Final Study Report Summary for PEI/BfArM (Abschlussbericht)

The ONE Study: A Unified Approach to Evaluating Cellular Immunotherapy in Solid Organ Transplantation - Natural Regulatory T-cells (nTregs) Clinical Trial

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Background / Rationale:

The ONE Study (www.onestudy.com) was a large-scale collaborative project funded by the Seventh Framework Program (FP7) of the European Commission under the work topic "Health: Translational research on cell-based immunotherapy", composed of a consortium of academic institutions with expertise in transplantation research and industrial partners providing biotechnological and regulatory support. The ONE Study aimed to test several distinct purified hematopoietic immunoregulatory cells as clinical therapies in solid organ transplantation in a series of independent clinical trials, based on the same general design, which allowed a post hoc inter-trial analysis. The studied investigational cell therapies were i) human regulatory macrophages, ii) tolerogenic dendritic cells, iii) natural regulatory T cells (nTreg), and iv) type I regulatory T cells. Within the clinical trial presented here, **The ONE Study nTregs Trial (protocol code: ONEnTreg13)**, safety and first hints of efficacy of autologous natural regulatory Tregs (nTregs) were investigated. ONEnTreg13 was an independent clinical trial and its interpretation does not depend on any other Cell Therapy Trial within The ONE Study.

Clinical Need for Alternative Immunosuppressive Approaches in SOT

Kidney transplantation is the only curative treatment for patients suffering from end-stage renal disease (ESRD) [1]. New generations of immunosuppressive drugs have decreased the incidence of acute rejection and improved the short-term prospects of renal transplant recipients so that many patients now achieve 1-year graft survival rates well above 90 %, with the highest cumulative incidence of first acute rejections episodes during the first year post solid organ transplantation (SOT) (**Figure 1A**) [2-4]. Unfortunately, the great efficacy of the immunosuppressive drug treatment is counter-balanced by undesired consequences for the transplant recipients. Apart from the intended therapeutic effect (suppressing transplant rejection), these drugs are also directly associated with serious non-immune toxicities (e.g. nephrotoxicity [5], metabolic disorders [6], cardiovascular disease [7]) and directly linked to life-threatening co-morbidities, such as infection [8] and malignancy [9]). Patients maintained long-term on conventional immunosuppressive treatment suffer significantly from consequences of drug toxicity, increased susceptibility to infections, and increased incidence of malignancy. Despite the considerable improvement in short-term graft survival achieved by immunosuppression, long-term graft survival has not significantly improved of the past decades [10]. Therefore, a primary objective in transplant medicine is to reduce the need for long-term pharmacological immunosuppression, while protecting the allograft from rejection. Achieving this goal would dramatically improve the outcome of transplantation, reduce comorbidities and reduce the high financial cost for the health care system.

Treg's as Therapeutic Agents: Scientific Rationale & Characterization

The immune system uses many mechanisms to maintain immunologic self-tolerance and to protect the host against hyper-inflammatory responses to foreign antigens. Regulatory T cells are one of the key immune modulators, which can control detrimental immune activation at multiple levels to counterbalance inflammatory and cytotoxic responses (**Figure 1B**) [11]. Shifting (Reshaping) the immune response of SOT recipients towards allograft acceptance by using Tregs is now becoming technically feasible and may provide a promising new clinical approach [12]. Animal models have already demonstrated that immunological tolerance can be induced with various purified preparations of immunoregulatory cells, such as different types of Tregs [13, 14]. This experimental proof-of-principle supports the idea that immunoregulatory cells may be used to promote tolerance in the clinical setting [15].

The reporting of studies on regulatory T cells (Tregs), first described by Gershon in 1972, started an exponential growth phase in 1995 (**Figure 1C**), when Sakaguchi et al. refined their definition as a subset of the CD4+ T cell compartment, characterized by high expression of CD25 (interleukin-2-receptor-α chain) and potent immunomodulatory function [16]. Additionally, in 2003, the fork-head family transcription factor FoxP3 was independently identified as a critical regulator of Treg development, function and homeostasis [17, 18]. These cells are described as thymus-derived "natural regulatory T cells" (nTregs), which are discriminated from the peripherally "induced regulatory CD4+ cells" (iTregs), characterized by a lower expression of CD25 and FoxP3 and detectable formation of several cytokines [19]. The nTreg's develop in the thymus and constitute between 3-10 % of the naïve peripheral CD4+ T cell population in humans. In contrast, CD4+CD25varFoxP3 iTregs are peripherally induced from conventional CD4+CD25-FoxP3- T cells. CD+4CD25+FoxP3+ nTregs play a critical role in the maintenance of immunological tolerance, by modulating the activation and proliferation of conventional T cells (Tconv), thereby controlling immune reactions towards self and non-self antigens [20]. A hallmark of thymus-derived nTregs is their functional stability, ensuring suppressive capacity independent from peripheral maturation. Definition of nTregs by phenotypic characteristics is complicated, considering that a marker or marker set that is exclusively expressed on nTregs has not yet been identified. Therefore, nTregs are often defined as e.g.: CD4+CD25+, CD4+CD25+FoxP3+, or CD4+CD25+CD127low T cells, since these markers highly correlate with their functional properties.

