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DISSERTATION

The TreaT-Assay – Determining the Immunological Risk after Kidney Transplantation by Measuring T cell Alloreactivity towards Urine-Derived Renal Transplant Cells

Das TreaT-Assay – Bestimmung des immunologischen Risikos nach Nierentransplantation durch Messung der T-Zell-Alloreaktivität gegen Transplantatzellen aus dem Urin

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1 Abstract in English and German

1.1 Abstract

In the past decades, the prognosis of kidney transplant patients improved significantly. This can be attributed to a prolonged graft survival due to the introduction of very effective immunosuppressive drugs and a strong reduction of rejection episodes. On the other hand, the immunosuppressive medication increases the risk of infectious, cardiovascular and malignant diseases, which are the main reason for death with a functioning graft. Personalized immunosuppressive therapy could minimize the risk of complications due to the therapy, while still providing long-term protection of the allograft. For the introduction of personalized medicine, however, a marker that reliably mirrors the risk of cellular rejection is needed to guide immunosuppressive therapy. The key players in cellular rejection are alloreactive T cells. Especially memory T cells targeting proteins of the transplant cells can limit the short- and long-term functionality of the graft. The aim of this thesis was therefore to investigate how the reactivity of T cells towards the allograft can be measured, and how these methods can be used in a test system for the risk of rejection episodes.

Cells of the organ donor are needed to activate alloreactive T cells and to determine their frequency. We were the first to describe the application of transplant cells derived from the urine of the transplant recipient for this matter. Therefore, we cultivated tubular epithelial cells from the urine and showed that these cells present HLA-proteins, the target molecules of alloreactive T cells. Alloreactive helper as well as cytotoxic T cells of transplant recipients reacted to the tubular epithelial cells of the kidney allograft donor. This reaction was even stronger than to splenocytes of the donor, an alternative source of stimulator cells. In a proof of concept study patients with a higher number of pre-transplant alloreactive T cells had a worse kidney function in follow-up after transplantation. Patients that experienced an acute rejection episode early after transplantation had more alloreactive T cells before transplantation compared to control patients.

These results illustrate that we developed a promising tool for the measurement of alloreactive T cells and therefore for the assessment of the immunological risk after kidney transplantation. The further development of this testing method shall improve the translatability in clinical routine diagnostics. Ultimately, the assay performance needs to be tested in larger trials.

1.2 Zusammenfassung

In den vergangenen Jahrzehnten hat sich die Prognose von Patienten und Patientinnen mit Nierentransplantat signifikant verbessert. Dies ist vor allem auf ein verlängertes Transplantatüberleben durch den Einsatz von sehr effektiven Immunsuppressiva und eine starke Verminderung von Abstoßungsreaktionen zurückzuführen. Allerdings erhöht die immunsuppressive Medikation das Risiko von infektiösen, kardiovaskulären und malignen Erkrankungen, welche die häufigsten Ursachen für das Versterben trotz funktionierendem Transplantat darstellen. Durch eine personalisierte immunsuppressive Therapie könnte Komplikationsrate durch die Immunsuppression möglichst gering gehalten werden, während gleichzeitig das Transplantat langfristig geschützt wird. Für die Einführung personalisierter Medizin wird allerdings ein Marker benötigt, welcher zuverlässig das Risiko einer zellulären Abstoßungsreaktion abbildet und so die immunsuppressive Therapie leiten kann. Die Hauptakteure der zellulären Rejektion sind alloreaktive T Zellen. Vor allem Gedächtnis-T Zellen, welche gegen Proteine auf den Transplantatzellen gerichtet sind, können die kurz- und langfristige Funktionalität des Transplantats vermindern. Das Ziel dieser Doktorarbeit war somit, zu erforschen, wie sich die Reaktivität der T Zellen gegen das Transplantat bestimmen lässt, um anhand dieser Ergebnisse einen Tests für das Risiko von Abstoßungsreaktionen zu entwickeln.

Um alloreaktive T Zellen zu aktivieren und ihre Frequenz zu bestimmen, werden Zellen des Organspenders für die Stimulation benötigt. Wir konnten als Erste den Einsatz von Transplantatzellen aus dem Urin des Empfängers für diesen Zweck beschreiben. Dazu kultivierten wir Tubulusepithelzellen aus dem Urin und konnten zeigen, dass diese HLA-Proteine, die Zielmoleküle für alloreaktive T Zellen, exprimieren. Sowohl alloreaktive T-Helferzellen als auch zytotoxische T Zellen von Transplantatempfängern reagierten auf die Tubulusepithelzellen des Nierenspenders, und taten dies sogar stärker als auf Milzzellen des Spenders, einer alternativen Quelle von Stimulatorzellen. In einer Proof of Concept Studie hatten Patienten, welche schon vor der Transplantation eine höhere Anzahl an gegen das Transplantat gerichteten T Zellen hatten, eine schlechtere Nierenfunktion im Verlauf nach der Transplantation. Patienten, welche eine frühe Abstoßungsreaktion nach der Transplantation entwickelten, hatten mehr alloreaktive T Zellen vor Transplantation als Kontrollpatienten.

Diese Ergebnisse zeigen, dass wir ein vielversprechendes Verfahren zur Messung von alloreaktiven T Zellen und damit der Bestimmung des immunologischen Risikos nach einer Nierentransplantation entwickeln konnten. In der weiteren Entwicklung des Testverfahrens soll die Anwendbarkeit in der Routinediagnostik von nierentransplantierten Patienten verbessert werden. Größere Studien müssen dann durchgeführt werden, um die diagnostische Leistungsfähigkeit zu testen.

2 Synopsis

In the following text, I will give a concise overview of the current scientific understanding of reasons for graft dysfunction and diagnostic options in a kidney transplant patient. Afterwards, I will present my own work and give an outlook on clinical applications and open research questions. This synopsis is based on the publication “The TreaT-Assay: A Novel Urine-Derived Donor Kidney Cell-Based Assay for Prediction of Kidney Transplantation Outcome” published in Scientific Reports, 2019.[1]

2.1 Current scientific knowledge

2.1.1 Introduction

The prevalence of chronic kidney diseases is rising with increasing age, and, given demographic changes, will become a growing challenge.[2] Although kidney functions can be partially taken over by dialysis, replacing failed kidneys with a transplant is the gold standard therapy in end-stage renal diseases. The numerous advantages of kidney transplantation over dialysis range from a lower mortality rate, higher life quality, and lower morbidity.[3,4] Throughout the history of transplantation medicine, alloimmunity has been the main hurdle for short- and long-term graft survival. The first kidney transplantation in humans was performed in 1933 by Ukrainian surgeon Yurii Voronoy, who implanted the kidney of a deceased donor into a young woman of a different blood group. Due to the immune reaction towards the allograft, the kidney was not functional and the patient died after two days.[5] Over 20 years later, in 1954, the US-American surgeon Joseph Murray performed the first successful kidney transplantation from a living donor to his identical twin. To overcome the reaction of the host’s immune system in non-identical individuals, immunosuppressive therapies were developed. Early strategies involved whole body irradiation or high dose steroids and azathioprine, but came with the prize of high toxicity and low success rates. The development of the calcineurin inhibitor cyclosporine A, a substance that mainly suppresses T cell activity, in the early 1980s revolutionized immunosuppressive therapy in transplantation medicine by substantially prolonging allograft survival.[5] Until today, the vast majority of kidney transplant recipients requires permanent immunosuppressive therapy. The immunosuppression has to be balanced between two extremes: Under-immunosuppression can lead to a destruction of the organ by the recipient’s immune system. In contrast, over-suppression of the immune system also inhibits its vital functions in fighting infections and cancer and leads to a higher cardiovascular risk, which are also the main reasons of death with a functioning allograft.[4,6] Currently, the protocols for immunosuppressive therapy after kidney transplantation employ a one-fits-all strategy, even though it is widely accepted that the immunological risk varies between patients.[4,7] This is mainly due to the lack of a convincing parameter that could reliably determine the individual patient’s immune reactivity towards the allograft and therefore the personalized need for immunosuppression. In the following paragraphs, I will give more

information on the immune system's reaction towards an allograft, present current strategies to monitor this reactivity and finally present our research hypothesis.

2.1.2 Alloimmunity

Immunity describes the adequate reaction to and control of potentially harmful events caused by foreign invaders such as microbes. It relies on the detection of such an event by the discrimination of self from foreign and danger signals by affected cells. Failures of this system can cause diseases, for example the inability to control pathogens or attacks against self instead of foreign structures as in the case of autoimmunity. Alloimmunity does not describe a failure of the system, as the detection of foreign proteins is essential for immunity; however, in the case of transplantation, this fight against non-self-structures is adverse. As described above, alloimmunity was the main obstacle on the way to a successful kidney transplantation, and remains a main reason for allograft dysfunction.[4]

This synopsis can only offer a very superficial overview of the highly complex immunological reactions towards an allograft. The immune system can be divided into two main parts: the innate and the adaptive immunity. Both are closely interacting and hold important functions in the transplantation setting. Innate immunity provides a broad line of defence and reacts to pathogenic or danger signals with limited specificity. Granulocytes and antigen-presenting cells like dendritic cells and macrophages are examples for its cellular components. In the transplantation setting, innate immune cells detect, for example, danger and damage signals after cell death due to ischemia reperfusion injury and drive inflammation. Antigen presentation by specialized cells, such as dendritic cells, and inflammatory signals can then attract and activate cells of the adaptive immune system. Neutrophil granulocytes and the complement system are examples of important effectors in rejection episodes and can be attracted by components of the adaptive immune system, in this example interleukin (IL)-17 and donor-specific antibodies, respectively.[8,9] The specific immune response is initiated by chemoattraction through chemokines and adhesion molecules, and by presentation of antigens and stimulatory signals by antigen-presenting cells to cells of the adaptive immune system, which will be discussed in greater detail later.[8,10] Adaptive immunity can be sub-divided into the humoral and the cellular arm. B lymphocytes are the main cells of the humoral adaptive immune system, and act mainly via production of antibodies. Donor-specific antibodies are the main element of hyperacute and acute antibody-mediated rejection episodes.[8] T cells are the major players of the so-called cell-mediated immune response and hold a central role in immune reactions towards a kidney allograft.[8,10] These cells recognize specific fragments of proteins (peptides) presented on major histocompatibility complex (MHC) molecules on other cells. In humans, these molecules are also called human leucocyte antigen (HLA). There are two main groups of MHC (HLA) molecules: MHC-class I (HLA-ABC) molecules are expressed by nearly all cells and present intracellular peptides. MHC-class II (HLA-DR) molecules present extracellular peptides and are expressed by professional antigen-presenting cells such as B

cells, dendritic cells, and macrophages, as well as on atypical antigen-presenting cells after induction by pro-inflammatory stimuli.[8,10,11] T cells randomly rearrange their T cell receptor during development to cover a wide variety of possible pathogens. The resulting T cell receptors are then selected in the thymus, and T cells that show a dysfunctional binding to their individual HLA molecules or recognize self-antigens are sorted out. The HLA molecules are a very heterogeneous group of proteins, and it is highly unlikely that two non-related individuals will share the same HLA genes. This diversity is beneficial for the population, as different variants of HLA genes have different abilities to present pathogens. Since pathogens are continuously evolving, a high variety of the HLA molecules in the population is important so that each pathogen can be presented at least by some individuals, which can subsequently acquire immunity. However, this evolutionary beneficial system has adverse effects for transplantation medicine: The donor's HLA molecules will very likely be different to those of the recipient and are therefore recognized by the recipient's T cells as foreign, since they are not presented in the patient's thymus, and may evoke an immune response.[8,10] Three pathways for this recognition have been described. In the direct pathway, HLA molecules are sensed on the donor's antigen-presenting cells. In the indirect pathway, the recipient's antigen-presenting cells take up and process donor HLA molecules, and present the donor HLA peptides on their own HLA molecules to T cells. The semi-direct pathway describes an uptake and presentation of whole donor HLA molecules by the recipient's antigen-presenting cells by various mechanisms.[10] The relative contribution of each pathway for rejection episodes is a matter of research. The direct pathway seems to be especially relevant in early acute rejection episodes, while the indirect pathway is assumed to be of primary importance in chronic rejection.[8,10] The T cells that attack the foreign cells are called alloreactive T cells, from the Ancient Greek word "állos" for "other". T cells are commonly identified by their expression of the T cell co-receptor cluster of differentiation (CD) 3. They can be classified into two main subsets: CD8 expressing cytotoxic T cells and CD4 expressing T helper cells. The CD8 molecule enables the T cell to bind to HLA-ABC molecules and thereby to recognize intracellular peptides, for example from viruses. If an intracellular pathogen is detected, the $CD8^+$ T cell can induce cell-death of the infected cell, for example by secreting the serin-protease granzyme B and by expression of the ligand for the death receptor Fas. The CD4 molecule enables the T cell to bind to HLA-DR molecules. For example, by secreting cytokines, the $CD4^+$ T cells can then attract further cells. The $CD4^+$ T cells are subdivided according to their cytokine production profile. TH1 cells, producing, among others, interferon γ, and TH17 cells, producing for example IL17, are prominent examples that play important roles in transplantation.[8,9] Naïve T cells are primed by interacting with cells that present a matching antigen in the above-described pathways and express the costimulatory molecules of the B7 family in the presence of other stimulating factors. After priming, they can form memory cell and are then able to react substantially faster to a second encounter with the same antigen. Interestingly, memory cells

directed against the allograft can be formed prior to transplantation, for example during pregnancy, blood transfusion or by cross-reactivity of T cells reacting against infectious pathogens. Memory T cells can act without co-stimulation and lymphoid tissues and therefore hold an important role in allograft rejection.[10,12] Despite the pathway of presentation (direct vs. indirect), there is also a scientific debate about the triggers of alloreactive T cell activation: One hypothesis regards the amino-sequences of the HLA molecule itself is the primary target, irrespective of the bound peptide. There is strong evidence, however, that the bound peptide plays a role and that the complex of peptide and HLA molecule triggers alloreactivity.[10,13] Supporting the latter hypothesis, experiments showed that kidney allograft infiltrating cells reacted to donor cells from the kidney allograft, but not to splenocytes from the same donor.[14] Considerable are also the regulatory mechanisms of the different components of the immune system, potentially counterbalancing detrimental effects and helping to maintain and tolerate the allograft.[8,10]

2.1.3 Other factors of kidney allograft dysfunction

It is important to keep in mind that alloimmunity is paramount, but it is not the only factor affecting the graft function after transplantation. After the immediate post-transplant period, where ischemia-reperfusion injury, hyperacute antibody-mediated rejection and surgical complications are the most common reasons for graft dysfunction, several conditions can present clinically similar to rejection episodes. Pre- (for example dehydration and renal artery stenosis) and post- (for example urinary obstruction) renal kidney injury can be diagnosed non-invasively, but for the differentiation of intrarenal conditions a biopsy is often inevitable.[4] Pyelonephritis, viral reactivation and nephrotoxicity are associated with a too strong immunosuppression. BK-virus nephropathy is a common complication after transplantation and is normally treated by reduction of immunosuppression. Calcineurin inhibitors are very potent in protecting the allograft from T cell alloreactivity, but are nephrotoxic themselves and a common reason for graft dysfunction. Recurrence or *de novo* kidney disease are also observed frequently.[4]

2.1.4 Biomarkers for the assessment of acute rejection

One of the most feared consequences of T cell activity against the allograft are acute rejection episodes, which can lead to a destruction and loss of function of the transplant. While treatment is very often successful, it is clinically challenging to differentiate acute rejection episodes from other reasons for graft dysfunction as described above. The current strategy involves measuring serum-creatinine as a marker for the kidney function and performing a kidney biopsy in case of allograft dysfunction.[4] This approach has several disadvantages:

- Serum-creatinine is a late marker and only elevated when the damage to the graft led to a deteriorated function. This has a negative impact on the long-term function of the allograft.[15]

- Kidney biopsies are an invasive procedure and can lead to complications such as haemorrhages.[4,16]
- In 10-30% the biopsies are not interpretable, as the relevant structures are missed.[16] The high interobserver variability of 30% means that the given diagnosis might not be reproducible.[17] Furthermore, the absence of signs of acute rejection in the biopsy does not mean that these are absent in the whole allograft.[18]
- The patient is normally hospitalized for at least one night for the performance of the biopsy, so the burden for the patient, the hospital and the health care system is high.

