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Pitfalls in the characterization of circulating and tissue-resident human $\gamma\delta$ T cells

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Short running title: Pitfalls in the characterization of $\gamma\delta$ T cells

Summary sentence: Overviewing the state-of-the-art and own experiences, highlighting
pitfalls with commercially-available reagents for $\gamma\delta$ T cell characterization by flow
cytometry, CyTOF, magnetic cell isolation or immunohistochemistry.

Keywords: Flow Cytometry, Mass Cytometry, Immunohistochemistry, Magnetic Cell
Isolation, Immunomonitoring

36 **Abbreviations**

- 37 ADCC: Antibody-dependent cellular cytotoxicity
- 38 AEC: Aminoethyl-carbazole
- 39 APC: Allophycocyanine
- 40 BV: Brilliant Violet
- 41 CMV: Cytomegalovirus
- 42 CyTOF: Mass cytometry
- 43 EBV: Epstein-Barr-Virus
- 44 EDTA: Ethylenediaminetetraacetic acid
- 45 EPCR: Endothelial protein C receptor
- 46 Er: Erbium
- 47 FITC: Fluorescein isothiocyanate
- 48 Gd: Gadolinium
- 49 IHC: Immunohistochemistry
- 50 Ir: Iridium
- 51 Monoclonal antibody: mAb
- 52 PBMC: Peripheral blood mononuclear cell
- 53 PBS: Phosphate-buffered saline
- 54 PE: Phycoerythrin
- 55 PHA-L: Phytohemagglutinin-L
- 56 PI: Positive isolation
- 57 PMA: Phorbol 12-myristate 13-acetate
- 58 Pr: Praseodymium
- 59 Sm: Samarium
- 60 TCR: T-cell receptor

Abstract

Dissection of the role and function of human $\gamma\delta$ T cells and their heterogeneous subsets in cancer, inflammation and auto-immune diseases is a growing and dynamic research field of increasing interest to the scientific community. Therefore, harmonization and standardization of techniques for the characterization of peripheral and tissue-resident $\gamma\delta$ T cells is crucial to facilitate comparability between published and emerging research. The application of commercially-available reagents to classify $\gamma\delta$ T cells, in particular the combination of multiple antibodies, is not always trouble-free, posing major demands on researchers entering this field. Occasionally, even entire $\gamma\delta$ T cell subsets may remain undetected when certain antibodies are combined in flow cytometric analysis with multicolor antibody panels, or might be lost during cell isolation procedures. Here, based on the recent literature and our own experience, we provide an overview of methods commonly employed for the phenotypic and functional characterization of human $\gamma\delta$ T cells including advanced polychromatic flow, mass cytometry, immunohistochemistry and magnetic cell isolation. We highlight potential pitfalls and discuss how to circumvent these obstacles.

Introduction

Knowledge of the orchestration of $\gamma\delta$ T cells in the ensemble of immunity is still limited, especially in humans. These “unconventional” T cells are a numerically minor population in peripheral blood, representing 1-10% of all T cells and are, unlike $\alpha\beta$ T cells, not MHC-restricted [1]. Knowledge of $\gamma\delta$ T cell receptor (TCR) ligands is sparse; only a few, structurally diverse molecules such as phosphoantigens, CD1, endothelial protein C receptor (EPCR) and other cell-surface structures have been identified [2], suggesting an immense potential for diversity. The $\gamma\delta$ T cell population comprises heterogeneous subsets with various functions including secretion of cytokines such as TNF, IFN- γ and IL-17 [3], cytotoxic activity via the granzyme-perforin axis and antibody-dependent cellular cytotoxicity (ADCC) by CD16 expressing cells [4], antigen-presentation functions [5] and interactions with B cells promoting immunoglobulin class switching [6]. V δ 1 $\gamma\delta$ T cells are the predominant T cell subset in some tissues, accounting for around 40% of all intra-epithelial lymphocytes in the large intestine, for example [7]. The V δ 1 TCR repertoire is often private, highly focused on a few clones, and displays features of the adaptive immune system [8]. On the other hand, V δ 2 $\gamma\delta$ T cells, which dominate the $\gamma\delta$ TCR repertoire in peripheral blood, have a semi-invariant TCR, a diverse public repertoire and mainly behave in an innate-like manner [9]. Due to their pleiotropic roles in immunity and implications in cancer, infectious and auto-immune diseases, $\gamma\delta$ T cells are of rapidly growing interest. Transcriptomic analyses have documented that intra-tumoral $\gamma\delta$ T cells may represent significant favorable prognostic immune cell populations in several different cancers [10] and Oberg et al. observed large numbers of different $\gamma\delta$ T cell subsets infiltrating isolated pancreatic and ovarian tumors *ex vivo* [11, 12, Oberg et al. this volume]. In areas other than cancer, $\gamma\delta$ T cells have been shown to

