A comparison of TRECs and flow cytometry for naïve T cell quantification

Stuart P. Adams[1*], Susanne Kricke[1], Elizabeth Ralph[2], Kimberly C. Gilmour[2]

[1]SIHMDS Haematology, Camelia Botnar Laboratories, Great Ormond Street Hospital for Children NHS Trust, London, WC1N 3JH, UK.

[2]Immunology, Camelia Botnar Laboratories, Great Ormond Street Hospital for Children NHS Trust, London, WC1N 3JH, UK.

[*]Correspondence: Stuart P. Adams, Principal Clinical Scientist, SIHMDS Haematology, Level 2 Camelia Botnar Labs, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London, WC1N 3JH, UK.

E-mail: stuart.adams@gosh.nhs.uk

Summary

Assessment of thymic output by measurement of naïve T cells is routinely carried out in clinical diagnostic laboratories, predominantly using flow cytometry with a suitable panel of antibodies. Naïve T cell measurements can also be made using molecular analyses to quantify TRECs (T cell receptor excision circle) levels in sorted cells from the peripheral blood. In this study we have retrospectively compared TRECs levels with CD45RA+CD27+ T cells and also with CD45RA+CD31+ T cells in 134 patient samples at diagnosis or during follow up. Both panels provide naïve T cell measurements that have a strongly positive correlation with TRECs numbers but CD45RA+CD31+ markers show the superior correlation with TRECs.

Introduction

Diagnosis of Primary Immunodeficiencies (PID), particularly Severe Combined Immunodeficiency (SCID), relies on laboratory assessment of basic lymphocyte measurements, lymphocyte subsets and increasingly naïve T cell assessment by flow cytometry. The European Society for Immunodeficiencies (ESID) have published online guidelines (https://esid.org/Working-Parties/Clinical/Resources/Diagnostic-criteria-for-PID2#Q12) for diagnosis of the most common PIDs and typically this requires the laboratory assessment of lymphocyte subset analysis as a minimum. In the US many states now routinely carry out newborn screening for SCID utilising the measurement of T cell receptor excision circles (TRECs) as a screening tool [1]. Thus laboratory testing to accurately measure thymic output is now a prerequisite for both screening and for diagnosis of PID. Additionally, monitoring TRECs levels has proved to be an essential tool to monitor T cell

immune reconstitution in haematopoietic stem cell transplant (HSCT), gene therapy and thymus transplant patients following treatment [2, 3, 4, 5].

Quantification of peripheral naïve and memory CD4+ and CD8+ T cells is routinely carried out in clinical diagnostic immunology laboratories, typically using combinations of cell surface markers such as CD4, CD8, CD45RA, CD45RO and CD27 antibodies with flow cytometric assessment [2, 6]. However, it has been well documented that, although useful in assessing T cell reconstitution, immunophenotyping using these markers may not be able to accurately measure thymic output [7]. An alternative marker, CD31 (platelet endothelial cell adhesion molecule-1 or PECAM-1), has been proposed as a more suitable target to quantify recent thymic emigrants (RTE) when used alongside CD45RA [7, 8]. The PECAM-1 protein was first cloned, named and characterised as a cell adhesion molecule belonging to the immunoglobulin gene superfamily [9, 10, 11]. Further studies have shown that CD31 is a differentiation antigen whose expression is lost after subsequent T cell receptor (TCR) engagement and during CD4 T-cell maturation into Th1 or Th2 effector cells [12, 13]. However, despite the advantages of using the combined expression of CD45RA and CD31 to measure RTE, it has been demonstrated that not every naïve T cell expressing CD31 is a newly formed T cell [14]. Thus using a second tool, such as quantification of TRECs, to measure the level of RTE can be useful [15]. Most TCRs are comprised from α and β chains with a small minority being formed of γ and δ chains. TRECs are formed during the ligation of the recombination signal sequences flanking the δ rec locus and the Ψ-Jα leading to the deletion of the TCRD locus from within the TCRA locus on the α-chain during the normal process of VDJ recombination (Fig.1). The resulting excised piece of DNA contains a unique signal joint sequence and thus is termed the sjTRECs [15]. This recombination event is identical in approximately 70% of $\alpha\beta$ T cells despite the enormous diversity generated during VDJ recombination [16]. The excised DNA subsequently forms an episomal circle from

which TRECs takes its name (T cell receptor excision circles). TRECs have proved useful in determining thymic output since they are stable and not easily degraded [17, 18]. In addition TRECs are not replicated during mitosis, are subsequently diluted during cell proliferation and can therefore be used as a measure of RTE [15]. TRECs can be measured using a real-time PCR approach [19] and are typically reported as TRECs per 10^6 cells [20].

