New flow-cytometry approaches for diagnosis of clonality and monitoring of T-cell CLPD

ESCCA 2023 Bridging the (cytometry) flows
UTRECHT, THE NETHERLANDS - 27-30 SEPTEMBER 2023

PAR14 - Validated approach to T-cell/B-cell lympho-proliferative disorders diagnosis and monitoring

ESCCA 2023 Utrecht

Disclosure commercial conflict of interest

The author declares no competing financial interests or any other conflict of interest

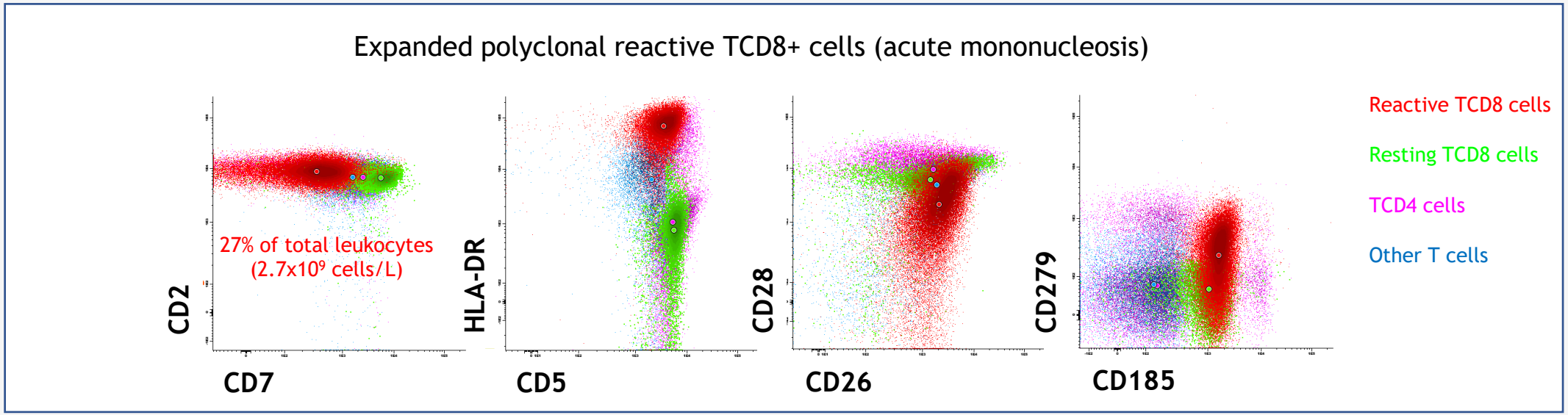
Diagnosis of mature T-cell neoplasms (T-CLPD)

EuroFlow

Diagnosis of T-CLPD is often challenging due to

.- Overlapping features of monoclonal T cells with reactive T cells

(i.e. morphologic and immunophenotypic similarities between clonal and reactive cells)



.- Lack of fast and reproducible routine diagnostic assays for T-cell clonality

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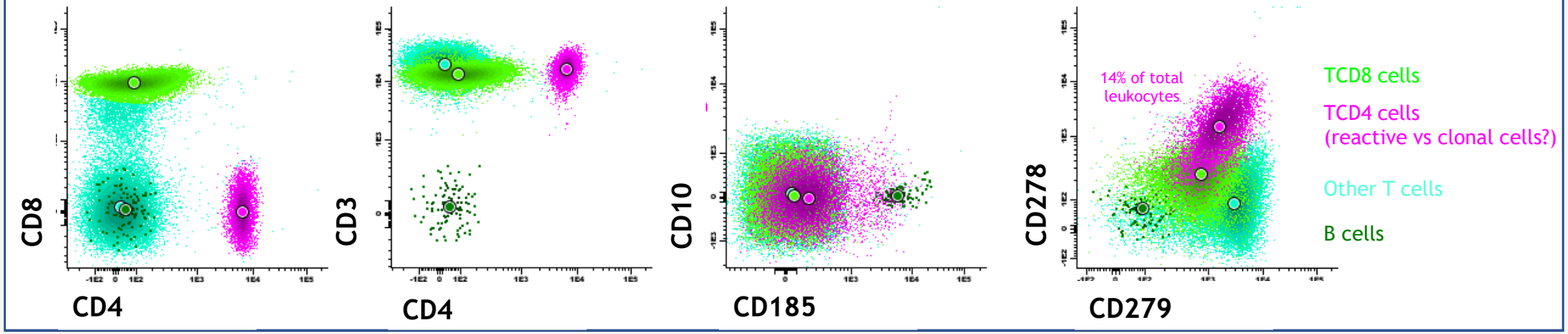
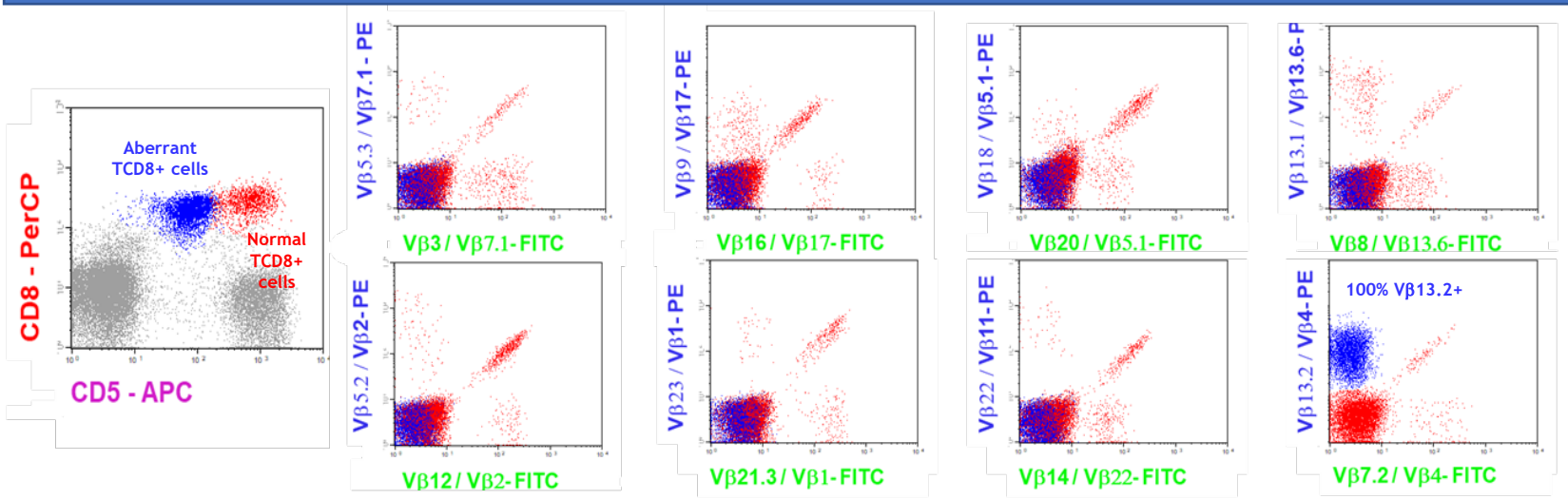
Patient diagnosed with nodal TFH-lymphoma, angioimmunoblastic type (WHO-HAEM5) with clinical suspicion of relapse



.- Lack of fast and reproducible routine diagnostic assays for T-cell clonality

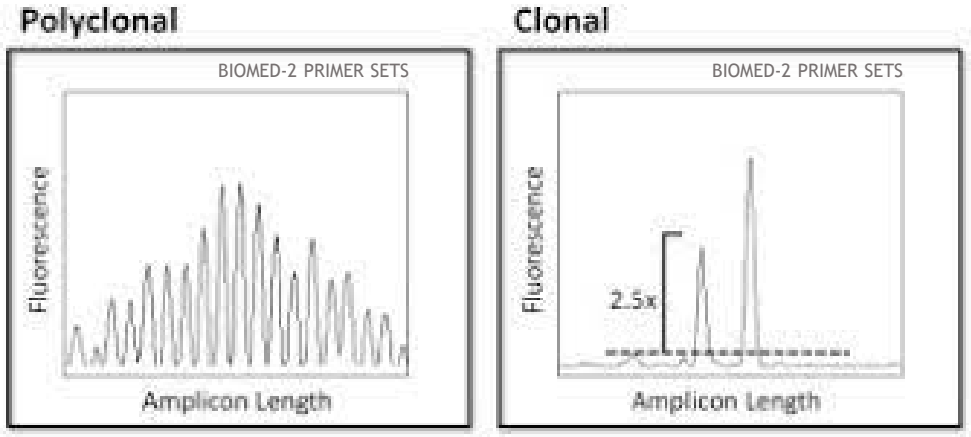
Clonality assessment for T-CLPD

TCRVß repertoire by FCM



Peruzzi B, Bencini S, Caporale R. TCR Vß Evaluation by Flow Cytometry. Methods Mol Biol. 2021;2285:99

PCR-based TRB and/or TRG gene rearrangement assays



Langerak et al, Blood 2001; Van Dongen et al, Leukemia 2003; Langerak et al, Leukemia 2012

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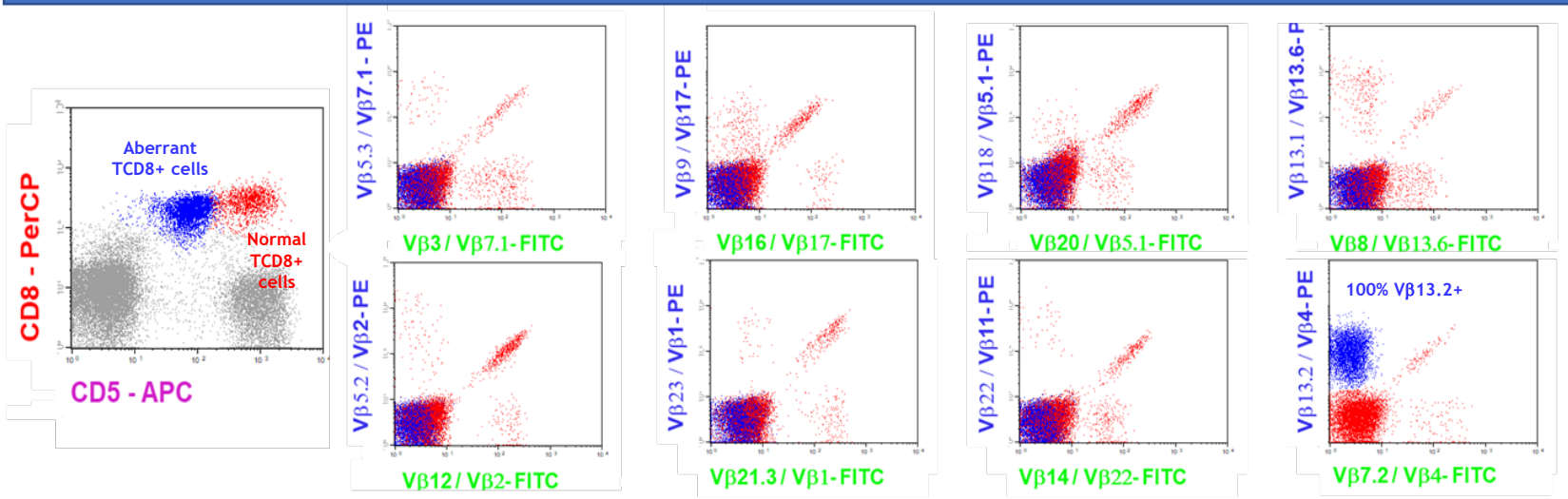

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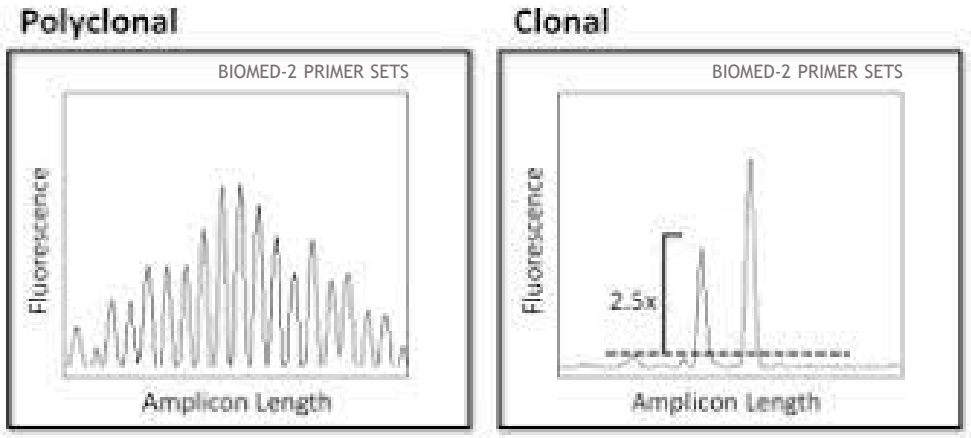
Limitations / disadvantages

Expensive
Labor-intense
Difficult to interpret
Limited sensitivity
Targeting ~ 70% of TCR-Vβ families
Restricted to Tαβ cells

Not available in many laboratories

PCR-based TRB and/or TRG gene rearrangement assays



Langerak et al, Blood 2001; Van Dongen et al, Leukemia 2003; Langerak et al, Leukemia 2012

Limitations / disadvantages

Restricted diversity of TCRγ
Complex
Lack of clone quantification
Incomplete coverage of VDJ rearrangements
High levels of background noise amplification
(requiring sorting of the suspicious clonal cell population)

Not available in many laboratories

Clonality assessment for T-CLPD

EuroClonality-NGS DNA capture panel as an integrated genomic tool for lymphoproliferative disorders

Current Molecular Testing for Lymphoproliferative Disorders

Genetic Alterations to Assess
- IG/TCR Rearrangements
- Translocations
- Copy number alterations
- Mutations

Separate/sequential tests
- PCR
- FISH
- Karyotyping/MLPA
- Sequencing

Limitations
- Multiple tests/laboratories
- Separate interpretations
- Increased staff time & cost
- Increased sample requirement

EuroClonality-NDC Assay Workflow for Lymphoproliferative Disorders

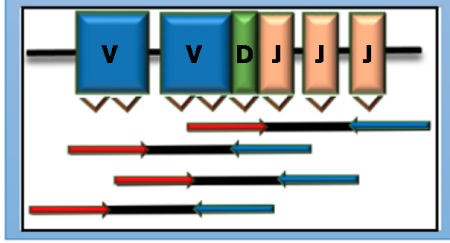
Assay Design

IG/TCR Probes
- Probes for all V, D and J genes

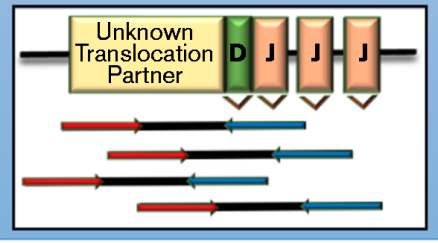
Additional Probes for
- IGH switch regions and recurrent translocation partners
- Clinically relevant copy number regions
- SNV/Indel detection in 72 genes

