Epigenetic Immune Cell Quantification For Diagnosis and Monitoring of Patients with Primary Immune Deficiencies and Immune Regulatory Disorders.

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Introduction

DNA-methylation analysis provides a unique approach to molecular immune cell quantification. Immune cell type-specific demethylated genomic regions are identified and quantified using real-time PCR. This allows immune cell profiling from small amounts of fresh and archived samples – e.g. whole blood, dried blood spots and tissue.

We established epigenetic immune cell quantification for **CD3⁺, CD4⁺, CD8⁺ T-cells, FOXP3⁺ Treg, B-, NK-cells, neutrophils and monocytes.**

Here we demonstrate:

1. **Equivalence of the epigenetic approach with flow cytometry** (Fig. 4)
2. **Identification of patients with SCID, XLA, IPEX and SCN** in dried blood spots (DBS) of newborns (Fig. 5 & 6)
3. **Identification of dysregulation of FOXP3⁺ Treg** in patients with **Primary Immune Regulatory Disorders** (Fig. 7)
4. **Earlier detection of immune cell reconstituion** after hematopoietic stem cell transplantation than using flow cytometry (Fig. 8)

Comparison of Immune Cell Quantification by Flow Cytometry and Epigenetic qPCR

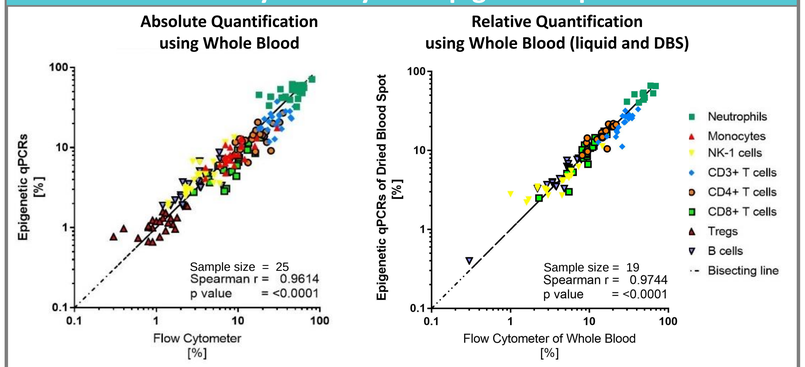

Figure 4: Equivalence of the epigenetic approach with flow cytometry

Epigenetic qPCR on Blood Samples from Patients with Primary Immune Regulatory Disorders or Immune Deficiencies.

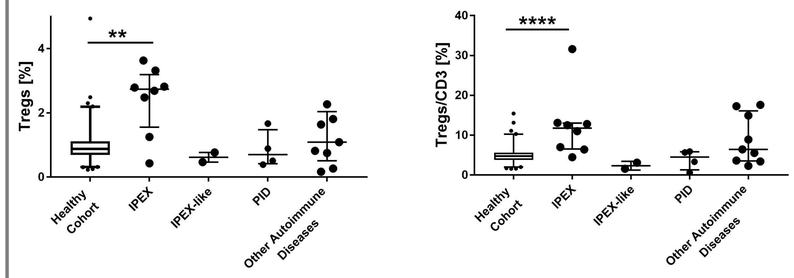

Figure 7: Tregs and Tregs/CD3+ T cell ratio was analyzed in male IPEX, IPEX-like and PID patients and compared with a healthy cohort (n=404) and a disease control cohort including patients with other autoimmune diseases. [2]

Method: DNA-Methylation Based Real-Time PCR Assays for the Quantification of Different Immune Cell Types

