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ARTICLE

Pitfalls in the characterization of circulating and tissue-resident human γδ T cells

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Abstract

Dissection of the role and function of human γδ 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 γδ T cells is crucial to facilitate comparability between published and emerging research. The application of commercially available reagents to classify γδ T cells, in particular the combination of multiple Abs, is not always trouble-free, posing major demands on researchers entering this field. Occasionally, even entire γδ T cell subsets may remain undetected when certain Abs are combined in flow cytometric analysis with multicolor Ab 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 γδ T cells including advanced polychromatic flow cytometry, mass cytometry, immunohistochemistry, and magnetic cell isolation. We highlight potential pitfalls and discuss how to circumvent these obstacles.

KEYWORDS

flow cytometry, immunohistochemistry, immunomonitoring, magnetic cell isolation, mass cytometry, γδ T cells

Abbreviations: Ab, antibody; ADCC, Ab-dependent cellular cytotoxicity; AEC, aminoethyl-carbazole; Ag, antigen; APC, allophycocyanine; BV, Brilliant Violet; CyTOF, mass cytometry; EPCR, endothelial protein C receptor; Er, erbium; Gd, gadolinium; IHC, immunohistochemistry; Ir, iridium; PBMC, peripheral blood mononuclear cell; PI, positive isolation; Pr, praseodymium; Sm, samarium.

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1 | 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 $\gamma\delta$ 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 Ab-dependent cellular cytotoxicity (ADCC) by CD16 expressing cells,[4] Ag-presentation functions,[5] and interactions with B cells promoting Ig class switching.[6] Vδ1 $\gamma\delta$ 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 Vδ1 TCR repertoire is often private, highly focused on a few clones, and displays features of the adaptive immune system.[8] On the other hand, Vδ2 $\gamma\delta$ 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 intratumoral $\gamma\delta$ 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 contribute to the immune response against CMV[13,14] and malaria,[15] and to be involved in various inflammatory conditions.[16] It is therefore important to harmonize and standardize techniques for the investigation of $\gamma\delta$ T cells in order to avoid potential pitfalls when using and combining commercially available Abs and comparing results between centers. Here, we aim to provide a basic framework for the phenotypic and functional characterization of peripheral and tissue-resident $\gamma\delta$ T cells, including magnetic cell isolation, advanced polychromatic flow cytometry, mass cytometry (CyTOF), and immunohistochemistry (IHC).

2 | MATERIALS AND METHODS

2.1 | 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).

2.2 | 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: Immunomagnetic negative selection cell isolation kit: TCRγ/δ+ T Cell Isolation Kit Human (Miltenyi Biotec) or EasySep™ Human Gamma/Delta T Cell Isolation Kit (STEMCELL Technologies) or, as we show in Fig. 1, customized versions of both kits were used, both omitting anti-CD16 Abs. Immunomagnetic positive selection was performed with a fluorochrome-labeled Vδ2-specific Ab (clone B6, BD) and for secondary labeling 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) was used.

2.3 | 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 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), Vδ1 TCR FITC, Vδ1 TCR PerCP-Vio700 and Vδ1 TCR APC (all clone REA173, Miltenyi Biotec), Vδ2 TCR PerCP (clone B6, Biolegend), Vδ2 TCR PE and Vδ2 TCR FITC (both clone 123R3, Miltenyi Biotec), Streptavidin-PE (Biolegend), F(ab')2-Fragment goat anti mouse Pacific Orange (Invitrogen). For characterization of established T cell clones, Abs against Vγ2/3/4 (clone 23D12),[17] Vγ3/5 (clone 56.3)[18] and pan-$\alpha\beta$ TCR FITC (clone IP26, Biolegend) were used. The generation of 56.3$^+$ T cell clones has been described previously. In brief, 56.3 positive cells were

