REGULATORY T CELLS and TH17 CELLS IN RENAL ISCHEMIA-REPERFUSION INJURY AFTER KIDNEY TRANSPLANTATION

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Abbreviations

AKI: acute kidney injury

AMP: adenosine monophosphate

APC: antigen presenting cell

ATP: adenosine triphosphate

AUC: area under the curve

BATF: basic leucine zipper transcription factor ATF-like

CIT: cold ischemic time

CFSE: carboxyfluorescein succinimidyl ester

CTLA-4: cytotoxic T-lymphocyte associated protein 4

DAMP: damage-associated molecular pattern

DBD: donor after brain death

DCD: donor after cardiac death

DGF: delayed graft function

DMS: N, N-dimethylsphingosine

ECD: expanded criteria donor

EPO-β: erythropoietin-β

FoxP3: forkhead box P3

GST: glutathione-S-transferase

H-FABP: heart-type fatty acid binding protein

HO-1: heme oxygenase-1

ICAM-1: intercellular adhesion molecule-1

ICOS: inducible T-cell costimulator

IDO: indoleamine 2,3-dioxygenase

IF/TA: interstitial fibrosis/tubular atrophy

IGF: immediate graft function

IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked

IRF4: interferon regulatory factor 4

IRI: ischemia-reperfusion injury

KIM-1: kidney injury molecule-1

KO: knockout

L-FABP: liver-type fatty acid binding protein

MyD88: myeloid differentiation primary response 88

NAC: N-acetylcysteine

NAG: N-acetyl-β-D-glucosaminidase

NGAL: neutrophil gelatinase-associated lipocalin

NK: natural killer cell

NKT: natural killer T cell

oATP: periodate-oxidized ATP

PBMC: peripheral blood mononuclear cell

PECAM-1: platelet-endothelial cell adhesion molecule-1

ROC: receiver operating characteristic

RORγt: retinoid acid receptor-related orphan receptor gamma

RORC2: retinoid acid receptor-related orphan receptor C2

rPSGL-Ig: recombinant P-selectin glycoprotein ligand immunoglobulin

S1P: sphingosine 1-phosphate

SGF: slow graft function

STAT: signal transducer and activator of transcription

Teff: effector T cell

TFH: T follicular helper

Th1: T helper 1

Th2: T helper 2

Th17: T helper 17

Treg: regulatory T cell

TLR: toll-like receptor

TNF: tumor necrosis factor

TNFR2: tumor necrosis factor receptor 2

TSDR: Treg-specific demethylation region

WT: wild type

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Contributions of authors

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Abstract

Acute kidney injury in the form of delayed or slow graft function is the most common complication after kidney transplantation and leads to adverse immunologic consequences. There is currently no clinically accepted biomarker to predict this ischemia-reperfusion related injury, and no clinically approved therapy to prevent it. Regulatory T cells (Tregs) are CD4+ T cells that play an essential role in the maintenance of transplant tolerance and are protective in murine renal ischemia-reperfusion injury. Their differentiation from naïve CD4+ T cells is closely related to the pro-inflammatory Th17 cells. The role of Tregs in ischemia-reperfusion related injury after human kidney transplantation is unknown. The long-term Treg and Th17 cell responses after renal ischemia-reperfusion injury and their effect on chronic kidney damage are also unknown. We first found that measuring pre-transplant recipient circulating regulatory T cell suppressive function was independently predictive of delayed and slow graft function after human kidney transplantation. Since the assay to measure Treg suppressive function is not clinically-friendly, we next found that the measurement of tumor necrosis factor receptor 2 (TNFR2) expression on Tregs correlated with Treg suppressive function in kidney transplant candidates, and that the measurement of pre-transplant recipient circulating TNFR2+ Tregs could replace Treg suppressive function as an independent predictor of delayed and slow graft function after human kidney transplantation. Since delayed and slow graft function increase the risk of acute rejection, we also found that the measurement of pre-transplant recipient circulating TNFR2+ Treg independently predicted acute rejection within 6 months

after human kidney transplantation. Finally, we complemented our human findings with murine experiments exploring the long-term Treg and Th17 cell responses after renal ischemia-reperfusion injury. We found that there is a sustained regulatory Treg and pro-inflammatory Th17 cell response following murine renal ischemia-reperfusion injury favoring the latter, and that blockade of the Th17 pathway led to worse ischemia-reperfusion related chronic kidney damage.

Résumé

L'insuffisance rénale aiguë sous la forme d'un délai de la fonction ou d'une fonction lente est la complication la plus fréquente après une greffe rénale et provoque des conséquences immunologiques négatives. Présentement, il n'y a aucun biomarqueur clinique pour prédire cette condition reliée à une blessure d'ischémie-reperfusion et aucune thérapie clinique pour la prévenir. Les lymphocytes T régulateurs (Tregs) sont des lymphocytes T CD4+ qui jouent un rôle essentiel pour maintenir la tolérance en transplantation et protègent contre la blessure d'ischémie-reperfusion chez les rongeurs. Leur différentiation à partir des lymphocytes T CD4+ naïfs est étroitement reliée aux lymphocytes Th17. Le rôle des Tregs pour protéger contre la blessure d'ischémie-reperfusion après la greffe rénale chez l'humain est inconnu. De plus, les réponses des Tregs et lymphocytes Th17 à long terme suivant une blessure d'ischémie-reperfusion ainsi que l'effet sur le développement d'une insuffisance rénale chronique sont inconnus. Premièrement, nous avons trouvé que la mesure de la fonction suppressive des Tregs dans la circulation des récipients avant la greffe rénale peut prédire d'une manière indépendante le délai de la fonction et la fonction lente suivant une greffe rénale chez l'humain. Puisque le test requis pour la mesure de la fonction suppressive des Tregs n'est pas pratique au niveau clinique, nous avons ensuite trouvé que la mesure de l'expression de tumor necrosis factor receptor 2 (TNFR2) chez les Tregs est en corrélation avec la fonction suppressive des Tregs chez les candidats pour une greffe rénale, et que la mesure des TNFR2+ Tregs dans la circulation des récipients avant la greffe rénale peut remplacer la

fonction suppressive des Tregs pour prédire d'une manière indépendante le délai de la fonction et la fonction lente suivant une greffe rénale chez l'humain. Puisque le délai de la fonction et la fonction lente augmentent le risque pour un rejet aigu, nous avons trouvé que la mesure des TNFR2+ Tregs dans la circulation des récipients avant la greffe rénale peut aussi prédire d'une manière indépendante le rejet aigu dans les 6 premiers mois après la greffe rénale chez l'humain. Finalement, nous avons complimenté nos résultats chez l'humain avec des expériences chez la souris pour déterminer les réponses à long terme des Tregs et lymphocytes Th17 suivant une blessure d'ischémie-reperfusion rénale. Nous avons trouvé qu'il y a une réponse soutenue des Tregs et lymphocytes Th17 favorisant ces derniers suivant la blessure d'ischémie-reperfusion chez la souris, et que le blocage des lymphocytes Th17 provoque une augmentation de l'insuffisance rénale chronique reliée à l'ischémie-reperfusion.

Chapter 1: Introduction and review of the literature

Kidney disease is a global health challenge affecting more than 750 million people worldwide, with more than 120,000 people progressing to end-stage renal disease yearly in the United States alone (1,2). Experimental attempts were made starting at the beginning of the 20th century to perform kidney transplantation as a cure to end-stage renal disease. This culminated to the first successful human kidney transplantation performed by Dr. Murray in 1954 between monozygotic twins (3). The technical aspects of the surgical procedure have not changed dramatically since then. Modern human kidney transplantation involves first the retrieval of an allograft from a living or deceased donor with a period of cold static preservation or cold machine perfusion preservation. This is followed by a warm ischemic period during which the donor renal allograft vein and artery are connected to the recipient's external iliac vein and artery respectively in a retroperitoneal fashion. After reperfusion, the donor ureter is then anastomosed to the recipient bladder for reconstitution of normal urine drainage. Concurrently, dialysis was introduced as a treatment for end-stage renal failure in the 1960s and addressed its life-threatening complications (4). Dialysis, however, is unable to totally replace the electrolyte homeostasis and metabolic functions of a well-functioning kidney.

With the discovery of cyclosporine in the 1970s and further advances in transplant immunology since then, outcomes of kidney transplantation dramatically improved. Currently, graft survival after kidney transplantation is above 90% at 1 year and

above 80% at 3 years (5,6). A landmark paper by Wolfe et al. showed that after the initial risk associated with surgery, kidney transplantation offers better long-term survival in comparison to dialysis in end-stage renal disease patients, and improves quality of life. In addition, kidney transplantation was also shown to be more cost-effective than dialysis (7,8). Kidney transplantation has thus become the preferred therapy for end-stage renal disease patients.

Despite the success of modern kidney transplantation and immunosuppressive regimen, long-term graft survival has not tremendously improved. Graft survival at 5- and 10-year after kidney transplantation is still in the 75% and 50% range respectively (5). One of the potential reasons for this lack of improvement in long-term graft survival could be related to the development of delayed (DGF) and slow (SGF) graft function after kidney transplantation and their associated immunological consequences.

1.1. Delayed and slow graft function after kidney transplantation

1.1.1. Definition and epidemiology

Kidney transplantation involves the procurement of a kidney graft from a donor, the preservation of the donor kidney graft ex-vivo, and the transplantation of the donor kidney graft into a recipient. Acute kidney injury (AKI) to the kidney graft inevitably occurs during this process. Although diagnostic and staging criteria exist for AKI in the non-transplantation context, the special circumstances surrounding kidney transplantation led to different terms and criteria to describe the spectrum of AKI that occurs in the immediate post-transplantation period.

Based on the severity of the injury, AKI is subdivided into two entities in the immediate post-transplantation period: 1) delayed graft function (DGF), the most severe form of AKI most commonly defined as the need for dialysis within a week after transplantation (9), and 2) slow graft function (SGF), a milder form of AKI without dialysis requirement. The exact definition of SGF varies between centers, and has been defined as a decrease in 24-hour serum creatinine of less than 20% after transplantation at McGill University (10,11). Recipients who suffer insignificant AKI after kidney transplantation and have prompt return of their kidney graft function are said to have immediate graft function (IGF). At McGill University, IGF has been defined as a decreased in 24-hour serum creatinine of more than 20% after transplantation (10,11).

Of note, the most common definition of DGF, which is based on dialysis within the first week after transplantation, is imperfect. Dialysis initiation after transplantation can be due to hyperkalemia or fluid overload, and does not always indicate significant kidney damage (12). The threshold to initiate dialysis is also subjective and physician-dependent (12). Thus, a kidney transplantation recipient with SGF could potentially have worse kidney damage than a recipient with DGF. It is therefore pertinent to consider DGF and SGF not only as two separate entities but also as a whole since SGF resembles DGF more than IGF.

Definitions of DGF based on serum creatinine or urine output have been proposed but remain imperfect (9). With regards to serum creatinine, a large amount of renal mass can be lost without a significant increase in serum creatinine. Dialysis prior to transplantation can also falsely decrease serum creatinine post-transplantation. With regards to urine output, some recipients, especially those who receive pre-emptive kidney transplantation, still make a significant amount of urine before transplantation. Recipients can also preserve their urine output despite suffering from severe AKI post-transplantation and have inadequate renal clearance (non-oliguric renal failure) (9). DGF defined as the need for dialysis within a week after transplantation remains the accepted definition by the kidney transplantation community, which allows inter-study and inter-transplant center outcome reporting and comparison.

The incidence of AKI (DGF and SGF) to the kidney graft from a cadaveric donor in the immediate post-transplantation period surpasses 50%, making it the most common complication after kidney transplantation. The incidence of the most severe form, DGF, approaches 30 % in most contemporary studies. Based on United States national registry data, it has been rising from 14.7% between 1985 – 1992 to 27.3% between 2003 – 2012 despite progress in organ preservation as well as donor and recipient management (13,14). This can be explained by the recent increased use of marginal grafts from expanded criteria donors (ECDs) and donors after cardiac death (DCDs) in the context of organ availability shortage (12). The incidence of the milder form of AKI, SGF, has not been as well studied but varies between 19 to 26% in single institutional studies (10,11,15-17).

1.1.2. Short- and long-term consequences

Historically considered as a benign and self-limited reversible phenomenon, there is now evidence supporting the fact that AKI in the form of DGF or SGF is a significant contributor to not only worse short- but also long-term outcomes after kidney transplantation.

1.1.2.1. Medical and economic burden

Kidney transplantation recipients who develop DGF suffer the inconvenience of a prolonged hospitalization, increased complexity of their post-transplantation care, and delayed recovery. In a retrospective study of 170 kidney transplant recipients, those who developed DGF stayed hospitalized on average 10 days longer than those who did not (18). A larger U.S. Renal Data System database study including 38,966 kidney transplant recipients confirmed the previous finding as those who had DGF had a median length of stay of 17 days compared to 10 days for those who did not develop DGF (13). DGF has also been shown to be the most important risk factor for a prolonged hospitalization beyond 10 days after kidney transplantation (19). Moreover, recipients with DGF often require multiple sessions of dialysis, undergo multiple diagnostic radiological studies and renal allograft needle core biopsies to rule out other etiologies of renal dysfunction, and require frequent monitoring of their immunosuppressive drug regimen (20). Altogether, this leads to a substantial increased cost of approximately $25,000 US to care for a recipient who develops DGF in comparison to one who does not (21). This economic burden gets even worse if DGF-related complications occur, such as acute rejection, chronic allograft nephropathy, and graft loss (21). The economic burden of SGF has not been directly studied, but can be estimated to be closer to a recipient with DGF than with IGF. Similarly to a recipient with DGF, recipients with SGF also require additional medical work-up and frequent monitoring of their immunosuppressive drug regimen. SGF is also associated with the same DGF-related complications, such as

acute rejection, chronic allograft nephropathy, and graft loss. The association between DGF or SGF and these complications will be touched upon next.

1.1.2.2. Acute and chronic rejection

Apart from the increased post-transplant medical management complexity, recipient inconvenience, and economic burden, AKI in the form of DGF or SGF has also been shown to have important immunological consequences after kidney transplantation.

Although some studies from the 1990s found no association between DGF and acute rejection (22,23), a meta-analysis by Yarlagadda et al. including data pooled from 11 studies during the same transplantation era reported that recipients with DGF had a significantly higher incidence of acute rejection (24). In this meta-analysis, the pooled incidence of having an acute rejection episode was 49% in recipients with DGF compared to 35% in recipients without DGF (relative risk of 1.38) (24). Since the 1990s, immunosuppressive strategies have improved to minimize the risk of acute rejection. Data from the current contemporary transplantation era still support the findings in Yarlagadda et al.'s meta-analysis. In a modern single center cohort of 645 deceased donor kidney transplant recipients between 2000 and 2011, Wu et al. reported that the cumulative probability of a biopsy-proven episode of acute rejection was 16%, 21.8%, and 22.6% in recipients with DGF at 1, 3, and 5 years post-transplant respectively. This was significantly higher than the 1-, 3-, and

5-year cumulative probabilities of 10.1%, 12.4%, and 15.7% reported in recipients without DGF (25). This higher probability of acute rejection in recipients with DGF remained significant in multivariate analysis correcting for recipient, donor, and transplant factors. The adjusted relative hazard for an episode of acute rejection in recipients with DGF was 1.55 (25). Similar adjusted relative hazards were observed when categorizing acute rejection episodes into T-cell and antibody-mediated (25).

These previous studies categorized SGF, the milder form of AKI after kidney transplantation, in the non-DGF control group, and did not examine the association between SGF and acute rejection. There is evidence suggesting that SGF, although to a lesser extent than DGF, increases the risk of acute rejection. In a single center study of 896 adult deceased donor kidney transplant recipients between 1984 and 1999, the incidence of acute rejection at 1 year post-transplant was highest with DGF at 44%, followed by SGF at 38% and IGF at 28% (15). Similarly, in another single center study of 972 adult deceased donor kidney transplant recipients between 1990 and 2001, SGF was also in the middle of the pack in terms of its association with acute rejection with an incidence of 16.2% in comparison to 26.5% with DGF and 14.2% with IGF (26). One study including 237 kidney transplant recipients between 1993 and 2001 aimed to compare the incidence of acute rejection directly between recipients with SGF and IGF. This study showed a significant increase in acute rejection with SGF, with an incidence of 24.8% compared to 8.6% with IGF (17).

Studies looking at the association between AKI (DGF or SGF) and chronic rejection (also more recently termed as chronic allograft nephropathy) are more limited, but also suggest an increased incidence of chronic rejection with both DGF and SGF. With regards to DGF, a single center study of 1,534 kidney transplant recipients between 1997 and 2005 demonstrated a relative risk of 6.087 for chronic rejection in recipients with DGF in comparison to recipients without (27). Similar to DGF, SGF has also been associated with chronic rejection. Humar et al. showed in their single center study of 896 deceased donor kidney transplant recipients that those with SGF and DGF had significantly higher rates of biopsy-proven chronic rejection (41.1% and 38.1% respectively) in comparison to recipients with IGF (21.5%) (15). Of note, recipients with SGF and DGF had the same rate of chronic rejection (15). As demonstrated above, there is ample evidence that DGF and SGF are associated with an increased risk of acute and chronic rejection.

1.1.2.3. Graft and patient survival

1.1.2.3.1. Graft survival

Debates exist about whether DGF and SGF have a direct effect on graft survival after kidney transplantation since there is a long time interval between the inciting event of AKI and the outcome. Moreover, since DGF and SGF are associated with an increased risk for rejection and rejection is associated with a decrease in graft

survival, there has been controversy regarding whether DGF and SGF are directly harmful to the kidney graft longevity in the absence of rejection. Evidence however suggests that DGF and SGF have a direct impact on kidney graft survival independent of rejection.

Although a smaller study including 589 deceased donor kidney transplant recipients showed no association between DGF and graft survival (28), multiple studies show the contrary. Sanfilippo et al. reported a significant association between DGF and graft loss in a multivariate cox regression analysis of 3,800 deceased donor kidney transplantations performed in 41 centers between 1977 and 1982 (29). Similarly, Ponticelli et al. demonstrated a relative risk of 1.45 for late graft failure in a multivariate analysis of 864 deceased donor kidney transplant recipients who suffered from DGF with a functioning graft at 1 year post-transplant (30). Furthermore, in a meta-analysis by Yarlagadda et al. including data pooled from 21 studies, the relative risk of graft loss in recipients with DGF was 1.41 compared to those without DGF (24).

Studies examining SGF separately from DGF and IGF demonstrate that it is also associated with poorer kidney graft survival to a lesser degree than DGF. 5-year death-censored graft survival was evaluated by Humar et al. in 896 kidney transplant recipients and was significantly different between DGF (67%), SGF (72%), and IGF (89%) recipients (15). Similarly, in a single-center study by Johnston et al. including 972 kidney transplant recipients, 5-year graft survival was

significantly different between DGF (48.5%), SGF (60.5%), and IGF (75%) recipients, with graft half-life of 4.9 years, 8.7 years, and 10.5 years respectively. A smaller study by Hassanain et al. including 583 patients from 1990 to 2007 also shows worse 5-year graft survival in DGF (54%) and SGF (76%) compared to IGF (85%) recipients (11).

All the previous studies presented above looked at the association between DGF or SGF with kidney graft survival without factoring in the possible confounding effect of acute rejection. Both DGF and SGF have been shown to increase the risk of acute rejection, and acute rejection by itself is known to be detrimental to graft survival. To control for the effect of acute rejection on graft survival, several studies have therefore looked at the influence of DGF or SGF on graft survival either as a sole entity or in combination with acute rejection. In a single-center study of 457 kidney transplant recipients, Troppmann et al. found that DGF had no effect on 5-year graft survival in the absence of acute rejection, and that DGF acts in synergy with acute rejection to decrease graft survival (31). Larger database studies, however, found that DGF was associated with a decrease in graft survival independent of acute rejection. In an U.S. Renal Data System database study including 37,216 kidney transplant recipients between 1985 and 1992, Ojo et al. demonstrated that recipients with DGF alone had worse 1- and 5-year graft survival (74% and 53% respectively) than recipients with no DGF and no rejection (88% and 66% respectively) in adjusted Cox regression analysis (13). Recipients who suffered from both DGF and acute rejection again fared the worst, with a dismal 1- and 5-year

graft survival of 56% and 35% respectively (13). Similarly, an United Network for Organ Sharing/Scientific Renal Transplant Registry study by Shoskes et al. including 27,096 kidney transplant recipients between 1994 and 1997 also showed that DGF negatively impacted graft survival independent of acute rejection (32). In comparison to recipients with no DGF and no rejection, DGF alone reduced 3-year graft survival from 89 to 84% and graft half-life from 14.2 to 9.7 years (32). In the presence of an acute rejection episode within the first 6 months post-transplant, DGF also decreased 3-year graft survival from 77 to 60% and graft half-life from 9.4 to 6.2 years (32). Studies looking at SGF, the milder form of AKI immediately after kidney transplantation, are more limited but suggest that it negatively impacts graft survival as well. In a single-center study of 237 kidney transplant recipients, Park et al. showed that 1- and 5-year graft survival decreased from 100 and 98.6% to 93% and 85.4% respectively when comparing IGF to SGF recipients (17). 5-year graft survival in recipients with SGF alone (without acute rejection) was still decreased at 87.2% when compared to recipients with IGF (17). Just as with previous studies examining DGF, acute rejection acted in synergy with SGF to result in the poorest 5-year graft survival at 74% (17). In a study of 367 pediatric and adolescent kidney transplant recipients between 1990 and 2012, Lim et al. also studied the impact of SGF separately from DGF and IGF on graft survival. After adjustments for the effect of acute rejection in Cox regression model analyses, the hazard ratios for overall and death censored graft loss were 2.08 and 2.09 respectively with DGF, and 2.60 and 2.49 respectively for SGF (33).

1.1.2.3.2. Patient survival

Just as in graft failure, the association between DGF and patient survival is a subject of debate due to the long time interval between the inciting event and the outcome. Most remote and contemporary studies however have found no direct effect of DGF on patient survival with the caveat of a relatively short follow-up. In a single-center study of 437 deceased donor kidney transplant recipients performed between 1986 and 1996, Marcen et al. found that 6-year patient survival was similar between DGF (85.8%) and non-DGF recipients (85.1%) (34). Similarly, Woo et al. found that patient survival was not affected by DGF in 589 kidney transplant recipients from 1984 to 1993 with a median follow-up of 7 years (28). A meta-analysis of 12 studies examining the association between DGF and patient survival supported the previous studies as no significant difference in patient survival was demonstrated when comparing recipients with DGF to those without at 5 years follow-up (24). Studies including patients from a more contemporary era have mixed findings. Contemporary single center studies corroborate the findings of the aforementioned remote era studies. Fonseca et al. did not find any association between DGF and patient death with a median follow-up of 9.8 years when analyzing data from 1,281 deceased donor kidney transplants performed at their center between 1983 and 2012 (35). Similarly, in their single-center experience with 1,784 kidney transplant recipients performed between 1983 and 2014, Chaumont et al. found no difference in 10-year patient survival between recipients who suffered from DGF (86.8%) and those who did not (84.5%) (36). Butala et al., however, found the contrary when

they used an instrumental variable statistical model to analyze data obtained from the Scientific Registry of Transplant Recipients on 80,690 kidney transplant recipients between 1997 and 2010. The authors reported that the probability of 1- and 5-year mortality was increased by 7.11 and 11.03% respectively in recipients suffering from DGF compared to those who did not (37). Instead of investigating overall patient mortality, Tapiawala et al. looked at risk of death with a functioning graft in 50,246 kidney transplant recipients included in the United States Renal Data System database from 1998 to 2004. With a median follow-up of 36.1 months, recipient mortality with a functioning graft was more likely in the presence of DGF with an adjusted relative risk of 1.53 (38). The influence of SGF on patient survival has not been extensively studied, as most studies did not differentiate SGF from IGF. In a single-center study including 516 kidney transplant recipients from 1990 to 2006, Smail et al. found no difference in 10-year patient survival between DGF, SGF, and IGF recipients (10).

Overall, the majority of the literature supports the finding that DGF and SGF increase the risk for acute rejection and negatively impact long-term graft survival after kidney transplantation in an independent fashion. DGF may also increase patient mortality with a functioning graft. The impact of DGF and SGF on overall patient survival, however, has not been demonstrated in most studies with the following caveats. The follow-up time in these studies, which varies between 5 to 10 years, is relatively short to assess mortality. Moreover, more than half of the patient

mortality after kidney transplantation is secondary to cardiovascular disease, which is unlikely to be linked to DGF and SGF (28).

1.1.3. Clinical risk factors

The development and severity of AKI in the immediate period after kidney transplantation is influenced by several factors related to the donor, the preservation of the kidney graft, and the recipient. Although the majority of the literature has focused on DGF, both DGF and SGF in reality represent a continuous spectrum of AKI that share the same risk factors for their development.

1.1.3.1. Donor factors

Several donor characteristics are known to affect the quality of the retrieved kidney allografts, and therefore influence whether DGF or SGF will develop in the intended kidney transplant recipient. First of all, the modality of organ procurement from the donor plays a major role in the risk stratification for DGF and SGF. Kidney allografts can be retrieved from deceased or living donors. Deceased donors are further categorized based on whether the kidney allograft recovery was performed after brain death or cardiac death. Kidney allografts from living donors are at the lowest risk of causing DGF, with a reported frequency varying between 1 – 10%, with an average of 5% (39-41). This is in contrast with an incidence approaching 30% for

deceased donors (25% for donation after brain death (DBD), 50% for donation after cardiac death (DCD)) (14,42). In comparison to a living donor, brain death in a deceased donor induces in itself a dynamic deranged physiological state that begins long before the surgical trauma of organ recovery. Due to an increase in intracranial pressure and the acute stress of brain death, nervous, hormonal, and inflammatory changes occur that negatively impact the future retrieved kidney graft quality. Following brain death and its associated intracranial hypertension, both the parasympathetic and sympathetic nervous system are deregulated. Once the brainstem becomes ischemic, a catecholamine storm ensues leading to an increase in vascular tone and resistance in an attempt to restore cerebral perfusion by increasing systemic arterial blood pressure above the elevated intracranial pressure. This compensatory mechanism unfortunately sacrifices renal blood flow, resulting in an ischemic damage to the potential renal allografts. When brain death is further prolonged, the spinal cord ultimately becomes ischemic leading to a deactivation of the sympathetic nervous system, relative hypotension, decrease in cardiac output, and systemic/renal hypoperfusion (43). Hormonally, there is a gradual decrease in adrenocorticotropic, anti-diuretic, and free-circulating triiodothyronine hormones. These hormonal changes lead to diabetes insipidus (anti-diuretic hormone deficiency) and hypovolemia, resulting again in a decrease in renal blood flow and an ischemic injury to the potential renal allografts (43). The acute stress of brain death also provokes an inflammatory state in which adhesion molecules and pro-inflammatory cytokines are upregulated, promoting leukocyte influx into the potential kidney allografts and increasing their propensity to DGF or

SGF (43). Moreover, brain death by itself was shown to produce AKI-related degenerative changes in the potential kidney graft structure on histologic examination of renal biopsies prior to organ retrieval (44). In comparison to DBD, the risk of DGF has been reported to be 3 times higher with DCD with a frequency surpassing 50% (42,45,46). The majority of DCD in the USA and Canada occurs in patients who have irreversible neurological injuries but do not meet all the criteria for brain death declaration (47). Consequently, DCDs have similar nervous, hormonal, and inflammatory changes than DBDs in addition to extra injury from the inherent warm ischemic time associated with the DCD organ retrieval process. The process of organ retrieval after cardiac death first involves withdrawal of care. This is followed by a period of hypotension, hypoxia and inadequate renal perfusion until complete cessation of peripheral blood flow. A mandatory stand off period (5 minutes in most jurisdiction) of warm pulseless ischemia is then respected before the organ retrieval surgery is initiated with the goal of rapid cold perfusion flushing of the intra-abdominal organs (47). This prolonged period of warm ischemia time inherent to the DCD process puts the potential kidney allografts at further risk of DGF and SGF than DBD.

Donor demographics also play an important role in the risk stratification for DGF and SGF. In an effort to circumvent the shortage in available organs for a growing kidney transplant waiting list, kidney allografts retrieved from donors considered marginal are increasingly being used. These marginal donors have been termed expanded criteria donors (ECDs). They are defined as donors either aged 60 years

and older, or aged 55 – 59 years with at least 2 of the following features: a history of hypertension, a terminal serum creatinine above 1.5 mg/dL (133 mmol/L), or a cerebrovascular etiology of death (48). These criteria were chosen based on an increased risk of graft failure by 70% relative to standard criteria donors (48). Recipients of an ECD kidney, nevertheless, benefit from a survival advantage over remaining on dialysis (49). The prevalence of DGF after receiving an ECD kidney (35%) is higher than for a standard criteria donor kidney (25%) (45). This is explained by the fact that several criteria defining ECDs have been shown to be some of the most significant risk factors associated with DGF. Donor age above 50 has been shown to increase by 2 to 3 times the risk for both DGF and SGF (15,39). Based on data from 24,653 adult kidney transplant recipients between 2003 and 2006, the odds ratio of developing DGF was 1.017 for each year increase in donor age starting at 16 years old (45). In the same study, donor terminal serum creatinine was also an important predictor for DGF. Each 1 mg/dL increase in donor terminal serum creatinine raised the odds of developing DGF by 69.3% (45). A donor history of hypertension and a cerebrovascular etiology of death also increased the risk for DGF, with odds ratio of 1.303 and 1.210 respectively (45). Other donor demographics that were predictive of DGF in that study were anoxia as the etiology of death and a lower donor weight (45). The donor's hemodynamic status was also shown to influence the rates of DGF. Marshall et al. found that recipients of a kidney allograft retrieved from a donor requiring inotropic support had lower rates of immediate graft function (58%) than those with a kidney allograft retrieved from a donor without inotropic support (83%) (50).

1.1.3.2. Preservation factors

Preservation factors, mainly the duration and methods of kidney allograft preservation, influence the rates of DGF and SGF after kidney transplantation as well. Cold ischemic time (CIT) is defined as the period between initiation of cold perfusion in the donor and reperfusion to a physiological temperature after implantation of the kidney allograft in the recipient. A prolonged CIT was shown to be one the most important factor associated with an increased incidence of DGF and SGF. In a U.S. Renal Data System study including 37,216 cadaveric kidney transplants between 1985 and 1992, every 6-hour increase in CIT was demonstrated to increase the risk of DGF by 23% (13). Similarly, CIT had an independent effect on DGF and SGF in a multivariate logistic regression analysis of 972 kidney transplant recipients between 1990 and 2001 (26). More recently, in the development of a risk prediction model for DGF using data from 24,653 kidney transplant recipients in the United Network for Organ Sharing/Organ Procurement and Transplantation Network between 2003 and 2006, Irish et al. found that each 1 hour increase in CIT heightened the risk of DGF by 4.1% (45). Moreover, in an analysis of 9,082 paired-deceased donor kidneys from the Scientific Registry of Transplant Recipients from 2000 to 2009, the odds ratio of developing DGF was 1.81 for a difference in CIT of more than 1 hour between 2 recipients receiving a kidney from the same donor. The odds ratio increased to 2.5, 3.3, and 4.4 when the difference in CIT was more than 5 hours, 10 hours, and 15 hours respectively (51).

There are currently two accepted clinical methods to preserve a future kidney allograft during the CIT: cold static storage or hypothermic pulsatile machine perfusion. In cold static storage, the future kidney allograft is stored ex-vivo in a bag of preservation fluid on ice after flushing it out of blood via the renal artery with chilled preservation fluid in situ and on the back table (52). On the other hand, in hypothermic pulsatile machine perfusion storage, after flushing out the blood from the future kidney allograft in situ and on the back table, the renal artery is cannulated and cold preservation fluid is administered in a pulsatile fashion within a pump system (52). The method selected to preserve the future kidney allograft has been shown to affect the risk of DGF, especially for marginal organs. In an European randomized control trial comparing 336 consecutive paired kidneys from the same deceased donor with one undergoing machine perfusion and the other cold static storage, machine perfusion was shown to significantly reduce the incidence of DGF from 26.5% to 20.8%, equaling to an adjusted odd ratio of 0.57 (53). For kidneys that suffered from DGF, machine perfusion also significantly reduced the duration of DGF from 13 to 10 days as compared to cold static storage (53). Similarly, in an American study analyzing the United Network for Organ Sharing database from 2005 to 2011, machine perfusion also reduced significantly the risk for DGF in both a propensity matched cohort analysis (13,293 patients in each group) and a paired kidney analysis from the same donor in which one underwent machine perfusion and the other cold static storage (2,290 pairs) (54). A meta-analysis including 1,475 kidneys from 7 randomized controlled trials further confirmed the above findings, as machine perfusion was shown to provide a relative

risk of 0.81 for DGF when compared to cold static storage (52). With regards to kidney allografts from higher risk donors specifically (ECDs and DCDs), hypothermic pulsatile machine perfusion has also been shown to provide a benefit in reducing DGF compared to cold static storage. In a meta-analysis of 7 studies including 2,374 machine perfused and 8,716 cold stored kidneys from ECDs only, machine perfusion reduced the risk of DGF by 41% when compared to cold static storage (55). Although individual small randomized controlled trials yielded conflicting results regarding the benefit of machine perfusion for kidneys retrieved from DCDs specifically, a meta-analysis of 4 randomized controlled trials showed that machine perfusion reduced the risk of DGF by 44% when compared to cold static storage (56).

1.1.3.3. Recipient factors

Recipient factors were also shown to influence whether DGF and SGF develop after kidney transplantation. The use and modality of maintenance dialysis prior to kidney transplantation are known great contributors to the development of DGF. In comparison to recipients who undergo kidney transplantation pre-emptively (prior to initiation of maintenance dialysis), recipients who are on maintenance dialysis prior to kidney transplantation had an adjusted odds ratio of 1.58 to develop DGF in a large multivariate paired kidney analysis where the 2 recipients from the same donor were discordant in the occurrence of DGF (57). Maintenance dialysis can be administered by 2 main techniques: peritoneal dialysis and hemodialysis. In a large

United Network for Organ Sharing registry study including 9,291 kidney transplant recipients between 1994 and 1995, hemodialysis was shown to be associated with a higher risk of DGF than peritoneal dialysis. DGF developed in 28.6% of recipients who were on maintenance hemodialysis as opposed to only 20% in recipients who were on maintenance peritoneal dialysis, yielding an odds ratio of 1.60 for developing DGF with hemodialysis (58).

Diabetes as the etiology of end-stage renal disease also has an important impact on the development of DGF after kidney transplantation. In a large paired analysis of 51,046 kidney transplant recipients in which one recipient had diabetes and the other one from the same donor did not, the odds ratio of developing DGF was 1.66 for male and 1.28 for female diabetic recipients (59). Similarly, Doshi et al. found that recipients with diabetes had an adjusted odds ratio of 1.37 to develop DGF in comparison to non-diabetic recipients (57). Although it is unclear how diabetes heightens the risk for DGF, diabetic recipients often have atherosclerotic vessels, leading to an increase in technical difficulty and anastomosis time during the kidney transplantation surgery. The potential longer warm ischemia time in diabetic recipients may explain a higher incidence of DGF. On the same line, due to atherosclerosis, diabetic recipients are at increased risk of peri-operative cardiac compromise, which may reduce adequate renal blood flow and jeopardize early kidney allograft function. Diabetes is also known to create a chronic inflammatory state, which could promote molecular mechanisms responsible for DGF (59).

Recipient obesity was also found to be important in the development of DGF after kidney transplantation. It is hypothesized that the large body habitus of the recipient tends to increase the technical difficulty and anastomosis time during the kidney transplantation surgery, leading to a potentially longer warm ischemia time and higher incidence of DGF. In an analysis of 51,927 kidney transplant recipients from the United States Renal Data System between 1988 and 1997, an obese body mass index of 36 kg/m^2 or above conferred a relative risk of 1.51 to develop DGF (60). A more recent analysis of the United Network of Organ Sharing database between 1995 and 2004 corroborate the above findings, as an obese body mass index of 30 kg/m^2 or above conferred an adjusted odds ratio of 1.41 to develop DGF (57). Similarly, another United Network of Organ Sharing database analysis between 2003 and 2006 found that each 1 kg/m^2 unit increase in body mass index was associated with an odds ratio of 1.043 to develop DGF (45).

The sensitization status of the recipient prior to kidney transplantation also has a significant impact on the development of DGF and SGF. Sensitization usually occurs when a recipient was previously exposed to foreign tissue, such as after a pregnancy, a previous transplant, or a blood transfusion, and produce antibodies directed against it. The panel reactive antibody is commonly used to assess sensitization level as it indicates the proportion of the population to which a recipient will react via pre-existing donor-specific antibodies. In a study of 124 kidney transplant recipients between 1991 and 1994, Monteiro et al. showed that a higher sensitization level assessed by the panel reactive antibody was associated

with DGF (61). The incidence of DGF was indeed 90%, 45%, and 27% for highly sensitized recipients with a panel reactive antibody above 50%, moderately sensitized recipients with a panel reactive antibody 10 to 50%, and non-significantly sensitized recipients with a panel reactive antibody less than 10% respectively (61). Similarly, Lopez-Hoyos et al. also showed that recipients with a higher panel reactive antibody had a higher incidence of both DGF and SGF compared to IGF (62). Larger database studies further corroborate these previous findings as Doshi et al. found that recipients with a panel reactive antibody above 50% had an adjusted odds ratio of 1.28 to develop DGF and Parekh et al. found that each 10% increase in panel reactive antibody increased the risk of DGF by 6% (57,59).

Multiple other recipient variables were also shown to influence DGF and SGF either to a lesser level or with lower level of evidence. These include male gender, African-American race, and a recipient-donor size mismatch. Recipient hypovolemia is also detrimental to early graft function as intra-operative fluid resuscitation of the recipient guided by measurement of their central venous pressure can reduce the occurrence of DGF (63,64).

In the current context of kidney transplantation, most of the aforementioned donor, preservation, and recipient factors that increase the risk for DGF and SGF are unfortunately not easily modifiable. For example, the ideal scenario to prevent DGF would be to perform a pre-emptive kidney transplantation using a young healthy living donor with minimum cold ischemic time in a non-diabetic non-obese and non-

sensitized recipient. This ideal scenario is however rare and most recipients will receive a deceased donor kidney allograft with an unpredictable amount of CIT due to organ sharing between geographically distant centers after they are already on dialysis. Understanding the cellular mechanisms underlying DGF and SGF in more details would therefore be useful. This would potentially allow the prediction, prevention or treatment of DGF and SGF, which have significant short- and long-term consequences after kidney transplantation.

1.2. Cellular mechanisms of injury involved in delayed and slow graft function after kidney transplantation

1.2.1. Cellular mechanisms of injury

The cellular mechanisms occurring during ischemia-reperfusion injury (IRI) are thought to be principally responsible for the development of AKI (DGF and SGF) after kidney transplantation (39). Renal IRI is intrinsic to the kidney transplantation process. Ischemia begins in the donor with impairment or interruption of blood flow to the future kidney allograft. Oxygen delivery to the kidney is dependent on the transport of hemoglobin from the peripheral circulation to the renal microvasculature (12). In the case of deceased DBD, hemodynamic disturbances that reduce blood flow to the kidney occur secondary to the physiologic changes of brain death in the donor (warm ischemia) prior to complete interruption of blood flow at aortic cross clamping and cold perfusion (cold ischemia). In the case of deceased DCD, there is a prolonged duration of warm ischemia with the hemodynamic disturbances occurring during withdrawal of care and the complete interruption of blood flow during the mandatory standoff period before quickly cannulating and flushing the aorta with cold perfusion solution to start the CIT. In both cases of deceased donation (DBD and DCD), cold ischemia continues during the storage of the retrieved kidney and transport to the recipient surgery center. In the case of a living donor, warm ischemia starts at the interruption of blood flow to the renal artery at the end of the living donor nephrectomy surgery. This is usually

followed by a much shorter period of cold ischemia once the kidney is flushed with cold perfusion solution and stored on ice since the recipient is most of the time at the same center as the donor. Regardless of donor type, upon finishing the kidney allograft renal vein and artery anastomoses to the recipient external iliac vein and artery, blood flow is reinstituted to the kidney and the reperfusion stage begins. Although not completely understood, several cellular mechanisms are altered first during the ischemia stage in which there is a reduction in oxygen and nutrient delivery leading to a switch from aerobic to anaerobic metabolism. These alterations in cellular mechanisms are further accentuated upon return to normoxia during the reperfusion stage. IRI is in fact a complex inflammatory syndrome classically involving the depletion of adenosine triphosphate, the generation of reactive oxygen species, the dysfunction of the renal microvascular system, the up-regulation of adhesion molecules, the production of cytokines/chemokines, the activation of the innate immunity and complement cascade, and the implication of cell death pathways (12,39).

1.2.1.1. Depletion of adenosine triphosphate

Early following renal ischemia, the cessation of blood flow to the kidney leads to oxygen deprivation and the subsequent switch from aerobic to anaerobic metabolism (65). Intracellular adenosine triphosphate (ATP) is quickly depleted with hypoxia, as anaerobic metabolism fails to meet the demand of the aerobic renal tissues. ATP is degraded to adenosine diphosphate and adenosine monophosphate,

and adenosine monophosphate is further metabolized to adenine nucleotides and hypoxanthine (66). Several molecular events resulting from the depletion of ATP contribute to kidney tissue damage starting during the ischemia phase and continuing during reperfusion.

In a hypoxic environment, anaerobic glycolysis initially replaces oxidative metabolism in an attempt to restore ATP levels for proper cellular function. Anaerobic glycolysis requires nicotinamide adenine dinucleotide, which is produced by the conversion of pyruvate to lactate. The excessive accumulation of lactate during anaerobic glycolysis subsequently decreases intracellular pH to acidotic levels. Normalizing the pH in previously ischemic acidotic cells upon reperfusion with oxygenated blood paradoxically promotes cell killing (65,67).

ATP depletion also leads to electrolyte disturbances that ultimately cause renal tissue damage. Due to a lack in ATP, the membrane-bound Na+/K+-ATPase pump becomes dysfunctional (68). This promotes cell edema due to a large increase in intracellular Na+ and water. In addition, there is an overload of intracellular calcium secondary to the inability of the dysfunctional Na+/K+-ATPase pump to extrude calcium into the extracellular space. The intracellular calcium overload is further exacerbated by an impaired calcium sequestration within the endoplasmic reticulum as calcium is released to promote actin myosin coupling (12,66). The accumulation of excessive intracellular calcium activates calcium-dependent proteases, phospholipases, and endonucleases that promote cell apoptosis (69).

Intracellular calcium overload also generates reactive oxygen species at the mitochondrial level, which contribute to cell damage (70).

Finally, the degradation of ATP down the road to hypoxanthine and adenine nucleotides also plays a role in renal tissue damage. The accumulation of hypoxanthine contributes to the generation of reactive oxygen species. On the other hand, adenine nucleotides indirectly play a role. During ischemia, adenine nucleotides freely diffuse out of the cells, and their depletion precludes the re-synthesis of intracellular ATP upon reperfusion with oxygenated blood (66).

1.2.1.2. Reactive oxygen species generation

As described in the previous section, there is an accumulation of hypoxanthine from the degradation of ATP secondary to oxygen deprivation during ischemia. Upon reperfusion with oxygenated blood, the accumulated hypoxanthine is key in the generation of reactive oxygen species at high concentrations in previously ischemic renal tissues (66). Free iron also accumulates during ischemia as the capacity to catabolize higher levels of volatile iron-containing cytochromes to inert compounds by heme oxygenase-1 (HO-1) is overcome and ferroportin loses its efficiency to transport free iron into the extracellular space. Free iron acts as a strong catalyst in reactions that generate free oxygen species (39,71).

Xanthine oxidase catalyzes the conversion of the accumulated hypoxanthine to xanthine. Xanthine subsequently generates hydrogen peroxide and superoxide. First, in the presence of free iron derived from injured cells, hydrogen peroxide forms the highly reactive and cytotoxic hydroxyl radical. Secondly, nitric oxide generated by ischemic tubular cells interacts with superoxide to form the reactive peroxynitrite. The end result of the generation of these reactive species is cell damage via oxidation of proteins, peroxidation of lipids, DNA damage, and induction of apoptosis (66). This is exacerbated by the fact that naturally occurring antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are present in the kidney to counteract the actions of reactive oxygen species under normal conditions. In the context of renal IRI, the antioxidant enzymatic system is however downregulated and antioxidant enzymes are overwhelmed by the rapid generation of reactive oxygen species in high concentrations (72,73).

1.2.1.3. Microvascular dysfunction

The endothelial cells lining the renal microvasculature play an important role in controlling vascular tone and smooth muscle responsiveness after renal IRI (74). Secondary to ischemia, endothelial cells are injured with a subsequent change in their structural integrity. They undergo a loss of their glycocalyx, a breakdown of their actin cytoskeleton, and a disruption in their junctional complexes. Ultimately, endothelial cell swelling, blebbing, death, and detachment occur. Sites of vascular

endothelium denudation have increased vascular permeability, consequently causing fluid losses in the renal interstitium (66). The resulting local edema exacerbates the heightened susceptibility of injured endothelial cells to vasoconstriction from an imbalance of local vasoactive substances described below.

As a matter of fact, injured endothelial cells release various vasoactive substances. Renal IRI induces the endothelial production of the vasoconstrictor endothelin-1, a cognate ligand for vascular-specific G protein coupled receptors. The excessive activation of these receptors by endothelin-1 released by the vascular endothelium provokes pathological vasoconstriction. At the same time, during IRI, the renal arterioles have increased reactivity to vasoconstrictor signals such as angiotensin II, thromboxane A2, prostaglandin H2, leukotrienes C4/D4, adenosine, and the sympathetic nervous stimulation. In parallel, there is a decreased in the production of the vasodilator substance nitric oxide by the injured endothelium during the reperfusion phase. Endothelial cells also have a reduced vasodilatory response to other substances such as acetylcholine and bradykinin. The final result is an imbalance favoring a vasoconstrictive over a vasodilatory effect (74-78).

1.2.1.4. Cell death pathways

Renal IRI causes a mismatch between oxygen and nutrient supply and waste product removal. When severe enough, it activates two main cell death pathways,

apoptosis and necrosis, which dictate the fate of injured renal cells, especially tubular epithelial cells (74).

Apoptosis is generally considered a normal physiologic process with the goal of removing damaged cells in a quiet and orderly fashion (66). In the context of IRI, apoptosis however contributes to renal dysfunction as it removes previously healthy cells as early as 6 hours after the insult (66). Two major apoptotic pathways are activated after renal IRI. The extrinsic apoptotic pathway results in the activation of caspase 8 after activation of the plasma membrane Fas receptor and signal transduction through the Fas-associated protein with death domain (66,79). The intrinsic apoptotic pathway is induced by the translocation of Bax to the mitochondria, which forms pores for the release of cytochrome c and the subsequent activation of caspase 9 (66). Bax is also induced by p53-dependent pathways and Bid activation (66,80). The later, Bid, also serves as a crosstalk between the extrinsic and intrinsic pathways (81). On the other hand, Bcl2 prevents the induction of Bax but is not upregulated in renal IRI (66,82). The final execution step of both pathways is the activation of caspase 3, which leads to a programmed cell demise typified by cytoplasmic and nuclear shrinkage, DNA fragmentation, and cell breakdown into apoptotic bodies that are cleared by phagocytosis (66). Caspases also can directly induce renal tissue inflammation and stimulate the immune system, thereby further enhancing a cycle of tissue damage after ischemia-reperfusion (65,83).

Necrosis usually occurs when renal tubular epithelial cells undergo a more severe ischemic injury than during apoptosis. As opposed to apoptosis, necrosis is a chaotic process that happens even in the absence of ATP and is characterized by loss of cell membrane integrity, cytoplasmic swelling, and cellular fragmentation. Necrosis and apoptosis are not mutually exclusive, and therefore the two cell death pathways can co-exist. The resulting sloughing of necrotic renal tubular epithelial cells produce intraluminal casts that can obstruct tubular flow and contribute to renal dysfunction (66). Just like caspases, the classical effectors of apoptosis, necrosis also contributes to increasing ischemia-reperfusion renal tissue damage by promoting inflammation and the activation of the immune system (65).

1.2.1.5. Up-regulation of adhesion molecules, production of cytokines/chemokines, and activation of the innate immune system

Renal IRI provokes a myriad of changes in endothelial and tubular epithelial cells that ultimately activate the innate immune system. The end result is an inflammatory milieu that promotes tissue damage.

On top of being involved in renal microvascular dysfunction, endothelial cells are important in attracting innate immune cells, especially neutrophils and macrophages, to the kidney. Following renal IRI, endothelial-leukocyte interactions are enhanced. The expression of the adhesion molecules P-selectin, intercellular adhesion molecule 1 (ICAM-1), and platelet-endothelial cell adhesion molecule 1

(PECAM-1), is increased on endothelial cells and plays an important role in the infiltration of leukocytes in the kidney. The upregulated P-selectin expression on endothelial cells first induces leukocyte rolling by its interaction with P-selectin glycoprotein 1 on leukocytes. Next, leukocyte adherence is promoted by the upregulated endothelial ICAM-1 that interacts with the leukocyte β2 integrins CD11a/CD18 and CD11b/CD18. Leukocyte transmigration into the renal interstitium is then facilitated by PECAM-1 (84,85). The up-regulation of the previous adhesion molecules therefore increase the infiltration of activated leukocytes in the kidney, which in turn increase tissue damage by several mechanisms including the obstruction of the microvasculature, the production of cytotoxic enzymes, the generation of reactive oxygen species, and the secretion of pro-inflammatory cytokines (74).