103 contribute to the immune response against CMV [13, 14] and malaria [15], and to be
104 involved in various inflammatory conditions [16]. It is therefore important to harmonize
105 and standardize techniques for the investigation of $\gamma\delta$ T cells in order to avoid potential
106 pitfalls when using and combining commercially-available antibodies and comparing
107 results between centers. Here, we aim to provide a basic framework for the phenotypic
108 and functional characterization of peripheral and tissue-resident $\gamma\delta$ T cells, including
109 magnetic cell isolation, advanced polychromatic flow cytometry, mass cytometry
110 (CyTOF) and immunohistochemistry (IHC).

111

112

Material and Methods

Study participants and sample acquisition

Blood samples were obtained from healthy adult volunteers at the Department of Hematology and Oncology, Children's Hospital, University of Tübingen (Project no. 38/2009BO2, 470/2013BO2, 673/2015BO2, 105/2017BO2 and 880/2017BO2) and from the biobank at the Interfaculty Institute of Cell Biology (IFIZ), Department of immunology, University of Tübingen (Project no. 156/2012BO1 and 633/2019BO2). Tissue samples from patients with EBV-associated Hodgkin's lymphoma (with pathological features assessed according to the WHO classification) and colon carcinoma (staged according to the UICC TNM classification system) were obtained at the University Hospital Schleswig-Holstein, Kiel (D430/09). Written informed consent was obtained from all blood and tissue donors. This study was conducted in accordance with the Declaration of Helsinki and applicable laws and regulations, and has been approved by the respective institutional review boards (Ethics Committees at the University Hospital Schleswig-Holstein in Kiel and at the University Hospital Tübingen).

Magnetic cell isolation

$\gamma\delta$ T cells were isolated from fresh peripheral blood mononuclear cells (PBMCs) via magnetic cell isolation using the following commercial kits: i) Immunomagnetic negative selection cell isolation kit: TCR $\gamma\delta$ + T Cell Isolation Kit Human (Miltenyi Biotec) or Immunomagnetic negative selection cell isolation kit: EasySep™ Human Gamma/Delta T Cell Isolation Kit (STEMCELL Technologies) or, as we show in Figure 1, customized versions of both kits were used, both omitting anti-CD16 antibodies. Immunomagnetic

positive selection was performed with a fluorochrome-labelled V δ 2-specific antibody (clone B6, BD) and for secondary labelling and anti-fluorochrome sorting, either anti-FITC Micro-beads (Miltenyi Biotec) or anti-PE MicroBeads Ultra Pure (Miltenyi Biotec) or an Immunomagnetic positive selection kit EasySep™ PE Positive Selection Kit II (STEMCELL Technologies) were used.

Polychromatic flow cytometry

Phenotypic and functional analysis followed standardized protocols on cryopreserved samples. For immunomonitoring studies, cryopreservation is still the gold standard to minimize bias introduced by batch to batch variance in polychromatic flow cytometry, although the expression pattern of some particular markers may be affected. In brief, peripheral blood was drawn and anticoagulated using EDTA, followed by Ficoll-Hypaque density gradient centrifugation to isolate PBMCs. After washing twice, the cells were cryopreserved in medium with 10% DMSO and 20% FCS in RPMI-1640 and stored at -196°C. Cryopreserved PBMCs were thawed, incubated with an Fc-receptor-blocking reagent (Gammunex, Grifols) and ethidium monoazide (EMA, Biotium) or LIVE/DEAD fixable red (Thermo Fisher Scientific) to label dead cells. Next, characteristic cell surface antigens of $\gamma\delta$ T cells were stained using the following monoclonal antibodies (mAbs) to illustrate various common panel compositions: CD3 Alexa Fluor 700 and CD3 BV510 (both clone UCHT1, Biolegend), pan- $\gamma\delta$ TCR Biotin (clone 11F2, Miltenyi Biotec), pan- $\gamma\delta$ TCR FITC (clone 11F2, BD), pan- $\gamma\delta$ TCR purified and pan- $\gamma\delta$ TCR PE (both clone IMMU510, Beckman Coulter), V δ 1 TCR FITC, V δ 1 TCR PerCP-Vio700 and V δ 1 TCR APC (all clone REA173, Miltenyi Biotec), V δ 2 TCR PerCP (clone B6, Biolegend), V δ 2

