Human Tubular Epithelial Cells and T-cell Alloreactivity

Martijn Demmers

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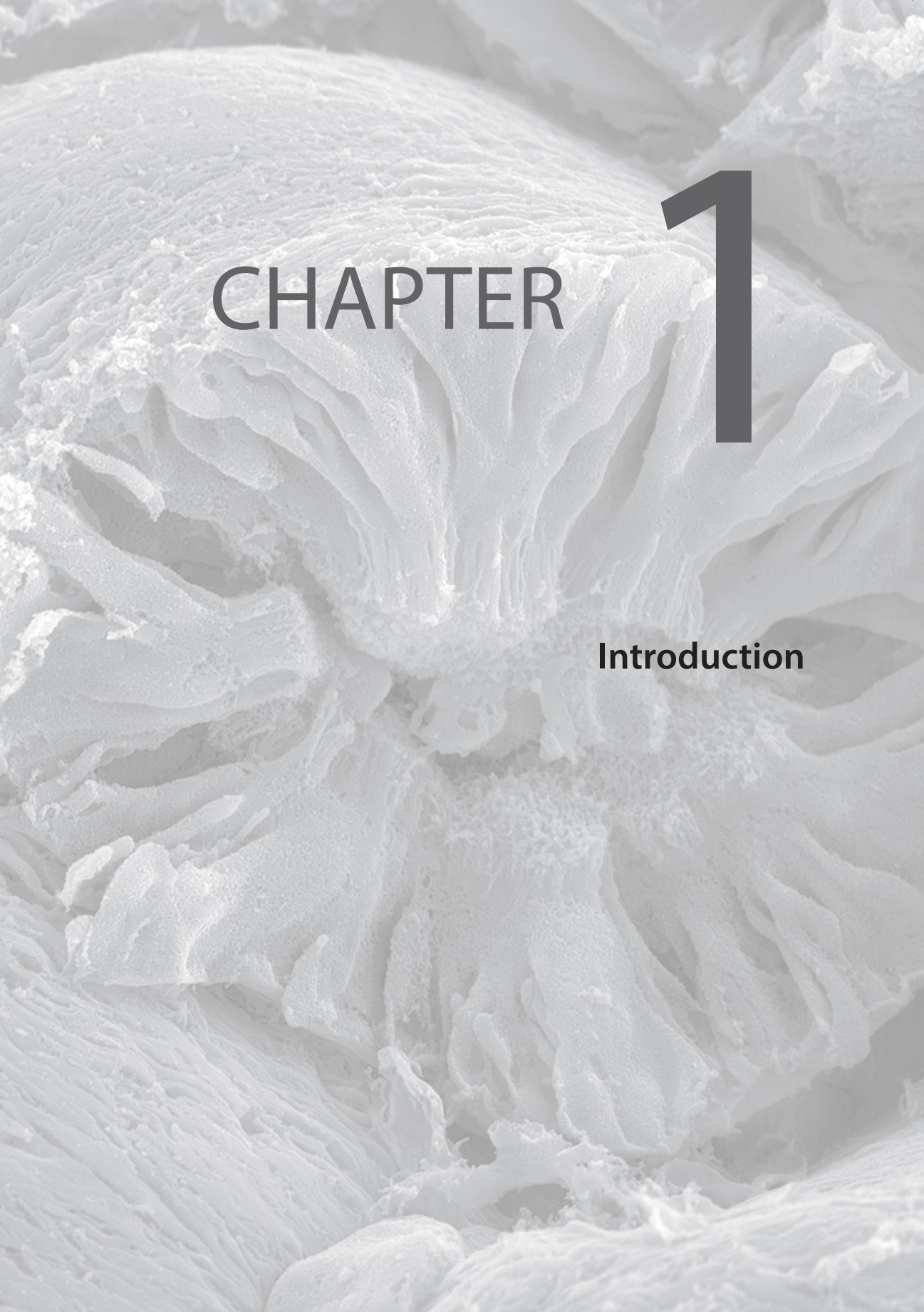
CONTENTS



CHAPTER 1

Introduction

INTRODUCTION

Kidney transplantation is the treatment of choice for patients with end-stage renal disease [1]. The first successful transplantation was performed in 1954 and acceptable renal allograft survival was achieved after introduction of azathioporine in combination with high dose of corticosteroids in the mid sixties of the 20th century [2-3]. Introduction of the immunosuppressant calcineurin inhibitor (CNI) cyclosporin A and later on tacrolimus significantly improved the outcome of kidney graft survival. Tacrolimus compared to cyclosporin diminished the acute rejection rate by 31% during the first year after transplantation [4], and this percentage was even more reduced using anti-CD25 mAb as induction treatment [5]. This success have shifted the problem of transplantation from short term graft survival to how to improve the long term graft survival. The 5-year allograft survival rate in the Netherlands is 83% for deceased donor kidneys and 92% for living donor kidney transplants (Nederlandse Transplantatie Stichting, 12th August 2013). In contrast, the overall European 5-year graft survival is 77% and the USA 5-year graft survival is even worse, representing only 71% graft survival [6].

Using the combination of calcineurin inhibitors, corticosteroids and anti-proliferative agents, transplant specialists target divers pathways of lymphocyte activation, proliferation and/or differentiation [7-9]. Calcineurin inhibitors (CNI) include cyclosporine A and tacrolimus. CNI block T-cell activation and proliferation. CNI inhibit the calcineurin-mediated dephosphorylation of nuclear factor of activated T cells transcription factor (NFAT), normally induced by calcium influx during T-cell activation. NFAT activation is essential for T-cell effector molecules and the transcription of IL-2 and other cytokines [10]. Antimetabolites like mycophenolate mofetil (MMF) decrease the availability of vital metabolites like nucleotides and are not cell-specific. MMF inhibits the vital enzyme inosine monophosphate dehydrogenase, which suppresses DNA synthesis in the S phase and results in cell-cycle arrest [11-13]. The mammalian target of rapamycin (mTOR) is involved in T-cell activation and metabolic regulation. mTOR inhibitors like sirolimus and everolimus block at the G1/S transition in the cell cycle and inhibit cellular proliferation [14]. Glucocorticoids target a broad range of cellular targets including T cells, NK cells and endothelial cells. Prednisolone is a synthetic glucocorticosteroid and a non-specific anti-inflammatory reagent inhibiting nuclear factor kB (NF-kB) activation [15].

So far, much attention has been paid to investigate the systemic effects of immunosuppressive drugs on circulating immune cells after renal transplantation. Of note, the outcome of allo-immune response is the balance between immunogenicity of the graft, the recipient's immune system and the level of immunosuppression. In this dissertation, we aim to explore and mimic the local effects of immunosuppressive drugs on immune cells within the allograft microenvironment by performing *in vitro* experiments focussing on the interaction between immune cells and renal resident parenchymal tubular epithelial cells.

Allo-immune response

In the lymphoid organs effector cells are activated via either direct or indirect antigen presentation [16]. Effector cells emerge from the lymphoid organs into the renal allograft and initiate an inflammatory response. In cellular mediated rejection, the allograft is infiltrated by activated mononuclear cells like macrophages, B cells, NK cells, T helper cells and cytotoxic T lymphocytes [17]. The diagnosis of T-cell mediated rejection (TCMR) is reflected by tubulitis and arteritis according to the Banff classification [7,18-19]. Antibody-mediated rejection (ABMR) is associated with complement 4d (C4d) deposition, donor specific antibodies, glomerulopathy, multilayering of capillary basement membranes, tubular atrophy, and with accumulation of mononuclear cells in peritubular capillaries [18-21].

T cells play a pivotal role in acute cellular allograft rejection recognized as a delayed type hypersensitivity response, in promotion of allo-antibody formation and/or in cytotoxic responses [22-23]. Upon allogeneic stimulation, alloreactive T cells rapidly express CD25, the high affinity receptor for IL-2. IL-2 acts at different stages of the immune response to promote clonal expansion, induction of cytolytic activity and activation induced cell death of allogenic stimulated T cells. Another main role of IL-2 is the generation of T regulatory cells, that can inhibit allograft rejection [24-26]. Regarding the function of cytotoxic T cells in allo-immunity, granzyme B positive (GrB^+) cytotoxic T lymphocytes (CTLs) are known to be frequently present in mononuclear infiltrates in renal transplant biopsy specimens from patients with deteriorating renal function. They have been designated as indicative for the existence of acute cellular rejection [27-28].

NK cells form part of the innate immunity, forming a crucial first line of defence against foreign antigens. They do not require priming and are able to kill target cells independently. The peak of NK activity occurs within 3 days of the initiation of the response, before T-cell activity reaches maturation. They have an established role in defense against tumors, viral infection, and the rejection of bone marrow transplant [29]. In the human, increased numbers of circulating NK cells have been demonstrated during acute rejection of renal allograft [30] and they have been shown to infiltrate cardiac [31] and renal allografts [32-33].

RENAL TUBULAR EPITHELIAL CELLS AND GRAFT MICROENVIRONMENT

Tubular epithelial cells & their physiological role

The kidney has multiple functions and plays a crucial role in the equilibrium of water and the concentration of electrolytes in the body. Reabsorption of water and electrolytes takes place in the proximal tubular epithelial cells (TECs) (Figure 1) [34-36]. Like many epithelial cells with a transport function, renal tubular epithelial cells have a apical (luminal) membrane and a basolateral membrane. Tubular epithelial cells have a number of transport carriers to allow solutes enter the cell from the luminal site, and the Na^+/K^+/ATPase pump and other ion channels to return filtered solutes into the circulation to maintain electrolyte concentrations in the body [37-38].

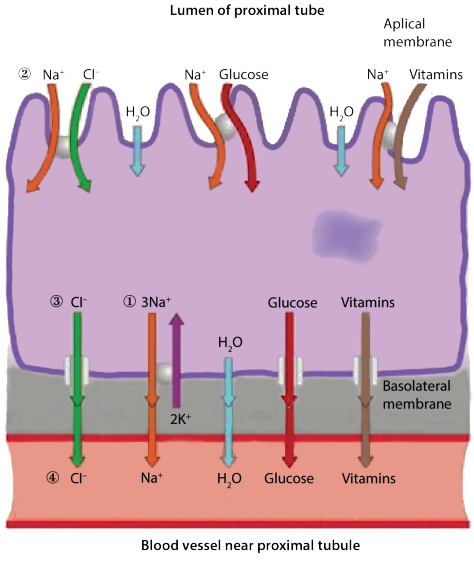


Figure 1 | Physiological function of a tubular epithelial cell.

Tubular epithelial cells & their pathophysiological role during inflammation

TECs play a central role in kidney allograft rejection [39-40]. Clinically, tubulitis denotes infiltration of the renal tubular epithelium by mononuclear cells (Figure 2). Tubulitis is one of the most reliable histopathological signs of cellular allograft rejection [39-42]. Uncontrolled tubulitis leads to structural graft damage and eventually to interstitial fibrosis and tubular atrophy (IF/TA) of the transplanted kidney and transplant failure. Tubulitis can be either a consequence of allo-incompatibility and T-cell mediated cytotoxicity against non-self donor antigens, or the consequence of direct tubular infections, mostly caused by viral pathogens [43-44].

In response to immune-stimulation, TEC upregulate HLA molecules class I [45] and class II [46], costimulatory molecules ICOS-L [47], CD40 [48] and ICAM-1 [49], and the inhibitory molecule PD-L1 [50]. Whether TECs express the costimulatory molecules CD80 and CD86 under inflammatory conditions which molecules are regarded as essential for the interaction with CD28 expressed by T cells, is still under debate [51-52].

Proinflammatory cytokines, chemokines, growth factors and extracellular matrix components which are locally released by divers types of immune cells amplify the parenchymal damage [53-55]. It is widely acknowledged that IFN-γ and TNF-α are upregulated and locally present during renal allograft rejection [56-57]. To dampen damage and inflammation, proliferating cells have a built-in regulatory mechanisms. One of the most studied mechanisms is indoleamine 2,3-dioxygenase (IDO) which has been identified as a rate-limiting enzyme degrading tryptophan, resulting in arrested alloreactive T cell proliferation [58-59]. Under inflammatory conditions and during renal allograft rejection, IDO has been shown to be expressed by TECs [60] suggesting that also activated graft-resident TECs have the capacity to control inflammatory responses during alloreactivity.

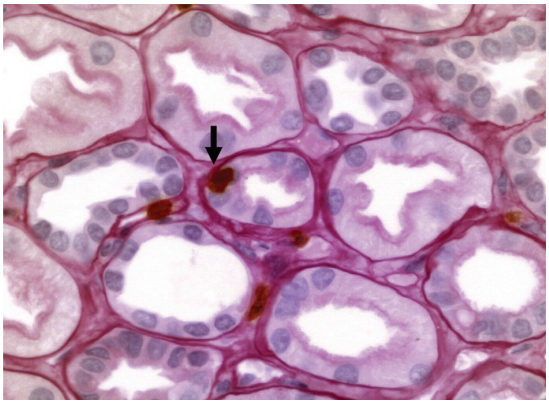


Figure 2 | A single T lymphocyte in the epithelial compartment of a tubule *(Elshafie et al. NDT 2011)*.

On the country, upon stimulationTECs produce cytokines like IL-6, TNF-α, IL-15, IL-17 and IL-18 [61]. Interestingly, studies in a glomerulonephritis murine model have revealed that locally produced TNF-α may contribute more to renal damage than infiltrating leukocyte-derived TNF-α [62]. With regard to chemokines, TECs produce Th1-related chemokines; CCL2, CCL5, CXCL8, CXLC9, CXCL10, CXC3L1 [61] as well as the Th17 associated chemokine CCL20 [63]. Transplant biopsy studies showed that TECs express the Th1 associated chemokines CCL2, CCL5 and CXCL10 during rejection using mRNA analysis [64-68]. Regulatory T cells (Treg) and Th17 cells express CCR6 and migrate towards the chemokine CCL20 which has been demonstrated to be upregulated during renal allograft rejection [63,69-70]. In contrast to experimental Th17 transplant models, human clinical studies provided limited evidence for the role of Th17 in renal alloreactivity so far [71]. Based upon the biological properties of TECs, it can be envisioned that TECs may play an essential role in the attraction of T-cell subsets.

Tubular epithelial cells & their interaction with immune cells

Tubular epithelial cells & CD4 T helper lymphocytes

$CD4^+$ T cells can be subdivided in several subtypes based on their transcription factor and cytokine production profile. The classical proinflammatory Th1 cell expressed the master regulator T-bet and the proinflammatory cytokine IFN-γ. In contrast, the proinflammatory Th17 cell expressed the transcription factor RORγt and the proinflammatory cytokine IL-17. The anti-inflammatory regulatory T cells (Treg) expressed the master regulator FoxP3 and the anti-inflammatory cytokine IL-10, IL-35 and TGF-β. Interestingly, the plasticity of T helper cells results in $CD4^+$ T cells that can change their profile based on the microenvironment [72]. Treg play an important role controlling immune responses. Regulatory T cells can inhibit the IL-2 production by effector T cells and/or can scavenge the cytokine IL-2. Production of anti-inflammatory cytokines IL-10, IL-35 and TGF-β are well known mechanisms of action exerted by T regs [73-74]. These $CD4^+$ T helper cells play essential roles in renal allograft rejection [75]. It has been demonstrated that under IFN-γ and TNF-α activated TECs

produce the chemokines CCL2 and CCL5 [76-77]. In addition, TECs also have the capacity to attract $CD4^+$ T cells expressing CCR2 and CCR5 from both the apical and the basolateral membrane site. Anti-CCL2 and anti-CCL5 significantly neutralized $CD4^+$ T-cell chemotaxis, while anti-TNF-α effectively abrogated CCL2 and CCL5 release by TECs [78]. Activated TECs possess higher cell surface expression levels of MHC II molecules and can support antigen-specific proliferation by $CD4^+$ T cells [46]. Liang et al. reported that rapamycin and tacrolimus alone completely to failed suppress proliferation and differentiation of alloreactive memory $CD4^+$ T cells in experimental transplant models [79]. Moreover, combined immunosuppression using rapamycin and Cyclosporin A allowed generation of memory $CD4^+$ and $CD8^+$ T cells and did not result in T-cell apoptosis [80-81]. Interestingly, memory T cells are more resistant to apoptosis due to the increased expression of antiapoptotic genes [82].

Tubular epithelial cells & Cytotoxic CD8 T lymphocytes

$CD8^+$ T cells are only attracted via the basolateral membrane site. Comparable with $CD4^+$ T cells, anti-CCL2 and anti-CCL5 significantly inhibited $CD8^+$ T-cell migration towards TECs [78]. Activated $CD8^+$ T cells produce cytotoxic molecules like perforins and granzyme B which directly lyse TECs [83]. Indeed, isolated and cloned cytotoxic T cells from renal biopsies taken during episodes of acute rejection showed specific lytic capacities against donor TECs [84]. Moreover, it was reported that TECs express tissue-specific antigens resulting in tissue specific lysis by $CD8^+$ T cells [84-85]. Immunohistochemical analysis revealed colocalization of IL-15 expressing TECs and proliferating $CD8^+$ T cells during rejection [86]. Antigen-specific effector memory $CD8^+$ T cells represent an important barrier for solid organ transplantation as they rapidly produce multiple proinflammatory cytokines and cytotoxic effector molecules [87-89]. These memory T cells might be less susceptible to the current prescribed immunosuppressive drugs.

Tubular epithelial cells & Natural Killer cells

NK cells are important cytotoxic lymphocytes of the innate immune system [90]. NK cells lyse target cells immediately without Ag presensitization. Activating and inhibitory molecules interact with HLA class-I or class I HLA-like ligands on target cells, which in turn control the reactivity of NK cells, becoming either inhibitory or stimulatory [91]. NK cells are known to rapidly infiltrate into injured kidneys and induce apoptosis in tubular epithelial cells. Already during ischemia reperfusion injury NK cells might play a pivotal role. Shortly after ischemia/reperfusion injury (IRI) NK cells infiltrate into the kidney. Consistent with creatinine levels, histology of kidneys demonstrated obvious tubular injury with extensive tubular necrosis/vacuolization. Depletion of the NK cells inhibits renal IRI resulting in significantly lower serum creatinine levels [92]. NK-cell mediated TEC apoptosis is regulated via osteopontin, playing an essential role in attracting and activating NK cells within a kidney [93]. In kidney transplantation, NK cells may be involved in both cellular and antibody mediated rejection [23,94-96].

OUTLINE OF THIS THESIS

We hypothesize that TECs modulate the outcome of a multicellular inflammatory process in a bi-directional way: directly by the production of pro- and anti-inflammatory cytokines to determine the environmental drive in further activation, differentiation and function of immune cells, and indirectly when TECs are themselves affected by cytokines produced by immune cells to enhance TEC self-injury. This introduction addresses the general background of the studies performed in this thesis. The interaction of TECs with mononuclear immune cells initiates an inflammatory response contributing to structural renal allograft damage. To date, data are scars on the interaction between $CD4^+$ T helper, cytotoxic $CD8^+$ T cells, NK lymphocytes, and graft resident TECs, and in particular how these interactions are affected by immunosuppressive drugs locally. The scope of this thesis is to explore these interactions mimicking local renal microenvironment *in vitro*.

In **Chapter 2,** we address whether IFN-γ/TNF-α stimulated TECs have the potential to attract Th1 and/or Th17 T-cell subsets. Chemokine and cytokine production by IFN-γ/TNF-α activated primary human TECs as well as their functional potential to attract $CD4^+$ T cell subsets will be studied.

In **Chapter 3,** we investigate the proliferative responses and the tissue damaging capacities of recipient $CD4^+$ T cells after co-culture with donor-derived TECs. In addition, we study their susceptibility to the immunosuppressant drugs tacrolimus, everolimus, prednisolone and mycophenolic acid in clinically relevant concentrations.

In **Chapter 4,** we investigate the proliferative and cytolytic responses of $CD8^+$ T cells and NK cells against TECs *in vitro* and questioned how these processes are affected by currently used immunosuppressive drugs.

In **Chapter 5,** we investigate how activated TECs might control T-cell proliferation, activation and apoptosis, and study immunosuppressive capacity of TECs.

In **Chapter 6,** we study the effect whether FoxP3 regulatory T cells inhibit tissue injury in deceased kidney donors.

In **Chapter 7,** we summarize and discuss the studies described in this thesis.

In **Chapter 8,** a Dutch summary is given.

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CHAPTER 2

Differential effects of activated human renal epithelial cells on T-cell migration

Martijn W.H.J. Demmers, Carla C. Baan, Els van Beelen, Jan N.M. IJzermans, Willem Weimar and Ajda T. Rowshani

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ABSTRACT

Background: Renal tubular epithelial cells (TECs) are one of the main targets of inflammatory insults during interstitial nephritis and kidney transplant rejection. While Th1 cells are know to be essential in the pathogenesis of rejection, the role of Th17 is still under debate. We hypothesize that TECs modulate the outcome of rejection process by production of distinct chemokines and cytokines that determine the attraction of different T-cell subsets. Therefore, we studied differential effects of activated human renal epithelial cells on T cell migration.

Methods: Human primary TECs were stimulated by IFN-γ and TNF-α *in vitro*. Chemokines and cytokines produced by activated TECs were measured using Luminex or ELISA. Chemotaxis assay was performed using activated peripheral blood mononuclear cells composed of $CD4^+CXCR3^+$ and $CD4^+CCR6^+$ T cells migrating towards stimulated and unstimulated TECs.

Results: While activated TECs secreted abundant amounts of the pro-inflammatory cytokines IL-6 and IL-8, the T helper cell differentiation cytokines IL-1β, IL-12p70, IL-23 or TGF-β1 were not produced. The production of Th1 chemokines CXCL9, CXCL10 and CCL5 were significantly upregulated after TEC stimulation. In contrast, Th17 chemokine CCL20 could not be detected. Finally, activated TECs attracted significantly higher numbers of $CD4^+CXCR3^+$ T cells as compared to unstimulated TECs. No migration of $CD4^+CCR6^+$ T-cells could be observed.

Conclusion: Activated primary renal tubular epithelial cells do not attract Th17 cells nor produce cytokines promoting Th17 cell differentiation in our experimental system mimicking the proinflammatory microenvironment of rejection.

INTRODUCTION

Tubular Epithelial Cells (TECs) comprise more than 75% of renal parenchymal cells. Their susceptibility and resistance to both inflammation and apoptosis directs the long-term function of kidney transplants, as tubular injury can be a major cause of nephron loss [1]. As such, tubulitis is the diagnostic hallmark of acute cellular rejection and can potentially lead to irreversible structural graft damage.

To date, important progress has been made in understanding the pathogenesis of rejection. So far, mechanisms of rejection entail a multi-cellular inflammatory process where local environment and multi-directional interplays between T cells and parenchymal cells such as tubular epithelial cells will determine the final outcome [2,3].

Classically, the proinflammatory Th1 cell is thought to represent one of the dominant immune cell types involved in induction and maintenance of acute cellular rejection [4,5]. On the contrary, the role of Th17 cells in the rejection process is not clear yet [6]. Th1 cells express IFN-γ and the transcription factor T-bet [7]. Th17 cells are defined by the expression of the cytokine IL-17 and the retinoic acid-related orphan receptor γ (RORγ) [8]. Distinct cytokines drive the differentiation of naïve $CD4^+$ T cells towards different T cell subsets. IL-12 and IFN-γ are considered to be the main T helper 1 associated cytokines and IL-1β, IL-6, IL-23 and TGF-β1 are T helper 17 associated counterparts [4].

At present, controversial data exist with regard to the involvement and the role of Th17 cells during kidney transplant rejection [6]. Despite some experimental murine data directly linking Th17 cells to rejection, most studies in clinical transplantation are limited to the detection of IL-17. IL-17 has been detected by immunofluorescent staining in biopsies from acutely rejecting transplants and not in pretransplant biopsy samples or healthy kidneys [9]. Increased IL-17 mRNA and protein expression levels in kidney biopsies and urine samples from patients with subclinical rejection underline the involvement of IL-17 in the process of rejection [10]. Yapici et al. reported that the majority of IL-17 producing cells in kidney transplant biopsies undergoing acute rejection are mast cells or neutrophils and not T-lymphocytes [11]. These data suggest that IL-17 may play a role during the rejection process.

Th1 cells preferentially express the chemokine receptors CCR5 and CXCR3 migrating towards CCL5 and CXCL10 which are expressed by TECs and present during kidney transplant rejection [12-16]. Th17 cells are characterized by the simultaneous expression of IL-17 and the chemokine receptor CCR6 directing the most robust chemotaxis towards CCL20 [17,18]. In a prospective biopsy-controlled study it was found that local CCL5 and CXCL10 produced by TECs led to the directional movement of activated CXCR3 and CCR5 bearing T cells into the kidney transplant mediating acute rejection [14]. Accordingly, CCL20 expression was shown by immunohistochemical staining of both infiltrating leucocytes and tubular epithelial cells in kidney biopsies obtained from patients experiencing rejection [19].

We hypothesize that TECs modulate the outcome of the inflammatory process by the production of distinct chemokines and cytokines that determine the attraction, activation and further differentiation of T-cell subsets. In the present study, we specifically aim to investigate whether TECs after stimulation by IFN-γ and TNF-α, which are instrumental inflammatory cytokines during

rejection, have the potential to attract Th1 and Th17 T-cell subsets. Chemokine and cytokine production by IFN-γ/TNF-α activated primary human TECs as well as their functional potential to attract $CD4^+$ T cell subsets were studied.

MATERIALS AND METHODS

Culture of primary tubular epithelial cells

Primary tubular epithelial cells were cultured from cortical tissue of human kidneys obtained at the time of transplantation as previously described [20,21]. TECs were cultured in serum-free Dulbecco's modified Eagle's medium and Ham F12 (BioWhittaker, Verviers, Belgium) in a 1:1 ratio, supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (40 pg/ml) and epidermal growth factor (10 ng/ml) (all from Sigma). Cell growth was maintained in a T75 cell culture flask (Greiner Bio-One, Essen, Germany). For passages of cell cultures, cells were washed twice with phosphate buffered saline (PBS) and trypsinized with 0.05% trypsin-EDTA at 37°C and then washed in Dulbecco's modified Eagle's medium and Ham F12 in a 1:1 ratio supplemented with 10% heat inactivated FBS. Cells were used between passages 2 and 6 of culture. The specific outgrowth of TEC was confirmed by morphologic appearance and immunofluorescence staining ($CD13^+$, $CD26^+$ and $CD90^-$) (data not shown).

TEC activation experiments

TECs were seeded in 24 well plates at a density of 2×10^5 TEC/ml in the above described medium. After one day medium was replaced with TEC culture medium with or without addition of cytokines. TECs were stimulated with 50ng/ml human recombinant IFN-γ (U-Cytech, Utrecht, the Netherlands) and/or 20 ng/ml human recombinant TNF-α (PeproTech, London, UK) for 24h, 48h and 72h. Supernatants were harvested and stored at -80°C until analysis.

Flow cytometry

TECs were harvested via trypsinization after 24, 48 and 72 hours. TECs washed with cold PBS supplemented with 0.5% albumin (PBSA 0.5%) and stained for CD13 PE-Cy7, CD26 FITC, CD86 FITC, HLA-I APC, HLA-DR APC-Cy7 (all BD Biosciences), CD40 PerCP-Cy5.5 (Biolegend), CD80 PE (Serotec), CD90 APC (R&D systems) at 4°C for 30 minutes. Cells were washed twice with cold PBSA 0.5% following flow cytometric analysis. Fifty thousand events were acquired from each tube by a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed with FACS Diva 6.1 software (BD Bioscience).

Quantitative real time PCR

Total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Almere, The Netherlands), according to the manufacturer's instructions. RNA concentrations were measured by using the Nanadrop ND-8000 Spectrophotometer (Isogen Life Science, De Meern, the Netherlands). First-strand complementary DNA (cDNA) reaction was performed from 500ng of the isolated RNA. A

quantitative RT-PCR was used to quantify the amount of MIP-3α in the samples. Assay-on-demand products for the detection and quantification of MIP-3α (hs00171125_m1) mRNAs were designed by Applied Biosystems (Foster City, CA). A 5 µL sample of cDNA was added to 20µL PCR mixture containing 12.5 µL Universal PCR Master Mix (Applied Biosystems), 0.625 µL of each specific primer and probe assay-on-demand mix, and 6.875 µL of water. The RT-PCR reaction was performed with the StepONEplus (Applied Biosystems). The amount of each target molecule was quantified by measuring the threshold cycles (C_t) on a TaqMan Real-Time PCR system (Applied Biosystems) and was transformed to the number of cDNA copies [$2^{(40-Ct)}$]. The absolute value of the number of MIP-3α mRNA copies was log transformed.

Cytokine analysis

Production of cytokines by unstimulated and 24h, 48h and 72h IFN-γ/TNF-α stimulated TECs were measured in supernatant using a Bio-Plex multiplex assay (Bio-Rad Laboraties, Veenendaal, the Netherlands) for IL-1β, IL-6, IL-12p70, IL-17, CXLC9 (MIG), CXLC10 (IP-10), CCL5 (RANTES), IL-8 and CCL2 (MCP-1). Samples were analyzed using a Bio-Plex Array Reader with Bio-Plex software. IL-23 (U-CyTech, Biosciences, Netherlands), TGF-β1 (eBioscience, San Diego, USA) and CCL20 (MIP-3α) (R&D Systems, Minneapolis, MN) were measured by ELISA. All immunoassays were performed according manufacturers guidelines.

Chemotaxis assay

Migration assays were performed using 3 µm pore membrane inserts (ThinCerts; Greiner Bio-One). For each experiment, TECs were seeded in a 24 well plate at a density of 5 x 10^3 cells (an optimized cell density; data not shown). Stimulation was performed with 50 ng/ml IFN-γ and 20 ng/ml TNF-α for 24 hours. PBMCs were activated to induce expression of the chemokine receptors CXCR3 and CCR6. PBMCs were stimulated with soluble anti-CD3 (1 µg/ml), anti-CD28 (1 µg/ml) and polyclonal goat anti-mouse Ig (1 µg/ml) (BD Pharmingen), IFN-γ (50 ng/ml, U-Cytech) and IL-2 (200 IU/ml Chiron BV, Amsterdam, the Netherlands) for 3 days. Anti-CD3/CD28 activated PBMCs were washed with medium without serum three times. After 4 hours of incubation at 37°C, migrated $CD4^+$ T cells were analyzed for CXCR3 and CCR6 expression by flow cytometry (BD FACS CantoII). Total cell numbers were determined by flow cytometric analysis, performed with a fixed time point and a fixed volume for standardization.

Ethical statement

This study was approved by the local medical ethical committee of the Erasmus MC – University Medical Center Rotterdam. All patients included in this study gave written informed consent. This study was conducted according to the principles of the Declaration of Helsinki.

Statistics

Results are expressed as mean ± SEM unless stated otherwise. Cytokines and chemokines present in supernatant were analyzed for statistical significance with GraphPad Prism 5.01 software (Graphpad Software, La Jolla, CA) using non-parametric Friedman test followed by Dunn's post hoc test.

Chemotaxis experiments were analyzed with the non-parametric Wilcoxon matched-pairs signed-rank test. P values of less than 0.05 were considered statistically significant.

RESULTS

IFN-γ and TNF-α stimulation upregulates expression of CD40, HLA-I and HLA-DR by TECs

TECs were cultured with medium alone and in the presence of IFN-γ/TNF-α. After 24 hours of stimulation, CD40, HLA-I and HLA-DR cell surface markers were upregulated by TECs and remained highly present for at least 72 hours as shown by a significantly higher mean fluorescence intensity (MFI) indicating that TECs are activated in this experimental system (P<0.001) (Figure 1A and Figure 1C). Additionally, we investigated the expression of some other TEC related (activation) markers. In line with earlier findings the co-stimulatory molecules CD80 and CD86 were not expressed by TECs irrespective of their stimulation state (Figure 1B).

