tissue [58,59].

In a murine model of IBD, γδ T cells that participate to chronic inflammation express the gut-homing molecules CD103 and α4β7 and produce IL-17 [60] which is known to sustain chronic inflammation in the intestine and contribute to cancer development [53, 61]. Moreover, a population of gut-tropic γδ T cells that express β integrin and produce TNF-α has been detected in the blood and in mucosa samples of IBD patients [62].

As γδ T cells may play a role in the regulation of the integrity of the mucosa barrier [63], it is important to understand whether γδ T cells participate to the pathogenesis of IBD and contribute to the transition from IBD to CRC.

In recent years we have been interested in studying γδ T cells and their Vδ1 and Vδ2 subsets in long-standing IBD patients (as a model of chronic gut inflammation) and in CRC patients [64,65], with the aim to establish their role in the pathogenesis of chronic IBD and CRC.

Results from a large number of tissue specimens have confirmed that Vδ1 T cells are the dominant γδ subset in normal colon tissue, while Vδ2 T cells are poorly represented. Patients with long standing (chronic) IBD have low frequencies of gut Vδ1 T cells but an increased frequency of Vδ2 T cells which correlates with the duration and severity of the inflammatory response [65].

From a functional point of view, gut Vδ1 T cells from healthy subjects and chronic IBD patients express relatively low percentages of IFN-γ and TNF-α. Gut Vδ2 T cells from healthy donors similarly produce low levels IFN-γ and TNF-α, but Vδ2 T cells from the gut of long lasting IBD patients produce more TNF-α and IL-17 [65]. These results indicate that Vδ2 T cells are recruited to the gut during chronic IBD and contribute to the whole TNF-α production. Moreover, our study identified a yet unappreciated role of Vδ2 T cells in contributing to IL-17 production in the gut of chronic IBD patients, and account for approximately one-third of all IL-17-producing cells. These results, together with the finding that the frequency of gut Vδ2 T cells significantly correlates with the severity of IBD, strongly support the view that Vδ2 T cells contribute to chronic inflammation in IBD patients (Figure 2).

These findings were confirmed by the analysis of a cohort of IBD transcriptomes showing that expression of the *TRDV2* or *CD3D* genes was significantly correlated with expression of *IFNG*, indicating that T cells in general and Vδ2 T cells in particular contribute to IFN-γ production in IBD samples [66]. Transcriptome analysis also revealed a significant correlation between *TRDV2* and *IL17A* gene expression but surprisingly, there was no significant correlation between *TRDV2* and *CD3D* gene expression, suggesting the existence of two diverse Vδ2 T cell populations, one CD3^{high} and one CD3^{low} (our unpublished results and [67]). Moreover, expression of the *TRDV2* gene did not correlate with expression of the *TRGV9* gene [63], suggesting the presence of two different Vδ2 T cell populations, one Vγ9^{+} and one Vγ9^{-}, that adopt divergent features, as above discussed [11].

Finally, we could not find any significant correlation between *TRDV2*, *TRGV9*, *CD3D* and *TNF* gene, indicating that *TNF* production arises from more heterogeneous cell sources than *IFNG* and *IL17*.

Chronic inflammation is the third highest risk condition for CRC [68]. Therefore, we analysed γδ T cells in CRC specimens with the expectation to find out similarities between chronic IBD and CRC [64]. Instead, by using both flow cytometry and transcriptomic we detected comprised a similar rate of γδ T cells (4% on average) in CRC tissue and normal adjacent tissue, with the majority expressing Vδ1. The majority of Vδ1 and Vδ2 T cells had an effector memory phenotype but had a very low, if any, production of IFN-γ and TNF-α and did not produce at all IL-17.

In an independent cohort of CRC trascriptomes, expression of *TRGV9*, *TRDV2* and *CD3D* genes was correlated with expression of the *IFNG* gene, suggesting that T cells in general and Vδ2 T cells in particular contribute to IFN-γproduction in CRC patients. In contrast, we did not find any significant correlation between *TRGV9* and *TRDV2* and *IL17A* gene expression, suggesting that IL-17 production arises from more diverse cell sources than IFN-γ [64]. This finding was unexpected, because another study in Chinese patients reported that γδ T cells are the main source of IL-17 in CRC [61]. Potential explanations to such apparently conflicting findings might be clinical scenarios, differences in the clinical cohorts focussed on, or alterations in microbiome.

5. IDENTIFICATION OF A HUMAN INTESTINAL INTRAEPITHELIAL Vδ1 T CELL SUBSET EXPRESSING NKP46 AND EQUIPPED WITH CYTOLYTIC AND ANTI-TUMOR ACTIVITY.

NKp46 is a member of the NCRs family of NK activating receptors that are involved in the recognition and killing of target cells, including tumor cells [69-72]. Although NCRs are not expressed by circulating T cells [35], expression of both NKp46 and NKp44 was detected on αβ intestinal intraepithelial lymphocytes (IELs) in patients affected by celiac disease [73]. In this context, NCR expression was induced by IL-15 and overexpressed in celiac patients [74]. These NCR^+αβ IELs had potent cytotoxic activity and an highly restricted TCR repertoire, likely suggesting that they had undergone to TCR-mediated clonal expansion [73].

While studying γδ T cells infiltrating CRC, we have identified a subset of Vδ1 T cells constitutively expressing NKp46, equipped with high cytolytic potential, and representing the most abundand γδ T cell subset in healthy human intestine [75]. Expression of NKp46 significantly contributes to the cytotoxic activity of NKp46$^+$ Vδ1 IELs freshly purified from the gut, since cytotoxicity is blocked by addition of a Mab to NKp46.

Additionally, we found that NKp46$^+$ Vδ1 IELs are not phylogenetically conserved across species, and are neither present in other human tissues nor in the blood. Therefore, they represent a unique population of gut-resident IELs.

The origin and development of IELs is still unclear, but by analogy with model of γδ T cell development, it is likely that NKp46$^+$γδ T cells develop in the thymus and migrate to the gut where IL-15, secreted by multiple cellular sources including intestinal epithelial cells [76] supports their growth [77,78]. In our *in vitro* studies, stimulation of thymocyte precursors with IL-2 or IL-15 promotes the development NKp46$^+$ Vδ1 IELs with the same phenotype and functions as those of their intestinal counterpart, and also induces the expression of CCR9 which, in turn, allows recruitment of NKp46$^+$ Vδ1 T cells to the gut [79]. However, and differently than IL-2 and/or IL-15–activated γδ thymocyte precursors, gut-resident NKp46$^+$ Vδ1 IELs lack the expression of NKG2A, an inhibitory receptor expressed by NK cells and specifically binding the nonclassical MHC Class I molecule HLA-E. One explanation for this apparent discrepance is that NKp46$^+$ Vδ1 T cells generated *in vitro* from thymic precursors do not interact with intestinal epithelial cells that constitutively express high levels of HLA-E [80]. Conversely, binding of HLA-E to NKG2A on NKp46$^+$ Vδ1 intestinal IELs might cause NKG2A downregulation and thus explain its low expression.