Benefits
- Single end-to-end workflow
- 100-200 ng input gDNA
- Bespoke bioinformatics pipeline

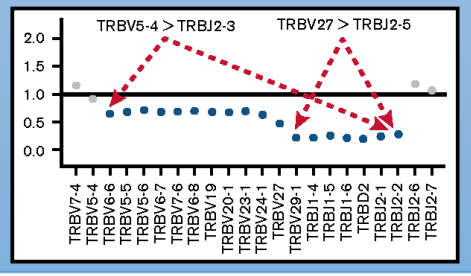
IG/TCR Rearrangement Detection



Translocation Detection



Copy Number Detection



Study Design

Multi-Site Validation

Sample Submission:
- **10** EuroClonality-NGS labs

Sequencing Centres:
- **7** EuroClonality-NGS labs

Proficiency Assessment:
- 14 cell lines assessed

Acceptance criteria:
- Mean unique coverage depth >500
- Expected rearrangements >95%

Validation:
- 280 samples from 10 labs
- 16 B/T cell malignancy entities
- 192 HMW, 88 FFPE
- Additional samples (n = 128)*

Performance:
- Limit of detection
- Precision
- Sensitivity
- Specificity

Advantages

More sensitive than conventional PCR
(high sensitivity for detection of TRA rearrangements)

It provides a single end-to-end workflow for simultaneous detection of B- and T-cell clonality, translocations, CNAs, and sequence variants

Disadvantages/Limitations

Expensive

Not suitable for minor clones present in <20% to 40% of total cells

Not validated in large series of T-CLPD

Not available in most laboratories

Stewart JP, et al. Validation of the EuroClonality-NGS DNA capture panel as an integrated genomic tool for lymphoproliferative disorders. *Blood Adv*. 2021;5:3188

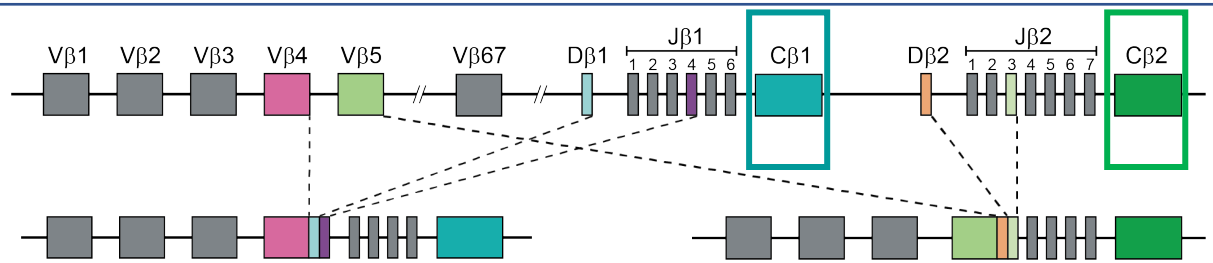
T-cell receptor ß chain constant region (TRBC1) reagent

A single monoclonal antibody (TRBC1, clone JOVI-1) has been recently identified as a potential flow cytometry marker for T-cell clonality assessment

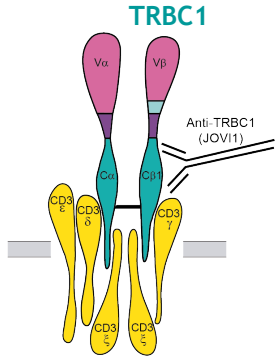
Rationale for using anti-TRBC1 Ab to detect T-cell clonality:

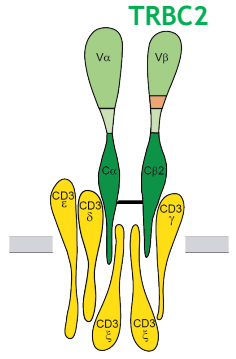
- It recognizes one of two mutually exclusive T-cell receptor β constant region (*TRBC)* genes randomly selected during *TCR* gene rearrangement (against TRBC1)

TRBC selection during TRB gene rearrangement in the thymus



Two resulting TRB complex structures, composed of either the TRBC1 or the TRBC2 proteins





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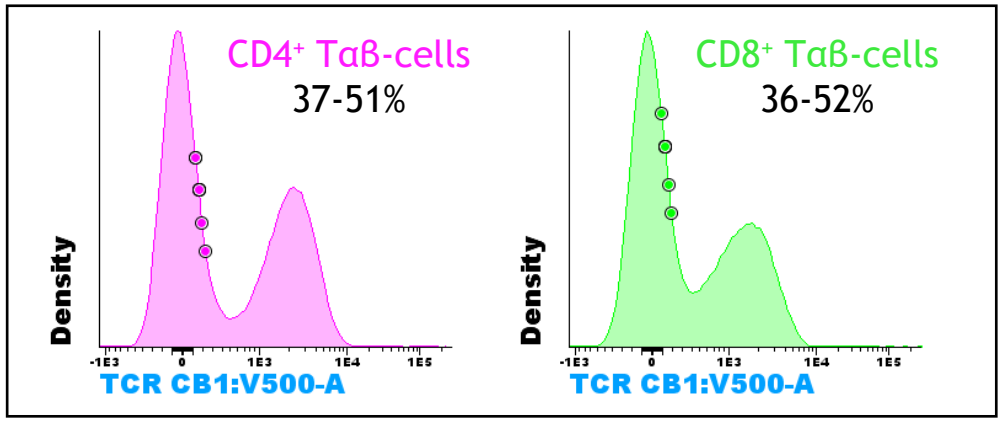
T-cell receptor ß chain constant region (TRBC1) reagent

Application of TRBC1 marker (clone JOVI-1) for T-cell clonality assessment by flow cytometry

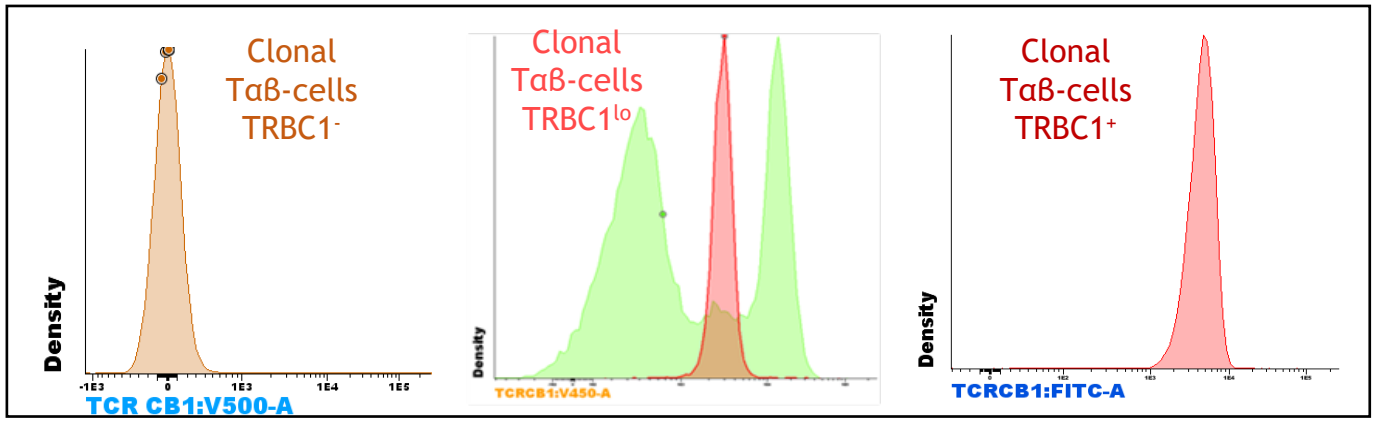
Normal and virus-specific (“reactive”) Tαß-cells:

Polytypic (or bimodal) TRBC1 expression



Monoclonal Tαß-CLPD:

Monotypic TRBC1 expression



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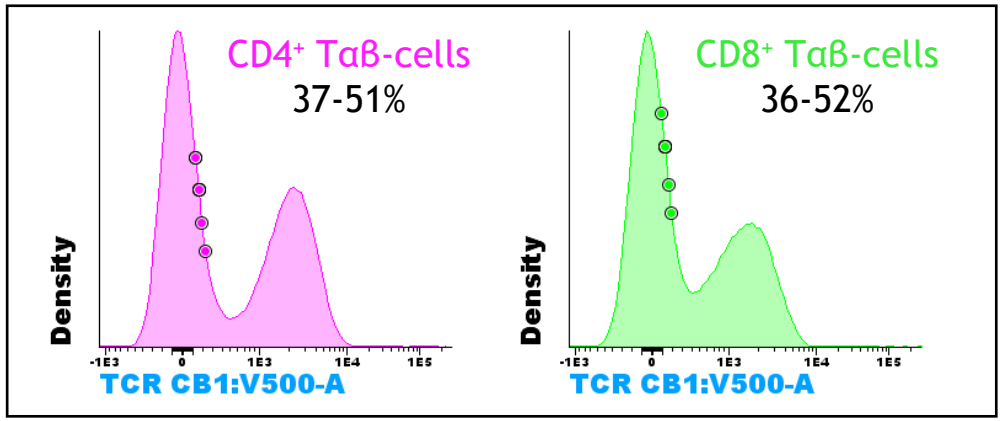

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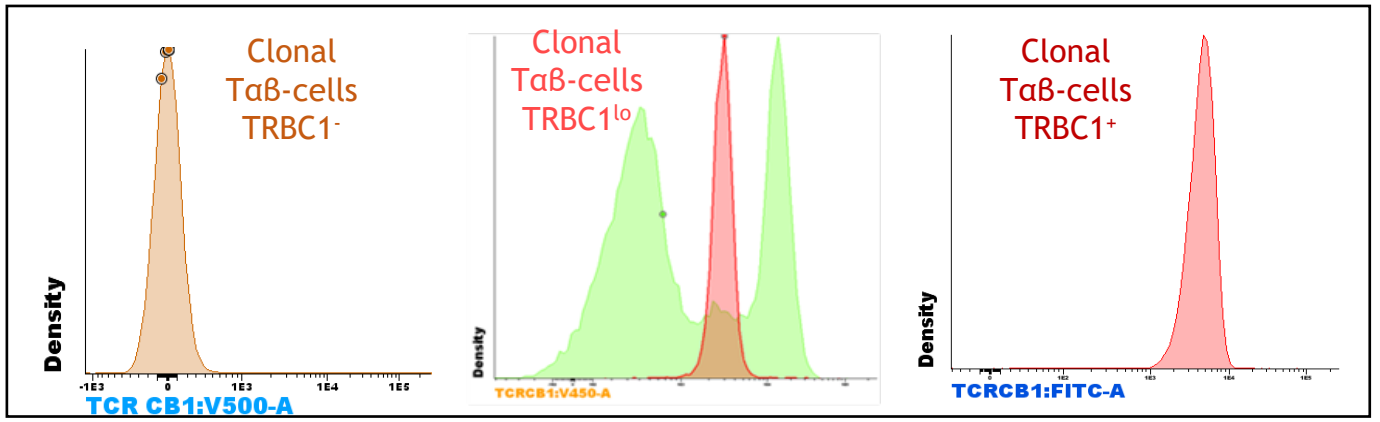

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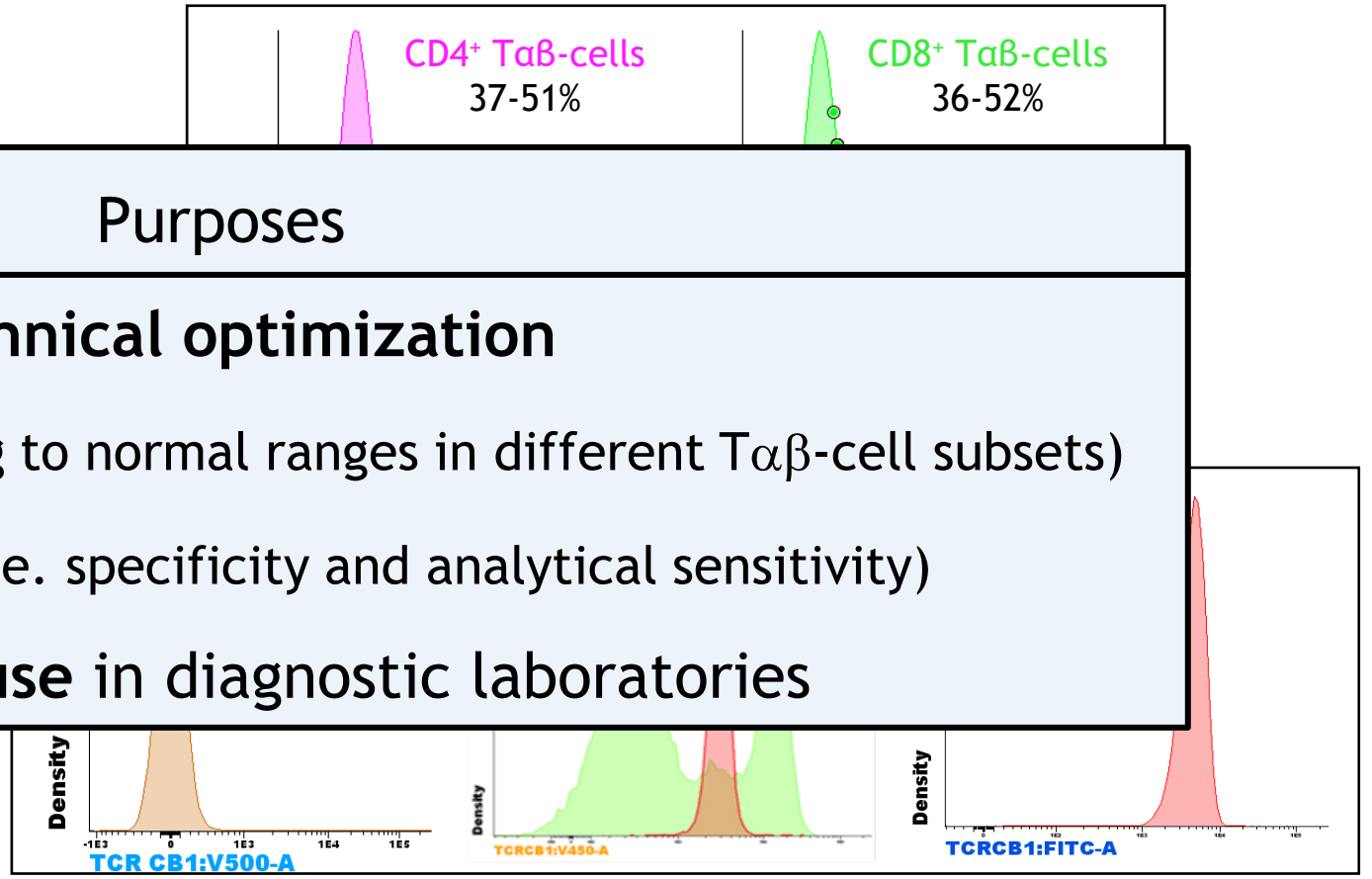