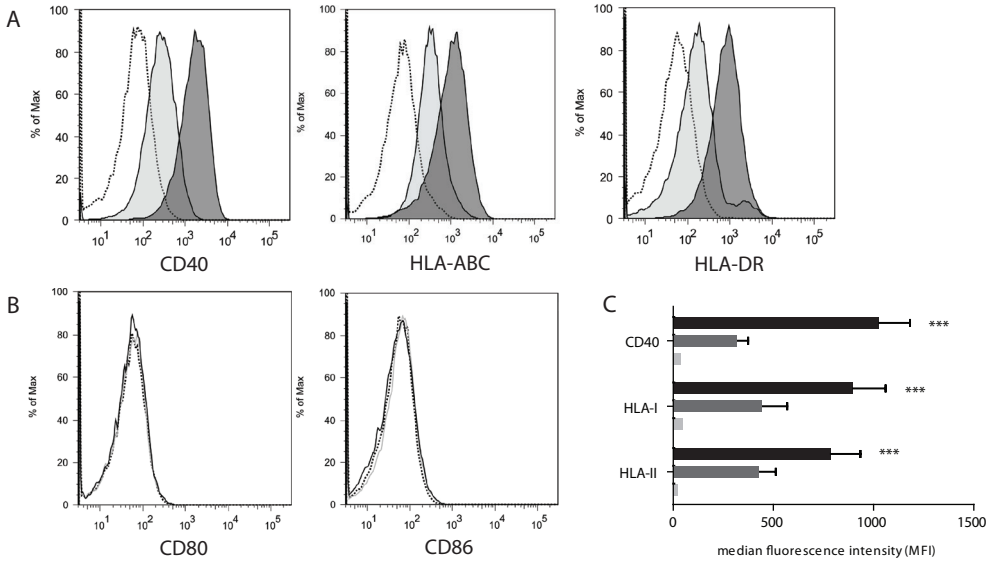

Figure 1 | IFN-γ and TNF-α stimulation upregulates expression of activation markers CD40, HLA-I and HLA-DR by TECs.

Primary TECs were stimulated with IFN-γ (50 ng/ml)/TNF-α (20 ng/ml) for 24, 48 and 72 hours. **(A)** Surface staining was performed for the activation markers CD40, HLA-I and HLA-DR. One representative out of 10 experiments is shown. The upregulation of activation markers was observed after 24h stimulation and still detectable after 72h stimulation (data not shown). **(B)** Primary TECs were also stained for the costimulatory molecules CD80 and CD86. The dotted line represents unstained control, grey bar/line represents unstimulated situation and the dark bar/line represents IFN-γ/TNF-α stimulation **(C)** Quantification of flow cytometric results (mean ± SEM, n=10) indicating the median fluorescence intensity of the activation markers CD40, HLA-I and HLA-II (n=3). White bars represent unstained control, grey bars represent unstimulated condition and black bars represent IFN-γ/TNF-α stimulation.

*** Significant increase (P<0.001).

TECs fail to secrete Th1 and/or Th17 differentiation cytokines after IFN-γ and TNF-α stimulation while producing abundant amounts of proinflammatory cytokines IL6 and IL8

To define whether TECs are capable of production of cytokines by which naïve $CD4^+$ T cells may differentiate into Th1 and / or Th17 cells or change the cytokine profile of Th1 and Th17 cells, we stimulated TECs (n=10) with IFN-γ/TNF-α and analyzed the cytokine profile of supernatants harvested under these stimulatory conditions compared to the unstimulated state. Unstimulated and IFN-γ/TNF-α activated TECs did not secrete IL-12p70. Th17 associated cytokines including IL-1β, IL-17, IL-23 and TGF-β1 were not produced either by unstimulated TECs or after IFN-γ/TNF-α stimulation (Table 1). In contrast, IL-6 and IL8 were produced in high concentrations by TECs after IFN-γ/TNF-α stimulation compared to unstimulated TECs (5 fold increase, Figure 2).

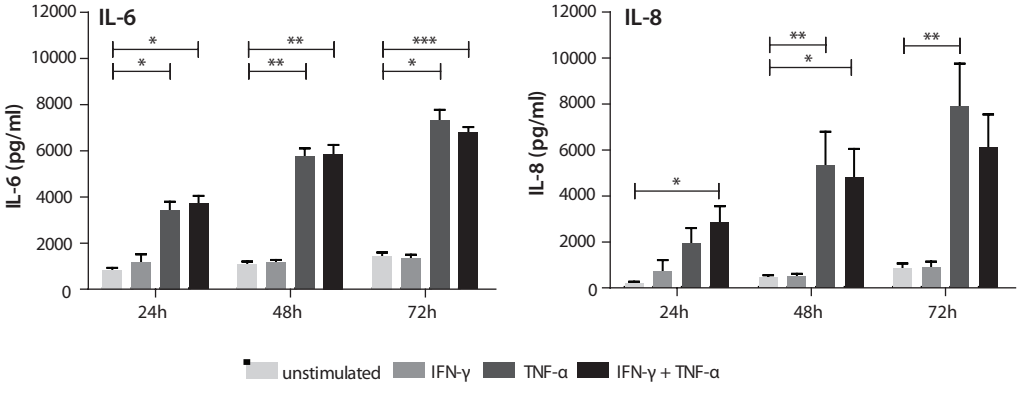

Figure 2 | TECs produce proinflammatory cytokines IL-6 and IL-8 after IFN-γ and TNF-α stimulation.

Cytokine production was tested using Bio-Plex multiplex assay after TEC stimulation with IFN-γ and TNF-α for 24, 48 and 72 hours (mean ± SEM, n=10). The proinflammatory cytokines IL-6 and IL-8 were found to be produced by stimulated TECs abundantly.

* Significant increase (P<0.05), ** Significant increase (P<0.01).

Table 1 | Th1 and Th17 differentiation cytokines after 72 hours of stimulation.

Cytokine	Unstimulated TEC	Stimulated TEC
IL-1β	N.D.	N.D.
IL-6	+	+++
IL-12p70	N.D.	N.D.
IL-17	N.D.	N.D.
IL-23	N.D.	N.D.
TGF-β1	N.D.	N.D.

N.D. not detectable; + cytokine present +++ significant upregulation; Stimulated TEC using combination of IFN-γ (50 ng/ml) and TNF-α (20 ng/ml).

Th1 associated chemokines are produced after IFN-γ and TNF-α stimulation

We investigated the production of Th1 associated chemokines CXCL9 (MIG), CXCL10 (IP-10), CCL5 (RANTES) and CCL2 (MCP-1) under non stimulatory and IFN-γ/TNF-α stimulatory conditions in a time dependent manner for 24, 48 and 72 hours. Combined stimulation with IFN-γ and TNF-α resulted in a synergistic induction of CXCL9, CXCL10 and CCL5. Compared to unstimulated condition CXCL10 showed a 65 fold increase after 24 hours stimulation (30 pg/ml vs. 1960 pg/ml; P<0.001). After 72 hours a 2.5 fold increased production of CXL10 was found compared to 24 hours (5064 pg/ml) (Figure 3). CCL5 was already significantly upregulated after 48 hours (47 pg/ml) and remained high after 72 hours (66 pg/ml) as compared to unstimulated condition, while CXCL9 could only be detected after 72 hours stimulation (114 pg/ml). CCL2 production by TECs was present constantly at all time points measured showing a rapid onset and reaching a high plateau level after 24 hours which was retained during 72 hours. In unstimulated condition and at 24 hours, TECs produced CCL2 at a concentration of 1018 pg/ml. IFN-γ alone failed to upregulate CCL2 production, while both TNF-α (2154 pg/ml) and IFN-γ/TNF-α (2550 pg/ml) stimulation significantly upregulated CCL2 production (Figure 3).

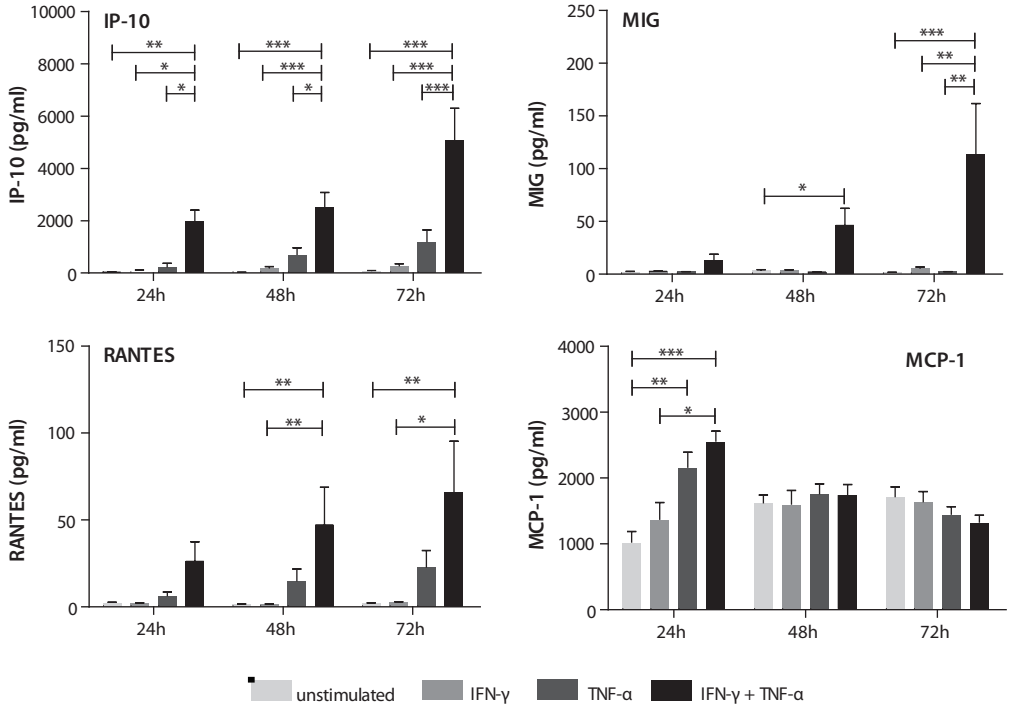

Figure 3 | Th1 associated chemokines are produced by TECs after IFN-γ and TNF-α stimulation.

TECs were stimulated with IFN-γ (50 ng/ml) and TNF-α (20 ng/ml) for 24, 48 and 72 hours. Supernatant was analyzed for the Th1 associated chemokines CXCL9, CXCL10, CCL2 and CCL5 using Bio-Plex multiplex assay technology. Data shown as mean ± SEM, n=10.

* Significant increase (P<0.05), ** significant increase (P<0.01), *** significant increase (P<0.001).

CCL20 is not produced by TECs after IFN-γ and TNF-α stimulation

We investigated whether tubular epithelial cells have the potential to produce CCL20 (MIP-3α) in our system. TECs did not upregulate CCL20 mRNA levels after IFN-γ and TNF-α stimulation. Accordingly, TECs were also not able to produce the Th17 associated chemokine CCL20 (Figure 4); neither unstimulated nor after 24, 48 and 72 hours of stimulation with IFN-γ and TNF-α.

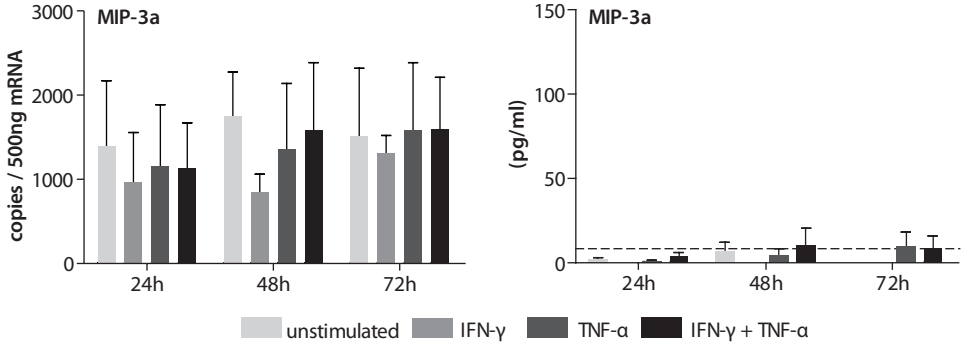

Figure 4| CCL20 is not produced by TECs after IFN-γ and TNF-α stimulation.

TECs were IFN-γ/TNF-α stimulated for 24, 48 and 72 hours. CCL20 mRNA expression by TECs was analyzed by PCR and the supernatant was analyzed for CCL20 protein using ELISA. The dotted line represents the lowest CCL20 detection limit (7.8 pg/mL). No significant CCL20 production could be detected after IFN-γ/TNF-α stimulation (n=8).

Th1 and not Th17 cells are attracted by IFN-γ and TNF-α stimulated TECs

Anti-CD3/CD28 activated PBMCs were added to the upper chamber of 3 μm pore membrane inserts. Unstimulated or IFN-γ/TNF-α stimulated TECs were tested for their capacity to attract $CD4^+CXCR3^+$ or $CD4^+CCR6^+$ T cells, representing respectively Th1 and Th17 containing cell pools (Figure 5A). After 4 hours of incubation, cells in the lower chamber were harvested and CXCR3 and CCR6 expression on $CD4^+$ T cells were determined. CD7 positive and CD16/CD56 negative lymphocytes were discriminated as anti-CD3/CD28 activated T lymphocytes (Figure 5B). The total numbers of migrated $CD4^+$ T cells (Figure 5C) were measured as readout. Although unstimulated TECs were able to attract $CD4^+CXCR3^+$ T cells to a low extent, IFN-γ/TNF-α activated TECs induced a significant migration of $CD4^+CXCR3^+$ T cells ($P<0.05$). No $CD4^+CCR6^+$ T cells were attracted by unstimulated TECs. In line with our chemokine data, IFN-γ/TNF-α activated TECs failed to induce migration of $CD4^+CCR6^+$ T cells. As such, activated primary renal tubular epithelial cells attract $CD4^+CXCR3^+$ T cells, but not $CD4^+CCR6^+$ T cells (Figure 5D and Figure 5E).

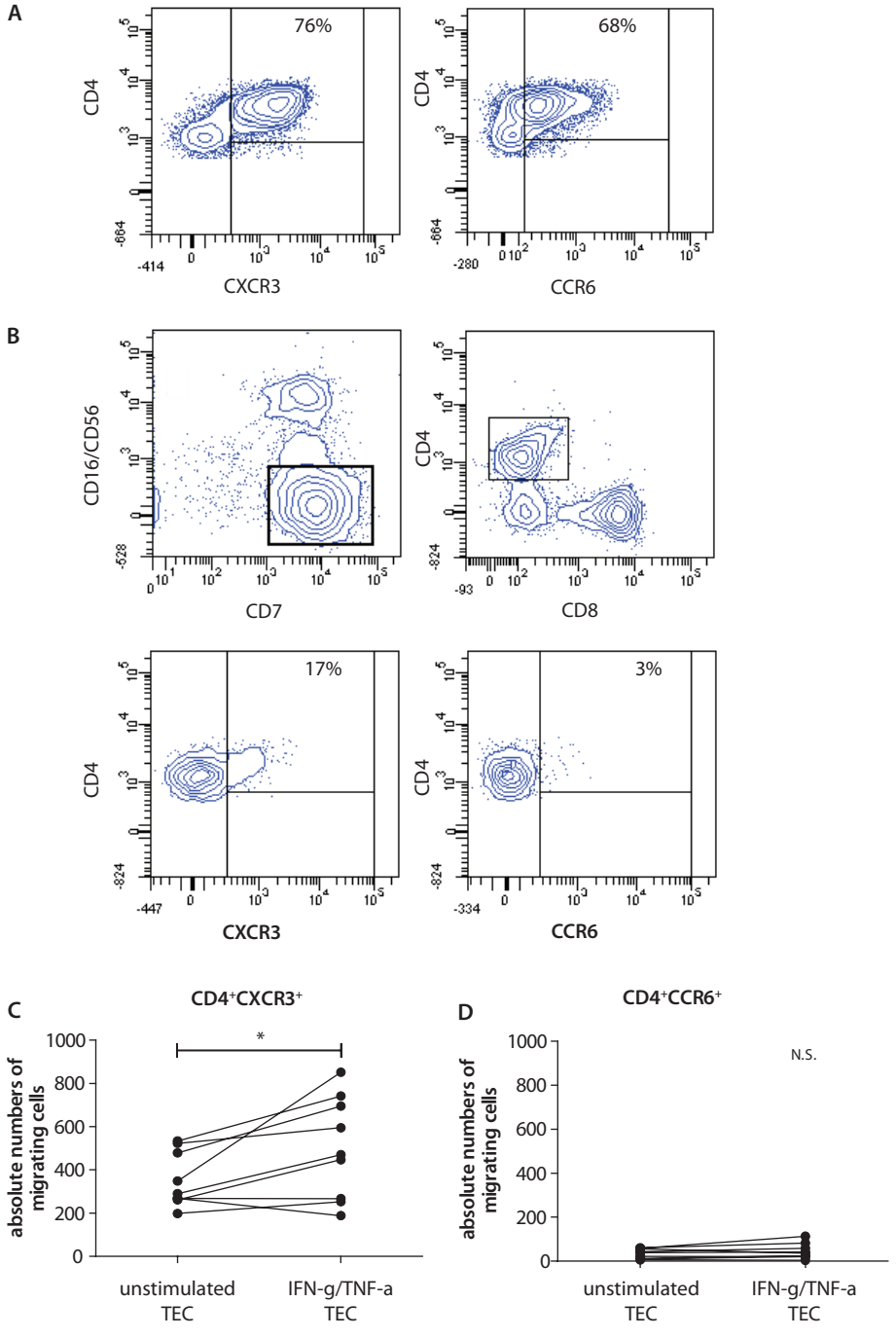

Figure 5 | Differential effects of renal tubular epithelial cells on T-cell migration.

(A) PBMCs were activated with anti-CD3/CD28, IL-2 and IFN-γ and placed in a 3.0μm pore transwell system upon unstimulated TECs or 24 hours IFN-γ/TNF-α stimulated TECs. Activated $CD4^+$ T cells expressed 76% CXCR3 and 68% CCR6. **(B)** Significant migration of $CD4^+CXCR3^+$ T cells, containing Th1 cell pool, by activated TECs was observed ($P<0.05$). No migration of $CD4^+CCR6^+$, containing Th17 cell pool, could be detected. $CD7^+$ and $CD16^-/CD56^-$ lymphocytes were discriminated as anti-CD3/CD28 activated T lymphocytes. **(C** and **D)** Transwell migration results of 9 individual experiments using unstimulated TECs vs. stimulated TECs are shown. The total cell number of migrated $CD4^+CXCR3^+$ and $CD4^+CCR6^+$ T cells are depicted on the Y-axis. Results were obtained by flow cytometry using fixed time point and fixed volume. * Significant increase ($P<0.05$).

DISCUSSION

In this study, we investigated the differential effects of activated human renal epithelial cells on T cell migration. We questioned whether TECs after stimulation by IFN-γ and TNF-α representing instrumental proinflammatory cytokines during rejection, have the potential to attract Th1 and Th17 T-cell subsets. Our data demonstrate that IFN-γ /TNF-α activated renal tubular epithelial cells primarily produce Th1-associated chemokines and not the Th17-associated chemokine CCL20. Functionally, only $CD4^+CXCR3^+$ T-cells and hardly any $CD4^+CCR6^+$ T-cells were attracted by activated TECs, when PBMC migration towards activated and resting TECs was tested using chemotaxis assays. Moreover, activated TECs do not produce cytokines promoting Th17 cell differentiation in our experimental system.

The recruitment of T cells from the peripheral circulation into the allograft is an essential event in acute kidney transplant rejection. Essential elements involved in this process are chemokines secreted in the tissue and the corresponding chemokine receptors present on T cells [14]. On the one side, transplantation procedure itself may have a direct effect on the chemokine profile within the graft. Following organ harvest, the intervening period of ischemia and subsequent reperfusion results in the release of various pro-inflammatory mediators like IL-1, TNF-α and IL-8 [22]. On the other side, rejection process can lead to release of chemokines which are mainly produced by inflamed parenchymal cells like tubular epithelial cells indicating a crucial role in the attraction of immune cells. It has been demonstrated that in kidney transplant biopsies from patients with acute rejection, expression of CCL2 and CCL5 is mainly found in TECs with mononuclear cell infiltration [23]. The production of chemokines like CCL2, CCL5 and IL-8 *in vitro* by TECs depends on cytokine activation. Various proinflammatory cytokines and CD40L stimulation resulted in a strong production of these chemokines by TECs [24,25]. Also IL-4 and IL-13 have a dose-dependent effect on CD40-induced CCL5 production by TECs [26]. Activation of TECs *in vitro* is assumed to mimic *in vivo* processes. In our *in vitro* experiments we stimulated the TECs with IFN-γ and TNF-α. This choice was made upon many *in vivo* and *in vitro* observations designating both IFN-γ and TNF-α as key regulator cytokines during kidney transplant rejection. A high TNF-α producer phenotype correlated with recurrent acute rejection episodes clinically [27] and also IFN-γ gene transcription is associated with high grade scores of tubulitis and the occurrence of rejection [28,29]. Not surprisingly, these proinflammatory cytokines are both upregulated during renal allograft rejection [30].

While Th1 differentiated cells preferentially express CXCR3 and CCR5, Th17 cells preferentially express the chemokine receptor CCR6 [12,17]. Increased expression of CXCR3 transcript levels were found in both subclinical and clinical acute kidney transplant rejection [31]. It is even suggested that CXCR3 is the most important chemokine receptor for the development of rejection, as $CXCR3^{-/-}$ mice showed an prolonged graft survival compared to wild type mice [29]. Although CXCR3 has been suggested to act as a potent chemokine receptor present during renal allograft rejection, the role of $CCR6^+$ T cells still needs to be elucidated. As we were interested in the attraction of Th1 and Th17 cells by TECs, we determined the total number of migrated $CD4^+CXCR3^+$ and $CD4^+CCR6^+$ T cells in our migration assay experiments. TECs were observed to have the potential to attract $CD4^+CXCR3^+$ T cells. Activation of TECs with IFN-γ and TNF-α increased the migration of $CD4^+CXCR3^+$ T cells, while

no migration of $CD4^+CCR6^+$ T cells could be detected. Our data confirm that activated TECs are capable of attracting Th1 cells while this is not the case for Th17 cells, assuming that the majority of Th1 cells are $CXCR3^+$ T cells and the majority of Th17 cells express CCR6 receptor.

CCR6 expression is of critical importance for Th17 migration and has been associated with aggravated experimental glomerulonephritis, graft-vershos-host disease (GVHD), inflammatory bowel disease and rheumatoid arthritis [32]. So far, Woltman et al. published the only report on CCL20 expression and $CCR6^+$ cells in kidney transplant rejection [19]. The authors reported CCL20 and $CCR6^+$ infiltrating cells in kidney graft biopsies during rejection and found that the attraction of immature dendritic cells by TECs depends on CCL20. In our study, the Th17 associated chemokine CCL20 was not produced by TECs after IFN-γ and TNF-α stimulation, while Woltman et al. found an increased CCL20 production after IL-1α and CD40L stimulation [19]. They also reported that after IFN-γ stimulation, a clear inhibitory effect on the IL-1α and CD40L-induced CCL20 production was found. Apparently, CCL20 produced by TECs depends on the type of stimulation.

Chemokines present in the allograft determine the attraction of specific T cell subsets recruited towards this allograft. CXCL9, CXCL10, CCL2 and CCL5 attract Th1 cells [14], while CCL20 attract Th17 cells [17]. We found a strong synergy between the cytokines IFN-γ and TNF-α on chemokine production by TECs. IFN-γ or TNF-α alone was not able to significantly increase CXCL9, CXCL10 or CCL5 at any time point tested, while a combination of IFN-γ and TNF-α increased the chemokine production by TECs profoundly. Synergistic effects of combined stimuli on TEC activation and cytokine/chemokine production have been observed before [24]. In our system, the production of CCL2 was strongly increased when IFN-γ and TNF-α were combined. IFN-γ alone was not able to upregulate CCL2 production while TNF-α could. During kidney transplant rejection intrarenal RNA expression of Th1-associated ligands CXCL10 and CCL5 have been documented [14], also increased urinary chemokines CXCL9 and CXCL10 were found during acute rejection compared to stable recipients [33]. In line, we found Th1-associated chemokines, while we were not able to document the production of the Th17-associated chemokine CCL20 by activated TECs. This indicates that the controversy in literature on CCL20 contribution to rejection probably depends on the dominant cytokines present in the microenvironment.

Local immunoregulation driven by cytokines during kidney graft rejection has been suggested by animal and clinical transplantation studies using graft biopsies and confirmed by *in vitro* studies using TEC cell lines. A variety of proinflammatory cytokines were shown to be produced during rejection like IL-6, TNF-α, IL-15 and IL-18 on both gene transcript and protein level [23]. In our study, proinflammatory cytokines like IL-1β, IL-12p70 and IL-23 were not produced by TECs in contrast to abundantly high IL-6 and IL-8 concentrations. Also active TGF-β1 could not be produced by both resting and activated TECs. As IL-12 is critical for the Th1 response [34] and IL-1β, IL-6, IL-23 and TGF-β1 are critical cytokines for Th17 cell differentiation and development [35], it can be argued whether under our experimental conditions, TEC associated cytokine profile might facilitate the Th1 and Th17 differentiation of naïve $CD4^+$ T cells. Dissection of the individual contributions of the $CD4^+$ T-cell subsets in transplant rejection is still a challenge for transplant immunologists because of redundancy and plasticity of the different effector pathways of $CD4^+$ T cells [36]. Under the influence of IL-12, Th17 cells can switch their phenotype to become Th1 cells, as Th17 express both IL-12 and

IL-23 receptors. In contrast, Th1 cells cannot undergo this phenotype switching [37]. The plasticity of the $CD4^+$ T-cell subsets on the one hand and the variety of locally produced inflammatory mediators like cytokines and chemokines *in vivo* on the other hand, challenge the balanced regulation between rejection and acceptance of the kidney graft in due time and make it susceptible for any inflammatory insult which can influence the determinants of this redundant process. We could not find any evidence for a stereotyped cytokine profile by TECs in our system that could direct any phenotype switching and reprogramming of $CD4^+$ T cells with regard to Th1- and Th17-associated pathways.

In summary, recruitment and infiltration of a kidney transplant by T cells, is one of the initial steps towards induction of structural graft damage. Our observations show that activated primary renal tubular epithelial cells do not attract Th17 cells nor produce cytokines promoting Th17 cell differentiation.

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CHAPTER 3

Substantial proliferation of human renal tubular epithelial cell-reactive $CD4^{+}CD28^{null}$ memory T cells, which is resistant to tacrolimus and everolimus

Martijn W.H.J. Demmers, Carla C. Baan, Malou Janssen, Nicolle H.R. Litjens, Jan N.M. IJzermans, Michiel G. Betjes, Willem Weimar and Ajda T. Rowshani

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ABSTRACT

Background: In spite of maintenance treatment with immunosuppressive drugs, tubulitis still occurs and can lead to structural kidney graft damage. We hypothesize that human renal tubular epithelial cells (TECs) trigger selective proliferation of recipient T-cell subsets with variable sensitivity to immunosuppressive drugs.

Methods: Recipient peripheral blood mononuclear cells were cocultured with donor-derived TECs for 7 days. The proliferation of the total $CD4^+$ T-cell pool was assessed. Next, we analysed which $CD4^+$ T-cell subset proliferated and how this response was affected by tacrolimus, everolimus, prednisolone and mycophenolic acid in clinically relevant concentrations.

Results: The $CD4^+$ T-cell proliferation upon TEC encounter was mainly executed by memory T-cells. Interestingly, 38 ± 7% of the proliferating $CD4^+$ T-cell pool showed a $CD28^{null}$ phenotype. These proliferating $CD4^+CD28^{null}$ memory T-cells produced high levels of IFN-γ, TNF-α, and the cytolitic protease granzyme B. TEC-reactive $CD4^+$ T-cell proliferation was significantly suppressed by tacrolimus, everolimus, prednisolone and mycophenolic acid ($P<0.05$). Surprisingly and in contrast to prednisolone and mycophenolic acid, neither tacrolimus nor everolimus could inhibit the $CD4^+CD28^{null}$ T-cell proliferative response.

Conclusion: Our data show substantial proliferation of TEC-reactive $CD4^+CD28^{null}$ memory T-cells which is resistant to tacrolimus and everolimus. This phenomenon might play an important mechanistic role during cellular rejection under full immunosuppression.

INTRODUCTION

Alloreactive memory T cells represent an important barrier for solid organ transplantation [1]. Memory T cells are less dependent on costimulation and have a reduced activation threshold. Rapid and enhanced production of cytotoxic effector molecules and proinflammatory cytokines are considered to be the common characteristics of this cell population [2]. Renal tubular epithelial cells (TECs) are one of the main targets of T cells during acute cellular rejection resulting in interstitial fibrosis/tubular atrophy and eventually chronic damage and graft loss [3].

Using a combination of calcineurin inhibitors, corticosteroids and anti-proliferative agents like mycophenolate mofetil, transplant specialists target divers pathways of T cell immunity simultaneously in order to dampen T-cell activation, proliferation and/or differentiation ([4-5]. Upon antigen stimulation memory T cells develop a quick allospecific effector response. However, conflicting data exist with regard to their responsiveness and/or resistance to calcineurin or mTOR inhibition [6-8].

$CD4^+CD28^{null}$ T cells form a unique subset of terminally differentiated effector memory $CD4^+$ T cells. Upon stimulation $CD4^+CD28^{null}$ T cells rapidly express high levels of IFN-γ, TNF-α and granzyme B [9]. While this subpopulation is rarely found in healthy individuals, the subset-frequency is slightly increased in elderly individuals [10] and largely expanded in patients with multiple sclerosis [11], rheumatoid arthritis [12] and acute coronary syndromes [13]. Various studies have shown an association between cytomegalovirus (CMV) infection and the increased numbers of circulating $CD4^+CD28^{null}$ T-cells in end-stage renal disease [9,14]. Down regulation of CD28 on CD4 T cells has been regarded as a marker for graft dysfunction in lung transplant recipients [15]. Interestingly, $CD4^+CD28^{null}$ T cells are less susceptible to suppression by $CD4^+CD25^{high}$ regulatory T-cells than the conventional $CD4^+CD28^+$ T cells [16]. In line, $CD4^+CD28^{null}$ T cells have upregulated levels of the anti-apoptotic protein Bcl-2 [10]. Overall, these data suggest that $CD4^+CD28^{null}$ T cells are difficult to control by the immune system and by immunosuppressive drugs.

At present, it is still unknown whether immunosuppressive drugs can directly inhibit the local proliferation of $CD4^+$ T cells in the graft-microenvironment and in the presence of TECs. We hypothesize that immunosuppressive drugs cannot sufficiently inhibit TEC-reactive memory $CD4^+$ T-cell responses. This may underlay the still occurring rejection processes. We investigated the proliferative responses and the tissue damaging capacities of recipient $CD4^+$ T cells after co-culture with donor-derived TECs. In addition, we studied their susceptibility to the immunosuppressants tacrolimus, everolimus, prednisolone and mycophenolic acid (MPA).

MATERIALS AND METHODS

Renal transplant recipients and kidney donors

Peripheral blood was obtained from kidney donors and their recipients (n=7) between 3 and 6 months post kidney transplantation. Patients were treated homogenously with prednisone, a calcineurin inhibitor (tacrolimus or cyclosporin A) and mycophenolate mofetil. IL-2R monoclonal

antibody basiliximab (Simulect® Novartis, Basel, Switzerland) was used as induction treatment. Allografts were stable at the time of blood collection. Written informed consent was obtained from all patients and donors as requested by the local Medical Ethical Committee of the Erasmus MC – University Medical Center Rotterdam the Netherlands.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation Ficoll-paque (density 1.077 g/ml, Amersham Pharmacia Biotech, Uppsala, Sweden), frozen in medium containing 10% DMSO and stored at -150°C until analysis.

Culture of primary renal tubular epithelial cells

TECs were cultured from cortical tissue of human donor kidneys obtained at transplantation as previously described [17-18]. Cells were used between passages 2 and 6 of culture. TEC outgrowth was confirmed by morphologic appearance and immunofluorescence staining ($CD13^+$, $CD26^+$ and $CD90^-$) (data not shown).

Mixed renal tubular epithelial cell lymphocyte coculture

TECs were seeded in 96 well flat-bottom culture plates (Corning Costar, Corning, NY) at least 1 day before coculture with PBMC. Defrosted PBMC were cultured in human culture medium (HCM); RPMI-Glutamax (Gibco) supplemented with 10% heat-inactivated human serum, 100I U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). PBMC were labelled with the fluorescent cell linker dye PKH-67 (Sigma, St. Louis, MO) to assess proliferation and mixed with primary TECs in a ratio of 150.000:20.000 [19]. Clinically relevant concentrations of immunosuppressive drugs were used. Tacrolimus (Astellas Pharma, Tokyo, Japan) (10 ng/ml), everolimus (Novartis Pharma, Basel, Switserland) (10 ng/ml), prednisolone (200 ng/ml) (Sigma) and mycophenolic acid (MPA) (Sigma) (10 µg/ml) were added. In addition, we performed dose-response experiments with different drug concentrations to assess whether our index concentrations could lead to maximal inhibition of T cell proliferation (data not shown). Untouched naïve $CD4^+$ T cells ($CD4^+CD45RA^+$) (Miltenyi Biotec, Bergisch Gladbach, Germany) and untouched memory $CD4^+$ T cells ($CD4^+CD45RO^+$) (Miltenyi Biotec) were isolated using indirect magnetic cell sorting (MACS) by DepleteS on the autoMACS (Miltenyi Biotec) from healthy volunteers (Sanquin Bloodbank, Rotterdam, the Netherlands). Purity of the isolated fractions was >96.6%. As CD57 is not included in the untouched naïve $CD4^+$ T cells ($CD4^+CD45RA^+$) kit of Miltenyi Biotec, the presence of T_{EMRA} could not be excluded. Proliferation analysis of the subsets was performed using ^{3}H-thymidine incorporation assay (0.5 µCi/well: Amersham Pharmacia Biotech, Roosendaal, The Netherlands) at day 7.