Preclinical and Clinical Evidence for Feasibility of nTreg Therapy

Cell types with immunosuppressive characteristics can be isolated and expanded *ex vivo* [21], and several research groups have already provided preclinical evidence supporting the use of regulatory cell populations to control auto- and allo-reactive immune responses in multiple treatment indications, although clinical studies remain scarce to date (**Figure 1C**) [22, 23]. Since the absolute amount of circulating nTregs is limited, *ex vivo* expansion is necessary to obtain sufficient cell numbers for autologous therapy. Phenotypically "normal" CD127- but CD4+CD25+FoxP3+ nTregs can be found in the blood of patients with ESRD [24], and purified by magnetic depletion of CD8+ T cells and subsequent enrichment of CD25+ cells from CD4+ cell fraction (as done in this study). Cell expansion for clinical products requires phenotypic and functional stability over the entire production process. Phenotypic stability can be monitored with flow cytometry, while functional characterization can be performed by determination of specific cell activation markers and effector molecule release in response to activating stimuli (e.g. pro/anti-inflammatory cytokine secretion), or by determination of nTreg's suppressive capacity in cell-based suppression assays (e.g. in allo-antigen- or mitogen-stimulated mixed lymphocyte reactions; MLRs) [25]. Both, *in vitro* and *in vivo* studies have confirmed the suppressive capacity of nTregs and first-in-man and small scale clinical trials have reported preliminary safety data and some first hints of clinical efficacy, e.g. in treatment of acute graft-versus-host disease (GvHD) [26-28].

In our ONEnTreg13 clinical trial (Figure 2), we tested autologous, polyclonal expanded CD4+CD25+FoxP3+ nTregs. They were produced at BCRT-GMP-facility by means of a new protocol developed in-house and administered seven days post SOT (Figure 2A) by intravenous infusion of three different cells doses (0.5×10^6, 1.0×10^6, and 3.0×10^6 cells/kg of body weight) as an adjunctive immunosuppressive therapy to living-donor renal transplant recipients, with the long-term goal of reducing immunosuppression (Figure 2B). The clinical trial follow-up was 60 weeks post- transplant.

Objectives and Hypothesis

Trial Objective and Main Trial Endpoints

The aim of this trial was to collect first-in-human trail evidence on the safety of administering our autologous CD4+CD45+FoxP3+ nTreg product to living-related renal SOT recipients. In addition, the study determined whether post-transplant nTreg infusion allows a careful tapering of conventional maintenance immunosuppression within 60 weeks after transplantation (**Figure 2A+B**), in appreciation of the occurrence and kind of adverse events (**Figure 3A**), as compared to a matched reference group. The primary clinical endpoint was the incidence of biopsy-confirmed acute rejection (BCAR) within 60 weeks after transplantation (**Figure 3B**). The guideline for BCAR was acute graft dysfunction combined with histological evidence of acute rejection.

Primary Objective - Safety

The primary safety endpoint regarding nTreg cell administration was defined as:

- Incidence of infectious complications associated with cell admirations.
- Incidence of embolic pulmonary complications and other embolic events.
- Incidence of immune responses resulting from anaphylactic reactions, cardiovascular compromise or other acute organ failure.
- Biochemical disturbances associated with cell infusion.
- Over-suppression of the immune system assessed by the incidence of opportunistic infections, especially CMV, EBV, and polyoma virus.
- Over-suppression of the immune system assessed by the incidence of neoplasia.

Secondary Objectives

Time to first acute rejection episode; severity of acute rejection episodes based on response to treatment and histological scoring; total immunosuppressive burden at the final trial visit; incidence of post-transplant dialysis; return to the transplant waiting list or re-transplantation following graft loss due to rejection (acute or chronic); incidence of adverse drug reactions.