In this study we compare results of RTE quantification between flow cytometric measurement (using CD45RA in combination with either CD27 or CD31 expression on both CD4+ and CD8+ T cells), and real-time PCR based TRECs quantification.

Materials and Methods

Patient samples

Blood (EDTA) was taken from patients for either routine diagnostic assessment or for routine follow up assessment following treatment and sent to the clinical laboratories for naïve T cell measurement as part of their standard care. Samples were collected between 2010-present and were analysed by flow Cytometry within 48 hours of collection. These patient samples were also cell-sorted on the same day as collection.

Flow Cytometry

Enumeration of lymphocyte populations was carried out by flow cytometric analysis. Whole blood was labelled with combinations of monoclonal antibodies conjugated with fluorescein isothionate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), or fluorochrome combinations with cyanines (PerCP-Cy5.5, APC-Cy7 and PE-Cy7) (BD Biosciences, UK.) Lymphocyte subsets were detected using a 6-colour multitest reagent containing CD3 FITC, CD16+56 PE, CD45 PerCP-Cy5.5, CD19 APC, CD4

PE-Cy7, and CD8-APC-Cy7 to which CD45RA V450 and CD27 V500 were added. Naïve, effector and memory T-cell populations were also detected using CD45RA FITC, CD31 PE, CD45 PerCP, and CD4 or CD8 APC. Post staining red cells were lysed (FACsLyse), samples washed (Cell Wash) and fixed (Cell Fix). 10,000 lymphocyte events were acquired on a FACsCanto II and analysed using FACs DIVA software.

Magnetic Bead Cell sorting

CD3+ T cells were isolated using magnetic bead cell sorting with Human Whole Blood CD3 MicroBeads on the autoMACs Pro Separator following the manufacturer's instructions (Miltenyi Biotec, Surrey, UK).

DNA Extraction

DNA was extracted directly from the cell sorted CD3+ T cells using the QIAamp DNA Blood Mini kit following the manufacturer's instructions (Qiagen UK). Eluted DNA was quantified using the Nanodrop 1000 spectrophotometer (Labtech International Ltd, UK).

Real-time quantitative PCR

TRECs were measured using a real-time quantitative assay as previously described [21]. Briefly, 5µl patient DNA was amplified in a 25µl total volume PCR solution containing primers and probes for the TRECs, KRECS and TRACs sequences with the Taqman Universal Mastermix (Life Technologies) in a 96-well plate on the Taqman 7500 Fast Real Time PCR System (Life Technologies). Standards for TRECs, KRECs and TRACs were prepared from a plasmid kindly provided by Sottini *et al*. [21], and this was also run in the assay to generate a standard curve. All patient DNA samples were run in triplicate alongside

no-template controls. TRECs levels for all patient samples were subsequently calculated per 10^6 CD3+ T cells.

Statistical Analyses

To accurately present the data a logarithmic adjustment of the TRECs counts was performed. This allows an accurate representation of the spread of TRECs values. In order to give an accurate representation of the relationship between TRECs counts and CD3/CD45RA/CD27 and CD3/CD45RA/CD31 percentages, patients' with TRECs counts of zero were not included in the graphs (Figs. 2-4). These 16 patients with TRECs values of 0 had a CD3/CD45RA/27 or CD3/CD45RA/CD31 percentage of less than 10%. All of these would be classified as SCID babies by either TRECs or immunophenotyping with markers of naïve T cells.

Results

A total of 134 patient samples were analysed using CD4+CD45RA+CD31+ and CD8+CD45RA+CD31+ panels to assess naïve T cell numbers. These samples also had absolute CD3+, CD4+ and CD8+ T cell counts measured. This enabled us to calculate the percentage of CD3+CD45RA+CD31+ T cells in each sample. These same samples also had CD4+CD45RA+CD27+ and CD8+ CD45RA+CD27+ cells quantified using flow cytometry at the same time. Again this permitted us to calculate the percentage CD3+CD45+CD27+ T cells in each sample. The remaining blood samples were then sorted into CD3+ T cells which were subsequently used to measure TRECs levels. Sorting for CD4+ and CD8+ cells was not undertaken as there was insufficient numbers of these cells in many samples. Thus the CD3+ T cells were isolated instead to maximise the potential for obtaining enough cells for TRECs analysis.