159 TCR PE and V δ 2 TCR FITC (both clone 123R3, Miltenyi Biotec), Streptavidin-PE
160 (Biolegend), F(ab')₂-Fragment goat anti mouse Pacific Orange (Invitrogen). For
161 characterization of established T cell clones, antibodies against V γ 2/3/4 (clone 23D12)
162 [17], V γ 3/5 (clone 56.3) [18] and pan- $\alpha\beta$ TCR FITC (clone IP26, Biolegend) were used.
163 The generation of 56.3⁺ T cell clones has been described previously. In brief, 56.3
164 positive cells were selected by MACS from PBMCs from healthy donors and were
165 cloned at 0.3 cells per well in the presence of irradiated feeder cells, PHA 0.5 μ g/mL and
166 IL-2 (50 IU) [18].

167 For intracellular staining, cryopreserved PBMCs that had been stimulated with PMA (20
168 ng/ml, Sigma) and Ionomycin (750 ng/ml, Merck) or Zoledronate (5 μ M, Hexal) and
169 incubated with Brefeldin A (GolgiPlug, BD), Monensin (GolgiStop, BD) and CD107a
170 Pacific Blue (clone H4A3, Biolegend) for 12h, were fixed and permeabilized using a
171 fixation/permeabilization solution kit (BD) and stained with the following antibodies: IFN-
172 γ PE-Cy7 (clone B27, Biolegend), IL-17A BV711 (clone BL168, Biolegend), TNF- α Alexa
173 Fluor 700 (clone Mab11, Biolegend). Proliferation in response to stimulation with PHA-L
174 (Roche) was tracked by labelling cells with CellTraceViolet (Thermo Fisher Scientific).
175 Optimal results were achieved when seeding 0.2×10^6 cells in 200 μ L medium per well in
176 96-well U bottom plates (Greiner). Proportional upscaling, e.g. seeding 0.6×10^6 cells in
177 600 μ L medium in a 48-well plate, was possible though not optimal, enabling analysis of
178 donors with low percentages of $\gamma\delta$ T cells, patient samples and subpopulations. Panels
179 for functional analysis and for tracking of proliferation are summarized in Table 1. Data
180 were acquired using a three laser LSR II (BD) with FACSDiva software V6.1.3 (BD) and
181 customized filter settings and data analysis was performed with FlowJo V10.5.3 (BD;
182 gating strategy, Supplementary Figure 1).

Mass cytometry

Mass cytometric analysis followed established protocols [19]. In brief, cryopreserved PBMCs were thawed, dead cells were stained with Rhodium 103 and cell surface Fc receptors were blocked. Samples were then incubated with an antibody cocktail for cell surface staining which contained antibodies against CD3 170Er (clone UCTH1, Fluidigm), pan- $\gamma\delta$ TCR 152Sm (clone 11F2, Fluidigm), V δ 2 TCR141Pr (clone B6, Biolegend; in house-conjugated, using Fluidigm's Maxpar antibody labelling kit; catalog no.: 201141B) and V δ 1 TCRFITC (clone TS8.2, Thermo Fisher Scientific) amongst others. Next, an anti-FITC 160Gd antibody (Fluidigm) was used to stain V δ 1 T cells. After fixation, permeabilization and staining of intracellular antigens, samples were incubated for at least 12 hours in a solution of 4% paraformaldehyde in PBS. Samples were stained in batches with 125Ir on the mornings of the respective days of data acquisition. Each sample was rebuffed in purified water directly before acquisition on a Helios system (Fluidigm) at King's College London. Data analysis was performed with FlowJo V10.5.3 (BD).

