Regulatory T-cells for minimising immune suppression in kidney transplantation: phase I/IIa clinical trial

SUPPLEMENTARY: TABLE OF CONTENTS

PART A – EXTENDED METHODS

Clinical Monitoring and Exploratory Biomarker Analysis - METHODS

The protocol-specific clinical assessment was designed to monitor treatment safety and hints of efficacy throughout the study. Clinical data were used to assess kidney graft function, the patients' general condition, and to classify trial subjects into immunological high-risk and low-risk groups. Importantly, the clinical monitoring was designed to detect declining renal function as an indicator of rejection or recurrent renal disease as early as possible.

1. Assessment of Biopsy-Confirmed Acute Rejection (BCAR)

The primary Clinical Endpoint was the incidence of biopsy-confirmed acute rejection (BCAR) within 60 weeks of organ transplantation. Histopathological grading of biopsy material was performed according to the Banff criteria (*1*). Patients with subclinical rejection detected by a protocol biopsy will not register a primary endpoint as long as the patient has a stable graft function with no signs of allograft rejection. The management of patients according to histopathological findings was done at the discretion of the responsible nephrologist. BCAR has been selected as the primary endpoint for the following reasons:

- The incidence of BCAR has become an accepted short-term surrogate marker for renal allograft survival and is widely used as a primary endpoint in renal transplantation trials of immunosuppressive therapies (*2, 3*).
- Allograft biopsy is the „gold-standard" for the evaluation of renal allograft dysfunction (*4, 5*) and the histopathological criteria for the diagnosis of acute rejection are standardized and unambiguous (*1*).
- BCAR represents the most pragmatic and testable primary endpoint for treatment efficacy within the time and resource constraints of a transplantation trial.

To assess BCAR, kidney graft biopsies were performed either as scheduled protocol biopsies, or as unscheduled for-cause biopsies. One protocol biopsy was mandatory at 36 weeks post-transplant (Visit 8) to guide the IS drug tapering. Additional, (optional) protocol biopsies were performed intermediately to identify early signs of graft pathologies. For-cause biopsies were performed in case an acute rejection episode was suspected.

- Visit 03 (02 weeks post-Tx) → Signs of early subclinical rejection (Optional)
- Visit 06 (12 weeks post-Tx) → Status before planned steroid withdrawal (Optional)
- Visit 08 (36 weeks post-Tx) → Status before planned MMF withdrawal (Mandatory)
- Visit 10 (60 weeks post-Tx) → Graft status at the final trial visit (Optional)

Whenever a graft biopsy was performed, a paired serum sample was intended to be collected for donor-specific antibody (DSA) testing. In case of a for-cause biopsy, DSA was measured according to the standard protocol. In case of protocol biopsies, the serum sample was collected and stored at the trial center. Only if histopathological findings were suspicious of antibody-mediated rejection (ABMR), the sample was tested for DSA. The DSA results were entered in the eCRF, regardless whether a for-cause biopsy or protocol biopsy was taken.

Considering biopsy material, as defined by the Banff criteria (*6*), an adequate specimen is defined as a biopsy with ten or more glomeruli and at least two arteries. A minimal sample

must contain at least seven glomeruli and one artery. For trial purposes, one core of allograft tissue was required from each biopsy. The trial centers were also allowed to examine biopsy tissue locally, before sending sufficient material to Prof. Ian Roberts (Oxford, Central Pathologist for The ONE Study). The results of the local histopathology were used to guide the clinical management of the patient, and the local histopathological analysis was recorded in the eCRF. The histopathological grade determined by the central pathologist was also entered into the eCRF on pages that have restricted access rights. The central pathologist's grading was used as the official result for the trial. In this way, assessments of rejection were standardized across all trials within The ONE Study, thus eliminating inter-center variability.

2. Clinical Laboratory Analysis and Biochemical Indices of Renal Function

These laboratory assessments include routine hematology, biochemistry, and urine analysis for kidney transplant recipients as listed in **Table S7**. All samples taken for clinical follow-up (as opposed to IM follow-up) will be processed in the local clinical chemistry laboratory of the trial center. The clinical laboratory must provide a current and approved list of reference ranges, including units, for each parameter. Additional laboratory values, if attained for medical reasons (*e.g.* laboratory tests initiated to investigate an AE experienced during the trial), should be recorded in the eCRF. The panel of biochemical measures and derived parameters included in the clinical follow-up have been chosen to assess glomerular function and integrity, renal tubular function, and acid-base homeostasis. Biochemical tests were performed as suggested by the KDIGO guidelines (*7*).

3. The Immune Monitoring (IM) Subproject

In addition to the recommended standard assessment, extensive exploratory immune monitoring (IM) was performed for all patients treated with nTregs in the ONEnTreg13 trial or the Charité reference group trial. The assays in the IM subproject were designed to assess the immune profile of all patients and surrogate markers that may indicate the safety and efficacy of the cell product. The IM schedule for patients enrolled in this trial was in accordance with the protocol of The ONE Study Reference Group Trial, which represents the comparative cohort receiving the standard of care treatment. In addition, in the nTreg cell therapy trial, we performed an additional IM assay for T-cell receptor repertoire sequencing (TCRseq) based on the next-generation sequencing (NGS) technology platform at the BCRT, to monitor the *in vivo* life span of the nTregs after infusion. The aims of the IM project were as follows: 1) Safety and 2) Pharmacodynamics / Pharmacokinetics. The data collected within the IM-subproject were generally not intended to provide any guidance for the clinical management of the patients within the trial. Nevertheless, the results from viral load assays were made available to the local clinicians, as this test is routinely used in standard clinical practice. Although the ONEnTreg13 was conducted as a monocenter study at the Charité Universitätsmedizin Berlin, "The ONE Study" consortium entailed multiple parallel clinical trials, involving the participation of several (international) trial sites, with the IM-subproject being common to all "The ONE Study" trial protocols. The Charité Universitätsmedizin Berlin has a dedicated immune monitoring facility and thus acted as the Central Immune Monitoring Laboratory (CIML) for the study, receiving samples and data from all participating centers, performing validated batch analysis. Here we report only on the data from the two Charité trial groups – nTreg and reference. According to **Table S7**, the IM follow-up included a panel of tests outlined in detail the following subsections.

3.1 Immune Monitoring Measures of Safety

3.1.1 Viral Load

The CMV, EBV and BKV tests, which were employed in this study, are accredited assays and routinely performed as standard post-transplant follow-up with direct consequences for the clinical management. The measurements were taken at Visits 04-06 using patient blood (2x 2.0 ml EDTA Vacutainer) and patient urine (1 x 1ml) using PCR in the Charité clinical routine laboratory. If anti-viral chemoprophylaxis was given during the first 12 weeks after transplantation (coinciding with viral testing specified at Visits 5 and 6), three viral load measurements were instead taken at four-week intervals after prophylaxis had ended.

3.1.2 HLA-DR Expression Levels on Circulating Monocytes

Quantification of HLA-DR expression on peripheral blood monocytic cells is a useful and reproducible surrogate marker of innate immune reactivity (*8*). Testing was done at Visits F01, F02, and V03 on patient blood (1x 2.0 ml EDTA Vacutainer) with flow cytometry at the Charité clinical routine laboratory. HLA-DR expression levels have been defined as follows:

- Normal healthy controls: >15,000 molecules per cell
- Immunodepression: 15,000 – 8,000 molecules per cell
- Immunoparesis: < 8,000 molecules per cell

3.1.3 Urinary IP-10 Levels as Marker of Intrarenal Inflammation

The presence of the chemokine interferon-inducible protein 10 (IP-10; CXCL10) in the urine is associated with impending acute rejection episodes. Elevated levels of urinary IP-10 within the first four postoperative weeks are predictive of graft function at 6 months even in the absence of acute rejection (*9*). The assay required samples from Visits F01, F02, and V02 – V10 (1 x 1 ml patient urine) for ELISA analysis at the CIML.

3.1.4 Systemic Cytokine Measurements in Whole Blood

In order to study the profile of systemic inflammatory cytokines in patient blood before and shortly after nTreg infusion (6 and 24 hour follow-up), we employed a validated Luminex assay for detection of TNF-alpha, IFN-gamma, IL-1, IL-6, IL-8, and IL-10 at Visits F01, F02 and V02 (1 x 3.0 EDTA Vacutainer) at CIML with validated multiplex technology (*10*).

3.2 Measures of (Allo-) Immune Reactivity

3.2.1 Gene Expression Profiling of Circulating PBMCs with qPCR

The expression of a defined set of genes that have been described to be associated with tolerance or rejection were profiled with qPCR at Visits V01, V03 – V10 using patient blood (2x 3.0 ml Tempus Tubes) at the CIML according to standard methods (*11*).

3.2.2 Leucocyte Immune Subset Profiling with Multi-Parameter Flow Cytometry

A large panel of leukocyte markers was assayed with flow cytometry to quantify multiple immune cell subpopulations in patient peripheral blood at Visits V01, V03 – V10, F1 and F2 using patient blood (1 x 3.0 ml EDTA Vacutainer) at the CIML according to previously validated methods (*12, 13*).

3.2.3 Donor-Specific Antibodies Indicative of Humoral Allo-Sensitization

Study patients were assessed for signs of humoral allo-sensitization prior to kidney transplant and after transplantation by conducting validated screening for anti-HLA antibody formation

at Visits Preoperative, V06 and V10 using patient blood (8.5 ml Serum-separating tube) with a validated Luminex panel reactive antibody (PRA)-screening platform followed by detailed testing of donor-recipient HLA-subtype specificities (in order to study anti-HLA class I and II antigen-specificity directed against the transplant) at the Charité Universitätsmedizin Berlin HLA laboratory.

3.3.4 Donor-Reactive T-cell Frequencies Indicative of Cellular Allo-Sensitization

Study patients were assessed for signs of cellular allo-sensitization prior to kidney transplant by conducting validated screening for cellular alloreactivity with IFN-gamma ELISpot after overnight stimulation to determine the frequency of donor-reactive memory/effector T-cells, indicating cellular sensitization against donor antigens. This assay required blood samples from the organ recipient at Visits V00, V03, V09 (2 x 10.0 ml Heparin Vacutainer) and PBMCs derived from the organ donor and was conducted at the CIML according to validated methods (*14*).

3.4 Pharmacokinetics

3.4.1 TCR-Repertoire Sequencing of nTregs and Data Analysis

Identification and tracking of even low-abundant T-cell clones has become feasible by Next-Generation Sequencing (NGS) (*15*). This method is based on identification of clone specific amino acid / or nucleotide sequences in the complementarity-determining regions (CDRs) of their antigen receptors (TCRs) (*16*), according to previously published procedures (*17-21*), by comparing the TCR-repertoire of the final nTreg product (day 23) to that of circulating Tregs in patient blood at Visit V04, V06, V08 and V10 (1 x 5.0 ml EDTA Vacutainer).

Sample preparation: GMP-produced nTregs were collected in lysis-buffer post expansion-bead-depletion at day 23 and patient-derived peripheral blood mononuclear cells (PBMCs) were isolated using LSM (Lonza, Basel Switzerland) gradient centrifugation and peripheral blood Tregs isolated with anti-human CD4 and CD25 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Library preparation and analysis: Genomic DNA (gDNA) and RNA were isolated using AllPrep DNA/RNA MiniKit (Qiagen, Hilden, Germany). The mRNA was reverse-transcribed using SuperScript III First-Strand-Synthesis-System for RT-PCR (Invitrogen, Carlsbad). The recombined TCR-beta locus was amplified as described earlier (*17*) and sequencing library preparation and sequencing performed with Illumina HiSeq Technology. Reads with a quality score <30 were excluded from the analysis. High quality reads were processed using IMSEQ (*18*). Each clonotype was assigned an ID, including V-beta- and J-beta-gene identity as well as CDR3 amino acid sequence. Equal clonotypes were clustered and further analyzed.

Secondary data analysis: Additional analysis of the TCR-repertoire was conducted as follows: **1)** Clonal space, **2)** Diversity over time (Renyi diversity profiles, Shannon and Berger-Parker comparison of diversity, and association between diversity and Treg frequency), and **3)** Overlap of clonotypes over time (Morisita-Horn similarity index, and comparison of Tregs at different time points *in vivo* to the infused GMP-produced nTreg product), according to previously described methods (*17-21*).

PART B - SUPPLEMENTARY RESULTS

Exploratory Biomarker Analysis

1. TCR-Repertoire Sequencing of Tregs

1.1.1 Initial Data Quality Control

We aimed to get a global overview of the samples and the general sample quality. The number of clonotypes (**Figure S5A**) gave us an impression of the size of the obtained repertoires. First of all, we saw a large range from 23 to 100387 clonotypes. We next inspected the number of accepted reads across the pooled samples (**Figure S5B**), and looked at the total number of Imseq accepted reads found in each sample in case the different runs are pooled. As a side-note, it is not surprising, if the total number of accepted reads is larger than the number of cells, because of the amplification process. For quality reasons, we removed samples with less than 20000 reads, which affected one sample of patient C5051 (V10 – 17602 reads / 393 clonotypes) and two samples of patient C5057-R (V08 – 4857 reads / 253 clonotypes and V10 – 333 reads / 23 clonotypes), while the other n=44 samples met our set quality criteria, thus only loosing 6% of our samples for analysis due to technical reasons.

1.1.2 Analysis of Clonal Space

We give here an extended description of the results from the corresponding figure in the main manuscript (**Figure 3B**). Briefly, we found a tendency for clonal expansion of the nTreg product derived clonotypes in the majority of the patients (Low dose: C5051, C5052, C5054, medium dose: C5058-R1B, and high dose: C5062, C5063-R and C5067), although some resisted this pattern (Low dose: C5053, medium dose: C5056-RIII and C5057-R1B, and high dose: C5059). Based on the sheer number of clonotypes, it is difficult to get an impression of the structure of the repertoire. The visualization in Figure 3B makes use of the division of clonotype frequencies into different groups (termed binning) and then to sum up the frequencies in each bin. The binning is essentially arbitrary, and here we used the bins 1, 0.1, 0.01, 0.001, and 0% (Please see the corresponding legend: TCR clone frequency). At some visits we observed hyper-expanded clonotypes, which can be related to as an artefact occurring due to the amplification process.. However, when we looked at the bins, in which we find less than 5 clonotypes, we see that the problem is relatively small, affecting only 5/47 samples (10%):

Patient	Timepoint	Group name	Bin	Sum frequency	Num clonotypes
5051	V08	Low dose	1 <= x < 100	4.42	4
5054	V06	Low dose	1 <= x < 100	1.00	1
5059	V04	High dose	1 <= x < 100	3.80	3
5059	V08	High dose	1 <= x < 100	3.45	3
5062	P	High dose	1 <= x < 100	1.01	1

1.1.3 Clonal Diversity Over Time

This section uses different mathematical expressions to describe the clonal diversity over time: either Renyi diversity profiles, or Shannon and Berger-Parker comparisons of diversity (**Figure S5C-H**) (*22-24*).

Diversity profile: The Renyi diversity profiles (*22*) combine different diversity measures to give a complete overview of the sample diversity. The x-axis indicates the weighing of clonotypes; at 0 all clonotypes are considered, and at "infinite" only the single most dominant clonotype is considered. Although somewhat complex, the interpretation is straightforward:

the sample that is always above all other samples is more diverse. If the lines of samples cross, their diversity cannot be distinguished. The diversity profiles here compare the different time points for each patient, first for all clonotypes (**Figure S5C**) and then for the top 500 clonotypes (**Figure S5D**). Overall, we did not find a particular pattern in diversity. No sample always had a different diversity compared to the others. This finding is not in direct contrast to the observation in Figure 3B, as we generally observe a decrease in diversity over time.

Diversity vs. cell infusion dose, rejection vs. non-rejection, and diversity vs. Treg frequency: We next compared the diversity to the number of cells originally infused, with the most commonly used Shannon and Berger-Parker diversity (*23, 24*). When comparing the Shannon diversity (**Figure S5E**) or the Berger-Parker index (**Figure S5F**) to the dose of cells infused at different time points, no association was indicated. We next compared rejection *vs*. non-rejection (**Figure S5G-H**). Four patients experienced signs of acute rejection or IgAN. To see if these events could be associated with diversity, we evaluated the Shannon and Berger-Parker diversity over time. There was no particular indication of association between diversity and rejection. We furthermore found no strong correlation between Treg frequency and sample diversity (**Figure S5J+K**). In most cases, the Treg frequency and Shannon diversity decrease over time, but in three cases (C5057-R, C5058-R, and C5059), the Treg frequency increased over time (**Figure S5K**).

1.1.4 Overlap of Clonotypes Over Time

Similarity index: Similarity indices measure the similarity of two populations considering not only the number of shared clonotypes but also take the clonotype count or percentage into account. One of the most popular indices is called Morisita-Horn similarity index (*25*). It is one of the first of the similarity indices, which can handle comparison of populations of unequal size (*e.g*. it is possible to compare a population of 100 clonotypes with a population of 20). The heatmaps of the similarity index are symmetric, there is the same number of columns and rows, and the upper triangle is identical to the lower triangle (**Figure S5L+M**). Numerically, the observed overlaps are small, but considering the potential repertoire being sampled, the chance of an overlap is very small. There is generally a larger overlap between the time points V04 and V06, than between any other time points. There are two exceptions, patient number C5053, with an overlap between V08 and V10, and patient 5067, with a stronger overlap between P and V04. The predominant overlap between V04 and V06 could be an indication of still settling homeostasis.

Comparison to product: To gain insight into the product-derived clonotypes, the total frequency of clonotypes found in the product and at later visits was evaluated (**Figure S5N**). A different question along the same line was how the product-derived clonotypes distribute across the visits (**Figure S5O**). For the low dose, there is a tendency to find more product-derived clonotypes at the later visits, whereas for the medium and high-dose group the earlier visits seem to dominate regarding product-derived clonotypes.

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PART D – SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Trial Reporting and Assessment of Adverse Events and Efficacy in Patients.

(A) CONSORT Transparent Reporting of Trials for: The ONEnTreg13 Charité natural thymus regulatory T-cell (nTreg) therapy trial and The ONErgt11 Charité reference group trial; and **(B)** Assessment of adverse events and efficacy in patients: Different types of adverse events (AE) are labeled in the Venn diagram, with events within the yellow box being "related" to the investigational medicinal product, and "serious" adverse events in the red box, and "unexpected" adverse events in the blue box not yet being documented in the product information of the investigator brochure (IB). The primary clinical endpoint (biopsy-confirmed acute rejection, BCAR) requires histological confirmation from a for-cause tissue biopsy. Italicized diagnoses correspond to histological categories of renal allograft pathology defined by the Banff criteria (please see IB). There were no significant differences in the incidence of Severe Adverse Effects (SAEs). In summary we registered 14 SAEs in 10/11 patients of the ONEnTreg13 group. These SAEs were not categorized as directly related to the nTreg infusion (one mixed acute rejection was indirectly related to the weaning strategy in the nTreg group but could be resolved – weaning failure). In the rONErgt11-CHA reference group 12 SAE´s were noted in 9/9 patients.

Figure S2: Supplementary Patient Data Clinical Monitoring.

(A) Assessment of additional kidney and liver function and inflammation parameters in both nTreg (n=11) and reference group (n=9): **(A)** Kidney (median +/- IQR, inter-quartile-range): serum albumin (g/L) and serum urea (mg/dL), **(B)** Liver (median +/- IQR): bilirubin (mg/dL), aspartate aminotransferase (ASAT, U/L), alanine transaminase (ALAT, U/L), and alkaline phosphatase (AP, U/L), and **(C)** Inflammation parameter: urinary interferon-gamma induced protein 10 (IP-10, pg/mL, median +/-IQR, ELISA),and hematological safety parameters (box plot, min-max range) with their respective typical norm-values: fibrinogen (1,6-4,0 g/L) and free hemoglobin (Free Hb <20,0 mg/dL) either 24-hours before or 6-hours and 24-hours post systemic nTreg infusion.

Figure S3: Case-by-Case Evaluations of Individual Patients.

The figure is composed of n=11 individual panels giving an overview of all patients treated with nTregs in the ONEnTreg13 trial (C5051, C5052, C5053, C5054, C5056, C5057, C5058, C5059, C5062, C5063, and C5067. The panels are structured as follows:

At the top of each panel, detailed informative clinical parameters are given on the individual patient background in the context of kidney transplantation (donor sex and age, recipient sex and age, recipient body mass index (BMI, kg/m^2), time on dialysis (months), HLA-mismatches (number), underlying disease, and any occurrence of complications of surgical, infectious immunological or other kind).

The center of each panel, shows the cell dose applied for each individual patient (either 0.5, 1.0, or 2.5-3.0 x 10^6 cells/kg) with a very brief summary of the clinical course (*e.g.* successful tapering of immunosuppression (IS) with stable creatinine) and a chart of the serum creatinine levels (mg/dL) over time (days after renal transplantation), together with the duration of IS (tacrolimus, mycophenolate-mofetil (MMF) and steroids) also indicating the tapering thereof.

The bottom of each panel, shows the results for the assessment of cellular and humoral allo-sensitization of the patient, with results of the IFN-gamma ELISpot assay (x spots/300,000 cells) being depicted on the left (IFN-gamma producing cells within donor- or patient-derived PBMCs, with indication of patient visits V00, V03, and V09, and tested antigen: either alloreactivity against donor, or antigen-reactivity against viral antigens pp65, IE-1, EBV, or SEB, see appreciations below) and the results of the panel-reactive antibody (PRA) screening being depicted to the right (positivity for HLA class I and II at patient visits V00, V03, V09, and V10, together with detailed results for specification indicated below, if positive), respectively.

Abbreviations Figure S3: ACR, acute cellular rejection and AMR, antibody-mediated rejection with Banff-grade I-III; CNI, calcineurin-inhibitor; DSA, donor-specific antibodies; IgA-N, IgA nephropathy; IVIG, intra-venous immunoglobulin; MMF, mycophenolate-mofetil; pp65 and IE-1, cytomegalovirus protein pp65 and IE-1 eliciting a CD8+ T-cell response; EBV, Epstein-Barr virus antigen; and SEB, staphylococcus enterotoxin B, a bacterial superantigen, and TNTC, to numerous to count (>max).

Figure S4: Flow Chart Manufacturing Process and Flow Cytometry Analysis of nTregs.

(A) Flow chart of the natural regulatory T-cell (nTreg) manufacturing process, Briefly, the process is composed of three major steps (left panel): 1) Preparation of the starting population on day 0, 2) Cell expansion under several bead (re)-stimulations at day 1-22, and 3) Product filling and application on day 23, entailing magnetic bead-depletion, washing, filling and application. All process steps are accompanied by various process-controls (right panel), and **(B)** GMP-process approved and validated flow cytometry gating and analysis strategy with representative scatterplots / histograms applied for the quality control and functional characterization of nTreg products. In a first step, cellular "duplets" are excluded by gating for "singlets" within the forward scatter (FSC INT / FSC TOF) profile, followed by setting the lymphocyte-gate in the forward-sideward scatter (FSC / SSC) profile, followed by gating of "living" cells with side-ward scatter live-dead discrimination (SSC / LD), which is then followed by stepwise phenotypic T-cell subset analysis (center panel) with identification of CD3+/CD4+/CD25+/FoxP3+ regulatory T-cells of naïve or memory phenotype (CD45RA positive or negative, respectively), with concomitant functional characterization by detection of cytokine production (IL-2 and IFN-gamma) following maximal PMA/ionomycin stimulation, demonstrating the absence or very low expression of the respective Th1-related effector mediators (histograms to the right).

Figure S5: Supplementary Data for nTreg TCR-Sequencing Analysis.

The following sequence of panels in **Figure S5A-O** gives further bioinformatics evaluation for the initial TCR-sequencing data quality control and the clonal diversity and overlap over time. Depending on the underlying analysis, data are grouped either according to patient visits (V04, V06, V08, V10, and "GMP" for the GMP-expanded nTreg product prior to infusion), and/or infused cell dose (low, medium and high) and/or patient rejection status ("R", all four patients with potential rejection event are marked: C5056R, C5057R, C5058R, and C5063R; C5063 only had IgAN, a rejection like pathology). In principle, there are three major aspects/questions answered in the following figures:

1) Initial Data Quality Control: **(A+B)** Number of clonotypes and of the accepted reads are sorted according to time points to check any negative bias from unacceptably low reads.

2) Diversity Over Time: **(C+D)** Renyi diversity profiles for each patient and time point depicted either for all clonotypes or for the top 500 clonotypes, with each line depicting one time point; (**E-F)** Shannon and Berger-Parker diversity for each infusion group (low, medium, and high cell dose) according to patient visits; **(G+H)** Shannon and Berger-Parker diversity over time of visits for each patient with distinction of rejection and non-rejection; and **(J+K)** Correlation of Shannon diversity to Treg frequency and association between Treg frequency and Shannon diversity).

3) Overlap of Clonotypes Over Time: (**L+M)** Morisita-Horn similarity of all or top 500 clonotypes between time points for each patients, with number 1 indicating identity (shown in white), while number 0 indicates complete dissimilarity (purple); and **(N+O)** Total frequency and distribution of product-derived clonotypes for each patient according to patient visits).

Figure S6: Supplementary Data for Multi-Parameter Immune Monitoring.

The figure shows a snapshot of the results obtained for the screening of about 80 exploratory biomarkers with multi-parameter flow cytometry (FACS) and quantitative real time polymerase chain reaction (qPCR) comparing either: **a)** nTreg-treated patients within the ONEnTreg13 trial (n=11) *versus* the reference group patients within the ONErgt11-CHA trial (n=9), or **b)** Dose-response relationship of nTreg therapy only in the ONEnTreg13 trial with evaluation of the different cell doses (0.5, vs. 1.0, vs. 3.0 x 10^6 cells/kg or mio/kg). The red frame indicates significant differences between the nTreg and reference group (details see Table S5. The figure is composed of three different parts:

Part 1) FACS data for the following immune subset parameters: #01) Blood differential counts, #02) Monocytes, #03) Dendritic cells, ##04) TCR-γ/δ T-cells, #05) Natural killer (NK) and T cells, #06-11) CD4 and CD8 T-cell subsets, activation, memory, CD4 regulatory T cells (Tregs), and Treg to Tconv ratios, and #12) B cells, all expressed either as absolute counts (cells/nl, left column) or cellular frequencies (% of parent, right column), for two different general comparisons: **a)** nTreg-treated *vs*. reference group (shown at the left), and **b)** nTreg dose-response analysis (shown to the right).

Part 2: Quantitative HLA-DR Expression per Monocyte: Marker of general immune competence. Normal range 18-40,000 molecules/cells, immunodepression 8-18,000 mol/cell, immunoparalysis <8,000 mol/cell with high risk of severe infections, as described previously: Docke W et al. Monitoring Temporary Immunodepression by Flow Cytometric Measurement of Monocytic HLA-DR Expression: A Multicenter Standardized Study . Clin Chem 2005 Dec;51(12):2341-7. doi: 10.1373/clinchem.2005.052639. Epub 2005 Oct 7.

Part 3: qPCR data of whole blood samples: Multiple gene expression markers (n=20 targets) were studied on mRNA transcript level with qPCR, as stratified in the study below, again for the same two general comparisons: a) nTreg-treated vs. reference group shown to the left, and b) nTreg dose-response analysis shown to the right. The establishment of the tolerance/rejection signature gene expression panel is described in the following reference: JClinInvest 2010 Jun 1; 120(6): 1848–1861. Published 2010 May 24. doi: 10.1172/JCI39922 PMID: 20501943 Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2877932/

PART E – SUPPLEMENTARY TABLES

Table S1: Primary and Secondary Study Objectives and Endpoints.

Primary Objectives
The primary objective of the ONEnTreg13 phase I/IIa trial was to assess safety and feasibility of the first-in-human application of our in-house developed autologous CD4+CD25+FoxP3+ nTreg product in living-donor KTx patients (n=11). Results were compared to the reference patients of our centre in the ONErgt11-CHA trial (n=9), conducted prior to the ONEnTreg13, to establish safety margins and biomarker panels for the study.
Primary Clinical Endpoints
Incidence of biopsy-confirmed acute rejection within 60 weeks of organ transplantation (histological grading according to the Banff criteria).
Primary Safety Endpoint
To assess the safety and feasibility of intravenous infusion of *ex vivo*-selected and *ex vivo*-expanded autologous natural Tregs in patients with solid organ transplantation. • adverse infusion-related effects • infections, response to anti-rejection therapy • graft function/failure
Secondary Objective
To evaluate the effect of nTregs on solid organ graft function (e.g. kidney graft) during tapering of immunosuppression as compared to the reference group receiving conventional triple maintenance immunosuppression.
Secondary Endpoints
• Surrogate markers of transplant function and surrogate immunologic markers related to general immune function. • Time to first acute rejection episode • Severity of acute rejection episodes based on response to treatment and histological scoring • Return to transplant waiting list or re-transplantation following graft-loss due to rejection (acute or chronic) • Incidence of adverse drug reactions • Incidence of post-transplant dialysis • Immunosuppressive burden at final visit

Table S2. Patient Clinical Study Eligibility Criteria.

Main (but not exhaustive) Inclusion Criteria
• Chronic renal insufficiency necessitating kidney transplantation and approved to receive a primary kidney allograft from a living donor. • Age 18 years or older and written informed consent.
Main (but not exhaustive) Exclusion Criteria
• Patient previously received a tissue or organ transplant other than the kidney graft. • Known contraindication to the protocol-specified treatment/medications. • Genetically identical to the prospective organ donor at the *HLA* gene loci *. • PRA grade >40 % within 6 months prior to enrolment. • Previous treatment with any desensitization procedure (with or without IVIG) • Concomitant malignancy or history or malignancy within 5 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. • HIV-positive, EBV-negative or suffering from chronic viral hepatitis. • Significant liver disease (persistently elevated AST and/or ALT levels > 2x ULN) • Malignant or pre-malignant hematological conditions.

Abbreviations: EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; IVIG, intravenous immunoglobulin; PRA, panel reactive antibodies; ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; and ULN, upper limit of normal in liver-function test. Asterisk : *was adapted to accelerate recruitment.

Table S3: Overview of the nTreg Manufacturing Process.