Mixed lymphocyte reaction

The effect of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml) on T cell proliferation was studied by mixed lymphocyte reaction (MLR). 1×10^5 T cells were incubated with irradiated (40 Gy) HLA-mismatched (2-2-2) PBMC (ratio 1:1). Proliferation was measured using ^{3}H-thymidine incorporation assay (0.5 µCi/well: Amersham Pharmacia Biotech, Roosendaal, The

Netherlands) at day 7. In order to find out what the frequency is of $CD4^+CD28^{null}$ T cells within the proliferative fraction after a classical MLR, we cultured the same PBMC obtained from patient 1, 2 and 7, which were used in our mixed renal tubular epithelial cell lymphocyte coculture, also as responder cells in classical MLR with irradiated (40 Gy) donor PBMC (ratio 1:1). We analysed then the proliferation by flow cytometry at day 7 using PKH labelling.

Flow cytometry

PBMC were harvested and washed at day 7. Cell surface staining was conducted with the following mAb: CD3-Amcyan (BD), CD4-APC-Cy7 (Biolegend, San Diego, USA), CD8-eFluor450 (eBioscience), $CD28^-$PE (BD Pharmingen, San Diego, USA), CD45RO-APC (Biolegend), CCR7-PE-Cy7 (BD Pharmingen) and 7-AAD (BD Pharmingen). Memory phenotypes were analysed based on CCR7 and CD45RO co-expression; naïve (T_N; $CCR7^+CD45RO^-$), central memory (T_{CM}; $CCR7^+CD45RO^+$), effector memory (T_{EM}; $CCR7^-CD45RO$) and terminally differentiated effectors (T_{EMRA}; $CCR7^-CD45RO^-$). Intermediate $CCR7^+CD45RO^+$ cells were included in the central memory population. Fifty thousand gated lymphocyte events were acquired from each tube by a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA). Fluorescence-minus-one (FMO) controls were used to determine positive or negative boundaries. Data were analysed using FlowJo software (Tree Star, San Carlos, CA). Flow cytometric analysis was performed with at least 100 events gated.

Intracellular cytokine staining

PBMC were restimulated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (1 µg/ml; Sigma-Aldrich) in the presence of Golgiplug (BD Biosciences) for at least 4 hours. Cells were stained with using CD3-Amcyan, CD4-APC, CD28-PerCP-Cy5.5, CD8-eFluor450. Cells were fixed and permeabilized using Perm Buffer II (BD Biosciences). Intracellular staining was performed with the following mAbs: IFN-γ-PE-Cy7, TNF-α-PE and granzyme B-PE.

Cytokine analysis

TEC-PBMC coculture supernatant was analysed using a Bio-Plex multiplex assay (Bio-Rad Laboraties, Veenendaal, the Netherlands) for IL-2, IL-7, IL-15, IL-17, IFN-γ and TNF-α. Samples were analysed using a Bio-Plex Array Reader with Bio-Plex software. Immunoassay was performed according to manufacturer's guidelines.

Statistics

Results are expressed as mean ± SEM. Data were analysed for statistical significance with GraphPad Prism 5.01 software (Graphpad Software, La Jolla, CA) using non-parametric Wilcoxon matched-pairs signed-rank test or Mann-Whitney U test. P values of less than 0.05 were considered significant.

RESULTS

TEC-reactive CD4$^+$ T-cell proliferation with a memory phenotype

Representative plots of proliferating CD4$^+$ T cells are shown in Figure 1. The mean TEC reactive CD4$^+$ T-cell proliferation was 4.3 ± 1.3% ($P<0.05$). The majority of the proliferating CD4$^+$ T cells were of the memory phenotype; 46 ± 3% of proliferating CD4$^+$ T-cell population showed the central-memory T-cell phenotype and 32 ± 6% of proliferating pool possessed the effector-memory T-cell phenotype. CD4$^+$CD45RO^{dim} T cells were also observed within the central-memory population. Although their existence has been described in literature previously, the biological importance still remains unclear [20]. As shown in Figure 1D, when magnetic cell sorting (MACS) isolated memory CD4$^+$ T cells and naïve CD4 T cells were cocultured with TECs, the isolated memory CD4$^+$ T cells preferentially proliferated upon TEC encounter as compared to naïve CD4$^+$ T cells ($P<0.05$).

TEC-reactive proliferation of CD4$^+$CD28^{null} T cells

Proliferating CD4$^+$ T cells were analysed for the expression of the costimulatory molecule CD28 (Figure 2). Interestingly, we found that 38 ± 7% of the proliferating CD4$^+$ T-cell pool had a CD28^{null} phenotype. This was significantly higher than the obtained proliferating CD28^{null} population using the same patient PBMC in a classical MLR (n=3: 10.7 ± 5%; $P<0.05$, Figure 2C). Prior to TEC/PBMC coculture or MLR, CD4$^+$CD28^{null} T cells composed only 4.5 ± 2% of the total CD4$^+$ T cell population. Intracellular staining for IFN-γ, TNF-α and granzyme B revealed a proinflammatory profile for TEC reactive CD4$^+$CD28^{null} T cells. 41 ± 2% of the CD4$^+$CD28^{null} T cells appeared to be double positive for the proinflammatory cytokines IFN-γ and TNF-α and 30 ± 3% of the CD4$^+$CD28^{null} T cells were double positive for IFN-γ and granzyme B (Figure 2D). In contrast, CD4$^+$CD28$^+$ T cells showed a substantially different profile than CD4$^+$CD28^{null} T cells with regard to production of IFN-γ and granzyme B as shown in Figure 2E.

Neither tacrolimus nor everolimus inhibit TEC-reactive CD4$^+$CD28^{null} T-cell proliferation

We measured the PKH labelled CD4$^+$ T-cell proliferation in the presence or absence of tacrolimus (10 ng/ml), everolimus (10 ng/ml), prednisolone (200 ng/ml) and MPA (10 μg/ml) after 7 days of TEC-coculture (Figure 3). MPA, everolimus and prednisolone showed the most powerful inhibitory effect; reducing the percentage of proliferating CD4$^+$ T cells to more than 66% (MPA; 1.0 ± 0.5%) and 55% (everolimus; 2.0 ± 0.5% and prednisolone; 1.8 ± 0.4%) compared to control (4.3 ± 1.3%) in the absence of the investigated drugs ($P<0.05$) (Figure 3A). Although in the presence of tacrolimus, the proliferation of CD4$^+$ T cells was statistically significantly reduced, this effect was less prominent leading to only 30% inhibition of proliferation (3.0 ± 1.4%).

The proliferation of CD4$^+$ T cells in TECs-coculture system was mainly due to a major proliferation of CD4$^+$ T cells with a CD28^{null} phenotype compartmentalizing up to 38 ± 7% of the proliferating CD4$^+$ T-cell pool (Figure 3B). This phenomenon prompted us to investigate the inhibitory effects of the tacrolimus, everolimus, prednisolone and MPA on the proliferation of this specific subset. Surprisingly, tacrolimus did not inhibit the proliferation of TEC-reactive CD4$^+$CD28^{null} T cells compared to the control coculture without addition of tacrolimus (52 ± 10%). Also everolimus failed

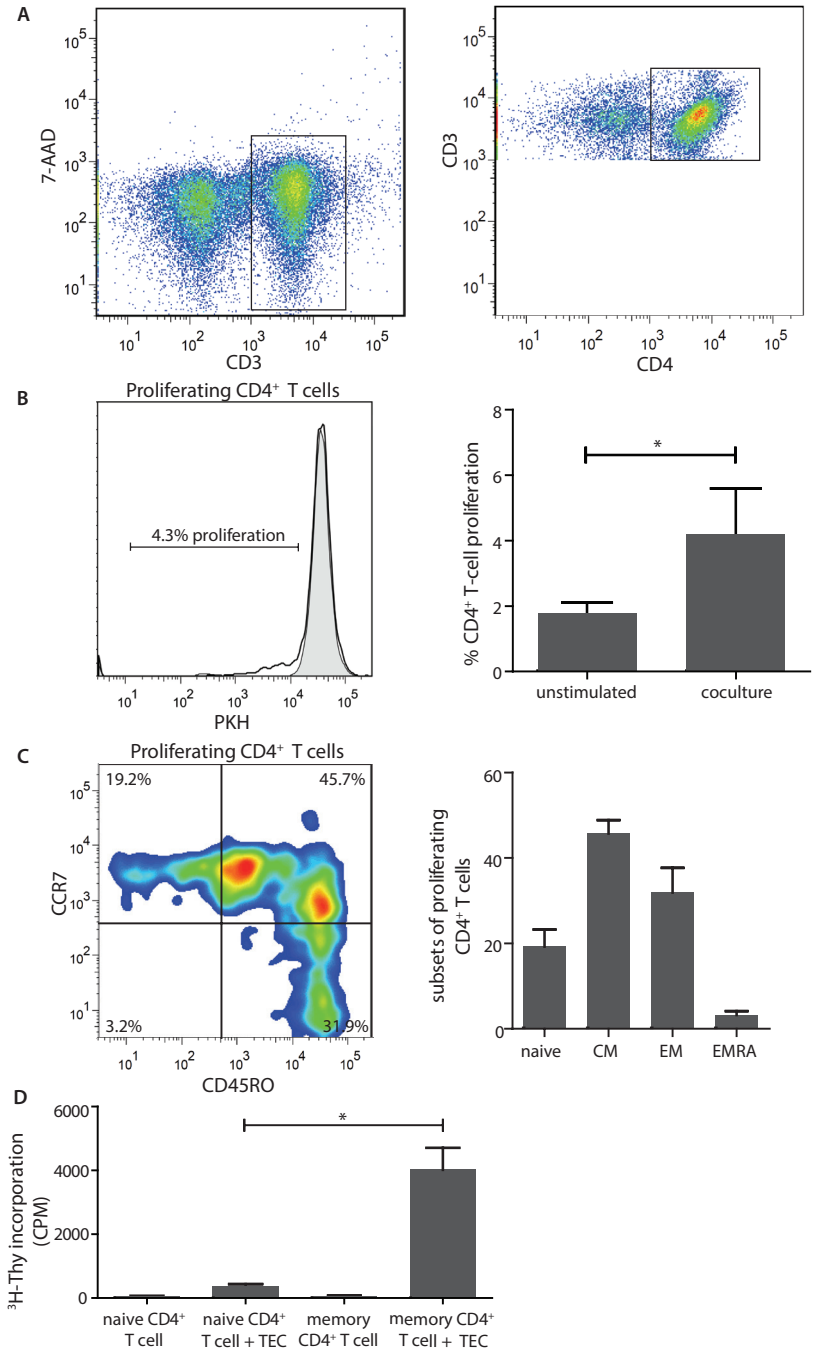

Figure 1 | TEC reactive CD4+ T-cell proliferation with a memory phenotype.

(A) Recipient PBMC were cocultured with donor TECs and harvested at day 7 (n=7). Viable CD3+ and CD4+ T cells were gated. **(B)** Subsequently, PKH losing/negative CD4+ T cells were marked as proliferating CD4+ T cells. Proliferative response of CD4 T cells were measured at 4.3 ± 1.3%. Comparisons were made by paired Wilcoxon signed-rank test (* P<0.05). **(C)** Proliferating CD4+ T cells were analyzed based on CCR7 and CD45RO expression. The percentages of T_N, T_{CM}, T_{EM} and T_{EMRA} are shown. Subsets of proliferating CD4 T cells resided mainly within memory cell pool. **(D)** MACS isolated naïve and memory CD4+ T cells (purity ≥96.6%) were cocultured with TECs (n=7). Proliferation was measured using ^{3}H-thymidine incorporation assay. Only memory CD4+ T-cell proliferation and no naïve CD4+ T-cell proliferation was observed.

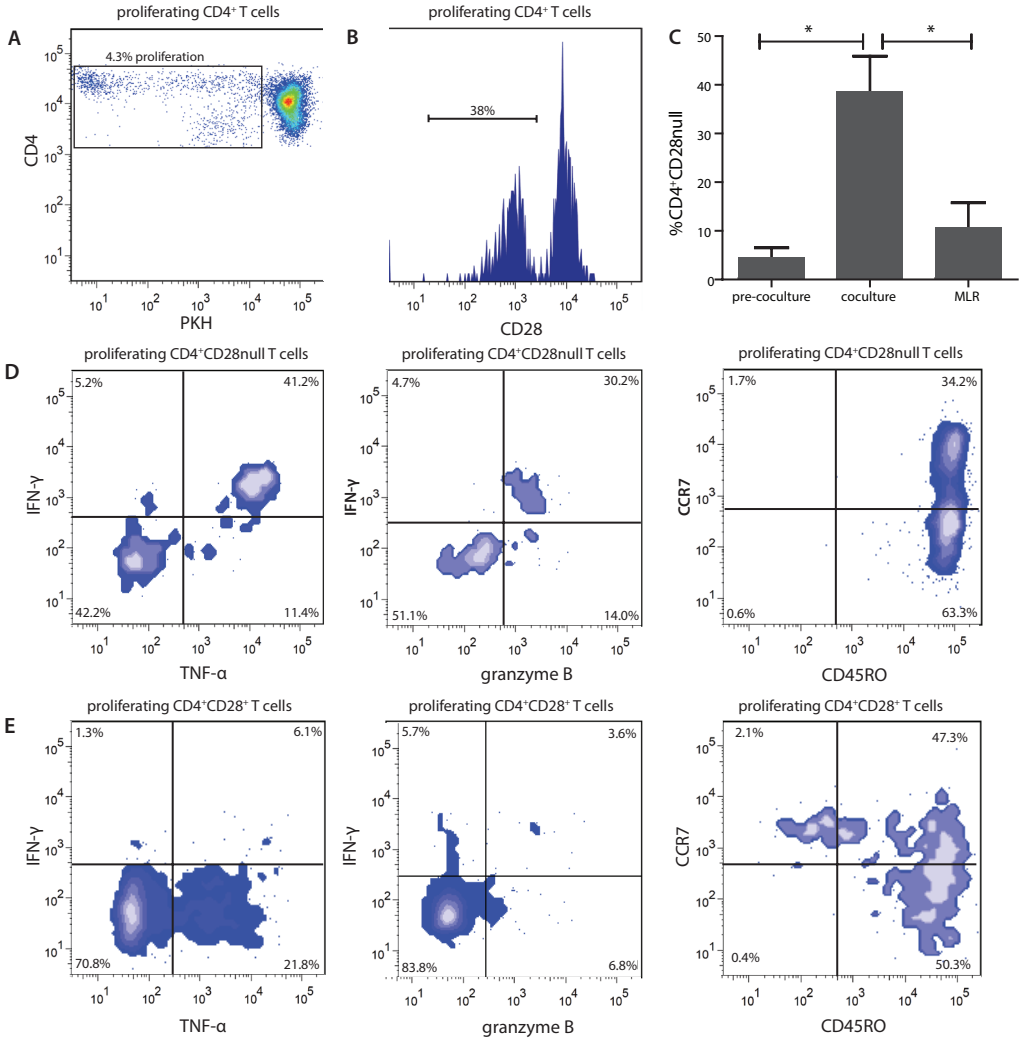

Figure 2 | TEC-reactive proliferation of CD4⁺CD28^{null} T cells.

(A-B) Recipient PBMC were cocultured with donor TECs and harvested at day 7 (n=7). Proliferating CD4⁺ T cells were analysed for CD28 expression. **(C)** Prior to TEC-PBMC coculture, CD4⁺CD28^{null} T cells compose 4.5% ± 2% of the total CD4⁺ T cell population. After 7 days TEC-PBMC coculture, CD4⁺CD28^{null} T cells compose 38.7 ± 7% of the proliferating CD4⁺ T cell compartment. After classical MLR (n=3), co-culturing responder PBMC obtained from same patients (patient 1, 2 and 7) which were also used in previous TEC-PBMC cocultures, with irradiated donor PBMC, CD4⁺CD28^{null} T cells compose only 10.7 ± 5% of the total CD4⁺ T-cell population.
(D-E) Proliferating PBMCs after 7 days TEC-PBMC coculture were restimulated with PMA/ionomycin in the presence of Golgiplug for 4 hours. **(D)** Proliferating CD4⁺CD28^{null} T cells and **(E)** Proliferating CD4⁺CD28⁺ T cells were intracellularly stained for IFN-γ, TNF-α and granzyme B. CD4⁺CD28^{null} T cells showed a potent proinflammatory and tissue damaging profile which was different from CD4⁺CD28⁺ T cells. In addition, proliferating CD4⁺CD28^{null} and CD4⁺CD28⁺ T cells were analysed for CCR7 and CD45RO expression. Results of seven experiments are shown (mean ± SEM). Comparison was made using paired Wilcoxon signed-rank test (* P<0.05). Non-parametric Mann-Whitney test was used to compare TEC coculture and MLR (* P<0.05).

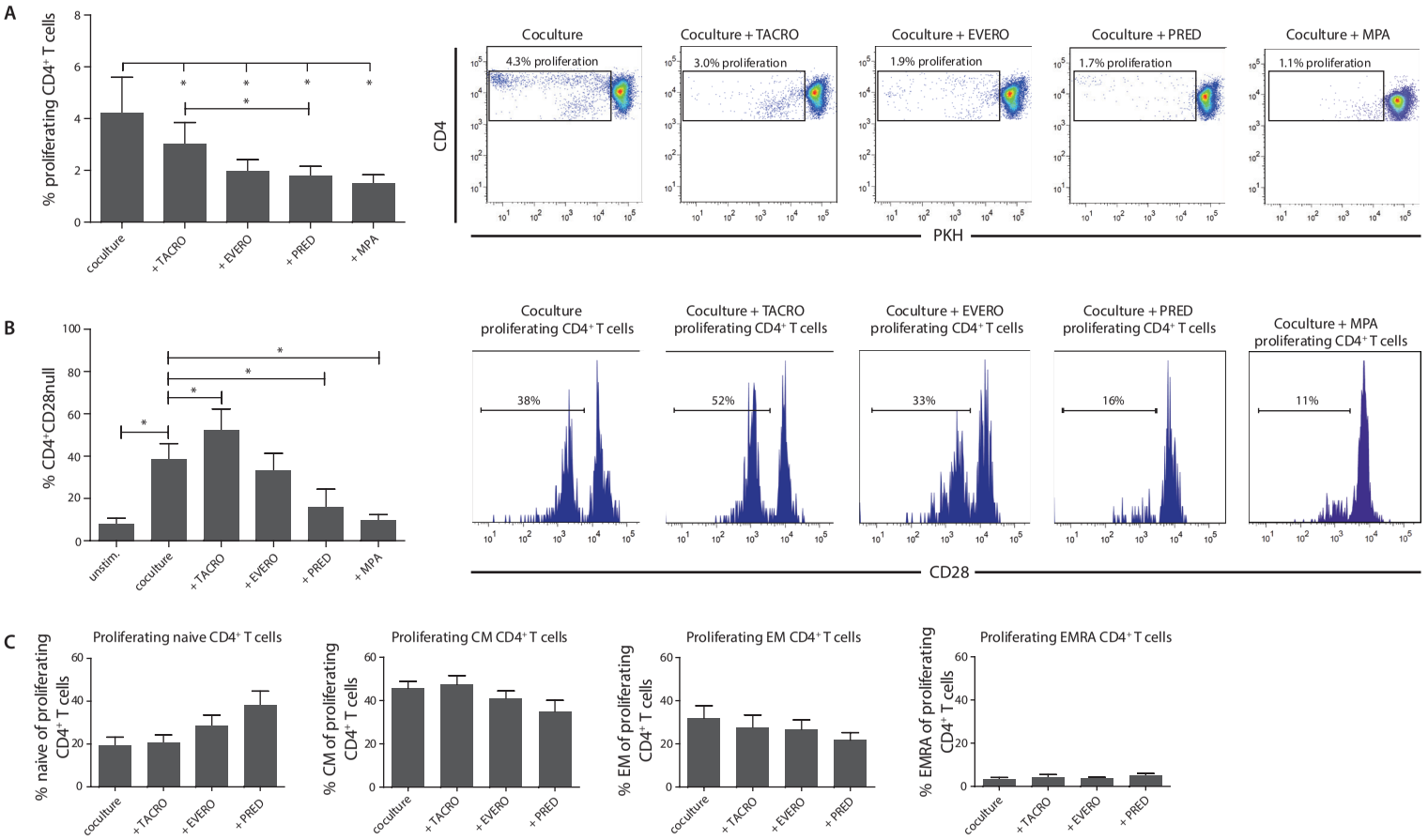

Figure 3 | Neither tacrolimus nor everolimus inhibit TEC-reactive CD4$^+$CD28^{null} T-cell proliferation.

(A) Recipient PBMC were cocultured with donor TECs and harvested at day 7 (n=7). CD4$^+$ T-cell proliferation was measured in the presence or absence of tacrolimus (10 ng/ml), everolimus (10 ng/ml), prednisolone (200 ng/ml) and MPA (10 µg/ml). Everolimus and prednisolone inhibited CD4$^+$ T cell proliferation up to 55%, while tacrolimus reduced this proliferation only up to 30%. MPA (n=4) abundantly inhibited CD4$^+$ T cell proliferation by maximally 66%. **(B)** Proliferating CD4$^+$ T cells were analysed for CD28 expression. In contrast to prednisolone, neither tacrolimus nor everolimus could inhibit TEC-reactive CD4$^+$CD28^{null} T cell proliferation. **(C)** Memory subset composition of proliferating CD4$^+$ T cells did not change in the presence of tacrolimus, everolimus and prednisolone. Results of seven experiments are shown (mean ± SEM). Comparison was made using paired Wilcoxon signed-rank test (* P<0.05). MPA (n=4) was compared with control using the non-parametric Mann-Whitney U test.

to show any inhibitory effect (33 ± 8%; ns). In contrary, prednisolone and MPA appeared to be the most powerful immunosuppressive drugs to inhibit the proliferation of this subset as compared to control (16 ± 8%; P<0.05) and (11 ± 3%; P<0.05).

As shown in Figure 3C the T_N, T_{CM} and T_{EM} cell subpopulation percentages remained constant in TEC/PBMC coculture in the presence or absence of immunosuppressive drugs of interest. MLR performed with clinically relevant concentrations of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml) showed significant inhibition up to 90% of base line (data not shown).

IL-2 and TNF-α production during TEC/PBMC coculture

We were interested in the concentrations of IL-2 and TNF-α in the supernatants of our TEC/PBMC cocultures as the production of these cytokines composes the main end-targets of tacrolimus, everolimus and prednisone as immunosuppressants. Moreover, it is unknown which cytokines are produced due to the interaction between TECs and PBMC, and what the effects of immunosuppressive drugs are on this interaction. In 6 out of 7 TEC/PBMC coculture-supernatants, we could analyse a panel of cytokines.

Significantly increased levels of the proinflammatory cytokines IL-2 (10 ± 5 vs. 3 ± 1 pg/ml; P<0.05) and TNF-α (9 ± 2 vs. 3 ± 1 pg/ml; P<0.05) were detected in our supernatants in comparison to control conditions (Figure 4). After addition of drugs a considerable decrease in IL-2 levels was detected; tacrolimus (4 ± 1 pg/ml), everolimus (4 ± 1 pg/ml) and prednisolone (1 ± 0.1 pg/ml) (Figure 4A). In contrast, higher TNF-α concentrations were measured in supernatants after addition of tacrolimus (15 ± 3 pg/ml; P<0.05) and everolimus (37 ± 15 pg/ml) (Figure 4B). Possible explanations might be consumption during coculture or TNF-α binding to the cell surface receptors and therefore not detectable in supernatant. Prednisolone led to a decrease in TNF-α production as expected but this decline did not reach statistical significance. The cytokines IL-7, IL-15, IL-17 and IFN-γ could not be detected in our coculture supernatants (data not shown). We also detected a higher concentration of TNF-α produced by TECs only as control which is in line with the known capability of TECs to produce TNF-α under stimulatory conditions [21]. In addition, it might be possible that human culture medium also stimulates TECs to produce TNF-α.

CMV antigen exposure is not related to the proliferation of TEC-reactive $CD4^+CD28^{null}$ T cells

Hypothetically, TECs may present CMV antigen as non-professional APC to T cells. We questioned whether CMV exposure could be responsible for proliferation of $CD4^+CD28^{null}$ T cells after TEC encounter. At transplantation, recipient 1, 2 and 3 and their donors were CMV naïve with no detectable CMV specific antibodies. Proliferating $CD4^+CD28^{null}$ T-cells percentage in their TEC/PBMC coculture system were respectively 60.8%, 55.5% and 30.3% (Table 1). In contrast, recipients 4, 5 and 6 and their donors were CMV seropositive with respectively 24.5%, 13.7% and 26.7% of proliferating $CD4^+CD28^{null}$ cells. These data show no relation between CMV infection and proliferation of $CD4^+CD28^{null}$ T cells upon TEC encounter.

Table 1 | CMV status and TEC-reactive $CD4^+CD28^{null}$ T-cell proliferation.

Patient index	Age/ Sex	Primary Kindey Disease	HLA-mismatch	Donor age	Donor Type	Recipient CMV status	Donor CMV status	Rejection	% TEC-reactive $CD4^+CD28^{null}$ T cells
1	25/M	Anti-GBM nephritis	1-1-1	52	LR	Negative	Negative	No	60.8
2	55/M	Unclear	1-1-1	56	LUR	Negative	Negative	No	55.5
3	49/F	Hypertensive nephropathy	1-2-2	52	LUR	Negative	Negative	Transplant Glomerulopathy (9 years after Tx)	30.3
4	61/M	Unclear	0-1-0	31	LR	Positive	Positive	No	24.5
5	58/F	PKD	1-0-0	30	LR	Positive	Positive	Acute cellular rejection with tubulitis and endothelialitis (Banff IIa, 3 weeks post Tx)	13.7
6	56/F	Glomerulonephritis	0-0-1	58	LUR	Positive	Positive	Transplant glomerulopathy with a chronic component of tubulointerstital nephritis (2 years after Tx)	26.7
7	25/F	Hypertensive nephropathy	2-1-2	23	LUR	Positive	Negative	No	58.9

Recipient and donor serum CMV-specific IgG levels were determined at the time of transplantation.
F, female; GBM, glomerular basement membrane; HLA mismatch, A-B-DR; M, male; LR, living related; LUR, living unrelated; PKD, polycystic kidney disease.

DISCUSSION

To date, the local effects of immunosuppressive drugs in the graft-microenvironment are poorly defined. As graft-infiltrating cell composition during rejection may be different in comparison to simultaneously observed alterations in peripheral blood [22], it can be envisioned that the immunosuppressive effects of currently used drugs could locally differ from their systemic effects. We hypothesize that TECs as graft resident parenchymal cells can modulate the outcome of donor-directed immune response by influencing the environmental drive in further activation, proliferation and function of TEC-reactive T cells.

In this presented study, we specifically investigated the TEC-reactive proliferative responses of the $CD4^+$ T-cell pool and studied the tissue damaging capacities of proliferating $CD4^+CD28^{null}$ T cells. In addition, we analysed their susceptibility to the immunosuppressive drugs tacrolimus, everolimus, prednisolone and MPA. We observed that TEC reactive proliferating $CD4^+$ T-cells were mainly consisted of memory phenotype. Interestingly, $CD4^+CD28^{null}$ T-cells were found to compose the major proliferating population producing IFN-γ, TNF-α and granzyme B. Importantly, proliferating $CD4^+CD28^{null}$ T-cells appeared not to be susceptible for inhibition by tacrolimus or everolimus.

The exact role of TEC/T-cell interaction during alloreactivity is not completely understood. Conflicting data exist whether activated TECs stimulate [23] or inhibit T-cell responses [24-25]. Proinflammatory cytokines upregulate co-stimulatory and co-inhibitory molecules on TECs [19,23, 26]. Wilson et al. documented T-cell proliferation after coculture with IFN-γ-activated allogeneic TECs in the presence of anti-CD28 antibody [23]. While epithelial cells were suggested to be unable to induce lymphocyte proliferation [24], others reported that addition of IL-2 restored the TEC anergized T-cell proliferation [25]. A study by Robertson et al. demonstrated intratubular proliferating $CD69^+$ T cells during acute renal allograft rejection [27]. Immunohistochemical analysis of rejecting grafts displayed memory T-cells infiltrating the epithelial barrier [28]. In our system we found proliferation of recipient memory $CD4^+$ T cells in response to donor derived TECs, and also showed in a pure experimental set-up using MACS isolated memory and naïve $CD4^+$ T cells in coculture with TECs that memory cells proliferated upon TEC encounter and not naïve $CD4^+$ T cells.

In our *in vitro* coculture model we analysed the effect of immunosuppressive drugs on proliferation of $CD4^+$ T-cell subsets. Tacrolimus, everolimus, prednisolone and MPA were able to reduce TEC-reactive $CD4^+$ T-cell proliferation. In our experiments the NF-κB inhibitor prednisolone and the antiproliferative drug MPA reduced the proliferation of $CD4^+$ T-cells more profoundly compared to tacrolimus, indicating that TEC-reactive proliferation of $CD4^+$ T cells favours inhibition via NF-κB or cell cycle arrest. MPA has a significant inhibitory effect on $CD4^+$ T-cell proliferation [29]. We found that tacrolimus and everolimus were not able to lower the frequency of $CD4^+CD28^{null}$ T cells indicating drug resistance. Vallejo et al. reported that $CD4^+CD28^{null}$ T-cells contain high amounts of the anti-apoptotic Bcl-2 protein [10]. Although tacrolimus is capable of inducing apoptosis in T cells, this drug is unable to change the activity of the anti-apoptotic protein Bcl-2 [30], while prednisone decreased Bcl-2 levels [31]. As such this may explain why tacrolimus is not able to inhibit the proliferation of the cytotoxic effector memory $CD4^+CD28^{null}$ T-cell subset.

It is known that the $CD4^+CD28^{null}$ T-cell population expands in various inflammatory disorders [11-13]. While pre-transplantation frequencies of $CD4^+CD28^{null}$ T cells were not related with renal allograft rejection [32], a higher frequency of circulating $CD4^+CD28^{null}$ T cells was described during chronic rejection [33]. Two explanations for these findings can be envisioned; either the local graft microenvironment is involved in the generation of the proliferative response of $CD4^+CD28^{null}$ T cells or these cells are attracted from the circulation into the graft.

CD28 downregulation might be caused by TNF-α production by cells of the allograft and by the T cell itself. T cells incubated with TNF-α showed a reduced cell-surface expression of CD28 [34]. In the presence of tacrolimus and everolimus we found increased levels of TNF-α as well as higher frequencies of $CD4^+CD28^{null}$ T cells. Although no data are available, anti-TNF-α therapy might be justified in renal transplant recipients, as TNF-α [35] and $CD4^+CD28^{null}$ [33] are both associated with renal allograft rejection. CD28 expression was reconstituted on circulating $CD4^+$ T cells of patients with RA and unstable angina who received anti-TNF-α treatment [36-37].

Another approach might be prevention of the migrating $CD4^+CD28^{null}$ T-cells towards TECs. CX_3CR1 drives $CD4^+CD28^{null}$ T-cell accumulation via fractalkine into the brain of MS patients [11]. TECs express also fractalkine after TNF-α stimulation *in vitro* [38]. Urinary fractalkine [39] and high frequencies of circulating $CD4^+CD28^{null}$ T-cells have been documented during rejection [33]. TECs might therefore have the capacity to attract CX_3CR1-positive $CD4^+CD28^{null}$ T cells.

Circulating $CD4^+CD28^{null}$ T cell pool has been associated with CMV exposure in the past [14]. As we could detect proliferating $CD4^+CD28^{null}$ T-cells upon TEC encounter irrespective of the CMV status of donor-recipient combinations, we have no support that intrarenal CMV antigen presentation by TECs [32,33] directly contribute to the proliferating $CD4^+CD28^{null}$ T-cell pool.

