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To cite this article: Marie Tosolini, Frédéric Pont, Mary Poupot, François Vergez, Marie-Laure Nicolau-Travers, David Vermijlen, Jean- Emmanuel Sarry, Francesco Dieli & Jean-Jacques Fournié (2017) Assessment of tumor-infiltrating TCRV γ 9V δ 2 $\gamma\delta$ lymphocyte abundance by deconvolution of human cancers microarrays, *Oncoimmunology*, 6:3, e1284723, DOI: 10.1080/2162402X.2017.1284723

To link to this article: <http://dx.doi.org/10.1080/2162402X.2017.1284723>

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Accepted author version posted online: 06 Feb 2017.
Published online: 06 Feb 2017.

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ORIGINAL RESEARCH

Assessment of tumor-infiltrating TCRV γ 9V δ 2 $\gamma\delta$ lymphocyte abundance by deconvolution of human cancers microarrays

Marie Tosolini ^{a,b,c,d,e,f}, Frédéric Pont^{a,b,e}, Mary Poupot^{a,b,c,d}, François Vergez^{a,b,f}, Marie-Laure Nicolau-Travers^f, David Vermijlen^g, Jean-Emmanuel Sarry^{a,b,d}, Francesco Dieli^h, and Jean-Jacques Fournié ^{a,b,c,d}

^aCentre de Recherches en Cancérologie de Toulouse (CRCT), Toulouse, France; ^bINSERM U1037-Université Paul Sabatier-CNRS ERL5294, Université de Toulouse, Toulouse, France; ^cLaboratoire d'Excellence TOUCAN, Toulouse, France; ^dProgramme Hospitalo-Universitaire en Cancérologie CAPTOR, Toulouse, France; ^ePôle Technologique du Centre de Recherches en Cancérologie de Toulouse (CRCT), Toulouse, France; ^fInstitut Universitaire du Cancer de Toulouse (IUCT), Toulouse, France; ^gCentral Laboratory for Advanced Diagnostics and Biomedical Research (CLADIBIOR), University of Palermo, Palermo, Italy; ^hDepartment of Biopharmacy - Institute for Medical Immunology (IMI), Université Libre de Bruxelles, Bruxelles, Belgium

ABSTRACT

Most human blood $\gamma\delta$ cells are cytolytic TCRV γ 9V δ 2⁺ lymphocytes with antitumor activity. They are currently investigated in several clinical trials of cancer immunotherapy but so far, their tumor infiltration has not been systematically explored across human cancers. Novel algorithms allowing the deconvolution of bulk tumor transcriptomes to find the relative proportions of infiltrating leucocytes, such as CIBERSORT, should be appropriate for this aim but in practice they fail to accurately recognize $\gamma\delta$ T lymphocytes. Here, by implementing machine learning from microarray data, we first improved the computational identification of blood-derived TCRV γ 9V δ 2⁺ $\gamma\delta$ lymphocytes and then applied this strategy to assess their abundance as tumor infiltrating lymphocytes ($\gamma\delta$ TIL) in ~10,000 cancer biopsies from 50 types of hematological and solid malignancies. We observed considerable inter-individual variation of TCRV γ 9V δ 2⁺ $\gamma\delta$ TIL abundance both within each type and across the spectrum of cancers tested. We report their prominence in B cell-acute lymphoblastic leukemia (B-ALL), acute promyelocytic leukemia (M3-AML) and chronic myeloid leukemia (CML) as well as in inflammatory breast, prostate, esophagus, pancreas and lung carcinoma. Across all cancers, the abundance of $\alpha\beta$ TILs and TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs did not correlate. $\alpha\beta$ TIL abundance paralleled the mutational load of tumors and positively correlated with inflammation, infiltration of monocytes, macrophages and dendritic cells (DC), antigen processing and presentation, and cytolytic activity, in line with an association with a favorable outcome. In contrast, the abundance of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs did not correlate with these hallmarks and was variably associated with outcome, suggesting that distinct contexts underlie TCRV γ 9V δ 2⁺ $\gamma\delta$ TIL and $\alpha\beta$ TIL mobilizations in cancer.

ARTICLE HISTORY

Received 22 December 2016
Revised 12 January 2017
Accepted 13 January 2017

KEYWORDS

Artificial intelligence; cancer; data mining; deconvolution; gamma delta lymphocyte; machine learning; microarray; transcriptome

Introduction

In healthy human adults, circulating T lymphocytes comprise ~1–3% of $\gamma\delta$ T cells, most of which express TCRV γ 9V δ 2, and mediate Th1 and cytolytic activities in response to stimulation by soluble phosphoantigens (PAg) or cancer cells.^{1–3} Hence, this subset of T lymphocytes represent promising effectors for cancer immunotherapy, but the few $\gamma\delta$ cell-based trials performed so far have proven difficult to interpret due to a lack of biomarkers for the stratification of patients.^{4–7} Notably, the detection of tumor infiltrating $\gamma\delta$ T lymphocytes ($\gamma\delta$ TIL) has been hampered by their controversial detection by immunohistochemical (IHC) methods due to their unreliable recognition by antibodies.

Alternative options would be to enumerate $\gamma\delta$ TIL from gene expression profiles (GEP), which is now possible due to recently developed computational methodologies that can

perform cell type-specific quantifications from the transcriptomic data of bulk samples (for review, see⁸). For example, the “deep deconvolution” CIBERSORT algorithm uses the transcriptome profile of reference leucocyte cell types to define a reference gene signature matrix which it then uses to deduce the leucocyte composition from the transcriptome of a tissue.⁹ Using CIBERSORT, the Alizadeh group were able to determine the relative proportions of 22 subsets of leucocytes that had infiltrated tumors from 30,000 transcriptomes representing 39 distinct malignancies, and identified $\gamma\delta$ TILs as the most significant favorable cancer prognostic cell population.¹⁰ However, we show that applying CIBERSORT to pure $\gamma\delta$ T cell transcriptomes failed to correctly differentiate these cells from $\alpha\beta$ T CD4⁺, CD8⁺ and natural killer (NK) cells. Here we optimized this deconvolution and applied it to enumerate $\gamma\delta$ TIL in human cancers.

Results

Optimization of CIBERSORT deconvolution for inferring $\gamma\delta$ T cell abundance from a transcriptome

We first applied the CIBERSORT algorithm and its LM22 gene signature to determine the fraction of $\gamma\delta$ T cells from the GEP of 12 well-characterized samples of TCRV γ 9V δ 2⁺ $\gamma\delta$ lymphocytes purified from healthy donor peripheral blood

mononuclear cells (PBMC) (purity >95%, assessed by staining for the TCRV γ 9 and TCRV δ 2 chains followed by FACS analysis).¹¹ CIBERSORT-LM22 detected 7–40% of $\gamma\delta$ cells and overestimated the CD4⁺, CD8⁺ and NK cell content of these samples (Fig. 1A). In addition, CIBERSORT-LM22 enumerated up to 55% of $\gamma\delta$ T cells in transcriptomes from purified CD4⁺, CD8⁺ and NK cells (Table S1). The GEP of these related cell subsets partly overlap¹¹ and the multicollinearity of this overlap