Process step	Description	Controls
Blood Collection and Testing	• For nTreg preparation ca. 50 ml patient blood is collected to Lithium-Heparin blood collection tubes by venipuncture. •	In-process
Cell Isolation	• Depletion of $CD8^+$ T cells and enrichment of $CD25^+$ cells from the CD8-depleted cell fraction.	. In-process
Cell Expansion	• After isolation, the cells are cultivated at 37°C in a 5% CO2 atmosphere. • The cell expansion phase is initiated and maintained by (re) stimulation with anti-CD3/anti-CD28 antibody coated GMP-grade Treg expansion beads. • Cell splitting and/or medium exchange is realized on the basis of visual culture inspection and cell counting. • The release-criteria-testing is carried out on the aliquots taken during manufacturing.	In-process
Cell Yield	• On day 23, when the target cell number is reached, cells are washed and resuspended in PBS. Beads are removed by LD-Columns.	end product
Filling of the Cell Product	• After bead-removal, cells are washed in saline. One batch will result in 50 ml cell-solution filled in a perfusor syringe. • The amount of cells depends on the body weight of the patient and the dose, which is defined in the clinical protocol.	end product

Table S4: Characteristics of Patients used for Clinical nTreg Production.

Patient ID with corresponding nTreg ID	Age at Inclusion (Years)	Donor Sex (M/F)	Cell donor on Hemodialysis (Months)	Body Weight (kg)	nTreg Total Dose (x10E6)	nTreg Dose / kg (x10E6)	nTreg Dose Group
OS Protocol nTregs Infused in Study							
C5051 / OS#01	33	F	**1,5 months**	53	26,5	0,5	**A**
C5052 / OS#02	36	F	**112 months**	56	28,0	0,5	**A**
C5053 / OS#03	56	M	None	69	34,5	0,5	**A**
C5054 / OS#04	36	F	**11 months**	61	30,5	0,5	**A**
C5056 / OS#06	45	M	None	82	82,0	1,0	**B**
C5057 / OS#07	57	M	**65 months**	108	108,0	1,0	**B**
C5058 / OS#08	35	M	None	85	85,0	1,0	**B**
C5059 / OS#09	33	M	**17 months**	80	240,0	2,5	**C**
C5062 / OS#12	41	F	None	102	255,5	2,5	**C**
C5063 / OS#13	35	M	**101 months**	81	200,0	3,0	**C**
C5067 / OS#17	52	F	**17 months**	82	244,5	3,0	**C**
OS Protocol nTregs Not Infused in Study							
C5055 / OS#05	38	M	None	N.A.	N.A.	N.A.	N.A.
C5060 / OS#10	28	M	None	N.A.	N.A.	N.A.	N.A.
C5061 / OS#11	46	F	None	N.A.	N.A.	N.A.	N.A.
C5064 / OS#17	60	M	None	N.A.	N.A.	N.A.	N.A.
C5065 / OS#15	58	M	**<1 month**	N.A.	N.A.	N.A.	N.A.
C5066 / OS#16	56	M	None	N.A.	N.A.	N.A.	N.A.
N=17 / Average All:	**45.5**	**11/6**					
Healthy Controls							
HC#01	45	M	None	N.A.	N.A.	N.A.	N.A.
HC#02	35	F	None	N.A.	N.A.	N.A.	N.A.
HC#03	40	F	None	N.A.	N.A.	N.A.	N.A.
HC#04	N.A	M	None	N.A.	N.A.	N.A.	N.A.
HC#05	N.A	M	None	N.A.	N.A.	N.A.	N.A.
HC#06	N.A	M	None	N.A.	N.A.	N.A.	N.A.
HC#07	N.A	M	None	N.A.	N.A.	N.A.	N.A.
HC#08	N.A	F	None	N.A.	N.A.	N.A.	N.A.
HC#09	N.A	M	None	N.A.	N.A.	N.A.	N.A.
N=09 / Average:	**40.0**	**6/3**					

Legend Table S4: Representative subjects recruited for autologous natural regulatory T-cell (nTreg) production according to The ONE Study protocol, are listed in order of enrollment, including n=17 solid organ transplant (SOT)-donors and n=9 healthy control donors. From the n=17 SOT-donors, n=11 were treated with a single dose nTregs (dose groups A-C, referring to 0.5, 1.0, and 2.5-3.0 million nTregs / kg of body weight) within The ONE Study. Patient C5060 was enrolled in the study, but was not transplanted, thus no nTregs were produced. **Abbreviations:** N.A., not available; HC, healthy controls; and OS, patient samples within The ONE Study.

Table S5: HLA-Typing of Kidney Donor and Recipient.

Patient ID	HLA Recipient	HLA Donor
Reference Group		
R5001	A2 A9 A24(9) B15 B40 CW3 CW7 DQ1 DQ3 DQ6(1) DR5 DR6 DR11(5) DR13(6)	A2 A3 B7 B40 B60(40) CW3 CW7 DQ1 DQ3 DQ6(1) DR2 DR5 DR11(5) DR15(2)
R5002 *	A1 A3 B8 B14 CW7 CW8 DQ1 DQ5(1) DR1 DR2 DR16(2)	A3 B14 CW8 DQ1 DQ5(1) DR1
R5003	A2 A10 A25(10) B7 CW7 DQ1 DQ5(1) DQ6(1) DR1 DR2 DR15(2)	A1 A2 B7 B12 B44(12) CW5 CW7 DQ3 DR4 DR7
R5004	A1 A10 A26(10) B8 CW7 DQ1 DQ2 DQ5(1) DR3 DR6 DR14(6)	A11 A19 A29(19) B12 B35 B44(12) CW4 CW16 DQ1 DQ5(1) DQ6(1) DR1 DR2 DR15(2)
R5005	A2 A9 A23(9) B16 B21 B39(16) B49(21) CW7 DQ1 DQ5(1) DR1	A2 A10 A25(10) B7 CW7 DQ1 DQ3 DQ5(1) DR1 DR5 DR11(5)
R5006	A2 A28 A68(28) B17 B40 B57(17) B61(40) CW5 CW6 DQ3 DR5 DR7 DR11(5)	A2 A28 A68(28) B15 B40 B61(40) B62(15) CW3 CW5 DQ1 DQ3 DQ6(1) DR5 DR6 DR11(5) DR13(6)
R5007	A19 A28 A30(19) A68(28) B12 B18 B44(12) CW5 DQ1 DQ3 DQ6(1) DQ7(3) DR2 DR4 DR15(2)	A1 B8 B17 B57(17) CW6 CW7 DQ2 DQ3 DR3 DR7
R5008	A2 B12 B15 B44(12) B62(15) CW3 CW5 DQ1 DQ3 DQ6(1) DQ7(3) DR2 DR5 DR12(5) DR15(2)	A2 A3 B7 B12 B44(12) CW5 CW7 DQ1 DQ3 DQ6(1) DR2 DR5 DR12(5) DR15(2)
R5009	A2 B12 B44(12) CW2 CW4 DQ3 DR4 DR5 DR11(5)	A2 A3 B5 B12 B51(5) B44(12) CW2 CW4 DQ1 DQ3 DQ5(1) DR1 DR5 DR11(5)
R5010	A2 A3 B7 B8 CW7 DQ2 DQ3 DR3 DR4	A3 B7 CW7 DQ1 DQ2 DQ6(1) DR2 DR3 DR15(2)
nTreg Group		
C5051	A2 A10 A26(10) B14 B35 B65(14) CW4 CW8 DQ1 DQ3 DQ5(1) DQ8(3) DR1 DR4	A2 B14 B27 B65(14) CW1 CW8 DQ1 DQ5(1) DR1
C5052	A2 B12 B27 B44(12) CW2 CW5 DQ1 DQ3 DQ5(1) DQ7(3) DR1 DR5 DR12(5)	A2 A3 B15 B27 B62(15) CW2 CW3 DQ1 DQ3 DQ5(1) DQ7(3) DR1 DR5 DR11(5)
C5053 *	A1 A2 B13 B21 B49(21) DQ1 DQ6(1) DR6 DR13(6)	A1 A2 B13 B21 B49(21) CW6 CW7 DQ1 DQ6(1) DR6 DR13(6)
C5054	A2 A9 A23(9) B5 B12 B51(5) B44(12) CW4 CW7 DQ1 DQ2 DQ6(1) DR6 DR7 DR13(6)	A9 A19 A23(9) A32(19) B12 B27 B44(12) CW2 CW4 DQ2 DQ3 DQ7(3) DR5 DR7 DR12(5)
C5056	A2 A10 A25(10) B17 B27 B58(17) CW2 CW7 DQ3 DR3 DR17(3)	A2 B22 B27 B56(22) CW1 CW2 DQ1 DQ5(1) DR1
C5057	A2 A19 A31(19) B7 B15 B62(15) CW4 CW15 DQ1 DQ2 DQ6(1) DR2 DR7 DR15(2)	A2 A28 A68(28) B13 B21 B50(21) CW6 DQ2 DR7
C5058	A19 A28 A31(19) A68(28) B12 B44(12) CW5 CW7 DQ3 DR4 DR5 DR11(5)	A9 A19 A24(9) A31(19) B12 B40 B44(12) B60(40) CW3 CW5 DQ3 DQ7(3) DR4 DR5 DR11(5)
C5059	A2 A19 A32(19) B15 B21 B50(21) B62(15) CW3 CW4 CW10(3) DQ1 DQ3 DQ5(1) DQ7(3) DR1 DR4	A11 A19 A32(19) B21 B22 B50(21) B55(22) CW3 CW4 CW9(3) DQ2 DQ3 DQ7(3) DR4 DR7
C5062	A1 A10 A26(10) B8 B17 B57(17) CW6 CW7 DQ1 DQ2 DQ6(1) DR2 DR3 DR15(2) DR17(3)	A3 A9 A24(9) B15 B18 B62(15) CW3 CW7 CW9(3) DQ1 DQ3 DQ6(1) DQ7(3) DR2 DR4 DR15(2)
C5063 *	A2 A3 B12 B17 B44(12) B57(17) CW5 CW6 DQ1 DQ3 DQ6(1) DQ9(3) DR6 DR7 DR13(6)	A2 A3 B12 B44(12) CW4 CW5 DQ1 DQ2 DQ6(1) DR6 DR7 DR13(6)
C5067	A2 A28 A68(28) B18 B35 CW4 CW7 DQ3 DQ7(3) DR5 DR11(5)	A10 A19 A26(10) A29(19) B7 B12 B44(12) CW7 CW16 DQ2 DQ3 DQ7(3) DR5 DR7 DR12(5)

Legend Table S5: There were no identical twins in our cohort, only standard HLA-typing-match in one and two transplant pairs in the reference group and nTreg group, respectively. In the nTreg group we did not observe any obvious correlation between long-term outcome and the degree of mismatch, considering the small number of cases. **Asterisk: *** no mismatch.

Table S6: ONE Study Statistical Subset Definitions and P-values.

To facilitate data-interpretation, all statistical subset definitions and statistical testing methods (described in more detail in the statistics sections of the main manuscript and published in the reference below) and the obtained P-values are summarized in the following overview tables:

Table S6A: Nonparametric analysis of time-course: clinical parameters

Table S6B: Overview of immune cell populations

Table S6C: Nonparametric analysis of time-course: absolute leukocyte subset counts

Table S6D: Nonparametric analysis of time-course: leukocyte subset frequencies

Table S6E: Nonparametric analysis of time-course: quantitative real-time PCR data

Table S6A: Longitudinal analysis of clinical parameters.

Changes over time (W01 to Y03)	*Within Group*		*Group:Time*
Clinical Parameter	**Reference** ONE-rgt11-CHA	**nTreg** ONEnTreg13	**Reference vs. nTreg**
Pharmacological Immunosuppression			
Tacrolimus trough concentration	**< 0.001**	**< 0.001**	0.303
Renal Allograft Function			
GFR	**0.041**	0.322	0.253
Creatinine	0.130	0.410	0.526
Proteinuria	0.096	**0.013**	0.466
Serum Albumin	**< 0.001**	**0.002**	0.270
Serum Urea	0.519	0.103	0.280
Liver Function			
Bilirubin	0.062	**< 0.001**	0.580
ASAT	0.250	0.075	0.312
ALAT	0.108	0.010	0.310
AP	0.086	**< 0.001**	0.183
Inflammation			
Systemic CRP	**0.026**	**0.038**	0.492
Urinary IP-10	**0.003**	**0.047**	0.586

Legend Table S6A: Shown are *P*-values of nonparametric repeated measures ANOVA-type testing for within-group time-dependent changes and for nonparallel response profiles between the two treatment groups (group:time interaction). Significant *P*-values in bold.

Table S6B: Descriptions and phenotypes of immune cell populations.

Cell subsets			
Description	***Phenotype***	***Parent population (%)***	***Figure S6***
Leucocytes	CD45+		#01
Granulocytes	CD45+ (SS high)	Leucocytes	#01
Monocytes	CD14+	Leucocytes	#01, #02
Classical Monocytes	CD14high CD16−	Monocytes	#02
Intermediate Monocytes	CD14high CD16+	Monocytes	#02
Nonclassical Monocytes	CD14low CD16high	Monocytes	#02
Plasmacytoid dendritic cells (pDC)	Lin− HLA-DR+ CD11c− CD123+ BDCA2+	Leucocytes	#03
Myeloid dendritic cells (mDC)	Lin− HLA-DR+ CD11c+	Leucocytes	#03
$CD16^+$ mDC	Lin− HLA-DR+ CD11c+ CD16+ BDCA3−	mDC	#03
MDC1	Lin− HLA-DR+ CD11c+ CD16− BDCA3−	mDC	#03
MDC2	Lin− HLA-DR+ CD11c+ CD16− BDCA3+	mDC	#03
Lymphocytes	CD45+ SS (low) FS (low)	Leucocytes	#01
NK-cells	CD3− CD56+	Lymphocytes	#01, #04
Mature NK-cells	CD3− CD56dim	NK-cells	#04
Immature NK-cells	CD3− CD56high	NK-cells	#04
T-cells	CD3+	Lymphocytes	#01, #04
$CD4^+$ T helper cells	CD3+ CD4+	$CD3^+$ T-cells	#04, #06
Naive CD4 $^+T_{Naive}$-cells	CD3+ CD4+ CCR7+ CD45RA+	$CD4^+$ T-cells	#06
Central Memory $CD4^+$ T_{CM}-cells	CD3+ CD4+ CCR7+ CD45RA−	$CD4^+$ T-cells	#06
Effector Memory $CD4^+$ T_{EM}-cells	CD3+ CD4+ CCR7− CD45RA−	$CD4^+$ T-cells	#06
Effector Memory CD45RA+ $CD4^+$ T_{EMRA}-cells	CD3+ CD4+ CCR7− CD45RA +	$CD4^+$ T-cells	#06
Activated $CD4^+$ T-cells	CD3+ CD4+ HLA-DR+ CD45RA+	$CD4^+$ T-cells	#07
Activated memory $CD4^+$ T-cells	CD3+ CD4+ HLA-DR+ CD45RA−	$CD4^+$ T-cells	#07
Differentiated/senescent $CD4^+$ T-cells	CD3+ CD4+ CD27−	$CD4^+$ T-cells	#07
Differentiated/senescent $CD4^+$ T-cells	CD3+ CD4+ CD28−	$CD4^+$ T-cells	#07
Differentiated/senescent $CD4^+$ T-cells	CD3+ CD4+ CD57+	$CD4^+$ T-cells	#07
$CD25^+CD127^{high}$ $CD4^+$ T-cells	CD3+ CD4+ CD25+ CD127high	$CD4^+$ T-cells	#07
$CD25^{high}$ $CD4^+$ T-cells	CD3+ CD4+ CD25high	$CD4^+$ T-cells	#08
Treg	CD3+ CD4+ CD25high CD127low (surface)	$CD4^+$ T-cells	#08
Naive Treg	CD3+ CD4+ CD25high CD127low CD45RA+	Treg	#08
Memory Treg	CD3+ CD4+ CD25high CD127low CD45RA−	Treg	#08
$CD25^{high}FoxP3^+$ Treg	CD3+ CD4+ CD25high FoxP3+ (intranuclear)	$CD4^+$ T-cells	#08
Treg : $CD4^+$ Effector Ratio	CD4+ CD25high CD127low / CD3+ CD4+ CCR7−	−	#09
Treg : $CD8^+$ Effector Ratio	CD4+ CD25high CD127low / CD3+ CD4+ CCR7−	−	#09
$CD25^{high}FoxP3^+$: $CD4^+$ Effector Ratio	CD4+ CD25high FoxP3+ / CD3+ CD4+ CCR7	−	#09
$CD25^{high}FoxP3^+$: $CD8^+$ Effector Ratio	CD4+ CD25high FoxP3+ / CD3+ CD8+ CCR7−	−	#09
$CD8^+$ Cytotoxic T-cells	CD3+ CD8+	$CD3^+$ T-cells	#04, #10
Naive $CD8^+$ T_{Naive}-cells	CD3+ CD8+ CCR7+ CD45+	$CD8^+$ T-cells	#10
Central Memory $CD8^+$ T_{CM}-cells	CD3+ CD8+ CCR7+ CD45−	$CD8^+$ T-cells	#10
Effector Memory $CD8^+$ T_{EM}-cells	CD3+ CD8+ CCR7− CD45−	$CD8^+$ T-cells	#10
Effector Memory CD45RA+ $CD8^+$ T_{EMRA}-cells	CD3+ CD8+ CCR7− CD45+	$CD8^+$ T-cells	#10
Activated $CD8^+$ T-cells	CD3+ CD8+ HLA-DR+ CD45RA+	$CD8^+$ T-cells	#11
Activated Memory $CD8^+$ T-cells	CD3+ CD8+ HLA-DR+ CD45RA−	$CD8^+$ T-cells	#11
Differentiated/senescent $CD8^+$ T-cells	CD3+ CD8+ CD27−	$CD8^+$ T-cells	#11
Differentiated/senescent $CD8^+$ T-cells	CD3+ CD8+ CD28−	$CD8^+$ T-cells	#11
Differentiated/senescent $CD8^+$ T-cells	CD3+ CD8+ CD57+	$CD8^+$ T-cells	#11
$CD25^+CD127^{high}$ $CD8^+$ T-cells	CD3+ CD8+ CD25+ CD127high	$CD8^+$ T-cells	#11
TCRγδ+ T-cells	TCRγδ+ CD3+	$CD3^+$ T-cells	#05
$TCR\gamma\delta^+CD4^+$ T-cells	TCRγδ+ CD3+ CD4+	$TCR\gamma\delta^+$ T-cells	#05
$TCR\gamma\delta^+CD8^+$ T-cells	TCRγδ+ CD3+ CD8+	$TCR\gamma\delta^+$ T-cells	#05
$TCR\gamma\delta^+CD4^-CD8^-$ T-cells	TCRγδ+ CD3+ CD4− CD8−	$TCR\gamma\delta^+$ T-cells	#05
B-cells	CD19+ CD3−	Lymphocytes	#01
Naive B-cells	CD19+ CD27− IgD+	B-cells	#12
Transitional B-cells	CD19+ CD27− CD38high IgM+ CD24+	B-cells	#12
Marginal Zone B-cells	CD19+ CD27+ IgD+	B-cells	#12
Class-switched B-cells	CD19+ CD27+ CD38dim IgD− IgM−	B-cells	#12
Non-switched B-cells	CD19+ CD27+ CD38dim IgM+	B-cells	#12
Plasmablasts	CD19+ CD27high CD38high IgD− IgM−	B-cells	#12
$CD21^{low}$ B-cells	CD19+ CD38low CD21low	B-cells	#12

Table S6C: Longitudinal analysis of absolute counts of leukocyte cell subsets.

Changes over time (W−04 to W60)	*Within Group*		*Group:Time*
Cell subset counts (cells/µL)	**Reference ONE-rgt11-CHA**	**nTreg ONEnTreg13**	**Reference vs. nTreg**
CD45⁺ Leucocytes	**< 0.001**	**< 0.001**	0.067
Granulocytes	**< 0.001**	**< 0.001**	0.138
CD14⁺ Monocytes	0.752	0.130	0.360
Classical	0.665	**0.032**	0.401
Intermediate	0.222	**0.037**	0.622
Nonclassical	**< 0.001**	**< 0.001**	0.593
pDC	**< 0.001**	**< 0.001**	0.492
mDC	**< 0.001**	**< 0.001**	0.250
CD16⁺	**< 0.001**	**< 0.001**	0.369
MDC1	**0.016**	0.423	0.540
MDC2	0.283	0.172	0.787
Lymphocytes	0.418	0.096	0.582
CD56⁺ NK-cells	**0.013**	**0.043**	0.240
CD56^{dim}	**0.005**	**0.049**	0.146
CD56^{high}	0.139	0.360	0.401
CD3⁺ T-cells	0.570	0.115	0.722
CD4⁺	0.202	0.026	0.437
HLA-DR⁺CD45RA⁺	0.371	0.549	0.520
HLA-DR⁺CD45RA⁻	0.098	**0.003**	0.127
Naive	0.146	**0.001**	0.301
CM	0.259	**0.031**	0.236
EM	0.422	0.211	0.565
TEMRA	0.385	0.779	0.806
CD27⁻	0.665	0.315	0.758
CD28⁻	0.139	0.208	0.316
CD57⁺	0.533	0.168	0.569
CD25⁺CD127^{high}	**< 0.001**	**0.009**	**< 0.001**
CD25^{high}	**< 0.001**	**0.030**	**< 0.001**
Treg	**< 0.001**	**0.003**	**< 0.001**
Naive	**< 0.001**	0.016	**< 0.001**
Memory	**< 0.001**	0.002	**< 0.001**
CD25^{high}FoxP3⁺ Treg	**< 0.001**	**< 0.001**	**< 0.001**
Treg : CD4⁺ Teff	**< 0.001**	**0.003**	**< 0.001**
Treg : CD8⁺ Teff	**< 0.001**	0.062	**< 0.001**
CD25^{high}FoxP3⁺ : CD4⁺ Teff	**< 0.001**	**0.015**	**< 0.001**
CD25^{high}FoxP3⁺ : CD8⁺ Teff	**< 0.001**	0.073	**< 0.001**
CD8⁺	0.947	0.366	0.863
HLA-DR⁺CD45RA⁺	0.675	0.243	0.629
HLA-DR⁺CD45RA⁻	0.442	0.785	0.611
Naive	0.348	**0.009**	0.327
CM	0.610	0.098	0.486
EM	0.671	0.602	0.698
TEMRA	0.399	0.297	0.694
CD27⁻	0.574	0.525	0.886
CD28⁻	0.487	0.438	0.853
CD57⁺	0.576	0.365	0.913
CD25⁺CD127^{high}	**0.008**	0.642	**0.012**
TCRγδ⁺	0.679	0.678	0.928
CD4⁺	0.227	**0.025**	0.459
CD8⁺	0.530	0.527	0.826
CD4⁻CD8⁻	0.647	0.747	0.863
CD19+ B-cells	**0.034**	**0.001**	0.608
Naive	0.176	**0.006**	0.457
Transitional	0.055	**< 0.001**	0.398
Marginal Zone	**0.016**	**< 0.001**	0.178
Class−switched	**0.019**	**< 0.001**	0.264
Non−switched	**0.043**	**< 0.001**	0.498
Plasmablasts	**0.012**	**< 0.001**	0.142
CD21^{low}	0.322	**< 0.001**	0.295

Legend Table S6C: Shown are *P*-values of nonparametric repeated measures ANOVA-type testing for within-group time-dependent changes in absolute blood subset cell counts and for nonparallel response profiles between the two treatment groups (group:time interaction). Significant *P*-values in bold.

Table S6D: Longitudinal analysis of leukocyte subset frequencies.

Changes over time (W−04 to W60)	*Within Group*		*Group:Time*
Cell subset frequency (%)	**Reference ONE-rgt11-CHA**	**nTreg ONEnTreg13**	**Reference vs. nTreg**
CD45+ Leucocytes	n.a.	n.a.	n.a.
Granulocytes	0.382	0.240	0.786
CD14+ Monocytes	**0.007**	**< 0.001**	0.169
Classical	**0.006**	**< 0.001**	0.242
Intermediate	0.183	**0.007**	0.563
Nonclassical	**< 0.001**	**< 0.001**	0.614
pDC	**< 0.001**	**< 0.001**	0.476
mDC	**< 0.001**	**< 0.001**	0.264
CD16+	0.145	**< 0.001**	0.753
MDC1	0.155	**< 0.001**	0.732
MDC2	**0.041**	**0.035**	0.811
Lymphocytes	0.563	0.378	0.867
CD56+ NK-cells	**0.026**	**0.002**	0.893
CD56dim	0.081	0.060	0.085
CD56high	0.081	0.060	0.085
CD3+ T-cells	0.401	0.229	0.742
CD4+	0.471	**0.010**	0.519
Naive	0.153	**< 0.001**	0.087
CM	0.134	**0.049**	0.464
EM	0.139	**0.011**	0.496
TEMRA	0.061	**0.004**	0.402
HLA-DR+CD45RA+	0.521	**0.019**	0.089
HLA-DR+CD45RA−	0.191	**< 0.001**	**0.018**
CD27−	0.109	**< 0.001**	0.326
CD28−	0.266	**0.008**	0.460
CD57+	0.518	**< 0.001**	0.149
CD25+CD127high	**< 0.001**	0.272	**< 0.001**
CD25high	**< 0.001**	0.854	**< 0.001**
Treg	**< 0.001**	0.125	**< 0.001**
Naive	n.a.	n.a.	n.a.
Memory	n.a.	n.a.	n.a.
CD25highFoxP3+ Treg	**< 0.001**	0.197	**0.005**
Treg : CD4+ Teff	n.a.	n.a.	n.a.
Treg : CD8+ Teff	n.a.	n.a.	n.a.
CD25highFoxP3+ : CD4+ Teff	n.a.	n.a.	n.a.
CD25highFoxP3+ : CD8+ Teff	n.a.	n.a.	n.a.
CD8+	0.331	**0.025**	0.637
Naive	**0.004**	0.090	0.604
CM	0.632	0.170	0.910
EM	0.072	0.244	0.527
TEMRA	**0.039**	0.103	0.480
HLA-DR+CD45RA+	0.510	**0.010**	0.218
HLA-DR+CD45RA−	0.150	**0.074**	0.366
CD27−	0.104	**0.046**	0.616
CD28−	0.190	**0.039**	0.713
CD57+	0.119	**0.001**	0.697
CD25+CD127high	**0.021**	0.743	0.081
TCRγδ+	0.269	**< 0.001**	0.551
CD4+	0.131	**< 0.001**	0.150
CD8+	0.273	**< 0.001**	0.483
CD4−CD8−	0.367	**< 0.001**	0.418
CD19+ B-cells	**0.004**	**0.009**	0.736
Naive	**0.040**	0.393	**0.039**
Transitional	**0.016**	**< 0.001**	0.540
Marginal Zone	0.083	0.060	**0.045**
Class−switched	0.065	**< 0.001**	0.313
Non−switched	**0.033**	0.103	0.230
Plasmablasts	**0.011**	**< 0.001**	0.250
CD21low	**0.013**	**< 0.001**	0.147

Legend Table S6D: Shown are *P*-values of nonparametric repeated measures ANOVA-type testing for within-group time-dependent changes and for nonparallel response profiles between the two treatment groups (group:time interaction). The vertical lines / indentions indicate parent populations used to calculate subset proportions. Significant *P*-values in bold.

Table S6E: Longitudinal analysis of gene expression levels of whole blood samples.

Changes over time (W−04 to W60)	*Within Group*		*Group:Time*
Genes	**Reference** ONE-rgt11-CHA	**nTreg** ONEnTreg13	**Reference vs. nTreg**
Tolerance-associated			
CD79B	**0.002**	0.089	0.126
FCRL1	0.176	**0.008**	0.189
FCRL2	**0.004**	0.367	0.569
HS3ST1	0.491	0.586	0.647
MAN1A1	**0.012**	0.113	0.312
MS4A1 (CD20)	**0.031**	**0.028**	0.348
PNOC	**0.023**	**< 0.001**	0.110
SH2D1B	**0.017**	**0.032**	0.553
TCL1A	0.537	0.305	0.819
Rejection-associated			
CD247	0.526	0.206	0.497
CXCL10 (IP-10)	0.581	**< 0.001**	0.162
HMMR	0.058	0.355	0.612
NAV3	**0.008**	0.077	0.387
SLC8A1	0.225	0.350	0.568
TMEM176B	0.074	**< 0.001**	**0.023**
TLR5	0.244	0.071	0.943
Co-inhibitory molecules			
LAG3	0.096	0.091	0.593
CD200	**0.049**	0.092	0.149
CD274	0.448	0.825	0.572
FoxP3			
mRNA	**0.042**	**0.003**	0.691

Legend Table S6E: Shown are *P*-values of nonparametric repeated measures ANOVA-type testing for within-group time-dependent changes and for nonparallel response profiles between the two treatment groups (group:time interaction). Significant *P*-values in bold.

Table S7: Summary of the Patient Assessment during the Trial (Table continued on next page).

	V00	V01	GMP	Tx	F01	V02	F02	V03	V04	V05	V06	V07	V08	V09	V10	EXX
Time points	**W-6**	**W-4**	**W-2**	**D0**	**D+6**	**W+1**	**D+8**	**W+2**	**W+4**	**W+8**	**W+12**	**W+24**	**W+36**	**W+48**	**W+60**	
Patient study inclusion and nTregs																
Eligibility checklist	X															
Patient trial identifier	X															
Pregnancy test result (1)	X															
Baseline characteristics		X														
Donor characteristics	X															
Blood collection for nTreg production			X													
nTreg infusion						X										
IIC monitoring (2)						X										
Clinical assessment																
Physical examination		X			X		X	X	X	X	X	X	X	X	X	
Body mass index (BMI) (3)		X			X				X	X	X	X	X	X	X	
Vital signs		X			X		X	X	X	X	X	X	X	X	X	
Immunosuppression					X		X	X	X	X	X	X	X	X	X	
Co-medication		X			X		X	X	X	X	X	X	X	X	X	
Protocol graft biopsy								X*			X*		X		X*	
Dermatological assessment		X													X	
Adverse event data	X	X			X		X	X	X	X	X	X	X	X	X	
Creatinine					X		X		X	X	X	X	X	X	X	X
Albumin					X				X	X	X	X	X	X	X	X
Urea / Blood urea nitrogen		X			X		X		X	X	X	X	X	X	X	X
Complete blood count (5)		X			X		X		X	X	X	X	X	X	X	X
Electrolytes (6)		X			X		X		X	X	X	X	X	X	X	
Liver function tests (7)		X			X		X		X	X	X	X	X	X	X	
Lipid profile (8)		X			X				X	X	X	X	X	X	X	
Parathyroid hormone		X														
C-reactive protein		X*			X		X		X*	X*	X*	X*	X*	X*	X*	X*
Tacrolimus trough levels					X	X**	X		X	X	X	X	X	X	X	X
D-dimer					X		X									
Cytokine array (9)					X	X**	X									
Total urine protein					X		X		X	X	X	X	X	X	X	
Urine dipstick test					X*		X*		X*	X*	X*	X*	X*	X*	X*	

Table S7: Summary of the Patient Assessment during the Trial (Table continued from previous page).