For these reasons, finding a reliable non-invasive biomarker that can diagnose or even predict acute rejection episodes is a primary goal in transplantation medicine.[17–19] There are several strategies that are currently investigated. Some prominent examples are:

- **Donor-specific antibodies** are part of clinical routine diagnostics and reflect the risk of an antibody-mediated-rejection. However, they do not allow the evaluation of T-cell-mediated-rejection episodes. These contribute a major part of acute rejection events and are discussed as a risk factor for antibody-mediated-rejections.[18,20]
- **Molecular markers in peripheral blood** rely on measurement of gene expression signatures or targeted single mRNA expression and the correlation to acute rejection episodes. These tests showed a good differentiation between undamaged grafts and grafts with complications. However, the differentiation of the reason for the kidney damage, for example BK-virus nephropathy or acute rejection, remains elusive. This might limit the applicability of these tests in clinical practice.[17,18,20]
- **Total or donor-specific cell-free DNA** can be measured in peripheral blood or urine and is a marker for graft damage. The advantage is that it can precede the elevation of serum-creatinine by several weeks. Again, however, the differentiation of the reason for the graft damage is limited.[20]
- The **analysis of urine** is another attractive strategy, considering it comes directly from the transplanted kidney and might therefore offer a more direct insight into the processes in the allograft. Examples are the analysis of **RNA** or **miRNA signatures**, the quantification of **chemokines** on the RNA or protein level, and broader methods such as **proteomics** and **metabolomics**. Some of these approaches are still very early in the development. A problem is the instability of the molecules in urine, which requires a fast processing. The markers that were tested so far also failed in showing that they can differentiate between infections and acute rejection episodes.[17,18,20]
- A very different approach is the analysis of immunological changes in the allograft by **imaging techniques** such as proton magnetic resonance spectroscopy and positron emission

tomography. The infiltration of leucocytes can be visualized and measured. The specific reason for the inflammation and the cause of graft damage, however, remains elusive.[18]

- In contrast to the above-mentioned approaches, **cell-based methods** directly measure memory donor-reactive T cells as the major players in rejection episodes.[20,21] Interferon γ-ELISPOT is a commonly chosen method to quantify alloreactive immune cells from the peripheral blood of the recipient after stimulation with stimulator-cells. Those stimulator-cells can come directly from the donor. For example, the spleen can be collected at the time of explantation of the kidney allograft from the deceased donor. The splenocytes consist of many antigen-presenting cells and are therefore seemingly ideal for the stimulation of alloreactive immune cells. The limited availability of the splenocytes is, however, problematic. Splenocytes cannot be obtained from every donor. If they can be procured, their number is limited and the source cannot be renewed, which limits multiple analyses in follow-up. Another type of stimulator-cells are HLA-bank cells. These cells of third-party donors are chosen to represent the most common HLA-types within the population. With this approximation, a measurement of the general alloreactivity can be achieved, but the reactivity towards the individual donor cannot be quantified. Studies showed a correlation of pre-transplant alloreactive T cells with the graft function after transplantation and acute rejection episodes. The limited availability of donor cells, the labour-intensive protocol, and the long incubation times hinder the introduction into routine diagnostic, however.[18,20]

2.1.5 Research hypothesis and approach

In this thesis, we hypothesized that urine-derived transplant tubular epithelial cells (TEC) can be used to mimic the reactivity of T cells towards the allograft *in vitro* and to quantify and characterize alloreactive T cells. The representation of the *in vivo* situation with this method is superior to similar approaches with other cell types. Our second hypothesis was that the number of pre-transplant alloreactive memory T cells measured with our approach correlates with the post-transplant kidney function and the occurrence of acute rejection episodes, as it has been shown with similar tests before.[18,20] To this end, we cultured cells from the urine of kidney transplant patients and characterized their cellular identity by flow cytometry. The expression of HLA molecules with and without induction by pro-inflammatory cytokines on the cultured cells was analysed to investigate their stimulatory capacity. Afterwards, the stimulation of alloreactive T cells by donor-derived allograft cells was tested and compared to the stimulation by donor-derived splenocytes. Finally, pre-transplant samples of kidney-transplant patients were analysed and the numbers of alloreactive T cells compared to the kidney function after transplantation and the occurrence of acute rejection episodes.

2.2 Methodology

2.2.1 Overview

In the following paragraphs, the methodology is summarized with a focus on the underlying principles. An overview about the TreaT-assay protocol is given in Figure 1. More details regarding materials and methodological procedures can be found in the method section of the attached publication.[1] A European patent has been applied (EP3203237A1).

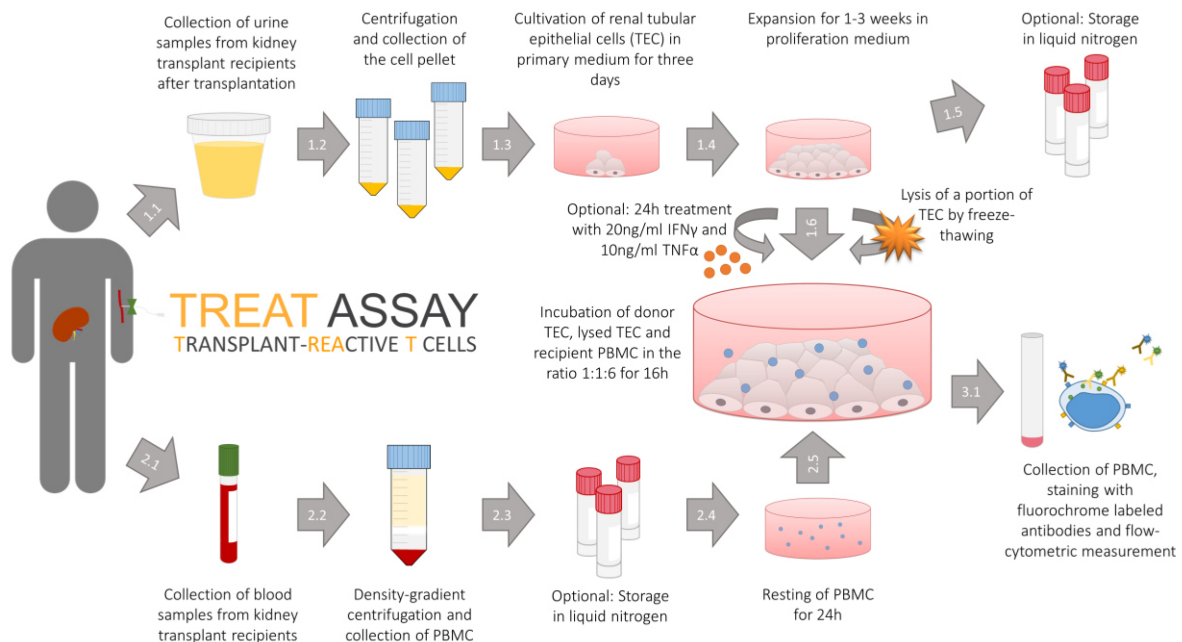

Figure 1: Overview of the methodology of the Transplant-Reactive T Cells (TreaT)-Assay. Adapted from Thieme et al. Sci Rep 2019.[1] Abbreviations: h= hours; IFNγ = interferon γ; PBMC = peripheral blood mononuclear cells; TEC = tubular epithelial cells; TNFα = tumour necrosis factor α

2.2.2 Study participants

We collected samples of four healthy volunteers and twenty-two kidney transplant patients at the Charité – Universtitätsmedizin Berlin after written informed consent and approval by the ethics commission of the Charité – Universitätsmedizin Berlin in accordance to the Declaration of Helsinki. Due to the exploratory character of the study and the different questions addressed, the numbers of patients in the distinct experiments differed. An overview of the number of patient samples that were used in the experiments is given in Table 1.

Table 1: Overview of the patients and samples analysed in the study. Green fields indicate that the individual patient has been included into the respective analysis. Numbers in the green fields indicate the numbers of blood samples used of the individual patient for the respective analysis. Asterisk indicates that the sample in the follow-up study was also included in the analysis of the alloreactivity measurement establishment. From Thieme et al. Sci Rep 2019.[1] Abbreviations: HLA = human leukocyte antigen; TEC = tubular epithelial cells

Patient number	TEC cultivation	HLA expression on TEC	Establishment of alloreactivity measurement (*number of samples*)	Pre transplant samples - follow up study (** = sample also used in establishment*)	Comparison donor-TEC vs donor-splenocytes (*number of samples*)
#1	■	■			
#2	■	■			
#3	■		2		
#4	■		1		
#5	■	■	4	*	
#6	■	■	4	*	
#7	■	■	2	*	
#8	■	■	4	*	
#9	■	■	3	*	
#10	■	■	1	*	
#11	■	■	1	*	
#12	■	■	1	*	
#13	■	■	2	■	
#14	■	■	3	■	
#15	■	■	2	■	
#16	■	■		■	
#17	■	■		■	
#18	■	■		■	
#19	■	■			4
#20	■	■			4
#21	■				4
#22	■				3
Total patients	**22**	**18**	**13**	**14**	**4**
Total blood samples			**30**	**14**	**15**

2.2.3 Cultivation of tubular epithelial cells

It has been shown before that exfoliated kidney cells can be isolated and cultivated from the urine, and that these cultures consist of functional tubular epithelial cells (TEC).[22] We performed the cultivation of TEC from the urine according to a previously published protocol.[23] Urine was collected from each study participant in a sterile container. If possible, the urine was transported immediately to the laboratory to ensure a sample processing time of under 4 hours to prevent a decrease in viability of the cells in the urine due to low pH, osmotic pressure and toxic metabolites.[23] By centrifugation, the cells were isolated from the urine and afterwards cultivated in primary medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher) and Ham's F12 Medium (Biochrom) mixed 1:1, 10% fetal calf serum (FCS, Biochrom) and Renal Epithelial Cell Growth Medium (REGM)

single quot kit (Lonza). The REGM single quot kit contains human epidermal growth factor, insulin, hydrocortisone, gentamicin, amphotericin B, transferrin, triiodothyronine and epinephrine and is designed to promote the expansion of renal epithelial cells and to prevent bacterial and fungal contamination.[23] Penicillin/streptomycin (P/S, Biochrom), ciprofloxacin (Fresenius), normocin (InvivoGene) and amphotericin B (Biochrom) were added to the primary medium, as bacterial or fungal contamination of urine, for example due to urinary tract infections, is frequent in immunosuppressed patients and requires high doses of antimicrobials. After three days the primary medium was exchanged by the proliferation medium, which is composed of renal epithelial basal medium (REBM), the REGM single quot kit, 10% FCS, P/S, non-essential amino acids (Thermo Fisher) and Glutamax (Thermo Fisher). The latter are required by proliferating cell cultures and increase the proliferation rate. Every two to three days, the proliferation medium was refreshed until the cells reached 80-90% confluency. The TEC cultures were then harvested using trypsin/EDTA solution (Biochrom). The harvested cells were either cryopreserved as described below and stored until later usage or directly characterized and used for stimulation of PBMC as described below.

2.2.4 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) are mainly composed of lymphocytes, monocytes and dendritic cells. They are a commonly used source to analyse lymphocytes in immunological studies. Therefore, they need to be separated from other constituents of the peripheral blood, namely erythrocytes, granulocytes, thrombocytes and plasma. As a starting material, heparinized blood was drawn from the study participants. Density gradient centrifugation was then used for isolation. In this method, Biocoll separating solution (Biochrom), containing polysaccharides with a specific density, is used to separate the cells by centrifugation. High-density cells such as erythrocytes and granulocytes accumulate under the Biocoll, while PBMC are directly above the separating solution. The plasma constitutes the upper fraction. An overview of the method is given in Figure 2. The PBMC can then be selectively taken out by using a Pasteur pipette. After isolation, the PBMC were cryopreserved or directly used for stimulation as described below.

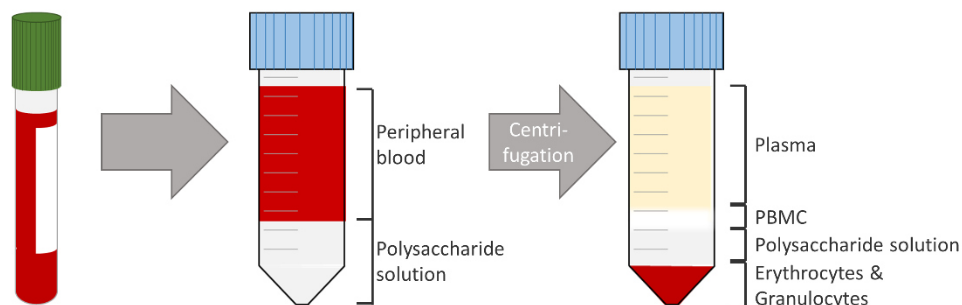

Figure 2: Schematic overview of the density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC) from peripheral blood.