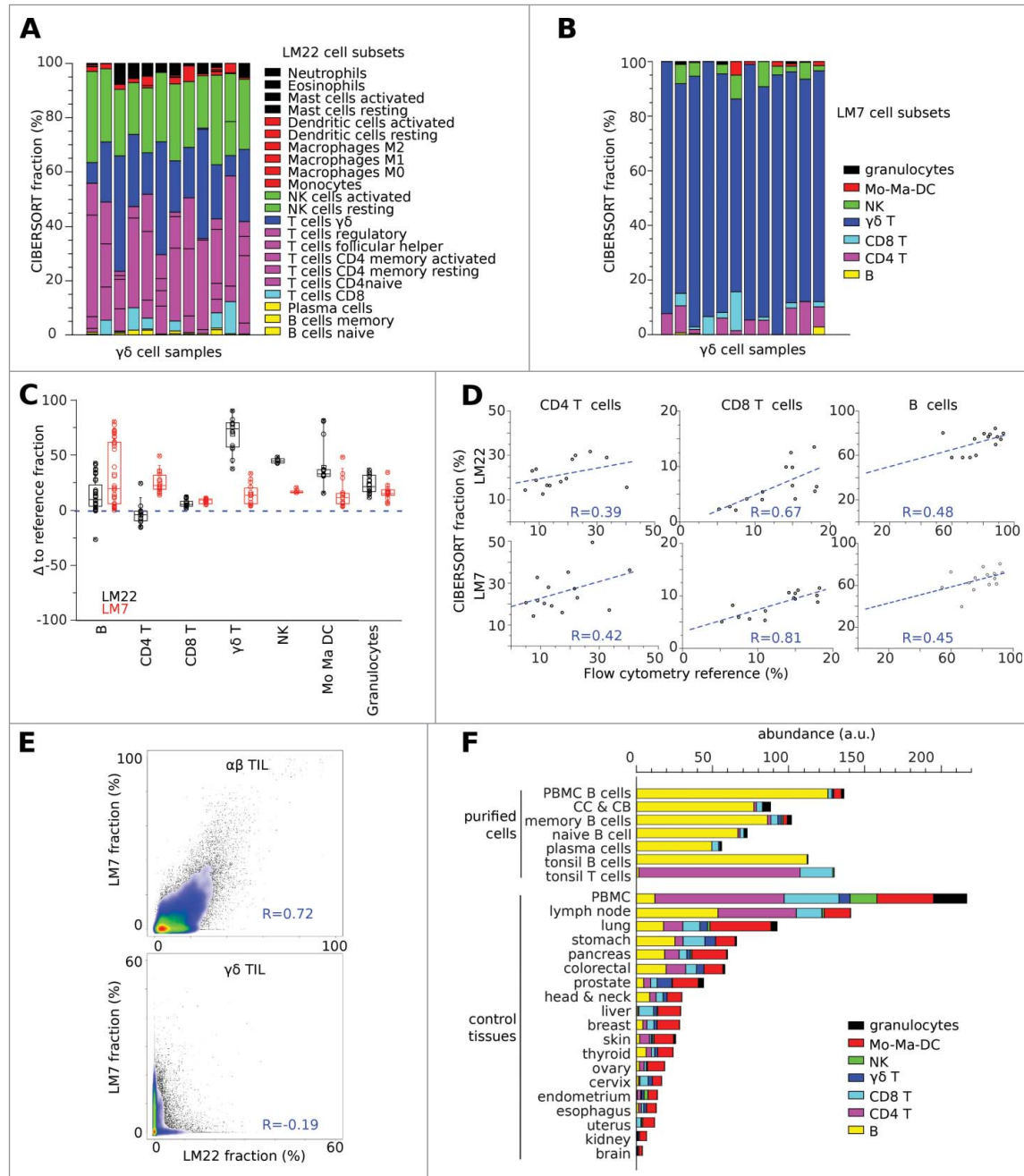


Figure 1. Deconvolution of microarrays from pure TCRV γ 9V δ 2⁺ $\gamma\delta$ T lymphocytes. (A) CIBERSORT detected only marginal rates of $\gamma\delta$ cells when the original reference matrix LM22 was applied to deconvolution of the transcriptomes from 12 samples of purified (> 90% TCRV γ 9V δ 2⁺) $\gamma\delta$ T lymphocytes. (B) Using the reference matrix LM7, CIBERSORT detected respectively 92%, 76%, 92%, 93%, 87%, 71%, 94%, 84%, 95%, 85%, 81% and 85% of TCRV γ 9V δ 2⁺ $\gamma\delta$ cells in the same samples as above. (C) Deviation of CIBERSORT results from the rates specified in each sample annotation file. With LM7, the deconvolution results were closer to most rates specified in the annotation file of control samples of known leucocyte composition, compared with LM22. (D) Pearson correlation of results from CIBERSORT-LM22 or CIBERSORT-LM7 with FACS-based reference rates of CD4⁺, CD8⁺ and B cells in a series of follicular lymphoma biopsies (GSE 65135). (E) Results from CIBERSORT-LM22 and CIBERSORT-LM7 yielded consistent rates of $\alpha\beta$ TILs but not of $\gamma\delta$ TILs upon the deconvolution of \sim 10,000 cancer data sets (see below). The rates of $\alpha\beta$ TILs were taken as the sum of CD4⁺ and CD8⁺ cells. Pearson correlation values are specified; the smaller scale of the $\gamma\delta$ TILs scatterplot reflects their lower abundance than $\alpha\beta$ TIL in the biopsies. (F) Mean leucocyte abundance in normal tissues from a validation series of ($n = 114$) transcriptomes.

is presumably what drives CIBERSORT to wrongly identify $\gamma\delta$ T cells as T CD4⁺, T CD8⁺ or NK cells and *vice versa*. Thus, these data demonstrated the limits of CIBERSORT's LM22 reference matrix for the rating of TCRV γ 9V δ 2⁺ $\gamma\delta$ cells. The LM22 reference gene signature matrix was developed by machine learning from Affymetrix HG-U133A microarrays (~8500 genes) from 113 reference samples downloaded from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) and which included only two samples annotated as “blood $\gamma\delta$ cell” whose purity and TCR phenotype, although most likely of the TCRV γ 9V δ 2⁺ subset, were not specified.¹² Furthermore, of the 58 genes in the LM22 gene signature which are aimed at identifying $\gamma\delta$ cells, the only TCR $\gamma\delta$ -specific gene *TRDC* was actually not expressed by any of the 12 purified TCRV γ 9V δ 2⁺ $\gamma\delta$ cell samples described above. These observations prompted us to seek to improve the deconvolution of tissue expression profiles for assessing TCRV γ 9V δ 2⁺ lymphocytes. We undertook this by increasing the CIBERSORT learning phase and reducing the number of cell subsets to discriminate between. The group of reference data sets was increased to 265 profiles, taken only from the Affymetrix HGU133 Plus 2.0 platform (which analyzes twice more genes than HGU133A microarrays), and included the above-depicted 12 samples of purified TCRV γ 9V δ 2⁺ $\gamma\delta$ T lymphocytes (Table S2). To optimize the CIBERSORT output, the number of subsets to determine was reduced from 22 to 7. These included B cells (pooling naïve, memory B cells and plasma cells), CD4⁺ cells (pooling CD4⁺ resting, CD4⁺ activated, regulatory T cells (Treg), and follicular helper T cells (TFH), CD8⁺ cells (resting and activated CD8⁺ cells), $\gamma\delta$ T cells (resting and activated TCRV γ 9V δ 2⁺ $\gamma\delta$ cells), NK cells (resting and activated NK cells), “MoMaDC” cells (pooling monocytes, macrophages and DC), and granulocytes (arbitrarily pooling mastocytes, eosinophils and neutrophils). These 265 reference data sets were curated, assembled, normalized using the robust multiarray average (RMA) method and then collapsed to construct a novel leucocyte gene signature matrix using the CIBERSORT learning algorithm.⁹ After the removal of genes expressed by non-hematopoietic tissues the resulting matrix, called LM7 (Table S3), comprised 375 genes, 134 of which were present in LM22. This learning identified 23 genes specifically upregulated in human $\gamma\delta$ T cells only: *BCOR*, *BTBD5*, *CCR5*, *CREM*, *CXCR3*, *CXCR6*, *FAM46C*, *FLJ25967*, *GPR132*, *GPR171*, *LEPREL1*, *LOC653506*, *LOC653725*, *METRNL*, *NR4A3*, *PDE4B*, *PDE4D*, *PRKX*, *PRKY*, *RORA*, *SYTL2*, *SYTL3* and *UPP1*, while it found that the TCR $\gamma\delta$ -encoding genes *TRD@*, *TRDV2*, *TRGC2*, *TRGV2* and *TRGV9* are strongly expressed in both $\gamma\delta$ T, $\alpha\beta$ T and NK cells (Table S3).

With the above reference data set ($n = 265$ samples), the relative leucocyte proportions predicted by CIBERSORT-LM7 and CIBERSORT-LM22 were concordant with the compositions annotated in most GEO sample record (GSM), with the exception of the above-depicted TCRV γ 9V δ 2⁺ $\gamma\delta$ samples whose values were still perfectible though significantly improved with LM7 (which calculated >90% $\gamma\delta$ cells on average, *versus* 31% with LM22) (Fig. 1B and Table S1). We then downloaded from GEO and processed a validation data set of tissues ($n = 114$) whose composition had previously been determined (Table S4). Deconvolution of this data set using

CIBERSORT-LM7 and CIBERSORT-LM22 matched the specified fractions of B cells and CD4⁺ cells, while the predicted levels of cytolytic (CD8⁺, NK, TCRV γ 9V δ 2⁺ $\gamma\delta$), myeloid (monocytes, macrophages and DC) and granulocyte subsets improved with LM7 (Fig. 1C, D). However, deconvolution with CIBERSORT-LM7 of ($n = 6$) transcriptomes from purified cord blood $\gamma\delta$ T cells, which encompass both TCRV γ 9V δ 2 and non-TCRV γ 9V δ 2 $\gamma\delta$ T cells dominated by TCRV γ 8V δ 1 cells in cytomegalovirus-infected samples,¹³ gave quite low results (detection of 24% $\gamma\delta$ cells on average; range 11–51%), which were better than with CIBERSORT-LM22 (detection of < 5% $\gamma\delta$ cells on average). Further, deconvolution of the transcriptomes of ~10,000 tumor biopsies of unknown composition (section below) by CIBERSORT-LM22 and CIBERSORT-LM7 was concordant for $\alpha\beta$ tumor-infiltrating lymphocytes (TILs) (taken as sum of CD4⁺ and CD8⁺ T cells, Pearson $r = 0.72$) but not for $\gamma\delta$ TILs (Pearson $r = -0.12$) (Fig. 1E), and was variably concordant for the other subsets: Mo-Ma-DC (Pearson $r = 0.8$), B lymphocytes (Pearson $r = 0.76$), NK (Pearson $r = 0.3$) and Granulocytes (Pearson $r = 0.2$) (data not shown).