Another objective was the analysis of exploratory biomarkers.

Methods

Clinical Study Design

This was a non-commercial, investigator-initiated, prospective, autologous natural Treg (nTreg) phase I/IIa dose-escalation mono-center clinical trial (ONEnTreg13, NCT02371434) for lowering immunosuppression and preventing graft-rejection in living-related SOT-patients. ONEnTreg13 was an independent clinical trial, conceptually linked to other trials within the framework of The ONE Study research consortium.

Investigational / Non-Investigational Medicinal Products (IMPs / NIMPs)

IMPs are nTregs (somatic cell therapy containing autologous CD4+CD25+FoxP3+ natural regulatory T cells) and Prednisolone + MMF + Tacrolimus (pharmacological suppression). The NIMPs are Paracetamol and anti-histamine (prophylactic therapy given with nTregs).Patient nTreg Treatment, Study

Population, and Target Recruitment

The trial was designed as a dose escalation study. The nTregs were aimed to be infused in an escalating dose of 0.5 x 10^6, 1.0 x 10^6, and 3.0 x 10^6 cells/kg of body weight in cohorts of three patients each. The product was administered by slow peripheral intravenous infusion on Day +7 (+/-2 days). Prophylactically, paracetamol and anti-histamines were administered before cell infusion. The nTreg therapy was added in conjunction to a standard immunosuppressive regimen (Prednisolone, MMF, and Tacrolimus) as an adjunctive treatment with the intention to reduce or taper the immunosuppression in accordance to patient status. The ONE Study was designed as a Phase I/IIa dose escalation study and followed a classical 3+3 design in patients scheduled for *de novo* living-related kidney transplantation. The nTreg cells were infused with an escalating dose, as outlined above. Each patient received one single intravenous injection of nTregs. In case no dose limiting toxicity (DLT) was observed in a cohort of three patients, the treatment was escalated to the next higher dose level for the next three patients. However, if one of the three patients in a cohort experienced DLT at a particular dose, additional three subjects were recruited to the same dose level cohort until a total of six subjects were treated at that dose level. Accordingly, the minimal number of patients in the study included at our center was nine, up to a maximal number of 18 patients.

Patient Eligibility Criteria

a) 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.

b) 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.

Statistical Evaluation

The ONE Study was designed as a Phase I/IIa study and followed a classical 3+3 design. The minimal number of patients treated in each group was nine and the maximal number was 18, with the trial being designed as a dose escalation study. Therefore a statistical evaluation of primary or secondary outcomes was not planned and neither feasible nor necessary. **Results**

Production of Clinical Grade nTreg Cell Products

Within the ONEnTreg13 study we have been successful in establishing a process for stable isolation, activation and expansion of human alloantigen-specific nTregs from patient peripheral blood derived cells after Treg expansion via repetitive bead-stimulation within our BCRT-Charité in-house GMP facility (**Figure 4A**). We could successfully complete manufacturing of clinical-grade nTreg products for all 17 patients, which have been enrolled in the ONEnTreg 13 trial since its start in March 2015.

We established a unique, robust production process by starting with a very low amount of peripheral blood: 40-50 mL, which is much lower than in all other trials (Ref: personal communication G. Lombardi, Kings College, by using 250-300 mL peripheral blood or leukopheresis). This small amount of blood was sufficient for consistently expanding large numbers of nTregs with high viability (Median 96 %) and high purity (Identified as CD4+ cells of the total product and CD4+CD25+FoxP3+ within CD4+ fraction, median 96 %, and range 92-98 %) (**Figure 4B**). Expansion to > $1x10^9$ could be repeatedly achieved for all n=17 patients within the tightly set time frame of only 23 days until harvest (**Figure 4C**). Of note, all of the donors in the ONE Study suffered from end-stage renal disease (ESRD) and half of the donors were on dialysis before kidney transplantation, with an average time on dialysis of 77 months (range between 0-289 month). We therefore compared the cell expansion kinetics for donors with and without previous dialysis (**Figure 4D**). The starting volume of blood was below the 50 mL threshold in both groups (**Figure 4D, right panel**). There was a slightly lower starting number of cells in the dialysis group compared to the non-dialysis group (P=0.02, Mean 2.9 vs. 4.8 x 10^6 cells), but similar nTreg yield at cell harvest (P=0.7, Mean 5.3 vs. 6.2 x 10^9 cells) and in the final product after bead depletion (P=0.6, Mean 3.0 vs. 3.8 x 10^9 cells) (**Figure 4D, left panel**).

