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T cell dynamics in peripheral blood in kidney transplantation

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Michael Strauch

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unter der Anleitung von

Priv. Doz. Dr. med. univ. Alexander Kirsch

und

Assoz. Prof. Priv. Doz. Dr. Kathrin Eller

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Graz, am 12. Februar 2021 *Michael Strauch, eh.*

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Glossary and abbreviations

ABMR	antibody-mediated rejection
APC	antigen-presenting cells
AZA	azathioprine
BMI	body mass index
CD	cluster of differentiation
CKD	chronic kidney disease
CMV	cytomegalovirus
CNI	calcineurin inhibitor
DGF	delayed graft function
ECD	expanded criteria donor
ESKD	end-stage renal disease
FACS	fluorescence- activated cell sorting
FoxP3	protein forkhead box P3
GARP	glycoprotein A repetitions predominant
GFR	glomerular filtration rate
HLA	human leukocyte antigen
HLA MM	human leukocyte antigen mismatch
IL-2	interleukine-2
iTreg	induced regulatory T cell
IVIG	intravenous immune globulin
KDIGO	Kidney Disease: Improving Global Outcomes
KDPI	kidney donor profile index
KPD	kidney paired donation
KTX	kidney transplantation
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
mmHg	millimetres of mercury
mTreg	memory regulatory T cell
NF κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nTreg	natural regulatory T cell
PAMP	pathogen-associated molecular pattern

PVAN	polyomavirus-associated nephropathy
RRT	renal replacement therapy
TCR	T cell receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell type 17
Tfh	follicular helper T cell
Treg	regulatory T cell

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Zusammenfassung

Hintergrund: Regulatorische T Zellen (Tregs) leisten einen wesentlichen Beitrag zur Immunabwehr und Immuntoleranz. Da Tregs bereits als vielversprechende Biomarker für Abstoßung oder Toleranz bei Organtransplantationen diskutiert werden, ist ein genaueres Verständnis solcher Zellen von großer Bedeutung. Wir untersuchten die Hypothese, dass sowohl zwischen Gesunden und Patient*innen mit dialysepflichtiger Niereninsuffizienz, als auch zwischen Patient*innen mit Nierentransplantation mit und ohne nachfolgender Abstoßungsreaktion unterschiedliche Zellzahlen von Tregs vorliegen.

Methoden: Vierundsiebzig gesunde Erwachsene und 74 Patient*innen mit chronischer Niereninsuffizienz, welche für eine Nierentransplantation gelistet waren, wurden in die Studie eingeschlossen und Patient*innen für ein Jahr klinisch nachverfolgt. Blutabnahmen wurden unmittelbar vor geplanter Transplantation durchgeführt. Tregs wurden mittels Durchflusszytometrie ausgewertet und in Form von Prozentzahlen als relative Werte miteinander verglichen.

Ergebnisse: Patient*innen mit terminaler Niereninsuffizienz zeigten keine signifikanten Unterschiede innerhalb $CD3^+CD4^+$ T-Zellen und $FoxP3^+$ Tregs im Vergleich zur gesunden Kontrollgruppe. Allerdings hatten Patient*innen signifikant mehr $CD127^+FoxP3^+$ Tregs (1.29±2.09 vs. 0.27±0.77 % von $CD3^+CD4^+$; $p<0.001$), mehr $CD25^+CD127^+$ Tregs (7.04±3.23 vs. 5.65±2.10 % von $CD3^+CD4^+$; $p<0.001$), mehr $CD25^+FoxP3^+$ Tregs (5.25±2.85 vs. 4.34±2.05 % von $CD3^+CD4^+$; $p<0.001$), mehr Th17 $CD161^+$ Zellen (2.94±1.94 vs. 1.79±1.47 % von $CD25^+CD127^{dim}$; $p<0.001$), mehr $CD161^-$Trans Tregs (34.47±19.90 vs. 28.37±10.14 % von $CD25^+CD127^{dim}$; $p=0.002$) aber weniger naive Tregs (30.20±12.47 vs. 34.69±12.53 % von $CD25^+CD127^{dim}$, $p=0.035$) als die gesunde Kontrollgruppe. Zwischen den Gruppen wurden keine signifikanten Unterschiede in effektor Tregs, effektor proliferative Tregs oder ruhenden Tregs gefunden, während mehr nicht-suppressive Tregs (2.92±1.53 vs. 2.59±1.61 % von $CD4^+$; $p=0.008$) und aktivierte Tregs (0.67±0.78 vs. 0.45±0.43 % von $CD4^+$; $p<0.001$) bei Patient*innen mit Niereninsuffizienz, gemessen wurden.

Insgesamt konnten wir bei 13 Patient*innen akute Abstoßungsreaktionen beobachten. Beim Vergleich von Patient*innen mit und ohne nachfolgender Abstoßung waren keine signifikanten Unterschiede zwischen den Gruppen zu finden, wenn Tregs vor der Transplantation analysiert wurden.

Conclusio: Mithilfe von Durchflusszytometrie konnten wir zeigen, dass sich die relative Anzahl von Tregs zwischen Patient*innen mit Niereninsuffizienz und Gesunden signifikant unterscheidet, während zwischen nierentransplantierten Patient*innen mit und ohne nachfolgender Abstoßung keine Unterschiede erkennbar sind. Da Vergleiche von verschiedenen Zellpopulationen von zahlreichen Faktoren wie Messtechnik sowie Art und Zeitpunkt der Messung abhängen, sind weitere Studien für das bessere Verständnis von Tregs notwendig.

Abstract

Introduction: Regulatory T cells (Tregs) play a crucial role in the avoidance of transplant rejection and achieving immunotolerance. The aim of this study was to evaluate Treg populations in patients with end-stage kidney disease (ESKD) before kidney transplantation was performed.

Methods: Seventy-four ESKD patients listed for kidney transplantation and 74 healthy controls were included. Peripheral blood samples for FACS analysis were drawn shortly before kidney transplantation was performed. Patients received long-term follow-up after transplantation to gain insight on rejection episodes. Treg subpopulations were evaluated using flow cytometry and cells were displayed as percent of their respective population.

Results: While there was no significant difference in $CD3^+CD4^+$ T cells and $FoxP3^+$ Tregs, significantly more $CD127^+FoxP3^+$ cells (1.29±2.09 vs. 0.27±0.77 % of $CD3^+CD4^+$; $p<0.001$), more $CD25^+CD127^+$ Tregs (7.04±3.23 vs. 5.65±2.10 % of $CD3^+CD4^+$; $p<0.001$) more FoxP3/CD25 Tregs (5.25±2.85 vs. 4.34±2.05 % of $CD3^+CD4^+$; $p < 0.001$), more Th17-type $CD161^+$ Tregs (2.94±1.94 vs. 1.79±1.47 % of $CD25^+CD127^{dim}$; $p<0.001$) more $CD161^-$ Trans Tregs (34.47±19.90 vs. 28.37±10.14 % of $CD25^+CD127^{dim}$; $p=0.002$), and less naïve Tregs (30.20±12.47 vs. 34.69±12.53 % of $CD25^+CD127^{dim}$, $p=0.035$) were measured in ESKD patients than in healthy controls. There were more non-suppressive T cells (2.92±1.53 vs. 2.59±1.61 % of $CD4^+$; $p=0.008$) and activated Tregs (0.67±0.78 vs. 0.45±0.43 % of $CD4^+$; $p<0.001$) in patients with ESKD, but there was no difference in resting Tregs, in effector Tregs and effector-proliferative Tregs between the groups.

During follow-up, 13 patients experienced biopsy proven rejection episodes. When Tregs were analysed before transplantation, we could not find significant alterations between patients of different rejection groups.

Conclusion: Based on flow cytometry of several Treg subsets, this study proved that healthy controls show different percentages of Tregs than ESKD patients, but no significant alterations of Tregs between patients with and without following rejection episodes could be found. Bearing in mind that Treg analysis is not only affected by cell definition, but also by timing and technical issues of evaluation, further trials at serial time points after transplantation are suggested for better Treg understanding.

1 Introduction

1.1 End-stage kidney disease

Chronic kidney disease (CKD) was defined by The Kidney Disease: Improving Global Outcomes (KDIGO) initiative as abnormalities of kidney structure or function, present for more than three months, with implications for health (1).
Furthermore, CKD is associated with multiple comorbidities. Progression of the CKD is one of the most serious outcomes. The last stage of CKD is kidney failure with severe symptoms that can only be treated by dialysis or transplantation. In contrast, the term end-stage kidney disease (ESKD) is often used synonymously, although it only refers to the level of kidney function at which a patient should initiate renal replacement therapy (RRT). Despite only one percent of patients suffering from CKD need to be treated with dialysis or a kidney transplant, CKD is still one of the most expensive chronic diseases and reduces lifespan dramatically. In countries, where health systems only allow limited or none of these therapeutic options, progressive CKD eventually results in death (1).
Moreover, latest meta-analyses have shown that CKD is a worldwide health burden. A systematic review from 2016 has estimated the mean global prevalence of CKD at 13.4% (2). The European Renal Care Providers Association states that in 2016 around 3.7million people worldwide are treated for ESKD (3). Meanwhile in Europe the overall unadjusted prevalence for renal replacement therapy (RRT) was 823 per million population (4).

1.1.1 Management of end-stage kidney disease

Once glomerular filtration rate of patients with CKD falls below a critical level, progression to ESKD is inevitable (5). As soon as ESKD is reached, pre-emptive planning for potential transplantation or for a permanent vascular access to support haemodialysis is essential (6).

1.2 Kidney transplantation

For advanced chronic renal failure, kidney transplantation (KTX) is the treatment of choice. It is superior to all others RRT regarding quality of life and furthermore, patients with kidney

transplants have a two to three times higher life expectancy. Due to these advantages KTX should be considered in all patients with ESKD (7).

Generally, KTX can be divided into two different groups: kidneys from living donors and kidneys from deceased donors, including donation after brain death or after circulatory death. Usually kidneys are implanted in the right or left iliac fossa. The renal artery is sutured to one of iliac arteries and the renal vein to the external iliac vein. Finally, the ureter is connected to the bladder. The immune system of the recipient recognises foreign material, including transplants. To avoid an immune reaction and immediate attack on the graft, immunosuppression is administered to reduce the chance of rejection, even though it increases the risk of infection and tumours (8).

1.2.1 Epidemiology

The first successful human kidney transplant was performed in 1954. Nowadays, kidney transplantations are no rarity anymore. Compared to all other organ transplants, KTX are by far the most common ones. The Global Observatory on Donation and Transplantation (GODT) is one of the most comprehensive sources on worldwide data of organ transplantation. In 2016, it refers to over 135,000 organ transplantations worldwide with over 40% of it being KTX (9). Meanwhile the European Renal Association – European Dialysis and Transplant Association (ERA-EDTA) reports of 22,046 kidney transplantations being performed in 2016 within European countries (4). In specific, the latest data provided by ÖBIG shows overall 414 KTX only for Austria in the year 2018 (10).

However, the data above underlines the importance of organ transplantation and there is still an enormous gap between the number of recipients and donors. Ever year the number of patients with ESKD is increasing and therefore the demand of kidney transplants also increases steadily. While in the year 2000, only 23,084 KTX had been registered, the number had risen to 78,867 in 2018 (11).

Meanwhile the number of potential donors remains almost the same. The official Eurotransplant statistics, which includes all data of transplantation from its eight member countries Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia, counted 2,074 deceased donors used in all countries in 2009 and 2,159 deceased donors in 2018 (12).

As a result, the increasing demand for kidney transplants and the limited supply of kidney grafts lead to a relatively long waiting list. In 2018, Eurotransplant counted in total only

4,806 KTX, but 10,791 candidates on the waiting list (12). In comparison, in the United States in 2015, there were less than 18,000 patients transplanted, even though 50,692 adults were on the waiting list. In Harrison's principles of internal medicine is stated that 'This imbalance is set to worsen over the coming years with the predicted increased rates of obesity and diabetes worldwide.' (13p2126). Consequently, the increasing number of candidates on the waiting list, combined with preserved transplant rates, lead to an average waiting time of more than 4.5 years in 2009 (14). Overall, the waiting period even seems to increase. The median time to transplant of waiting candidates has not been calculated, because half of the listed patients of the Scientific Registry of Transplant Recipients and Organ Procurement Transplantation Network SRTR/OPTN Annual Data Report 2017 have not undergone transplantation yet (15). Nevertheless, the overall mortality for patients on the waiting list ranges from 1.4 to 9.4 per 100 patient years across different donation service areas and generally decreased over the past ten years (15).

However, due to the growing discrepancy between the numbers of patients waiting for transplantation and available organs, Expanded Criteria Donors (ECD) allografts became interesting.

1.2.2 Expanded criteria donors

At the turn of the millennium, numerous studies tried to gain more information of risk factors of marginal organ grafts, to possibly broaden the donor pool. The aim was to get an increasing number of cadaveric kidney transplants with organs from donors who would have been considered unusable before.

Already in 2001, Ojo et al. compared life expectancy of patients on waiting lists for KTX with people receiving a so-called marginal donor kidney. They concluded that even the transplantation of a marginal kidney leads to a survival benefit of 5 years compared with maintenance dialysis. In this study, marginal donor kidneys were defined as donor age over 55 years, donor history of hypertension longer than 10 year duration, donor history of diabetes mellitus for more than 10 years, non-heart beating cadaver donor (NHBD), and cold preservation time over 36 hours (16). Based on these ground-breaking observations, more of these marginal organs could be used, which could help to reduce the organ shortage.