In summary, $CD4^+CD28^{null}$ T-cells compose the major proliferating memory population upon TEC encounter with a proinflammatory and cytotoxic profile, resistant to inhibition by tacrolimus and everolimus. These $CD4^+CD28^{null}$ T-cells may participate in the pathogenesis of renal allograft rejection occurring under maintanance immunosuppression. Once in the renal allograft or locally produced upon TEC encounter, $CD4^+CD28^{null}$ T-cells may release proinflammatory and cytolytic mediators. Our report provides supportive evidence that local graft-microenvironment can significantly influence the immunosuppressive effects of tacrolimus or everolimus.

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CHAPTER 4

Limited efficacy of immunosuppressive drugs on CD8$^+$ T-cell and NK-cell mediated lysis of human renal tubular epithelial cells

Martijn W.H.J. Demmers, Sander S. Korevaar, Michiel G.H. Betjes, Willem Weimar, Ajda T. Rowshani* and Carla C. Baan*

*equal dual principal investigators

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ABSTRACT

Background: Although $CD8^+$ T-cell and NK-cell mediated cytotoxicity against renal tubular epithelial cells (TECs) play a crucial role during rejection, the degree of inhibition of these lytic immune responses by immunosuppressive drugs are unknown. We investigated the CD8 T-cell and NK-cell responses induced by TECs *in vitro* and questioned how these processes are affected by immunosuppressive drugs.

Methods: Donor-derived TECs were cocultured with recipient PBMC. Proliferation of $CD8^+$ T cells and NK-cell subsets was assessed using PKH dilution assay. CD107a degranulation and europium-release assay were performed to explore $CD8^+$ and NK-cell mediated TEC lysis. Experiments were conducted in absence or presence of tacrolimus (10 n g/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml).

Results: TECs induce significant $CD8^+$ T-cell and NK-cell proliferation. All immunosuppressive drugs significantly inhibited TEC-induced $CD8^+$ T-cell proliferation. Interestingly, prednisolone was the most powerful inhibitor of NK-cell proliferation. CD8 and NK-cell mediated early lytic responses were marked by strong degranulation after encounter of unstimulated TECs, represented by a high cell surface expression of CD107a. However, using IFN-γ/TNF-α activated TECs, the NK degranulation response was significantly reduced, and CD8 degranulation response was even more enhanced ($P<0.05$). TEC-induced CD8 degranulation and CD8 mediated TEC lysis were preferentially inhibited by tacrolimus and prednisolone, and not by everolimus. While tacrolimus showed the most inhibitory effect on degranulation of NK cells, NK-cell mediated TEC lysis was efficiently inhibited by prednisolone ($P<0.05$).

Conclusion: Overall, our data point to limited efficacy of immunosuppressive drugs on $CD8^+$ T-cell and NK-cell mediated lysis of human renal tubular epithelial cells.

INTRODUCTION

Renal tubular epithelial cells (TECs) are one of the main targets of immunological attack during kidney transplant rejection. Disruption and apoptosis of TECs results in organ damage and eventually in nephron loss [1-2]. The local effects of tacrolimus, everolimus and prednisolone on lymphocyte-mediated cytotoxicity in the graft-microenvironment are poorly defined. Transplant specialists target divers pathways of T-cell immunity; by inhibiting calcineurin using tacrolimus, inhibiting the mTOR pathway using everolimus, and inhibiting the NF-κB pathway using prednisolone [3]. Emerging evidence shows that graft-infiltrating cell composition and function during rejection could differ from simultaneously observed alterations in peripheral blood [4-5]. These findings led to the question whether the immunosuppressive effects of currently used drugs could locally differ from their systemic effects. In line, we have recently shown a substantial proliferation of TEC-reactive $CD4^+CD28^{null}$ memory T cells, which is resistant to tacrolimus and everolimus [6].

While $CD8^+$ T cells are primarily associated with T-cell mediated rejection [2], recent studies show that NK cells are mainly associated with late antibody-mediated rejection [7-9]. The questions are whether TEC mediated lysis by $CD8^+$ T cells and NK cells are equally and/or completely affected by immunosuppressive drugs *in vitro*. These insights might provide a rational to use predefined combinations of immunosuppressive drugs in combatting a special type of rejection.

Granule exocytosis is one of the mechanisms by which cytotoxic $CD8^+$ T cells (CTLs) and NK cells induce cell death [10]. Upon target recognition CTLs and NK cells release effector mediators like granzyme B and perforin [1]. Immunohistochemical analysis of kidney transplant rejection biopsies showed abundant expression of perforin and granzyme B by infiltrating T lymphocytes and also by sparse present NK cells [12-13]. In line, increased urinary granzyme B and perforin mRNA may predict rejection [13-14]. Granzyme A mRNA, another cytolytic molecule, has been shown to be a diagnostic marker of cellular (sub)clinical kidney graft rejection [13]. Isolated graft-infiltrating T lymphocytes from a donor kidney showed powerful cytolytic responses towards the same donor TECs [15]. Simultaneous release of granzymes and perforin is accompanied by increased cell surface expression of CD107a which can be used to measure the degree of cytolytic activity [16-17]. CD107a expression correlated both with $CD8^+$ T-cell cytotoxicity [18] and NK-cell cytotoxicity [19-20]. Decreased cytolytic activity executed by tacrolimus pretreated NK cells against K562 cell line was shown by parallel experiments using both methods; ^{51}Cr release cytotoxicity assay and CD107a degranulation assay [21].

In the present study, we hypothesized that immunosuppressive drugs cannot sufficiently inhibit the TEC-induced $CD8^+$ T-cell and NK-cell alloreactivity. We questioned whether $CD8^+$ T-cell and NK-cell mediated lysis of TECs are differentially affected by immunosuppressive drug, and whether the activation state of TECs could influence these processes. Both overt clinical and subclinical rejection still occur while patients are treated with potent combinations of immunosuppressive drugs experiencing undesirable systemic effects such as infection and malignancy. Besides clinical experiences, biological evidence is needed to rationalize the different minimization treatment protocols. To our knowledge, biological evidence and specially *in vitro* experiments focussing on the immune interaction between graft resident parenchymal cells like tubular epithelial cells and

T – or NK cells are sparse if not lacking. We investigated the TEC-induced proliferative responses, degranulation and lytic capacities of $CD8^+$ T cells and NK cells *in vitro*. In addition, we studied their susceptibility to lysis in the presence or absence of tacrolimus, everolimus and prednisolone in clinically relevant drug concentrations.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) of transplant recipients (3 months post transplantation) and healthy volunteers (Blood Bank Sanquin, Rotterdam, the Netherlands) were isolated by density gradient centrifugation Ficoll-paque (density 1.077 g/ml, Amersham Pharmacia Biotech, Uppsala, Sweden), frozen in medium containing 10% DMSO and stored at -150°C until analysis.

Culture of primary renal tubular epithelial cells

TECs were cultured from cortical tissue of human donor kidneys obtained at transplantation as previously described [22]. Cells were used between passages 2 and 6 of culture. TEC outgrowth was confirmed by morphologic appearance and immunofluorescence staining ($CD13^+$, $CD26^+$ and $CD90^-$). TECs were stimulated with 50 ng/ml human recombinant IFN-γ (U-Cytech, Utrecht, the Netherlands) and 20 ng/ml human recombinant TNF-α (PeproTech, London, UK) for upregulation of HLA classes as described before [22]. Unstimulated and stimulated TECs were stained for HLA-ABC and HLA-DR expression.

Proliferation assay

TECs were seeded in 96 well flat-bottom culture plates (Corning Costar, Corning, NY) 1 day before coculture with PBMC with at least 1 HLA-mismatch at HLA-A, B and DR. PBMC were cultured in human culture medium (HCM); RPMI-1640 containing Glutamax (GibcoBRL, Paisley, Scotland) supplemented with 10% heat-inactivated human serum, 100 IU/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). PBMC were labelled with the fluorescent cell linker dye PKH-67 (Sigma, St. Louis, MO) to assess proliferation and cocultured with primary TECs in a ratio of 150.000:20.000 [23]. At day 7 PBMC were cell surface stained with the following mAb: CD3-Amcyan, 7-AAD, CD16-PE, CD56-PE-Cy7 and CD19-APC (all BD Pharmingen), CD4-APC-Cy7 (Biolegend, San Diego, CA) and CD8-eFluor450 (eBioscience). Events were acquired by a FACSCanto II (BD Biosciences). Clinically relevant concentrations of immunosuppressive drugs were used. Tacrolimus (Astellas Pharma, Tokyo, Japan) (10 ng/ml), everolimus (Novartis Pharma, Basel, Switserland) (10 ng/ml) and prednisolone (200 ng/ml) (Sigma) were added. Transwell experiments were performed using 0.4 µm pores membranes (ThinCerts; Greiner Bio-One). TECs were seeded in a 24 well plate at a density of 5×10^3 cells, PKH-labelled PBMC were seeded on top of the 0.4 µm pores membranes and harvested at day 7. Fluorescence-minus-one (FMO) controls were used to determine positive or negative boundaries. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Flow cytometric analysis was performed with at least 100 events gated.

Generation of TEC-specific CD8+ T cells

In a 96 well flat-bottom culture plate, 1.5 x 10^5 PBMC and TECs (HLA-mismatch A-B-DR 2-2-2) were cocultured with HCM in a ratio of 1.5 x 10^5:2 x 10^4 [23]. Coculture was performed in the presence of 200 IU/ml IL-2 (Proleukin, Chiron, Amsterdam, the Netherlands) and 10 ng/ml IL-15 (PeproTech, Rocky Hill, NJ) for 7 days. We aimed to examine the effects of tacrolimus, everolimus and prednisolone on the effector phase and not on the priming phase, so during the 7 days no drugs were present in our system. Subsequently, cells were harvested and stained with the following mAb: CD3-Amcyan (BD Pharmingen), CD8-eFluor450 (eBioscience), CD16-PE (BD Pharmingen), CD56-PE (BD Pharmingen) and the viability marker 7-AAD (BD Pharmingen). $CD8^+$ T cells were defined as $CD3^+CD8^+CD4^-$ cells and NK cells were defined as $CD3^-CD16^+CD56^+$ cells. $CD8^+$ T cells and NK cells were isolated by fluorescence-activated cell sorting (BD FACSAria II, BD Biosciences). The isolated $CD8^+$ T cells and NK cells were >97% pure. Isolated cells were either used immediately or kept overnight at 37°C in HCM in the presence of 200 IU/ml IL-2 and 10 ng/ml IL-15.

Degranulation assay

$CD8^+$ T cells and NK cells were isolated by FACS sorting. Effector cells were incubated in the absence or presence of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml) for 3 hours in 96-well plate. Subsequently, generated TEC-specific $CD8^+$ T cells and NK cells were cocultured with 25 x 10^3 TECs (4:1). Monensin 10 µl (1:10) (Golgistop, BD Biosciences) was added 1 hour after coculture to prevent internalization of CD107a. Subsequently, effector cells were harvested after 1 hour and kept on ice during assay. Harvested cells were surface stained with the following mAb: CD8-eFluor450 (eBioscience), CD16-PE, CD56-PE, CD107a-APC and 7-AAD (all BD Pharmingen).

Cytotoxicity-mediated lysis assay

$CD8^+$ T cells and NK cells were isolated by FACS sorting. Cytotoxicity-mediated lysis assay of TECs was performed using europium release assay as described previously [24]. In brief, 10^4 TECs were kept overnight in a flat-bottom 96-wells plate for adherence. TEC-specific effector cells were incubated in the absence or presence of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml) for 3 hours in a round bottom 96-well plate before coculture. TEC-specific CD8 or NK effector cells were added to europium labelled TEC for 4 h. Effector:target (E:T) ratios ranged from 40:1 to 0:1 for $CD8^+$ T cells and 20:1 to 0:1 for NK cells based on the available cell numbers. Released europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, LKB-Wallac, Turku, Finland). Fluorescence was expressed in counts per second (cps). Maximum release of europium by target cells was measured by incubation of labelled target cells with 1% triton (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 4 h. Spontaneous release of europium by target cells was measured by incubation of labelled target cells without effector cells. Percentage leakage was calculated as: (spontaneous release/maximum release) × 100%. Mean percentage of leakage was 26 ± 6%. The percentage of cytotoxicity-mediated lysis was calculated as: % lysis = [(measured lysis – spontaneous release)/(maximum release – spontaneous release)] × 100%.

Statistics

Results are expressed as mean ± SEM. Data were analyzed using GraphPad Prism 5.01 software (Graphpad Software, La Jolla, CA). Wilcoxon matched-pairs signed-rank test or paired sample T-test were applied to test the statistical significance. P values of less than 0.05 were considered significant.

RESULTS

TEC-induced proliferative responses of $CD8^+$ T cells and NK cells in the presence or absence of immunosuppressive drugs

TECs significantly induce $CD8^+$ T-cell proliferation as 5.3 ± 2.4% of cells proliferated upon coculture with TECs after 7 days (Figure 1A). Transwell experiments revealed that $CD8^+$ T-cell proliferation is cell-cell contact dependent as no proliferation was detected in the transwell experiments (Figure 1B). All immunosuppressive drugs tested appeared to be potent inhibitors of TEC-induced $CD8^+$ T-cell proliferation; i.e. inhibition up to 75% by tacrolimus (1.6 ± 0.3%), everolimus (1.8 ± 0.5%,) and prednisolone (1.8 ± 0.4%) (Figure 1A, lower panel; $P<0.05$).

We observed that 27.8 ± 4.7% of the proliferative NK cells possessed $CD56^{high}/CD16^{dim}$ phenotype [25-26], and 18.6 ± 8.3% of proliferative NK-cell fraction showed the $CD56^{dim}/CD16^{high}$ NK-cell phenotype (Figure 1C). Tacrolimus inhibited the proliferation of NK cells effectively, reducing the proliferative $CD56^{high}$ NK cell fraction to 13.9 ± 6.6% and proliferating $CD56^{dim}$ NK cells fraction to 7.6 ± 2.0% (Figure 1D). Similar results were obtained when everolimus was added to the coculture resulting in 11.3 ± 0.9% of $CD56^{high}$ NK cells and 4.1 ± 1.5% of $CD56^{dim}$ NK cells within the proliferative fraction (Figure 1E). In sharp contrast, prednisolone was the most powerful drug and significantly inhibited both $CD56^{high}$ and $CD56^{dim}$ NK-cell proliferation (Figure 1F).

Immunological activation state of TECs determines the degree of TEC-specific CTL and NK cell mediated lysis

Figure 2A shows the degranulation degree of TEC-specific CTLs and NK cells as measured by CD107a expression after 7 days of TEC coculture using unstimulated TECs and IFN-γ/TNF-α activated TECs. To these ends, we used highly purified sorted TEC-specific CTLs generated in separate cocultures using the same TEC targets. When cytokine-activated TECs were used, we observed a significant increase in lytic activity of CTLs as expressed by highly elevated CD107a cell surface expression of significantly increased percentage of degranulated CTLs up to 23.5 ± 5.3% whereas only 6.2 ± 1.1% degranulated upon TEC encounter when TECs were left unstimulated ($P=0.018$). Completely reversed effects were found using NK cells with similar TEC conditions with the same experimental set-up. While 33.5 ± 5.7% of NK cells expressed CD107a after encounter of unstimulated TECs, the lytic activity was decreased if TECs were activated by IFN-γ/TNF-α. We observed that 21.3 ± 3.1% of the NK cells expressed CD107a; i.e. almost 40% decrease in the percentage of degranulated NK cells ($P<0.05$).

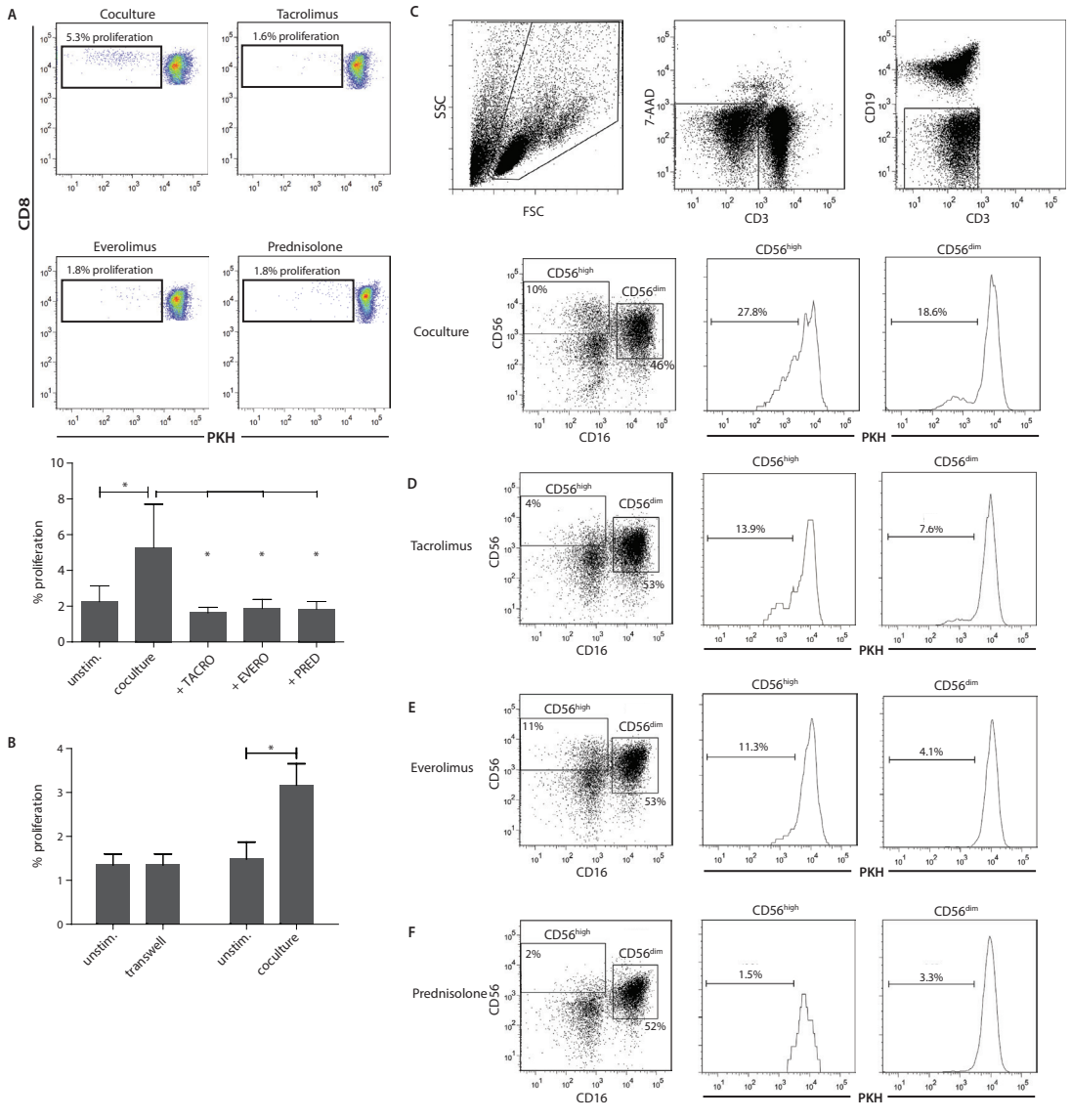

Figure 1 | TEC-induced CD8+ T-cell and NK-cell proliferative responses in the presence or absence of immunosuppressive drugs.

(A) Recipient PBMC were cocultured with donor-derived TECs and harvested at day 7 (n=7 different donors). PKH-labelled CD8+ T cells proliferated significantly upon TEC encounter ($P<0.05$). Proliferation was measured in the presence or absence of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml). Immunosuppressive drugs significantly inhibited CD8+ T-cell proliferation. Results of seven experiments are shown (mean ± SEM). **(B)** Transwell and coculture experiments were performed simultaneously and revealed that CD8+ T-cell proliferation was cell-cell contact dependent as in transwell experiments no CD8+ T-cell proliferation was found. Unstimulated represents the negative control CD8+ T-cells without TECs. Results of seven experiments are shown (mean ± SEM). Comparison was made using paired Wilcoxon singed-rank test (* $P<0.05$). **(C)** PBMC were cocultured with donor-derived TECs and harvested at day 7 (n=3 different donors). NK-cell proliferation was measured using PKH dilution assay in the absence or presence of tacrolimus, everolimus and prednisolone. Within the proliferative fraction, NK-cell subsets were defined as $CD56^{high}(CD56^{++}CD16^{+/-})$ and $CD56^{dim}(CD56^{+}CD16^{+})$. The use of **(D)** tacrolimus (10 ng/ml), **(E)** everolimus (10 ng/ml) and **(F)** prednisolone (200 ng/ml) resulted in differential effects on the proliferative responses on NK-cell subsets as depicted and as detailed in section Results.

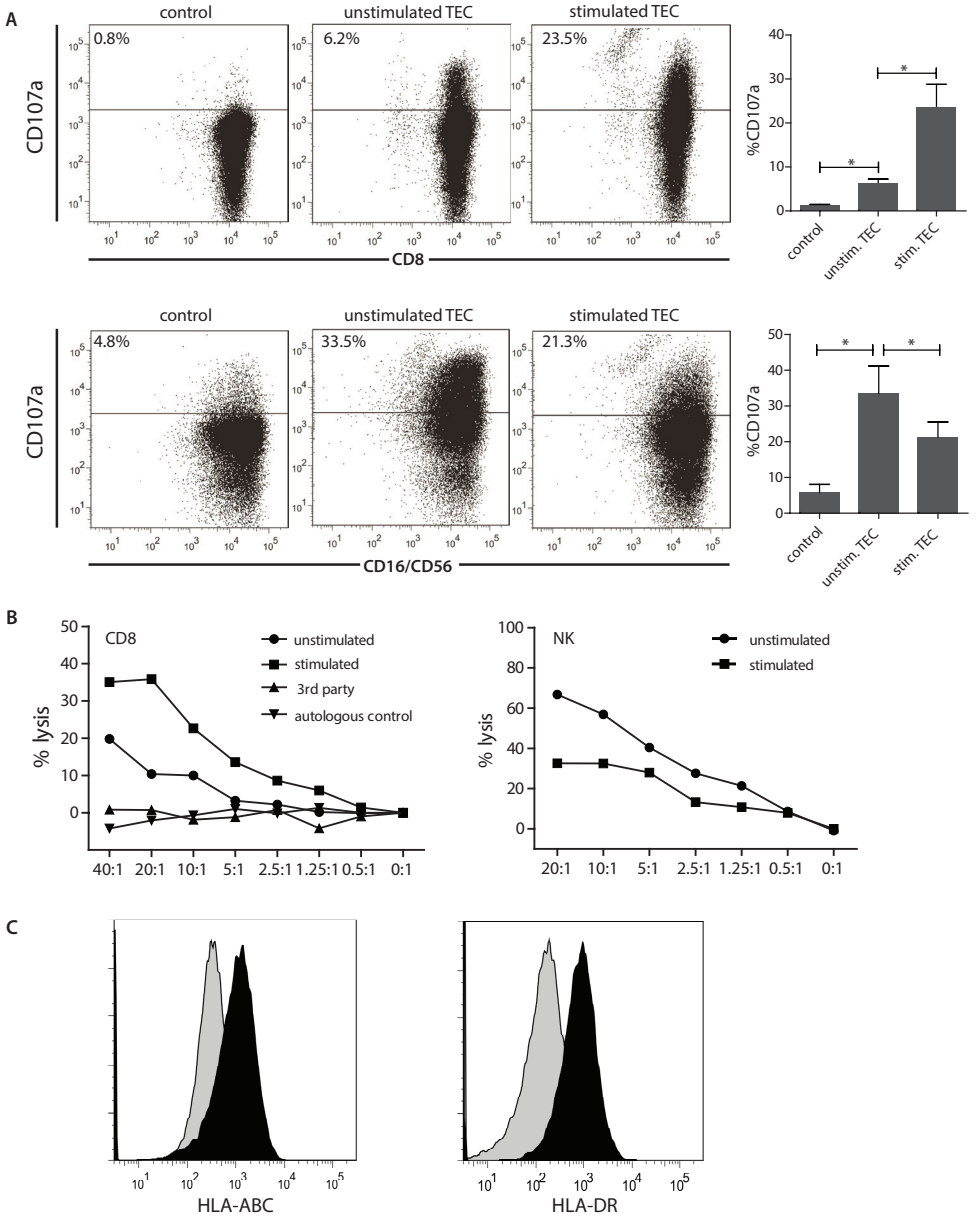

Figure 2 | Immunological activation state of TECs determines the degree of TEC specific CTL and NK-cell mediated lysis.

Healthy donor PBMC were cocultured with TEC (HLA-mismatch A-B-DR 2-2-2) and harvested at day 7 (n=6 different donors). FACS sorted TEC specific $CD8^+$ T cells and NK cells were re-cultured with either unstimulated TECs or IFN-γ/TNF-α activated TECs in the following experiments; (**A**) $CD8^+$ T cells and NK cells were harvested after 2 hours of TEC coculture and stained for CD107a as a marker for degranulation (n=6). While cytokine activation of TECs led to significantly increased degranulation of CTLs, NK-cell degranulation was dimished compared to unstimulated condition. (**B**) Europium release assay representative experiments are shown which were simultaneously and in parallel performed with degranulation assays. We found an increased net-lytic effect of CTLs and a diminished net-lytic effect of NK-cells under proinflammatory conditions (n=3 different donors). 3rd party TECs were not lysed by TECs, confirming that lysis was TEC specific (n=2 different donors). Control experiments showed that TECs were not lysed by autologous CTLs (n=2 different donors). (**C**) Unstimulated TECs (grey) and IFN-γ/TNF-α activated TECs (black) were stained for HLA-I and HLA-II expression. Significantly increased HLA I and II expression by TECs was observed after stimulation (n=10 different donors).

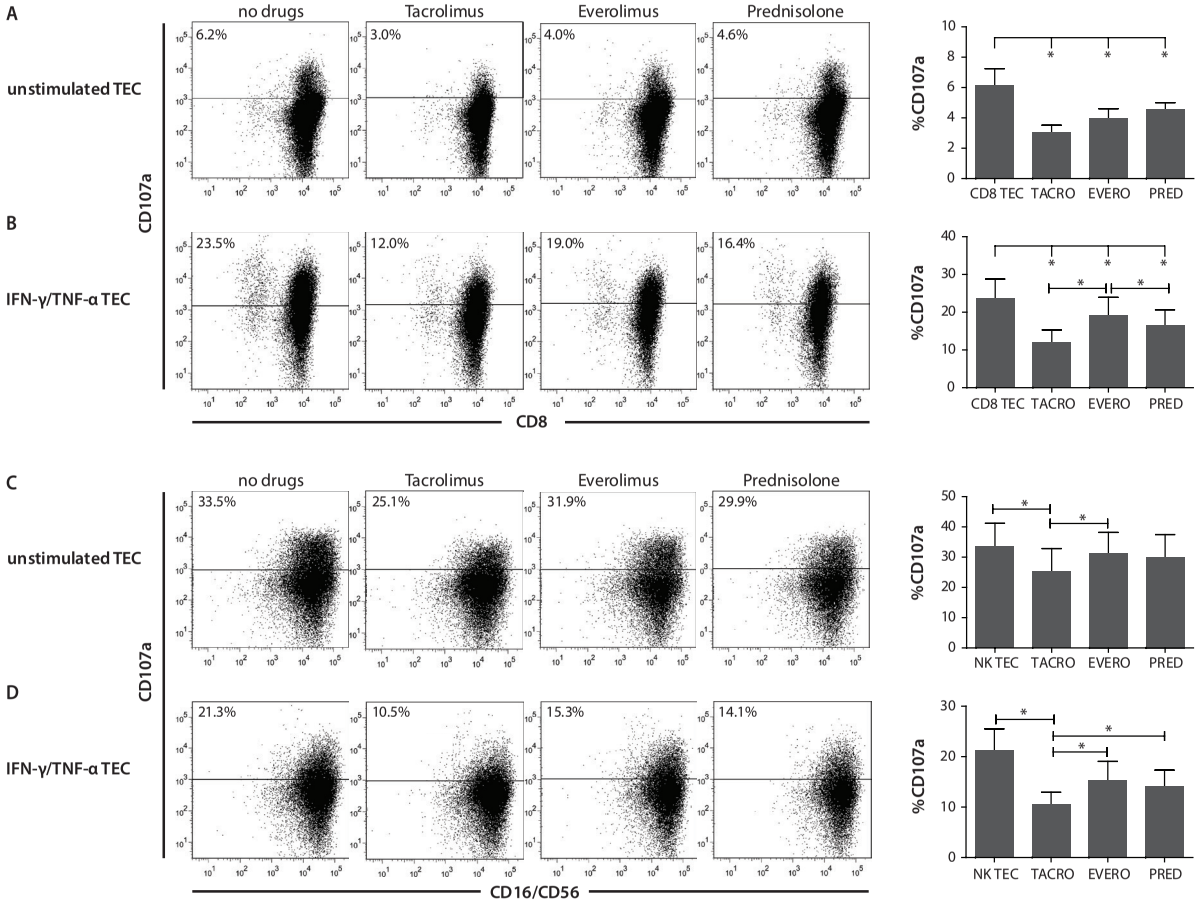

Figure 3 | Limited efficacy of immunosuppressive drugs on CTL- and NK-cell degranulation.

Healthy donor PBMC were cocultured with TEC (HLA-mismatch A-B-DR 2-2-2) and harvested at day 7 (n=6 different donors). FACS sorted TEC-specific $CD8^+$ T cells and NK cells were re-cultured with either unstimulated TECs or IFN-γ/TNF-α activated TECs in the following experiments. (**A-B**) $CD8^+$ T cells and (**C-D**) NK cells were re-cultured in the absence or the presence of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml) and stained for the degranulation marker CD107a. All immunosuppressive drugs significantly inhibited $CD8^+$ T-cell degranulation. Only tacrolimus significantly reduced NK-cell degranulation. Results of six experiments are shown (mean ± SEM). Comparison was made using paired Wilcoxon singed-rank test (* $P<0.05$).

Next, we questioned whether these differences in lytic degranulation activity of effectors cells would be reflected by the eventual lysis of europium uploaded TEC targets confirming the same results. IFN-γ/TNF-α activated TECs were indeed lysed more effectively by $CD8^+$ T cells as compared to unstimulated TECs (Figure 2B; 35 ± 7.5% vs. 20 ± 1.8% TEC lysis at 40:1 E/T ratio). NK cells were the most potent killers leading to 67 ± 7% TEC lysis of the unstimulated TECs with a significant decrease to 33 ± 3.9% TEC lysis when cytokine activated TECs were used (20:1 E/T ration). In addition, we used in parallel 3rd party TECs (HLA mismatch A-B-DR: 2-2-2) to demonstrate the efficacy of our system to generate TEC specific CTLs as these generated effector cells could not lyse 3rd party TECs. In addition we performed control experiments using autologous CTLs which did not lyse TECs. In Figure 2C we show that IFN-γ/TNF-α activated TECs significantly upregulated HLA-ABC and HLA-DR molecules.

Limited efficacy and differential effects of immunosuppressive drugs on $CD8^+$ T-cell and NK-cell mediated lysis of TECs

Tacrolimus inhibited CTL degranulation potently by 50% (Figure 3A: 3.0 ± 0.5% vs. 6.2 ± 1.1%) whereas the range of inhibition by everolimus and prednisolone was; 33% inhibition by everolimus (4.0 ± 0.6%) and 23% inhibition by prednisolone (4.6 ± 0.4%). Interestingly and in line with obtained data from described experiments in previous section, IFN-γ/TNF-α activation again resulted in a strongly increased degranulation of 23.5 ± 5.3% vs. 6.2 ± 1.1%. Again 50% CTL degranulation inhibition was achieved by tacrolimus (Figure 3B; 12.0 ± 3.3%). Everolimus and prednisolone showed comparable efficacy inhibiting CTL degranulation of activated TECs (19.0 ± 5.0% vs. 16.4 ± 4.3% respectively). In line, IFN-γ/TNF-α activation resulted in decreased degranulation of 21 ± 4.2% NK cells as compared to unstimulated TEC condition with 33.5 ± 7.6%. Only tacrolimus could diminish NK-cell degranulation to a modest degree up to 20-50% in case of unstimulated and activated TECs respectively (Figure 3C and 4D: 25.7 ± 7.8% and 10.5 ± 2.4% respectively).