The median CD3+CD45RA+CD31+ naïve T cell level detected was 13% (range 0-77%) with a mean level of 21% naïve T cells. The median CD3+CD45RA+CD27+ naïve T cell level detected was 18% (range 0-91%) with a mean level of 25% naïve T cells. The median TRECs level detected was 3107 per million CD3+ T cells (range 0-66073) with a mean TRECs level of 6642 per million CD3+ T cells. *The TRECs levels were plotted against CD3+CD45RA+CD31+ naïve T cell levels to assess the overall correlation between the two (Fig. 2, correlation coefficient 0.76). Similarly the TRECs levels were plotted against CD3+CD45RA+CD27+ naïve T cell levels (Fig. 3, correlation coefficient 0.75). There is a very minor difference between their correlation coefficients of less than 0.015. This is insignificant. This is in contrast with the calculated correlation coefficient of 0.40 between TRECs and CD3+ T cell levels (Fig. 4). This suggests that it is not possible to estimate/predict the TRECs count from a total CD3 percentage.*

Discussion

Since most of the samples received in the diagnostic laboratory were from children with primary immunodeficiencies, or from children who had recently received a haematopoietic stem cell transplant, gene therapy or thymus transplant, it was expected that most would have low naïve T cell numbers and TRECs levels. This was borne out by the results obtained.

As expected the relationship between TRECs levels and flow cytometric measurement of naïve T cells using either panel of antibodies (CD3+CD45RA+CD27+ or CD3+CD45RA+CD31+) was very linear with high levels of correlation as assessed using Spearman's rank order correlation. *There was no significant difference between the use of either panels when comparing to TRECs levels. Thus, there is no evidence to suggest that CD31 is a more appropriate cell surface marker of naïve T cells than CD27 when used with CD45RA. Unsurprisingly the use of either naïve T cell markers showed a much stronger correlation with TRECs than by using just CD3+ T cells alone.*

For highly accurate assessment of thymic output other measurements are still required since he use of CD45RA+CD31+ (or CD45RA+CD27+) alone is still partially flawed. Krenger *et al*. [14] has shown that not every naïve T cell expressing CD31 is a newly formed T cell. Other studies have shown that the nuclear protein, Ki67, a proliferation marker expressed from late stage G1 through to the end of mitosis [22], can be used in conjunction with TRECs measurement to form a highly comprehensive model calculating thymic output [23, 24]. However, although modelling thymic output with Ki67 and TRECs is more accurate, there are logistical problems with incorporating this into the routine diagnostic setting. Most routine clinical labs operate with a high throughput of samples, often having to perform a number of different tests on small blood samples from young infants. To incorporate non-cell surface nuclear protein markers into routine working practice is prohibitive to workflow. Thus the use of CD31 or CD27 aligned with CD45RA may prove to be the flow cytometric panel of choice, especially if allied with TRECs measurement.

This retrospective study of naïve T cell assessment has showed that the widely used flow cytometry panel of CD4+ or CD8RA+ with CD45+CD27+ correlates strongly with TREC levels in paediatric samples. We have also shown that there is little to be gained by using CD45RA+CD31+ instead of CD45RA+CD27+ to measure naïve T cells. However there is a considerable advantage in using at least one of these naïve T cell panels since the CD3+ marker alone is not suitable for accurately quantifying naïve T cell numbers. It is therefore suggested that CD45RA+ should be used with either CD27+ or CD31+ for enumerating naïve T cells in routine diagnostic clinical laboratories.

For clinical laboratories where TRECs measurements may not be routinely available, either panel is sufficient for detecting the presence of naïve T cells post therapy or absence/low levels of them in patients with SCID or other primary immunodeficiencies.

Acknowledgements

Disclosure

None disclosed by all authors.

Author Contributions

The study was designed by K.G and S.A. The TRECs assay was developed at GOSH by S.A. The TRECs assay runs were performed by S.A and S.K. The flow cytometry assays were performed by E.R under the supervision of K.G. Data was collated by S.A and S.K and analysed by S.A. The manuscript was written by S.A and edited by K.G. The final manuscript was reviewed by all authors.