Altogether, our results indicate that the biological properties of the NKp46$^+$ Vδ1 IELs are dictated both by distinctive features of human Vδ1 thymocytes and by the gut microenvironment.

6. CLINICAL RELEVANCE OF TUMOR-INFILTRATING γδ T CELLS.

The clinical relevance of tumor-infiltrating γδ T lymphocytes is still unclear because of contrasting results. In fact, in different studies that have utilized either immunohistochemistry or flow cytometry, the frequency of tumor-infiltrating γδ T cells, has been reported to correlate with tumor remission, or with tumor progression, or even failed to correlate with prognosis [49,50]. These findings are strongly suggestive of the fact that γδ T cells at the tumor site may play opposite (anti- or pro-tumor) functions, and thus might show positive or negative correlation with prognosis depending on the γδ T cell subset infiltrating the tumor site (Figure 3).

Recent studies suggest that the differential expression of IFN-γ and IL-17 by tumor infiltrating γδ T cells observed in several tumor mouse models, may also extend to cancer patients [81]. Thus, the percentage of IL-17-producing γδ T cells amongst tumor-infiltrating lymphocytes was significantly higher in patients with squamous cell carcinoma at advanced stage, while the percentage of IFN-γ-producing γδ T cells in the tumor tissue was higher in patients at early stage. Along these lines, other studies have demonstrated that the frequency of either total γδ T cells or γδ T cells expressing IFN-γ correlate with better survival [48,49,81], while γδ T cells expressing IL-17 are associated with bad prognosis in patients with gallbladder [82] and colon [61] cancers.

In the colon cancer study by Wu et al. [61], γδ T cells constitute the major source of IL-17 in tumor biopsies, and the frequency of γδ T cells expressing IL-17 at the tumor site positively correlates with overall tumor progression. This is in sharp contrast with a subsequent study by our group [64] showing that in colon cancer patients an high frequency of tumor-infiltrating γδ T cells significantly correlated with higher 5-year disease-free survival rate. Unlike murine γδ T cells, human γδ T cells have a Th1-type signature (they produce IFN-γ and TNF) and the presence of γδ T cell subsets making IL-17 in humans has been more controversial and is less generally accepted. This suggests that accumulation of γδ T cells making IL-17 at the tumor site may be the consequence of tumor-associated inflammation. This is consistent with our findings in children with bacterial meningitis, where γδ T cells expressing IL-17 were detected in the cerebrospinal fluid but were absent from the circulation [23]. It is likely that as with mouse γδ T cells, IL-1β, IL-6, IL-23, and TGF-β account for γδ T cell polarization to IL-17-production also in humans [23].

In our study in human CRC, we initially investigated whether intratumoral γδ T cells had any clinical relevance, by data mining transcriptomes and clinical files from a large cohort of CRC samples [64]. The leucocyte deconvolution of this dataset demonstrated a link between genetic and molecular markers of CRC and their abundance of TILs, whether of the αβ or γδ T cell subtype. For instance, the KRAS mutation status made no difference for these criteria, but the abundance of both subsets of αβ or γδ TILs was significantly higher in mismatch repair-deficient (MMR-D) than -proficient (MMR-P) tumors, in BRAFmutated versus BRAFwt, and in TP53wt versus TP53 mutated tumors. A higher content of αβ or γδ TILs was also apparent in tumors positive for the CPG island methylator phenotype (CIMP) as compared to their negative counterparts, and in tumors negative for the chromosomal instability phenotype (CIN) as compared to their positive counterparts. Importantly, each of these prognostic factors influenced in the same direction the abundance of both αβ and γδ TILs [64].

We then correlated the relative abundance of γδ T cells with clinical features by analyzing the independent dataset of those CRC patients which follow-up was available. Across the whole cohort, those patients with more abundant γδ TILs had a better disease-free survival (DFS), as confirmed by stratifying patients according to expression of the *TCRGV9* gene.

Patients with higher expression of the *IFNG* gene also had a higher DFS, whereas those with higher expression of the *IL17A* gene had a significantly lower DFS [64]. This γδ- and IFN-γ-associated favorable pattern was still maintained when reducing the whole cohort to those patients without lymph node invasion. By contrast, the IL17-dependent unfavorable pattern was rather observed in patients with lymph node metastasis. Stratifying the entire cohort according to both γδ TIL abundance and *IFNG* gene expression produced groups with strikingly different DFS, and demonstrated most notably that γδ TIL abundance overweighted *IFNG* gene expression in contributing to better patient's outcome [64]. This suggests the antitumor activity of γδ T cells amongst TILs might be attributable mostly to their cytotoxic activity, rather than to IFN-γ production. The role of Vδ1 T cells could not be investigated likewise using the microarray data set which lacks the *TRDV1* gene and in which the correlated levels of *TRGV9* and *TRDV2* genes indicated presence of TCR Vγ9Vδ2 T lymphocytes.

In another study, the frequencies of γδ T cells among TILs of squamous cell cancer (SCC) patients did not substantially change with disease stage but the frequencies of IFN-γ-producing Vδ1 and Vδ2 T cells were higher in SCC patients at stages I and II and with favorable outcome (absence of lymph node metastasis, cancer relapse and overall survival), but significantly decreased in patients with advanced disease (stages III and IV) [81]. The percentages of γδ T cells among TILs of melanoma patients did not change similarly, but the frequencies of Vδ2 T cells that expressed IFN-γ decreased in parallel with disease progression [83].

Therefore, a common feature of the three cancer types studied so far (CRC, SCC and melanoma) is that frequencies of IFN-γ-producing Vδ2 T cells amongst TIL are significantly correlated with favourable prognosis.

Moreover, it also emerged from our studies, that a reduced frequency of Vδ1 T cells among TILs was positively associated (although not always significantly), with the severity of melanoma, SSC, and CRC [81,64]. This include also the NKp46$^+$ Vδ1 IELs subset which was substantially scarce in the intestinal tissues surrounding the tumor mass in patients with CRC and the low frequency of NKp46$^+$ Vδ1 IELs correlated with rapid tumor progression toward metastatic disease [75]. Interestingly, a recent report showed that NKp46-mediated production of IFN-γ by NK cells is able to control tumor progression and decreases metastasis *in vivo* [84].

γδ T cells may play a pro-tumor role not only by producing IL-17, but also by the adoption of suppressive functions that impair dendritic cell maturation and functions. For instance, an immunochemistry study on breast cancer specimens [85] has revealed massive infiltration by γδ T cells that correlates positively with advanced tumor stages and the presence of lymph node metastasis, and negatively with patient survival.
Moreover, in a murine model of pancreatic ductal cancer, γδ T cells in the tumor tissue express the programmed death ligand 1 (PD-L1) and suppress CD4$^+$ and CD8$^+$ T cell recruitment and function [86]. It remains to be established whether PD-L1 expression by γδ T cells is exclusive to the pancreatic microenvironment or extended to other tumor types. PD-1 is upregulated by *in vitro* activated Vδ2 T cells and modulates IFN- production [87] , but it is unclear how long is PD1 expression maintained [88,89].
Therefore, there is a urgent need to in deep characterize human γδ T lymphocytes at the site of tumor and in adjacent healthy tissue in order to understand their impact on cancer progression.

