Material and methods:

Patients, study cohorts – The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks approved the study. All patients who participated in this study gave informed consent. A total of 65 age-matched kidney transplant patients were included in the study (summary of clinical data in Table I). Criteria for operational tolerance have been described in detailed elsewhere (1). Briefly renal allograft recipients were separated in three groups: (i) tolerant recipients defined as patients with a well functioning graft (<150µM/L and proteinuria <1g/24h) without immunosuppression for more than one year (TOL, n=13); (ii) patients defined as patients with a well functioning graft (creatinine <150µM/L and proteinuria <1g/24h) under classic immunosuppressive therapy (STA, n=33); (iii) patients with signs of graft rejection and functional degradation (creatinine >150µM/L and proteinuria >1g/24h) (RC, n=19). (iv) Finally an additional group of healthy volunteers with no known history of infection or other pathologies was included as a control (HV, n=15). Clinical and demographical data of these patients are summarized in Table 1.

Antibodies, flow cytometry and cell sorting - The following antibodies and reagents were used for flow cytometry and cell sorting: CD45-PO (Caltag, Invitrogen, Darmstadt, Germany), CD3-AlexaFluor-700, anti-CD45-PE and CD4-PB (both from BD Biosciences, Heidelberg, Germany), CD3 Brillant-Violet-605, CD4 APC-Cy7, CD45RA-PeCy7, CCR7-PE, CD25 Brillant-Violet-421, CD127-AlexaFluor-647 (Biolegend, San Diego, USA) Foxp3-PercpCy5.5, GITR-APC, CD39-APC, Lag3-Pe and CTLA4-Pe, (eBioscience, San Diego, USA) anti-CD4-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-CD25-Alexa Fluor 647 (anti-CD25 from Immunotech, Marseille, France). Live/Dead Fixable Aqua stain was used to exclude dead

cells. Thawed PBMC samples were stained in PBS with 5% SVF and 2mM EDTA for 15' at 4°C in the dark. For Foxp3 intranuclear staining, cells were fixed and permeabilized with the Foxp3 staining Kit (eBisocience, San Diego, USA) according to the manufacturer's instructions. Flow cytometry was performed on a BD LSR II cytometer (BD Bioscience, San Diego, USA) and cell sorting was performed on a FACSAriaIII (BD Biosciences, San Diego, USA). Compensations were set using BD compbeads (BD Biosciences, San Diego, USA). For Sorting, DAPI was used to exclude dead cells. Effector T cells were sorted based on the $CD3^+$ $CD4^+$ $CD25^-$ phenotype, total regulatory T cells were sorted based on the $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype and mTregs based on the phenotype $CD3^+$ $CD4^+$ $CD25^{hi}$ $CD45RA^-$. Post-sort analysis revealed a purity of > 95%.