	V00	V01	GMP	Tx	F01	V02	F02	V03	V04	V05	V06	V07	V08	V09	V10	EXX
Time points	**W-6**	**W-4**	**W-2**	**D0**	**D+6**	**W+1**	**D+8**	**W+2**	**W+4**	**W+8**	**W+12**	**W+24**	**W+36**	**W+48**	**W+60**	
Immune Monitoring (IM) Subproject**																
1.1: Viral Load (PCR)									X	X	X					
1.2: HLA-DR (Flow Cytometry)					X		X	X								
2.1: Gene Expression (qPCR)		X						X	X	X	X	X	X	X	X	X
2.2: Leucocyte Profiling (Flow Cytometry)		X			X		X	X	X	X	X	X	X	X	X	X
2.3: Anti-donor antibodies (Unspecified)											X				X	
2.4: T-cell Frequencies (IFN-g ELISPOT)	X							X						X		
2.5: Urinary IP-10 (ELISA)					X	X	X	X	X	X	X	X	X	X	X	
2.6: Cytokine Profiling (Luminex)					X	X	X									
3.1: Gene expression (Microarray)					X		X	X								
3.2: nTreg Lifespan in vivo (NGS)									X		X		X		X	
Total blood volume (ml) for the IM subproject	40,0	11,0	74,0	0,0	11,0	0,0	11,0	59,0	60,0	55,0	68,5	51,0	56,0	51,0	24,5	9,0
Kidney donor blood collection		X														
HEC Subproject																
KTQ-34 questionnaire (11)									X				X		X	
EQ-5D questionnaire (11)		X							X			X		X	X	
SF-36 questionnaire (11)		X										X			X	
Resource use (PRU-Log) (11)									X			X		X	X	
Initial hospitalization												X				
Hospital re-admissions (11)																
Extras																
Drug dose adjustments	**As indicated**															
For-cause graft biopsy																
Dialysis episodes																
SAE reporting																

Legend Table S7: Asterisks and numbers denote: * optional assessment, and ** to be performed 6 hours after cell infusion; 1) Only for female patients; 2) Heart rate, blood pressure, and temperature measurements, with heart rate and blood pressure being monitored after 5 min of rest and according to local trial center routines; 3) Patient mass (kg) and height (m) should be measured to calculate the BMI: formula = mass / $(height)^2$; 4) ICC (International Intensive Care) monitoring of the patient for nTreg cell infusion includes ECG, blood pressure, respiratory rate, SaO_2 and temperature; 5) Red blood cells, hemoglobin, hematocrit, mean cell volume, white blood cells (with differential count) and platelets; 6) Na^+, K^+, Ca^{2+}, PO_4^{3-} and glucose; 7) Bilirubin, aminotransferase and alkaline phosphatase; 8) Cholesterol, triacylglyceride and high-density lipoprotein; 9) TNF-alpha, IFN-gamma, IL-1, IL-6, IL-8, IL-10, Fibrinogen and free hemoglobin; 10) If proteinuria, hematuria or leucocytes detected, microscopic examination of a urinary sediment smear should be undertaken; and 11) To be completed by the study nurse.

Abbreviations: V, trial visit; F, time point for sample collection in the immune monitoring (IM) subproject; D, day; W, week(s); SAE, serious adverse events; EXX, acute rejection episode.

2020-10-12 Supplemental Figures and Tables

Figure S1A: CONSORT Transparent Reporting of Trials for the ONEnTreg13 Charité Cell Therapy Trial.

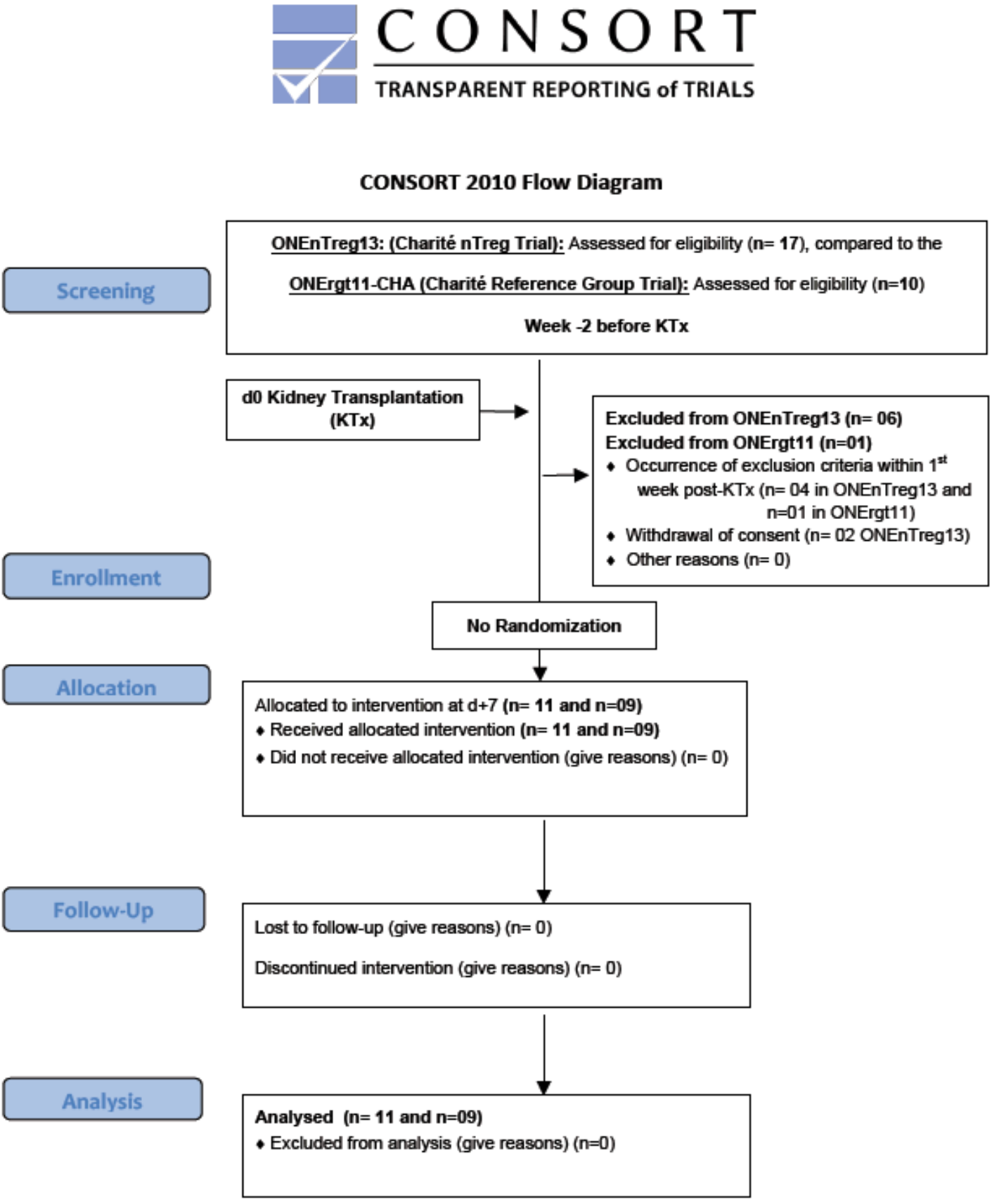
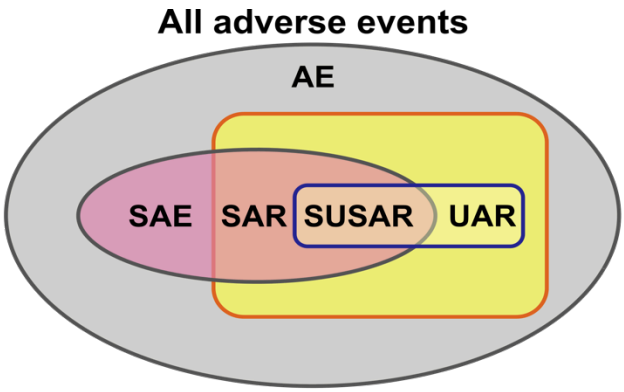


Figure S1B: Assessment of Adverse Events and Efficacy in Patients.



Evaluation of adverse events (Venn diagram shown to the left) and clinical efficacy by primary endpoint decision tree based on histopathology and clinical criteria (below).

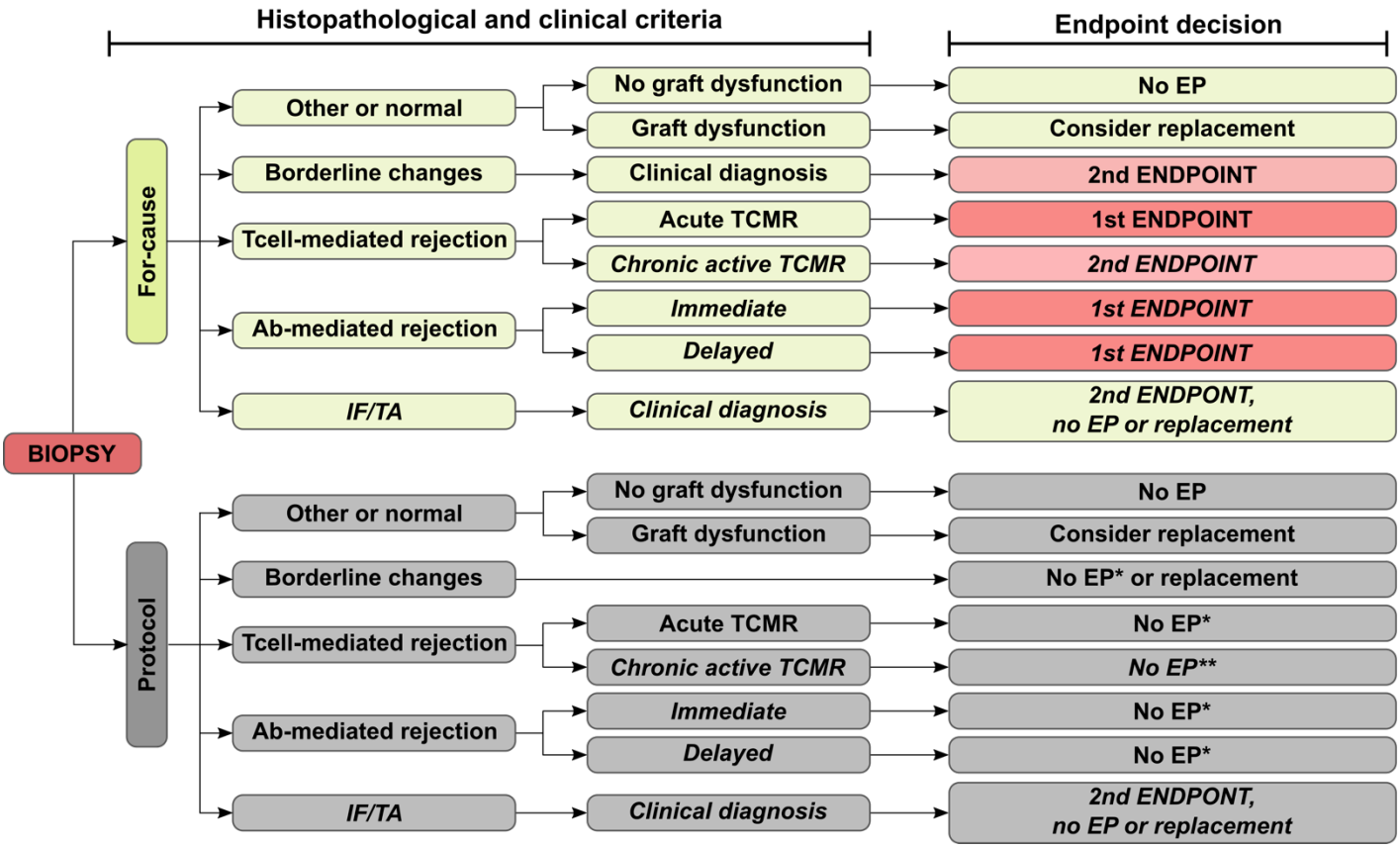


Figure S2: Supplementary Patient Data Clinical Monitoring.

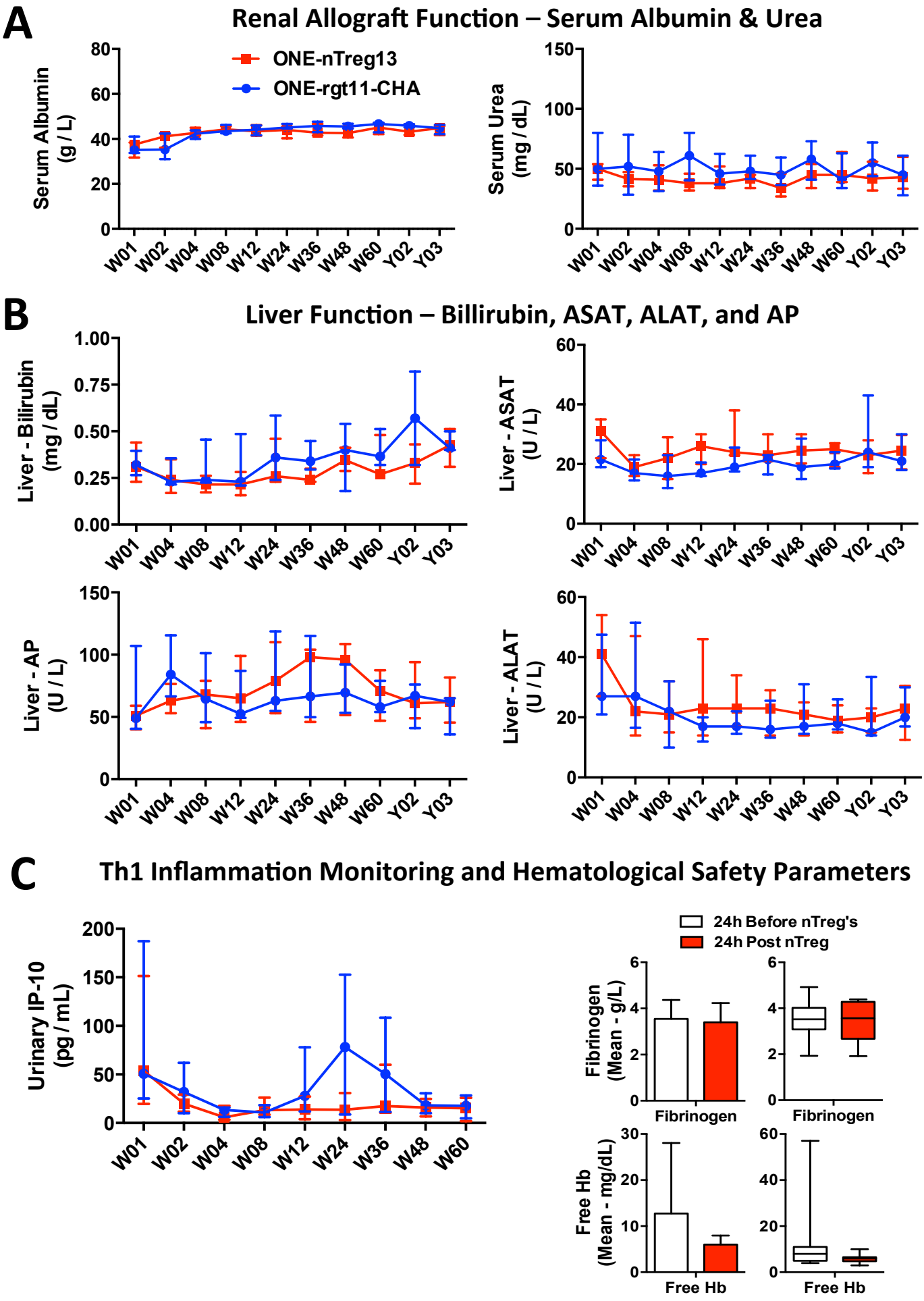
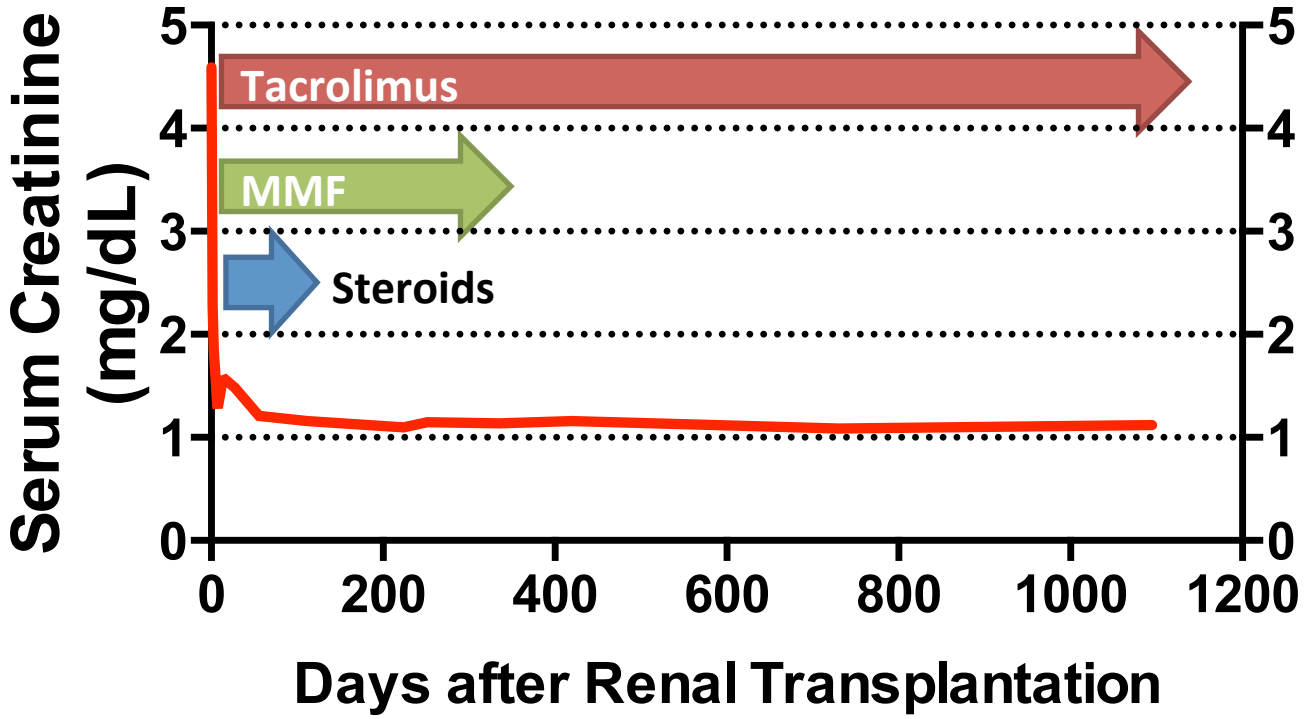


Figure S3: Case-by-Case Evaluation: Patient #01 (C5051)

Cell Product: 26.5x10E6 T-cells					
Recipient sex:	Female	**Recipient age:**	33 years	**Surgical complications:**	None
Underlying disease:	Diabetes type 1	**BMI:**	20.2 kg/m2	**Infectious complications:**	None
Time on dialysis:	none	**HLA-mismatches**	1	**Immunological complications:**	None
Donor sex:	Female	**Donor age:**	60 years	**Other complications:**	None

Low dose nTregs (0.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
pre KTx	0	1	334	1	7	TNTC
w2	0	1	441	3	23	TNTC
w48	2	0	393	3	35	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
pre KTx	**Positive 19%**	negative
w2	**Positive 2%**	negative
w48	**Positive 2%**	negative
w60	**Positive 11%**	negative

Panel Reactive Antibody Screening: HLA antibodies class I were present prior to transplantation and lost activity over the time course. The patient never developed HLA class II .
HLA class I specification:

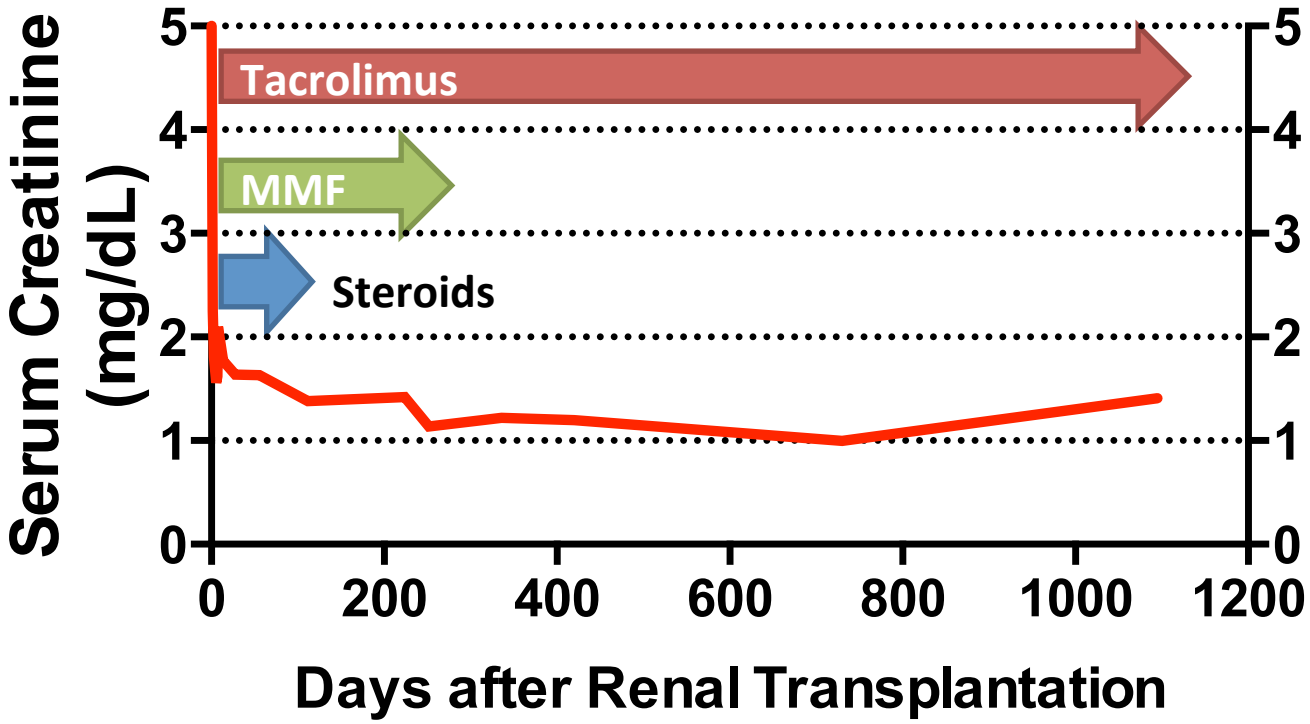
- Preoperative: A30, A31, B63, B67, CW1, CW12, CW15
- Postoperative at w60: B63, CW1, CW12, CW15
- DSA CW1

Pathology: not available (patient declined biopsy)

Figure S3: Case-by-Case Evaluation: Patient #02 (C5052)

Cell Product: 28.0x10E6 T-cells					
Recipient sex:	Female	**Recipient age:**	36 years	**Surgical complications:**	None
Underlying disease:	Interstitial nephritis	**BMI:**	18.9 kg/m2	**Infectious complications:**	None
Time on dialysis:	105 month	**HLA-mismatches**	2	**Immunological complications:**	None
Donor sex:	Female	**Donor age:**	63 years	**Other complications:**	None

Low dose nTregs (0.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	2	5	1	2	16	TNTC
w2	1	1	1	1	12	TNTC
w48	1	1	1	0	10	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
Pre KTx	negative	negative
w2	negative	negative
w48	negative	negative
w60	negative	negative

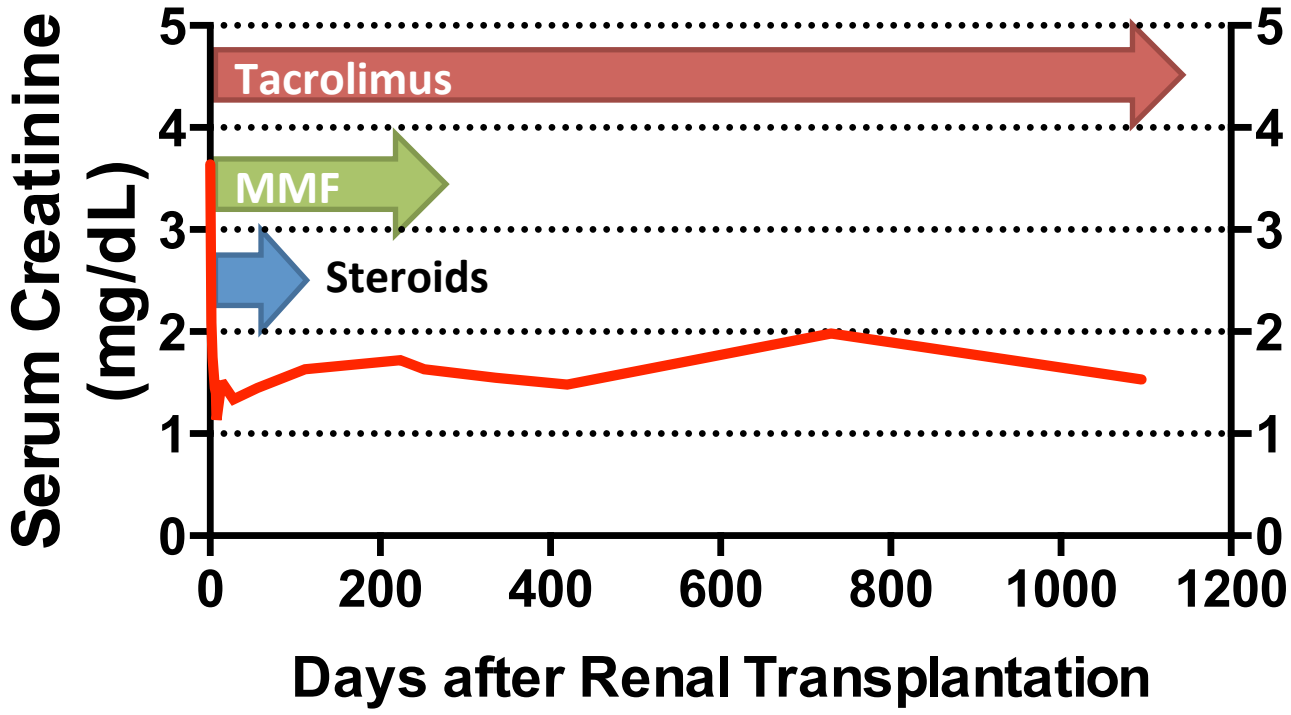
Panel Reactive Antibody Screening: negative

Pathology: no acute cellular rejection, interstitial lymphocyte infiltration (<5%) v0 i1 t0 g0 cg0 mn0 ptc0 ah0-1

Figure S3: Case-by-Case Evaluation: Patient #03 (C5053)

Cell Product: 34.5x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	56 years	**Surgical complications:**	Formation of ileal conduit
Underlying disease:	Uropathy	**BMI:**	22.8 kg/m2	**Infectious complications:**	Recurrent UTI's , BK viruria
Time on dialysis:	None	**HLA-mismatches**	0	**Immunological complications:**	None
Donor sex:	Male	**Donor age:**	55 years	**Other complications:**	None

Low dose nTregs (0.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	0	0	2	1	6	TNTC
w2	2	1	1	4	1	739
w48	1	1	1	2	3	530

Panel reactive antibody screening

Visits	HLA class I	HLA class II
Pre KTx	negative	negative
w2	negative	negative
w48	negative	negative
w60	negative	negative

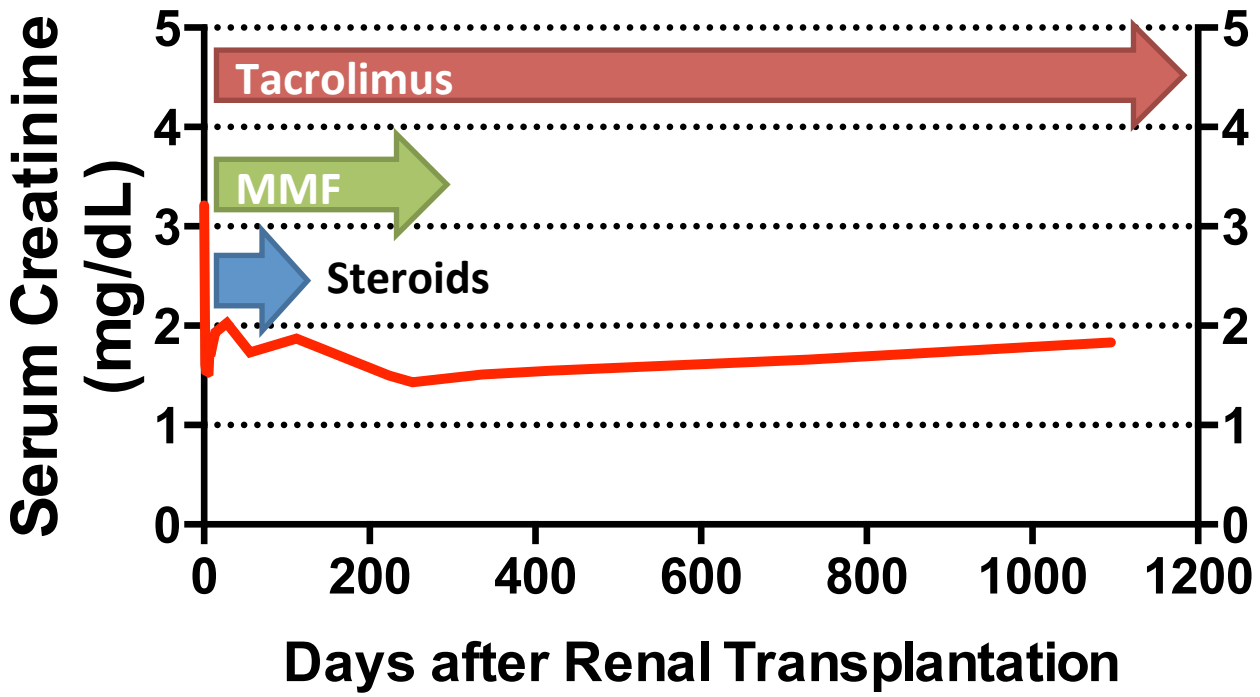
Panel Reactive Antibody Screening: negative

