Epigenetic Counting of γδ-T Cells for Immune Monitoring in Clinical Settings

Udo Baron[1], Kati Bourquain[1], Isabell Janack[1], Stefan Kärst[1], Deborah Phippard[2], Angelina Bisconte[2], Ciro Novaes Lino Rosa[1], Laura Lozza[1], Sven Olek[1]

[1] Precision for Medicine GmbH, Berlin, Germany, [2] Precision for Medicine, Frederick, MD, USA

Introduction

Human gamma delta T cells (γδ-T cells) are non-conventional T lymphocytes expressing a TCR heterodimer comprised of gamma (γ) and delta (δ) chains. They constitute a part of the innate immune system playing a pivotal role at the frontline of host defense against pathogens. γδ-T cells have received particular attention as they exert multiple functions under non-infectious settings, most notably in carcinogenesis, where they can initiate a potent HLA-independent anti-tumor activity. γδ-T cells appear to be a promising lead for designing cell-based anti-cancer immunotherapies. For such strategies - which involve manipulation of γδ-T cells, e.g., by activation, tumor-targeting, or genetic modification - accurate and quantitative cell-counting tools are indispensable. The gold standard for immune cell quantification to date is flow cytometry (FCM).

However, to circumvent logistic issues and the need of flow cytometry specialty labs, epigenetic approaches might be recommended for cell counting to support large studies and when limited amount of biological material is available.

Objective

In recent years, evidence has been accumulated demonstrating epigenetic qPCR (Epiontis ID®) as a powerful methodology for cell quantification and immune monitoring and which is comparable to flow cytometry. Here, we employed this approach to the establishment of a (validated), real-time PCR-based assay for the quantification of γδ-T cells in blood, fresh and formalin-fixed and paraffin embedded (FFPE) tissue samples for facilitated immune monitoring in clinical settings.

Summary & Conclusion

We present a robust, sensitive and specific epigenetic qPCR-based assay for the quantification of γδ-T cells. We believe that this assay represents a useful tool for immune monitoring in clinical settings and may support the development of innovative γδ-T cell-based immunotherapies combatting cancer. Its applicability in cancerous samples remains to be confirmed.

Results

I) Unmethylated CpG sites in the TRGC1 gene represent a biomarker for the identification of γδ-T cells.

Differentially methylated CpG sites were identified in the gene encoding the constant region of the T cell receptor gamma chain (TRGC1). This marker region was entirely unmethylated in γδ-T cells but fully methylated in conventional CD4⁺ T cells, in CD56⁺ NK and CD19⁺ B cells, in CD14⁺ monocytes and CD15⁺ granulocytes. The observed incomplete methylation of the CD8⁺ T cell population may be due to a co-purification of unmethylated CD8⁺ γδ-T cells.

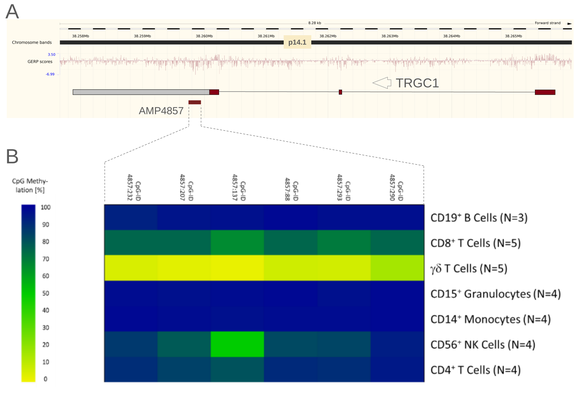

Figure 1: A) Illustration of the chromosomal position of the marker region (AMP4857) in the TRGC1 locus.

B) Bisulfite sequencing data of marker region AMP4857 in selected immune cell populations (left, Y axis) are shown. CpG sites (X axis; indicated by their ID) are represented by boxes with the color reflecting the methylation status according to the color bar at the left (full methylation (100%) marked in blue; complete demethylation (0%) displayed in yellow). CpG methylation values are averaged from multiple measurements; sample number is indicated in parenthesis.

