Supplementary material

Supplementary methods

Flow cytometry

Measurements were done locally at each study site upon training by central immune monitoring lab personnel and interlab comparisons. Blood samples collected into EDTA tubes were stained within 4 hours and analysed by flow cytometry using the previously published antibody panels and protocols (Kverneland Cytometry A 2016). Briefly, 100 µl EDTA blood were directly stained with prepared panel antibody mixes and incubated before lysing erythrocytes with lyse-fix solution composed of Versa Lyse™ and IOTest® Fixative Solution (Beckman Coulter GmbH). For the B cell panel (panel 4) 300 µl EDTA blood was first lysed with Red Blood Cell Lysis Solution (Miltenyi Biotec GmbH) prior to antibody staining. The dendritic cell panel was prepared twice and combined after staining. Samples were measured on a 10 colour Navios flow cytometer (Beckman Coulter). Calibration with "Flow-Set Pro Beads" and "Flow Check Pro Beads" (both Beckman Coulter) was performed daily. Acquired LMD files were centrally analysed by central immune monitoring lab personnel. Analysis of LMD files was done with Kaluza version 1.2 (Beckman Coulter). To calculate absolute cell numbers of all reported immune cell subsets, leucocyte cell count was obtained from the local clinical chemistry and related to the $CD45^+$ count within each panel. The corresponding proportions of all reported immune cell subsets were calculated in Excel.

Real-time quantitative reverse transcription PCR and TSDR-demethylation analysis

Patient blood samples were collected in Tempus Blood RNA Tubes (Thermo Fisher Scientific, Schwerte, Germany) and stored at -20°C until shipment as batches into central immune monitoring lab. RNA was isolated using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Thermo Fisher Scientific). Up to 1000 ng RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Hypothesis-driven expression of genes whose expression have been previously shown to be increased in samples from immunosuppression-free operationally-tolerant kidney transplant patients, such as HS3ST1, SH2D1B, CD79B, MS4A1, PNOC, TCL1A, FCRL1 and FCRL2, or in patients with rejection, such as HMMR, TLR5, SLC8A1 and VAV3 (Sagoo et al., *J Clin Invest* 2010, PMID: 20501943; Sawitzki et al., *Am J Transpl* 2007, PMID: 17456197; Viklicky et al., *Transplantation* 2013, PMID: 23222918; and Krepsova et al., *BMC Nephrol* 2015, PMID: 26286066) was measured using TaqMan Gene Expression Assays (Thermo Fisher Scientific, *Hypoxanthine-guanine phosphoribosyltransferase (HPRT)* = Hs02800695_m1*, beta-2-microglobulin (B2M)* = Hs00984230_m1*, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* = Hs99999905_m1*, (HMMR) = , toll-like receptor 5 (TLR5*) = Hs01019558_m1, *heparan sulfate-glucosamine 3-sulfotransferase 1 (HS3ST1)* = Hs01099196_m1, *solute carrier family 8 member A1 (SLC8A1)* = Hs01062258_m1, *SH2 domain containing 1B (SH2D1B)* = Hs01592483_m1, *neuron navigator 3 (NAV3)* = Hs00372108_m1, *forkhead box P3 (FOXP3)* = Hs00203958_m1, *CD200* = Hs01033303_m1, *lymphocyte activating 3 (LAG3)* = Hs00158563_m1, *CD274* = Hs01125301_m1, *CD79B* = Hs00236881_m1, *membrane spanning 4-domains A1 (MS4A1)* = Hs00544818_m1, *prepronociceptin (PNOC)* = Hs00918595_m1, *T cell leukemia/lymphoma 1A (TCL1A)* = Hs00172040_m1, *Fc receptor like 1 (FCRL1)* = Hs00957541_m1, *Fc receptor like 2 (FCRL2)* = Hs00229156_m1), microfluidic cards and TaqMan Universal Master Mix (Thermo Fisher Scientific) on the ViiA7 Real Time PCR System (Thermo Fisher Scientific). Reactions were run in duplicates using 384-well microfluidic Custom TaqMan® Array Cards and obtained data were analyzed applying the respective ViiA7 Software v 1.2.2. Gene expression was calculated relative to median expression of three reference genes (*HPRT, B2M, GAPDH)* using the $2^{-\Delta\Delta Ct}$ method.

TSDR Analysis was done centrally at the central immune monitoring lab in batches upon shipment of frozen EDTA blood samples.