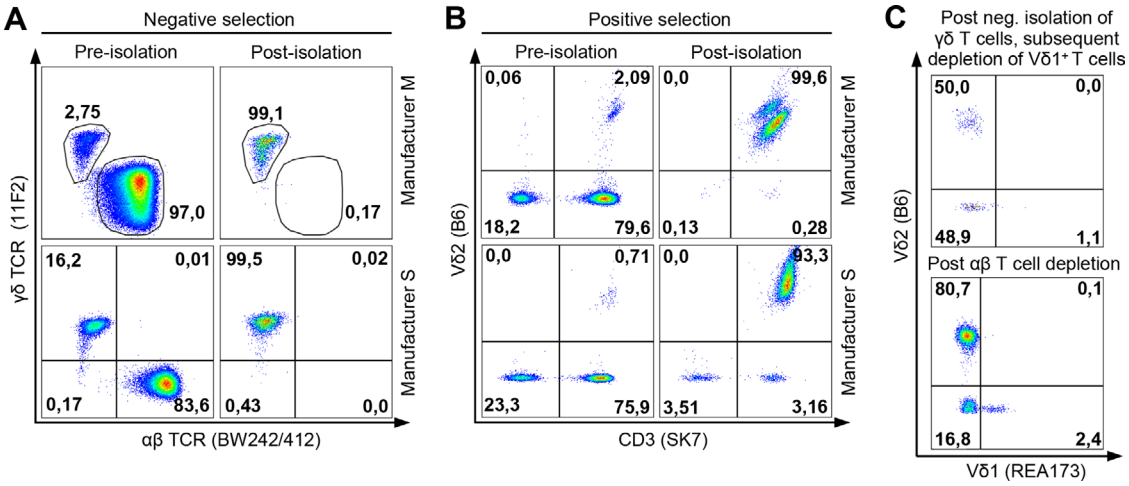

FIGURE 1 **Magnetic isolation of $\gamma\delta$ T cells from PBMCs.** (**A**) Representative FACS plots showing the CD3 compartment pre and post isolation of untouched $\gamma\delta$ T cell preparations. Presented data reflect the reproducibly high purities that are achieved using kits from either Miltenyi 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**) $V\delta1^-V\delta2^-$ 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 $V\delta3$ Ab these donors cannot be used for the isolation of negatively selected pure $V\delta2$ $\gamma\delta$ T cells

selected by MACS from PBMCs from healthy donors and were cloned at 0.3 cells per well in the presence of irradiated feeder cells, PHA 0.5 μg/mL and IL-2 (50 IU).[18]

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

TABLE 1 Monoclonal Ab 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
$V\delta1$	PerCP-Vio700	REA173
$V\delta2$	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
$V\delta1$	APC	REA173
$V\delta2$	FITC	123R3

2.4 | 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 Ab cocktail for cell surface staining which contained Abs against CD3 170Er (clone UCTH1, Fluidigm), pan-$\gamma\delta$ TCR 152Sm (clone 11F2, Fluidigm), $V\delta2$ TCR 141Pr (clone B6, Biolegend; in house-conjugated, using Fluidigm´s Maxpar Ab labeling kit) and $V\delta1$ TCR FITC (clone TS8.2, Thermo Fisher Scientific) among others. Next, an anti-FITC 160Gd Ab (Fluidigm) was used to stain $V\delta1$ T cells. After fixation, permeabilization, and staining of intracellular antigens, samples were incubated for at least 12 h 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 rebuffered 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).

2.5 | Immunohistochemistry

Immunostaining with mAbs against $\gamma\delta$ TCR (clone γ3.20, Thermo Fisher Scientific), Vγ9 TCR (clone 7A5),[20] Vγ2/3/4 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 Ag retrieval was performed in Bond Epitope Retrieval Solution 1 (citrate buffer pH 6.0; Leica-Menarini). Additionally, immunostaining with Vγ2/3/4 TCR (clone 23D12), Vδ1 TCR (clone R3.12, Beckman Coulter), or mouse IgG1 isotype control of cryopreserved sections from patients with colon carcinoma was done after acetone fixation and blockade with 4% BSA. As second step, Ab EnVision mouse HRP (DAKO) was used. The substrate reaction was performed using the AEC substrate for peroxidase (DAKO). Finally, sections were stained with hematoxylin and embedded in glycerine gelatine (Merck).

3 | RESULTS AND DISCUSSION

3.1 | 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; Fig. 1A and B). In addition to beads directly coupled to a $\gamma\delta$ TCR-targeting Ab (Miltenyi Biotec), secondary labeling 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 Vγ/Vδ elements from PBMCs (Fig. 1B), and $\gamma\delta$ T cells from intratumoral 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 Ab-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 Ab complexes and dextran-coated magnetic particles by retaining them inside a tube using a strong magnetic field. Whereas labeled 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 an "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 labeling and purification are avoided.