Although CIBERSORT-LM7 was able to determine the leucocyte composition of tissues arrayed with Affymetrix HGU133 Plus 2.0 microarrays, it was possible that the use of other platforms which lack some of the LM7 genes could alter its results. We estimated this bias by evaluating the LM7-generated leucocyte subset fractions from the training data set with and without the genes missing from the other platforms (Fig. S1) and found that the incomplete data introduced negligible (< 5%) deviations from the original deconvolution results (Fig. S2).

CIBERSORT determines the relative fractions of leucocytes within a sample, and not their abundance, whereas the sample enrichment score (SES) of leucocytes (SES-LM7) (see Materials and methods) reflects the abundance of leucocytes in each sample¹⁴ and parallels CD45 gene expression levels (Fig. S3). Thus, the abundance of each leucocyte subset in each sample was obtained by computing [(SES-LM7) \times (CIBERSORT-LM7 fraction)] using the DeepTIL algorithm (see Materials and methods) which yields abundance results expressed as arbitrary units. The leucocyte abundance estimates determined by this method from HGU133 Plus 2.0 microarrays of normal tissues ($n = 494$) were consistent with the annotations of purified cell samples and the composition of normal tissues in that leucocytes were found to be most abundant in PBMC, lymph nodes and lung tissue while scarce in normal brain and kidney tissue (Fig. 1F). This validated the DeepTIL algorithm as a useful tool for obtaining more accurate quantification of leucocyte subset abundance, particularly the blood-derived TCRV γ 9V δ 2⁺ $\gamma\delta$ T cells.

Abundance of tumor infiltrating leucocytes in a spectrum of cancers

We then investigated the abundance of TCRV γ 9V δ 2⁺ $\gamma\delta$ T cells and other LM7-defined leucocyte subsets determined by this computational method from a compendium of ~10,000 bulk tumor biopsies from 16 hematological and 30 solid types of cancers (listed in Table S6). This showed that the hematological malignancies had a distinct leucocyte abundance and

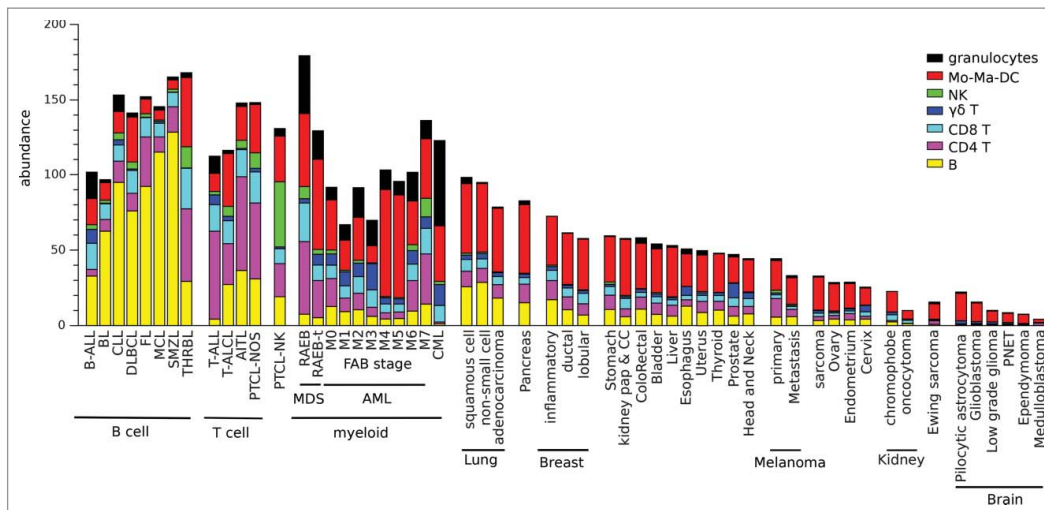


Figure 2. Abundance of TILs across 55 types of hematological and solid cancers. Shown are the mean leucocyte composition of each specified cancer type. Blood malignancies were found to be as “leucocyte-rich” as normal PBMC or lymph nodes, whereas solid tumors included less leucocytes. Lung carcinomas displayed the most abundant leucocyte infiltrates, whereas brain tumors had a particularly low leucocyte composition.

composition dominated by the cell-of-origin lineage (COO), except for Hodgkin’s lymphoma (HL) and T-cell/histiocyte rich B cell lymphoma (*THRL*) whose composition reflected the tumor microenvironment.⁹ In follicular lymphoma (FL), CIBERSORT-LM7 detected less $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs (on average 0.22 a.u. corresponding to 0.5% of leucocytes) than previously reported ($\sim 5\%$ of leucocytes infiltrating FL and diffuse large B cell lymphoma (DLBCL) according to¹⁰). Our result (Fig. 2) are in agreement with earlier IHC and FACS studies showing significantly less $\gamma\delta$ T cells in FL lymph nodes than in inflammatory adenitis lymph nodes.¹⁵ Of note, deconvolution of the same FL and DLBCL transcriptomes by CIBERSORT-LM-7 detected 10-fold less $\gamma\delta$ cells than CIBERSORT-LM22, illustrating its more stringent identification of $\gamma\delta$ cells (Fig. 1C, E). Besides a few cases with a $\gamma\delta$ COO,^{16,17} most peripheral T cell lymphoma (PTCL) and the other T cell malignancies did not present abundant $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ cell infiltrates, whereas the cohorts of B-ALL, CML and AML included in this study revealed an abundant $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TIL compartment (corresponding to $\sim 10\%$ of leucocytes). This result is higher than has previously been reported for AML samples,^{9,18,19} and even for normal bone marrow ($\sim 2.5\%$ of leucocytes,²⁰). Among the eight subtypes of AML defined by the French–American–British (FAB) classification, acute megakaryoblastic leukemia (FAB-M7 subtype of AML) presented the greatest infiltration of total leucocytes and acute promyelocytic leukemia (FAB-M3 subtype of AML) presented the highest abundance of $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs. Solid tumors generally presented less abundant leucocyte infiltration than blood malignancies, and half of their infiltrated content was consistently of myeloid lineage. Quantitatively, these infiltrates were found to be most important in lung, pancreas, prostate, breast, stomach, renal (papillary and clear cell subtypes), colorectal, bladder, liver, esophagus, uterus, liver, head and neck carcinoma, melanoma, thyroid, uterus, endometrium, cervix, and ovary carcinoma tissues (ranked by decreasing magnitude). The least abundant infiltrates were found in cancers carrying the lowest number of somatic mutations, such as kidney chromophobe carcinoma,

Ewing sarcoma, kidney oncocytoma and indeed in central nervous system (CNS) cancers. The infiltrates of brain cancers are mostly of myeloid lineage, probably the monocyte-related microglia.²¹ The abundance of $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs varied across the different cancers, being quite high in prostate, esophagus, cervix, head and neck, lung, pancreas and breast carcinoma. Both CD8^+ and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs were abundant in inflammatory breast carcinoma, whose samples generally consisted of triple negative breast cancers. In melanoma, both $\alpha\beta$ and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs were also found to be more abundant in primary lesions than in metastases (Fig. 2). Overall, the extent of leucocyte infiltration was quite heterogeneous across cancers and the average abundance of $\alpha\beta$ and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs varied likewise between cancer types (Table S5). This large inter-individual fluctuation (ranging across several orders of magnitude) was also seen in FACS analysis of TILs from small cohorts of AML and prostate cancer patients (not shown). Plotting the individual abundance of $\alpha\beta$ TILs at the level of patients and ranking cancer types on this criterion confirmed that the abundance of immune infiltrates fluctuated strongly among patients both inside and across cancer types. The abundance of $\text{TCRV}\gamma 9\text{V}\delta 2^+ \alpha\beta$ TILs among patients was also found to be highly variable, though they were consistently less abundant than $\alpha\beta$ TILs. Strikingly however, the individual abundance of $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs did not appear to correlate with the abundance of the $\alpha\beta$ TILs neither within nor across the different types of cancer (Fig. 3).