First, genomic DNA was isolated from EDTA blood using the QIAamp DNA Mini Kit (Qiagen). Up to 2 µg DNA were used for bisulfite treatment (EpiTect, Qiagen). Real-time PCR was done in a final reaction volume of 20 µl with 10 µl FastStart Universal Probe Master (ROX, Roche Diagnostics, Mannheim, Germany), 100 ng Lamda DNA (NEB, Frankfurt a.M., Germany), 5 pmol methylation or non-methylation specific probe, 30 pmol methylation or non-methylation specific primers and at least 15 ng bisulfite-treated DNA or plasmid standard (all Epiontis GmbH, Berlin, Germany). Samples were analyzed in triplicates on an ABI 7500 Cycler (Thermo Fisher Scientific). The percentage of $CD4^+$ T cells with demethylated TSDR was calculated by division of non-methylated by total genomic FoxP3 copy-number and normalization to the proportion of total $CD3^+CD4^+$ T cells as determined by flow cytometry.

IFNg EliSpot

Local immune monitoring labs performed isolation and cryopreservation of donor and recipient peripheral blood mononuclear cells (PBMCs). For donor PBMCs isolation RosetteSep Human CD3 Depletion Cocktail (Stemcell) was added to collected citrate blood prior to Ficoll (Ficoll-Paque Plus, GE Healthcare Life Sciences) gradient centrifugation to remove T cells.

EliSpot analyses were done centrally by central immune monitoring lab. Stimulation (24h) was done using the EliSpot Interferon-gamma Assay Kit (AID, Strassberg, Germany). For anti-donor responses $3x10^5$ recipient PBMCs were stimulated with $3x10^5$ T cell-depleted PBMCs in triplicates. Anti-CMV were quantified upon stimulation of $3x10^5$ recipient PBMCs with a CMV pp65 peptide pool (1.25µg/ml; Jerini peptide Technologies, Berlin, Germany) in duplicates. Unstimulated PBMCs served as controls.

Supplementary Tables

CBMP	TRIAL LOCATION	CELL ORIGIN	DESCRIPTION	REFERENCE TO CBMP CHARACTERISTICS
Regulatory T cell-derived products				
pTreg-1	London/Oxford	Autologous	Polyclonal regulatory T cells	[7] Fraser H et al.
pTreg-2	Berlin	Autologous	Polyclonal regulatory T cells	[8] Landwehr-Kenzel S et al.
darTreg-sBC	San Francisco	Autologous	Donor antigen-reactive Treg stimulated with donor B cells	[10] Putnam AL et al.
darTreg-CSB	Boston	Autologous	Donor antigen-reactive Treg generated under costimulatory blockade	[9] Guinan EC et al.
Monocyte-derived cell products				
ATDC	Nantes	Autologous	Autologous tolerogenic dendritic cells	[11] Moreau A et al.
Mreg	Regensburg	Allogeneic (organ donor)	Regulatory macrophages	[12] Hutchinson JA et al.

Table S1: ***Overview of CBMPs used in the ONE Study CTG trials.*** CBMP = cell-based medicinal product.

ORGAN RECIPIENT	ORGAN DONOR
MAIN INCLUSION CRITERIA	
• Chronic renal insufficiency necessitating kidney transplantation and approved to receive a primary kidney allograft from a living donor • Age ≥ 18 years • Signed and dated written informed consent	• Eligible for live kidney donation • Age ≥ 18 years • Signed and dated written informed consent
MAIN EXCLUSION CRITERIA	
• Any previous tissue or organ transplant • Genetically identical to the prospective organ donor at the HLA loci (0-0-0 mismatch) • PRA grade > 40% within six months prior to enrolment • Previous treatment with any desensitisation procedure (with or without IVIg) • HIV-positive, EBV-negative or chronic viral hepatitis • Significant liver disease, defined as persistently elevated AST and/or ALT levels > 2 x upper limit of normal range • Malignant or pre-malignant haematological conditions • Concomitant malignancy or history of malignancy within five years prior to planned study entry (excluding successfully-treated non-metastatic basal/squamous cell carcinoma of the skin) • Evidence of significant local or systemic infection • Ongoing treatment with systemic immunosuppressive drugs at study entry • Known contraindication to the study medications • Female patients with a positive pregnancy test at enrolment • Female patients who are breast-feeding • Female patients of child-bearing potential, unless the patient maintains a highly effective method of birth control or the career, lifestyle, or sexual orientation of the patient ensures that there is no risk of pregnancy • Patients unable to freely give informed consent (e.g. individuals under legal guardianship)	• Genetically identical to the prospective organ recipient at the HLA loci (0-0-0 mismatch) • Exposure to any investigational product at the time of kidney donation, or within 28 days prior to kidney donation • Subjects unable to freely give informed consent (e.g. individuals under legal guardianship)

Table S2: ***Core eligibility criteria applied to the selection of donor-recipient pairings enrolled in The ONE Study clinical trials.*** Each CTG trial additionally applied a set of customised exclusion criteria specific to the infused cell product. HLA = human leukocyte antigen; PRA = panel reactive antibody; IVIg = intravenous immunoglobulin; HIV = human immunodeficiency viruses; EBV = Epstein-Barr virus; AST = aspartate transaminase; ALT = alanine transaminase.

