Detection of regulatory T cells, a comparison of flow cytometry and epigenetic qPCR immunophenotyping using healthy donor and oncology patient blood samples

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Abstract# 1739

Introduction

Human regulatory T cells (Tregs) are part of the adaptive immune response and play a pivotal role in suppressing immune responses and also cause unwanted tumor protection. Infiltration of Tregs into the tumor microenvironment has been associated with poor clinical prognosis and increased peripheral Treg frequency is associated with disease progression. Monitoring Tregs in both the blood and in the tumor of patients is being used to evaluate clinical prognosis and response to treatment in immuno-oncology studies. The key challenge is the ability to detect Tregs with high accuracy and precision.

Currently, the gold standard for immune cell quantification is flow cytometry, which provides flexibility in terms of cell types to be measured. However, flow cytometry is inherently limited as it requires fresh and/or intact cell material. This imposes high demands on sample management as time to analysis may influence quality of results. Standardization remains a challenge, too, due to biological, technical, and operational variations, and universal protocols remain to be established.

In recent years, evidence has been accumulated demonstrating epigenetic qPCR (Epiontis ID®) as a powerful methodology for cell quantification and a viable alternative to immune monitoring by flow cytometry. Epiontis ID® does not require fresh samples or intact cells, as DNA can be extracted from fresh or frozen blood which substantially simplifies the requirements for sample management.

Here, Epiontis ID® was employed for the quantification of total and regulatory T cells in peripheral blood samples and compared with the gold standard flow cytometry. Further, its applicability for immune monitoring was tested in blood and PBMC samples from cancer patients.

Objective

Epigenetic qPCR (Epiontis ID®) was employed as a method to detect and quantify Tregs and total T cells in peripheral whole blood and data were compared with flow cytometry. For method comparison, 143 whole blood samples from healthy donors were analyzed. The method was shown to be equivalent and there is a strong correlation between epigenetic qPCR and flow cytometry-based data, indicating that epigenetic qPCR is a robust and sensitive DNA-based method for Treg quantification.
Further, T cell and Treg levels in blood and PBMC samples from patients with solid tumor cancer were investigated by epigenetic qPCR to demonstrate the clinical applicability and relevance for immune monitoring.

Summary and Conclusion

Epigenetic qPCR-based cell counting demonstrates good correlation to flow cytometry, the gold standard for immunophenotyping. Its applicability for the analysis of regulatory T cells in tumor samples was demonstrated. It was observed that the ratio of Treg to T cells appears to be increased in the periphery of the tumor patients tested. A diagnostic/prognostic potential could not be derived due to the limited number of samples and remains to be assessed in follow-up studies.

As a DNA-based and well standardized technology, Epiontis ID® mitigates the operational and technical challenges of monitoring Tregs in immuno-oncology studies which is traditionally performed by flow cytometry. Epiontis ID® provides the sensitivity and accuracy needed for determining patient response in progressive disease assisting current immune diagnostics.

Method

Principle of epigenetic immune cell counting

Quantification of immune cells based on DNA methylation (epigenetic qPCR) provides relative and absolute immune cell counts and can be used on a variety of specimens (including fresh/frozen blood and tissue, dried blood spots, FFPE and other body fluids) regardless of preservation status. The signals are digital, indicating either a positive or negative value per cell, rather than arbitrary thresholds for "positivity" as in flow cytometry. Epigenetic qPCR can be performed in an automated and operator-independent manner, reducing susceptibility to reagent variability, and standardization is easy to achieve.

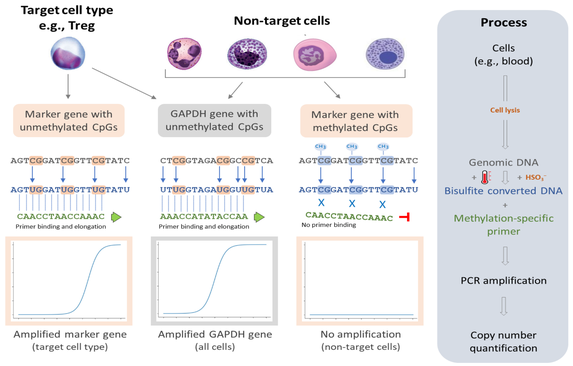

Figure 2: Principle of epigenetic qPCR analysis (Epiontis ID®).
Gene loci with cell type specifically unmethylated CpGs are used for analysis. Upon cell lysis, genomic DNA is chemically treated with bisulfite. In this reaction, unmethylated cytosine is converted to uracil (and then, in PCR, replaced by thymidine (T)), whereas methylated cytosine (C) remains unchanged. Methylated cytosines occur exclusively in the context of CpG dinucleotides. By means of the described chemical treatment, differential methylation is translated into primary sequence information. Using oligonucleotides that base-pair with converted UpGs, only the marker gene in the target cell type is PCR amplified. The corresponding sequence in non-target cells (i.e., with unconverted CpGs) is not amplified. To quantify DNA copies from all cells, a locus in the GAPDH (Glycerinaldehyd-3-phosphat-Dehydrogenase) gene is used, which is constitutively unmethylated. This latter epigenetic marker serves as a reference to determine the copy number of all DNAs and thus to quantify all cells in a sample.
(Adapted from Schildknecht K. et al. (2023) Clin Chem Lab Med. 21;62(4):615-626)

FOXP3 TSDR methylation is a highly specific biomarker for detection and quantification of regulatory T cells.