No correlation between $\alpha\beta$ TIL and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TIL abundance across the cancer spectrum

The above results suggested that tumor infiltration by the $\alpha\beta$ and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs occur in distinct immunological contexts. To characterize these contexts, we computed SES¹⁴ of all the gene sets from the MSigDB²² for the 10,000 cancer samples, and determined whether these correlated with the abundance of $\alpha\beta$ and $\text{TCRV}\gamma 9\text{V}\delta 2^+ \gamma\delta$ TILs. This approach revealed significant differences between the TIL subsets. Across

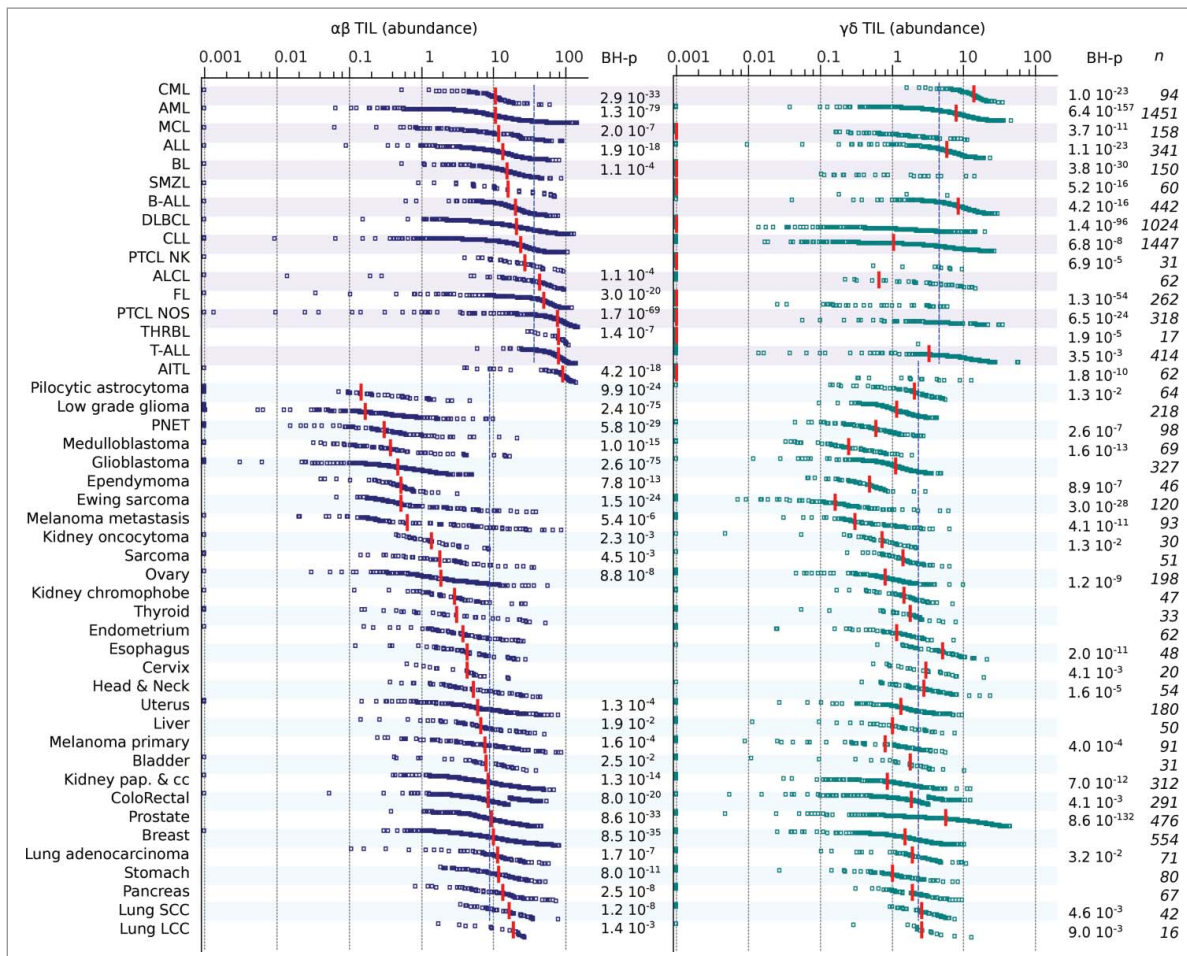


Figure 3. Individual abundances of tumor-infiltrating $\alpha\beta$ T and TCRV γ 9V δ 2+ $\gamma\delta$ T lymphocytes across 55 types of hematological and solid cancers. Individual abundances of $\alpha\beta$ and TCRV γ 9V δ 2+ $\gamma\delta$ TILs across cancer types. Blood and solid cancers are successively presented in order of increasing $\alpha\beta$ TIL abundance. Each dot is a sample, red bars show the median of each cancer type, blue dotted lines show the mean abundance of the specified TILs across hematological (top) and solid (bottom) cancers. For each type of cancer, the Wilcoxon test was used to calculate the statistical significance of deviation from the mean abundance of TIL in hematological or solid cancers. The Benjamini–Hochberg (BH) correction was applied to determine the FDR for multiple comparisons, only the significant BH-corrected p -values are specified (right).

all cancers, the abundance of $\alpha\beta$ TILs positively correlated with the enrichment of “TCR signaling” (Spearman $r = 0.78$), “Toll-like receptor signaling pathway” (Spearman $r = 0.45$) and related gene sets, whereas the abundance of TCRV γ 9V δ 2+ $\gamma\delta$ TILs did not correlate with these (Spearman $r = -0.02$ and -0.03 , respectively). These distinct features were best exemplified within specific cancer types. For example, in colorectal carcinoma, the abundance of $\alpha\beta$ TILs correlated with the enrichment of the “Response to IFN α ”, “Inflammatory response”, “Antigen processing and presentation”, “T cell activation” and “Cytolytic activity” gene sets, as well as with the abundance of Mo-Ma-DC cells (Fig. 4A). We found that all of these metrics also cross-correlated, and patients with “ $\alpha\beta$ TIL-high” tumors presented significantly higher overall survival (OS) rates than those with “ $\alpha\beta$ TIL-low” tumors (Fig. 4B). This result is in accordance with the well-known association of $\alpha\beta$ TIL density with the outcome of colorectal cancer patients.^{23,24}

In contrast, the abundance of TCRV γ 9V δ 2+ $\gamma\delta$ TILs did not correlate with any of these transcriptomic readouts (Spearman $r < 0.12$), since correlation with these reference MSIGDB gene sets was the same as with a collection of random gene sets (RGS, data not shown). Nevertheless, colorectal cancer patients with “TCRV γ 9V δ 2+ $\gamma\delta$ TIL-high” tumors presented better OS

rates than those with “TCRV γ 9V δ 2+ $\gamma\delta$ TIL-low” tumors, irrespective of their abundance of $\alpha\beta$ TIL. Hence, the presence of TCRV γ 9V δ 2+ $\gamma\delta$ TIL was like $\alpha\beta$ TIL, associated with a favorable outcome in colorectal cancer, even though these lymphocytes are recruited through a distinct immunological context. The same conclusions were drawn from the analyses of additional cohorts of CLL, AML and prostate cancer patients (Fig. 4B). These and the above results (Fig. 3) indicate that the recruitment of $\alpha\beta$ and TCRV γ 9V δ 2+ $\gamma\delta$ TILs occurs in different immunological contexts.

Discussion

We report here the application of an emerging method of machine learning for microarray deconvolution to assess the abundance of leucocyte infiltration in tumor biopsies. Since its first application in the deconvolution of blood transcriptomes from patients with systemic lupus erythematosus through iterative linear least-squares fitting,²⁵ this field has rapidly expanded and has progressively validated the use of computational deconvolution to identify the individual component cells from RNA mixtures. The current *in silico* approaches for characterizing cellular heterogeneity from GEP encompass either the