The nTregs were characteristically low in Th1 cytokine expression upon both T cell receptor (TCR)-dependent (e.g. CD2/CD3/CD28-activation beads) and TCR-independent (Phorbol myristate acetate (PMA) and ionomycin) cell activation. Upon stimulation, the formation of the effector cytokines IL-2 and IFN-γ remained well below the set < 10 % GMP process validation threshold in all ONE study nTreg cell lines, with mean values of 2.8 (range 0.2-6.0 %) and 2.9 % (range 0.2-7.0 %) of IL-2 and IFNγ positive cells , respectively (**Figure 4E, left panel**), with the values for both cytokines being in a similar range as the corresponding healthy cell lines from the initial four process validation runs shown in parallel. In contrast, the levels of TNF-α mostly exceeded the set < 20 % threshold with a mean value of 30 % positive cells (Range 5.9-41 %) within the ONE Study cohort, although all four cells from healthy donors tested in the validation runs were well-below both the < 20 % threshold defined for TNF-α and the < 10 % threshold defined for the other two cytokines. None of the three cytokine parameters showed a positive correlation with progressing time of patient on dialysis (**Figure 4E, right**

panel), thus excluding any major progressing negative influence of dialysis and the associated donor blood environment on the nTreg cytokine expression profile, but rather hinting at a general influence of the ESRD-associated patient comorbidities on cellular phenotype.

Both, the values we had collected in the validation runs and the basic immunological knowledge at the time suggested that with a cut-off of < 20 % TNF-α producers within the final nTreg product, we would be within the range to be reached for product release. The values obtained with the 4 validation runs were 4.74 %, 4.55 %, 9.15 % and 1.15 % and thus clearly below the cut-off of < 20 %. In the further course of the study, we had already conducted test runs with three dialysis patients as part of the clinical protocol design and found that the TNF-α values were significantly higher than in the prior healthy patients (0.4 %; 25.3 % and 22.1 %). Since no reference values were available (neither national nor international), we assumed at that time that we would be able to realize a release with our cut-off. In the course of the concrete production and implementation of the EU project, however, it turned out that this TNF-α value was realistic for very few patients only. This is essentially due to the fact that we had moved in an autologous setting - i.e. working with patient material. Importantly, the scientific findings of multiple renowned international groups and our own data and practical experience with SOT patient-derived material indeed suggest that nTregs are able to produce TNF-α, of course to a much lesser extent than for Teff cells, but measurable and even stronger when Tregs are activated by *ex vivo* stimulation. This fits to our patient observations. Thus, we have now also justified and interpreted the revised evaluation in a deviation procedure (internal deviation №. 74), namely caused by the strong artificial stimulation of the Treg cells.

Recent research data show that the functional stability of nTreg is not only determined by the expression of FOXP3 protein, but by the demethylation status of the Treg-specific demethylated region (TSDR) within the FoxP3 promoter/enhancer, and that all nTreg, even if fully demethylated in this region, still produce significant amounts of TNF-α in a very individual way (this is not yet well understood). We and other research groups have been steadily working intensively on this topic, due to the observation described above, and can therefore summarize from our own clinical and experimental experience that TNF-α is not relevant as a release criterion. Irrespective of this, however, the question remains to be answered as to whether "TNF-α producing nTregs" could mean a safety risk for the patient(s). In a risk-benefit analysis, we came to the conclusion that this is not the case. We would like to justify this part as follows: The patients are associated with a high level of inflammation due to their underlying disease, the surgical trauma and the allo-antigen recognition processes that take place during transplantation, including elevated TNF-α plasma levels. The quantity of the infused cells alone and then the much smaller part within the entire product of TNF-α producers was not a risk in the logic of the distribution volume, so that undesired reactions were not to be expected.

In fact, we carried out a bedside test, after 4-6 hours and after a 24-hour follow-up of nTreg infusion, for a comprehensive panel of relevant systemic pro-inflammatory cytokines, in order to quickly detect any potential signals that might suggest a hazard for the patient. The above points have already shown, that a theoretical hazard was not very likely. Nonetheless, this was actually confirmed by our clinical monitoring of patient and laboratory data. In all cases, we demonstrated that post nTreg infusion the inflammation level was rather downregulated within the first few hours, or at least remained stable depending on the initial patient situation. In summary, we incorporated these findings into the documentation and changed the specification

accordingly. Recent data suggested that nTreg stability is better characterized by the TSDR-demethylation of the promotor region. In conclusion, TNF-α was not well suited as a release criterion and has already been removed from the nTreg product release criteria.