However, specific risk factors had to be identified to simultaneously minimise graft loss. As a result of further research, four major risk factors besides HLA mismatches that could

predict graft loss could be identified. These factors included donor age, cerebrovascular event as cause of donor death, renal insufficiency and history of hypertension (17, 18).
Finally, the United Network for Organ Sharing (UNOS) precisely defined expended criteria donor (ECD) kidneys as any kidney whose relative risk of graft failure exceeded 1.7 when compared to a reference group of ideal donor kidneys (19).
Those inclusion criteria were:

- Donors over the age of 65
- Donors aged 50-59 with at least two of the three criteria:
    - History of hypertension
    - Creatinine > 1.5mg/dl
    - Cerebrovascular accident as cause of death

However, there is an even newer allocation system that has recently replaced the ECD category in the United States. The kidney donor profile index (KDPI) is a modified version of the approach by Rao and associates (20). The KDPI score uses a single pool of kidneys and calculates the risk of graft failure based on 10 donor characteristics. Expressed as percentile score, grafts are graduated from 0 to 100 percent, implying perfect or marginal organ quality. Deceased donor kidneys with a KDPI of less than 20% and excellent quality will be allocated to candidates with the highest post transplantation life expectancy. Meanwhile donor kidneys with a high KDPI, implying higher risk of graft failure, will be allocated to older candidates, for example, who earlier would have agreed to receive an ECD graft, to avoid long waiting time which often represented a barrier to transplantation for those candidates (21). Besides the new matching system, even those kidneys scoring a high KDPI showed a survival benefit for patients compared to candidates waiting for a better graft with lower associated risk. However, it is well known that all these benefits regarding life expectancy and long-term mortality only emerge after some time. Still mortality hazard increases during the perioperative period and only had survival benefit after some months, depending on the KDPI of the graft (22). Therefore, it is still necessary to provide strict indications for transplantation.

1.2.3 Indication for transplantation

Only a few attributes in recipients are contraindications to renal transplantation. Usually, the transplant procedure is relatively non-invasive and recipients without perioperative

complications can often be discharged within days or weeks from hospital. Besides neoplasms and a few infectious diseases as hepatitis B, C, HIV or tuberculosis, only immunologic factors are seen as absolute contraindications (13p2126). However, finding indications for a transplantation is a much more complicated topic.

As mentioned before, KTX is the treatment of choice for ESKD, which may be a result of acute kidney injury or CKD. Usually, before transplantation is needed, peritoneal- or in most cases haemodialysis is started. Nevertheless, the point of initiating dialysis is widely controversial and shall be discussed elsewhere (6, 23).

1.2.4 Individual approaches on KTX

In general, kidney grafts can be distinguished in kidneys from living and deceased donors. Furthermore, deceased donors are categorised in donation after brain death or donation after circulatory death. Compared to deceased donor kidneys, live donor kidney transplantation from relatives or volunteers is still superior. In comparison one-year survival of grafts from deceased donors is about 92%, meanwhile the one of living donor kidneys is about 97%. Furthermore, life expectancy of a living donor graft is around 14 years and that of a deceased donor graft about 10 years (13). Besides better allograft survival, recipients of living donor kidneys wait less time for transplantation, have lower risk of rejection and longer life (24). All this may be because living donor kidneys are of better general donor health and cold ischemia time is shorter than in deceased donors. Another important advantage for the recipient of living donor kidney transplantation is avoiding the growing waiting time for deceased donor transplantation. There seems to be a direct relation between the amount of time spent on dialysis before transplantation and increased mortality. Therefore, pre-emptive transplantation before dialysis offers significant advantages. In contrast, too early transplantation may waste residual renal function and subsequently half-life of the graft must be wisely considered. However, until now studies have raised a lot of questions on timing of living donor transplantation. (25-27).

Another important fact is that, of course, donation is not without risk, but could be minimised in the last years. Surgical risk of donation equals standard elective surgery and for donors, long-term risks of ESKD and mortality are equivalent to or even lower than those of general population. More precisely, voluntary donors have lower risk of ESKD compared with the general population, but compared with a healthy non-donor control group, living donors have higher chances of developing ESKD (27).

However, besides the many advantages, living kidney donation is even more affected by one major problem than deceased kidney donation: AB0 incompatibility.

1.2.5 AB0 incompatible kidney transplantation

The AB0 blood group consist of four different categories: A, B, AB and 0 with corresponding isohaemagglutinins against the missing antigens. Antigens are expressed on red blood cells, lymphocytes, platelets, epithelial and endothelial cells. Thus, like in blood transfusions, AB0 blood groups define pathophysiological fundament for compatible and incompatible organ transplantation. Major incompatibilities are recipient antibodies against blood group B and subgroup A1. The antigenic expression of antigen A2 is quantitative and qualitatively less than that of A1. Therefore, the risk for antibody-mediated rejection (ABMR) following ABO incompatible transplantation is A1>B>A2. That is why minor incompatibility constellations against A2 antigen can be successfully transplanted into recipients with low pretransplant anti-A titers without the use of desensitisation. However, initially in AB0 incompatible KTX, hyperacute rejection was a significant issue, today graft survival times are equal to AB0 compatible graft recipients. This is due to strict protocols including extracorporeal elimination of isohaemagglutinins, typically with plasmapheresis or immunoadsorption, depletion of B Cell population responsible for AB0 antibody production and intensified immunosuppression pre- and postoperatively (28).

Unfortunately, up to 54% of otherwise appropriate live-donor kidney transplantations, are incompatible due to AB0 blood groups or pre-existing donor human leukocyte antigen (HLA) antibodies (29). Another approach to bypass these constellations was kidney paired donation (KPD). Kidney transplant candidates with willing but incompatible living donors can join a registry of other incompatible pairs, to find a potentially compatible transplant solution. In other words, KPD is the exchange of kidney between two or more immunologic incompatible living donor recipient pairs so that all recipients receive compatible organs from strangers. Even though KPD was initially proposed as an exchange between two pairs, nowadays KPD can be arranged with multiple pairs. These unique advantages have led to develop many different national KPD programs around the world (29).

In the broader sense, KPD programs even enable benefits for AB0 compatible recipients or highly sensitised patients. By using optimised matching algorithms, better HLA concordance can be achieved, resulting in better graft and patient survival following even a cost advantage for public health (30).

1.3 Immunosuppressive drugs for kidney transplantation

Still the biggest challenge of allograft transplantation is preventing graft rejection with little as possible side effects. Currently, immunosuppressive therapy still suppresses all immune responses with undesired consequences of immunodeficiency (infection or cancer) and nonimmune toxicity to other tissues. Therefore, little as possible medication is needed without increasing rejection rates.
For better understanding of the mechanism of immunosuppressive agents, alloimmune response will be illustrated shortly.

1.3.1 Alloimmune response

As soon as the graft is reconnected to recipient blood vessels, a broad array of immune responses starts. For identification of foreign material and mediation of graft rejection, lymphocytes are the main actors (31). Generally, lymphocytes can be distinguished in B cells and thymus-dependent T cells. Among many authors, Halloran, who published one of the best-known reviews about immunosuppressive drugs in The New England Journal of Medicine in 2004 (32), described T cell response as a three-signal model:

Antigen-presenting cells (APC) become activated in the graft and its surrounding tissue. Among many different types of APC, mature dendritic cells are seen as the most potent antigen presenting cells (31). The antigen on the surface of dendritic cells triggers T cells with cognate T cell receptors (TCR), which leads to “signal 1”. Simultaneously, the costimulatory “signal 2” is delivered when dendritic cells engage CD28 on T cells. These two signals result in the activation of another three additional signal transduction pathways: the calcium–calcineurin pathway, the RAS-mitogen activated protein (MAP) pathway and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. These pathways activate transcription factors leading to the production of surface molecules and cytokines, like interleukine-2 (IL-2). Together with other cytokines, IL-2 leads to “signal 3”, the stimulation of T cell proliferation via mammalian target of rapamycin pathway (mTOR). This proliferation and differentiation leads to a large availability of effector T cells (32).
Apart from effector T cells, B cells are activated after antigen engagement to their antigen receptor, leading to alloantibody production against donor HLA antigens. Antibodies cause target cell destruction either by complement dependent or complement independent

mechanisms. In this way, immune response mediates rejection through effector T cells and alloantibodies within days (32).

1.3.2 Classification of immunosuppressive drugs

In general, immunosuppressive strategies are often classified into induction and maintenance therapy. Induction therapy is the rapid achievement of immunosuppression, usually administered at the time of transplantation. Induction is often achieved by so-called depleting agents, which work by eliminating alloreactive lymphocytes. In contrast, maintenance immunosuppression provides a continuous prophylaxis of rejection. By the use of a combination of oral agents of different drug categories with synergistic or additive immunosuppressive effects, dosage can be decreased after time (21).
However, for better understanding of the immune system, a detailed explanation of the working mechanism of immunosuppressive agents is necessary. Thus, following the review of Wiseman AC in the Clinical Journal of the American Society of Nephrology in 2016 (33), it seems easier to categorise immunosuppressive agents by immune cell target:

- **T cell directed therapy**
  - Calcineurin inhibitors (Cyclosporin, Tacrolimus)
  - Costimulatory signal blockers (Belatacept)
- **B cell directed therapy**
  - Anti-CD20 targeting (Rituximab)
- **Agents targeting cytokines**
  - Nonspecific cytokine inhibition (Corticosteroids)
  - IL-2 receptor antagonist (Basiliximab)
- **Polyclonal antibodies**
  - Intravenous immune globulins (IVIG)
  - Polyclonal antithymocyte globulin (ATG)
- **Antiproliferative agents**
  - mTOR inhibitors (Sirolimus, Everolimus)
- **Antimetabolites–inhibition of DNA synthesis**
  - Azathioprine
  - Mycophenolate
  - Leflunomide

1.3.3 T cell directed therapy

As mentioned before, T lymphocytes play a major part in the initiation of rejection. Therapeutic agents that target T cells can be distinguished in either interfering with 'signal 1'- interaction of APC with TCR- or costimulatory 'signal 2'-additional APC – T cell interaction. Typical agents of the first group are calcineurin inhibitors (CNI), while Belatacept is a famous 'signal 2' inhibitor.

1.3.3.1 Calcineurin inhibitors

By the combination of TCR binding and a costimulatory signal, cytosolic influx of calcium and a downstream activation of calcineurin is triggered. Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT), which then translocates to the nucleus. There, calcineurin-dependent gene transcription is activated, resulting in cytokine production (i.e. IL-2) which further leads to T cell expansion (21).

Cyclosporin and Tacrolimus both prevent calcineurin-dependent gene transcription. The introduction of cyclosporin in the early 1980s transformed the field of transplantation dramatically. Lower rejection rates and the superiority over standard immunosuppression regimen of azathioprine and steroids lead to ongoing development of cyclosporin. Still, due to numerous side effects and complex pharmacology, dosing is critical and monitoring blood levels two hours after ingestion is necessary (21).

In the late 1990s, tacrolimus was introduced in kidney transplantation and soon showed more efficacy in reducing rejection rates compared to cyclosporin. Even if tacrolimus resembles cyclosporine in its mechanism of actions, its toxicity profile is slightly different. Both agents may lead to CNI-induced vascular constriction contributing to hypertension and decreased renal perfusion (nephrotoxic). Additional side effects of cyclosporin are hyperlipidemia, gingival hyperplasia, hypertrichosis and tremors, meanwhile tacrolimus is associated with greater risk of neurotoxicity, higher incidence of posttransplantation diabetes mellitus (PTDM) and gastrointestinal toxicity (21).

With the intention of avoiding CNI toxicity, multiple studies tried CNI sparing protocols. One of the most famous trials was the ELITE-Symphony study in 2007, which came to the following conclusion: A regimen of daclizumab, mycophenolate mofetil, and corticosteroids in combination with low-dose tacrolimus may be advantageous compared with regimens containing daclizumab induction plus either low-dose cyclosporine or low-dose sirolimus or with standard-dose cyclosporine without induction (34). However, the topic of CNI minimisation still is an enormous challenge. Numerous authors compared multiple

immunosuppression protocols, meanwhile the only joint conclusion was that CNI minimisation might be beneficial in different aspects, but still, long-term outcomes are not clear enough (35-41).

1.3.3.2 Costimulatory signal blockers

For optimal T cell activation, the costimulatory 'signal 2' between APC and T cells is necessary. Belatacept is a selective blocker of the interaction of CD80/CD86 on APC with CD28 on T cells. However, the benefit of Belatacept is controversial. Studies showed that Belatacept may provide better long-term survival with the cost of higher acute rejection rates (21). Especially in comparison with CNI, a Cochrane review of 2014 concluded, that Belatacept might be an alternative with fewer side effects (42).

1.3.4 B cell directed therapy

Besides inhibiting humoral immune response, B cell inhibition affects APC function and furthermore B/T cell interactions that lead to T cell activation and proliferation (33). Even though nowadays there are numerous agents targeting B cells, only a few of them are used in kidney transplantation.

1.3.4.1 Anti-CD20 targeting

B lymphocytes present the specific transmembrane protein CD20, which is not expressed on other cell lines. CD20 is essential for B cell differentiation and regulation of activation for cell cycling. Rituximab was the first agent targeting CD20. It is a chimeric anti-CD20 cytolytic monoclonal antibody interfering with humoral alloresponse by leading to B cell depletion. Rituximab is used in desensitisation protocols or for the treatment of acute humoral rejection. However, Rituximab is still associated with side effects like fever, hypotension and bronchospasm, based on cytokine release due to the chimeric nature of the antibody (21, 33).

1.3.5 Agents targeting cytokines

Cytokines are proteins, produced by a variety of cell types, including APC, T and B lymphocytes. These proteins function as growth, activation and differentiation factors, up-

and down regulating the immune response. Thus, by targeting specific cytokines, immune response is expected to be reduced (21, 33).

1.3.5.1 Nonspecific cytokine inhibition

Corticosteroids are classified as nonspecific cytokine inhibitors and have a broad array of effects. The immune response is modulated by corticosteroids through regulation of gene expression. By blocking transcriptions factors such as NF-κB, all cytokine transcription, including IL-2, is inhibited. As a result, "signal 3" for immune response is blocked, leading to several downstream effects, such as T-cell depletion, induction of apoptosis, apoptosis of eosinophils and dysfunction of macrophages. Thus, the many effects on immune cells of corticosteroids not only accounts for its efficacy, but also for a broad array of side effects. Diabetes, hypertension, hyperlipidemia, truncal obesity, osteoporosis, avascular necrosis and cataract are just a few complications of chronic steroid use (21, 33).