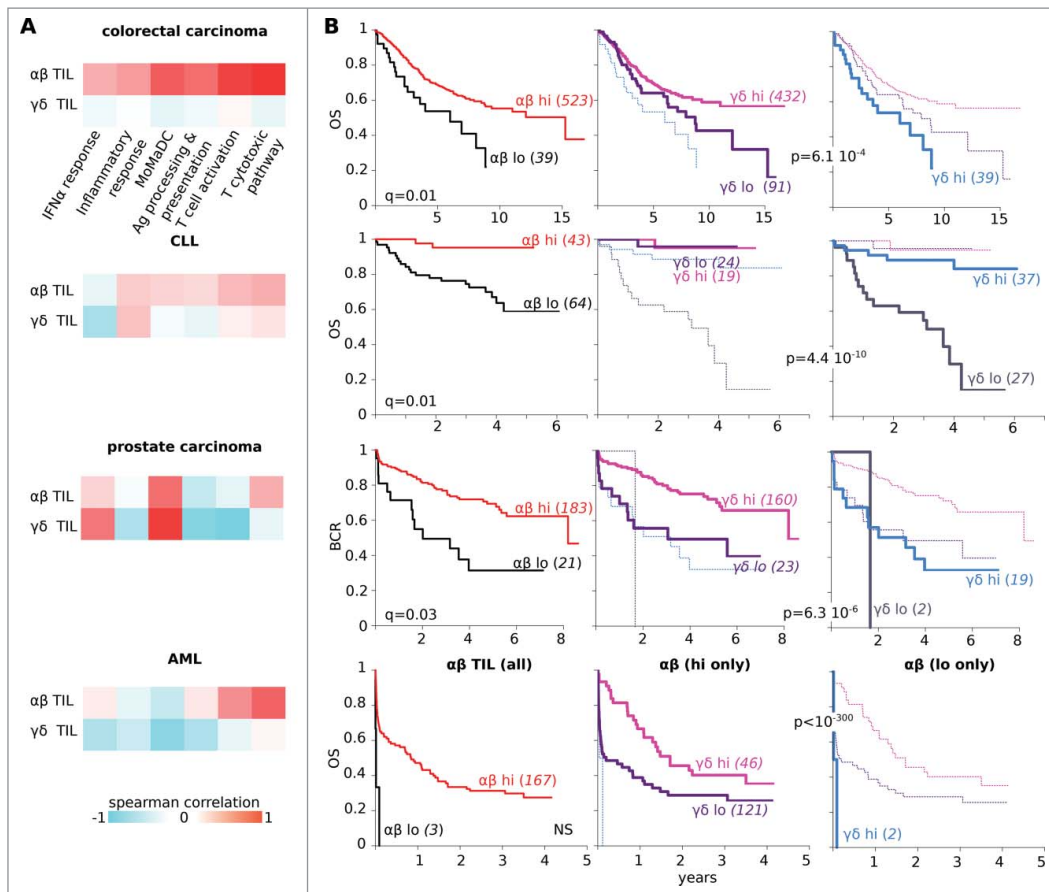


Figure 4. Different correlates of $\alpha\beta$ and TCRV γ 9V δ 2⁺ $\gamma\delta$ TIL abundance. (A) In the specified groups of cancer patients, heatmaps of spearman correlation between abundance of $\alpha\beta$ TILs (top) or TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs (bottom) with SES for the gene sets indicated. The abundance of $\alpha\beta$ TILs never shows the same correlation profile as the abundance of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs. (B) Kaplan–Meier plots for the overall survival of colorectal carcinoma, CLL and AML patients or for the biochemical relapse (BCR) of prostate cancer patients, according to the abundance of the $\alpha\beta$ TILs only (left), the TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs superimposed on the $\alpha\beta$ TILs-low only (middle) and the TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs on $\alpha\beta$ TILs-high only (right). Abundance of both subsets of TILs is associated to favorable outcome in these cohorts of cancer patients.

analysis of cell type-specific genes and gene set enrichments, or the direct algorithmic deconvolution of transcriptomes to deduce cell-type compositions (reviewed in⁸). These both now yield results for the rapid profiling of TILs that match those achieved by conventional flow cytometry (FACS) and IHC techniques, but without the biases due to the mechanical dissociation of samples or the antibodies used for reliable detection of $\gamma\delta$ TILs by IHC of formalin-fixed paraffin-embedded tumor biopsies. Optimizing the discrimination of ontologically related cell types is not trivial, however, as it requires the multicollinearity of their highly similar transcriptomes be addressed. Several techniques of automated deconvolution have recently been developed toward this aim (reviewed in²⁶), among which the most robust and performant is currently the machine learning algorithm CIBERSORT.⁹ CIBERSORT applies nu-support vector regression to enumerate 22 leucocyte subsets from bulk tumor biopsies, and has allowed the identification of genes and leucocyte predictors of survival in a meta-analysis of thousands of cancers.¹⁰ More recently, CIBERSORT also enabled the successful leucocyte enumeration by deconvolution of microarray data sets from PBMC,²⁷ glioma,²⁸ lung carcinoma²⁹ and triple negative breast cancers.³⁰ Here, however, we found that CIBERSORT-LM22 does not discriminate between $\gamma\delta$ T cells and the closely related T CD4⁺, T CD8⁺ and NK lymphoid subsets due to insufficient training from only two $\gamma\delta$ T cell transcriptomes

assessed on first-generation microarrays. The improved learning from more than 260 second-generation microarrays (encompassing \sim 21,000 genes) and the reduced output of only 7 instead of 22 subtypes of leucocytes was expected to reduce the multicollinearity of their individual gene profiles, and thereby improve output reliability. Our report shows that CIBERSORT-LM7 detects TCRV γ 9V δ 2⁺ $\gamma\delta$ T lymphocytes more accurately than CIBERSORT-LM22, but it has learnt this from 12 microarrays of purified, resting and activated polyclonal $\gamma\delta$ T cells expressing only TCRV γ 9V δ 2, raising the question of whether it detects as efficiently the other (non-TCRV γ 9V δ 2) subsets of $\gamma\delta$ T lymphocytes. So far, the scarce publicly available microarrays for such purified $\gamma\delta$ T cells do not allow to address this point. Nevertheless, the less performant deconvolution of transcriptomes for $\gamma\delta$ cell bulks highly purified from fetal blood,¹³ although far better with LM7 than LM22, indicates that the current learning process enabled CIBERSORT-LM7 to reliably identify as $\gamma\delta$ cells those cells sharing the gene signature of blood TCRV γ 9V δ 2 $\gamma\delta$ T cells. Future integration of other $\gamma\delta$ T cell subsets purified from normal tissues or tumor samples and profiled on microarray or RNA Seq will enlarge the learning of CIBERSORT-LM7 to empower its identification capacities. However, since learning from more than one hundred CD4⁺ and CD8⁺ microarrays does not empower CIBERSORT to discriminate between the

various TCR-defined subsets of $\alpha\beta$ T cells, the learning from smaller sampling data sets might set limits for performing TCR-defined $\gamma\delta$ T cell identification.

The overall output of CIBERSORT-LM7 matches that of CIBERSORT-LM22 but with a better reliability for the detection of $\gamma\delta$ T cells, which were previously mistakenly identified as CD4⁺ (31%) or NK cells (23%), while 8% of CD8⁺ T cells were mistakenly classified as $\gamma\delta$ T lymphocytes (Table S4). As a consequence, the association of $\gamma\delta$ cells with a favorable outcome across a large number of cancer patients should be taken with caution.¹⁰ Our study reports TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs levels that are fully consistent with the body of literature about various types of cancers (³¹⁻³⁴ and see below). For B-ALL, however, this result could reflect either the actual recruitment of $\gamma\delta$ TILs or the bi-phenotypic profile of such leukemia, which frequently co-express rearranged Ig and $\gamma\delta$ TCR genes.^{35,36} In CML, bone marrow-infiltrating TCRV γ 9V δ 2⁺ $\gamma\delta$ T lymphocytes have not been reported, but the reported average of 8% CD3, 0% CD4⁺ and <4% CD8⁺ T cells in such tissues³⁷ is compatible with our results. In AML, the few studies which have quantified TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs in bone marrow biopsies have found them to be at similar levels as in the blood, but these figures might differ across patients and FAB subtypes.^{19,38} In solid tumors, independent studies have mentioned an abundance of $\gamma\delta$ TILs in cancers of the pancreas,³⁴ breast,³¹ head and neck,³³ colorectal³⁹ and lung,⁴⁰ but there have been no reports for prostate, cervix and esophagus cancers. Moreover, beside of the pioneering CIBERSORT studies which sub-optimally detect $\gamma\delta$ cells,^{9,10} there are currently no published meta-analyses of TCRV γ 9V δ 2⁺ $\gamma\delta$ TIL across different human cancers.