Normal and virus-specific ("reactive") Tαß-cel

Polytyp

Monoclo

Monoty

Purposes
Technical optimization
interpretation (according to normal ranges in different Tαβ-cell subsets)
and validation (i.e. specificity and analytical sensitivity)
for routine use in diagnostic laboratories

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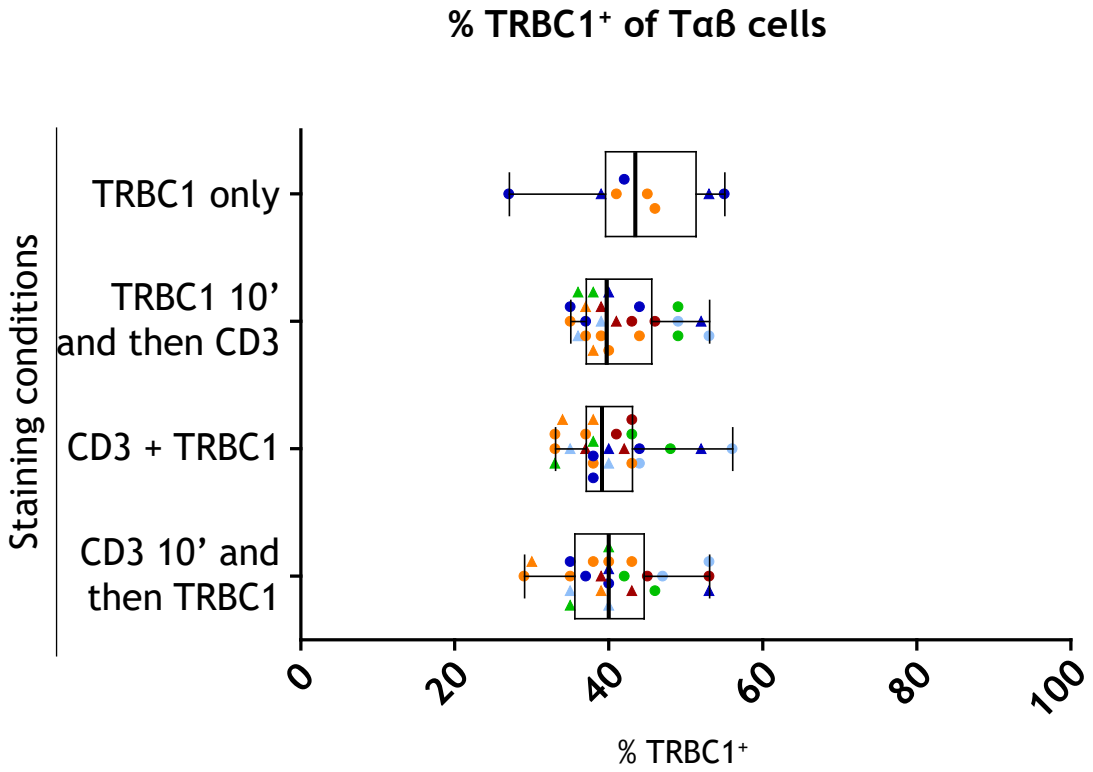
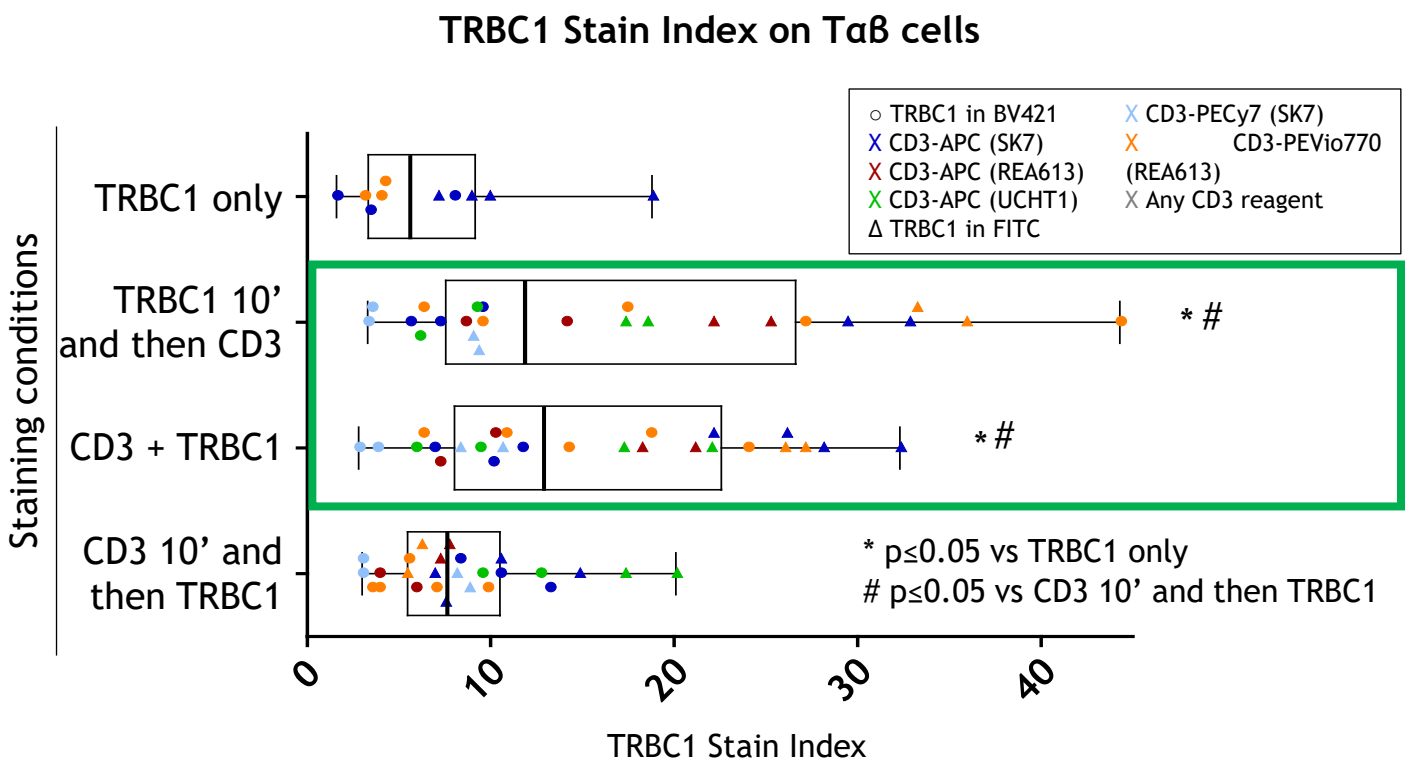
Optimization and validation of the TRBC1-FCM approach for detecting clonal Tαß-cells (n=211 normal, reactive and pathological samples)

EuroFlow

Optimization of the approach





TRBC1 labeling significantly improved in the presence of CD3; the best resolution to identify TRBC1⁺ cells was achieved by adding CD3 either simultaneously or after TRBC1

Muñoz-García N et al, Cancers (Basel). 2021;13:4379, on behalf of EuroFlow

Optimization and validation of the TRBC1-FCM approach for detecting clonal Tαβ-cells

TRBJ gene rearrangements of FACS-sorted TRBC1 positive and/or TRBC1 negative Tαβ⁺ cell populations

TRBC1-FCM approach	Clonality Status of cell populations*	TRBJ rearrangement	
		JB1	JB1+JB2
TRBC1 +	Monoclonal (n=4)	4	0
	Oligoclonal (n=3)	3	0
	Polyclonal (n=40)	37	3
	TOTAL	**44/47 (94%)**	3/47 (6%)
TRBC1 -	Monoclonal (n=3)	0	3
	Oligoclonal (n=4)	0	4
	Polyclonal (n=41)	0	41
	TOTAL	0	**48/48 (100%)**

97% concordance (92/95 cell populations)

All FACS-sorted cell populations had a purity ≥95% and a clear expression of CD3 on the cell surface membrane by FCM with optimal PCR amplification of the *TRBJ* gene product.
*The clonal nature (mono vs. oligo vs. polyclonality) of each purified cell population was assessed by *TCRVB* gene rearrangement analysis

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EuroFlow

Ranges for Polyclonal (Normal and Reactive) Tαß-Cells and Major Tαß-Cell Populations in blood

Tαβ-cell subset	% TRBC1⁺ cells*		TRBC1⁺/TRBC1⁻ ratio		Probability (%) of Finding A Clonal Tαβ Expansion When TRBC1⁺/TRBC1⁻ Ratio is Outside the Range Mean ± 3 SD (ρ-Value)
	Mean ± 1 SD	Range (Mean ± 3 SD)	Mean ± 1 SD	Range (Mean ± 3 SD)	
Tαβ cells	40 ± 6.7	20–60	0.66 ± 0.071	0.25–1.4	99.73% (<0.001)
Tαβ CD4⁺	43 ± 6.3	24–62	0.75 ± 0.067	0.31–1.6	
Tαβ CD8⁺	35 ± 8.8	8.3–61	0.53 ± 0.096	0.091–1.6	
Tαβ DP	36 ± 12	1.6–71	0.57 ± 0.13	0.016–2.5	
Tαβ DN	29 ± 10	0-61	0.41 ± 0.12	0–1.5	

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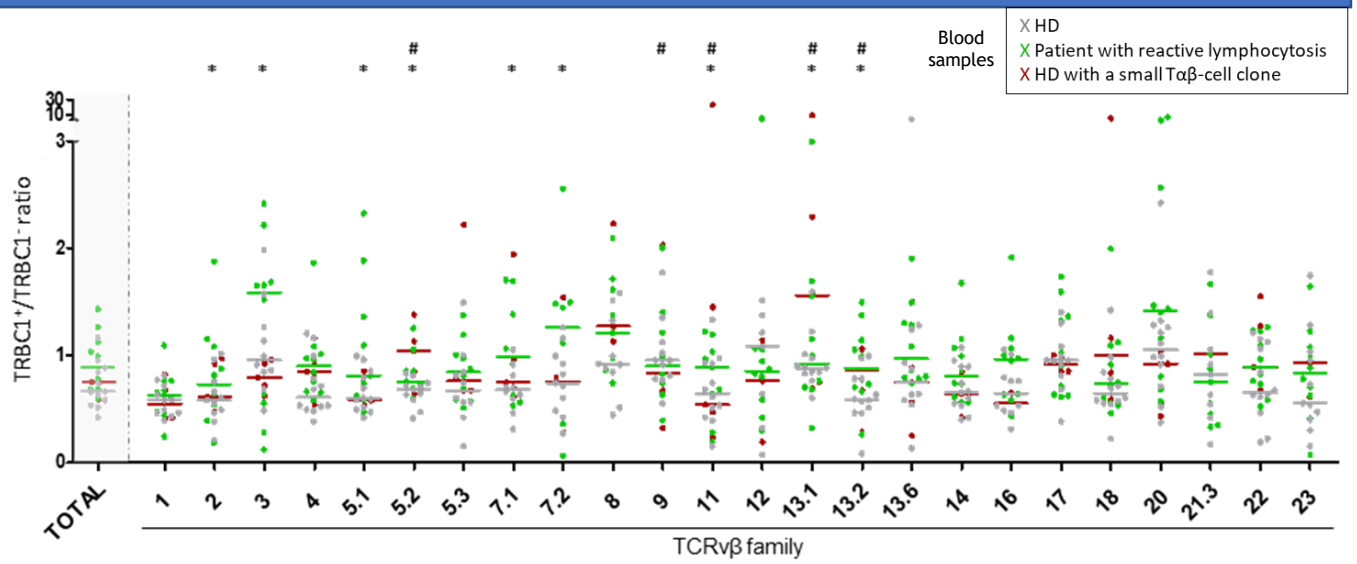
Bimodal distribution of TRBC1 for every TCRVß subset of polyclonal Tαß-cells



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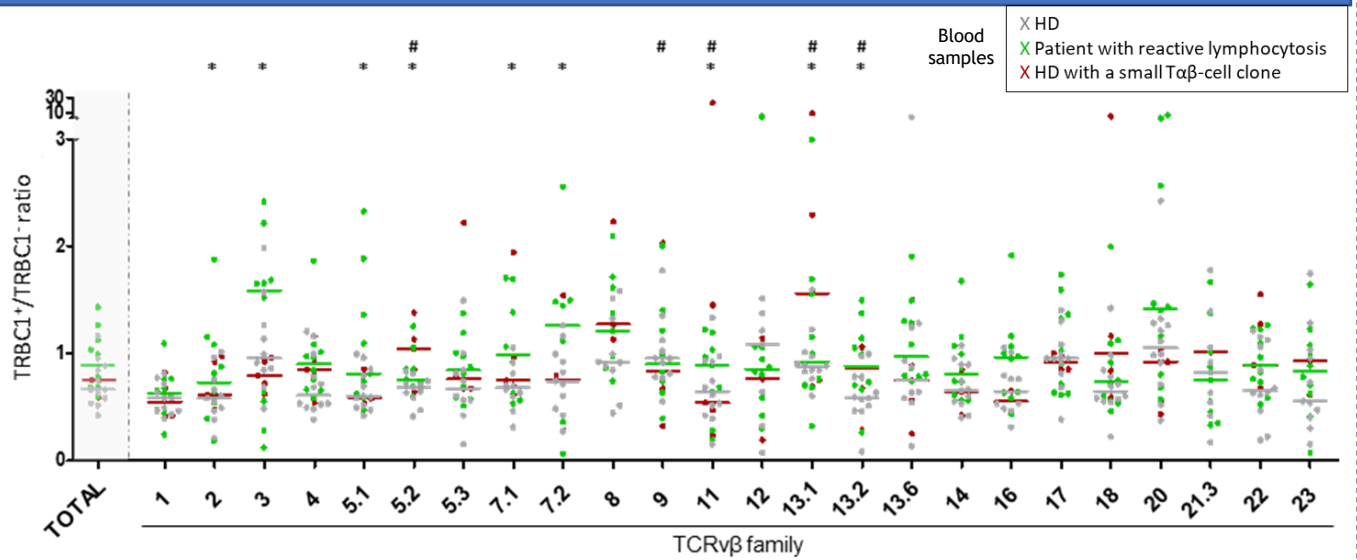
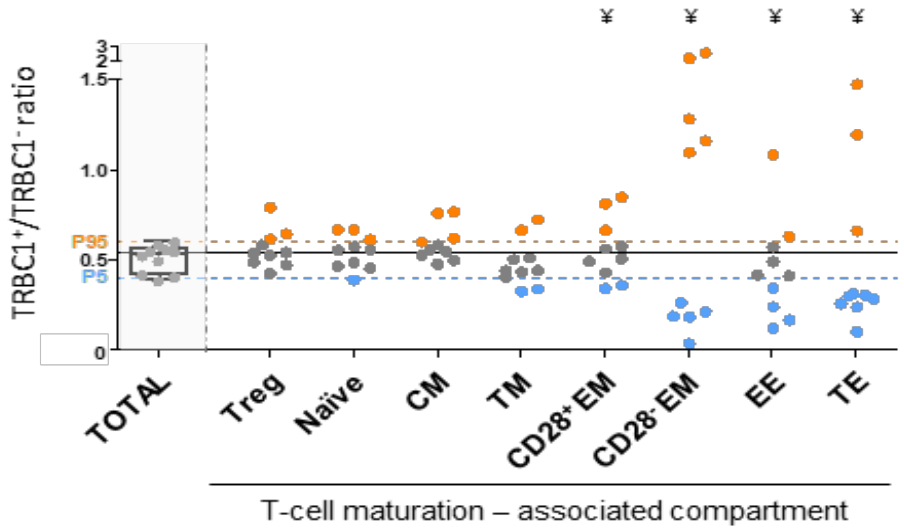