Furthermore, we evaluated the TCR repertoire of nTregs by Next-Generation Sequencing approach before/following expansion (**Figure 4F**). We found that primary nTregs freshly isolated and enriched from donor blood, possess a broad unbiased TCR repertoire, which was preserved during *in vitro* expansion, when using our protocol (heterogenous clonotypes, no hints for oligoclonality) [29]. As a control, we stimulated the expanded nTreg product by an alloantigen-challenge and found an oligoclonal TCR-repertoire with a distinct clonotype from Tconv, confirming the sensitivity of the assay. To further characterize the nTreg end product and assess its immunosuppressive properties mixed lymphocyte reactions with polyclonal *in vitro* expanded nTregs and freshly isolated PBMCs were performed. Co-cultures were stimulated with lethally irradiated allogeneic cells for a time frame of 7 hours, and subsequently stained and analysed by flow cytometry for expression of CD154 and CD69 on both CD4+ and $CD8^+$ effector T cells. Polyclonal expanded nTregs effectively suppressed the activation of $CD4^+$ and $CD8^+$ Tconv, as determined by surface fluorescence intensity of CD154 and CD69 (Data not shown).

ONEnTreg13 Clinical Trial Results Summary

The ONE Study nTreg cell therapy trial was approved in November 2014 and initiated immediately thereafter. Recruitment at the Berlin site is completed and the final study visits concluded. All patients have excellent graft function and there had been no SAEs, no reported side effects or complications arising directly from the cell infusion**.** The clinical results of the patients enrolled in the nTreg trial (n=17 were enrolled, and n=11 were successfully treated with nTreg therapy and concomitant tapering of immunosuppression) were compared to The ONE Study matched reference group of n=10 patients also enrolled at the same center at the Charité, receiving standard of care.

In total, 11 patients have been successfully treated at low, medium, and high cell dose (0.5, 1.0, 2.5 and 3 $x10^6$ cells/kg). In detail, 4 patients received $0{,}5x10^6$ (1 patient was identified as HLA full-house, see Exclusion Criteria), 3 patients were treated with the dose of $1x10^6$, 2 patients with $2{,}5x\ 10^6$ and the last 2 patients with $3x10^6$ cells/kg body weight. The dose of 3 $x10^6$ cells/kg could not be obtained in 2 patients due to high cell loss during the bead depletion procedure. A total of 6 nTreg products were not administered due to health-related complications of the patients before day7 post-Tx (the day of nTreg infusion). There was no apparent dose relationship in any of the parameters investigated. As summarized in **Table 1**, there was no significant differences between nTreg-treated patients or reference group for any of the following parameters: recipient age (P=0,8, mean 36 vs. 44 years), recipient male sex (P=0,7, n=4 vs. n=5), kidney donor age (P=0,4, mean 57 vs. 53 years), delayed graft function (P=1, n=1 each), acute graft rejection (P=0,7, n=3 vs. n=4), HLA class I and II panel-reactive antibody (PRA)-positivity, (P=0,6**,** n=3 vs. n=1), infectious complications, such as CMV-infection (P=0,6, n=1 vs. and n=2), EBV-viremia (P=0,5, n=0 vs. n=1), BK-viremia / BKVN (P=1, n=1 each), septic events (P=0,5, n=0 vs. n=1), and cancer (P=1, n=0 each). When comparing nTreg-treated (irrespective of cell dose) vs. control group, there was no apparent difference in tacrolimus levels between the two groups (**Figure 5A**), and kidney function parameters (**Figure 5B**), such as estimated glomerular filtration rate, proteinuria, serum creatinine and serum urea; liver function parameters (**Figure 5C**), such as aspartate

aminotransferase (ASAT), alanine transaminase (ALAT), bilirubin and alkaline phosphatase (AP); and inflammation and other blood safety indicative parameters (**Figure 5D-G**), such as C-reactive protein (CRP), fibrinogen, free hemoglobin, and cytokine levels before, 6 hours and 24 hours after nTreg infusion. Importantly, we did not detect any substantial systemic increase in typical pro-inflammatory cytokines like IFN-γ, TNF-α, IL-1 or IL-6, post cell infusion.