Pathology: no acute cellular rejection, mild interstitial lymphocyte infiltration (<5%) i1 t0 g0 cg0 mn0 ptc0 ah0

Figure S3: Case-by-Case Evaluation: Patient #04 (C5054)

Cell Product: 30.5x10E6 T-cells					
Recipient sex:	Female	**Recipient age:**	36 years	**Surgical complications:**	None
Underlying disease:	Hemorrhagic shock	**BMI:**	21.4 kg/m2	**Infectious complications:**	None
Time on dialysis:	11 months	**HLA-mismatches**	3	**Immunological complications:**	None
Donor sex:	Female	**Donor age:**	57 years	**Other complications:**	None

Low dose nTregs (0.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	1	3	587	496	10	TNTC
w2	0	1	423	308	10	TNTC
w48	1	3	450	229	6	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
Pre KTx	negative	negative
w2	negative	negative
w48	negative	negative
w60	negative	negative

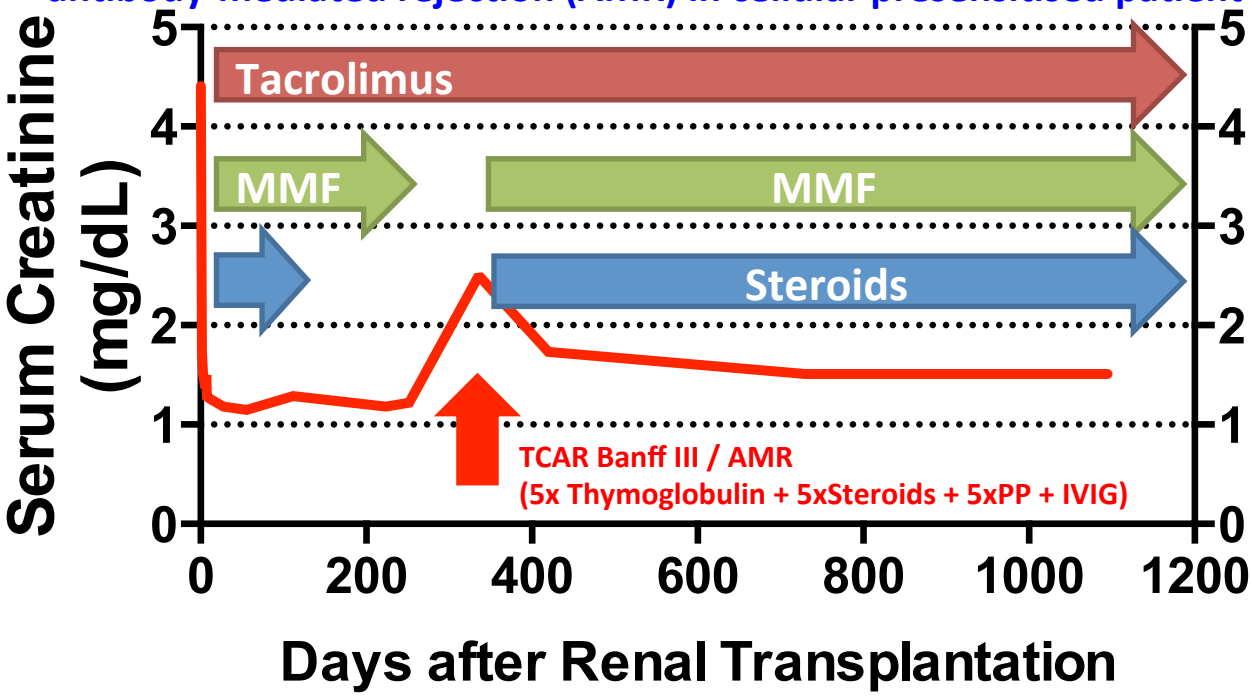
Panel Reactive Antibody Screening: negative

Pathology: not available (patient declined biopsy)

Figure S3: Case-by-Case Evaluation: Patient #05 (C5056)

Cell Product: 82.0x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	45 years	**Surgical complications:**	None
Underlying disease:	ADPKD	**BMI:**	27.5 kg/m2	**Infectious complications:**	CMV viremia
Time on dialysis:	None	**HLA-mismatches**	2	**Immunological complications:**	ACR Banff III and AMR +355 days
Donor sex:	Female	**Donor age:**	45 years	**Other complications:**	None

Medium dose nTregs (1.0 mio/kg):
Failure of weaning - mixed T cell mediated acute rejection (TCAR) and antibody-mediated rejection (AMR) in cellular presensitised patient



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
pre KTx	9	47	6	9	37	TNTC
w2	1	2	2	3	12	301
w48	1	6	486	101	7	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
pre KTx	negative	negative
w2	negative	negative
w48	**positive 28%**	**positive 93%**
w60	**positive 01%**	**positive 71%**

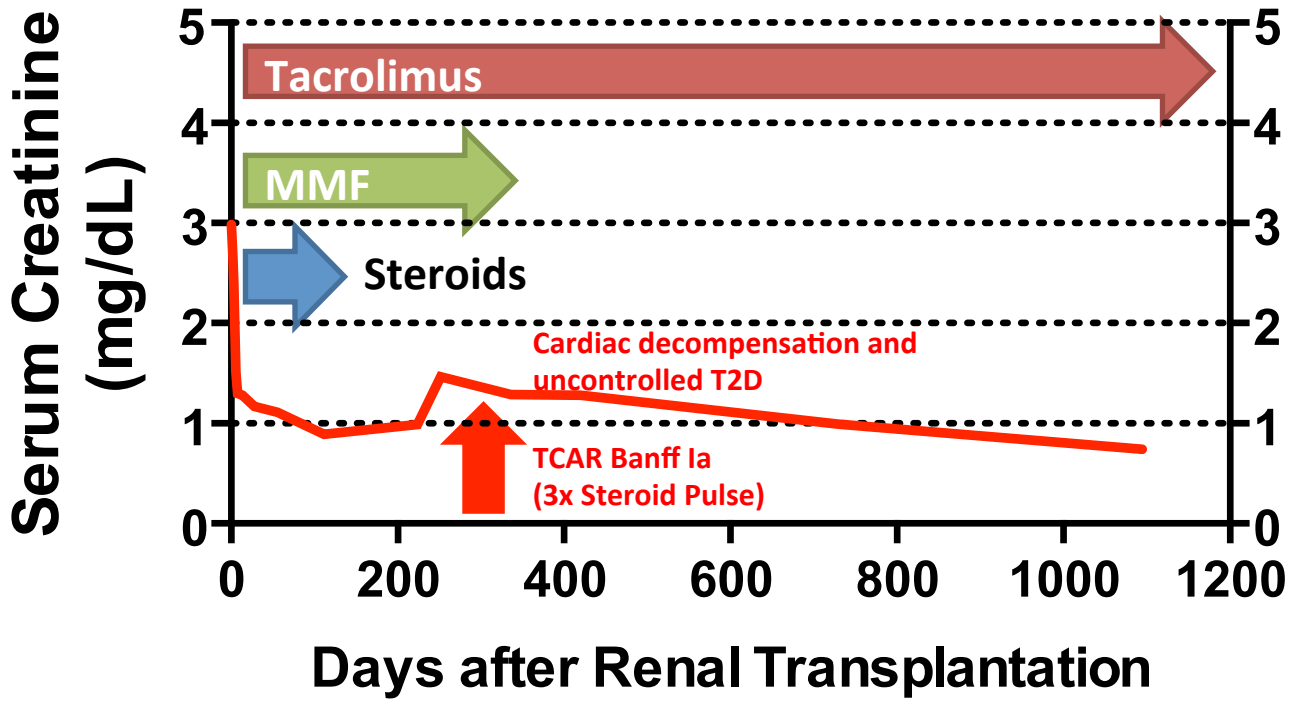
Panel Reactive Antibody Screening: HLA class I (Specification: B56, B42, B55, B62, B76, CW1, CW9, CW10) and HLA class II (Specification: DP10, DP11, DP14, DP17, DP18, DP2, DP20, DP28, DP3, DP6, DP9, DQ4, DQ5, DQ6, DQ8, DQ9, DR1, DR9, DR10, DR11, DR103,DR51) were present at day 305 (11months) after transplantation. Donor specific-antibodies (DSA) HLA Class I developed against B56 and CW1 and DSA HLA-Class II against DR1 and DQ5. After having received rejection therapy the patient presented a reduced panel reactivity, DSA and specification of HLA class I and II antibodies.

Pathology: patient with T-cell mediated acute rejection (TCAR) Banff grade III and antibody-mediated rejection (AMR), v3 31 t3 g0 cg0 mm0 ptc2 ah0 C4d+ at 3 months post minimization (CNI monotherpay)

Figure S3: Case-by-Case Evaluation: Patient #06 (C5057)

Cell Product: 108.0x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	57 years	**Surgical complications:**	None
Underlying disease:	IgA Nephropathy	**BMI:**	33.3 kg/m2	**Infectious complications:**	None
Time on dialysis:	64 months	**HLA-mismatches**	3	**Immunological complications:**	ACR Banff Ia
Donor sex:	Female	**Donor age:**	53 years	**Other complications:**	Cardiac decompensation, liver zirrhosis

Medium dose nTregs (1.0 mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	2	3	187	9	3	TNTC
w2	1	2	2	3	12	301
w48	1	6	486	101	7	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
Pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w60	neg	neg

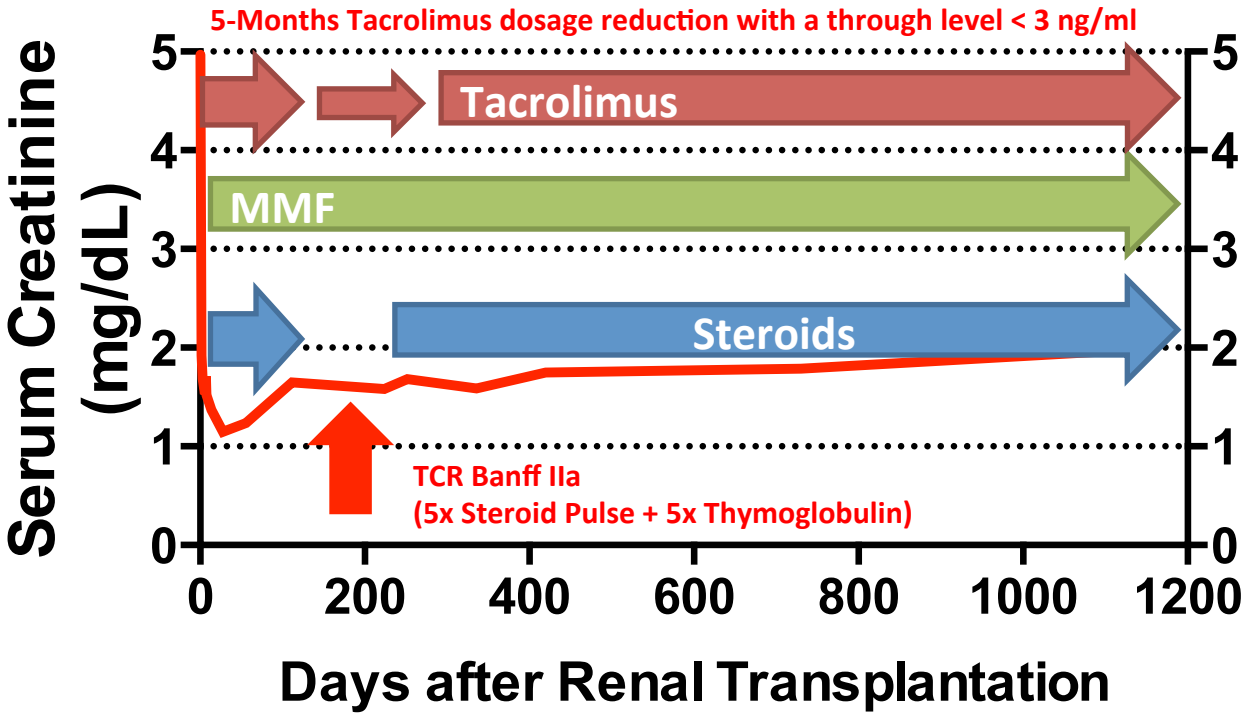
Panel Reactive Antibody Screening: negative

Pathology: patient with T-cell mediated acute rejection (TCAR) Banff grade Ia, i2 t2 g0 cg0 mm0 ptc0 ah1 C4d0
Temporary graft deterioration probably rather due to cardial/T2D decompensation than TCAR

Figure S3: Case-by-Case Evaluation: Patient #07 (C5058)

Cell Product: 85.0x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	35 years	**Surgical complications:**	None
Underlying disease:	FSGS	**BMI:**	26.5 kg/m2	**Infectious complications:**	None
Time on dialysis:	None	**HLA-mismatches**	2	**Immunological complications:**	ACR Banff IIa +197 days
Donor sex:	Female	**Donor age:**	57 years	**Other complications:**	Posterior reversible encephalopathy syndrome (PRES)

Medium dose nTregs (1.0 mio/kg):
No weaning due to early PRES with need for strong reduction of Tac for 3 months and development of TCAR before weaning to monotherapy



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
pre KTx	0	0	0	2	23	TNTC
w2	0	0	2	0	23	TNTC
w48	0	0	0	0	42	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w60	**pos 63%**	neg

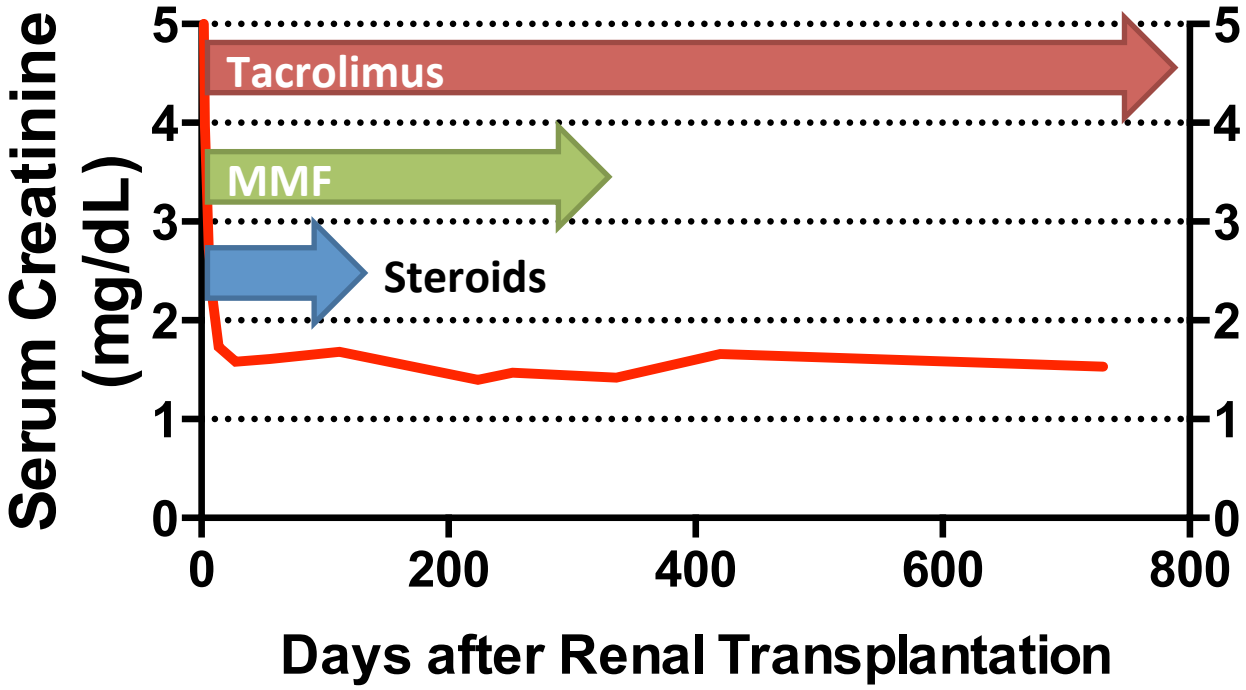
Panel Reactive Antibody Screening: HLA class I (Specification: A23, A24, B46, B60, B61, B78, B8, B81) were present at day 305 (11 months) after transplantation. Donor specific-antibodies (DSA) HLA Class I developed against A24 and B60. HLA class II antibodies remained negative.

Pathology: patient with T-cell mediated acute rejection TCAR Banff grade IIa, v1 i3 t2 cv2g0 cg0 mn0 ptc0 ah0 C4d0

Figure S3: Case-by-Case Evaluation: Patient #08 (C5059)

Cell Product: 240.0x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	33 years	**Surgical complications:**	None
Underlying disease:	Hypertension	**BMI:**	23.4 kg/m2	**Infectious complications:**	None
Time on dialysis:	16 months	**HLA-mismatches**	3	**Immunological complications:**	None
Donor sex:	Female	**Donor age:**	56 years	**Other complications:**	None

High dose nTregs (2.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine.



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	1	5	4	0	128	TNTC
w2	0	1	3	0	64	TNTC
w48	1	1	2	0	63	TNTC

Panel reactive antibody screening

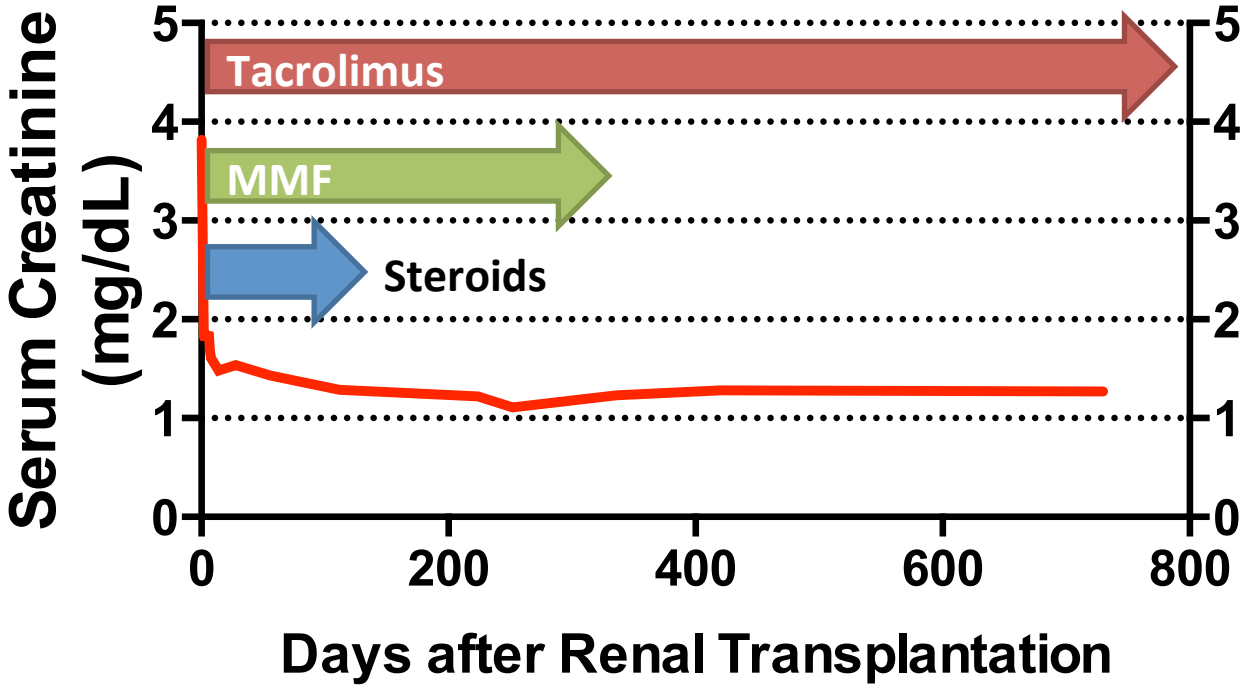
Visits	HLA class I	HLA class II
Pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w90	neg	**pos 34%**

Panel Reactive Antibody Screening: At week 60 the patient developed HLA class II antibodies with donor specificity against DQ2.
Pathology: no acute cellular rejection, interstitial lymphocyte infiltration v0 i1 t0 g0 cg0 mn0 ptc0 ah0

Figure S3: Case-by-Case Evaluation: Patient #09 (C5062)

Cell Product: 255.5x10E6 T-cells					
Recipient sex:	Female	**Recipient age:**	42 years	**Surgical complications:**	None
Underlying disease:	ADPKD	**BMI:**	34.7 kg/m2	**Infectious complications:**	None
Time on dialysis:	None	**HLA-mismatches**	5	**Immunological complications:**	None
Donor sex:	Male	**Donor age:**	43 years	**Other complications:**	None

High dose nTregs (2.5 mio/kg):
Successful tapering of immunosuppression with stable creatinine.



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
pre KTx	1	7	92	226	30	TNTC
w2	0	1	49	72	33	TNTC
w48	1	0	52	209	69	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w60	neg	neg

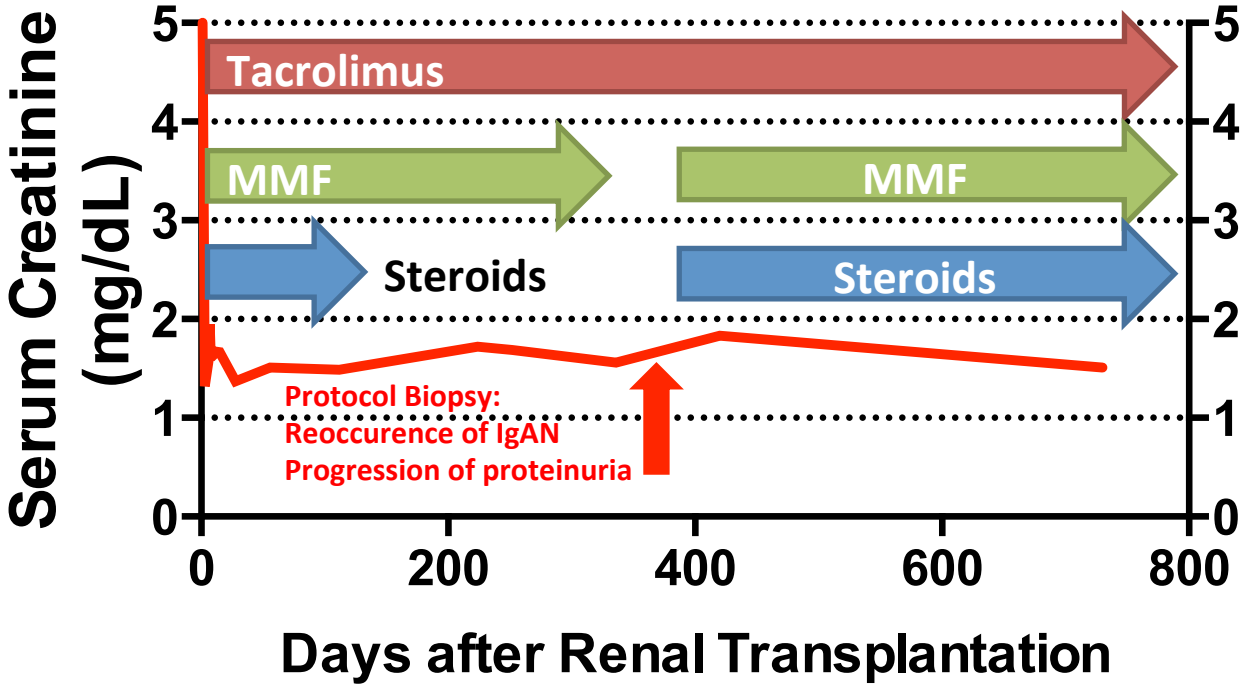
Panel Reactive Antibody Screening: In January 2019 the patient developed HLA class II antibodies with donor specificity against DQ7.

Pathology: no acute cellular rejection, interstitial lymphocyte infiltration v0 i0 t0 g0 cg0 mn0 ptc0 ah0

Figure S3: Case-by-Case Evaluation: Patient #10 (C5063)

Cell Product: 200.5x10E6 T-cells					
Recipient sex:	Male	**Recipient age:**	35 years	**Surgical complications:**	None
Underlying disease:	GN	**BMI:**	25.8 kg/m2	**Infectious complications:**	None
Time on dialysis:	93 months	**HLA-mismatches**	0	**Immunological complications:**	None
Donor sex:	Male	**Donor age:**	33 years	**Other complications:**	Reoccurence of underlying renal disease

High dose nTregs (3.0mio/kg):
Failure of weaning due to reoccurrence of IgA Nephropathy



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
pre KTx	1	13	TNTC	37	1	TNTC
w2	0	0	522	3	1	TNTC
w48	0	0	660	6	0	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w60	neg	neg

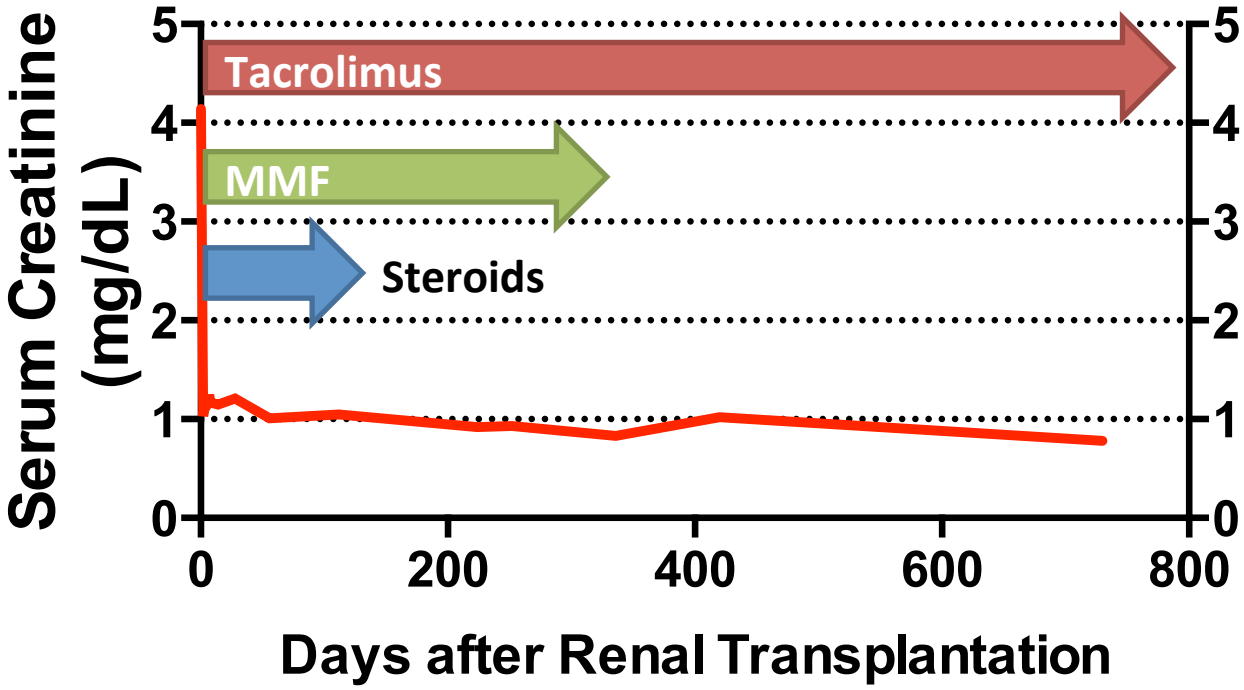
Panel Reactive Antibody Screening: negative

Pathology: no rejection, reoccurrence of underlying renal disease, IgA Nephropathy (IgAN) Oxford Classification M0E0S0T0, biopsy pathology confirmed by 2nd biopsy

Figure S3: Case-by-Case Evaluation: Patient #11 (C5067)

Cell Product: 244.5x10E6 T-cells					
Recipient sex:	Female	**Recipient age:**	52 years	**Surgical complications:**	None
Underlying disease:	MPGN	**BMI:**	27.3 kg/m2	**Infectious complications:**	None
Time on dialysis:	17 months	**HLA-mismatches**	5	**Immunological complications:**	None
Donor sex:	Male	**Donor age:**	56 years	**Other complications:**	None

High dose nTregs (3.0mio/kg):
Successful tapering of immunosuppression with stable creatinine



IFNg ELISpot Assay

Visits	neg	allo	pp65	IE-1	EBV	SEB
Pre KTx	0	4	656	106	107	TNTC
w2	5	21	854	110	69	TNTC
w48	11	0	TNTC	676	110	TNTC

Panel reactive antibody screening

Visits	HLA class I	HLA class II
Pre KTx	neg	neg
w2	neg	neg
w48	neg	neg
w60	neg	neg

Panel Reactive Antibody Screening: negative

Pathology: not available (patient declined biopsy)

Figure S4: Flow Chart Manufacturing Process and Flow Cytometry Analysis of nTregs Products.

S4A) Flow Chart Manufacturing Process.

	Process-Steps	Process-Controls
Day 0	**Preparation of Start-Population** • Blood Donation • CD8-Depletion and CD25-Enrichment (CliniMACS)	**Initial Blood Testing:** HLA Typing; HIV-1, -2; Hepatitis B & C; *Treponema pallidum* **Safety:** Sterilty, Endotoxin
Day 1 – Day 22	**Cell Expansion** • Bead-Stimulation (CD3/CD28 –Beads) • Several Bead Re-Stimulations and Re-Supplementations (Rapamycin, IL-2)	**Recurrent:** Cell No.; Visual Inspec. **Release-Tests:** I) Safety (Day 18 +/- 1): Mycoplasma; Endotoxin; HLA Typing II) Flowcytometry (Day 21): Identity, Cytokines
Day 23	**Final Filling and Application** • Magnetic Bead-Depletion • Washing • Filling • Application	**Release-Tests:** III) Endproduct-Control (Day 23): Cell No; Appearance; Viability; Yield; Sterility

S4B) Flow Chart Flow Cytometry Analysis of nTreg Products.