More mature stages of Tαß-cells were outside the normal range of TRBC1⁺/TRBC1⁻ ratio observed for total Tαß-cells



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TRBC1-FCM approach for detecting clonal Tαß-cells

SUMMARY : Technical optimization and normality ranges

- The optimal **TRBC1** resolution is achieved when CD3 antibody is **added, either simultaneously or after** (but not before) **TRBC1**
- High degree of correlation between the **pattern of TRBC1 protein expression** and the **TRBJ1 vs. TRBJ1+TRBJ2 gene rearrangement** profile
- **Reference TRBC1+/TRBC1− ranges** have been established, including both normal and reactive polyclonal Tαß cells for all major Tαß-cell populations in blood, also according to TCRVß family expression
- **More heterogeneous and skewed TRBC1+/TRBC1− ratios** were detected in more mature T-cell populations, consistent with an increasingly higher degree of oligoclonality as maturation progresses

Validation of the TRBC1-FCM approach for detecting clonal Tαß-cells

TRBC1-FCM Assay vs. TCRVß-FCM and/or Molecular Techniques for Assessment of Tαß-Cell Clonality

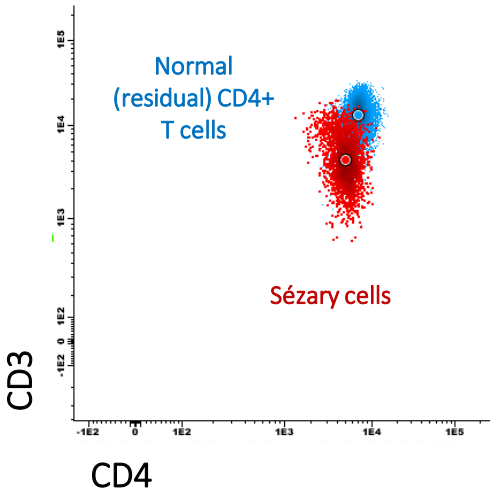
EuroFlow

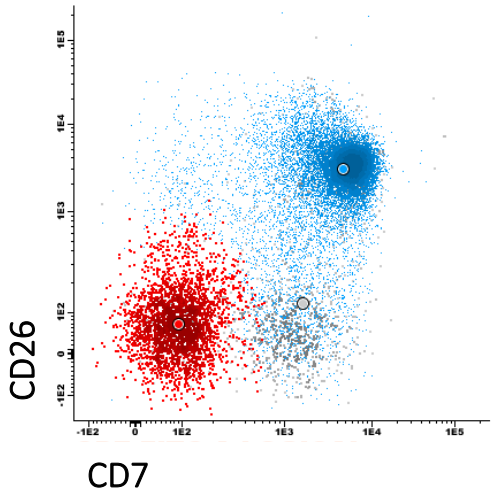
Clonality status by other techniques	TRBC1 expression pattern		P-value
	Polytypic (n=23)	**Monotypic (n=94)**	
Poly/oligoclonal (n=24)	**21/24 (87%)**	3/24 (13%)	<0.0001
Monoclonal (n=93)	2/93 (2%)	**91/93 (98%)**	

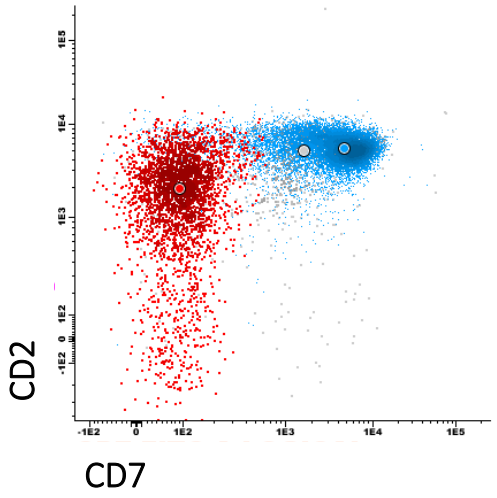
112/117 (96%) concordance

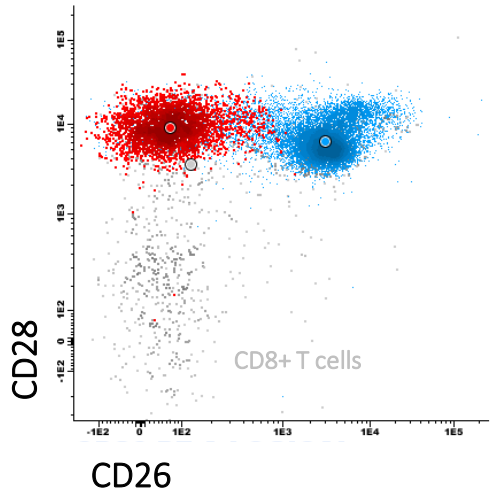
Muñoz-García N et al, Cancers (Basel). 2021;13:4379, on behalf of EuroFlow

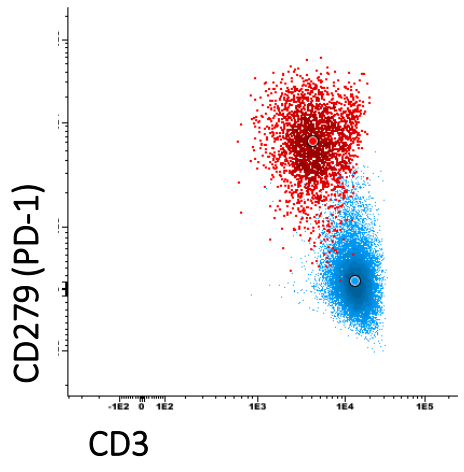
Immunophenotypic identification of Sézary cells in blood: additional value of a T-cell clonality marker (TRBC1)

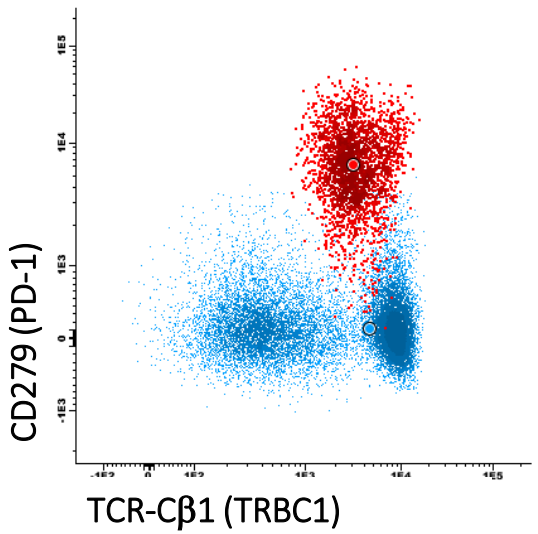


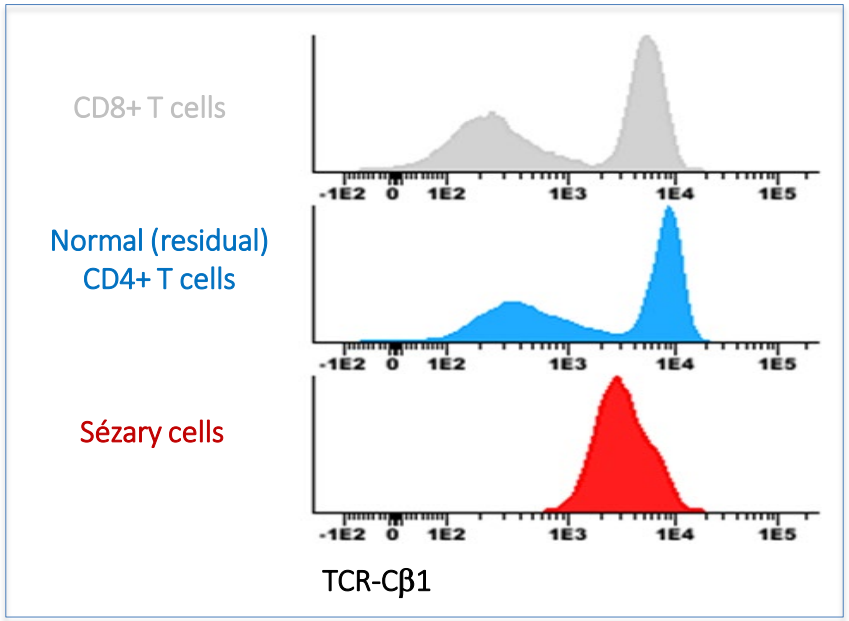


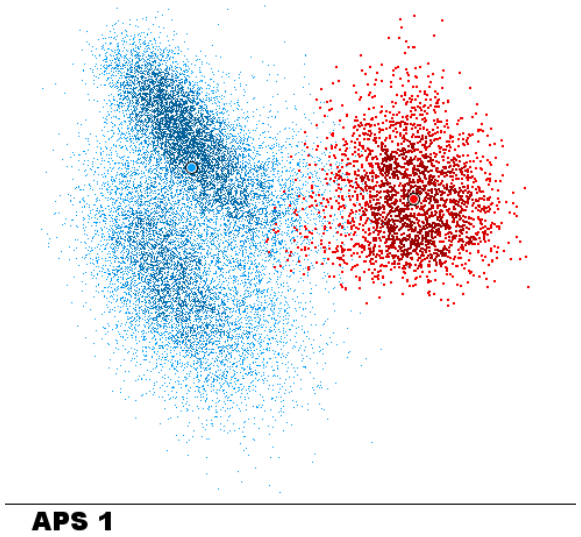












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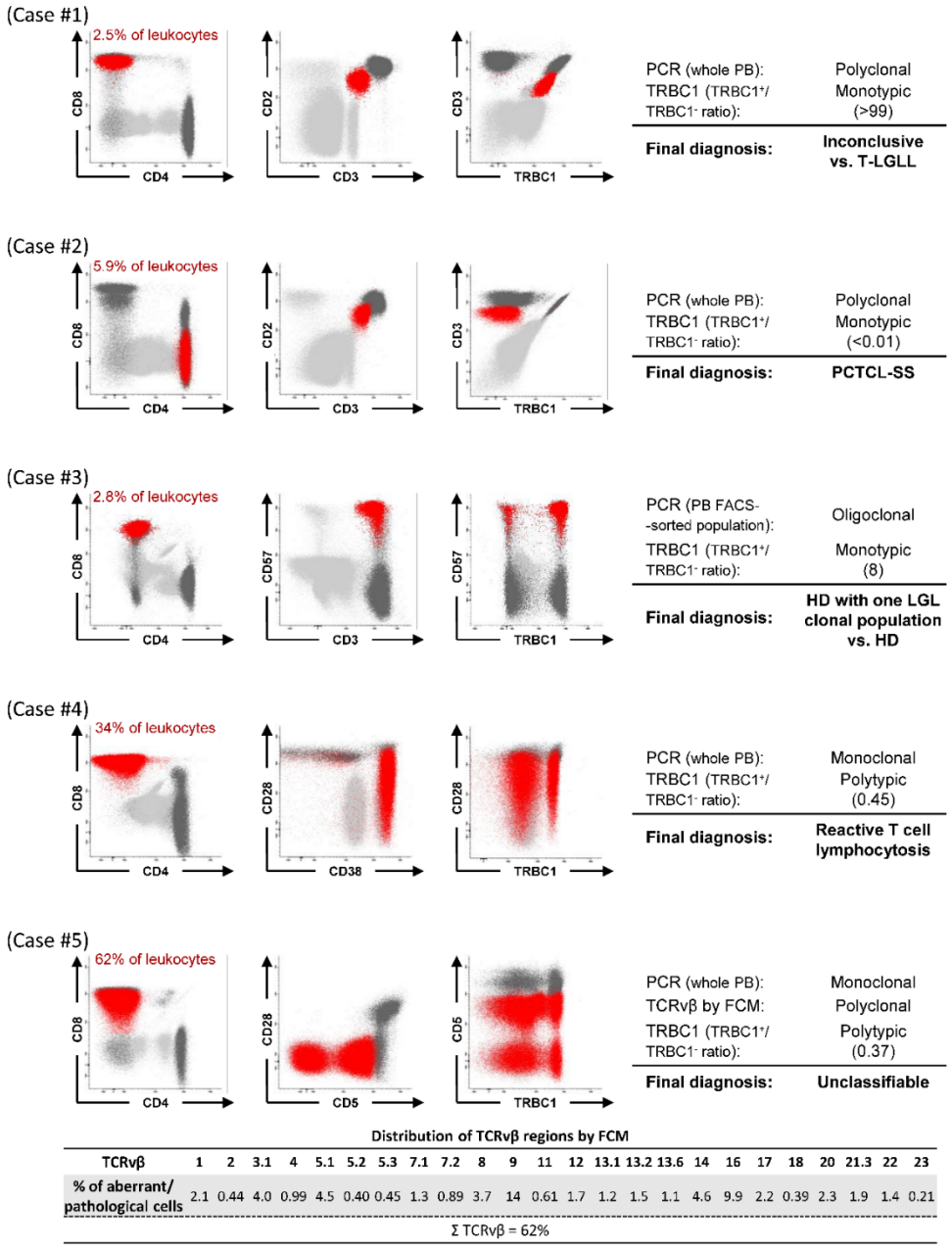
Monoclonal (n=93)	2/93 (2%)	91/93 (98%)	