First analysis of exploratory biomarkers revealed a tendency for short-term engraftment / detection of circulating nTregs ($CD4+CD25^{high}CD127^{low}$) up to week 5 post infusion, again with no apparent dose-response relationship (**Figure 5H**). Interestingly, nTreg infusion was generally associated with a significant increase or normalization of marginal zone-like B cell (CD19+IgM+IgD+CD27+) from prior significantly reduced pre-transplant levels, which was not apparent in the control cohort (**Figure 5I**).

Case-by-Case evaluation (**Table 2 and Figure 6: individual description of case #01 to case #11**) demonstrated successful long-lasting weaning of steroids in 8/11 patients (C5051, C5052, C5053, C5054, C5057, C5059, C5062, C5067). Only 3/11 patients were continued on steroids after experiencing either a rejection episode (C5056, pre-sensitized patient presenting with acute cellular rejection Banff grade III at 3 months post initiation of mono-therapy), or due to other underlying transplant harming pathology unrelated to nTreg-treatment (C5058, PRES-syndrome patient, which is related to calcineurin-inhibitor therapy and the late development of de-novo PRA at week 60 under triple drug therapy is also not related to the nTreg infusion, and C5063, no rejection case, but reoccurrence of the underlying IgA nephropathy). Importantly, patient C5057 showed graft deterioration from uncontrolled diabetes mellitus, cardiac decompensation and a histological picture of acute rejection, but could successfully be weaned to mono-therapy following nTreg therapy. Furthermore, successful weaning of MMF was achieved in the same 8/11 patients (C5051, C5052, C5053, C5054, C5057, C5059, C5062, C5067), while one patient got MMF all the way (C5058, the PRES-syndrome patient) and two were continued on MMF after rejection or other pathology (C5056, acute rejection; and C5063, IgA nephropathy).

In summary: Patients treated with nTreg have no increased risk regarding the relevant primary endpoints. This not only applies to the observation period of 60 weeks (15 months), but can now also be stated for a period of more than 3 years. There is no increased safety risk for the observation period compared to standard immunosuppression.

Regarding the secondary endpoints, we cannot identify any risk regarding the safety of the transplants or the success of the transplantation. Only two out of the 11 nTreg treated patients experienced an acute rejection episode, while the rejection rate at the Berlin site for the reference trial was 4 out of 10 patients. The observed 2 relevant rejections in the nTreg group could be treated well. These patients show a very good graft function in the long term. There was no cell dose dependency.

At the time of the biopsy patient C5057 suffered from severe hyperglycemia and as a result herewith presented a decline in renal graft function. The histological findings showing a lymphocytic infiltration can also be seen under severe hyperglycemic conditions, thus making the diagnosis of an acute rejection debatable. The patient remained negative for

alloreactive T-cell and anti-HLA-antibody. The patient was treated accordingly to the protocol and the immunosuppression was successfully tapered.

The observation of T-cell infiltrates in the kidney transplants indicates a homing of the cells and complicates histological diagnosis in patients receiving such therapies. As a short notice: The same observation was also made by colleagues in Oxford. The effectiveness of the nTreg therapy was not correlated with the administered cell dose.

Exploratory biomarkers revealed no significant differences between nTreg and reference group in a broad panel of phenotypical and functional immune parameters. Most importantly, we did not see any drop in monocytic HLA-DR expression and antiviral T-cell response as markers for risk of bacterial/fungal and viral infection, respectively, in the cell therapy vs. reference group. This was in line with the absence of any infection problems.

Discussion

The main trial objective / endpoint was to assess the feasibility of CD4+CD45+FoxP3+ nTreg infusion to living-related renal transplant recipients. In addition, the study aimed to determine whether post-transplant nTreg infusion allowed a tapering of maintenance immunosuppression within 60 weeks after transplantation in line with primary and secondary objectives, most importantly the occurrence of biopsy confirmed rejection. We therefore chose a classical 3+3 design with dose escalation, emphasizing that although statistically evaluation was neither planned nor feasible with these small groups, we conducted a general comparison to a well-matched control / reference group conducted prior to the nTreg group, which gave us first hints on the safety of the treatment.