	RGT (N = 66)	CTG (N = 38)
Recipient age (years)		
20 – 29	6 (9·1 %)	2 (5·3 %)
30 – 39	15 (22·7 %)	10 (26·3 %)
40 – 49	17 (25·8 %)	11 (28·9 %)
50 – 59	15 (22·7 %)	8 (21·1 %)
60 – 69	10 (15·2 %)	6 (15·8 %)
70 – 79	3 (4·5 %)	1 (2·6 %)
Mean ± SD	47·5 ± 13·1	47·6 ± 12·7
Median	47·3	45·3
Min - Max	23·3 – 72·6	24·4 – 71·3
Recipient sex		
Female	18 (27·3 %)	11 (28·9 %)
Male	48 (72·7 %)	27 (71·1 %)
Recipient race		
White	59 (89·4 %)	36 (94·7 %)
Asian	6 (9·1 %)	1 (2·6 %)
Other	1 (1·5 %)	1 (2·6 %)
Renal replacement therapy (RRT)		
No RRT (pre-emptive transplantation)	27 (40·9 %)	18 (47·4 %)
Haemodialysis	30 (45·5 %)	16 (42·1 %)
Peritoneal dialysis	9 (13·6 %)	4 (10·5 %)
RRT time on dialysis (months)		
< 6	12 (18·2 %)	5 (13·2 %)
6 – 12	9 (13·6 %)	4 (10·5 %)
12 – 24	6 (9·1 %)	4 (10·5 %)
24 – 36	4 (6·1 %)	3 (7·9 %)
36 – 48	1 (1·5 %)	0 (0·0 %)
> 48	6 (9·1 %)	4 (10·5 %)
Unknown	1 (1·5 %)	0 (0·0 %)
Underlying CKD diagnosis		
Polycystic kidney disease	16 (24·2 %)	8 (21·1 %)
IgA nephropathy	13 (19·7 %)	8 (21·1 %)
Diabetic nephropathy	8 (12·1 %)	2 (5·3 %)
Hypertensive nephrosclerosis	5 (7·6 %)	3 (7·9 %)
Idiopathic focal segmental glomerulosclerosis	3 (4·5 %)	2 (5·3 %)
Congenital obstructive uropathy	2 (3·0 %)	2 (5·3 %)
Chronic pyelonephritis (incl. reflux nephropathy)	2 (3·0 %)	1 (2·6 %)
Alport syndrome	2 (3·0 %)	1 (2·6 %)
Membranoproliferative glomerulonephritis	2 (3·0 %)	0 (0·0 %)
Membranous glomerulonephritis	2 (3·0 %)	0 (0·0 %)
Henoch-Schonlein purpura	1 (1·5 %)	0 (0·0 %)
Toxic or drug-related tubulointerstitial disease	1 (1·5 %)	0 (0·0 %)
Idiopathic tubulointerstitial disease	1 (1·5 %)	0 (0·0 %)
Thrombotic microangiopathy	0 (0·0 %)	1 (2·6 %)
Other disease	8 (12·1 %)	10 (26·3 %)

Table S3: ***Baseline characteristics of organ recipients in The ONE Study.*** RGT = Reference Group Trial; CTG = Cell Therapy Group trials; CKD = chronic kidney disease; SD = standard deviation; RRT = renal replacement therapy. Age calculated at time of transplantation, dates of birth with incomplete day information set to the first of the respective month. All categorical variables shown as number (%) by group.

	RGT (N = 66)	CTG (N = 38)
Donor age (years)		
20 – 29	3 (4·5 %)	5 (13·2 %)
30 – 39	8 (12·1 %)	4 (10·5 %)
40 – 49	14 (21·2 %)	8 (21·1 %)
50 – 59	25 (37·9 %)	13 (34·2 %)
60 – 69	16 (24·2 %)	7 (18·4 %)
70 – 79	0 (0·0 %)	1 (2·6 %)
Mean ± SD	51·8 ± 11·3	49·2 ± 12·8
Median	53·3	51·1
Min - Max	23·8 – 69·0	23·8 – 70·7
Donor sex		
Female	41 (62·1 %)	22 (57·9 %)
Male	25 (37·9 %)	16 (42·1 %)
Donor race		
White	58 (87·9 %)	36 (94·7 %)
Asian	6 (9·1 %)	1 (2·6 %)
Other	2 (3·0 %)	1 (2·6 %)
Donor's relationship to recipient		
Mother	10 (15·2 %)	7 (18·4 %)
Father	10 (15·2 %)	1 (2·6 %)
Daughter	3 (4·5 %)	0 (0·0 %)
Son	1 (1·5 %)	3 (7·9 %)
Sibling	16 (24·2 %)	6 (15·8 %)
Niece / Nephew / First cousin	2 (3·0 %)	0 (0·0 %)
Spouse / Unrelated	24 (36·4 %)	21 (55·3 %)

Table S4: ***Baseline characteristics of organ donors in The ONE Study.*** **RGT** = Reference Group Trial; CTG = Cell Therapy Group trials; SD = standard deviation. Age calculated at time of transplantation, dates of birth with incomplete day information set to the first of the respective month. All categorical variables shown as number (%) by group.