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Fig. 1. Following deletion of the TCRδ locus from the TCRα locus, a signal joint TREC is formed. Real-time PCT can then be used to quantify the sjTRECs as a measure of thymic output.
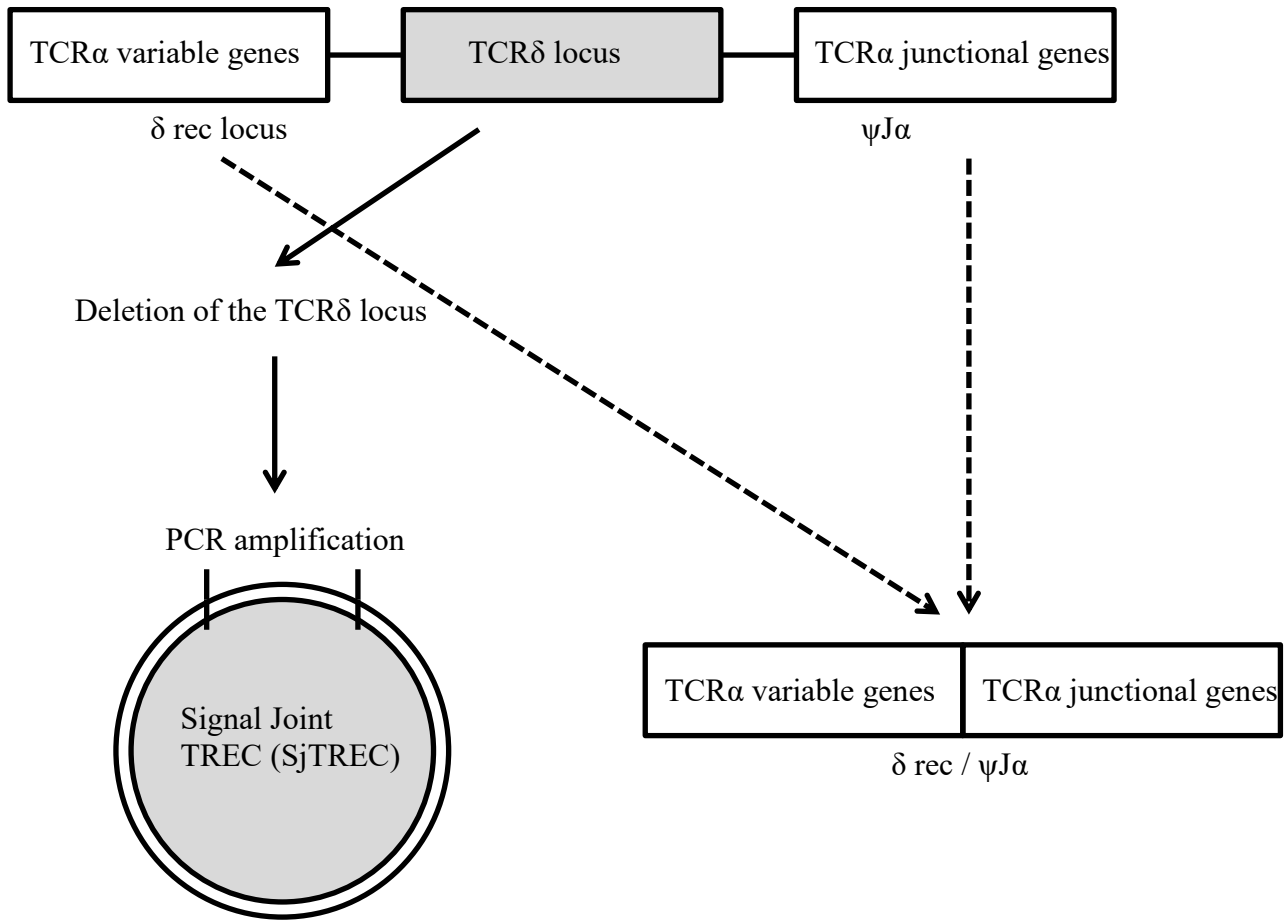
Fig. 2. TRECs (per 10^6 CD3+ T cells) plotted against CD3+CD45RA+CD31+ naïve T cells (percentage of overall CD3+ T cells).