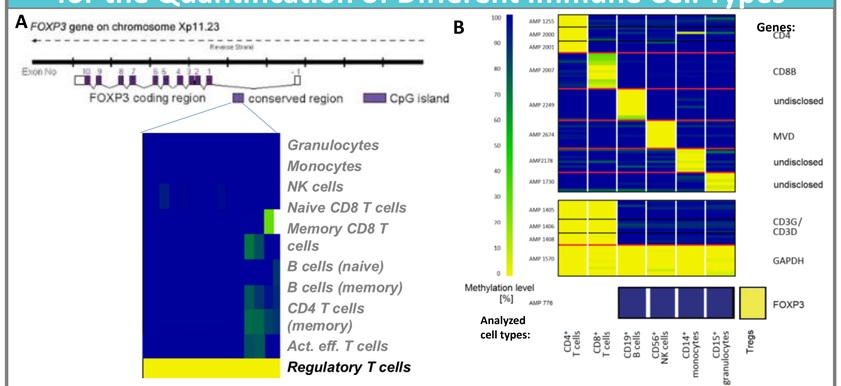

Figure 1: Identification of demethylated regions specific for individual immune cell types. A) TSDR within the FOXP3 gene is specifically demethylated in regulatory T cells. B) Development of a marker panel for various immune cells. [1]

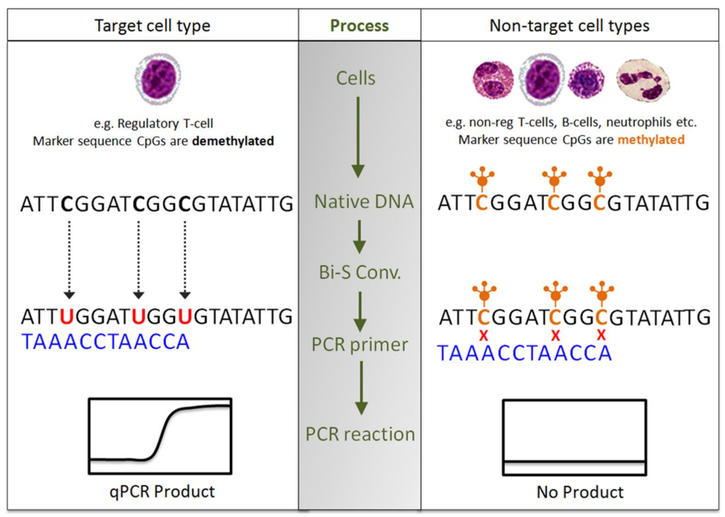

Figure 2: Development of demethylation-specific real-time PCR assays for the quantification of different immune cells. Methylation pattern is transferred into the DNA sequence by bisulfite conversion. Primers and probes match only converted, demethylated target sequence. In parallel, housekeeping gene GAPDH is analyzed. [1,3]

Identification of SCID and XLA Patients on Dried Blood Spots using Epigenetic qPCR

Disease Description				TREC/KREC Newborn Screening		Epigenetic qPCR Analysis			
Classification	Gene Defect	Loss of Function Type	TREC[1] Positive [yes/no]	KREC[2] Positive [yes/no]	Screening Classification	(CD3 G/D, GAPDH)[3] Conspicuous [yes/no]	(MVD, GAPDH)[3] Conspicuous [yes/no]	(LRP5, GAPDH)[3] Conspicuous [yes/no]	Screening Classification
SCID	*ADA*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
SCID	*ADA*	amorph	no	yes	correctly identified	yes	yes	yes	correctly identified
DO-SCID[4]	*ADA*	hypomorph	no	yes	correctly identified	no	yes	yes	correctly identified
DO-SCID[4]	*ADA*	hypomorph	no	yes	correctly identified	yes	yes	yes	correctly identified
SCID	*AK2*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
SCID	*AK2*	amorph	yes	yes	correctly identified	yes	yes	no	correctly identified
SCID	*Artemis*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
SCID	*CD3D*	amorph	yes	no	correctly identified	yes	yes	no	correctly identified
SCID w ME[5]	*IL2RG*	amoprh	yes	no	correctly identified	no	no	no	not identified
SCID	*IL2RG*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	no	no	correctly identified
SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
SCID	*IL7RA*	amorph	yes	no	correctly identified	yes	yes	yes	correctly identified
DO-SCID[4]	*JAK3*	hypomorph	no	no	not identified	yes	yes	yes	correctly identified
SCID	*PNP*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
SCID	*PNP*	amorph	yes	yes	correctly identified	yes	yes	yes	correctly identified
SCID	*RAG1*	hypomorph	yes	yes	correctly identified	yes	yes	no	correctly identified
SCID	*RAG1*	amorph	yes	yes	correctly identified	no	yes	yes	correctly identified
SCID	*RAG2*	amorph	yes	yes	correctly identified	yes	no	yes	correctly identified
XLA	*BTK*	amorph	no	yes	correctly identified	yes	no	yes	correctly identified
XLA	*BTK*	amorph	no	yes	correctly identified	no	no	yes	correctly identified
XLA	*BTK*	amorph	no	yes	correctly identified	no	no	yes	correctly identified
XLA	*BTK*	amorph	no	yes	correctly identified	yes	yes	yes	correctly identified
XLA	*BTK*	hypomorph	no	no	not identified	no	yes	yes	correctly identified