7. TUMOR MICROENVIRONMENT IMPARTS DISTINCT FUNCTIONAL FEATURES ON γδ T CELLS.

The mechanism responsible for the massive reduction of Vδ1 TILs in CRC patients remains unknown. Possible hypothesis includes terminal differentiation and exhaustion of the Vδ1 T cell subset due to repeated stimulation by the Vδ1 TCR ligands, MICA/MICB, which are overexpressed in colon cancer cells. Other possibilities include, activation-induced Vδ1 T cell anergy and death by neglect drive by inadequate signals delivered during activation, or Vδ1 T cell intrinsic features. Finally, alterations in the normal microbiota as occurs in CRC [61], influenced by many factors, and/or increases in immunosuppressive cytokines in the CRC microenvironment, can result in loss of Vδ1 T cells at mucosal level (reviewed in [90]).

To investigate the reciprocal interactions between the tumor environment and γδ T cells present in TILs, we purified colon cancer stem cells (CSC) and colon cancer-associated fibroblasts (CAF) from tissue samples of CRC patients, cultured the in medium and tested the effect of the 48-hrs culture supernatants on polyclonal γδ T cell lines (containing both Vδ1 and Vδ2 T cells) obtained from the peripheral blood of healthy donors [64]. We found that supernatants from colon CSCs caused significant inhibition of γδ T cell proliferation and IFN-γ production, whereas supernatants from CAF had very limited suppressive ability. The colon CSC supernatants also inhibited IFN-γ production by CD4 and CD8 αβ T cells, clearly indicating that inhibitory molecules produced by colon CSC have a profound immunosuppressive effect on both adaptive αβ and γδ T cell subset.

We do not have evidence on the nature of the responsible molecule(s) and our attempts to its/their identification have been inconclusive so far. By comparing molecules which were differentially overexpressed in the inhibitory supernatants form CSC and in the non inhibitory supernatants from CAF, we found out only 3 such differentially expressed cytokines IL-8, IL-12 and VEGF. By definition, IL-12 does not inhibit T cell proliferation and IFN-γ production, which eventually leaves IL-8 and VEGF as potential candidates of the immunosuppressive activities of the colon CSC secretome [64]. However, neither IL-8 and VEGF alone, or in combination, were capable to inhibit proliferation and IFN-γ production by γδ T cell (our unpublished resuults), which leaves open the question of the nature of the suppressive molecule(s) in the CRC microenvironment. Moreover, it may also be possible that in the *in vivo* setting, suppressive molecules in the CRC microenvironment may target other cell types like dendritic cells, myeloid-derived suppressor cells, M2 macrophages and Treg cells [91,92] and thus indirectly inhibit immune responses. Finally, it is also possible that molecules/elements like prostaglandins [93], kynurenins [94] or potassium [95] may be responsible for the immunosuppressive activity.

The immunosuppressive tumor microenvironment may also explain the loss of $NKp46^+$ Vγδ1 T cells around the tumor mass in CRC [75]. In fact, incubation of $NKp46^+$ Vγδ1 T cells with the colon cancer cell line Caco2 or TGF-β (overproduced in CRC) [96] significantly inhibited their expansions.

8. WHAT HAVE γδ T CELL-BASED CLINICAL TRIALS IN CANCER IMMUNOTHERAPY TAUGHT US ?

The participation of γδ T cells to stress surveillance immune responses [96] and their capacity to exert numerous effector functions relevant to tumor immunology, has led to a great interest in exploring their potential use in novel immunotherapies in cancer.

A remarkable boost to the utilization of γδT cells in cancer immunotherapy came from the discovery that aminobisphosphonates (N-BP), like Zoledronate, which inhibit FPPS the downstream enzyme of the MVA pathway, cause accumulation of endogenous PAgs and together with IL-2 promote activation and differentiation of human γδT cells. Assessing that N-BPs were already widely used in the clinic, in the early 2000s the Wilhelm group in Wuerzburg [98] and our group [99] conducted for the first time phase I clinical studies in hematologic and solid malignancies, respectively, using N-BPs and IL-2 *in vivo* to intentionally activate γδT cells.

Several small scale clinical trials have been conducted in patients with different types of cancer, which have used either PAgs or n-BPs and IL-2 to activate Vγ9Vδ2T cells *in vivo* or adoptive T cell therapy with Vγ9Vδ2 T cell taken from patients and expanded *ex vivo*[31].

Despite a systematic assessment of safety and efficacy which includes the most recent clinical studies is missing, a meta-analysis we performed on data available up to 2014 showed that in both settings γδ T cell therapy was safe and gave some clinical benefit in the short term [100].

However, more recent results rather suggest very low objective response rates which implies the need to carry out larger and double-blinded clinical studies.

Nevertheless, the results of these clinical trials, although generally disappointing, have proven useful because they have provided us several possible explanations for the long-term limited efficacy of the γδ T cell therapy and how to overcome them.

First, there are clinical reasons related to the heterogeneity of the studies (low number of patients enrolled, advanced disease, previous chemotherapy, different tumor types, different protocols used to expand γδ T cells *in vivo* or *ex vivo*, different treatment schedules). [31].

Second, it is known that *in vivo* administration of PAgs or N-BPs and IL-2 causes a progressive loss of γδ T cell number and response and this is a critical factor in cancer patients, especially the older ones,which have significantly reduced γδ T cell numbers and responses [101]. The loss of the γδ T cell response is likely to be due to activation-induced exhaustion and cell death, and this in turn poses the question of whether IL-2, which has pro-apoptotic effects, is the right cytokine to achieve γδ T cells *in vivo*. Accordingly, IL-15 in contrast to IL-2, promotes γδ T cell activation in the absence of apoptosis [102]. In addition, IL-15 sustains those γδ T cells with cytotoxic activity [78] and induces the differentiation of antigen-presenting capacity γδ T cells [102] that activate classical CD8 cytotoxic T lymphocytes. Therefore, it would be desirable in future trials of γδ T cell therapy to compare the effects of IL-2 and IL-15.

Third, peripheral blood neutrophils internalize N-BPs and produce hydrogen peroxide that inhibits γδ T cell activation [103].

Fourth, and concerning the adoptive transfer therapy, a major issue is how to sustain *ex vivo*-expanded γδ T cells, because results of few studies have been inconsistent so far [104-107].

Fifth, despite effective in activating γδ T cells in the peripheral blood *in vivo*, we have no evidence until now that the administration of PAgs or N-BPs and IL-2 also promotes activation of γδ T cells in the tumor microenvironment. Similarly, we lack robust evidence that adoptively-transferred γδ T cells (that are usually injected intravenously) are capable to localize to the tumor site.