Differentially methylated CpG sites were identified in the gene encoding the Forkhead-Box-Protein P3 (FOXP3). These 15 CpGs are entirely unmethylated in regulatory T cells but fully methylated in all other immune cell types such as CD15⁺ granulocytes, CD14⁺ monocytes, CD56⁺ NK, CD8⁺ T, CD19⁺ B, CD4⁺ T and activated effector T cells. This marker region has been designated as TSDR (Treg specifically demethylated region) and constitutes the basis for the development of a highly specific PCR-based assay for the quantification of regulatory T cells.

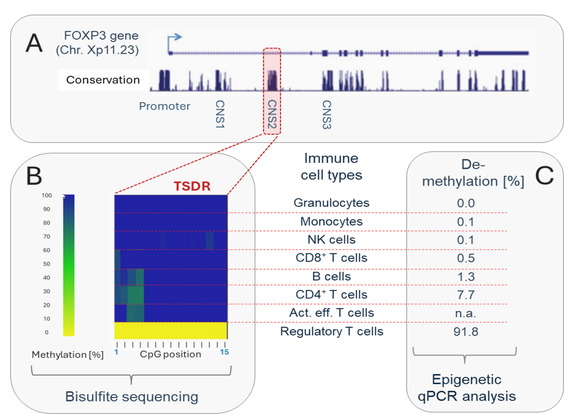

Figure 1: FOXP3, a DNA methylation marker for Treg.

A) Illustration of the chromosomal position of the marker region (TSDR) in the FOXP3 locus. B) Bisulfite sequencing data of the TSDR in selected immune cell populations. 15 CpG sites are represented by colored boxes reflecting the methylation status according to the color bar at the left (full methylation (100%) marked in blue; complete de-methylation (0%) displayed in yellow). C) Data from epigenetic qPCR analysis of selected immune cell populations are shown. Calculated de-methylation values correlate with data from B).

Results

Comparison of cell counting methods epigenetic qPCR (Epiontis ID®) and flow cytometry

Method comparison was performed using 143 peripheral blood samples from adult healthy donors which were analyzed in parallel for CD3⁺ T and FOXP3⁺ Treg cells by epigenetic qPCR and flow cytometry. Data (CD3⁺ T cells and Treg, as well as the ratio of Treg to T cells) from both methods were plotted as relative cell counts (i.e., %) in a scatter plot showing good correlation (Pearson's correlation coefficients (r) of >0.74 at $P < 0.001$). Bland-Altman analysis, which determines relative systematic biases and precision, was used to compare CD3⁺ T and FOXP3⁺ T cell counting between the two methods. For CD3⁺ T cells, data show a high degree of method agreement, with a minor bias (-4.6) and high precision (<20%).

For Treg, and thus also the ratio of Treg to T cells, the systematic bias between the two methods is 41.4 and 42.6%, which is also evident in the scatterplots in the different slopes of the bisectrix and the regression line. This indicates that the epigenetic analysis of Treg - although highly correlative to cytometry - yields slightly higher measured values.