II) Development and characterization of an epigenetic qPCR assay targeting TRGC1.

For the identified marker region in the TRGC1 gene, bisulfite-specific and CpG overlapping oligonucleotides (i.e., primer and probe) were designed to specifically amplify and detect unmethylated template DNA. For quantification of amplified target sequences, a plasmid standard was constructed that contain the TRGC1 marker region (as well as a fragment of the GAPDH gene, which is universally unmethylated and thus serves as a quantifier of all leukocytes (data not shown)).

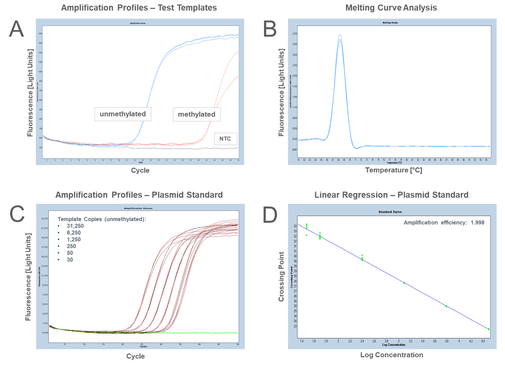

Figure 2: A) shows amplification profiles of the qPCR assay specific for the unmethylated, bisulfit-converted TRGC1 sequence after amplification of unmethylated target (blue) and methylated control templates (red), respectively. Discriminatory power (ΔCP) is 16.6 cycles. NTC (gray curve) corresponds to no template control.

B) illustrates data from a melting curve analysis after amplification of the target sequence. The graph shows a unique peak without any by-product.

C) displays amplification profiles (brownish curves) of a serially diluted plasmid-based quantification standard containing the TRGC1 target sequence. Template copies used are indicated and profile of a No Template Control is shown as a green curve.

D) shows the linear regression calculated from the measured crossing point (CP) values after amplification of the plasmid standard dilution series. The calculated amplicon efficiency of the PCR system is 1.998.

III) Linearity and cell type-specificity of epigenetic qPCR.

The developed qPCR assay was characterized for linearity and cell type specificity. Analysis of mixtures of methylated and unmethylated test templates shows a linear measurement range between 0% and 100% methylation (Figure 3). To demonstrate the cell type specificity, demethylation of the TRGC1 locus was quantified in sorted immune cell preparations. Results are in agreement with bisulfite sequencing data (Fig. 1). γδ-T cells appear to be completely demethylated, whereas control populations analyzed were strongly methylated. CD8⁺ T, NK and CD4⁺ T cells display some background (Table 1).

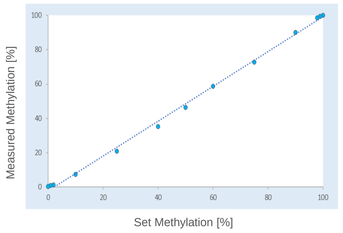

Figure 3. Artificially methylated and unmethylated test templates were mixed to simulate methylation levels between 0% and 100% and analyzed using the TRGC1-specific qPCR assay. Measured methylation values (mean of triplicates measurements) were plotted against the set methylation levels.

Tested Immune Cell Population		Measured TRGC1 De-Methylation [%]
T helper cells	CD3⁺ CD4⁺	2.2
Memory CD4⁺ T cells	CD4⁺ CD45RO⁺	3.3
Naive CD4⁺ T cells	CD4⁺ CD45RA⁺	3.0
Cytotoxic T cells	CD3⁺ CD8⁺	8.6
Memory CD8⁺ T cells	CD8⁺ CD45RO⁺	11.1
Naive CD8⁺ T cells	CD8⁺ CD45RA⁺	3.3
NK cells	CD3⁻ CD56⁺ CD16⁺	5.1
Monocytes	CD14⁺	0.4
Neutrophils	CD15⁺	0.4
B cells	Lin.⁻ CD19⁺	0.7
γδ-T cells	CD3⁺ TCRγ/δ⁺	99.7

Table 1. Sorted immune cell populations were analyzed in parallel by the TRGC1-specific qPCR assay, and a GAPDH-specific assay designed to quantify all cells present in each sample. In the table, the calculated percentage TRGC1 de-methylation is shown as the mean of three measurements.