In addition, we investigated the net effect of immunosuppressive drugs on lysis of cytokine activated TECs performing europium release assays in a separate set of experiments. $CD8^+$ T-cell mediated kill was significantly inhibited by tacrolimus (33%) and prednisolone (40%) (Figure 4A: $P<0.05$). With regard to NK cells, prednisolone appeared to be the most potent inhibitor of NK-cell mediated lysis of activated TECs resulting in 45% reduction in cytoxicity (Figure 4B: $P<0.05$). Tacrolimus reduced the lytic capacity of NK cells by 22% and everolimus showed no inhibitory effect on IFN-γ/TNF-α activated NK cell mediated TEC lysis.

Of note, although de europium release assay data and degranulation results showed the same pattern and follow the same trends, they are not completely resulting in the same degree of inhibition which is rationale because these assays represent different steps of the lysis process, looking at the different time points and different read out parameters. The fact that degranulation of effector cells does not necessarily have to overlap completely with the degree of net-lysis of target cells has been reported before [27-28].

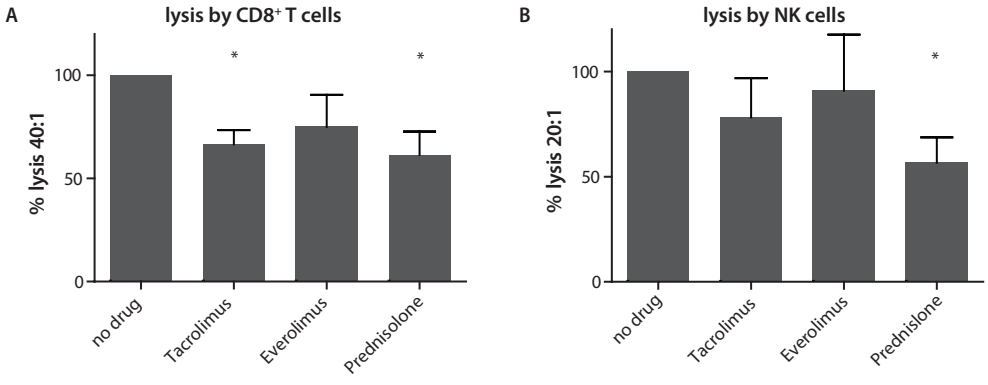

Figure 4 | Limited efficacy of immunosuppressive drugs to control TEC lysis by CTLs and NK cells.

Healthy donor PBMC were cocultured with TEC (HLA-mismatch A-B-DR 2-2-2) and harvested at day 7 (n=4 different donors). CD8+ T cells and NK cells were isolated by FACS sorting and re-cultured with IFN-γ/TNF-α activated TECs in the following experiments (**A**) CD8+ T cells and (**B**) NK cells were recultured with TECs in the absence or presence of tacrolimus (10 ng/ml), everolimus (10 ng/ml) and prednisolone (200 ng/ml). TEC lysis was measured using europium assay after 4 hours incubation. Tacrolimus and prednisolone showed the strongest inhibitory effect on CTL mediated TEC lysis. NK-cell mediated lysis was only inhibited by prednisolone. Normalized data of four independent experiments are shown. Comparison was made using paired T-test (* P<0.05).

DISCUSSION

In spite of potent immunosuppression, kidney allograft rejection still occur [29-31]. Moreover, several studies show that ongoing inflammation such as subclinical cellular and/or subclinical antibody mediated tissue injury eventually lead to the development of irreversible graft damage and dysfunction [32-34]. The local effects of immunosuppressive drugs within the graft microenvironment are poorly defined. At the moment, a paucity of data exist regarding effective treatment and/or preventive strategies to combat the occurrence of subclinical injury eventually leading to irreversible structural changes on the long term.

In the present study, we found that primary human TECs induce significant CD8+ T-cell proliferation, which was potently inhibited by tacrolimus, everolimus and prednisolone. Majority of proliferative NK cells possessed $CD56^{high}/CD16^{dim}$ phenotype. We observed differential inhibitory effects of immunosuppressive drugs tested on NK-cell subset proliferation. Prednisolone was the most powerful drug inhibiting both $CD56^{high}$ and $CD56^{dim}$ NK-cell proliferation. In addition, all immunosuppressive drugs inhibited CTL degranulation to various degrees while tacrolimus appeared to be the only immunosuppressive drug inhibiting NK-cell degranulation in our system. Both tacrolimus and prednisolone inhibited TEC lysis by CTLs, while only prednisolone was able to significantly inhibit NK mediated TEC lysis. It is also suggested that immunosuppressive drugs that impair lytic capacities of effector cells, simultaneously decrease the cytokine production and in this way contribute to protection of graft against effector cells [20-21]. Here, we showed that immunological activation state of TECs determines the degree of TEC-specific CTLs and NK cell mediated lysis and report on the reverse effects of cytokine activated TECs on CD8 vs. NK-cell

mediated lysis as measured by both methods: CD107a degranulation assay and europium release assay. TEC activation leads to enhanced susceptibility for degranulation and net lysis by TEC-specific CTLs, while NK-cell degranulation and NK mediated net-lysis is decreased. Significantly increased expression levels of MHC I and II molecules by TECs after activation and stimulation with IFN-γ and TNF-α could be one of possible explanations. According to the missing 'self' hypothesis, MHC I expression inhibit NK cell activation and degranulation providing mechanistic rationale for our findings. Whether differential expression of stimulatory or inhibitory NK cell ligands by TECs after activation contributes to this phenomenon is an interesting subject for further studies. NK-cell receptor ligands were shown to be expressed by TECs under inflammatory conditions. They directly influence NK-cell granule and cytokine release [35-36]. Moreover, elevated levels of NKG2D mRNA were also associated with acute rejection [37]. Our results indicate the relative importance of distinct immune cells during different types of rejection and the major importance of environmental drive in the definite outcome of alloreactivity pointing to CTLs as mediators of cellular rejection. NK-cell immunity could be inferior in this regard because of high expression of MHC I and II by TECs during overt inflammation [22].

Limited efficacy of immunosuppressive drugs on $CD8^+$ T-cell and NK-cell mediated TEC lysis *in vitro* could explain why despite the use of potent systemic combinations of immunosuppressive drugs, clinical and subclinical tissue injury still occurs [13]. Apparently the intra-graft immunosuppressive effects of drug do not represent their systemic effects. Differential effects of immunosuppressive drugs on both type of immune effector cells investigated in this study indicate tacrolimus and prednisolone as the most powerful combination in combatting both CD8 and NK mediated immune reactions. Though no randomized controlled data are available to determine the most effective treatment modality for antibody-mediated rejection, most treatment regiments consist of plasmapheresis, IVIg, rituximab, eculizimab and/or bortezemib, and don't include prednisolone [38]. Moreover, NK cells are increasingly associated with late antibody-mediated rejection [7-9]. Obtained results from our study may provide a scientific rational for the use of steroids as the corner stone in the treatment of each type of rejection, including the antibody mediated type.

The effects of immunosuppressive drugs on an immune response could be partly determined by the type of target cells involved. We report in this study on differential effects of drugs on immune interaction between CD8 and NK cells, and TECs. Several studies describe differential effects of immunosuppressive drugs on human Epstein-Barr virus (EBV)-specific effector CTLs [39-40] and NK-cell cytotoxicity [21,26]. While tacrolimus is capable of 90% inhibition of lymphocyte proliferation in a mixed lymphocyte reaction [41], the inhibitory effect of tacrolimus on EBV specific CTLs appeared to be limited. EBV-CTLs retained their cytotoxic activity and EBV-antigen specificity to a high degree using tacrolimus [40]. Controversial data were found regarding NK cells, cyclosporine A did not affect IFN-γ production by NK cells and ^{51}Cr release of K562 target cells [26], while tacrolimus pretreated NK cells resulted in decreased CD107a expression and ^{51}Cr release of K562 target cells [21]. The reasons for this phenomenon are not clearly elucidated. Possibly, differential effects of drugs on pro- and anti-apoptotic intracellular pathways may play a role as tacrolimus did not change the activity of the anti-apoptotic protein Bcl-2 [42], while prednisone did decrease Bcl-2 levels of lymphocytes for example in patients with autoimmune hepatitis [43]. Different T-cell subsets can express the

apoptosis-related proteins to varying degrees as effector memory CD8+ T cells express higher levels of Bcl-2 than naïve T cells turning them less sensitive to immunosuppressive drugs [44].

Of note, to stay close to the clinical set up we decided to use bulk PBMC and regarded the setup with purified T cells and NK cells as beyond the scope of this study. We considered bulk PBMC essential as they contain CD4+ T cells and/or DCs producing cytokines which are crucial for the activation of alloreactive cytotoxic CD8+ T cells. As IL-2 and IL-15 are essential cytokines for both CD8+ T-cell and NK-cell cytotoxicity *in vitro* and assumingly present in the renal allograft microenvironment during rejection [45-46], we used these cytokines also overnight to maintain fully activated effector cells, so we cannot exclude the possibility of any non-specific activation.

In summary, our data point to reversed susceptibility of cytokine activated TECs to be killed by CTLs and NK cells, limited efficacy of immunosuppressive drugs on lymphocyte mediated TEC lysis *in vitro*, and differential inhibitory effects of immunosuppressive drugs on CTL- and NK-cell mediated TEC lysis. These phenomena may underlay the silent chronic subclinical inflammation in the graft under full systemic immunosuppression eventually leading to graft fibrosis.

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CHAPTER 5

Human renal tubular epithelial cells suppress alloreactive T-cell proliferation

Martijn W.H.J. Demmers, Carla C. Baan , Marieke Roemeling-van Rhijn,
Thierry P.P van den Bosch, Martin J. Hoogduijn,
Michiel G.H. Betjes, Willem Weimar, Ajda T. Rowshani

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Abstract

Introduction: Renal tubular epithelial cells (TECs) are one of the main targets of alloreactive T-cells during acute rejection. We hypothesize that TECs modulate the outcome of allo-immunity by executing immunosuppressive effects in order to dampen the local inflammation. We studied whether TECs possess immunosuppressive capacities and if indoleamine 2,3-dioxygenase (IDO) might play a role suppressing T-cell alloreactivity.

Materials and Methods: CD3/CD28$^-$ and allo-activated peripheral blood mononuclear cells were cocultured with IFN-γ/TNF-α activated TECs for 3 days. We analysed CD4$^+$ T-cell and CD8$^+$ T-cell proliferative response and apoptosis in the absence or presence of IDO inhibitor 1-L-MT. Further we examined whether inhibition of T-cell proliferation was cell-cell contact dependent using transwell membrane experiments.

Results: We found that TECs dose-dependently inhibited CD4$^+$ and CD8$^+$ T cell proliferation (TEC:PBMC ratio 1:2.5; $P<0.05$). Cytokine activated TECs showed significantly increased IDO mRNA expression and IDO activity, determined by L-kynurenine measurements compared to the unstimulated condition. Suppressed CD4$^+$ and CD8$^+$ T-cell proliferation was only partly or not restored using 1-L-MT respectively. Activated TECs increased early and late apoptosis of proliferating CD4$^+$ and CD8$^+$ T-cells. Addition of 1-L-MT reduced the percentage of apoptotic T cells. Interestingly, transwell experiments revealed that TEC-mediated immunosuppression is cell-cell contact dependent.

Discussion: Our data show that TECs suppress both CD4 and CD8 T cell proliferation contact dependently. Increased TEC induced apoptosis of T cells is probably mediated by IDO immunosuppressive activity.

INTRODUCTION

Renal tubular epithelial cells (TECs) represent 75% of the parenchymal cells of the kidney and are one of the main targets of T cells during tubulointerstitial inflammation like acute cellular rejection of the kidney graft. Consequences are huge and will eventually result in structural TEC damage, nephron disruption and graft loss [1-2]. The final outcome is partially determined by the local micro-environmental drive and the interaction between tubular epithelium and the T cells [3-4]. In our previous work, we reported on the proinflammatory role of the tubular compartment directing selective migration of Th1 and Th17 $CD4^+$ T cells *in vitro* [5]. Furthermore, we investigated distinct proliferation of recipient T-cell subsets after TEC encounter with variable sensitivity to immunosuppressive drugs. Our data show substantial proliferation of TEC-reactive $CD4CD28^{null}$ memory T cells, which are resistant to tacrolimus and everolimus [6]. Previously, we and others have shown that TECs can resist granzyme B mediated apoptosis induced by allo-reactive cytotoxic T cells through upregulation of cytosolic SERPINB9 during rejection [7-8]. Moreover, we showed that TEC-derived SERPINB9 is also high during viral infections like CMV, EBV or BK pointing towards a general mechanism of self-defence [9-10]. Altogether, these findings led to the question whether other bi-directional pathways of TEC mediated defence resulting in suppression of T cell mediated allo-immunity exist, and whether TECs are capable of abrogating T-cell activation and/or proliferation in order to limit the burdens of inflammation and the eventual damage.

TECs might possess immune regulatory capacities like other parenchymal cells with well-established immunomodulatory capacities such as mesenchymal stem cells [11]. Indoleamine 2,3-dioxygenase (IDO) is a cell survival related rate-limiting enzyme which leads to the degradation and depletion of the essential amino acid tryptophan along the kynurenine pathway [12]. IDO can be directly activated by a number of proinflammatory cytokines like IFN-γ and TNF-α, and is involved in regulating immune responses [13-14]. Tryptophan catabolism leads to production of L-kynurenine derivatives and O_2 free radicals thereby regulating T-cell proliferation and survival. Tryptophan starvation inhibits T-cell activation and induces cell cycle arrest by blocking their entry into the S phase eventually leading to T-cell apoptosis [15-18].

TECs express IDO upon IFN-γ/TNF-α stimulation resulting in tryptophan depletion, which can be reversed using the IDO inhibitor 1-methyl-L-tryptophan (1-MT) [14]. In an experimental glomerulonephritis model, IDO inhibition resulted in acceleration of kidney damage and accumulation of $CD4^+$ T cells, suggesting a protective role for IDO [19]. Interestingly, strong immunohistochemical cytoplasmic IDO expression was documented in tubular epithelial cells from kidney transplant recipients with acute rejection compared to non-rejectors. Serum and urine kynurenine/tryptophan ratios of rejecting patients were significantly elevated compared to uncomplicated transplant recipients [20]. Even though no functional data are available yet regarding the immunosuppressive capacities of TECs, we speculate that proliferation of alloreactive T cells within the transplanted organ might also be suppressed via renal TECs with a focus on local TEC-derived IDO activity.

In the present study, we hypothezise that donor-derived TECs possess immunosuppressive capacities and that IDO might play a pivotal role suppressing T-cell alloreactivity. Furthermore, we

questioned whether inflammatory conditions influence the immunomodulatory functions of TECs via IDO controlled mechanisms. To these ends, we investigated the differential immunosuppressive effects mediated by TECs on CD4 T-cell and CD8 T-cell proliferation and apoptosis.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) of transplant recipients and healthy volunteers (Blood Bank Sanquin, Rotterdam, the Netherlands) were isolated by density gradient centrifugation Ficoll-paque (density 1.077 g/ml, Amersham Pharmacia Biotech, Uppsala, Sweden), frozen in medium containing 10% DMSO and stored at -150°C until analysis.

Culture of primary renal tubular epithelial cells and mesenchymal stem cells used as controls

TECs were cultured from cortical tissue of human kidney nephrectomies due to tumors as previously described [5]. Cells were used between passages 2 and 6 of culture. TEC outgrowth was confirmed by morphologic appearance and immunofluorescence staining ($CD13^+$, $CD26^+$ and $CD90^-$). To mimic rejection and proinflammatory intragraft conditions, TECs were stimulated with 50ng/ml human recombinant IFN-γ (U-Cytech, Utrecht, the Netherlands) and 20 ng/ml human recombinant TNF-α (PeproTech, London, UK) [5]. As mesenchymal stem cells (MSC) are well-established parenchymal cells with proven immunomodulatory properties, we used MSCs as the positive control cell line in our experimental set-up. Human MSCs were isolated from adipose tissue fat as described previously [21-22]. MSC were activated using 50 ng/ml human recombinant IFN-γ (U-Cytech) and used for experiments between passages 2 and 4. Both TECs and MSCs were kept at 37°C, 5% CO_2 and 95% humidity. Unstimulated and IFN-γ/TNF-α activated TECs were analyzed for the surface marker expression of ICAM-1 (CD54, BD Biosciences, San Jose, USA) and PD-L1 (CD274, Biolegend, San Diego).

IDO mRNA expression and IDO activity

To assess the IDO activity in TECs, 75.10^3 TECs were stimulated in a 24 well culture plate for 24 hours with IFN-γ/TNF-α, in the absence or presence of the IDO inhibitor; 1-methyl-L-tryptophan (1-L-MT) (Sigma, St. Louis, MO) at a concentration of 50μM. Fresh stock 1-L-MT concentrations of 10mM was prepared for each experiment. 1-L-MT was dissolved in 0.1M NaOH and neutralized using an equal volume of 0.1M HCl. TEC mRNA was stabilized using RNA*later* RNA Stabilization Solution (Ambion, Austin, TX). The culture plate was stored for 48 hours at 4°C and subsequently at -20°C until analysis. mRNA expression was measured as previously described [5]. Briefly, a 500 ng mRNA quantitative real-time RT-PCR containing universal PCR mix (Invitrogen) was used to quantify the amount of IDO in samples. Assay-on-demand products for the detection and quantification of IDO (Hs00158627. m1) mRNAs were designed by Applied Biosystems (Foster City, CA).

L-Kynurenine accumulation reflecting IDO activity was measured in the supernatants of 24 hours cytokine activated TECs. Briefly, 30% trichloroacetic acid was added to samples at a 1:3 ratio and incubated at 50°C for 30min. Samples were centrifuged at 12,000rpm for 5 min. Supernatants were 1:1 diluted in Ehrlich reagent 200 mg 4-dimethylaminobenzaldehyde (Sigma) in 10 ml of glacial acetic acid. Then, supernatants were measured in duplicate in a 96-well flat bottom plate. Absorbance was determined at 490 nm using a multilabel plate reader (VersaMax™, Molecular Devices, Sunnyvale, CA). L-Kynurenine (Sigma) diluted in unconditioned medium was used as standard control [23].

Mixed TEC lymphocyte coculture

0.5×10^5 PBMC were incubated with irradiated (40 Gy) HLA-mismatched (A-B-DR: 2-2-2) PBMC (ratio 1:1) in a mixed lymphocyte reaction (MLR). Both MLR- and anti-CD3/CD28 activated lymphocytes were added to IFN-γ (50 ng/ml)/TNF-α (20 ng/ml) activated TECs in TEC:PBMC ratios 120.10^3:300.10^3 (1:2.5), 60.10^3/300.10^3 (1:5) and 30.10^3/300.10^3 (1:10). PBMC proliferation was measured using ^{3}H-thymidine incorporation assay (0.5 µCi/well: Amersham Pharmacia Biotech, Roosendaal, The Netherlands) at day 7 for the MLR and at day 3 for the CD3/CD28 stimulation conditions. T cells were activated using 1 ug/ml anti-CD3, 1 ug/ml anti-CD28 and 2 ug/ml polyclonal antibody goat anti-mouse (BD). In addition to the above described experiments proliferation was also measured after 3 days of coculture using CFSE dilution assay (Sigma). As positive controls, MSC cell lines were used. MLR- and anti-CD3/CD28-derived activated lymphocytes were added to IFN-γ (50 ng/ml) activated MSC in MSC:PBMC ratios 1:2.5, 1:5 and 1:10. Results were analysed as described for TEC co-cultures before.

To investigate the role of IDO, we performed TEC lymphocyte cocultures in de presence or absence of IDO inhibitor and measured the T cell proliferation using CFSE dilution method. TECs (120.10^3) were seeded in 24 well flat-bottom culture plates (Corning Costar, Corning, NY) and activated for 3 days with IFN-γ (50 ng/ml)/TNF-α (20 ng/ml) in the absence or presence of 50 µM 1-L-MT (Sigma). CFSE-labelled anti CD3/CD28 activated PBMC (300.10^3) were cocultured with TECs in human culture medium (HCM); RPMI-Glutamax (Gibco) supplemented with 10% heat-inactivated human serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. At day 3, T cells were harvested and proliferation was analysed using flow cytometry.

TEC lymphocyte transwell experiments

IFN-γ/TNF-α activated TECs (120.10^3) were seeded in 24 well plates in the absence or presence of 50µM 1-L-MT. After 24hours IFN-γ/TNF-α stimulation, 0.4µm pore membranes (ThinCerts; Greiner Bio-One) were placed above the TECs. CFSE-labelled anti CD3/CD28 activated PBMC (300.10^3) were placed upon the membrane. As control anti-CD3/CD28 activated PBMC were placed upon a membrane without TECs. PBMC were harvested at day 3 and analyzed for proliferation and subset analysis using CFSE dilution.

Subset analysis of proliferating T cells using flowcytometry

Anti-CD3/CD28 activated T cells were harvested at day 3. Cell surface staining was conducted with the following mAbs: CD7-eFluor450 (eBioscience), CD4-APC-Cy7, CD8-BV510 (Biolegend, San Diego,

USA), CD25-PE-Cy7, CD69 PE, 7-AAD and Annexin V-APC (BD). Twenty thousand gated lymphocyte events were acquired from each tube by a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA). Fluorescence-minus-one (FMO) controls were used to determine positive or negative boundaries. Data were analysed using FlowJo software (Tree Star, San Carlos, CA). Flow cytometric analysis was performed with at least 100 events gated.

Statistics

Results are expressed as mean ± SEM. Data were analysed for statistical significance with GraphPad Prism 5.01 software (Graphpad Software, La Jolla, CA) using non-parametric Wilcoxon matched-pairs signed-rank test. P values less than 0.05 were considered significant.

RESULTS

Cytokine activated renal tubular epithelial cells suppress T-cell proliferation

In order to investigate the inhibitory capacity of TECs on T-cell proliferation, TECs were pre-activated by proinflammatory cytokines IFN-γ and TNF-α to mimic the intra-graft inflammatory microenvironment. IFN-γ activated MSCs were used as the positive control cell line. ^{3}H-Thymidine incorporation experiments revealed that IFN-γ/TNF-α activated TECs dose-dependently inhibit T-cell proliferation induced by anti-CD3/CD28 stimulation (Figure 1A, n=3). In line, allogeneic stimulation in a mixed lymphocyte reaction (MLR) revealed similar immunosuppressive effects of activated TECs. IFN-γ/TNF-α activated TECs dose-dependently inhibit T-cell proliferation induced by MLR (Figure 1B, n=3).

Subsequently, we analysed proliferation of anti-CD3/CD28 activated CD4 and CD8 T cell subsets by CFSE dilution using flow cytometry. Polyclonal anti-CD3/CD28 stimulation resulted in a proliferation rate of 85 ± 0.8% of $CD4^+$ T-cell and 68.4 ± 3.4% of $CD8^+$ T-cell pool (n=6). Both $CD4^+$ T-cell and $CD8^+$ T-cell proliferation was dose-dependently suppressed by TECs. Activated TECs significantly inhibited T-cell proliferation down to a proliferation rate of 34.1 ± 5.9% of $CD4^+$ T-cells, and to 31.6 ± 10.8% of $CD8^+$ T-cell pool at a TEC:PBMC ratio 1:2.5. This means 60% suppression of $CD4^+$ T-cell proliferation and 54% suppression of CD 8 T-cell proliferation ($P<0.05$; Figure 1C and Figure 1D).

Cytokine activated renal tubular epithelial cells express IDO mRNA

Twenty-four hours IFN-γ/TNF-α activated TECs express indoleamine 2,3-dioxygenase (IDO) mRNA in contrast to the unstimulated TECs (Figure 2A). Harvested supernatants were used to measure the concentration of L-Kynurenine as a sign of functional IDO activity showing the catalytic degree of tryptophan. IFN-γ/TNF-α activated TECs showed abundant IDO enzyme activity after 24 hours stimulation as shown by significantly increased L-Kynurenine concentration up to 22.2 ± 2.6 μM compared to 0.5 ± 0.2 μM in unstimulated condition ($P<0.05$). In addition, the IDO inhibitor 1-L-MT significantly inhibited IDO enzyme activity of IFN-γ/TNF-α activated TECs resulting in 12.5 ± 2.0 μM L-Kynurenine ($P<0.05$) (Figure 2B).

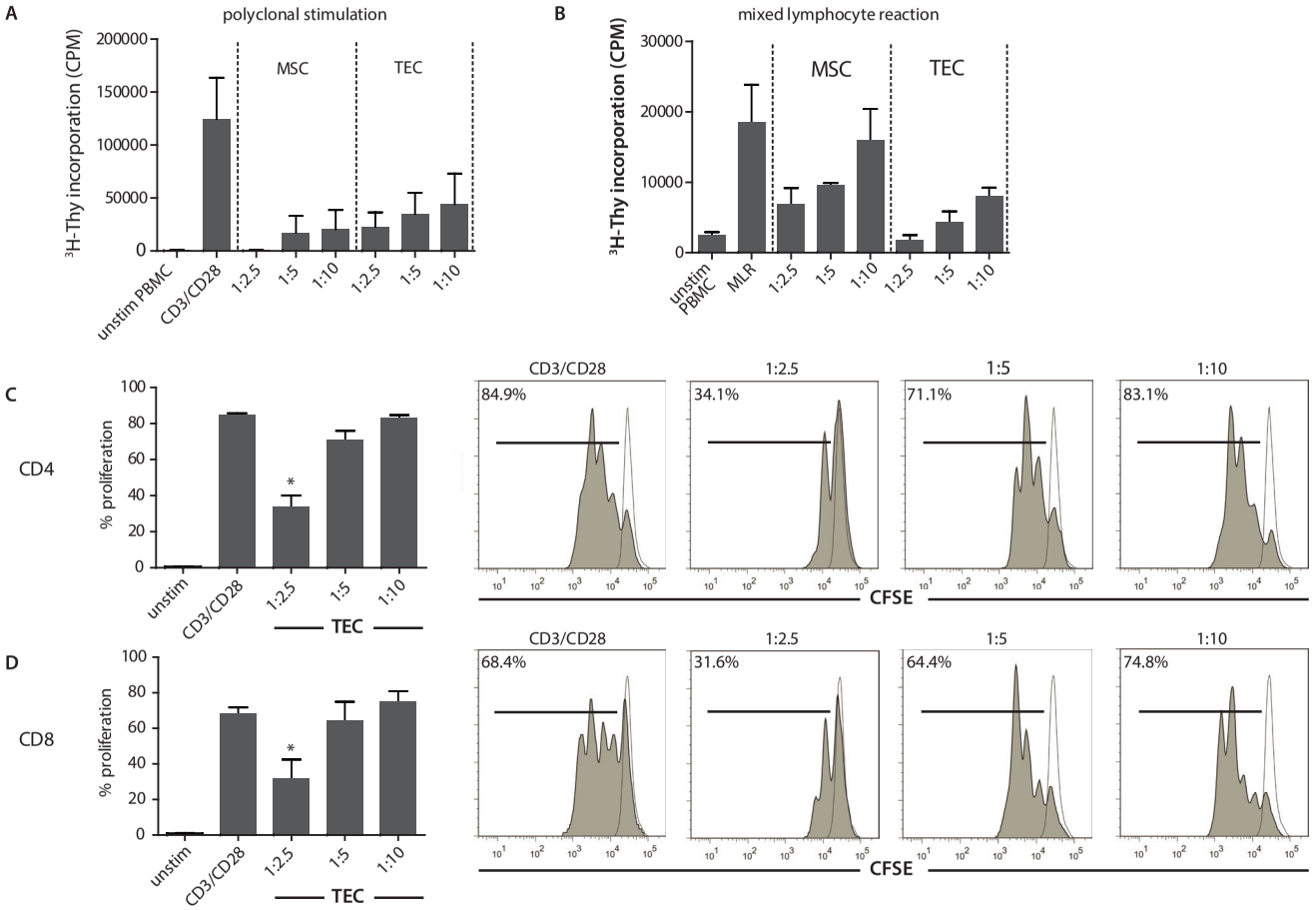

Figure 1 | Cytokine activated renal tubular epithelial cells suppress T-cell proliferation.

(**A-B**) IFN-γ/TNF-α stimulated TECs (n=3) inhibit both polyclonal anti-CD3/CD28 and MLR induced antigen specific T-cell proliferation using ^{3}H-Thymidine incorporation. IFN-γ activated mesenchymal stem cells (MSC) were used as positive controls (n=3). (**C-D**) IFN-γ/TNF-α activated TECs were cocultured with anti-CD3/CD28 stimulated PBMC according to TEC:PBMC ratios 1:2.5, 1:5 and 1:10. $CD4^+$ T-cell and $CD8^+$ T-cell proliferation was significantly inhibited at the TEC:PBMC ratio 1:2.5.

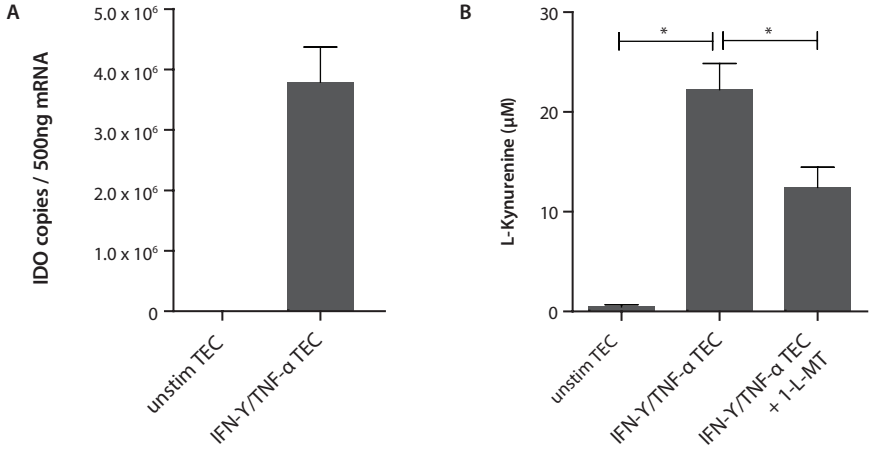

Figure 2 | Cytokine activated renal tubular epithelial cells express IDO mRNA and IDO activity.

TECs were cultured for 24 hours in absence or presence of IFN-γ/TNF-α and in the presence of the IDO inhibitor 1-L-MT (n=6). **(A)** IFN-γ/TNF-α activated TECs express IDO mRNA. **(B)** In order to quantify IDO activity, L-kynurenine was measured in supernatant. IFN-γ/TNF-α stimulation significantly upregulated L-kynurenine that was in turn significantly reduced using 1-L-MT.

Cytokine activated renal tubular epithelial cells suppress CD4⁺ T-cell proliferation in a cell -cell contact dependent manner, partially mediated by IDO

In a separate set of 6 experiments, we examined whether IFN-γ/TNF-α activated TECs inhibit T-cell proliferation via IDO and whether IDO inhibition results in recovery of T-cell proliferation. Polyclonal stimulation resulted in a proliferating rate of 79 ± 1.8% of $CD4^+$ T cell and 64.5 ± 3.3% of $CD8^+$ T cell. Cytokine activated TECs significantly inhibited the proliferation of $CD4^+$ T-cell down to 27.5 ± 6.5% and of $CD8^+$ T cells up to 31.2 ± 8.3%, meaning 65% and 52% suppression at a 1:2.5 ratio. IDO inhibitor 1-L-MT only partly restored $CD4^+$ T-cell proliferation by 38%; increasing $CD4^+$ T-cell proliferation up to 37.9 ± 8.6% ($P<0.05$, Figure 3A). Surprisingly, we could not detect a significant recovery of $CD8^+$ T-cell proliferation using 1-L-MT (34.8 ± 8.6% vs. 31.2 ± 8.3%, Figure 3B). Despite statistical significance, the recovery of $CD4^+$ but also $CD8^+$ T cell proliferation is only partially or not affected by the addition of 1-L-MT.

Interestingly, transwell experiments revealed that the observed immunomodulatory effects of TECs on proliferating T cell subsets are cell-cell contact dependent as separated TECs do not inhibit $CD4^+$ T-cell and $CD8^+$ T-cell proliferation in a transwell system. Also 1-L-MT added to the TECs from the beginning did not affect $CD4^+$ T-cell or $CD8^+$ T-cell proliferation compared to the transwell experiments without the IDO inhibitor (Figure 3C). This suggests that TECs need to use cell-surface molecules suppressing $CD4^+$ T-cell and $CD8^+$ T-cell proliferation. Flow cytometric analysis revealed indeed that the cell-surface molecules ICAM-1 and programmed death ligand 1 (PD-L1) are significantly upregulated after IFN-γ/TNF-α stimulation known for their activities to inhibit T-cell proliferation [24-25] (Figure 3D).