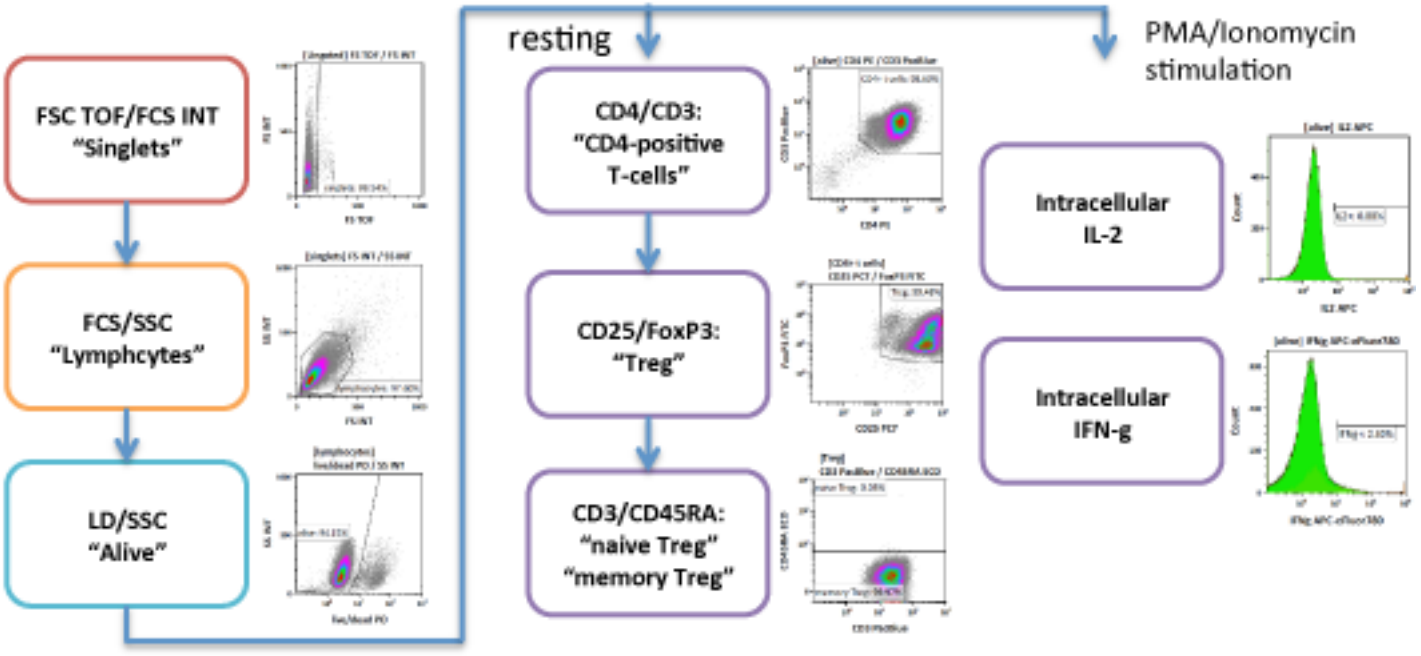
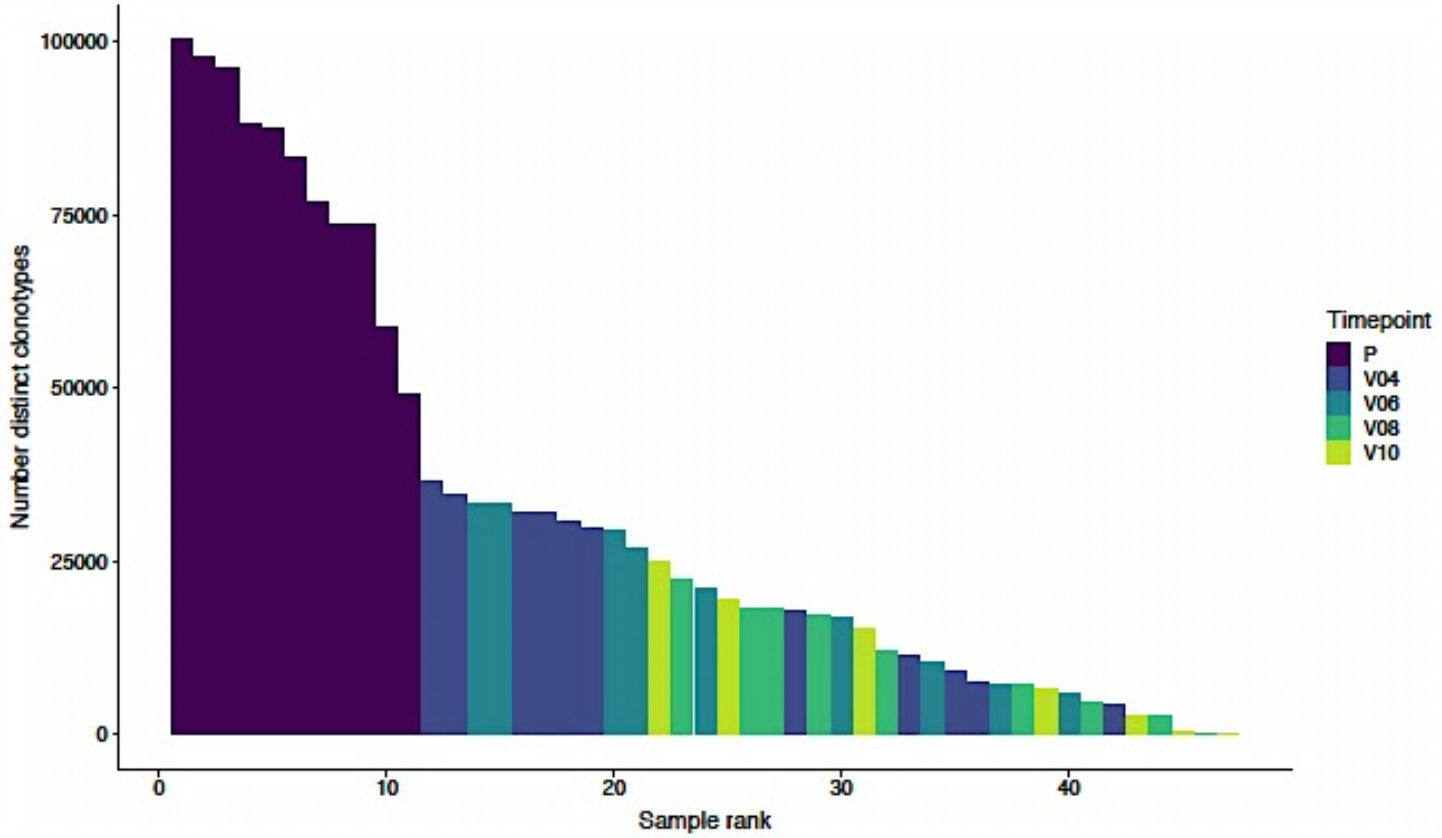


Figure S5A+B: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5A) Initial Data Quality Control: Number of distinct clonotypes according to time points.



S5B) Initial Data Quality Control: Number of accepted reads according to time points.

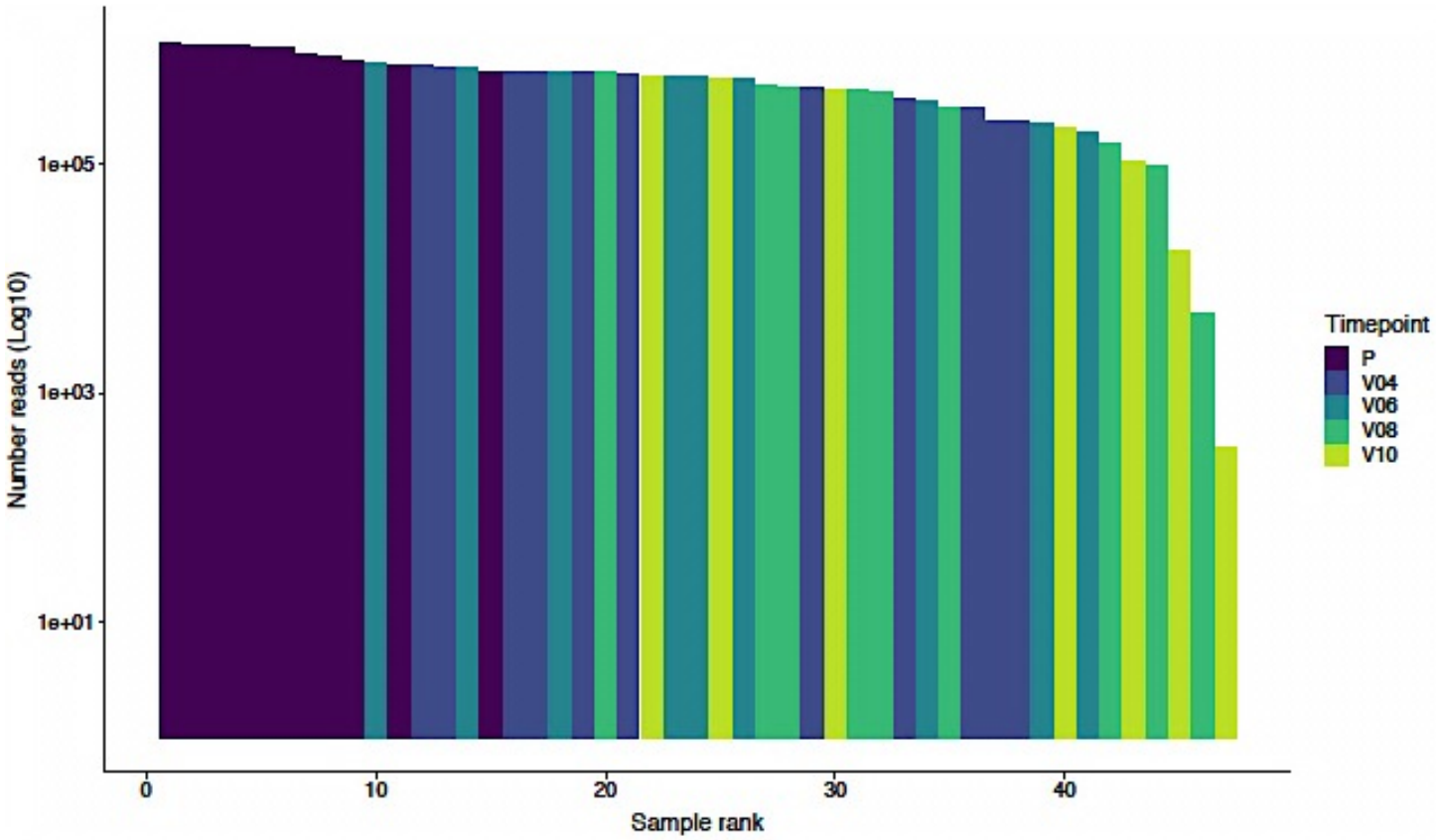
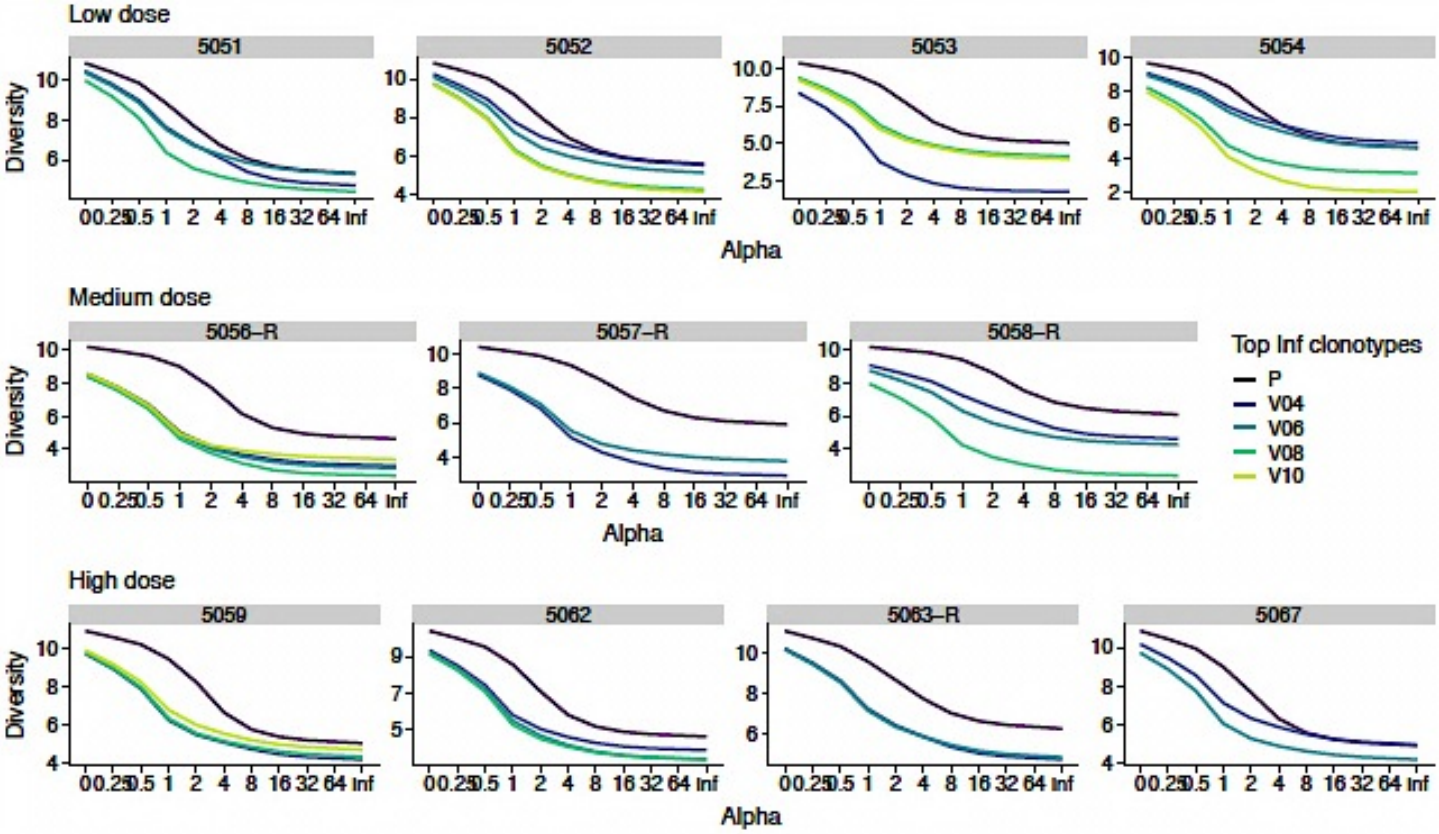


Figure S5C+D: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5C) Renyi diversity profile for each patient and time point, for all clonotypes. (Each line is a time point)



S5D) Renyi diversity profile for each patient and time point, for the top 500 clonotypes. (Each line is a time point)

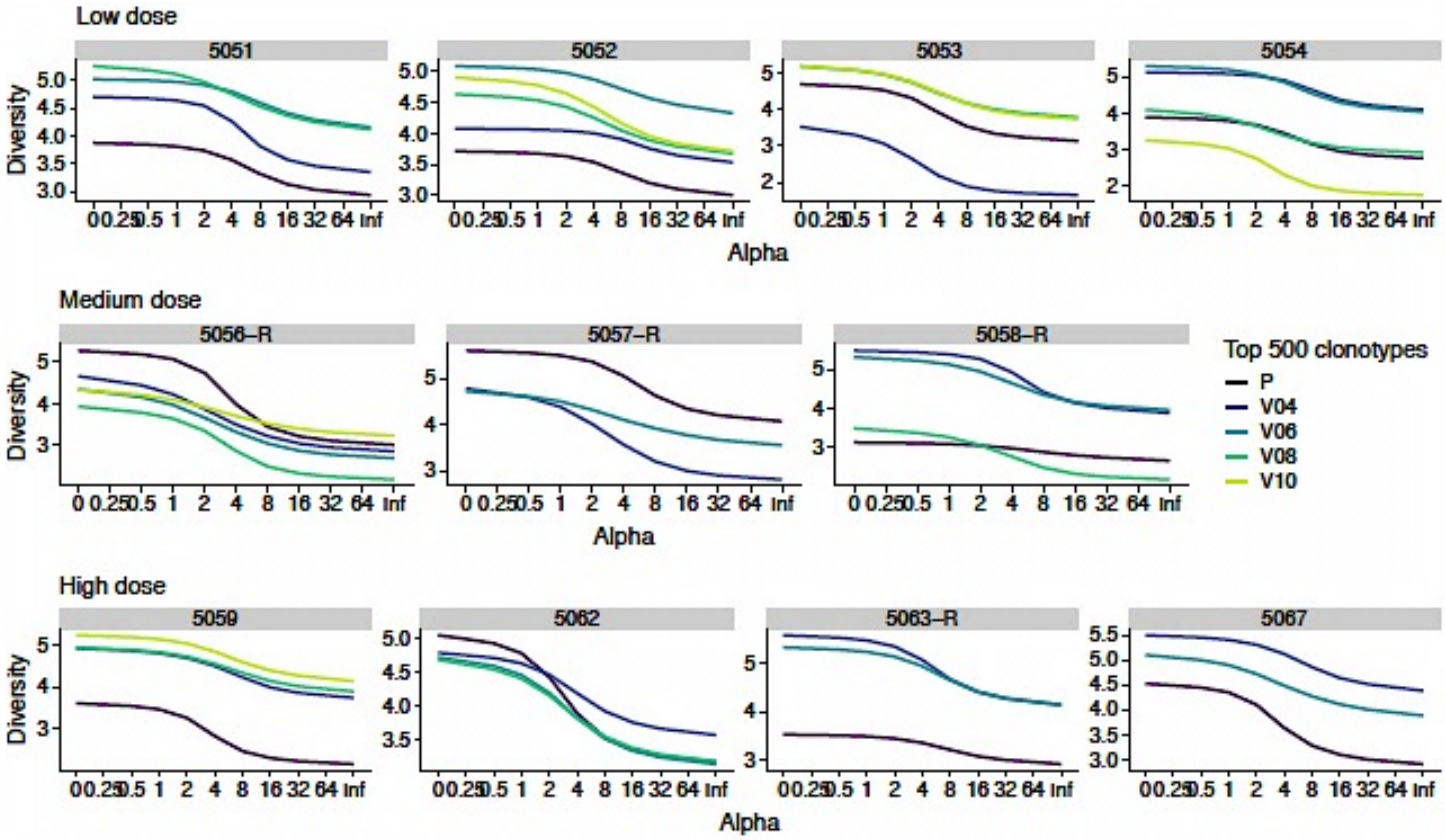
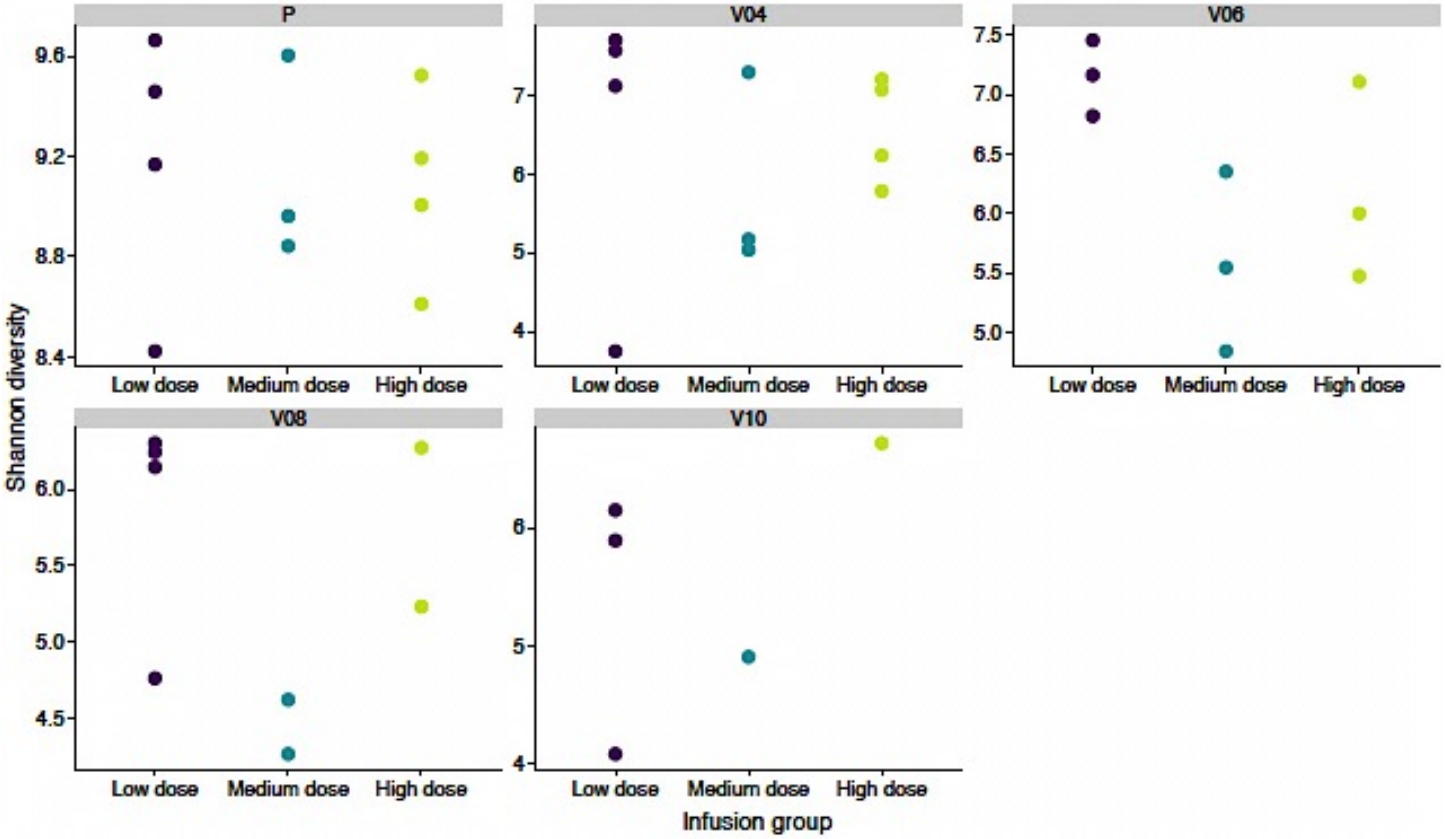


Figure S5E+F: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5E) Shannon diversity for each infusion group (low, medium, and high cell dose).



S5F) Berger-Parker diversity for each infusion group (low, medium, and high cell dose).

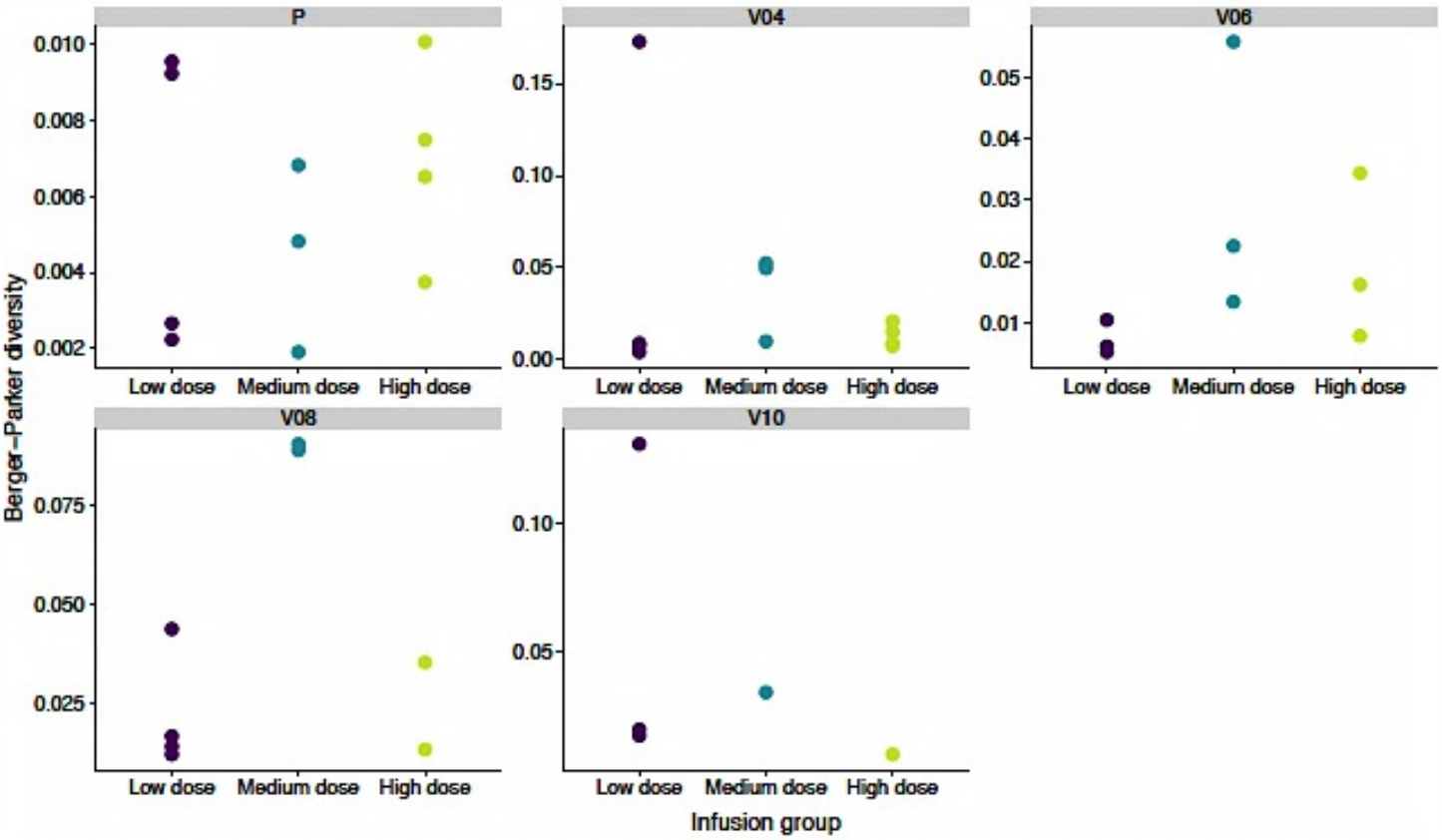
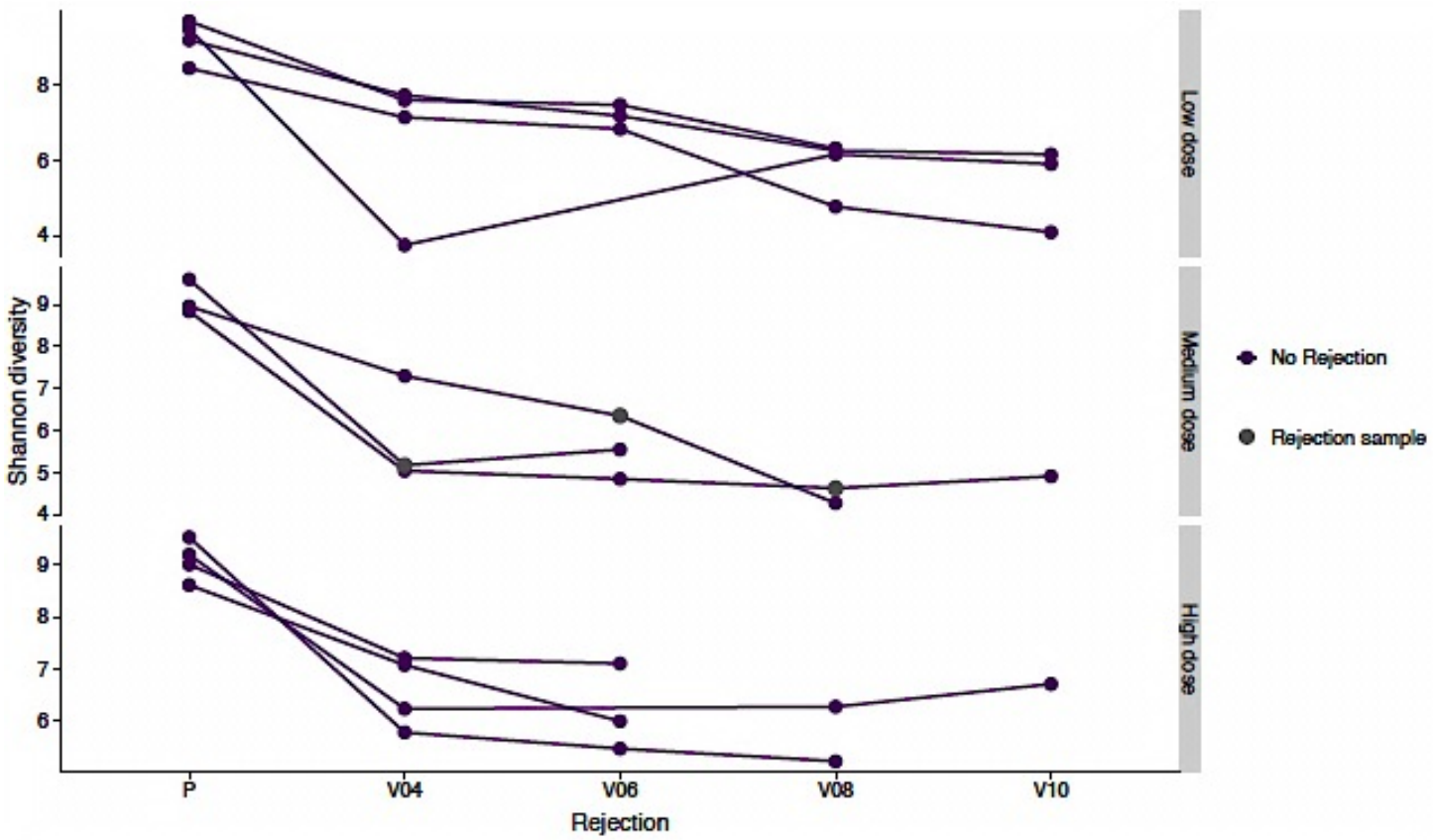


Figure S5G+H: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5G) Shannon diversity over time for each patient (Sample closest to rejection marked).