Damaged renal tissue secondary to an ischemic injury also massively releases damage-associated molecular patters (DAMPs) such as hyaluronic acid, fibronectin, heat shock proteins, and DNA (86). In parallel, renal tubular epithelial cells increase their constitutive expression of toll-like receptors (TLRs) after ischemia-reperfusion, especially TLR2 and TLR4 (87-89). TLRs are also expressed on neutrophils and macrophages (90,91). TLRs are an evolutionary conserved family of transmembrane pattern recognition receptors that recognize not only exogenous microbial products, but also DAMPs, which are endogenous ligands released from damaged tissue. The activation of TLRs by DAMPs provokes the activation of cell death signaling pathways. Moreover, activation of TLRs results in the production of

multiple pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, CCL2, MIP-2, and keratinocytes-derived chemokines. This occurs via signal transduction of adaptive proteins, mainly myeloid differentiation primary response 88 (MyD88), and the subsequent activation of the transcription factor NF-κB (86). The activation of NF-κB is also involved in the generation of reactive oxygen species and apoptotic signaling (92,93). The increased in pro-inflammatory cytokines and chemokines secondary to the activation of TLRs is accompanied by an increased infiltration of leukocytes, consequently causing inflammation and damage to the kidney after IRI (86).

The leukocytes involved in the infiltration of the kidney and causing damage after ischemia-reperfusion are traditionally thought to be part of the innate arm of the immune system. These innate immune cells contributing to renal IRI mainly include neutrophils, macrophages, dendritic cells, and natural killer T cells.

Neutrophils were shown to accumulate first in the kidney after reperfusion of the kidney. In both animal and human IRI, they infiltrate as early as 30 minutes after reperfusion, especially in the peritubular capillary of the outer medulla and the interstitium. This occurs following rolling, adhesion, and transmigration secondary to their enhanced interaction with endothelial cells as discussed above. Neutrophils are responsible for reducing endothelial and tubular epithelial cell integrity by the generation of proteases, myeloperoxidase, reactive oxygen species, and pro-inflammatory cytokines. They can also cause capillary plugging and interfere with

restitution of blood flow upon reperfusion of the kidney in the context of IRI (66,86,94).

Both macrophages and dendritic cells derive from a common precursor, the monocytes. Monocytes patrol the endothelium and are recruited to the injured kidney where they differentiate into macrophages in the presence of IFN-γ and IL-6 or dendritic cells in the presence of TNF-α, IL-4, and IL-15. Following renal IRI, macrophages infiltrate the kidney after neutrophils. Their number starts to increase at 1 hour after reperfusion, peaks at 24 hours, and persists for 7 days (95). Their transmigration from the peripheral blood to the renal interstitium is guided by several chemokines, especially CX3CL1 and CCL2. After renal IRI, endothelial cells increase their expression of CX3CL1. CX3CL1 is important in macrophage transmigration to the injured kidney, as it is the ligand for CX3CR1, a membrane-bound receptor expressed in abundance on macrophages. CCL2 is released following the activation of TLRs on tubular epithelial cells, and bind to the receptor CCR2 expressed on macrophages to guide their transmigration to the kidney. In murine models of renal IRI, CCR2 and CX3CR1 deficiencies were protective against kidney damage (86,96-98). Depending on the local environment, macrophages can differentiate into inflamed 'M1' macrophages or pro-repair 'M2' macrophages. The inflamed 'M1' macrophages are responsible for causing tissue damage after renal IRI by producing a large amount of reactive oxygen as well as a wide array of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1α, and IL-12. This was demonstrated in a murine model in which depletion of macrophages prior to renal

IRI using liposomal clodronate decreased acute kidney damage while adoptive transfer of macrophages restored the kidney damage. Depletion of macrophages later after renal IRI, however, was detrimental, as pro-repair 'M2' macrophages are removed (99-104). Dendritic cells are professional antigen presenting cells that are activated during renal tubular injury. They are the most abundant leukocyte subset in the kidney (105). Once activated following renal IRI, dendritic cells contribute to kidney damage by in turn activating naïve T cells through antigen presentation, co-stimulation, and cytokine production. Dendritic cells also in turn activate natural killer T (NKT) cells by presenting them with glycolipids via CD1d molecules and by making contact with them via CD40 – CD40L interaction. In mice transgenic for the human diphtheria toxin receptor, injection of diphtheria toxin prior to renal IRI primarily killed dendritic cells and resulted in less severe renal tubular cell necrosis as well as a lower rise in plasma creatinine compared to control mice (86,95).

NKT cells are a unique population of lymphocytes that share properties of both natural killer (NK) cells and T cells. NKT cells express both the NK cell-associated marker NK1.1 and a T cell receptor that does not recognize peptides presented by MHC I or MHC II molecules. They recognize lipids and glycolipids that are presented by CD1d molecules (106,107). NKT cells start to infiltrate the kidney 30 minutes following reperfusion and cause damage by linking the innate and adaptive immunity via the amplification of dendritic cell function and the production of pro-inflammatory cytokines. In renal IRI, blockade of NKT cells using anti-CD1d or anti-NK1.1 antibodies protected against acute kidney damage (108).

The complement cascade, which is an integral part of innate immunity, also contributes to kidney damage after renal IRI. The complement cascade can be activated by ischemic renal cells and cells undergoing apoptosis. Three pathways of complement activation have been described, the classical pathway, the lectin pathway, and the alternate pathway. The classical pathway was the first discovered and is triggered by antibodies. The lectin pathway, on the other hand, is activated by lectin-type proteins that bind to carbohydrates on the surface of pathogens. The alternate pathway, which has been described in the microbiology context as being activated by the presence of a pathogen alone, is the predominant complement cascade pathway activated during renal IRI (109,110). Renal tubular epithelial cells express the complement inhibitor Crry, which regulates the C3/C5 convertase step of the complement cascade. During renal IRI, Crry is redistributed away from the basolateral membrane of renal tubular epithelial cells. This alteration in Crry localization permits activation of the complement cascade via the alternate pathway and subsequent C3 deposition in the kidney (111). C3 participates in the maturation of dendritic cells (112). Moreover, completion of the complement cascade leads to the formation of the membrane attack complex (C5b-9) and C5a. The membrane attack complex damage renal cells by creating pores in cell membranes and causing cell lysis. Additionally, C5a acts as a chemotactic agent (113). Chemotaxis of neutrophils and macrophages to the injured kidney is further enhanced by the complement-mediated stimulation of tubular epithelial cells to produce chemokines and the complement-mediated upregulation of adhesion molecules on endothelial cells (114). Selective inhibition of the alternate

complement pathway in a murine model of renal IRI confirmed its role in promoting renal tissue damage (110).

1.2.2. Experimental diagnostic tests and therapeutic interventions based on cellular mechanisms of injury involved in DGF and SGF

1.2.2.1. Experimental diagnostic tests based on cellular mechanisms of injury involved in DGF and SGF

The current diagnosis of DGF and SGF after kidney transplantation relies on the requirement for post-transplant dialysis and the serial measurement of post-transplant serum creatinine. The use of these factors as the accepted standard for the diagnosis of DGF and SGF is however problematic. The threshold to initiate dialysis post-transplant is subjective as it can be physician- and center-dependent. Moreover, in certain recipients, dialysis is initiated post-transplant for reasons unrelated to renal IRI such as pulmonary edema and hyperkalemia. For recipients already on pre-transplant dialysis, the timing of their last dialysis treatment prior to their kidney transplantation can also influence their need for dialysis post-transplant. With regards to serum creatinine, it is the clinical gold standard test to assess kidney function and estimate glomerular filtration rate since the mid 1900s in all patients regardless of transplant status (115). Nevertheless, serum creatinine remains imperfect for multiple reasons. First, various patient factors are known to

alter serum creatinine levels including gender, hydration status, diet, medications, and muscle mass. A change in serum creatinine therefore does not necessarily reflect renal tubular injury. Secondly, the timing between actual kidney injury and an elevation in serum creatinine level is asynchronous. An elevation in serum creatinine is in fact only observed when more than 50% of the kidney function fails (116). Due to the aforementioned limitations of dialysis requirement and serum creatinine in assessing kidney injury, there has been interest in discovering novel biomarkers to predict or diagnose DGF and SGF after kidney transplantation using either blood or urine samples. A 'shotgun' gene microarray approach has yielded promising biomarkers involved in the cellular mechanisms of renal IRI for the prediction of DGF and SGF, but none are performing well enough or consistently reproducible to be included in standard clinical practice (117-119).

Neutrophil gelatinase-associated lipocalin (NGAL) is the most promising and studied biomarker to date for DGF, although it lacks the accuracy and reproducibility for standard clinical practice. From murine gene microarray evaluation, it is the most upregulated gene in the kidney after an ischemic renal injury (120). NGAL is excreted by the epithelial cells of the ascending loop of Henle and connecting tubules, and subsequently accumulates in the urine and blood after renal IRI (116,121). NGAL is also produced by activated neutrophils, which infiltrate the kidney after ischemia-reperfusion and are involved in mediating injury (122). Although the mechanism of action of NGAL is not completely understood, NGAL is known to inhibit apoptosis (123). NGAL also prevents the formation of radical

oxygen species by sequestering iron via siderophores (121). In the kidney transplantation setting, NGAL staining of kidney allograft protocol biopsies taken 1 hour after reperfusion was most intense in recipients eventually developing DGF (124). As a biomarker, urine and plasma/serum NGAL from either the recipient or donor have been studied with various results. With regards to recipient urine NGAL, Parekh et al. first described in a small cohort of 53 kidney transplant recipients its promising utility when measured within the first post-transplant day in the prediction of DGF with an area under the curve (AUC) of 0.9 in receiver operating characteristic (ROC) curve analysis (125). In a larger multicenter cohort study of 91 deceased donor kidney transplant recipients initiated by the same group, the accuracy of recipient urine NGAL at post-operative day 1 to predict DGF remained, but dropped to the moderate range (AUC = 0.82) (126). A larger single center study of 176 kidney transplant recipients corroborated these more modest results, as post-transplant day 1 recipient urine NGAL predicted DGF with an AUC of 0.75 in ROC curve analysis (127). Moreover, Pajek et al. observed that post-transplant day 1 recipient urine NGAL was also predictive of AKI (DGF and SGF) after kidney transplantation (AUC = 0.82) in a cohort of 71 kidney transplant recipients (128). Nevertheless, Buemi et al. failed to show that post-transplant recipient urine NGAL had a predictive value for DGF in a cohort of 97 kidney transplant recipients (129). Instead, Buemi et al. showed that post-transplant day 1 and 2 recipient plasma NGAL levels were predictive of DGF with AUCs of 0.80 and 0.85 respectively in ROC curve analysis (129). In a smaller study of 41 kidney transplant recipients, Bataille et al. also showed that recipient plasma NGAL, this time measured 12 hours after

kidney transplantation, predicted DGF with an AUC of 0.9 in ROC curve analysis (130). The evaluation of recipient plasma NGAL as a biomarker for DGF in these previous two studies, however, lacked a multivariate analysis component to correct for confounding variables. Additionally, Pianta et al. failed to show that post-transplant recipient plasma NGAL was predictive of DGF in a cohort of 81 kidney transplant recipients (131). With regards to donor-derived measurement of NGAL, there is a lack of evidence for the utility of both donor urine and plasma/serum NGAL as predictors of DGF (129,132). Overall, recipient-derived NGAL measured in the urine or plasma after kidney transplantation shows promise as a biomarker for DGF but lacks the diagnostic accuracy and reproducibility required for clinical applicability. Other markers that are released by renal tubular epithelial cells following ischemic injury or participate in creating a pro-inflammatory response exist such as IL-18, clusterin, liver-type fatty acid binding protein (L-FABP), kidney injury molecule-1 (KIM-1), TLR4, and MyD88. They have been studied as potential predictors of DGF but to a lesser extent than NGAL.

IL-18 is activated by cleavage of caspase-1, and mediates ischemic kidney injury via its pro-inflammatory properties that are independent of neutrophils (133,134). In a cohort of 53 kidney transplant recipients, recipient urine IL-18 measured within the first 24 hours after kidney transplantation was predictive of DGF with an AUC of 0.9 on ROC curve analysis (125). The same group of investigators further supported these findings in a multicenter study including 91 kidney transplant recipients, although with a lower predictive accuracy (AUC = 0.82). Another group of

investigators, however, reported a more modest predictive accuracy for DGF and AKI (DGF and SGF) using recipient urine IL-18 measured at 4 hours after kidney transplantation in a cohort of 81 kidney transplant recipients (AUC = 0.70) (131).

Clusterin is released by injured kidney cells, and has a role in protecting from ischemic injury and fibrosis via anti-apoptotic mechanisms and the maintenance of intercellular and cell-matrix interactions (135-137). Recipient urine clusterin measured 4 hours post-transplant was moderately predictive of DGF and AKI (DGF and SGF) in a single study comprising of 81 kidney transplant recipients with an AUC in the 0.70 range on ROC curve analysis (131).

L-FABP is another candidate biomarker that is expressed in proximal renal tubular cells and released into the urine following tubular injury. L-FABP protects from renal IRI by reducing oxidative cellular damage via the transport of oxidized fatty acid metabolites from the cytosole to the renal tubular lumen (138-140). In a cohort of 71 kidney transplant recipients, post-transplant day 2 recipient urine L-FABP was predictive of AKI (DGF and SGF) with an AUC of 0.85 on ROC curve analysis (128). Post-transplant day 1 serum L-FABP was also observed to be predictive of DGF in recipients of DCD kidneys (141).

KIM-1 is a gene that is highly upregulated in proximal renal tubules and is shed into the tubular lumen after renal ischemic injury. It facilitates the clearance of apoptotic debris and mediates the phagocytosis of necrotic cells and oxidized lipoproteins

(74). KIM-1 was thought to be a promising candidate biomarker for DGF, but evidence points to the contrary. Pianta et al. observed that recipient urine KIM-1 measured 4 hours after kidney transplantation only had a moderate predictive value for DGF and AKI (DGF and SGF) with an AUC in the 0.7 range on ROC curve analysis (131). In their multicenter study including 91 kidney transplant recipients, Hall et al., however, failed to demonstrate that post-transplant day 1 recipient urine KIM-1 had a predictive value for DGF (142). Donor urine KIM-1 and pre-transplantation kidney graft KIM-1 mRNA and protein levels were also investigated but had limited predictive value for DGF (143,144).

TLR4 and MyD88 are involved in the production of pro-inflammatory cytokines and chemokines. This occurs following the recognition of DAMPs, which are released by ischemic kidney tissue, by TLR4 via downstream MyD88 signaling. In a cohort of 80 kidney transplant recipients, TLR4 and MyD88 mRNA levels measured in the recipient's peripheral blood within the first day after kidney transplantation were predictive of prolonged DGF (dialysis requirement lasting more than 14 days) with AUCs of 0.88 and 0.79 respectively on ROC curve analysis, but not of the standard DGF definition. This analysis lacked a multivariate component to take into consideration confounding variables. Also, TLR4 and MyD88 mRNA levels in pre-transplantation kidney graft biopsies or post-transplant urine samples were not predictive of DGF (145).

Apart from urinary, peripheral blood, and graft biopsy samples, the preservation fluid used during the CIT to store the kidney graft on a machine perfusion has also been investigated as a potential source for biomarkers in the prediction of DGF. Moers et al. analyzed preservation fluid samples from 306 kidney grafts that were immediately put on the machine perfusion after procurement until transplantation. They investigated potential markers that are released from renal tubular cells following an ischemic injury. They found that the enzymes glutathione-S-transferase (GST) and N-acetyl-β-D-glucosaminidase (NAG) as well as the lipid-binding protein heart-type fatty acid binding protein (H-FABP) measured at the end of machine perfusion were independent predictors of DGF. The predictive accuracies were however low, with AUCs of 0.67, 0.64, and 0.64 on ROC curve analysis for GST, NAG, and H-FABP respectively (146).

Although the previously mentioned biomarkers show promise for the prediction of DGF or SGF, they all lack the diagnostic accuracy and reproducibility necessary to become part of a physician's toolbox to diagnose DGF and SGF in clinical practice. Moreover, the source and timing of these previously mentioned biomarkers are intrinsically problematic for a useful prediction of DGF and SGF. The ideal biomarker should be able to predict which recipients will develop DGF and SGF prior to the renal IRI insult, which occurs immediately upon reperfusion of the kidney graft and causes irreversible tissue damage. Consequently, the use of post-transplant samples is flawed since irreversible damage already occurred to the kidney graft by the time a prediction of DGF and SGF is made. The use of post-

transplant biomarkers therefore precludes the implementation of potential interventions to prevent or reduce tissue damage related to renal IRI.

The use of a urine sample to measure a biomarker is also suboptimal since kidney transplant recipients who develop DGF or SGF may be anuric and have no urine samples to provide. A recipient biomarker is also preferred to a donor biomarker since it avoids the ethical considerations associated with collecting a sample from a donor. With regards to preservation fluid biomarkers from machine perfusion, they are limited by the inconsistency of the use of machine perfusion in the context of national organ sharing. Currently, not every kidney graft is being connected to machine perfusion after procurement. Even when the kidney graft is put on machine perfusion, it does not necessarily occur immediately after procurement and does not necessarily stay connected continuously until transplantation. The resulting preservation fluid samples therefore have potentially different length of discontinuous exposure to the kidney graft. The optimal scenario would be to find a biomarker for DGF and SGF that would be collected from the recipient's peripheral blood prior to kidney transplantation.

1.2.2.2. Experimental therapeutic interventions based on cellular mechanisms of injury involved in DGF and SGF

Despite DGF and SGF being the most common complications after kidney transplantation, there are currently no clinically accepted therapeutic interventions

to treat or prevent it. Potential therapies have been investigated in the kidney transplant recipient or their donor, but remain at best experimental at the moment. These experimental therapies target one or multiple known cellular mechanisms involved in renal IRI, mainly oxidative stress, apoptosis, and adhesion molecules.

With regards to anti-oxidant therapies, dopamine is a HO-1 inducer which has the ability to inhibit the generation of free oxygen radicals by delaying ATP degradation and intracellular calcium accumulation (147-149). Pre-treatment of DBD donors with a low dose dopamine infusion was investigated as a preventative measure to reduce the incidence of DGF in the recipient after kidney transplantation. In a randomized controlled trial including 264 DBD donors and 487 kidney transplant recipients, dopamine infusion at 4 μg/kg/minute for a median time of 344 minutes in the donor significantly reduced the incidence of DGF from 35.4% to 24.7% in the recipient after kidney transplantation (150). The pre-treatment of donor, however, is limited due to ethical reasons and the potential adverse effects to other retrieved organs from the same donor. N-acetylcysteine (NAC) is another anti-oxidant that has the ability to scavenge oxygen free radicals. NAC was investigated in a study in which 74 kidney transplant recipients were randomized to a group receiving 600 mg of oral NAC daily for 7 days post-transplant (n = 38) and a control group (n = 36). The incidence of DGF was significantly lower in the NAC group (55.3% vs. 72.2%) (151). It is unknown if these results would be reproducible in another cohort since the incidence of DGF was abnormally high in this study and the sample size was very small.

Investigations of potential therapies targeting the upregulation of adhesion molecules have also been undertaken but with disappointing results in the prevention of DGF after kidney transplantation. Enlimomab is an inhibitor of the adhesion molecule ICAM-1 and prevents the infiltration of leukocytes during renal IRI. Although ICAM-1 inhibition reduced tissue damage in a murine model of renal IRI, a randomized double-blind placebo-controlled trial including 262 kidney transplant recipients failed to corroborate the murine results in humans. Recipients who received enlimomab for 6 days post-transplant had a similar rate of DGF than recipients who received a placebo (152). Recombinant P-selectin glycoprotein ligand Ig (rPSGL-Ig) fusion protein is another drug that has been studied which inhibits leukocyte-endothelial interactions by reducing the availability of the adhesion molecule P-selectin by binding to it. In a multicenter phase 2a safety trial including 59 kidney transplant recipients, there was no statistically significant difference in the incidence of DGF between recipients receiving rPSGL-Ig (41%) and those receiving a placebo (20%) (153).

Anti-apoptotic agents have also been investigated in the prevention of DGF after kidney transplantation. Erythropoietin has anti-apoptotic properties and decreases renal tubular hypoxia by increasing hypoxia-inducible factor 1-α. Although erythropoietin was shown to be nephroprotective in a murine model of renal IRI, its ability to prevent DGF and SGF was not demonstrated in kidney transplantation. In a multicenter randomized control study including a total of 104 kidney transplant recipients, 51 recipients received recombinant human erythropoietin-β (EPO-β)

infusions at a dose of 30,000 UI at days 0, 1, 7, and 14 post-transplant and were compared to 53 control recipients. There was no significant difference in DGF and SGF rates between the group receiving EPO-β and the control group (154). Other anti-apoptotic agents, on the other hand, show promise in the prevention of DGF after kidney transplantation but trials are still in the preliminary phases and ongoing. QPI-1002 is a synthetic chemically modified small interfering RNA that inhibits p53 RNA and protein levels, thus delaying cell death by apoptosis and protecting renal tubular cells after IRI. Preliminary results from a randomized double-blind placebo-controlled phase 2 clinical trial including 327 kidney transplant recipients were presented as an abstract at the World Transplant Congress 2014 and showed that QPI-1002 was effective at reducing the incidence of DGF with a relative risk reduction of 30% for grafts coming from ECDs and 15% for grafts coming from all donors (12). Diannexin is a recombinant human annexin V homodimer that inhibits apoptotic signaling and prevents cell death. Preliminary results presented at the American Transplant Congress 2010 suggest that kidney transplant recipients receiving a single 400 μg/kg dose of diannexin 15 minutes after reperfusion (n = 21) had a decreased incidence of DGF from 56% to 33% compared to those receiving a placebo (12). Phase 3 trials focusing on the efficacy of QPI-1002 and diannexin rather than their safety profiles are still ongoing to confirm these encouraging but preliminary results.

The complement cascade is another cellular mechanism involved in renal IRI that has been investigated as a target to prevent DGF. Eculizumab is a recombinant

humanized monoclonal antibody against the terminal complement component C5. Currently unpublished results however suggest that eculizumab does not confer any advantage compared to placebo to prevent DGF after kidney transplantation. Complement cascade blockage with other agents such as C1-inhibitor and soluble complement receptor 1 are still in ongoing trials (65).

Vascular dysfunction and chemokine upregulation are other promising therapeutic targets in animal renal IRI but have not been tested in human kidney transplantation. Endothelin receptor antagonist, nitrite, and carbon monoxide all promote vascular vasodilation and were shown to be beneficial in animal models of kidney transplantation but are still untested in the human setting (155-158). Similarly, the chemokine receptor inhibitors meraxin and repertaxin are successful at dampening renal IRI in murine kidney transplantation but their ability to prevent DGF and SGF in human kidney transplantation is unknown (159-161).

Despite encouraging results mainly from anti-apoptotic and anti-oxidant agents as described above, none of these therapies have made the transition from the experimental to the clinical realm. Ideally, a therapy for DGF and SGF should be administered to the recipient prior to kidney transplantation in order to avoid the ethical issues associated with donor therapy and to prevent kidney graft damage prior to irreversible renal IRI. There is therefore still a high need to find innovative cellular targets to mitigate DGF and SGF after kidney transplantation.

Traditionally, it has been thought that only the innate immune response, especially neutrophils, was the sole immunologic mediator of renal IRI, and thus DGF and SGF after kidney transplantation. The experimental blockade of neutrophils, however, has not consistently resulted in an improvement in injury after renal IRI (162,163). A possible explanation is that neutrophils have recently been found to also have immunosuppressive functions by directly suppressing pro-inflammatory cytokine production and weakening the interaction of dendritic cells with naïve T cells (164). Moreover, other type of immune responses, especially adaptive immunity, could also be implicated in the pathophysiology of renal IRI.

The adaptive immune response comprises T and B cells. In classic immunological models, it usually takes 3 to 5 days for these cells to get activated in an antigen-dependent fashion (165). In that context, the adaptive immune system is not expected to play a role in the rapid damage occurring after renal IRI. Counter-intuitively, there is now more recent evidence that the adaptive immune response can be activated in an antigen-independent fashion early after renal IRI. Particularly, there is direct evidence that T lymphocytes are important modulators of kidney damage in experimental and clinical models of renal IRI.

1.3. Adaptive immune system and renal ischemia-reperfusion injury

The immune system is compartmentalized into 2 main lines of defense: the innate and the adaptive immune responses (166). Innate immunity is the first line of defense against foreign antigens. Upon encountering an antigen, the innate immune response gets activated within minutes to hours. Its mechanism of action is, however, non-specific and lacks memory as it acts in an antigen-independent fashion and is unable to recognize the same antigen if exposed to it again in the future (167). As discussed in the previous section, due to the rapidity and non-specificity of its action, cells categorized as part of innate immunity, mainly neutrophils and macrophages, have been implicated in the pathophysiology of renal IRI. Experimental blockade of innate immunity, however, has failed to consistently improve the damage resulting from renal IRI. Consequently, researchers also started looking at the other arm of the immune system, the adaptive immune response, as a potential mediator of renal IRI.

As opposed to innate immunity, adaptive immunity is classically thought to act slower, taking 3 to 5 days to get fully activated after exposure to a foreign antigen. This lag time in activation is related to the fact that adaptive immunity is usually a specific and antigen-dependent response. Adaptive immunity also has the capacity for memory, which allows it to mount a rapid response when exposed to the same antigen again in the future (167). The adaptive immune system comprises 2 types of cell, the T lymphocyte and the B lymphocyte. Both T and B lymphocytes originate

from hematopoietic stem cells in the bone marrow. T lymphocytes develop in the thymus and are activated through their interaction with antigen presenting cells (APCs). B lymphocytes, on the other hand, develop in the bone marrow, recognize antigens directly, and are the key players in the production of antibodies against foreign antigens (167,168). As we will discuss later, evidence is now challenging the classic dogma that the adaptive immune response is only antigen-dependent, and suggests that it can also be activated in an antigen-independent context such as renal IRI. Particularly, experimental studies that will be discussed later in this chapter show that T lymphocytes are the adaptive immune cells playing a role in mediating renal IRI. We therefore will focus the remaining of this chapter on reviewing T lymphocytes and their known links to renal IRI.

1.3.1. Overview of T lymphocytes

1.3.1.1. T lymphocyte development

The development of T lymphocytes is a process that begins in the bone marrow and continues in the thymus. Progenitors to T lymphocyte derive from multipotent hematopoietic stem cells in the bone marrow. These progenitor cells subsequently migrate via the peripheral blood from the bone marrow to the thymus where they are instructed to commit to the T cell lineage through Notch signaling (168). After up to a week undergoing a complex developmental and selection process, only 2 to

4% of 5 x 10^7 progenitor cells entering the thymus daily will become mature T lymphocytes in the end, while the remaining die by apoptosis (169).

Precursor cells lack the surface markers that characterize T cells when they enter the thymus via the peripheral blood from the bone marrow. Their interaction with the thymic stroma is critical and sets the stage for their commitment to the T cell lineage (170). As a matter of fact, individuals suffering from DiGeorge syndrome who lack a thymus due to deletion of their chromosome 22q11 as well as thymus-deficient nude mice secondary to a defect in their Foxn1 gene fail to produce T lymphocytes (171). Upon interaction with the thymic stroma, precursor cells proliferate and start expressing a first surface marker specific for T cells, CD2 (172). At this point, these cells still lack the CD3 – T cell receptor complex which is essential for T cell activation and function. They also lack the characteristic T cell co-receptors CD4 and CD8, and thus are defined as immature double-negative thymocytes. Of note, a small proportion of double-negative thymocytes include 2 mature minority T cell lineages, the γδ T cell and the NKT cell (173-175). A detailed discussion regarding these 2 minority T cell lineages is outside the scope of this overview.

1.3.1.1.1. Double-negative thymocytes

The development of immature double-negative thymocytes is subdivided into 4 stages based on their expression of Kit, CD44, and CD25 (176,177). During double-

negative stage 1, double-negative thymocytes express Kit and CD44, but not CD25. At this stage, the genes encoding the 2 chains of the T cell receptor (α and β chains) are still in their germline configuration. As maturation continues during double-negative stage 2, double-negative thymocytes begin to express CD25 along with Kit and CD44 on their surface. Re-arrangement of the β-chain locus of the T cell receptor begins in stage 2 as well. During double-negative stage 3, double-negative thymocytes reduce their expression of Kit and CD44. In this stage, the assembly of the pre-T cell receptor occurs. The expressed β-chains pair with pre-T cell receptor α chains to form pre-T cell receptors. These pre-T cell receptors are expressed on the cell surface in a complex with CD3 to provide the signaling machinery of the eventual T cell receptors. In addition, expression of pre-T cell receptors stops the rearrangement of the β-chain locus and induces the progression to the double-negative stage 4. During double-negative stage 4, double-negative thymocytes rapidly proliferate. These cells then transition briefly through an immature CD8 single positive stage before eventually expressing both characteristic T cell co-receptors CD4 and CD8 along with low levels of the T cell receptor (176-178). At the end of double-negative stage 4, these cells therefore become double-positive thymocytes, as they express both CD4 and CD8 (178,179). Rearrangement of the T cell receptor α chain locus continues in the double-positive thymocytes until a self-peptide – self-MHC molecular complex is recognized. The fate of double-positive thymocytes and their further maturation is in fact linked to the strength of their interaction with self-peptide – self-MHC molecular complexes, as we will discuss in the following paragraph.

1.3.1.1.2. Double-positive thymocytes

The development, maturation, and survival of double-positive thymocytes depend on their ability to interact with and their affinity to self-peptide – self-MHC molecular complexes in the thymus (179). As a matter of fact, only 10-30% of double-positive thymocytes can recognize self-peptide – self-MHC molecular complexes after final gene rearrangement of their T cell receptors, and those undergo a selection process that will determine their survival or programmed cell death (180). This selection process occurs via two methods in the thymus based on the affinity of the T cell receptor to self-peptide – self-MHC molecular complex: positive selection and negative selection (178). Low affinity interactions between the T cell receptor and self-peptide – self-MHC molecular complex leads to positive selection, in which the thymocyte is rescued from programmed cell death by neglect and eventually will mature into a T cell. On the other hand, high affinity interactions between the T cell receptor and self-peptide – self-MHC molecular complex leads to negative selection, in which the thymocyte undergoes programmed cell death via apoptosis. The negative selection process serves as a mechanism to eliminate the unwanted development of potential self-reactive T cells. The determination of positive versus negative selection is also mediated by the mitogen-activated protein kinase Erk, as low affinity interactions between the T cell receptor and self-peptide – self-MHC molecular complex result in increased activation of Erk and positive selection while high affinity interactions lead to a transient activation of Erk and negative selection (178). Since the likelihood of a weak interaction between the T

cell receptor and self-peptide – self-MHC molecular complex is higher than a strong one, a larger repertoire of thymocytes is in the end positively rather than negatively selected.

In addition to ensuring the survival of thymocytes, positive selection also determines the phenotype and function of the future mature T cell. When a double-positive thymocyte recognizes with low affinity a self-peptide – self-MHC molecular complex and is positively selected, it starts to express higher levels of the T cell receptor. Simultaneously, a program is initiated for it to become a single-positive thymocyte as expression of one of the two characteristic T cell co-receptors, CD4 or CD8, ceases. At the beginning of positive selection, downregulation of both CD4 and CD8 occurs in the double-positive thymocytes. CD4 is then re-expressed, resulting in a CD4+CD8^{low} cell that will ultimately develop into either the single-positive CD8 or single-positive CD4 lineage based on the interaction of its T cell receptor with peptides bound to class I or class II self-MHC molecules and Lck signaling (181,182). If the interaction is with a MHC class II molecule, the re-expression of CD4 is stronger secondary to Lck signaling and leads to further differentiation into the CD4 lineage with complete loss of CD8. On the contrary, if the interaction is with a MHC class I molecule, re-expression of CD4 is weaker due to the absence of Lck signaling, with a subsequent loss of CD4 expression, re-expression of CD8, and further differentiation into the CD8 lineage (183).

At the end of positive and negative selection, only about 2% of double-positive thymocytes survive the dual screening and mature into either a CD4 or CD8 single-positive T cell. The single-positive mature T cell is then exported from the thymus to peripheral lymphoid organs. This exportation of mature T cell is dependent on the lipid molecule sphingosine 1-phosphate (S1P) gradient between the thymus and the periphery. At the end of their maturation, single-positive thymocytes express the S1P receptor 1 which recognizes S1P. Since S1P is present in higher concentration in the blood and lymph, single-positive thymocytes are attracted to it and therefore immigrate out of the thymus to the periphery (184,185). The whole developmental process to create a mature T cell takes about 3 weeks from the time a precursor cell enters the thymus until it is exported into the periphery (186).

1.3.1.2. T lymphocyte activation

Once mature T cells exit the thymus and are exported into the periphery, they are called naïve T cells since they have not yet encountered a specific foreign antigen. In general, for a naïve T cell to be involved in an immune response, it must meet its specific antigen presented as a peptide – MHC complex on the surface of an APC and subsequently differentiate into what is called an effector T cell (Teff) that has acquired specialized functions that participate in the removal of the antigen (187). Recently, it was shown that naïve T cells could also be activated into Teff in an antigen-independent manner (165). We will first discuss the traditional antigen-

dependent activation of T cells, and then the more recently described antigen-independent activation of T cells.

1.3.1.2.1. Antigen-dependent T lymphocyte activation

For the typical antigen-dependent T lymphocyte activation to happen, a naïve T cell must encounter its specific antigen presented by an APC in a peripheral lymphoid organ. The specific antigen can either be taken up by the APC in the peripheral blood and transported to the lymphoid organ or directly in the lymphoid organ (187,188). Most commonly, APCs are dendritic cells, but can also be macrophages or B cells (189). Of special interest, in the unique context of transplantation, three pathways of antigen presentation exist based on the origin of the APC, the direct pathway, the indirect pathway, and the semi-direct pathway (190-192). A transplanted organ, such as a kidney allograft, carries with it APCs of donor origin that contribute to the transplant adaptive immune response. In the direct pathway of antigen presentation, donor APCs leave the allograft and migrate to peripheral lymphoid organs where they activate the recipient's naïve T cells by presenting allogeneic antigens. In contrast, in the indirect pathway of antigen presentation, the recipient's own APCs present the allogeneic antigens and consequently activate the recipient's naïve T cells. In the semi-direct pathway of antigen presentation, allogeneic major histocompatibility complex is transferred from donor to recipient APC either via direct cell-to-cell contact or via the release of exosomes. The recipient APC thus becomes chimeric to both donor and recipient major histocompatibility complex

and can stimulate naïve T cells via both direct and indirect pathway mechanisms (190-192). The end result of the direct, indirect, or semi-direct pathways is the differentiation of naïve T cells into activated alloreactive Teffs that have the potential to damage the allograft. The antigen-dependent activation of a naïve T cell and its differentiation into Teff requires 3 signals, and these will be described next.

The activation of naïve T cells and their eventual differentiation into Teffs are dependent on 3 signals that are commonly referred as signal 1, signal 2, and signal 3 (193,194). Signal 1 consists of the antigen-specific activation of the T cell receptor as described in the previous paragraph. Signal 1 is, however, insufficient to ensure the survival and proliferation of naïve T cells into Teffs. Signal 2, which is the interaction of co-stimulatory molecules on both APCs and T cells, is also required. Without signal 2, the naïve T cell becomes anergic and undergoes functional inactivation or clonal deletion. The prototypical co-stimulatory molecules that deliver signal 2 are the B7 (CD80/CD86) ligand on the APC and the CD28 receptor on the T cell. The binding of B7 to CD28, in addition to antigen recognition by the T cell receptor (signal 1), leads to the production of the cytokine IL-2 by the T cell along with the expression of the α chain of the IL-2 receptor on its surface. The autocrine action of IL-2 is essential to the proliferation and activation of the T cell. The potential excessive proliferation and activation of T cell through the B7 – CD28 costimulatory signal is auto-regulated by cytotoxic T-lymphocyte associated protein 4 (CTLA-4). The activation of naïve T cell induces the expression of CTLA-4 on their surface as a regulatory mechanism. CTLA-4 binds the B7 molecules more avidly than CD28 and

competes with CD28 for the interaction with the B7 molecules. As opposed to the B7 – CD28 interaction, the binding of CTLA-4 to B7 molecules inhibits rather than activates T cell by restricting IL-2 production (195). Other co-stimulatory molecules belonging either to the CD28 or tumor necrosis factor (TNF) family of receptors also participate in signal 2. Inducible T-cell costimulator (ICOS) is a receptor present on T cells that belongs to the CD28 family and binds to ICOS ligand (ICOSL) on APCs. The ICOS – ICOSL interaction drives T cell proliferation by inducing the cytokines IL-4 and IFN-γ rather than IL-2 (196-198). Interactions between co-stimulatory molecules belonging to the TNF family present on T cells and APCs such as CD27 – CD70, CD40 ligand – CD40, 4-1BB – 4-1BB ligand, and OX40 – OX40 ligand also participate in driving naïve T cell proliferation (199,200). Signal 3 pertains primarily to the CD4 class and not the CD8 class of naïve T cells and constitutes the various cytokines that control CD4 naive T cell differentiation into several subsets with distinct functions (194). The main functional subsets of CD4 T cells are T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), T follicular helper (TFH), and regulatory T (Treg) cells. Each subset is associated with unique transcription factors and cytokines that forge their identity. This will be discussed in more details later in this chapter.

1.3.1.2.2. Antigen-independent T lymphocyte activation

Although the presence of an antigen has traditionally been viewed as essential to the activation of T lymphocytes, there is recent evidence demonstrating that T

lymphocyte activation can occur in a totally antigen-independent fashion. This discovery was initiated by observations that T lymphocytes play a role in mediating injury in disease processes that typically do not involve antigens, such as renal IRI. The mechanisms behind the antigen-independent activation of T lymphocytes are not as clearly delineated and not completely understood, but include the following.

The chemokine RANTES, which is upregulated in renal IRI, has been shown to directly activate T cells (201). Similarly, oxygen free radicals, which are also generated in renal IRI as described in a previous section, have been shown to be capable of activating T cells (202). Naïve T cells exposed to hypoxic renal epithelial cells can also become activated, likely via the action of inflammatory cytokines produced by the hypoxic cells (203). Moreover, hypoxic endothelial cells can also participate in the activation of naïve T cells by providing co-stimulatory signals and thus taking on the role of an APC (204).

1.3.1.2. T lymphocytes and renal ischemia-reperfusion injury

T lymphocytes have traditionally not been considered as a causative factor in the pathophysiology of renal IRI. Evidence in both murine and human studies, however, challenged this dogma and suggested otherwise. Renal IRI is thought to be an antigen-independent event. Naïve T lymphocytes were therefore ignored as participants in mediating renal IRI, as they require the encounter of an antigen to become activated and take 3 to 5 days to proliferate and differentiate into functional

Teffs. More recent evidence, however, shows that T cells can become activated even in the absence of an antigen. This thus opened the door to investigating their role in mediating renal IRI.

Studies utilizing either knockout mice lacking T lymphocytes or drugs altering the number or activation status of T lymphocytes showed that T lymphocytes were important in mediating both warm and cold renal IRI. Double CD4/CD8 knockout mice, which lack T lymphocytes, were protected from warm renal IRI in a model in which the renal vascular pedicle was clamped for 30 minutes bilaterally. Compared to wild type mice, double CD4/CD8 knockout mice had less tubular injury and better serum creatinine after warm renal IRI (203). Athymic nu/nu mice, which also lack T lymphocytes, were similarly protected in the same warm renal IRI model. Adoptive transfer of wild-type T lymphocytes back into these nu/nu mice restored kidney damage (205). The use of $S1P_1$-selective agonists, which sequester T lymphocytes to secondary lymphoid organs and consequently reduce the number of circulating T lymphocytes, is also protective against renal dysfunction as evidenced by a decrease in tubular damage and absence of serum creatinine elevation in the murine warm renal IRI model as well as in a model of murine isograft kidney transplantation (206-208). The implication of T lymphocytes in pure cold renal IRI was also demonstrated. In a model in which the right kidney was removed and the left kidney was clamped following in situ perfusion of cold University of Wisconsin solution, the inhibition of T cell activation by blocking the B7-CD28 co-stimulatory pathway using CTLA-4 Ig prevented the development of renal dysfunction in the clamped left

kidney as demonstrated by an absence in serum creatinine elevation and proteinuria development (209). Indirect evidence of T lymphocyte involvement in the human kidney transplantation setting also exists. Thymoglobulin, a potent induction immunosuppressive agent that significantly reduces T cell counts and that is used to prevent rejection, was also observed to serendipitously reduced the rate of DGF after kidney transplantation in some studies (210,211).

1.3.1.3. CD8 and CD4 T lymphocytes in renal ischemia-reperfusion injury

As previously discussed in the section on T lymphocyte development, T lymphocytes can be divided into two main classes based on whether they express the co-receptor CD8 or CD4 on their cell surface. Naïve CD8 T cells are activated by the recognition of antigens presented by MHC class I molecules, and all differentiate into cytotoxic CD8+ Teffs. Cytotoxic CD8+ Teffs directly kill their target cells by inducing them to undergo programmed cell death. This occurs mainly via the release and action of the preformed cytotoxic effector proteins perforin and granzymes. Perforin creates pores in the cell membrane, and allows the delivery of granzymes into the cytosol of the target cell. Granzymes then trigger apoptosis of the target cell by activating caspases. Cytotoxic CD8+ Teffs also release pro-inflammatory cytokines such as IFN-γ and TNF-α which further contribute to killing target cells by upregulating MHC class I molecules and the recruitment of macrophages. Cytotoxic CD8+ Teffs are thus usually considered essential in the host defense against intracellular pathogens, especially viruses (212,213). On the other hand, naïve CD4 T cells are activated by

the recognition of antigens presented by MHC class II molecules. As opposed to naïve CD8 T cells, naïve CD4 T cells differentiate into a diverse repertoire of effector and regulatory subsets. The different subsets of CD4+ Teffs have distinct functions that contribute to amplify the immune response, for example by further activating APCs and naïve CD8 T cells. On the contrary, the regulatory subsets of CD4+ T cells act as a counterbalance to the CD4+ Teff subsets and limit the extent of the immune response (214,215).

In the context of renal IRI, it was demonstrated that T lymphocytes play a role in mediating kidney damage. As T lymphocytes are divided into two main classes, CD8 T cells and CD4 T cells, the next step was to determine if both classes of T lymphocytes or only one of them were involved in renal IRI. In the murine model of warm renal IRI, CD4-deficient but not CD8-deficient mice were protected from renal damage. Reconstitution of CD4-deficient mice with wild-type functional CD4+ T cells restored renal damage. This demonstrated the specific importance of CD4+ T cells in renal IRI (205). Since only the CD4 but not the CD8 class of T lymphocytes was shown to be involved in renal IRI, the remaining of this chapter will focus on CD4 T cells, particularly the different effector and regulatory subsets as well as their known implications in renal IRI and DGF/SGF after kidney transplantation.

1.3.2. CD4 T lymphocytes

Upon encountering an antigen (signal 1) in the presence of co-stimulatory signals (signal 2), naïve CD4 T lymphocytes become activated and proliferate. Depending on the cytokine environmental milieu (signal 3), these naïve CD4 T lymphocytes then differentiate into distinct subsets that each has their own phenotype and function. For the initial two decades after their discovery, CD4 T lymphocytes or CD4 T helper cells were thought to differentiate into only two subsets, the Th1 and Th2 cells. Newer evidence, however, has shown the existence of additional subsets of CD4 T lymphocytes. These include the Th17 cells, the Treg cells, and the TFH cells (216). We will discuss in more details the characteristics defining the different subsets of CD4 T lymphocytes as well as their potential contribution to renal damage following ischemia-reperfusion in animal models and human kidney transplantation. TFH cells, one of the most recently recognized subset of CD4 T helper cells that provides help to B cells in secondary lymphoid organs, will be omitted in our discussion as their differentiation process has not been clearly established yet and no literature exists regarding their role in renal IRI and DGF/SGF after kidney transplantation (217,218).

1.3.2.1. Th1 and Th2 cells: the original CD4 T helper cell dichotomy

Following a landmark paper by Coffman and Mosman in the 1980s, CD4 T helper cells were originally thought to differentiate into only two distinct subsets for about

two decades, the Th1 and the Th2 cells. The fate of naïve CD4 T helper cells into a Th1 or Th2 subset is based on the cytokine environmental milieu and the participation of different transcription factors specific to each subset (219).

1.3.2.1.1. Overview of Th1 cells

Th1 cells are characterized by the production of their signature cytokine IFN-γ. Th1 cells are also identified by the transcription factors STAT4 and T-BET. The presence of the cytokine IL-12 in the environmental milieu, which is produced by innate immune cells such as dendritic cells and macrophages, is critical to the differentiation of naïve CD4 T helper cells into Th1, as IL-12 acts via STAT4 to drive Th1 differentiation. The production of the Th1 signature cytokine IFN-γ is promoted by the activation of T-BET, which turns on the genes for both IFN-γ and the IL-12 receptor (220). In the infectious context, Th1 cells are thought to be essential in the defense against various pathogens, in particular intracellular organisms (220,221).

1.3.2.1.2. Overview of Th2 cells

As opposed to Th1 cells, Th2 cells are characterized by the production of their signature cytokine IL-4. They are also identified by the transcription factors STAT6 and GATA3. The presence of IL-4 in the environmental milieu, which can also be produced by eosinophils, basophils, and mast cells, is important for the

differentiation of naïve CD4 T helper cells into Th2 cells (220,222). IL-4 signaling leads to the activation of STAT6, which in turn promotes the expression of GATA3 in naïve CD4 T helper cells. GATA3 then turns on the genes for the signature Th2 cytokine IL-4, and further induces its own expression to stabilize the Th2 phenotype during the differentiation process (220). Via the secretion of their signature cytokine IL-4, Th2 cells are viewed as essential in class switching of B cells to IgE antibody production (223). Consequently, Th2 cells are classically thought to be important in the control of parasitic infections, especially helminthes, which relies on the IgE antibody response (221,223). Th2 cells are also classically involved in the pathogenesis of asthma and allergies, which also relies on the IgE antibody response (223).

1.3.2.1.3. Th1 and Th2 cells in experimental renal ischemia-reperfusion injury and DGF/SGF after kidney transplantation

Since CD4 T helper cells were discovered to play an integral role in renal IRI, the role of Th1 and Th2 cells were further dissected initially. In a murine model of warm renal IRI in which bilateral renal pedicles were clamped for 35 minutes, renal function and post-ischemic tubular injury were compared between Th1-deficient STAT4 knockout mice, Th2-deficient STAT6 knockout mice, and wild-type mice. Compared to wild-type mice, STAT6 knockout mice with a defective Th2 phenotype had increased serum creatinine and acute tubular injury on histology after renal IRI while STAT4 knockout mice with a defective Th1 phenotype had mildly improved

serum creatinine and acute tubular injury on histology (224). These results suggest that tipping the balance towards Th2 cells is protective and tipping the balance towards Th1 cells is detrimental in renal IRI. In the human kidney transplantation context, Loverre et al. reported data suggesting that Th1 cells are also potentially detrimental in recipients who suffered from severe renal IRI in the form of DGF. Recipients with DGF (n=40) had an increase in expression of the Th1 prototypical transcription factor T-BET in post-transplant kidney allograft biopsy samples compared to recipients who had acute tubular damage from calcineurin inhibitor toxicity (n=12) (225).

The CD4 T cell dichotomy of Th1 and Th2 cells, however, has been questioned due to the fact that it incompletely explained autoimmune disease (226). This led to the discovery of two additional subsets of CD4 T helper cells that have interconnected developmental pathways, the Th17 and the Treg cells. We will now discuss in more details the characteristics defining Th17 and Treg cells, as well as their known implications in renal IRI and DGF/SGF after kidney transplantation.

1.3.2.2. Th17 cells

1.3.2.2.1. Overview of Th17 cells

Th17 cells were discovered in response to the fact that the traditional separation of CD4 T cells into Th1 and Th2 cells did not fully explain the pathogenesis of autoimmune disease (226,227). As a matter of fact, mice deficient in the prototypical Th1 cytokine IFN-γ or its receptor were not protected from experimental autoimmune encephalitis (228,229). On the other hand, blockade of IL-12, a key cytokine in the differentiation of Th1 cells, was sufficient to prevent experimental autoimmune encephalitis (230,231). IL-12 is a heterodimeric cytokine formed by the subunits p35 and p40. It was later found that the subunit p40 also forms a heterodimer with the subunit p19, and this novel cytokine at the time was named IL-23 (232). Previous blockade of IL-12 was therefore also blocking IL-23, which is an essential cytokine in the expansion of Th17 cells, as we will discuss later. The specific blockade of IL-23 via knocking out the p19 subunit improved experimental autoimmune encephalitis whereas the specific blockade of IL-12 via knocking out the p35 subunit did not, thus indicating that another subset of CD4 T cells distinct from Th1, later identified as Th17, was involved in this autoimmune process (233,234).