The ONE Study RGT and CTG
PER-PROTOCOL CRITERIA

• No major violation of trial eligibility criteria (e.g. HLA 0-0-0 mismatch)
• Treatment with cell infusion (CTG only)
• Treatment with at least one dose of basiliximab (RGT only)
• Duration of treatment with oral prednisolone not exceeding 20 weeks post-transplantation
  ▪ Concomitant therapy with steroids taken for other indications (prior to or after 20 weeks) is permitted
  ▪ Concomitant therapy with topical / inhaled steroids is permitted
  ▪ Any steroid treatment given for anti-rejection prophylaxis after 20 weeks is not permitted
• MMF/MPA and tacrolimus (or acceptable substitutes) dosed continuously until study end / BCAR / drop out (interruptions of <2 months duration permitted)
• CTG only: MMF/MPA (or acceptable substitute) dosed continuously until study end / BCAR / drop out or until deliberate dose tapering starting from 30 weeks (interruptions of <2 months duration permitted)
• No high-dose immunosuppressive agents or potent anti-inflammatory treatments given as supplementary therapy in the absence of a confirmed primary endpoint

Table S5: ***Criteria applied to the per-protocol analysis set***. Patients who registered a primary endpoint of BCAR or dropped out prematurely were included in the per-protocol set if compliant with these criteria up to the date of first BCAR diagnosis / date of withdrawal. RGT = Reference Group Trial; CTG = Cell Therapy Group trials; HLA = human leukocyte antigen; MMF = mycophenolate mofetil; MPA = mycophenolic acid; BCAR = biopsy-confirmed acute rejection.

	RGT (N=66; PSY=72.34)			CTG (N=38; PSY=43.87)		
	Total Events	**Events per 100-PSY**	**Patients (%) with events**	**Total Events**	**Events per 100-PSY**	**Patients (%) with events**
All Body Systems						
Any serious adverse event (SAE)	66	91·2	36 (54·5 %)	31	70·7	16 (42·1 %)
Any adverse event (AE)	1168	1614·6	65 (98·5 %)	637	1452·0	38(100·0 %)
Any SAE possibly related* to immunosuppression	21	29·0	15 (22·7 %)	7	16·0	7 (18·4 %)
Any AE possibly related* to immunosuppression	210	290·3	53 (80·3 %)	119	271·3	33 (86·8 %)

Table S6: ***Summary of all treatment-emergent (S)AEs in*** **The ONE Study.** Treatment-emergent (S)AEs are events with onset date equal to or after first dose of any study drug (i.e. basiliximab, prednisolone, MMF or tacrolimus in RGT; cell therapy, prednisolone, MMF or tacrolimus in CTG). All events coded to the MedDRA PT: "transplant rejections" are excluded, since rejection was measured as the primary efficacy endpoint. Incidence rates are expressed as "Events per 100-PSY" and use the cohort-specific cumulative patient-time observed in the RGT (72.34) and CTG (43.87) as denominator. * Relationship to immunosuppression was assessed by the reporting Investigator and defined as a reasonable possibility of a causal relationship to any of the study drugs. RGT = Reference Group Trial; CTG = Cell Therapy Group trials; PSY = patient study years.

MedDRA System Organ Class (SOC) MedDRA Preferred Term (PT)	**Number of events**
Blood and lymphatic system disorders	
Lymphopenia	1
SOC SUB-TOTAL	**1**
General disorders and administration site conditions	
Feeling hot	1
SOC SUB-TOTAL	**1**
Investigations	
Alanine aminotransferase increased	1
Blood creatinine increased (SAE)	1
C-reactive protein increased	1
Donor-specific antibody present	1
Gamma-glutamyltransferase increased	1
SOC SUB-TOTAL	**5**
Musculoskeletal and connective tissue disorders	
Muscle spasms	1
SOC SUB-TOTAL	**1**
Nervous system disorders	
Dysgeusia	1
Headache	1
Paraesthesia	1
SOC SUB-TOTAL	**3**
Skin and subcutaneous tissue disorders	
Pruritus	1
SOC SUB-TOTAL	**1**
TOTAL:	**12**

Table S7: ***All (S)AEs assessed as possibly related to a cell product in The ONE Study CTG trials.*** 12 events were assessed by the reporting investigator as having a reasonable possibility of a causal relationship with the cell product. Events are categorised by primary SOC and coded with MedDRA version 20·1. SOC = System Organ Class; PT = preferred term.