To minimise major adverse effects, steroid sparing protocols have been a goal in organ transplantation for years. First systematic reviews showed that glucocorticoid sparing regimes had no effect on patient mortality (43) and already in 2009, KDIGO guidelines for kidney transplant recipients recommend the possibility of maintenance therapy without corticosteroids (44, 45). Later in 2016, based on the HARMONY study by Oliver Thomusch and colleagues (46), many authors drew the conclusion that rapid corticosteroid withdrawal is safe in kidney transplant recipients with a low immunologic profile (47).

1.3.5.2 IL-2 receptor antagonist

As mentioned before, IL-2 plays an important role in the immune response as 'signal 3'. It is produced by activated T cells and leads to further T cell proliferation. Basiliximab is a chimeric monoclonal antibody directed against the alpha chain of the IL-2 receptor (CD25 antigen), which is expressed on activated T lymphocytes. By blocking the IL-2 receptor, Basiliximab inhibits T cell proliferation and reduces acute rejection rates in KTX with little toxic effect (21, 33).

1.3.6 Polyclonal antibodies

1.3.6.1 Intravenous immune globulins

Intravenous immune globulin is a product that is rich in Immune globulin G (IgG) extracted from pools of several thousand plasma donors. In general, IVIG acts immune modulatory

and anti-inflammatory in high dose therapy. Reported mechanisms are direct binding to natural antibodies, immunomodulatory proteins such as cytokines, superantigens and pathogens, and inhibition of complement fixation on target tissue. In KTX IVIG is primarily used to support desensitisation of recipients and for treatment of antibody mediated rejection (33).

1.3.6.2 Polyclonal antithymocyte globulin

Antithymocyte globulins (ATGs) are polyclonal antibodies against human T cells, created by humanising animals with human lymphoid cells. The currently available Thymoglobulin and ATG Fresenius are purified globulins derived from rabbits immunised with human thymocytes. These agents target more than 20 different T cell epitopes leading to complement-dependent lysis and T cell depletion. As non-humanised immunoglobulin, all ATG products share side effects of cytokine release, such as fever, chills, hypotension and usually mild cardiovascular events (21). Thus, these agents are only used in treatment and prevention of acute kidney graft rejection (33).

1.3.7 Antiproliferative agents

In lymphoid cells, activation of mammalian target of rapamycin (mTOR) pathway leads to proliferation. Target of rapamycin inhibitors block 'signal 3' by preventing cytokines from activating the cell cycle. As a result, mTOR inhibitors such as Sirolimus or Everolimus, inhibit T and B cell proliferation and reduce antibody production. Side effects of mTOR inhibitors include hyperlipidemia, thrombocytopenia and impaired wound healing. Furthermore, in combination with CNI Sirolimus increases nephrotoxicity. Therefore, mTOR inhibitors may be an alternative to reduce nephrotoxicity in combination with CNI by withdrawing one of the agents (21, 32).

1.3.8 Antimetabolites- inhibition of DNA synthesis

These agents share similar mechanisms of interfering with either purine or pyrimidine synthesis and thus DNA synthesis. T and B cells rely exclusively on purine *de novo* synthesis because they are lacking a purine salvage pathway. Consequently, purine antimetabolites are relatively lymphocyte-specific and prevent B and T cell replication leading to reduced immune response (21).

1.3.8.1 Azathioprine

Azathioprine (AZA) is a prodrug and analog of 6-mercaptopurine whose metabolites have several effects. First, incorporation of these metabolites acting as purine analogs damages DNA and RNA, leading to cell death. Second metabolites inhibit *de novo* purine synthesis and third, block costimulatory signals via Rac1, resulting in T cell apoptosis (32, 33).

The most important side effect of AZA is myelosuppression. Due to its pharmacology of a prodrug, individuals with reduced enzyme activity are more prone to its toxicity, which is affecting 10% of the population (21).

However, AZA has now been replaced by mycophenolate in most new immunosuppressive protocols. Except in the setting of pregnancy: Azathioprine is not associated with teratogenicity, unlike mycophenolate (33).

1.3.8.2 Mycophenolate

Mycophenolate, accurately mycophenolate acid, is an inhibitor of inosine monophosphate dehydrogenase, which is required for purine *de novo* synthesis. There are two formulations available for immunosuppression: enteric coated mycophenolate sodium and mycophenolate mofetil (MMF), a prodrug releasing mycophenolate acid. These drugs have largely replaced azathioprine due to its effectiveness in combination with other agents, simple use without drug monitoring and better side effects. Besides many studies favouring MMF over AZA (48-51), a Cochrane review of 2015 showed that MMF was more effective than AZA for reducing graft loss by 20 % and acute rejection by 30 % (52). Furthermore, MMF is more tolerable in combination with mTOR inhibitors than AZA and its nonimmune toxicity is restricted to gastrointestinal and haematologic problems. Still cost is significantly greater than AZA (33).

1.4 Complications

Even if immunosuppression has advanced over the years and protocols have become more and more efficient, still organ transplantation comes at many risks. On the one hand immunosuppressive medication leads to severe infections, development of cancer and adverse effects. On the other hand, graft rejection and graft dysfunction are the main concerns of nephrologists. Due to this broad field of challenges, we will only have a look on a few of them.

1.4.1 Infections

Infections are a predictable risk in organ transplantation. Specific risk factors for infections are higher recipient age, deceased donor, higher number of HLA mismatches and higher risk for CMV disease (53). To reduce infection rates, surgical technique, antiviral and antibiotic prophylaxis, vaccination and avoidance of excessive immunosuppression seem to be cornerstones. But still, infections under immunosuppression remain a significant risk for transplantation outcome. Especially in the first year after transplantation, infections remain the leading cause of death, with around 40% (53). Furthermore, diagnosis of infection is complicated. Fever and physical signs are often diminished, laboratory or radiographic abnormalities are subtler. In up to 40% of infections fever is absent and about 22% of fevers are of non-infectious origin (54).

However, the risk of infection for the recipient at any time after transplantation is determined by two factors (21, 54):

1. Epidemiologic exposures of the recipient and donor: viruses, bacteria, parasites and fungus.
2. Net state of immunosuppression: all factors that contribute to the risk of infections like cumulative amount of immunosuppression, recipient comorbidities, infection with viruses that effect the immune system and the integrity of the mucocutaneous barriers.

Because of standardised immunosuppressive protocols, infections occur at a relatively specific pattern of time after transplantation. This pattern reflects the changing risk factors over time and changes with alterations in medication. Using prophylactic antimicrobial agents, for example, the “normal” appearance of infections will be delayed but not eliminated (54).

However, by dividing time after transplantation in three different categories of risk, Fishman, who has published several articles dealing with infections in organ transplantation (54-56), was able to create a timeline of post-transplant related infections. In the context of infection in transplant recipients, this timetable may be helpful for establishing a differential diagnosis, identifying environmental risks, over- and under-immunosuppression and for designing preventive microbial strategies (54).

1.4.1.1 Early post transplantation period (<4 weeks)

In the first months, infections are usually a result of surgery complications, as they would be seen in non-transplant patients after invasive procedures. However, differentiation of donor derived, pre-existing recipient or nosocomial infections is difficult. Furthermore, fevers might be associated with antibodies, transfusions, drug reaction or graft rejection. Still, early opportunist infections are uncommon since immunosuppression takes time to reach its full level (54).

1.4.1.2 Intermediate post transplantation period (1-12 months)

In this period, opportunistic infections due to immunosuppression are the main risk. Common causes are cytomegalovirus (CMV), Epstein-Barr virus (EBV), Listeria monocytogenes, Pneumocystis jiroveci and many Nocardia species. Preventive methods include antiviral prophylaxis that should prevent most human Herpesviridae for the duration of therapy and usually a combination of antibiotic agents (54). Recent KDIGO clinical practice guideline for the care of kidney transplant recipients suggest daily trimethoprim-sulfamethoxazole medication for at least 6 months after transplantation to avoid urinary tract infections and Pneumocystis jiroveci pneumonia (44). Again, besides opportunistic infections, there are multiple differential diagnosis such as graft rejection, lingering chronic infections and viral infections that had been in a state of latency before immunosuppression (54).

1.4.1.3 Late post-transplant period (>12 months)

By reducing maintenance immunosuppression, the risk of infection decreases subsequently. Recipients with good ongoing graft function and gradual reduction of immunosuppression are affected by all kinds of community-based exposures. In contrast, those with necessary maintenance therapy often suffer recurrent infections and develop progressive colonisation with drug-resistant pathogens (54).

1.4.1.4 CMV

Despite generally effective antiviral therapies, CMV remains an important pathogen in transplantation. About two thirds of all adults are latently infected with CMV. In transplant recipients, CMV infection may arise from reactivation of latent virus in the recipient, primary infection with donor-derived virus or reactivation of latent virus in the graft (21). The greatest risk for CMV infection occurs in the first 1-6 months after transplantation.

Especially primary infection of seronegative recipients with a graft from a seropositive donor, which represent up to 25% of all renal transplant recipients, is the highest risk (57).
In general, CMV infections lead to direct and indirect effects. Direct effects are CMV disease, which can either present as a CMV syndrome, characterised by flu like symptoms with neutropenia, or as tissue invasive disease, presenting as inflammation of specific organs with subsequent symptoms. Each of these conditions can be diagnosed by high levels of CMV viremia. Therefore, viral load surveillance with polymerase chain reaction (PCR) of CMV DNA in blood is widely used. (58).
Indirect effects are more complicated and are a result of the influence of the virus on the host's immune system. These mechanisms are complex and not caused by high viral load, but rather by low-level viral replication that seem to be affecting immune response. However, indirect effects of CMV seem to be responsible for secondary fungal and bacterial infections, development of cancer, development of post-transplant diabetes mellitus and decreased graft and patient survival (58).
The association of CMV infection and graft rejection in kidney transplantation has been proven and resulted in antiviral prophylaxis (54, 58-60). Recent KDIGO guidelines suggest all kidney transplant recipients with risk for infection should be administered chemoprophylaxis with oral ganciclovir or valganciclovir for at least 3 months (44). Furthermore, treatment of CMV infection with intravenous ganciclovir should be continued until CMV or pp65 antigen is no longer detectable (44, 61).

1.4.1.5 Polyomaviruses

Of the family of polyomaviruses especially BK and JC viruses are affecting transplant recipients. They are widely distributed in general population and tend to latency in urogenital epithelial cells (62). However, diseases are restricted to immunocompromised hosts. In transplant recipients, polyomaviruses are associated with several pathologies, such as tubulointerstitial nephritis and nephropathy, condylomata and even malignancies.
BK virus infection occurs in 1-10% of renal recipients and originates from the allograft. Infection is associated with viruria, viremia, ureteric stenosis, ureteric ulceration and polyoma-associated nephropathy (54). Due to polyomavirus BK 15% of kidney transplant recipients are in danger of graft loss (63).
However, diagnosis is difficult and patients with polyomavirus-associated nephropathy (PVAN) often present with diminishing graft function. Plasma BK viral load may be helpful, but biopsy with immunohistology is needed to distinguish PVAN from graft rejection or drug toxicity. Furthermore, there is no specific treatment for polyomaviruses other than

reducing immunosuppressive medication. On the one hand reducing immunosuppression may diminish PVAN, but risks graft rejection. On the other hand, symptoms of PVAN may resemble graft rejection requiring higher immunosuppression, but which would lead to worsening of PVAN and graft loss. Thus, diagnosis and therapy are crucial (54).
Therefore, screening of all kidney transplant recipients for BK virus with quantitative plasma nucleic acid tests is recommended and reduction of immunosuppression is suggested when viral plasma loads are very high (44, 63).

1.4.2 Delayed graft function

The most common definition of delayed graft function (DGF) is requirement of dialysis within one week after transplantation. DGF is a very common complication with an incidence of 3%, 23% and 31% for living donor, standard deceased donor and ECD, respectively (21). DGF results from immunologic and non-immunologic events during the transplantation process. Usually it is a consequence of ischemia and reperfusion injury leading to acute tubular necrosis (64).
Risk factors for DGF include cold ischemia time, recipient`s BMI, recipient gender, recipient`s residual diuresis < 500ml per day, perioperative saline loading, deceased cardiac donor, donor age, donor`s terminal creatinine and quality of pre-kidney procurement care (64, 65).
However, diagnosis of DGF is difficult because reperfusion injury and acute rejection are both, the main causes for DGF and hard to distinguish from each other (65). Even if dialysis requirement or persistent high serum creatinine are cornerstones for detection, biopsy is necessary for a definitive diagnosis (66). Additional standard ultrasonography is often used to assess potential surgical complications and with duplex sonography elevated resistive index might suggest intrarenal graft dysfunction (21).
Furthermore, there is no established treatment for DGF and in general patient management remains supportive. Despite the fact that uncomplicated acute tubular necrosis usually resolves spontaneously (21), several studies showed that recipients with DGF experience significantly reduced graft survival (65, 67, 68). Therefore biomarkers predicting DGF might be important (69) and until then, prevention seems to be a cornerstone. Reducing cold ischemia time and using hypothermic machine perfusion instead of cold storage lead to better outcomes and reduced DGF incidence (64).

1.4.3 Rejection

The biggest fear of nephrologists regarding dysfunction of the kidney graft is rejection. Hyperacute rejection is the earliest form of it and can often already be seen intraoperative. Preformed recipient antibodies react with antigens on the endothelium of the graft. This leads to immediate activation of complement and coagulation cascade which can be seen in cyanotic discoloration (7). These antibodies are usually directed against antigens of the AB0 blood group system or HLA class 1. HLA class 1 antibodies are formed in previous transplantation, blood transfusions or pregnancy. The only treatment for hyperacute rejection is transplant nephrectomy. However, modern tissue typing technologies, antibody screening and cross matches have ensured that hyperacute rejection is uncommon nowadays (21).