In line with the main trial objective we could confirm that nTreg infusion was feasible, well tolerated and safe, as there was no difference in occurrence of any adverse events compared to the control group, most importantly, biopsy-confirmed rejection. Attempts for tapering the immunosuppression to the less-toxic option of a monotherapy with tacrolimus (necessitating the step-wise weaning of commonly used steroids and MMF) showed stable long-term success in 8 of the 11 patients, which is a great success for this first attempt of substituting conventional immunosuppression with single-shot cellular therapy. Most importantly, our sophisticated and detailed accompanying patient safety and immunomonitoring approach / program (in particular the results from the ELISPOT assays and panel-reactive allo-antibody monitoring) enabled us to measure the allo-presensitized status of the patients. We were therefore able to develop a "risk-score" identifying patients at risk for rejection in conjunction with or without nTreg therapy.

The safety of nTreg therapy could be furthermore confirmed by meeting all primary safety endpoints, when compared to a well-matched control group: in particular no increased incidence of infectious complications neither bacterial, viral or fungal, no increase in embolic pulmonary embolism or other embolic events, no altered immune response with regards to anaphylactic reactions and cardiovascular compromise or acute organ failure, no biochemical disturbance of any kind. We did not observe any signal of over-immunosuppression which is classically linked to very early events of neoplasia or repetitive reactivation of CMV, EBV or polyoma virus.

Feasibility of an nTreg cell therapy approach in SOT must not only address safety (and potential efficacy at later stages), but also a robust and reproducible production process in the GMP environment, providing enough affordable product in a meaningful time-scale for effective treatment in the given treatment indication. We demonstrated that we were able to produce sufficient amounts of highly pure, well-characterized nTregs meeting the general release criteria, with the exception of only one disputable parameter, that we discussed in greater detail below. Our group finished all testing, validations and re-qualifications required for obtaining the manufacturing license of the Treg cell product with the following characteristics: Phenotypic characterization: > 70 % viability and nTreg-content defined as $CD4^+CD25^+FoxP3^+$ cells, and several functional parameters.

The capacity of nTregs to suppress Tconv activation and proliferation can be assessed by mixed lymphocyte reactions. We found that the assessment of Tconv ($CD4^+CD25^-$ and $CD8^+$) proliferation in the presence or absence of nTregs is prone to errors and underlies a high intra- and inter-assay variability, but CD69 and CD154 have recently been described as useful surrogate markers to assess nTreg suppressive capacities. We confirmed CD154 and CD69 as surface activation markers up-regulated in response to TCR activation and found in-line with previously published data that this process can be suppressed by nTregs.

Most importantly, functional assessment was also based on the perception that nTregs are characteristically low in inflammatory cytokine expression upon both TCR dependent (i.e. CD2/CD3/CD28-activation beads) and independent (i.e. PMA and ionomycin) cell activation (see introduction). Upon strong stimulation, the formation of the effector cytokines IL-2, IFN-γ and TNF-α were defined to be low to negative . Although this could be confirmed for IL-2 and IFN-γ, which were both consistently expressed at only very levels well-below 10 %, we found weakly elevated baseline levels of TNF-α expression, which clearly exceeded with an average of 30 % the set validation threshold of < 20 %. We here propose to discuss this maker with the authorities and potentially exempt this marker from our quality assessment as discussed below.

We are now aware that TNF as a marker must/should be revaluated and the production procedure and its corresponding "positivity" threshold adjusted accordingly, as this definition can't be accomplished in the given setting according to our experience. Importantly, this appears not related to the starting material of transplant donors *per-se*, e.g. since we could not detect any influence of dialysis-length (and accompanying uremia/dialysis). More importantly, our very detailed patient *in vivo* monitoring program did not find any signs of acute TNF-α release (or any other proinflammatory cytokine release) in any patients that would indicate toxicity or cytokine storm, and no increased incidence of immunological complications or graft failure, thus documenting no direct harm for the patient associated by this deviation from the "norm" (defined by us).

In addition, absence of TGFß and IL-10 in our cell product confirm that our nTreg cells are distinct from iTreg.

One of the major concept innovations in the safety, functionality and potency assessment of ACTs/ATMPs was the recent suggestion for the introduction of an combinatorial assay matrix approach (e.g. [25]), which may also apply to Treg-based ATMPs. This method suggests to incorporate / integrate multiple ACT/ATMP production process parameters, which may be a

selection of either regulatory- or self-imposed surrogate markers indicative of product quality safety and efficacy, and most importantly specific potency assays (tailed to a specific target indication), which are suggested to be indicative or relevant for a positive clinical outcome.