The results presented here detected highly heterogeneous amounts of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs in cancers, and their relative abundance in the above solid cancers as well as in prostate and esophagus carcinoma. The high level of the TCR γ gene expression by prostatic epithelial cells⁴¹ might set pitfalls to identify $\gamma\delta$ TILs in prostate cancer by conventional methods, but not to the deconvolution depicted here which takes in account all the LM7 genes for this aim. So far however, the abundance of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs in prostate cancer was not reported, so this study brings further rationale to harness these cells for immunotherapeutic purposes.⁴²⁻⁴⁵ This work also reports abundant TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs in breast carcinoma, a finding in line with earlier studies demonstrating that these $\gamma\delta$ cells are abundant but associated with immunosuppressive activity, tumor progression, lymph node metastases and correlate with poor outcome.³¹ Likewise, the stroma of ductal adenocarcinoma was reported to comprise abundant $\gamma\delta$ TIL with unfavorable role,³⁴ supporting our result for this disease. Actually, a detrimental role is reportedly associated to $\gamma\delta$ TILs in breast, colorectal and pancreas cancer,^{31,34,39} raising the question of whether their abundance generally predicts a favorable or unfavorable outcome. The results presented here illustrate an association of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs with favorable clinical evolution in four cohorts of patients with different types of cancer (Fig. 4). This seemingly contrasts with an earlier study depicting promotion of colorectal cancer in patients with abundant Th17-producing $\gamma\delta$ TILs.³⁹ Nevertheless, the clinical significance of $\gamma\delta$ TILs abundance in cancer most probably depends on the TCR subsets of $\gamma\delta$ cells,⁷ their maturation and

cytokine profile⁴⁶ and the tumor contexts as demonstrated in AML,⁴⁷ breast,³¹ colorectal³⁹ and pancreatic³⁴ cancers.

The abundance of $\alpha\beta$ and TCRV γ 9V δ 2⁺ $\gamma\delta$ TIL across the spectrum of cancers tested varied considerably. The ordering of cancers according to their abundance of $\alpha\beta$ TILs presented a similar pattern as that seen when they are ordered by mutational load.^{48,49} This was illustrated by children's cancers, which typically have few mutations and here presented the least $\alpha\beta$ TILs whereas cancers associated with durable exposure to carcinogens had the greatest number of these cells (Fig. 3). This is in agreement with the finding that the diversity and amount of $\alpha\beta$ TIL clonotypes increases with the amount of non-synonymous mutations within tumors.⁵⁰ Nevertheless, we detected a different distribution of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs, suggesting that their incentive for infiltrating tumors is not mutational as for $\alpha\beta$ TILs. The lack of identification of transcriptomic correlates for the abundance of TCRV γ 9V δ 2⁺ $\gamma\delta$ TILs across the cancer spectrum could be due to several diverse reasons: the screen for correlates involved only MSIGDB-predefined human gene sets not unknown ones, this human transcriptome-based strategy cannot detect associations with most viral⁵¹ or microbial contexts,^{52,53} this strategy might be intrinsically unable to detect the non-transcriptional hallmarks of cancers such as specific somatic mutations or the accumulation of endogenous (onco) metabolites. Due to the metabolic and microbial origin of the PAg that activate most human blood TCRV γ 9V δ 2⁺ $\gamma\delta$ lymphocytes,² such hypotheses deserve further investigation. Future research exploiting the increasing body of RNASeq and metabolomics data sets from human cancers will certainly help in addressing these issues.

Materials and methods

Signature matrix

Public raw data of ($n = 265$) transcriptomes from purified cell types obtained using Affymetrix HGU133 Plus 2.0 microarrays were downloaded from <https://www.ncbi.nlm.nih.gov/gds>, normalized together and collapsed to HUGO gene symbols using chipset definition files available from the NCBI GEO. These are referred to as reference data (Table S2). As a filtration step, genes with enriched expression (mean log₂ (expression level) >7) in all the normal non-hematopoietic tissues from the Human Body Index (study GSE7307) were removed. We then defined seven leucocyte subsets (B cells, CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, Mo-Ma-DC cells and granulocytes) which were imported into the CIBERSORT software (<https://cibersort.stanford.edu/>)⁹ to build the LM7 matrix (Table S3). For validation, CIBERSORT-LM7 was applied to a variety of external normalized public data sets (Table S4) containing purified leucocyte cells subsets including non-TCRV γ 9V δ 2⁺ $\gamma\delta$ T cells isolated from human cord blood⁵⁴ or mixed populations of known composition.

Abundance calculations

Abundances were calculated from the CIBERSORT results and the SES, as depicted in the text. The SES¹⁴ were computed by applying the open source software AutoCompare-SES (<https://>

sites.google.com/site/fredsoftwares/products/autocompare_ses) with normalized settings. Then, the open source software DeepTIL (<https://sites.google.com/site/fredsoftwares/products/deeptil>) was used to automatically compute the abundance of the seven leucocyte subsets in the samples. DeepTIL was written using the cross-platform Julia programming language⁵⁵; its typical computation time is 3–5 sec per sample. A parallel version of DeepTIL is also available for computing data tables simultaneously. From CIBERSORT and SES calculations, DeepTIL computes the following steps: (1) selection of LM7 genes with a mean log₂ (expression level) >11.5 in each leucocyte type to constitute its defining gene set. In each sample, the SES of the seven leucocyte defining gene sets are computed against the testing alternative “greater than”.¹⁴ The SES of each type of leucocyte is also computed for each of the seven pure types of leucocyte to determine the seven corresponding correcting factors. (2) a linear combination of the seven leucocyte type SESs weighted by their respective correcting factors is computed for each sample. (3) for each sample, CIBERSORT fractions are finally multiplied by the linear combination of SES to produce the abundance of each leucocyte subset. The mean abundance of each leucocyte subset in ~10,000 samples of human cancers is summarized (Table S6).

Statistical analysis

Assessments of leucocyte fractions from the specified transcriptomes were performed using CIBERSORT (<https://cibersort.stanford.edu/>)⁹ with 500 Monte Carlo iterations and the matrix indicated in the text. Comparisons of TIL ($\alpha\beta$ and $\gamma\delta$) abundances across cancers (Fig. 3) were performed by Wilcoxon tests, assessing the abundance of TIL in the specified cancer type comparatively to the same variable across the whole set of hematological or solid cancers. The Benjamini–Hochberg (BH) correction⁵⁶ was applied for multiple comparisons. The functionally defined collection of gene sets was downloaded from MSIGDB (<http://software.broadinstitute.org/gsea/msigdb>)²² while for control experiments, we generated a collection of 500 random gene sets of the same size as MSIGDB, referred to as RGS. For the transcriptome samples specified in the text, the SES of each gene set was computed as depicted¹⁴ and their respective correlations (Pearson and Spearman) with the abundance of the specified cell types were automatically computed using the “Correlations by lines in list” software (<https://sites.google.com/site/fredsoftwares/products/correlation-by-lines-in-list>). For Kaplan–Meier plots, optimal cutoffs were determined with the survival R package⁵⁷ and Log-Rank *p*-values were corrected using the BH method.⁵⁶

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.



Acknowledgments

We acknowledge JFF’s team members for their stimulating comments and Kelly Thornber from Scientific Scripts for English editing of the text.

Funding

This work was funded by the Institut National de Santé et de la Recherche Médicale (INSERM), the Université Toulouse III: Paul Sabatier, the Center National de la Recherche Scientifique (CNRS), the Laboratoire d’Excellence Toulouse Cancer (TOUCAN) (contract ANR11-LABX) and the Program Hospitalo-Universitaire en Cancérologie CAPTOR (contract ANR11-PHUC0001).

ORCID

Marie Tosolini  <http://orcid.org/0000-0001-5278-5952>
Jean-Jacques Fournié  <http://orcid.org/0000-0001-6542-6908>