To be successful, γδ T cell-based cancer immunotherapies certainly will require updated protocols and strategies. Thus γδ T cells can be redirected to the cancer cell using bispecific antibodies or other immunoligands, in which one site binds to a tumor-specific antigen and the other binds to CD3 or the γδ TCR; such bispecific antibodies have shown efficacy in preclinical mouse models[108]. Another approach is a chimeric molecule in which the variable regions of a γδ TCR is linked to the Fc fragment of human IgG1[109]. This chimeric construct binds to several ovarian cancer cellsand promotes the killing of the cells *via* ADCC mediated by binding of the Fc region of the chimeric construct to CD16. Moreover, it is also possible to engage γδ T cells independently on the γδ TCR: for instance chimeric immunoligands composed by a tumor antigen-specific single-chain variable fragment (scFv) joined to MICA or ULBP2[110] bind to NKG2D consitutively expressed by γδ T cells (and other cells expressing NKG2D). Similarly, a bispecific antibody recognizing HER2, a tumor antigen, and CD16 redirects γδ T cells (and other cells) which express CD16, to the tumor cell and enhance their cytotoxic activity[111].

An alternative strategy consists of the utilization of γδ-based CARs such as γδ T cells transduced with a CD19-specific CAR which confers to the γδ T cells the capability to kill CD19 leukemia cells[112].Other approaches consist in transducing peripheral CD3 lymphocytes (including both both γδ and αβ) with a high-affinity γδ TCR[113-115]or introduce a tumor-specific αβ TCR into γδ T cells[116-118].

Some of the above approaches have already entered clinical trials typically in patients with hematologic malignancies and may represent attractive strategies to promote tumor cytotoxicity of γδ T cells.

9. CONCLUSION

Despite some recent study has documented elevated percentages of γδ T lymphocytes infiltrating different types of cancer, more recent evidence indicate that γδ are detected in most human cancer types but at an average of ~ 4% of TILs.
Moreover, the nature of this association and the underlying mechanisms remain unknown to date, and whether or not the presence of tumor-infiltrating γδ T lymphocytes is a definite prognostic factor similarly remains controversial.

The reciprocal interaction between tumor cells and the tumor microenvironment is a crucial topic in cancer research and therapy, as recently demonstrated by the finding that targeting stromal factors could improve efficacies of chemotherapy and prevent metastasis.
Our studies on tumor-infiltrating γδ T lymphocytes from well controlled cohorts of patients with different types of solid tumors clearly show that both γδ abundance and *IFNG* gene expression positively associate with better patient outcome in three different cancers , namely CRC, NMSC and melanoma. Moreover in CRC samples, this association appears significantly influenced by the tumor microenvironment, through yet unidentified suppressive molecules released from tumor cells and capable to inhibit γδ T cell proliferation and IFN-γ production.

γδ T cell-based immunotherapy represents an emerging therapeutic option for patients afflicted with different cancers, despite so far disappointng results in different small-scale clinical trials. It is possible that the tumor microenvironment contributes to limit their efficacy. Therefore, the identification of immunosuppressive factors in the tumor microenvironment is of crucial importance when designing γδ T cell-based therapy, either alone or in combination with other immunotherapies.

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CONFLICT OF INTEREST

All authors declare no conflict of interest with this review.

REFERENCES

1. Hayday AC. γδ T cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol.*2000; **18**: 975-1026.doi: 10.1146/annurev.immunol.18.1.975.

2. Fournié JJ, Bonneville M.Stimulation of γδ T cells by phosphoantigens.*Res Immunol*. 1996;**147**:338-347.doi:10.1016/0923-2494(96)89648-9.

3.Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H. Microbial isoprenoid biosynthesis and human γδ T cell activation. *FEBS Lett*. 2003; **544**: 4-10.doi: 10.1016/s0014-5793(03)00483-6.

4. Harly C, Guillaume Y, Nedellec S, et al. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood*. 2012; **120**: 2269-2279.doi: 10.1182/blood-2012-05-430470.

5. Vavassori S, Kumar A, Wan GS, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human γδ T cells. *Nat Immunol*. 2013; **14**: 908-916.doi: 10.1038/ni.2665.

6. Sandstrom A, Peigné CM, Léger A, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells. *Immunity*. 2014; **40**: 490-500.doi: 10.1016/j.immuni.2014.03.003.

7. Karunakaran MM, Willcox CR, Salim M, et al. Butyrophilin-2A1 directly binds germline-encoded regions of the Vγ9Vδ2 TCR and is essential for phosphoantigen sensing. *Immunity*. 2020;**52**: 487-498.e6. doi: 10.1016/j.immuni.2020.02.014.

8. Rigau M, Ostrouska S, Fulford TS, et al. Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. *Science*. 2020;**367**: pii: eaay5516. doi: 10.1126/science.aay5516.

9. Ravens S, Schultze-Florey C, Raha S, et al. Human γδ T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. *Nat Immunol.* 2017; **18**: 393-401.doi: 10.1038/ni.3686.

10. Davey MS, Willcox CR, Joyce SP, et al. Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance. *Nat. Commun.*2017; **8**: 14760.doi: 10.1038/ncomms14760.

11. Davey MS, Willcox CR, Hunter S, et al. The human Vδ2^{+} T-cell compartment comprises distinct innate-like Vγ9^{+} and adaptive Vγ9^{-} subsets. *Nat Commun.*2018; **9**:1760.doi: 10.1038/s41467-018-04076-0.

12. Xu B, Pizarro JC, Holmes MA, et al. Crystal structure of a γδ T cell receptor specific for the human MHC class I homolog MICA. *Proc Natl Acad Sci USA*. 2011; **108**: 2414-2419.doi: 10.1073/pnas.1015433108.

13. 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 MICA and MICB.*Proc Natl Acad Sci USA.* 1999; **96**: 6879-6884. doi: 10.1073/pnas.96.12.6879.

14. Uldrich AP, Le Nours J, Pellicci DG, et al. CD1d lipid antigen recognition by the γδ TCR.*Nat Immunol.* 2013; **14**: 1137-1145. doi: 10.1038/ni.2713.

15. Luoma AM, Castro CD, Mayassi T, et al. Crystal structure of Vδ1 T cell receptor in complex with CD1d sulfatide shows MHC like recognition of a self-lipid by human γδ T cells. *Immunity*. 2013; **39**: 1032-1042.doi: 10.1016/j.immuni.2013.11.001.

16. Mangan BA, Dunne MR, O'Reilly VP, et al. CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vδ3 T cells. *J Immunol*. 2013; **191**: 30-34. doi: 10.4049/jimmunol.1300121.

17. Marlin R, Pappalardo A, Kaminski H, ei al. Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2. *Proc Natl Acad Sci USA*. 2017; **114**: 3163-3168.doi: 10.1073/pnas.1621052114.

18. Dieli F, Poccia F, Lipp M, et al. Differentiation of effector/memory Vδ2 T cells and migratory routes in lymph nodes or inflammatory sites.*J Exp Med*. 2003.**198**: 391-397. doi: 10.1084/jem.20030235.