112/117 (96%) concordance

Muñoz-García N et al, Cancers (Basel). 2021;13:4379, on behalf of EuroFlow

5/117 (4%) discordant cases



Validation of the TRBC1-FCM approach for detecting clonal Tαß-cells

EuroFlow

TRBC1-FCM Assay vs. TCRVß-FCM and/or Molecular Techniques for Assessment of Tαß-Cell Clonality

Clonality status by other techniques	TRBC1 expression pattern		P-value
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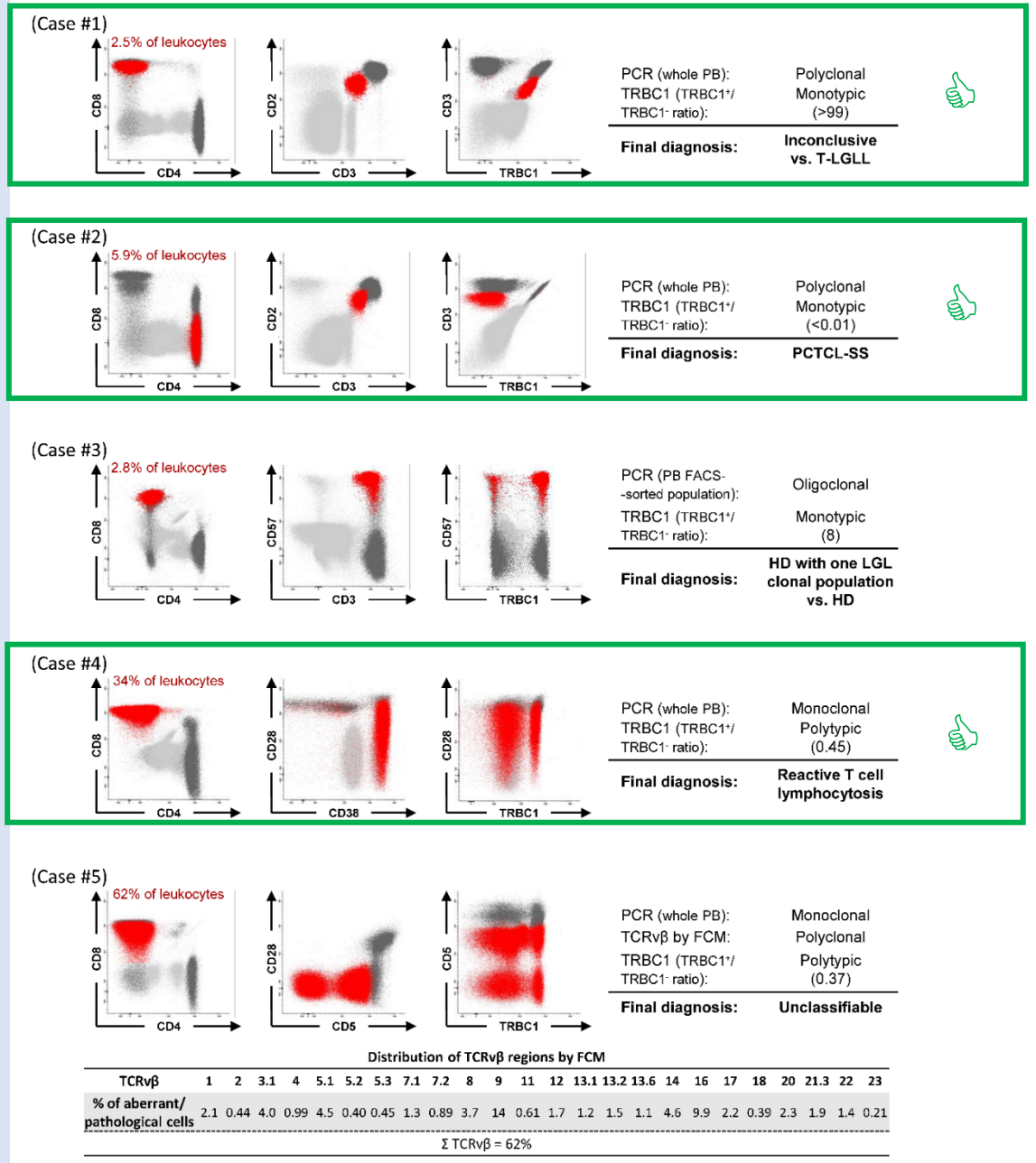

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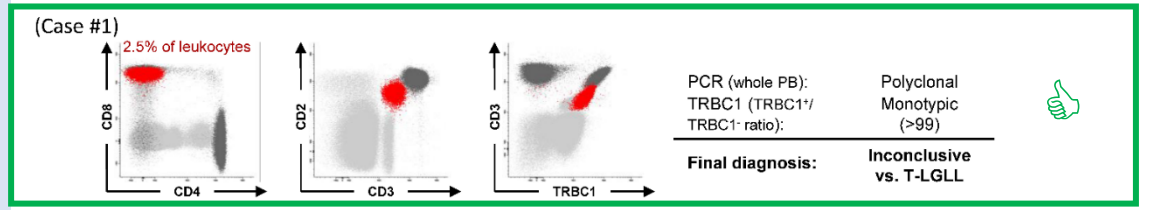
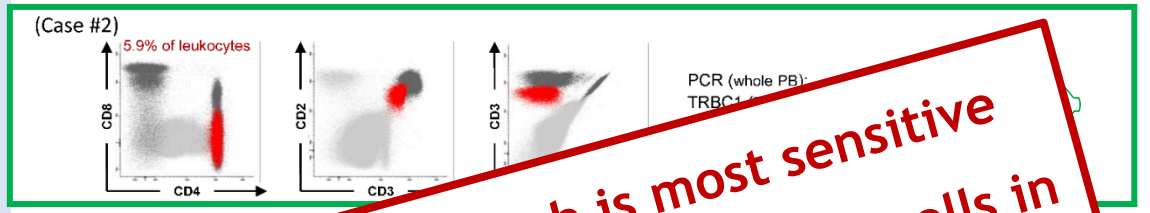
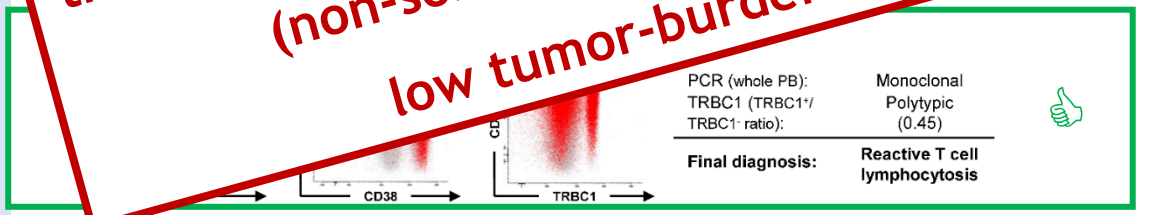
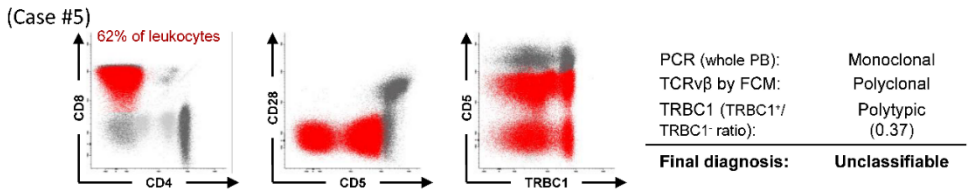




(Case #3)



TRBC1-FCM approach is most sensitive than PCR for detecting clonal Tαß-cells in (non-sorted) samples with low tumor-burden



Distribution of TCRvβ regions by FCM

TCRvβ	1	2	3.1	4	5.1	5.2	5.3	7.1	7.2	8	9	11	12	13.1	13.2	13.6	14	16	17	18	20	21.3	22	23
% of aberrant/ pathological cells	2.1	0.44	4.0	0.99	4.5	0.40	0.45	1.3	0.89	3.7	14	0.61	1.7	1.2	1.5	1.1	4.6	9.9	2.2	0.39	2.3	1.9	1.4	0.21

Σ TCRvβ = 62%

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EuroFlow

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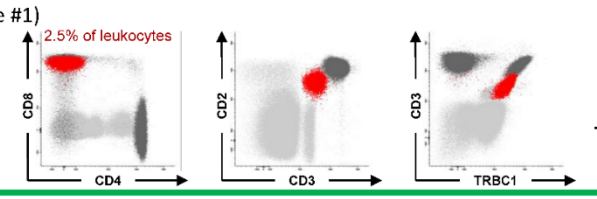
Waldron D, et al. Lab Med. 2022 Jul 4;53(4):417-425

Capone M, et al, Transl Oncol. 2022 Sep 30;26:101552

Dexter T, et al. Clin Lymphoma Myeloma Leuk. 2022 Oct;22 Suppl 2:S401

5/117 (4%) discordant cases

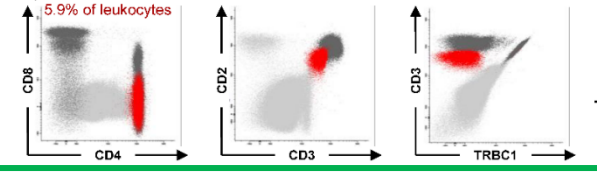
(Case #1)



PCR (whole PB): TRBC1 (TRBC1⁺/TRBC1⁻ ratio): Polyclonal Monotypic (>99)

Final diagnosis: Inconclusive vs. T-LGLL

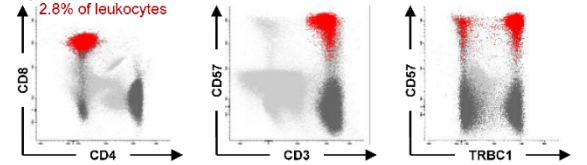
(Case #2)



PCR (whole PB): TRBC1 (TRBC1⁺/TRBC1⁻ ratio): Polyclonal Monotypic (<0.01)

Final diagnosis: PCTCL-SS

(Case #3)

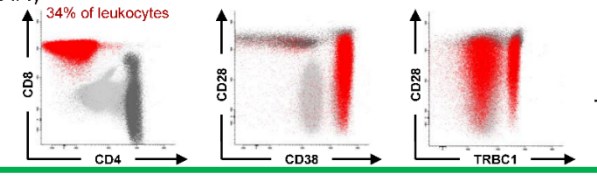


PCR (PB FACS-sorted population): Oligoclonal

TRBC1 (TRBC1⁺/TRBC1⁻ ratio): Monotypic (8)

Final diagnosis: HD with one LGL clonal population vs. HD

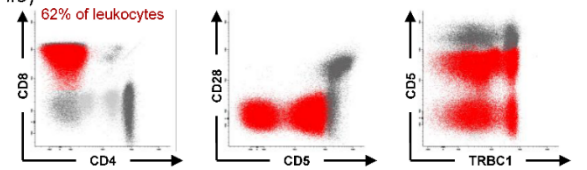
(Case #4)



PCR (whole PB): TRBC1 (TRBC1⁺/TRBC1⁻ ratio): Monotypic Polytypic (0.45)

Final diagnosis: Reactive T cell lymphocytosis

(Case #5)



PCR (whole PB): Monoclonal

TCRvβ by FCM: Polyclonal

TRBC1 (TRBC1⁺/TRBC1⁻ ratio): Polytypic (0.37)

Final diagnosis: Unclassifiable

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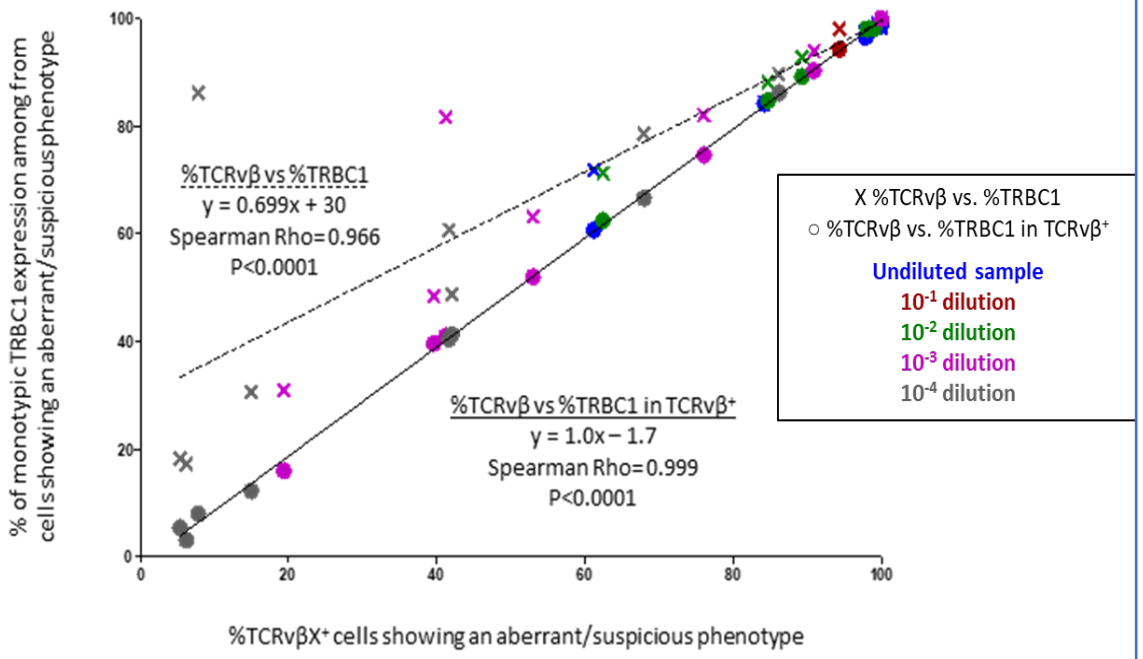
Validation of the TRBC1-FCM approach for detecting clonal Tαß-cells

Sensitivity of TRBC1-FCM assay for Detection of Clonal Tαß-Cells

Serial dilutional experiments
(of PB pathological Tαß cells in normal blood cells)



Sensitivity level for detecting clonal Tαß cells (identified among cells displaying an aberrant/suspicious phenotype) was of at least 10^{-4} in 7/8 T-CLPD cases tested