Immunohistochemistry

Immunostaining with mAbs against $\gamma\delta$ TCR (clone γ 3.20, Thermo Fisher Scientific), V γ 9 TCR (clone 7A5) [20], V γ 2/3/4 TCR (clone 23D12) [17, 18] or mouse IgG1 isotype control (Thermo Fisher) of serial paraffin-embedded tissue sections from patients with EBV-associated Hodgkin's lymphoma was carried out after deparaffinization with the fully automated Bond Max-system using the Bond Polymer Refine Detection Kit (Leica-Menarini). Automated antigen retrieval was performed in in Bond Epitope Retrieval

206 Solution 1 (citrate buffer pH 6.0; Leica-Menarini). Additionally, immunostaining with
207 V γ 2/3/4 TCR (clone 23D12), V δ 1 TCR (clone R3.12, Beckman Coulter) or mouse IgG1
208 isotype control of cryopreserved sections from patients with colon carcinoma was done
209 after acetone fixation and blockade with 4% bovine serum albumin. As second step,
210 antibody EnVision mouse horseradish peroxidase (DAKO) was used. The substrate
211 reaction was performed using the AEC substrate for peroxidase (DAKO). Finally,
212 sections were stained with hematoxylin and embedded in glycerine gelatine (Merck).

Results and Discussion

Magnetic isolation of $\gamma\delta$ T cells and subsets

Isolation of $\gamma\delta$ T cells is a critical procedure and the methodology of choice needs to be adapted to the design of each particular experiment. The total $\gamma\delta$ T cell compartment can be positively or negatively selected using kits from commercial suppliers (e.g. Miltenyi Biotec and STEMCELL Technologies) (Figure 1A, 1B). In addition to beads directly coupled to a $\gamma\delta$ TCR-targeting antibody (Miltenyi Biotec), secondary labelling strategies allow sorting and discriminating $\gamma\delta$ T cell subsets. Multi-sort beads facilitate multiple rounds of positive selection because these labels can be removed. Positive isolation (PI) is suitable for separating aminobisphosphonate-expanded $\gamma\delta$ T cells or $\gamma\delta$ T cells expressing particular V γ /V δ elements from PBMCs (Figure 1B), and $\gamma\delta$ T cells from intra-tumoral lymphoid compartments, e.g. for TCR sequence analysis, offering the advantage of sparing other (infiltrating) cells for further isolation/analysis. On the other hand, for studies examining cell activation status, receptor signaling, cytokine expression and/or cytotoxicity, $\gamma\delta$ T cells should be negatively selected to avoid antibody-cross-linking of the $\gamma\delta$ TCR. Although some manufacturers claim that immune cells experience no activation through their PI procedure, $\gamma\delta$ T cells do upregulate CD69 after PI [21] and show significant functional bias compared to negatively selected cells from the same donor. The EasySep™ Human Gamma/Delta T Cell Isolation Kit from STEMCELL Technologies removes non- $\gamma\delta$ T cells with tetrameric antibody complexes and dextran-coated magnetic particles by retaining them inside a tube using a strong magnetic field. Whereas labelled cells remain attached to the tube wall, the remaining (negatively-selected) cells can be poured into a separate tube. Similarly, Miltenyi

Biotec's biotin-conjugated depletion cocktail eliminates non- $\gamma\delta$ T cells via a secondary magnetic label that retains them on a "MACS® Column" in a magnetic field. High purities – above 98.5% $\gamma\delta$ T cells – are routinely achieved with both methods independent of the initial content of $\gamma\delta$ T cells and obtained isolates do not contain any undesired $\alpha\beta$ T cells (Fig. 1A). The "untouched" negatively-sorted cells are not activated. The STEMCELL Technologies procedure is faster and yields remain quantitative, even when drastically downscaling initial cell numbers (due to zero dead space volume). Moreover, physiological stressors such as mechanical stress, centrifugal forces, and extensive incubation at unphysiologically low temperatures during labelling and purification are avoided.

Pitfalls that may be encountered, when using either of these negative selection procedures are: i) depleted cells are heavily labelled and can not be used for further downstream applications; ii) depletion cocktails often contain antibodies targeting molecules also expressed by subpopulations of $\gamma\delta$ T cells, e.g. CD16, which may severely bias subsequent (functional) studies, such as ADCC or gene expression analyses. We therefore suggest the use of anti-NKp46 (a lineage marker of NK cells) and anti-NKp30 instead of anti-CD16 for NK-cell elimination during negative $\gamma\delta$ T cell isolation. NKp46 and NKp30 are expressed on peripheral NK cells but not on $\gamma\delta$ T cells in the peripheral blood. However, it should be noted that long-term activation of $\gamma\delta$ T cells induces the expression of NKp46, NKp30 and also of Nkp44 in certain subsets (Supplementary Figure 2) [22]. Until commercial kits that exclude anti-human CD16 mAbs become available customized kits are an option.