Pitfalls that may be encountered, when using either of these negative selection procedures are: (i) depleted cells are heavily labeled and cannot be used for further downstream applications; (ii) depletion cocktails often contain Abs targeting molecules also expressed by subpopulations of $\gamma\delta$ T cells, for example, 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 Fig. 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 Vδ2 $\gamma\delta$ T cells from PBMCs, Vδ1 and other $\gamma\delta$ T cell subsets such as Vδ3 $\gamma\delta$ T cells must be removed (via PI) before $\gamma\delta$ T cell negative selection yields the Vδ2 T cell subset isolate. Unless an anti-human Vδ3 mAb is available, individuals with high numbers of Vδ1$^-$Vδ2$^-$ $\gamma\delta$ T cells are not suitable for Vδ2 negative selection (Fig. 1C). A potential candidate for this approach might be the monoclonal anti-human T cell receptor Vδ3 Ab (clone P11.5B), which was previously distributed by Gentaur and Coulter. However, at the time of writing, the commercial availability of this Ab, its format, and thus its suitability for magnetic cell isolation remain unclear.

3.2 | Phenotypic and functional analysis via polychromatic flow cytometry

When designing an Ab 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 Ab clone 11F2 was the only tested Ab able to detect all $\gamma\delta$ T cells when

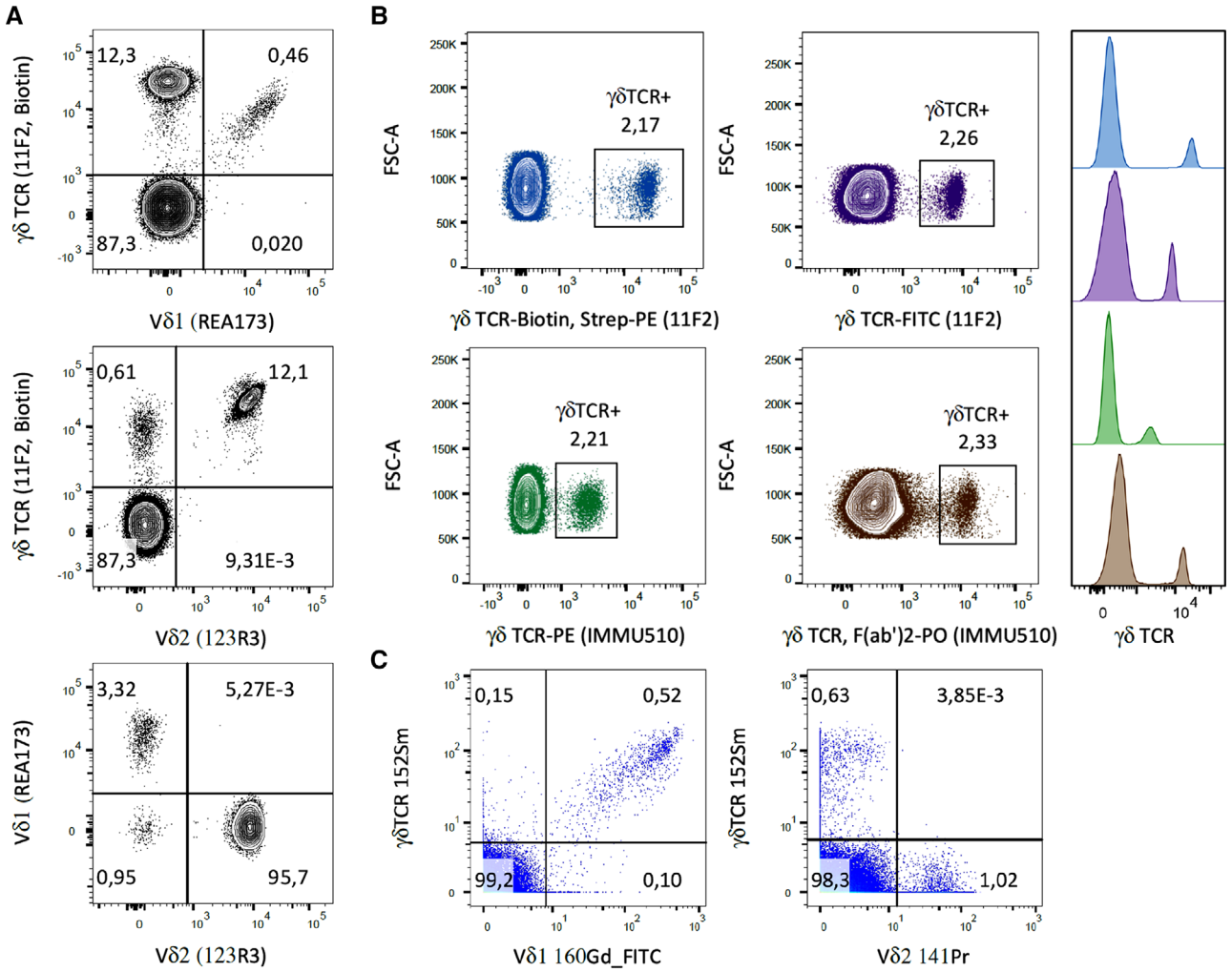

FIGURE 2 **Phenotypic characterization of $\gamma\delta$ T cells via flow cytometry and CyTOF.** (**A**) Phenotypic characterization of the Vδ1 and Vδ2 sub-populations via flow cytometry. Gating on peripheral, viable, CD3$^+$, lymphocytes showed that the $\gamma\delta$ TCR Ab (clone 11F2) recognized all Vδ1$^+$ (clone REA173) and Vδ2$^+$ (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 Vδ1$^+$ (clone TS8.2 FITC a Gd160), but none of the Vδ2$^+$ (clone B6; Pr141) $\gamma\delta$ T cells