Muñoz-García N et al, Cancers (Basel). 2021;13:4379

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EuroFlow

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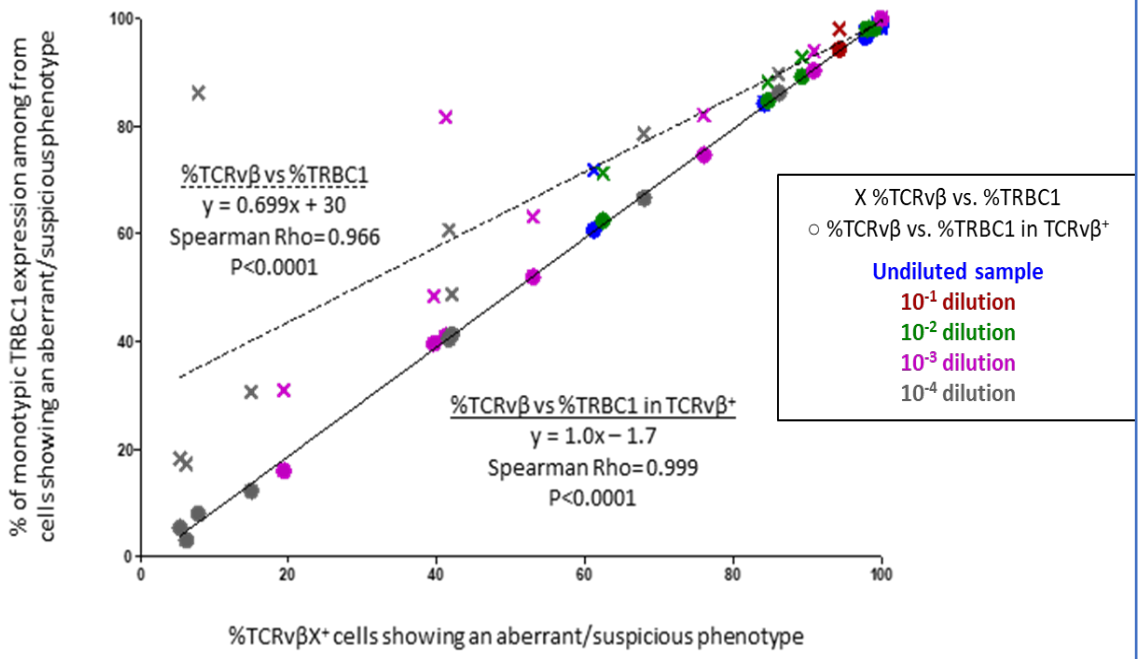

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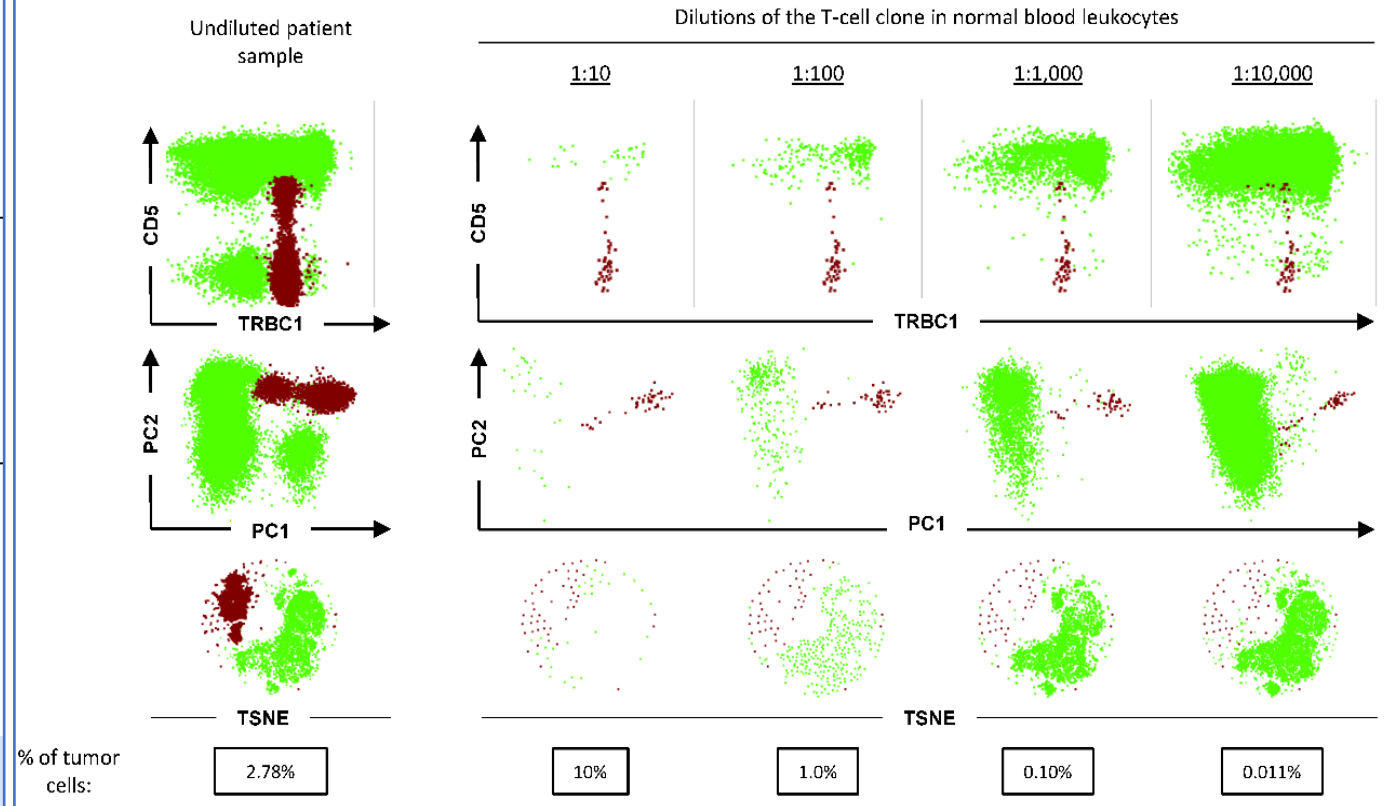
Dilutional experiment of clonal Tαß-cells from a T-LGLL patient in normal blood



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TRBC1-FCM approach for detecting clonal Tαß-cells

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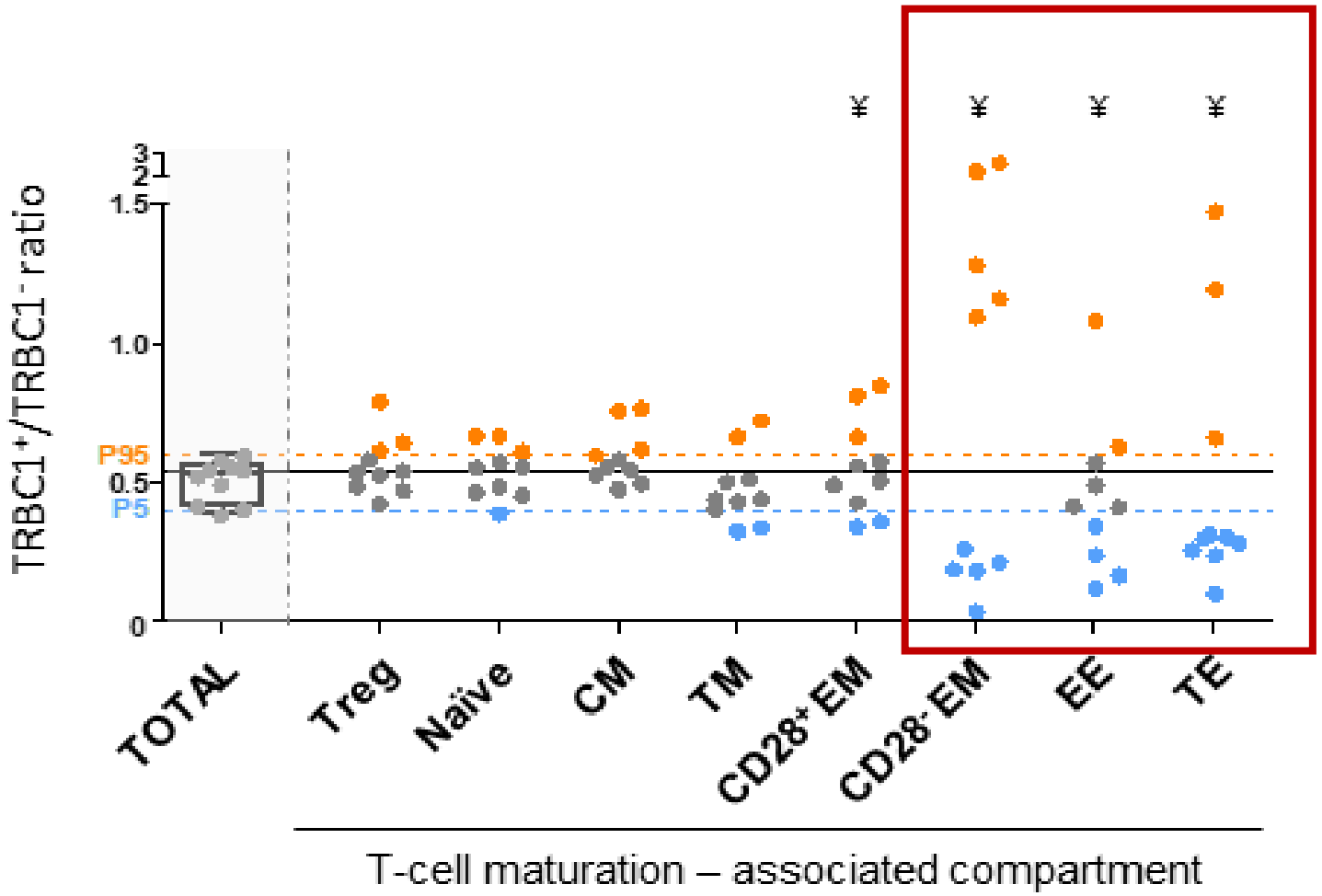
- **TRBC1-FCM approach shows a high specificity**
  - 96% of cases concordant with the gold standard (PCR), validated in the longest series of samples
- **Analytical sensitivity / level of detection of at least 10^{-4}** can be reached when cells show an aberrant phenotype (which improves when combined with TCRVß)
  - TRBC1-based FMC approach is more sensitive than PCR-based TRB and/or TRG gene rearrangement assays detecting clonal Tαß-cells in samples with low tumor-burden (e.g., <5%)
  - This assay could be useful for MRD evaluation in T-CLPD, but still it could be verified in larges series with “real” MRD samples

Validation of TRBC1-based flow cytometric assessment of T-cell clonality in Tαβ-large granular lymphocytic leukemia

More mature stages of normal Tαβ cells are outside the normal range of $TRBC1^+/TRBC1^-$ ratio observed for total Tαβ cells



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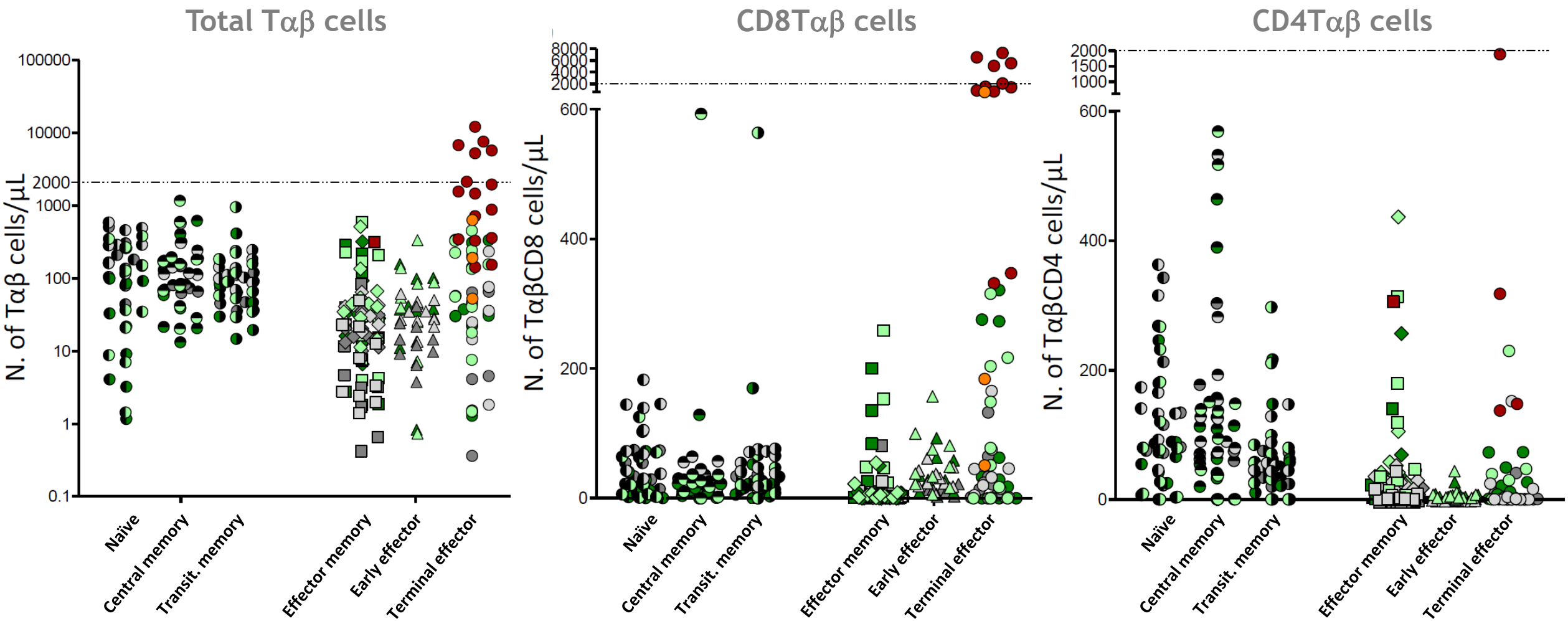
EuroFlow

TRBC1 expression profile (Abs. N. cells/uL) of T-LGLL (n=20) and HDc (n=3) with that of polyclonal EM-EE-TE T cells from healthy donors (n=11) and patients with reactive conditions (n=8)