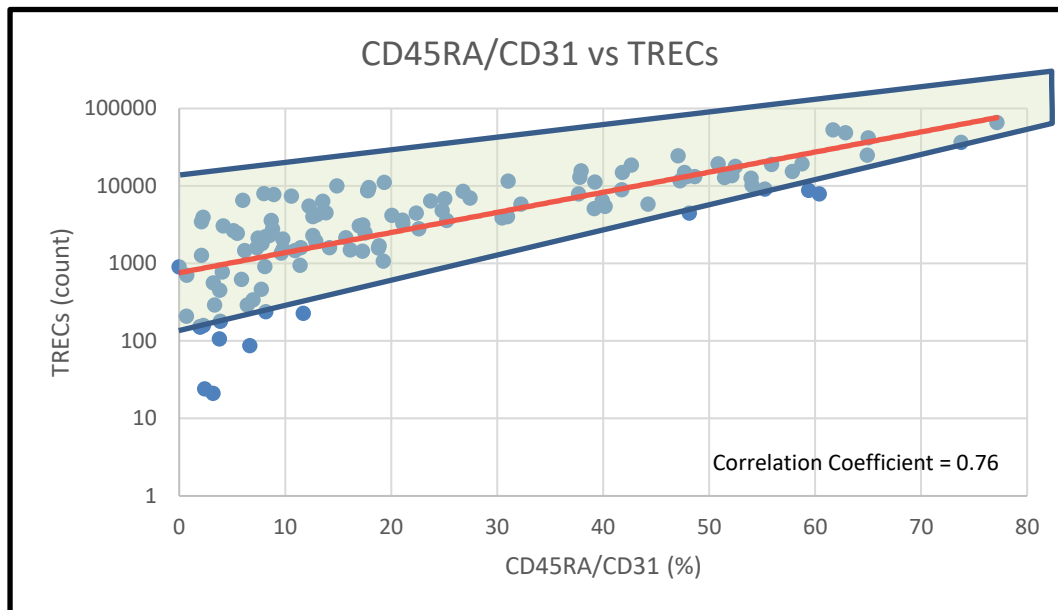
Fig. 3. TRECs (per 10^6 CD3+ T cells) plotted against CD3+CD45RA+CD27+ naïve T cells (percentage of overall CD3+ T cells).
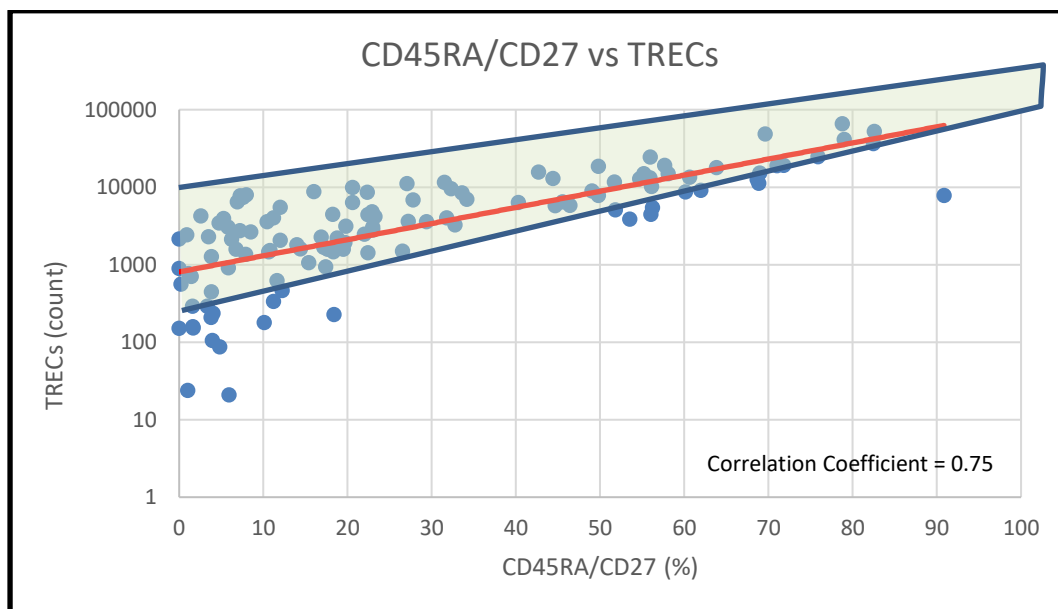
Fig. 4. TRECs (per 10^6 CD3+ T cells) plotted against CD3+ T cells (percentage of overall lymphocytes).