1.4.3.1 Acute rejection

Acute rejection is a clinical syndrome caused by either a cellular or humoral immune response against a transplanted organ. It is characterised as decline in a kidney function and usually occurs in the first 6 months after transplantation. Incidence in the first 12 months is about 10%. In clinical practice, rejection is often only detected through surveillance monitoring of graft function, but symptoms like fever, oliguria and graft pain or tenderness might occur. Since differential diagnosis, such as infection, drug nephrotoxicity and delayed graft function, are difficult to distinguish but require directly opposed therapy, biopsy is needed (21).

Treatment of graft rejection is based upon histological finding. For cellular rejection initial treatment consists of additional prednisone and in steroid resistant cases lymphocyte depleting antibodies (44). Antibody mediated rejection has poorer prognosis than cellular rejection and is more difficult to treat. Strategies include combinations of plasma exchange, IVIG, anti-CD20 antibodies and lymphocyte-depleting antibodies (21, 44, 70). Therefore, strict histological classification for biopsies is needed. The Banff Classification of allograft pathology is the international standard, updated on regular basis by international committees. It provides diagnostic criteria for active and chronic antibody mediated rejection, borderline rejection and acute and chronic T cell mediated rejection of different grades by histologic, serologic and molecular evidence (71).

However, acute rejection rates are generally associated with a reduction in long term allograft survival, but completely reversed episodes to baseline function don`t have to influence allograft outcomes (72). Risk factors for acute rejection seem to be donation after

circulatory death, ECD, DGF, older donor age, African American ethnicity and immunological risk factors such as HLA mismatch, HLA antibodies and donor specific antibodies (73-75).

1.4.3.2 Chronic rejection

Chronic rejection refers to slow but progressive decline in graft function. Histological findings are characterised by interstitial fibrosis and tubular atrophy, which is seen in almost all kidney grafts over time. Even though acute and chronic rejection seem to have the same effector mechanisms, it is thought that delayed type hypersensitivity and alloantibodies are particularly important for chronic rejection. Clinical prevention of chronic rejection consists of minimisation of CNI nephrotoxicity, control of blood pressure and blood glucose levels (76).

1.5 Immune system

1.5.1 The immune system-an overview

The human immune system consists of a variety of effector cells and molecules with the function of protecting the body from foreign and infectious agents. Most cells of the immune system develop and mature in the bone marrow including its main actors, the white blood cells, called leucocytes. Mature immune cells can reside in peripheral tissue, circulate in the blood stream or circulate in the lymphatic system. The lymphatic system is a specialised drainage from peripheral tissue through lymph nodes and into the thoracic duct, back to empty in the left subclavian vein. Its main purpose is to carry loaded antigen presenting cells to lymph nodes and lymphocytes back into the blood. (77p2-3)
In general, the vertebrate immune system can be divided in innate and adaptive immunity.

1.5.2 Innate immune system

If anatomic and chemic barriers of the host are breached by pathogens, the innate immune system is the first line of defence. Innate immune responses occur rapidly speaking in terms of hours, in contrast to adaptive immune responses, which takes rather days to develop. The

innate immune system consists of several cell types with different functions: macrophages, granulocytes (neutrophils, eosinophils and basophils), mast cells, natural killer cells and dendritic cells. The main purpose of these cells is recognition of foreign materials known as pathogen-associated molecular patterns (PAMPs) and induction of specific immune responses. To achieve a broad array of responses, immune cells either specialised in killing pathogens or in attracting and activating other cells. Especially sensor cells such as macrophages and dendritic cells can amplify the immune response by producing inflammatory mediators. These inflammatory mediators are secreted proteins important for cell signalling that are called cytokines and chemokines. Cytokines affect the behaviour of nearby cells that bear suitable receptors. More than 60 different cytokines have been identified until now. Chemokines are chemotactic cytokines. This subgroup of secreted proteins attracts cells bearing chemokine receptors. In general cytokines and chemokines act to direct cells from the bloodstream into the infected tissue resulting in inflammation (77p6-10).

Another component of innate immunity is the so-called complement system, which combines direct recognition of microbes with a complex effector system. In specific, it is a group of 30 different plasma proteins that interact with each other to form several different pathways of complement activation. These serum factors “compliment” the activity of antibodies, but can also target foreign organisms in the absence of specific antibodies. Its main purpose is to target pathogens for lysis and phagocytosis and furthermore inducing inflammatory responses that help fighting the infection. Even if the complement system is often described as humoral innate immunity, it contributes to both innate and adaptive responses (77p49,p73).

1.5.3 Adaptive immune system

Usually, when the innate defence mechanisms are overwhelmed by a pathogen, the adaptive immune system gets activated by innate immune mechanisms. Most pathogens require this recruitment of the adaptive immunity and only few infections can be handled by innate immunity alone. The adaptive immune response consists of several different activities of its two main players: B lymphocytes and T lymphocytes (often referred to as B and T cells). T cell responses lead to cellular immunity, while B cell responses lead to humoral, antibody-mediated immunity (77p345).

As described before, lymphocytes recirculate between bloodstream and secondary lymphoid tissue. Mature recirculating T cells that haven't encountered antigens yet are called naïve T cells. To participate in an immune response, naïve T cells must be presented antigens as a peptide-MHC complex on the surface of an antigen presenting cell. This induces differentiation and proliferation into progeny called effector T cells that are fully functional contributing to antigen removal (77p345).

However, there are several types of T cells characterised by their type of T cell receptor and by the expression of different markers. The two main classes of T cells express either a transmembrane glycoprotein called cluster of differentiation 8 (CD8) or another one called cluster of differentiation 4 (CD4). These surface proteins recognise different regions of MHC molecules and thus, are known as co-receptors (77p29). CD8 recognises MHC class I molecule and is expressed by cytotoxic T cells that kill infected cells. Meanwhile CD4 recognises MHC class II and is expressed by T cells whose function is to activate other cells. Naïve CD4 cells can differentiate into effector subsets with different immunological functions. The main CD4 effector subsets are T helper cell type 1 (Th1), T helper cell type 2 (Th2), T helper cell type 17 (Th17) and follicular helper T cell (Tfh), which activate their target cells. Recent discoveries identified an additional type of T cell, so-called regulatory T cells (Tregs), that restrain immune response (77p346).

1.6 Regulatory T cells

Regulatory immune cells are considered to play an important immunoregulatory role in preventing autoimmune disease and in alloreactive immune response in graft rejection. About 10% of all CD4 cells in circulation are Tregs. They can be classified in different groups defined by their different developmental origins and functions (77p379). Natural regulatory T-cells (nTregs) develop in Thymus and are CD4 positive that constitutively express CD25 and high levels of the L-selectin receptor CD62L and of CTLA-4 (CD152). In contrast, induced Tregs (iTregs) originate from peripheral naïve CD4 T cells, but express CD25 and CTLA-4 just like nTregs. A characteristic attribute of both natural and induced Tregs is expression of the transcription factor FoxP3, which is essential for preventing IL-2 production. In general, Tregs control immune response by preventing adequate co-stimulation of naïve T cells or by producing immunosuppressive cytokines. The major function of iTregs is prevention of inflammatory immune response to the commensal

microbiota. In the intestines, for example, iTregs seem to be the main source of IL-10, deficiency of which causes inflammatory bowel disease (77p379).

1.6.1 Treg identification

The first group to prove the importance of Tregs was Sakaguchi and colleagues. They described these at this time called CD25 positive T cells to be responsible for maintaining self-tolerance and preventing autoimmune diseases (78). Furthermore, the suppressor function of Tregs seems to work antigen-nonspecific and expansion can be induced with IL-2 and anti-CD3 (79). However, molecules expressed on the surface that define human Tregs are not fully identified. The so-called protein forkhead box P3 (FoxP3) seemed to be the most promising marker for Tregs, but due to its function as transcription factor, it's only found intracellular and therefore can't be used for Treg isolation (80). As a result, other cell markers expressed by Tregs have been found. The expression of FoxP3 has been proven to correlate with low levels of CD127 (cell surface expression of IL-7 receptor) and high levels of CD25 (81).

1.6.2 Treg subsets

In combination with CD45RA, and CD45RO three main types of T cell subsets can be distinguished: naïve/resting Tregs staining for $CD25^{+}$ $FoxP3^{+}$ $CD45RA^{+}$ $CD45RO^{-}$ $CD127^{low}$; memory Tregs (mTregs) staining for $CD25^{high}$ $FoxP3^{high}$ $CD45RA^{-}$ $CD45RO^{+}$ $CD127^{low}$; and activated/effector Tregs with the highest expression of $CD25^{+++}$ $FoxP3^{high}$ $CD45RA^{-}$ (82, 83). The name of naïve/resting Tregs attributes to the fact, that these cells have not encountered their specific antigen yet and are waiting for their activation. Meanwhile effector/activated Tregs have already received antigen stimulation and have activated their suppressive effect (84). Despite effector and resting Tregs are both of suppressive function, other subsets of Tregs mirror conventional T cells in their purpose. The population of $CD25^{+}$ $FoxP3^{low}$ $CD45RA^{-}$ cells, for example has been proven to produce inflammatory cytokines like IL-17 (83). Due to their similar function to Th17 cells, these Tregs are often referred to as non-suppressive Tregs. In addition, the surface marker CD161, can not only be found on Tregs, but also on Th17 cells, associated with proinflammatory potential (85). Furthermore, conversion of Tregs into Th17 cells has been discussed before (86, 87).

Anyways, cell markers not only allow identification of separate Treg lineages, but they also bear information of different assets of Tregs. By detection of the nuclear protein Ki-67, effector Tregs can be assessed on their potential of proliferation (83). The cell marker CD15s and the transmembrane protein called glycoprotein A repetitions predominant (GARP) identify effector Tregs with very high suppressive function (88, 89). Moreover, GARP is expressed only for limited time after activation of Tregs and restricted to FoxP3 population, allowing more precise Treg identification (89).

However, even if FoxP3 seems to be one of the most prominent cell markers, research suggests that it's neither necessary nor sufficient for Treg specification (90). After the discovery of the important role of FoxP3, it was demonstrated that non-Tregs can acquire both FoxP3 and the regulatory function associated with it (91). The fact that naïve conventional T cells can be induced to express FoxP3 by a variety of methods, raises the question if Tregs are rather a distinct lineage or one of several states into which naïve T cells can differentiate (90).

1.6.3 Tregs in kidney transplantation

By further understanding and characterisation of the Treg population, manipulating these cells effectively to treat diseases became interesting. In transplantation the number of Treg cells was higher in tolerant recipients than in those with rejection, indicating their ability of maintaining tolerance (92). Research showed that transferred Tregs protect recipient mice from undergoing graft rejection (92). Several *in vivo* experiments showed that by adaptive tolerance induction, naïve CD4 T cells can undergo conversion into functional Tregs with the capacity to prevent allograft rejection (93). This offers the possibility of converting alloreactive CD4 T Cells into graft protective Tregs in clinical transplant recipients without the need for large scale T cell depletion.

The ONE study was one of the first clinical trials in renal transplantation investigating cellular therapy (94). Even if results are eagerly awaited, its most important aspect might be the representation of the start of cellular therapy in solid organ transplantation. Several reviews have discussed this enormous potential of Treg administration as well as practicality issues and other valuable lessons of these first trials (80, 95, 96).

Until cellular therapy becomes available, analysis of regulatory cell population in transplant patients may be essential for new therapeutic strategies. For example, a higher frequency of circulating mTregs in tolerant recipients compared with nontolerant ones has been reported

(97). Furthermore, possible biomarkers predicting graft survival, function or rejection are already being evaluated (96, 98).

1.7 Fluorescence activated cell sorting

To study specific immune cell populations, special equipment is needed. By using a laser beam, a flow cytometer can detect and measure individual cells. Fluorescence activated cell sorting (FACS) takes this technique even further by separating identified cells with the use of antibodies. First, cells are mixed with antibodies against the desired cell markers. To make these antibodies detectable, either the antibodies itself are combined with fluorescent dyes or they are tagged themselves with fluorescent anti-immunoglobulin antibodies. Finally, this mixture of cells and antibodies is put through a fine nozzle, so that only one cell at a time is able to pass in front of a laser beam. As each single cell is hit by the laser, two things happen simultaneously: laser light is scattered and detected by photomultiplier tubes, giving information about size and granularity of cells; at the same time the fluorescent dye emits light allowing conclusion on level of expression of cell markers (77p767-768).

With the use of a computer, the cell sorter can identify each single cell: At the moment the stream is broken up into droplets of single cells, each droplet is given an electrical charge. Thereafter, as the stream passes between plates of opposite charges, repulsion of like charges and attraction of unlike charges is used for separating cells into tubes. By this method, specific cell populations can be separated from a mixture of cells into different tubes by the use of antibodies (77p768).

Furthermore, with the use of several lasers and antibodies at the same time, flow cytometry allows examination of large amounts of T cell subsets bearing different molecules, such as T cell receptor associated molecule CD3 (77p769).

2 Materials and methods

2.1 Study design

In this study we conducted a prospective monocentric pilot trial to evaluate differences in immune population in 74 patients, who underwent kidney transplantation. Data gathering started in January 2017 and ended in January 2019.
The study has been approved by the Ethics Committee of the Medical University of Graz, Austria (28-514 ex 15/16). Informed consent has been obtained and all the collected data has been anonymised for analysis.