Th17 cells are characterized by the production of their signature cytokine IL-17A (commonly referred as IL-17 only) (227). Th17 cells exist in both mouse and human,

with minor inter-species disparities regarding the requirement for their differentiation from naïve CD4+ T cells and their signature transcription factor (235). In the mouse, the combination of the cytokines TGF-β and IL-6 are essential for the induction of Th17 cells from naïve CD4+ T cells (236-238). Th17 differentiation is also driven by the expression of the signature transcription factor retinoid acid receptor-related orphan receptor gamma (RORγt), which induces the IL-17 gene in naïve CD4+ T cells (239,240). Mice reconstituted with bone marrow from RORγt deficient mice have impaired Th17 cell differentiation (239). In humans, initial studies challenged the requirement for TGF-β in the induction of Th17 cells from naïve CD4+ T cells. It was thought that the cytokines IL-1β and IL-6 were sufficient for the differentiation of naïve CD4+ T cells into Th17 cells (241,242). These initial studies, however, were biased since endogenous sources of TGF-β contained in serum or platelets were not properly controlled for (235). Later studies that eliminated endogenous sources of TGF-β showed that TGF-β is essential in the differentiation of naïve CD4+ T cells into Th17 cells in humans in combination with the cytokines IL-6, IL-21, and/or IL-1β (243,244). Human Th17 cell differentiation is driven by the expression of the retinoid acid receptor-related orphan receptor C2 (RORC2), which is the human counterpart of RORγt (245,246).

Apart from their signature cytokine IL-17A, Th17 cells also secrete other key cytokines in both mice and humans. The IL-17 family of cytokines includes IL-17A, B, C, D, E, and F (247,248). Th17 cells also secrete IL-17F. Both IL-17A and IL-17F exert their function through the IL-17 receptors A and C, and play an essential role

in the recruitment, activation, and migration of neutrophils to sites of inflammation (249). It remains unclear whether IL-17A and IL-17F have overlapping and/or differential functions, as both cytokines are largely co-expressed in CD4+ T cells but can also be produced in isolation. In murine autoimmune disease, there are suggestions that IL-17A rather than IL-17F is required to induce experimental autoimmune encephalitis. On the contrary, IL-17F rather than IL-17A seems to be required for the induction of airway neutrophilia as well as colitis induced by dextran sulfate sodium (250). Another key cytokine secreted by Th17 cells is IL-21. IL-21 is part of the IL-2 family of cytokine and serves to amplify Th17 differentiation in combination with TGF-β in an independent fashion from IL-6. IL-21 further increases its own production in an autocrine loop fashion and up-regulates the IL-23 receptor (251-253). This paves the way for IL-23, a member of the IL-12 family of cytokine as previously described, which is mainly produced by activated dendritic cells and macrophages (254). IL-23 acts to stabilize, expand, and sustain the Th17 phenotype (255). As opposed to IL-6 and IL-21, IL-23 cannot induce the initial differentiation of naïve CD4+ T cells into Th17 cells, as its receptor is not expressed in naïve CD4+ T cells but only following their differentiation into Th17 cells (237). IL-22, a member of the IL-10 family, is also produced by Th17 cells in response to IL-23 (256). IL-22 plays an important role in the immune barrier defense mechanisms against klebsiella in the bronchus and citrobacter in the gut (257,258).

Although RORγt in mice and RORC2 in humans are thought to be the master transcription factors for Th17 cells, other transcription factors are also crucial in

controlling Th17 differentiation (259). As a matter of fact, mice lacking RORγt have residual Th17 cells, and can still develop autoimmune diseases linked to the Th17 phenotype such as experimental autoimmune encephalitis (259,260). Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is an important player in the IL-6 mediated differentiation of naïve CD4 T cells into Th17 cells. The induction of RORγt also appears to be dependent on STAT3, as STAT3-deficient T cells have decreased expression of RORγt. STAT3-deficient T cells also have decreased production of IL-17A, IL-17F, IL-22, and IL-23R, whereas overexpression of STAT3 increases IL-17A production (260-262). Interferon regulatory factor 4 (IRF4) is another transcription factor that was shown to be important in Th17 cell differentiation. IRF4 is part of the interferon regulatory factor family of transcription factor, and plays a role in TLR signaling, IFN-1 production, and T helper cell differentiation. In the specific case of Th17 development, IRF4 is required for the in vitro generation of Th17 cells using the cytokines IL-6 and TGF-β. IRF4 is also required for the action of IL-21 in the amplification of the Th17 lineage. IRF4 likely acts upstream of RORγt as IRF4-deficient T cells had markedly reduced expression of RORγt (263,264). The basic leucine zipper transcription factor ATF-like (BATF) has also been shown to be essential in Th17 differentiation. BATF is a member of the activator protein 1 transcription factor family, and is considered an inhibitor of activator protein 1 activity. BATF-deficient mice failed to produce IL-17A in their CD4 T cells and were protected from the Th17-mediated experimental autoimmune encephalitis. BATF appears independent from the transcription factor STAT3, as IL-6 and IL-21-

mediated phosphorylation of STAT3 was intact in BATF-deficient T cells while Th17 development was impaired. Although RORγt is initially induced in BATF-deficient T cells under Th17 polarizing conditions, BATF-deficient T cells fail to maintain RORγt expression. Retroviral re-expression of both RORγt and BATF in BATF-deficient T cells is necessary for normal IL-17A production, suggesting that RORγt and BATF have a synergistic role in Th17 differentiation (265).

Apart from experimental autoimmune encephalitis, a model of multiple sclerosis in the mouse, Th17 cells have been shown to have a variety of important roles in human infectious and autoimmune diseases. The Th17 lineage is important in the clearance of pathogens that require a massive inflammatory response and that are not adequately addressed by the Th1 or Th2 lineages. These bacteria and fungi include but are not limited to klebsiella pneumoniae, citrobacter rodentium, pseudomonas aeruginosa, mycobacterium tuberculosis, pneumocystis carinii, candida albicans and aspergillus fumigatus (226,266-271). With regards to human autoimmune diseases, Th17 cells have been implicated in multiple sclerosis, rheumatoid arthritis, psoriasis, asthma, and inflammatory bowel disease (272-277). In the context of transplantation, the role of Th17 cells has not been as fully elucidated, but there is evidence pointing towards their implication in both acute and chronic rejection. With regards to acute rejection, urine IL-17A mRNA was found to be elevated in kidney transplant recipients with early acute rejection compared to those with urinary tract infections or normal biopsies (278). In another study, Th17 cells defined by CD4+IL-17A+ were increased in graft biopsy samples of

kidney transplant recipients with acute T cell mediated rejection compared to other causes of graft dysfunction (225). In addition, IL-17A was increased in tubular epithelial cells of kidney transplant recipients with acute antibody-mediated rejection (225). Th17 cells were also increased in acutely rejecting cardiac allografts in a mouse model of heart transplantation (279). With regards to chronic rejection, Th17 cells have been shown to be the primary mediators of chronic allograft vasculopathy in murine heart transplantation in the absence of the Th1 lineage (280). Th17 cells have also been shown to be involved in bronchiolitis obliterans syndrome in both murine and human lung transplantation (281). In human kidney transplantation, the presence of Th17 cells in the explanted kidney allograft correlated with faster progression to chronic rejection (282).

1.3.2.2.2. Th17 cells in experimental renal ischemia-reperfusion injury and DGF/SGF after kidney transplantation

The role of Th17 cells in renal IRI remains unclear. IL-17A, the signature cytokine of Th17 cells, is also produced by other immune cells such as neutrophils and γδ T cells (283,284). Murine studies in which bilateral warm renal IRI was induced demonstrated an important role for IL-17A in the acute phase of kidney damage at 24 hours following reperfusion. In a study in which mice underwent 45 minutes of bilateral warm renal IRI, IL-17A increased in the plasma and kidney at 24 hours compared to controls. Administration of anti-IL-17-A monoclonal antibody either 30 minutes prior or after renal IRI decreased kidney damage as demonstrated by a

reduction in plasma creatinine and histopathologic acute tubular injury scores (285). Similarly, in another study in which mice underwent 28 minutes of bilateral warm renal IRI, blockade of the IL-17/IL-23 signaling pathway using IL-17A knockout, IL-17R knockout, IL-23p40 knockout and IL-23p19 knockout mice also protected against kidney damage at 24 hours. Moreover, blockade of the IL-17/IL-23 signaling pathway also reduced the IL-12/IFN-γ signaling pathway, suggesting that it acts upstream of it. IL-17A secretion was, however, identified as coming from neutrophils rather than CD4 T cells in this study (286). Nevertheless, there is some indirect evidence that Th17 cells have a potential role in mediating murine renal IRI. First, wistar rats fed a Vitamin D-free diet and undergoing bilateral warm renal IRI for 45 minutes had worse kidney damage than those fed a standard diet. This Vitamin D deficiency mediated injury was associated with an elevation in the Th17 to Treg ratio in these rats' kidneys (287). Secondly, mice lacking the NF-κB kinases IKK2 or NEMO in their lymphocytes had increased kidney damage after bilateral warm renal IRI for 55 minutes, and this was associated with an increase in kidney-infiltrating Th17 cells (288). In human kidney transplantation, the cytokine IL-17A and Th17 cells were increased in kidney biopsy samples of recipients suffering from DGF (n = 40) compared to pre-transplant kidney biopsy samples (n = 20) from recipients who had IGF (n = 20) or calcineurin inhibitor nephrotoxicity post-transplant (n = 12) (289). No studies have looked at the specific implications of Th17 cells with SGF after human kidney transplantation.

1.3.2.3. Regulatory T cells

1.3.2.3.1. Regulatory T cells discovery and traditional identification markers

The presence of thymus-derived lymphocytes suppressing the effector immune response and maintaining self-tolerance was first described in 1970 (290). These lymphocytes were later refined as CD4+ T cells constitutively expressing the surface marker CD25 (IL-2RA) in the mouse in 1995 and in humans in 2001, and named Tregs (291-297). In 2003, the transcription factor forkhead box P3 (FoxP3) further characterized Tregs. FoxP3 was shown to be a master control gene for the development and function of Tregs in mice and subsequently in humans (298-300). Scurfy mice, which have a spontaneous mutation in the FoxP3 gene, develop a lethal lymphoproliferative disease causing early death by 4 weeks of age (301). Similarly, humans with the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome have mutations in their FoxP3 gene that lead to severe autoimmune diseases (refractory enteritis, type 1 diabetes, thyroiditis) and allergy (302,303). In 2006, using gene microarray, CD127 (IL-7R) was further identified as a useful alternative or adjunct surface marker to CD25 for the identification of Tregs. CD4+ T cells with FoxP3 expression and suppressive ability were found to express low levels of CD127 (304,305). Tregs are therefore traditionally described as CD4+ T cells expressing the transcription factor FoxP3, high levels of the surface marker CD25, and low levels of the surface marker CD127.

1.3.2.3.2. Mechanisms of action of regulatory T cells

Tregs constitute only about 5 to 10% of CD4+ T cells, and are key players in the maintenance of immune homeostasis and self-tolerance by controlling excessive effector immune responses (306). Tregs have been shown to be able to suppress the activation, proliferation, and effector function of a multitude of adaptive and innate immune cells both in vitro and in vivo. These immune cells include CD4+ T helper cells and CD8+ cytotoxic T cells, but also NK cells, NKT cells, B cells, APCs, neutrophils, and macrophages (307). Tregs exert their immunosuppressive activities via either direct cell-to-cell contact mechanisms or via the modulation of the local microenvironment (summarized in Figure 1).

Direct cell-to-cell contact mechanisms

Tregs have several direct cell-to-cell contact mechanisms to prevent stable and prolonged interactions between naïve T cells and APCs. Naïve T cells are thus unable to proliferate and differentiate into Teffs in the presence of Tregs. A major direct cell-to-cell contact mechanism used by Tregs to exert their suppressive function is by their expression of CTLA-4, and consequently the inhibition of the CD28 – CD80/CD86 co-stimulatory signal necessary for T cell activation. Mice with Treg-specific CTLA-4 deficiency have an uncontrolled Teff response, and develop lymphoproliferative and autoimmune diseases (308). In contrast to CD28 expressed on T cells, the affinity of CTLA-4 expressed on Tregs for the ligands CD80/CD86

present on APCs is much higher. CTLA-4 expressed on Tregs therefore outcompetes CD28 expressed on T cells for CD80/CD86 on APCs, thus preventing co-stimulatory signaling and T cell activation (309,310). The expression of CD80/CD86 on APCs is also directly diminished by Tregs in a CTLA-4-dependent manner (311,312). CTLA-4 expressing Tregs can degrade the CD80/CD86 ligands by trans-endocytosis (308,313,314). Moreover, when CTLA-4 expressing Tregs interact with APCs, specifically dendritic cells, the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) is produced by dendritic cells. This up-regulation in IDO leads to an arrest in T cell proliferation via local tryptophan deprivation and via apoptosis induced by tryptophan catabolites (315-317). Apart from CTLA-4, adhesion molecules also play a role in mediating the direct cell-to-cell contact suppressive function of Tregs. In comparison to naïve T cells, Tregs have a higher expression of the adhesion molecules lymphocyte function-associated antigen-1 and ICAM-1. This higher expression of adhesion molecules on Tregs gives them the upper hand on naïve T cells for interactions with APCs, thereby preventing the activation, proliferation, and differentiation of naïve T cells (318).

Modulation of local microenvironment

In addition to direct cell-to-cell contact mechanisms, Tregs also possess several mechanisms to control the local cytokine microenvironment. Tregs can either restrict the availability of cytokines essential in pathogenic immune responses or release cytokines with immunosuppressive properties.

IL-2 is produced by activated T cells, and is an essential cytokine for their proliferation and differentiation (319). On the other hand, Tregs are low producers of IL-2, and have high expression of the IL-2 receptor (320). IL-2 plays an essential role in the suppressive function of Tregs in two manners. First, Tregs are highly dependent on exogenous IL-2 produced by activated T cells for their proliferation, stability, and survival (321,322). IL-2 signaling via the IL-2 receptor on Tregs lead to the downstream phosphorylation of STAT5, which in turn is indispensable for the induction of the Treg master transcription factor FoxP3 (320). Secondly, since IL-2 is being taken up by Tregs as mentioned above, this limits the availability of IL-2 in the local milieu for the proliferation and differentiation of activated T cells (323,324). Tregs therefore use IL-2 to enhance their proliferation and survival, while simultaneously suppressing the action of T cells by consuming and limiting the availability of IL-2.

ATP released in the extracellular space from the lysis of damaged cells is known to create a local pro-inflammatory microenvironment by acting via P2 receptors on the surface of immune cells. ATP can be degraded to adenosine, and the latter has the reverse effect of creating an anti-inflammatory milieu via its action through the P1 (adenosine) receptors on the surface of immune cells. The CD39/CD73 pathway has been shown to be crucial in modulating the balance between ATP and adenosine in the extracellular space (325). CD39 is an ectonucleotidase that converts ATP into adenosine monophosphate (AMP). CD73, another ectonucleotidase, then converts AMP into adenosine (325). Tregs were shown to express both CD39 and CD73, and

use these ectonucleotidases to exert their immunosuppressive function (326-328). The increased expression of CD39 in concert with CD73 on Tregs allows their entry into pro-inflammatory microenvironments where they reduce extracellular levels of ATP by degrading it to adenosine (329). This Treg-driven adenosine generation first acts through the A_{2A} receptor (subtype of P1 adenosine receptor) on Teffs, consequently decreasing NF-κB activation and thereby decreasing the release of pro-inflammatory cytokines and chemokines. The Treg-driven adenosine generation also has an inhibitory effect on neutrophils, which are activated by ATP. Simultaneously, the Treg-driven adenosine generation also acts on A_{2A} receptors present on Tregs. This creates a positive auto-feedback loop in which expansion of Tregs is further promoted (325,330).

TNF-α is produced by a variety of immune cells, and is typically thought to have a pro-inflammatory effect (331). In T cells, TNF-α binds to either TNFR2 or TNFR1, and acts via NF-κB to promote their activation (332). Interestingly, TNFR2, a cytokine receptor lacking an intracellular death domain that is more restricted to lymphocytes as compared to TNFR1, is predominantly express in Tregs (333,334). Tregs with expression of TNFR2 are thus able to outcompete T cells for TNF-α, thus limiting their activation and pathogenic function (335). Tregs were also shown to be able to shed soluble TNFR2, further limiting the availability of TNF-α for T cells (336). Simultaneously, TNF-α-TNFR2 interactions on Tregs increase their survival and suppressive function, further controlling excessive T cell responses (333-335).

In contrast to the above mechanisms, Tregs also possess cytokines that have direct immune suppressive activities. TGF-β and IL-10 are the two main ones. TGF-β has been shown to have an inhibitory effect on T cell proliferation and differentiation. It appears to have a role in the suppressive function of Tregs (337,338). TGF-β, after conversion to its active form following liberation from the latency-associated protein, first binds to the TGF-β receptor II. This consequently phosphorylates and activates TGF-β receptor I. This receptor complex then phosphorylates and activates SMAD2/3, which in turn heteromerizes with SMAD4. This SMAD complex translocates to the nucleus and regulates transcription (339). CD4 T cells lacking the TGF-β receptor II are refractory to Treg-mediated suppression, pointing to a role for TGF-β in this process (340-342). Similarly, the use of anti-TGF-β antibody also cancelled the immunosuppressive effects of Tregs in an in vivo model of colitis (343). In another study using RAG1 knockout mice, TGF-β also had an important role in mediating Treg suppressive function as co-transfer of TGF-β-deficient Tregs with wild type naïve CD4 T cells resulted in worse colitis than co-transfer of wild type Tregs with wild type naïve CD4 T cells (344). IL-10 is another immune suppressive cytokine that can be produced by Tregs. Mice with Tregs deficient in IL-10 have an increase in colitis, lung hyperreactivity, and skin hypersensitivity, suggesting a role for IL-10 in mediating the suppressive function of Tregs (345,346).

1.3.2.3.3. Development of regulatory T cells: thymus-derived or peripherally induced

The source of Tregs to prevent excessive immune responses can come from two origins. First, Tregs can develop in the thymus and migrate to the periphery, and these are called natural or thymic Tregs. Secondly, Tregs can also develop in the periphery from the differentiation of naïve CD4 T cells into a phenotype that acquire regulatory functions, and these are called induced or adaptive Tregs. Both natural/thymic or induced/adaptive Tregs are important in self-tolerance and controlling excessive immune responses. It is unclear if they serve different roles, and there are no universally accepted and specific markers to differentiate them.

Natural/thymic Tregs are generated during the neonatal phase in the mouse and this generation starts even earlier in utero in humans (215,347). In the thymus, auto-reactive T cells are eliminated by negative selection as a mechanism to prevent self-reactivity and autoimmune diseases. An additional mechanism for prevention of self-reactivity is the development of Tregs in the thymus. Their development requires high affinity T cell receptor interactions with self-antigens – MHC II complexes presented by thymic epithelium or dendritic cells (348-350). Co-stimulatory signals are also necessary for thymic Treg development as loss of the CD80/86 – CD28 and CD40 – CD40L pathways decrease the number of Tregs generated in the thymus (215,307). Moreover, the cytokines IL-2 and IL-7 are required in the thymus for the development of natural/thymic Tregs (351). The

generation of Tregs in the thymus appears to happen in the CD4+CD8- single positive stage of T cell development, at the same time as positive and negative selection (352). Once generated in the thymus, natural/thymic Tregs are exported to peripheral tissues in a fully functional state and are thought to have stable expression of the master transcription FoxP3 (353,354).

As opposed to natural/thymic Tregs, induced/adaptive Tregs derive from the differentiation of naïve CD4+ T cells already present in the periphery. The decision to differentiate into induced/adaptive Tregs depends on the local microenvironment. In vitro experiments have shown that CD4+CD25- naïve T cells are able to convert to CD4+CD25hiFoxP3+ Tregs upon T cell receptor stimulation when cultured in the presence of both IL-2 and TGF-β (338,355). IL-2 is known to activate the transcription factor STAT5, which subsequently binds to the FoxP3 gene to promote Treg differentiation. Mice lacking IL-2 or IL-2 receptors all had a decrease in Tregs and developed autoimmune diseases. Similarly, mice with STAT5 deficiency also had a severe decrease in their Tregs (356-358). The co-presence of TGF-β with IL-2 was shown to be essential in pushing naïve CD4+ T cells to differentiate into induced/adaptive Tregs. As a matter of fact, SMAD3-deficient T cells, which are unable to respond to TGF-β, have a marked reduction in their differentiation into induced/adaptive Tregs (359). In contrast to natural/thymic Tregs, it is thought that induced/adaptive Tregs have a less stable expression of the master transcription factor FoxP3, and are susceptible to the microenvironment for maintaining their phenotype and suppressive function.

In the category of induced/adaptive Tregs, other non-FoxP3 regulatory cell types can also develop in the periphery. For example, IL-10 secreting CD4+ T cells have been described as Tr1 cells and were shown to have a role in preventing autoimmune colitis (360). Another example is the TGF-β secreting CD4+ T cells that have been termed Th3 cells, and were shown to have a role in oral tolerance (361). These other non-FoxP3 regulatory cell types will not be discussed further in this thesis as they are not extensively studied, appear to have limited roles in specific conditions, and lack an identified master transcription factor. Our focus will be on the conventional FoxP3+ Tregs.

1.3.2.3.4. Regulatory T cells plasticity and their special relationship with Th17

Although Tregs have suppressive function following their generation, it has been postulated that Tregs can lose their suppressive function and gain effector functions. This is thought to be a mechanism to adapt to diverse and changing microenvironments to ensure an adequate balance between regulatory and effector responses (362). This plasticity in the Treg lineage appears to be dependent on the local milieu. In particular, lymphopenic and inflammatory microenvironments can potentially transform Tregs into Teffs by re-programming their gene expression (363-365).

Approximately 50% of purified Tregs adoptively transferred into T cell-deficient mice lost their expression of the master transcription factor FoxP3 after 4 weeks,

and this was not due to the outgrowth of contaminated FoxP3- T cells. These purified Tregs that lost their FoxP3 expression in a lymphopenic environment also had low expression of other Treg signature molecules such as CD25 and CTLA-4, while secreting pro-inflammatory cytokines such as IFN-γ, IL-17, and IL-2. They also lost their suppressive function (366).

In non-lymphopenic conditions, it is also thought that inflammatory signals could transform Tregs into various subtypes of Teffs. In the presence of classic effector T helper cell polarizing cytokines in vitro, purified Tregs can lose their FoxP3 expression and take on the corresponding T helper cell characteristics. In some circumstances, Tregs can also retain their FoxP3 expression while acquiring a hybrid effector T helper-like phenotype. When purified Tregs are activated in the presence of IL-4, this resulted in their transformation into IL-4-secreting Th2 cells (367,368). In the presence of the Th1 polarizing cytokine IL-12, this led to their transformation into IFN-γ-secreting Th1 cells, although FoxP3 expression was maintained. This was similar in some in vivo murine models of infection or autoimmune diseases where FoxP3+IFN-γ+ T cells were detected (362,369-371). In the presence of the Th1 signature cytokine IFN-γ, however, other studies have reported that Tregs have a limited ability to expand and lose their expression of the prototypical marker CD25 and the master transcription factor FoxP3 (372). In the presence of the Th17 polarizing cytokine IL-6, this generated a transformation into IL-17-secreting Th17 cells (367,368). FoxP3+IL-17+ cells, however, have been identified in humans and mice in vivo (240,373,374). When a hybrid situation

occurs where both FoxP3 and an effector T helper cell-like phenotype co-exists, it remains unclear what role these cells have. It appears that suppressive function is generally maintained, and that this effector phenotype might be used for the mediation of suppressive function in specific circumstances.

A special relationship to note is the one that exists between Treg and Th17 cells with regards to their differentiation requirement in the periphery. The difference in cytokine requirements for their generation is minimal, and an inflammatory microenvironment can tip the differentiation of naïve T cells from a regulatory Treg phenotype to an effector Th17 phenotype. As a matter of fact, the differentiation of naïve CD4+ T cells into Tregs generally requires IL-2 and TGF-β. The addition of a pro-inflammatory cytokine such as IL-6 or IL-1β to TGF-β, however, promotes the differentiation of naïve CD4+ T cells into Th17 cells (355). The balance between Treg and Th17 cells could therefore be slanted towards Th17 in inflammatory milieus, such as renal IRI in the context of this thesis.

1.3.2.3.5. Number and suppressive function of regulatory T cells in immune-driven diseases

Tregs are indispensable in the maintenance of self-tolerance and prevention of allergies. As a matter of fact, an impairment in Tregs have been implicated in various human autoimmune diseases, including type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis (375-378). Tregs also play an

important role in maintaining transplant allograft and fetal-maternal tolerance during pregnancy. On the contrary, an excess of Tregs in oncologic diseases suppresses anti-tumor immune responses and favors cancer progression (215,379). Two factors come into play when analyzing the role of Tregs in immune-driven diseases. First, a decrease in the number of Tregs can tip the balance towards Teffs, and promote excessive immune responses. Secondly, even if the number of Tregs is adequate, excessive immune responses can still occur if the Tregs have a defect in their suppressive function.

There is ample evidence pointing towards the fact that Treg number, Treg suppressive function, or both are involved in immune-driven disease processes. Although the data is clearer in mice, it is more conflicting in humans. For example, with regards to type 1 diabetes, it was shown that the number and suppressive function of Tregs were both decreased in the NOD mouse model of the disease (380-382). Overall, the data from studying human subjects with type 1 diabetes point towards a defect in Tregs, but are more conflicting. Several groups have shown that the number of Tregs is similar to healthy controls, but Tregs suppressive function is decreased with type 1 diabetes (375,383). There are, however, studies showing that Treg frequency is decreased or unaltered compared to healthy controls (384,385). Another example of an autoimmune disease in which Treg number and function have been studied is multiple sclerosis. In the mouse model of multiple sclerosis termed experimental autoimmune encephalitis, a deficiency in Treg number and a defect in the suppressive mechanism of Tregs were shown in different studies to

exacerbate experimental autoimmune encephalitis (386-389). In humans, patients suffering from multiple sclerosis seem to have no change in their Treg numbers compared to healthy controls (376,390,391). Their Treg suppressive function, however, was reported to be defective (376). Similarly to the previous examples, systemic lupus erythematosus is another autoimmune disease in which Treg number and function were both shown to have a potential role in the disease process, again with conflicting results. The majority of studies reported that patients with systemic lupus erythematosus have a decrease in Treg number compared to healthy controls (392-399). Treg suppressive function was also found to be defective in patients with systemic lupus erythematosus in various studies (396,399,400). Both Treg number and suppressive function inversely correlated with disease activity (401). Nevertheless, a minority of studies exist that contradict the above findings and show either an increase in Treg number or no difference in Treg suppressive function in patients with systemic lupus erythematosus (401-403). In the context of patients with end-stage renal disease who could eventually become kidney transplant candidates, there is evidence that they can also have a defect in their Treg number and suppressive function. In a comparative study between 80 end-stage renal disease patients and 17 healthy controls, Treg number was lower in patients with end-stage renal disease. Moreover, end-stage renal disease patients also had a defect in their Treg suppressive function, and this was more pronounced in those who already required dialysis (hemodialysis or peritoneal dialysis) (404). Another smaller study comparing 14 hemodialysis patients with 14 healthy controls, however, showed that Treg number was similar

between both groups. Nevertheless, although number was similar, suppressive function was also defective in the hemodialysis group (405).

The conflicting results between different studies regarding the association between Treg number or function and immune-driven diseases are likely explained by several factors. First, the identification of Tregs by the conventional markers CD25, CD127, and FoxP3 is imperfect in humans. Similarly to Tregs, recently activated Teffs can have transient expression of the surface marker CD25 and the transcription factor FoxP3, as well as downregulation of CD127 expression (215,406,407). The measurement of Tregs using these markers could therefore be contaminated by a Teff component. With regards to measuring Treg suppressive function, this involves the in vitro co-culture of purified Tregs with activated Teffs, and the determination of Teff proliferation inhibition by Tregs. Different methods used to purify Tregs (different cell markers, magnetic bead isolation versus flow cytometry cell sorting) and determine Teff proliferation (carboxyfluorescein succinimidyl ester [CFSE] versus 3H-thymidine) could affect the measurement of Treg suppressive function (408). Moreover, it is unknown if the in vitro quantification of Treg suppressive function reflects what actually happens in vivo (215). It has also become recently evident that Tregs are not simply a homogeneous population characterized solely by the conventional markers CD25, CD127, and FoxP3. In fact, Tregs appear to be a heterogeneous population that could have specific additional markers leading them to have enhanced suppressive function in special circumstances.

1.3.2.3.6. Regulatory T cells heterogeneity and novel identification markers

Traditionally, Tregs have been thought to be a homogeneous CD4+ T cell population that can be identified by the high expression of the surface marker CD25 and the master transcription factor FoxP3, as well as the low expression of the surface marker CD127. As discussed in the previous section, a disparity in the results obtained from the measurement of Treg number and suppressive function in human diseases led to the idea that Tregs are a heterogeneous population. Recent evidence in fact suggests that Tregs can be divided into different subsets that can have distinct suppressive functional properties adjusted to different microenvironments (409).

Tregs progress through different stages of maturity as evidence by their expression of the naïve T cell marker CD45RA and the memory T cell marker CD45RO. Miyara et al. has shown that the expression of CD45RA along with the traditional markers CD25 and FoxP3 can divide Tregs into three distinct subpopulations with different functional properties: resting CD25++FoxP3^{lo}CD45RA+ Tregs, activated CD25+++FoxP3^{hi}CD45RA- Tregs, and cytokine-secreting CD25++FoxP3^{lo}CD45RA- Tregs. Both resting and activated Tregs had in vitro suppressive activity, while cytokine-secreting Tregs did not. The activated Tregs appeared to derive from the resting Treg population, and was short-lived (410). It remains unclear, however, whether suppressive activity is limited to Tregs expressing the naïve T cell marker CD45RA as other studies showed that both naïve CD45RA+ and memory CD45RO+

subsets of Treg are equally suppressive (411,412). HLA-DR is another marker of maturity that is usually present on activated T cell in humans. The expression of HLA-DR on Tregs has been shown to delineate a distinct subpopulation of Treg with higher suppressive activity. As a matter of fact, HLA-DR+ Tregs were shown to be highly suppressive by carrying out early contact-dependent suppression, while HLA-DR- Tregs were less suppressive by utilizing an early IL-10-dependent mechanism followed by a later suppression by a contact-dependent mechanism (413). CD62L, a member of the selection adhesion molecule family, is also another marker of maturity that distinguishes a highly suppressive population of Treg. CD62L plays an essential role in the homing of lymphocytes to peripheral lymph nodes. In Teffs, CD62L expression is used to divide memory T cells into CD62L+ central memory T cells that migrate to peripheral lymphoid organs and require further stimulation and CD62L- effector memory T cells that display immediate effector functions. With regards to Tregs, their expression of CD62L suggests that they are not fully activated and have a more naïve phenotype. Studies have shown that CD62L+ Tregs more potently suppress Teffs in vitro by 3 to 4 fold compared to CD62L- Tregs (414). Moreover, CD62L+ Tregs appear to mediate their suppression via contact-dependent mechanisms (415).

The origin of Tregs is also a potential determinant of suppressive activity. Thymic/natural Tregs are thought to have stable expression of the master transcription FoxP3 that is essential for suppressive activity. In comparison, recently activated Teffs only transiently express FoxP3. The stable expression of

FoxP3 in thymic/natural Tregs requires epigenetic DNA-methylation based regulation. It was found in both mice and humans that a CpG dinucleotide-rich non-coding conserved region within the FoxP3 gene, termed Treg-specific demethylation region (TSDR), is constitutively fully demethylated in thymic/natural Tregs and thus open to transcription factors. On the other hand, this region is fully methylated in Teffs and only partially demethylated in induced/adaptive Tregs (416-418). Assessing the methylation status of the TSDR can therefore identify truly suppressive Tregs with stable FoxP3 gene expression. Quantifying the TSDR methylation status, however, requires fixation and permeabilization of the cell. This destructive process thus does not allow the use of epigenetic quantification in isolating highly suppressive Tregs for cell-based therapy (409).

Other markers of Tregs that can identify subpopulations of highly suppressive Tregs while maintaining cell integrity exist and are based on mechanisms of suppression used by Tregs. These markers include CD39, CTLA-4, ICOS, and TNFR2. CD39 is a member of the ectonucleoside triphosphate diphosphohydrolase family, and plays a role in controlling ATP-mediated inflammation by hydrolyzing extracellular ATP to adenosine. The resulting effect is a decrease in pro-inflammatory ATP and increase in anti-inflammatory adenosine. Several studies have shown that the expression of CD39 on Tregs identifies a highly suppressive subset in vitro and in vivo (329,419-421). In particular, CD39+ Tregs appear to be important in inflammatory environments and in suppressing Th17 responses (419,421). CTLA-4 is critical in the function of Tregs and provides a negative feedback loop to prevent excessive T

cell immune responses by inhibiting the CD28 – CD80/CD86 co-stimulation pathway. It was in fact shown that Treg-specific CTLA-4 deficiency impaired in vitro suppressive function of Tregs as well as in vivo suppressive function of Tregs. Mice with CTLA-4 deficiency in their Tregs developed spontaneous systemic lymphoproliferation and fatal T-cell mediated autoimmune disease (308). ICOS is another co-stimulatory molecule that can identify highly suppressive Tregs in both mice and humans. ICOS is usually present on activated T cells and interact with the ICOS-ligand on APCs to enhance Teff response. It was found that ICOS is also expressed on Tregs, and that ICOS+ Tregs were more potently suppressive than ICOS- Tregs both in vitro and in vivo in mice (422,423). Similarly, in humans, ICOS+ Tregs isolated from melanoma-infiltrating lymphocytes showed superior suppressive function than ICOS- Tregs (424). TNFR2 is a cytokine receptor lacking an intracellular death domain that is mostly restricted to lymphocytes and mediates the biological function of TNF-α (425). By binding TNF-α, TNFR2 is involved in the proliferation of Teffs (426). TNFR2, however, is predominantly expressed on Tregs as compared to Teffs (333). It has been shown that the interaction between TNF-α and TNFR2 on Tregs is critical in the generation, proliferation, and suppressive function of Tregs in both mice and humans. TNFR2 was shown to be a better marker than CD25 to identify suppressive Tregs, as CD4+TNFR2+ cells are 4 times more suppressive than CD4+CD25+ cells in the mouse (334). Moreover, CD4+FoxP3+ or CD4+CD25+ cells lacking TNFR2 expression only had minimal suppressive function (334,426). Similar results were found in humans where CD4+CD25+TNFR2+ Tregs expressed the highest levels of the master transcription factor FoxP3 and had the

most potent suppressive function (333). Due to the traditional pro-inflammatory nature of TNF-α, it is thought that TNFR2 expression might be particularly important for the suppressive function of Tregs at inflammatory sites (335). TNFR2+ Tregs have an increase production of the anti-oxidative molecule thioredoxin-1 and thus have increased resistance to the oxidative stress caused by inflammation (427).

1.3.2.3.7. Regulatory T cells in experimental renal ischemia-reperfusion injury and DGF/SGF after kidney transplantation

Several studies have demonstrated a role for Tregs in preventing murine renal IRI. Both Treg number and suppressive function prior to and/or after renal IRI appeared to have a role in reducing kidney damage in murine models. These studies used either unilateral or bilateral renal pedicle clamping followed by reperfusion as a model to mimic DGF/SGF after kidney transplantation. These models are however imperfect as they lack the CIT associated with kidney transplantation.

1.3.2.3.7.1. Regulatory T cell number prior to murine renal ischemia-reperfusion injury

In a mouse model of bilateral renal pedicle clamping for 24 minutes followed by reperfusion for 24 hours, Kinsey et al. showed that the number of Tregs prior to renal IRI is important in dampening damage. Partial depletion (approximately 50%)

of Tregs with anti-mouse CD25 monoclonal antibody prior to renal IRI resulted in more severe kidney damage compared to controls as evidence by higher plasma creatinine, worse acute tubular necrosis, and increased intra-renal accumulation of neutrophils, macrophages, and pro-inflammatory cytokines. In addition, RAG-1 knockout mice lacking T and B cells reconstituted with lymph node cells from FoxP3-deficient Scurfy mice had significantly worse renal IRI than RAG-1 knockout mice reconstituted with lymph node cells from wild type mice. As a further proof of concept, co-transfer of isolated wild type CD4+CD25+ Tregs with lymph node cells from FoxP3-deficient Scurfy mice at a 1 to 5 ratio reversed the heightened renal IRI associated with Treg deficiency (428). In another study from the same group, Tregs were found to play a mechanistic role in the protective effect of ischemic preconditioning on renal IRI. Mice underwent ischemic preconditioning with bilateral renal pedicle clamping for 24 minutes, allowed to recover for 7 days, and then underwent renal IRI with bilateral renal pedicle clamping for 28 minutes. Ischemic preconditioning significantly decreased loss of kidney function, acute tubular necrosis, and the accumulation of neutrophils and macrophages in the subsequent renal IRI compared to sham control, and this was mediated by a significant increase in intra-renal CD4+CD25+FoxP3+ Tregs following ischemic preconditioning. Partial depletion of Tregs using an anti-CD25 monoclonal antibody following ischemic preconditioning reversed its protective effect on the subsequent renal IRI. Additionally, the adoptive transfer of isolated CD4+CD25+ Tregs prior to renal IRI instead of ischemic preconditioning also decreased loss of kidney function,

acute tubular necrosis, and the accumulation of neutrophils and macrophages in a dose-dependent manner (429).

Pharmacologic strategies to increase the number of Tregs in the mouse prior to renal IRI have also shown a protective effect. Lai et al. treated mice with N, N-dimethylsphingosine (DMS) prior to 30 minutes of bilateral renal pedicle clamping. DMS is a sphingosine kinase inhibitor with an effect on T cell trafficking and without a direct effect on renal function or histopathology. The injection of DMS led to a 10-fold increase in CD4+FoxP3+ Treg trafficking to the kidneys, and its injection prior to renal IRI resulted in a significant reduction in serum BUN/creatinine, acute tubular necrosis, and neutrophil infiltration at 24 hours following reperfusion. The protective effect of DMS was reversed by the co-administration of the depleting Treg agents anti-CD25 (50% Treg depletion) or anti-CTLA-4 (25% Treg depletion) monoclonal antibodies (430). Kim et al. induced murine in vivo Treg expansion with IL-2C, an IL-2/anti-IL-2 complex. Treatment with IL-2C prior to 28 minutes of bilateral renal pedicle clamping resulted in an increase in kidney-infiltrating CD4+FoxP3+ Tregs in the kidneys as well as a decrease in serum BUN/creatinine, acute tubular injury, apoptosis, and accumulation of neutrophils and macrophages between 1 and 3 days after reperfusion (431). The use of periodate-oxidized ATP (oATP), a P2X7 receptor antagonist, is known to suppress Teffs and innate immunity while inducing Treg expansion by interfering with response to the pro-inflammatory effects of extracellular ATP. Koo et al. showed that the injection of oATP prior to 27 minutes of bilateral renal pedicle clamping increased the number

of kidney-infiltrating CD4+FoxP3+ Tregs. This was also associated with improvements in renal function and histological injury at 1 day following reperfusion, as well as a decrease in kidney-infiltrating dendritic cells, neutrophils, and macrophages (432). Stremska et al. created the novel hybrid cytokine IL233, which bears the activities of both IL-2 and IL-33. The maintenance of the Treg population is dependent on IL-2, and the addition of IL-33 to create this hybrid cytokine further increased the in vivo expansion of Tregs in mice. The administration of IL233 prior to 26 minutes of murine bilateral renal pedicle clamping led to an improvement in plasma creatinine and acute tubular necrosis as well as a reduction in kidney-infiltrating leukocytes at 24 hours post-reperfusion (433).

1.3.2.3.7.2. Regulatory T cell suppressive function prior to murine renal ischemia-reperfusion injury

Several murine studies also show that the suppressive function of Tregs rather than only the number of Tregs prior to renal IRI also impact kidney damage following reperfusion. These studies either created a defect in a mechanism of suppression used by Tregs, or pharmacologically enhanced their suppressive function.

Kinsey et al. showed that a Treg-specific deficiency in IL-10 abolished the protective effect of Tregs in renal IRI. Using RAG-1 knockout mice (T and B cell deficient), the adoptive transfer of CD4+CD25+ Tregs from IL-10 knockout mice prior to 28

minutes of bilateral renal pedicle clamping had no protective effect on kidney damage as opposed to the adoptive transfer of wild-type CD4+CD25+ Tregs (428). The same group further showed that a Treg-specific defect in adenosine signalling also abolished the protective effect of Tregs in renal IRI. The ability for Tregs to generate or respond to adenosine has been shown to play a role in mediating their suppressive function. CD73-deficient Tregs are not able to generate adenosine, while A_{2A}R-deficient Tregs are unresponsive to adenosine. The adoptive transfer of CD73-deficient Tregs or A_{2A}R-deficient Tregs prior to 28 minutes of murine bilateral renal pedicle clamping offered no renal protection at 18 and 48 hours following reperfusion in contrast to the renal protection offered by the adoptive transfer of wild type Tregs (434).

Using pharmacologic strategies, other groups have increased Treg suppressive function in addition to number prior to murine renal IRI and showed a protective effect. oATP, a P2X7 receptor antagonist, does not only expand Tregs but also increased their suppressive function as demonstrated by their positive effect on Tregs at inhibiting Teff proliferation in vitro. The injection of oATP prior to 27 minutes of murine bilateral renal pedicle clamping ameliorated kidney function and histological injury at 24 hours post-reperfusion as previously mentioned (432). The hybrid cytokine IL233 described in the previous section not only expanded Tregs but also increased the ability of Tregs to suppress CD4+ T cell proliferation in vitro. Its administration prior to 26 minutes of murine bilateral renal pedicle clamping

ameliorated kidney function, histological injury, and the infiltration of leukocytes at 24 hours post-reperfusion as previously mentioned (433).

1.3.2.3.7.3. Regulatory T cell number after murine renal ischemia-reperfusion injury

Other studies have looked at the potential of Tregs to contribute to the repair process during the healing phase following renal IRI. Gandolfo et al. showed that there was a significant increase in CD4+CD25+FoxP3+ Treg trafficking in the ischemic kidneys at 3 and 10 days following 45 minutes of murine unilateral renal pedicle clamping. Depletion of Tregs using an anti-CD25 monoclonal antibody at 24 hours following unilateral murine renal IRI increased renal tubular damage, reduced tubular proliferation, and increased pro-inflammatory cytokine production by infiltrating T cells in the ischemic kidney at 3 and 10 days post-reperfusion compared to the contralateral control kidney. On the other hand, the adoptive transfer of CD4+CD25+ Tregs at 24 hours following unilateral renal IRI markedly reduced tubular damage and pro-inflammatory cytokine generation by kidney-infiltrating T cells in the ischemic kidney (435). The same group also showed that the administration of the anti-metabolite mycophenolate mofetil starting at 48 hours following 45 minutes of unilateral murine renal pedicle clamping worsened kidney tubular damage at 10 days post-reperfusion, and this was associated with a marked reduction in kidney-infiltrating CD4+CD25+FoxP3+ Tregs (436). The pharmacologic agents IL-2C, oATP, and IL233 described in the previous sections were also administered after murine renal IRI. These agents were shown to increase

Treg numbers even when administered after murine renal IRI, and this was associated with an improvement in kidney damage. IL-2C administered starting at day 1 following 28 minutes of murine bilateral renal pedicle clamping for 3 consecutive days was shown to enhance Treg number and improve renal function (431). Similarly, the administration of oATP starting at day 1 following 27 minutes of murine bilateral renal pedicle clamping for 4 consecutive days also enhanced kidney-infiltrating Tregs as well as improved renal function and histologic damage post-reperfusion (432). Moreover, IL233 administered starting at 2 hours following 26 minutes of murine bilateral renal pedicle clamping for 5 consecutive days had the same effect (433).

1.3.2.3.7.4. Regulatory T cell suppressive function after murine renal ischemia-reperfusion injury

The previously described pharmacologic agents oATP and IL233 have a positive effect on both the number and suppressive function of Tregs. The administration of these agents following murine renal IRI improved kidney damage as mentioned above. In addition, Chen et al. used a different strategy to suggest that Treg suppressive function also plays a role in dampening kidney damage when increased after renal IRI. As a matter of fact, the in vitro stimulation of isolated Tregs with rapamycin has been shown to promote their expansion and suppressive function. The adoptive transfer of rapamycin-treated CD4+CD25+ Tregs at 24 hours following

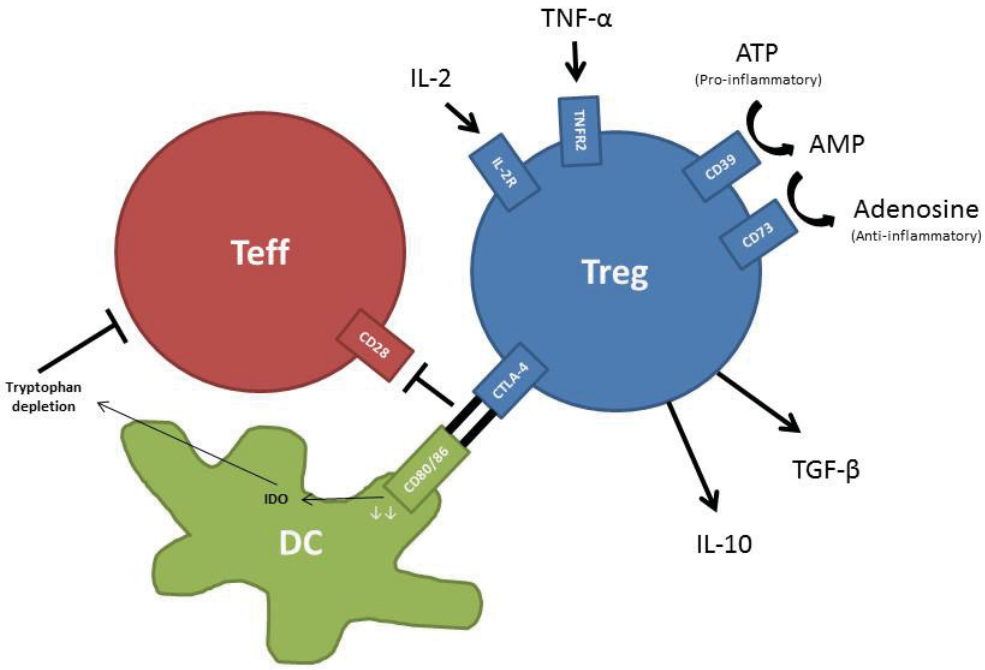
22 minutes of murine bilateral renal pedicle clamping improved renal function at day 3 post-reperfusion as well as histological damage (437).

1.3.2.3.7.5. Regulatory T cell and DGF/SGF after kidney transplantation

Although multiple murine studies have suggested an important role for Tregs in dampening renal IRI, the models of unilateral or bilateral renal pedicle clamping do not perfectly mimic the injury occurring during kidney transplantation leading to DGF/SGF. These models are limited to a period of warm ischemic time. In contrast, the IRI in kidney transplantation is the result of a period of CIT followed by warm ischemic time prior to reperfusion. There have been no studies looking specifically at Tregs and its potential contribution to DGF/SGF after kidney transplantation.

Figure 1.

Summary of direct cell-to-cell contact and microenvironment modulatory mechanisms used by Tregs to exert their immune suppressive function presented in section 1.3.2.3.2. Mechanisms of action of regulatory T cells. ATP, adenosine triphosphate; AMP, adenosine monophosphate; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; Teff, effector T cell; Treg, regulatory T cell.



Hypothesis and specific aims

Since there is a protective role for Tregs in murine renal IRI, we hypothesized that Tregs also have a role in renal IRI in the form of DGF/SGF after human kidney transplantation. Specifically, our first aim was to study whether pre-transplant Treg number and/or suppressive function were predictive of DGF/SGF and its associated immunologic injury (acute rejection) after human kidney transplantation (Chapters 2 – 4). Although Tregs are protective and the Th17 signature cytokine IL-17 detrimental to early damage after murine renal IRI, it remains unknown what is the long-term Treg-Th17 balance after murine renal IRI and whether this influences chronic kidney damage. Our secondary aim was thus to study the long-term Treg and Th17 responses following murine renal IRI and whether this impacted the development of chronic kidney damage manifested as fibrosis or tubular atrophy (Chapter 5).

Preface to Chapter 2

Since there is a paucity of literature regarding the role of Tregs in DGF/SGF after human kidney transplantation, the first objective of this thesis was to investigate whether the murine data regarding the role of Tregs in renal IRI could be translated to the human setting. More specifically, we sought to determine in Chapter 2 whether Treg number, Treg suppressive function, or the balance between Treg and Teff were associated with the development of DGF/SGF after human kidney transplantation.