2.2.5 Isolation of splenocytes

Pieces of spleen from deceased kidney transplant donors with a diameter of at least six centimetres were taken by the transplant surgeon and sent to our laboratory. The samples were stored in Roswell Parm Memorial Institute-1640 medium (RPMI) (Biochrom) at 4°C for a maximum of 4 hours to maintain viable cells. Sterile forceps (Hartmann) and scalpel (Feather) were used to remove connective tissue and to cut the spleen into smaller pieces. To obtain a single cell suspension the pieces were minced through cell strainer sieves (Becton Dickinson). Density gradient centrifugation was used as described above to isolate mononuclear cells from the suspension. These cells were then cryopreserved until further usage.

2.2.6 Cryopreservation and thawing of cells

After isolation or harvesting, the PBMC, splenocytes or TEC were suspended in RPMI with 60% fetal bovine serum (FBS) (Biochrom) and 10% dimethylsulfoxid (DMSO) (Sigma-Aldrich) and immediately frozen at -80°C in a freezing box (ThermoFisher) with isopropylalcohole (Sigma-Aldrich). After one day, the vials were stored in liquid nitrogen until further usage. When frozen under ideal conditions, the functionality of the immune cells is preserved.[24] After thawing, the cells were left resting for 24h, as it has been shown that this improves antigen-specific T cell responses.[25]

2.2.7 Characterization of tubular epithelial cells

To analyse the origin of cells cultivated from the urine, we used phase-contrast microscopy and flow cytometry. Phase-contrast microscopy uses changes in the phase of light in addition to the brightness to make objects visible without requiring staining of cells. This is particularly useful to study viable cells, as staining often results in cell death. To analyse specific proteins expressed by the cells we used flow cytometry, as described below. After harvesting, we stained the cells with antibodies directed against cytokeratin (Becton Dickinson), CD90, CD13, and CD326 (all BioLegend). Those markers were identified after extensive literature research, since culturing can change the expression of some proteins normally found in TEC isolated directly from the kidney. Therefore, we analysed markers that are stably expressed under cell culture conditions. Cytokeratins are cytoskeleton proteins found in epithelial cells. We used antibody clone CAM5.2 directed against cytokeratin-7 and -8, which can be found in TEC.[26] CD90 (Thy-1) is a cell-surface protein widely expressed on mesenchymal cells, among those fibroblasts.[27,28] Alanine aminopeptidase (CD13) is expressed by renal proximal TEC.[29] Epithelial cell adhesion molecule (EpCAM, CD326) can be found on distal TEC, but also on regenerating proximal TEC.[30] Both CD13 and CD326 are not expressed by cells in the bladder. The combination of these markers allows therefore differentiation of proximal and distal TEC from contaminating fibroblasts and epithelial cells from other origins than the kidney.

2.2.8 Induction of HLA expression on tubular epithelial cells

Interferon γ (IFNγ) and tumour necrosis factor α (TNFα) are proinflammatory cytokines that are produced by innate and adaptive immune cells as a response to danger signals, for example during ischemia-reperfusion injury after transplantation.[31] We used these cytokines (both Miltenyi) to mimic inflammatory conditions in our cell culture system. It has been shown before that TEC respond to these stimuli by upregulation of HLA molecules.[32] HLA-ABC and HLA-DR are the ligands for the T cell receptors of allogeneic $CD8^+$ and $CD4^+$ T cells, respectively, and therefore the target of direct allorecognition.[10] By flow cytometric evaluation of HLA molecule expression (anti-HLA ABC and anti-HLA DR antibodies from BioLegend), we assessed the ability of the cultured TEC to induce direct allorecognition.

2.2.9 Alloantigen assay

The main goal of this project was to set up an assay to quantify alloreactive T cells using donor-derived TEC as stimulators. During initial experiments, we compared several experimental conditions to optimize the stimulation. Factors that we evaluated were well size, well geometry (round bottom versus flat bottom), low- versus high-attachment culture vessels, coating of well surface with gelatine, cell numbers, cell ratios and incubation time, among others. In addition, we evaluated lysis of a fraction of TEC before stimulation to facilitate the uptake and presentation of peptides by antigen-presenting cells. As an initial read-out parameter we used proliferation of T cells measured by carboxyfluorescein succinimidyl ester (CFSE) staining, but due to more robust experimental procedures and shorter incubation time we decided to evaluate activation marker expression instead, as described below. In the final assay we used flat bottom, tissue-culture treated 24-well plates without gelatine. We compared stimulation by TEC treated for 24h with IFNγ and TNFα to untreated TEC. 0.25×10^6 TEC were seeded into the wells and lysates of 0.25×10^6 TEC were added. Then 1.5×10^6 PBMC of the corresponding recipient were incubated together with the TEC for 16h. Brefeldin-A (Sigma) and monensin (BD) were added to prevent protein secretion and thus enable intracellular detection of cytokines. After incubation, we harvested the PBMC to stain them for flow cytometry. PBMC without stimulus and with phorbol 12-myristate 13-acetate (PMA, Sarstedt) and ionomycin (Iono, ThermoFisher) were used as negative and positive control, respectively.

2.2.10 Flow cytometric analysis

A flow cytometer enables the multiparameter-analysis of suspended single cells using lasers of different wavelengths, a complex filter and mirror system and sensors. The cells can be analysed according to their relative size, granularity, and fluorescence. Prior to analysis, the cells can be stained with fluorescent dyes or fluorochrome-coupled antibodies to enable the specific analysis for example of proteins expressed by the cell. Cell-surface proteins can be stained directly, but for staining of intracellular components fixation and permeabilization of cells is necessary. The fluorochromes are excited by lasers of the corresponding excitation wavelength and emit light, which is then filtered so

that only light of a specific wavelength is detected by photomultiplier tubes or avalanche photodiodes. These convert the energy of the photons into an electrical signal. The amplification of this signal can be adjusted by fine-tuning the voltage or gain applied to the detectors. We used a LSR II Fortessa (BD) and a Cytoflex LX (Beckman Coulter) cytometer. The antibodies we used for the analysis of T cells and the significance of their respective antigen can be found in Table 2. The antibodies for the analysis of TEC have been described above.

Table 2: Antibodies for flow cytometric analysis of alloreactive T cells		
Antibody/ fluorescent dye	Manufacturer	Significance in this study
Surface		
Live dead fixable dead cell stain kit blue	Thermo Fisher	Permeates the cell membrane of dead cells and binds to intracellular amines enabling differentiation of living and dead cells
Anti-human CD161 (KLRB1)	BioLegend	Surface marker of T helper (TH) 17 cells[33]
Intracellular staining after fixation and permeabilization (Thermo Fischer)		
Anti-human CD3	BD	Marker of T cells
Anti-human CD4	BioLegend	Marker of T helper cells
Anti-human CD8	BioLegend	Marker of cytotoxic T cells
Anti-human CD137 (4-1BB)	BD	Activation marker of T cells[34]
Anti-human CD154 (CD40L)	BioLegend	Activation marker of T helper cells[35]
Anti-human granzyme B	BD	Effector molecule of cytotoxic T cells

To control the specific detection of the antigen of interest, several controls of the flow cytometric staining are essential. Therefore, we titrated antibody concentration and measured unstained cells, single stains and fluorescence-minus-one controls. These controls are important to adjust voltages or gains as described above, adjust the compensation matrix and control gating strategies. Compensation is a mathematical process in which unspecific signals from spectral overlap of other fluorochromes are subtracted from the channel of interest. This is important to avoid false positive or negative results. A common strategy to analyse the data obtained by flow cytometry is the so-called gating of the cells. In this process, a cell-population of interest is chosen by certain characteristics and further analysed. An example can be found in Figure 3. Apart from the frequency of cells in the sub-population of interest, the median fluorescence intensity (MFI) is a way of quantifying flow cytometric data. When instrumental settings and compensation are identical, this parameter can be used to compare the fluorescence intensity between cells and thereby the expression of the analysed marker between samples. The fluorescence intensity correlates with the amount of the respective protein expressed by the cell. The analysis of flow cytometric data was performed with FlowJo version 10 (FlowJo LLC).

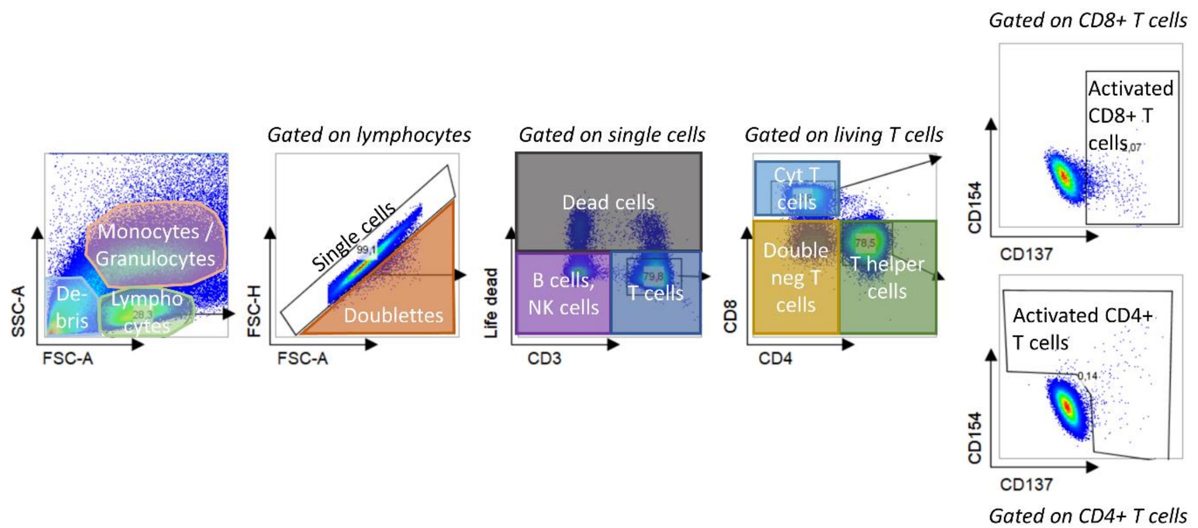

Figure 3: Example of a gating strategy for the analysis of flow cytometric data. Adapted from Thieme et al. Sci Rep 2019.[1] Abbreviations: -A = area; CD = cluster of differentiation; Cyt = cytotoxic; FSC = forward scatter; -H = height; neg = negative; NK = natural killer; SSC = sideward scatter

2.2.11 Statistical analysis

Gates with a count of less than two cells were set zero to exclude unspecific events. Basal activation and granzyme B production in the negative control were subtracted from alloantigen stimulated T cells to account for alloreactive T cells where appropriate. Statistical analysis was performed with Prism 7 (GraphPad). Gaussian distribution was tested before statistical comparison by D'Agostino & Pearson and Shapiro-Wilk normality tests, and parametric or non-parametric tests were used accordingly in the further analysis. The specific tests are indicated in the results section. When sample sizes were small, no statistical comparison was performed.

2.3 Main results

2.3.1 Overview

My PhD project was focused on establishing an assay to measure allograft reactive T cells to assess the risk of the patient for immunological complications after transplantation. The process of establishment can be divided into four steps: (1) Characterization of urinary cell cultures, (2) Measurement of alloantigen-reactive T cells, (3) Comparison of different sources of stimulating cells for alloreactive T cells and (4) Proof-of-principle study. The results regarding these goals will be summarized here briefly. For more details and the published figures, please consult the attached publication.[1]

2.3.2 Characterization of urinary cell cultures

The urinary cell cultures were characterized microscopically and by flow cytometry. In phase-contrast microscopy, the cultured cells showed an epithelial phenotype and formed domes, also described as hemicysts, which are characteristic for TEC.[22] Flow cytometric evaluation of markers for epithelial cells (cytokeratin), proximal and distal TEC (CD13 and EpCam)[29,30] and fibroblasts (CD90)[27,28] showed that the cultures consist mainly of kidney-derived TEC (Table 3).

Table 3: Cellular composition of urinary cell cultures (*n*=22)

Cell type	Median (%)	25th-75th percentiles	*P*	Statistical test
Tubular epithelial cells (TEC) Cytokeratin^{+} CD90^{-}	81.1	68.9 – 89.1	<0.0001	Wilcoxon matched-pairs signed rank test for non-parametric data
Fibroblasts Cytokeratin^{-} CD90^{+}	1.1	0.2 – 2.1		

It has been described before that TEC can upregulate HLA-ABC and HLA-DR molecules under inflammatory conditions.[32,36] The cultivated cells from the urine showed basal unstimulated HLA-ABC expression, but very little expression of HLA-DR. Simulation of the inflammatory environment in kidney transplants, present for example after ischemia-reperfusion injury or during rejection, was performed by treatment with IFNγ and TNFα resulting in a significant upregulation of both HLA-ABC and HLA-DR molecules (Table 4). This implies that cultivated TEC can present antigen to T cells and are therefore a possible interaction partner and a target for both CD4^{+} and CD8^{+} memory T cells in pro-inflammatory conditions.[12]

Table 4: HLA expression of tubular epithelial cell cultures with and without 20ng/ml interferon γ and 10ng/ml tumour necrosis factor α treatment (*n*=18)				
	Median	25th-75th percentiles	*P*	Statistical test
HLA-ABC median fluorescence intensity				
Without treatment	1670	1019 – 4072	<0.0001	Wilcoxon matched-pairs signed rank test for non-parametric data
With treatment	5346	3527 – 22444		
Fold change (With treatment / Without treatment)	4.22	2.77 – 4.96		
HLA-DR median fluorescence intensity				
Without treatment	32	15 – 43	<0.0001	Wilcoxon matched-pairs signed rank test for non-parametric data
With treatment	361	230 – 558		
Fold change (With treatment / Without treatment)	10	6.73 – 16.37		

2.3.3 Measurement of alloantigen-reactive T cells

Testing donor-reactive cellular immunity using donor stimulator cells from other organs than the transplant (i.e. blood or spleen) was first introduced 20 years ago.[21] To translate this approach to a novel source of stimulator cells, the transplant-derived TEC, was the next aim in this project. Therefore, we stimulated PBMC of kidney transplant recipients with their respective donor's TEC and assessed the activation by flow cytometry. As markers for activation we analysed the expression of CD137 (4-1BB), a member of the tumor necrosis factor receptor superfamily,[34] on $CD4^+$ and $CD8^+$ T cells, and CD154 (CD40L) on $CD4^+$ T cells.[35] $CD8^+$ T cells showed a significant upregulation of CD137 upon stimulation with TEC. This could be further enhanced by treatment of the TEC with IFNγ and TNFα prior to stimulation. $CD4^+$ T cells upregulated activation markers CD137 and / or CD154 when stimulated with TEC treated with IFNγ and TNFα, but not when stimulated with untreated TEC (Table 5). The lack of HLA-DR expression can explain the failure of untreated TEC to stimulate alloreactive $CD4^+$ T cells.