Supplementary figures

Figure S1

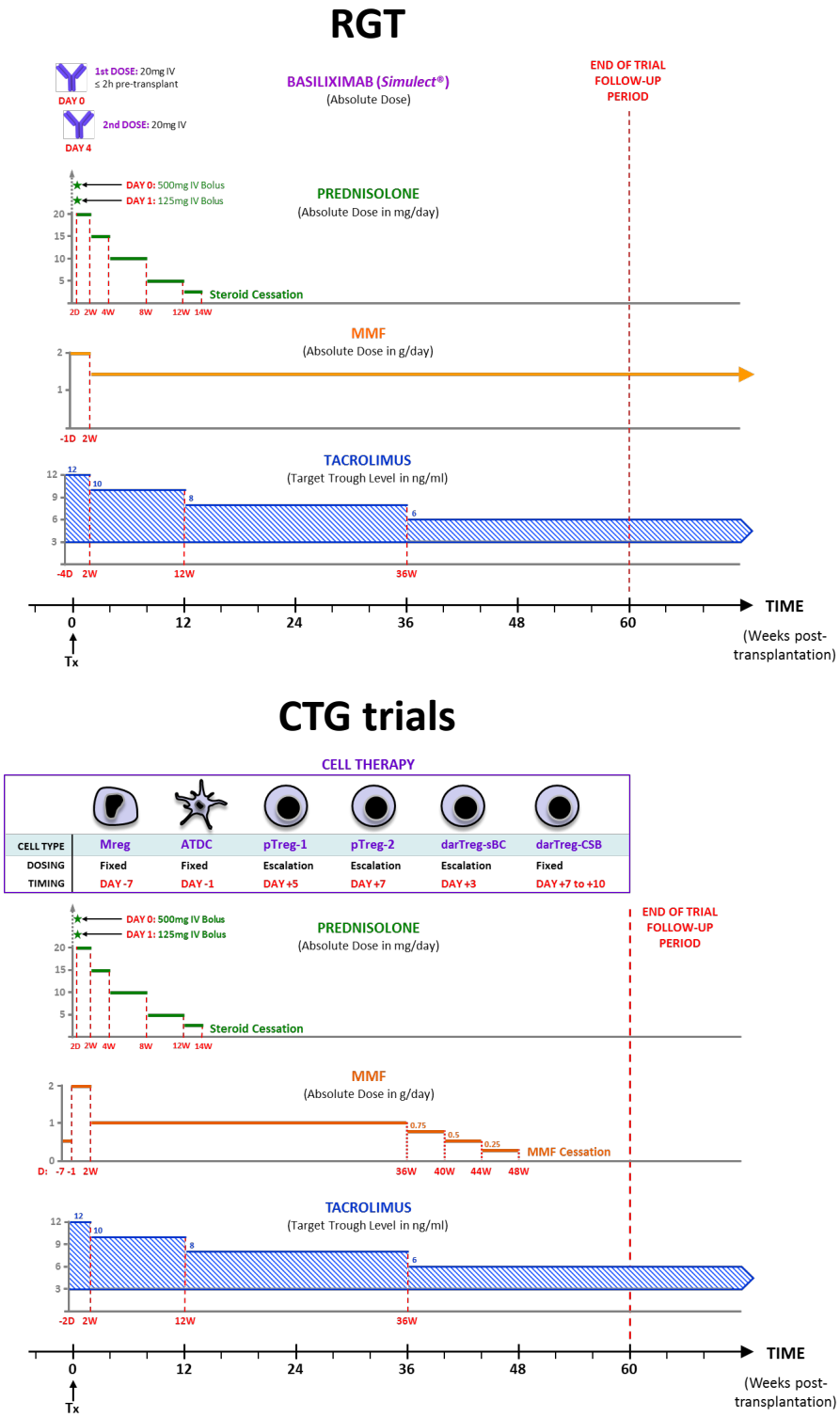

Fig. S1: ***Treatment protocol for the RGT and CTG trials.*** IV = intravenous, D = day; W = week; MMF = mycophenolate mofetil; Tx = transplantation.