2.2 Study procedures

Every study participant had five study visits. Patients were first asked to participate in this study on the day transplantation was planned. All following study visits took place during a standard procedure visit at the outpatient care of the Division of Nephrology Graz.
Planned study visits:

- Study visit 1: Before the planned kidney transplantation
- Study visit 2: 7-10 days after kidney transplantation
- Study visit 3: 56 days after kidney transplantation
- Study visit 4: 6 months after kidney transplantation (without flow cytometry)
- Study visit 5: 1 year after kidney transplantation (without flow cytometry)

2.3 Patient population

Patients undergoing kidney transplantation and healthy control participants were recruited by clinicians involved in this study, working at the Division of Nephrology, Department of Internal Medicine at the Medical University of Graz, Austria.
Inclusion criteria:

- Age between 18-80 years
- Patients receiving their first kidney transplant
- Induction therapy with anti-thymocyte globulin (ATG)
- Signed informed consent

Exclusion criteria:

- Pregnancy
- Immunosuppression within the past 3 months
- Patients with AB0-incompatible kidney transplantation
- Patients with repeated kidney transplantation

2.4 Data collection

After informed consent has been obtained, socio-demographic information, information about medical history, medication and dietary habits were collected. Sources were case report forms for each participant, hospital records, laboratory records and correspondence. Table 1 sums up all gathered information

Demographics: date of birth, gender, race, smoking status, alcohol consumption
Medical history and standardised questionnaire on fertility outcomes
Concomitant medication
Vital signs: resting pulse, blood pressure
Blood samples for FACS analysis at study visit 1,2 and 3
Urine samples at all study visits, stored at -70°C for further evaluation
Outcome parameters such as estimated GFR at study visits 4 and 5

Table 1: Data collected of all subjects

Fluorescence activated cell sorting (FACS); glomerular filtration rate (GFR).

2.5 Laboratory measurements

Routine laboratory measurements including blood count, CRP-, serum creatinine- and urea-levels, were conducted at every study visit. Additionally, urine samples were collected and stored at -70°C.

The cellular expression of biomarkers was quantified using fluorescence- activated cell sorting (FACS) technology. Peripheral mononuclear cells are isolated from freshly donated whole blood. For the quantification of distinct biomarkers, cells will be purified and stained with selected monoclonal antibodies. Table 2 shows all monoclonal antibodies used for the

staining of surface and intracellular markers of specific leucocytes (purchased mainly from BD Biosciences, USA).

Panel 1	**Panel 2**	**Panel 3**	**Panel 4**	**Panel 5**
CD3	CD3	CD3	CD27	CD3
CD4	CD4	CD4	IgM	CD4
CD127	CD127	CD127	IgD	CD25
CD25	CD25	CD25	CD19	FoxP3
FoxP3	CD45RA	CD45RA	CD86	CD45RA
CD45RA	CD95	CD8	CD5	CD8
GARP	CD147	CD28	CD38	CD73
CD15S	CD307c	CD39	CD20	CD152
Ki-67	CD366	CD49b	CD43	CD274
FVS	PI-16	CD223	CD1d	CD279
CD161	LAP	CD226	CD24	FVS
	HLA-DR		CD131	pSTAT5

Table 2: Cell markers used for staining

Five different panels of antibodies were used in the clinical trial for flow cytometry of Treg subpopulations. This thesis focused on Tregs identified only using panel 1.

Using panel 1, different Treg subpopulations will be studied. This diploma thesis only focused on Tregs evaluated with Panel 1. Other cell population analysis using Panel 2-5 are not included. Table 3 shows a list of the Tregs of interest, including their phenotype and how their measured values will be displayed.

If needed, additional controls for the quantification of positive signals were used, such as isotype controls and fluorochrome minus one (FMO) method. Standardisation of the FACS system (BD LSR Fortessa) was controlled by day-to-day cytometer performance checks using BD Cytometer Setup, Tracking Beads and standardised assay specific settings, which were daily checked by BDOneFlow setup beads (BD Bioscences, USA).

Reported marker	Tregs displayed as percent of	Phenotype
FoxP3 Treg	Total $CD3^+$ $CD4^+$	$CD3^+CD4^+FoxP3^+$
CD127 FoxP3	Total $CD3^+$ $CD4^+$	$CD3^+CD4^+FoxP3^+CD127^+$
CD25 /CD127 Treg	Total $CD3^+$ $CD4^+$	$CD3^+CD4^+CD25^+CD127^{dim}$
FoxP3 /CD25 Treg	Total $CD3^+$ $CD4^+$	$CD3^+CD4^+CD25^{high}FoxP3^+$
Treg naïve	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^+$ $CD15S^-$
Treg effector	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^+$
Treg effector-proliferative	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $Ki\text{-}67^+$
Trans Treg 161^-	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^-$ $CD161^-$
Th17 type Treg 161^+	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^-$ $CD161^+$
GARP Tregs	$CD25^+$ $CD127^{dim}$ Tregs	$CD3^+CD4^+CD25^+CD127^{dim}$ $GARP^+$
activated Treg	Total $CD4^+$	$CD3^+CD4^+CD45RA^-$ $FoxP3^+$
non-suppressive T cells	Total $CD4^+$	$CD3^+CD4^+CD45RA^-$ $FoxP3^{low}$
resting Treg	Total $CD4^+$	$CD3^+CD4^+CD45RA^+$ $FoxP3^{low}$

Table 3: List of evaluated cell markers and their presentation.

First column shows all reported cell markers that were studied. Second column lists the corresponding group of total cells of which Tregs are displayed as percent. Third column shows the exact phenotype of each Treg population.

2.6 Statistical analysis

The statistical analysis for this thesis was done in November 2020 using *IBM SPSS Statistics Version 25* and *Version 26.* The data was checked for normal distribution using Shapiro-Wilk and Kolmogorov-Smirnov test. Normally distributed data were presented by means and standard deviation, non-normally distributed data by medians and interquartile range. For analysis of differences among two groups, Students t-test was used for parametric data, Mann-Whitney U-test was used for nonparametric data. For comparison of multiple groups,

one-way analysis of variances was used for normally distributed data and Kruskal- Wallis test for non-parametric data. To establish further differences between multiple groups, Bonferroni correction was applied. A two-sided p value < 0.05 was considered statistically significant.

3 Results

3.1 Baseline characteristics

3.1.1 Control group vs. end-stage kidney disease

Of the 74 healthy study participants, 23 were males and 51 females. The mean age was 46.0 ± 15.88 years.

Seventy-four ESKD patients listed for kidney transplantation were included. Fifty transplant candidates were male and 24 were female with a mean age of 55.5 ± 11.93years.

The baseline characteristics of all study subjects sorted by group are listed in Table 4. KTX candidates were significantly older (55.5 ± 11.93 years vs 46.0 ± 15.88 years; $p < 0.001$), had higher weight (80.0 ± 17.07 kg vs. 73.3 ± 14.61 kg; $p = 0.008$), body mass index (26.6 ±7.5 kg/m² vs 23.9 ±6.2 kg/m²; $p = 0.008$) and systolic blood pressure (143± 24.4 mmHg vs. 126 ± 15.3 mmHg; $p = 0.965$) than their healthy control group. No significant differences were seen regarding height (171.0±9.07 cm vs. 170.3 ± 8.28cm; $p = 0.442$) and diastolic blood pressure (80 ± 16 mmHg vs. 82 ± 15mmHg; $p = 0.965$).

Demographics and recipient characteristics	Group		p-values
	Healthy	ESKD	
Total Number	74 (100%)	74 (100%)	
Male N (% of group)	23 (31%)	50 (68%)	
Female N (% of group)	51 (69%)	24 (32%)	
Age [years] M (SD)	46.0 (15.88)	55.5 (11.93)	**0.000**
Height [cm] M (SD)	170.3 (8.28)	171.0 (9.07)	0.442
Weight [kg] M (SD)	73.3 (14.61)	80.0 (17.07)	**0.008**
BMI [kg/m²] MD (IQR)	23.9 (6.2)	26.6 (7.5)	**0.008**
BP Sys [mmHg] M (SD)	126 (15.3)	143 (24.4)	**0.000**
BP Dias [mmHg] MD (IQR)	82 (15)	80 (16)	0.965

Table 4: Baseline characteristics sorted by groups.

Comparison of healthy control group (Healthy; n=74) and end-stage kidney disease group (ESKD; n=74). Data are given as numbers (N), means (M), standard deviation (SD), median (MD) or interquartile range (IQR).

3.2 Biopsy proven rejection

Throughout follow-up, 13 patients experienced biopsy proven rejection.
Rejection was categorised with Banff classification. 2 patients had borderline rejection, 3 patients Banff 1B rejection, 5 patients Banff 2A rejection, 1 patient with Banff 2B rejection and 1 patient with Banff 3 rejection. No Banff 1A rejections were observed. In total 3 humoral mediated rejection episodes were seen: one of these showed simultaneous signs of Banff 3, the other one had signs of Banff 2A and one of them without criteria of Banff classification. Rejection time was measured as time of days between transplantation and biopsy proven rejection. Median rejection time was 8 (interquartile range: 50 days). All results are presented in Table 5.

Rejection characteristics	Rejection numbers
BANFF	
Borderline	2 (16.7%)
Banff 1A	0
Banff 1B	3 (25%)
Banff 2A	5 (41.7%)
Banff 2B	1 (8.3%)
Banff 3	1 (8.3%)
Total	***12 (100%)***
Humoral rejection	3 (2 of which are Banff)
Rejection Time [days] MD (IQR)	8.0 (50.00)

Table 5: Rejection categories and rejection time.
Data are given as absolute numbers, percent among rejection category, median (MD) or interquartile range (IQR). Rejection time represents time of days between transplantation and biopsy proven rejection.

3.3 Baseline characteristics regarding rejection

During follow-up, 13 of all 74 kidney transplant recipients showed biopsy proven rejection. As shown in Table 6, there were no significant differences in terms of age, height, weight, BMI, blood pressure and number of HLA mismatches (HLA MM) between those who

experienced biopsy proven rejection episodes and those who did not. Interestingly recipients with biopsy proven rejection had spent significantly less time on dialysis than patient without rejection (27.5 ± 10.21 months vs. 36.8 ± 20.67 months; p = 0.018).

Demographics and recipient characteristics	Group		p-values
	No Rejection	Rejection	
Number of KTX	61 (100%)	13 (100%)	
Male N (% of group)	43 (70.5%)	7 (53.5%)	
Female N (% of group)	18 (29.5%)	6 (46.2%)	
Age [years] M (SD)	55.7 (11.85)	54.0 (12.87)	0.445
Height [cm] M (SD)	171.2 (9.39)	170.0 (7.60)	0.876
Weight [kg] M (SD)	81.4 (17.87)	72.4 (13.38)	0.125
BMI [kg/m²] MD (IQR)	27.1 (7.76)	25.6 (3.88)	0.091
BP Sys [mmHg] M (SD)	141.3 (24.90)	151.9 (22.58)	0.230
BP Dias [mmHg] MD (IQR)	80.0 (15)	84.0 (15)	0.227
Dialysis vintage [months] M (SD)	36.8 (20.67)	27.5 (10.21)	**0.018**
HLA MM MD (IQR)	4.0 (1.00)	4.0 (2.00)	0.717

Table 6: Baseline characteristics sorted by rejection group.

Data are given as numbers (N), means (M), standard deviation (SD), median (MD) or interquartile range (IQR). Kidney transplant recipients (KTX); body mass index (BMI); blood pressure systolic (BP Sys); blood pressure diastolic (BP diastolic); HLA mismatches (HLA MM).

3.3.1 HLA mismatch

The number of HLA mismatches was evaluated using Eurotransplant.org. All data was gathered anonymously. Differences in HLA qualities between donor and recipient were transformed in numbers of HLA mismatches. Among patients with rejection, no patient had full mismatch or a full- house match. A detailed view of all HLA mismatches is presented in Table 7.

Number of HLA MM	Total of ESKD	No rejection	rejection
0	1 (1.4%)	1 (1.6%)	0
1	5 (6.8%)	3 (4.9%)	2 (15.4%)
2	5 (6.8%)	4 (6.6%)	1 (7.7%)
3	21 (28.4%)	18 (29.5%)	3 (23.1%)
4	26 (35.1%)	22 (36.1%)	4 (30.8%)
5	15 (20.3%)	12 (19.7%)	3 (23.1%)
6	1 (1.4%)	1 (1.6%)	0
Total	***74 (100%)***	***61 (100%)***	***13 (100%)***

Table 7: Numbers of HLA-MM sorted by groups

Data are given as absolute numbers and percent of each group. HLA mismatch (HLA MM), End-stage kidney disease patients (ESKD).

3.3.2 Primary diseases

Renal diseases were categorised in 5 different groups: glomerulonephritis, hereditary polycystic kidney disease, diabetic nephropathy, hypertensive nephropathy and other kidney diseases. The absolute numbers and differences among groups are presented in Table 8.
Furthermore, dialysis modality was evaluated. In total, two patients underwent pre-emptive kidney transplantation without prior dialysis, 60 patients had previously been treated with haemodialysis and 12 patients with peritoneal dialysis. No live donations were observed. Table 8 shows a detailed view of all observations.

	Total of ESKD	No rejection	Rejection
Renal disease			
Diabetic Nephropathy	11 (14.9%)	10 (16.4%)	1 (7.7%)
Hypertensive nephropathy	5 (6.8%))	5 (8.2%)	0 (0%)
Glomerulonephritis	16 (21.6%)	14 (23.0%)	2 (15.4%)
Hereditary polycystic KD	9 (12.2%)	6 (9.8%)	3 (23.1%)
Other Kidney diseases	32 (43.2%)	26 (42.6%)	6 (46.2%)
Total number within group	***74 (100%)***	***61 (100%)***	***13 (100%)***
Therapy			
PD therapy	12 (16.2%)	10 (16.4%)	2 (15.4%)
HD therapy	60 (81.1%)	50 (82.0%)	10 (76.9%)
Pre-emptive KTX	2 (2.7%)	1 (1.6%)	1 (7.7%)
Total number within group	***74 (100%)***	***61 (100%)***	***13 (100%)***
Live donation	0	0	0

Table 8: Primary diseases, type of therapy and live donations sorted by groups.
Data are given as absolute numbers and percent of each group. End-stage kidney disease (ESKD), Kidney transplantation (KTX).