Chapter 2. Pre-transplant recipient Treg suppressive function predicts delayed and slow graft function after kidney transplantation

Minh-Tri JP Nguyen, Elise Fryml, Sossy K Sahakian, Shuqing Liu, Rene P Michel, Mark L Lipman, Istvan Mucsi, Marcelo Cantarovich, Jean I Tchervenkov, and Steven Paraskevas. Transplantation 2014; 98: 745 – 753.

Abstract

Background: Delayed (DGF) and slow (SGF) graft function are a continuous spectrum of ischemia-reperfusion related acute kidney injury (AKI) that increase the risk for acute rejection and graft loss after kidney transplantation. Regulatory T cells (Tregs) are critical in transplant tolerance and attenuate murine AKI. In this prospective observational cohort study, we evaluated whether pre-transplant peripheral blood recipient Treg frequency and suppressive function are predictors of DGF/SGF after kidney transplantation.

Methods: Deceased donor kidney transplant recipients (n=53) were divided into AKI (n=37; DGF, n=10; SGF, n=27) and immediate graft function (IGF, n=16) groups. Pre-transplant peripheral blood CD4+CD25^{hi}FoxP3+ Treg frequency was quantified by flow cytometry. Treg suppressive function was measured by suppression of autologous effector T cell proliferation by Treg in co-culture.

Results: Pre-transplant Treg suppressive function, but not frequency, was decreased in AKI recipients ($p<0.01$). In uni- and multivariate analyses accounting

for the effects of cold ischemic time and donor age, Treg suppressive function discriminated DGF from IGF recipients in multinomial logistic regression (odds ratio=0.77, $p<0.01$), accurately predicted AKI in receiver operating characteristic curve (AUC=0.82, $p<0.01$), and predicted 14-day estimated glomerular filtration rate in linear regression ($p<0.01$).

Conclusion: Our results indicate that recipient peripheral blood Treg suppressive function is a potential independent pre-transplant predictor of DGF/SGF.

2.1. Introduction

Acute kidney injury (AKI) related to ischemia-reperfusion is inevitable after kidney transplantation (12,39). It is severe enough in 20% of recipients to cause delayed graft function (DGF), which manifests as the need for dialysis within the first week after transplantation (9,12,39). A substantial number of recipients also suffer a milder form of AKI without dialysis requirement and are described as having slow graft function (SGF) (15,16,438). Both DGF and SGF independently increase the risk for long-term graft loss and acute rejection, while DGF also increases the risk for chronic allograft dysfunction (11,13,24,26,27,438). Diagnosis relies on post-transplant diuresis, serum creatinine, and need for dialysis, and is made after damage already occurred to the graft (9). Immunological measures of risk for DGF/SGF have not yet been identified, and could assist in preventing these important complications.

Regulatory T cells (Tregs) are CD4+ T lymphocytes most commonly identified by their expression of the surface molecule CD25 and their upregulation of the transcription factor forkhead box P3 (FoxP3) (299). These cells are essential in maintaining immune homeostasis by suppressing excessive immune responses via cell-cell contact mechanisms and release of soluble mediators. A deficiency in the frequency or a dysfunction in the suppressive function of Tregs is sufficient to break self-tolerance in healthy subjects (439). In the context of murine AKI, kidney-infiltrating Tregs were shown to be protective by modulating neutrophils, macrophages, and pro-inflammatory cytokine production by effector T cells (Teffs) (428,429,435). The role of Tregs in AKI after kidney transplantation is, however, unknown. We conducted a prospective observational cohort study of deceased donor kidney transplant recipients to investigate whether pre-transplant recipient peripheral blood Treg frequency and suppressive function predicted AKI (DGF/SGF) and subsequent short-term outcomes after kidney transplantation.

2.2. Results

Patient characteristics

Consecutive consenting adult deceased donor kidney transplant recipients (n=53) were enrolled into the study. Recipients were prospectively divided into 1) DGF group (n=10), defined as recipients requiring dialysis within 7 days of transplantation, 2) SGF group (n=27), defined as recipients with a decrease in 24-

hour serum creatinine <20% and without requiring dialysis within 7 days of transplantation, and 3) immediate graft function (IGF) group (n=16), defined as recipients with a decrease in 24-hour serum creatinine ≥20% (11). Since SGF and DGF are a continuous spectrum of ischemia-reperfusion injury, these two groups were also combined to form an AKI group (n=37) (16,26). Decision to initiate post-transplant dialysis was made independently by the treating physicians. Recipient, donor, and organ procurement information were collected prospectively (Table 1, S1). None of the recipients, including those with an autoimmune disease or a previous transplant, were on immunosuppressive therapy for at least 180 days prior to transplantation. AKI and IGF recipients received similar immunosuppressive regimen (detailed in Table 1). Significant differences in cold ischemic time (CIT), donor age, and use of expanded criteria donors (ECDs) were observed between AKI and IGF recipients. Histological allograft quality was similar between AKI and IGF recipients. Acute rejection episodes were more frequent in recipients with DGF.

Similar pre-transplant Teff and Treg frequencies between AKI (DGF/SGF) and IGF recipients

There were no significant differences in pre-transplant CD4+CD25- Teff frequencies, CD4+CD25^{hi}FoxP3+ Treg frequencies, FoxP3 expression on CD4+CD25^{hi} Tregs, and Treg to Teff ratio between DGF, SGF, and IGF recipients ($p>0.05$; Figure 1A-D).

Similar results to the ones reported above were found when comparing AKI and IGF recipients ($p>0.05$; Figure 1A-D).

Lower pre-transplant Treg suppressive function in AKI (DGF/SGF) recipients

We verified that inter-recipient variability in purities of enriched CD4+CD25- Teffs and CD4+CD25+ Tregs did not correlate with proliferation ($r=0.11$, $p=0.54$; Figure S1A) or suppressive function ($r=-0.06$, $p=0.73$; Figure S1B) respectively. Variability in percentage of FoxP3 expression in CD4+CD25+ Tregs enriched from different recipients also did not correlate with suppressive function ($r=0.26$, $p=0.13$; Figure S1C). None of the recipient baseline characteristics with immunomodulatory potential, including age (440,441), gender (442), body mass index (443,444), autoimmune disease diagnosis (398,445-447), vitamin D supplementation (448), statin therapy (449), previous blood transfusion (450), sensitization (451), and dialysis modality/duration (404), were predictive of pre-transplant Teff proliferation (Table S2) or Treg function (Table S3) in linear regression analysis.

No significant differences were found in pre-transplant Teff proliferation between DGF, SGF, and IGF recipients ($p=0.15$) or between AKI and IGF recipients ($p=0.06$; Figure 1E-F). Pre-transplant Treg suppressive function, however, was significantly lower in DGF (3.86 ± 1.86%) and SGF (11.71 ± 2.11%) in comparison to IGF (27.33 ± 5.00%) recipients ($p<0.01$). Treg function was also significantly lower in AKI in comparison to IGF recipients ($p<0.01$; Figure 1G-H).

Pre-transplant Treg suppressive function independently distinguishes DGF from IGF recipients

Since pre-transplant Treg function was decreased in DGF and SGF recipients, we examined whether it can distinguish recipients who will have DGF or SGF rather than IGF post-transplant. Using the IGF recipients as reference group, each percentage increase in pre-transplant Treg function decreased the odds of being in the DGF or SGF group by 23% and 10% respectively in univariate multinomial logistic regression analysis. CIT, donor age, and ECD category were the other significant variables in the univariate analysis. In a multivariate analysis accounting for CIT and donor age, Treg function remained a significant variable distinguishing DGF from IGF recipients (Table 2). We excluded ECD category and retained donor age in this multivariate analysis as well as all further ones below since a strong correlation existed between the two variables (Figure S2A) and donor age is the main determinant of ECD categorization (48). No or weak correlations existed among the other significant variables in the univariate analysis, including Treg function (Figure S2B-F).

Pre-transplant Treg suppressive function independently predicts AKI

Since DGF and SGF represent a continuum of injury (16,26), we investigated whether pre-transplant Treg function also predicts AKI (combined DGF/SGF group) after kidney transplantation. Receiver operating characteristic (ROC) curve analysis

showed that pre-transplant Treg function was accurate at predicting AKI with an area under the curve (AUC) of 0.82 (95% confidence interval: 0.65 – 1.00, $p<0.01$; Figure 2A). The optimal cut-off point for Treg function as a marker of AKI was determined by the largest sums of sensitivity and specificity. A pre-transplant Treg function <13% was thus chosen as the optimal cut-off point to predict AKI (sensitivity=75.0%, specificity=88.9%, positive predictive value=95.5%, negative predictive value=53.3%). Other variables that were significant predictors of AKI in ROC curve analysis were CIT >9.5 hours (Figure 2B), donor age >47 years old (Figure 2C), and ECD category (Figure 2D). Multivariate binary logistic regression accounting for CIT and donor age showed that a pre-transplant Treg function <13% remained a significant predictor of AKI with an adjusted odds ratio of 21.86 (Table 3).

Pre-transplant Treg suppressive function independently predicts 14-day graft function

We then sought to determine if pre-transplant Treg function predicted better short-term graft function regardless of AKI (DGF/SGF) or IGF grouping. Univariate linear regression analysis showed that each percentage increase in pre-transplant Treg function improved estimated glomerular filtration rate (eGFR) (452) by 0.53 – 0.82 mL/min/1.73m^2 up to 180 days post-transplant. CIT, donor age, and ECD category were the only other variables that also predicted eGFR up to 180 days post-transplant in the univariate analysis. In a multivariate analysis accounting for CIT

and donor age, Treg function remained a significant predictor of eGFR only at 14 days post-transplant, while both donor age and CIT remained significant predictors at 90 and 180 days post-transplant (Table S4).

2.3. Discussion

We are reporting a novel association between pre-transplant peripheral blood recipient Treg function and AKI (DGF/SGF) in deceased donor kidney transplant recipients. Although previous studies suggest that pre-transplant pro-inflammatory cytokines are associated with acute tubular necrosis after kidney transplantation (453), we did not find an increase in pre-transplant Teff responses in recipients with AKI. Instead, we found that recipients with AKI had a lower pre-transplant Treg function, and this was not related to recipient characteristics with immunomodulatory capacity. Additionally, our findings suggest that Treg function is a potential independent novel recipient-based peripheral blood immune marker for AKI (DGF/SGF) when measured prior to transplantation.

Previous candidate markers for AKI (DGF/SGF) have been previously studied in donor urine (132), machine perfusion fluid (146), and early post-transplant recipient urine samples (126,127,142). Measurement of pre-transplant recipient peripheral blood Treg function, however, has the following advantages. As opposed to donor and machine perfusion fluid markers, it allows guidance in the donor allocation process. In comparison to early post-transplant recipient markers, it

allows timely prediction of AKI prior to kidney transplantation and onset of graft damage, and the identification of recipient candidates at risk for AKI. In contrast to urine markers, measuring a peripheral blood-based marker is not limited by oliguria in the context of renal failure.

Although CIT significantly distinguished DGF from IGF recipients in univariate multinomial logistic regression and predicted AKI in univariate logistic regression, this variable was no longer significant in our multivariate analyses including Treg function. A possible explanation is that nearly all of our deceased donor grafts are preserved with machine perfusion, which has been shown to diminish the association between long CIT and the development of AKI (454). Furthermore, although both donor age and Treg function were significant variables in predicting AKI in uni- and multivariate analyses, our results suggest that Treg function might be more important than donor age in predicting recipients who will develop the most severe form of ischemia-reperfusion related graft injury. As a matter of fact, only Treg function significantly distinguished DGF from IGF recipients in multivariate multinomial logistic regression.

Previous mechanistic studies in murine ischemic AKI models support our finding that pre-transplant Treg function is crucial in determining immediate graft outcome regardless of donor and organ procurement variables. AKI after kidney transplantation is at first an inflammatory and antigen-independent event (455). Peripheral Tregs are known to home to areas of inflammation, and in the context of

murine ischemic AKI, were shown to traffic to the kidney, decrease infiltration of innate immune cells, inhibit production of pro-inflammatory cytokines by Teffs, and promote healing (428,435,456). Removal of the suppressive functional mechanisms of these trafficking peripheral Tregs prior to ischemia-reperfusion reversed their protective effect. In fact, Tregs depleted of their ability to suppress effector immune responses via CTLA-4 or via the secretion of soluble factors (adenosine, IL-10) were unable to protect from murine ischemic AKI in vivo (428,430,434). It is therefore plausible that kidney transplant recipients with less potently suppressive peripheral Tregs prior to transplantation are more susceptible to AKI after transplantation and that targeted therapies to enhance recipient Treg function could reduce the risk for AKI. Although still in the experimental phase, promising therapies currently exist, including transfusion of ex-vivo expanded highly suppressive Tregs and pharmacologic modulation of in vivo Treg function with protein kinase C-theta, glycogen synthase kinase-3β or histone deacetylase inhibitors (457-460).

We also found that pre-transplant Treg function predicted 14-day eGFR, while donor age and CIT were more important predictors of 90- and 180-day eGFR. Although donor age and CIT have traditionally been associated with worse long-term graft outcomes after kidney transplantation, this notion has been recently disputed. A large retrospective study of deceased donor kidney transplant recipients in fact showed that using older donor age grafts did not worsen 5-year graft survival (461). Additionally, a Scientific Registry of Transplant Recipients database analysis demonstrated that a longer CIT in paired donor recipients did not influence 8-year

graft survival (51). Further studies of pre-transplant Treg function with longer follow-up would be required to demonstrate a role for regulatory mechanisms in promoting long-term graft survival, potentially by dampening the pro-fibrotic effect of initial ischemia-reperfusion injury (39,462,463).

We acknowledge that the results of our study are limited by a small sample size. Nevertheless, it was sufficient to identify similar risk factors (CIT, donor age, ECD category) for AKI (DGF/SGF) as larger database studies (13,15). The Treg suppressive function assay in its current state also has its own limitations for clinical applicability in deceased donor kidney transplantation as it is time-consuming, labour-intensive, requires a large amount of recipient blood to isolate a scarce population of Tregs (<10% of total CD4+ T lymphocytes in healthy individuals) (464), and is not standardized between research groups with regards to Treg and Teff purification techniques (magnetic bead-based vs. flow sorting), Teff stimulation techniques (plate-bound vs. bead-coated anti-CD3/CD28), and Teff proliferation detection (H3-thymidine incorporation vs. CFSE dilution) (408). Due to concerns of anemia prior to surgery, the maximum amount of blood we were permitted to draw by our institutional ethics board yielded insufficient peripheral blood mononuclear cells (PBMCs) for flow-sorting enrichment of Teffs and Tregs. Flow sorting is also a technology that is not currently widely available in the emergency setting of transplantation. We therefore chose to enrich Treg and Teff cells solely by magnetic bead-based technique, which yielded a lower FoxP3 purity (37%) in the enriched CD4+CD25+ Tregs than expected with flow sorting. We

recognize that this could negatively affect the percentage suppression of Teff proliferation by Treg in the assay, and consequently the optimal cut-off point for Treg function as a predictor of AKI. Nevertheless, this has minimal impact on our findings since the assay was performed identically in all recipients. Lastly, we did not assess the stability of Treg function in the pre-transplant period by serial measures, although we acknowledge that this would be an important goal of a separate study. We could also not follow early post-transplant Treg function since all but one recipient received lymphodepleting induction immunosuppressive therapy. Although our findings are limited by the above conditions, the fundamental observation that Treg function is an important donor-independent pre-transplant recipient variable in the prediction of post-transplant graft injury is a novel concept in recipient risk stratification. Standardization, improvement, and external validation of this measure could ultimately be useful in redefining organ allocation schemes, guiding peri-transplant clinical decisions, as well as developing pre-transplant immunotherapy to specific measures of Treg function.

In conclusion, we found that pre-transplant recipient Treg function predicted AKI (DGF/SGF) after kidney transplantation. Measurement of recipient pre-transplant immune regulatory capacity, without prior knowledge of donor and organ procurement characteristics, could potentially indicate recipients at risk for AKI and graft damage prior to transplantation, guide peri-transplant clinical decisions, and identify recipients in whom development of novel immunotherapeutic strategies against AKI could be tested.

2.4. Materials and Methods

The study was approved by the McGill University Health Centre research ethics board, registered on ClinicalTrials.gov (NCT01232816), and conducted in adherence with the declarations of Helsinki and Istanbul.

Blood sample collection and mononuclear cell isolation

Peripheral blood (40 mL) was collected in heparin-coated tubes prior to induction immunosuppression and skin incision. PBMCs were isolated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ).

Treg and Teff frequencies

PBMCs were surface stained with anti-human CD4 FITC and CD25 PE antibodies, fixed and permeabilized with the FoxP3 staining buffer set as per the manufacturer's protocol, and stained intracellularly with anti-human FoxP3 PerCP-Cy5.5 antibody (all purchased from eBioscience, San Diego, CA). Flow cytometry acquisition was performed on the FACScan (BD Biosciences, San Jose, CA), and data analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Lymphocytes were gated on based on forward and side scatters. Treg and Teff frequencies were determined by the percentage of CD4+CD25^{hi}FoxP3+ and CD4+CD25- cells in the lymphocyte gate respectively (Figure S3).

Teff proliferation and Treg suppressive function assays

CD4+CD25+ Tregs and CD4+CD25- Teffs were enriched from PBMCs by magnetic bead isolation using the EasySep Human CD4+CD25+T Cell Isolation Kit as per the manufacturer's protocol (StemCell Technologies, Vancouver, Canada). Sufficient enriched cells were obtained in seven DGF, 21 SGF, and nine IGF recipients to perform Teff proliferation and Treg suppressive function assays. Purities of enriched CD4+CD25+ Tregs and CD4+CD25- Teffs were respectively 86 ± 1% (37 ± 2 % FoxP3+) and 85 ± 2% (3 ± 1% FoxP3+). Teffs were labeled with 5 μm/mL carboxyfluorescein succinimidyl ester (CFSE, Life Technologies, Burlington, Canada) at 37°C for 5 minutes, then washed with ice-cold 10% fetal bovine serum in phosphate buffered saline (408). In all assays, 4 x 10^4 CFSE-labeled Teffs were co-cultured with Tregs at a 1:0 or 1:1 ratio for 5 days in 200 μL of X-VIVO 15 media (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Sigma-Aldrich, Oakville, Canada) in the presence of anti-CD3/CD28-coated beads (1 bead/cell, Life Technologies). Using flow cytometry analysis, the unlabeled CFSE negative Tregs were excluded. Teff proliferation (1:0 ratio) was determined by CFSE dilution (% divided function in FlowJo software). Treg suppressive function was calculated as followed: 100 – (((% divided 1:1 Teff:Treg ratio) / (%divided 1:0 Teff:Treg ratio)) x 100). Titration of Teff:Treg ratio (1:1, 1:0.5, 1:0.125) in a subset of 32 kidney transplant recipients confirmed that the enriched CD4+CD25+ Tregs suppressed CD4+CD25- Teff proliferation in a dose dependent manner (Figure S4).

Statistical analysis

Data are expressed as mean ± standard error of the mean. Analyses were performed using SPSS 20 (IBM, Armonk, NY) and considered significant at a 0.05 level. Categorical data comparisons between DGF, SGF, and IGF groups or AKI and IGF groups were made using chi-square test. Continuous data comparisons between three groups were made using one-way ANOVA followed by Tukey HSD post-hoc analysis while comparisons between two groups were made using Student's t-test. Correlations were performed with Pearson's correlation coefficient. Multinomial logistic regression was performed to assess predictors of DGF or SGF from IGF. ROC curve analysis and binary logistic regression were performed to assess the predictive accuracy of recipient, donor, and organ procurement characteristic variables for AKI. ROC curve analysis was also performed to determine the optimal cut-off point of each significant variable as a marker for AKI based on the largest sums of sensitivity and specificity. Linear regression was performed to assess recipient, donor, and organ procurement characteristic variables as predictors of post-transplant eGFR and to assess baseline recipient characteristics as predictors of pre-transplant Teff proliferation and Treg function. All significant variables in univariate analyses were considered for the multivariate analyses. We excluded ECD category from all multivariate analyses since this variable was strongly collinear with donor age (Figure S2A) and donor age is the main determinant of ECD categorization (48).

Table 1.

TABLE 1. Clinical information regarding recipient, donor, and organ procurement

	IGF	SGF	DGF	P^a	AKI	P^b
Recipient characteristics						
n	16	27	10		37	
Age, yr	60±3	58±2	56±4	0.61	57±2	0.41
Male	11	22	8	0.61	30	0.33
African American race	2	2	2	0.55	4	0.86
Diagnosis						
SLE	0	1	0	0.61	1	0.51
GN	3	10	2	0.35	12	0.31
DM2	4	9	2	0.68	11	0.73
HTN	0	1	1	0.43	2	0.34
Other	9	6	5	0.06	11	0.07
BMI, kg/m^2	27±1	28±1	28±2	0.75	28±1	0.47
PRA>50%	2	2	2	0.55	4	0.86
Previous Tx	0	2	4	<0.01	6	0.09
HLA mismatches	3.2±0.3	3.4±0.2	3.2±0.4	0.75	3.4±0.2	0.59
Pre-Tx dialysis	13	24	10	0.26	34	0.14
Time on dialysis, yr	3.6±0.6	3.3±0.6	6.0±1.6	0.08	4.0±0.6	0.69
Pre-Tx eGFR, mL/min/1.73 m^2	11±1	12±1	10±1	0.24	11±1	0.75
Immunosuppression regimen						
ATG Tac, MMF, steroid[c]	10	17	5	0.54	22	0.28
Alemtuzumab Tac, MMF[d]	5	10	5		15	
Daclizumab Tac, MMF, steroid[c]	1	0	0		0	
Donor characteristics						
Age, yr	36±3	56±3	54±4	<0.01	56±2	<0.01
ECD	1	16	6	<0.01	22	<0.01
DBD	16	26	9	0.43	35	0.34
DCD	0	1	1		2	
Terminal eGFR, mL/min/m^2	116±12	136±15	128±15	0.60	134±11	0.28
Kidney biopsy						
ATN	3	4	1	0.89	5	0.67
GS	1	1	0	0.75	1	0.59
IF/TA	0	1	0	0.60	1	0.48
Procurement information						
CIT, hr	11±1	16±1	20±3	<0.01	17±1	<0.01
Machine perfusion	14	24	7	0.34	31	0.73

[a] *P* value for comparisons among DGF, SGF, and IGF groups.
[b] *P* value for comparisons between AKI and IGF groups.
[c] Maintenance immunosuppression starting on day 1 with tacrolimus (trough levels 4–8 ng/mL), MMF, and corticosteroid tapering protocol.
[d] Maintenance immunosuppression starting on day 1 with tacrolimus (trough levels 4–8 ng/mL) and MMF.

AKI, acute kidney injury; ATG, antithymocyte globulin; ATN, acute tubular necrosis; CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after cardiac death; DGF, delayed graft function; DM2, diabetes mellitus type 2; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; F, female; GN, glomerulonephritis; GS, glomerulosclerosis; HTN, hypertension; IGF, immediate graft function; IF, interstitial fibrosis; TA, tubular atrophy; M, male; MMF, mycophenolate mofetil; PCKD, polycystic kidney disease; PRA, panel reactive antibody; SGF, slow graft function; SLE, systemic lupus erythematosus; Tac, tacrolimus; Tx, transplant; BMI, body mass index; HLA, human leukocyte antigen.

Table 2.

TABLE 2. Multinomial logistic regression analysis to predict DGF or SGF with IGF as reference group

	Univariate analysis			Multivariate analysis		
Variables	**OR**	**95% CI**	***P***	**Adjusted OR**	**95% CI**	***P***
Treg function, %						
DGF	0.77	0.64–0.93	<0.01	0.79	0.65–0.97	0.03
SGF	0.90	0.83–0.98	0.01	0.90	0.80–1.00	0.06
IGF	1.00			1.00		
CIT, hr						
DGF	1.30	1.09–1.54	<0.01	1.08	0.79–1.50	0.62
SGF	1.21	1.04–1.40	0.01	0.97	0.72–1.30	0.84
IGF	1.00			1.00		
Donor age, yr						
DGF	1.08	1.02–1.15	0.01	1.05	0.96–1.15	0.30
SGF	1.10	1.04–1.15	<0.01	1.08	1.01–1.17	0.03
IGF	1.00			1.00		
ECD						
DGF	22.50	2.07–244.84	0.01			
SGF	21.82	2.50–190.12	<0.01			
IGF	1.00					

CI, confidence interval; CIT, cold ischemic time; DGF, delayed graft function; ECD, expanded criteria donor; IGF, immediate graft function; OR, odds ratio; SGF, slow graft function; Treg, regulatory T cell.

Table 3.

TABLE 3. Logistic regression analysis to predict AKI

	Univariate analysis			Multivariate analysis		
Variables	**OR**	**95% CI**	***P***	**Adjusted OR**	**95% CI**	***P***
Treg function<13%	24.00	2.54–227.24	<0.01	21.86	1.25–381.89	0.04
CIT	1.23	1.06–1.42	<0.01	0.98	0.74–1.31	0.91
Donor age	1.09	1.04–1.15	<0.01	1.07	1.01–1.15	0.04
ECD	22.00	2.62–184.75	<0.01			

AKI, acute kidney injury; CI, confidence interval; CIT, cold ischemic time; ECD, expanded criteria donor; OR, odds ratio; Treg, regulatory T cell.

Figure 1.

Pre-transplant Treg suppressive function, but not Teff frequency, Treg frequency, %FoxP3 expression in Treg, Treg to Teff ratio, or Teff proliferation, was significantly lower in DGF, SGF, and AKI recipients in comparison to IGF recipients. Comparisons of pre-transplant A) CD4+CD25- Teff frequency, B) CD4+CD25hiFoxP3+ Treg frequency, C) %FoxP3 expression in CD4+CD25hi Treg, and D) CD4+CD25hiFoxP3+ Treg to CD4+CD25- Teff ratio were performed between DGF, SGF, and IGF recipients or AKI and IGF recipients. Comparison of pre-transplant E) CD4+CD25- Teff proliferation was performed between DGF, SGF, and IGF recipients or AKI and IGF recipients with F) representative pre-transplant Teff proliferation assays in an IGF, SGF, and DGF recipient. Comparison of pre-transplant G) CD4+CD25+ Treg suppressive function was performed between DGF, SGF, and IGF recipients (*: one-way ANOVA, $p<0.01$, followed by Tukey HSD post-hoc analysis, $p<0.01$ versus IGF group) or AKI and IGF recipients (#: Student's t test, $p<0.01$ versus IGF group) with H) representative pre-transplant Treg suppression function assays in an IGF, SGF, and DGF recipient.

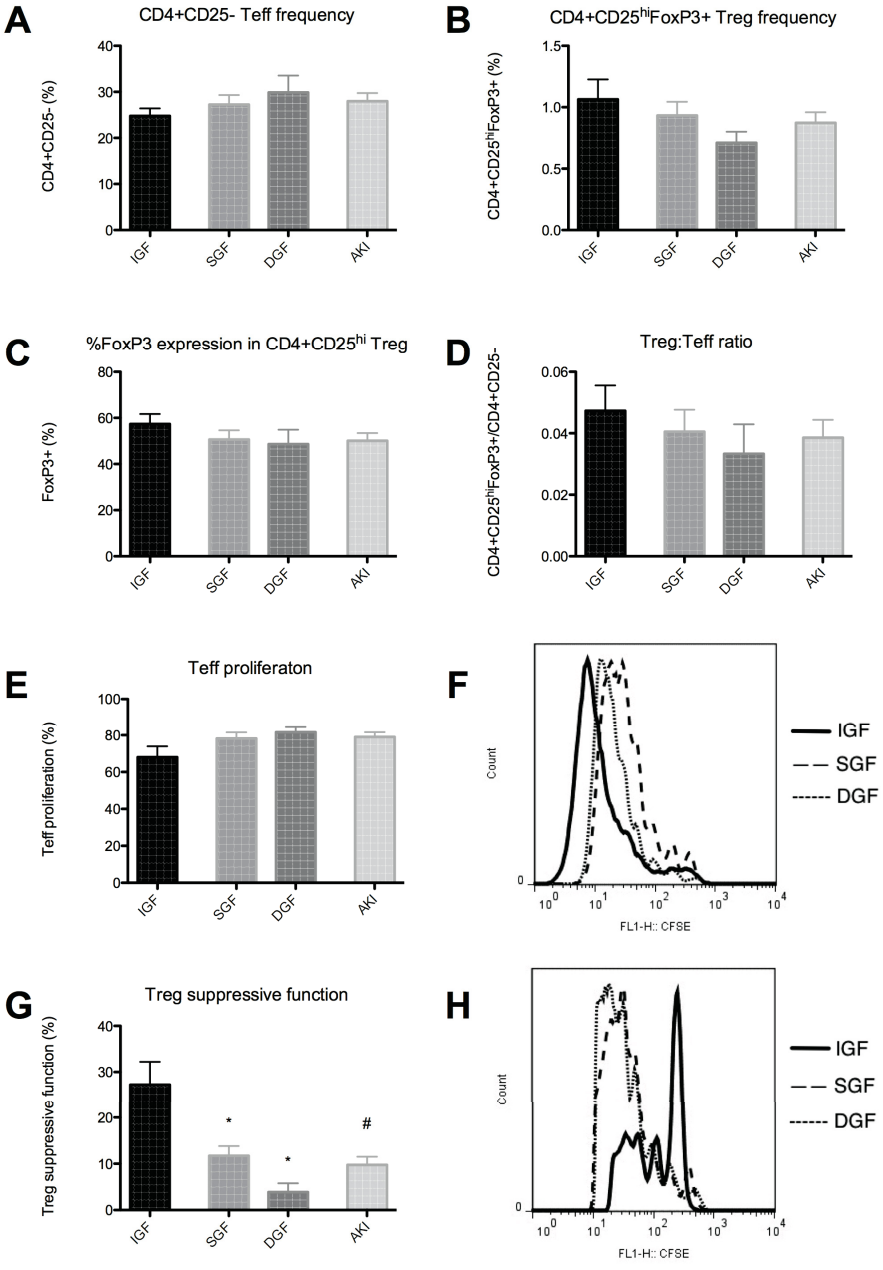

A
CD4+CD25- Teff frequency
CD4+CD25- (%)
40
30
20
10
0
IGF
SGF
DGF
AKI
B
CD4+CD25hiFoxP3+ Treg frequency
CD4+CD25hiFoxP3+ (%)
1.5
1.0
0.5
0.0
IGF
SGF
DGF
AKI
C
%FoxP3 expression in CD4+CD25hi Treg
FoxP3+ (%)
80
60
40
20
0
IGF
SGF
DGF
AKI
D
Treg:Teff ratio
CD4+CD25hiFoxP3+/CD4+CD25-
0.06
0.04
0.02
0.00
IGF
SGF
DGF
AKI
E
Teff proliferaton
Teff proliferation (%)
100
80
60
40
20
0
IGF
SGF
DGF
AKI
F
Count
0
10^0
10^1
10^2
10^3
10^4
FL1-H:: CFSE
IGF
SGF
DGF
G
Treg suppressive function
Treg suppressive function (%)
40
30
20
10
0
*
*
#
IGF
SGF
DGF
AKI
H
Count
0
10^0
10^1
10^2
10^3
10^4
FL1-H:: CFSE
IGF
SGF
DGF

Figure 2.

Significant predictors of AKI in ROC curve analysis are shown. A) Pre-transplant recipient peripheral blood Treg suppressive function (AUC = 0.82, 95% confidence interval (95% CI) = 0.65 – 1.00, $p<0.01$; optimal cut-off point = 13%, sensitivity = 75.0%, specificity = 88.9%, positive predictive value (PPV) = 95.5%, negative predictive value (NPV) = 53.3%) B) cold ischemic time (AUC = 0.75, 95% CI = 0.61 – 0.88, $p<0.01$; optimal cut-off point = 9.5 hours, sensitivity = 91.9%, specificity = 37.5%, PPV = 77.3%, NPV = 66.7%), C) donor age (AUC = 0.85, 95% CI = 0.74 – 0.96, $p<0.01$; optimal cut-off point = 47 years old, sensitivity = 83.8%, specificity = 81.2%, PPV = 82.5%, NPV = 69.2%), and D) use of expanded criteria donor (AUC = 0.77, 95% CI = 0.64 – 0.90, $p<0.01$) accurately predict AKI in ROC curve analysis.

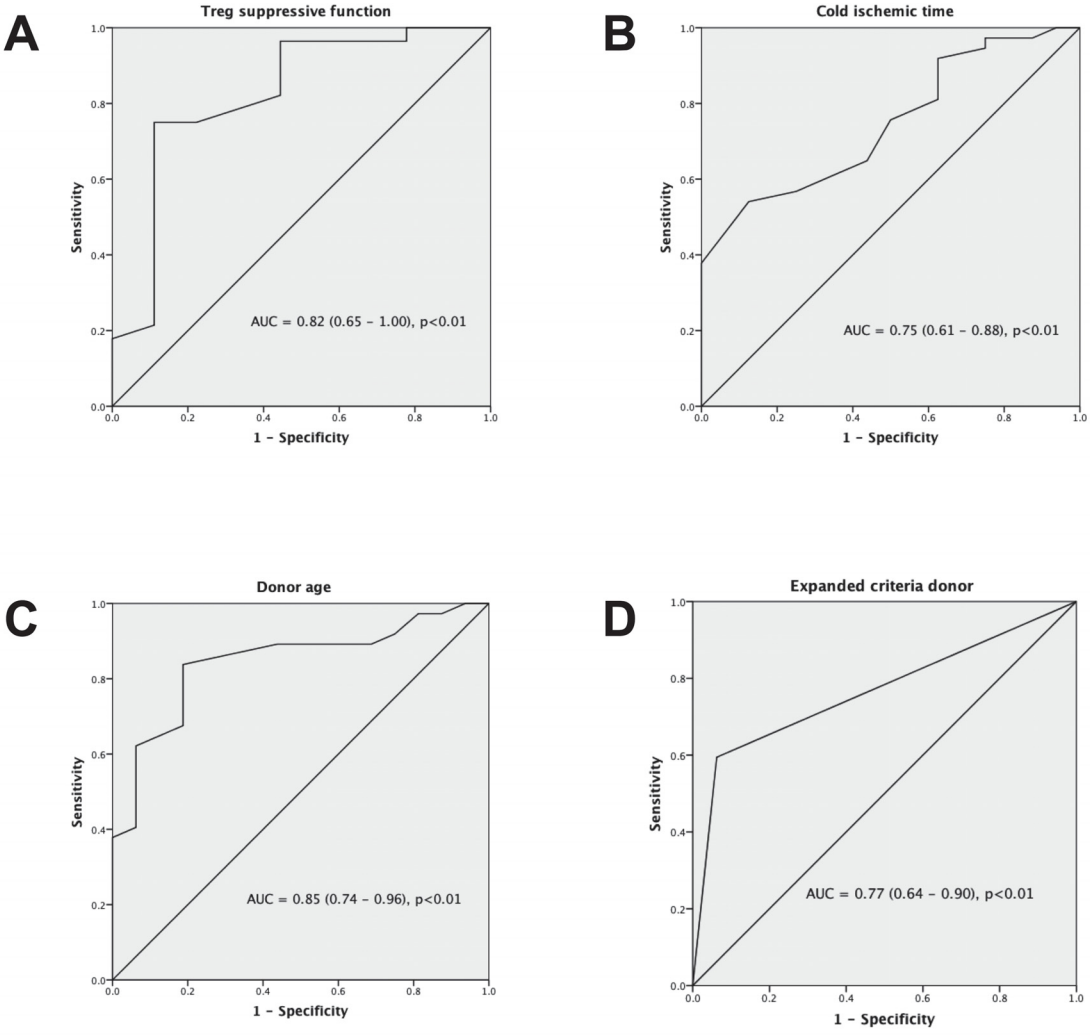

A
Treg suppressive function
Sensitivity
1 - Specificity
AUC = 0.82 (0.65 - 1.00), p<0.01
B
Cold ischemic time
Sensitivity
1 - Specificity
AUC = 0.75 (0.61 - 0.88), p<0.01
C
Donor age
Sensitivity
1 - Specificity
AUC = 0.85 (0.74 - 0.96), p<0.01
D
Expanded criteria donor
Sensitivity
1 - Specificity
AUC = 0.77 (0.64 - 0.90), p<0.01

Table S1.

TABLE S1. Additional clinical information regarding recipient						
	IGF	**SGF**	**DGF**	**p***	**AKI**	**p#**
Recipient characteristics						
Pre-tx Vit D hx	5	3	2	0.34	5	0.21
Pre-tx statin therapy	11	17	5	0.93	22	0.41
Blood transfusion hx	4	6	4	0.37	10	0.65
PRA class I (%)	7.4 ± 4.5	8.1 ± 3.9	9.1 ± 8.3	0.92	8.4 ± 3.6	0.69
PRA class II (%)	3.5 ± 2.8	7.6 ± 4.5	8.8 ± 8.4	0.74	7.9 ± 3.9	0.44
Pre-tx dialysis	13	24	10	0.26	34	0.14
Hemodialysis	9	21	9	0.30	30	0.12
Peritoneal dialysis	4	3	1		4	
Recipient outcomes						
eGFR at day 1 (mL/min/1.73m^2)	15 ± 2	12 ± 1	9 ± 1	0.02	11 ± 1	0.04
eGFR at day 7 (mL/min/1.73m^2)	52 ± 6	30 ± 2	11 ± 1	<0.01	24 ± 2	<0.01
eGFR at day 14 (mL/min/1.73m^2)	62 ± 4	39 ± 3	18 ± 3	<0.01	33 ± 3	<0.01
eGFR at day 30 (mL/min/1.73m^2)	58 ± 5	41 ± 2	32 ± 5	<0.01	38 ± 2	<0.01

eGFR at day 90 (mL/min/1.73m^2)	65 ± 3	44 ± 2	40 ± 8	<0.01	43 ± 3	<0.01
eGFR at day 180 (mL/min/1.73m^2)	66 ± 5	44 ± 3	46 ± 9	<0.01	44 ± 3	<0.01
AR within 180 days	1	2	4	0.02	6	0.33

*: p value for comparisons between DGF, SGF, and IGF groups.

#: p value for comparisons between AKI and IGF groups.

AKI, acute kidney injury; AR, acute rejection; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; IGF, immediate graft function; hx, history; PRA, panel reactive antibody; SGF, slow graft function; tx, transplant; Vit, vitamin.

Table S2.

TABLE S2. Linear regression to predict pre-transplant Teff proliferation from recipient characteristics			
Recipient characteristics	**Univariate analysis**		
	B	**95% CI**	**p**
Age	-0.38	-0.78 – 0.03	0.07
Sex	2.27	-10.84 – 15.38	0.73
Autoimmune dx	-14.98	-46.27 – 16.32	0.34
BMI	0.79	-0.12 – 1.70	0.09
Pre-tx Vit D hx	-2.63	-16.06 – 10.80	0.69
Pre-tx statin therapy	-5.25	-15.56 – 5.07	0.31
Blood transfusion hx	-2.55	-14.15 – 9.05	0.66
PRA class I (%)	-0.08	-0.30 – 0.15	0.50
PRA class II (%)	-0.06	-0.27 – 0.16	0.58
Previous tx	-3.27	-18.27 – 11.73	0.66
Pre-tx dialysis	-1.19	-23.93 – 21.55	0.92
Hemodialysis	5.80	-13.21 – 24.82	0.54
Time on dialysis	-0.65	-2.04 – 0.75	0.36
Pre-tx eGFR	-0.16	-1.52 – 1.20	0.81

BMI, body mass index; CI, confidence interval; dx, diagnosis; eGFR, estimated glomerular filtration rate; hx, history; PRA, panel reactive antibody; tx, transplant; Vit, vitamin.

Table S3.

TABLE S3. Linear regression to predict pre-transplant Treg suppressive function from recipient characteristics			
Recipient characteristics	**Univariate analysis**		
	B	**95% CI**	**p**
Age	0.27	-0.09 – 0.63	0.14
Sex	-0.56	-11.94 – 10.82	0.92
Autoimmune dx	2.55	- 6.93 – 12.03	0.59
BMI	-0.32	-1.14 – 0.49	0.43
Pre-tx Vit D hx	1.77	-9.92 – 13.46	0.76
Pre-tx statin therapy	5.76	-3.10 – 14.63	0.20
Blood transfusion hx	-3.46	-13.50 – 6.59	0.49
PRA class I (%)	0.10	-0.09 – 0.29	0.30
PRA class II (%)	0.07	-0.12 – 0.25	0.48
Previous tx	-4.89	-17.82 – 8.05	0.45
Pre-tx dialysis	2.14	-17.56 – 21.84	0.83
Hemodialysis	-6.41	-22.97 – 10.15	0.44
Time on dialysis	0.50	-0.72 – 1.71	0.41
Pre-tx eGFR	0.76	-0.39 – 1.91	0.19

BMI, body mass index; CI, confidence interval; dx, diagnosis; eGFR, estimated glomerular filtration rate; hx, history; PRA, panel reactive antibody; tx, transplant; Vit, vitamin.

Table S4.

TABLE S4. Linear regression analysis to predict eGFR						
Variables	Univariate analysis			Multivariate analysis		
	B	95% CI	p	B	95% CI	p
eGFR 7						
Treg function	0.53	0.05 – 1.01	0.03	0.35	-0.21 – 0.90	0.21
CIT	-1.06	-1.84 – -0.27	<0.01	-0.46	-1.58 – 0.66	0.41
Donor age	-0.45	-0.77 – -0.14	<0.01	-0.23	-0.64 – 0.18	0.27
ECD	-15.11	-25.63 – -4.58	<0.01			
eGFR 14						
Treg function	0.82	0.34 – 1.30	<0.01	0.59	0.05 – 1.12	0.03
CIT	-1.18	-2.01 – -0.35	<0.01	-0.51	-1.63 – 0.60	0.36
Donor age	-0.54	-0.87 – -0.20	<0.01	-0.32	-0.73 – 0.10	0.13
ECD	-17.18	-28.15 – -6.20	<0.01			
eGFR 30						

Treg function	0.71	0.22 – 1.19	0.01	0.43	-0.11 – 0.97	0.12
CIT	-1.11	-1.81 – -0.41	<0.01	-0.65	-1.65 – 0.35	0.20
Donor age	-0.51	-0.79 – -0.22	<0.01	-0.29	-0.65 – 0.07	0.11
ECD	-16.38	-25.76 – -6.99	<0.01			
eGFR 90						
Treg function	0.64	0.11 – 1.18	0.02	0.19	-0.32 – 0.71	0.45
CIT	-1.20	-1.89 – -0.51	<0.01	-1.05	-2.02 – -0.09	0.03
Donor age	-0.61	-0.89 – -0.33	<0.01	-0.53	-0.89 – -0.16	0.01
ECD	-17.27	-26.78 – -7.76	<0.01			
eGFR 180						
Treg function	0.81	0.20 – 1.42	0.01	0.21	-0.33 – 0.76	0.43
CIT	-1.62	-2.40 – -0.84	<0.01	-1.31	-2.33 – -0.30	0.01
Donor age	-0.69	-1.02 – -	<0.01	-0.67	-1.05 – -	<0.01

		0.35			0.29	
ECD	-18.35	-29.74 – -6.96	<0.01			

CI, confidence interval; CIT, cold ischemic time; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; Treg, regulatory T cell.

Figure S1.

No correlations were found between purity of enriched Teff and proliferation or enriched Treg and suppressive function. Correlations between A) enriched CD4+CD25- Teff purity and their proliferation, B) enriched CD4+CD25+ Treg purity and their suppressive function, and C) percentage expression of FoxP3 in enriched CD4+CD25+ Tregs and their suppressive function, are shown.

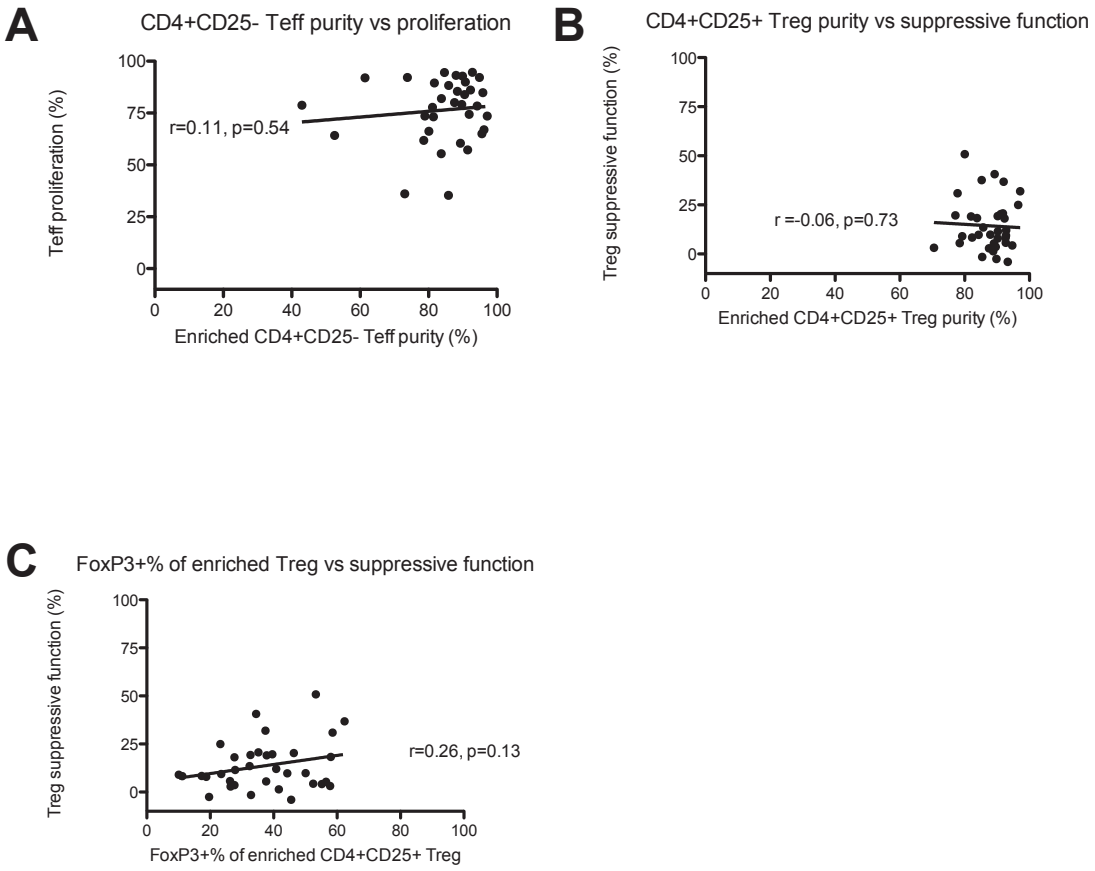


Figure S2.

A strong correlation was found between donor age and ECD category, while no or weak correlations were found among the other significant variables to predict DGF/AKI/eGFR in the univariate analyses. Correlations between A) donor age and use of ECDs, B) Treg suppressive function and CIT, C) Treg suppressive function and donor age, D) Treg suppressive function and ECD category, E) donor age and CIT, and F) CIT and ECD category, are shown.