[1]TREC values ≤ 6 copies per dot were considered positive; [2]KREC values ≤ 4 copies per dot were considered positive;
[3]Values outside the joint 99% reference range were considered conspicious, see Fig. 5; [4] Delayed onset SCID; [5] SCID with maternal engraftment

Figure 5: 24 DBS from PID-diagnosed newborns were analyzed. Reference ranges for T-, B- and NK cells were established using DBS from healthy neonates (n=250). [1]

Epigenetic qPCR on DBS from Newborns with IPEX or Severe Congenital Neutropenia (SCN)

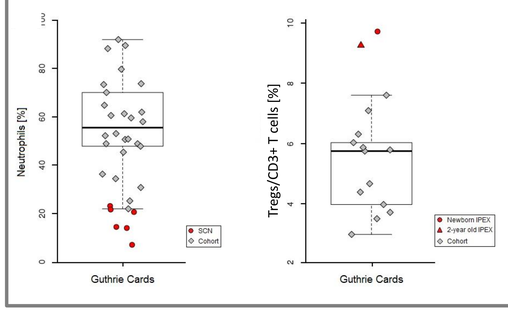

Figure 6: DBS from healthy controls and newborns with SCN or IPEX were subjected to epigenetic qPCR. This finding opens the possibility to identify patients already at birth using epigenetic qPCR in newborn screening. [1]

Highly Sensitive qPCRs are Suitable for Early Immune Cell Quantification of HSCT patients

	Flow Cytometry	Epigenetic qPCR	
Immune cell types	**First valid test result (days post Tx)**	**First valid test result (days post Tx)**	**P value**
Leukocytes	6 (6-12)	9 (5-11)	0.9047
CD3+ T cells	29 (20-40)	19 (13-20)	0.0011
CD3+CD4+ T cells	29 (20-40)	19 (13-20)	0.0003
CD3+CD8+ T cells	29 (20-40)	19 (14-26)	0.0014
CD19+ B cells	47 (34-72)	20 (19-30)	<0.0001
CD56dimCD16+ NK cells	29 (20-40)	19 (17-25)	0.0009

Figure 8: Whole blood samples of 21 pediatric HSCT recipients were sent to clinical laboratory and analyzed in parallel with epigenetic qPCRs. The median day after transplantation (median; interquartile range) was calculated where the respective technology provided first valid immune cell counts..

Conclusion

Epigenetic immune cell quantification offers substantial benefits for broad immune cell profiling where a fresh blood sample in sufficient quality and quantity is difficult or impossible to obtain.

Here we show applicability of the method to identify SCID and XLA patients **in dried blood spots from newborns**. This could be the basis for an expanded PID/PIRD newborn screening that includes immune regulatory disorders presenting with severe autoimmunity as well as congenital neutropenia and autoinflammatory diseases.

We also show that epigenetic immune cell profiling is **more sensitive than flow cytometry** in detecting immune cell reconstitution in HSCT patients. This supports application of epigenetic immune cell profiling in routine patient monitoring using DBS sampling in a near patient setting without the need for phlebotomy at dedicated facilities.

References

[1]**Baron et al.**, Epigenetic immune cell counting in human blood samples for immunodiagnostics**.** Sci. Transl. Med. 2018 Aug; 10 –pp1-11

[2]**Barzaghi et al.,** Demethylation analysis of the FOXP3 locus shows quantitative defects of regulatory T cells in IPEX-like syndrome. J Autoimmun. 2012 Feb;38(1):49-58

[3]**Wieczorek et al.,** Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res. 2009 Jan 15;69(2):599-608