References

1. Fisch P, Malkovsky M, Braakman E, Sturm E, Bolhuis RL, Prieve A, Sosman JA, Lam VA, Sondel PM. Gamma/delta T cell clones and natural killer cell clones mediate distinct patterns of non-major histocompatibility complex-restricted cytotoxicity. *J Exp Med* 1990; 171:1567–79; PMID:2185329; <http://dx.doi.org/10.1084/jem.171.5.1567>
2. Fournie JJ, Bonneville M. Stimulation of gamma delta T cells by phosphoantigens. *Res Immunol* 1996; 147:338–47; PMID:8876063; [http://dx.doi.org/10.1016/0923-2494\(96\)89648-9](http://dx.doi.org/10.1016/0923-2494(96)89648-9)
3. Poccia F, Malkovsky M, Gougeon ML, Bonneville M, Lopez-Botet M, Fournie JJ, Colizzi V. Gammadelta T cell activation or anergy during infections: the role of nonpeptidic TCR ligands and HLA class I molecules. *J Leukoc Biol* 1997; 62:287–91; PMID:9307066
4. Fournie JJ, Sicard H, Poupot M, Bezombes C, Blanc A, Romagne F, Ysebaert L, Laurent G. What lessons can be learned from gamma delta T cell-based cancer immunotherapy trials? *Cell Mol Immunol* 2013; 10:35–41; <http://dx.doi.org/10.1038/cmi.2012.39>
5. Meraviglia S, Lo Presti E, Dieli F, Stassi G. gammadelta T cell-based anticancer immunotherapy: progress and possibilities. *Immunotherapy* 2015; 7:949–51; PMID:26569071; <http://dx.doi.org/10.2217/imt.15.68>
6. Kobayashi H, Tanaka Y. gammadelta T Cell Immunotherapy-A Review. *Pharmaceuticals (Basel)* 2015; 8:40–61; PMID:25686210; <http://dx.doi.org/10.3390/ph8010040>
7. Silva-Santos B, Serre K, Norell H. gammadelta T cells in cancer. *Nat Rev Immunol* 2015; 15:683–91; PMID:26449179; <http://dx.doi.org/10.1038/nri3904>
8. Shen-Orr SS, Gaujoux R. Computational deconvolution: extracting cell type-specific information from heterogeneous samples. *Curr Opin Immunol* 2013; 25:571–8; PMID:24148234; <http://dx.doi.org/10.1016/j.coi.2013.09.015>
9. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M, Alizadeh AA. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015; 12:453–7; PMID:25822800; <http://dx.doi.org/10.1038/nmeth.3337>
10. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, Nair VS, Xu Y, Khuong A, Hoang CD et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med* 2015; 21:938–45; PMID:26193342; <http://dx.doi.org/10.1038/nm.3909>
11. Pont F, Familiades J, Dejean S, Fruchon S, Cendron D, Poupot M, Poupot R, L’faqihi-Olive F, Prade N, Ycart B et al. The gene expression profile of phosphoantigen-specific human gammadelta T lymphocytes is a blend of alphabeta T-cell and NK-cell signatures. *Eur J Immunol* 2012; 42:228–40; PMID:21968650; <http://dx.doi.org/10.1002/eji.201141870>
12. Chtanova T, Newton R, Liu SM, Weininger L, Young TR, Silva DG, Bertoni F, Rinaldi A, Chappaz S, Sallusto F et al. Identification of T cell-restricted genes, and signatures for different T cell responses, using a comprehensive collection of microarray datasets. *J Immunol* 2005; 175:7837–47; PMID:16339519; <http://dx.doi.org/10.4049/jimmunol.175.12.7837>
13. Vermijlen D, Brouwer M, Donner C, Liesnard C, Tackoen M, Van Ryselberge M, Twité N, Goldman M, Marchant A, Willems F et al.

- Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J Exp Med* 2010; 207:807–21; PMID:20368575; [hiip://dx.doi.org/10.1084/jem.20090348](http://dx.doi.org/10.1084/jem.20090348)
14. Tosolini M, Alkans C, Pont F, Ycart B, Fournie JJ. Large scale microarray profiling reveals four stages of immune escape in non-Hodgkin's lymphomas. *Oncoimmunology* 2016; 5(7):e1188246; in press; PMID:27622044; [hiip://dx.doi.org/10.1080/2162402X.2016.1188246](http://dx.doi.org/10.1080/2162402X.2016.1188246)
 15. Braza MS, Caraux A, Rousset T, Lafaye de Micheaux S, Sicard H, Squiban P, Costes V, Klein B, Rossi JF. gammadelta T lymphocytes count is normal and expandable in peripheral blood of patients with follicular lymphoma, whereas it is decreased in tumor lymph nodes compared with inflammatory lymph nodes. *J Immunol* 2010; 184:134–40; PMID:19949101; [hiip://dx.doi.org/10.4049/jimmunol.0901980](http://dx.doi.org/10.4049/jimmunol.0901980)
 16. Gaulard P, de Leval L. Pathology of peripheral T-cell lymphomas: where do we stand? *Semin Hematol* 2014; 51:5–16; PMID:24468311; <http://dx.doi.org/10.1053/j.seminhematol.2013.11.003>
 17. Przybylski GK, Wu H, Macon WR, Finan J, Leonard DG, Felgar RE, DiGiuseppe JA, Nowell PC, Swerdlow SH, Kadin ME et al. Hepatosplenic and subcutaneous panniculitis-like gamma/delta T cell lymphomas are derived from different Vdelta subsets of gamma/delta T lymphocytes. *J Mol Diagn* 2000; 2:11–9; PMID:11272897; [doi.org/10.1016/S1525-1578\(10\)60610-1](http://dx.doi.org/10.1016/S1525-1578(10)60610-1)
 18. Gertner-Dardenne J, Castellano R, Mamessier E, Garbit S, Kochbati E, Etienne A, Charbonnier A, Collette Y, Vey N, Olive D. Human Vgamma9Vdelta2 T cells specifically recognize and kill acute myeloid leukemic blasts. *J Immunol* 2012; 188:4701–8; PMID:22467661; [hiip://dx.doi.org/10.4049/jimmunol.1103710](http://dx.doi.org/10.4049/jimmunol.1103710)
 19. Gertner-Dardenne J, Fauriat C, Vey N, Olive D. Immunotherapy of acute myeloid leukemia based on gammadelta T cells. *Oncoimmunology* 2012; 1:1614–6; PMID:23264912; [hiip://dx.doi.org/10.4161/onci.21512](http://dx.doi.org/10.4161/onci.21512)
 20. Dean J, McCarthy D, Golden-Mason L, O'Farrelly C. Trepine biopsies are enriched for activated T/NK cells and cytotoxic T cells. *Immunol Lett* 2005; 99:94–102; PMID:15894117; [hiip://dx.doi.org/10.1016/j.imlet.2005.01.008](http://dx.doi.org/10.1016/j.imlet.2005.01.008)
 21. Jack AS, Lu JQ. Immune cell infiltrates in the central nervous system tumors. *Austin Neurosurgery: Open access* 2015; 2(1):30
 22. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005; 102:15545–50; PMID:16199517; [hiip://dx.doi.org/10.1073/pnas.0506580102](http://dx.doi.org/10.1073/pnas.0506580102)
 23. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; 313:1960–4; PMID:17008531; [hiip://dx.doi.org/10.1126/science.1129139](http://dx.doi.org/10.1126/science.1129139)
 24. Mlecnik B, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, Church SE, Lafontaine L, Fischer M, Fredriksen T et al. Integrative analyses of colorectal cancer show immunoscore is a stronger predictor of patient survival than microsatellite instability. *Immunity* 2016; 44:698–711; PMID:26982367; [hiip://dx.doi.org/10.1016/j.immuni.2016.02.025](http://dx.doi.org/10.1016/j.immuni.2016.02.025)
 25. Abbas AR, Wolslegel K, Seshasayee D, Modrusan Z, Clark HF. Deconvolution of blood microarray data identifies cellular activation patterns in systemic lupus erythematosus. *PLoS One* 2009; 4:e6098; PMID:19568420; [hiip://dx.doi.org/10.1371/journal.pone.0006098](http://dx.doi.org/10.1371/journal.pone.0006098)
 26. Newman AM, Alizadeh AA. High-throughput genomic profiling of tumor-infiltrating leukocytes. *Curr Opin Immunol* 2016; 41:77–84; PMID:27372732; [hiip://dx.doi.org/10.1016/j.coi.2016.06.006](http://dx.doi.org/10.1016/j.coi.2016.06.006)
 27. Karpinski P, Frydecka D, Sasiadek MM, Misiak B. Reduced number of peripheral natural killer cells in schizoprenia but not in bipolar disorder. *Brain Behav Immun* 2016; 54:194–200; PMID:26872421; [hiip://dx.doi.org/10.1016/j.bbi.2016.02.005](http://dx.doi.org/10.1016/j.bbi.2016.02.005)
 28. Wang QH, Muller F, Kim H, Squatrito M, Millelsen T, Scarpace L, Barthel F, Lin YH, Satani N, Martinez-Ledesma E et al. Tumor evolution of glioma intrinsic gene expression subtype associates with immunological changes in the microenvironment. *bioRxiv* 2016; <http://dx.doi.org/hiips://doi.org/10.1101/052076>
 29. Araujo JM, Prado A, Cardenas NK, Zaharia M, Dyer R, Doimi F, Bravo L, Pinillos L, Morante Z, Aguilar A et al. Repeated observation of immune gene sets enrichment in women with non-small cell lung cancer. *Oncotarget* 2016; 7:20282–92; PMID:26958810; [hiip://dx.doi.org/10.18632/oncotarget.7943](http://dx.doi.org/10.18632/oncotarget.7943)
 30. Vinayak S, Gray RJ, Adams S, Jensen KC, Manola J, Afghahi A, Goldstein LJ, Ford JM, Badve SS, Telli ML. Association of increased tumor-infiltrating lymphocytes (TILs) with immunomodulatory (IM) triple-negative breast cancer (TNBC) subtype and response to neoadjuvant platinum-based therapy in PreCOG0105. *J Clin Oncol* 2014; 32:suppl. abstract 1000
 31. Ma C, Zhang Q, Ye J, Wang F, Zhang Y, Wevers E, Schwartz T, Hunchburg P, Varvares MA, Hofstetter DF et al. Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer. *J Immunol* 2012; 189:5029–36; PMID:23034170; [hiip://dx.doi.org/10.4049/jimmunol.1201892](http://dx.doi.org/10.4049/jimmunol.1201892)
 32. Kowalczyk D, Skorupski W, Kwias Z, Nowak J. Flow cytometric analysis of tumour-infiltrating lymphocytes in patients with renal cell carcinoma. *Br J Urol* 1997; 80:543–7; PMID:9352689; <http://dx.doi.org/10.1046/j.1464-410X.1997.00408.x>
 33. Bas M, Bier H, Schirlau K, Friebe-Hoffmann U, Scheckenbach K, Balz V, Whiteside TL, Hoffmann TK. Gamma-delta T-cells in patients with squamous cell carcinoma of the head and neck. *Oral Oncol* 2006; 42:691–7; PMID:16527515; [hiip://dx.doi.org/10.1016/j.joroloncol.2005.11.008](http://dx.doi.org/10.1016/j.joroloncol.2005.11.008)
 34. Daley D, Zambirinis CP, Seifert L, Akkad N, Mohan N, Werba G, Barilla R, Torres-Hernandez A, Hundeyin M, Mani VR et al. gammadelta T cells support pancreatic oncogenesis by restraining alphabeta T cell activation. *Cell* 2016; 166:1485–99 e15; [hiip://dx.doi.org/10.1016/j.cell.2016.07.046](http://dx.doi.org/10.1016/j.cell.2016.07.046)
 35. Goorha R, Bunin N, Mirro J, Jr, Murphy SB, Cross AH, Behm FG, Quertermous T, Seidman J, Kitchingman GR. Provocative pattern of rearrangements of the genes for the gamma and beta chains of the T-cell receptor in human leukemias. *Proc Natl Acad Sci U S A* 1987; 84:4547–51; PMID:2955409; [hiip://dx.doi.org/10.1073/pnas.84.13.4547](http://dx.doi.org/10.1073/pnas.84.13.4547)
 36. Buccheri V, Matutes E, Dyer MJ, Catovsky D. Lineage commitment in biphenotypic acute leukemia. *Leukemia* 1993; 7:919–27; PMID:8501986
 37. Bachireddy P, Wu CJ. Understanding anti-leukemia responses to donor lymphocyte infusion. *Oncoimmunology* 2014; 3:e28187; PMID:25340010; [hiip://dx.doi.org/10.4161/onci.28187](http://dx.doi.org/10.4161/onci.28187)
 38. Aswald JM, Wang XH, Aswald S, Lutynski A, Minden MD, Messner HA, Keating A. Flow cytometric assessment of autologous gamma-delta T cells in patients with acute myeloid leukemia: potential effector cells for immunotherapy? *Cytometry B Clin Cytom* 2006; 70:379–90; PMID:16977635; [hiip://dx.doi.org/10.1002/cyto.b.20115](http://dx.doi.org/10.1002/cyto.b.20115)
 39. Wu P, Wu D, Ni C, Ye J, Chen W, Hu G, Wang Z, Wang C, Zhang Z, Xia W et al. gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived suppressor cells in human colorectal cancer. *Immunity* 2014; 40:785–800; PMID:24816404; [hiip://dx.doi.org/10.1016/j.immuni.2014.03.013](http://dx.doi.org/10.1016/j.immuni.2014.03.013)
 40. Zocchi MR, Ferrarini M, Migone N, Casorati G. T-cell receptor V delta gene usage by tumour reactive gamma delta T lymphocytes infiltrating human lung cancer. *Immunology* 1994; 81:234–9; PMID:8157272
 41. Essand M, Vasmatzis G, Brinkmann U, Duray P, Lee B, Pastan I. High expression of a specific T-cell receptor gamma transcript in epithelial cells of the prostate. *Proc Natl Acad Sci U S A* 1999; 96:9287–92; PMID:10430935; [hiip://dx.doi.org/10.1073/pnas.96.16.9287](http://dx.doi.org/10.1073/pnas.96.16.9287)
 42. McNeel DG, Malkovsky M. Immune-based therapies for prostate cancer. *Immunol Lett* 2005; 96:3–9; PMID:15585302; [hiip://dx.doi.org/10.1016/j.imlet.2004.06.009](http://dx.doi.org/10.1016/j.imlet.2004.06.009)
 43. Dieli F, Vermijden D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, Roberts A, Buccheri S, D'Asaro M, Gebbia N et al. Targeting human {gamma}delta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res* 2007; 67:7450–7; PMID:17671215; [hiip://dx.doi.org/10.1158/0008-5472.CAN-07-0199](http://dx.doi.org/10.1158/0008-5472.CAN-07-0199)