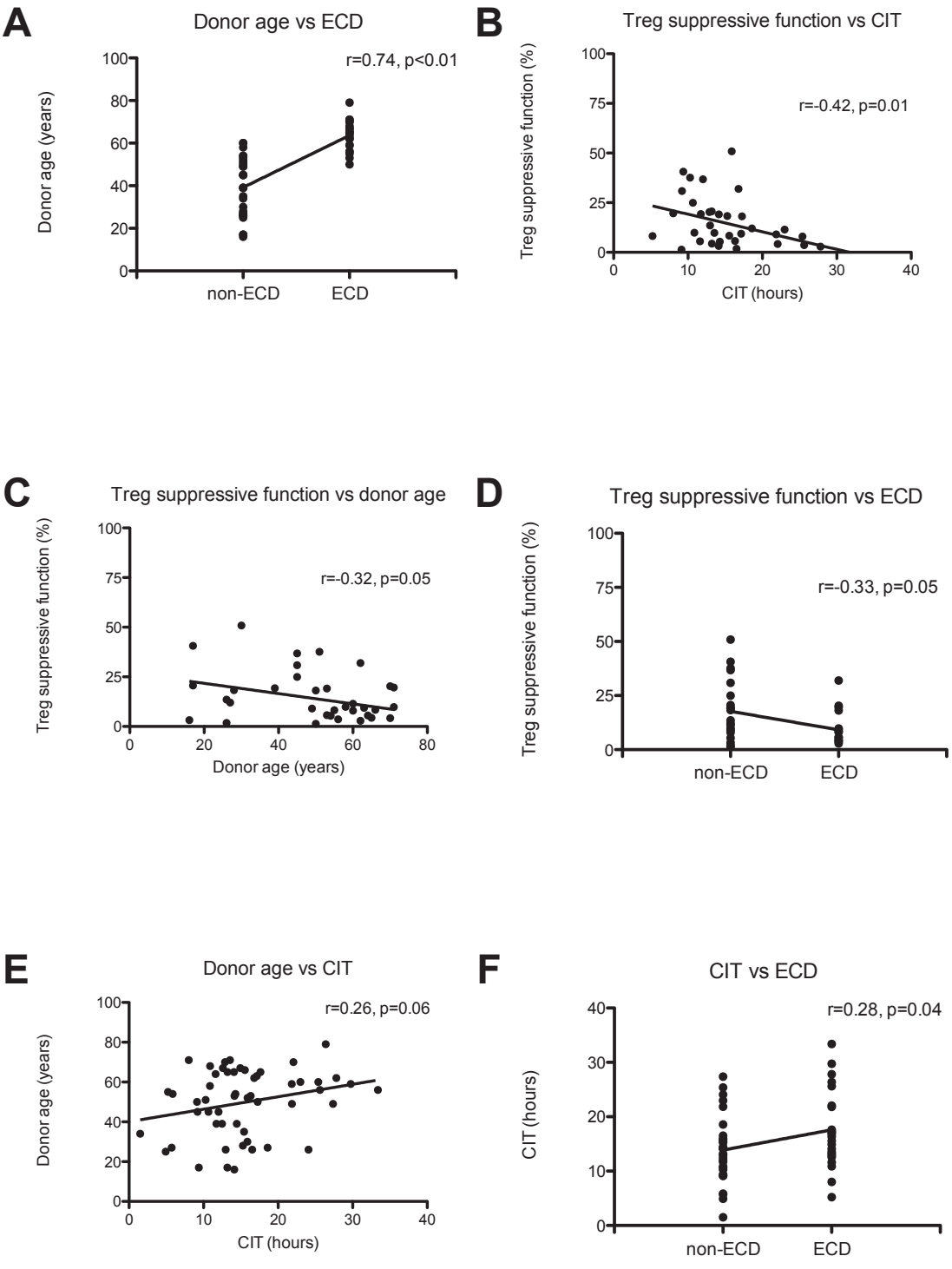

A
Donor age vs ECD
r=0.74, p<0.01
Donor age (years)
non-ECD
ECD
B
Treg suppressive function vs CIT
r=-0.42, p=0.01
Treg suppressive function (%)
CIT (hours)
C
Treg suppressive function vs donor age
r=-0.32, p=0.05
Treg suppressive function (%)
Donor age (years)
D
Treg suppressive function vs ECD
r=-0.33, p=0.05
Treg suppressive function (%)
non-ECD
ECD
E
Donor age vs CIT
r=0.26, p=0.06
Donor age (years)
CIT (hours)
F
CIT vs ECD
r=0.28, p=0.04
CIT (hours)
non-ECD
ECD

Figure S3.

Representative flow cytometry analysis of CD4+CD25^{hi}FoxP3+ Treg frequency, %FoxP3 expression in CD4+CD25^{hi} Treg, and CD4+CD25- Teff frequency in a kidney transplant recipient prior to transplantation. A) A lymphocyte gate was created based on forward and side scatters. B) CD4+CD25^{hi} and CD4+CD25- gates were created on cells from the lymphocyte gate. C) FoxP3+ gate was created on cells from the CD4+CD25^{hi} gate.

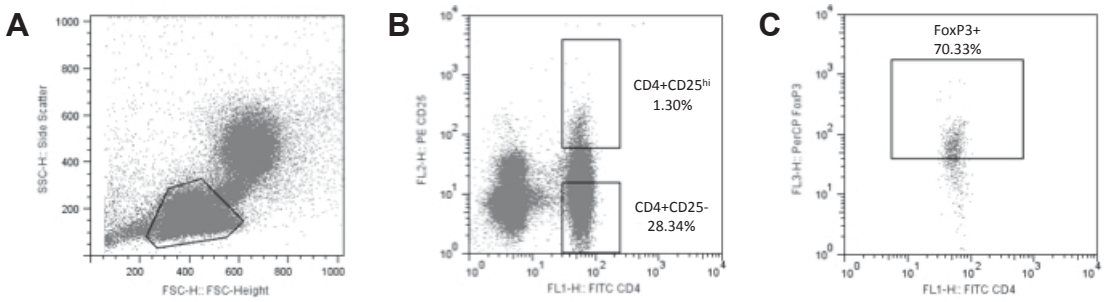
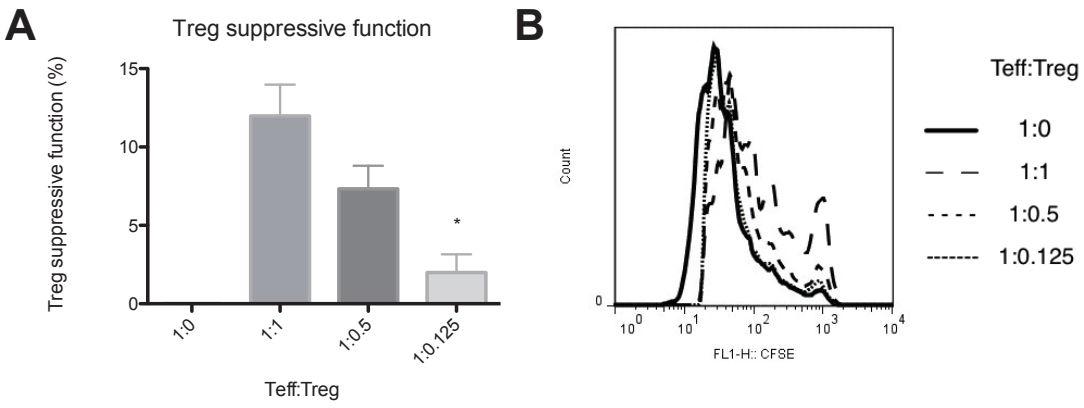


Figure S4.

Enriched Tregs suppressed Teff proliferation in a dose-dependent manner. A) Percentage suppression of enriched CD4+CD25- Teff proliferation by enriched CD4+CD25+ Treg was measured at 1:1, 1:0.5, and 1:0.125 Teff to Treg ratios in a subset of 32 kidney transplant recipients relative to 1:0 Teff to Treg ratio (*: $p<0.05$). B) Representative example of suppression of CFSE-labeled Teff proliferation by Treg at different Teff:Treg ratios (1:0, 1:1, 1:0.5, 1:0.125) in a kidney transplant recipient is shown.



Preface to Chapter 3

In this chapter, we showed that pre-transplant recipient circulating Treg suppressive function, measured by an in vitro assay of enriched recipient Tregs co-cultured with stimulated autologous Teffs, was predictive of DGF/SGF in deceased donor kidney transplant recipients (465). The clinical applicability of this in vitro assay as a pre-transplant biomarker to predict DGF/SGF in kidney transplant recipients is however limited by the fact that it is labor intensive, time-consuming, costly, and non-standardized between centers (408). A solution to transform Treg suppressive function into a rapid and clinically applicable biomarker would be to identify and quantify a highly suppressive subset of Tregs using phenotypic surface markers.

As previously described in chapter 3, Tregs are CD4+ T cells traditionally identified by their expression of the transcription factor FoxP3 and the surface markers CD25 and CD127 (215). The sole use of FoxP3, CD25, and CD127 in the clinical setting is however problematic. Since FoxP3 is an intracellular protein, the fixation and permeabilization step required prior to its staining lengthens the Treg quantification process by flow cytometry and does not allow its use for Treg isolation and potential future cellular therapy (304,466). Although CD25 is a surface marker, only the CD4+ T cells with the highest CD25 expression are suppressive Tregs in humans, and the flow cytometry gating strategy to identify these CD4+CD25hi cells is variable (304,467). The downregulation of CD127 on CD4+ T

cells was shown to be an equivalent surface marker alternative to CD25hi for the identification of suppressive Tregs, but also suffer from the fact that it can be downregulated on recently activated Teff (215,304).

In the context of an inflammatory environment such as DGF/SGF after kidney transplantation, TNFR2 is a recently discovered surface marker that is particularly interesting for the identification of the most potently suppressive subset of Tregs. As described in Chapter 3, TNFR2 is a cytokine receptor that mediates many of the biological functions of TNF-α. TNFR2 expression is mostly restricted to lymphocytes, and preferentially expressed on Tregs as compared to Teffs (335). Although TNF-α is traditionally thought to be pro-inflammatory in renal IRI, it could also have an immunoregulatory role via its interaction with kidney-infiltrating Tregs (468). Recent murine and human studies showed that TNF-α signaling through TNFR2+ Tregs increased their survival, proliferation, and suppressive function (335). Moreover, Tregs can also shed soluble TNFR2 to act as decoy to TNF-α and limits its biological functions on Teffs (336).

The next objective in this thesis was therefore to investigate whether the measurement of TNFR2 expression on Tregs could serve as a surrogate of Treg suppressive function in kidney transplant candidates. Subsequently, we aimed to test whether pre-transplant circulating TNFR2+ Tregs could replace the traditional Treg suppressive function assay as a rapid biomarker to predict DGF/SGF after kidney transplantation.

Chapter 3. Pretransplant Recipient Circulating CD4+CD127lo/− Tumor Necrosis Factor Receptor 2+ Regulatory T Cells: A Surrogate of Regulatory T Cell–Suppressive Function and Predictor of Delayed and Slow Graft Function After Kidney Transplantation

Minh-Tri JP Nguyen, Elise Fryml, Sossy K Sahakian, Shuqing Liu, Marcelo Cantarovich, Mark Lipman, Jean I Tchervenkov, Steven Paraskevas. Transplantation 2016; 100: 314 – 324.

Abstract

Background. Delayed graft function (DGF) and slow graft function (SGF) are ischemia-reperfusion–associated acute kidney injuries (AKI) that decrease long-term graft survival after kidney transplantation. Regulatory T (Treg) cells are protective in murine AKI, and their suppressive function predictive of AKI in kidney transplantation. The conventional Treg cell function coculture assay is however time-consuming and labor intensive. We sought a simpler alternative to measure Treg cell function and predict AKI.

Methods. In this prospective observational cohort study, pretransplant recipient circulating CD4+CD25+CD127lo/− and CD4+CD127lo/− tumor necrosis factor receptor 2 (TNFR2)+ Treg cells were measured by flow cytometry in 76 deceased donor kidney transplant recipients (DGF, n = 18; SGF, n = 34; immediate graft function [IGF], n = 24). In a subset of 37 recipients, pretransplant circulating Treg

cell–suppressive function was also quantified by measuring the suppression of autologous effector T-cell proliferation by Treg cell in coculture.

Results. The TNFR2+ expression on CD4+CD127lo/− T cells correlated with Treg cell–suppressive function ($r = 0.63$, $P < 0.01$). In receiver operating characteristic curves, percentage and absolute number of CD4+CD127lo/−TNFR2+ Treg cell predicted DGF from non-DGF (IGF + SGF) with area under the curves of 0.75 and 0.77, respectively, and also AKI (DGF + SGF) from IGF with area under the curves of 0.76 and 0.72, respectively ($P < 0.01$). Prediction of AKI (DGF + SGF) from IGF remained significant in multivariate logistic regression accounting for cold ischemic time, donor age, previous transplant, and pretransplant dialysis modality.

Conclusions. Pretransplant recipient circulating CD4+CD127lo/−TNFR2+ Treg cell is potentially a simpler alternative to Treg cell function as a pretransplant recipient immune marker for AKI (DGF + SGF), independent from donor and organ procurement characteristics.

3.1. Introduction

Delayed graft function (DGF) and slow graft function (SGF) are a continuous spectrum of ischemia-reperfusion related acute kidney injuries (AKI) that occur in more than 20% of kidney transplant recipients (12,16,39). DGF, the most severe form of AKI, is highly detrimental to kidney transplant recipients as it increases the risk for both acute and chronic rejection as well as long-term graft loss (13,26,27).

Although SGF is a milder form of AKI, it behaves similarly to DGF, and also increases the risk for acute rejection and long-term graft loss (26,438).

Regulatory T cells (Tregs) are essential in maintaining immune homeostasis in healthy individuals. A defect in their frequency or function has been implicated in autoimmune diseases, transplant rejection, and more recently AKI (215). Murine studies demonstrated that decreasing Treg frequency or suppressive function prior to renal ischemia-reperfusion injury increased the severity of AKI (428,429,435). We subsequently showed that a lower pre-transplant recipient circulating Treg suppressive function, measured by an *in vitro* assay of enriched recipient Tregs co-cultured with stimulated autologous effector T cells (Teffs), was predictive of AKI (DGF+SGF) in deceased donor kidney transplant recipients (465). The clinical applicability of this *in vitro* assay as a pre-transplant immune marker to predict AKI in kidney transplant recipients is however limited by the fact that it is labor-intensive, time-consuming, costly, and non-standardized (408). A solution to transform Treg suppressive function into a clinically applicable immune marker would be to identify and quantify the most potently suppressive subset of Tregs with phenotypic surface markers.

Tregs are CD4+ T cells most commonly identified by their expression of the transcription factor forkhead box P3 (FoxP3) and the surface markers CD25 and CD127 (215). The use of these markers in the clinical setting is however problematic. Since FoxP3 is an intracellular protein, the fixation and

permeabilization step required prior to its staining lengthens the Treg quantification process by flow cytometry and does not allow its use for Treg isolation and potential cellular therapy (304,466). Although CD25 is a surface marker, only the CD4+ T cells with the highest CD25 expression are suppressive Tregs in humans, and the flow cytometry gating strategy to identify these CD4+CD25^{hi} cells is variable (304,467). Similarly, the downregulation of CD127 on CD4+ T cells was shown to be an equivalent surface marker alternative to CD25^{hi} for the identification of suppressive Tregs, but cannot exclude recently activated Teffs (215,304).

In the context of an inflammatory environment such as AKI after kidney transplantation, tumor necrosis factor receptor 2 (TNFR2) is another recently discovered surface marker that is particularly interesting for the identification of the most potently suppressive subset of Tregs. TNFR2 is a cytokine receptor mostly restricted to lymphocytes and endothelial cells (468). It is preferentially expressed on Tregs as compared to Teffs, and mediates many of the biological functions of tumor necrosis factor-alpha (TNF-α) (335). TNF-α blockade has indeed been shown to both dampen and exacerbate autoimmune diseases (468). Although TNF-α is traditionally thought to be upregulated and pro-inflammatory in AKI, it could also have an immunoregulatory role via its interaction with kidney-infiltrating Tregs (469). Recent murine and human studies showed that TNF-α signaling through TNFR2+ Tregs increased their survival, proliferation, and suppressive function (335).

Based on previous literature, we therefore hypothesized that TNFR2 expression on circulating Tregs could serve as a surrogate phenotypic surface marker of pre-transplant Treg suppressive function in patients awaiting a kidney transplantation. Moreover, based on our previous finding that pre-transplant recipient Treg suppressive function predicted AKI (DGF+SGF) after kidney transplantation, we hypothesized that pre-transplant recipient TNFR2+ Tregs could predict those who will suffer from AKI (DGF+SGF).

3.2. Materials and Methods

Our prospective observational cohort study was approved by the McGill University Health Centre Research Ethics Board, registered on ClinicalTrials.gov (NCT01232816), and conducted in adherence with the declarations of Helsinki and Istanbul.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ and CD4+CD25+CD127lo/- Treg frequencies

Peripheral blood was drawn in heparin-coated tubes in the operating theater, prior to induction immunosuppression and skin incision. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ). PBMCs were surface stained with anti-human CD4 FITC (clone OKT4, eBioscience, San Diego, CA), CD127 PE-Cy5 (clone eBioRDR5,

eBioscience), and CD25 PE (clone BC96, eBioscience) or TNFR2 PE (clone TR75-89, BD Biosciences, San Jose, CA) for 30 minutes in the dark at 4°C, then washed with PBS (Wisent, St-Bruno, Canada). Flow cytometry acquisition was performed on the FACScan (BD Biosciences), and data analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Forward and side scatters were used to gate on lymphocytes. Treg frequency was determined by the percentage of CD4+CD25+CD127lo/- or CD4+CD127lo/-TNFR2+ cells in the lymphocyte gate. Gating strategies for CD4, CD127, CD25 and TNFR2 were based on single-color compensation. Using the same peripheral blood sample, lymphocyte count was obtained from our clinical hematology laboratory and absolute Treg cell counts were calculated using the following formula: % cells in lymphocyte gate x lymphocyte count.

Pre-transplant recipient Treg suppressive function assay

In a subset of 37 kidney transplant recipients, pre-transplant Treg suppressive function was quantified as previously described (465). Briefly, CD4+CD25+ Tregs and CD4+CD25- Teffs were enriched from PBMCs by magnetic bead isolation (EasySep Human CD4+CD25+T Cell Isolation Kit, StemCell Technologies, Vancouver, Canada). Purities of enriched CD+CD25+ Tregs and CD4+CD25- Teffs were 86 ± 1% and 85 ± 2% respectively. Teffs were labeled with 5 μm/mL CFSE (Life Technologies, Burlington, Canada) at 37°C for 5 minutes, then washed with ice cold 10% fetal bovine serum in PBS. In all assays, 4 x 10^4 CFSE-labeled Teffs were co-cultured with Tregs at a 1:0 or 1:1 ratio for 5 days in 200 μL of X-VIVO 15 media

(Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Sigma-Aldrich, Oakville, Canada) in the presence of anti-CD3/CD28-coated beads (1 bead/cell, Life Technologies). Unlabeled CFSE-Teffs were excluded by flow cytometry analysis gating. Teff proliferation was measured by CFSE dilution (% divided function, FlowJo software). Treg suppressive function was then calculated using the following formula: $100 - \frac{\%\ divided\ 1{:}1\ Teff{:}Treg\ ratio}{\%\ divided\ 1{:}0\ Teff{:}Treg\ ratio} \times 100$.

Statistical analysis

Data is presented as mean ± standard error of the mean. Statistical analyses were performed using SPSS 20 (IBM, Armonk, NY) and considered significant if $p \leq 0.05$. Categorical data was compared using chi-square test. Continuous data between three groups was compared using one-way analysis of variance followed by Tukey's honestly significant difference post-hoc analysis, while continuous data between two groups was compared using Student's t test. Correlations were made with Pearson's correlation coefficient. Linear regression was performed to assess recipient baseline characteristics as predictors of pre-transplant circulating CD4+CD127lo/-TNFR2+ Tregs. Logistic regression and receiver operating characteristic (ROC) curve were performed to assess recipient, donor, and organ procurement variables as predictors of DGF from non-DGF (IGF+SGF) or AKI (DGF+SGF) from IGF. ROC curve was also used to determine a range of cut-off values for each significant variable that predicted DGF or AKI (DGF+SGF), as well as an optimal cut-off value based on the largest sums of sensitivity and specificity.

Multivariate logistic regression was used to assess the independent predictive ability of CD4+CD127lo/-TNFR2+ Treg for DGF and AKI (DGF+SGF) in models containing variables that were significantly different between our outcomes of interest with the exception of ECD category as this variable was strongly collinear with donor age and donor age is the main determinant of ECD categorization (48). The multivariate logistic regression models were internally validated by generating 95% confidence intervals for the odds ratios using the bootstrap technique with 1000 replicates.

3.3. Results

Patient characteristics

Seventy-six consecutive consenting adult deceased-donor kidney transplant recipients were enrolled and prospectively divided into 1) DGF (n=18), 2) SGF (n=34), and 3) IGF (n=24) groups. IGF and SGF groups were combined into a non-DGF group (n=58). DGF and SGF were also combined into an AKI group (n=52) since these two groups are a continuous spectrum of renal ischemia-reperfusion injury (16). DGF recipients were defined as those requiring dialysis within 7 days post-transplant. Recipients not requiring dialysis post-transplant were defined as having SGF if their 24-hour serum creatinine decreased by less than 20%, and IGF if it decreased by more than 20% (11,465). Initiation of post-transplant dialysis was decided independently by the treating physicians.

Recipient, donor, and organ procurement characteristics were collected prospectively (Tables 1, S1). Donor age and cold ischemic time (CIT) were significantly higher in DGF and SGF recipients. The use of expanded criteria donors (ECDs) and the presence of a previous transplant were also significantly more frequent in DGF and SGF recipients. The use of pre-transplant dialysis was significantly different between AKI (DGF+SGF) and IGF, but not DGF and non-DGF (IGF+SGF) recipients. Induction and maintenance immunosuppressive regimen were similar between groups (details in Tables 1, S1). None of the recipients, especially those with an autoimmune etiology of end-stage renal disease, were on immunosuppressive therapy within 6 months preceding their transplant. As expected, estimated glomerular filtration rate was significantly lower in DGF and SGF recipients up to 6 months post-transplant (Tables 1, S1).

Expression of TNFR2 on CD4+CD127lo/- T cells is a surrogate phenotypic surface marker of Treg suppressive function

Based on previous literature, we first sought to verify whether expression of the surface marker TNFR2 is a surrogate marker of Treg suppressive function in a subset of 37 deceased donor adult kidney transplant recipients. Expression of TNFR2 on CD4+CD127lo/- T cells positively correlated with Treg suppressive function ($r=0.63$, $p<0.01$; Figure 1). Identification of Treg using the CD4+CD127lo/-TNFR2+ markers positively correlated with the CD4+CD25+CD127lo/- markers

(r=0.40, p<0.01), and yielded a larger cell population (4.39±0.30% or 59.95x10^6/L instead of 2.54%±0.12% or 34.31x10^6/L, p<0.01).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were lower in DGF and SGF recipients

We then examined whether the percentage and absolute number of pre-transplant circulating CD4+, CD4+CD127lo/-, CD4+CD25+CD127lo/-, and CD4+CD127lo/-TNFR2+ T cells were different between DGF, SGF, and IGF recipients. Only the percentage and absolute number of CD4+CD127lo/-TNFR2+ Tregs were significantly lower in DGF (3.08±0.59%, 39.70±10.45 x10^6/L) and SGF (3.95±0.35%, 54.83±5.34 x10^6/L) in comparison to IGF (6.05±0.57%, 82.72±10.99 x10^6/L) recipients (p<0.05; Figures 2, 3, S1). The same results were observed when comparing DGF to non-DGF or AKI to IGF recipients (p=0.02; Figures 2, 3, S1).

Recipient baseline characteristics were not predictive of pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs

Certain recipient baseline characteristics, such as age (440,441), gender (442), autoimmune etiology of end-stage renal disease (398,445-447), body mass index (443,444), vitamin D supplementation (448), statin therapy (449), blood transfusion history (450), sensitization (451), and dialysis modality/duration (404), were shown to have immunomodulatory potential. We therefore investigated whether the

aforementioned variables influenced the percentage or absolute number of pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs. In our cohort of recipients, none of the recipient baseline characteristics was predictive of CD4+CD127lo/-TNFR2+ Tregs (Table S2).

Prediction of DGF or AKI based on pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs

Univariate analyses

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted DGF

Since pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were significantly decreased in DGF recipients, we investigated whether they could predict if a recipient will have DGF or not post-transplant. In logistic regression analysis, each percentage increase in CD4+CD127lo/-TNFR2+ Treg decreased the odds of having DGF by 30% ($p=0.02$), while each $1x10^6$/L increase decreased the odds by 2% ($p=0.03$; Table 2). Predictive accuracy for DGF was assessed in ROC curve analysis, in which area under the curves (AUCs) of 0.75 and 0.77 were obtained for CD4+CD127lo/-TNFR2+ Treg percentage and absolute number respectively ($p<0.01$; Figure 4A). The performance of various cut-off values for the previous two variables with regards to sensitivity, specificity, and predictive value is presented in Table 3. Identifying Tregs with CD4+CD127lo/- or

CD4+CD25+CD127lo/- was not predictive of DGF (Figure S2). CIT was the only other significant variable in the prediction of DGF (AUC=0.75, p<0.01), while donor age and previous transplant were not (Figure 4B).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted AKI

Because DGF and SGF represent a continuous spectrum of renal ischemia-reperfusion injury (16), we also examined whether pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted post-transplant AKI (combined DGF and SGF group). In logistic regression analysis, each percentage increase in CD4+CD127lo/-TNFR2+ Treg decreased the odds of having AKI by 31% (p<0.01), while each $1x10^6$/L increase decreased the odds by 2% (p<0.01; Table 4). Predictive accuracy for AKI was assessed in ROC curve analysis, in which AUCs of 0.76 and 0.72 were obtained for CD4+CD127lo/-TNFR2+ Treg percentage and absolute number respectively (p<0.01; Figure 5A). The performance of various cut-off values for the previous two variables with regards to sensitivity, specificity, and predictive value is presented in Table 3. Identifying Tregs with CD4+CD127lo/- or CD4+CD25+CD127lo/- was not predictive of AKI (Figure S3). Other significant variables in the prediction of AKI included donor age (AUC=0.81, p<0.01), ECD category (AUC=0.71, p<0.01), and pre-transplant dialysis modality (AUC=0.67, p=0.02; Figure 5B).

Multivariate analyses

For our multivariate analyses, we considered all variables that were significantly different between our outcomes of interest. Since a strong correlation existed between ECD category and donor age (Table S3), we excluded ECD category and retained donor age in our multivariate analyses because donor age is the main determinant of ECD categorization (48). No or weak correlations existed among the other aforementioned variables included in our multivariate analyses (Table S3).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were not independently associated with DGF

In multivariate logistic regression analysis adjusting for the effects of CIT, donor age, and previous transplant, CD4+CD127lo/-TNFR2+ Treg percentage and absolute number did not remain significant variables in predicting which recipients will have DGF or not (Table 2).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted AKI

In multivariate logistic regression analysis, CD4+CD127lo/-TNFR2+ Treg percentage and absolute number remained significant predictors of AKI when adjusting for the effects of CIT, donor age, previous transplant, and pre-transplant dialysis modality, as demonstrated by odds ratios of 0.72 (p=0.02) and 0.98 (p=0.02) respectively (Table 4). This analysis was internally validated by generating

95% confidence intervals for the odds ratios with the bootstrap re-sampling technique using 1000 replicates (Table S4).

CD4+CD127lo/-TNFR2+ Treg-based logistic regression model to predict AKI

Since pre-transplant dialysis modality and donor age were also significant variables in the prediction of AKI in multivariate logistic regression and are known risk factors for AKI (45,48,58), we incorporated these variables with pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs to create a logistic regression model that could be used pre-transplant to guide organ allocation by estimating the probability of a recipient to develop AKI post-transplant (probability of AKI = $\frac{1}{1+e^{-z}}$ with z = -4.28 + (-0.34 x CD4+CD127lo/-TNFR2+ Treg %) + (0.09 x donor age) + (0.70 x peritoneal dialysis [0/1]) + (2.96 x hemodialysis [0/1]). We chose CD4+CD127lo/-TNFR2+ Treg percentage instead of absolute number in the model since it had the highest AUC of the two in ROC curve analysis. This logistic regression model improved the prediction accuracy for AKI on ROC curve analysis with an AUC of 0.90 ($p<0.01$) in comparison to using CD4+CD127lo/-TNFR2+ Treg percentage, donor age, or dialysis modality alone (Figures 5, 6). Its prediction accuracy, however, did not differ significantly from a logistic regression model using only the clinically available variables donor age and pre-transplant dialysis modality (AUC=0.88; Figure 6). Nevertheless, at the optimal cut-off probability value based on the best sums of sensitivity and specificity, the model including CD4+CD127lo/-TNFR2+ Treg had a better specificity and PPV while maintaining a similar sensitivity and NPV (Table 5).

3.4. Discussion

We previously found that pre-transplant recipient Treg suppressive function was predictive of AKI (DGF+SGF) after kidney transplantation. Due to its labor-intensive and time-consuming nature, the conventional assay to measure Treg suppressive function by suppression of autologous Teff proliferation by Treg in co-culture is however impractical for widespread clinical use as an immune marker for AKI (DGF+SGF). This is especially true in deceased donor kidney transplantation, which often occurs in a semi-urgent fashion and during off-hours. We found that the measurement of circulating recipient CD4+CD127lo/-TNFR2+ Tregs prior to transplantation could be a suitable rapid and simple alternative to the conventional Treg suppressive function assay as an immune marker for AKI (DGF+SGF).

Although Tregs are currently conventionally identified with the surface markers CD4, CD25, and CD127, this combination failed to predict DGF or AKI (DGF+SGF) in our study. On the other hand, the combination of CD4, CD127, and TNFR2, which partially correlated with CD4+CD25+CD127lo/-, was able to predict DGF and AKI (DGF+SGF). This reflects the known heterogeneity of Tregs (409), and perhaps both populations identify Tregs with distinct functional capacities in AKI. Previous murine and healthy subjects literature reported that TNFR2 identifies a highly suppressive subset of Tregs independent of CD25. TNFR2+ T cells were shown to contain a substantial portion of CD25- cells, while CD25+ T cells that were TNFR2- had minimal to no suppressive activity (333-335,470). Our results also suggest that

upregulation of TNFR2 on CD4+CD127lo/- T cells could identify highly suppressive Tregs in uremic patients awaiting a deceased donor kidney transplant, as it correlated with the conventional Treg suppressive function assay.

In an inflammatory environment such as AKI where TNF-α is upregulated (469), the presence of TNFR2 on Tregs could be essential for their maximal suppressive function via several mechanisms. First, TNF-α binding to TNFR2+ Tregs serves as a negative feedback loop to prevent excessive effector immune responses by stimulating Treg activation/expansion (470). Secondly, interaction between TNF-α and TNFR2+ Tregs also increases their resistance to oxidative stress by upregulation of the antioxidant thioredoxin-1, thereby increasing their survival in an inflammatory environment (427). Thirdly, Tregs can shed soluble TNFR2, which then acts as a decoy to decrease the availability of TNF-α to exert its pro-inflammatory activities (336). Circulating TNFR2+ Tregs could therefore home to the ischemia-reperfusion injured transplanted kidney and subsequently decrease damage by directly suppressing effector immune activity or by inhibiting the pro-inflammatory functions of TNF-α.

Similarly to the conventional pre-transplant Treg suppressive function assay, the pre-transplant measurement of the most potently suppressive subset of circulating CD4+CD127lo/-TNFR2+ Tregs was independently predictive of AKI (DGF+SGF), but with a lower accuracy, specificity, and PPV (465). The addition of CD4+CD127lo/-TNFR2+ Treg to known clinical risk factors for AKI to form a predictive model

improved the specificity and PPV, but did not improve the accuracy compared to using a model without CD4+CD127lo/-TNFR2+ Treg. Although clinical variables such as donor age are undoubtedly strong predictors of AKI, this could also be attributed to the fact that we limited our identification of the most potently suppressive subset of Tregs to only three surface markers. This was done with the intentional purpose of optimizing cost-effectiveness, rapidity, and simplicity. The measurement of circulating CD4+CD127lo/-TNFR2+ Treg, from blood draw to flow cytometry analysis, can be done within 2 hours using a basic flow cytometer that is widely available for dedicated use in pre-transplant immune monitoring and does not require complex fluorescence compensation. With the wider availability of multi-color flow cytometers and automatic compensation, identification of the most potent subset of Tregs with additional described markers such as CD45RA (410), CTLA-4 (471), HLA-DR (413), ICOS (424), and CD62L (414) could increase the correlation between phenotypically identified potent Tregs and the conventional Treg suppressive function assay without compromising cost-effectiveness, and potentially improve the predictive value for AKI.

CD4+CD127lo/-TNFR2+ Treg, however, was not significantly different between DGF and SGF recipients, and could not independently predict DGF from non-DGF (IGF/SGF) recipients. Although DGF is considered the most severe form of ischemia-reperfusion injury, evidence suggests that the contribution of SGF to immunological outcomes and graft survival is more similar to DGF than IGF (15,16,438). Moreover, the classification of graft function into DGF or SGF is based on a subjective decision

to dialyze a recipient within the first week post-transplant. It is therefore possible that for two recipients with the same severity of ischemia-reperfusion injury, one was classified as DGF since the clinical decision was to initiate dialysis but graft function was about to recover while the other was classified as SGF since dialysis requirement was imminent but graft function recovered in time to avoid dialysis.

Our study is limited by the fact that it was conducted in a single institution with a small sample size. This could explain why CIT, donor age, and previous transplant were not predictors of both DGF and AKI in multivariate analyses. With regards to pre-transplant dialysis modality, it was only predictive of AKI but not DGF. Recipients undergoing a pre-emptive transplant rarely develop DGF (472), but could have a very limited residual native kidney function still making them susceptible to developing SGF. Although the 24% rate of DGF in our cohort was on par with the literature, we had a higher rate of SGF at 45% (15). This could be related to the fact that almost 50% of our grafts are from ECDs. Since we recruited consecutive consenting patients in a prospective manner before transplantation at a time when their graft outcome was unknown, it is also possible that, by chance, more patients volunteering to participate in our study developed SGF. This volunteer effect could influence our results. Due to the urgent nature of deceased donor kidney transplantation, we also did not test the stability of circulating CD4+CD127lo/-TNFR2+ Tregs by serial measures in the pre-transplant setting. We acknowledge that this would be an important goal of a separate study.

Despite the limitations of our study, our results nevertheless indicate that circulating CD4+CD127lo/-TNFR2+ Treg is a potential novel immune marker for AKI. This is in fact the second study supporting the concept that a decreased pre-transplant recipient circulating Treg suppressive function is linked to AKI. External validation of circulating CD4+CD127lo/-TNFR2+ Treg as a predictor of AKI could eventually guide organ allocation and therapeutic interventions aimed at individual specific targets of Treg. For example, pre-transplant infusion of maximally suppressive Tregs isolated based on CD127 and TNFR2 expression could potentially decrease ischemia-reperfusion injury, and decrease the risk for AKI. TNFR2+ Tregs could also be expanded ex- or in-vivo with specific TNFR2 agonists (473,474). Another therapeutic avenue would be to simulate soluble TNFR2 shedding by Tregs with etanercept (fusion protein composed of the extracellular domain of TNFR2 and the hinge and Fc domains of human IgG1), which has been shown to dampen renal ischemia-reperfusion injury in mice (475). This is in contrast with strong clinical predictors of AKI such as donor age, which cannot be manipulated especially in the current context of organ demand and supply mismatch.

In conclusion, our findings suggest that pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg is a potential alternative to the conventional Treg suppressive function assay as an immune marker for AKI (DGF+SGF), independent of donor and organ procurement characteristics. Measuring pre-transplant circulating CD4+CD127lo/-TNFR2+ Tregs could therefore allow identification of recipients at risk for AKI pre-transplant, and consequently guide organ allocation

and AKI-targeted immunotherapies to specific measures of CD4+CD127lo/-TNFR2+ Tregs.

Table 1.

TABLE 1.

Recipient, donor, and organ procurement characteristics

	Non-DGF (IGF + SGF)	DGF	P[a]	IGF	AKI (SGF + DGF)	P[b]
Recipient						
n	58	18		24	52	
Age, y	57 ± 2	57 ± 3	0.85	57 ± 3	57 ± 2	0.95
Male	42	15	0.35	15	42	0.09
African American race	6	4	0.19	3	7	0.91
Diagnosis						
SLE	1	0	0.73	0	1	0.77
GN	14	7		5	16	
DM2	16	4		6	14	
HTN	2	1		1	2	
Other	25	6		12	19	
BMI, kg/m^2	28 ± 1	29 ± 1	0.54	27 ± 1	28 ± 1	0.27
PRA > 50%	8	2	0.66	5	5	0.14
PRA class I, %	13 ± 3	5 ± 5	0.24	16 ± 5	9 ± 3	0.22
PRA class II, %	8 ± 3	5 ± 5	0.61	10 ± 6	6 ± 3	0.54
Previous transplant	3	5	<0.01	1	7	0.22
Blood transfusion history	15	4	0.82	5	14	0.40
HLA mismatches	3.5 ± 0.1	3.9 ± 0.3	0.27	3.6 ± 0.3	3.6 ± 0.2	0.86
Pretransplant vitamin D	19	6	0.83	10	15	0.42
Pretransplant statin	29	8	0.68	13	24	0.52
Pretransplant dialysis						
None	5	1	0.34	4	2	<0.01
PD	11	1		7	5	
HD	40	15		12	43	
Time on dialysis, y	3.8 ± 0.4	5.0 ± 1.0	0.19	4 ± 1	4 ± 1	0.81
Pretransplant eGFR, mL/min per 1.73 m^2	11 ± 1	9 ± 1	0.11	10 ± 1	11 ± 1	0.63
Immunosuppression regimen						
ATG Tac, MMF, steroid[c]	27	5	0.29	10	22	0.33
Alemtuzumab Tac, MMF[d]	30	13		13	30	
Daclizumab Tac, MMF, steroid[c]	1	0		1	0	
Posttransplant outcomes						
Day 1 eGFR, mL/min per 1.73 m^2	13 ± 1	9 ± 1	0.01	14 ± 1	11 ± 1	0.09
Day 7 eGFR, mL/min per 1.73 m^2	37 ± 3	14 ± 2	<0.01	51 ± 5	23 ± 2	<0.01
Day 14 eGFR, mL/min per 1.73 m^2	47 ± 2	23 ± 3	<0.01	58 ± 3	32 ± 2	<0.01
Day 30 eGFR, mL/min per 1.73 m^2	48 ± 2	31 ± 3	<0.01	58 ± 4	37 ± 2	<0.01
Day 90 eGFR, mL/min per 1.73 m^2	52 ± 2	38 ± 5	<0.01	63 ± 3	41 ± 2	<0.01
Day 180 eGFR, mL/min per 1.73 m^2	53 ± 3	41 ± 6	0.04	65 ± 4	44 ± 3	<0.01
AR within 180 d	7	6	0.04	3	9	0.59
Donor						
Age, y	49 ± 2	58 ± 3	0.01	38 ± 4	57 ± 2	<0.01
ECD	23	11	0.11	4	30	<0.01
DBD	57	17	0.38	24	50	0.33
DCD	1	1		0	2	
Terminal eGFR, mL/min per 1.73 m^2	121 ± 8	117 ± 11	0.79	118 ± 10	120 ± 9	0.86
Procurement						
CIT, h	15 ± 1	20 ± 2	<0.01	14 ± 1	17 ± 1	0.05
Machine perfusion	49	14	0.61	21	42	0.67

[a] *P* value for comparisons between DGF and non-DGF groups.
[b] *P* value for comparisons between AKI and IGF groups.
[c] Maintenance immunosuppression starting on day 1 with tacrolimus (through levels, 4-8 ng/mL), MMF, and corticosteroid tapering protocol.
[d] Maintenance immunosuppression starting on day 1 with tacrolimus (through levels, 4-8 ng/mL) and MMF.
AR, acute rejection; ATG, antithymocyte globulin; BMI, body mass index; DBD, donor after brain death; DCD, donor after cardiac death; DM2, type 2 diabetes mellitus; eGFR, estimated GFR; GN, glomerulonephritis; HD, hemodialysis; HTN, hypertension; MMF, mycophenolate mofetil; PD, peritoneal dialysis; PRA, panel-reactive antibody; SLE, systemic lupus erythomatosus; Tac, tacrolimus.

Table 2.

TABLE 2.

Logistic regression analysis to predict DGF

Univariate Analysis			
Variables	**OR**	**95% CI**	***P***
CD4+CD127lo/–TNFR2+ Treg, %	0.70	0.52-0.95	0.02
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.96-0.99	0.03
CIT, h	1.15	1.04-1.26	<0.01
Donor age, y	1.04	0.99-1.08	0.06
Previous transplant	7.05	1.49-33.35	0.01
Multivariate analysis with CD4+CD127lo/–TNFR2+, %			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg, %	0.73	0.51-1.05	0.09
CIT, h	1.14	1.03-1.27	0.02
Donor age, y	1.01	0.97-1.05	0.58
Previous transplant	8.40	1.20-58.88	0.03
Multivariate analysis with CD4+CD127lo/–TNFR2+, 10^6/L			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg (10^6/L)	0.98	0.96-1.01	0.10
CIT, h	1.16	1.04-1.29	<0.01
Donor age, y	1.01	0.97-1.06	0.60
Previous transplant	7.16	1.11-46.25	0.04

95% CI, 95% confidence interval; OR, odds ratio.

Table 3.

TABLE 3.

Sensitivity, specificity, and predictive value for predicting DGF or AKI using CD4+CD127lo/–TNFR2+ Treg

	Cutoff Value	Sens, %	Spec, %	PPV, %	NPV, %
Prediction of DGF					
CD4+CD127lo/–TNFR2+ Treg, %	1.49	27.8	96.5	71.4	80.9
	3.32[a]	77.8	71.9	46.7	91.1
	6.19	94.4	24.6	28.3	93.3
CD4+CD127lo/–TNFR2+, 10^6/L	19.26	33.3	96.5	75.0	82.1
	37.13[a]	77.8	78.9	53.8	91.8
	72.65	94.4	33.3	30.9	95.0
Prediction of AKI					
CD4+CD127lo/–TNFR2+ Treg, %	2.76	36.5	95.7	95.2	40.7
	4.27[a]	71.2	69.6	84.1	51.6
	6.79	94.2	39.1	77.8	75.0
CD4+CD127lo/–TNFR2+, 10^6/L	31.67[a]	38.5	95.7	95.2	40.7
	43.26	55.8	78.3	85.3	43.9
	110.35	94.2	26.1	74.2	66.7

[a] Optimal cutoff value based on the best sums of sensitivity and specificity.
NPV, negative predictive value; Sens, sensitivity; Spec, specificity.

Table 4.

TABLE 4.

Logistic regression analysis to predict AKI

Univariate Analysis			
Variables	**OR**	**95% CI**	***P***
CD4+CD127lo/–TNFR2+ Treg, %	0.69	0.55-0.86	<0.01
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.97-0.99	<0.01
CIT, h	1.09	1.01-1.18	0.05
Donor age, y	1.08	1.04-1.12	<0.01
ECD	6.82	2.04-22.78	<0.01
Previous transplant	3.58	0.42-30.86	0.25
Pretransplant dialysis			
None	1.00		
PD	1.43	0.18-11.09	0.73
HD	7.17	1.17-43.97	0.03
Multivariate analysis with CD4+CD127lo/–TNFR2+ (%)			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg (%)	0.72	0.55-0.94	0.02
CIT, h	1.08	0.94-1.23	0.27
Donor age, y	1.09	1.04-1.15	<0.01
Previous transplant	2.67	0.14-49.48	0.51
Pretransplant dialysis			
None	1.00		
PD	2.16	0.15-31.19	0.57
HD	22.54	1.67-303.43	0.02
Multivariate analysis with CD4+CD127lo/–TNFR2+, 10^6/L			
Variables			
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.97-0.99	0.02
CIT, h	1.09	0.95-1.23	0.22
Donor age, y	1.10	1.04-1.17	<0.01
Previous transplant	1.80	0.09-36.24	0.70
Pretransplant dialysis			
None	1.00		
PD	1.69	0.12-23.98	0.70
HD	20.14	1.40-289.36	0.03

Table 5.

TABLE 5.

Sensitivity, specificity, and predictive value for predicting AKI using logistic regression models

Models	AUC (95% CI), *P*	Optimal Cutoff Value	Sens	Spec	PPV	NPV
Donor age + pretransplant dialysis modality	0.88 (0.79-0.98), <0.01	0.74	80.0	82.6	90.9	65.5
CD4+CD127lo/−TNFR2+ Treg (%) + donor age + pretransplant dialysis modality	0.90 (0.81-0.99), <0.01	0.76	78.0	95.7	97.5	66.7

Figure 1.

Pre-transplant recipient circulating Treg suppressive function correlates with TNFR2+ expression on CD4+CD127lo/- T cells. Representative examples of circulating Treg suppressive function measured by the suppression of stimulated autologous CFSE-labeled Teff by Treg in co-culture and the corresponding TNFR2 expression on CD4+CD127lo/- T cells are shown for three recipients prior to transplantation.

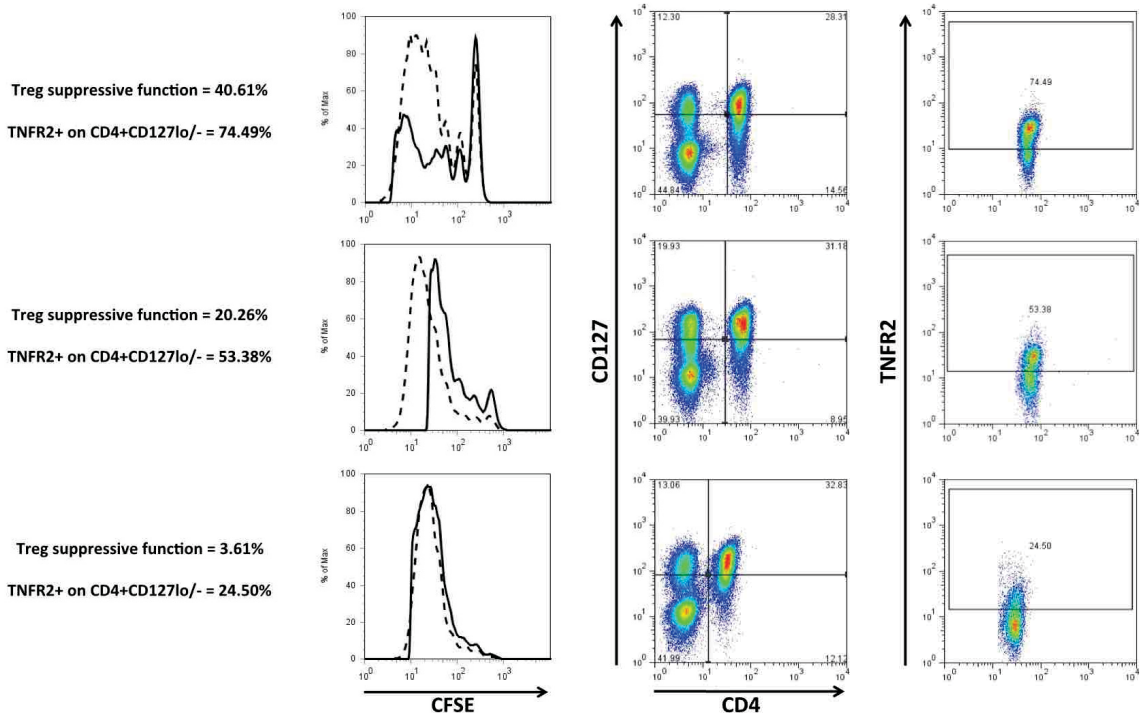


Figure 2.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were lower in DGF and SGF in comparison to IGF recipients. Representative CD4+CD127lo/-TNFR2+ and CD4+CD25+CD127lo/- flow cytometry analyses for a DGF, SGF, and IGF recipient are shown.

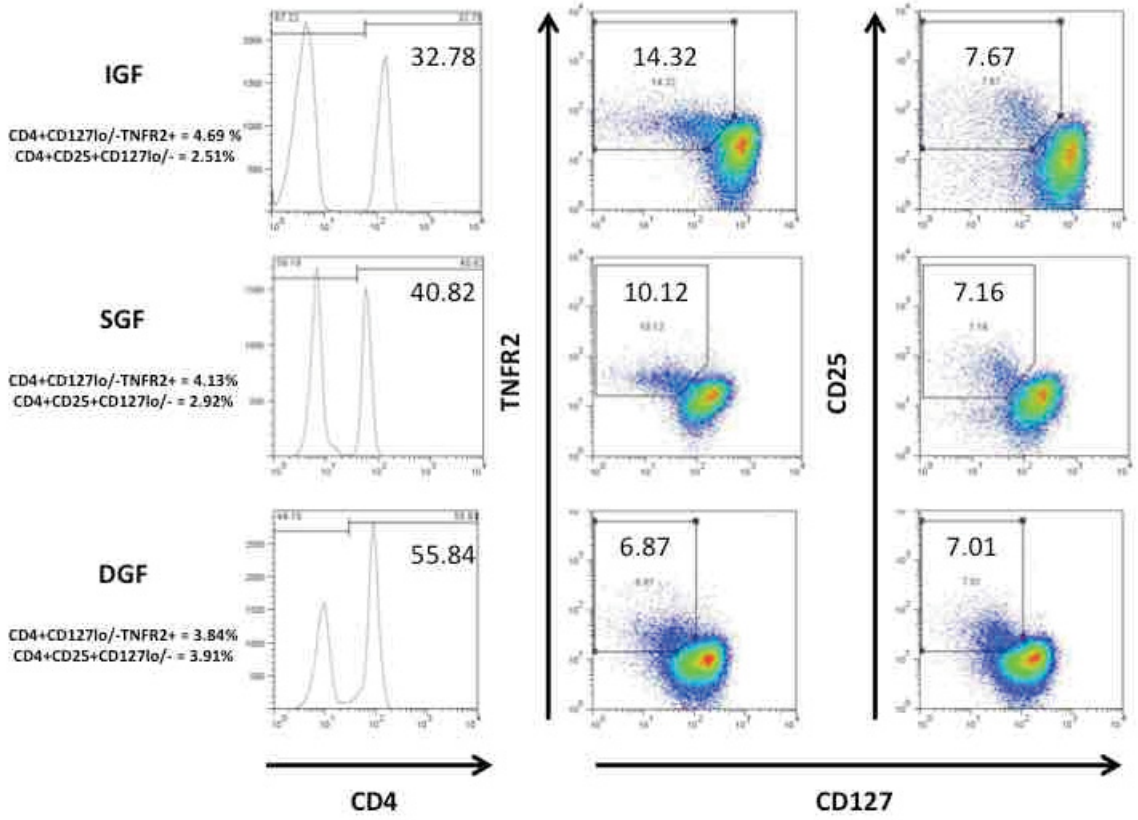


Figure 3.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg **A)** percentage and **B)** absolute number, but not CD4+CD25+CD127lo/- Treg **C)** percentage and **D)** absolute number, were significantly lower in DGF and SGF in comparison to IGF, significantly lower in DGF in comparison to non-DGF (IGF+SGF), and significantly lower in AKI (DGF+SGF) in comparison to IGF recipients.