When aiming to negatively isolate specific subsets of $\gamma\delta$ T cells, a combination of strategies is required. For selecting untouched V δ 2 $\gamma\delta$ T cells from PBMCs, V δ 1 and

other $\gamma\delta$ T cell subsets such as V δ 3 $\gamma\delta$ T cells must be removed (via PI) before $\gamma\delta$ T cell negative selection yields the V δ 2 T cell subset isolate. Unless an anti-human V δ 3 mAb is available, individuals with high numbers of V δ 1⁺V δ 2⁻ $\gamma\delta$ T cells are not suitable for V δ 2 negative selection (Figure 1C). A potential candidate for this approach might be the monoclonal anti-human T cell receptor V δ 3 antibody (clone P11.5B), which was previously distributed by Gentaur and Coulter. However, at the time of writing, the commercial availability of this antibody, its format and thus its suitability for magnetic cell isolation remain unclear.

Phenotypic and functional analysis via polychromatic flow cytometry

When designing an antibody panel for multicolor flow cytometry a few considerations need to be taken into account, in order to avoid certain $\gamma\delta$ T cell subsets remaining undetected, and thus biasing subsequent analysis. Below, we aim to highlight the commonest problems and to provide a framework for the flow cytometric analysis of $\gamma\delta$ T cells. Furthermore, we briefly present panels designed for the investigation of phenotypic markers, as well as functional and proliferative properties.

We previously reported that the unconjugated pan- $\gamma\delta$ TCR antibody clone 11F2 was the only tested antibody able to detect all $\gamma\delta$ T cells when combined with a V δ 2 antibody (B6, IMMU389), while the conjugated forms of the commercially-available clones 11F2, B1/B1.1 do not always stain 100% of the $\gamma\delta$ T cell population [23]. Moreover, clone B1/B1.1 is unsuitable for multicolor flow cytometry panels including a CD3 antibody due to interference between these two antibodies [23]. The recently-developed generation of $\gamma\delta$ TCR subset-specific antibodies, namely V δ 1 clone REA173 and V δ 2 clone 123R3 in combination with pan- $\gamma\delta$ TCR antibodies (11F2, IMMU510) seems to offer a standard for

285 flow cytometric characterization of $\gamma\delta$ T cells (Figure 2A), overcoming the above-
286 mentioned issues (Supplementary Figure 3A). We tested directly fluorophore-labelled
287 and secondarily detected pan- $\gamma\delta$ TCR antibodies (11F2 and IMMU510) to achieve an
288 optimal balance between a rapid and straightforward staining protocol, low background
289 signal and a high staining index (Figure 2B). Separation of the $\gamma\delta$ T cell population via
290 directly-labelled pan- $\gamma\delta$ TCR antibodies (11F2, IMMU510) can be problematic in the
291 detection of V δ 1 T cells with low surface TCR expression levels (Supplementary Figure
292 3B) as seen in patient samples or in *in vitro* culture systems. Detection of the purified
293 formats of these antibodies via fluorophore-labelled anti-mouse antibodies is generally
294 not preferable due to prolonged staining procedures and high background signals. We
295 identified the biotinylated 11F2 clone as an optimal choice (Figure 2A, 2B), because the
296 biotin/streptavidin detection system combines signal amplification with simultaneously
297 low background, resulting in improved separation of the target population, especially in
298 fixed cells (Supplementary Figure 3C). The prolonged staining protocol (1) pan- $\gamma\delta$ TCR,
299 (2) streptavidin conjugate, (3) surface antibody cocktail can be reduced by integration of
300 the streptavidin conjugate into the antibody master mix for extracellular staining. In
301 general, use of PE-conjugates for pan $\gamma\delta$ T cell antibodies is recommended, because
302 PE itself is recognized by certain $\gamma\delta$ TCRs and thus stains a small percentage of
303 peripheral $\gamma\delta$ T cells [24]. This should also be kept in mind when tandem conjugates
304 containing PE (e.g. PE-Cy7, PE-Cy5.5 etc.) are included in the antibody panel. As stated
305 above, the V δ 2 123R3 antibody is, in contrast to clone B6, compatible with fluorophore-
306 conjugated pan- $\gamma\delta$ TCR antibodies. Of note, clone B6 might be specific for the V γ 9V δ 2
307 TCR pairing, because it has been reported that detection of the V γ 9⁻V δ 2⁺ population is

not possible with clone B6, but clone 123R3 can be used as an alternative to also detect the rare population of $V\gamma 9^- V\delta 2^+$ T cells [9].