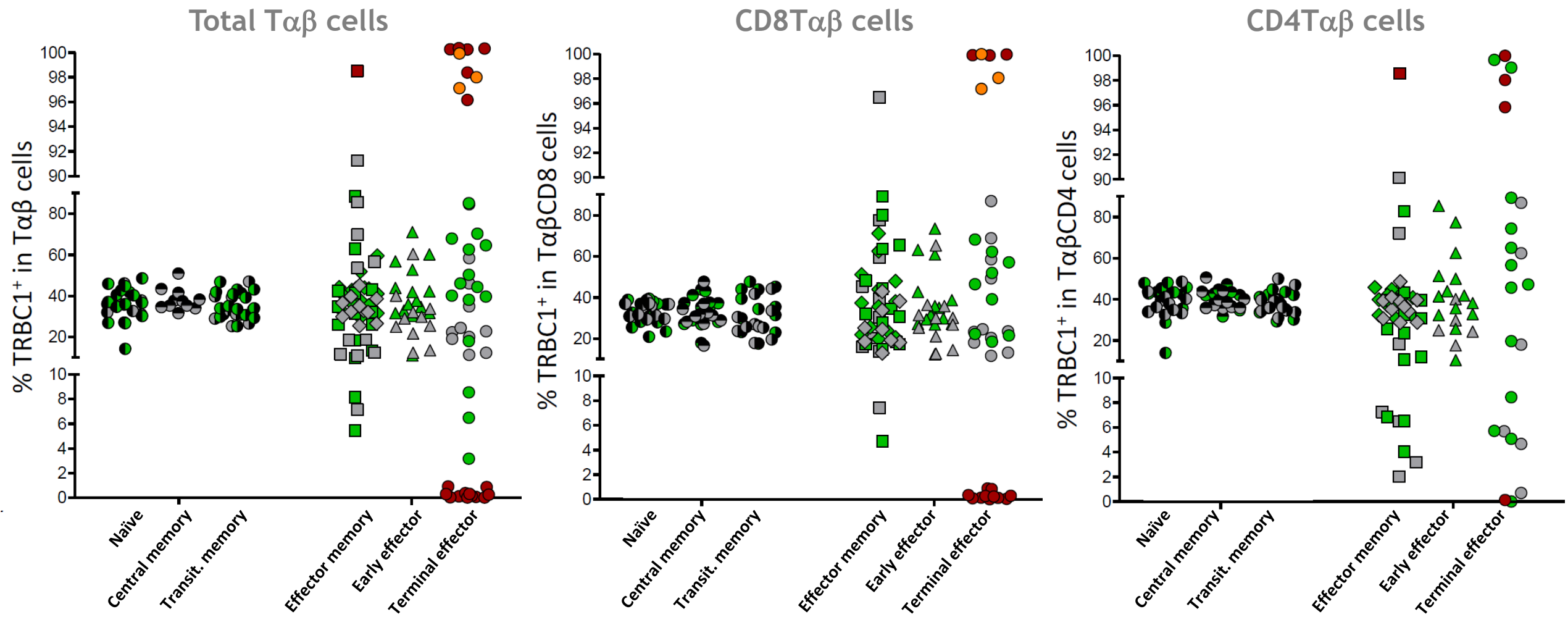


Muñoz-García N et al, Cancers (Basel). 2022;14:408

Validation of TRBC1-based flow cytometric assessment of T-cell clonality in Tαβ-large granular lymphocytic leukemia

EuroFlow

% TRBC1⁺ cells in T-LGLL (n=20) and HDc (n=3) with that of polyclonal EM-EE-TE T cells from healthy donors (n=11) and patients with reactive conditions (n=8)



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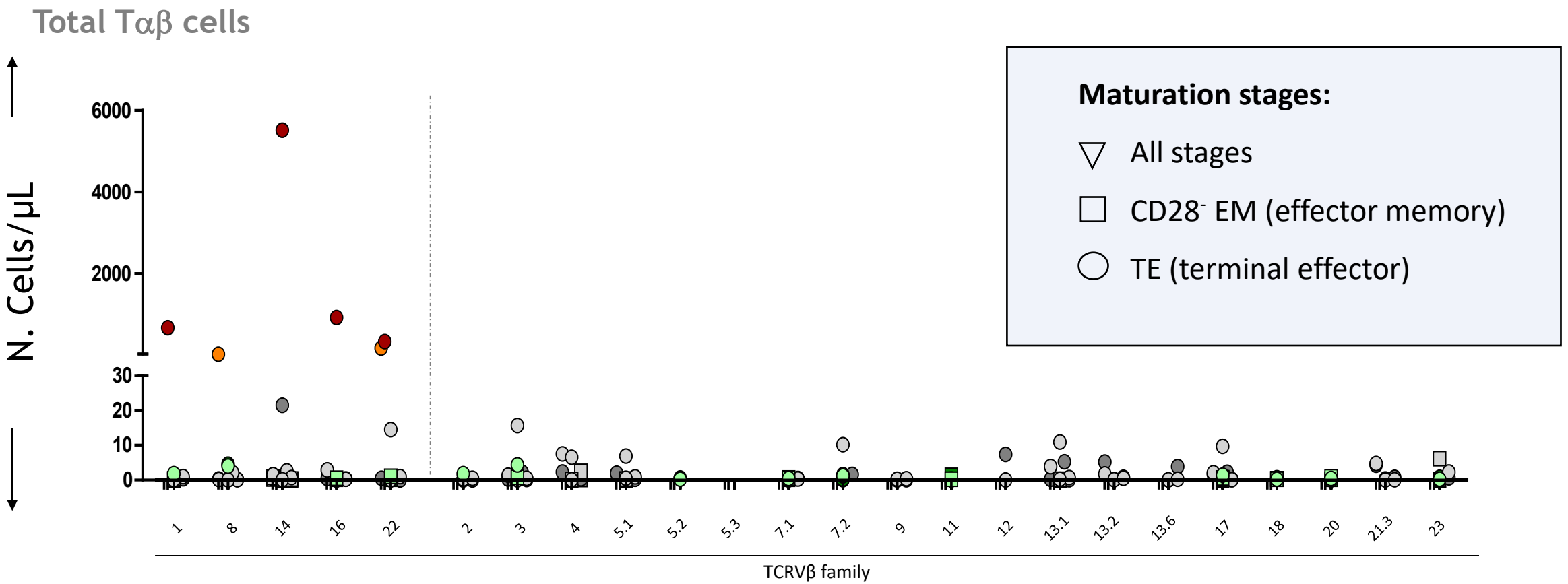
Absolute counts of cells identified by TRCB1 per TCR-Vβ family among the more mature subsets (CD28-EM and TE) of normal (n=6) and reactive (n=1) polyclonal cells vs. T-LGLL (n=4) and HDc (n=2)



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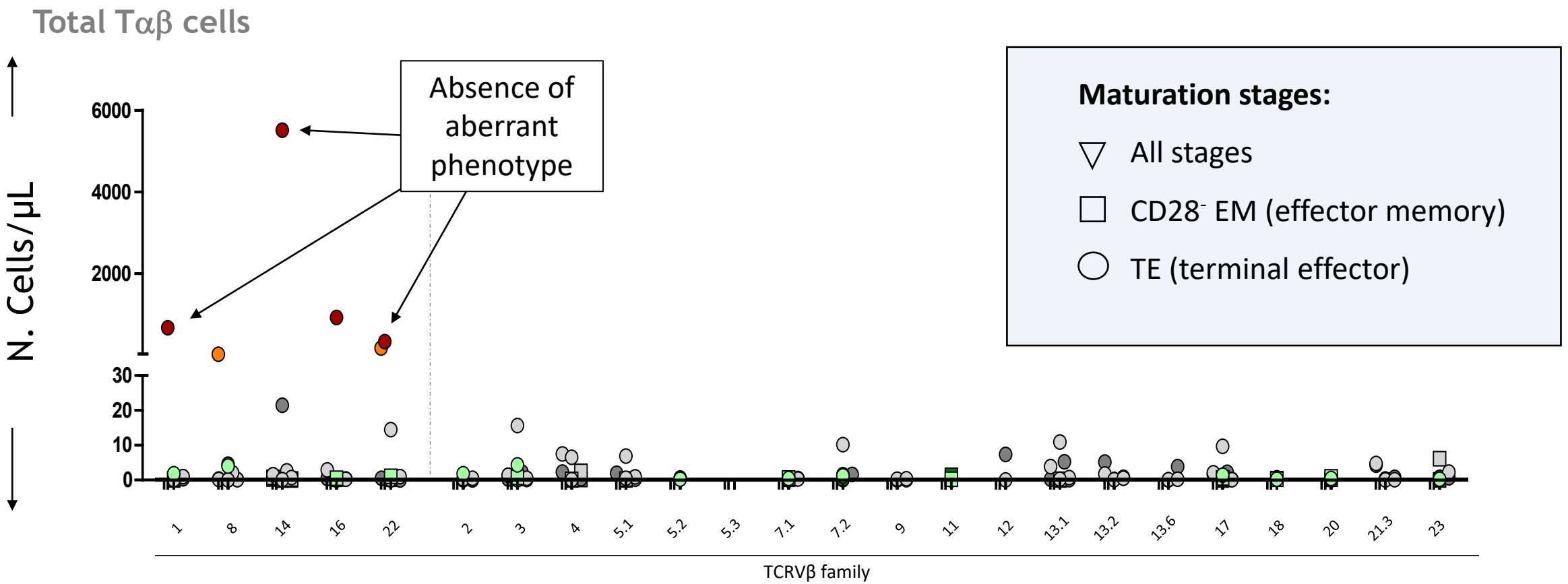

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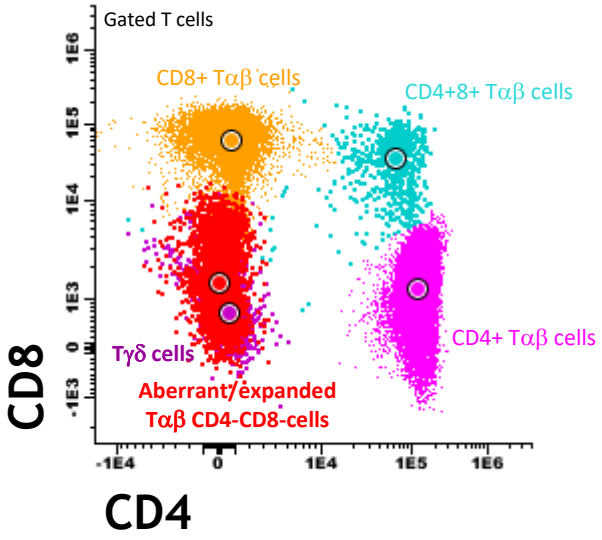
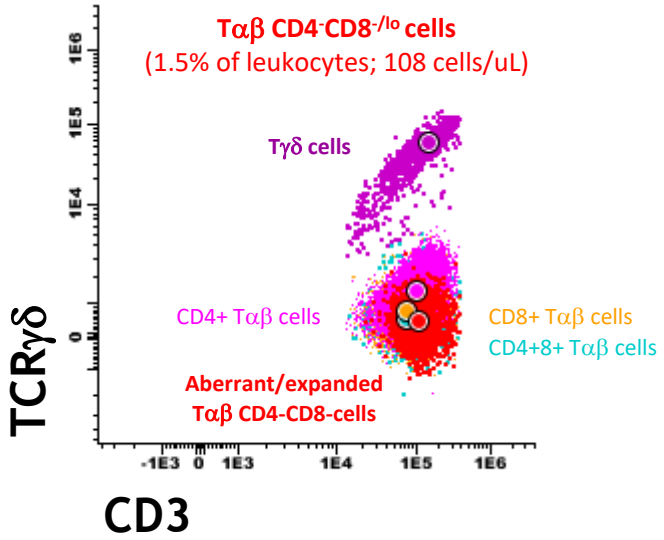
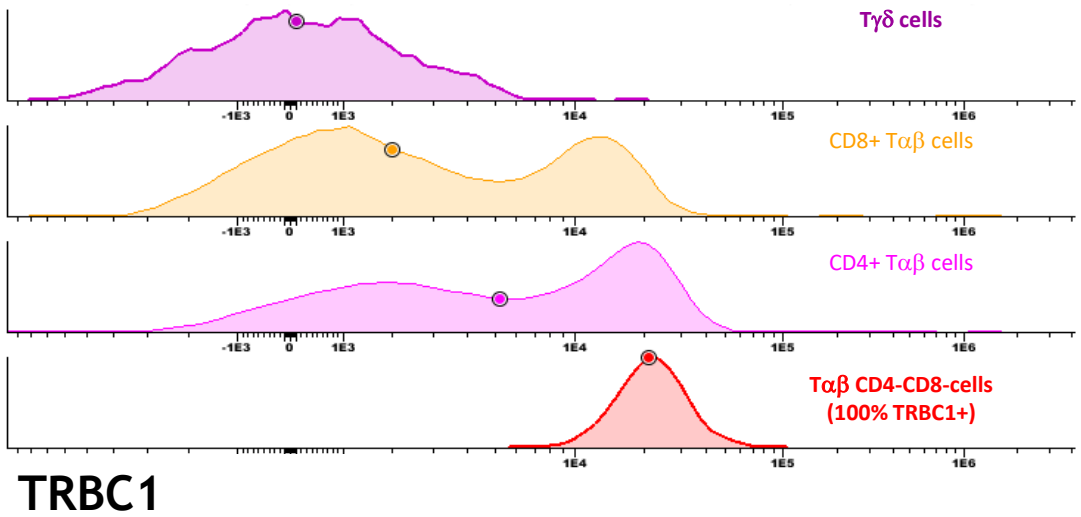
Detection of clonal T cells in healthy donors from the general population using LST together with TRBC1







Blood donor, Female 47y (# 67719 (USAL).)