combined with a Vδ2 Ab (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 Ab due to interference between these two Abs.[23] The recently developed generation of $\gamma\delta$ TCR subset-specific Abs, namely Vδ1 clone REA173 and Vδ2 clone 123R3 in combination with pan-$\gamma\delta$ TCR Abs (11F2, IMMU510) seems to offer a standard for flow cytometric characterization of $\gamma\delta$ T cells (Fig. 2A), overcoming the above-mentioned issues (Supplementary Fig. 3A). We tested directly fluorophore-labeled and secondarily detected pan-$\gamma\delta$ TCR Abs (11F2 and IMMU510) to achieve an optimal balance between a rapid and straightforward staining protocol, low background signal, and a high staining index (Fig. 2B). Separation of the $\gamma\delta$ T cell population via directly labeled pan-$\gamma\delta$ TCR Abs (11F2, IMMU510) can be problematic in the detection of Vδ1 T cells with low surface TCR expression levels (Supplementary Fig. 3B) as seen in patient samples or in in vitro culture systems. Detection of the purified formats of these Abs via fluorophore-labeled anti-mouse Abs is generally not preferable due to prolonged staining procedures and high background signals. We identified the biotinylated 11F2 clone as an optimal choice (Fig. 2A and B), because the biotin/streptavidin detection system combines signal amplification with simultaneously low background, resulting in improved separation of the target population, especially in fixed cells (Supplementary Fig. 3C). The prolonged staining protocol (1) pan-$\gamma\delta$ TCR, (2) streptavidin conjugate, and (3) surface Ab cocktail can be reduced by integration of the streptavidin conjugate into the Ab master mix for extracellular staining. In general, use of PE-conjugates for pan $\gamma\delta$ T cell Abs is recommended, because