We understood, that the specific predictive / prospective value of any one of the parameters integrated into the combinatorial assay matrix can only be truly verified in advanced well-controlled phase III studies (measured vs. a blinded placebo group), and must thus be reevaluated as science progresses. Any single assessment of a given parameter, proposed to be indicative of ACT/ATMP potency, is still largely hypothesis-driven and thus experimental in its nature. In our case we had decided for a stringent approach to exclude the expression of IL-2 and two potential proinflammatory cytokines IFN-γ and TNF-α, with optional assessment of IL10 and TGF-β as potential suppressor candidates, frequently referred to in the literature. Irrespectively of the discussion of specific parameters to be integrated into the combinatorial assay matrix for product safety and potency assessment, the concerns and guidance of the Paul-Ehlich-Institute (PEI) regulatory authority in Germany has a great influence on the validation efforts of the/our nTreg ATMP manufacturing process. Thus, validation proceeded as follows to guarantee the best possible production process and its documentation:

Validation of bead detection and depletion from the product: Flow cytometric methods were set up allowing a precise detection of beads within the cell suspension. Beads in different concentrations were measured with the Navios (Beckman Coulter) and the MACSQuant (Miltenyi) flow cytometer to determine detection limits, recovery rates, linearity and precision/accuracy. Due to a high variance, the method was not applicable for validation purposes. As an alternative method, the CASY cell counter was tested. Particles can be measured using an electrical current exclusion method. This method reached high accuracy and reproducibility. However, this was not true for the beads. Bead detection was performed with a high content screener Opera (Perkin Elmer) combining fluorescence microscopy and imaging software. Because the results obtained by this method were accurate and highly reproducible, the detection of beads by high content screening was chosen as the method of choice and validated successfully. With a robust method at hand, validation of the bead depletion was carried out and gave evidence that beads could be acceptably depleted.

Requalification of cleanroom and process equipment: To ensure compliance of the GMP site and validity of the data collected during validation runs, the cleanroom and process equipment underwent a complete requalification.

Validation of safety release criteria and aseptic handling: Release of a cell therapeutic product requires a standard release test that covers safety aspects. These included tests for endotoxin, mycoplasma and microbiological control. To ensure that the applied tests work in a valid manner with the test matrix, all tests were validated. Aseptic handling was validated successfully by each operator beforehand and media fills were repeated biannual by each operator.

Flow cytometry panel: In parallel a flow cytometry panel was established and used to determine purity and cytokine levels. The panel was set up on the basis of a flow cytometry panel used for monitoring patients. For purity the product was checked for $CD4^+/CD25^+/FoxP3^+$ cells. The cytokine level was determined for safety reasons. IL-2, IFN-γ and TNF-α were chosen to track

contaminating T-effector cells. The panel was validated with respect to precision/accuracy, inter- and intra-assay variance and inter-operator variance.

Process validation: With the prerequisites fulfilled, the process validation was performed. Four consecutive runs were carried out to show that the GMP unit could manufacture a product that meets defined criteria. The product was microbiologically clean, endotoxin free (below detection limit) and free of mycoplasma. The purity was > 90 %. Nonetheless, we are aware that cytokine level of IL-2 and IFN-γ can be met, but that the defined safety threshold for the assessment of TNF-α expression in nTreg product needs to be reevaluated.

Manufacturing authorization: The GMP unit applied for the manufacturing authorization for nTreg cells and the manufacturing license for nTregs was obtained in August 2014 and the use of the product within T*he ONE Study* clinical trials was approved in November 2014.

Conclusions

We here conclude that the administration of nTreg's within the ONEnTreg13 study was safe and well tolerated and met all the set primary and secondary objectives of the clinical trial. We documented first evidence that tapering of immunosuppression to monotherapy with tacrolimus may be feasible in conjunction with employing nTreg-based ATMPs in conjunction with SOT. We could demonstrate that nTreg production practically is feasible at our in-house GMP facility, although some very minor adjustments in specific release criteria, in particular the flow cytometry based TNF-α expression after strong *ex vivo* stimulation within the final product, may be advisable and needs to be reevaluated with the corresponding authorities. We also learned that it does not make sense at present to define unclear biological parameters as release criteria in the product context and that the findings of basic science can only be applied here to a limited extent if they have not been obtained from patient material. Nonetheless, we could demonstrate that our in-depth immunomonitoring approach can give critical clues to improve the outcome of SOT irrespective of nTreg therapy, but also give decisive information on the safety and mode of action of innovative ATMP-based approaches, to develop safe and effective therapies for patients.

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