44. Dieli F, Caccamo N, Meraviglia S. Advances in immunotherapy of castration-resistant prostate cancer: bisphosphonates, phosphoantigens and more. *Curr Opin Investig Drugs* 2008; 9:1089–94; PMID:18821471
45. Lang JM, Wallace M, Becker JT, Eickhoff JC, Buehring B, Binkley N, Staab MJ, Wilding G, Liu G, Malkovsky M et al. A randomized phase II trial evaluating different schedules of zoledronic acid on bone mineral density in patients with prostate cancer beginning androgen deprivation therapy. *Clin Genitourin Cancer* 2013; 11:407–15; PMID:23835291; [hiip://dx.doi.org/10.1016/j.clgc.2013.04.029](http://dx.doi.org/10.1016/j.clgc.2013.04.029)
46. Rei M, Pennington DJ, Silva-Santos B. The emerging Protumor role of gammadelta T lymphocytes: implications for cancer immunotherapy. *Cancer Res* 2015; 75:798–802; PMID:25660949; [hiip://dx.doi.org/10.1158/0008-5472.CAN-14-3228](http://dx.doi.org/10.1158/0008-5472.CAN-14-3228)
47. Jin Z, Luo Q, Lu S, Wang X, He Z, Lai J, Chen S, Yang L, Wu X, Li Y. Oligoclonal expansion of TCR Vdelta T cells may be a potential immune biomarker for clinical outcome of acute myeloid leukemia. *J Hematol Oncol* 2016; 9:126; PMID:27863523; [hiip://dx.doi.org/10.1186/s13045-016-0353-3](http://dx.doi.org/10.1186/s13045-016-0353-3)
48. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Børresen-Dale AL et al. Signatures of mutational processes in human cancer. *Nature* 2013; 500:415–21; PMID:23945592; [hiip://dx.doi.org/10.1038/nature12477](http://dx.doi.org/10.1038/nature12477)
49. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 2013; 499:214–8; PMID:23770567; [hiip://dx.doi.org/10.1038/nature12213](http://dx.doi.org/10.1038/nature12213)
50. Li B, Li T, Pignon JC, Wang B, Wang J, Shukla SA, Dou R, Chen Q, Hodi FS, Choueiri TK et al. Landscape of tumor-infiltrating T cell repertoire of human cancers. *Nat Genetics* 2016; 48:725–32; PMID:27240091; [hiip://dx.doi.org/10.1038/ng.3581](http://dx.doi.org/10.1038/ng.3581)
51. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015; 160:48–61; PMID:25594174; [hiip://dx.doi.org/10.1016/j.cell.2014.12.033](http://dx.doi.org/10.1016/j.cell.2014.12.033)
52. Zitvogel L, Ayyoub M, Routy B, Kroemer G. Microbiome and Anti-cancer Immunosurveillance. *Cell* 2016; 165:276–87; PMID:27058662; [hiip://dx.doi.org/10.1016/j.cell.2016.03.001](http://dx.doi.org/10.1016/j.cell.2016.03.001)
53. Zitvogel L, Galluzzi L, Viaud S, Vetizou M, Daillere R, Merad M, Kroemer G. Cancer and the gut microbiota: an unexpected link. *Sci Transl Med* 2015; 7:271ps1; PMC4690201; [hiip://dx.doi.org/10.1126/scitranslmed.3010473](http://dx.doi.org/10.1126/scitranslmed.3010473)
54. Dimova T, Brouwer M, Gosselin F, Tassignon J, Leo O, Donner C, Marchant A, Vermijlen D. Effector Vgamma9Vdelta2 T cells dominate the human fetal gammadelta T-cell repertoire. *Proc Natl Acad Sci U S A* 2015; 112:E556–65; PMID:25617367; [hiip://dx.doi.org/10.1073/pnas.1412058112](http://dx.doi.org/10.1073/pnas.1412058112)
55. Bezanson J, Karpinski S, Shah VB, Edelman A. Julia: A fast dynamic language for technical computing. eprint arXiv:12095145 2012.
56. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. *Stat Med* 1990; 9:811–8; PMID:2218183; [hiip://dx.doi.org/10.1002/sim.4780090710](http://dx.doi.org/10.1002/sim.4780090710)
57. Therneau TM, Grambsch PM. Modeling survival data: Extending the cox model. New York: Springer, 2000.