On the basis of this framework for detection of $\gamma\delta$ T cells, we developed several polychromatic antibody panels: i) a phenotypic $\gamma\delta$ T cell panel including markers for differentiation state [23] ii) a panel to monitor functionality of $\gamma\delta$ T cells including the degranulation marker CD107a and the cytokines TNF, IFN- γ and IL-17A (Figure 3A) and iii) a panel to track $\gamma\delta$ T cell proliferative capacity using CellTraceViolet (Figure 3B). The above-described basic phenotypic markers (pan- $\gamma\delta$ TCR, V δ 1 and V δ 2) also worked well after fixation and permeabilization.

Using a combination of available mAbs, flow cytometry is also useful to monitor the entire expressed human V γ repertoire [18]. Such an analysis is based on antibodies detecting V γ 9 (e.g., clone 7A5) [20], V γ 2/3/4 (clone 23D12) [17, 25], V γ 3/5 (clone 56.3) [18] and V γ 8 (clone R4.5.1) [26, 27]. As an example, the combination of mAb 56.3 and 23D12 unequivocally identifies $\gamma\delta$ T cell clones expressing V γ 3 (56.3 $^+$ 23D12 $^+$) and V γ 5 (56.3 $^+$ 23D12 $^-$) (Figure 4A, 4B). Moreover, such antibodies are useful for detecting rare $\alpha\beta$ T cells with a trans-rearranged TCR [13, 28]. As shown in Figure 4C, the 56.3-positive clone (established by positive selection of 56.3-positive cells from PBMC) stains with a pan- $\alpha\beta$ T cell antibody (clone IP26) but not with a pan- $\gamma\delta$ antibody (clone 11F2). These cells carry an in-frame V γ 5-J β -C β trans-rearrangement [18].

Identification of immune signatures via mass cytometry

A good choice of markers for basic identification of cell populations intended to be divided into numerous subsets is essential for mass cytometric analysis, as currently up

to 40 channels can be acquired in parallel and multidimensional, automated data analysis is performed. At the time of writing, the only commercially-available pan $\gamma\delta$ T cell antibody suitable for mass cytometry derives from the 11F2 clone. We identified even greater problems in mass cytometry than those we faced in polychromatic flow cytometry when using the 11F2 TCR $\gamma\delta$ 152 Sm antibody in combination with custom-made B6 TCR V δ 2 141 Pr and TS8.2 V δ 1 FITC antibodies (detected via an anti-FITC 160 Gd). Large proportions of V δ 1 T cells were stained with the pan- $\gamma\delta$ TCR antibody, but none or only a fraction of the V δ 2 T cells (Figure 2C). Steric hindrance caused by close proximity of the recognized epitopes and size and nature of the antibody tags might account for these observations. Further testing of the combinations of V δ 1 REA173 and V δ 2 123R3 with the pan- $\gamma\delta$ 11F2 antibodies that achieved good resolution in polychromatic flow cytometry is also warranted in mass cytometry.

Immunohistochemical detection of tissue-associated $\gamma\delta$ T cells

IHC-based tissue analysis enables tissue-infiltrating ($\gamma\delta$) T cell subsets to be analyzed in the context of their native surroundings, thereby providing a complementary approach to the above-discussed flow cytometry experiments. In the cancer setting, monitoring the abundance of tumor-infiltrating $\gamma\delta$ T cells and the localization of distinct $\gamma\delta$ T cell subsets can provide a more comprehensive assessment of the tumor status [11]. The best choice for analysis of the $\gamma\delta$ TCR expression was the anti-TCR γ clone γ 3.20. As we previously reported, IHC staining of consecutive paraffin-embedded sections of pancreatic ductal adenocarcinoma tissue revealed that a large proportion of the CD3⁺CD8⁺ T cells in the ductal epithelium were $\gamma\delta$ T cells [11, 12]. Unfortunately the

clone $\gamma 3.20$ is no longer available, but Jungbluth et al. recently reported that the TCR δ antibody clone H-41 (SC-100289, Santa Cruz) is an alternative for the detection of $\gamma\delta$ T cells in paraffin-embedded tissue [29]. To visualize the distribution of different $\gamma\delta$ T cell-subsets, serial tissue sections were stained with our in-house V $\gamma 9$ and V $\gamma 2/3/4$ antibodies. Analysis of sections from patients with EBV-associated Hodgkin's lymphoma showed that most of the $\gamma\delta$ T cells from these patients expressed V $\gamma 9$, whereas $\gamma\delta$ T cells expressing V $\gamma 2$, 3 or 4 were nearly absent (Figure 5A). Furthermore, staining of cryosections obtained from patients with colon adenocarcinoma using the V $\delta 1$ and V $\gamma 2/3/4$ antibodies revealed that these $\gamma\delta$ T cells are enriched in respective malignancies, as shown for one representative patient (Figure 5B). This indicates that the V $\gamma 2/3/4$ clone 23D12 is also suitable for staining cryosections.