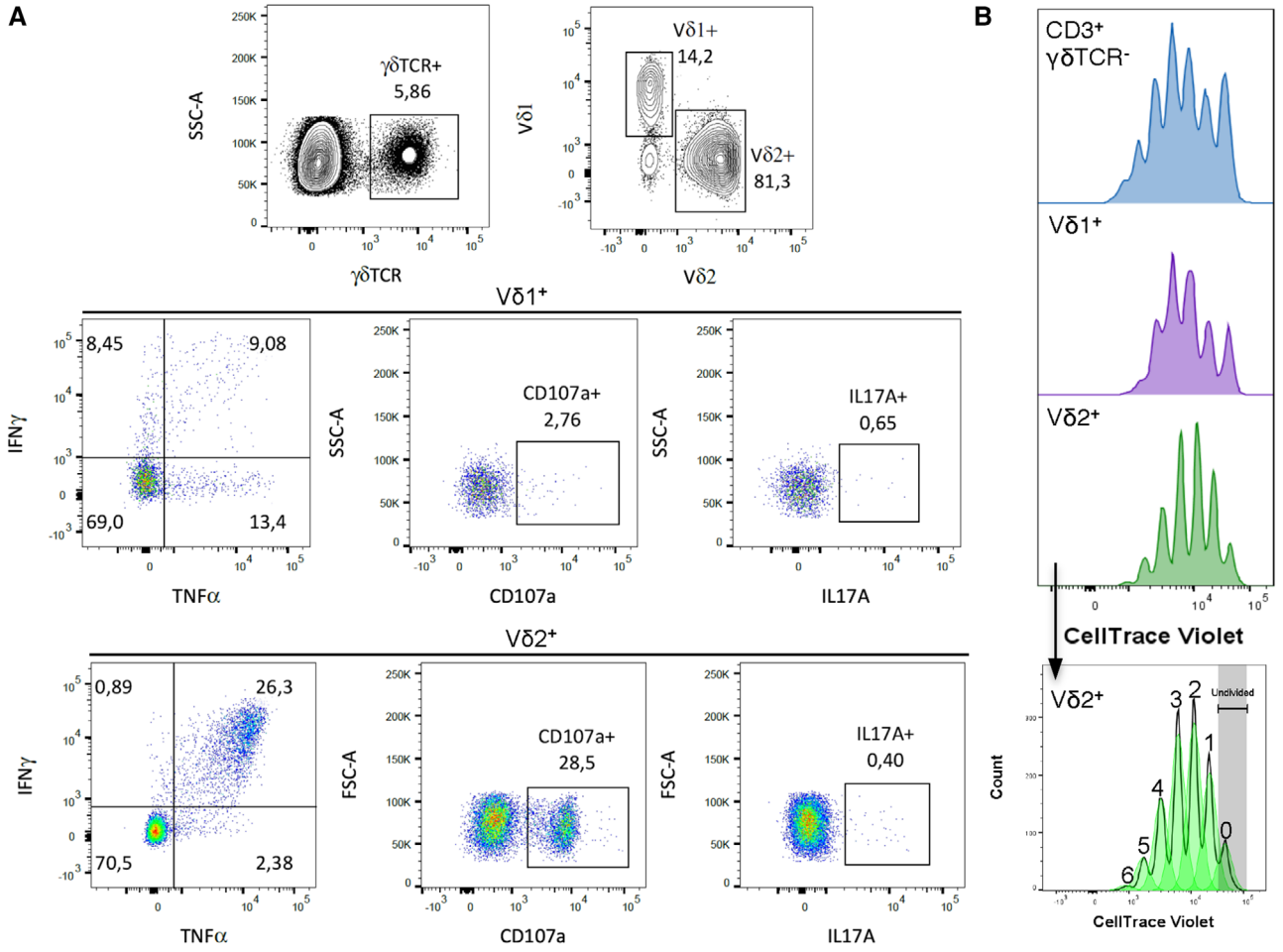

FIGURE 3 **Functional characterization of $\gamma\delta$ T cells via flow cytometry.** (**A**) Analysis of cytokine expression in fixed PBMC samples after stimulation with Zoledronate (Vδ2$^+$) or PMA/Ionomycin (Vδ1$^+$). Vδ1$^+$ and Vδ2$^+$ cells were gated on viable, CD3$^+$, $\gamma\delta$ TCR$^+$ lymphocytes. (**B**) Proliferation of CD3$^+$$\gamma\delta$TCR$^-$, V$\delta$1$^+$ and Vδ2$^+$ 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

PE itself is recognized by certain $\gamma\delta$ TCRs and thus stains a small percentage of peripheral $\gamma\delta$ T cells.[24] This should also be kept in mind when tandem conjugates containing PE (e.g., PE-Cy7, PE-Cy5.5, etc.) are included in the Ab panel. As stated above, the Vδ2 123R3 Ab is, in contrast to clone B6, compatible with fluorophore-conjugated pan-$\gamma\delta$ TCR Abs. Of note, clone B6 might be specific for the Vγ9Vδ2 TCR pairing, because it has been reported that detection of the Vγ9$^-$Vδ2$^+$ population is not possible with clone B6, but clone 123R3 can be used as an alternative to also detect the rare population of Vγ9$^-$Vδ2$^+$ T cells.[9]

On the basis of this framework for detection of $\gamma\delta$ T cells, we developed several polychromatic Ab 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 (Fig. 3A), and (iii) a panel to track $\gamma\delta$ T cell proliferative capacity using CellTraceViolet (Fig. 3B). The above-described basic phenotypic markers (pan-$\gamma\delta$ TCR, Vδ1, and Vδ2) also worked well after fixation and permeabilization.