19. Caccamo N, Todaro M, Sireci G, Meraviglia S, Stassi G, Dieli F.. Mechanisms underlying lineage commitment and plasticity of human γδ T cells.*Cell Mol Immunol*. 2013; **10**: 30-34.doi: 10.1038/cmi.2012.42.

20. Sireci G, Champagne E, Fournié JJ, et al. Patterns of phosphoantigen stimulation of human Vγ9Vδ2 T cell clones include Th0 cytokines. *Hum Immunol*. 1997; **58**: 70-82.doi: 10.1016/s0198-8859(97)00211-5.

21. Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood γδ T cells toward Th1- or Th2-phenotype. *Cell Immunol*. 2001; **212**: 110-117.doi: 10.1006/cimm.2001.1850.

22. Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vγ9Vδ2 T cells. *J Immunol*. 2010; **184**: 7268-7280. doi: 10.4049/jimmunol.1000600.

23. Caccamo N, La Mendola C, Orlando V, et al. Differentiation, phenotype and function of interleukin-17-producing human Vγ9Vδ2 T cells. *Blood*. 2011;**118**: 129138. doi: 10.1182/blood-2011-01-331298.

24. Fenoglio D, Poggi A, Catellani S, et al. Vδ1 T lymphocytes producing IFN-γ and IL-17 are expanded in HIV-1-infected patients and respond to *Candida albicans*. *Blood*. 2009; **113**: 6611-6618.doi: 10.1182/blood-2009-01-198028.

25. Peters C, Häsler R, Wesch D, Kabelitz D. Human Vδ2 T cells are a major source of interleukin-9. *Proc Natl Acad Sci USA*. 2016; **113**: 12520-12525.doi: 10.1073/pnas.1607136113.

26. Caccamo N, Battistini L, Bonneville M, et al. CXCR5 identifies a subset of Vγ9Vδ2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J Immunol.*2006; **177**: 5290-5295.doi: 10.4049/jimmunol.177.8.5290.

27. Bansal RR, Mackay CR, Moser B, Eberl M. IL-21 enhances the potential of human γδ T cells to provide B-cell help. *Eur J Immunol.* 2012; **42**: 110-119. doi: 10.1002/eji.201142017.

28. Caccamo N, Todaro M, La Manna MP, Sireci G, Stassi G, Dieli F. IL-21 regulates the differentiation of a human γδ T cell subset equipped with B cell helper activity. *PLoS One*. 2012; **7**: e41940. doi: 10.1371/journal.pone.0041940.

29. Peters C, Oberg HH, Kabelitz D, Wesch D. Phenotype and regulation of immunosuppressive Vδ2-expressing γδT cells. *Cell Mol Life Sci*. 2014; **71**: 1943-1960. doi: 10.1007/s00018-013-1467-1.

30. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by γδ T cells.*Science*. 2001; **294**: 605-609. doi: 10.1126/science.1063916.

31. Lo Presti E, Pizzolato G, Gulotta E, et al. Current advances in γδ T cell-based tumor immunotherapy. *Front Immunol.*2017; **8**: 1401.doi: 10.3389/fimmu.2017.01401.

32. Das H, Groh V, Kuijl C, et al. MICA engagement by human Vγ9Vδ2 T cells enhances their antigen dependent effector function. *Immunity*. 2001; **15**: 83-93.doi: 10.1016/s1074-7613(01)00168-6

33. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T.. Activation of Vγ9Vδ2 T cells by NKG2D. *J Immunol.* 2005; **175**: 2144-2151. doi: 10.4049/jimmunol.175.4.2144.

34. Lanca T, Correia DV, Moita CF, et al. The MHC class Ib protein ULBP1 is a non redundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity. *Blood.*2010; **115**: 2407-2411.doi: 10.1182/blood-2009-08-237123.

35. Hudspeth K, Silva-Santos B, Mavilio D. Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. *Front Immunol*. 2013; **4**: 69.doi: 10.3389/fimmu.2013.00069.

36. Dieli F, Troye-Blomberg M, Ivanyi J, et al. Granulysin dependent killing of intracellular and extracellular *Mycobacterium tuberculosis* by Vγ9Vδ2 T lymphocytes. *J Infect Dis*. 2001; **184**: 1082-1085. doi: 10.1086/323600.

37. Dalton JE, Howell G, Pearson J, Scott P, Carding SR. Fas-Fas ligand interactions are essential for the binding to and killing of activated macrophages by γδ T cells. *J Immunol*. 2004. **173**: 3660-3667. doi: 10.4049/jimmunol.173.6.3660.

38. Chen HC, Dieli F, Eberl M. An unconventional TRAIL to cancer therapy.*Eur J Immunol*.2013; **43**: 3159-3162.doi: 10.1002/eji.201344105.

39. Todaro M, D’Asaro M, Caccamo N, et al. Efficient killing of human colon cancer stem cells by γδ T lymphocytes.*J Immunol*. 2009; **182**: 7287-7296. doi: 10.4049/jimmunol.0804288.

40. Todaro M, Orlando V, Cicero G, et al. Chemotherapy sensitizes colon cancer initiating cells to Vγ9Vδ2 T cell-mediated cytotoxicity. *PLoS One*. 2013; **8**: e65145. doi: 10.1371/journal.pone.0065145.

41. Angelini DF, Borsellino G, Poupot M, et al. FcγRIII discriminates between 2 subsets of Vγ9Vδ2 effector cells with different responses and activation pathways. *Blood*. 2004; **104**: 1801-1807.doi: 10.1182/blood-2004-01-0331.

42. Maniar A, Zhang X, Lin W, et al. Human γδ T lymphocytes induce robust NK cell-mediated anti tumor cytotoxicity through CD137 engagement. *Blood*. 2010; **116**: 1726-1733.doi: 10.1182/blood-2009-07-234211.

43. Conti L, Casetti R, Cardone M, et al. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated γδ T cells: role of CD86 and inflammatory cytokines. *J Immunol*. 2005; **174**: 252-260.doi: 10.4049/jimmunol.174.1.252.

44. Devilder MC, Maillet S, Bouyge-Moreau I, Donnadieu E, Bonneville M, Scotet E. Potentiation of antigen-stimulated Vγ9Vδ2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. *J Immunol*. 2006; **176**: 1386-1393.doi: 10.4049/jimmunol.176.3.1386.

45. Brandes M, Willimann K, Moser B. Professional antigen-presentation function by human γδ T cells. *Science*. 2005; **309**: 264-268.doi: 10.1126/science.1110267.

46. Himoudi N, Morgenstern DA, Yan M, et al. Human γδ T lymphocytes are licensed for professional antigen presentation by interaction with opsonized target cells. *J Immunol*. 2012; **188**: 1708-1716.doi: 10.4049/jimmunol.1102654.