Figure S2

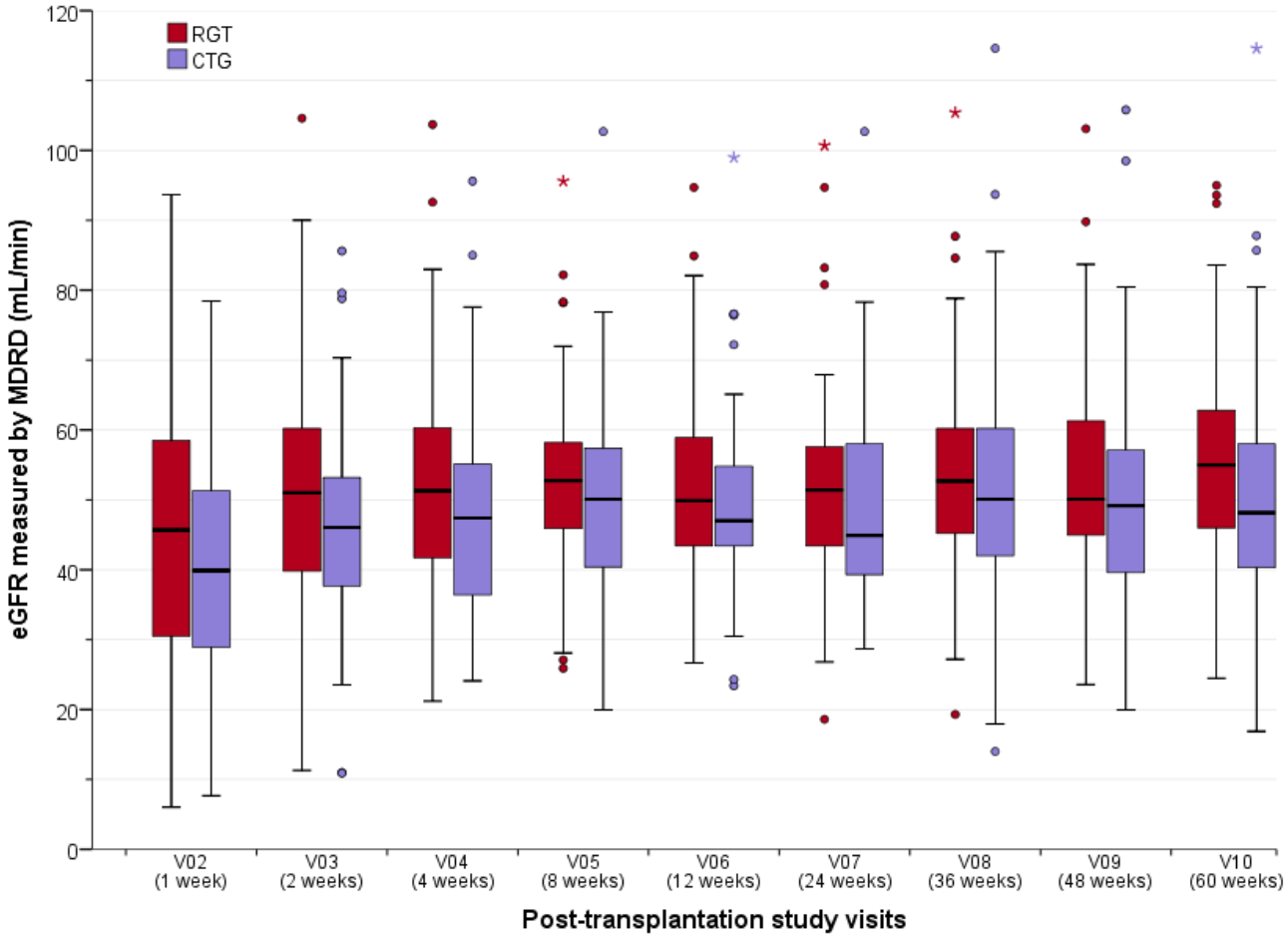

Fig. S2: ***ONE Study kidney function post-transplantation.*** MDRD eGFR measured in the RGT and CTG trials at each study visit post-transplantation. Points mark outliers beyond inner fences set at 1.5 x IQR (interquartile range); asterisks mark extreme outliers beyond outer fences set at 3 x IQR. RGT = Reference Group Trial; CTG = Cell Therapy Group trials; eGFR = estimated glomerular filtration rate; MDRD = Modification of Diet in Renal Disease formula; V = Visit.