Using a combination of available mAbs, flow cytometry is also useful to monitor the entire expressed human Vγ repertoire.[18] Such an analysis is based on Abs detecting Vγ9 (e.g., clone 7A5),[20] Vγ2/3/4 (clone 23D12),[17,25] Vγ3/5 (clone 56.3),[18] and Vγ8 (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 Vγ3 (56.3$^+$23D12$^+$) and Vγ5 (56.3$^+$23D12$^-$; Fig. 4A and B). Moreover, such Abs are useful for detecting rare $\alpha\beta$ T cells with a trans-rearranged TCR.[13,28] As shown in Fig. 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 Ab (clone IP26) but not with a pan-$\gamma\delta$ Ab (clone 11F2). These cells carry an in-frame Vγ5-Jβ-Cβ trans-rearrangement.[18]

3.3 | 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 Ab

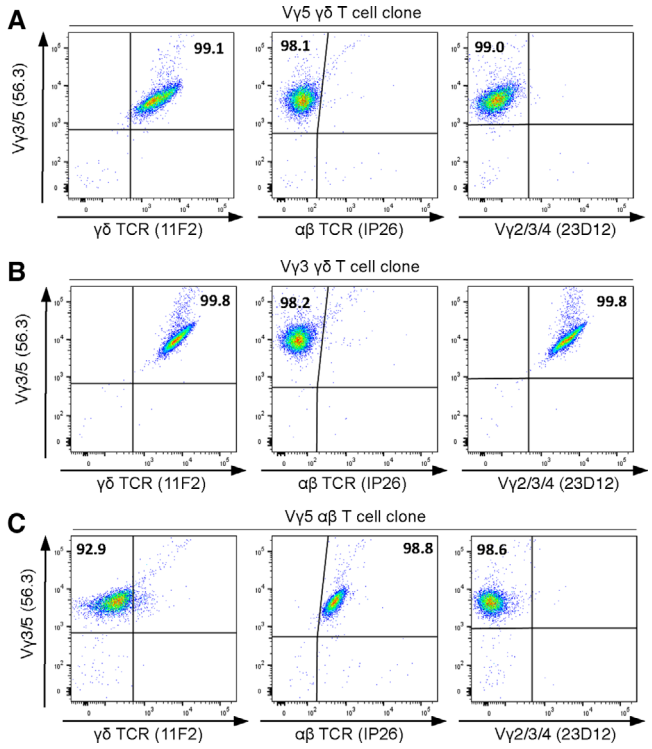

FIGURE 4 **Identification of Vγ3 and Vγ5 $\gamma\delta$ and $\alpha\beta$ T cell clones.** Viable cells were discriminated by gating on lymphocytes (FSC vs. SSC) and by near infra-red live/dead-staining. $\gamma\delta$ and $\alpha\beta$ T cell clones stained by mAb Vγ3/5 (clone 56.3) were co-labeled with the mAbs recognizing the $\alpha\beta$ TCR (clone IP26), $\gamma\delta$ TCR (clone 11F2), Vγ2/3/4 (clone 23D12). (**A**) Vγ5 $\gamma\delta$ clone (IP26$^-$,11F2$^+$,56.3$^+$,23D12$^-$); (**B**) Vγ3 $\gamma\delta$ clone (IP26,11F2$^+$,56.3$^+$,23D12$^+$); (**C**) Vγ5 $\alpha\beta$ clone (IP26$^+$,11F2$^-$, 56.3$^+$,23D12$^-$)

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 $\gamma\delta$ 152Sm Ab in combination with custom-made B6 TCR Vδ2 141Pr and TS8.2 Vδ1 FITC Abs (detected via an anti-FITC 160Gd). Large proportions of Vδ1 T cells were stained with the pan-$\gamma\delta$ TCR Ab, but none or only a fraction of the Vδ2 T cells (Fig. 2C). Steric hindrance caused by close proximity of the recognized epitopes and size and nature of the Ab tags might account for these observations. Further testing of the combinations of Vδ1 REA173 and Vδ2 123R3 with the pan-$\gamma\delta$ 11F2 Abs that achieved good resolution in polychromatic flow cytometry is also warranted in mass cytometry.