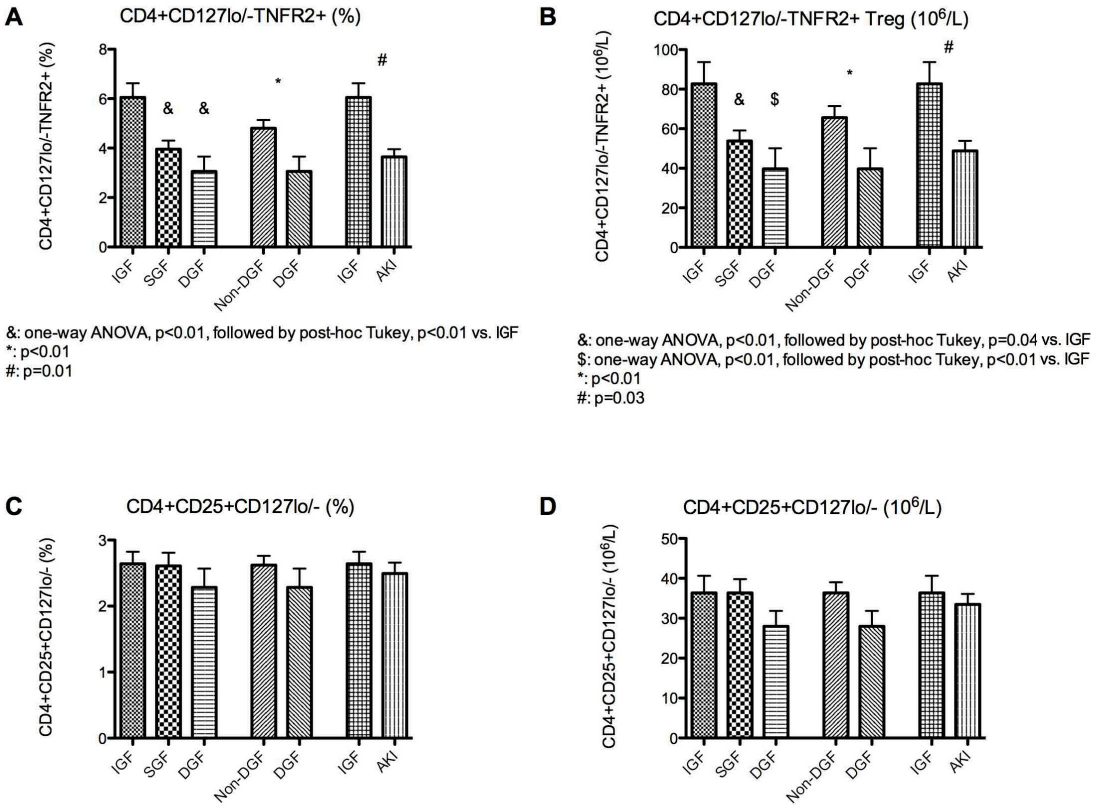


Figure 4.

Predictors of DGF in ROC curve analysis. **A)** Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg percentage (AUC=0.75, 95% CI: 0.61 – 0.88, p<0.01; optimal cut-off value=3.32%, sensitivity=77.8%, specificity=71.9%, PPV=46.7%, NPV=91.1%) and absolute number (AUC=0.77, 95% CI: 0.64 – 0.90, p<0.01; optimal cut-off value=37.13x10^6/L, sensitivity=77.8%, specificity=78.9%, PPV=53.8%, NPV=91.8%) predicted DGF in ROC curve analysis. **B)** Cold ischemic time (AUC=0.75, 95% CI: 0.63 – 0.88, p<0.01; optimal cut-off value=14.5 hours, sensitivity=88.9%, specificity=56.9%, PPV=39.0%, NPV=94.3%) predicted DGF in ROC curve analysis, while donor age (AUC=0.65, 95% CI: 0.52 – 0.77, p=0.07) and previous transplant (AUC=0.61, 95% CI: 0.45 – 0.78, p=0.15) did not.

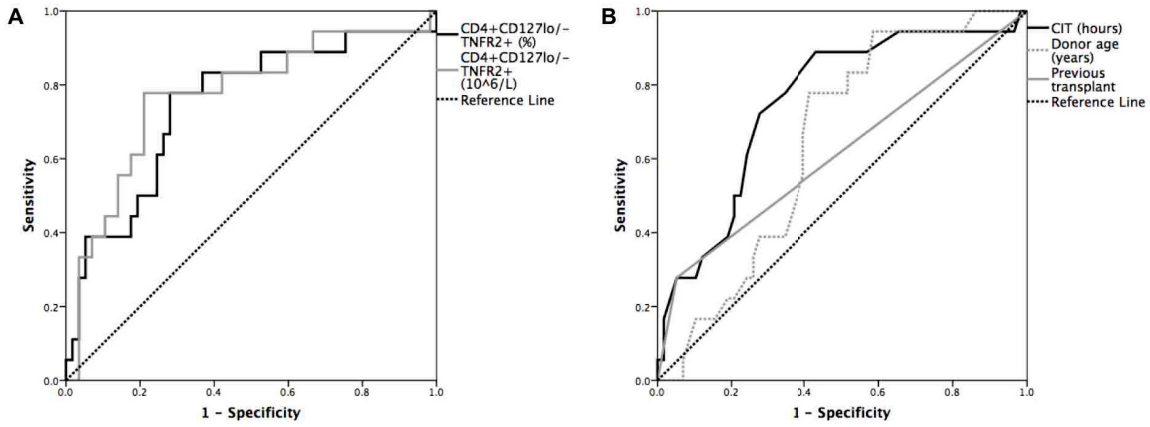


Figure 5.

Predictors of AKI in ROC curve analysis. **A)** Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg percentage (AUC=0.76, 95% CI: 0.64 – 0.87, p<0.01; optimal cut-off value=4.27%, sensitivity=71.2%, specificity=69.6%, PPV=84.1%, NPV=51.6%) and absolute number (AUC=0.72, 95% CI: 0.60 – 0.84, p<0.01; optimal cut-off value=31.67x10^6/L, sensitivity=38.5%, specificity=95.7%, PPV=95.2%, NPV=40.7%) predicted AKI in ROC curve analysis. **B)** Donor age (AUC=0.81, 95% CI: 0.69 – 0.93, p<0.01; optimal cut-off value=46.5 years, sensitivity=88.5%, specificity=75.0%, PPV=88.4%, NPV=75.0%), expanded criteria donor category (AUC=0.71, 95% CI: 0.58 – 0.83, p<0.01; sensitivity=57.7%, specificity=83.3%, PPV=88.2%, NPV=45.5%), and pre-transplant dialysis modality (AUC=0.67, 95% CI: 0.53 – 0.81, p=0.02; sensitivity=86.0%, specificity=47.8%, PPV=78.2%, NPV=61.1%) predicted AKI in ROC curve analysis, while cold ischemic time (AUC=0.63, 95% CI: 0.49 – 0.76, p=0.08) and previous transplant (AUC=0.55, 95% CI: 0.41 – 0.68, p=0.52) did not.

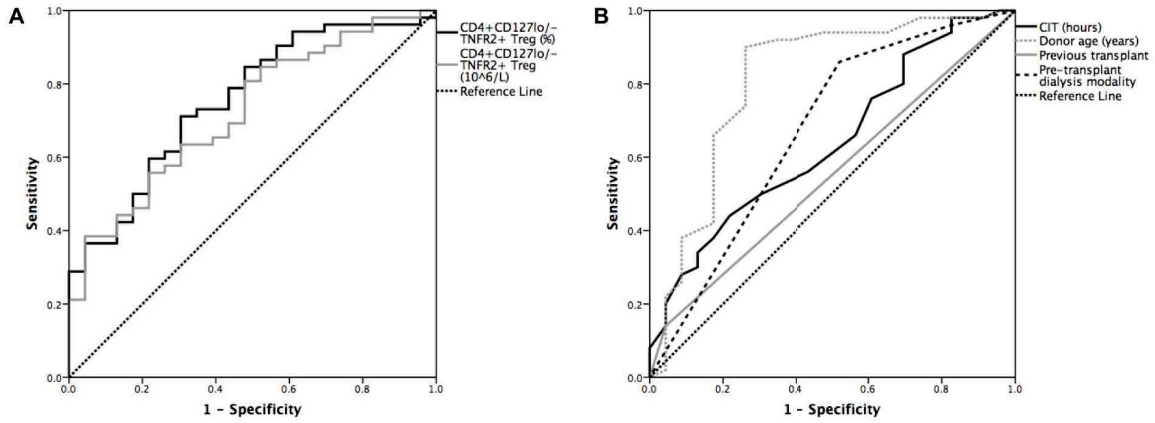


Figure 6.

Prediction of AKI in ROC curve analysis using logistic regression models based on clinical variables alone (AUC=0.88, 95% CI: 0.79 – 0.98, p<0.01) or in combination with CD4+CD127lo/-TNFR2+ Treg percentage (AUC=0.90, 95% CI: 0.81 – 0.99, p<0.01).

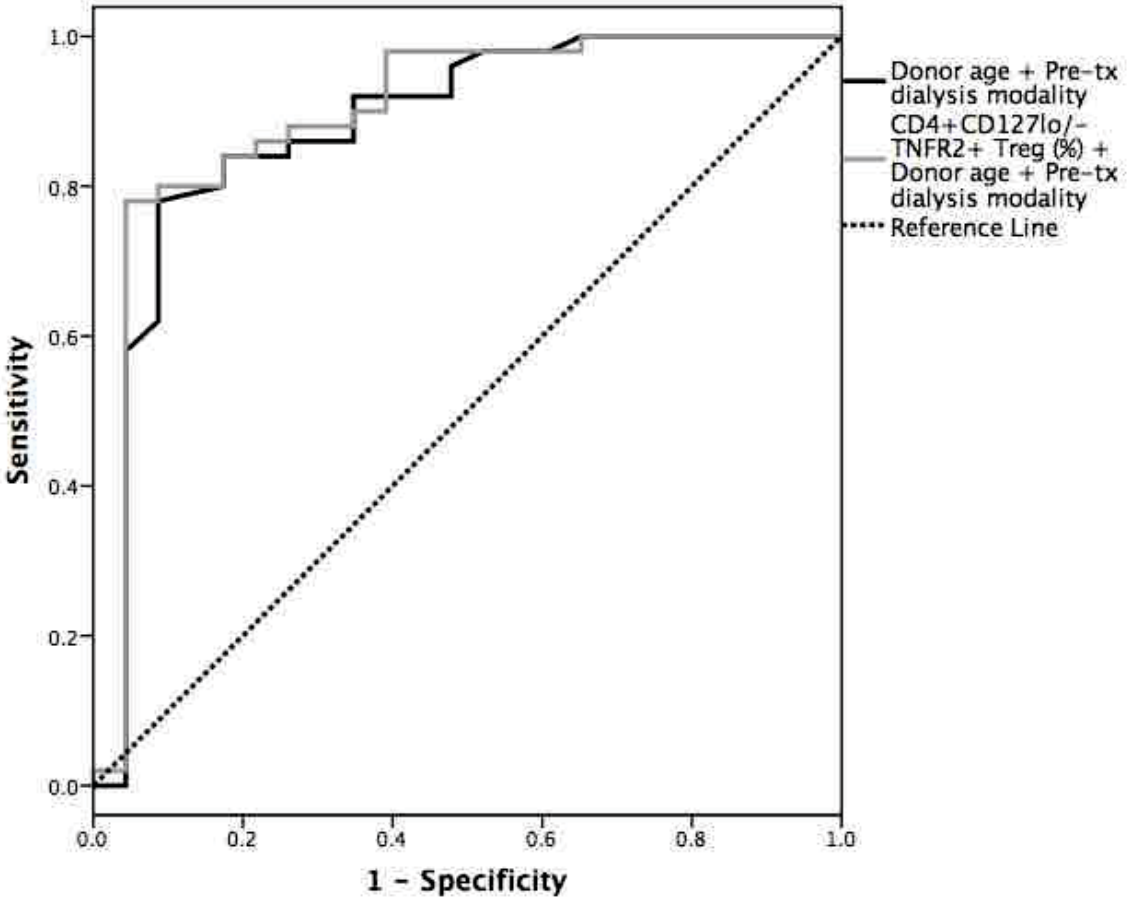


Table S1.

Table S1. Recipient, donor, and organ procurement characteristics				
	IGF	**SGF**	**DGF**	**p**
Recipient				
n	24	34	18	
Age (years)	57±3	57±2	57±3	0.98
Male	15	27	15	0.22
African-American race	3	3	4	0.39
Diagnosis				
SLE	0	1	0	0.88
GN	5	9	7	
DM2	6	10	4	
HTN	1	1	1	
Other	12	13	6	
BMI (kg/m^2)	27±1	28±1	29±1	0.54
PRA>50%	5	3	2	0.33
PRA class I (%)	16±5	10±4	5±5	0.35
PRA class II (%)	10±6	7±4	5±5	0.79
Previous tx	1	2	5	0.02
Blood transfusion history	5	10	4	0.70
HLA mismatches	3.6±0.3	3.5±0.2	3.9±0.3	0.53

Pre-transplant Vit D	10	9	6	0.61
Pre-transplant statin	13	16	8	0.80
Pre-tx dialysis				
None	4	1	1	0.04
PD	7	4	1	
HD	12	28	15	
Time on dialysis (years)	4±1	4±1	5±1	0.41
Pre-transplant eGFR (mL/min/1.73m^2)	10±1	11±1	9±1	0.14
Immunosuppression regimen				
ATG Tac, MMF, steroid[a]	10	17	5	0.33
Alemtuzumab Tac, MMF[b]	13	17	13	
Daclizumab Tac, MMF, steroid[a]	1	0	0	
Post-transplant outcomes				
Day 1 eGFR (mL/min/1.73m^2)	14±1	12±1	9±1	0.02
Day 7 eGFR	51±5	28±2	14±2	<0.01

(mL/min/1.73m^2)				
Day 14 eGFR (mL/min/1.73m^2)	58±3	38±3	23±3	<0.01
Day 30 eGFR (mL/min/1.73m^2)	58±4	41±2	31±3	<0.01
Day 90 eGFR (mL/min/1.73m^2)	63±3	43±2	38±5	<0.01
Day 180 eGFR (mL/min/1.73m^2)	65±4	45±3	41±6	<0.01
AR within 180 days	3	3	6	0.06
Donor				
Age (years)	38±4	57±2	58±3	<0.01
ECD	4	19	11	<0.01
DBD	24	33	17	0.53
DCD	0	1	1	
Terminal eGFR (mL/min/1.73m^2)	118±10	123±13	117±11	0.92
Procurement				
CIT (hours)	14±1	15±1	20±2	<0.01
Machine perfusion	21	28	14	0.73

a: maintenance immunosuppression starting on day 1 with tacrolimus (through levels 4-8 ng/mL), MMF, and corticosteroid tapering protocol

b: maintenance immunosuppression starting on day 1 with tacrolimus (through levels 4-8 ng/mL) and MMF

AR, acute rejection; ATG, anti-thymocyte globulin; BMI, body mass index; CIT, cold ischemic time; DBD, donor after brain death; DCD, donor after cardiac death; DGF, delayed graft function; DM2, type 2 diabetes mellitus; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; HD, hemodialysis; HLA, human leukocyte antigen; HTN, hypertension; IGF, immediate graft function; MMF, mycophenolate mofetil; PD, peritoneal dialysis; PRA, panel reactive antibody; SGF, slow graft function; SLE, systemic lupus erythomatosus; TAC, tacrolimus; tx, transplant; Vit D, vitamin D.

Table S2.

Table S2. Linear regression analysis to predict pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg from recipient characteristics						
Recipient characteristics	**Prediction of CD4+CD127lo/-TNFR2+ Treg (%)**			**Prediction of CD4+CD127lo/-TNFR2+ Treg (10^6/L)**		
	B	**95% CI**	**p**	**B**	**95% CI**	**p**
Age	0.00	-0.05 – 0.05	1.00	-0.38	-1.26 – 0.49	0.39
Sex	-0.03	-1.43 – 1.37	0.97	-1.11	-24.83 – 22.61	0.93
Autoimmune disease	0.23	-5.08 – 5.54	0.93	-22.66	-112.46 – 67.14	0.62
BMI	0.02	-0.10 – 0.14	0.78	0.06	-1.95 – 2.07	0.95
Pre-transplant Vit D hx	0.57	-0.78 – 1.92	0.41	5.57	-17.25 – 28.39	0.63
Pre-transplant statin hx	1.02	-0.18 – 2.21	0.09	11.19	-9.28 – 31.66	0.28
Blood transfusion hx	-0.16	-1.63 – 1.30	0.83	-12.08	-34.48 – 10.32	0.29
PRA class I (%)	-0.01	-0.04 – 0.02	0.59	-0.10	-0.54 – 0.35	0.67
PRA class II (%)	0.01	-0.03 – 0.03	0.83	-0.04	-0.48 – 0.40	0.87
Previous transplant	-0.72	-2.69 – 1.24	0.47	-19.93	-53.03 – 13.17	0.23

Pre-transplant dialysis						
None	-0.34	-2.62 – 1.93	0.76	4.74	-33.62 – 43.10	0.81
Peritoneal dialysis	1.07	-0.59 – 2.74	0.20	17.37	-10.77 – 45.52	0.22
Hemodialysis	-0.65	-2.10 – 0.79	0.37	-14.77	-38.98 – 9.43	0.23
Time on dialysis	0.08	-0.11 – 0.27	0.41	-0.61	-3.89 – 2.67	0.71
Pre-transplant eGFR	-0.11	-0.27 – 0.06	0.20	-2.42	-5.11 – 0.28	0.08

CI, confidence interval; eGFR, estimated glomerular filtration rate; hx, history; PRA, panel reactive antibody; Treg, regulatory T cell; Vit D, vitamin D.

Table S3.

Table S3. Correlations between variables that were significantly different between DGF and non-DGF or AKI and IGF recipients			
Correlations between		**r**	**p**
CD4+CD127-TNFR2+ Treg (%)	CIT	-0.16	0.17
CD4+CD127-TNFR2+ Treg (%)	Donor age	-0.32	<0.01
CD4+CD127-TNFR2+ Treg (%)	ECD	-0.32	<0.01
CD4+CD127-TNFR2+ Treg (%)	Previous transplant	-0.09	0.47
CD4+CD127-TNFR2+ Treg (%)	Pre-transplant dialysis	-0.06	0.62
CD4+CD127-TNFR2+ Treg (10^6/L)	CIT	-0.05	0.68
CD4+CD127-TNFR2+ Treg (10^6/L)	Donor age	-0.26	0.02
CD4+CD127-TNFR2+ Treg (10^6/L)	ECD	-0.25	0.03
CD4+CD127-TNFR2+ Treg (10^6/L)	Previous transplant	-0.14	0.23
CD4+CD127-TNFR2+ Treg (10^6/L)	Pre-transplant dialysis	-0.11	0.35
CIT	Donor age	0.23	0.05
CIT	ECD	0.30	<0.01
CIT	Previous transplant	0.08	0.51

CIT	Pre-transplant dialysis	0.04	0.77
Donor age	ECD	0.73	<0.01
Donor age	Previous transplant	0.04	0.72
Donor age	Pre-transplant dialysis	0.07	0.56
ECD	Previous transplant	0.04	0.76
ECD	Pre-transplant dialysis	-0.05	0.67
Previous transplant	Pre-transplant dialysis	0.19	0.12

AKI, acute kidney injury; CIT, cold ischemic time; DGF, delayed graft function; ECD, expanded criteria donor; IGF, immediate graft function; SGF, slow graft function; Treg, regulatory T cell.

Table S4.

Table S4. Logistic regression analysis to predict AKI		
Univariate analysis		
Variables	**OR**	**Bootstrap 95% CI**
CD4+CD127lo/-TNFR2+ Treg (%)	0.69	0.48 – 0.85
CD4+CD127lo/-TNFR2+ Treg (10^6/L)	0.98	0.96 – 0.99
CIT (hours)	1.09	1.01 – 1.22
Donor age (years)	1.08	1.04 – 1.16
ECD	6.82	2.39 – 40.98
Previous transplant	3.58	0.59 – 1.18x10^9
Pre-transplant dialysis		
None	1.00	
PD	1.43	0.03 – 1.94x10^9
HD	7.17	0.65 – 7.91x10^9
Multivariate analysis with CD4+CD127lo/-TNFR2+ Treg (%)		
Variables	**OR**	**Bootstrap 95% CI**
CD4+CD127lo/-TNFR2+ Treg (%)	0.72	0.21 – 0.93
CIT (hours)	1.08	0.92 – 1.81
Donor age (years)	1.09	1.04 – 1.46

Previous transplant	2.67	$0.27 – 5.74x10^{8}$
Pre-transplant dialysis		
None	1.00	
PD	2.16	$4.11x10^{-8} – 8.30x10^{9}$
HD	22.54	$1.59 – 7.65x10^{11}$
Multivariate analysis with CD4+CD127lo/-TNFR2+ Treg (10^{6}/L)		
Variables	**OR**	**Bootstrap 95% CI**
CD4+CD127lo/-TNFR2+ Treg (10^{6}/L)	0.98	0.93 – 0.99
CIT (hours)	1.09	0.95 – 1.85
Donor age (years)	1.10	1.05 – 1.52
Previous transplant	1.80	$0.15 – 3.56x10^{8}$
Pre-transplant dialysis		
None	1.00	
PD	1.69	$2.46x10^{-8} – 6.85x10^{9}$
HD	20.14	$1.16 – 7.07x10^{11}$

AKI, acute kidney injury; CI, confidence interval; CIT, cold ischemic time; ECD, expanded criteria donor; HD, hemodialysis; OR, odds ratio; PD, peritoneal dialysis; Treg, regulatory T cell.

Figure S1.

Pre-transplant recipient circulating CD4+ T cell **A)** percentage and **B)** absolute number and CD4+CD127lo/- T cell **C)** percentage and **D)** absolute number were not significantly different among DGF, SGF, and IGF recipients, between DGF and non-DGF (IGF+SGF) recipients, or between AKI (DGF+SGF) and IGF recipients.

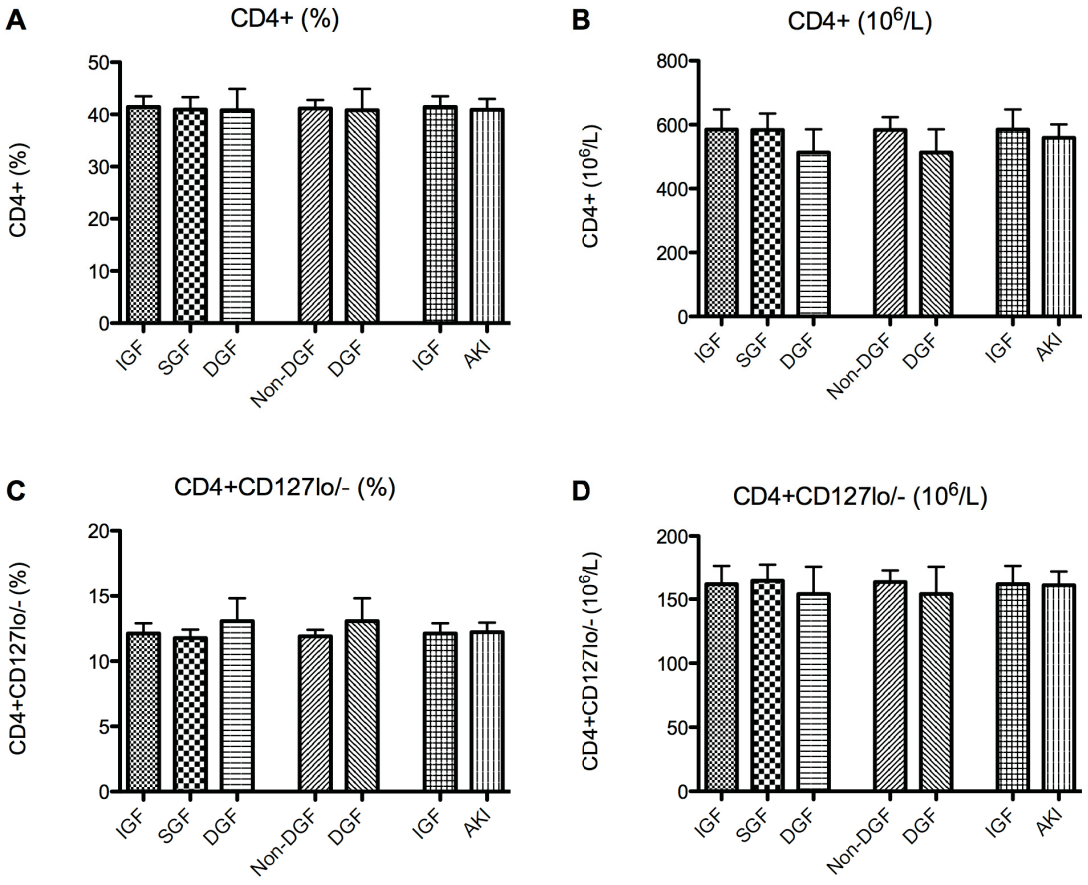


Figure S2.

CD4+CD127lo/- percentage (AUC=0.46, 95% CI: 0.29 – 0.64, p=0.65) and absolute number (AUC=0.54, 95% CI: 0.38 – 0.70, p=0.60) as well as CD4+CD25+CD127lo/- percentage (AUC=0.56, 95% CI: 0.39 – 0.72, p=0.47) and absolute number (AUC=0.60, 95% CI: 0.45 – 0.76, p=0.19) were not predictors of DGF on ROC curve analysis.

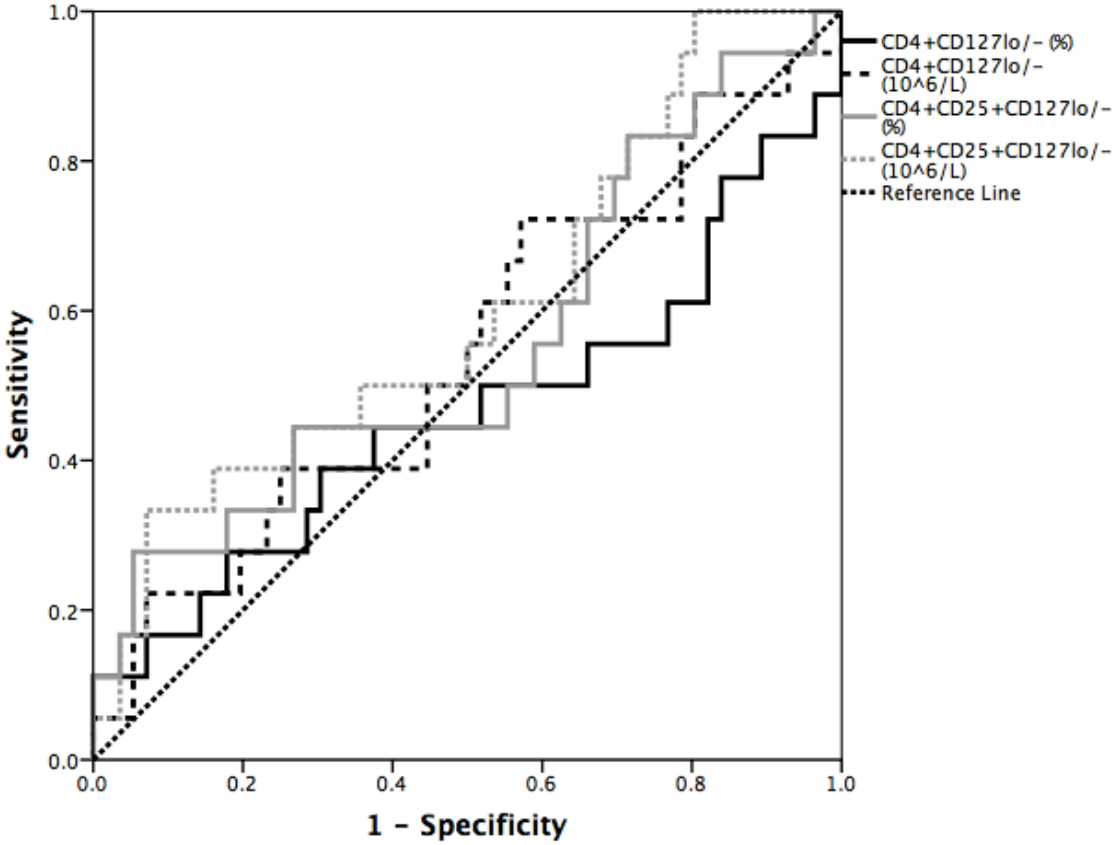
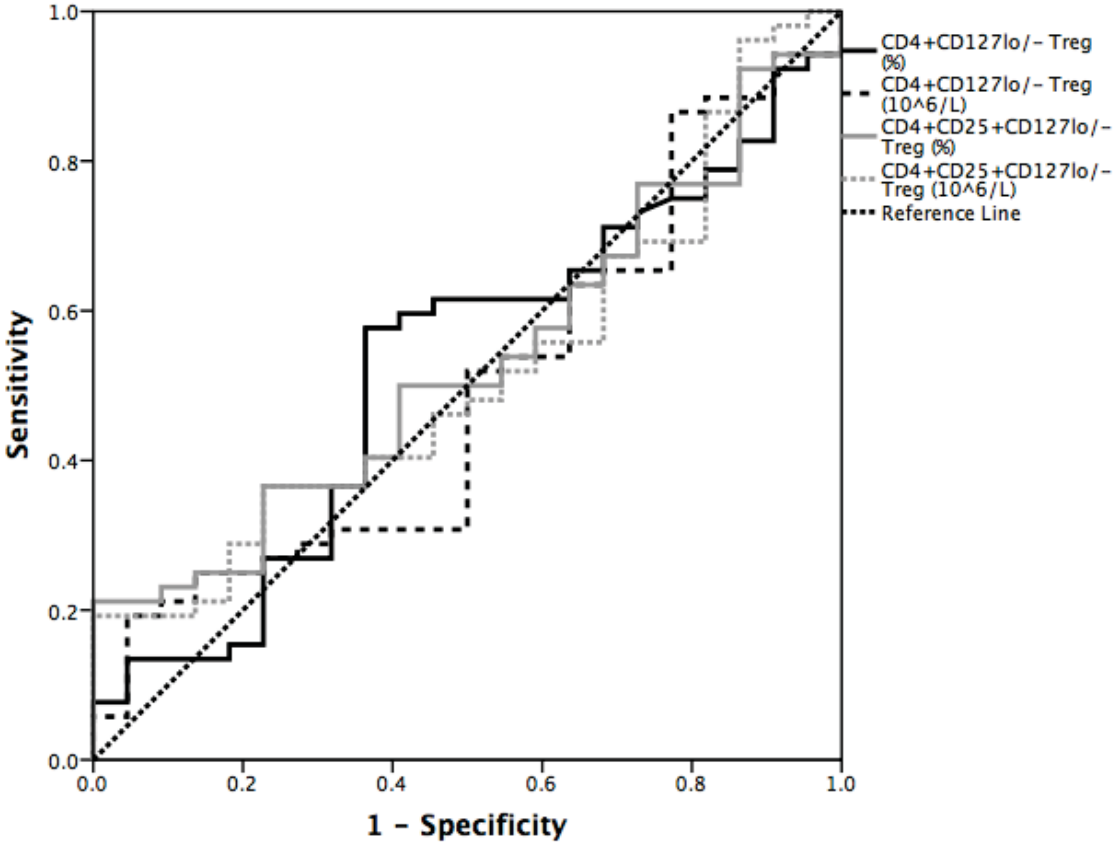


Figure S3.

CD4+CD127lo/- percentage (AUC=0.52, 95% CI: 0.38 – 0.66, p=0.79) and absolute number (AUC=0.49, 95% CI: 0.35 – 0.63, p=0.84) as well as CD4+CD25+CD127lo/- percentage (AUC=0.53, 95% CI: 0.40 – 0.67, p=0.67) and absolute number (AUC=0.52, 95% CI: 0.38 – 0.66, p=0.80) were not predictors of AKI (DGF+SGF) on ROC curve analysis.



Preface to chapter 4

In Chapter 3, we found that TNFR2 expression on Tregs could serve as a surrogate for the Treg suppressive function assay in kidney transplant candidates. Moreover, we showed that measuring pre-transplant recipient circulating TNFR2+ Tregs could replace Treg suppressive function as an independent predictor of DGF/SGF after kidney transplantation (476). Since DGF and SGF are known risk factors for acute rejection after kidney transplantation (12,438), we sought to determine whether pre-transplant circulating TNFR2+ Tregs could also predict the development of acute rejection within the first 6 months after kidney transplantation using the same cohort of patients (minus 1 patient due to unavailable acute rejection data). Since not all DGF/SGF recipients develop acute rejection, we also explored in a subgroup analysis including only DGF/SGF recipients whether TNFR2+ Tregs could differentiate immunologically relevant from immunologically benign DGF/SGF.

Chapter 4. Pre-transplant recipient circulating TNFR2+ regulatory T cells predict acute rejection within the first 6 months after kidney transplantation

Abstract

Background: Acute rejection most often occurs during the first 6 months after kidney transplantation and contributes importantly to graft loss. Diagnosis relies on changes in serum creatinine, but histological damage occurs prior. Regulatory T cells (Tregs) are critical in transplant tolerance. Their utility in the peri-transplant period to predict acute rejection remains controversial due to the heterogeneity in their identification markers and immune suppressive function. Tumor necrosis factor receptor 2 (TNFR2) identifies a subset of maximally immune suppressive Tregs. We investigated whether pre-transplant circulating TNFR2+ Tregs predict acute rejection within the first 6 months after kidney transplantation.

Methods: 75 deceased donor kidney transplant recipients were recruited. 12 developed biopsy-proven acute rejection (11 acute cellular rejections [5 borderline, 3 1A, 2 1B], 1 antibody-mediated rejection). Donor, organ procurement, and recipient characteristics were similar between acute rejection and non-rejector recipients except for cold ischemic time (CIT) and sensitization. CD4+CD127lo/- TNFR2+ Tregs were quantified by flow cytometry in pre-transplant recipient peripheral blood.

Results: Pre-transplant circulating TNFR2+ Tregs were significantly decreased in recipients who developed acute rejection (2.74 ± 0.28%) compared to non-rejectors (4.70 ± 0.34%, p=0.02). In univariate logistic regression, each percentage increase in TNFR2+ Tregs decreased the odds of developing acute rejection by 38% (p=0.02). TNFR2+ Tregs also accurately predicted acute rejection in receiver operating characteristic analysis with an area under the curve (AUC) of 0.75 (p=0.01). In multivariate logistic regression adjusting for the effects of CIT and sensitization, TNFR2+ Tregs remained a significant predictor of acute rejection (OR=0.58, p=0.04). Combining TNFR2+ Tregs with CIT and sensitization in a model improved the predictive accuracy for acute rejection (AUC=0.86, p<0.01).

Conclusion: Pre-transplant circulating TNFR2+ Tregs predict acute rejection during the first 6 months after kidney transplantation independently or in combination with CIT and sensitization.

4.1. Introduction

Acute rejection is most frequent in the first 6 months after kidney transplantation with an incidence of up to 20% (477). It remains a major risk factor for graft loss despite the advent of modern immunosuppressive regimen (478-481). Since acute rejection is generally asymptomatic, surveillance with serial serum creatinine is currently the monitoring method of choice in clinical practice. Serum creatinine is however a late marker of acute rejection and is unable to differentiate acute

rejection from other etiologies of post-transplant kidney dysfunction (482). Diagnosis requires a kidney allograft biopsy, which is invasive and is associated with low but potentially severe risks of bleeding, arterio-venous fistulas, ureteral obstruction, and graft loss (483-485). Novel non-invasive methods are needed to predict and provide early diagnosis of acute rejection after kidney transplantation.

Regulatory T cells (Tregs) are CD4+ T cells that have an essential role in self and transplant tolerance by suppressing excessive adaptive and innate immune responses. They have conventionally been identified by their high expression of CD25, low expression of CD127, and their expression of the transcription factor FoxP3 (215). Due to its role in transplant tolerance, several groups have attempted to measure Tregs mainly post-transplant in peripheral blood, urine, or biopsy samples in order to predict the development of acute rejection with conflicting results (486). These conflicting results could be due to the fact that Tregs are not a homogeneous population. Recent evidence suggests that markers beyond the conventional ones exist for the identification of Tregs, and their expression could identify subsets with differing suppressive function capacity (409). One such marker is tumor necrosis factor receptor 2 (TNFR2).

TNFR2 is a cytokine receptor that is preferentially expressed on Tregs as compared to effector T cells (Teffs) and mediates the biological function of the cytokine TNF-α. Although TNF-α generally promotes inflammation, it also possesses immunoregulatory functions when it interacts with TNFR2+ Tregs. In both mice and

humans, TNF-α signaling increased the survival, proliferation, and suppressive function of TNFR2+ Tregs. TNFR2+ Tregs also decrease the bioavailability of TNF-α to perform its pro-inflammatory functions (335). In the specific context of kidney transplant candidates, we showed that TNFR2 expression on Tregs correlated with their suppressive function (476). Moreover, we demonstrated that the measurement of pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs was independently predictive of delayed (DGF) and slow (SGF) graft function after kidney transplantation (476). Interestingly, DGF/SGF is known to increase the risk for acute rejection (12,438). We therefore hypothesized that pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs could also predict acute rejection within the first 6 months after kidney transplantation.

4.2. Materials and methods

Our prospective observational cohort study was approved by the McGill University Health Centre Research Ethics Board and registered on ClinicalTrials.gov (NCT01232816). The study was conducted in adherence with the declarations of Helsinki and Istanbul.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg frequency

Peripheral blood was drawn in heparin-coated tubes in the operating room prior to induction immunosuppression and skin incision. Peripheral blood mononuclear

cells (PBMCs) were isolated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ). The PBMCs were surface stained with anti-human CD4 fluorescein isothiocyanate (clone OKT4; eBioscience, San Diego, CA), CD127 PE-Cy5 (clone eBioRDR5; eBioscience), and TNFR2 PE (clone TR75-89; BD Biosciences) for 30 minutes in the dark at 4°C, then washed with phosphate-buffered saline (Wisent, St-Bruno, Canada). Flow cytometry acquisition was performed on the FACScan (BD Biosciences), and data analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Forward and side scatters were used to gate on lymphocytes. The Treg cell frequency was determined by the percentage of CD4+CD127lo/−TNFR2+ cells in the lymphocyte gate. Gating strategies for CD4, CD127, and TNFR2 were based on single-color compensation.

Histopathological diagnosis of acute rejection

Renal allografts biopsies were performed post-transplant for renal dysfunction associated with a clinical suspicion for acute rejection. A renal pathologist analyzed the biopsies as part of clinical practice. Acute rejection diagnosis and grading were performed according to Banff criteria (487).

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using SPSS 20 (IBM, Armonk, NY) and considered significant if p value ≤

0.05. Categorical data were compared using Chi-Square test. Continuous data between 2 groups was compared using Student t test. Logistic regression and receiver operating characteristic (ROC) curve were performed to assess recipient, donor, and organ procurement variables as predictors of acute rejection within the first 6 months after kidney transplantation. ROC curve was also used to determine an optimal cutoff value for the prediction of acute rejection based on the largest sums of sensitivity and specificity. Multivariate logistic regression was used to assess the independent predictive ability of CD4+CD127lo/−TNFR2+ Tregs for acute rejection in models containing variables that were significantly different between our outcomes of interest. A subgroup analysis of DGF/SGF recipients only was also performed using the previous statistical analyses.

4.3. Results

Patient characteristics

Seventy-five consecutive consenting adult deceased-donor kidney transplant recipients were enrolled into the study. Recipients were prospectively divided into acute rejection (n = 12) and no acute rejection (n = 63) groups based on a diagnosis of biopsy-proven acute rejection within the first 6 months after kidney transplantation according to Banff criteria (487). Within the acute rejection group, 11 had acute cellular rejection (5 borderline, 3 Banff 1A, 2 Banff 1B) and 1 had antibody-mediated rejection. Recipient, donor, and organ procurement characteristics were collected prospectively (Table 1). Sensitization status (panel

reactive antibody (PRA) > 50%) and cold ischemic time (CIT) were significantly higher in kidney transplant recipients who developed acute rejection within the first 6 months. Pre-transplant dialysis status as well as induction and maintenance immunosuppressive regimen were similar between the 2 groups (Table 1). None of the recipients, including those with an autoimmune etiology of end-stage renal disease, were on immunosuppressive therapy within 6 months preceding their kidney transplant. Estimated glomerular filtration rate was significantly lower in recipients who developed acute rejection at 3 and 6 months after kidney transplantation (Table 1).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were lower in the acute rejection group

We first sought to determine whether the percentage of pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs was different between the acute rejection and non-rejector groups. The percentage of CD4+CD127lo/-TNFR2+ Tregs was significantly lower in kidney transplant recipients who developed acute rejection within the first 6 months (2.74 ± 0.28%) compared to those who had no acute rejection (4.70 ± 0.34%; Figure 1). We previously showed that recipient baseline characteristics did not influence the frequency of pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs (476).

Prediction of acute rejection within the first 6 months after kidney transplantation based on pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted acute rejection within the first 6 months after kidney transplantation on univariate analysis

Since pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were significantly lower in kidney transplant recipients who developed acute rejection, we examined whether its measurement could predict if a recipient will develop acute rejection within the first 6 months after kidney transplantation. In logistic regression analysis, each percentage increase in CD4+CD127lo/-TNFR2+ Treg decreased the odds of developing acute rejection by 38% ($p = 0.02$; Table 2). Using ROC curve analysis, predictive accuracy of CD4+CD127lo/-TNFR2+ Treg for acute rejection was further assessed and an area under the curve (AUC) of 0.75 was obtained ($p = 0.01$; Figure 2). The optimal cut-off point for CD4+CD127lo/-TNFR2+ Treg as a marker for acute rejection was determined based on the largest sums of sensitivity and specificity. A pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg value of 3.41% was thus chosen as the optimal cut-off point to predict acute rejection within 6 months after kidney transplantation, with a sensitivity of 83.3%, specificity of 65.1%, positive predictive value of 31.3%, and negative predictive value of 95.3%. Two other variables were also predictive of acute

rejection within the first 6 months after kidney transplantation in logistic regression. These variables were a PRA > 50% and CIT (Table 2).

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs predicted acute rejection within the first 6 months after kidney transplantation on multivariate analysis

Using multivariate logistic regression, pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs remained a significant predictor of acute rejection within 6 months after kidney transplantation. When adjusting for the effect of sensitization (PRA > 50%) and CIT, CD4+CD127lo/-TNFR2+ Tregs had an adjusted odds ratio of 0.58 ($p = 0.04$; Table 2) for the prediction of acute rejection.

CD4+CD127lo/-TNFR2+ Treg-based logistic regression model to predict acute rejection within the first 6 months after kidney transplantation

Since sensitization (PRA > 50%) and CIT were also significant variables in the prediction of acute rejection within the first 6 months after kidney transplantation on multivariate analysis (Table 2), we incorporated these variables with pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg to create a logistic regression model that could be used to predict prior to kidney transplantation the probability of developing acute rejection (probability of acute rejection = $1/(1 + e^{-z})$ with $z = -3.83 + (-0.54 * \text{CD4+CD127lo/-TNFR2+ Treg}) + (0.19 * \text{CIT}) + (2.90 *$

PRA>50%(1))). This logistic regression model improved the predictive accuracy for acute rejection on ROC curve analysis with an AUC of 0.86 ($p < 0.01$), as compared to using CD4+CD127lo/-TNFR2+ Treg alone (Figure 3).

Subgroup analysis of kidney transplant recipients who developed DGF/SGF after kidney transplantation

Since DGF/SGF is a known risk factor for the development of acute rejection after kidney transplantation (12,438), we performed a subgroup analysis that included only the 53 recipients who developed DGF (n = 18) or SGF (n = 34). In this subgroup, a total of 9 recipients developed acute rejection, 6 who had DGF and 3 who had SGF. The frequency of CD4+CD127lo/-TNFR2+ Treg was significantly lower in DGF/SGF recipients who developed acute rejection (2.58 ± 0.34%) compared to those who did not (3.87 ± 0.36%; $p = 0.01$; Figure 4). Predictive analyses for acute rejection in this subgroup did not yield statistically significant results.

4.4. Discussion

Based on our previous finding that pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs were predictive of DGF/SGF and that DGF/SGF is a risk factor for acute rejection, we found that pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs could also be a predictive immune marker for the development of acute rejection within 6 months after deceased donor kidney

transplantation. In our study, pre-transplant circulating CD4+CD127lo/-TNFR2+ Tregs were lower in recipients who eventually developed acute rejection. Our results are diverging from the limited literature available. In a study including 75 kidney transplant recipients with 6 developing acute rejection within the first month, San Segundo et al. showed that pre-transplant circulating CD4+CD25hiCD62L+CD45RO+ Tregs were higher in recipients who had acute rejection, and that CD4+CD25hiCD62L+CD45RO+ Tregs were predictive of acute rejection in univariate analysis (488). The discrepancy with our study is likely related to the measurement of different functional subpopulations of Treg.

The CD4+CD25hiCD62L+CD45RO+ Tregs measured by San Segundo et al. are thought to be highly functional activated Tregs that are short-lived (410). These short-lived activated Tregs measured pre-transplant are unlikely to be present anymore by the time acute rejection occurs post-transplant. On the other hand, we measured a different subpopulation of Tregs expressing TNFR2. TNFR2 is a cytokine receptor preferentially expressed on Tregs that mediates the biological function of TNF-α (335,468). TNF-α is a pro-inflammatory cytokine that is upregulated during acute rejection and enhances effector immune responses (489). At the same time, the binding of TNF-α to TNFR2+ Tregs has been shown to promote their generation, proliferation, and suppressive function as well as limit the bioavailability of TNF-α to promote effector immune responses (335). Moreover, TNFR2+ Tregs can shed soluble TNFR2, which further acts as a decoy to limit the bioavailability of TNF-α to promote effector immune responses (336). It is thus plausible that kidney

transplant recipients with higher pre-transplant TNFR2+ Treg frequency have a better ability to prevent effector immune responses against their kidney allograft post-transplant.

Since DGF/SGF puts recipients at risk for acute rejection and not all recipients who have DGF/SGF develop acute rejection, we performed a subgroup analysis including only recipients who developed DGF/SGF. Although pre-transplant circulating CD4+CD127lo/-TNFR2+ Tregs were lower in DGF/SGF recipients who developed acute rejection compared to those who did not, we were not able to show that CD4+CD127lo/-TNFR2+ Tregs could discriminate DGF/SGF leading to immunological consequences. This subgroup analysis of DGF/SGF recipients however remain exploratory due to its small sample size, and a future larger study will be needed to investigate whether CD4+CD127lo/-TNFR2+ Tregs can identify immunologically-relevant DGF/SGF.

Our study is limited by several factors. First, it is a single-center study with an overall small sample size. This limits the number of variables that can be included in multivariate analyses. Secondly, we only had 12 patients who developed acute rejection within the first 6 months. We were therefore unable to perform sub-analyses regarding the role of pre-transplant recipient circulating TNFR2+ Tregs across different severity of acute rejection or whether it predicted response to treatment of acute rejection. Finally, our study lacks external validation, and it will be important to confirm our findings in a multi-center trial.

Despite the above limitations, we nevertheless found that measuring pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Tregs is a potential novel independent predictive immune marker for acute rejection within the first 6 months. The combination of CD4+CD127lo/-TNFR2+ Tregs with other pre-transplant variables (sensitization status, CIT) further enhanced the predictive accuracy for acute rejection. The pre-transplant measurement of CD4+CD127lo/-TNFR2+ Tregs could ultimately help transplant clinicians predict which recipients are at risk for acute rejection prior to any immunologic injuries. This would allow proper allocation of kidney allografts at risk for acute rejection and pre-emptive tailoring of immunosuppressive regimen in the recipient.

Table 1.