S5H) Berger-Parker diversity over time for each patient (Sample closest to rej. marked).

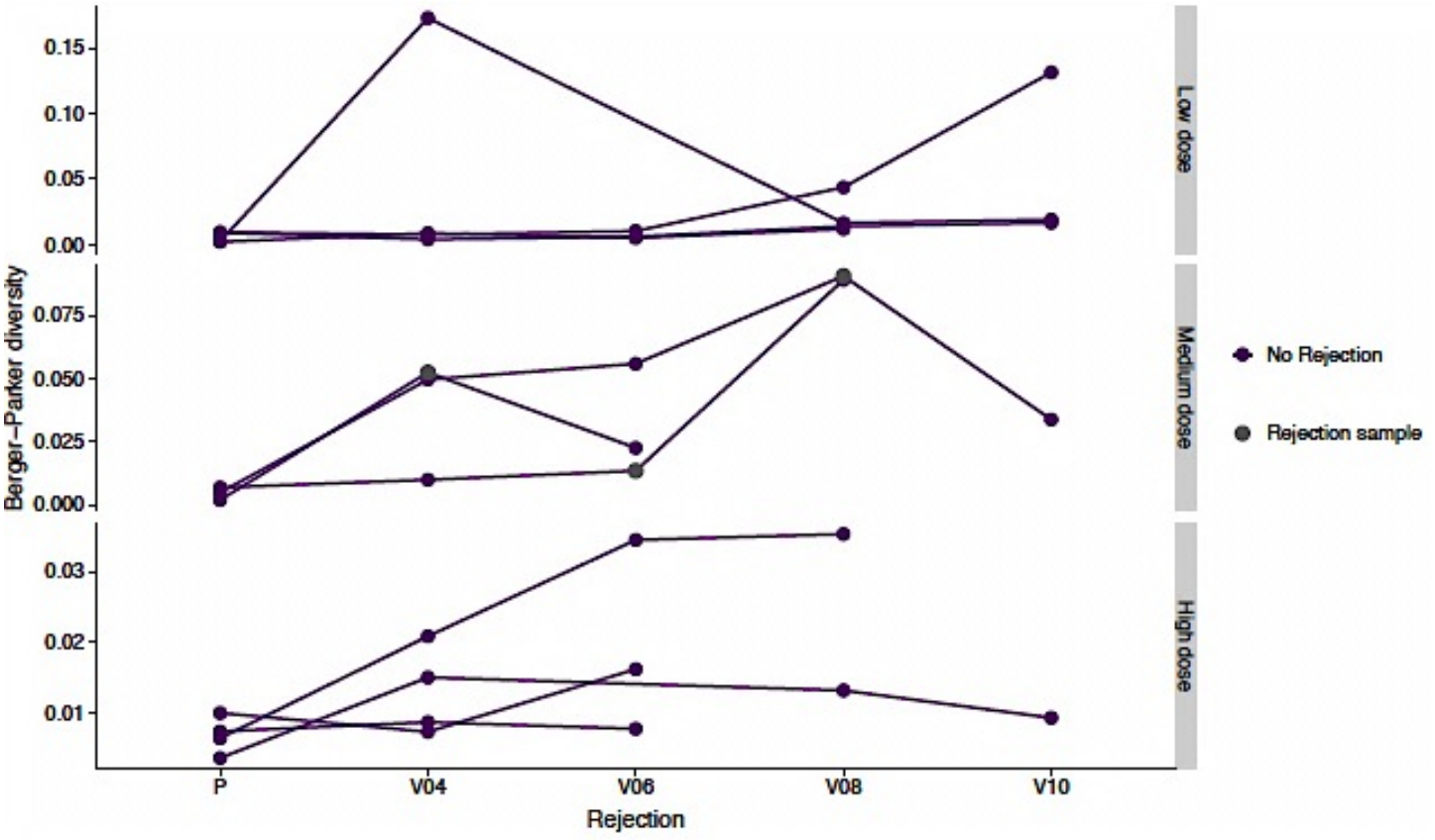
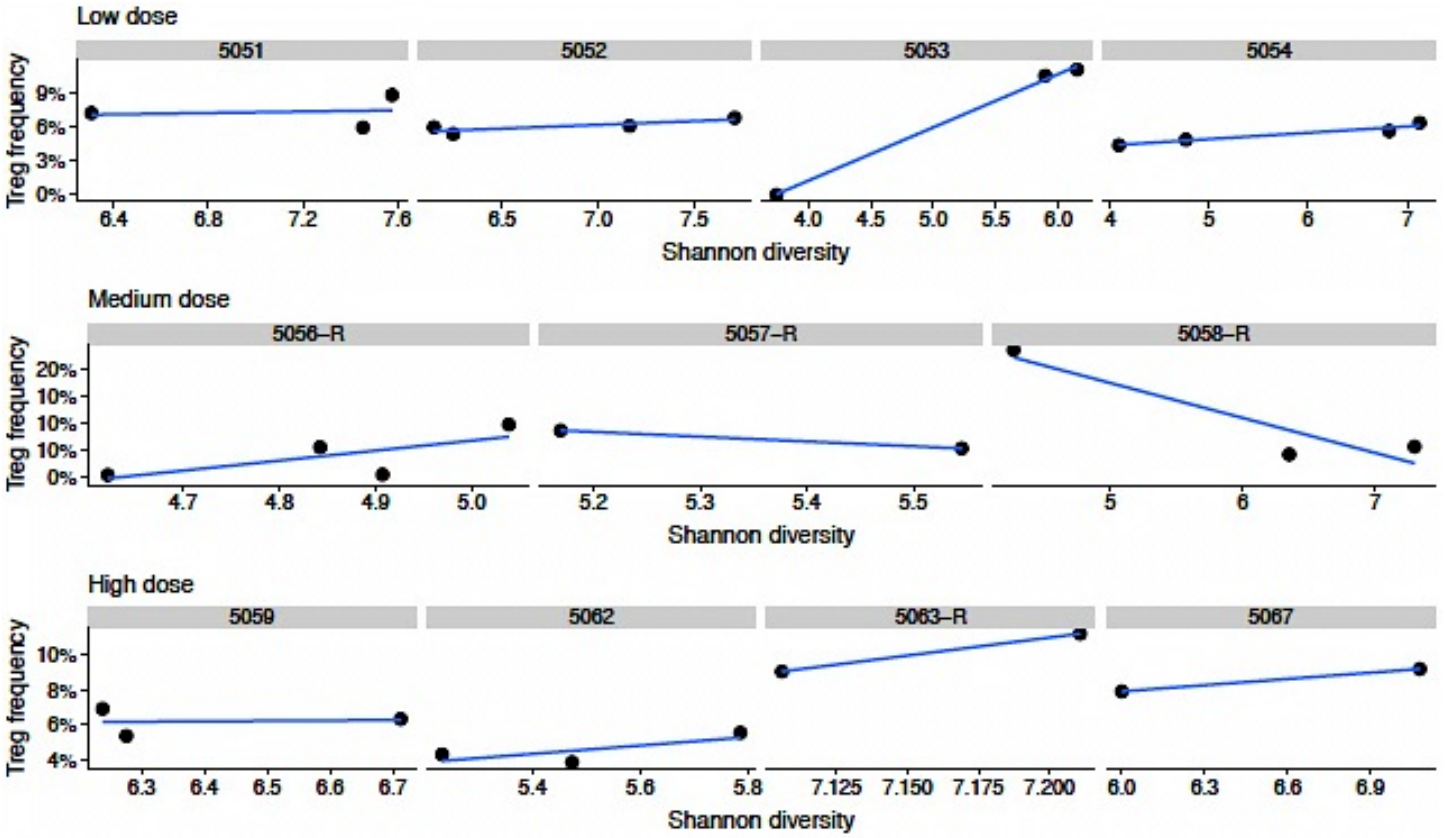


Figure S5J+K: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5J) Correlation of Shannon diversity to Treg frequency.



S5K) Association between Treg frequency and Shannon diversity.

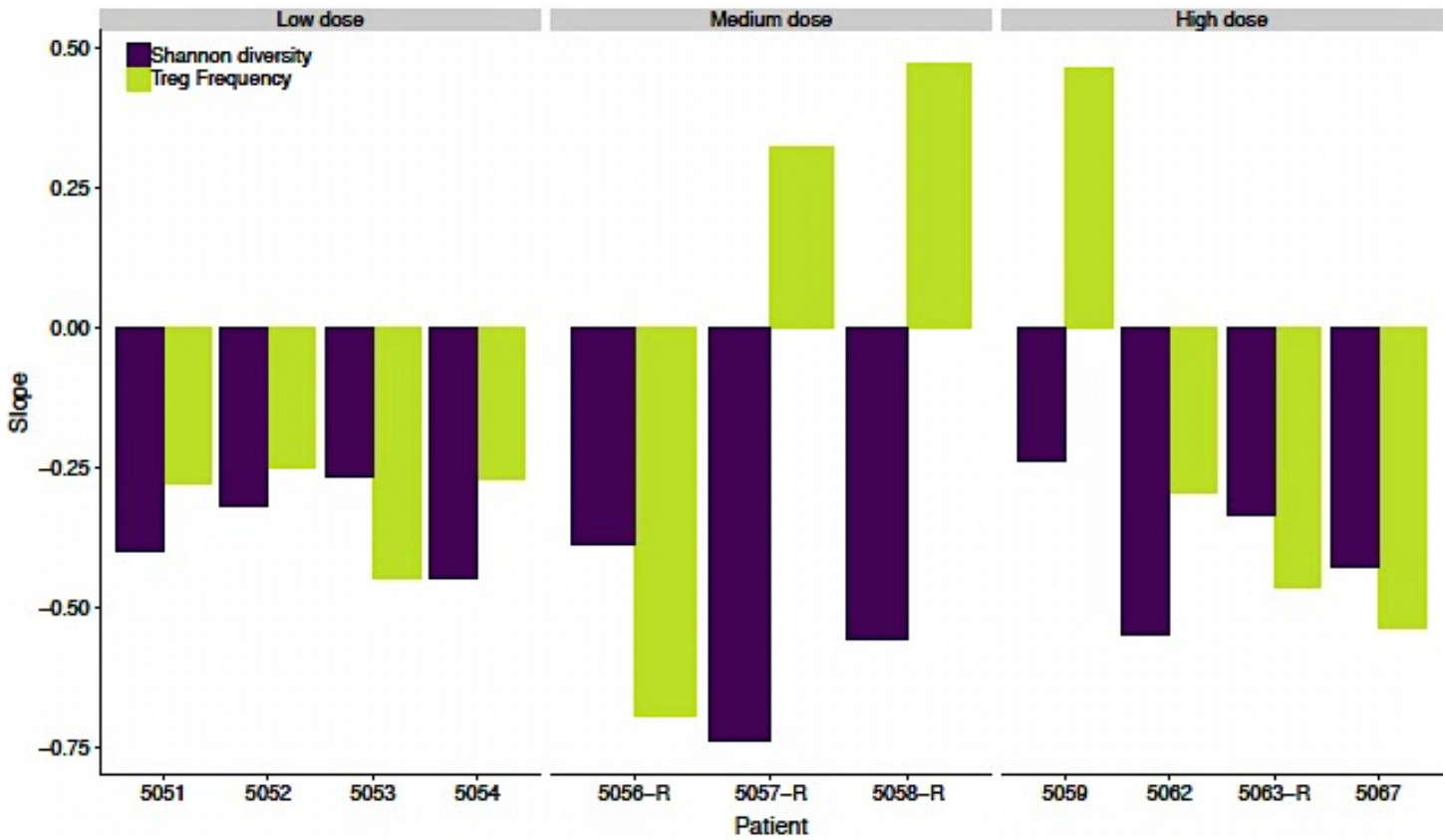
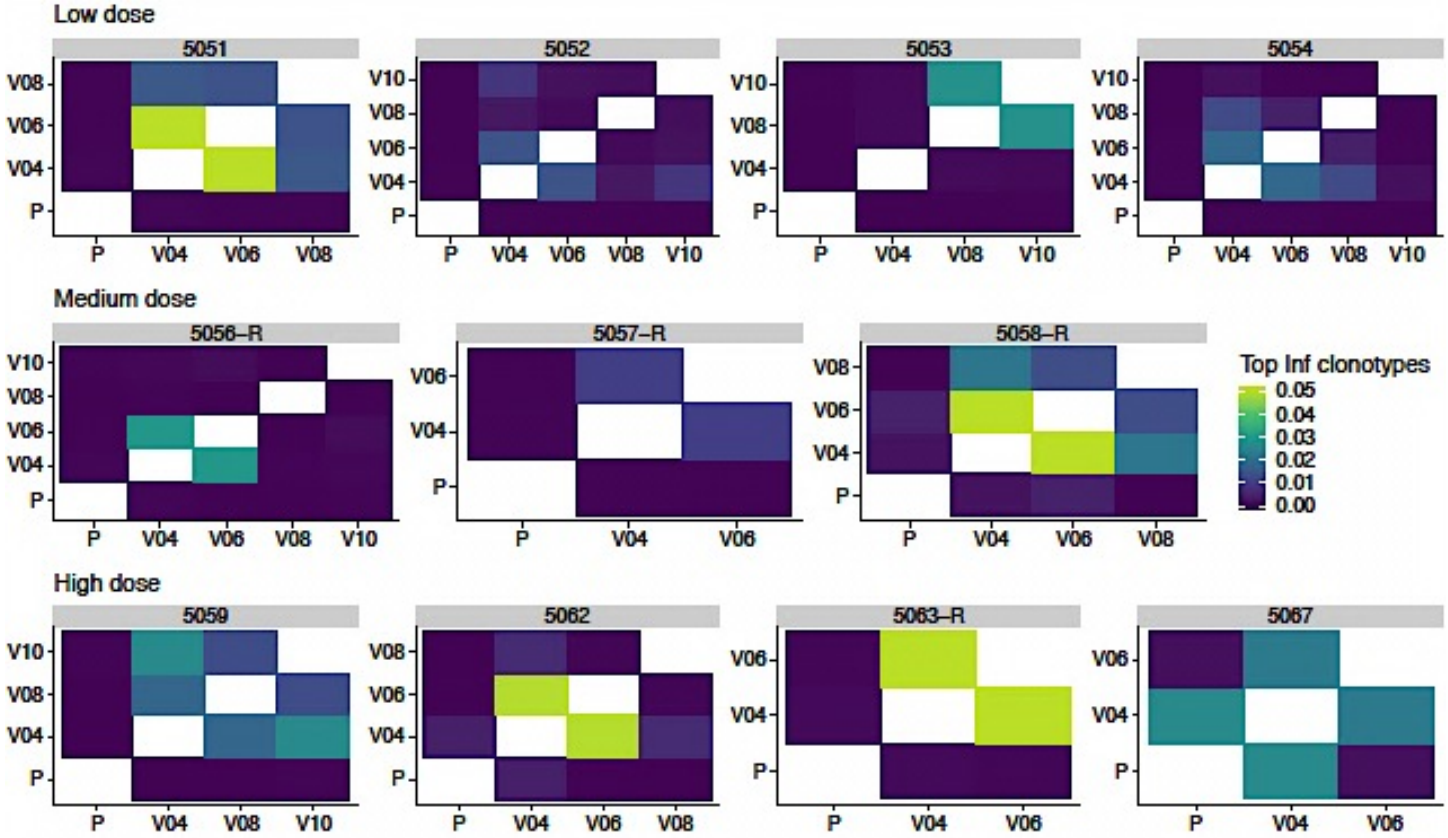


Figure S5L+M: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5L) Morisita-Horn similarity of all clonotypes between time points for each patient.
(The number 1 indicates identity (white), while number 0 indicates complete dissimilarity)



S5M) Morisita-Horn similarity between time points for each patient, top 500 clonotypes.
(The number 1 indicates identity (white), while number 0 indicates complete dissimilarity)

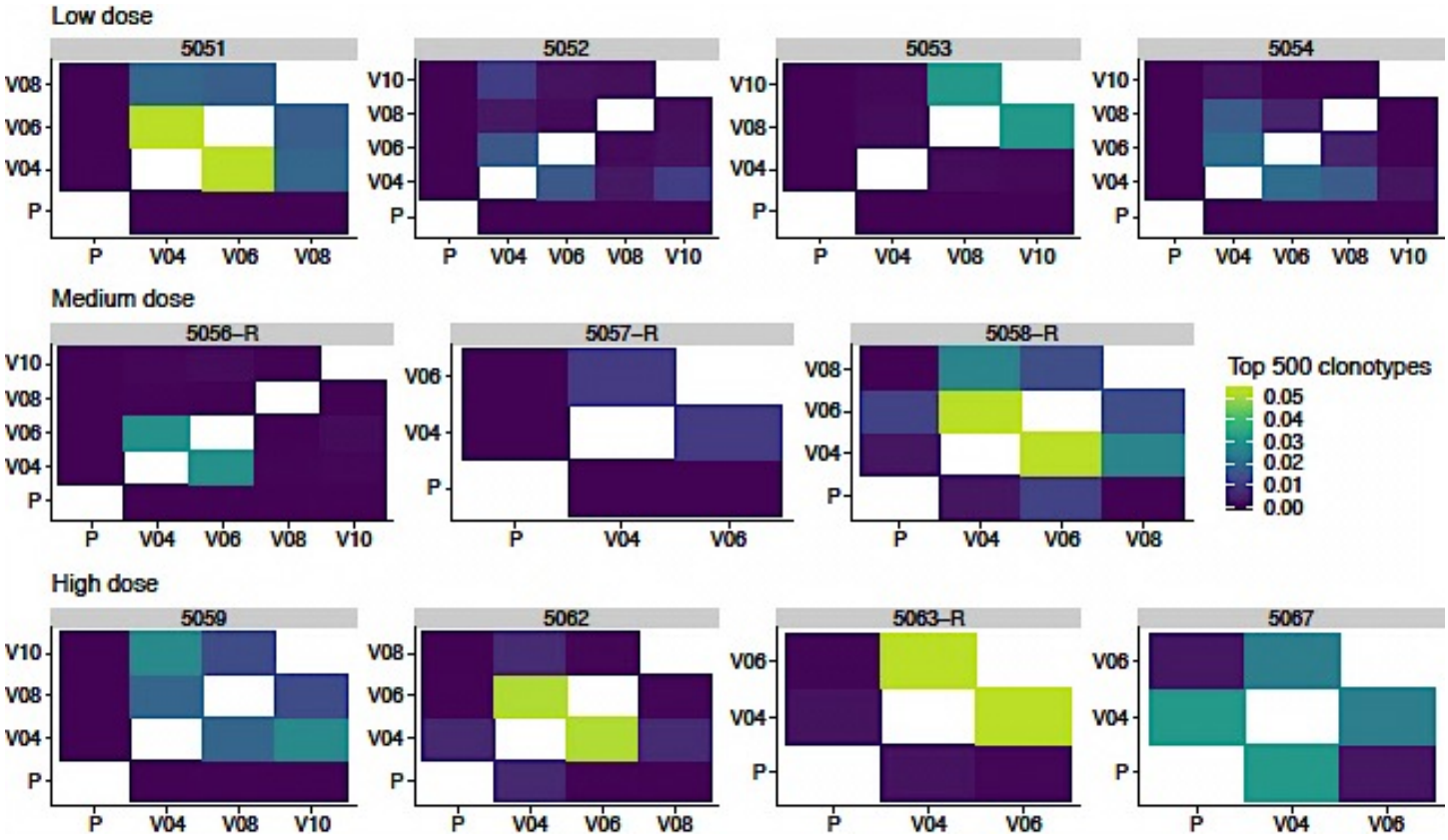
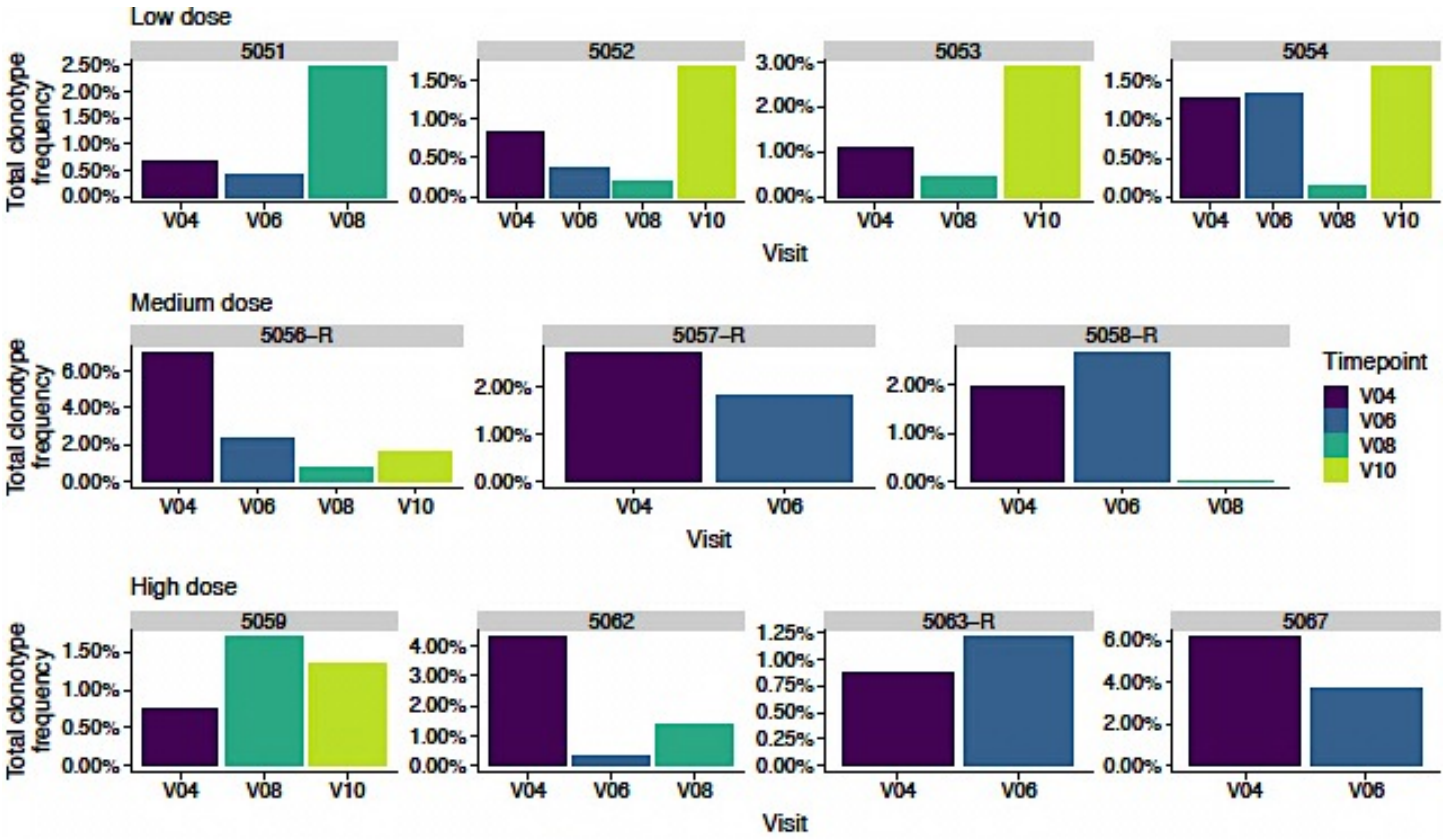


Figure S5N+O: Supplementary Data for nTreg T-cell Receptor Repertoire Analysis by TCR-NGS sequencing.

S5N) Total frequency of product derived clonotypes for each patient.



S5O) The distribution of product derived clonotypes for each patient.

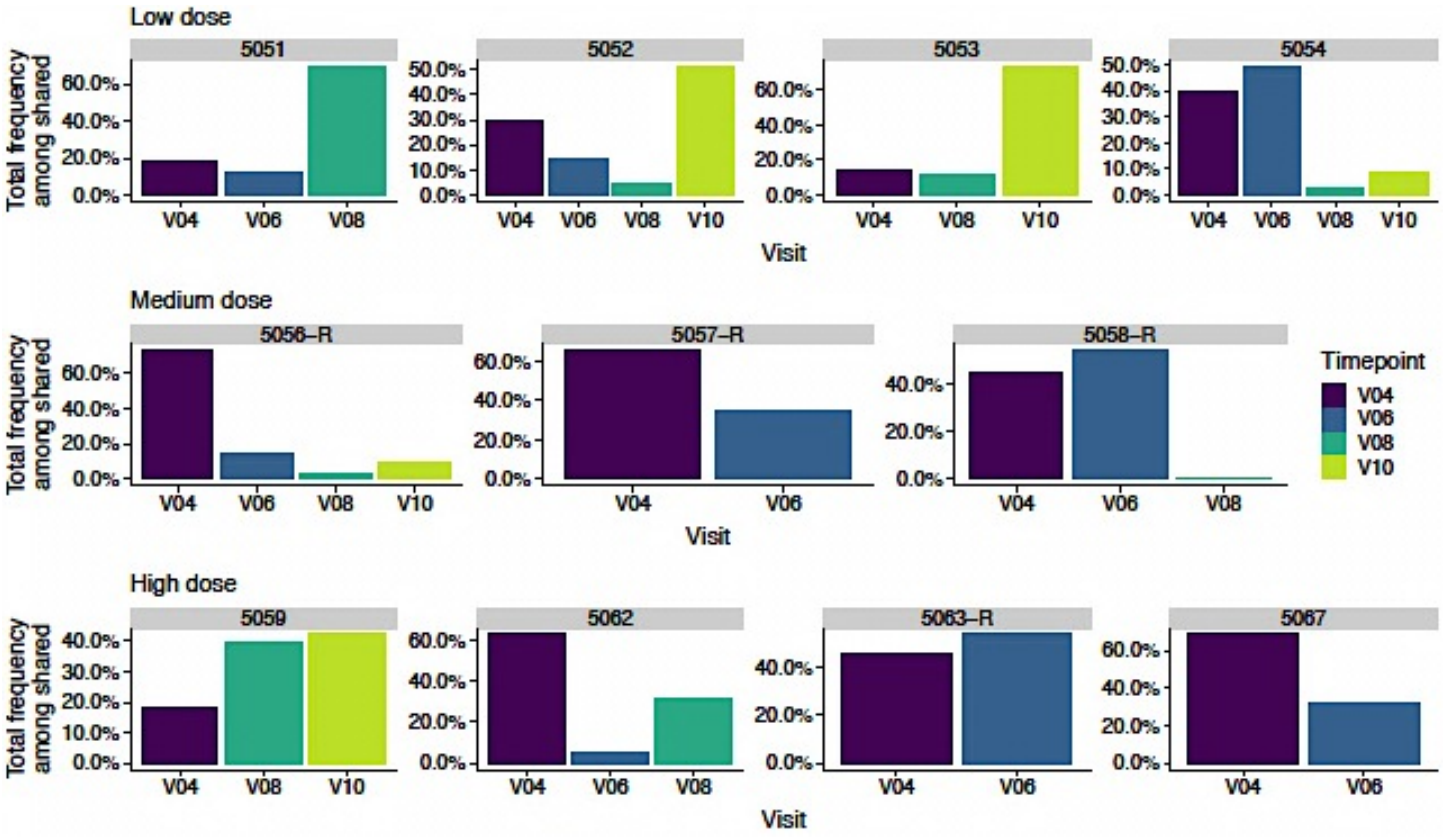


Figure S6: Supplementary Data for Multi-Parameter Immune Monitoring.

- **Short Summary of Figure S6 (next 15 pages):**

The following pages show the results for the screening of more than n=80 biomarkers with multi-parameter flow cytometry (FACS) and quantitative real-time polymerase chain reaction (qPCR) comparing either:

a) nTreg-treated patients within the ONEnTreg13 (n=11) vs. the reference group patients within the ONErgt11-CHA trial (n=09),

b) Dose-response relationship of nTreg therapy only in the ONEnTreg13 trial with evaluation of the different cell doses (0.5 vs. 1.0 vs. 3.0 mio/kg)

→ **For a statistical summary on significant differences please see Table S5**

- **Part 1: FACS Immune Cell Subsets for the Following Parameters:**

#01) Blood differential count, #02) Monocytes, #03) Dendritic cells, #04) Natural killer (NK) and T cells, #05) TCR-γ/δ T-cells, #06-11) CD4-Tregs and CD4/CD8 T-cell subsets, activation status, and Treg to Tconv ratios, and #12) B cells,
all expressed as absolute counts (cells/ul, left column) or cellular frequencies (% of parent population, right column), for the two different general comparisons:

a) nTreg-treated vs. reference group shown to the left, and

b) nTreg dose-response analysis shown to the right

- **Part 2: Quantification of HLR-DR Expression on Monocytes: (Safety Marker for Overimmunosuppression)**

Marker of general immune competence. Normal range 18-40,000 molecules/cells, immunodepression 8-18,000 mol/cell, immunoparalysis <8,000 mol/cell with high risk of severe infections, as described previously:

Docke W et al. Monitoring Temporary Immunodepression by Flow Cytometric Measurement of Monocytic HLA-DR Expression: A Multicenter Standardized Study. Clin Chem 2005 Dec;51(12):2341-7. doi: 10.1373/clinchem.2005.052639. Epub 2005 Oct 7.