IV) Normal range of γδ-T cells in whole blood from normal donors

EDTA anti-coagulated whole blood from 41 normal donors was analyzed with the γδ-T cell-specific epigenetic qPCR assay. The results showed an average of 3.4% and a median of 2.9% γδ-T cells with a standard deviation of +/- 1.5%. The lowest cell content was 1.0% and the highest 6.4%. The epigenetically assessed γδ-T cell values lie above the expected normal range (about 1%), indicating a moderate measurement bias.

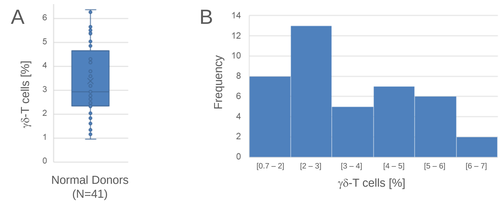

Figure 4: A) illustrates a boxplot distribution and B) a histogram from the analysis of 41 EDTA anti-coagulated whole blood samples from normal donors as analyzed with the epigenetic γδ-T cell-specific qPCR assay.

V) Accuracy of the epigenetic γδ-T cell-specific qPCR assay

Accuracy was addressed by comparing the epigenetic qPCR assay with corresponding data from flow cytometry, an established method for γδ-T cell quantification. A PBMC sample, serving as background, was spiked with increasing amounts of γδ-T cells and mixtures were analyzed on both measurement platforms.

The analysis of spiked samples showed linearity down to the range of about 3% γδ-T cells while higher dilutions (< 3%) could not be accurately quantified because the unspiked PBMC sample showed a background of γδ-T cells masking quantification. Epigenetic measurement values were higher (28%) than results from flow cytometry indicating a systematic bias. Correlation of both results showed a Pearson correlation coefficient of 1 (Spearman's Rho: 0.87).

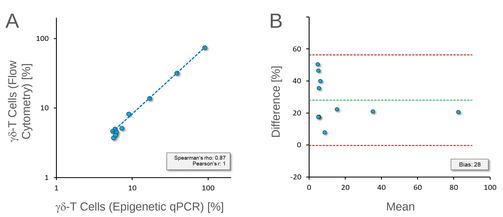

Figure 5: Comparison of γδ-T cell quantification by flow cytometry and epigenetic qPCR. A) Purified γδ-T cells were spiked into PBMCs and measured with epigenetic qPCR (x axis) and flow cytometry (y axis). Relative immune cell counts are shown as percentage of total cells. The blue dotted line indicates the regression line; correlation coefficients are indicated. B) shows a Bland-Altman plot of the data. Averaged γδ-T cell counting of the respective analyses (x axis) were plotted over their relative difference (y axis). Red lines reflect limit of agreement, the central green line indicates the systematic bias of the method comparison.

VI) Intra and Inter Assay Precision and LLoQ

Validation experiments were performed in accordance with internal quality standards based on DIN EN ISO 17025. The structure of the validation process is based on the ICH Guideline for Validation of Analytical Procedures.

Precision of the γδ-T cell assay was investigated by measuring a whole blood sample at four different input amounts, each analyzed in 4 replicates in 5 independent repetitions. Data was used to calculate the lower limit of quantification (LLoQ) by conducting trendline analysis, determining the exact CP value at which Epiontis ID standard precision requirements are breached.

Epiontis ID® standard precision requirements are defined by

- an intra assay coefficients of variation (C.V.) of less than **15%**
- an inter assay C.V. of less than **20%**
- and an average deviation of less than **15%** when comparing lowest and highest input.

Assay	CP Value Maximum Limit	Target Copy Number Minimum Limit (LLoQ)
TRGC1-specific qPCR Assay for the quantification of γδ-T cells	33.07	20

Table 2. Calculated lower limit of quantification from the γδ-T cell assay range validation