Concluding remarks

The variety of available reagents for the characterization of $\gamma\delta$ T cells is, as the research field itself, dynamic and growing, but currently still very limited. Here, we outlined a framework for the phenotypic and functional characterization of human $\gamma\delta$ T cells based on currently available reagents. We are certainly aware that the above-described antibodies and antibody combinations still have room for improvement. On the part of the manufacturers, a broader spectrum of fluorophore-antibody conjugates, preferably of smaller size to minimize potential steric hindrance problems, would be appreciated. One must also be aware that rare $\alpha\beta$ T cells harbor a TCR trans-rearrangement and thus may express a V γ rather than a V β element. Such $\alpha\beta$ T cells stain with anti-V γ antibodies as shown here for V $\gamma 5$, and this has been reported previously also for V $\gamma 4$

and V γ 9 [25, 28]. When characterizing $\gamma\delta$ T cells in tissue samples the following issues should be taken into account. First, one should carefully select the enzymes used for tissue dissociation in order to avoid the loss of certain cell surface markers. Besides that, sample size and in some cases the low abundance of $\gamma\delta$ T cells may be limiting factors. For $\gamma\delta$ T cell immunomonitoring within tumor-infiltrating lymphocytes or tumor-ascites lymphocytes by flow cytometry, the additional use of an anti-CD45 mAb in a multicolor panel is recommended for precise analysis of the different $\gamma\delta$ and $\alpha\beta$ T cell subsets surrounded by many other tumor-associated immune cells and tumor cells (Oberg et al. this volume). Furthermore, the development of antibodies suitable for staining paraffin-embedded tissue sections, and antibodies compatible with the fluorescence microscopy-based MACSima imaging system or the mass cytometry-based Hyperion imaging system could take $\gamma\delta$ T cell research to a new level, supporting the various promising attempts to exploit these remarkable cells for treating infectious and non-infectious diseases as well as malignancies.

Authorship

Conceptualization: NB, DW, HO, GP, SK, KS, KWH

Investigation: NB, DW, HO, JB, BW, CG, SS, CR, HH, CP, PN, SK, KS, KWH

Data curation - formal analysis: NB, DW, HO, JB, SS, CR, HH, PN, SK, KS, KWH

Writing - original draft: NB, DW, KS, KWH

Writing - review, editing, and revision: NB, DW, HO, GP, MWL, SK, DK, KS, KWH

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Conflict of Interest Disclosure

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Figure legends

Figure 1: Magnetic isolation of $\gamma\delta$ T cells from PBMCs **(A)** Representative FACS plots showing the CD3 compartment pre and post isolation of untouched $\gamma\delta$ T cell preparations. Presented data reflect the reproducibly high purities that are achieved using kits from either Miltenyi Biotec (Manufacturer M) or STEMCELL Technologies (Manufacturer S). Viability is reproducibly above 99%. **(B)** Representative FACS plots of cell isolates gained with positive selection strategy using kits from Miltenyi Biotec or STEMCELL Technologies. Shown are target cells in the CD3⁺ gate. Viability is reproducibly above 98%. **(C)** V δ 1⁻V δ 2⁻ cell fractions from two healthy adult donors are presented (gated on CD3⁺ T cells, no contaminating $\alpha\beta$ T cells are present). Without the availability of an anti-human V δ 3 antibody these donors cannot be used for the isolation of negatively selected pure V δ 2 $\gamma\delta$ T cells.