- **Part 3: qPCR Data of Whole Blood Samples: (Rejection/Tolerance Gene Profile)**

Multiple hypothesis-driven gene expression markers (n=20 targets) were studied on mRNA transcript level with qPCR, as stratified in the study below, again for the same two general comparisons:

a) nTreg-treated vs. reference group shown to the left, and

b) nTreg dose-response analysis shown to the right

J Clin Invest 2010 Jun 1; 120(6): 1848–1861. Published online 2010 May 24. doi: 10.1172/JCI39922
PMID: 20501943 **Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans** https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2877932/

Figure S6: Part 1 FACS#01: Blood Differential Counts

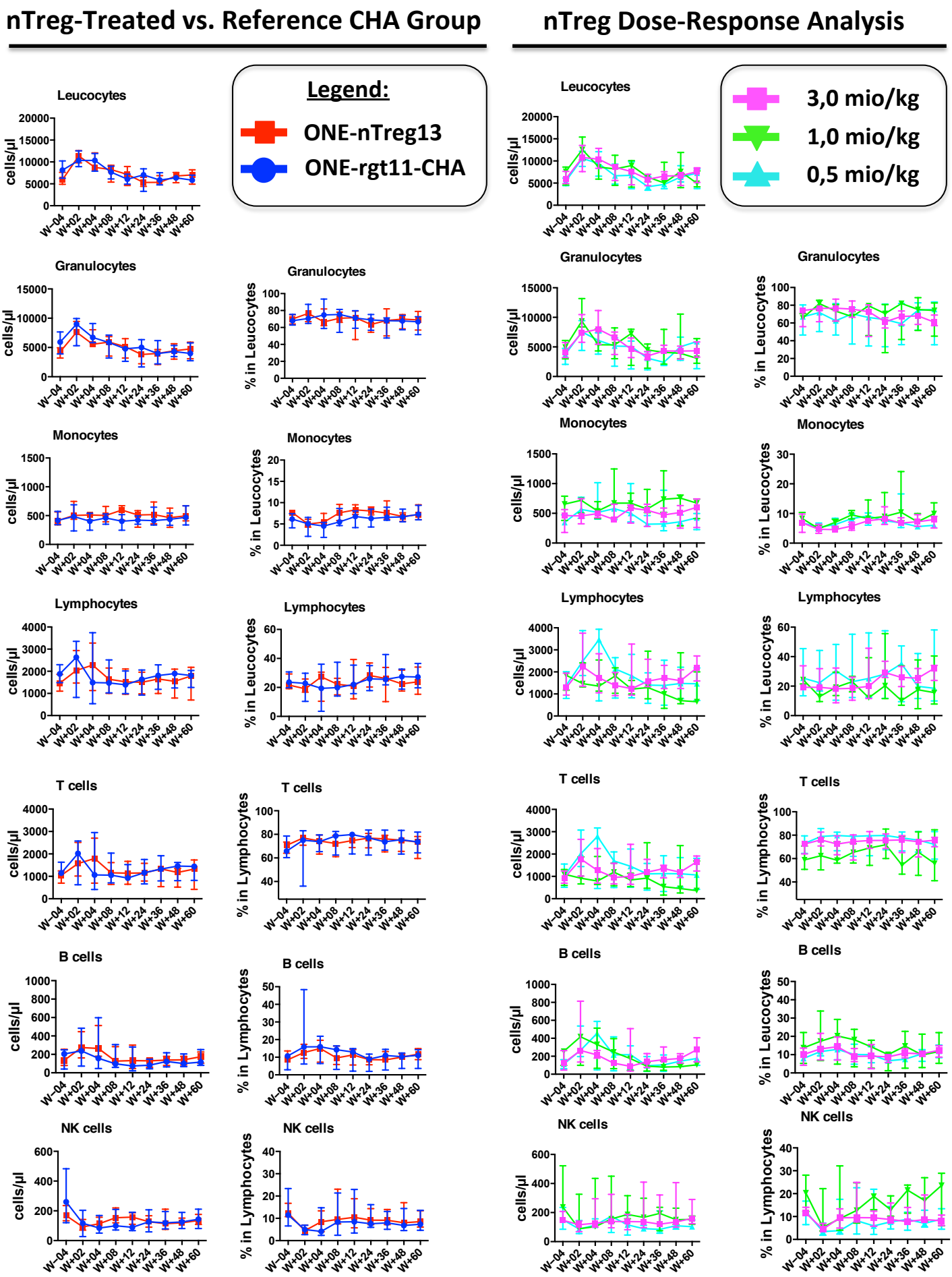


Figure S6: Part 1 FACS#02: Monocytes

nTreg-Treated vs. Reference CHA Group

nTreg Dose-Response Analysis

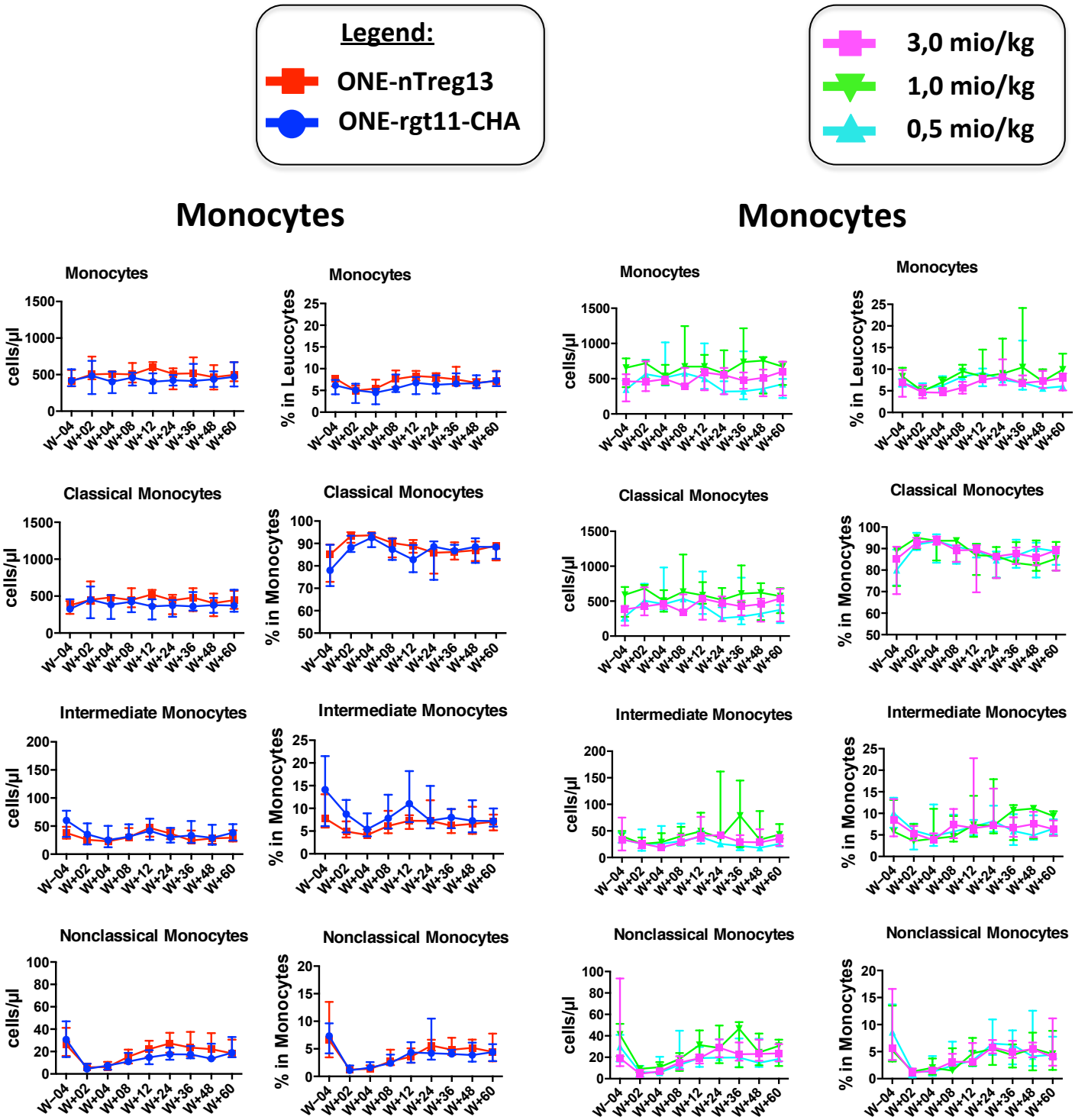


Figure S6: Part 1 FACS#03: Dendritic Cells

nTreg-Treated vs. Reference CHA Group

nTreg Dose-Response Analysis

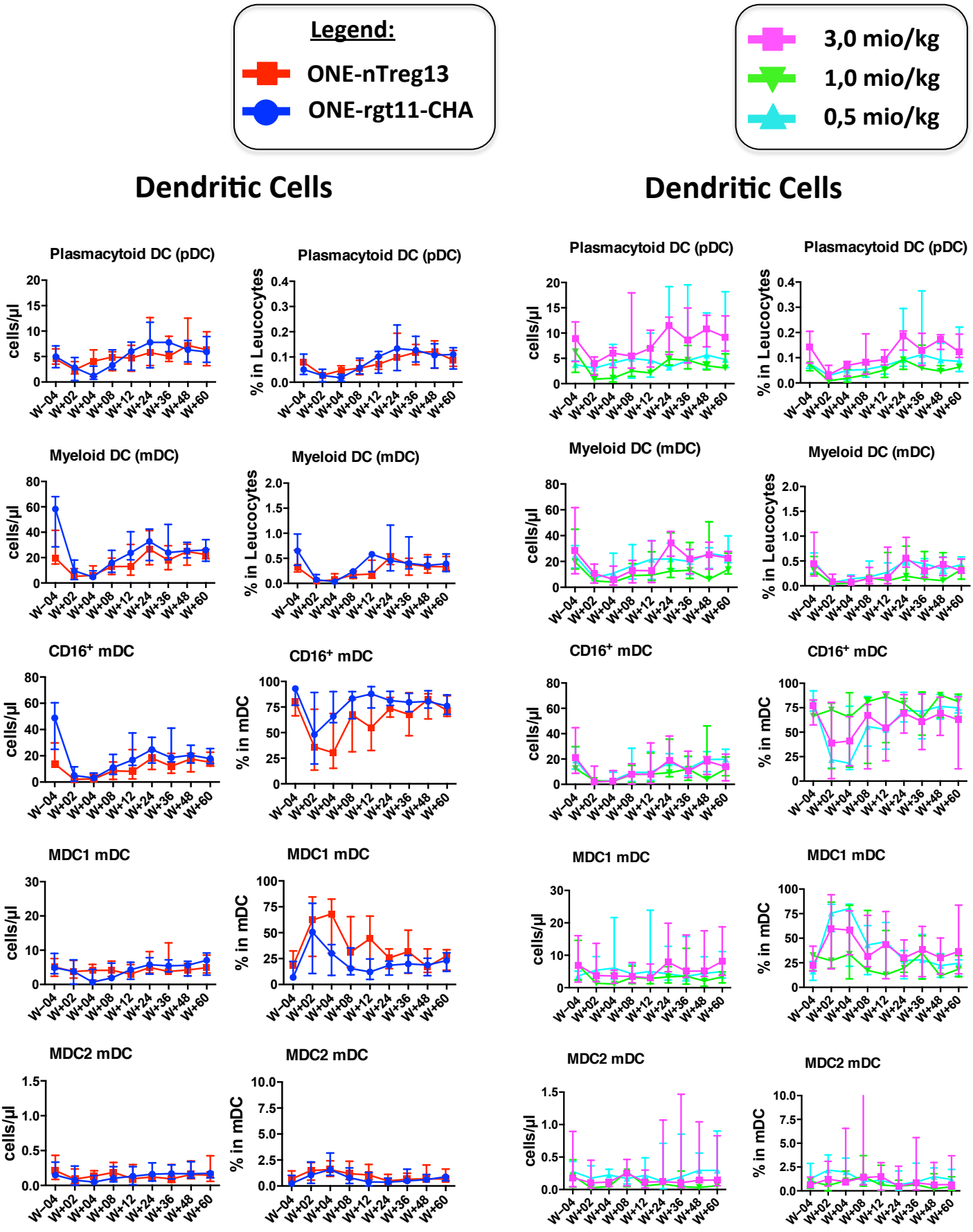
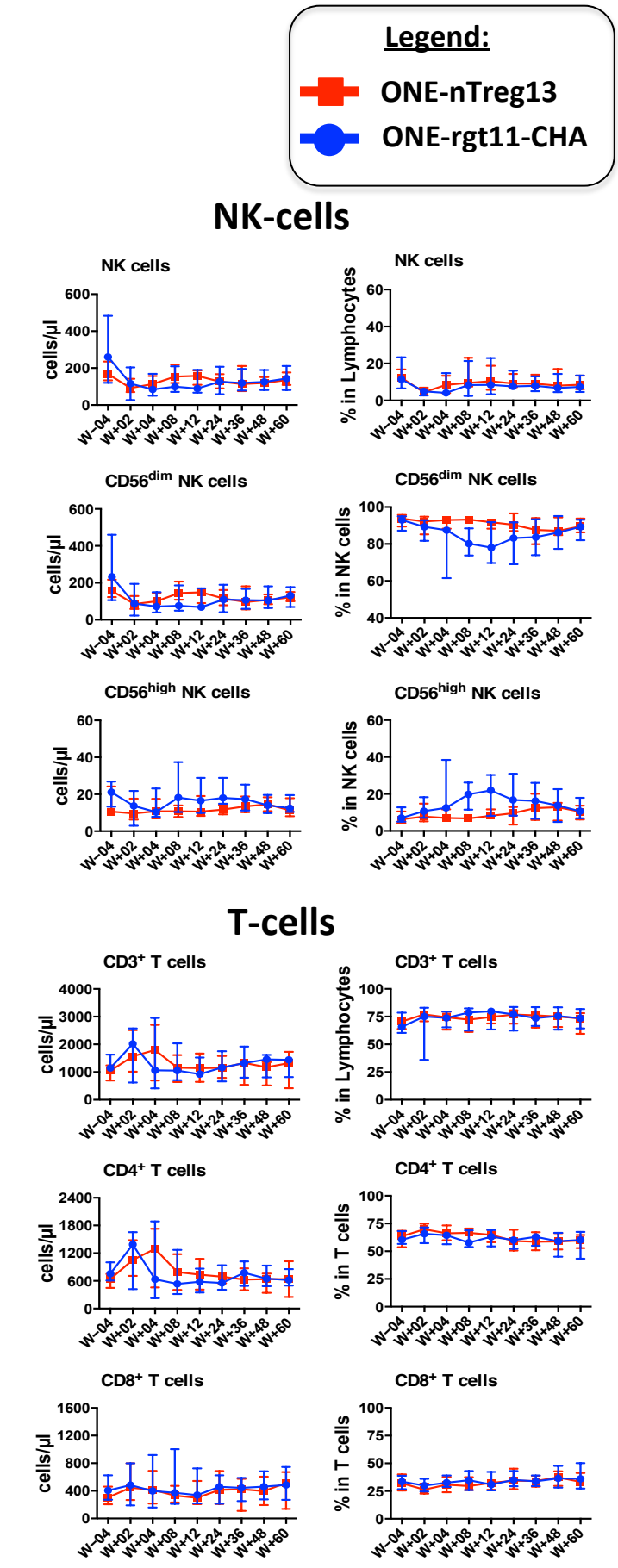


Figure S6: Part 1 FACS#04: NK- and T-cells

nTreg-Treated vs. Reference CHA Group



nTreg Dose-Response Analysis

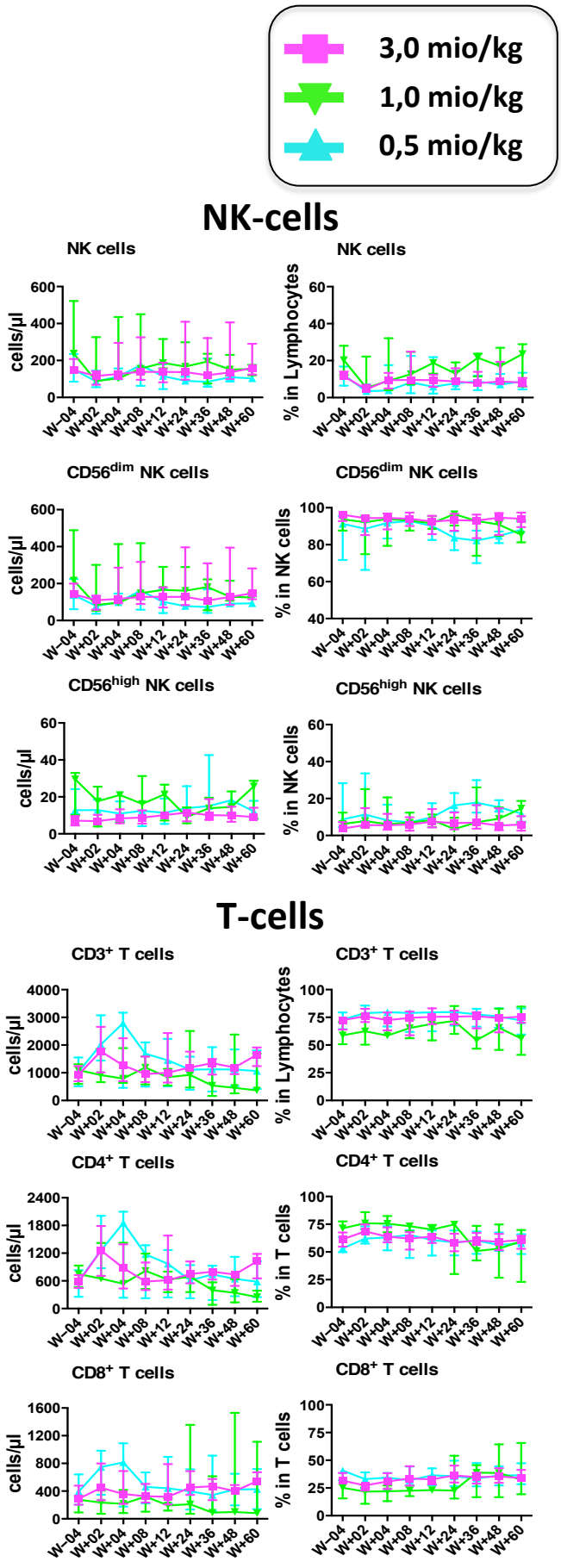
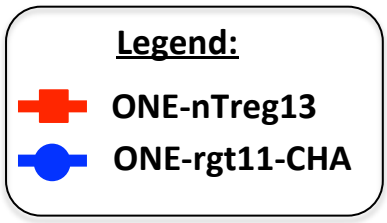
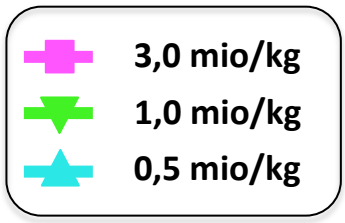