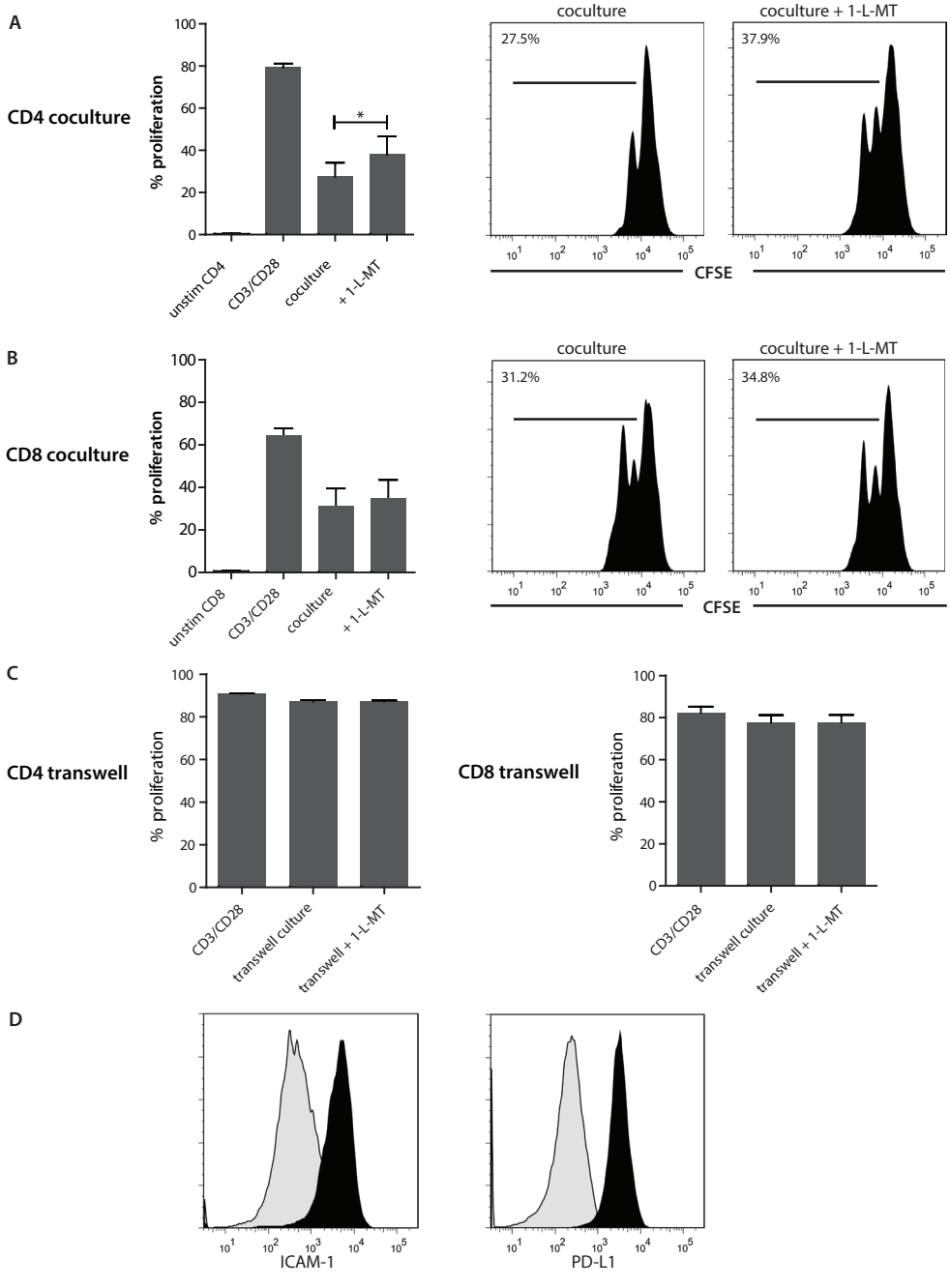

Figure 3 | Cytokine activated renal tubular epithelial cells inhibit CD4⁺ and CD8⁺ T-cell proliferation in a cell –cell contact dependent irrespective of TEC derived-IDO activity.

IFN-γ/TNF-α TEC were cocultured with anti-CD3/CD28 activated PBMC (TEC:PBMC ratio 1:2.5, n=6). (**A**) CD4⁺ T-cell proliferation was significantly inhibited by activated TECs. 1-L-MT recovered CD4⁺ T-cell proliferation only partially. (**B**) Proliferating CD8⁺ T cells were significantly inhibited by activated TECs. 1-L-MT did not recover CD8⁺ T-cell proliferation at all. (**C**) IFN-γ/TNF-α TECs were cultured with anti-CD3/CD28 activated PBMC using transwell membranes (TEC:PBMC ratio 1:2.5, n=6). Simultaneous transwell cultures in the absence or presence of 1-L-MT were performed. Activated TECs did not affect CD4⁺ T-cell proliferation and CD8⁺ T-cell proliferation in transwell experiments. 1-L-MT did not affect CD4⁺ and CD8⁺ T-cell proliferation. (**D**) TECs were stained for ICAM-1 and PD-L1 under unstimulated conditions (grey) and after IFN-γ/TNF-α stimulation (black). Stimulation of TECs resulted in significant upregulation of ICAM-1 and PD-L1 cell surface expression levels (n=3).

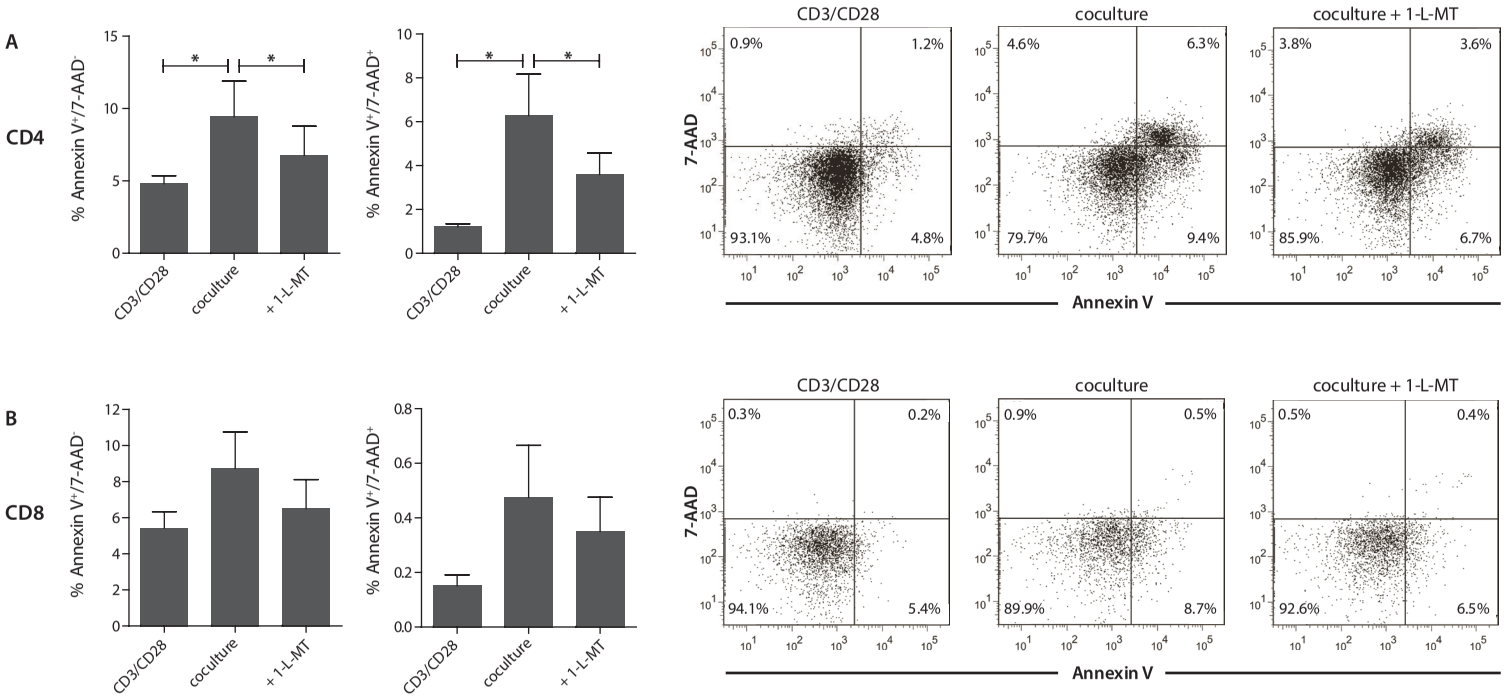

Figure 4 | Cytokine activated renal tubular epithelial cells induce T-cell apoptosis probably mediated via IDO.

IFN-γ/TNF-α stimulated TECs were cocultured with anti-CD3/CD28 activated PBMC (TEC:PBMC ratio 1:2.5, n=6). (**A**) Proliferating $CD4^+$ T cells and (**B**) $CD8^+$ T cells were analysed for markers indicating early apoptosis (Annexin $V^+/7\text{-}AAD^-$) and late apoptosis (Annexin $V^+/7\text{-}AAD^+$). (**A**) Activated TECs significantly increased $CD4^+$ T-cell early and late apoptosis. IDO inhibitor 1-L-MT recovered $CD4^+$ T-cell early and late apoptosis partly. (**B**) Same trends were observed for TEC induced $CD8^+$ T-cell early and late apoptosis.

Cytokine activated renal tubular epithelial cells induce T-cell apoptosis probably mediated by IDO

Early apoptotic cells are characterized by expression of Annexin V^+/7-AAD^-, and late apoptosis can be designated as Annexin V^+/7-AAD^+ cells. Anti-CD3/CD28 activated T-cells showed a low degree of $CD4^+$ T-cell early (4.8 ± 0.5%) and late apoptosis (1.2 ± 0.1%) (Figure 4A) at day 3. However, cytokine activated TECs significantly increased $CD4^+$ T-cell early apoptosis (9.4 ± 2.4%) and late apoptosis (6.3 ± 1.9%) at day 3 co-culture ($P<0.05$). IDO inhibition using 50 μM 1-L-MT inhibited $CD4^+$ T-cell early apoptosis (6.7 ± 2.0%) and late apoptosis (3.6 ± 0.9%). $CD8^+$ T-cell early apoptosis (5.4 ± 0.9%) and late apoptosis (0.2 ± 0.04) increased after incubation with activated TECs (8.7 ± 2.0% and 0.5 ± 0.2%). Likewise, 1-L-MT showed the same trend in reduction of $CD8^+$ T-cell early apoptosis (6.5 ± 1.6%) and late apoptosis (0.4 ± 0.1%) suggesting that IDO immunosuppressive effects could be mediated via targeting apoptotic pathways.

DISCUSSION

In this study, we investigated whether TECs possess immunosuppressive capacities and if IDO might play a role suppressing T-cell alloreactivity. We observed that activated TECs have the capacity to suppress both $CD4^+$ T-cell and $CD8^+$ T-cell allo-reactive proliferation. Transwell experiments showed that TEC-mediated immunosuppression is cell-cell contact dependent. IFN-γ/TNF-α activated TECs express high levels of mRNA IDO, and produce abundant amounts of L-kynurenine reflecting a high degree of IDO activity. However, IDO inhibition resulted merely in a partial recovery of $CD4^+$ T-cell proliferation and apoptosis, suggesting a role for IDO in the regulation of TEC $CD4^+$ T-cell immune interaction. We could not detect any effect of IDO activity on TEC $CD8^+$ T-cell immune regulation. Addition of IDO inhibitor to the transwell system did not result in a significant change in proliferative response of CD4 and CD8 T cells, again attenuating an important role for IDO with regard to observed immunosuppressive capacities of TECs.

Generally, the role of IDO in inhibition of immune responses has been widely acknowledged [12,15]. Immunohistochemical studies have previously shown a significant upregulation of IDO expression by TECs in rejecting kidney grafts compared to stable grafts [20]. Brandacher et al. suggested that IDO activity might offer a novel non-invasive means of immunomonitoring of renal allografts [20]. We studied whether IDO expressing TECs also affect lymphocyte responses. IDO is an intracellular rate-limiting enzyme resulting in tryptophan deprivation. The tryptophan catabolite L-kynurenine is involved in T-cell apoptosis, as it has been demonstrated that tryptophan deprivation results in blocking entry into the S phase and stagnation of T-cell cycle progression at G0/G1 level. In this way the T cell is unable to start DNA synthesis [16,26-27]. Interestingly, Fallarino et al. found differential susceptibility to cell death by murine Th1 and Th2 clones. T-cell exposure to L-kynurenine resulted in a significant degree of apoptosis of Th1 cell as demonstrated by propidium iodide staining in contrast to Th2 cells [28]. Of notable interest, Forouzandeh et al. found a differential sensitivity in cell proliferation between two subsets of human $CD8^+$ T cells and $CD4^+$ T cells. IDO-expressing fibroblasts were co-cultured with human $CD4^+$ and $CD8^+$ T cells. When grown in the same IDO-

induced tryptophan deficient microenvironment, $CD8^+$ T cells were more sensitive for IDO enzyme activity than $CD4^+$ T cells showing less $CD8^+$ T-cell proliferation by 3H-Thymidine incorporation [29]. Remarkably, Sørensen et al. described the effects of IDO-specific cytotoxic $CD8^+$ T cells against IDO enzyme active human cancer cell lines. IDO-specific cytotoxic T cells were capable of killing these cancer cell lines. The authors suggested that IDO-specific T cells might be crucial for an effective immune response during cancer [30]. In an IDO active experimental rat lung allograft model, Lui et al. suggested that infiltrating $CD8^+$ T cells remained viable but the cytotoxic function was affected accompanied by defects in production of granule cytotoxic proteins [31]. In line but not totally comparable, we documented a partial recovery of $CD4^+$ T-cell proliferation and inhibition of $CD4^+$ T-cell apoptosis using the IDO inhibitor 1-L-MT. The question remains whether this statistically significant result is biologically relevant. In contrast, neither recovery of $CD8^+$ T-cell proliferation nor $CD8^+$ T-cell apoptosis could be detected after addition of IDO inhibitor. To our surprise, the transwell experiments revealed the absolute necessity of cell-cell contact in order to suppress the T-cell proliferation that was not affected by the addition of IDO inhibitor to the system. This suggests that in a simplified IDO active renal tubular microenvironment model both $CD4^+$ and $CD8^+$ T-cells remain fully activated as not all cells can be in contact with tubular barrier and will probably mount an effective immune response against allo-antigens.

In response to immune-stimulation, TECs upregulate HLA class I and II [32] and class II [33], costimulatory molecules ICOS-L [34], CD40 [35] and ICAM-1 [36], and the inhibitory molecule PD-L1 [35]. In line, activated TECs have been shown to execute inhibitory effects on T-cell alloreactivity via PD-L1 pathway. IFN-γ stimulation leads to a strong dose-dependent increase of PD-L1 cell surface expression. In a TEC T-cell coculture system using anti-CD3/CD28 activated T cells, blockade of PD-L1 resulted in a significant increase of $CD4^+$ T-cell proliferation and not $CD8^+$ T-cell proliferation [24]. In an *in vitro* model using an OVA specific $CD8^+$ T cells and TECs, the PD-L1 pathway significantly inhibited $CD8^+$ T-cell effector function [37]. We also detected high levels of PD-L1 on IFN-γ/TNF-α activated TECs. Because we could only detect a partial recovery of $CD4^+$ T-cell proliferation using IDO blocking 1-L-MT, we suggest that blocking both PD-L1 and IDO might result in a more pronounced recovery of $CD4^+$ T-cell proliferation. Parenchymal cells exert their immunosuppressive effects in a cell-cell contact dependent manner as supernatant experiments did not reveal any inhibitory effect of IDO [38-39]. Likewise, ICAM-1 and VCAM-1 are required for lymphocyte-MSC adhesion. ICAM-1 and VCAM-1 were also critical for MSC-mediated immunosuppression. MSC-mediated immunosuppression was significantly reversed *in vitro* and *in vivo* when the adhesion molecules were genetically deleted or functionally blocked [25]. We found significant inhibitory properties of activated TECs using cell-cell contact dependent experiments. We also documented high cell surface expression levels ICAM-1 on IFN-γ/TNF-α activated TECs in our systems, which might indeed facilitate cell-cell contact necessary for suppression of T-cell proliferation.

In summary, our data show that TECs exert immunosuppressive effects on $CD4^+$ and $CD8^+$ T-cell proliferation, and lead to enhanced T cell-apoptosis. This would mean that in renal microenvironment, T cells in contact with TEC barrier are exposed to more inactivation and death by TECs. Infiltrating T cells in renal interstitial compartment will still be able to mount effective immune responses against alloantigens. In this light, significantly serum and urine kynurenine/tryptophan ratios of rejecting

patients might reflect the degree of tubular damage during rejection rather than an active repair mechanism aiming to dampen the local inflammation. Whether drug strategies aimed to target cell-cell contact and to modulate the expression level of co-stimulatory molecules on TECs will be efficient to prevent overt tubular disease and eventual structural kidney damage remain to be investigated.

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CHAPTER 6

FoxP3 T Cells and the pathophysiologic effects of brain death and warm ischemia in donor kidneys

Carla C. Baan, Annemiek M.A. Peeters, **Martijn W.H.J. Demmers**, Wendy M. Mol, Karin Boer, Janneke N. Samsom, Ajda T. Rowshani, Jan N.M. Ijzermans, Willem Weimar

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ABSTRACT

Background and objectives: Forkhead box P3 regulatory T cells control inflammatory responses, but it remains unclear whether they inhibit brain death-initiated inflammation and tissue injury in deceased kidney donors.

Design, setting, participants, & measurement: To study the actions of regulatory T cells at various stages of the donation and transplantation procedure, forkhead box P3, regulatory and inflammatory cytokine expression, and tissue injury markers were determined in time 0 kidney biopsies from deceased and living donors. Additionally, the interaction between forkhead box $P3^+$ T cells and kidney injury molecule-1 by activated primary tubular epithelial cells was studied.

Results: After cold storage, the deceased donor kidneys expressed the higher mRNA levels of kidney injury molecule-1 and CD3ε. In these samples, the inflammatory cytokines IL-8 and IFN-γ and markers associated with regulation (forkhead box P3, TGF-β, and IL-10) were highly expressed compared with living donor kidneys. Correlations were found between mRNA expression levels of forkhead box P3 and kidney injury molecule-1 and forkhead box P3 and IFN-γ. Immunohistochemical analysis confirmed the presence of forkhead box $P3^+$ T cells in donor kidneys. Renal function (analyzed by serum creatinine levels) at the first week posttransplantation correlated with kidney injury molecule-1 and forkhead box P3 mRNA levels. *In vitro* studies showed that kidney injury molecule-1 expression by primary tubular epithelial cells was 63% (mean) lower when cocultured with regulatory T cells compared with control T cells.

Conclusions: These results show that donor forkhead box $P3^+$ T cells infiltrate the deceased donor kidney, where they may control inflammatory and injury responses.

INTRODUCTION

In transplantation, ischemic graft injury is an unavoidable process that occurs at key stages during the donation and transplantation procedure. Of note, tissue injury is induced by the pathophysiologic events in brain death donors even before organ retrieval. Brain death is associated with a storm of inflammatory cytokines, and infiltrates can be found in the peripheral tissues, which together with other cardiovascular instability, results in organ damage [1-4]. This brain death-induced donor kidney damage is associated with upregulated kidney injury molecule-1 (KIM-1) expression in the kidney [5]. Furthermore, in donor organs, ischemia/reperfusion injury induces additional IFN-γ and IL-8 upregulation in grafted parenchymal cells followed by recruitment of inflammatory cells of both the innate and adaptive immune system. This aggressive immune response is considered as an important cause of tissue injury in the first phase after transplantation [6,7]. However, tissue injury itself perturbs immune homeostasis by inducing compensatory anti-inflammatory responses [8]. For instance it was shown that $CD4^+CD25^+$forkhead box P3 (FoxP3) +IL-10+ regulatory T cells (Tregs) control inflammatory responses after burn injury [9]. Evidence that Tregs participate in tissue injury comes from experimental AKI models. Depletion of Tregs increased renal tubular damage, whereas infusion of these T cells reduced IFN-γ production and improved tissue repair [10]. The finding of a counterinflammatory mechanism in AKI prompted us to study whether Tregs play a role in controlling inflammatory responses that are present in deceased donor kidneys. These Tregs share a complex relationship with IL-17-producing cells, major players in induction of inflammation, because differentiation into IL-17 CD4 T cells and Tregs is directed by TGF-β. When naive CD4 T cells are exposed to TGF-β and antigen, they differentiate into Tregs, whereas in the presence of the proinflammatory cytokines IL-6 and IL-23, they differentiate into Th17 cells [11].

Here, we analyzed whether tissue damage characteristic for deceased donor kidneys initiates a compensatory reaction by $FoxP3^+$ Tregs. For that purpose we studied time 0 biopsies of kidneys from deceased donors with brain death, warm ischemia, and prolonged cold ischemia times and living donors. Biopsies were taken at the end of cold storage and after reperfusion. In these samples, IL-8, IFN-γ, IL-17, FoxP3, and Treg-associated molecules and tissue injury markers were measured. Additionally, the inhibitory potential of $FoxP3^+$ T cells on KIM-1 expression by activated primary tubular epithelial cells (PTECs) was studied [5,12].

MATERIALS AND METHODS

Donor and patient characteristics

A total of 50 kidney biopsies were obtained for analysis from 11 deceased heart-beating donors (mean age=44 years; range=28–57 years) and 14 living donors (mean age=44 years; range=26–66 years). Biopsies were studied of 25 donors who enrolled in the study over a period of 1 year. Donor characteristics are shown in Table 1. Biopsies were taken at the end of cold storage and 20–30 minutes after reperfusion (Figure 1). Of the deceased donors, six were female; cause of brain death was cerebrovascular in six cases and trauma/other in the other five donors. Nine donors were

treated with vasoactive drugs. Preoperative warm ischemia time was comparable among living and deceased donors (range=15–90 minutes). In living donor kidneys, the time of cold ischemia was 3.0 hours (median; range=2.1–4.2 hours), and for the deceased donor kidneys, the time of cold ischemia was 19.0 hours (median; range=14.4–32.5 hours; P<0.001). The mean ages were 43 years (range=25–55 years) and 43 years (range=23–60 years) for recipients of kidneys from living and deceased donors, respectively. Patients (n=25) received maintenance immunosuppressive therapy consisting of tacrolimus, mycophenolate mofetil, and prednisone. Morning recipient serum creatinine levels were used to assess graft function. The study was approved by the Erasmus Medical Center review board (no. 196.927/2000/235), and informed consent of the patients was obtained.

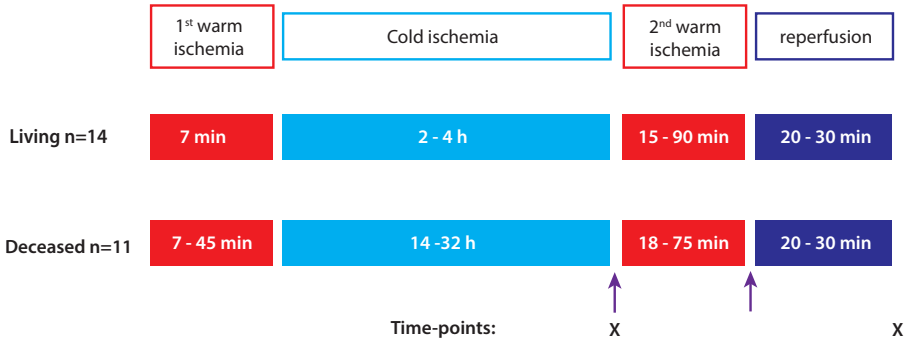

Figure 1 | Schematic overview of biopsy sampling.

Schematic overview of biopsy sampling. Per donor kidney, two biopsies were obtained. The first biopsy was taken after cold ischemia of the kidney, and the second sample was taken after 20–30 minutes of reperfusion. Sampling times points are marked by X.

Quantitative real-time PCR

mRNA extraction from the biopsies, cDNA transcription, and amplification were performed as described before [13]. Measurements were performed using Assays on Demand (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in each sample to control sample-to-sample variations in mRNA concentrations. The amount of target molecule was quantified by measuring cycle threshold (*Ct*), which was transformed to the number of cDNA copies [$2^{(40 - Ct)}$]. The relative concentrations were normalized to the relative concentration of the housekeeping gene GAPDH present in each sample and multiplied by 10^6 because of the lower concentration of the target gene compared with the concentration of GAPDH.

Immunohistochemistry

Protein expression of FoxP3 and CD3 was studied by immunohistochemistry. Negative control experiments for both antigens were performed by omitting the incubation with the primary antibody. In brief, paraffin-embedded sections were deparaffinized, rehydrated, and incubated in H_2O_2 diluted in PBS to block the endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment in citrate buffer. Sections were washed with PBS and incubated for 30 minutes in 10% normal human serum. Hereafter, sections were incubated with the primary antibody CD3

(DAKO, Glostrup, Denmark) or FoxP3 (eBiosciences, San Diego, CA) overnight at 4°C. Biotinylated donkey anti-rat or goat anti-rabbit secondary antibody combined with the Vectastain ABC Elite Kit was used to visualize the immunoreactivity; 3,3'-diaminobenzidine was used as chromogen, and sections were counterstained with hematoxylin.

PTECs treg interaction studies

PTECs were obtained from nephrectomy specimens operated for various reasons and cultured from fragments of histologic normal kidney tissue. Outgrowth of PTEC was confirmed by morphologic appearance $CD13^+$ $CD26^+$ staining. Cells were used between passages 3–5 of culture. PTECs were plated overnight in 96-well plates and induced with recombinant IFN-γ (50 ng/ml) and TNF-α (20 ng/ml). After 24 hours, the PTECs were cocultured with 5×10^4 FACS-sorted T cell populations ($CD4^+CD45RO^+CD25^{high+}$ $CD127^{-/lo}$ Treg cells and CD4 $CD25^-CD45RO^-$ control T cells) in RPMI1640 supplemented with 10% heat inactivated human serum. The Treg cells and control T cells were isolated by FACSAria (BD Biosciences) and stimulated with soluble αCD3 (0.3 μg/ml) and αCD28 (0.4 μg/ml) antibodies for 3 days in the presence of 2000 IU recombinant IL-2.The sorted T cells were obtained from peripheral blood of blood bank donors that was stained with monoclonal antibodies for CD3-Amcyan, CD4-pacific blue, CD25 PE-Cy7, CD45RO-APC, and CD127-PE (BD Biosciences). Intracellular staining with the FoxP3 (e-Bioscience) antibody showed that $CD4^+CD45RO^+CD25^{high+}$ $CD127^{-/lo}$ Treg cells expressed FoxP3 (>95%), which was not detectable in the $CD25^-CD45RO^-$ control T cells. Activated Treg and control T cells were cocultured with activated PTECs for 3 days. To measure gene expression by the PTEC and T cell subsets, the T cells were separated from the PTEC layer by trypsinization procedures followed by Ficoll gradient centrifugation. KIM-1 and IL-10 mRNA expression by the PTECs, Tregs, and control T cells and the PTEC cocultures were determined by quantitative PCR as described above.

Statistical analyses

A log transformation was performed on the mRNA levels of the analyzed markers to reduce the positive skew of the distribution. For determination of levels of statistical significance, two-sided probability values were used according to the *t* test if the data had a normal distribution; otherwise, groups were compared using Mann-Whitney U test for continuous variables and Fisher exact test for dichotomous variables. The relationships between variables were assessed by Spearman correlation and univariate linear regression analysis. A value of $P<0.05$ was considered significant. Statistical analysis was performed using Prism software.

RESULTS

Clinical data

Delayed graft function requiring dialysis occurred in 4 of 11 deceased donor kidneys and 2 living donor kidneys; 3 of 11 patients who received a deceased donor and 4 of 14 patients transplanted with a kidney from a living donor experienced an acute rejection episode within the first 3 months

after transplantation, and 3 of 25 kidneys were rejected within 1 year after transplantation (2 were deceased donor kidneys and one was a living donor kidney) (Table 1).

Table 1 | Donor and patient characteristics.

	Living donor kidneys (n=14)	Deceased donor kidneys (n=11)	P value
Donor			
mean age in years (range)	44 (26–66)	44 (28–57)	0.62
sex (male/female)	8/6	5/6	
mean cold ischemia time in hours (range)	3.0 (2.1–4.2)	19.0 (14.4–32.5)	<0.001
Recipient			
mean age in years (range)	43 (25–55)	43 (23–60)	0.47
sex (male/female)	10/4	7/4	1.00
HLA-A mismatch (mean ± SD)	1.0 ± 0.7	0.81 ± 0.9	0.56
HLA-B mismatch (mean ± SD)	1.4 ± 0.7	0.7 ± 0.6	0.04
HLA-DR mismatch (mean ± SD)	1.0 ± 0.7	0.6 ± 0.5	0.17
delayed graft function	2/14	4/11	0.35
acute rejection	4/14	3/11	1.00
graft failure within 1 year	1/14	2/11	0.56

KIM-1, cytokine, retinoid-related orphan receptor-γc, and Foxp3 expression in deceased and living donor kidneys

To examine the mRNA expression levels in deceased and living donor kidneys, biopsies were analyzed that were taken after donation and cold storage (Figure 1). In biopsies taken after both cold storage and reperfusion, the mRNA expression levels of KIM-1 were higher in deceased kidney donors than in biopsies of living donor kidneys (P<0.001 and P=0.01, respectively) (Figure 2A). In contrast to the deceased donor kidneys, reperfusion affected the KIM-1 expression in living donor kidneys. After reperfusion, significantly higher KIM-1 mRNA levels were found compared with living donor kidneys after cold storage (P=0.02). The proinflammatory cytokine IL-8 was expressed at higher levels by the deceased donor kidneys after cold storage compared with the living donors (P=0.02) (Figure 2B). This finding was not observed in specimens taken after reperfusion (P=0.85). Reperfusion affected the mRNA expression levels of IL-8 in living but not diseased donor kidneys. In living donor kidneys, a 30% higher mRNA expression level was measured after reperfusion compared with the samples taken after cold storage (P<0.01).

Next, we studied the contribution of CD3 T cells in brain death and renal ischemia reperfusion injury and the involvement of Tregs in the modulation of this response. In the deceased donor kidneys, the highest mRNA level of CD3ε was measured (Figure 3A) (after cold storage, P=0.02; after reperfusion, P=0.01). In samples from deceased donor kidneys, high levels of IFN-γ mRNA were also found (Figure

3B). mRNA levels of IL-17A were hardly detectable. After cold storage in 27% (3/11) of the deceased donor kidneys and 21% (3/14) of the living donor kidneys, IL-17A gene expression was found, which was not influenced by reperfusion (Figure 3C). To explain the absence of IL-17-producing cells in kidney from deceased and living donor kidneys, we measured the mRNA expression levels of the transcription factor retinoid-related orphan receptor-γc (RORγc) important for the differentiation into Th17 cells [14]. Both in the cold storage and after reperfusion specimens, RORγc mRNA was expressed at lower levels in deceased than living donor kidneys (Figure 3D) (P<0.001). However, these low RORγc expression levels were not the result of shortage of the Th17 priming cytokines, because ample mRNA for IL-6 and IL-23 was detectable (Figure 3, E and F).

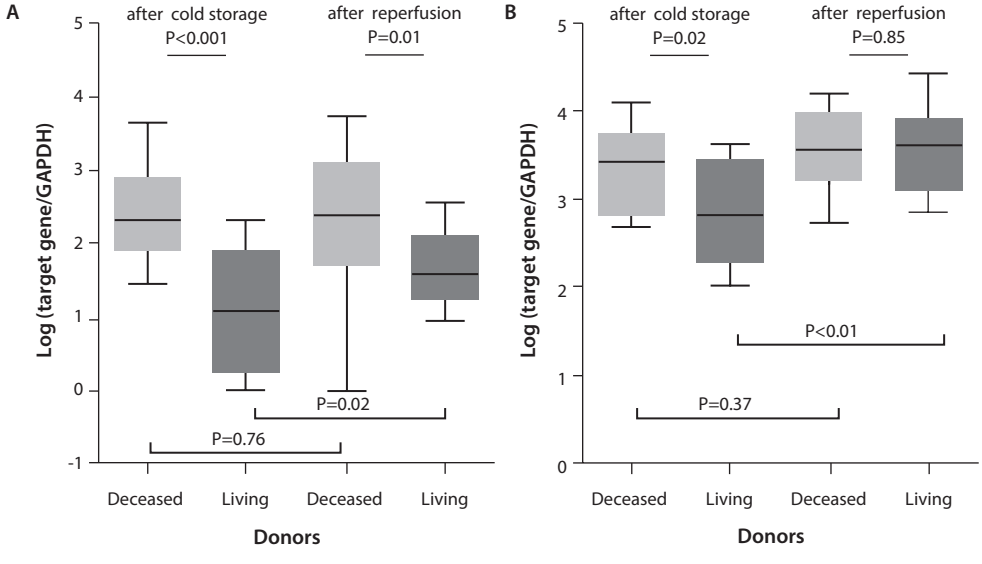

Figure 2 | Kidney injury molecule-1 (KIM-1) and IL-8 mRNA expression levels in donor kidney biopsies.