Recipient, donor, and organ procurement characteristics

	No AR	AR	p
Recipient variables			
n	63	12	
Age (years)	58 ± 2	55 ± 2	0.44
Male:Female	48:15	8:4	0.49
African-American race	7/63 (11%)	3/12 (25%)	0.20
BMI (kg/m^2)	28 ± 1	29 ± 1	0.43
Diagnosis			
Autoimmune disease	1/63 (2%)	0/12 (0%)	0.19
Diabetes mellitus	20/63 (32%)	0/12 (0%)	
Glomerulonephritis	15/63 (24%)	5/12 (42%)	
Hypertension	2/63 (3%)	1/12 (8%)	
Other	25/63 (39%)	6/12 (50%)	
PRA > 50%	6/59 (10%)	4/11 (36%)	0.02
PRA class I (%)	8 ± 2	26 ± 10	0.12
PRA class II (%)	6 ± 3	13 ±9	0.37
HLA mismatches	3.6 ± 0.1	3.4 ± 0.4	0.56
Previous transplant	6/63 (10%)	2/12 (17%)	0.46
Blood transfusion history	15/53 (28%)	4/8 (50%)	0.22
Vitamin D use	21/59 (36%)	3/11 (27%)	0.59

Statin use	31/63 (49%)	6/12 (50%)	0.96
Pre-transplant dialysis			
None (pre-emptive)	5/61 (8%)	1/12 (8%)	0.68
PD	9/61 (15%)	3/12 (25%)	
HD	47/61 (77%)	8/12 (67%)	
Time on dialysis (years)	4.0 ± 0.4	4.7 ± 1.1	0.54
Immunosuppression			
ATG	29/63 (46%)	3/12 (25%)	0.34
Alemtuzumab	33/63 (52%)	9/12 (75%)	
Basiliximab	1/63 (2%)	0/12 (0%)	
Donor variables			
Age (years)	51 ± 2	54 ± 3	0.37
ECD	28/63 (44%)	6/12 (50%)	0.72
DBD	62/63 (98%)	11/12 (92%)	0.18
DCD	1/63 (2%)	1/12 (8%)	
Terminal eGFR (mL/min/m^2)	121 ± 7	110 ± 22	0.55
Weight (kg)	77 ± 2	80 ± 3	0.48
Procurement variables			
Cold ischemic time	15 ± 1	19 ± 2	0.04
Machine perfusion	53/63 (84%)	9/12 (75%)	0.07
Recipient outcomes			
eGFR day 7	32 ± 2	27 ± 7	0.42

eGFR day 14	42 ± 2	31 ± 6	0.08
eGFR day 30	45 ± 2	34 ± 6	0.06
eGFR day 90	50 ± 2	36 ± 7	0.01
eGFR day 180	53 ± 3	35 ± 7	0.01
IGF	20/63 (32%)	3/12 (25%)	0.07
SGF	31/63 (49%)	3/12 (25%)	
DGF	12/63 (19%)	6/12 (50%)	

AR, acute rejection; ATG, anti-thymocyte globulin; BMI, body mass index; DBD, donor after brain death; DCD, donor after cardiac death; DGF, delayed graft function; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; HD, hemodialysis; HLA, human leukocyte antigen; IGF, immediate graft function; PD, peritoneal dialysis; PRA, panel reactive antibody; SGF, slow graft function.

Table 2.

Logistic regression to predict acute rejection within 6 months after kidney transplantation.

Logistic regression to predict acute rejection within 6 months			
Univariate analysis			
Variables	**OR**	**95% CI**	**p**
CD4+CD127lo/-TNFR2+ Treg (%)	0.62	0.41 – 0.94	0.02
Cold ischemic time (hours)	1.10	1.01 – 1.22	0.05
PRA > 50%	5.05	1.14 – 22.41	0.03
Multivariate analysis			
Variables	**OR**	**95% CI**	**p**
CD4+CD127lo/-TNFR2+ Treg (%)	0.58	0.35 – 0.98	0.04
Cold ischemic time (hours)	1.21	1.04 – 1.40	0.01
PRA > 50%	18.17	2.17 – 151.86	<0.01

CI, confidence interval; PRA, panel reactive antibody; Treg, regulatory T cell.

Figure 1.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg frequency was significantly lower in recipients who developed acute rejection within the first 6 months after kidney transplantation. AR, acute rejection; Tregs, regulatory T cells.

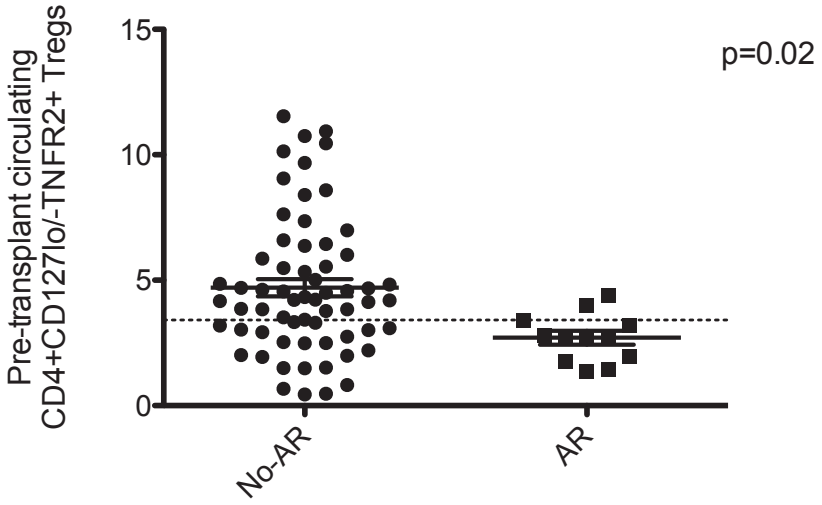


Figure 2.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg frequency is a predictor of acute rejection within 6 months after kidney transplantation in ROC curve analysis. AUC, area under the curve; TNFR2, tumor necrosis factor receptor 2; Treg, regulatory T cell.

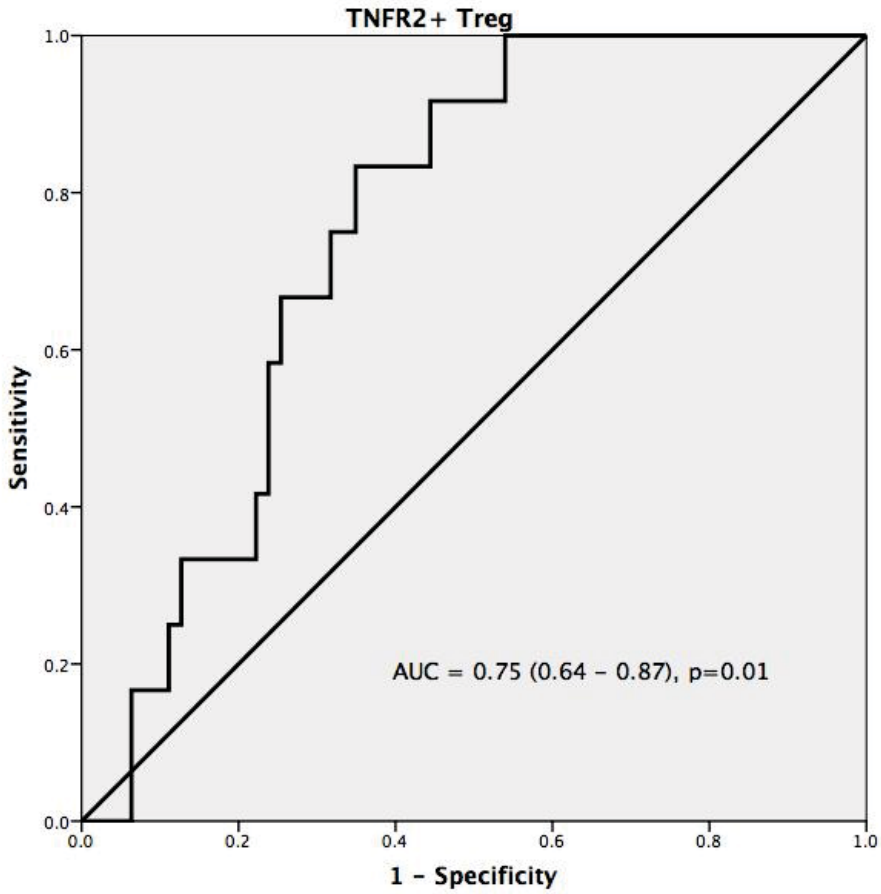


Figure 3.

Logistic regression model combining pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg frequency with sensitization status and cold ischemic time is a predictor of acute rejection within 6 months after kidney transplantation in ROC curve analysis. AUC, area under the curve; CIT, cold ischemic time; TNFR2, tumor necrosis factor receptor 2; Treg, regulatory T cell.

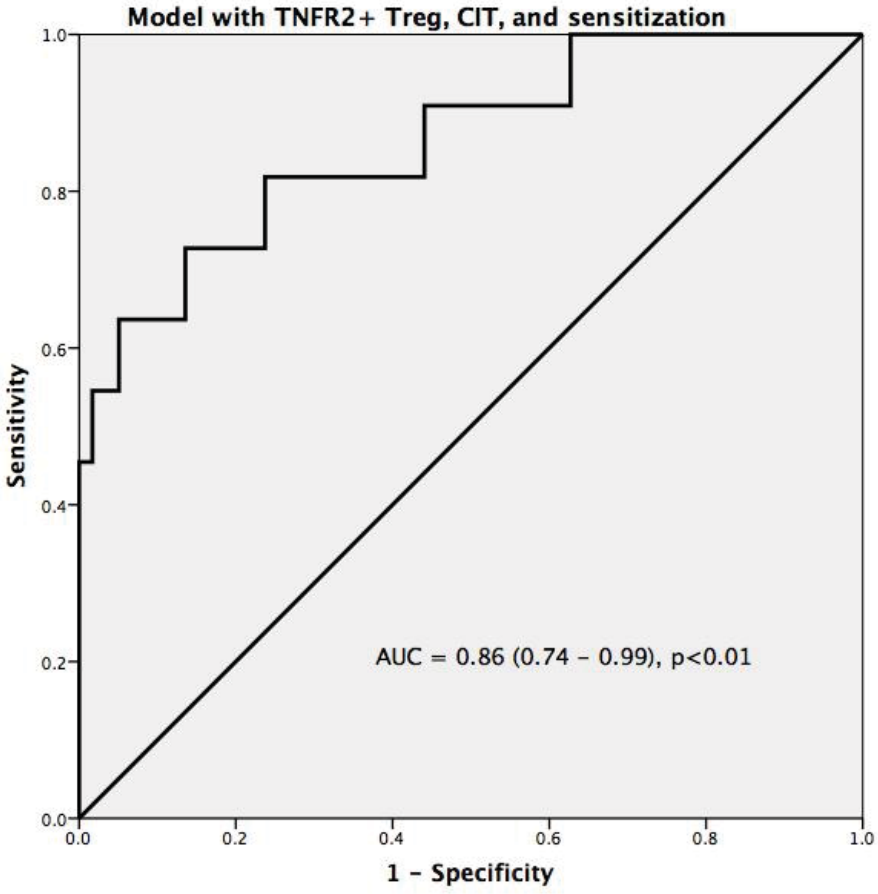
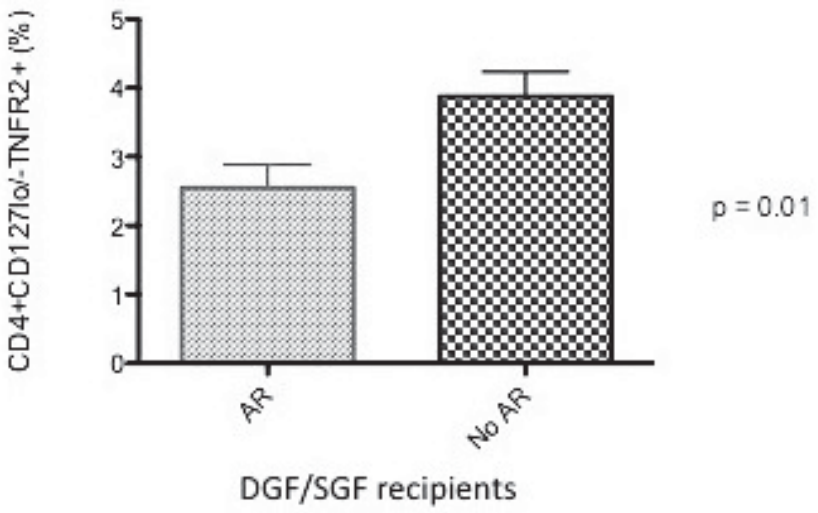


Figure 4.

Pre-transplant recipient circulating CD4+CD127lo/-TNFR2+ Treg frequency was significantly lower in delayed and slow graft function recipients who developed acute rejection within the first 6 months after kidney transplantation. AR, acute rejection; DGF, delayed graft function; SGF, slow graft function; Tregs, regulatory T cells.



Preface to Chapter 5

As described in chapter 1, Treg and Th17 cells have a special developmental relationship that depends on the local microenvironment. In the presence of TGF-β only, naïve CD4+ T cells differentiate into Tregs. On the other hand, in an inflammatory environment like renal IRI containing pro-inflammatory cytokines such as IL-6 and IL-1β, the differentiation of naïve CD4+ T cells is diverted from a regulatory Treg phenotype towards a pro-inflammatory Th17 phenotype (355). Although there have been studies investigating the early beneficial effect of Tregs and early detrimental effect of the Th17 signature cytokine IL-17A in renal IRI, it remains unknown if there is a sustained Treg or Th17 response chronically after renal IRI, and if this have an influence on chronic kidney damage. In the next chapter, we used a murine model of unilateral renal IRI to investigate further the above questions.

Chapter 5. A defect in the Th17 pathway worsens interstitial fibrosis and tubular atrophy after renal ischemia-reperfusion injury

Abstract

Background: Delayed graft function after kidney transplantation, which is due to ischemia-reperfusion injury (IRI), is associated with the detrimental development of chronic allograft nephropathy manifested histologically as interstitial fibrosis/tubular atrophy (IF/TA). IL-17A, the signature cytokine of Th17 cells, is implicated in mediating early kidney damage after renal IRI. It remains however unclear what is the role of the Th17 pathway in the progression to IF/TA.

Methods: Wild-type (WT), IL-17A knockout, and BATF knockout (Th17 lineage deficient) mice on a C57BL/6 background underwent unilateral renal pedicle clamping for 30 minutes, followed by reperfusion for up to 42 days. Ischemic and control kidneys were digested in collagenase, and lymphocytes isolated by density gradient. Phenotypic analysis of Th17, Th1, Th2, and Treg cells was performed by flow cytometry. IF/TA was quantified by a blinded pathologist on Masson's Trichrome and hematoxylin and eosin kidney sections.

Results: CD4+IL-17A+ Th17 and CD4+FoxP3+ Treg cells progressively infiltrated the ischemic but not the control kidneys in WT mice, peaking at 14 days after reperfusion. The Th17 cell response was earlier and more predominant. The

majority of CD4+IL-17A+ Th17 cells co-expressed the Th17-specific transcription factor RORγt, and co-secreted IL-17F. At 42 days after reperfusion, significant IF/TA developed in the ischemic kidney of WT mice, while the control kidney was IF/TA-free. In comparison to WT mice, IL-17A knockout had worse IF/TA, while deficiency in the Th17 cell lineage (BATF knockout) only worsened tubular atrophy. IL-17F did not explain worse fibrosis as its expression in IL-17A knockout was similar to WT mice. There was no difference in the expression of IFN-γ (Th1), IL-4 (Th2), or FoxP3 (Treg) by intrarenal CD4+ T cells after reperfusion between WT and knockout mice.

Conclusion: Following renal IRI, there is a long-lasting infiltration of Th17 and Treg cells in the ischemic kidney with a Th17 predominance. A defect in the Th17 pathway is fibrogenic after renal IRI, and worsens tubular atrophy. Targeting the Th17 pathway to reduce early acute damage after renal IRI could therefore have the unwanted effect of worsening chronic injury.

5.1. Introduction

Renal ischemia-reperfusion injury (IRI) is the main pathophysiological mechanism leading to delayed graft function after kidney transplantation (12,39). Delayed graft function has important implications after kidney transplantation due to its poor prognostic significance. On top of its economic burden caused by prolonging hospital stay and increasing post-transplant management complexity, delayed graft

function also increases the risk for acute rejection, chronic allograft nephropathy, and long-term graft loss (13,26,27).

Th17 cells are a subtype of pro-inflammatory CD4+ T cells that are identified by the secretion of their signature cytokine IL-17A (355). Th17 cells also secrete the cytokine IL-17F, which shares an amino acid sequence identity of 50% and serves overlapping function with IL-17A (226,490). The differentiation of naïve CD4+ T cells into Th17 requires the cytokines TGF-β and IL-6, and is driven by the transcription factors retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγt) and basic leucine zipper transcriptional factor ATF-like (BATF) (265,355). Removing IL-6, however, skews the differentiation of naïve CD4+ T cells into regulatory T cells (Tregs) instead (355). Tregs are a subtype of CD4+ T cells identified by their expression of the master transcription factor forkhead box P3 (FoxP3), and are essential in maintaining self- and transplant tolerance (215).

Investigations regarding the role of Th17 cells and their differentiation link with Treg cells in renal IRI have mostly focused on early kidney damage. Murine studies have shown that blockade of the Th17 cell signature cytokine IL-17A improves kidney damage 24 hours after renal IRI (285,286). In humans, an increase in IL-17A was also found in early kidney allograft biopsy samples of recipients who had delayed graft function after kidney transplantation (289). IL-17A secretion is however not restricted to Th17 cells, and there is no direct evidence that Th17 cells themselves are implicated in renal IRI (226). On the other hand, murine studies

have shown a protective role for Tregs in the first 10 days after renal IRI (428,435). In humans, a decrease in pre-transplant circulating highly suppressive Tregs was also found in recipients who had delayed graft function after kidney transplantation (476). It remains unclear whether Th17 cells rather than solely IL-17A are elevated after renal IRI and whether they have a role in mediating the ischemia-reperfusion associated chronic kidney damage manifesting as interstitial fibrosis and tubular atrophy (IF/TA). It is also unclear whether there is a sustained Treg response after renal IRI.

In this study, we first investigated the long-term pattern of kidney-infiltrating Th17 and Treg cells after murine renal IRI. We then explored the role of Th17 cells in chronic kidney damage manifested as IF/TA after renal IRI using wild type (WT), IL-17A knockout (KO), and BATF KO mice.

5.2. Materials and methods

Animals

C57BL/6 mice and BATF KO mice on a C57BL/6 background were purchased from Charles River Laboratories (Wilmington, MA, USA) and Jackson Laboratory (Bar Harbor, ME, USA) respectively. IL-17A KO mice on a C57BL/6 background were obtained from Professor Iwakura. (Tokyo, Japan). Animals were maintained in the

animal facility at the McGill University Health Centre and treated in compliance with the Canadian Council on Animal Care guidelines and policies.

Unilateral renal ischemia-reperfusion injury

Mice underwent midline laparotomy under isoflurane anesthesia. The left renal vessels were identified and clamped for 30 minutes using an atraumatic vascular clamp (100 g closing pressure; Roboz Surgical Instrument, Gaithersburg, MD, USA). The left (ischemia-reperfusion injured) and right (control) kidneys were harvested on post-operative day 1, 3, 7, 14, 30, and 42 after exsanguination.

Kidney lymphocyte isolation

Kidneys were minced and digested for 45 minutes in Collagenase D (2 mg/mL, Roche Applied Science, Laval, QC, Canada) at 37^{o} Celsius. The digested kidneys were washed in Hank's balanced salt solution (Wisent, St-Bruno, QC, Canada) and lymphocytes were isolated by Histopaque (1.083 g/mL; Sigma, St. Louis, MO, USA) density gradient centrifugation.

Surface and intracellular staining of kidney lymphocytes

Kidney lymphocytes were labeled with anti-mouse CD4 antibody (eBioscience, San Diego, CA, USA), fixed and permeabilized, and labeled with anti-mouse FoxP3

antibody (eBioscience) for phenotypic analysis of intrarenal Treg cells by flow cytometry. Concurrently, kidney lymphocytes were stimulated with phorbol myristate acetate (50 ng/μL; Sigma) and ionomycin (1 μg/μL; Sigma) for 5 hours in the presence of monensin (eBioscience). Lymphocytes were then labeled with anti-mouse CD4 antibody (eBioscience), fixed and permeabilized, and labeled with anti-mouse IL-17A antibody (eBioscience) for phenotypic analysis of intrarenal Th17 cells by flow cytometry. Fixed and permeabilized stimulated lymphocytes were also labeled with anti-mouse IFN-γ, IL-4, RORγt, and IL-17F (all from eBioscience).

Histology

At 42 days after IRI, kidneys were harvested, and immediately formalin-fixed and paraffin-embedded. Kidney sections (4μm) were stained for either Masson's trichrome or hematoxylin and eosin for quantification of IF/TA by a blinded transplant pathologist.

Statistical analysis

Data are expressed as mean ± standard error of the mean. Mann-Whitney U test and Kruskal-Wallis test followed by Mann-Whitney U test post-hoc analysis were used for comparisons between two and multiple groups respectively.

5.3. Results

Increased intrarenal CD4+ T cells after renal IRI

We first studied the infiltration of CD4+ T cells in the kidney after IRI. Consistent with previous literature implicating CD4+ T cells as mediators of renal IRI, we observed a progressive increase in CD4+ T cells in the ischemic kidney only, peaking at day 14 after IRI and declining back down by day 30. At their peak at day 14 after IRI, the frequency of intrarenal CD4+ T cells was significantly higher in the ischemic kidney (41.09 ± 3.08%) in comparison to the control contralateral kidney (17.12 ± 1.97%; $p<0.01$).

Increased intrarenal effector T cell after renal IRI

We then dissected out the CD4+ T cell compartment and examined if the pathogenic CD4+FoxP3- effector T cell (Teff) subtype was also elevated in the kidney after IRI. Teff cells infiltrated the ischemic kidney similarly to CD4+ T cells, peaking at day 14 after IRI and declining back down close to baseline by day 30. At their peak at day 14 after IRI, the frequency of intrarenal CD4+FoxP3- Teff cells was significantly higher in the ischemic kidney (38.66 ± 2.94%) in comparison to the control contralateral kidney (16.87 ± 1.97%; $p<0.01$).

Increased intrarenal Th17 cells after renal IRI

We further studied if CD4+IL-17A+ Th17 cells, a subset of pro-inflammatory Teff cells, were also increased in the kidney after IRI. Th17 cells increased significantly starting at day 7, peaked at day 14, and declined back down by day 30 after IRI (Figure 1). Additionally, Th17 cells were significantly elevated in the ischemic kidney compared to the control contralateral kidney at all time points after IRI (Figure 1), the most remarkable differences occurring at day 7 and 14 ($p<0.01$). We further characterized the intrarenal CD4+IL-17A+ Th17 cells at day 7 and 14 after renal IRI. We confirmed that the majority of CD4+IL-17A+ cells co-expressed the master transcription factor RORγt (Figure 2). The secretion of IL-17F by CD4+ T cells was mostly restricted to CD4+IL-17A+ Th17 cells, while only a minimal proportion of CD4+ T cells co-expressed both IL-17A and prototypic Th1 cytokine IFN-γ, prototypic Th2 cytokine IL-4, and Treg master transcription factor FoxP3 (Figure 3).

Increased intrarenal Tregs after renal IRI

In the context of an increase in intrarenal Th17 cells after renal IRI, we then sought to determine its influence on intrarenal CD4+FoxP3+ Treg cells. Previous in vitro literature suggests that in the presence of a pro-inflammatory milieu (such as renal IRI), naïve CD4+ T cell differentiation is skewed towards a Th17 rather than a Treg phenotype, and that Tregs can be reprogrammed into Th17 cells (259,355). On the

other hand, Tregs are also known to home to areas of inflammation in order to suppress excessive immune responses. In fact, in the context of renal IRI, Tregs trafficked to the kidney and suppressed early innate immune mediators of injury (428). We observed an increase in intrarenal CD4+FoxP3+ Treg cells after renal IRI. Following a similar timecourse than Th17 cells, Tregs increased significantly starting at day 7, peaked at day 14, and declined back down by day 30 after IRI (Figure 4). However, Tregs were significantly elevated in the ischemic kidney compared to the control contralateral kidney later than Th17 cells, starting at day 3 (Figure 4). Similar to Th17 cells, the most remarkable differences occurred at day 7 and 14 after IRI ($p<0.01$), but Th17 cell frequency was more predominant.

Abrogation of the Th17 pathway leads to worse chronic kidney damage after renal IRI

Our finding that there is a predominantly prolonged and long-lasting elevation of Th17 cells in the ischemic kidney after reperfusion led us to investigate its role in causing chronic kidney damage, mainly IF/TA. As a matter of fact, in the context of kidney transplantation, the most severe form of IRI termed delayed graft function has been associated with an increased risk of chronic allograft nephropathy (27). We induced unilateral renal pedicle clamping in WT, IL-17A KO, and Th17 lineage deficient (BATF KO) mice to study the effect of the Th17 pathway on IF/TA after renal IRI.

In comparison to the control contralateral kidney, the ischemic kidney suffered from significant IF/TA 42 days after reperfusion in WT mice. Deficiency in the Th17 signature cytokine IL-17A worsened IF/TA in the ischemic kidney compared to WT mice, while deficiency in the Th17 lineage (BATF KO) worsened tubular atrophy only (Figure 5).

Since Th17 cells not only secrete the cytokine IL-17A but also the cytokine IL-17F, we then explored whether there was a compensatory secretion of IL-17F in the absence of IL-17A in IL-17A KO mice explaining worse fibrosis compared to Th17 lineage-deficient BATF KO mice. CD4+IL-17F+ T cells were similar in the ischemic kidneys of IL-17A KO mice and WT mice (Figure 6). As expected, CD4+IL-17F+ T cells were absent in the Th17-lineage deficient BATF KO mice (Figure 6). Since IL-17F expression in the ischemic kidney was similar between IL-17A KO and WT mice, IL-17F did not explain the worse fibrosis experienced by IL-17A KO mice.

We further investigated whether there was a difference in intrarenal Th1, Th2, or Treg cells after reperfusion in the ischemic kidneys of IL-17A KO, BATF KO, and WT mice as an explanation to worse IF/TA in IL-17A KO mice and worse tubular atrophy in BATF KO mice. There was no difference in the expression of IFN-γ secreting (Th1), IL-4 secreting (Th2), or FoxP3 expressing (Treg) kidney-infiltrating CD4+ T cells after reperfusion in the ischemic kidneys of IL-17A KO, BATF KO, or WT mice (Figure 7). The worse chronic kidney damage in the KO mice was thus not

due to an enhancement or reduction of other effector or regulatory CD4 T cell lineages.

5.4. Discussion

In our study, we first found that there is a long-lasting progressive infiltration of both Th17 and Treg cells in ischemic kidneys peaking at 14 days after renal IRI. Compared to control kidneys, Th17 cells were significantly elevated earlier than Tregs in the ischemic kidneys. Moreover, the increased frequency of Th17 cells was more prominent than that of Tregs in the ischemic kidneys. The pro-inflammatory environment of renal IRI, which is known to contain high levels of IL-6, thus promotes both Th17 and Treg expansion with a larger effect on Th17 cells (491). This is in line with the fact that a pro-inflammatory milieu skews the differentiation of naïve CD4+ T cells into Th17 rather than Treg cells (355).

Previous studies have looked at Th17 or Treg infiltration in solitary rather than concomitantly into the kidney after IRI. With regards to Th17 cells, Mehrota et al. has shown an elevation in intrarenal CD4+IL-17A+ Th17 cells only at 1 and 3 days after unilateral renal pedicle clamping in a rat model (492). The discrepancy with our findings could be related to the use of rat instead of mouse. Moreover, the duration of renal pedicle clamping was not described, and their inflicted renal IRI might have been less severe than in our study. With regards to Tregs, our findings are consistent and expand on a study by Gandolfo et al. showing an increase in

intrarenal CD4+CD25+FoxP3+ Tregs at 3 and 10 days after mouse unilateral renal pedicle clamping for 45 minutes (435). The study by Gandolfo et al. did not look at intrarenal Tregs beyond 10 days. On the other hand, our findings are different from Kinsey et al. showing an increase in intrarenal CD4+CD25+FoxP3+ Tregs only at 7 days after mouse bilateral renal pedicle clamping for 24 minutes. There was no difference in intrarenal Tregs at 3 or 14 days after reperfusion (429). The discrepancy in the results of Kinsey et al. in comparison to our results and those of Gandolfo et al. is likely related to the use of bilateral instead of unilateral mouse renal pedicle clamping.

Since the Th17 response was highest after renal IRI, we focused on the Th17 pathway and found that a complete deficiency in IL-17A and Th17 cells worsened chronic kidney damage at 42 days after IRI. More specifically, a pure deficiency in IL-17A worsened IF/TA, while Th17 cell deficiency via the abolition of the transcription factor BATF worsened tubular atrophy only. We verified that the worse chronic kidney damage with IL-17A deficiency was not due to a compensatory secretion of IL-17F. IL-17F expression in the ischemic kidneys of IL-17A KO was similar to its expression in the ischemic kidneys of WT mice, thus not explaining the worse fibrosis. There was also no skewing of other CD4+ T cell effector or regulatory subsets, as no difference was found in the expression of IFN-γ (Th1), IL-4 (Th2), or FoxP3 (Treg) by intrarenal CD4+ T cells after IRI in the ischemic kidneys of WT, IL-17A KO and BATF KO mice. Our findings therefore

suggest a protective role of the Th17 pathway mainly via IL-17A in chronic kidney damage mediated by IRI.

Previous studies regarding the role of IL-17A or Th17 in fibrosis have yielded conflicting results in non-renal organs. On one hand, Th17 or IL-17A responses have been shown to be pro-fibrotic in chronic rejection after lung and heart transplantation as well as in hepatitis (493-496). On the other hand, IL-17A responses had an anti-fibrotic effect in scleroderma by decreasing connective tissue growth factor and α1(I)collagen (497). With regards to renal-specific studies, Mehrota et al. showed that an increased Th17 cell response in rats via a high salt diet following acute kidney injury was associated with fibrosis, and this was partially reversed with the angiotensin II type 1 receptor antagonist losartan. Losartan, however, also reduced Th1 and Th2 cell responses and did not completely deplete Th17 cells (492). Their differing findings could be due to an effect of Th1 or Th2 cells rather than Th17 cells. Moreover, it is possible that complete depletion of the Th17 pathway is detrimental in chronic kidney damage, while reduction of the Th17 pathway is protective. Since Th17 and Treg cells are plastic and have a close differentiation link, it is possible that some of the Th17 cells transform into Tregs or take on a regulatory role during the recovery process. Complete rather than partial depletion of Th17 cells could therefore be detrimental in chronic kidney damage. Another possibility is that a complete deficiency of the Th17 pathway could cause an imbalance in the TGF-β pathway, which is known to have pro-fibrotic effects (498).

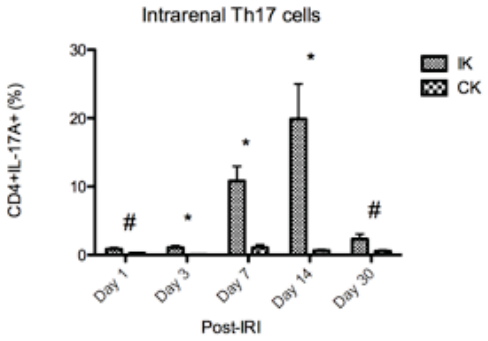
In the context of mimicking the effect of delayed graft function after kidney transplantation, our study has limitations. First, we used a murine model of unilateral renal pedicle clamping for 30 minutes to mimic IRI occurring during kidney transplantation. We therefore lack the obligatory cold ischemic time that occurs in kidney transplantation prior to the average 30 minutes warm ischemic time for performing the vascular anastomosis. Secondly, our model does not include the effect of induction and maintenance immunosuppression routinely used during and after kidney transplantation. Alterations in immune profile by immunosuppression after IRI could lead to differing effects on chronic kidney damage.

Despite the above limitations, our study is a first step to study the infiltration of Th17 and Treg cells into ischemic kidneys following pure renal IRI. We found that there was an increase in both intrarenal Th17 and Tregs cells with renal IRI, with the Th17 component being earlier and more prominent. Although blocking the pro-inflammatory IL-17A or Th17 cells was previously shown to decrease acute kidney injury after renal IRI, this led to worse chronic kidney damage manifested as IF/TA. Caution will therefore need to be taken in strategies targeting the Th17 pathway for dampening early acute kidney injury as this may lead to an unwanted detrimental long-term effect.

Figure 1.

Increased intrarenal Th17 cells after renal IRI. **A)** Comparison of intrarenal CD4+IL-17A+ Th17 cells between ischemic (IK) and control controlateral (CK) kidneys at day 1, 3, 7, 14, and 30 after IRI (*:p<0.01, #: p<0.05, n=4-5 per time point). **B)** Representative flow cytometry analysis of intrarenal CD4+IL17-A+ Th17 cells (right upper quadrant) in IK and CK at day 1, 3, 7, 14, and 30 after IRI.

A



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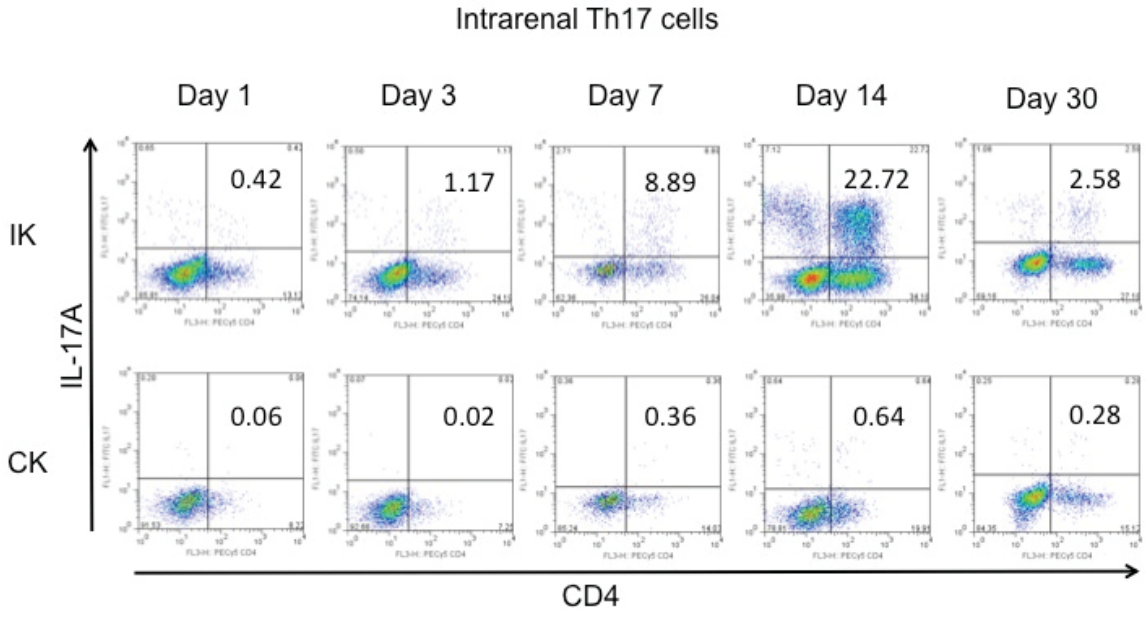
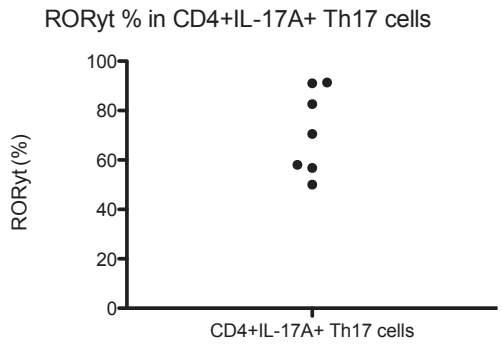


Figure 2.

The majority of CD4+IL-17A+ Th17 cells co-expressed the master transcription factor RORγt. **A)** RORγt expression by CD4+IL-17A+ Th17 cells at 7 days after renal IRI in the ischemic kidney (n=7). **B)** Representative flow cytometry analysis in which percentage of RORγt (92.72%) was quantified inside the CD4+IL-17A+ gate (16.23%) at 7 days after renal IRI in the ischemic kidney.

A



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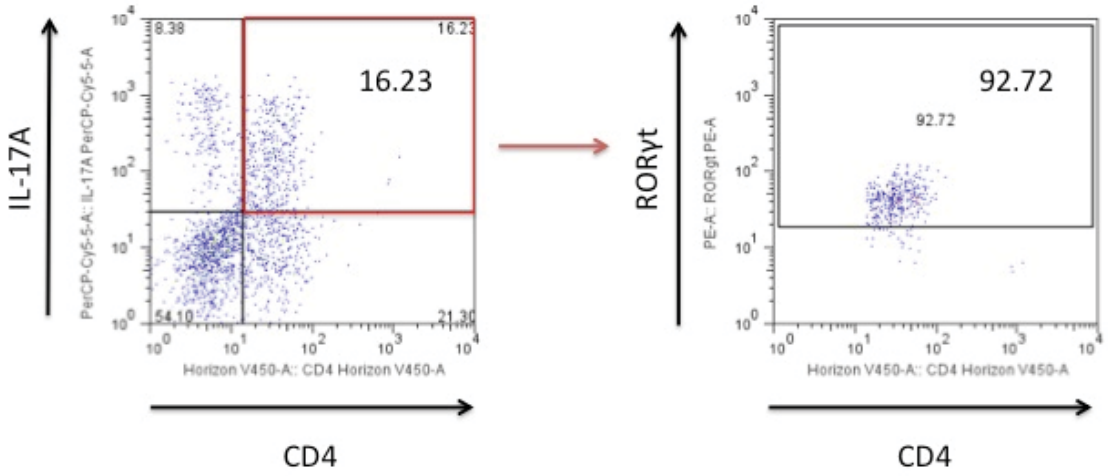


Figure 3.

Secretion of IL-17F was mostly restricted to CD4+IL-17A+ Th17 cells, while IFN-γ and IL-4 were not co-secreted by CD4+IL-17A+ Th17 cells. CD4+IL-17A+ Th17 cells also did not co-express FoxP3. Percentage expression of IL-17A, IL-17F, IFN-γ, IL-4, and FoxP3 was quantified inside the CD4+ gate at 7 days after renal IRI in the ischemic kidney (n=3).

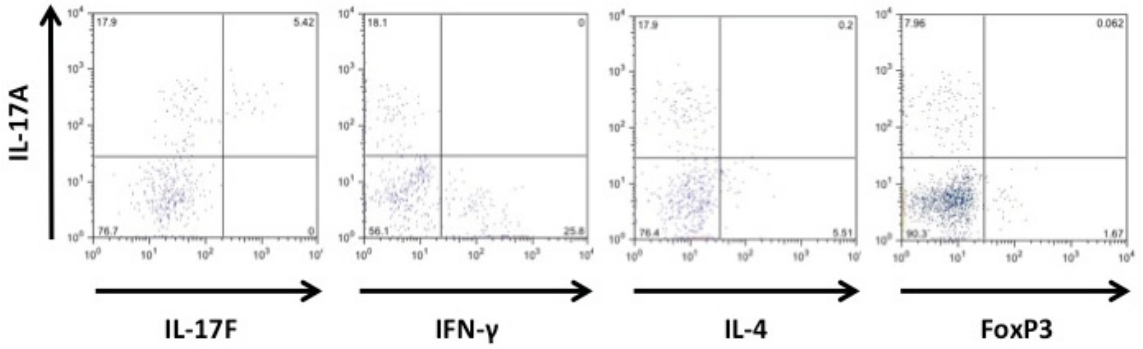
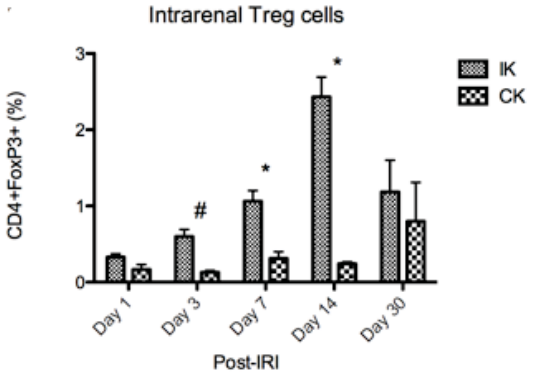


Figure 4.

Increased intrarenal Treg cells after renal IRI. **A)** Comparison of intrarenal CD4+FoxP3+ Treg cells between ischemic (IK) and control contralateral (CK) kidneys at day 1, 3, 7, 14, and 30 after IRI (*:p<0.01, #: p<0.05, n=4-5 per time point). **B)** Representative flow cytometry analysis of intrarenal CD4+FoxP3+ Treg cells (right upper quadrant) in IK and CK at day 1, 3, 7, 14, and 30 after IRI.

A



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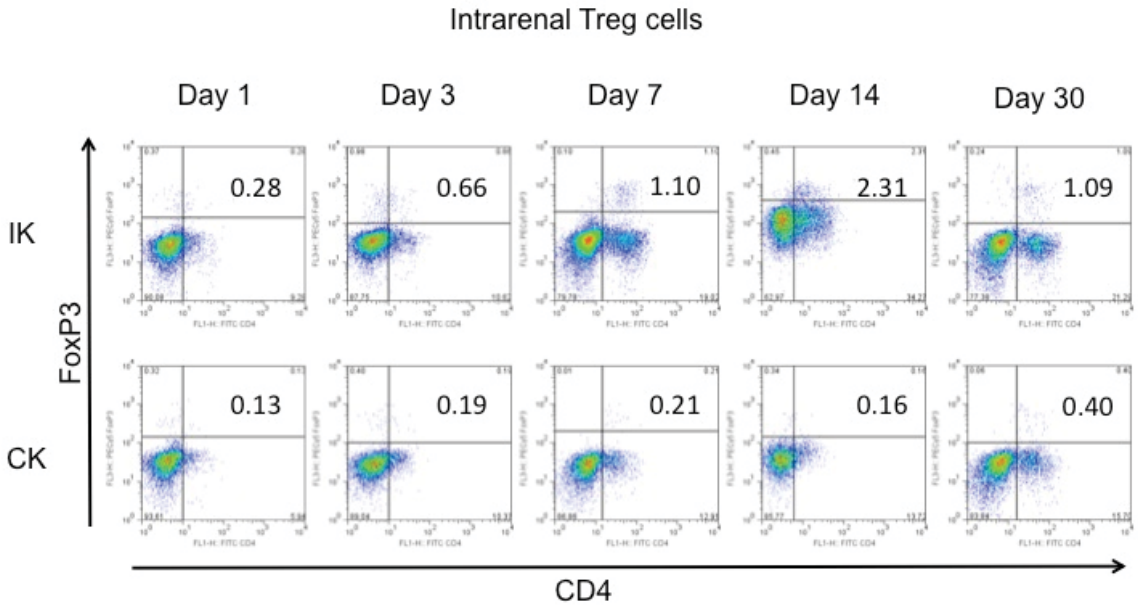
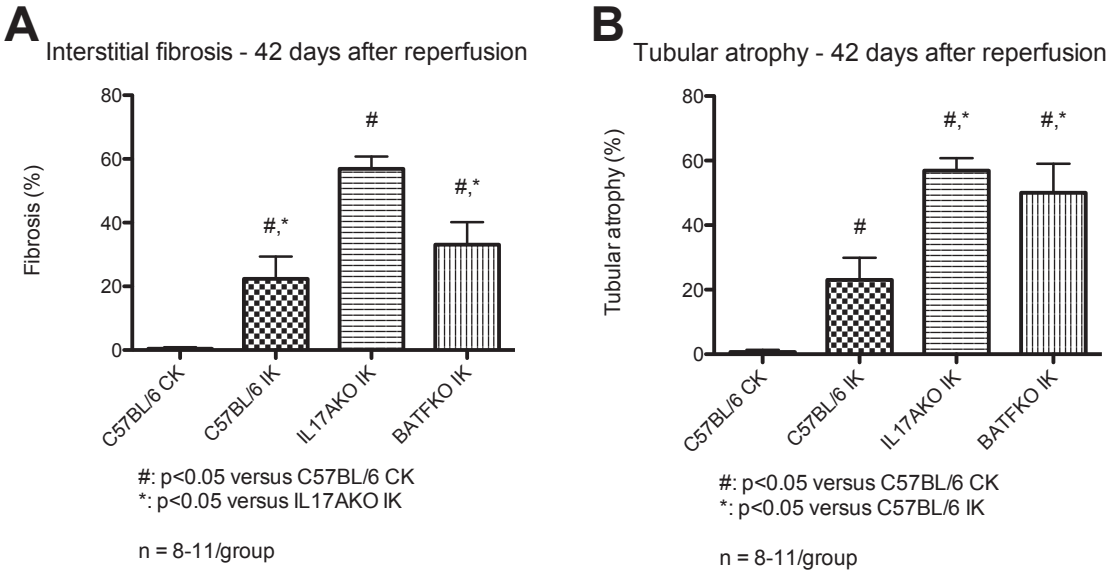
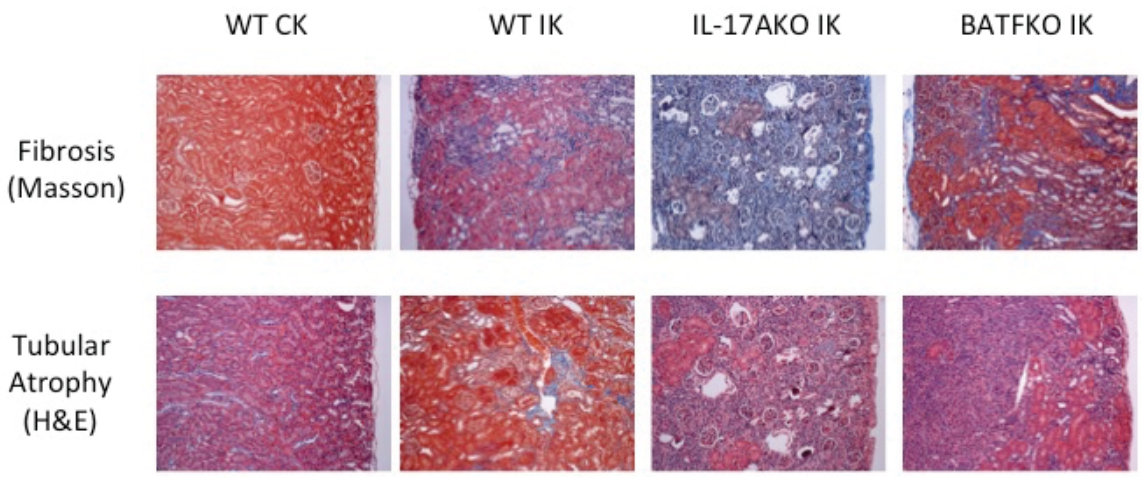


Figure 5.

Chronic kidney damage was assessed in kidney sections 42 days after renal IRI in C57BL/6 wild-type, IL-17A KO, and BATF KO mice. **A)** Interstitial fibrosis was assessed in kidney sections stained by Masson's Trichrome (n=8-11 per group). **B)** Tubular atrophy was assessed in kidney sections stained by hematoxylin and eosin (n=8-11 per group). IK, ischemic kidney; CK, control contralateral kidney. **C)** Representative Masson's trichrome and hematoxylin and eosin kidney section staining of C57BL/6 wild-type, IL-17A KO, and BATF KO mice 42 days after renal IRI.



C



IK = ischemic kidney
CK = control kidney

100x

Figure 6.

Intrarenal CD4+IL-17F+ Th17 cells at 7 days after renal IRI in C57BL/6, IL-17A KO, and BATF KO mice (n=3-4 per group). B6, C57BL/6; CK, contralateral kidney; IK, ischemic kidney.