Figure S6: Part 1 FACS#05: TCR-γ/δ T-cells

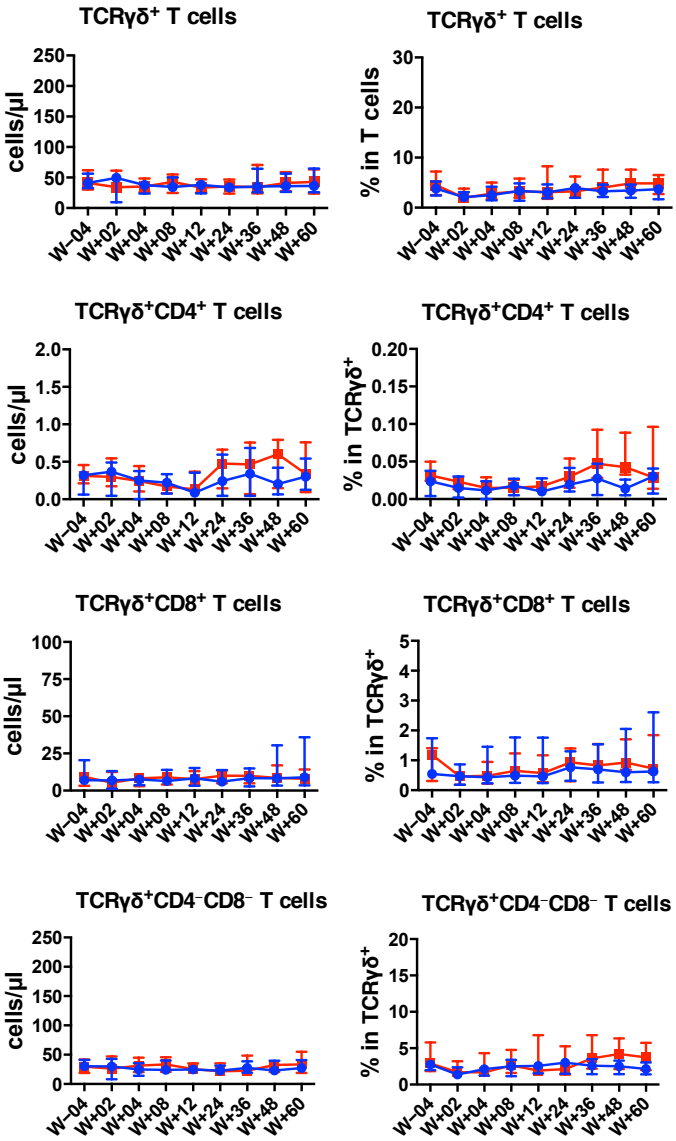
nTreg-Treated vs. Reference CHA Group

nTreg Dose-Response Analysis





TCR-γ/δ T-cells



TCR-γ/δ T-cells

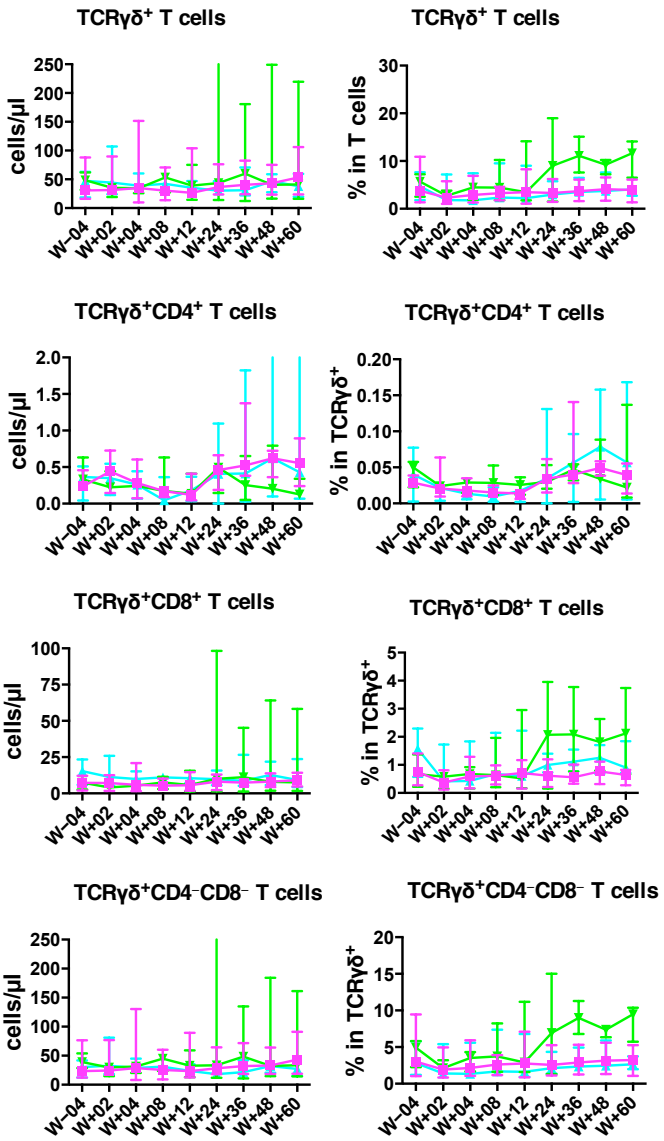


Figure S6: Part 1 FACS#06: CD4 T-cell Subsets

nTreg-Treated vs. Reference CHA Group

nTreg Dose-Response Analysis

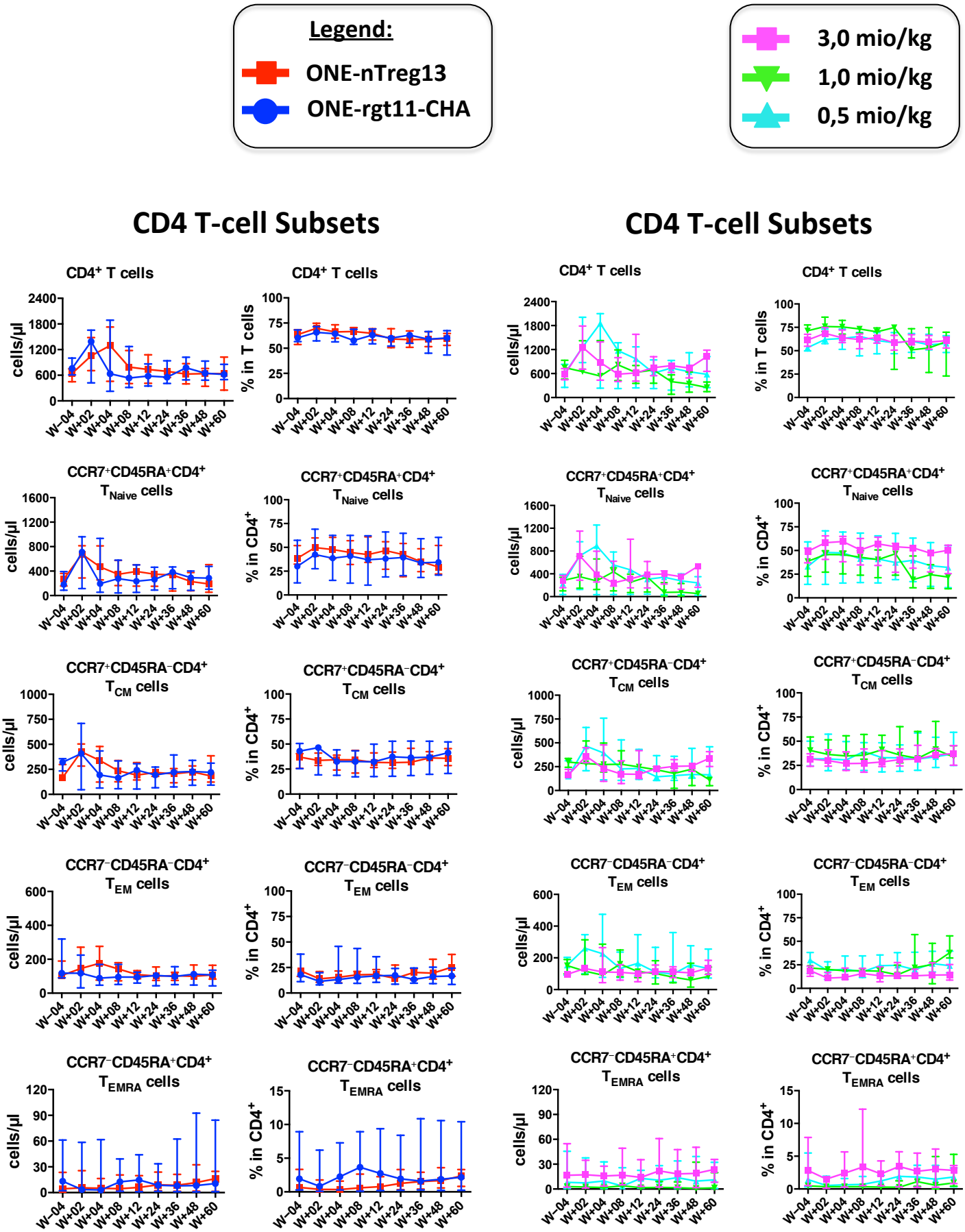


Figure S6: Part 1 FACS#07: CD4 T-cell Activation

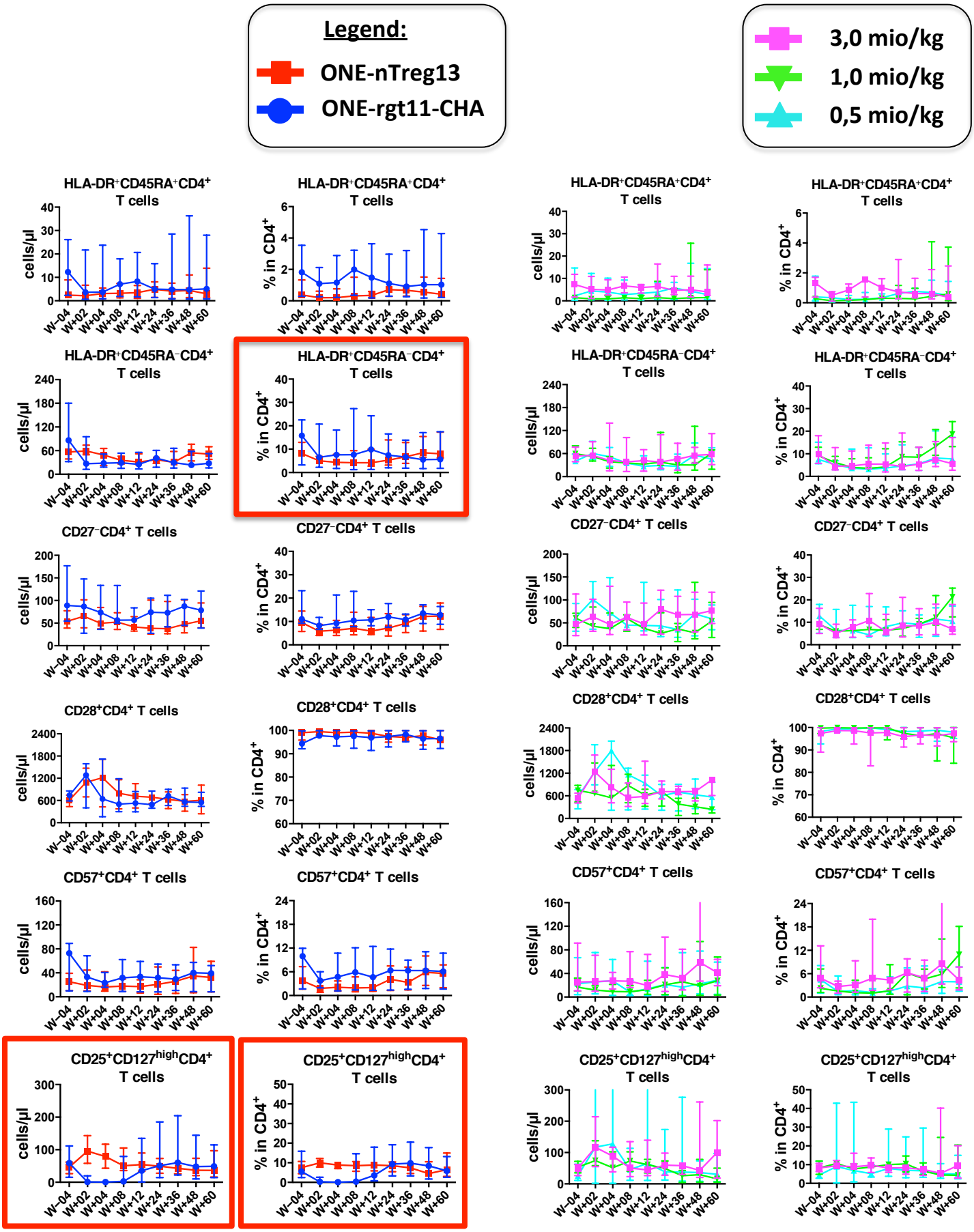


Figure S6: Part 1 FACS#08: CD4 Tregs

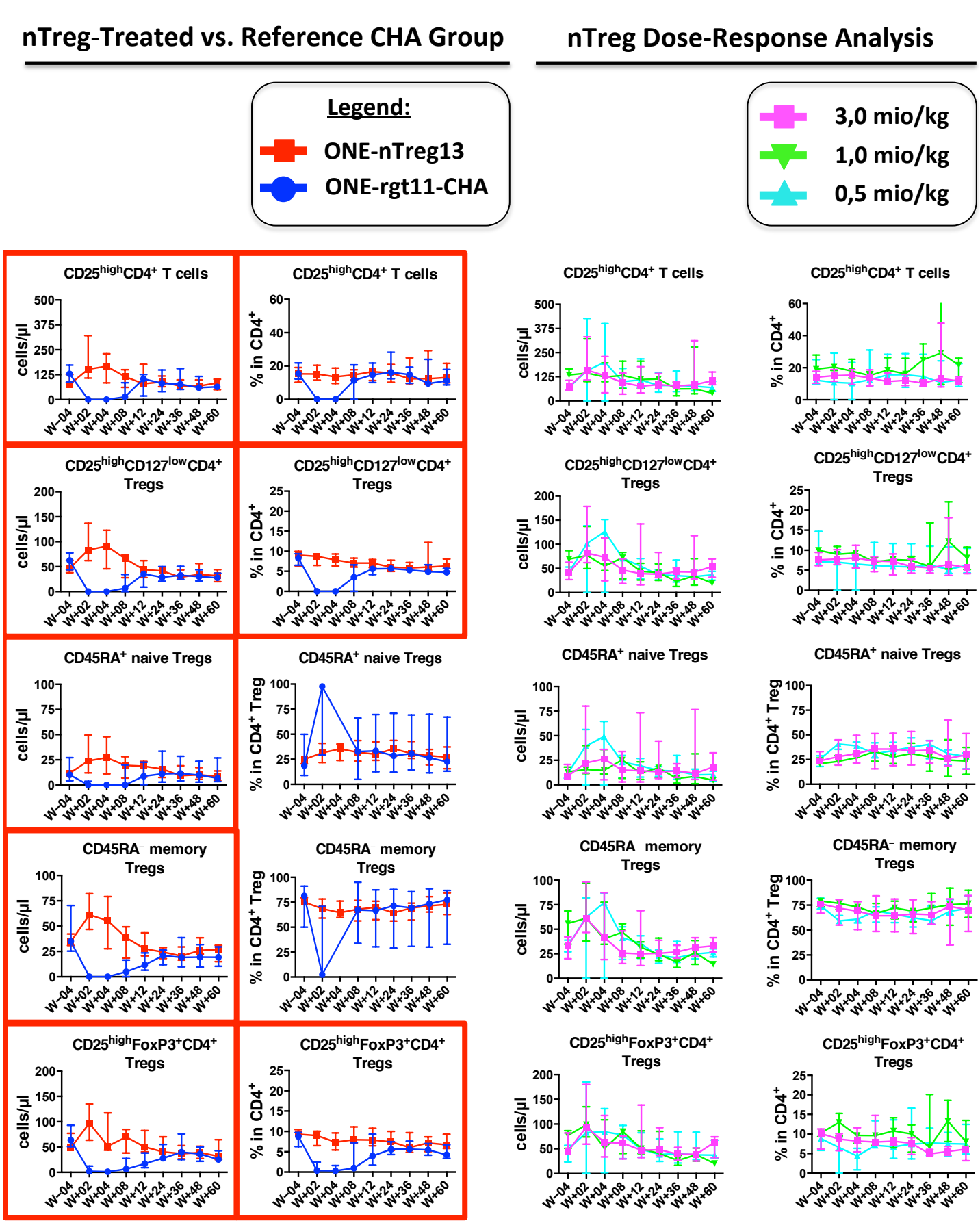
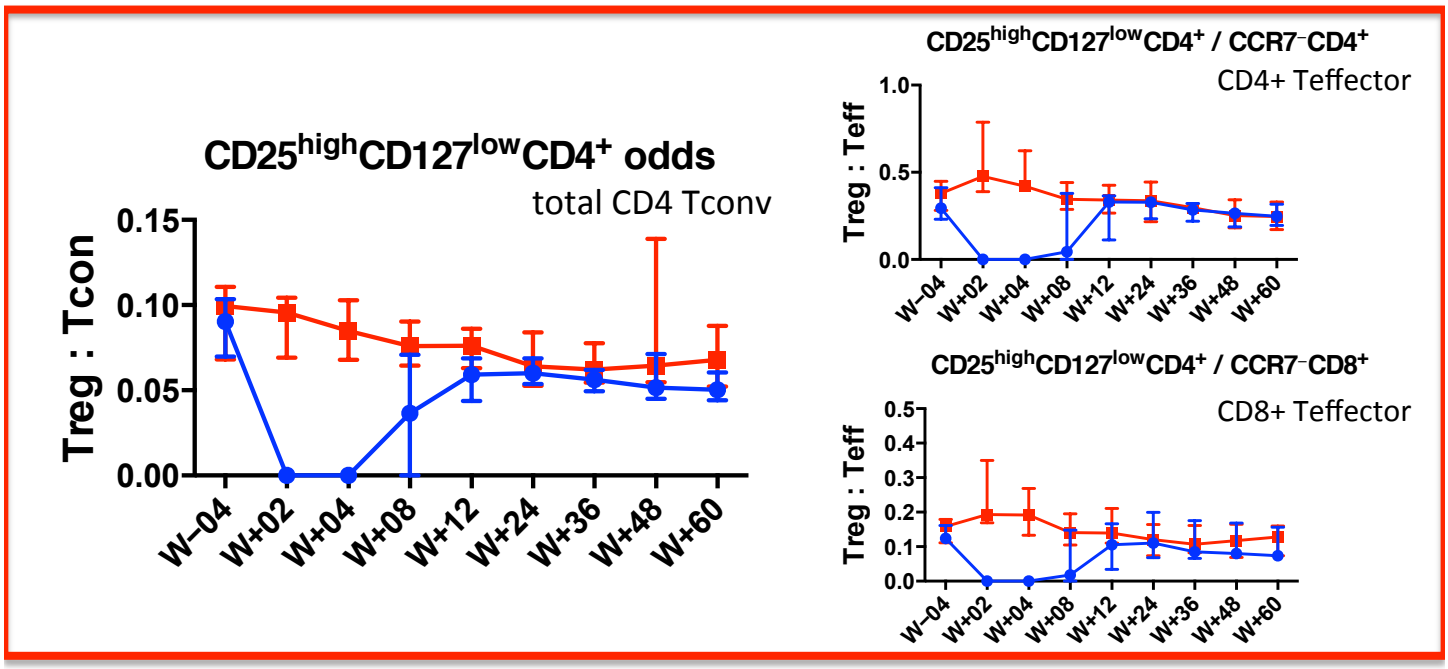


Figure S6: Part 1 FACS#09: CD4 Treg/Teff ratios

nTreg-Treated vs. Reference CHA Group

Legend:
- ONE-nTreg13
- ONE-rgt11-CHA



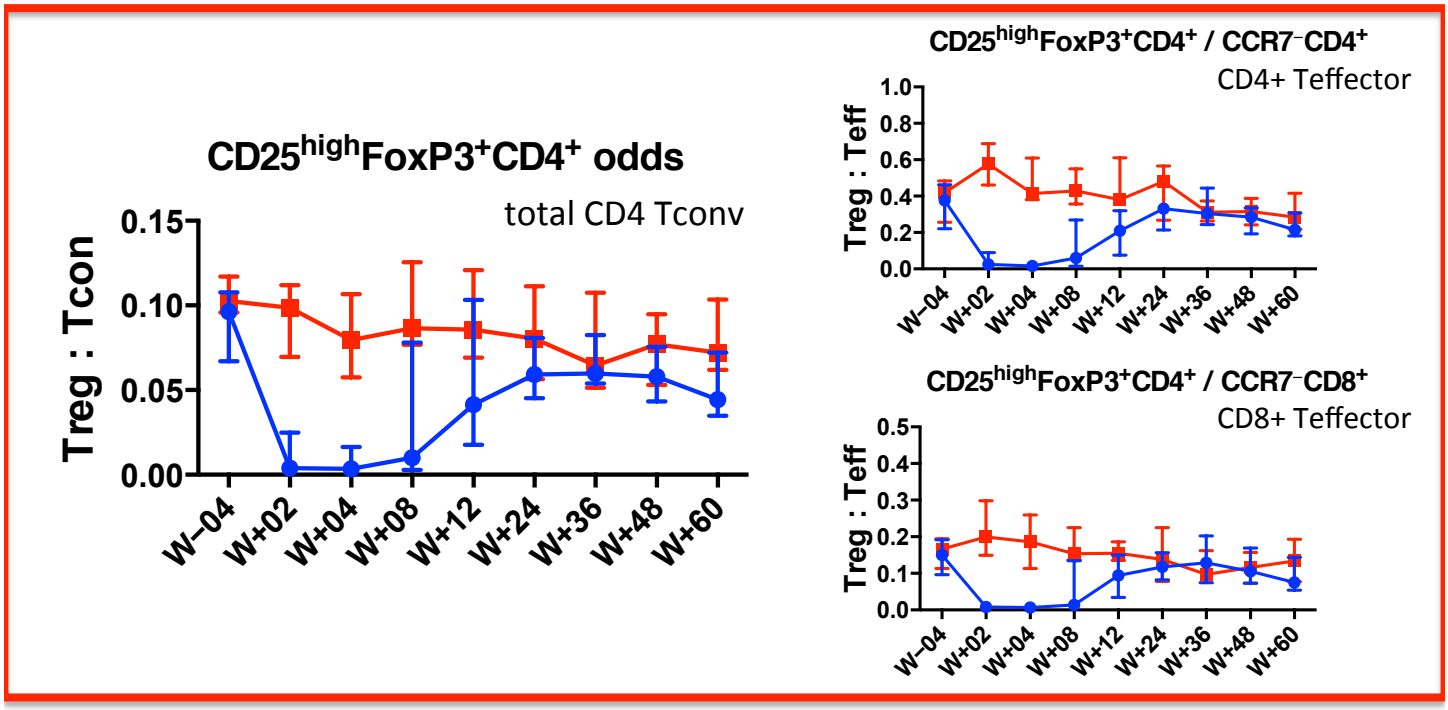


Figure S6: Part 1 FACS#10: CD8 T-cell Subsets

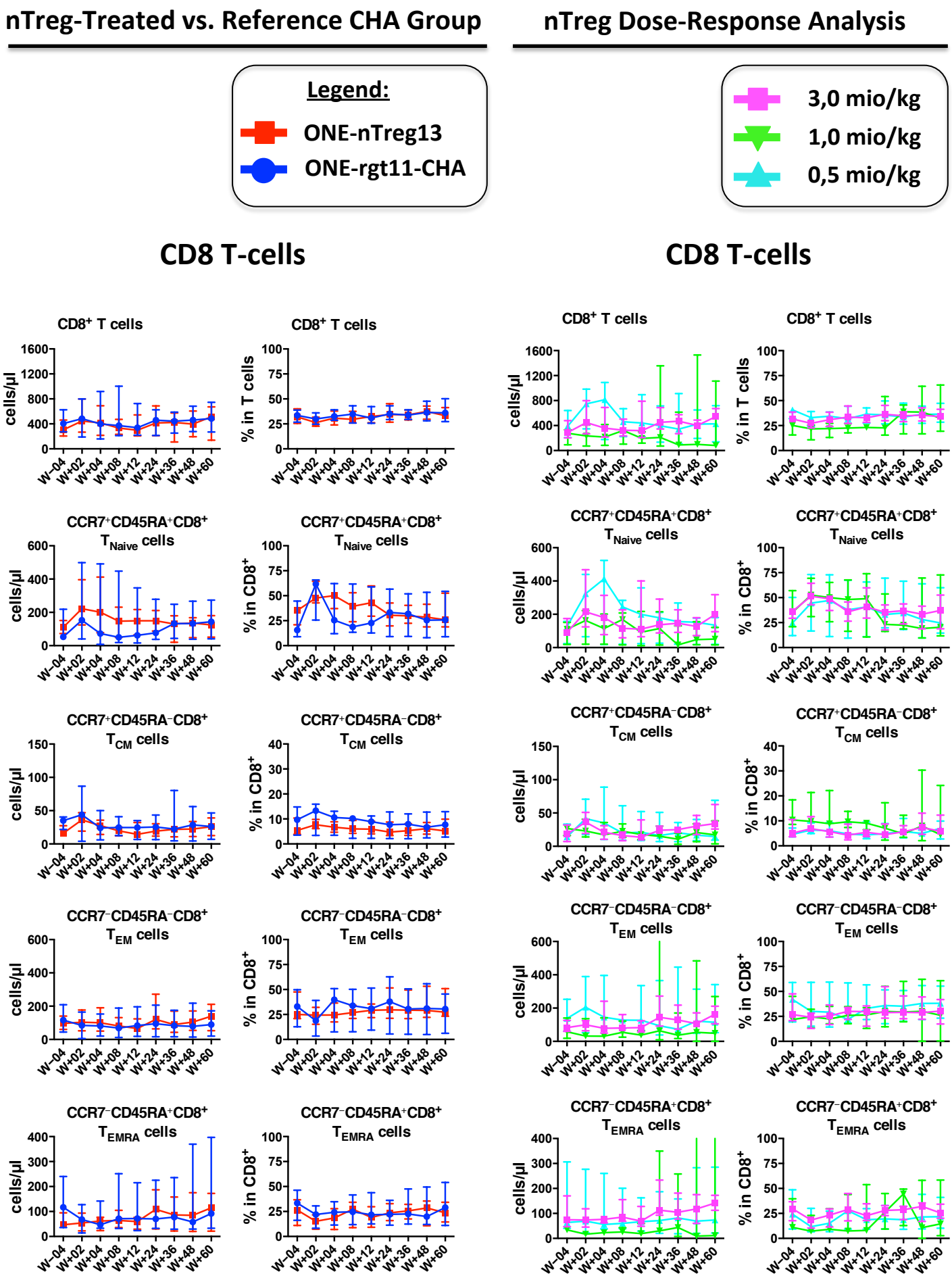


Figure S6: Part 1 FACS#11: CD8 T-cell Activation

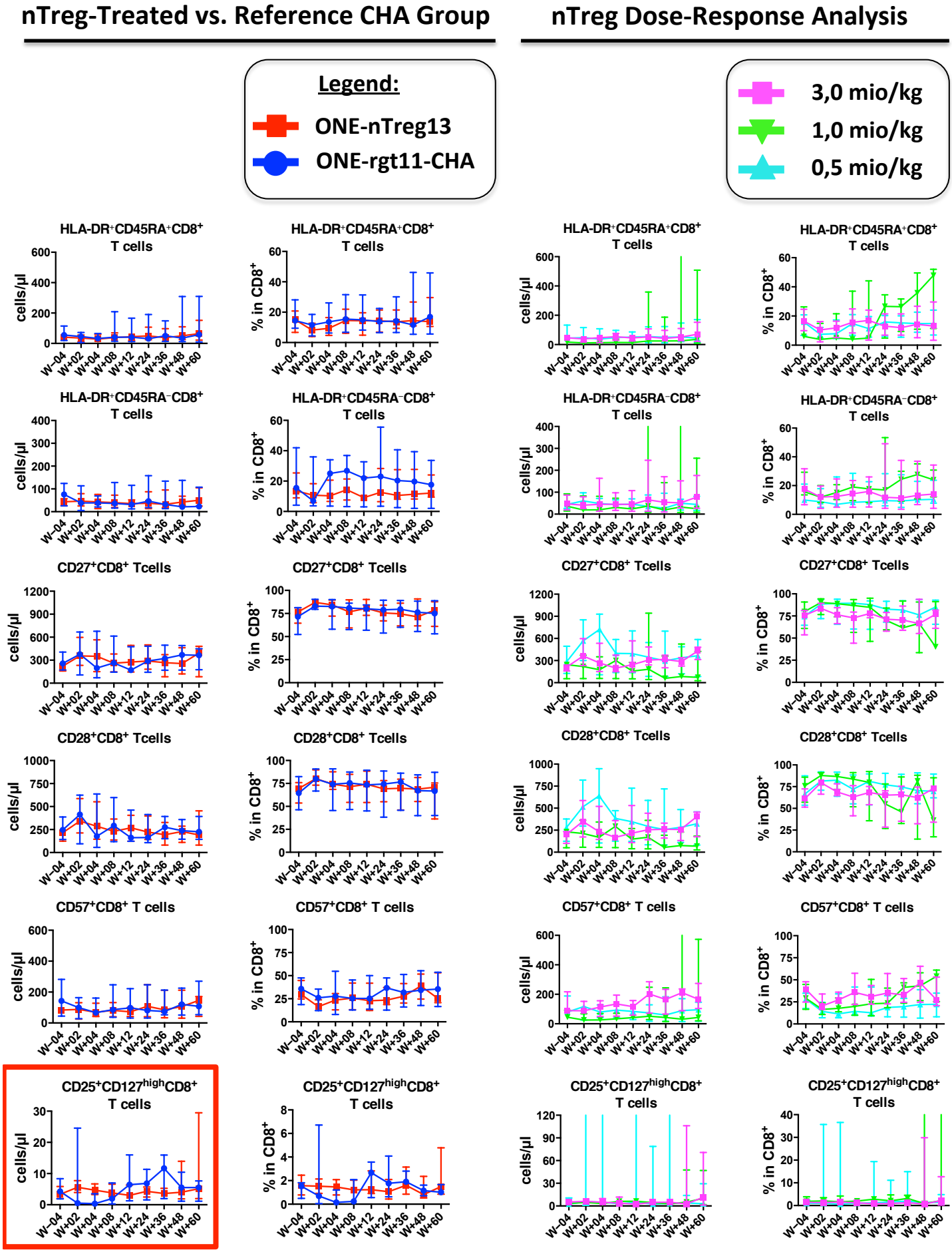


Figure S6: Part 1 FACS#12: B-cell Subsets

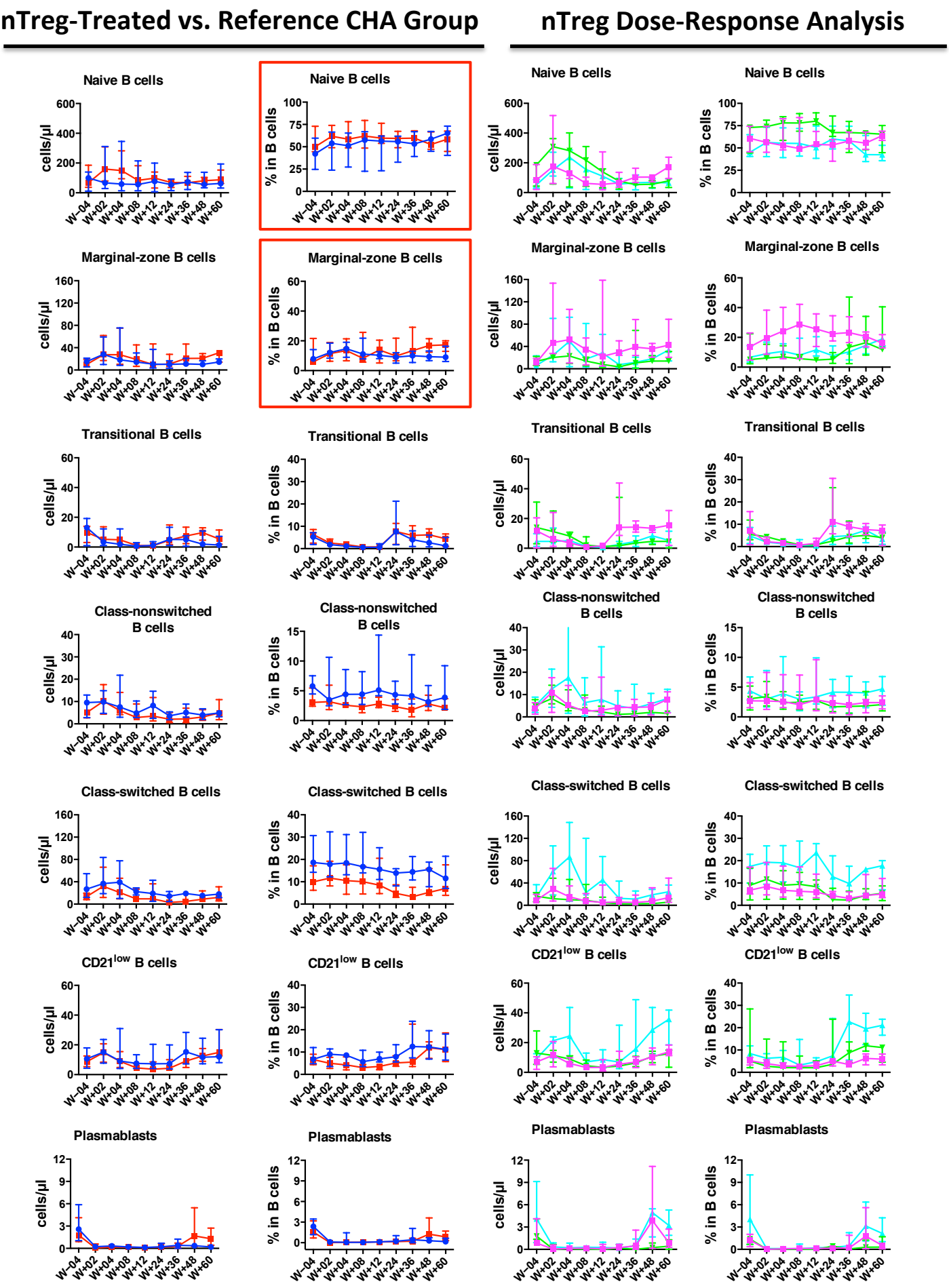
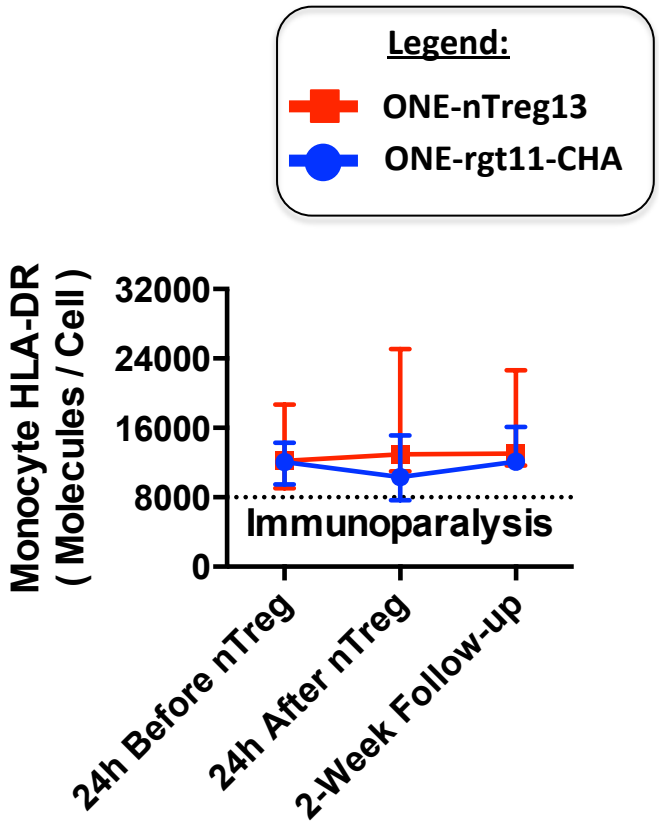


Figure S6: Part 2 HLA-DR Expression per Monocyte

nTreg-Treated vs. Reference CHA Group



nTreg Dose-Response Analysis

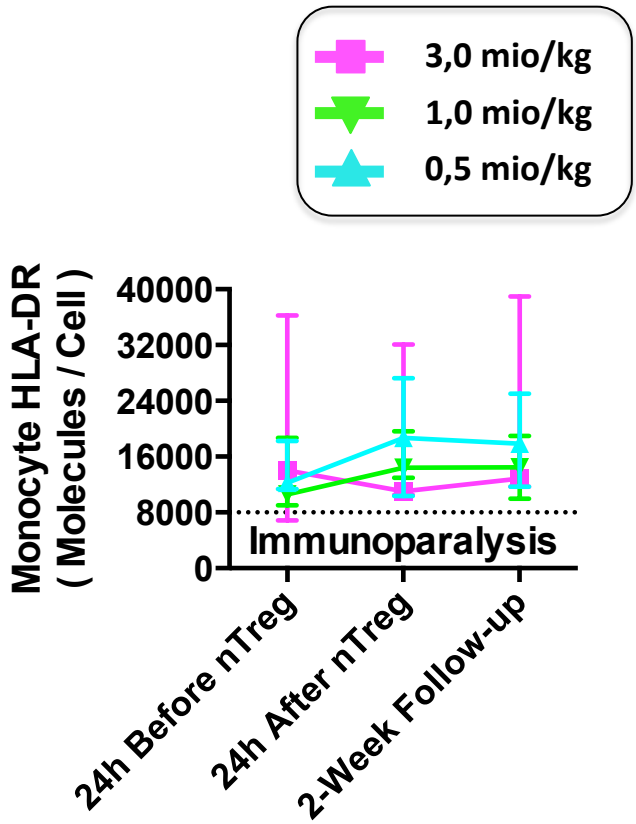


Figure S6: Part 3 QPCR#01: Targets 01-10

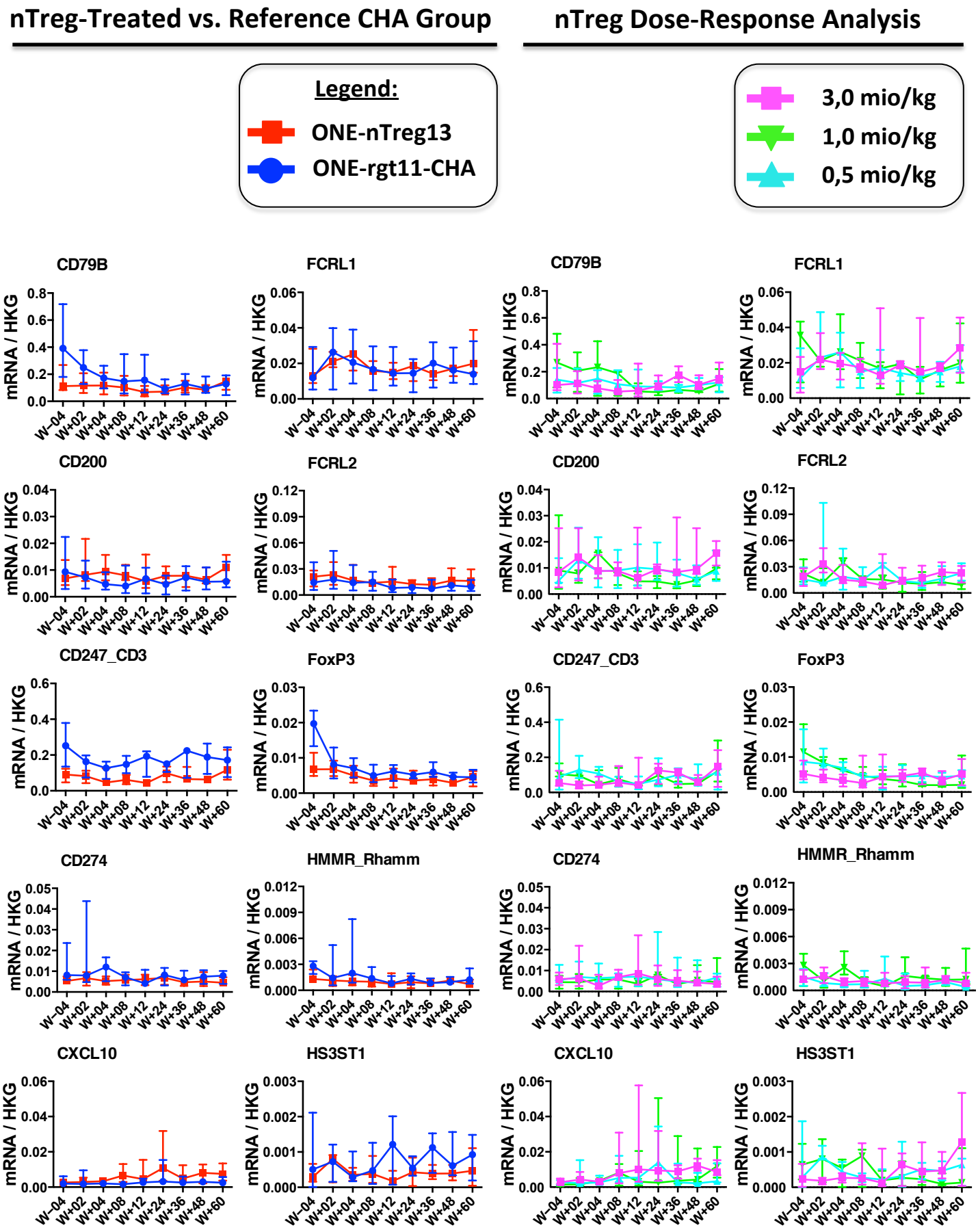


Figure S6: Part 3 QPCR#01: Targets 11-20