Table 5: T cell activation with and without donor-derived tubular epithelial cells (TEC) stimulation with and without interferon γ and tumour necrosis factor α pre-treatment (*n*=30)				
	Median	25th-75th percentiles	*P* Compared to negative control	Statistical test
Activated ($CD154$ / $CD137^+$) cells per 10^6 $CD4^+$ T cells				
Recipient PBMC alone	404	249 – 557	Reference	Friedman test for non-parametric data with Dunn's multiple comparison test (comparison with recipient PBMC alone)
Recipient PBMC + donor TEC	447	335 – 771	0.3934	
Recipient PBMC + IFNγ and TNFα treated donor TEC	712	380 – 1085	0.0004	
Activated ($CD137^+$) cells per 10^6 $CD8^+$ T cells				
Recipient PBMC alone	801.8	574 – 1802	Reference	Friedman test for non-parametric data with Dunn's multiple comparison test (comparison with recipient PBMC alone)
Recipient PBMC + donor TEC	1811	907 – 2757	0.0008	
Recipient PBMC + IFNγ and TNFα treated donor TEC	2212	1175 – 4748	0.0001	

To assess the specificity of the assay to measure alloreactive T cells, we cultivated TEC of healthy individuals and used them to stimulate autologous and allogenic PBMC. While the T cells reacted to allogeneic TEC, there was only negligible T cell activation towards autologous TEC, demonstrating that allogenicity is the factor that drives T cell stimulation by TEC (Table 6). Taken together, these experiments show that the specific assessment of donor-reactive T cells using our approach is feasible.

Table 6: Healthy donor T cell activation after stimulation with autologous or allogeneic tubular epithelial cells (TEC) (*n*=4 PBMC donors)		
	Median	Range
Activated ($CD154$ / $CD137^+$) cells per 10^6 $CD4^+$ T cells		
Allogeneic PBMC + IFNγ and TNFα treated TEC	403	304 – 502
Autologous PBMC + IFNγ and TNFα treated TEC	66	59 – 73
Activated ($CD137^+$) cells per 10^6 $CD8^+$ T cells		
Allogeneic PBMC + IFNγ and TNFα treated TEC	854	704 – 1004
Autologous PBMC + IFNγ and TNFα treated TEC	111	69 – 152

2.3.4 Comparison of different sources of stimulating cells for alloreactive T cells

Various trials have used donor-splenocytes to stimulate and assess donor-specific T cells and demonstrated promising results.[17,18,20] Therefore, we aimed to directly compare both stimulator cell types. Comparison of HLA expression on splenocytes and IFNγ and TNFα treated TEC revealed that HLA expression is higher on splenocytes (Table 7). Nevertheless, the number of $CD4^+$ T cells reacting to the donor-TEC was higher as compared to T cells reacting to donor-splenocytes (Table 8). This was surprising, however it has been described before that alloreactive T cells can be tissue-specific,

depending on the allopeptides that are presented on the HLA molecules, which might explain our findings.[10,13]

Table 7: HLA expression of splenocytes and interferon γ and tumour necrosis factor α treated tubular epithelial cell cultures (n=4)		
	Median (%)	25th-75th percentiles
HLA-ABC expressing cells		
Splenocytes	99.4	98.6 – 99.8
Tubular epithelial cells	95.5	88.7 – 97.5
HLA-DR expressing cells		
Splenocytes	76.8	72.5 – 90.8
Tubular epithelial cells	12.6	11.4 – 21.2

Table 8: T cell activation after stimulation with donor-derived splenocytes or interferon γ (IFNγ) and tumour necrosis factor α (TNFα) treated tubular epithelial cells (TEC) (*n*=15)				
	Median	25th-75th percentiles	*P*	Statistical test
Activated (CD154 / $CD137^{+}$) cells per 10^6 $CD4^{+}$ T cells				
Recipient PBMC + splenocytes	80	0 – 350	0.0001	Wilcoxon matched-pairs signed rank test for non-parametric data
Recipient PBMC + IFNγ and TNFα treated donor TEC	670	400 – 930		

2.3.5 Proof-of-principle study

Numerous previous studies showed a correlation between pre-transplant alloreactive memory T cells measured by IFNγ-ELISPOT and kidney graft function after transplantation, as well as higher rates of pre-transplant positive IFNγ-ELISPOT counts in patients developing acute rejection.[17,18,20] Therefore, we tested our new method in a cohort of 14 kidney transplant patients. The design of the study is outlined in Figure 4.

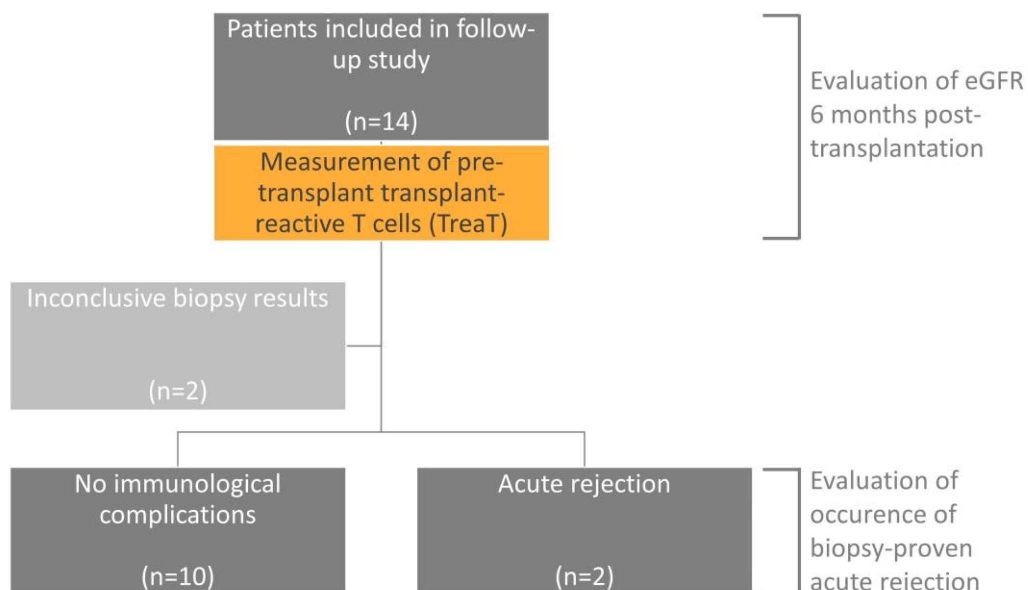

Figure 4: Flow diagram of the proof-of-principle study (eGFR = estimated glomerular filtration rate)

The most important parameter for the assessment of kidney function is the glomerular filtration rate (GFR). In clinical practice, the typically used way to determine the GFR is to calculate it from the creatinine-levels in the peripheral blood, taking into account several factors depending on the formula. In the case of the commonly used CKD-EPI formula, these factors are the age, sex, skin colour, and a factor for the creatinine-level of the patient. The resulting estimated glomerular filtration rate (eGFR) is more sensitive to kidney function impairments than the serum-creatinine alone.[2] eGFR values above 90 are considered normal, and values beneath 15 are considered end-stage renal failure.[2] We could demonstrate that the number of pre-transplant alloreactive $CD4^+$ and $CD8^+$ T cells stimulated with kidney transplant TEC negatively correlated with the eGFR at 6 months after transplantation (Table 9). This is in line with results from multiple other working groups that used the IFNγ-ELISPOT assay with splenocytes for the stimulation of pre-transplant alloreactive T cells and could also demonstrate a correlation with the eGFR 6 or 12 months after transplantation.[20]

Table 9: Correlation of estimated glomerular filtration rate (eGFR, calculated with CKD-EPI) 6 months post transplantation and T cell activation after interferon γ (IFNγ) and tumour necrosis factor α (TNFα) treated donor-derived tubular epithelial cell (TEC) stimulation (*n*=14)

	Median	25th-75th percentiles	*r*	95% confidence interval	*P*	Statistical test
Activated (CD154 / $CD137^+$) cells per 10^6 $CD4^+$ T cells vs eGFR 6 months post-transplantation						
$CD4^+$ T cells	150	0 – 504	-0.5566	-0.8393 – -0.03698	0.0387	Pearson correlation coefficient
eGFR	52	33 – 56				
Activated ($CD137^+$) cells per 10^6 $CD8^+$ T cells vs eGFR 6 months post-transplantation						
$CD8^+$ T cells	390	0 – 3801	-0.6397	-0.8737 – -0.1652	0.0138	Pearson correlation coefficient
eGFR	52	33 – 56				

In addition, we analysed the clinical course of the patients with regard to the development of acute rejection episodes (Figure 4). Ten patients showed no signs of immunological complications after transplantation and were included in the control group. Two patients developed graft dysfunction after transplantation, but the reason remained elusive despite several biopsies and they were not included in the further analysis. Two patients developed a delayed graft function and showed signs of acute rejection in a subsequent biopsy. They were included in the acute rejection group.

Eight of the control patients were male and two female. Their median age was 64.5 (range 31-77). They spent a median time of five years on dialysis before transplantation (range 3-11 years). The patients in the acute rejection group were both male. One was 73, the other 74 years old. Both spent 12 years on dialysis before transplantation. Concerning immunological risk factors, the control and the acute rejection group were comparable. The current panel reactive antibody level was zero percent in all patients. The median HLA-mismatch was four (range 0-6) in the control patients and five (range 4-6) in

the acute rejection patients. The cold-ischemia time was below 24 hours in all patients. However, the median donor age was lower in the control patients with 59 years (range 23-74), compared to 74 and 76 years in the acute rejection patients. One control patient had a previous kidney transplant and one a transplant of another organ. One acute rejection patient had a previous kidney transplant. All patients received an induction therapy with anti-IL2-receptor-antibody (basiliximab). Maintenance immunosuppression consisted of tacrolimus, mycophenolate-mofetil and methylprednisolone in all patients except for one control patient, who received cyclosporine, everolimus, and methylprednisolone.

We measured the numbers of alloreactive T cells with our novel assay using donor-derived TEC. Patients that had an acute rejection episode after transplantation showed higher values of allograft reactive T cells prior to transplantation compared to patients without immunological complications. Analysis of alloreactive T cell subsets revealed that $CD4^+$ T cells expressing CD161, a hallmark of TH17 cells[33], and $CD8^+$ T cells producing the cytotoxic effector molecule granzyme B, are especially numerous in patients developing an acute rejection. Comparing these subsets therefore allowed for a clearer distinction of the two patient groups (Table 10).

Table 10: Pre-transplant T cell activation after donor-derived interferon γ (IFNγ) and tumour necrosis factor α (TNFα) treated donor-derived tubular epithelial cell (TEC) stimulation in acute rejection and control patients (*n*=12)

	Control patients (*n*=10)		Acute rejection patients (*n*=2)	
T cell subset	Median	Range	Median	Range
Activated (CD154 / $CD137^+$) cells / 10^6 $CD4^+$ T cells	74	0 – 490	796	547 – 1044
$CD161^+$ activated (CD154 / $CD137^+$) cells / 10^6 $CD4^+$ T cells	0	0 – 73	459	397 – 520
Activated ($CD137^+$) cells / 10^6 $CD8^+$ T cells	70	0 – 3494	5406	4828 – 5984
Granzyme B^+ activated ($CD137^+$) cells / 10^6 $CD8^+$ T cells	0	0 – 105	1028	452 – 1605

All in all, these experiments provide first evidence that the TreaT-assay can help in the identification of patients developing immunological complications after transplantation due to a high number of alloreactive memory T cells.

2.4 Clinical implications and open research questions

In our study, we investigated the usage of urine-derived kidney cells to study alloreactive T cells and demonstrated the quantification and characterization of alloreactive T cells in kidney transplant patients. In a translational approach, a novel assay based on these results was developed. A proof-of-principle study showed that the assay could possibly be used in the prediction of acute rejection episodes and the post-transplant allograft function. An extensive discussion of the results can be found in the attached publication.[1] In this outlook, I will focus on the steps that have to be addressed in follow-up in order to develop a test that can be applied in clinical routine monitoring.

2.4.1 Simplification of the assay, reduction of time to first results and standardization

In its current form, the protocol is very labour-intensive. Therefore, a simplification of the assay is necessary in order to implement it in routine diagnostic procedures. In a following and ongoing research project, we aim to modify several parts of the protocol, for example the current read-out with flow cytometry, in order to facilitate execution, analysis and standardization. In line with this, the costs per test and the availability of the required material are important to consider.

Another important factor for an improved applicability is the time needed between the collection of urine and blood samples and the first results. Currently, two to three weeks are necessary to cultivate sufficient numbers of TEC from a single urine sample. Therefore, in the current form, the test could only deliver results after the very early post-transplant period. Collection of multiple urine samples could increase the number of colonies and shorten the time needed to obtain enough TEC. Furthermore, a downscaling of the number of TEC to stimulate immune cells can shorten the time until first results are available. In living-donations, a collection of urine from the donor before transplantation can assure on-time availability of TEC. In deceased-donor transplantation, the remaining gap between transplantation and first results with our assay can be bridged with stimulator cells from other sources, such as HLA-bank cells (for example TEC from healthy donors) or donor-splenocytes. Currently, approximately 48h are needed for the test performance, which is comparable to the IFNγ ELISPOT test[37] and to the time until biopsy samples are analysed and results reported. After drawing of blood, the most time-consuming steps are isolation of PBMC, (optional) cryopreservation and resting of PBMC after thawing, the treatment of TEC with IFNγ and TNFα for 24h, the stimulation of PBMC with TEC for 16h as well as the staining procedure, flow-cytometric recording of samples and sample analysis. Therefore, a modification of these factors, for example shortening incubation times and using a different read-out parameter has the potential to facilitate and shorten the assay procedure.

Standardization across different laboratories is inevitable to enable a broad application of the assay. This involves the development of standard operating procedures (SOP), comparison of different

laboratory equipment and reagents, sample collection, training of personnel and assessment of the intra- and interlaboratory variability, among others.[37] Therefore, it is important to design the assay in an easily standardisable way, to facilitate the translation into patient care.