3.4 Laboratory results

Basic blood work of all patients was collected at day of transplantation. Differences among patients with rejection and no rejection were evaluated. No significant differences were observed among Na^+, Ka^+, Ca^{++}, Cl^-, Phosphate, CRP, albumin, total bilirubin and creatinine. All data can be seen in Table 9.

Complete blood count and differential counts were evaluated. Haemoglobin levels, haematocrit, leukocytes, lymphocytes, neutrophils and thrombocytes numbers are shown in Table 10. There were no significant alterations of cell counts between groups.

Lab category	Group		p-values
	No Rejection	Rejection	
Na^+ [mmol/l] M (SD)	138.5 (2.99)	139.8 (2.79)	0.143
K^+ [mmol/l] MD (IQR)	4.48 (1.100)	4.80 (0.650)	0.477
Ca^{++} [mmol/l] M (SD)	2.25 (0.171)	2.32 (0.207)	0.179
Cl^- [mmol/l] MD (IQR)	100 (6.0)	98 (14.0)	0.5210
Phosphate [mmol/l] M(SD)	5.46 (1.795)	5.60 (1.428)	0.796
CRP [mg/dl) MD (IQR)	3.1 (5.90)	5.4 (5.45)	0.826
Albumin [g/dl] MD (IQR)	4.2 (0.40)	4.4 (0.50)	0.145
Bilirubin [mg/dl] Total MD (IQR)	0.3 (0.20)	0.3 (0.17)	0.678
Creatinine [mg/dl] M (SD)	8.27 (2.896)	7.40 (2.239)	0.317

Table 9: Baseline laboratory sorted by groups

Data are given as means (M), standard deviation (SD), median (MD) or interquartile range (IQR). C- reactive protein (CRP).

Lab category	Group		p-value
	No Rejection	Rejection	
Hb [g/dl] M (SD)	11.5 (1.41)	12.0 (1.53)	0.251
Haematocrit [%] M (SD)	34.3 (4.31)	35.8 (4.61)	0.264
Leukocytes MD (IQR)	6.6 (3.10)	6.5 (2.54)	0.878
Lymphocytes MD (IQR)	1.3 (0.83)	1.5 (1.10)	0.388
Neutrophils M (SD)	4.8 (1.65)	4.6 (1.85)	0.641
Thrombocytes M (SD)	220.0 (63.33)	203.8 (36.21)	0.378

Table 10: Blood work sorted by groups.

Data are given as numbers (N), means (M), standard deviation (SD), median (MD) or interquartile range (IQR), cells are presented as x 10^9 /L unless otherwise indicated. Haemoglobin (Hb).

3.5 Treg comparison

3.5.1 Tregs in control group

Using previously described panel 1 in Table 2, cell markers for distinct regulatory cell populations of 74 healthy controls were evaluated. The frequency of each population is listed in Table 11 sorted by groups.

3.5.2 Tregs in transplant candidates

All blood samples of transplant candidates were drawn just before kidney transplantation was performed. Blood samples of kidney transplant recipients were evaluated for distinct lymphocyte subpopulations. Furthermore, differences between patients and healthy controls were analysed and the results are presented in Table 11.

While there was no significant difference in $CD3^+$ $CD4^+$ T cells (44.31 ± 9.47 vs. 46.06 ± 8.90 % of lymphocytes; p = 0.076), and $FoxP3^+$ Tregs (5.70 ±3.20 vs. 5.09 ± 1.95 % of $CD3^+CD4^+$; p = 0.076), significantly more $CD127^+$ $FoxP3^+$ cells (1.29± 2.09 vs. 0.27 ± 0.77 % of $CD3^+CD4^+$; p < 0.001), more $CD25^+$ $CD127^+$ Tregs (7.04 ± 3.23 vs. 5.65 ± 2.10 % of $CD3^+CD4^+$; p < 0.001) and more FoxP3/CD25 Tregs (5.25 ± 2.85 vs. 4.34± 2.05 % of $CD3^+CD4^+$; p < 0.001) were measured in ESKD patients.

Within $CD25^+$ $CD127^{dim}$ Tregs there were significantly more Th17 type $CD161^+$ Tregs (2.94 ± 1.94 vs. 1.79 ± 1.47 % of $CD25^+$ $CD127^{dim}$; p< 0.001) and more $CD161^-$ Trans Tregs (34.47 ± 19.90 vs. 28.37 ± 10.14 % of $CD25^+$ $CD127^{dim}$; p = 0.002), while less naïve Tregs (30.20 ± 12.47 vs. 34.69± 12.53 % of $CD25^+$ $CD127^{dim}$, p = 0.035) were found in patients waiting for transplantation than in healthy controls. There was no significant difference in effector Tregs (30.61 ± 10.04 vs. 32.60±11.28 % of $CD25^+$ $CD127^{dim}$; p = 0.224) and effector-proliferative Tregs in $CD25^+$ $CD127^+$ Treg (4.64± 3.46 vs. 4.64± 3.49 % of $CD25^+$ $CD127^{dim}$; p = 0.766) among groups.

Among all $CD4^+$ T cells, there were more non-suppressive T cells (2.92 ± 1.53 vs. 2.59 ± 1.61 % of $CD4^+$; p = 0.008) and activated Tregs (0.67 ± 0.78 vs. 0.45 ± 0.43 % of $CD4^+$; p < 0.001) in patients with ESKD, but there was no difference in resting Tregs (1.93 ± 1.21 vs. 1.87 ± 1.40 % of $CD4^+$; p = 0.823). All outcomes of statistical significance are presented in Figure 1.

Cell population	Tregs displayed as percent of	Group		p-value
		Healthy	ESKD	
$CD3^+$ $CD4^+$ M (SD)	Total lymphocytes	46.06 (8.90)	44.31 (9.47)	0.076
FoxP3 Treg MD (IQR)	Total $CD3^+$ $CD4^+$	5.09 (1.95)	5.70 (3.20)	0.059
$CD127^+$ $FoxP3^+$ Treg MD (IQR)	Total $CD3^+$ $CD4^+$	0.27 (0.77)	1.28 (2.09)	**0.000**
$CD25^+$ $CD127^{dim}$ Treg MD (IQR)	Total $CD3^+$ $CD4^+$	5.65 (2.10)	7.02 (3.23)	**0.000**
$FoxP3^+$ CD25high Treg MD (IQR)	Total $CD3^+$ $CD4^+$	4.34 (2.05)	5.26 (2.85)	**0.000**
Treg effector M (SD)	$CD25^+$ $CD127^{dim}$ Tregs	32.60 (11.28)	30.61 (10.04)	0.224
Treg naïve M (SD)	$CD25^+$ $CD127^{dim}$ Tregs	34.69 (12.53)	30.20 (12.47)	**0.035**
Treg effector-proliferative MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	4.64 (3.49)	4.61 (3.46)	0.766
Th17 type Treg $CD161^+$ MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	1.79 (1.47)	2.94 (1.94)	**0.000**
Trans Tregs $CD161^-$ MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	28.37 (10.14)	34.47 (15.90)	**0.002**
non-suppressive T cells MD (IQR)	Total $CD4^+$	2.59 (1.61)	2.92 (1.35)	**0.008**
resting Treg MD (IQR)	Total $CD4^+$	1.87 (1.40)	1.93 (1.21)	0.823
activated Treg MD (IQR)	Total $CD4^+$	0.45 (0.43)	0.67 (0.78)	**0.000**

Table 11: Percent of Tregs sorted by group

Treg comparison of healthy control group (Healthy, n= 74) and end-stage kidney disease group (ESKD, n= 74). Data are given as percent of respective cell population. Results are displayed as means (M), standard deviation (SD), median (MD) or interquartile range (IQR).

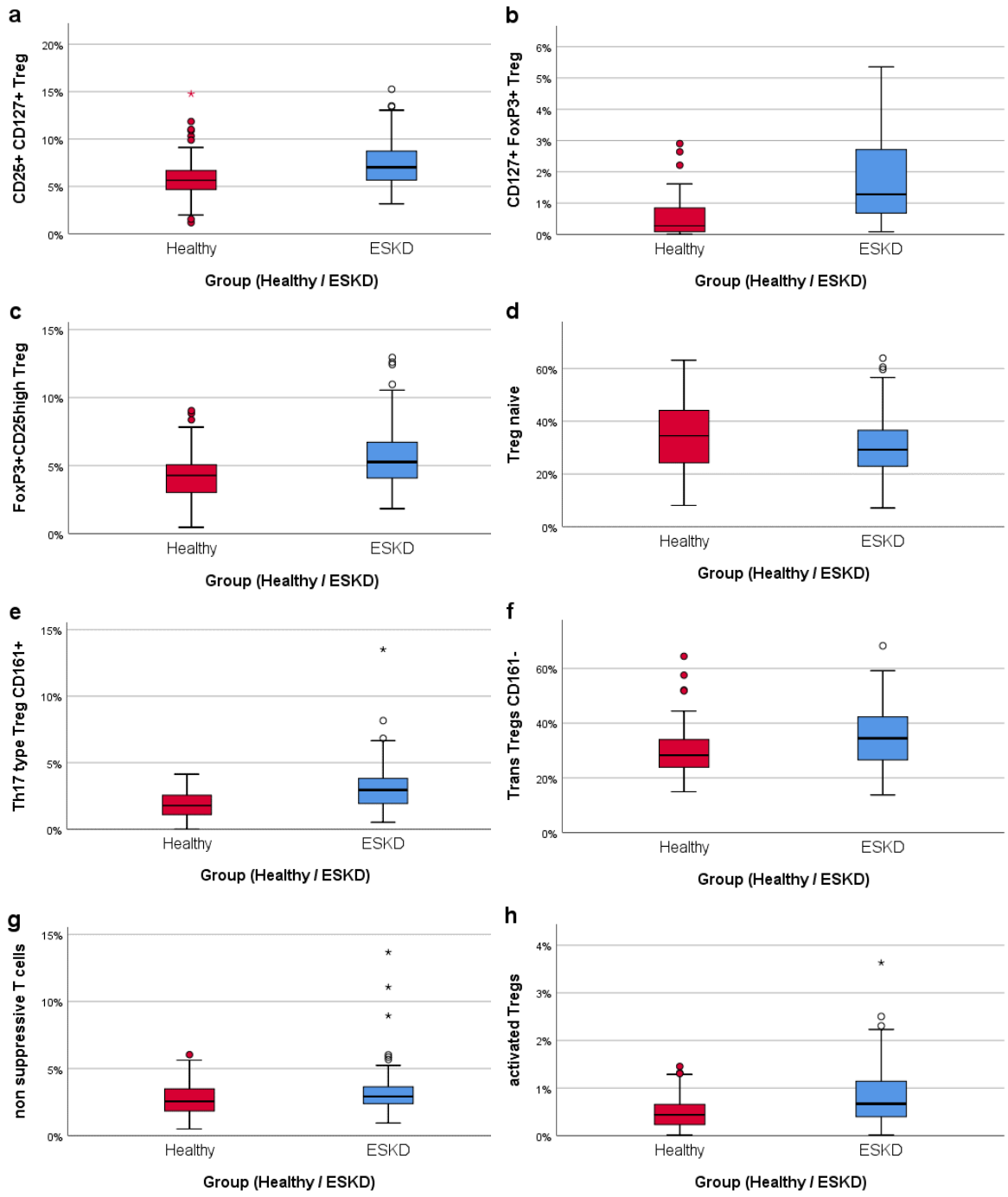

Figure 1: Tregs of transplant candidates and healthy controls

Blood samples of patients listed for kidney transplantation (ESKD; n=74) were compared to healthy controls (Healthy; n=74). Only statistically significant results ($p < 0.05$) are presented. Treg naïve defined as $CD3^+CD4^+$ $CD25^+$ $CD127^{dim}$ $CD45RA^+$ $CD15S^-$ cells; Trans Tregs $CD161^-$ defined as $CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^-$ $CD161^-$; Th17 type Tregs defined as $CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^-$ $CD161^+$; non-suppressive T cells defined as $CD3^+CD4^+CD45RA^-$ $FoxP3^{low}$; activated Tregs defined as $CD3^+CD4^+CD45RA^-$ $FoxP3^+$. Numbers refer to percent of Tregs among given cell population: percent of $CD3^+$ $CD4^+$ cells (**a-c**); percent of $CD25^+$ $CD127^{dim}$ Tregs (**d-f**); percent of total $CD4^+$ cells (**g,h**).

3.6 Treg comparison regarding rejection

Kidney transplant candidates were separated into two groups depending on biopsy-proven rejection episodes during follow-up. Patients with rejection showed significant alterations among $CD3^+$ $CD4^+$ cells in all lymphocytes (48.5 ± 10.39 vs.42.5 ± 9.01 % of lymphocytes; p = 0.039), as seen in Figure 2 and Table 12.

There were no significant differences among $FoxP3^+$ Tregs (5.5 ± 1.94 vs. 5.9 ± 2.98 % of $CD3^+CD4^+$; p = 0.657), $CD127^+$ $FoxP3^+$ Tregs (2.2 ± 2.71 vs. 1.2 ± 2.10 % of $CD3^+CD4^+$; p = 0.554), $CD25^+$ $CD127^+$ Tregs (7.0 ± 3.20 vs. 7.1 ± 3.11 % of $CD3^+CD4^+$; p = 0.334), $FoxP3^+$ CD25 Tregs (4.3 ± 3.66 vs. 5.6 ± 2.33 % of $CD3^+CD4^+$; p = 0.175). Within $CD25^+$ $CD127^{dim}$ Tregs no significant differences in effector Tregs (p = 0.957), in naive Tregs (p = 0.665), in effector-proliferative Tregs (p = 0.158), in Th17 type $CD161^+$ Treg (p = 0.252) or in $CD161^-$Trans Tregs (p = 0.509) was found. Further no significant alterations in non-suppressive T cells (p = 0.052), in resting Tregs (p = 0.196) or in activated Treg (p = 0.564) among $CD4^+$ T cells could be seen between rejecting and non-rejecting groups.

A detailed list of all Tregs is presented in Table 12.