Figure S3

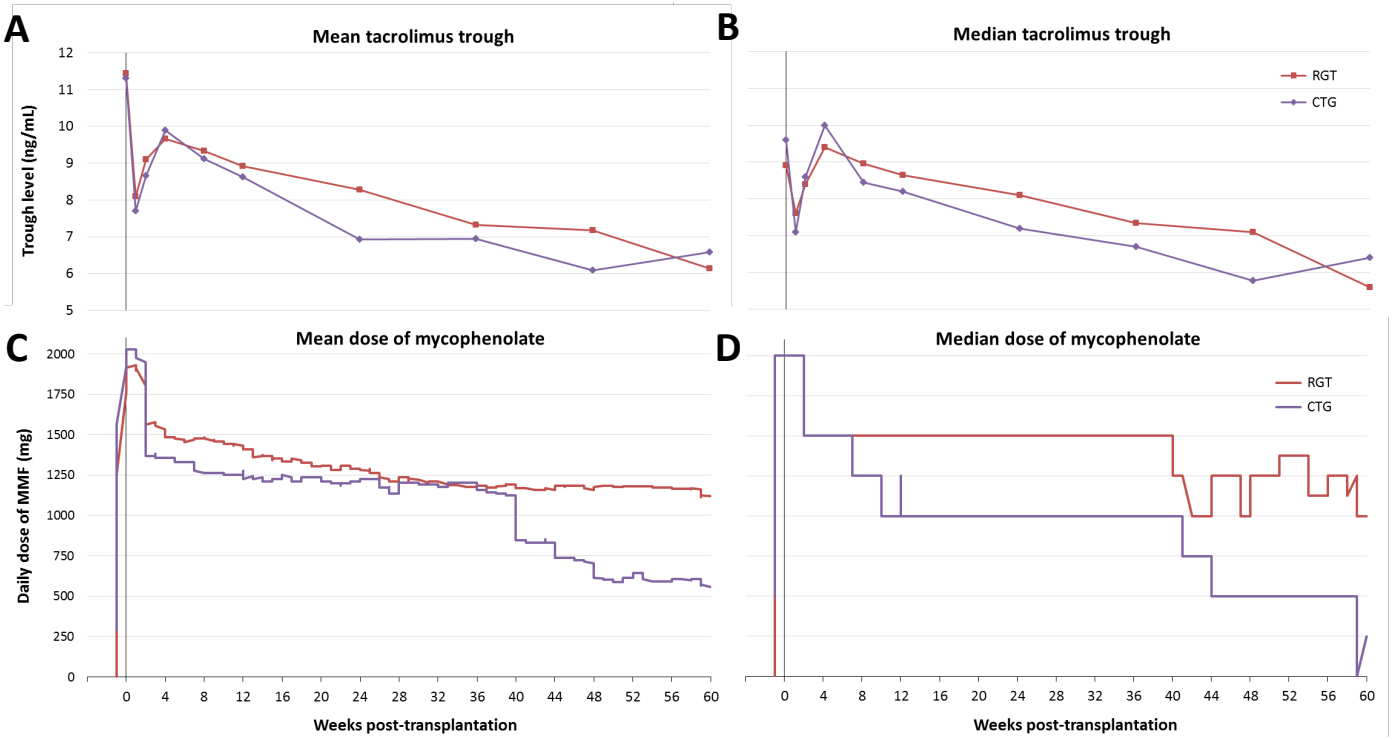

Fig. S3: ***Immunosuppressive burden of tacrolimus and mycophenolate over time.*** Mean (S3A) and median (S3B) blood trough levels of tacrolimus calculated at 10 time points using all available readings within the following time windows: day 0 (± 1 day), 7 (± 1), 14 (± 1), week 4 (± 1 week), 8 (± 1), 12 (± 1), 24 (± 1), 36 (± 1), 48 (± 1) and 60 (± 1); total number of data points = 771 (RGT) and 496 (CTG). Mean (S3C) and median (S3D) daily doses of mycophenolate calculated continuously from one week pre-transplantation to 60 weeks post-transplantation. Doses of mycophenolate sodium converted to biologically equivalent doses of mycophenolate mofetil. Patients switched to azathioprine were censored at the time of last dose of mycophenolate; if mycophenolate was discontinued permanently or temporarily without switching to an alternative anti-proliferative agent, the dose was set to zero for the period during which mycophenolate was withheld. Incomplete or unknown start / stop dates were imputed to the mid-point of two sequential doses (if in the middle of a dosing regimen) or to the protocol-specified start date (for the very first dose). RGT = Reference Group Trial (N=66); CTG = Cell Therapy Group trials (N=38); MMF = mycophenolate mofetil.