2.4.2 Application scenarios

Biomarkers for acute rejection can have different functions and can be used for diverse purposes. The evaluation of different usage scenarios has important implications for the marker and study design. It is therefore important to consider aspects of medical value, feasibility and commercial aspects for each possible application.

My thesis focused on the use as a **prognostic marker**, a marker that is used to stratify the patient's risk for developing an acute rejection. For example, a patient identified to have a high risk can be treated with higher doses of immunosuppressants and monitored more closely for complications.[17,19,38]

A **diagnostic marker** can be used to diagnose or rule out acute rejection episodes. The current reference standard, a kidney transplant biopsy, has several disadvantages, as it has been discussed in the introduction. Most importantly, it is an invasive procedure with the attached risks, costs and time and is therefore a burden for the patient, the performing physician and the health care system. A non-invasive test system for the diagnosis of acute rejection would thus be of great value for transplantation medicine.[17] A combination with other markers can be beneficial, for example in a triage test. The result of our assay can rule out or rule in acute rejection, and a biopsy can be performed accordingly only in the patients with a high probability.[39] In slightly different designs, such a combined approach is also possible in the other usage scenarios. A diagnostic marker can also be used to classify a condition into subtypes with consequences regarding prognosis and treatment.[19]

A **monitoring marker** is a measurement for the current risk or disease status of acute rejection and can change with time, for example in response to treatment.[19]

A **predictive marker** indicates the reaction of the patient to a specific therapeutic intervention. It can be used to identify patients that have a high or low probability of benefiting from an intervention and should therefore receive or not receive this specific therapy, respectively.[19]

A marker that can reliably predict or is strongly associated with a clinical endpoint can be of use as a **surrogate endpoint** for clinical trials. An intervention or treatment could then be evaluated based on this marker.[17]

Depending on the intended clinical role, the requirements for diagnostic accuracy as described below vary.

2.4.3 Expansion of clinical data and assessment of assay performance

The assay performance for the above mentioned application scenarios needs to be tested in clinical studies. An important factor in the design of the study is the selection of patients. To obtain reliable assay parameters, the cohort needs to be representable of the patients that will be tested later. Exclusion of patients with certain clinical conditions or other features might lead to a selection bias. The intended sample size needs to be calculated in a power-analysis based on experiences and estimates regarding sensitivity and specificity, the mean difference between the groups and the variance and with regard to the specific hypothesis and objectives of the study. Important parameters for the diagnostic accuracy are sensitivity, specificity, positive and negative predictive value and the area under receiver-operating-characteristics (ROC) curves. The relationship between sensitivity, specificity, positive and negative predictive value can be depicted in a fourfold table (Figure 5). In this table, the numbers of patients tested positive or negative in the novel test are entered and compared to the results of the reference standard, in this case transplant biopsies. In a perfect study, the positive and negative predictive value can be calculated directly from the study results as depicted in Figure 5. However, in order to reduce the number of study participants, in most studies there is a selection of patients that have a higher risk of developing the target condition (for example by prospective sampling and retrospective analysis). As the positive and negative predictive value are strongly associated with the prevalence, the values need to be corrected for the prevalence using Bayes Theorem in order to avoid base rate fallacy.[17] The consequences of false positive and false negative results have to be evaluated regarding the different application scenarios. In the example of a diagnostic test, very low rates for both errors are important in order to base a treatment decision solely on the test results. A test with a very high negative predictive value, but a lower positive predictive value could still be used to rule out patients that do not have acute rejection, however. In a prognostic scenario, a test with a high positive predictive value could be used to identify patients that need to be monitored more closely than patients with a negative test result.[18]

		Biopsy result (reference standard)		
		Acute rejection	No acute rejection	
TreaT test result	TreaT positive	True positive	False positive (Type 1 error)	Positive predictive value $\frac{\sum \text{True positive}}{\sum \text{TreaT positive}}$
	TreaT negative	False negative (Type 2 error)	True negative	Negative predictive value $\frac{\sum \text{True negative}}{\sum \text{TreaT negative}}$
		Sensitivity $\frac{\sum \text{True positive}}{\sum \text{Biopsy acute rejection positive}}$	Specificity $\frac{\sum \text{True negative}}{\sum \text{Biopsy acute rejection negative}}$	

Figure 5: Fourfold table (adapted from Lo et al., Nat Rev Nephrol, 2014)[17]

2.4.4 Conclusion

It is a long road to implementation of a novel assay into the clinical routine. This thesis provides the first milestone on this way. A reliable, non-invasive test for the prediction or diagnosis of rejection episodes after kidney transplantation can reduce costs and burdens for the patient and the health care system and enable personalized immunosuppressive medicine. Therefore, a continuation of the efforts along this long road might prove of great value in the future of kidney transplantation medicine.

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3 Declaration of own contribution

Constantin Thieme contributed the following to the below listed publication:

Thieme, C. J., Weist, B. J. D., Mueskes, A., Roch, T., Stervbo, U., Rosiewicz, K., Wehler, P., Stein, M., Nickel, P., Kurtz, A., Lachmann, N., Choi, M., Schmueck-Henneresse, M., Westhoff, T. H., Reinke, P. & Babel, N. **The TreaT-Assay: A Novel Urine-Derived Donor Kidney Cell-Based Assay for Prediction of Kidney Transplantation Outcome**. Scientific Reports (2019)

CT was a main contributor to all aspects of the study. A detailed description can be found below.

Assay establishment: NB had the initial assay idea and entrusted **CT** with the topic. **CT** had a major influence on conceptualization and design of the assay, together with NB and BW. **CT** planned, carried out and analysed experiments for the assay as well as for the flow cytometry panels, initially under supervision by BW and later mostly independently.

Literature research: Literature research was done mostly independently by **CT** with some suggestions by his supervisors.

Sample collection: **CT** and AM collected the transplant patient samples with help by Anett Sefrin after the patients have been informed about the study by PN, MC and PR. Documentation of patient characteristics was done by **CT** and AM with help by PN and MC.

Experiments:

- **Figure 1**: **CT** designed and drew the figure.
- **Figure 2**: **CT** identified and chose the markers for characterization of tubular epithelial cells and fibroblasts by literature research and based on experiences in the workgroup. Establishment of the culture of tubular epithelial cells from the urine was done mostly by KR, together with **CT** and BW. Isolation of cells from patients' urine samples and cultivation of the cells was done by AM and **CT**. Experiments were designed by **CT** together with his supervisors. Experiments, data analysis and presentation were carried out by **CT**.
- **Figure 3 a-c**: **CT** designed the experiments together with his supervisors. Culture of TEC and isolation of PBMC from patients was performed by **CT** and AM. Experiments, data analysis and presentation were carried out by **CT**.
- **Figure 3 d-e**: **CT** designed the experiments together with his supervisors. Culture of TEC and isolation of PBMC from healthy donors were done by PW. Experiments and data analysis were carried out by TR and PW. **CT** prepared the figures.
- **Figure 4**: Splenocytes were isolated and frozen by MS. **CT** designed the experiments together

with his supervisors. Experiments, data analysis and presentation were carried out by **CT**.

- **Figure 5**: **CT** designed the experiments together with his supervisors. Experiments, data analysis and presentation were carried out by **CT**. Figure 5f was conceptualized by US.

Preparation of manuscript: **CT** wrote the first draft of the complete publication (abstract, introduction, material and methods, results and discussion) and finalized the publication after detailed discussion and suggestions of the co-authors, especially NB and TR. He carried out corrections suggested during peer-review together with NB and TR.

____

Signature, date and stamp of first supervising university professor / lecturer

____

Signature of doctoral candidate

4 Statutory Declaration

"I, Constantin Thieme, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic

The TreaT-Assay – Determining the Immunological Risk after Kidney Transplantation by Measuring T cell Alloreactivity towards Urine-Derived Renal Transplant Cells

Das TreaT-Assay – Bestimmung des immunologischen Risikos nach Nierentransplantation durch Messung der T-Zell-Alloreaktivität gegen Transplantatzellen aus dem Urin

independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

5 Journal Summary List

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1	NATURE	745,692	43.070	1.285010
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3	National Science Review	1,842	13.222	0.006500
4	Science Advances	21,901	12.804	0.110010
5	Nature Communications	243,793	11.878	1.103290
6	Nature Human Behaviour	1,230	10.575	0.006550
7	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	661,118	9.580	1.022190
8	Science Bulletin	3,569	6.277	0.009840
9	Scientific Data	3,240	5.929	0.015610
10	Frontiers in Bioengineering and Biotechnology	1,994	5.122	0.006540
11	Journal of Advanced Research	2,691	5.045	0.004780
12	Research Synthesis Methods	1,932	5.043	0.005420
13	GigaScience	2,674	4.688	0.012510
14	Annals of the New York Academy of Sciences	46,385	4.295	0.025840
15	Scientific Reports	302,086	4.011	1.061540
16	Journal of the Royal Society Interface	12,933	3.224	0.029190
17	NPJ Microgravity	203	3.111	0.000670
18	PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A-MATHEMATICAL PHYSICAL AND ENGINEERING SCIENCES	19,227	3.093	0.028200

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The TreaT-Assay: A Novel Urine-Derived Donor Kidney Cell-Based Assay for Prediction of Kidney Transplantation Outcome

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Donor-reactive immunity plays a major role in rejection after kidney transplantation, but analysis of donor-reactive T-cells is not applied routinely. However, it has been shown that this could help to identify patients at risk of acute rejection. A major obstacle is the limited quantity or quality of the required allogenic stimulator cells, including a limited availability of donor-splenocytes or an insufficient HLA-matching with HLA-bank cells. To overcome these limitations, we developed a novel assay, termed the TreaT (Transplant reactive T-cells)-assay. We cultivated renal tubular epithelial cells from the urine of kidney transplant patients and used them as stimulators for donor-reactive T-cells, which we analyzed by flow cytometry. We could demonstrate that using the TreaT-assay the quantification and characterization of alloreactive T-cells is superior to other stimulators. In a pilot study, the number of pre-transplant alloreactive T-cells negatively correlated with the post-transplant eGFR. Frequencies of pre-transplant CD161$^+$ alloreactive CD4$^+$ T-cells and granzyme B producing alloreactive CD8$^+$ T-cells were substantially higher in patients with early acute rejection compared to patients without complications. In conclusion, we established a novel assay for the assessment of donor-reactive memory T-cells based on kidney cells with the potential to predict early acute rejection and post-transplant eGFR.

Kidney transplantation is the standard therapy for end-stage renal diseases. Acute (AR) or chronic rejection are among the biggest challenges in transplantation medicine, and donor reactive immunity is an important factor that counters allograft acceptance[1]. Immunosuppressive medication is given to control or prevent immune reactions; however, this medication has serious side effects. Many previous studies report heterogeneous risk profiles with respect to post-transplant complications such as AR or infections, suggesting introduction of personalized immunosuppressive therapy regimen[2–4]. For such individual therapy biomarkers that allow the discrimination between patients with different risk profiles are required[2,4–6]. The presence of donor-reactive T cells pre- and

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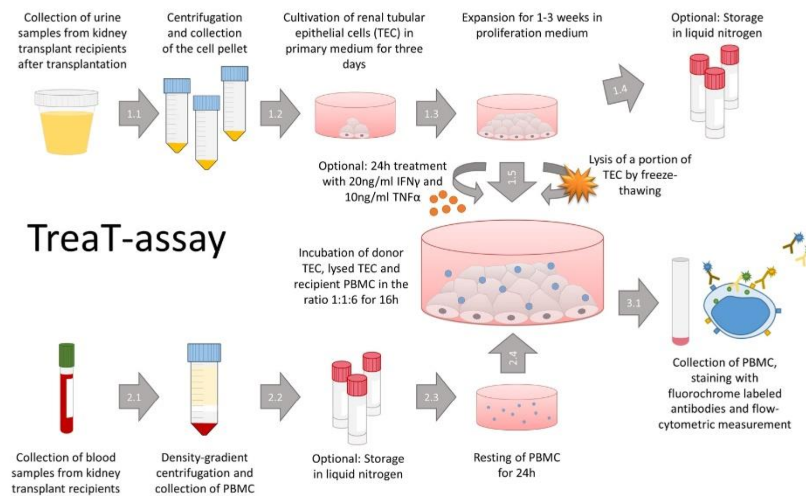

Figure 1. Methodological overview of the TreaT-assay. For a detailed description please consult the materials and methods section of the main text.

post-kidney-transplantation has been demonstrated to be associated with AR and reduced allograft survival[1,7,8]. Therefore, T cell allosensitization assays have been developed and successfully tested as predictors of the transplant outcome[7,9–12]. In these assays, the recipient's peripheral blood cells are stimulated by alloantigen sources presenting foreign human leukocyte antigen (HLA) molecules. Currently, two sources of stimulator cells are used. One source are donor splenocytes collected during removal of the donor kidney[12,13]. However, the availability of splenocytes is limited and only feasible from deceased donors. The second source of allogenic cells is a cell bank representing the most common HLA-types[10,13]. Unfortunately, these cells often lack the adequate quality as the matching between donor and recipient HLA-types is frequently insufficient. Furthermore, since both sources are not allograft-derived, the peptides they present do not exactly resemble the tissue specific peptides presented by renal tubular epithelial cell (TEC), the main target during AR[14]. Another limitation of the currently used methods is the lack of a broad phenotypic and functional characterization of the detected alloreactive cells[3]. These underscore the need for a specific and sensitive assay for the monitoring and in-depth characterization of allograft-reactive T cells. We therefore developed the Transplant reactive T cell (TreaT)-assay by means of donor-derived renal cells obtained from recipient´s urine, a handy and renewable antigenic source for stimulation. Using multiparameter flow cytometry, we showed that our assay has the potential to predict the graft function post-transplantation and early episodes of AR and could thus serve as an important tool for guiding precision immunosuppressive regimens in kidney transplant patients. Furthermore, the characterization of alloreactive T cells has the potential to reveal new molecules involved in the process of AR.

Materials and Methods

An overview of the TreaT-assay setup can be found in Fig. 1.