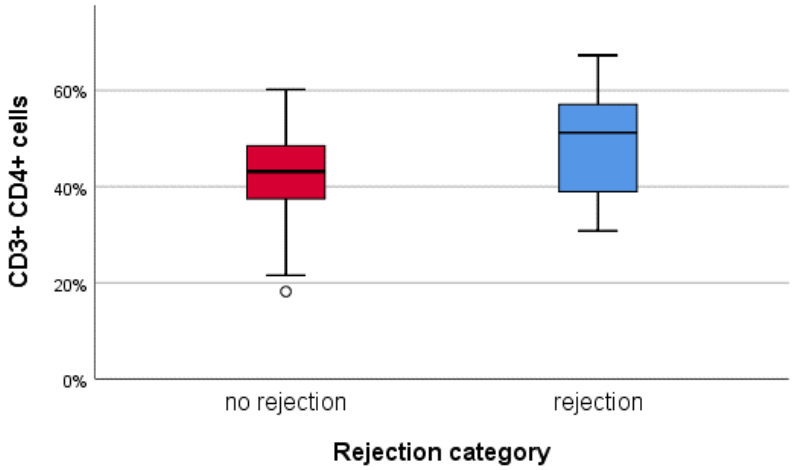

Figure 2: T cells of transplant candidates with and without rejection

When $CD3^+$ $CD4^+$ cells of transplant candidates were compared, patients with acute rejection episodes during follow-up (n= 13) had significant (p = 0.039) higher levels of $CD3^+$ $CD4^+$ cells than patients without biopsy proven rejection (n=61). Numbers represent percent of $CD3^+$ $CD4^+$ cells among all viable lymphocytes.

Cell population	Tregs displayed as percent of	Group		p-value
		No rejection	Rejection	
$CD3^+$ $CD4^+$cells M (STD)	Total lymphocytes	42.51 (9.01)	48.47 (10.39)	**0.039**
$FoxP3^+$ Tregs M (STD)	Total $CD3^+$ $CD4^+$	5.89 (2.98)	5.50 (1.94)	0.657
$CD127^+$ $FoxP3^+$ Tregs MD (IQR)	Total $CD3^+$ $CD4^+$	1.20 (2.10)	2.24 (2.71)	0.554
$CD25^+$ $CD127^+$ Tregs MD (IQR)	Total $CD3^+$ $CD4^+$	7.12 (3.11)	7.02 (3.20)	0.334
$FoxP3^+$ $CD25^+$ Tregs MD (IQR)	Total $CD3^+$ $CD4^+$	5.59 (2.33)	4.31 (3.66)	0.175
Treg effector M (STD)	$CD25^+$ $CD127^{dim}$ Tregs	30.58 (9.56)	30.75 (12.45)	0.957
Treg naive Treg MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	29.28 (14.64)	28.86 (19.44)	0.665
Treg effector-proliferative MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	4.95 (3.71)	4.05 (2.34)	0.158
Th17 type Treg $CD161^+$ MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	3.16 (2.79)	2.58 (1.91)	0.252
Trans Tregs $CD161^-$ M (STD)	$CD25^+$ $CD127^{dim}$ Tregs	35.24 (11.04)	33.03 (9.54)	0.509
non-suppressive T cells MD (IQR)	Total $CD4^+$	3.05 (1.39)	2.48 (1.20)	0.052
resting Tregs M (STD)	Total $CD4^+$	1.91 (1.01)	2.34 (1.29)	0.196
activated Tregs MD (IQR)	Total $CD4^+$	0.74 (0.88)	0.61 (0.31)	0.564

Table 12: Treg levels sorted by rejection class

Comparison of different Treg subpopulations among kidney transplant candidates with and without biopsy proven rejection during follow-up. Numbers display Tregs as percent of given population at the time before transplantation. Data are given as percent of respective cell population. Results are displayed as means (M), standard deviation (SD), median (MD) or interquartile range (IQR). Groups: no rejection n= 61; biopsy proven rejection n= 13.

3.7 Treg analysis according to rejection severity

To establish further differences among kidney transplant recipients with and without rejection, analysis of variances was performed. Patients undergoing kidney transplantation

were categorised into three different groups using Banff classification: “No Rejection” group 1, including all patients without rejection. “Mild rejection” including patients with borderline rejection and Banff category type 1 rejection. “Severe rejection” including all patients of higher rejection categories, respectively Banff categories 2 and 3. Table 13 shows the distribution of Tregs among those three rejection categories.

Cell population	Tregs displayed as percent of	Rejection group		
		No	Mild	Severe
$CD3^+$ $CD4^+$ M (SD)	Total lymphocytes	42.7 (9.02)	45.3 (10.50)	50,2 (11.31)
FoxP3 Treg MD (IQR)	Total $CD3^+$ $CD4^+$	5.9 (3.72)	5.9 (2.81)	5.5 (1.36)
$CD127^+$ $FoxP3^+$ Treg MD (IQR)	Total $CD3^+$ $CD4^+$	1.2 (2.09)	2.5 (2.64)	2.1 (2.42)
$CD25^+$ $CD127^+$ Treg MD (IQR)	Total $CD3^+$ $CD4^+$	7.1 (3.11)	7.0 (3.48)	5.4 (3.68)
$FoxP3^+$ CD25high Treg MD (IQR)	Total $CD3^+$ $CD4^+$	5.6 (2.29)	5.1 (4.44)	3.6 (3.79)
Treg effector M (SD)	$CD25^+$ $CD127^{dim}$ Tregs	30.4 (9.55)	26.3 (14.55)	33.0 (12.16)
Treg naïve MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	29.4 (14.57)	30.4 (32.35)	24.7 (17.97)
Treg effector-proliferative MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	4.9 (3.84)	4.1 (5.08)	4.3 (2.24)
Th17 type Treg $CD161^+$ MD (IQR)	$CD25^+$ $CD127^{dim}$ Tregs	3.1 (2.76)	2.8 (2.28)	2.6 (2.23)
Trans Tregs $CD161^-$ M (STD)	$CD25^+$ $CD127^{dim}$ Tregs	35.2 (11.01)	24.0 (4.13)	39.3 (7.56)
non-suppressive T cells MD (IQR)	Total $CD4^+$	3.1 (1.36)	2.2 (0.81)	2.5 (1.43)
resting Treg MD (IQR)	Total $CD4^+$	1.9 (1.14)	3.1 (2.34)	1.6 (0.83)
activated Treg MD (IQR)	Total $CD4^+$	0.8 (0.88)	0.6 (0.27)	0.6 (0.36)

Table 13: Treg distribution among three rejection categories

Kidney transplant candidates were separated into three different groups, based on BANFF category of biopsy proven rejection during follow-up: no rejection n=61; mild rejection n=5; severe rejection n=7. Numbers display Tregs as percent of given population at the time before transplantation. Data are given as percent of respective cell population. Results are displayed as means (M), standard deviation (SD), median (MD) or interquartile range (IQR).

3.7.1 One-way analysis of variance

Further evaluation of groups was performed. One-way analysis of variances was used for normally distributed data and Kruskal- Wallis test for non-parametric data. Among CD 25^{+} $CD127^{dim}$ cells, there were significant differences in Trans Tregs $CD161^{-}$ ($p = 0.041$), while no significant alterations were seen among other cell populations. Table 14 presents all results of differences between groups.

Cell population	Tregs displayed as percent of	p-value	
		One-way ANOVA	Kruskal–Wallis test
$CD3^{+}$ $CD4^{+}$,	Total lymphocytes	0.127	
$FoxP3^{+}$ Tregs	Total $CD3^{+}$ $CD4^{+}$		0.678
$CD127^{+}$ $FoxP3^{+}$	Total $CD3^{+}$ $CD4^{+}$		0.933
$CD25^{+}$ $CD127^{+}$ Treg	Total $CD3^{+}$ $CD4^{+}$		0.391
$FoxP3^{+}$ $CD25^{+}$ Treg	Total $CD3^{+}$ $CD4^{+}$		0.293
Treg effector	$CD25^{+}$ $CD127^{dim}$ Tregs	0.780	
Treg naive	$CD25^{+}$ $CD127^{dim}$ Tregs		0.119
Treg effector-proliferative	$CD25^{+}$ $CD127^{dim}$ Tregs		0.518
Th17 type Treg $CD161^{+}$	$CD25^{+}$ $CD127^{dim}$ Tregs		0.580
Trans Tregs $CD161^{-}$	$CD25^{+}$ $CD127^{dim}$ Tregs	**0.041**	
non-suppressive T cells	Total $CD4^{+}$		0.057
resting Treg	Total $CD4^{+}$		0.145
activated Treg	Total $CD4^{+}$		0.601

Table 14: P-values for variance analysis of different Tregs

Kidney transplant candidates were separated into three different groups, based on BANFF category of biopsy proven rejection during follow-up: no rejection n=61; mild rejection n=5; severe rejection n=7. One-way analysis was used for comparison of groups with normally distributed data, Kruskal- Wallis test for groups with non-parametric Treg distribution.

3.7.2 Multiple comparison between groups

Bonferroni correction was used for evaluation of differences in between groups with significant alterations in variances. Within $CD25^{+}CD\ 127^{dim}$ cells, analysis of $CD\ 161^{-}$ trans Tregs cells showed significant distinctions between groups (Table 15). Patients with severe rejection had significant more $CD161^{-}$ trans Tregs than patients with borderline or Banff 1 rejection ($p = 0.046$). All results are presented in Table 15, significant differences are visualised in Figure 3.

(A) Rejection category	(B) Rejection category	Mean Difference (A-B)	p-value	95% Confidence interval Lower Bound	Upper Bound
no rejection	mild rejection	11.20592	0.074	-0.7506	23.1625
	severe rejection	-4.04233	1.000	-14.2994	6.2148
mild rejection	no rejection	-11.20592	0.074	-23.1625	0.7506
	severe rejection	-15.24826	**0.046**	-30.2984	-0.1981
severe rejection	no rejection	4.04233	1.000	-6.2148	14.2994
	mild rejection	15.24826	**0.046**	0.1981	30.2984

Table 15: Trans Tregs CD161$^-$ analysis among groups of rejection severity .

Kidney transplant candidates were separated into three different groups, based on BANFF category of biopsy proven rejection during follow-up: no rejection n=61; mild rejection n=5; severe rejection n=7. Means of each rejection group were compared with Bonferroni correction. Numbers refer to percent of Trans Treg CD3$^+$CD4$^+$CD25$^+$CD127^{dim} CD45RA$^-$ CD15S$^-$ CD161$^-$ in CD25$^+$ CD127^{dim} Tregs. Mean difference, p-value and 95% confidence interval are displayed for each group comparison.

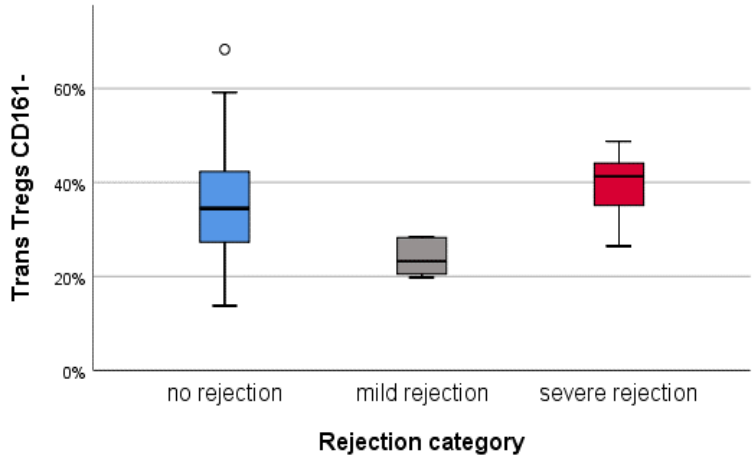

Figure 3: Trans Tregs CD161$^-$ comparison among groups of rejection severity.

Kidney transplant candidates were separated into three different groups, based on BANFF category of biopsy proven acute T cell mediated rejection: no rejection n=61; mild rejection (borderline or BANFF 1) n=5; severe rejection (BANFF 2 or 3) n=7. Numbers refer to percent of Trans Tregs CD161$^-$ cells in CD25$^+$CD127^{dim} Tregs.

4 Discussion

End-stage kidney disease is worldwide health burden affecting millions of people. Besides livelong dialysis therapy, there is only one therapeutic strategy: kidney transplantation. Still, in order to avoid rejections, immunosuppressive medications are needed, which comes at cost of various side effects, especially increased risk of cancer and severe infections. In search of new strategies to lower immunosuppression without increasing risk of rejection, biomarkers predicting immunological risk seem to be a promising step for personalized treatment in renal transplant recipients. As regulatory T cells play a crucial role in the avoidance of rejection and achieving immunotolerance, specific subpopulations of these Tregs might represent promising candidates for future biomarkers. By further analysis and understanding of these cells, new therapeutic strategies for kidney transplant recipients come one step closer.

The aim of this study was to evaluate regulatory T cell populations in patients with end-stage kidney disease before kidney transplantation was performed. To gain insights on Treg levels in health, disease and immunotolerance, Tregs of healthy controls were compared to ESKD patients. This thesis was part of a prospective monocentric pilot trail and due to its large extent, the thesis only focused on a smaller set of regulatory T cells.

4.1 Differences of Tregs among ESKD and health

When comparing Tregs of healthy controls with Tregs of kidney transplant recipients before transplantation, we found no significant differences of $CD3^+$ $CD4^+$ cells among lymphocytes. These findings slightly differ from previous literature, as Landwehr-Kenzel and colleagues found more $CD4^+$ cells among $CD3^+$ lymphocytes in healthy individuals, than in patients with ESKD (99). Taking a closer look at this study, differences seem to be reasonable. While Landwehr-Kenzel and colleagues found 30% CD 4^+ among total $CD3^+$ T cells in healthy controls, we found 46%. This might be due to the fact that there were strong differences in the staining, gating and analysis strategy employed. Thus, while our serial measurements are highly reproducible and comparable, the general caveat of limited comparability between studies reporting relative frequencies in flow cytometry remains.