Figure S4

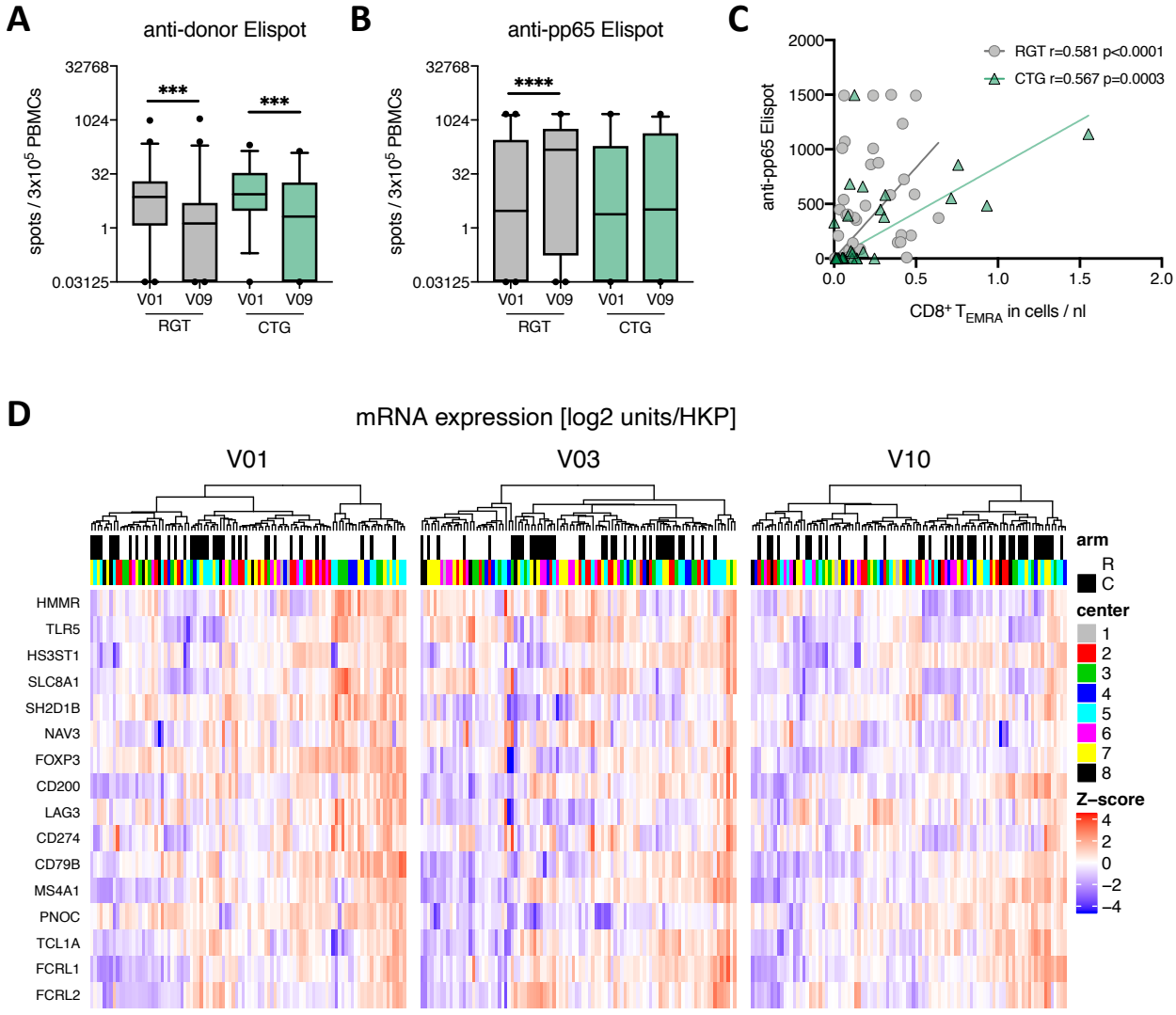

Fig. S4: ***Anti-donor and anti-CMV IFNg EliSpot analyses as well as gene expression analyses.***
A) Anti-donor IFNg EliSpot analysis prior to transplantation (V01) and 12 months post-transplantation (V09) of PBMCs from RGT (n=45) and CTG (n=33) patients. B) Anti-CMV (pp65) IFNg EliSpot analysis prior to transplantation (V01) and 12 months post-transplantation (V09) of PBMCs from RGT (n=45) and CTG (n=33) patients. C) Correlation between anti-CMV (pp65) Elispot spot counts and absolute numbers of $CD8^+$ T_{EMRA} ($CD8^+CD45RA^+CCR7^-$ T cells) at 12 months post-transplantation (V09). Statistical analysis by Wilcoxon matched-pairs signed rank test and Spearman's correlation. *** $p<0.001$, **** $p<0.0001$
D) Heat maps upon unsupervised clustering summarizing gene expression results of previously defined tolerance- (HS3ST1, SH2D1B, CD79B, MS4A1, PNOC, TCL1A, FCRL1, FCRL2) and rejection-associated (HMMR, TLR5, SLC8A1, VAV3) genes as well as FOXP3 and co-inhibitory molecules (CD200, LAG3, CD274) measured by qRT-PCR of whole blood samples from RGT patients (n=60, arm R, white bars) and CTG patients (n=38, arm C, black bars) collected pre-transplant (V01), two weeks post-transplant (V03) or 60 weeks post-transplant (V10).