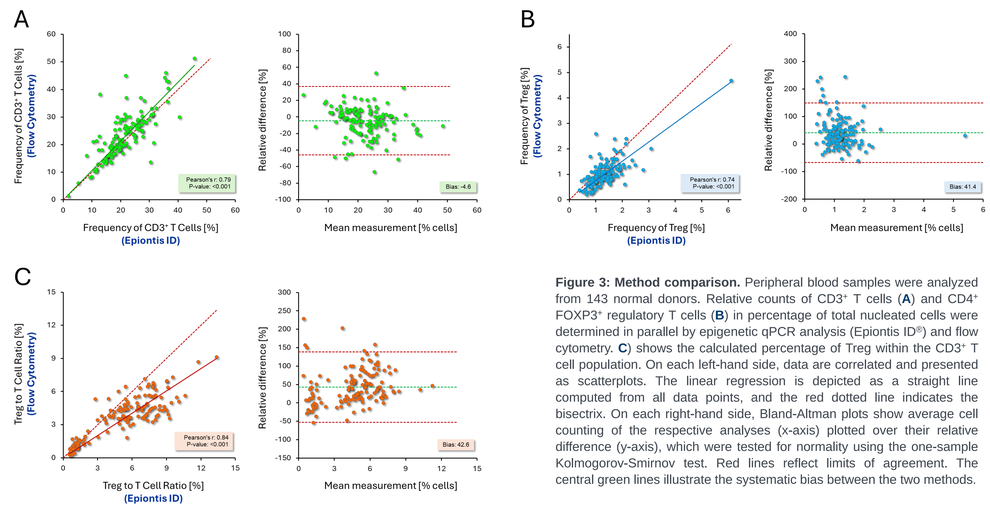

Figure 3: Method comparison. Peripheral blood samples were analyzed from 143 normal donors. Relative counts of CD3⁺ T cells (**A**) and CD4⁺ FOXP3⁺ regulatory T cells (**B**) in percentage of total nucleated cells were determined in parallel by epigenetic qPCR analysis (Epiontis ID®) and flow cytometry. **C**) shows the calculated percentage of Treg within the CD3⁺ T cell population. On each left-hand side, data are correlated and presented as scatterplots. The linear regression is depicted as a straight line computed from all data points, and the red dotted line indicates the bisectrix. On each right-hand side, Bland-Altman plots show average cell counting of the respective analyses (x-axis) plotted over their relative difference (y-axis), which were tested for normality using the one-sample Kolmogorov-Smirnov test. Red lines reflect limits of agreement. The central green lines illustrate the systematic bias between the two methods.

Applicability of epigenetic immune cell counting in blood and PBMC samples from cancer patients.

Epigenetic counting of CD3⁺ T and FOXP3⁺ Treg cells was applied to peripheral blood and PBMC samples from cancer patients.

- Blood samples (ambient, >48hrs old): breast (N=3), colon (N=4), head and neck (N=3), melanoma (N=4)
- PBMC samples (cryo-conserved): breast (N=6), colon (N=6), head and neck (N=6)

- In comparison to controls, blood samples of the tumor patients show no significant difference in Treg frequency (Fig. 4A) (only breast tends to have elevated values). However, the calculated Treg to T cell ratio appears to increase in breast and colon (Fig. 4B).
- This observation is supported by the analysis of the PBMC samples (Fig. 4D): tumor samples also show an increased Treg to T cell ratio (although Treg frequencies appear to be lower than the PBMC controls).
- Pearson's correlation between the data from the epigenetic and cytometric analysis of the PBMC samples were 0.698 (p=0.012) for Treg and 0.634 (p=0.007) for the Treg to T cell ratio (data not shown). In contrast to epigenetic analysis, cytometric analysis of the 14 blood samples was not possible due to prolonged storage (>48hrs) at ambient temperature.
- It should be noted that the informative value of this analysis is limited due to the small number of samples and must be confirmed by a more comprehensive analysis.

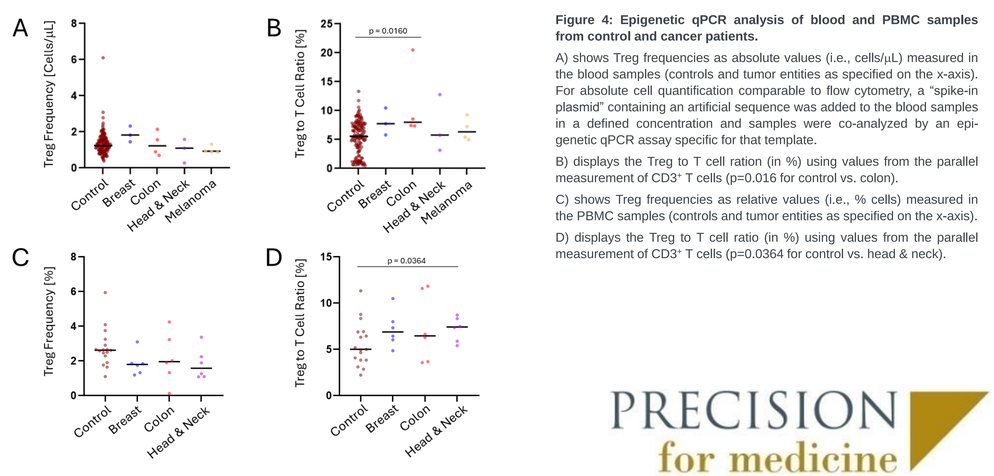

Figure 4: Epigenetic qPCR analysis of blood and PBMC samples from control and cancer patients.

A) shows Treg frequencies as absolute values (i.e., cells/µL) measured in the blood samples (controls and tumor entities as specified on the x-axis). For absolute cell quantification comparable to flow cytometry, a "spike-in plasmid" containing an artificial sequence was added to the blood samples in a defined concentration and samples were co-analyzed by an epigenetic qPCR assay specific for that template.

B) displays the Treg to T cell ration (in %) using values from the parallel measurement of CD3⁺ T cells (p=0.016 for control vs. colon).

C) shows Treg frequencies as relative values (i.e., % cells) measured in the PBMC samples (controls and tumor entities as specified on the x-axis).

D) displays the Treg to T cell ratio (in %) using values from the parallel measurement of CD3⁺ T cells (p=0.0364 for control vs. head & neck).