Kidney injury molecule-1 (KIM-1) and IL-8 mRNA expression levels in donor kidney biopsies. Box plots show the minimum to maximum and 25th, 50th (median), and 75th percentiles for levels of mRNA for KIM-1 (**A**) and IL-8 (**B**) in biopsies from living (n=14) and deceased donor kidneys (n=11) after cold ischemia and reperfusion. In the biopsies taken after both cold storage and reperfusion, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) -normalized, log-transformed mRNA levels of KIM-1 and IL-8 were significantly higher in the deceased than living donor kidneys. The mRNA expression levels of KIM-1 and IL-8 did not significantly change after reperfusion in the deceased donor kidneys in contrast to the living donor kidneys (P=0.02 and P<0.01, respectively). Because of shortage of material, the mRNA levels of KIM-1 could not be tested in four biopsies.

In the deceased donor kidneys, FoxP3 mRNA was present at high levels, suggesting that FoxP3$^+$ T cells are present in donor kidneys after cold storage and before transplantation (P=0.01) (Figure 4A). Also, IL-10, a key cytokine for Treg function, and TGF-β mRNA were expressed at higher levels by deceased than living donor kidneys (P=0.02 and P=0.004, respectively) (Figure 4, B and C). Moreover, FoxP3$^+$-expressing infiltrating cells were detected in specimens of donor kidneys after reperfusion (Figure 5).

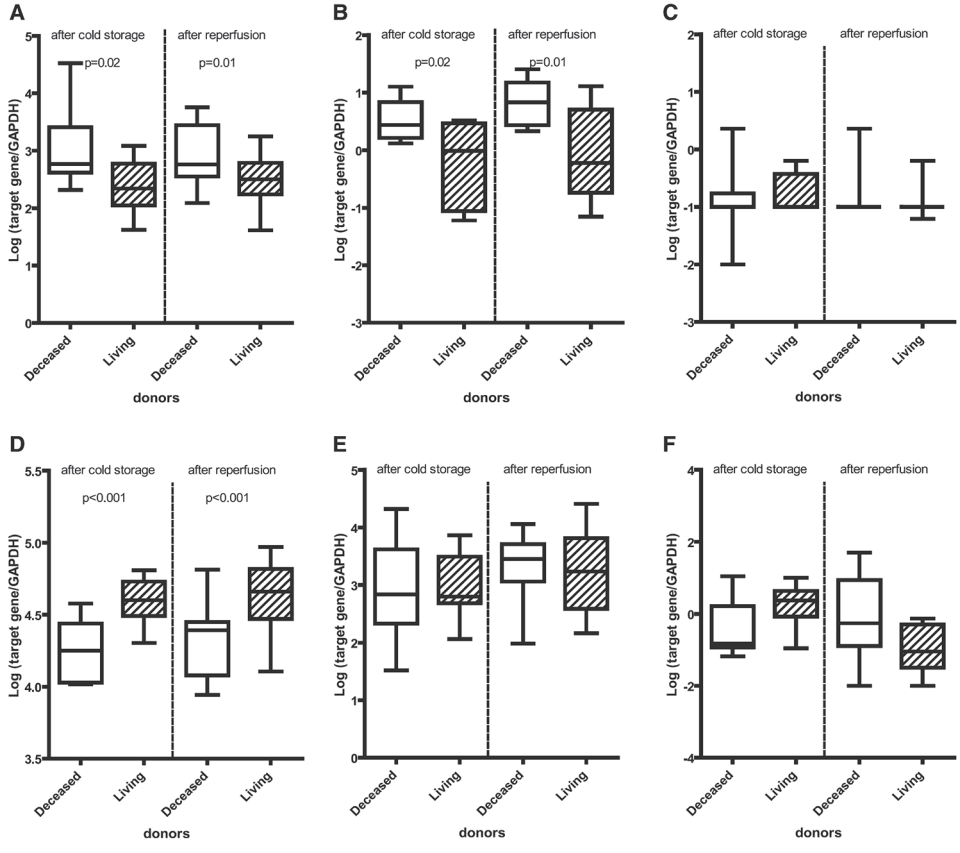

Figure 3 | mRNA expression levels of CD3 ε, cytokines, and transcription factors in donor kidney biopsies.

mRNA expression levels of CD3ε, cytokines, and transcription factors in donor kidney biopsies. Box plots show the minimum to maximum and 25th, 50th (median), and 75th percentiles for GAPDH-normalized, log-transformed mRNA levels of CD3ε (**A**), IFN-γ (**B**), IL-17A (**C**), Retinoid-related orphan receptor-γc (RORγc) (**D**), IL-6 (**E**), and IL-23 (**F**) in biopsies from living (n=14) and deceased (n=11) donor kidneys after cold ischemia and reperfusion. The mRNA levels of CD3ε and IFN-γ were expressed significantly higher in deceased than living donor kidneys, whereas the highest mRNA levels for RORγc were measured in the living donor kidneys. The difference between deceased and living donor kidneys was significant in the specimens taken after cold storage as well as specimens taken after reperfusion. Because of shortage of material, the mRNA levels of IFN-γ could not be tested in six biopsies.

To determine if the FoxP3 mRNA levels were associated with tissue damage and inflammation, we studied the correlation with KIM-1 and IFN-γ. Correlations were found between mRNA expression levels for FoxP3 and KIM-1 (r_s=0.58, P=0.006) and FoxP3 and IFN-γ (r_s=0.48, P=0.04). The FoxP3 mRNA expression levels inversely correlated with RORγc mRNA expression levels, suggesting that FoxP3$^+$ T cells control the response of inflammatory IL-17 T cells by repressing RORγc (r_s=–0.46, P=0.02).

The analyzed mRNA expression levels were not associated with the cause of brain death, treatment with vasoactive drugs, or occurrence of acute rejection or graft failure (all P>0.05).

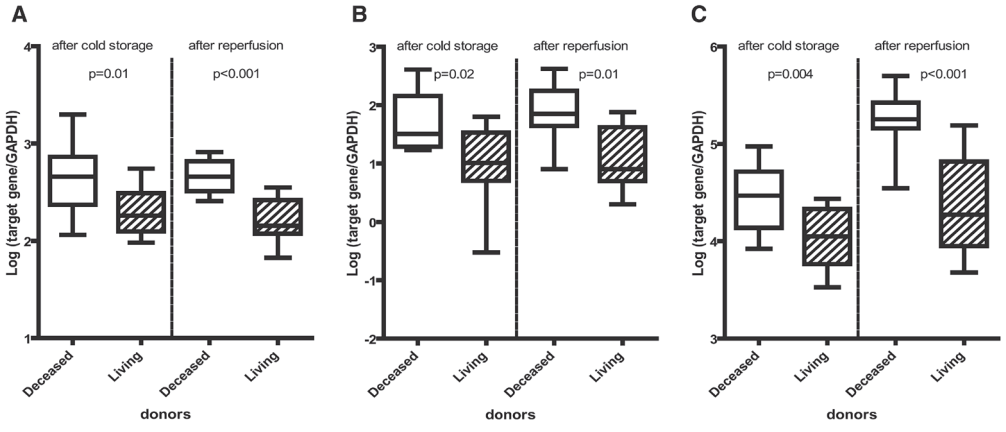

Figure 4 | mRNA expression levels of forkhead box P3 (FoxP3), IL-10, and TGF-b in donor kidney biopsies.

mRNA expression levels of forkhead box P3 (FoxP3), IL-10, and TGF-β in donor kidney biopsies. Box plots show the minimum to maximum and 25th, 50th (median), and 75th percentiles for levels of GAPDH-normalized, log-transformed mRNA levels of FoxP3 (**A**), IL-10 (**B**), and TGF-β (**C**) in biopsies from living (n=14) and deceased (n=11) donor kidneys after cold ischemia and reperfusion. The mRNA levels of FoxP3, IL-10, and TGF-β were expressed significantly higher in deceased than living donor kidneys. The difference between deceased and living donor kidneys was significant in the specimens taken after both cold storage and reperfusion.

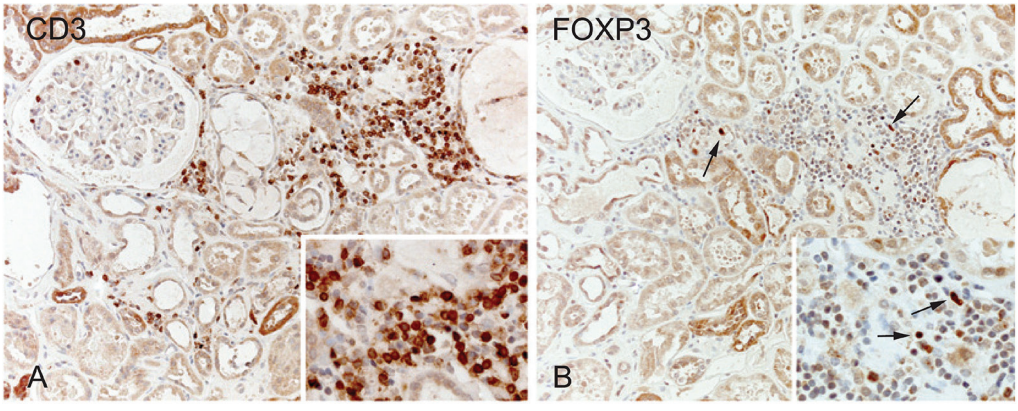

Figure 5 | Immunohistochemical staining of CD3 and FoxP3.

Immunohistochemical staining of CD3 and FoxP3. Example of infiltrating CD3 and FoxP3-positive cells in the kidney of a deceased donor (**A**). Marked expression of FoxP3 (arrows in B) was found in the cellular infiltrate in the kidney of a deceased donor (**B**). Sections are counterstained with hematoxylin. Original magnification, 200×; 400× in Insets.

Graft function and mRNA intragraft expression

We determined whether kidney function shortly after transplantation correlated with intragraft mRNA expression levels. For the patient group studied, the median serum creatinine levels at day 7 after transplantation were 161 µmol/L (range=73–1546 µmol/L). No correlation between serum

creatinine levels and mRNA expression levels was found for all samples analyzed (Figure 6, A and B). However, when we focused on the samples of patients with impaired kidney function at day 7 (>161 µmol/L, median of the study), a correlation between KIM-1, RORγc, and FoxP3 was found (r_s=0.66, P=0.02; r_s=0.68, P=0.02, respectively) (Figure 6, C and D).

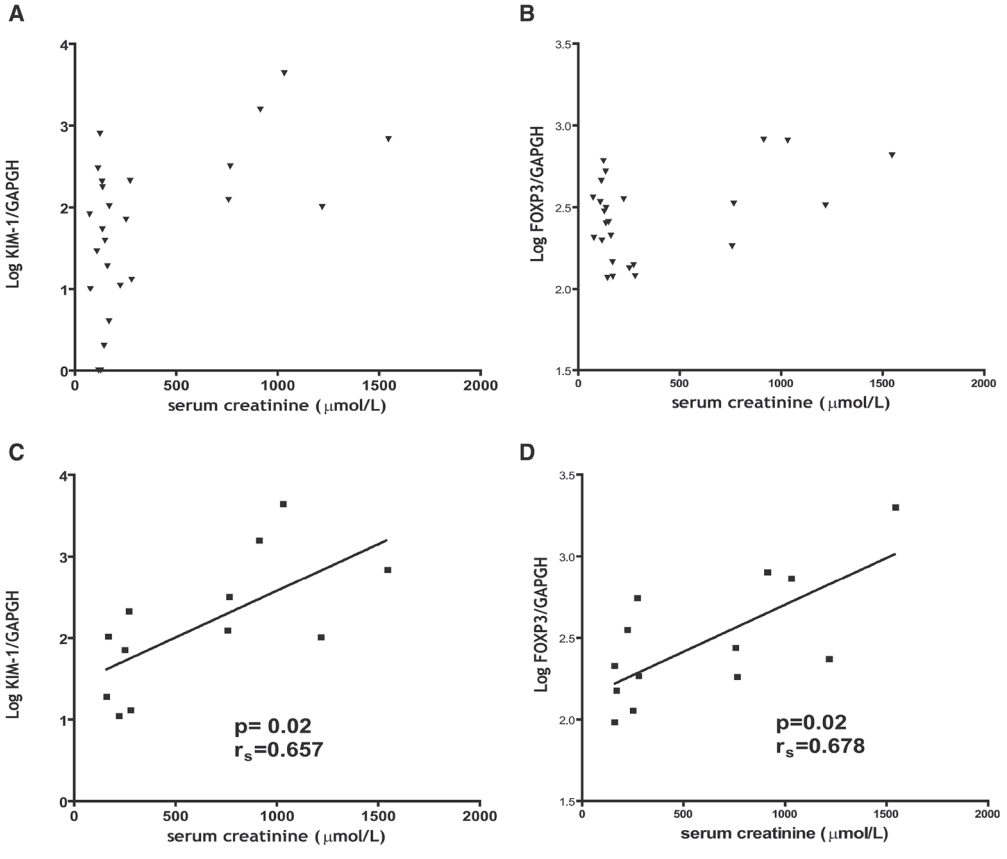

Figure 6 | Correlation between KIM-1 and FoxP3 mRNA expression levels and kidney function.

Correlation between KIM-1 and FoxP3 mRNA expression levels and kidney function. (**A** and **B**) No correlation between mRNA expression levels of KIM-1 and FoxP3 in all studied kidney biopsies from deceased and living donor kidneys obtained after cold ischemia was found. (**C** and **D**) Kidney KIM-1 and FoxP3 mRNA expression levels correlated with impaired kidney function at day 7 (>161 µmol/L, median of the study, n=12).

FoxP3$^+$ T Cells and PTECs

To investigate whether FoxP3$^+$ T cells do have the potential to suppress the KIM-1 expression by PTEC, FACS sorted αCD3/αCD28/IL-2–activated FoxP3$^+$Treg cells and control FoxP3−control T cells were cocultured for 3 days with TNF-α/IFN-γ–activated PTEC. Figure 7A shows the gating strategy of the sorted T cell populations. Tregs highly expressed IL-10 message and were negative for KIM-1, whereas PTECs expressed IL-10 at low levels and were strongly positive for KIM-1 (Figure 7, B and C).

In three sets of experiments, we consistently found that KIM-1 expression by activated PTECs was 63% (mean) lower when cocultured with Tregs compared with control T cells (P=0.05) (Figure 7C).

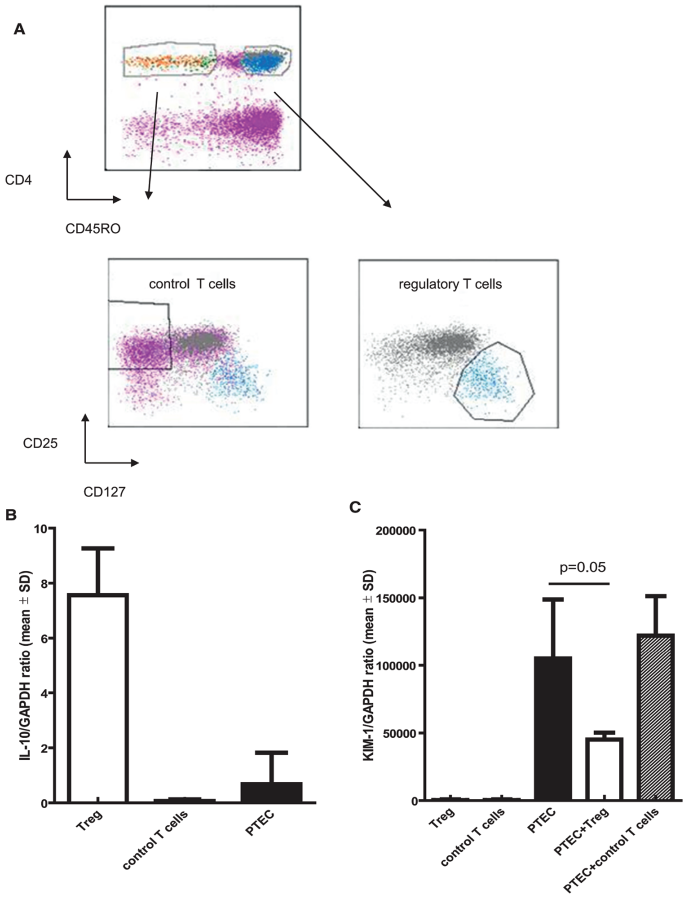

Figure 7 | $CD4^+CD45RO^+CD25^{high}CD127^-FoxP3^+$ T cells but not $CD4^+CD25^-CD45RO^-$ T cells inhibit KIM-1 expression by proximal tubulus epithelial cells (PTECs).

$CD4^+CD45RO^+CD25^{high+}CD127^{-/lo}FoxP3^+$ T cells but not $CD4^+CD25^-CD45RO^-$ T cells inhibit KIM-1 expression by proximal tubulus epithelial cells (PTECs). (**A**) shows the gating strategy of the sorted T cell populations. (**B**) depicts IL-10 mRNA expression levels of activated PTECs, activated $CD4^+CD45RO^+CD25^{high+}CD127^{-/lo}FOXP3^+$ T regulatory (Treg) cells, and $CD4^+CD25^-CD45RO^-$ T (control) cells. (**C**) shows the results of the coculture experiments. T cells were activated by αCD3/αCD28 and IL-2 and were cocultured with activated PTECs for 3 days. To measure gene expression by the PTEC and T cell subsets, the T cells were separated from the PTEC layer by standard trypsinization procedures followed by Ficoll gradient centrifugation. KIM-1 expression by PTEC was inhibited by 63% (mean) when cocultured with Treg cells compared with control T cells.

DISCUSSION

We investigated regulatory and T-helper 17 cell-associated markers in the donor kidneys from deceased and living donors. Our study shows a marked overexpression of the kidney injury markers KIM-1, CD3ε, IFN-γ, IL-8, IL-10, TGF-β, and FoxP3 in deceased donor kidneys. In addition, hardly any IL-17A mRNA was detectable, whereas RORγc, the transcription factor for Th17 cells, was highly expressed in living donor kidneys.

The role of Th17 in tissue injury caused by the events of the donation and transplantation procedures is unknown. It has been speculated that, because of its chemoattractant function for neutrophils, IL-17 would be important in ischemia reperfusion injury [15-18]. In samples taken after brain death and warm ischemia followed by a median cold ischemia time of 19 hours and 20-30 minutes reperfusion, we did not find evidence for the involvement of Th17 in kidney injury. This finding could be the result of sample timing. The work by Loong et al. [18] showed that IL-17-infiltrating cells are present in allografts at day 5 posttransplantation. The role of Th17 cells in human kidney injury is restricted to studies in renal autoimmune diseases and rejection after kidney transplantation [19,20]. These studies show that Th17 cells can infiltrate the kidney and that these cells contribute to injury. In our study, the IL-17 transcription factor RORγc inversely correlated with the FoxP3. We hypothesize that FoxP3$^+$ T cells inhibit IL-17 production or prevent the differentiation of activated T cells into Th17 cells. In mouse cells, FoxP3 suppresses transcriptional activity of RORγt by direct interaction, thereby inhibiting Th17 differentiation [21].

Here, we found that, during inflammation and tissue injury reflected by respectively high IFN-γ and KIM-1 mRNA levels, FoxP3 T cells infiltrate the kidney and that this finding mainly occurs in the donor as a result of brain death and warm ischemia-related events. Importantly, a biologic mechanism was found for FoxP3$^+$ T cells, because they inhibited KIM-1 mRNA expression by PTECs in contrast to control FoxP3$^-$ T cells. Studies analyzing inflammatory responses after brain damage caused by cerebral ischemia reported an increased presence of FoxP3$^+$ Tregs in blood and spleen [22]. It is known that enhanced regulatory T cell activity is an element of the host response to tissue injury [9,23]. These Tregs control the inflammatory immune response characteristic for burn injury [9]. In our study, the positive correlation between FoxP3 and KIM-1 mRNA levels suggests a responsive reaction by Tregs. These Tregs may accumulate in the kidney subsequent to inflammation and injury. Data in the work by Veronese et al. [24] suggest an interaction between Tregs and PTECs and support a role for FoxP3$^+$ T cells in tissue injury. Immunohistochemical analysis showed that, during allograft rejection, the proportion of CD4$^+$ cells in tubules expressing FoxP3 is significantly higher than in the interstitium. To show the biologic activities of FoxP3$^+$ IL-10–producing T cells directly on parenchymal cell function, we preformed coculture experiments and found that the expression of molecular marker for injury KIM-1 by PTECs was inhibited. Thus, FoxP3$^+$ T cells have the potential to modulate KIM-1 through an IL-10–mediated mechanism. In line with this finding, it is tempting to speculate that Tregs have beneficial activities in repair processes in immunosuppressed patients. It has been shown that immunosuppressive agents like mTOR inhibitors and rabbit antithymocyte globulins help to expand or augment the activity of Tregs, whereas at the same time, these agents target effector T cells [25,26]. Therefore, treatment of the organ donor, the harvested organ, and/or the

recipient with these agents may beneficially contribute to the recovery of the organ. We speculate that this finding will lead to less tissue injury and improved kidney function after transplantation. The outcome of KIM-1 and FoxP3 measurements in urine samples of recipients of deceased donor kidneys who are treated with rabbit antithymocyte globulins induction therapy may support this hypothesis.

Proof that Treg function is important in tissue repair processes comes from Treg depletion studies. In mouse models of ischemic AKI, depletion of FoxP3 T cells potentiated kidney damage [27]. In addition, it was shown that $FoxP3^+$ T cells infiltrate the ischemic reperfused transplanted kidney during the healing process and promote repair by inhibiting the T cell production of IFN-γ and TNF-α [10].

In conclusion, our results show that donor $FoxP3^+$ T cells infiltrate the deceased donor kidney, where they may control inflammatory and injury responses.

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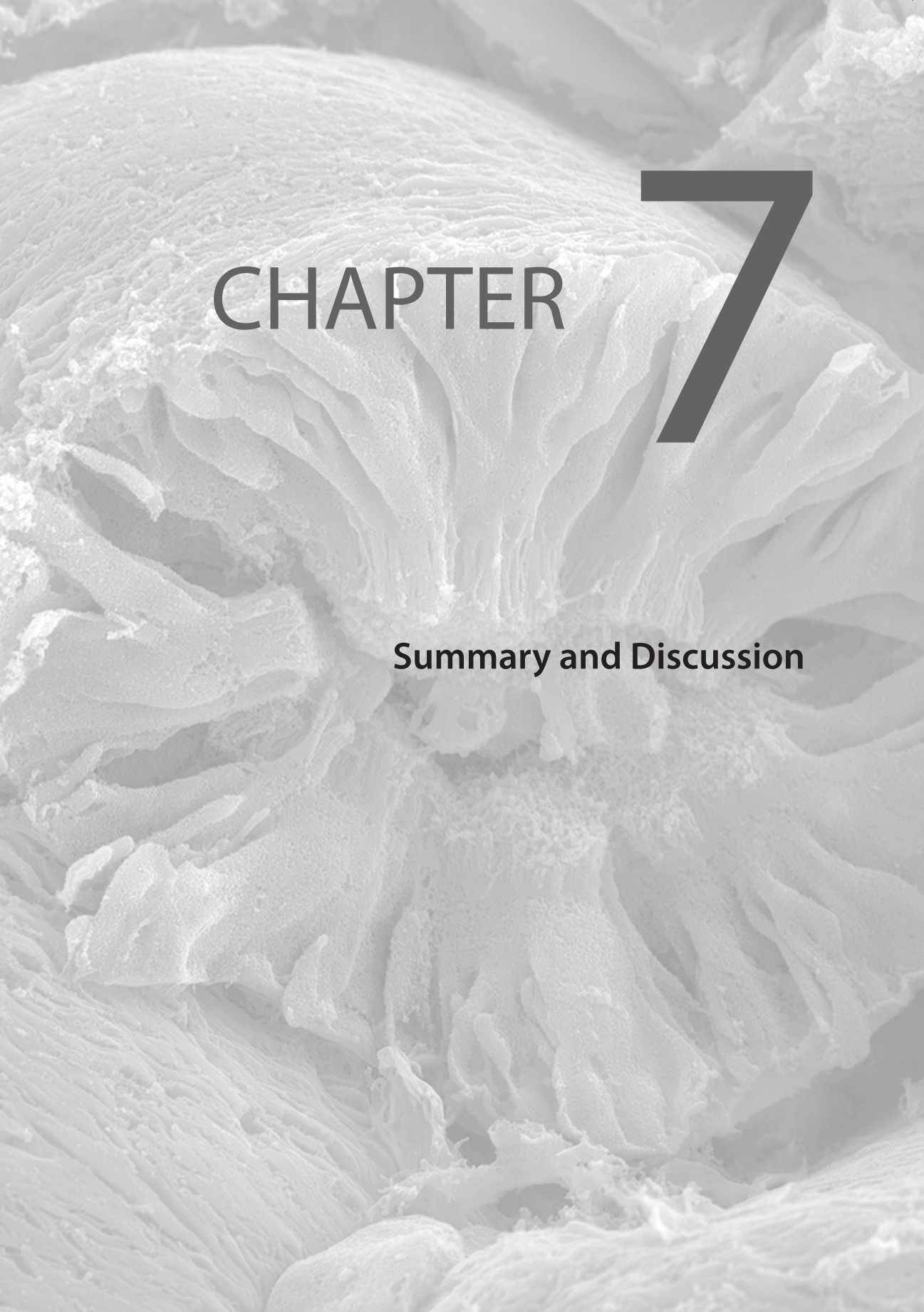

CHAPTER 7

Summary and Discussion

SUMMARY AND GENERAL DISCUSSION

In this thesis, we hypothesized that the renal tubular epithelial cells (TECs) affect the attraction, activation, differentiation and proliferation of allo-reactive immune cells, and that the local effects of immunosuppressive drugs on these immune-interactions may differ from systemic effects of drugs in circulation. TECs produce pro- and anti-inflammatory cytokines and/or growth factors contributing to *"the renal microenvironmental drive"* determining the end-effects of locally and systemically initiated allo-immune responses. TECs are non-professional antigen presenting cells expressing HLA molecules class I [1] and class II [2], costimulatory molecules ICOS-L [3], CD40 [4] and ICAM-1 [5]. TECs have been shown to protect themselves against inflammatory and apoptotic hits by expression of negative co-stimulatory molecules like PDL-1 [6] and anti-apoptotic SERPINB9 which specifically inhibits the effects of cytotoxic effector molecule granzyme B [7-8]. The central aim of this thesis was to elucidate the immune interactions between human TECs, T-cell subsets and NK cells *in vitro* mimicking transplantation-related inflammatory conditions like rejection. Furthermore, we aimed to investigate the effects of immunosuppressive drugs on outcome measures of the studied interactions.

Kidney transplant rejection results in the induction of chemokine expression by divers cell sources in the kidney [9], and is denoted by the infiltration of immune cells into the tubulo-interstitium [10]. Differential T-cell subsets have been described. Isolated graft-infiltrating T lymphocytes, mostly $CD8^+$ T cells, from a donor kidney showed powerful cytolytic responses towards the same donor TECs [11]. $CD4^+$ T helper cells have been shown to play an essential role in renal allograft rejection and TECs have the capacity to attract $CD4^+$ T cells [12-13]. Interestingly, the role of the $CD4^+$ T cell subset Th17 cells in renal allograft rejection is still under debate [14]. Even though IL-17 is present during renal allograft rejection, this does not imply Th17 cells playing a central role in renal alloreactivity [15-16]. While the chemokine CCL20 is essential for Th17 attraction, distinct cytokines; IL-1β, IL-6, IL-23 and TGF-β1 drive the differentiation of naïve $CD4^+$ T cells towards Th17-cell development. Interestingly, as these cytokines are known to be present during renal allograft rejection [17-19], one can assume that Th17 cells are detrimental in graft-related immunity. In **Chapter 2,** we studied the differential effects of activated human renal epithelial cells on Th17 and Th1 migration. We questioned whether TECs modulate the outcome of the inflammatory process by the production of distinct chemokines and cytokines that determine the attraction of T-cell subsets. We analyzed TEC derived cytokine and chemokine production under IFN-γ and TNF-α inflammatory conditions which are shown to be essential during rejection [20]. After IFN-γ and TNF-α stimulation, TECs produced abundant amounts of IL-6 and IL-8. Importantly, no Th17 differentiation related cytokines; IL-1β, IL-23 or TGF-β1 were produced by cytokine activated TECs. On the contrary, Th1 related chemokines CCL2, CCL5, CXCL9 and CXCL10 were highly produced after cytokine stimulation of TECs, however the Th17 related chemokine CCL20 could not be detected at both gene and protein level. Even though high numbers of migrating $CD4^+CXCR3^+$ T cells towards activated TECs could be observed, no migration of $CD4^+CCR6^+$ T cells could be detected using transwell experiments. Thus, renal tubular epithelial cells do not attract Th17 cells nor produce cytokines promoting Th17 cell differentiation in the renal microenvironment under these inflammatory conditions. In contrast to TECs, endothelial cells have

the capacity to generate Th17 cells. Taflin et al. cocultured $CD4^+$ T cells with activated endothelial cells and found a significant and selective expansion of Th17 cells which was dependent on the cytokine IL-6 and STAT-3 phosphorylation [21]. In patients with acute renal allograft rejection significantly increased expression of CXCR3 and CCR5 $CD4^+$ T cells was found in the renal tubulo-interstitium compared to controls. In line, in situ hybridization revealed corresponding CXCL10 and CXCL11 expression in the tubulo-interstitium in the whole biopsy [22]. Further *in vitro* experiments revealed a powerful chemotactic response using IFN-γ/TNF-α activated TECs, this chemotactic response was significantly abrogated using anti-CCR5 and anti-CXCR3 antibodies [23].

Targeting T-cell migration into the renal allograft might be of potential interest. Two options can be envisioned, (i) either inhibiting the intrarenal inflammatory response and thus the cytokine/chemokine release by TECs or (ii) inhibiting the migrating capacities of the infiltrating T cells by blocking chemokine receptors. We found that IFN-γ/TNF-α activated TECs attract Th1 cells strongly. Anti-TNF-α treatment is being used in several autoimmune diseases like rheumatoid arthritis and Crohn's disease [24]. Although not yet being extensively used in the renal transplantation setting, anti-TNF-α treatment was applied in posttransplant recurrent FSGS and resulted in significantly decreased proteinuria levels [25]. Treatment of ulcerative colitis after renal transplantation using anti-TNF-α therapy did not increase serum creatinine and did not affect eGFR [26]. Although theoretically plausible, increased infection rates and malignancies are afraid side effects hampering the use of TNF blockade in solid organ transplantation.

Another potential therapeutic strategy is disruption of the chemokine and/or chemokine receptor network inhibiting the migration of activated T cells to the graft. Renal allograft rejection is associated with increased frequencies of intrarenal $CCR5^+$ T cells [22]. T-cell migration towards IFN-γ/TNF-α activated TEC was abrogated using anti-CCR5 and anti-CXCR3 antibodies using Boyden chambers [23]. Interestingly, blockade of CCR5 could prevent Graft-Versus-Host-Disease (GVHD), inhibited lymphocyte chemotaxis and led to lower incidences of liver and gut GVHD [27]. However, CCR5 blockade as the therapeutic strategy in patients with active RA could not be supported [28]. The CCR5 antagonist used in GVHD might be useful in solid organ transplantation as well. The development of effective drugs controlling chemokine release and T-cell trafficking into the graft composes a very interesting treatment option in the future.