Patients. Four healthy volunteers as well as in total twenty-two patients transplanted in the Charité - Universitätsmedizin Berlin were recruited and analyzed in a cross-sectional study for the assay establishment at different time-points pre- and post-transplantation. No organs were procured from prisoners. The number of patients and samples analysed in the study differed in dependence from the aim addressed in the study. Thus, 22 patients were recruited for the cultivation procedure of TEC. For analysis of HLA expression on TEC, samples of 18 patients were used. For the assay establishment, samples of 13 patients were collected. The prediction of eGFR were performed using the samples of 14 patients, and 12 patients were followed up clinically for the prediction of acute rejection. For the comparison of stimulatory capacity between splenocytes and TEC, samples of 4 patients were collected (Supplementary Table S1). All patients gave written informed consent and the study was approved by the ethics commission of the Charité – Universitätsmedizin Berlin, Germany in accordance with the declaration of Helsinki.

Collection of peripheral blood mononuclear cells (PBMCs). Blood was collected from each kidney transplant recipient recruited into the study at least once and up to four times in Vacutainers containing lithium heparin (Beckton Dickinson (BD), Franklin Lakes, US-NJ). The PBMCs were isolated by density gradient centrifugation Ficoll-Paque (Biochrom, Berlin, Germany). The cells were frozen in CryoPure tubes (Sarstedt, Nümbrecht, Germany) in Roswell Parm Memorial Institute-1640 medium (RPMI, Biochrom) with 60% fetal bovine serum (FBS, Biochrom) and 10% dimethylsulfoxid (DMSO; Sigma-Aldrich, Munich, Germany) at −80 °C

in a freezing box (ThermoFisher, Waltham, US-MA) containing isopropylalcohole (Sigma-Aldrich) and stored in liquid nitrogen until further usage. Upon defrosting, the cells were left resting for 24 hours.

Cultivation of tubular epithelial cells. Up to 300 ml of urine were collected in sterile flasks (Corning Falcon, Corning, US-NY) within the first week after kidney transplantation. To ensure the donor origin of urine cells, urine was collected from mono pigtail stents in patients with existing residual diuresis. The samples were then processed further according to a previously published protocol[15]. Briefly, the urine sediment was washed with phosphate buffered saline (PBS, ThermoFisher) and seeded in primary medium, containing Dulbecco's modified Eagle medium (DMEM, ThermoFisher) and Ham's F12 (Biochrom) in a 1/1 ratio, 10% FBS, renal epithelial growth medium (REGM) SingleQuot kit (Lonza Clonetics), 100 U/ml penicillin/streptomycin (P/S, Biochrom), 2.5 µg/ml amphotericin B (Biochrom), 100 µg/ml normocin (InvivoGene) and 10 µg/ml ciprofloxacin (Fresenius Kabi Austria). After three days, the medium was replaced by the proliferation medium, containing renal epithelial basal medium (REBM, Lonza, Basel, Swiss), REGM SingleQuot kit, 10% FBS, 2.5 mM GlutaMAX (ThermoFisher), 1% non-essential amino acids (ThermoFisher) and 100 U/ml / 100 µg/ml P/S. Mycoplasma contamination was monitored in random samples (MycoAlert, Lonza). The cells were cultivated until confluency, harvested using trypsin/EDTA-solution (Biochrom), and frozen as described above until further usage.

Induction of HLA expression. Frozen tubular epithelial cells (TEC) were thawed, seeded in two cell-culture flasks of 75 cm^2 (Coning Falcon), and cultivated in proliferation medium until confluency. Then, the flasks were incubated with RPMI containing 10% FBS and 100 U/ml P/S with or without supplementation of 20 ng/ml interferon γ (IFNγ) and 10 ng/ml tumor necrosis factor α (TNFα) (both Miltenyi Biotec, Bergisch-Gladbach, Germany) to enhance or induce surface HLA-ABC and -DR expression. After 24 h, the TEC were harvested. A fraction of the cells was analysed by flow cytometry and the rest was used as stimulators in an alloantigen-assay.

Cell lysis. To mimic tissue damage and to facilitate presentation of alloantigens by antigen presenting cells, a fraction of TEC were lysed by centrifugation with 4500 g, vortexing and repeated freeze-thawing at −20 °C in RPMI until further usage.

Preparation of donor splenocytes. Pieces of at least 6 cm of spleen from kidney-transplant donors were used for further preparation. Splenic connective tissue was removed with sterile forceps and scalpel (Feather, Osaka, Japan) and minced through a 100 µm and a 40 µm sterile cell strainer sieve (BD) with PBS. Mononuclear cells were isolated by Ficoll gradient and frozen as described above. Frozen splenocytes were thawed in RPMI containing 1% DNAse (Roche Diagnostics, Rotkreuz, Swiss) 12 h prior to use in the alloantigen-assay. A fraction of the splenocytes was lysed as described above. To discriminate recipient's PBMC and donor splenocytes in flow cytometry, the remaining splenocytes were labelled with 5 µM carboxyfluorescein succinimidyl ester (CFSE, Sigma) one hour before cultivation in the alloantigen-assay.

Alloantigen-assay. 0.25×10^6 TEC treated with or without IFNγ- and TNFα were seeded into 24-well-plates (Corning Falcon). 1.5×10^6 recipient PBMCs and lysates of 0.25×10^6 TEC were added (ratio TEC:lysed TEC:PBMC = 1:1:6). For the experiments comparing autologous with allogenic stimulation, TEC isolated from two healthy volunteers were incubated with fresh, not cryopreserved PBMCs of the same or a different donor. PBMCs seeded into wells without TEC and lysed TEC served as the negative control. The positive control was treated with phorbol 12-myristate 13-acetate (PMA, Sarstedt) and ionomycin (Iono, ThermoFisher). All wells were incubated with 1 µg/ml brefeldin A (Sigma) and 1 µl/ml protein transport inhibitor containing monensin (BD). Additional specimen with 0.25×10^6 CFSE labelled kidney transplant donor-derived splenocytes and 0.25×10^6 lysed splenocytes as stimulators for 1.5×10^6 recipients' PBMCs were set. After 16 hours of incubation the PBMCs were harvested and stained for flow cytometry.

Flow cytometry staining of TEC and PBMCs. Cell surface of PBMCs and splenocytes was stained with anti-CD161 Brilliant Violet (BV) 510 (clone HP-3G10, Biolegend, San Diego, US-CA) and Live/Dead Blue (ThermoFisher) to exclude dead cells. Intracellular staining of PBMCs was performed with FoxP3-Permeabilization Buffer (ThermoFisher) and with anti-CD3 Brilliant Ultraviolet 737 (clone UCHT1, BD), anti-CD4 BV650 (clone OKT4, Biolegend), anti-CD8 allophycocyanin (APC)/Cy7 (clone RPA-T8, Biolegend), anti-CD137 phycoerythrin (PE)/Cy5 (clone 4B4-1, BD), anti-CD154 peridinin-chlorophyll-protein (PerCP)/Cy5.5 (clone 24-31, Biolegend) and anti-granzyme B Alexa Fluor 700 (clone GB11, BD).

Cell surface of TEC was stained with Live/Dead Blue, anti-CD13 APC (clone WM15, Biolegend), anti-CD90 PerCP/Cy5.5 (clone 5E10, Biolegend), anti-CD326 (EpCam) FITC (9C4, Biolegend), anti-HLA-ABC PE (clone 311506, Biolegend) and anti-HLA-DR APC/Cy7 (clone L243, Biolegend). Anti-cytokeratin BV421 (clone CAM5.2, BD) was administered for intracellular staining. Anti-HLA-ABC PE and anti-HLA-DR APC/Cy7 were also used for staining of splenocytes.

Patient samples were measured using LSRFortessa (BD) while healthy donors were measured using CytoFlex LX (Beckman Coulter, Brea, US-CA). Gating and analysis was performed with FlowJo version 10 (FlowJo LLC, Ashland, US-OR). Analyses of patient samples and healthy blood donors by two different flow cytometers were not critical, since the data of both populations were not directly compared to each other in the study.

Statistical analysis and graphical representation. Gating of flow cytometry data was performed with unstained, untreated, and adequate fluorescence minus one (FMO) controls according to standard flow cytometry guidelines[16]. HLA expression on TEC was analysed as median fluorescence intensity (MFI). $CD137/CD154^+$ $CD4^+$ and $CD137^+$ $CD8^+$ T cells and their subsets were analysed as cells per 10^6 $CD4^+$ and $CD8^+$ T cells, respectively. Counts of less than two cells per gate were excluded. To account for specifically activated cells and their

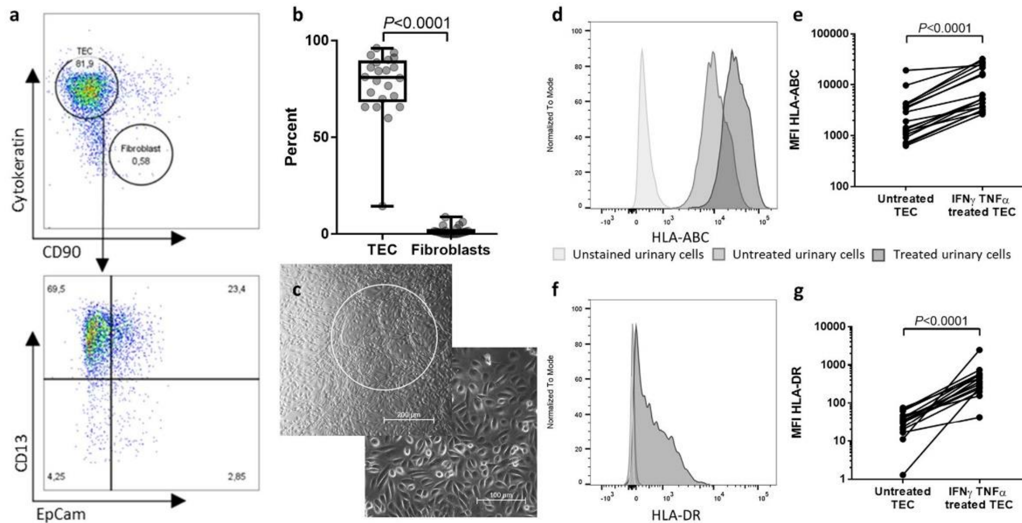

Figure 2. Urinary culture cells mainly consist of tubular epithelial cells (TEC) and present HLA-ABC and -DR-molecules upon IFNγ and TNFα treatment. (**a**) Representative flow cytometric characterization of urinary culture cells. Urine was collected from kidney-transplant patients after transplantation and the cell pellet was seeded in culture media after centrifugation. The colonies were then expanded in proliferation media for 1–3 weeks. After harvesting, the cells were stained for epithelial cell marker cytokeratin, proximal and distal renal tubular cell markers CD13 and EpCam, and for fibroblast marker CD90. One representative example of 22 individual donors is demonstrated. (**b**) Quantification of TEC (cytokeratin$^+$ CD90$^-$) and Fibroblasts (cytokeratin$^-$ CD90$^+$) among living urinary culture cells ($n = 22$). Cells were analysed after 3–6 weeks in culture. Statistical comparison was done with Wilcoxon matched-pairs signed rank test for not normally distributed samples. (**c**) Representative phase-contrast microscopy of two samples of urinary cell cultures. The upper picture shows the characteristic dome formation of TEC (white circle). The cell morphology shown in the lower picture indicates epithelial cells. Magnification 20 × (left) and 10 × (right). A representative example for 22 individual donors is shown. (**d–g**) Expression levels indicated by median fluorescence intensity (MFI) of HLA-ABC (**d**,**e**) and HLA-DR ((**f**,**g**) on urine-derived TEC with and without addition of 20 ng/ml IFNγ and 10 ng/ml TNFα for 24 h ($n = 18$). Harvested cells were analysed by flow cytometry. Histograms are representative for n = 18. Statistical analysis was done with Wilcoxon matched-pairs signed rank test for not normally distributed samples.

phenotype, the corresponding population in the negative control was subtracted, except for the experiments to show the specificity of the assay where the negative control is depicted for a better illustration of the background T cell activity. Statistical analysis was performed with Prism 7 (GraphPad Software, San Diego, US-CA). Gaussian distribution was assessed using D'Agostino & Pearson normality test and Shapiro-Wilk normality test. Parametric or non-parametric statistical tests were used accordingly as indicated. In detail, comparison of HLA expression as well as the reactivity of recipient PBMC to donor UC and splenocytes was done with Wilcoxon matched-pairs signed rank test for not normally distributed samples. To test the ability of UC to stimulate alloantigen-reactive T cells, non-parametric statistics was done with Friedman-Test and Dunn's multiple comparison test. As sphericity was not assumed, the Greisser-Greenhouse correction was applied. Correlation of eGFR (calculated with CKD-EPI) and pre-transplant alloreactive T cells was calculated with Pearson's correlation coefficient.

Results

Generation of urine-derived tubular epithelial cells and induction of HLA-ABC and –DR expression. In total, four healthy individuals and twenty-two kidney-transplant patients were included in a cross-sectional study in order to establish our novel methodological approach. Cells were cultured from all twenty-two patients and two healthy individuals. Flow cytometric expression of cytokeratin, CD13, and epithelial cell adhesion molecule (EpCam), which define tubular epithelial cells (TEC)[17,18], as well as CD90 as a marker for contaminating fibroblasts[19] was analysed. We found that the vast majority of the cultivated cells consists of TEC (median 81%, IQR 69–89%), mainly proximal (CD13$^+$) and to a lesser extent distal (EpCam$^+$) TEC (Fig. 2A,B). Microscopical evaluation confirmed the epithelial phenotype and the dome formation that is characteristic for TEC (Fig. 2C). We will therefore refer to the cells cultured from the urine as TEC in the following paragraphs.