Screening of clonal lymphoid populations in an otherwise healthy donor from general population: detection of a suspected TabCD4-CD8-CD5+ population showing monomodal expression of TRBC1 (+)

Clonal TCRgamma rearrangement confirmed by PCR on sorted cells

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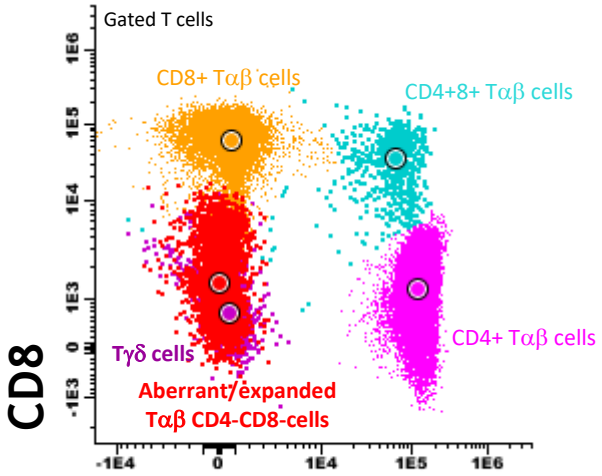

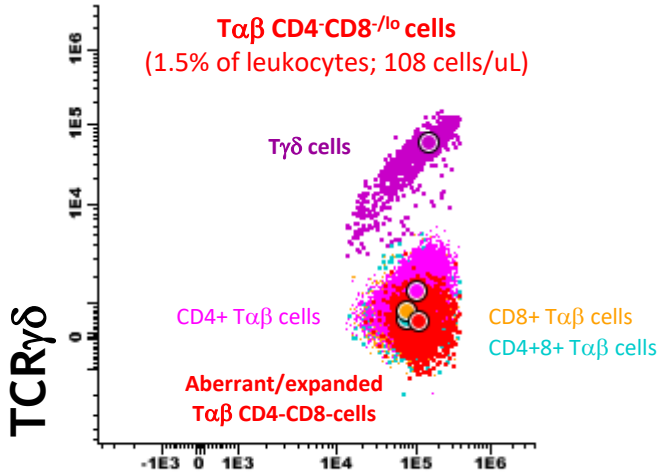

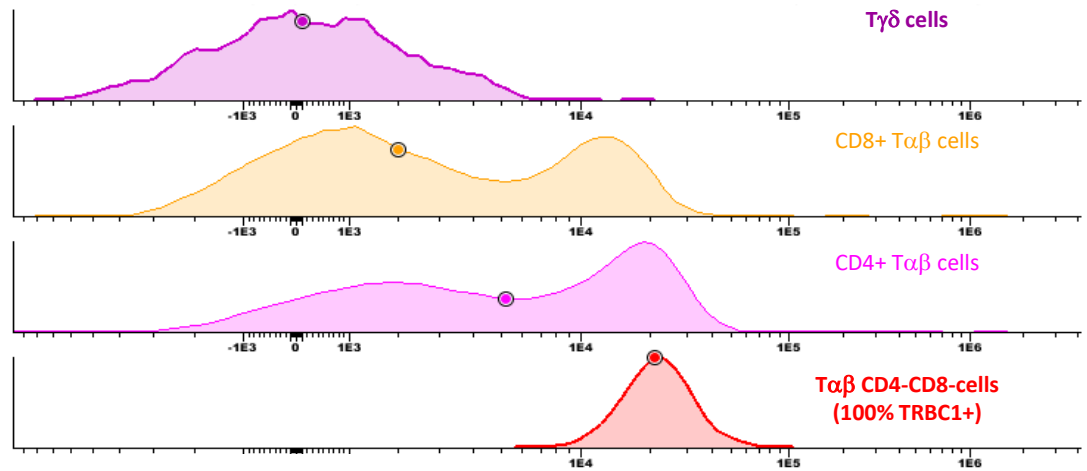

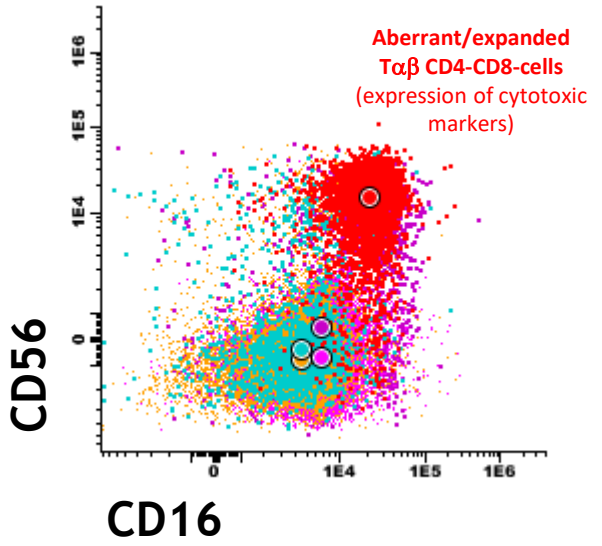

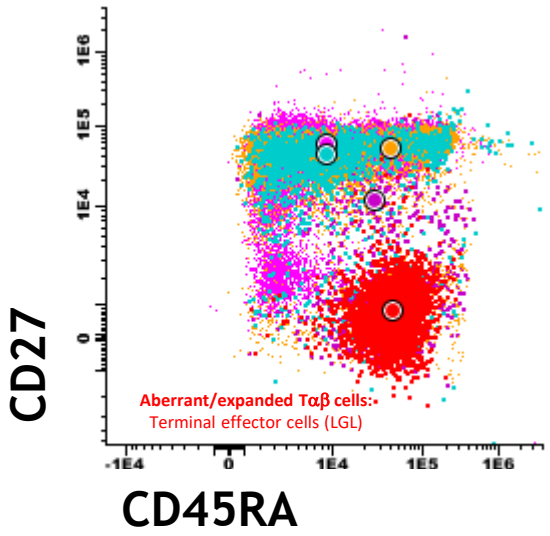

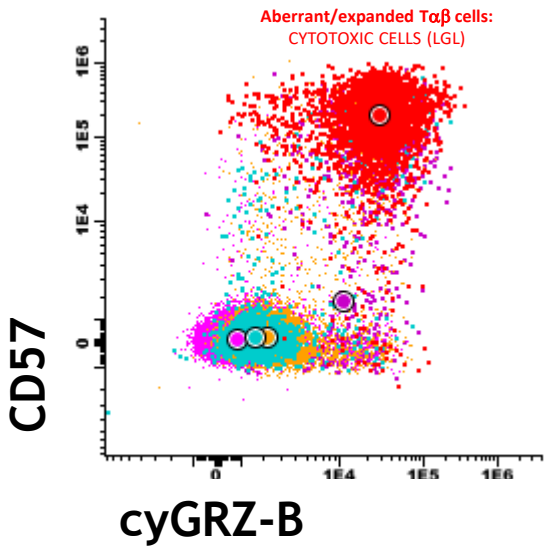

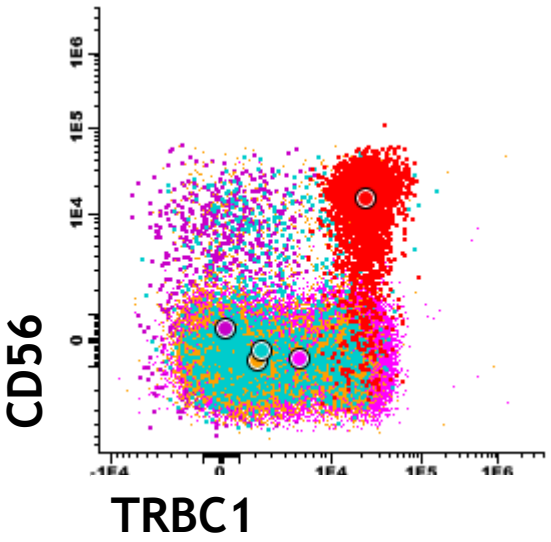

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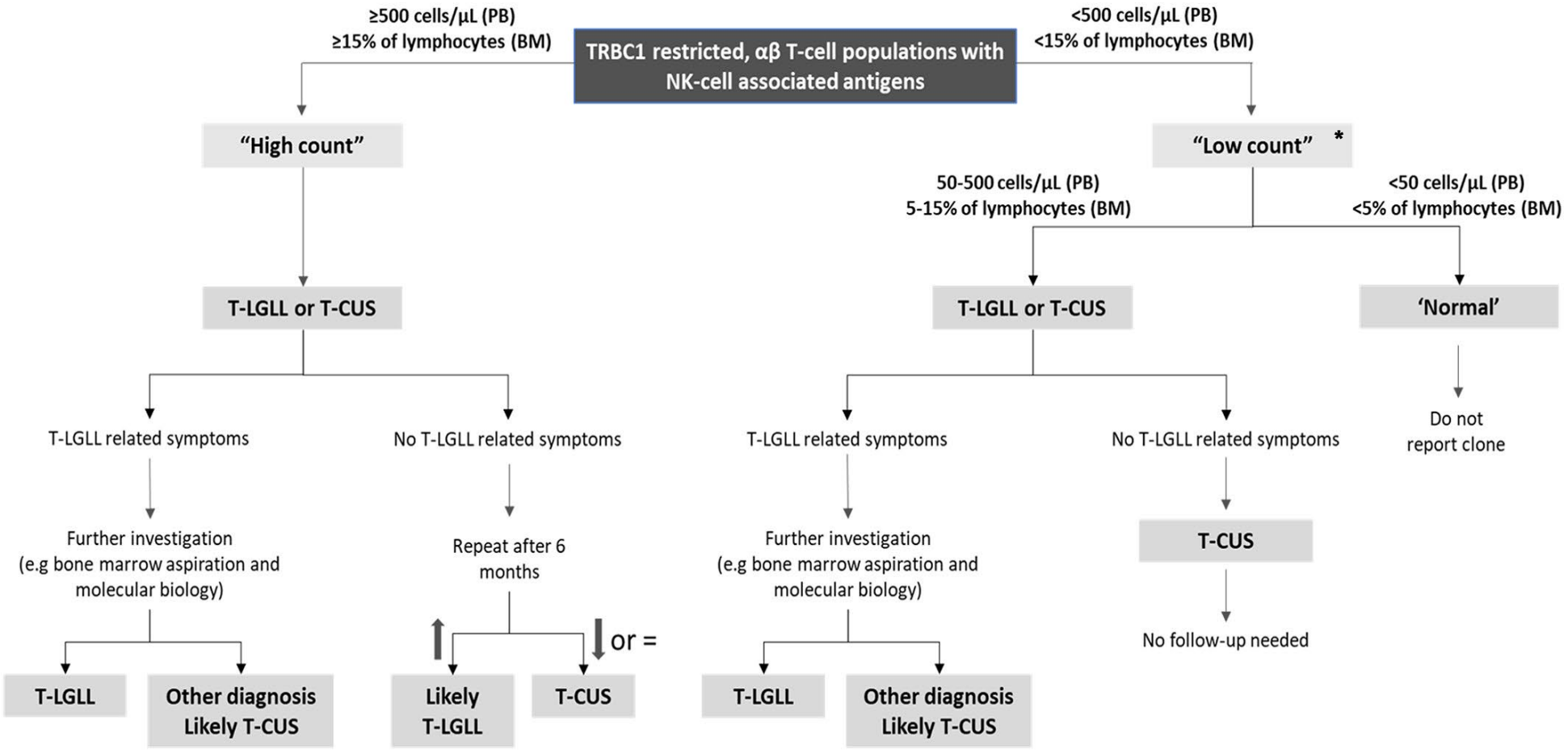
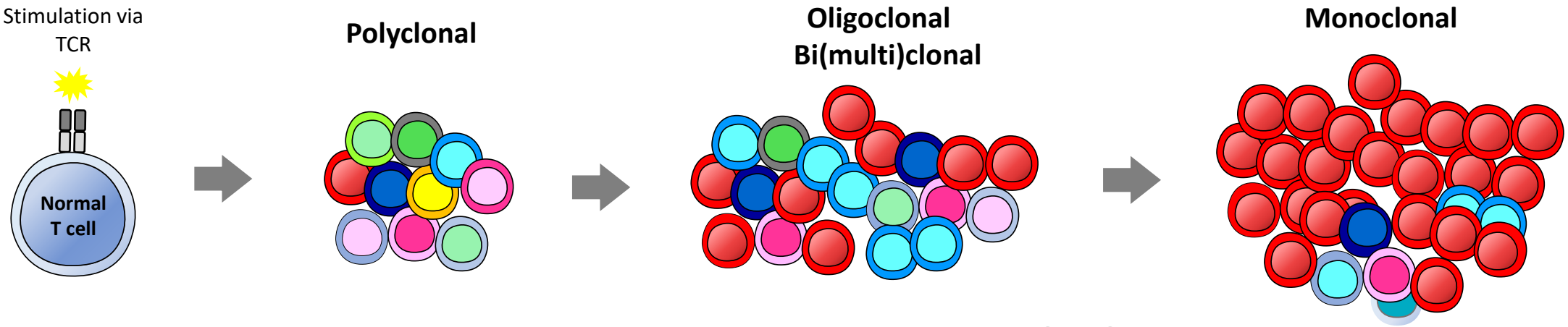
Proposal of a flowchart for the assessment of monotypic T-cell populations detected by a "lymphocyte screening tube" *plus* TRBC1



**** 9% of samples/cases referred for flow cytometric investigation of hematological malignancy carried small T-cell clones***

Blomme et al. Int J Lab Hematology 2023; 45: 496-505 (https://doi.org/10.1111/ijlh.14045)

T-LGL has been proposed to result from an antigen-driven leukemogenesis, transitioning from poly/oligo/monoclonality responses to malignant T-LGLL occurring gradually



Emergence and sequential selection and expansión of clones

LGL disorders are at the intersection of clonal lymphoproliferative diseases, immune dysregulation, chronic inflammation, and bone marrow failures and related conditions

Small T-cell clonal expansions have been reported in non-malignant conditions (autoimmune disorders, viral infections, non-T lymphoid malignancies, immunodeficiency, immune reconstitution following HSCT, "immunosenescence") and otherwise healthy donors:

T-cell clones of uncertain significance (T-CUS)

Prevalence of T-CUS and relationship with T-LGLL remains unknown

Lima et al, Am J Pathol 2001; Langerak et al, BJH 2003; Singleton et al, Am J Clin Pathol 2015; Shi et al, Clinical Cytometry 2020; Semenzato & Zambello Blood 2022

TRBC1-FCM approach for detecting clonal Tαß-cells

EuroFlow

SUMMARY : Validation for routine use

- **TRBC1-FCM approach shows a high specificity** (96% of cases concordant with the gold standard (PCR), validated in the longest series of samples; and
- **Analytical sensitivity / level of detection of at least 10^{-4}** can be reached when cells show an aberrant phenotype (which improves when combined with TCRVß)
- **TRBC1-FCM is a valid assay for the diagnosis of Tαβ-LGLL** based on altered (increased or decreased) % of TRBC1+ Tαß cells of LGL lymphocytosis; in the absence of lymphocytosis (or in TαßCD4-LGLL), detection of increased absolute counts of **more precisely defined subpopulations of T-LGL** expressing individual TCRVß families is required
- **Routine implementation of TRBC1-FCM at the screening stage** has identified a **high frequency of T-CUS**. Many questions remain regarding the clinical significance of T-CUS, which need to be answered in large follow-up studies

CONCLUSIONS

TRBC1-FCM approach for detecting clonal Tαß-cells

The TRBC1 approach is a useful, simple and fast FCM assay for accurate Tαß-cell clonality assessment in patients with suspicious T-CLPD, including Tαβ-LGLL

When used in combination with aberrant phenotypes, the approach has high specificity and sensitivity

Our results (combined with other recent studies evaluating TRBC1 assessment) strongly support the use of single-antibody TRBC1 in the reliable screening of T-cell clonality in routine clinical settings to diagnose and monitoring T-cell neoplasms (and T-CUS)

Acknowledgments

Classification and monitoring of T/NK-CLPD (WP L&L-10)

WP Leaders: **Julia Almeida** (USAL, Spain) and **Margarida Lima** †(CHP Porto, Portugal)

Participants: Neus Villamor (UB, Barcelona, Spain), Paula Fernández (KSA, Aarau, Switzerland), Matthias Ritgen (University of Schleswig-Holstein, Kiel, Germany), Anton W Langerak (Erasmus MC, RT, The Netherlands)

EuroFlow Leaders: **Jacques van Dongen and Alberto Orfao**

EuroFlow

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