Once immune cells are attracted from the peripheral blood into the renal allograft, the tubular epithelial cells can modulate the outcome of donor-directed immune response by influencing the environmental drive in further activation, proliferation and function of TEC-reactive T cells. In **Chapter 3**, we questioned how immunosuppressive drugs affect TEC-induced proliferation of different $CD4^+$ T-cell subsets. Upon TEC encounter, memory $CD4^+$ T cells composed the major proliferating $CD4^+$ T cell population. Interestingly, we detected substantial proliferation of TEC-reactive $CD4^+CD28^{null}$ memory T cells producing high levels of proinflammatory cytokines IFN-γ, TNF-α, and the cytolitic protease granzyme B. Even though the TEC-reactive $CD4^+$ T-cell proliferation was significantly suppressed by tacrolimus, everolimus, prednisolone and mycophenolic acid in clinically relevant concentrations, the proliferation of aforementioned TEC-reactive $CD4^+CD28^{null}$ T-cell sub-population was not inhibited by tacrolimus and everolimus; this in contrast to prednisolone and mycophenolic

acid. Differential sensitivity of proliferating TEC reactive T cells *in vivo* might play an important mechanistic role during cellular rejection under full systemic immunosuppression.

$CD4^+CD28^{null}$ T cells are a unique effector memory $CD4^+$ T cell subset expressing high levels of IFN-γ, TNF-α and granzyme B [29]. This subset frequency is upregulated in the circulation of patients with multiple sclerosis [30], rheumatoid arthritis [31] and acute coronary syndromes [32]. This subpopulation is rarely found in healthy individuals and slightly increased in elderly individuals [33]. Even though a higher frequency of circulating $CD4^+CD28^{null}$ T cells was described during chronic rejection [34], pre-transplantation frequencies of $CD4^+CD28^{null}$ T cells did not correlate with renal allograft rejection [35]. Interestingly, CX_3CR1 appeared to drive cytotoxic $CD4^+CD28^{null}$ T cells into the brain of multiple sclerosis patients. The authors found simultaneous expression of the $CD4^+CD28^{null}$ related chemokine fractalkine and its receptor CX_3CR1 on $CD4^+$ T cells *in vitro* [30]. TECs express fractalkine after TNF-α stimulation *in vitro* [36] supporting a suitable milieu for proliferating $CD4^+CD28^{null}$ T cells to exert cytotoxicity resistant to tacrolimus and everolimus. In contrast, prednisolone and mycophenolate mofetil (MMF) could inhibit these cells efficiently providing rational not to lower the dose or withdraw these drugs at least in patients experiencing rejection or in patients with high immunological risk profile before transplantation.

The local effects of tacrolimus, everolimus and prednisolone on lymphocyte-mediated cytotoxicity in the graft-microenvironment are poorly defined. Transplant specialists target divers pathways of T-cell immunity; by inhibiting calcineurin using tacrolimus, inhibiting the mTOR pathway using everolimus, and inhibiting the NF-κB pathway using prednisolone [37]. Emerging evidence shows that graft-infiltrating cell composition and function during rejection could differ from simultaneously observed alterations in peripheral blood [38-39]. These findings led to the question whether the immunosuppressive effects of currently used drugs could locally differ from their systemic effects. With regard to the measured immunosuppressive drug concentrations present within the renal allograft vs. peripheral blood, Noll et al. showed that tacrolimus levels are approximately 20 times higher in the kidney than in whole blood using liquid chromatography tandem mass spectrometry method [40]. Here, we questioned in **Chapter 4** whether TEC-mediated lysis by $CD8^+$ T cells and NK cells are differentially affected by immunosuppressive drugs *in vitro*. Intragraft granzyme B producing cytotoxic T cells and NK cells are associated with rejection [41]. While $CD8^+$ T cells are primarily associated with cellular mediated rejection [42], recent studies showed that NK cells are mainly associated with (late) antibody-mediated rejection [43-45]. We found that cocultured TECs induced significant $CD8^+$ T-cell proliferation that could be inhibited by tacrolimus, everolimus and prednisolone in clinically relevant concentrations. Significant NK-cell proliferation was observed after TEC-coculture. We found differential effects of immunosuppressive drugs of interest on proliferation of $CD56^{high}$ and $CD56^{dim}$ NK-cell subsets, and observed that prednisolone is the most powerful drug inhibiting NK-cell proliferation. Upon TEC encounter $CD8^+$ T cells and NK cells degranulated as documented by a higher expression of CD107a on their cell surface. Using IFN-γ/TNF-α activated TECs, $CD8^+$ T cell degranulation response was significantly enhanced compared to non-stimulated TECs, while the NK cell degranulation was reduced vs. non-stimulated condition. TEC-induced $CD8^+$ T-cell degranulation and $CD8^+$ T-cell mediated TEC lysis were preferentially inhibited by tacrolimus and prednisolone, and not by everolimus. Even though tacrolimus was the

only drug able to inhibit NK-cell degranulation, NK-cell mediated TEC lysis was only inhibited by prednisolone. Overall, our data point towards a limited efficacy of immunosuppressive drugs on $CD8^+$ T-cell and NK-cell mediated lysis of human renal tubular epithelial cells. From a clinical point of view, these experiments illustrate nicely the dilemma of each transplant physician when treating the rejection with a higher dose of immunosuppressive drugs. The dilemma of therapy being effective or ineffective in the light of redundancy of divers immune activated cell types involved, differences in cell-type specific sensitivity to drugs together with the issue of intra-graft vs. systemic effects.

Despite the use of potent systemic combinations of immunosuppressive drugs clinical and subclinical tissue injury still occurs [46]. Because chronic use of calcineurin inhibitors (CNI) is associated with nephrotoxicity, an effective treatment not relying on CNI might provide a novel kidney transplant strategy [47]. Non-nephrotoxic immunosuppressive agents, like mammalian target of rapamycin (mTOR) inhibitors are therefore considered of potential interest. In a randomized study, primary de novo renal transplant recipients were directly treated either with a CNI-free regimen including sirolimus or with a cyclosporine A regimen. In the sirolimus arm, the overall biopsy-confirmed acute rejection was significantly higher than in the CNI arm [48]. In line, we found that activated TEC-specific $CD8^+$ T cells and NK cells were not affected by everolimus in lysis experiments. This phenomenon might underlay the higher rejection rate under everolimus regimens used as both induction and maintenance treatment in kidney transplantation [48]. Off note, conversion of CNI to mTOR inhibitor later post-transplant may represent an appropriate strategy for maintenance therapy in renal transplant recipients, assuming that the late phase after transplantation is associated with significantly lower donor-reactive T cell frequencies. Van Besouw et al. nicely demonstrated that kidney transplant patients with low donor-specific cytotoxic T lymphocyte precursor frequencies (CTLpf) could be safely reduced in their immunosuppression without the occurrence of rejection [49]. In line, biopsy-proven acute rejection was similar between the CNI group and mTOR group at 24 months [50]. To date, renal allografts with late antibody-mediated rejection (ABMR) have a poor survival [51]. Using molecular diagnostic markers late ABMR is marked by the presence of NK cell transcripts [43-45]. The currently used drugs and interventions for the management of ABMR may consist of plasmapheresis, IVIG, rituximab, eculizmab and bortezomib, and don't include steroids in many transplant centres [52]. Our *in vitro* experiments showed that prednisolone was the only immunosuppressive drug inhibiting NK-cells mediated TEC lysis as measured by europium release assay. Of note, CD8 mediated TEC lysis was also significantly inhibited by steroids. Therefore, we would like to suggest to use "the old drug prednisolone as the high potential new drug" in the treatment of ABMR in order to improve the poor clinical outcome.

The outcome of local alloimmunity is the net result of a bi-directional way executing a pro-inflammatory allograft response and the local immunosuppressive effects of TECs; strategies used by TECs to dampen the local inflammation. In our previous work, we reported on the proinflammatory role of the tubular compartment directing selective migration of Th1 and Th17 $CD4^+$ T cells *in vitro* [53]. Furthermore, our data show substantial proliferation of TEC-reactive $CD4CD28^{null}$ memory T cells, which are resistant to tacrolimus and everolimus [54]. Previously, we and others have shown that TECs can resist granzyme B mediated apoptosis induced by allo-reactive cytotoxic T cells through upregulation of cytosolic SERPINB9 during rejection [7-8]. TECs might possess immune

regulatory capacities like other parenchymal cells such as mesenchymal stem cells [55]. Indoleamine 2,3-dioxygenase (IDO) is a cell survival related rate-limiting enzyme which leads to the degradation and depletion of the essential amino acid tryptophan along the kynurenine pathway [56]. We questioned whether TECs can supress alloreactive immunity and whether inflammatory conditions influence the immunomodulatory functions of TECs via IDO controlled mechanisms. To these ends, we investigated the differential immunosuppressive effects mediated by TECs on CD4 T-cell and CD8 T-cell proliferation and apoptosis. In **Chapter 5**, we found that TECs dose-dependently inhibited $CD4^+$ and $CD8^+$ T-cell proliferation. Cytokine activated TECs showed significantly increased IDO mRNA expression and IDO activity, determined by L-kynurenine measurements compared to the unstimulated condition. Suppressed $CD4^+$ and $CD8^+$ T-cell proliferation was only partly or not restored using IDO inhibitor; 1-L-MT respectively. Activated TECs increased early and late apoptosis of proliferating $CD4^+$ and $CD8^+$ T-cells. Addition of 1-L-MT reduced the percentage of apoptotic T cells. Interestingly, transwell experiments revealed that TEC-mediated immunosuppression is cell-cell contact dependent.

Overall, our data point to other mechanisms underlying the immunosuppressive capacity of TECs. It seems that TECs primarily inhibit an alloreactive T-cell response via cell-cell contact. It has been reported that programmed death ligand 1 (PD-L1) plays a pivotal role in the modulation of T-cell responses by TECs. De Haij et al. showed that TECs were able to promote IL-10 production by activated T cells, which was accompanied by inhibition of T-cell proliferation and IFN-γ production [3]. Interestingly, Starke et al. showed significant upregulation of PD-L1 in biopsies of patients with renal allograft rejection compared to the respective levels found in the pre-transplant biopsies. Blockade of PD-L1 resulted in a significant increase in $CD4^+$ T-cell proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells *in vitro* [6]. Interestingly, Nosov et al. studied the role of lentivirus-mediated overexpression of PD-L1 in corneal allograft survival. They found that local PD-L1 gene transfer in cultured corneas is a promising approach for the survival of the corneal allograft [57]. As we could find limited evidence for TEC-derived indoleamine 2,3-dioxygenase mediated immune regulation and could show overexpression of PD-L1 after cytokine stimulation by TECs, the role of PD-L1 as a potential local renal allograft immune regulator remains to be investigated.

The Holy Grail in solid organ transplantation is the induction of tolerance towards the allograft. This would mean that the recipient immune system accepts the renal allograft without the need for long-term immunosuppressive therapy. Regulatory T cells (T_{regs}) have been proposed to suppress alloimmunity via several mechanisms. Using CTLA-4 Tregs can suppress $CD4^+$ T-cell and $CD8^+$ T-cell activation, proliferation and differentiation directly via cell-cell contact [58-59]. Indirectly, T_{regs} also secrete cytokines like IL-10, IL-35 and TGF-β to dampen T-cell responses [60-62]. T_{regs} derived perforin and granzyme B have been proposed to suppress T-cell activation by killing effector T-cells [63]. Veronese et al. nicely demonstrated the presence of intratubular $CD4^+FOXP3^+$ T cells. The significance of this "Treg tubulitis" is not established yet, but it is hypothesized that this might be beneficial for graft acceptance [64]. Whether these intratubular $CD4^+FOXP3^+$ T cells are also immunosuppressive remains to be investigated, because it has been demonstrated that activated nonsuppressive $CD4^+$ T cells also are capable of expressing FOXP3 [65]. In **Chapter 6,** we discussed the role of regulatory T cells with regard to their potential capacity in controlling tissue injury. Kidney injury molecule-1

(KIM-1) is an emerging biomarker for tubular injury. It is expressed and released by TECs upon injury [66]. We found that renal function as measured by serum creatinine levels at the first week post-transplantation correlated with KIM-1 and FoxP3 mRNA expression levels measured in kidney biopsies. *In vitro* studies showed that KIM-1 expression by TEC was 63% lower when cocultured with T_{regs} compared with control T cells. This means that T_{regs} may control intrarenal tissue injury and inflammation.

Preclinical data from Sakaguchi et al. showed that T_{regs} from naive mice prevented rejection of allogeneic skin grafts in T cell-deficient nude mice given $CD25^-$ T cells [67]. The first adoptive transfer of human T_{regs} in graft-versus-host disease (GVHD) demonstrated that T_{regs} were well tolerated by patients [68]. In a Phase I/II study Di Lanni et al. enrollled patients who underwent HLA-haploidentical hematopoietic stem-cell transplantation. They nicely showed that adoptive transfer of Tregs prevented GVHD in the absence of immunosuppression [69]. T_{reg} therapy is well tolerated and will be tested in solid organ transplantation soon as a part of The One Study [70-71]. The question remains how the transferred T_{regs} will execute their immunosuppressive function in the renal allograft. T_{regs} migration depends on chemokine receptor expression CCR6 by T_{regs} and the secretion of the chemokine CCL20. As discussed in **Chapter 2**, IFN-γ/TNF-α activated TECs do not secrete the T_{regs} chemokine CCL20. Whether regulatory T cell therapy will eventually lead to "T_{regs} tubulitis", discussed by Veronese et al. [64], and execute their immunosuppressive function in the renal allograft by direct interfering with tissue injury remains to be elucidated. Regulatory T cell therapy should eventually allow the clinician to minimize or withdrawal immunosuppressive drugs.

CONCLUDING REMARKS

It can be envisioned that the renal microenvironment plays a pivotal role in the pathogenesis of renal allograft rejection, and that the renal microenvironment directs the outcome of the transplantation-related immunity. We studied TEC-immune cell interactions and the effects of conventional immunosuppressive therapy on migration, proliferation and cytolytic activity of T cells and NK cells after TEC encounter. We identified several findings that could be important when it comes to the adjustment of nowadays available conventional immunosuppressive therapy, or when it comes to new concepts underlying the development of new specific drugs targeting early and late graft injury.

In **Chapter 2**, we found that renal tubular epithelial cells attract Th1 cells, but do not attract Th17 cells. Blockade of the Th1 chemokine receptor CCR5 could be of clinical interest.

In **Chapter 3**, we found TEC-reactive $CD4^+CD28^{null}$ T cell proliferation which is resistant to tacrolimus and everolimus. In contrast, prednisolon and mycophenolate mofetil (MMF) could inhibit these proliferative T cell response efficiently providing rational not to lower the dose or withdraw these drugs at least in patients experiencing rejection, or in patients with high immunological risk profile before transplantation.

In **Chapter 4**, we found limited efficacy of immunosuppressive drugs on $CD8^+$ T-cell and NK-cell mediated lysis of human renal tubular epithelial cells. This study indicated prednisolone as the most powerful drug in combination with tacrolimus in combatting both CD8 and NK mediated immune reactions.

In **Chapter 5**, we show that TECs can protect themselves by supressing TEC reactive proliferation of both CD4 and CD8 T cells that are in contact with them. Increased TEC induced apoptosis of T cells is probably mediated by IDO immunosuppressive activity.

In **Chapter 6**, we discussed the role of regulatory T cells with regard to their potential capacity in controlling tissue injury. The use of regulatory T cells is a novel concept of cell therapy in human clinical organ transplantation which is under investigation at this moment in the context of The One Study, a European study to evaluate cellular immunotherapy in solid organ transplantation.

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CHAPTER 8

Nederlandse Samenvatting

Patiënten van wie de nieren steeds minder goed gaan werken en die dus hun nierfunctie verliezen, zijn op een gegeven moment aangewezen op dialyse totdat een nieuwe nier van een donor beschikbaar is. Transplantatie is de beste manier om de nierfunctie van de patiënt te herstellen. Het probleem na een transplantatie is dat het vreemde orgaan kan worden aangevallen door het afweersysteem van de patiënt. Om dit te voorkomen, worden er sterkte medicijnen gegeven die het afweersysteem remmen, waaronder de T cel, een van de belangrijkste soorten van afweer cellen. De medicijnen zijn erg effectief, 5 jaar na transplantatie heeft 85% van de getransplanteerde nieren het overleefd, dit betekent dat 15% van de nieren is afgestoten. De nier bestaat uit verschillende onderdelen: de glomerulus filtert het bloed zodat er geen bloed en eiwitten de urine in gaan, de tubulus epitheelcellen (TECs) zorgen dat allerlei voedingsstoffen terug het lichaam in kunnen en dat afvalstoffen het lichaam verlaten via de urine. Deze TECs zijn daarnaast een doelwit van het afweersysteem, onder andere T cellen zijn zeer goed in staat om hen aan te vallen en medicijnen kunnen dit niet altijd voorkomen.

In dit proefschrift beschrijven wij of TECs afkomstig van de donornier immuuncellen van de patiënt kunnen aantrekken, activeren, laten delen en differentiëren. Daarnaast vragen wij ons af hoe afweerremmende (immunosuppressieve) medicijnen invloed kunnen hebben op de interactie tussen de TECs en de afweer (immuun)cellen. TECs produceren zowel pro- als anti-inflammatoire ontstekingseiwitten genaamd cytokines en groeifactoren die bijdragen aan het nier micromilieu. TECs brengen eiwitten tot expressie op hun cel membraan die immuuncellen kunnen activeren en binden, zoals HLA moleculen, CD40 en ICAM-1. Daarnaast zijn TECs ook in staat om zichzelf te beschermen tegen immuuncellen door PD-L1 en SERPINB9 tot expressie te brengen. Het **doel** van dit proefschrift is om de immuun interactie tussen TECs met T-cellen en natural killer (NK) cellen (andere soort immuun cellen) te onderzoeken. Deze interactie wordt onderzocht onder afstoting gerelateerde cytokines. Daarnaast onderzoeken wij de effecten van immunosuppressieve medicijnen op de bestudeerde interacties.

Na niertransplantatie worden er door niercellen chemokines aangemaakt die immuuncellen aantrekken. Immuuncellen infiltreren naar het interstitium tussen de tubuli. Er zijn verschillende T-cellen beschreven die een rol spelen bij afstoting van de nier. Infiltrerende $CD8^+$ T-lymfocyten kunnen de donornier ernstig beschadigen door TECs te lyseren. Daarnaast kunnen TECs $CD4^+$ T-lymfocyten aantrekken die ook een essentiële rol spelen bij het afstoten van de nier. Het is op dit moment onbekend wat de rol van interleukine (IL)-17 producerende T-helper (Th17) cellen daadwerkelijk is bij afstoting. De Th17 cellen spelen een normaal gezien belangrijke rol bij infecties. Naast de chemokine CCL20, die Th17 cellen aantrekt, zijn er ook cytokines die van een naïeve $CD4^+$ T-cel een Th17 cel kunnen maken. Dit gebeurt onder invloed van IL-1β, IL-6, IL-23 en TGF-β1. Omdat bekend is dat deze cytokines aanwezig zijn ten tijde van afstoting, kan men veronderstellen dat Th17 cellen betrokken zijn bij afstoting van de nier. In **Hoofdstuk 2** onderzoeken wij verschillende effecten van geactiveerde TECs op de migratie van Th1 en Th17 cellen. Wij vroegen ons af of TECs het afweersysteem aanstuurt via de productie van pro-inflammatoire cytokines en chemokines die deze T-cellen kunnen aantrekken. Omdat IFN-γ en TNF-α een essentiële/belangrijke rol hebben in het afstotingsproces hebben wij geanalyseerd welke cytokines en chemokines geproduceerd worden door IFN-γ/TNF-α geactiveerde TECs. Na activatie produceerden TECs de ontstekingscytokines IL-6

en IL-8. Belangrijk om te weten is dat de Th17 cel gerelateerde cytokines IL-1β, IL-23 en TGF-β1 niet werden geproduceerd door cytokine geactiveerde TECs. Ook de Th17 chemokine CCL20 werd niet geproduceerd. Daarentegen werden de Th1 chemokines CCL2, CCL5, CXCL9 en CXCL10 wel geproduceerd die de $CD4^+CXCR3^+$ T-cellen kunnen aantrekken. Dus, de TECs trekken geen Th17 cellen aan, noch produceren cytokines die Th17 differentiatie stimuleren.

In **Hoofdstuk 3** is getracht uit te zoeken hoe T cellen reageren op TECs. Daarnaast is onderzocht of immunosuppressieve medicijnen de proliferatie van TEC-reactieve T-cellen remmen. Wij vonden dat voornamelijk memory $CD4^+$ T-cellen delen als deze cellen in direct /binden contact komen met TECs. Het interessante is dat een groot gedeelte van de TEC-reactieve T-cellen het fenotype $CD4^+CD28^{null}$ heeft. Deze cellen produceren veel pro-inflammatoire cytokines, zoals IFN-γ, TNF-α en protease granzyme B. Alhoewel tacrolimus, everolimus, prednisolon en mycofenolzuur (immunosuppressiva) de totale populatie van TEC-reactive proliferende $CD4^+$ T-cel remmen, was de $CD4^+CD28^{null}$ T-cel proliferatie niet geheel te stoppen met tacrolimus en everolimus. Prednisolon en mycofenolzuur remden deze T-cel populatie wel in belangrijke mate. Dit verschil in gevoeligheid voor deze medicijnen kan belangrijk zijn bij afstoting.

De effecten van tacrolimus, everolimus en prednisolon op door lymfocyten veroorzaakte cytotoxiciteit in de donornier zijn niet goed gedefinieerd. Ten tijde van afstoting worden verschillen in samenstelling en activatie-graad van immuuncellen waargenomen in de donornier ten opzichte van het perifere bloed. Deze bevinding leidde tot de vraag of immunosuppressieve medicijnen andere effecten hebben in een nier micromilieu ten opzichte van hun systemische effecten in het perifere bloed. Het interessante is dat tacrolimus concentraties tot 20 maal hoger gevonden worden in de nier ten opzichte van het perifere bloed. In **Hoofdstuk 4** staan de effecten van immunosuppressieve medicatie op de functie van $CD8^+$ T cellen en NK cellen centraal. Wij vroegen ons af hoe TEC lysis door $CD8^+$ T-cellen en NK cellen voldoende geremd wordt door deze immunosuppressieve medicijnen. Granzyme B producerende cytotoxische $CD8^+$ T-cellen en NK cellen zijn geassocieerd met afstoting. Terwijl $CD8^+$ T-cellen vooral geassocieerd zijn met cellulaire afstoting, zijn NK cellen vooral geassocieerd met late antilichaam gemedieerde afstoting, een andere zeer ongunstige vorm van afstoting. Wij vonden dat tacrolimus, everolimus en prednisolon TEC-reactieve $CD8^+$ T-cel proliferatie konden remmen. Daarnaast bleek dat prednisolon het sterkst de NK cel proliferatie remde. Na cel-cel contact met TECs brachten $CD8^+$ T-cellen en NK cellen de degranulatie marker CD107a tot expressie. Door TECs te stimuleren met IFN-γ/TNF-α werd ook op CD8 T-cellen meer CD107a tot expressie gebracht, terwijl de CD107a expressie van NK cellen juist lager was. Tacrolimus en prednisolon, maar niet everolimus, waren in staat om zowel de $CD8^+$ T-cel degranulatie als de lysis van TECs door $CD8^+$ T-cellen te inhiberen. Tacrolimus was het enige medicijn dat NK cel degranulatie kon inhiberen, maar was niet goed in staat om de uiteindelijke lysis van TECs te remmen. Prednisolon was het enige medicijn dat het lyseren van TECs door NK cellen kon tegengaan. Concluderend kunnen wij zeggen dat immunosuppressieve medicijnen vaak een beperkt effect hebben op het voorkomen van TEC lysis. Deze resultaten laten zien voor welk dilemma transplantatie artsen vaak staan; medicijnen hebben verschillende immuuncel specifieke sensitiviteit.

De lokale alloimmuniteit in de nier wordt mede bepaald door de pro-inflammatoire response op het transplantaat in combinatie met de lokale immunosuppressieve effecten van TECs en andere

weefselcellen. Het idee is dat niercellen zoals TECs zichzelf proberen te beschermen op verschillende manieren tegen ontsteking veroorzakende immuuncellen. In de voorgaande hoofdstukken hebben wij beschreven dat tubuli selectief Th1 cellen aantrekken en dat er een substantiële proliferatie plaatsvond van TEC reactieve $CD4^{+}CD28^{null}$ T-cellen die ongevoelig waren voor tacrolimus en everolimus. Daarnaast vonden wij en anderen ook dat TECs zich kunnen beschermen tegen door cytotoxische T-cellen gereguleerde granzyme B geïnduceerde celdood; TECs beschermden zich door SERPINB9 tot expressie te brengen. TECs hebben dus mogelijk immuun regulerende capaciteiten, net zoals mesenchymale stamcellen. Indoleamine 2,3-dioxygenase (IDO) activiteit (een enzym) zorgt voor het degenereren van het essentiële aminozuur tryptofaan, waardoor cellen in apoptose gaan. Wij vroegen ons af of TECs alloreactieve immuniteit kunnen onderdrukken en dus zichzelf beschermen door middel van IDO activiteit. Wij onderzochten verschillende immunosuppressieve effecten van TECs op $CD4^{+}$ en $CD8^{+}$ T-cel proliferatie en apoptose. In **Hoofdstuk 5** beschrijven wij dat TECs op een dosisafhankelijke manier de CD4 en de CD8 T-cel deling remmen. Cytokine geactiveerde TECs lieten hoge IDO mRNA expressie en IDO activiteit zien. Door gebruikt te maken van de IDO remmer 1-L-MT werd de geremde CD4 T-cel deling gedeeltelijk hersteld, maar de CD8 T-cel deling niet. Geactiveerde TECs brachten delende CD4 en CD8 T-cellen in apoptose, en 1-L-MT verminderde het aantal apoptotische cellen. Het interessante resultaat van transwel experimenten was dat TECs door middel van cel-cel contact de door ons beschreven immuun remmende capaciteiten kunnen uitoefenen. Samenvattend laat onze data zien dat er andere mechanismen zijn die de immunosuppressieve capaciteiten van TECs kunnen verklaren. TECs remmen de alloreactieve T-cel respons via cel-cel contact.

De heilige graal in orgaantransplantatie is het accepteren van het getransplanteerde orgaan. Dit betekent dat het ontvangende immuunsysteem de nieuwe nier accepteert zonder het langdurige gebruik van immunosuppressieve medicatie en dat de overige immunreactiviteit behouden blijft die je bijvoorbeeld nodig hebt om infecties te bestrijden. De immunonderdrukkende, regulatoire T-cellen (Tregs) kunnen de alloreactiviteit remmen via verschillende mechanismen. Tregs remmen effector T cel functies via cel-cel contact waardoor de CD4 en CD8 T cellen niet meer delen en differentiëren. Ook de cytokines IL-10, IL-35 en TGF-β, en de eiwitten perforine en granzyme zijn hierbij betrokken. Intratubulaire $CD4^{+}FOXP3^{+}$ T-cellen zijn gevonden, maar wat hun functie is, weet men nog niet. Er wordt gedacht dat dit verschijnsel, "Treg tubulitis", mogelijk voordeling zou kunnen zijn voor de donornier. In **Hoofdstuk 6** bediscussiëren wij de rol van Treg en hun capaciteit om weefselschade te beperken. $FoxP3^{+}$ T cellen werden voornamelijk aangetoond in nierweefsels met veel schade. Ook vonden wij dat de nierfunctie na de eerste 3 maanden na transplantatie correleerde met de hoogte van FOXP3 mRNA expressie in nierweefsel welke was afgenomen direct na donatie. Onze *in vitro* studie toonde aan dat de expressie van TEC schade marker KIM-1 verminderd tot expressie gebracht wordt in aanwezigheid van Tregs. Dit betekent dat Tregs schade in de nier en directe schade aan TECs mogelijk kunnen beperken.

CONCLUSIE

Men kan zich voorstellen dat het nier micromilieu een belangrijke rol speelt in het afstoting proces en dat juist het nier micromilieu de transplantatie gerelateerde immunologie gedeeltelijk bepaalt. Wij bestudeerden de interactie tussen TECs en T cellen, daarnaast de effecten van immunosuppressieve medicijnen op migratie, proliferatie en cytotoxische activiteiten van T-cellen en NK cellen na het tegenkomen van TECs. Onze bevindingen kunnen een belangrijke rol spelen bij het aanpassen van de dagelijkse conventionele immunosuppressieve medicijnen, daarnaast heeft ons onderzoek geleid tot inzichten om nieuwe behandelopties toe te passen met het doel vroege en late nierschade te voorkomen.

ADDENDUM

Curriculum Vitae

PhD portfolio

Dankwoord

CURRICULUM VITAE AUCTORIS

Martijn Demmers werd geboren op 23 februari 1984 te Deventer. Na het afronden van het VWO op het Etty Hillesum Lyceum te Deventer, ging hij Bewegingswetenschappen studeren in Maastricht. Na het voltooien van de bachelor begon Martijn met de researchmaster Cardiovascular Biology and Medicine, onderdeel van het onderzoeksinstituut Cardiovascular Research Institute Maastricht (CARIM). In 2008 begon hij met zijn seniorstage bij de afdeling Klinische en Experimentele Immunologie van het AZM onder leiding van dr. Marielle Thewissen en prof. dr. J.W. Cohen Tervaert. Hij rondde zijn masteropleiding af met een thesis: Effects of RAGE and its ligands on T-cell function, om vervolgens in september 2009 te starten aan zijn promotieonderzoek bij het Transplantatielaboratorium van de afdeling Inwendige Geneeskunde in het Erasmus MC te Rotterdam. Onder begeleiding van dr. Ajda T. Rowshani, prof. dr. Carla Baan en prof. dr. Willem Weimar onderzocht hij de interactie tussen nier tubulus epitheelcellen en alloreactieve lymfocyten. Zowel tijdens zijn studie als promotietijd heeft Martijn zich ingezet voor zowel studenten als promovendi door zitting te nemen in de faculteitsraad en het bestuur van de promovendiverenigingen Promeras en EPAR. Momenteel werkt hij als klinisch chemicus in opleiding bij het Ziekenhuis Gelderse Vallei en het RadboudUMC.

PhD PORTFOLIO

Summary of PhD training and teaching activities

Name PhD candidate: Martijn W.H.J. Demmers **PhD period:** 2009-2013

Erasmus MC Department: Internal Medicine **Research School:** Molecular Medicine

Promotor: Prof.dr. W. Weimar

Copromotor: Dr. A.T. Rowshani en Prof.dr. C.C. Baan

Research and general academic skills

Course Molecular Medicine		2010
Methodologie van patiëntgebonden onderzoek		2010
Stralingshygiëne niveau 5b		2010
Statistical Course (Classical Methods of Datan Anylysis, CC02, NIHES)		2010
Advanced Course of Molecular Immunology		2011
Course English Biomedical Writing and Communication		2012

(Inter)national conferences and presentations

Bootcongres Rotterdam – Nederlandse Transplantatie Vereniging		2010
Bootcongres Amsterdam – Nederlandse Transplantatie Vereniging	oral	2011
Joint Basic Science Meeting Cape Cod USA – ESOT and TTS	poster	2011
ESOT Transplantation Meeting – Glasgow Scotland	oral	2011
Scientific Dutch Immunology Meeting – NVVI	poster	2011
Bootcongres Maastricht – Nederlandse Transplantatie Vereniging	oral	2012
American Transplant Congres – Boston USA	poster	2012
The Transplantation Society – Berlin Germany	oral/poster	2012
Bootcongres Nijmegen – Nederlandse Transplantatie Vereniging	oral	2013
American Transplant Congres – Seattle USA	poster	2013
ESOT Transplantation Meeting – Vienna Austria	poster	2013

Seminars and Workshops

Internal Medicine Erasmus MC Seminar Antwerp		2009
Klinisch Review Symposium NTV Utrecht		2009
Internal Medicine Erasmus MC Seminar Antwerp	poster	2010
Klinisch Review Symposium NTV Utrecht		2010
Molecular Medicine Day Erasmus MC	poster	2011
Internal Medicine Erasmus MC Seminar Antwerp	oral/poster	2011
Klinisch Review Symposium NTV Utrecht		2011
Internal Medicine Erasmus MC Seminar Antwerp	poster	2012

Grants

Travel grant Novartis ESOT	Glasgow	2011
Travel grant Dutch Society of Immunology	TTS Berlin	2012
Travel grant Erasmus Trustfonds	ESOT Vienna	2013

Scientific Award

Internal Medicine Erasmus MC Seminar – Poster price	2011
American Transplant Congres 2012 – Poster of Distinction	2012

Organizations

President Board of Promeras – PhD organization Erasmus Medical Center	2011-2013
Member Board of EPAR – PhD organization Erasmus University Rotterdam	2011-2013
Erasmus MC PhD Committee	2011-2013
Erasmus MC PhD day	2011-2013

Teaching activity

Supervision of a medical student for one year	2010
Supervision of a technician (HLO) student for three months	2012

DANKWOORD

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martijn