47. Altvater B, Pscherer S, Landmeier S, et al. Activated human γδ T cells induce peptide-specific CD8$^+$ T-cell responses to tumor-associated self-antigens. *Cancer Immunol Immunother*. 2012; **61**: 385-396.doi: 10.1007/s00262-011-1111-6.

48. Lo Presti E, Dieli F, Meraviglia S. Tumor-infiltrating γδ T lymphocytes: pathogenicrole, clinical significance, and differential programing in the tumor microenvironment. *Front Immunol*. 2014; **5**: 607.doi: 10.3389/fimmu.2014.00607.

49. Silva-Santos B, Serre K, Norell H. γδT cells in cancer.*Nat Rev Immunol*. 2015; **15**: 683-691.doi: 10.1038/nri3904.

50. Jungbluth AA, Frosina D, Fayad M, et al. Immunohistochemical detection of γ/δ T lymphocytes in formalin-fixed paraffin-embedded tissues. *Appl Immunohistochem Mol Morphol*.2019 ;**27**: 581-583. doi: 10.1097/PAI.0000000000000650.

51. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods*. 2015; **12**: 453-457.doi: 10.1038/nmeth.3337.

52. Gentles AJ, Newman AM, Liu CL, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers.*Nat Med*. 2015; **21**: 938-945. doi: 10.1038/nm.3909.

53. Tosolini M, Pont F, Poupot M, et al. Assessment of tumor-infiltrating TCR Vγ9Vδ2 γδ lymphocyte abundance by deconvolution of human cancers microarrays. *Oncoimmunology.* 2017;**6**: e1284723. doi: 10.1080/2162402X.2017.1284723.

54. Pont F, Familiades J, Déjean S, et al. The gene expression profile of phosphoantigen-specific human γδ T lymphocytesis a blend of αβ T-cell and NK-cell signatures.*Eur J Immunol*.2012; **42**: 228-240.doi: 10.1002/eji.201141870.

55. Pizzolato G, Kaminski H, Tosolini M, et al. Single-cell RNA sequencing unveils the shared and the distinct cytotoxic hallmarks of human TCRVδ1 and TCRVδ2 γδT lymphocytes. *Proc Natl Acad Sci USA*. 2019; **116**: 11906-11915.doi: 10.1073/pnas.1818488116.

56. Danese S, Fiocchi C. Ulcerative colitis. *N Engl J Med*. 2011; **365**:1713-1725. doi: 10.1056/NEJMra1102942.

57. Cunningham, D. Atkin W, Lenz HJ, et al. Colorectal cancer. *Lancet*. 2010; **375**: 1030-1047.doi: 10.1016/S0140-6736(10)60353-4.

58. McCarthy NE, Hedin CR, Sanders TJ, et al. Azathioprine therapy selectively ablates human Vγδ2$^+$ T cells in Crohn's disease. *J Clin Invest*. 2015; **125**: 3215-3225.doi: 10.1172/JCI80840.

59. Andreu-Ballester JC, Amigó-García V, Catalán-Serra I, et al. Deficit of γδ T lymphocytes in the peripheral blood of patients with Crohn's disease.*Dig Dis Sci*. 2011; **56**: 2613-2622. doi: 10.1007/s10620-011-1636-8.

60. Do JS, Kim S, Keslar K, et al. γδ T cells coexpressing gut homing α4β7 and αE integrins define a novel subset promoting intestinal inflammation. *J Immunol*. 2017; **198**: 908-915.doi: 10.4049/jimmunol.1601060.

61. Wu P, Wu D, Ni C, et al. γδT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity*. 2014; **40**: 785-800.doi: 10.1016/j.immuni.2014.03.013.

62. Mielke LA, Jones SA, Raverdeau M, et al. Retinoic acid expression associates with enhanced IL-22 production by γδ T cells and innate lymphoid cells and attenuation of intestinal inflammation. *J Exp Med*. 2013; **210**: 1117-1124. doi: 10.1084/jem.20121588.

63. McCarthy NE, Eberl M. Human γδ T-cell control of mucosal immunity and inflammation. *Front Immunol*. 2018; **9**: 985.doi: 10.3389/fimmu.2018.00985.

64. Meraviglia S, Lo Presti E, Tosolini M, et al. Distinctive features of tumor-infiltrating γδ T lymphocytes in human colorectal cancer. *Oncoimmunology*. 2017;**6**: e1347742. doi: 10.1080/2162402X.2017.1347742.

65. Lo Presti E, Di Mitri R, Mocciaro F, et al. Characterization of γδ T cells in intestinal mucosa from patients with early-onset or long-standing inflammatory bowel disease and their correlation with clinical status. *J Crohns Colitis*. 2019; **13**: 873-883.doi: 10.1093/ecco-jcc/jjz015.

66. Lo Presti E, Mocciaro F, Di Mitri R, et al. Analysis of colon-infiltrating γδ T cells in chronic inflammatory bowel disease and in colitis-associated cancer. *J Leukoc Biol*. 2020.doi: 10.1002/JLB.5MA0320-201RR. [Epub ahead of print].

67. Dunne PJ, Maher CO, Freeley M, et al. CD3ε expression defines functionally distinct subsets of Vδ1 T cells in patients with human immunodeficiency virus infection. *Front Immunol*. 2018; **9**: 940.doi: 10.3389/fimmu.2018.00940.

68. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer.*Gastroenterology*. 2010; **138**: 2044-2058.doi: 10.1053/j.gastro.2010.01.054.

69.Glasner A, Ghadially H, Gur C, et al. Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. *J Immunol*. 2012; **188**: 2509-2515.doi: 10.4049/jimmunol.1102461.

70. Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? *Nat Immunol*. 2002; **3**: 6-8.doi: 10.1038/ni0102-6.

71. Lakshmikanth T, Burke S, Ali TH, et al. NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines *in vitro* and *in vivo*. *J Clin Invest*. 2009; **119**: 1251-1263.doi: 10.1172/JCI36022.

72. Halfteck GG, Elboim M, Gur C, Achdout H, Ghadially H, Mandelboim O. Enhanced *in vivo* growth of lymphoma tumors in the absence of the NK-activating receptor NKp46/NCR1*. J Immunol*. 2009; **182**: 2221-2230.doi: 10.4049/jimmunol.0801878.

73.Meresse B, Curran SA, Ciszewski C, et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J Exp Med*. 2006; **203**: 1343-1355. doi: 10.1084/jem.20060028.

74. Meresse B, Chen Z, Ciszewski C, et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity*. 2004; **21**: 357-366.doi: 10.1016/j.immuni.2004.06.020.

75. Mikulak J, Oriolo F, Bruni E, et al. NKp46-expressing human gut-resident intraepithelial Vδ1 T cell subpopulation exhibits high antitumor activity against colorectal cancer. *JCI Insight*. 2019; **4**.pii: 125884. doi: 10.1172/jci.insight.125884.

76. Qiu Y, Wang W, Xiao W, Yang H. Role of the intestinal cytokine microenvironment in shaping the intraepithelial lymphocyte repertoire. *J Leukoc Biol.* 2015; **97**: 849-857. doi: 10.1189/jlb.3RU1014-465R.