To mimic the inflammatory environment during rejection and enhance HLA expression, the TEC were treated with IFNγ and TNFα for 24h[20]. The flow cytometric analysis of the HLA expression confirmed an

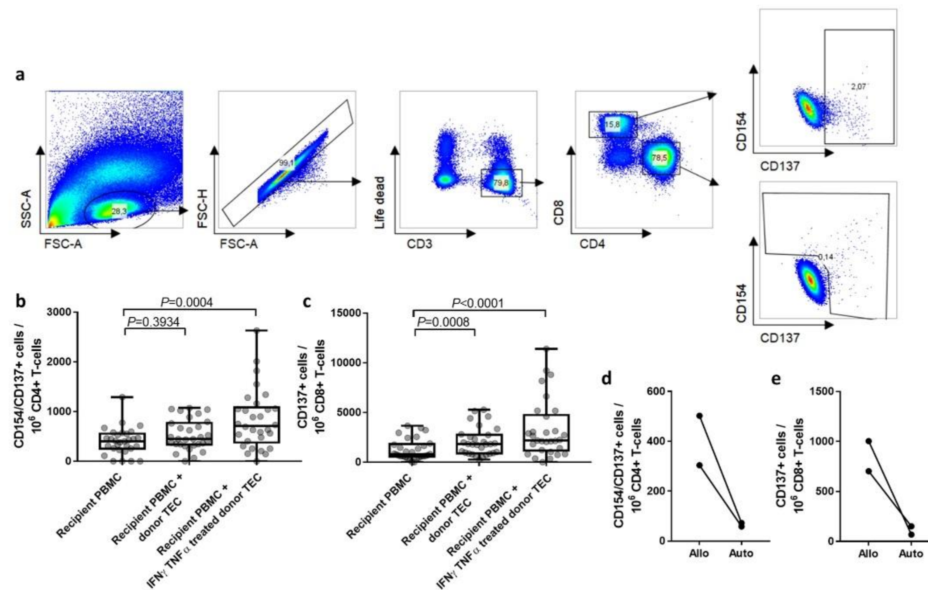

Figure 3. Alloreactive T cells can be monitored with urine-derived donor TEC. (**a**) Representative flow cytometry plots illustrating the gating strategy to identify single living alloreactive $CD3^+$ $CD4^+$ $CD154^+$/$CD137^+$ and $CD8^+$ $CD137^+$ lymphocytes. From left to right: Scatter plot of PBMCs distinguishing lymphocytes from debris and non-lymphocytes; doublet exclusion; discrimination of live $CD3^+$ T cell from dead and non-T cells; discrimination of $CD4^+$ and $CD8^+$ T cells; identification of reactive T cells according to CD137 and CD154 expression. (**b–c**) Urine-derived donor TEC can elicit a donor-reactive activation of recipients' $CD4^+$ (**b**) and $CD8^+$ (**c**) T cells. PBMC of 13 recipients obtained at different time points ($n=30$) were co-cultivated for 16 h with lysed and intact urine-derived donor TEC or with no further stimuli (negative control). The specific stimulation was further compared between donor TEC treated with 20 ng/ml IFNγ and 10 ng/ml TNFα for 24 h or untreated TEC. Activation of T cells was assessed by flow cytometric determination of $CD4^+$ $CD137^+$/$CD154^+$ and $CD8^+$ $CD137^+$ T cells as described in (**a**). Statistical comparison between the three experimental groups was performed with Friedman test and Dunn's multiple comparisons test. (**d–e**) Urine-derived TEC do not elicit an activation of autologous $CD4^+$ (**d**) and $CD8^+$ (**e**) T cells. TEC of two healthy donors were cultivated, treated with 20 ng/ml IFNγ and 10 ng/ml TNFα, partially lysed and incubated with autologous (auto) or randomly selected allogenic (allo) PBMCs ($n=4$ PBMC donors). T cell activation was assessed as described above.

increase of HLA-ABC on TEC when compared to the untreated sample (Fig. 2D,E; $P<0.0001$, $n=20$). Similarly, HLA-DR on TEC significantly increased after TNFα and IFNγ treatment (Fig. 2F,G; $P<0.0001$, $n=20$). Taken together, as it has been shown before for TEC derived directly from donor kidneys[21], our data portray the ability of urine-derived TEC to act as atypical antigen-presenting cells.

Alloreactive T cells can be stimulated with urine-derived TEC. To examine whether we can induce a specific activation of alloantigen-reactive T cells, we analysed the activation marker profile of T cells following 16 h of co-cultivation of recipient PBMC with respective donor TEC. Lysed TEC were added to intact TEC to facilitate presentation by antigen presenting cells and to mimic ischemic cell death that can occur during transplantation. Recipient PBMC alone were used to define the T cell baseline activation. For the assay establishment, PBMCs from 13 patients were obtained, some of them at different time points, so 30 samples were analysed in total.

Activated $CD4^+$ T cells were defined as activation marker CD154 and CD137 positive cells (Fig. 3A). Compared to the untreated controls we observed a significantly higher number of activated $CD4^+$ T cells in cultures where the TEC have been stimulated with IFNγ and TNFα (Fig. 3B; $P=0.0004$, $n=30$). Of note, untreated TEC did not induce specific activation of $CD4^+$ T cells ($P=0.3934$, $n=30$).

With regard to $CD8^+$ T cells, we observed an even higher proportion of alloreactive cells compared to the $CD4^+$ T cells. Cytokine-treated TEC induced a significantly higher number of $CD137^+$ $CD8^+$ T cells compared to the negative control (Fig. 3C; $P<0.0001$, $n=30$). In contrast to $CD4^+$ T cells, they could also be activated by untreated TEC ($P=0.0008$, $n=30$), although the magnitude of the response was lower.

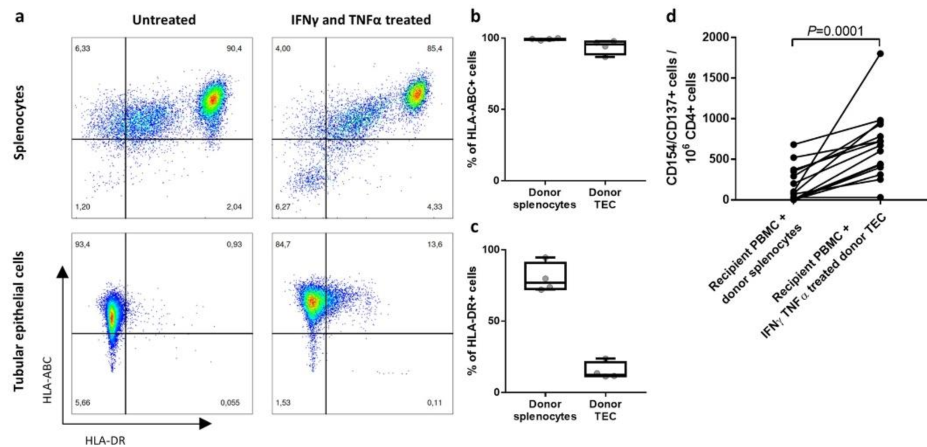

Figure 4. Assessment of alloreactive T cells using urine-derived donor TEC-stimulation shows higher sensitivity compared to donor splenocytes-stimulation. (**a**) Splenocytes express higher levels of HLA-DR than TEC. Representative plots demonstrating flow cytometric analysis of HLA-ABC and HLA-DR expression on untreated and 24 h IFNγ and TNFα treated urine-derived TEC or splenocytes. Both cell types were derived from the same deceased donor. (**b**,**c**) Frequencies of HLA-ABC (**b**) and HLA-DR (**c**) expressing living splenocytes and treated living TEC determined by flow cytometry (n = 4). (**d**) TEC elicit a higher alloreactivity than splenocytes from the same donor. PBMCs of four kidney-allograft recipients of different time points (n = 15) were stimulated with IFNγ and TNFα treated TEC and lysed TEC or splenocytes and lysed splenocytes, both derived from the kidney-transplant donor. Specific activation was assessed by flow cytometric measurements of the activation marker CD154 and CD137 expression on $CD4^+$ T cells. Statistical analysis was done with Wilcoxon matched-pairs signed rank test.

To exclude that the observed reactivity reflects an unspecific reaction to cultivated and lysed TEC, we performed additional experiments with randomly selected healthy individuals ($n = 4$), where we used cytokine-treated TEC of two of these individuals as stimulators for either PBMCs of the same donor (auto) or of an allogenic donor (allo) (Fig. 3D,E). The healthy volunteers were randomly selected without specific HLA recruitment criteria as previously published[22]. While the frequencies of autologous activated $CD4^+$ and $CD8^+$ T cells were negligible, allo-specific $CD4^+$ and $CD8^+$ T cells were detectable (304–502.7 for $CD4^+$ and 703.5–1004.2 for $CD8^+$ alloreactive T cells/10^6 T cells). Collectively, these data show that donor-reactive T cells can be detected using the assay based on urine-derived TEC of kidney transplant patients.

Donor-TEC demonstrate superior stimulatory capacity compared to donor-splenocytes. Usage of donor-splenocytes for monitoring alloreactive T cells is a well-established and elegant method, but only applicable for deceased donations. Urine-derived TEC offer the advantages of an unlimited availability of cells directly from the transplanted organ. We performed our assay in four kidney-transplant patients with both stimulator cell types from the corresponding donor to compare the amount of detectable alloantigen-reactive T cells. Analysis of the HLA-ABC and -DR expression on splenocytes showed a high expression of both molecules. After treatment with IFNγ and TNFα the expression slightly decreased (Fig. 4A, upper row), which might be due to a decreased viability (not shown). In comparison, the basal expression of HLA-DR on the TEC of the same donor was very low and inducible by IFNγ and TNFα treatment, but stayed lower in comparison to splenocytes (Fig. 4A–C). To compare the stimulatory capacity of the TEC, as a novel source of stimulator cells directly from the kidney allograft, to splenocytes from the same donor as the most commonly used source, we analysed the respective allograft-reactive T cell responses. We lysed a fraction of the TEC and splenocytes and incubated them together with the respective intact cells to facilitate presentation by antigen presenting cells and to mimic ischemic cell death that can occur during transplantation. The analysis was performed in four patients at different time points pre- and post-transplantation, making a total of 15 samples. We detected on average 8 times higher median frequencies of activated $CD4^+$ T cells in samples stimulated with donor TEC compared to donor-derived splenocytes (Fig. 4D; $P = 0.0001$, $n = 15$). These data thus show that TEC can elicit a stronger donor-specific $CD4^+$ T cell response than splenic antigen presenting cells can.

Characteristics of patients in follow up-study. It has been shown before that measurement of pre-transplant donor-reactive T cells can predict the post-transplantation kidney-function and AR[13,23–26]. To confirm the capacity of our method to quantify donor-reactive T cells and predict the post-transplant clinical course we collected PBMCs of fourteen patients before transplantation. Thereafter, the patients were clinically followed up for 6 months after transplantation. Two patients developed biopsy-proven early acute rejection (AR

Parameter		Control patients (No DGF, no AR) $n=10$	AR-patients $n=2$
Recipient	Age *year, median (range)*	64.5 (31–77)	73.5 (73–74)
	Sex *male/female*	8/2	2/0
	Underlying renal disease	Glomerulonnephritis ($n=3$), diabetic nephropathy ($n=1$), hypertensive nephropathy ($n=1$), chronic pyelonephritis ($n=1$), interstitial nephritis ($n=1$), CNI-toxicity after previous lung-tx ($n=1$) and unknown ($n=2$)	Autosomal dominant polycistic kidney disease ($n=1$), glomerulonephritis ($n=1$)
	Previous kidney transplant	10%	50%
	Previous transplant other than kidney	10%	0%
	Time on dialysis before Tx *years, median (range)*	5 (3–11)	12 (12)
	Current PRA	0%	0%
	Induction immunosuppression	Basiliximab	Basiliximab
	Maintenance immunosuppression	Tacrolimus, mycophenolat-mofetil, methylprednisolone ($n=9$) Cyclosporine, everolimus, methylprednisolone ($n=1$)	Tacrolimus, mycophenolate-mofetil, methylprednisolone
Donor-recipient	HLA-mismatches broad *median (range)*	4 (0–6)	5 (4–6)
	Cold ischemia time *hours, median (range)*	7 (4–17)	12 (9–15)
Donor	Age *year, median (range)*	59 (23–74)	75 (74–76)
	Sex *male/female*	4/6	1/1

Table 1. Characteristics of control and acute rejection (AR) patients in follow-up TreaT-assay.

group). Ten patients showed a stable graft function and no signs for delayed graft function, AR or other relevant post-transplant complications (control group). One patient showed multiple surgical and infectious complications post-transplantation. Another patient showed a good transplant function initially but a deterioration of kidney function two weeks after transplantation. Both patients had single transplant-biopsies with inconclusive findings regarding immunological complications, so they were not included into the analysis. The demographic and clinical characteristics of the 10 control patients and the 2 AR patients are presented in Table 1. Briefly, eight control patients were male and two female. Their age at transplantation was 64.5 (32–77) years (median, range). The immunosuppression regimen of nine control patients consisted of tacrolimus, mycophenolat-mofetil and methylprednisolone, one patient received a regimen with everolimus, cyclosporine and methylprednisolone. Two of the twelve monitored patients developed a biopsy-proven cellular AR at early stage after transplantation, confirmed via biopsy. They were both male and their median age at transplantation 73.5 (74–76) years (median, range). Their therapeutic regimen after transplantation consisted of a maintenance immunosuppression with tacrolimus, mycophenolate-mofetil, and methylprednisolone.

Pre-transplant alloreactive T cells measured with the TreaT-assay correspond with the post-transplant outcome. To determine phenotypical and functional subsets of alloreactive T cells we applied multi-color flow cytometry (Fig. 5A). The pre-transplant numbers of alloreactive T cells correlated negatively with the eGFR (CKD-EPI) 6 months post-transplantation. In detail, this could be seen for alloreactive $CD4^+$ ($r=-0.5566$, $P=0.0387$, $n=14$) as well as $CD8^+$ ($r=-0.6397$, $P=0.0138$, $n=14$) T cells (Fig. 5B,C).

To confirm that the established assay can be used to predict early AR episodes, twelve transplant patients were grouped into a control group without immunological complications ($n=10$) and an AR group with biopsy proven rejection ($n=2$) and analysed in a pilot study. The frequencies of alloreactive T cells were measured in samples obtained immediately before transplantation. The number of alloreactive $CD4^+$ and $CD8^+$ T cells before transplantation was higher in the two AR patients compared to controls. Comparing these results by a Mann-Whitney test resulted in significant differences ($P=0.0152$ in both subsets), but as applying statistical tests in experiments with low sample numbers is controversial we refrained from depicting the P-value (Fig. 5B,C). The number of allograft-reactive $CD154^+$ and/or $CD137^+$ $CD161^+$ $CD4^+$ T cells and of $CD137^+$ granzyme B producing $CD8^+$ T cells could clearly distinguish between patients that developed an early AR and patients with an uncomplicated course after transplantation (Fig. 5D). CD161 is a marker for TH17 cells[27], which are important players in the T cell reaction towards the allograft[28]. Granzyme B has been introduced as a marker for AR after kidney transplantation before[29].

Discussion

T cell-mediated rejection is a common threat after kidney transplantation[1]. Donor-reactive T cells suggested to be involved in pathogenesis of AR can be generated prior to transplantation, for example during pregnancies, blood transfusions, previous transplantations or as cross-reactive T cells during infections[30]. They are of special interest, since they may allow the prediction of the immediate post-transplant clinical course[8]. $CD4^+$ and $CD8^+$ memory T cells can act without secondary lymphoid organs[31], which enables monitoring their reactivity *ex vivo* in a short-term stimulation approach[32]. Previously, we and others could show that the number of pre-transplant donor-reactive IFNγ-producing cells measured by ELISPOT correlates with post-transplant glomerular filtration rate[23–25] and predicts early AR[13,26,33,34]. However, stimulator cells applied in these assays pose several limitations.