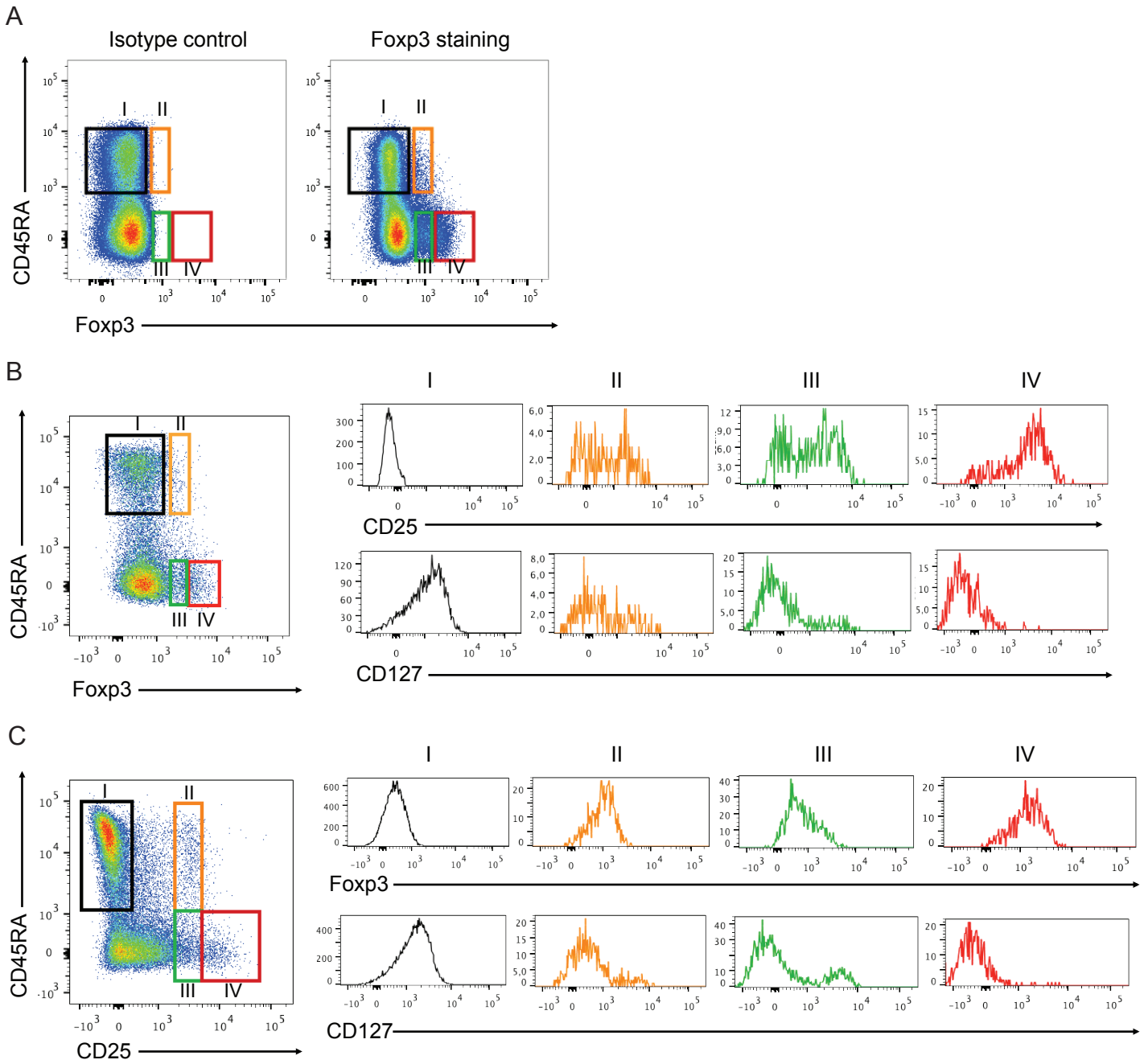
Foxp3 TSDR quantification - Genomic DNA was isolated from PBMC samples, purified CD4+ T cells and FACS-sorted regulatory T cells using the QIAamp^R DNA Blood Mini kit (Qiagen, Hilden, Germany). 500ng eluted genomic DNA was given a subsequent bisulfite treatment (EpiTect^R, Qiagen, Hilden, Germany). A minimum of 60ng bisulfite-treated genomic DNA was than used in a Realtime-PCR to quantify the Foxp3 TSDR. Real time-PCR was performed with a final reaction volume of 20 µl containing 10 µl FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany), 50ng/µl Lamda DNA (New England Biolabs, Frankfurt, Germany), 5 pmol/µl methylation or non-methylation specific probe, 30 pmol/µl methylation or non-methylation specific primer and 60 ng bisulfite-treated DNA or the same amount of plasmid standard. The samples were analyzed in triplicate in an ABI 7500 Cycler using the following cycling schedules: 1 cycle of 10min 95°C and 45 cycles of 15s 95°C followed by 1 min 61°C. % Foxp3 TSDR content was then calculated by dividing the non-methylated copy number by the total genomic Foxp3 copy number. When PBMC samples

were used, the results obtained were adjusted to the individual % $CD4^+$ T cells (Flow Cytometry). For female patients the results were doubled. For detailed instruction see also (24).