77. Chu CL, Chen SS, Wu TS, Kuo SC, Liao NS. Differential effects of IL-2 and IL-15 on the death and survival of activated TCR $\gamma\delta^+$ intestinal intraepithelial lymphocytes. *J Immunol*. 1999; **162**: 1896-1903.

78. Caccamo N, Meraviglia S, Ferlazzo V, et al. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets. *Eur J Immunol*. 2005; **35**:1764-1772. doi: 10.1002/eji.200525983.

79. Kabelitz D, Wesch D. Features and functions of γδT lymphocytes: focus on chemokines and their receptors. *Crit Rev Immunol.* 2003; **23**: 339-370. doi: 10.1615/critrevimmunol.v23.i56.10.

80. Perera L, Shao L, Patel A, et al. Expression of nonclassical class I molecules by intestinal epithelial cells. *Inflamm Bowel Dis.* 2007; **13**: 298-307. doi: 10.1002/ibd.20026.

81. Lo Presti E, Toia F, Oieni S, et al. Squamous cell tumors recruit γδ T cells producing either IL17 or IFN-γ depending on the tumor stage. *Cancer Immunol Res*. 2017; **5**: 397-407. doi: 10.1158/2326-6066.CIR-16-0348.

82. Patil RS, Shah SU, Shrikhande SV, et al. IL17 producing γδ T cells induce angiogenesis and are associated with poor survival in gallbladder cancer patients. *Int J Cancer*.2016; **139**: 869-881.doi: 10.1002/ijc.30134.

83. Toia F, Buccheri S, Anfosso A, et al. Skewed differentiation of circulating Vγ9Vδ2 T lymphocytes in melanoma and impact on clinical outcome. *PLoS One*. 2016; **11**: e0149570. doi: 10.1371/journal.pone.0149570.

84. Glasner A, Levi A, Enk J, et al. NKp46 receptor-mediated IFN-γproduction by natural killer cells increases fibronectin 1 to alter tumor architecture and control metastasis. *Immunity*. 2018; **48**: 107-119. doi: 10.1016/j.immuni.2017.12.007.

85. Ma C, Zhang Q, Ye J, et al. Tumor-infiltrating γδ T lymphocytes predict clinical outcome in human breast cancer. *J Immunol*. 2012; **189**: 5029-5036.doi: 10.4049/jimmunol.1201892.

86. Daley D, Zambirinis CP, Seifert L, et al. γδT cells support pancreatic oncogenesis by restraining αβ T cell activation. *Cell*. 2016; **166**:1485-1499.doi: 10.1016/j.cell.2016.07.046.

87. Hoeres T, Holzmann E, Smetak M, Birkmann J, Wilhelm M. PD-1 signaling modulates interferon-γ production by gamma delta (γδ) T-cells in response to leukemia. *Oncoimmunology*.2019;**8**: 1550618. doi: 10.1080/2162402X.2018.1550618.

88. Iwasaki M, Tanaka Y, Kobayashi H, et al. Expression and function of PD-1 in human γδ T cells that recognize phosphoantigens. *Eur J Immunol*.2011; **41**: 345-355.doi: 10.1002/eji.201040959.

89. Hsu H, Boudova S, Mvula G, et al. Prolonged PD1 expression on neonatal Vδ2 lymphocytes dampens proinflammatory responses: role of epigenetic regulation. *J Immunol*. 2016; **197**: 1884-1892.doi: 10.4049/jimmunol.1600284.

90. Lo Presti E, Pizzolato G, Corsale AM, et al. γδ T cells and tumor microenvironment: from immunosurveillance to tumor evasion. *Front Immunol*. 2018; **9**:1395. doi: 10.3389/fimmu.2018.01395.

91. Liu Q, Li A, Tian Y, et al. The CXCL8-CXCR1/2 pathways in cancer. *Cytokine Growth Factor Rev.* 2016; **31**: 61-71. doi: 10.1016/j.cytogfr.2016.08.002.

92. Voron T, Marcheteau E, Pernot S, et al. Control of the immune response by pro-angiogenic factors. *Front Oncol.* 2014; **4**: 70; doi: 10.3389/fonc.2014.00070.

93. Basingab FS, Ahmadi M, Morgan DJ. IFNγ-dependent interactions between ICAM-1 and LFA-1 counteract prostaglandin E2-mediated Inhibition of antitumor CTL responses. *Cancer Immunol Res.* 2016; **4**: 400-411. doi: 10.1158/2326-6066.CIR-15-0146.

94. Zhai L, Spranger S, Binder DC, et al. Molecular pathways: targeting IDO1 and other tryptophan dioxygenases for cancer immunotherapy. *Clin Cancer Res.* 2015; **21**: 5427-5433. doi: 10.1158/1078-0432.CCR-15-0420.

95. Eil R, Vodnala SK, Clever D, et al. Ionic immune suppression within the tumour micro-environment limits T cell effector function. *Nature.* 2016; **537**: 539-543. doi: 10.1038/nature19364.

96. Tauriello DVF, Batlle E. Targeting the microenvironment in advanced colorectal cancer. *Trends Cancer*. 2016; **2**: 495-504. doi: 10.1016/j.trecan.2016.08.001.

97. Hayday AC. γδ T cells and the lymphoid stress-surveillance response. *Immunity*. 2009;**31**:184-196. doi: 10.1016/j.immuni.2009.08.006.

98. Wilhelm M, Kunzmann V, Eckstein S, et al. γδ T cells for immune therapy of patients with lymphoid malignancies.doi: 10.1182/blood-2002-12-3665.

99. Dieli F, Gebbia N, Poccia F, et al. Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients *in vivo*. *Blood*. 2003; **102**: 2310-2311.doi:10.1182/blood-2003-05-1655.

100. Buccheri S, Guggino G, Caccamo N, Li Donni P, Dieli F. Efficacy and safety of γδ T cell-based tumor immunotherapy: a meta-analysis. *J Biol Regul Homeost Agents*.2014; **28**: 81-90.

101. Oberg HH, Kellner C, Peipp M, et al. Monitoring circulating γδ T cells in cancer patients to optimize γδ T cell-based immunotherapy. *Front Immunol*. 2014;**5**: e643.doi: 10.3389/fimmu.2014.00643.

102. Van Acker HH, Campillo-Davo D, Roex G, Versteven M, Smits EL, Van Tendeloo VF..The role of the common gamma-chain family cytokines in γδT cell-based anti-cancer immunotherapy.*Cytokine Growth Factor Rev*. 2018;**41**:54-64.doi: 10.1016/j.cytogfr.2018.05.002.

102. Tyler CJ, McCarthy NE, Lindsay JO,Stagg AJ, Moser B, Eberl M.. Antigen-presenting human γδT cells promote intestinal CD4$^+$T cell expression of IL-22 and mucosal release of calprotectin.*J Immunol*. 2017;**198**:3417-3425.doi:10.4049/jimmunol.1700003.