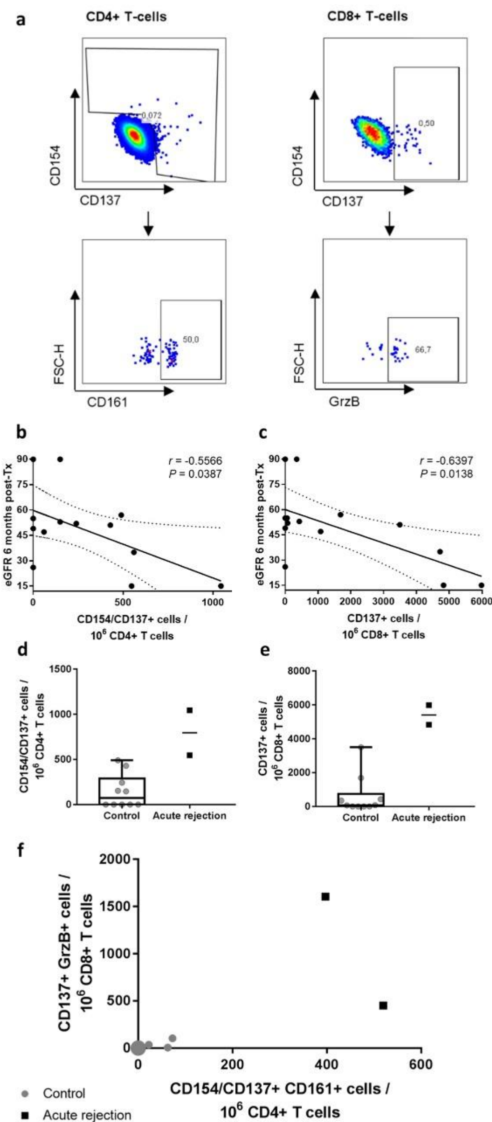

Figure 5. Pre-transplant alloreactive T cells measured with the TreaT-assay correspond with the post-transplant outcome. (**a**) Representative gating strategy to identify allograft-reactive T cell subsets. (**b**,**c**) Analysis of donor-reactive T cells collected before transplantation ($n = 14$ patients). Recipient's PBMC were stimulated with the corresponding urine-derived donor TEC for 16 h. Before incubation, the TEC were treated with 20 ng/ml IFNγ and 10 ng/ml TNFα for 24 h and a fraction of the TEC lysed to facilitate presentation by antigen presenting cells. After incubation, the PBMC were analyzed by flow cytometry for frequencies of alloreactive T cells. Correlation with the eGFR (CKD-EPI) at 6 months post-transplantation with pre-transplant alloreactive $CD4^+$ (**b**) and $CD8^+$ (**c**) T cells was calculated with Pearson's correlation coefficient. Dotted lines show 95% confidence bands. (**d**–**e**) Two patients of the previously described cohort developed biopsy-proven early acute rejection (AR) in clinical follow-up, and the frequencies of their pre-transplant alloreactive $CD4^+$ (**d**) and $CD8^+$ (**e**) T cells were compared to ten patients without immunological complications in the first six months after transplantation (control group). Whiskers show minimal and maximal values. (**f**) Further characterization

of alloreactive CD4$^+$ T cells by the expression of TH17 marker CD161 and of alloreactive CD8$^+$ T cells by production of Granzyme B. Gray dots show control patients ($n = 10$), the large gray dot conjoins 7 individual patient data. Black squares show patients with biopsy-proven early AR ($n = 2$).

They are either of restricted availability (splenocytes) or lack sufficient matching (HLA-bank cells). Furthermore, functional and phenotypic analysis of alloreactive cells with ELISPOT applied in previous studies is very limited[3]. Here, we present the Transplant reactive T cells (TreaT)-Assay, a novel multi-parameter flow cytometry-based diagnostic tool using an easily accessible and renewable urine-derived donor-specific source of stimulator cells for monitoring allograft-specific T cells. Compared to the currently used sources our model has the advantages of high quantity, availability, and quality.

The cultivation process is easy to perform. The outgrowth of cells from the urine worked for all patients included in our study. As we could show, the majority of cells in the cultures are TEC. Since the urine was collected from a pigtail catheter, the allograft origin of TEC can be ensured. Therefore, this method offers a non-invasive way to procure kidney allograft cells.

Regarding the quality, urinary cells have been shown earlier to be fully functional renal tubular cells[35]. Most important in our setting is their stimulatory capacity. We could show that urine-derived TEC, like TEC from other sources[21,36], up-regulate both HLA-ABC and –DR molecules in a pro-inflammatory environment. These cells can therefore act as atypical antigen presenting cells and activate memory T cells[37,38]. The specificity of alloreactive T cells and the influence of pro-inflammatory conditions on the stimulatory capacity of TEC is displayed by the differing reactivity of CD4$^+$ and CD8$^+$ T cells. Homeostatic HLA-ABC expression was sufficient to trigger a CD8$^+$ T cell response, while CD4$^+$ T cells only reacted on inflammatory treated TEC with upregulated HLA-DR molecules. Further, comparing autologous with allogenic stimulation, we could demonstrate that the activation of T cells followed by TEC stimulation was due to allogenic capacity of TEC and not due to unspecific cytokine pre-treatment of TEC. Thus, T cells of healthy volunteers show little to no reaction towards autologous TEC, while allogenic TEC could elicit a measurable reactivity. Taken together, these experiments show that TEC have the ability to induce a specific T cell alloreaction without provoking significant unspecific reactivity.

Knowing that we can specifically monitor TEC-induced donor-reactive T cells, we assessed the sensitivity of our assay in comparison to splenocytes, currently the most commonly used stimulator source. Previously, some authors stated the existence of tissue-specific alloreactivity by HLA-molecules presenting kidney cell specific peptides[39]. Accordingly, splenocytes would only monitor a fraction of the alloreactive T cells as their HLA-molecules do not bind the peptides present in the kidney-allograft. The existence of tissue-specific T cells in the kidney-transplantation setting was already shown more than two decades ago by demonstrating that some clones of graft-infiltrating T cells lyse TEC, but not splenocytes isolated from the corresponding donor[39–45]. In line with these results, we observed a significantly lower reactivity upon stimulation with donor-splenocytes as compared to the donor-derived TEC, despite a higher expression of HLA-molecules on the splenocytes. This underscores the superiority of our TEC-based alloreactivity-assay and suggests that it may reflect donor- and tissue-specific reactivity as well as the intragraft situation more accurately than currently used sources for stimulator cells.

To prove the clinical utility of the established assay, we performed a proof-of-principle study on the correlation of pre-transplant alloreactive T cells measured by the TreaT-assay and the post-transplant GFR as well as early AR. As it has been shown also for the IFNγ-ELISPOT-assay[23–25], pre-transplant alloreactive CD4$^+$ and CD8$^+$ T cells inversely correlated with the eGFR at 6 months post transplantation. The prediction of AR with IFNγ-ELISPOT showed differing results in clinical trials[13,23–26,33,34]. Comparing patients with very early AR to patients with a stable graft function in our assay, AR patients showed a higher number of alloreactive CD4$^+$ and CD8$^+$ T cells and a clear distinction between these two groups can be drawn when the numbers of alloreactive CD161$^-$ CD4$^+$ T cells and of granzyme B producing CD8$^+$ T cells are compared. CD161 is a marker restricted to memory phenotype and a hallmark of TH17 cells and interleukin-17 production[27,46]. Our data are in line with the results by numerous authors demonstrating the involvement of TH17 cells in alloreaction[28]. In addition, Kim *et al*. demonstrated very recently a significant increase of CD161$^+$CD4$^+$ T cells in patients with antibody-mediated rejection confirming thereby our data on the role of this cell subset in the alloreactivity[47]. The involvement of granzyme B in AR has also been intensely studied and demonstrated before[29]. Taken together, the detection of CD161$^-$CD4$^+$ and granzyme B in our patients with early AR reported to be relevant in other *ex vivo* and *in vivo* studies provides evidence for the clinical relevance of the data collected.

Our study has some limitations. Similar to other studies performed on peripheral blood cells, our *ex vivo* analyses do not necessarily reflect the intragraft situation. Therefore, in future studies a comparison with the biopsy findings in follow-up would be needed. However, this is challenging due to ethical reasons. Furthermore, the numbers of some alloreactive T cell subsets were relatively low. Detection of very low numbers of antigen-specific T cells in kidney transplant patients has been demonstrated before for virus-specific and vaccine-specific T cells[48–50]. Quality controls for data acquisition and analyses including subtraction of unspecific background and adequate fluorescence-minus-one controls as applied in our studies enable however reliable detection of these cells. On the other hand, analysis of a higher cell number would facilitate identification and in-depth characterization of alloreactive T cells, especially with regard to cytokines or quantification of rare subpopulations. Therefore, drawing higher amounts of blood (e.g. 20 mL) will be advantageous and can be drawn unproblematicly in most cases. Moreover, the number of patients analysed in our proof-of-concept pilot study is insufficient to draw final conclusions on the applicability and prognostic power of our assay and further prospective studies will be required to confirm and extend our data. Additionally, so far we did not perform a direct comparison with similar assays using IFNγ-ELISPOT with donor-splenocytes or HLA-bank cells. Nonetheless, our results are

in line with similar assays measuring allograft-reactive T cells in comparable patient cohorts. Further, the time needed for the first results is a critical point in a clinical setting. In order to achieve sufficient numbers of TEC, up to two weeks are necessary, depending on the amount of urine and the sediment quality. However, a combination of the aforementioned sources, such as splenocytes, could bridge this diagnostic window. Another option is down scaling of TEC numbers for stimulation, which is also envisioned for future studies.

Taken together, the TreaT-assay offers a donor-specific measurement of allograft reactive T cells. Compared to previous assays it has the advantages of an unlimited availability and a superior performance. In a pilot study, we were able to obtain encouraging data on the applicability of our assay in patients with early AR and on the prediction of post-transplant GFR. Furthermore, our approach allows deep personalized insights into the biology of alloreactive immune cells and their interaction with donor TEC. Therefore, it might help to guide personalized therapy in the future of kidney transplantation.

Data availability

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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Author contributions

C.T. collected patient samples, designed and performed experiments, analyzed and interpreted data, and wrote the paper. B.W. designed and performed experiments and analyzed and interpreted data. A.M. collected patient samples and performed experiments. T.R. performed experiments and edited the manuscript. U.S. analyzed data. K.R. established the protocol for the UC cultivation. P.W. collected patient samples and performed experiments. M.S. processed donor spleens. M.C. and P.N. took care of the patients in the transplantation ambulance, collected clinical data and patient samples. N.L. performed and analyzed experiments. A.K., M.S.-H., T.W. and P.R. supervised and helped design the study. N.B. took clinical care of the kidney transplant patients, designed the study and the experiments, interpreted data and wrote the paper. All authors approved the final version of the manuscript.

Competing interests

C.T., B.W., P.R. and N.B. applied for a European patent (EP3203237A1). The other authors declare no potential conflict of interests.

Additional information

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8 Complete list of publications

Research articles and Abstracts

1. **Thieme CJ,** Weist BJD, Mueskes A, Roch T, Stervbo U, Rosiewicz K, Wehler P, Stein M, Nickel P, Kurtz A, Lachmann N, Choi M, Schmueck-Henneresse M, Westhoff TH, Reinke P, Babel N: **The TreaT-Assay: A Novel Urine-Derived Donor Kidney Cell-Based Assay for Prediction of Kidney Transplantation Outcome.** *Sci Reports*. 2019 Dec 13;9(1):19037. (Impact Factor JCR 2018: 4.011)

2. **Thieme C,** Schulz M, Roch T, Wehler P, Amini L, Choi M, Viebahn R, Schmueck-Henneresse M, Reinek P, Westhoff T, Babel N**: EBV-Associated Post-Transplant Lymphoproliferative Disorder: In Vitro Model for a Rational Modification of Immunosuppression.** *Transpl Int.* 2019:32 (Suppl. 2), 403–433 (Impact Factor JCR 2018: 3.526)

3. **Thieme C**, Weist B, Müskes A, Reinke P, Westhoff T, Babel N: **Assessment of Donor-Reactive T-Cell Immunity by the Novel Urine Cell-Derived Alloantigen Assay Allows Prediction of Acute Rejection in Renal Transplant Patients.** *Am J Transplant.* 2017; 17 (suppl 3). (Impact Factor JCR 2017: 6.493)

4. **Thieme C**, Weist B, Schmück M, Reinke P, Babel N: **Urine-Derived Cell Lines as a Novel Tool for Monitoring Allo-Reactivity in Kidney Transplant Patients**. *Am J Transplant.* 2016;16 (suppl 3). (Impact Factor JCR 2016: 6.165)

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Patents

1. Babel N, Reinke P, **Thieme C,** Weist B (2016): **Urine-derived epithelial cell lines for diagnosis and therapy of an anti-bk-virus or anti-graft immune response,** European Patent Application No EP3203237A1

Presentations

1. **Thieme C**, Weist B, Rocht T, Müskes A, Westhoff T, Reinke P, Babel N.: **A Novel Urine-Derived Donor Kidney Cell-Based Assay for Prediction of Acute Kidney Rejection**
   e:Med Meeting on systems medicine, Berlin (09/18)

2. **Thieme C**, Weist B, Müskes A, Reinke P, Westhoff T, Babel N.: **Assessment of Donor-Reactive T-Cell Immunity by the Novel Urine Cell-Derived Alloantigen Assay Allows Prediction of Acute Rejection in Renal Transplant Patients**
   25. Jahrestagung der Deutschen Transplantationsgesellschaft (DTG), Essen (08/2016)

3. **Thieme C**, Weist B, Westhoff T, Reinke P, Babel N.: **Urine-Derived Cells As Novel Tools For Monitoring Allo-Reactivity In Kidneytransplant Patients**
   24. Jahrestagung der DTG, Dresden (10/2015)

4. **Thieme C**, Weist B, Westhoff T, Reinke P, Babel N.: **Urine-Derived Cells As Novel Tools For Monitoring Allo-Reactivity In Kidneytransplant Patients**
   17th Congress of the European Society for Organ Transplantation (ESOT), Brüssel (Belgien) (09/2015)

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