3.4 | 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 γ3.20. 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 γ3.20 is no longer available, but Jungbluth et al. recently reported that the TCR δ Ab 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 Vγ9 and Vγ2/3/4 Abs. Analysis of sections from patients with EBV-associated Hodgkin's lymphoma showed that most of the $\gamma\delta$ T cells from these patients expressed Vγ9, whereas $\gamma\delta$ T cells expressing Vγ2, 3, or 4 were nearly absent (Fig. 5A). Furthermore, staining of cryosections obtained from patients with colon adenocarcinoma using the Vδ1 and Vγ2/3/4 Abs revealed that these $\gamma\delta$ T cells are enriched in respective malignancies, as shown for one representative patient (Fig. 5B). This indicates that the Vγ2/3/4 clone 23D12 is also suitable for staining cryosections.

4 | 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 Abs and Ab combinations still have room for improvement. On the part of the manufacturers, a broader spectrum of fluorophore–Ab 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 Vγ rather than a Vβ element. Such $\alpha\beta$ T cells stain with anti-Vγ Abs as shown here for Vγ5, and this has been reported previously also for Vγ4 and Vγ9.[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 Abs suitable for staining paraffin-embedded tissue sections, and Abs 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.

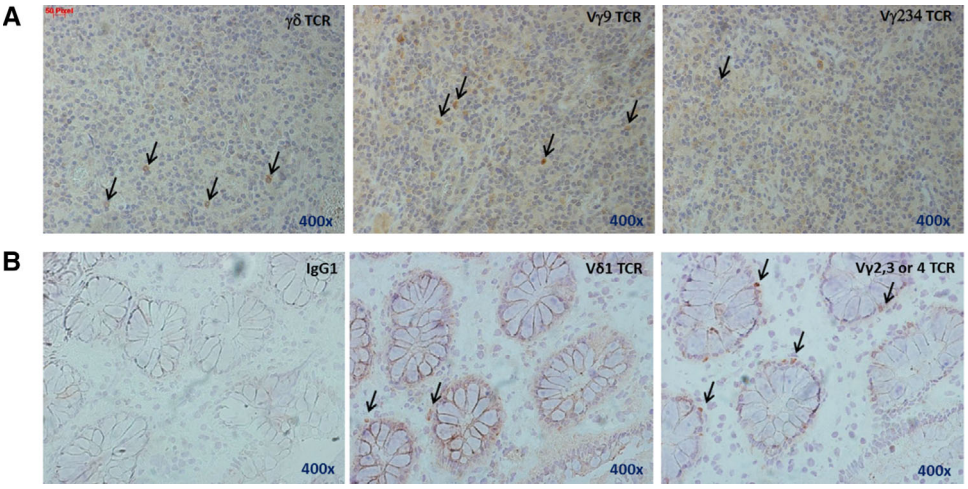

FIGURE 5 **Distribution of different T cell subsets in EBV-associated Hodgkin's lymphoma tissues and colon adenocarcinoma tissue.** (**A**) Serial paraffin-embedded tissue sections from patients with EBV-associated Hodgkin´s lymphoma were stained with $\gamma\delta$ TCR (clone γ3.20), Vγ9 (clone 7A5), Vγ2/3/4 (clone 23D12) mAbs as indicated in one representative donor. (**B**) Serial cryosections obtained from colon adenocarcinoma patients were stained with IgG control, Vδ1 (clone R3.12), Vγ2/3/4 (clone 23D12) mAbs as indicated in one representative patient. IHC staining was performed as described in the Materials and Methods section

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AUTHORSHIP

N.B., D.W., H.O., G.P., S.K., K.S., K.W.H. were associated with study conceptualization. N.B., D.W., H.O., J.B., B.W., C.G., S.S., C.R., H.H., C.P., P.N., S.K., K.S., K.W.H. performed the investigations. N.B., D.W., H.O., J.B., S.S., C.R., H.H., P.N., S.K., K.S., and K.W.H. were associated with data curation and formal analysis. N.B., D.W., K.S., and K.W.H. wrote the original draft. N.B., D.W., H.O., G.P., M.W.L., S.K., D.K., K.S., and K.W.H. reviewed, edited, and revised the manuscript.

CONFLICT OF INTEREST DISCLOSURE

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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