103. Kalyan S, Chandrasekaran V, Quabius ES, Lindhorst TK, Kabelitz D.. Neutrophil uptake of nitrogen-bisphosphonates leads to the suppression of human peripheral blood γδ T cells. *Cell Mol Life Sci*. 2014; **71**: 2335-2346. doi:10.1007/s00018-013-1495-x.

104. Kobayashi H, Tanaka Y, Yagi J, Minato N, Tanabe K.. Phase I/II study of adoptive transfer of γδ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma. *Cancer Immunol Immunother*. 2011; **60**: 1075-1084.doi: 10.1007/s00262-011-1021-7.

105. Wada I, Matsushita H, Noji S, et al. Intraperitoneal injection of *in vitro* expanded Vγ9Vδ2 T cells together with zoledronate for the treatment of malignant ascites due to gastric cancer. *Cancer Med*. 2014; **3**: 362-375.doi:10.1038/ng.2984.

106. Wilhelm M, Smetak M, Schaefer-Eckart K, et al. Successful adoptive transfer and *in vivo* expansion of haploidenticalγδ T cells. *J Transl Med*. 2014; **12**: 45.doi:10.1186/1479-5876-12-45.

107. Nussbaumer O, Gruenbacher G, Gander H, Essential requirements of zoledronate-induced cytokine and T cell proliferative responses. *J Immunol*. 2013; **191**: 1346-1355.doi: 10.4049/jimmunol.1300603.

108. Oberg HH, Kellner C, Gonnermann D, et al. γδ T cell activation by bispecific antibodies. *Cell Immunol*. 2015; **296**: 41-49.doi: 10.1016/j.cellimm.2015.04.009.

109. Zheng J, Guo Y, Ji X, Cui L, He W.A novel antibody-like TCR γδ-Ig fusion protein exhibits antitumor activity against human ovarian carcinoma. *Cancer Lett*. 2013; **341**: 150-158.doi:10.1016/j.canlet.2013.07.036.

110. Peipp M, Wesch D, Oberg HH, et al. CD20-specific immunoligands engaging NKG2D enhance γδ T cell-mediated lysis of lymphoma cells. *Scand J Immunol*.2017; **86**: 196-206.196-206. doi: 10.1111/sji.12581.

111. Oberg HH, Kellner C, Gonnermann D, et al. Tribody [(HER2)2xCD16] is more effective than trastuzumab in enhancing γδ t cell and natural killer cell cytotoxicity against her2-expressing cancer cells.*Front Immunol*. 2018;**9**: 814. doi:10.3389/fimmu.2018.00814.

112. Deniger DC, Switzer K, Mi T, et al. Bispecific T-cells expressing polyclonal repertoire of endogenous γδ T-cell receptors and introduced CD19-specific chimeric antigen receptor. *Mol Ther*. 2013; **21**: 638-647.doi: 10.1038/mt.2012.267.

113. Marcu-Malina V, Heijhuurs S, van Buuren M, et al. Redirecting αβ T cells against cancer cells by transfer of a broadly tumor-reactive γδ T-cell receptor. *Blood*. 2011; **118**: 50-59.doi: 10.1182/blood-2010-12-325993.

114. Zhao H, Xi X, Cui L, He W. CDR3δ-grafted γ9δ2 T cells mediate effective antitumor reactivity. *Cell Mol Immunol*. 2012; **9**: 147-154.doi:10.1038/cmi.2011.28.

115. Straetemans, T. et al. GMP-grade manufacturing of T cells engineered to express a defined γδTCR. *Front Immunol*. 2018; **9**: 1062.doi:10.3389/fimmu.2018.01062.

116. Van der Veken LT, Hagedoorn RS, van Loenen MM, et al. αβ T-cell receptor engineered γδ T cells mediate effective anti-leukemic reactivity. *Cancer Res*. 2006; **66**: 3331-3337.doi:10.1158/0008-5472.CAN-05-4190.

117. Van der Veken LT, DiezCampelo M, van der Hoorn MA, Falkenburg JH, Schumacher TN, Heemskerk MH..αβ T cell receptor transfer to γδ T cells generates functional effector cells without mixed TCR dimers *in vivo*. *J Immunol*. 2009; **182**: 164-170.doi: 10.4049/jimmunol.182.1.164.

118. Hiasa A, Nishikawa H, Hirayama M, et al. Rapid αβ TCR-mediated responses in γδ T cells transduced with cancer-specific TCR genes. *Gene Ther*. 2009; **16**: 620-628.doi: 10.1038/gt.2009.6.

LEGEND TO FIGURES

FIGURE 1.

The γδ T lymphocytes in t-SNE map of a scRNAseq dataset from human PBMC. Cell are clustered according to transcriptomic similarity, constituting clouds of cell from the same lineage and subset. PBMC datasets typically comprise a group of moreless continuous T cell clusters neighboured by an NK cell cluster, a myeloid cluster encompassing monocytes and DC s, as well as a separate but compact cluster of B lymphocytes. Minor clusters of plasma cells, plasmacytoid DC, and scarce megakaryocytes or erythroid precursors are eventually observed in larger datasets. In t-SNE maps from such samples, most γδ T lymphocytes are embedded between TCD4, TCD8, and NK cells, although with slight differences between cells of the TCRVδ1 and TCRVδ2 subset from CMV$^+$ individuals. For visual clarity, the γδ T cells are shown with larger symbols.

FIGURE 2.

The anti- and pro-tumor activities of γδ T lymphocytes. The figure shows several different studies on the anti- or pro-tumor activities of human γδ T lymphocytes in different types of cancer. Numbers inside each circle indicates refer to the to the reference number, while the size of the circle indicates the number of citation each study has received: for an estimation, see the lower box on the right hand side of the figure.

FIGURE 3.

γδ T lymphocytes in the progression from IBD to CRC. Vδ1 T cells are the major γδ T cell population in normal gut and a large fraction of intraepithelial Vδ1 T cells express NKp46. They express IFN-γ and TNF-α and are equipped with cytolytic and anti-tumor activity.

Chronic IBD patients have a reduced frequency of gut-resident Vδ1 T cells which still maintain their effector functions, but in the gut of CRC patients Vδ1 T cells are almost completely depleted (including the NKp46+ population) and those few remaining Vδ1 T cells have lost the capacity to produce IFN-γ and TNF-α.

Gut Vδ2 T cells from healthy donors are less abundant than Vδ1 T cells, but similarly produce IFN-γ and TNF-α. Conversely, but Vδ2 T cells from the gut ofchronic IBD patients show increased production of TNF-α and also produce IL-17. Finally, and similarly to Vδ1 T cells, gut Vδ2 T cells from CRC patients are massively depleted and have very limited cytokine-production capability.

The figure also illustrates the hypothesis that yet unknown soluble factors produced ny several cells in the tumor microenvironment (including CAF and CSC) profoundly inhibit proliferation and cytokine production by tumor-infiltrating γδ T cells.