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Supporting Information

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Roberto Spreafico, Maura Rossetti, Theo van den Broek, Nicolaas J.G. Jansen, Hong Zhang, Maryam Moshref, Berent Prakken, Jorg van Loosdregt, Femke van Wijk and Salvatore Albani

A sensitive protocol for *FOXP3* epigenetic analysis in scarce human samples

Supporting information

Samples

Adult blood was collected at the TSRI Healthy Blood Donor Service under SBMRI IRB approval upon written informed consent, and processed within 2 hours from withdrawal. Matched blood-thymus pairs from thymectomized infants undergoing heart surgery within their first 2 months of life were obtained at the Wilhelmina Children's Hospital. Neonatal samples were processed immediately after acquisition and FACS sorted the same day.

Flow cytometry and cell sorting

Treg cells ($CD3^+CD4^+CD25^{high}CD127^{low/-}$ or $CD3^+CD4^+CD8^-CD25^+CD127^-FOXP3^+$) and Tconv cells ($CD3^+CD4^+CD25^{low/-}$ or $CD3^+CD4^+CD8^-CD25^-FOXP3^-$) were sorted from either thawed adult PBMCs or neonatal fresh paired PBMCs and thymocytes using a FACSAria II (BD Biosciences). Intracellular staining was performed using the anti-human FOXP3 staining set from eBioscience, following manufacturer's instructions. Fluorochrome-conjugated antibodies were from Biolegend, BD Biosciences and eBioscience. Analysis was performed with FlowJo (Treestar).

TSDR methylation

Large samples, for which DNA yields are not a concern, were processed using a two-step protocol: gDNA was first isolated with the ZR-Duet DNA/RNA MiniPrep kit (fresh cells) or the ZR FFPE DNA MiniPrep (fixed cells), then

bisulphite conversion was performed with EZ DNA Methylation-Gold kit (all from Zymo Research). Smaller samples (10^3 to 10^5 cells) were processed using the single-step EZ DNA Methylation-Direct kit (Zymo Research) to avoid gDNA loss due to separate bisulphite conversion. Sorting cells directly into lysis buffer maximizes DNA yields. Digestion with the Proteinase K provided by the kit was performed for 20 min (fresh cells) or 4 hrs (fixed cells). The TSDR sequences of methylated and unmethylated TSDR were synthesized by AITbiotech and cloned into a pUC57 plasmid.

The TSDR of the *FOXP3* locus was amplified by touchdown nested PCR using the Taq PCR core kit (Qiagen). The primary PCR was performed using the following primers: f: GTTTGTGGTTATTTTTGAAGT; r: CAAATAAACATCACCTACCAC. The secondary PCR was performed with modified Amp5 primers[5]: f: ACCAAC-TGTTTGGGGGTAGAGGATTT; r: AGTGGT-TATCACCCCACCTAAACCAA. The modified Amp5 primers have a 6-base tail at the 5' end (followed by a hyphen in the sequence reported above).

For the primary PCR, bisulfite-converted gDNA was amplified in a 20-µl reaction, using 1 U Taq enzyme, 1X buffer containing 1.5 mM $MgCl_2$, 100 nM each primer, 0.2 mM dNTPs. Cycling conditions were as follows: 1) Initial denaturation: 94°C, 5 min; 2) Touchdown amplification: 7 cycles of 94°C, 30 sec; 58.5°C, 1 min (touchdown -0.5°C/cycle); 72°C, 30 sec; 3) Regular amplification: 25 cycles of 94°C, 30 sec; 55.5°C, 1 min; 72°C, 30 sec; 4) Final extension: 72°C, 5 min; 5) Hold: 4°C.

For the secondary PCR, 1 µl of 10-fold diluted primary PCR was amplified in a 20-µl final volume, using 1 U Taq enzyme, 1X buffer containing 1.5 mM $MgCl_2$, 200 nM each primer, 0.2 mM dNTPs. Cycling conditions were as follows: 1) Initial denaturation: 94°C, 3 min; 2) Touchdown amplification: 5 cycles of 94°C, 30 sec; 60°C, 45 sec (touchdown -0.5°C/cycle); 72°C, 30 sec; 3) Preliminary amplification: 5 cycles of 94°C, 30 sec; 58°C, 45 sec; 72°C, 30 sec; 4) Regular amplification specific to full-length tailed primers: 25 cycles of 94°C, 30 sec; 68°C, 30 sec; 72°C, 30 sec; 5) Final extension: 72°C, 5 min; 6) Hold: 4°C.

Successful amplification was confirmed by running 5 µl PCR products in a 2% agarose gel. PCR products were cleaned up enzymatically using ExoSAP-IT (Affymetrix) according to the manufacturer's instructions, then sequenced using the following HPLC-grade primer: ACCAACTGTTTGGGGGTA. The sequencing reaction was performed in 10 µl final volume using 5 ng cleaned PCR product, BigDye Terminator v3.1 (Life Technologies) at 1/8 strength in 1x buffer (1 µl BigDye, 1.5 µl 5x buffer), and 0.3 µM primer. Cycling conditions were as follows: 1) Initial denaturation: 96°C, 1 min; 2) Cycle sequencing: 25 cycles of 96°C, 10 sec; 50°C, 2 min; 3) Hold: 4°C. The reaction was cleaned up using the BigDye XTerminator kit (Life Technologies) according to the manufacturer's instructions, and then loaded on a Genetic Analyzer (Applied Biosystems). We observed that best results were obtained when the KB basecaller was used on traces generated by the 3100 Genetic Analyzer with the POP4 polymer, or when the SR basecaller was used on traces generated by the 3730 DNA Analyzer with the POP7 polymer. Electropherograms were

analyzed with ESME v3.2.5[11], obtained from www.epigenome.org/index.php?page=download. ESME was executed on a 64-bit Linux Mint distribution, with the following packages installed: ia32-libs, lib32stdc++6, libc6-i386, lsb-core. A Linux script to consolidate ESME output from multiple trace files and the FOXP3 TSDR reference file are available upon request to the corresponding authors.