To our surprise, no significant alterations were found when we studied $CD3^+$ $CD4^+$ $FoxP3^+$ Tregs. This differs from a study from 2010, that showed lower frequencies of $FoxP3^+$ mRNA

transcripts, which they associated with lower Treg numbers, in patients with haemodialysis than in healthy volunteers (100).

However, when Tregs were more closely characterised by using additional and more recently described cell markers, our study showed intriguingly different results. Here, ESKD patients showed significantly ($p<0.001$) more $CD3^+$ $CD4^+$ $CD127^+$ $FoxP3^+$ cells, more $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs and more $CD3^+$ $CD4^+$ $FoxP3^+$ $CD25^{high}$ Tregs as percent of total $CD3^+$ $CD4^+$ than their healthy counterparts.

Regarding $CD3^+CD4^+$ $FoxP3^+$ $CD25^{high}$ Tregs, literature is controversial. Hu and colleagues reported no significant differences in the number of $CD4^+$ $FoxP3^+$ $CD25^+$ cells between ESKD patients and healthy controls (101). Meanwhile, results from a study from Poland showed lower pretransplant levels of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs in dialysis patients with or without subsequently occurring acute rejection compared to healthy controls (102). However, a much larger trial by Landwehr-Kenzel and colleagues reported similar results to ours, with only a slight but non-significant increase in $CD4^+FoxP3^+$ $C25^+$ cells in dialysis patients before transplantation in comparison to healthy volunteers (99).

The heterogeneity of the literature in this regard may be due to one of two main reasons: first, the study by Hu and colleagues had a much smaller sample size of only 20 healthy controls and 28 ESKD patients, which may indicate that this study was underpowered to detect small, yet potentially clinically significant alterations. Second, ongoing research on Treg biology has increased the number of distinct Treg subpopulations, that are progressively well defined, for example other studies did not differentiate between $CD25^{high}$ and $CD25^{low}$ cells (101). This impact of Treg definition can also easily be seen by comparing exact levels of these Tregs: Hu and colleagues reported a mean of 8.26% of $CD4^+$ $CD25^+$ FoxP3 Tregs among $CD4^+$ cells and T lymphocytes, meanwhile our data shows a mean of 4.34% of $CD3^+$ $CD4^+$ $CD25^{high}$ FoxP3 among all $CD3^+$ $CD4^+$ cells (102). A systematic review from 2016 underlines this thesis and resonates with our results. This meta-analysis of Caprara et al mainly focused on $CD25^+$ $FoxP3^+$ Tregs and delivered similar results to ours, as Tregs were increased in ESKD patients compared to healthy counterparts, but results did not always reach statistical significance (103).

When comparing $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs, we found significantly more ($p < 0.001$) Tregs among patients on dialysis than in healthy subjects. Within $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{dim}$ Tregs, there were significantly more ($p < 0.001$) $CD45RA^-$ $CD15S^-$ $CD161^+$ Th17 type Tregs and more ($p = 0.002$) $CD45RA^-$ $CD15S^-$ $CD161^-$ Trans Tregs, while less ($p = 0.035$) naïve $CD45RA^+$ $CD15S^-$ Tregs were found in patients on the waitlist than in healthy

controls. There was no significant difference ($p = 0.224$) in effector Tregs and no difference ($p = 0.766$) in effector-proliferative Tregs in $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{dim}$ Treg among groups.

Out of all $CD4^+$T cells, we recorded more ($p = 0.008$) non-suppressive T cells and more ($p < 0.001$) activated Tregs, but there was no difference ($p = 0.823$) in resting Tregs.

Only limited data has been published on these specific subpopulations of Tregs in ESKD patients. A study from Sweden could not find such significant difference among $CD25^+$ $CD127^{dim}$ Tregs (104). Afzali and colleagues reported no differences in $CD4^+$ $CD25^{high}$ $CD127^{low}$ Tregs and naïve Tregs between healthy controls and patients on haemodialysis (105). Furthermore, they found significantly less effector Tregs in haemodialysis patients than in their control group (105). These results must be compared cautiously, because in the latter study, naïve Tregs were defined as $CD4^+$ $CD25^{high}$ $CD127^{low}$ $CD45RA^+$ and effector Tregs were referred as $CD4^+$ $CD25^{bright}$ $CD127^{low}$ $CD45RA^-$, which differs from our definition. These differences also mirror in the reported percentage in $CD4^+$ $CD25^{high}$ $CD127^{low}$ Tregs as the mean percent of effector Tregs was 4.7% in the study of Afzali et al, while we observed a mean percent of 32.6% of $CD3^+CD4^+CD25^+CD127^{dim}$ $CD45RA^-$ $CD15S^+$ effector Tregs.

The study of San Segundo and colleagues also focused on activated Tregs and compared patients with and without acute rejection. They found a similar increase in activated Tregs before transplantation in patients of their acute rejection group, even if activated Tregs were defined as $CD4^+$ $CD25^{high}$ $CD45RO^+$ Tregs in this trial (106).

While, based on our data, we cannot make a definitive statement as to why these differences in distinct Treg subpopulations between healthy controls and dialysis patients occur, it is tempting to speculate as follows: patients with advanced CKD, and especially patients treated with haemodialysis have repeatedly been shown to display chronic, low-grade inflammation (107-113). This low-grade inflammation is known to not only be an epiphenomenon of haemodialysis treatment, but to be a product of the retention of various uremic toxins and to be at least partially causal for the huge toll of cardiovascular morbidity and mortality in these patients (114). It is known that inflammatory stimuli trigger anti-inflammatory, regulatory counteractions. It has been proven that in the process of antigen recognition, naïve Tregs convert to activated Tregs, by losing CD45RA (115). Thus, this low-grade inflammation seen in ESKD patients may be an environment in which naïve Tregs, the only subpopulation we observed to be significantly reduced compared to healthy

controls in ESKD, differentiate to activated Tregs. This very well provides an explanation for the increased frequency of activated Tregs in our study: In this context, our results, where we found less naïve Tregs and more activated Tregs in ESKD patients than in healthy individuals, would support the thesis of plastic Treg differentiation in vivo from naïve to activated Tregs in inflammatory environment.

However, plasticity of Tregs is a widely discussed topic in research, especially the relationship between Tregs and Th17 cells (87). Th17 $CD161^+$cells have been associated with autoimmune diseases by exhibiting pathogenic features such as proinflammatory cytokine production and thereby impairing Treg function (116). Furthermore, Th17 cells are discussed to play an important role in allograft rejection (117). In patients with previous graft dysfunction, a link between lower frequency of Tregs and more Th17 cells has been proven (118). Despite the opposing function, Tregs have been observed to convert into Th17 like cells when exposed to an inflammatory environment (86). Recent studies suggested, that a similar $CD161^+$ Treg lineage plays an important role in autoimmune disease, indicating a hybrid function of these Tregs with both inflammatory and suppressive potentials (85). However, in our study, besides the higher numbers of conventional Tregs, we also found a much higher number of Th17 type $CD161^+$ $CD25^+$ $CD127^{dim}$ Tregs in ESKD, allowing us to further speculate on Treg plasticity in the uremic milieu. With this aspect in mind, our data supports the thesis of Tregs differentiation into divergent linages with contrary function to create a balance of the net state of immunity. This would be of enormous importance, because disbalance between effector T cells and Tregs has been proven to disfavour graft tolerance (119) and studies suggest benefits by tilting the equilibrium towards Tregs in Treg - Th17 cells ratio (120).

4.2 No differences among rejection

When we compared ESKD patients, who are about to receive a renal allograft and subsequently experience biopsy proven rejection during follow-up, to those without rejection, the differences in T cell subpopulations at baseline were marginal. Patients with subsequent rejection showed significantly more $CD3^+$ $CD4^+$ cells among total lymphocytes ($p = 0.039$). There were no significant differences among $FoxP3^+$ Tregs, $CD127^+$ $FoxP3^+$ Tregs, $CD25^+$ $CD127^+$ Tregs, $FoxP3^+CD25^+$ Tregs. Within the $CD25^+$ $CD127^{dim}$ Treg population, no significant alterations in effector Tregs, in naïve Tregs, in effector-proliferative Tregs, in Th17 type $CD161^+$ Treg or in $CD161^-$trans Tregs was found. Further

no significant differences in non-suppressive T cells, in resting Tregs or in activated Tregs among $CD4^+$ T cells could be seen between rejecting and non-rejecting groups.
Finally, we divided allograft patients with subsequent rejection episodes during follow-up into two groups based on rejection severity as assessed by the updated BANFF classification. Patients with "severe rejection" (a collective term employed for episodes of BANFF 2A, 2B and 3 T cell mediated rejection) showed significantly more $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{dim}$ $CD45RA^-$ $CD15S^-$ $CD161^-$ trans Tregs ($p = 0.046$) than patients with "mild rejection" (a collective term used for borderline and Banff 1 T cell mediated rejection). Still, we need to be careful in interpreting these results due to low n-numbers in the subgroups.

There is only a very limited study which looked at the frequency of Treg subpopulations at baseline in relation to the subsequent occurrence of biopsy-proven acute rejection. In 2014, a multicentre trial found significantly more activated Tregs in the group with acute rejection than in rejection free recipients at the time before transplantation (106). We did not find such a difference among groups, which might be caused by their definition of $CD4^+$ $CD25^{high}$ $CD62L^+$ $CD45RO^+$ activated Tregs.
However, previously published data suggests that analysis focused on Tregs at the time after transplantation leads to more results. Even if before transplantation no differences appeared, frequency of Tregs has been shown to decrease after transplantation and at later stage, even significant differences between patients with stable graft function and those with rejection were identified (97, 121). Moreover, a connection of Treg numbers and acute rejection rates or stable graft function has been described several times (122, 123). Still, timing of Treg evaluation seems to be crucial. Several studies reported Treg dynamics shortly after transplantation or around clinical rejection (124, 125). Considering that most rejections occurred in first months after transplantation, we eagerly look forward evaluating our other time points after transplantation.

Nonetheless, taking into account that Tregs are not only constitutive for immune tolerance (78), but also that their number plays an important role in graft rejection (97, 101), new directions in research of immune tolerance are ahead. Thus, if studies succeed in definitely showing that the frequency of certain cell populations at certain time points after transplantation reliably predict the presence or absence of later rejection episodes, Tregs may become an important biomarker to guide immunosuppressive therapy in patients after kidney transplantation (126, 127) Activated Tregs have been evaluated in this respect for predicting

acute rejection (106). Furthermore, not only circulating Tregs have been studied for predicting graft survival (123, 125), also the presence of intragraft Tregs in biopsies correlates with better graft function (128, 129). Even a relationship between Tregs and estimated GFR has already been proven (97, 130). In the scope of this thesis, we did not evaluate such biomarkers for predicting graft survival or function, but we showed that immediately prior to transplantation our broad array of Treg subpopulations does not differ between rejecting and non-rejecting groups.

4.3 Limitations

The present study has several strengths: First, to our knowledge this is one of the largest analyses of ESKD patients with long term follow-up after kidney transplantation. Stringently stream-lined and standardized flow cytometric analysis allows for good comparability between different measurements performed at different points in time in different patients.
Still the main limitation of this study is the relatively small population size of patients with clinical rejection. While the rejection frequency – 13 episodes of biopsy-proven acute rejection over a period of one year after transplantation in a study population of 74 kidney transplants – was adequate, the present analysis has only limited power to detect small, yet potentially clinically important differences in the Treg frequencies. Therefore, findings of our study can only be cautiously generalized to larger populations.
Another small limitation of the study is its design being part of a pilot trial. Prior studies often focused only on fewer or different types of Tregs or on Tregs in relation to other fields of expertise like cancer research, autoimmune diseases or liver transplantation, which makes comparison of research findings difficult. Especially analysis of Tregs before transplantation in regard of biomarkers for rejection has not been studied to full extent.
Furthermore, comparison of Tregs is challenging. Due to its new research typology and the complexity of human Tregs, no consistent classification of regulatory T cells has been defined. Often papers use classification of Sakaguchi's group, who used CD45RA, Foxp3 and CD25 expression and defined $CD45RA^{+}$ $FoxP3^{low}$ CD4 Tregs as resting (rTregs), whereas $CD45RA^{-}$ $FoxP3^{high}$ $CD4^{+}$ were classified as activated Tregs, and $CD45RA^{-}$ $FoxP3^{low}$ $CD4^{+}$ as non-Treg cells (83). In our study, resting Tregs were defined as $CD3^{+}$ $CD4^{+}$ $CD45RA^{+}$ $FoxP3^{low}$, activated Tregs as $CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $FoxP3^{+}$ and non-suppressive Tregs as $CD3^{+}$ $CD4^{+}$ $CD45RA^{-}$ $FoxP3^{low}$. While our panel design and choice of

Treg subpopulations studies is well-founded in the published evidence, differences in these study characteristics, make direct comparison difficult.
Still, due to the broad field of analysed Tregs, the large population and follow-up evaluation, this study offers many new insights in regulatory T cell populations in kidney transplant recipients.

4.4 Conclusion

The main aim of this thesis was to analyse differences of regulatory T cell subpopulations among ESKD patients and healthy controls, plus differences among renal allograft recipients at baseline with and without subsequently occurring acute graft rejection. Based on flow cytometry of several Treg subsets, we proved that healthy controls show different levels of Tregs than ESKD patients. We could not find significant alterations between rejection groups of kidney transplant recipients, when Tregs were analysed before transplantation. Bearing in mind that Tregs analysis seems to be not only affected by cell definition, but also by time and technique of evaluation, further multicentre trials with standardised evaluation of Tregs in kidney graft recipients after transplantation are suggested for better Treg understanding. Additional analysis of Tregs shortly after kidney transplantation and before rejection may bear biggest benefits for clinical research helping to find a predictor of kidney graft function, to guide therapy and ultimately to improve patient care.

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