Suppression assay - RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100µg/mL streptomycin and L-glutamine was used for T cell culture (Life technologies, Carlsbad, CA). 96-well round-bottom plates were coated with 0.5µg/mL of anti-CD3 (OKT3 mAb) and then 1 x 10^5 $CD4^-$ irradiated feeders cells were added to the plates. $CD4^+CD25^-$ responder T cells were labeled with Cell Trace Violet (Invitrogen, Cergy Pontoise, France) following the manufacturer's instructions and 1 x 10^4 cells were seeded. Suppressive activity was assessed by the addition of 1 x 10^4 or 2 x 10^4 $CD4^+CD25^{hi}CD45RA^-$ unlabeled mTreg cells per well. After 96 hours of culture the cells were stained with the anti-CD4-FITC antibody and the proliferation of $CD4^+$ Cell Trace Violet labeled cells was assessed by flow cytometry using an LSRII cytometer and FACSDiva™ software (BD Parmingen, Mountain View, CA).

Statistical analysis – We compared subject group characteristics using chi-square testing as appropriate. The non-parametric Kruskal Wallis test was used for comparison of more than 2 groups. Differences were defined as statistically significant when $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Correlations were analyzed by linear regression. All statistical analysis was performed in GraphPad Prism v6.

Supplemental Figure 1



Supplemental Figure 1: Phenotyping of Foxp3-expressing T cell subsets. Isotype control for Foxp3 staining (A). Three distinct subsets of Foxp3 expressing-cells are defined by the expression of CD45RA, Foxp3, CD25 and CD127: Orange line (II), naïve Tregs (Foxp3lo CD45RA+ CD25+ CD127lo cells); Green line (III), non-Tregs (Foxp3lo CD45RA- CD25+ CD127lo cells); Red line (IV), memory Tregs (Foxp3hi CD45RA- CD25hi CD127lo) (B, C).

		Age (years)	Gender	Donor (living vs deceaded)	Number of HLA mismatches	Previous episode of acute rejection	lymphoma status, cancer	Episode of acute infection (CMV)	Time between graft and analysis (months)	Creatinemia (µmol/L)	Proteinuria g/24h	medical treatment	Time between immunosuppression withdrawal and analysis (years)	reason of immunosuppression withdrawal	Banff/Histology	C4d staining	Ab aHLA	Donor specific Abs	Allograph glomerulopathy
Tolerant	1	65	M	NLD	4	0	0	1	283	108	ND	0	10	Non-compliance	ND	ND	0	0	ND
	2	84	F	NLD	3	1	1	1	230	83	ND	0	17	Initial renal deagradation	ND	ND	1	anti-class II (DQ7)	ND
	3	45	M	LD	0	0	0	0	145	116	ND	0	10	Non-compliance	ND	ND	0	0	ND
	4	57	F	LD	0	0	0	1	310	63	0,02	0	8	Non-compliance	ND	ND	0	0	ND
	5	48	F	NLD	1	0	0	0	115	88,5	0,88	0	8	Non-compliance	ND	ND	1	0	ND
	6	55	M	NLD	5	0	1	0	184	104,8	ND	0	8	Non-compliance	ND	ND	1	anti-class II (DQ5)	ND
	7	84	M	NLD	3	0	0	1	226	134	0	0	6	Non-compliance	ND	ND	0	0	ND
	8	40	F	NLD	2	NA	1	1	267	164,4	0,26	0	8	PTLD	ND	ND	0	0	ND
	9	55	F	NLD	4	0	1	1	235	68	ND	0	2	PTLD	ND	ND	1	0	ND
	10	48	M	NLD	4	1	0	0	347	83	0,05	0	15	Non-compliance	ND	ND	0	0	ND
	11	58	F	NLD	4	NA	0	1	114	77	0,15	0	5	Non-compliance	ND	ND	0	0	ND
	12	30	M	NLD	0	0	0	0	156	216	0,48	0	4	meningio-encephalitis	ND	ND	0	0	ND
	13	13	M	NLD	3	0	1	0	211	119	1,69	0	10	PTLD	Fibrose intimale Grade I	ND	0	0	0,00

Supplemental Table 1: Clinical characteristics of operationally tolerant patients.