Figure 2: Phenotypic characterization of $\gamma\delta$ T cells via flow cytometry and CyTOF **(A)** Phenotypic characterization of the V δ 1 and V δ 2 sub-populations via flow cytometry. Gating on peripheral, viable, CD3⁺, lymphocytes showed that the $\gamma\delta$ TCR antibody (clone 11F2) recognized all V δ 1⁺ (clone REA173) and V δ 2⁺ (clone 123R3) cells. Sufficient separation of the sub-populations, gated on $\gamma\delta$ TCR⁺ T cells, was achieved. **(B)** Direct and indirect staining of the $\gamma\delta$ TCR with the clones 11F2 and IMMU510. The best separation with the lowest background signal in flow cytometry was achieved with the biotinylated 11F2 clone. The population was gated on viable, CD3⁺ lymphocytes. **(C)** Characterization of $\gamma\delta$ T cell sub-populations by CyTOF in a fixed and permeabilized representative sample. Both plots display the same population that was gated on viable,

CD45⁺, CD14⁻, CD33⁻, CD20⁻, CD3⁺ T cells. $\gamma\delta$ TCR (clone 11F2; Sm152) stained the vast majority of the indirectly stained V δ 1⁺ (clone TS8.2 FITC a Gd160), but none of the V δ 2⁺ (clone B6 ; Pr141) $\gamma\delta$ T cells.

Figure 3: Functional characterization of $\gamma\delta$ T cells via flow cytometry. (A) Analysis of cytokine expression in fixed PBMC samples after stimulation with Zoledronate (V δ 2⁺) or PMA/Ionomycin (V δ 1⁺). V δ 1⁺ and V δ 2⁺ cells were gated on viable, CD3⁺, $\gamma\delta$ TCR⁺ lymphocytes. (B) Proliferation of CD3⁺ $\gamma\delta$ TCR⁻, V δ 1⁺ and V δ 2⁺ T cells after stimulation with PHA-L was tracked on the basis of dye dilution using CellTrace Violet. $\gamma\delta$ T cell subpopulations were gated on viable, CD3⁺, $\gamma\delta$ TCR⁺ lymphocytes. The proliferation modeling tool included in the FlowJo software enabled a more in-depth analysis of the proliferative properties.

Figure 4: Identification of V γ 3 and V γ 5 $\gamma\delta$ and $\alpha\beta$ T cell clones. Viable cells were discriminated by gating on lymphocytes (FSC vs. SSC) and by near infra-red live/dead-staining. $\gamma\delta$ and $\alpha\beta$ T cell clones stained by mAb V γ 3/5 (clone 56.3) were co-labeled with the mAbs recognizing the $\alpha\beta$ TCR (clone IP26), $\gamma\delta$ TCR (clone 11F2), V γ 2/3/4 (clone 23D12). (A) V γ 5 $\gamma\delta$ clone (IP26⁻,11F2⁺,56.3⁺,23D12⁻) (B) V γ 3 $\gamma\delta$ clone (IP26,11F2⁺,56.3⁺,23D12⁺); (C) V γ 5 $\alpha\beta$ clone (IP26⁺,11F2⁻,56.3⁺,23D12⁻).

Figure 5: Distribution of different T cell subsets in EBV-associated Hodgkin's lymphoma tissues and colon adenocarcinoma tissue. (A) Serial paraffin-embedded

568 tissue sections from patients with EBV-associated Hodgkin's lymphoma were stained
569 with $\gamma\delta$ TCR (clone γ 3.20), V γ 9 (clone 7A5), V γ 2/3/4 (clone 23D12) mAbs as indicated in
570 one representative donor. **(B)** Serial cryosections obtained from colon adenocarcinoma
571 patients were stained with IgG control, V δ 1 (clone R3.12), V γ 2/3/4 (clone 23D12) mAbs
572 as indicated in one representative patient. IHC staining was performed as described in
573 the Materials and Methods section.

Table 1: Monoclonal antibody panels for the functional characterization and for tracking the proliferation of $\gamma\delta$ T cells via flow cytometry

Specificity	Fluorophore	Clone
Functional characterization		
Dead cells	Fixable red	
CD3	BV510	UCHT1
$\gamma\delta$ TCR	Biotin + Streptavidin-PE	11F2
V δ 1	PerCP-Vio700	REA173
V δ 2	FITC	123R3
IFN- γ	PE-Cy7	B27
TNF- α	Alexa Fluor 700	Mab11
IL17A	BV711	BL168
CD107a	Pacific Blue	H4A3
Tracking of proliferation		
Dead cells	Fixable red	
CD3	Alexa Fluor 700	UCHT1
$\gamma\delta$ TCR	Biotin + Streptavidin-PE	11F2
V δ 1	APC	REA173
V δ 2	FITC	123R3

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5