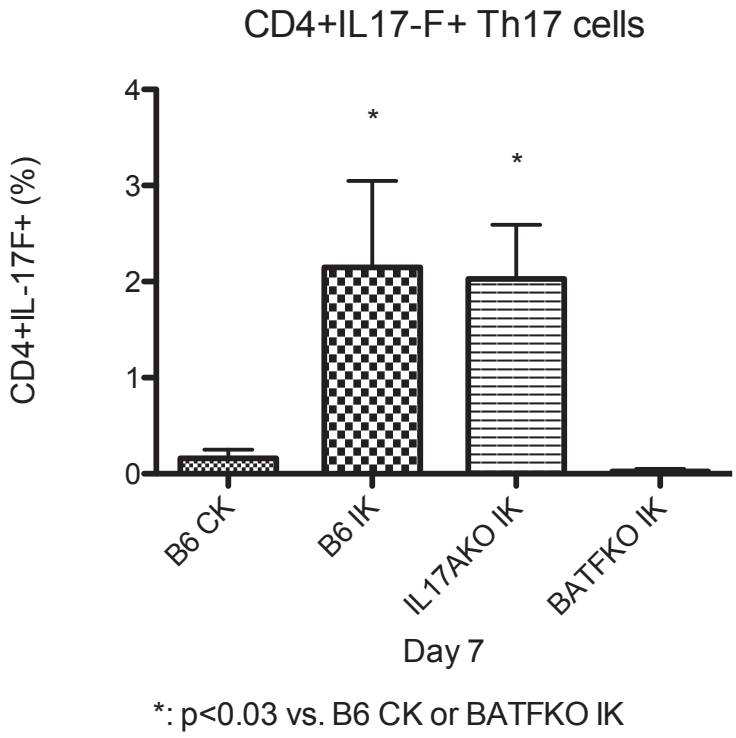
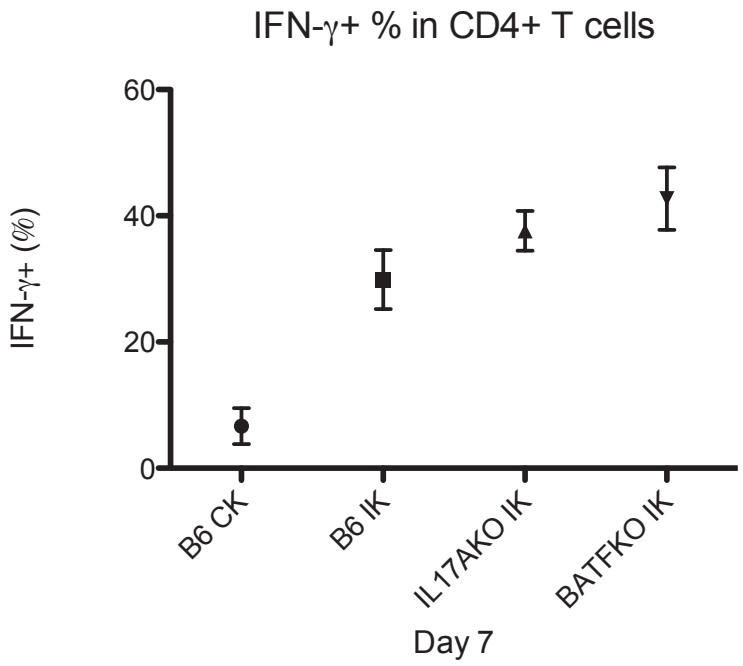


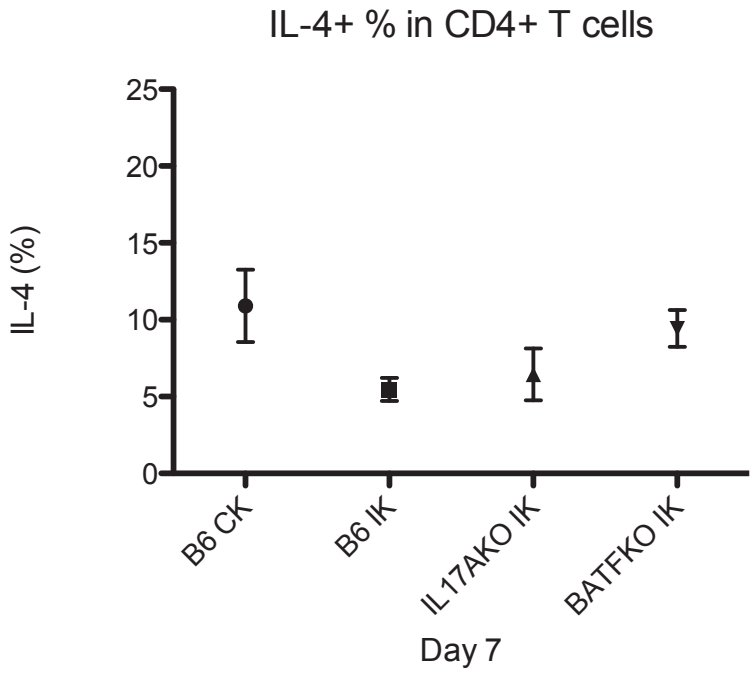
Figure 7.

No difference in the expression of **A)** IFN-γ secreting (Th1), **B)** IL-4 secreting (Th2), or **C)** FoxP3 expressing (Treg) CD4+ T cells 7 days after renal IRI in the ischemic kidneys of C57BL/6 wild-type, IL-17A knockout, and BATF knockout mice (n=3-4 per group).

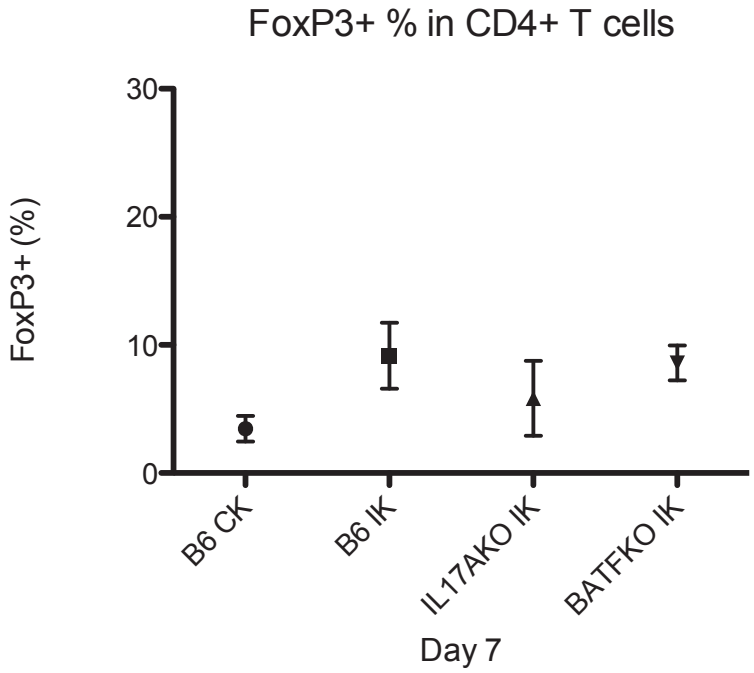
A



B


IL-4+ % in CD4+ T cells
IL-4 (%)
25
20
15
10
5
0
B6 CK
B6 IK
IL17AKO IK
BATFKO IK
Day 7

C


FoxP3+ % in CD4+ T cells
FoxP3+ (%)
30
20
10
0
B6 CK
B6 IK
IL17AKO IK
BATFKO IK
Day 7

Chapter 6. Technical caveats and future directions

Despite the results presented in this thesis in chapters 2-5, we acknowledge that the cellular mechanisms leading to renal IRI, DGF/SGF after kidney transplantation, and their downstream immunologic consequences (acute rejection, IF/TA) remain complex, multifactorial, and beyond the sole contribution of Tregs or Th17 cells. This thesis nevertheless presented findings that support an important contribution of Treg and Th17 cells to renal IRI, with an emphasis on the use of Treg suppressive function as a potential predictor of ischemia-reperfusion related outcomes after human kidney transplantation. In particular, the finding of this thesis with the most promise for clinical applicability is that measurement of a pre-transplant circulating recipient highly suppressive subtype of Treg (TNFR2+ Treg) is a potential rapid biomarker for DGF/SGF and acute rejection after kidney transplantation. Due to the semi-urgent nature of human kidney transplantation, which often occurs at off hours when experimental laboratory resources and equipment are limited, we first discuss in this chapter some resulting technical caveats in our measurement of pre-transplant Treg suppressive function and TNFR2+ Tregs that will need to be addressed to confirm that an intrinsic Treg suppressive function defect exists pre-transplant in recipients at risk for DGF/SGF and acute rejection. We also propose potential strategies to address these technical caveats in the future. Secondly, we discuss potential future directions based on our finding that Treg suppressive function or TNFR2+ Tregs can serve as potential pre-transplant biomarkers for DGF/SGF and acute rejection after kidney transplantation.

6.1. Technical caveats

Since kidney transplant candidates are often anemic secondary to their end-stage renal disease and were about to undergo surgery just prior to peripheral blood collection in the human studies presented in this thesis, this limited the amount of blood that we were permitted to collect by our institutional ethics review board (40 cc) prior to transplantation to isolate PBMCs for two purposes: 1) measurement of pre-transplant circulating Treg suppressive function via a co-culture assay and 2) measurement of pre-transplant Treg frequency based on phenotypic markers by flow cytometry. In order to yield sufficient PBMCs to perform both the Treg suppressive function assay and phenotypically identify Tregs by flow cytometry, the limited amount of blood collected from each patient had to be processed immediately fresh often at off hours as opposed to being cryopreserved for batch processing during daytime work hours. In that context, only magnetic bead as opposed to flow cytometry cell-sorter was consistently available for purification of Tregs and Teffs to perform the Treg suppressive function assay. Moreover, only a three-color flow cytometer for phenotypic identification of Treg was consistently available. Consequently, technical caveats arose due to the above circumstances that hampered our ability to fully experimentally confirm that a pre-transplant intrinsic defect in Treg suppressive function exists in recipients at risk of developing DGF/SGF and acute rejection after kidney transplantation.

With regards to the Treg suppressive function assay used in chapters 2-3, the percentage suppression of recipient autologous circulating Teff by Treg was lower than expected. Several factors could be contributing to this phenomenon. First, purities of enriched CD4+CD25+ Tregs from recipients prior to kidney transplantation via magnetic bead isolation were on average 86%, with only on average 37% expressing the Treg master transcription factor FoxP3. Contamination of the enriched CD4+CD25+ Treg fraction by Teff was thus present. This contamination contributed to the lower than expected percentage suppression of recipient autologous circulating Teff by Treg. In order to address this technical caveat, future studies recruiting kidney transplant candidates on the waitlist rather than just prior to their kidney transplantation surgery could allow collection of a larger amount of peripheral blood during regular workday hours. The higher PBMC isolation yield from a larger amount of peripheral blood in addition to access to a flow cytometry cell-sorter during regular workday hours would allow for enrichment of a Treg fraction with higher purity for the Treg suppressive function assay. Secondly, rather than an intrinsic Treg suppressive function defect, it is possible that kidney transplant recipients at risk for DGF/SGF or acute rejection have Teffs that are resistant to Treg suppression. We were not able to study this possibility in the studies presented in this thesis due to the aforementioned experimental constraints. In future studies, the use of third party Teff instead of autologous Teff in the Treg suppressive function assay could confirm whether an intrinsic Treg suppressive function defect or Teff resistance to Treg exist in kidney

transplant recipients at risk for DGF/SGF or acute rejection after kidney transplantation.

With regards to the measurement of TNFR2+ Treg frequency in Chapters 3-4, we based our identification of Tregs on CD4, CD127, and TNFR2 only. Since we needed to use fresh PBMCs immediately often at off hours in order to perform the Treg suppressive function assay simultaneously, we only had access to a three-color flow cytometer to phenotypically identify Tregs. We were therefore not able to use all traditional Treg surface markers (CD4, CD25, CD127) in conjunction with TNFR2 to phenotypically identify and measure TNFR2+ Tregs. Moreover, since we used fresh PBMCs to measure the frequency of CD4+CD127lo/-TNFR2+ Tregs, flow cytometry analysis was done for each recipient separately based on single color compensation rather than collectively in batch. Thus, slight difference in gating occurred when analyzing the flow cytometry data between different recipients based on individual single color compensation. These slight gating differences were however unlikely to be consequential as flow cytometry data were analyzed in a blinded fashion from recipient outcomes (DGF/SGF, acute rejection). In future studies focusing solely on the identification of highly suppressive Tregs as a biomarker for DGF/SGF or acute rejection after kidney transplantation without simultaneous performance of a Treg suppressive function assay, PBMCs isolated from peripheral blood could be cryopreserved to circumvent the above technical caveats. This would allow the use of a more sophisticated multi-color flow cytometer in order to use all traditional

surface markers of Tregs in addition to TNFR2. Moreover, this would allow batching of recipient samples for flow cytometry analysis with identical gating strategies.

Despite the above technical caveats in the measurement of Treg suppressive function and TNFR2+ Treg frequency, these measurements were performed in a similar manner in all kidney transplant recipients and analyzed in a blinded fashion from recipient outcomes (DGF/SGF, acute rejection). Moreover, both the pre-transplant recipient circulating Treg suppressive function assay and the identification of highly suppressive TNFR2+ Treg were able to accurately and independently predict DGF/SGF after kidney transplantation. The fact that two methods used to measure Treg suppressive function were able to predict DGF/SGF after kidney transplantation reinforce the validity of our concept that pre-transplant measurement of an intrinsic defect in Treg suppressive function is a promising biomarker for this common complication after kidney transplantation.

6.2. Future directions

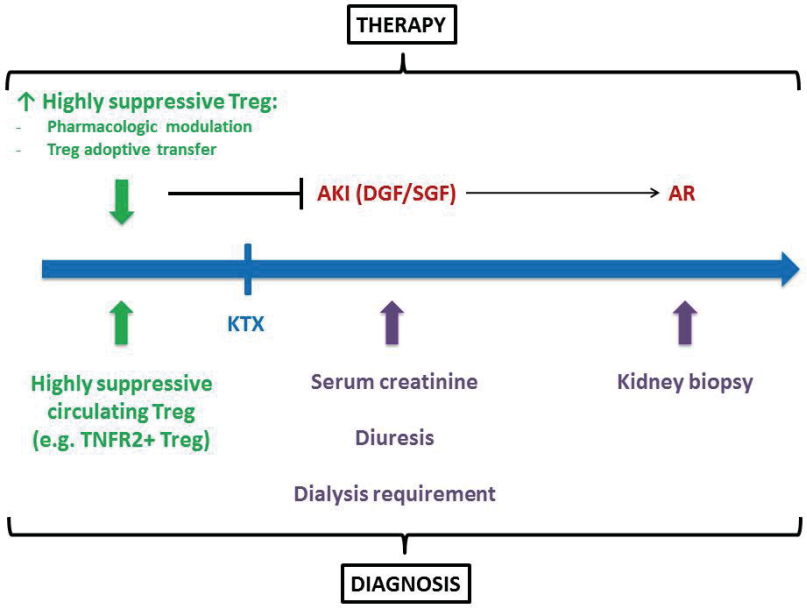
Apart from addressing the aforementioned technical caveats, future multi-center studies with a larger sample size will be needed for external validation prior to considering moving the measurement of highly suppressive Tregs (e.g. TNFR2+ Tregs) as a biomarker for DGF/SGF or acute rejection to the clinical realm. Moreover, its diagnostic accuracy will need to be improved, possibly with the addition of other Treg markers of potent suppressive function such as HLA-DR,

CTLA-4, CD45RA, ICOS, and CD39. If external validation and increased diagnostic accuracy are achieved, this would open the door for the use of highly suppressive Tregs in the prediction of DGF/SGF and acute rejection in kidney transplantation prior to irreversible kidney allograft damage instead of using the imperfect post-transplant assessment of serum creatinine, diuresis, and need for dialysis for DGF/SGF or the invasive kidney allograft biopsy for acute rejection (Figure 1). Highly suppressive Tregs could also be used in the prediction of other clinical outcomes such as IRI in other solid organ transplantations as well as autoimmune disease. For example, in a study published shortly after ours, TNFR2 was part of a Treg gene signature in the prediction of new onset type 1 diabetes (499). Finally, pharmacologic or cell therapies aimed at targeting or enhancing Treg suppressive function prior to transplantation could then potentially dampen or prevent the development of DGF/SGF and acute rejection after kidney transplantation prior to irreversible kidney allograft damage (Figure 1). For example, etanercept is a genetically engineered fusion protein composed of the extracellular domain of TNFR2 linked to the hinge and Fc domains of human IgG1 (500). It would be interesting to study whether its use prior to kidney transplantation could mimic the release of soluble TNFR2 by Tregs as a mechanism to limit the availability of TNF-α for Teffs, and thereby prevent DGF/SGF and acute rejection after kidney transplantation in the future. Multiple trials are also currently ongoing that investigate the use of Treg cell-based therapy for the induction of clinical tolerance in kidney and liver transplantation, suppression of graft versus host disease after bone marrow transplantation, and treatment of type 1 diabetes (409). None of these

studies isolated Tregs for cell therapy based on TNFR2. It would be interesting to study whether the adoptive transfer of isolated TNFR2+ Tregs prior to kidney transplantation could prevent DGF/SGF and acute rejection after kidney transplantation in the future. Another option would be to stimulate and expand ex-vivo isolated Tregs with a TNFR2 agonist prior to adoptive transfer, as this strategy was shown to produce a phenotypically homogeneous and functionally superior subset of Tregs (473).

Figure 1.

Following further validation, pre-transplant highly suppressive Tregs such as TNFR2+ Tregs could have a diagnostic and therapeutic role in DGF/SGF and acute rejection after kidney transplantation. The measurement of highly suppressive Tregs prior to kidney transplantation could predict before irreversible kidney allograft damage which recipient is at risk for DGF/SGF or acute rejection in isolation or conjunction with current standard diagnostic methods for DGF/SGF (serum creatinine, diuresis, dialysis need) and acute rejection (kidney allograft biopsy). Increasing the frequency of highly suppressive Tregs prior to kidney transplantation by pharmacologic modulation or adoptive transfer could also prevent DGF/SGF or acute rejection prior to irreversible kidney allograft damage. AKI, acute kidney injury; DGF, delayed graft function; KTX, kidney transplantation; SGF, slow graft function; Treg, regulatory T cell; TNFR2, tumor necrosis factor receptor 2.



Conclusion

AKI in the form of DGF or SGF remains the most common complication after kidney transplantation, and results in significant downstream adverse immunologic outcomes. There is currently a gap in the clinical realm with regards to biomarkers to predict and therapies to prevent DGF/SGF after kidney transplantation. Based on previous murine findings that Tregs have a protective role in renal IRI, this thesis explored the potential role of Treg as a biomarker to predict DGF/SGF after human kidney transplantation. This thesis first presented the novel finding that pre-transplant circulating Treg suppressive function is an independent predictor of DGF/SGF after kidney transplantation. Since the measurement of pre-transplant circulating Treg suppressive function requires the use of an assay that is tedious, time-consuming, and non-standardized between centers, we next sought to determine whether there was a quicker way to identify a subset of Tregs with the most potent suppressive function in order to have a rapid and clinically applicable biomarker for the prediction of DGF/SGF after kidney transplantation. Based on previous literature showing that TNFR2 is expressed on Tregs with potent suppressive function, this thesis presented the second novel finding that TNFR2 expression on Tregs correlated with the traditional Treg suppressive function assay in kidney transplant candidates. Moreover, the use of pre-transplant recipient circulating TNFR2+ Tregs could replace the Treg suppressive function assay as an independent predictor of DGF/SGF after kidney transplantation. Since DGF/SGF is associated with an increased risk of acute rejection, the use of TNFR2+ Tregs as a

predictor of acute rejection was next explored. This thesis presented the third novel finding that pre-transplant recipient circulating TNFR2+ Treg was an independent predictor of acute rejection within 6 months after kidney transplantation. Lastly, since the regulatory Treg and the pro-inflammatory Th17 cell have a special differentiation relationship, we explored in a murine model if there is a sustained Treg or Th17 response after renal IRI and what is the effect on chronic kidney damage. This thesis presents the fourth novel finding that there is a concomitant sustained regulatory Treg and pro-inflammatory Th17 cell response after murine renal IRI in favor of the latter, and that blockade of the Th17 pathway leads to a counterintuitive increase in chronic kidney damage in the form of IF/TA.

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Appendix

Pretransplantation Recipient Regulatory T cell Suppressive Function Predicts Delayed and Slow Graft Function after Kidney Transplantation

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Background. Delayed graft function (DGF) and slow graft function (SGF) are a continuous spectrum of ischemia-reperfusion–related acute kidney injury (AKI) that increases the risk for acute rejection and graft loss after kidney transplantation. Regulatory T cells (Tregs) are critical in transplant tolerance and attenuate murine AKI. In this prospective observational cohort study, we evaluated whether pretransplantation peripheral blood recipient Treg frequency and suppressive function are predictors of DGF and SGF after kidney transplantation.

Methods. Deceased donor kidney transplant recipients (n=53) were divided into AKI (n=37; DGF, n=10; SGF, n=27) and immediate graft function (n=16) groups. Pretransplantation peripheral blood $CD4^+CD25^{hi}FoxP3^+$ Treg frequency was quantified by flow cytometry. Regulatory T-cell suppressive function was measured by suppression of autologous effector T-cell proliferation by Treg in co-culture.

Results. Pretransplantation Treg suppressive function, but not frequency, was decreased in AKI recipients ($P<0.01$). In univariate and multivariate analyses accounting for the effects of cold ischemic time and donor age, Treg suppressive function discriminated DGF from immediate graft function recipients in multinomial logistic regression (odds ratio, 0.77; $P<0.01$), accurately predicted AKI in receiver operating characteristic curve (area under the curve, 0.82; $P<0.01$), and predicted 14-day estimated glomerular filtration rate in linear regression ($P<0.01$).

Conclusion. Our results indicate that recipient peripheral blood Treg suppressive function is a potential independent pretransplantation predictor of DGF and SGF.

Keywords: Delayed graft function, Treg, Acute kidney injury, Kidney transplantation.

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Acute kidney injury (AKI) related to ischemia-reperfusion is inevitable after kidney transplantation (*1*, *2*). It is severe enough in 20% of recipients to cause delayed graft function (DGF), which manifests as the need for dialysis within the first week after transplantation (*1–3*). A substantial number of recipients also experience a milder form of AKI without dialysis requirement and are described as having slow graft function (SGF) (*4–6*). Both DGF and SGF independently increase the risk for long-term graft loss and acute rejection, whereas DGF also increases the risk for chronic allograft

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dysfunction (*6–11*). Diagnosis relies on posttransplantation diuresis, serum creatinine, and need for dialysis and is made after damage already occurred to the graft (*3*). Immunologic measures of risk for DGF and SGF have not yet been identified and could assist in preventing these important complications.

Regulatory T cells (Tregs) are $CD4^+$ T lymphocytes most commonly identified by their expression of the surface molecule CD25 and their up-regulation of the transcription factor forkhead box P3 (FoxP3) (*12*). These cells are essential in maintaining immune homeostasis by suppressing excessive immune responses by means of cell-cell contact mechanisms and release of soluble mediators. A deficiency in the frequency or a dysfunction in the suppressive function of Tregs is sufficient to break self-tolerance in healthy subjects (*13*). In the context of murine AKI, kidney-infiltrating Tregs were shown to be protective by modulating neutrophils, macrophages, and proinflammatory cytokine production by effector T cells (Teffs) (*14–16*). The role of Tregs in AKI after kidney transplantation is, however, unknown. We conducted a prospective observational cohort study of deceased donor kidney transplant recipients to investigate whether pretransplantation recipient peripheral blood Treg frequency and suppressive function predicted AKI (DGF and SGF) and subsequent short-term outcomes after kidney transplantation.

RESULTS

Patient Characteristics

Consecutive consenting adult deceased donor kidney transplant recipients (n=53) were enrolled into the study. Recipients were prospectively divided into the following: (1) DGF group (n=10), defined as recipients requiring dialysis within 7 days of transplantation; (2) SGF group (n=27), defined as recipients with a decrease in 24-hr serum creatinine by less than 20% and without requiring dialysis within 7 days of transplantation; and (3) immediate graft function (IGF) group (n=16), defined as recipients with a decrease in 24-hr serum creatinine by 20% or greater (*7*). Because SGF and DGF are a continuous spectrum of ischemia-reperfusion injury, these two groups were also combined to form an AKI group (n=37) (*4, 9*). The decision to initiate posttransplantation dialysis was made independently by the treating physicians. Recipient, donor, and organ procurement information were collected prospectively (Table 1, **Table S1, SDC,** http://links.lww.com/TP/A992). None of the recipients, including those with an autoimmune disease or a previous transplantation, were on immunosuppressive therapy for at least 180 days before transplantation. The AKI and IGF recipients received similar immunosuppressive regimen (detailed in Table 1). Significant differences in cold ischemic time (CIT), donor age, and the use of expanded criteria donors (ECDs) were observed between AKI and IGF recipients. Histologic allograft quality was similar between the AKI and IGF recipients. Acute rejection episodes were more frequent in the recipients with DGF.

Similar Pretransplantation Teff and Treg Frequencies Between AKI (DGF and SGF) and IGF Recipients

There were no significant differences in pretransplantation $CD4^+CD25^-$ Teff frequencies, $CD4^+CD25^{hi}FoxP3^+$ Treg frequencies, FoxP3 expression on $CD4^+CD25^{hi}$ Tregs, and Treg-to-Teff ratio among DGF, SGF, and IGF recipients ($P>0.05$, Fig. 1A–D). Similar results to the ones reported earlier were found when comparing AKI and IGF recipients ($P>0.05$, Fig. 1A–D).

Lower Pretransplantation Treg Suppressive Function in AKI (DGF and SGF) Recipients

We verified that interrecipient variability in purities of enriched $CD4^+CD25^-$ Teffs and $CD4^+CD25^+$ Tregs did not correlate with proliferation ($r=0.11$, $P=0.54$; **Figure S1A, SDC,** http://links.lww.com/TP/A992) or suppressive function ($r=-0.06$, $P=0.73$; **Figure S1B, SDC,** http://links.lww.com/TP/A992), respectively. Variability in percentage of FoxP3 expression in $CD4^+CD25^+$ Tregs enriched from different recipients also did not correlate with suppressive function ($r=0.26$, $P=0.13$; **Figure S1C, SDC,** http://links.lww.com/TP/A992). None of the recipient baseline characteristics with immunomodulatory potential, including age (*17, 18*), sex (*19*), body mass index (*20, 21*), autoimmune disease diagnosis (*22–25*), vitamin D supplementation (*26*), statin therapy (*27*), previous blood transfusion (*28*), sensitization (*29*), and dialysis modality or duration (*30*) were predictive of pretransplantation Teff proliferation (**Table S2, SDC,** http://links.lww.com/TP/A992) or Treg function (**Table S3, SDC,** http://links.lww.com/TP/A992) in linear regression analysis.

No significant differences were found in pretransplantation Teff proliferation among DGF, SGF, and IGF recipients ($P=0.15$) or between AKI and IGF recipients ($P=0.06$, Fig. 1E and F). Pretransplantation Treg suppressive function, however, was significantly lower in DGF (3.86%±1.86%) and SGF (11.71±2.11%) in comparison with IGF (27.33%±5.00%) recipients ($P<0.01$). Treg function was also significantly lower in AKI in comparison with IGF recipients ($P<0.01$, Fig. 1G and H).

Pretransplantation Treg Suppressive Function Independently Distinguishes DGF From IGF Recipients

Because pretransplantation Treg function was decreased in DGF and SGF recipients, we examined whether it can distinguish recipients who will have DGF or SGF rather than IGF after transplantation. With the use of the IGF recipients as reference group, each percentage increase in pretransplantation Treg function decreased the odds of being in the DGF or SGF group by 23% and 10%, respectively, in univariate multinomial logistic regression analysis. Cold ischemic time, donor age, and ECD category were the other significant variables in the univariate analysis. In a multivariate analysis accounting for CIT and donor age, Treg function remained a significant variable distinguishing DGF from IGF recipients (Table 2). We excluded ECD category and retained donor age in this multivariate analysis as well as all further ones later because a strong correlation existed between the two variables (**Figure S2A, SDC,** http://links.lww.com/TP/A992) and donor age is the main determinant of ECD categorization (*31*). No or weak correlations existed among the other significant variables in the univariate analysis, including Treg function (**Figure S2B–F, SDC,** http://links.lww.com/TP/A992).

TABLE 1. Clinical information regarding recipient, donor, and organ procurement

	IGF	SGF	DGF	P^a	AKI	P^b
Recipient characteristics						
n	16	27	10		37	
Age, yr	60±3	58±2	56±4	0.61	57±2	0.41
Male	11	22	8	0.61	30	0.33
African American race	2	2	2	0.55	4	0.86
Diagnosis						
SLE	0	1	0	0.61	1	0.51
GN	3	10	2	0.35	12	0.31
DM2	4	9	2	0.68	11	0.73
HTN	0	1	1	0.43	2	0.34
Other	9	6	5	0.06	11	0.07
BMI, kg/m^2	27±1	28±1	28±2	0.75	28±1	0.47
PRA>50%	2	2	2	0.55	4	0.86
Previous Tx	0	2	4	<0.01	6	0.09
HLA mismatches	3.2±0.3	3.4±0.2	3.2±0.4	0.75	3.4±0.2	0.59
Pre-Tx dialysis	13	24	10	0.26	34	0.14
Time on dialysis, yr	3.6±0.6	3.3±0.6	6.0±1.6	0.08	4.0±0.6	0.69
Pre-Tx eGFR, mL/min/1.73 m^2	11±1	12±1	10±1	0.24	11±1	0.75
Immunosuppression regimen						
ATG Tac, MMF, steroid[c]	10	17	5	0.54	22	0.28
Alemtuzumab Tac, MMF[d]	5	10	5		15	
Daclizumab Tac, MMF, steroid[c]	1	0	0		0	
Donor characteristics						
Age, yr	36±3	56±3	54±4	<0.01	56±2	<0.01
ECD	1	16	6	<0.01	22	<0.01
DBD	16	26	9	0.43	35	0.34
DCD	0	1	1		2	
Terminal eGFR, mL/min/m^2	116±12	136±15	128±15	0.60	134±11	0.28
Kidney biopsy						
ATN	3	4	1	0.89	5	0.67
GS	1	1	0	0.75	1	0.59
IF/TA	0	1	0	0.60	1	0.48
Procurement information						
CIT, hr	11±1	16±1	20±3	<0.01	17±1	<0.01
Machine perfusion	14	24	7	0.34	31	0.73

[a] *P* value for comparisons among DGF, SGF, and IGF groups.
[b] *P* value for comparisons between AKI and IGF groups.
[c] Maintenance immunosuppression starting on day 1 with tacrolimus (trough levels 4–8 ng/mL), MMF, and corticosteroid tapering protocol.
[d] Maintenance immunosuppression starting on day 1 with tacrolimus (trough levels 4–8 ng/mL) and MMF.

AKI, acute kidney injury; ATG, antithymocyte globulin; ATN, acute tubular necrosis; CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after cardiac death; DGF, delayed graft function; DM2, diabetes mellitus type 2; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; F, female; GN, glomerulonephritis; GS, glomerulosclerosis; HTN, hypertension; IGF, immediate graft function; IF, interstitial fibrosis; TA, tubular atrophy; M, male; MMF, mycophenolate mofetil; PCKD, polycystic kidney disease; PRA, panel reactive antibody; SGF, slow graft function; SLE, systemic lupus erythematosus; Tac, tacrolimus; Tx, transplant; BMI, body mass index; HLA, human leukocyte antigen.

Pretransplantation Treg Suppressive Function Independently Predicts AKI

Because DGF and SGF represent a continuum of injury (*4, 9*), we investigated whether pretransplantation Treg function also predicts AKI (combined DGF and SGF groups) after kidney transplantation. Receiver operating characteristic (ROC) curve analysis showed that pretransplantation Treg function was accurate at predicting AKI with an area under the curve (AUC) of 0.82 (95% confidence interval, 0.65–1.00; $P<0.01$; Fig. 2A). The optimal cutoff point for Treg function as a marker of AKI was determined by the largest sums of sensitivity and specificity. A pretransplantation Treg function less than 13% was thus chosen as the optimal cutoff point to predict AKI (sensitivity, 75.0%; specificity, 88.9%; positive predictive value, 95.5%; negative predictive value, 53.3%). Other variables that were significant predictors of AKI in ROC curve analysis were CIT greater than 9.5 hr (Fig. 2B), donor age greater than 47 years (Fig. 2C), and ECD category (Fig. 2D).

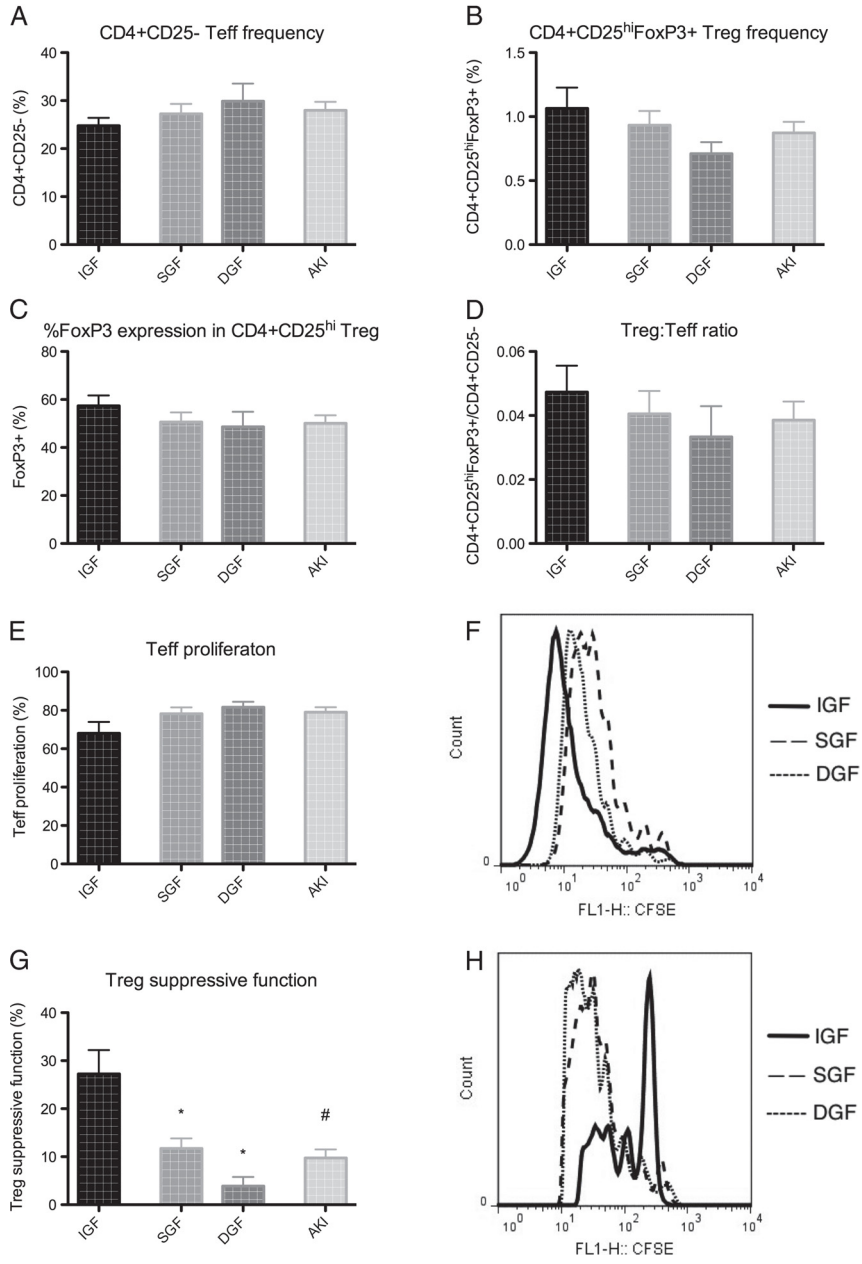

FIGURE 1. Pretransplantation Treg suppressive function, but not Teff frequency, Treg frequency, %FoxP3 expression in Treg, Treg-to-Teff ratio, or Teff proliferation, was significantly lower in DGF, SGF, and AKI recipients in comparison with IGF recipients. Comparisons of pretransplantation (A) $CD4^+CD25^-$ Teff frequency, (B) $CD4^+CD25^{hi}FoxP3^+$ Treg frequency, (C) %FoxP3 expression in $CD4^+CD25^{hi}$ Treg, and (D) $CD4^+CD25^{hi}FoxP3^+$ Treg–to–$CD4^+CD25^-$ Teff ratio were performed among DGF, SGF, and IGF recipients or AKI and IGF recipients. Comparison of pretransplantation (E) $CD4^+CD25^-$ Teff proliferation was performed among DGF, SGF, and IGF recipients or AKI and IGF recipients with (F) representative pretransplantation Teff proliferation assays in an IGF, SGF, and DGF recipients. Comparison of pretransplantation (G) $CD4^+CD25^+$ Treg suppressive function was performed among DGF, SGF, and IGF recipients (*one-way ANOVA, $P<0.01$, followed by Tukey's HSD post hoc analysis, $P<0.01$ vs. IGF group) or AKI and IGF recipients (#Student's t test, $P<0.01$ vs. IGF group) with (H) representative pretransplantation Treg suppression function assays in an IGF, SGF, and DGF recipient. Teff, effector T cell; FoxP3, forkhead box P3; Treg, regulatory T cell; DGF, delayed graft function; SGF, slow graft function; AKI, acute kidney injury; IGF, immediate graft function; ANOVA, analysis of variance; HSD, honestly significant difference.

Multivariate binary logistic regression accounting for CIT and donor age showed that a pretransplantation Treg function less than 13% remained a significant predictor of AKI, with an adjusted odds ratio of 21.86 (Table 3).

Pretransplantation Treg Suppressive Function Independently Predicts 14-Day Graft Function

We then sought to determine if pretransplantation Treg function predicted better short-term graft function

TABLE 2. Multinomial logistic regression analysis to predict DGF or SGF with IGF as reference group

	Univariate analysis			Multivariate analysis		
Variables	**OR**	**95% CI**	***P***	**Adjusted OR**	**95% CI**	***P***
Treg function, %						
DGF	0.77	0.64–0.93	<0.01	0.79	0.65–0.97	0.03
SGF	0.90	0.83–0.98	0.01	0.90	0.80–1.00	0.06
IGF	1.00			1.00		
CIT, hr						
DGF	1.30	1.09–1.54	<0.01	1.08	0.79–1.50	0.62
SGF	1.21	1.04–1.40	0.01	0.97	0.72–1.30	0.84
IGF	1.00			1.00		
Donor age, yr						
DGF	1.08	1.02–1.15	0.01	1.05	0.96–1.15	0.30
SGF	1.10	1.04–1.15	<0.01	1.08	1.01–1.17	0.03
IGF	1.00			1.00		
ECD						
DGF	22.50	2.07–244.84	0.01			
SGF	21.82	2.50–190.12	<0.01			
IGF	1.00					

CI, confidence interval; CIT, cold ischemic time; DGF, delayed graft function; ECD, expanded criteria donor; IGF, immediate graft function; OR, odds ratio; SGF, slow graft function; Treg, regulatory T cell.

regardless of AKI (DGF and SGF) or IGF grouping. Univariate linear regression analysis showed that each percentage increase in pretransplantation Treg function improved estimated glomerular filtration rate (eGFR) (*32*) by 0.53 to 0.82 mL/min per 1.73 m^2 up to 180 days after transplantation. Cold ischemic time, donor age, and ECD category were the only other variables that also predicted eGFR up to 180 days after transplantation in the univariate analysis. In a multivariate analysis accounting for CIT and donor age, Treg function remained a significant predictor of eGFR only at 14 days after transplantation, whereas both donor age and CIT remained significant predictors at 90 and 180 days after transplantation (**Table S4, SDC,** http://links.lww.com/TP/A992).

DISCUSSION

We are reporting a novel association between pretransplantation peripheral blood recipient Treg function and AKI (DGF and SGF) in deceased donor kidney transplant recipients. Although previous studies suggest that pretransplantation proinflammatory cytokines are associated with acute tubular necrosis after kidney transplantation (*33*), we did not find an increase in pretransplantation Teff responses in recipients with AKI. Instead, we found that recipients with AKI showed a lower pretransplantation Treg function, and this was not related to recipient characteristics with immunomodulatory capacity. In addition, our findings suggest that Treg function is a potential independent novel recipient-based peripheral blood immune marker for AKI (DGF and SGF) when measured before transplantation.

Previous candidate markers for AKI (DGF and SGF) have been previously studied in donor urine (*34*), machine perfusion fluid (*35*), and early posttransplantation recipient urine samples (*36–38*). Measurement of pretransplantation recipient peripheral blood Treg function, however, has the following advantages. As opposed to donor and machine perfusion fluid markers, it allows guidance in the donor allocation process. In comparison with early posttransplantation recipient markers, it allows timely prediction of AKI before kidney transplantation and onset of graft damage and the identification of recipient candidates at risk for AKI. In contrast to urine markers, measuring a peripheral blood-based marker is not limited by oliguria in the context of renal failure.

Although CIT significantly distinguished DGF from IGF recipients in univariate multinomial logistic regression and predicted AKI in univariate logistic regression, this variable was no longer significant in our multivariate analyses including Treg function. A possible explanation is that nearly all of our deceased donor grafts are preserved with machine perfusion, which has been shown to diminish the association between long CIT and the development of AKI (*39*). Furthermore, although both donor age and Treg function were significant variables in predicting AKI in univariate and multivariate analyses, our results suggest that Treg function might be more important than donor age in predicting recipients who will develop the most severe form of ischemia-reperfusion–related graft injury. As a matter of fact, only Treg function significantly distinguished DGF from IGF recipients in multivariate multinomial logistic regression.

Previous mechanistic studies in murine ischemic AKI models support our finding that pretransplantation Treg function is crucial in determining immediate graft outcome regardless of donor and organ procurement variables. Acute kidney injury after kidney transplantation is at first an inflammatory and antigen-independent event (*40*). Peripheral Tregs are known to home to areas of inflammation and, in the context of murine ischemic AKI, were shown to traffic to

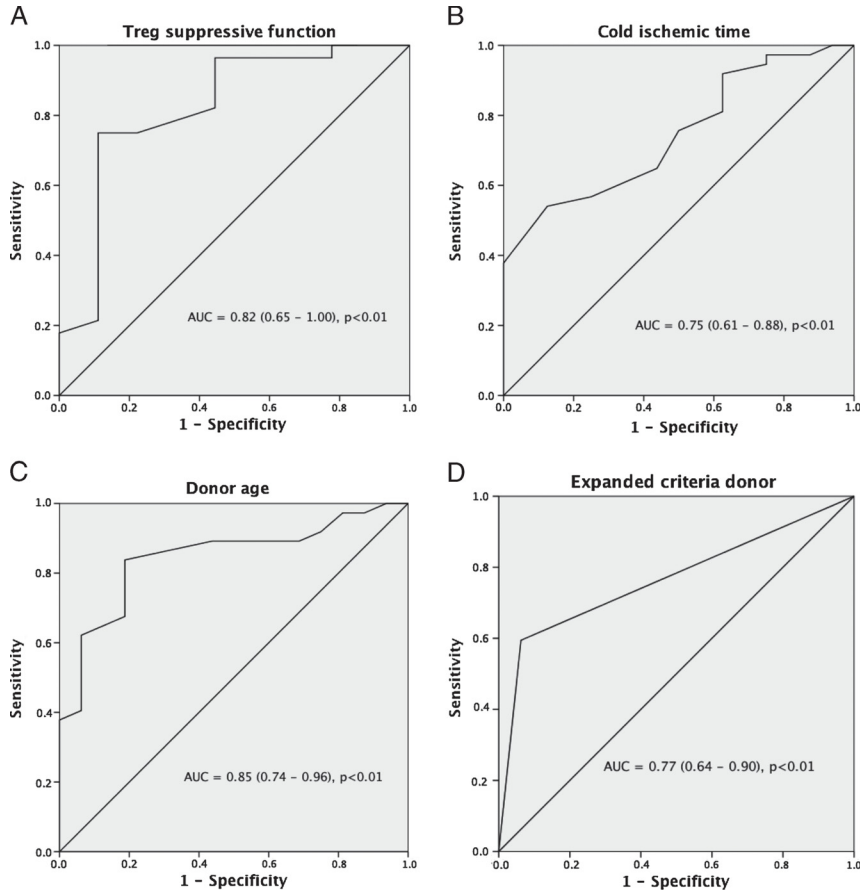

FIGURE 2. Significant predictors of AKI in ROC curve analysis. A, pretransplantation recipient peripheral blood Treg suppressive function (AUC, 0.82; 95% CI, 0.65–1.00; $P<0.01$; optimal cutoff point, 13%; sensitivity, 75.0%; specificity, 88.9%; PPV, 95.5%; NPV, 53.3%), (B) CIT (AUC, 0.75; 95% CI, 0.61–0.88; $P<0.01$; optimal cutoff point, 9.5 hr; sensitivity, 91.9%; specificity, 37.5%; PPV, 77.3%; NPV, 66.7%), (C) donor age (AUC, 0.85; 95% CI, 0.74–0.96; $P<0.01$; optimal cutoff point, 47 years; sensitivity, 83.8%; specificity, 81.2%; PPV, 82.5%; NPV, 69.2%), and (D) use of expanded criteria donor (AUC, 0.77; 95% CI, 0.64–0.90; $P<0.01$) accurately predict AKI in ROC curve analysis. AKI, acute lung injury; ROC, receiver operating characteristic; Treg, regulatory T cell; AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

the kidney, decrease infiltration of innate immune cells, inhibit production of proinflammatory cytokines by Teffs, and promote healing (*14, 15, 41*). Removal of the suppressive functional mechanisms of these trafficking peripheral Tregs before ischemia-reperfusion reversed their protective effect. In fact, Tregs depleted of their ability to suppress effector immune responses by means of CTLA-4 or by means of the secretion of soluble factors (adenosine, interleukin-10) were unable to protect from murine ischemic AKI in vivo (*14, 42, 43*). It is therefore plausible that kidney transplant recipients with less potently suppressive peripheral Tregs before transplantation are more susceptible to AKI after transplantation and that targeted therapies to enhance recipient Treg function could reduce the risk for AKI. Although still in the experimental phase, promising therapies currently exist, including transfusion of ex vivo expanded highly suppressive Tregs and

TABLE 3. Logistic regression analysis to predict AKI

	Univariate analysis			Multivariate analysis		
Variables	**OR**	**95% CI**	***P***	**Adjusted OR**	**95% CI**	***P***
Treg function<13%	24.00	2.54–227.24	<0.01	21.86	1.25–381.89	0.04
CIT	1.23	1.06–1.42	<0.01	0.98	0.74–1.31	0.91
Donor age	1.09	1.04–1.15	<0.01	1.07	1.01–1.15	0.04
ECD	22.00	2.62–184.75	<0.01			

AKI, acute kidney injury; CI, confidence interval; CIT, cold ischemic time; ECD, expanded criteria donor; OR, odds ratio; Treg, regulatory T cell.

pharmacologic modulation of in vivo Treg function with protein kinase C-theta, glycogen synthase kinase-3β, or histone deacetylase inhibitors (*44–47*).

We also found that pretransplantation Treg function predicted 14-day eGFR, whereas donor age and CIT were more important predictors of 90-day and 180-day eGFR. Although donor age and CIT have traditionally been associated with worse long-term graft outcomes after kidney transplantation, this notion has been recently disputed. A large retrospective study of deceased donor kidney transplant recipients in fact showed that using older donor age grafts did not worsen 5-year graft survival (*48*). In addition, a Scientific Registry of Transplant Recipients database analysis demonstrated that a longer CIT in paired donor recipients did not influence 8-year graft survival (*49*). Further studies of pretransplantation Treg function with longer follow-up would be required to demonstrate a role for regulatory mechanisms in promoting long-term graft survival, potentially by dampening the profibrotic effect of initial ischemia-reperfusion injury (*1, 50, 51*).

We acknowledge that the results of our study are limited by a small sample size. Nevertheless, it was sufficient to identify similar risk factors (CIT, donor age, ECD category) for AKI (DGF and SGF) as larger database studies (*5, 8*). The Treg suppressive function assay in its current state also has its own limitations for clinical applicability in deceased donor kidney transplantation because it is time consuming, is labor intensive, requires a large amount of recipient blood to isolate a scarce population of Tregs (<10% of total $CD4^+$ T lymphocytes in healthy individuals) (*52*), and is not standardized among research groups with regard to Treg and Teff purification techniques (magnetic bead-based vs. flow sorting), Teff stimulation techniques (plate bound vs. bead-coated anti-CD3/CD28), and Teff proliferation detection (H3-thymidine incorporation vs. carboxyfluorescein succinimidyl ester [CFSE] dilution) (*53*). Because of concerns of anemia before surgery, the maximum amount of blood we were permitted to draw by our institutional ethics board yielded insufficient peripheral blood mononuclear cells (PBMCs) for flow-sorting enrichment of Teffs and Tregs. Flow sorting is also a technology that is not currently widely available in the emergency setting of transplantation. We therefore chose to enrich Tregs and Teffs solely by magnetic bead-based technique, which yielded a lower FoxP3 purity (37%) in the enriched $CD4^+CD25^+$ Tregs than expected with flow sorting. We recognize that this could negatively affect the percentage suppression of Teff proliferation by Treg in the assay and, consequently, the optimal cufoff point for Treg function as a predictor of AKI. Nevertheless, this has minimal impact on our findings because the assay was performed identically in all recipients. Lastly, we did not assess the stability of Treg function in the pretransplantation period by serial measures, although we acknowledge that this would be an important goal of a separate study. We could also not follow early posttransplantation Treg function because all but one recipient received lymphodepleting induction immunosuppressive therapy. Although our findings are limited by the previously mentioned conditions, the fundamental observation that Treg function is an important donor-independent pretransplantation recipient variable in the prediction of posttransplantation graft injury is a novel concept in recipient risk stratification. Standardization, improvement, and external validation of this measure could ultimately be useful in redefining organ allocation schemes, guiding peritransplantation clinical decisions, as well as developing pretransplantation immunotherapy to specific measures of Treg function.

In conclusion, we found that pretransplantation recipient Treg function predicted AKI (DGF and SGF) after kidney transplantation. Measurement of recipient pretransplantation immune regulatory capacity, without previous knowledge of donor and organ procurement characteristics, could potentially indicate recipients at risk for AKI and graft damage before transplantation, guide peritransplantation clinical decisions, and identify recipients in whom development of novel immunotherapeutic strategies against AKI could be tested.

MATERIALS AND METHODS

The study was approved by the McGill University Health Centre Research Ethics Board, registered on ClinicalTrials.gov (NCT01232816), and conducted in adherence with the declarations of Helsinki and Istanbul.

Blood Sample Collection and Mononuclear Cell Isolation

Peripheral blood (40 mL) was collected in heparin-coated tubes before induction immunosuppression and skin incision. Peripheral blood mononuclear cells were isolated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ).

Treg and Teff Frequencies

Peripheral blood mononuclear cells were surface stained with anti-human CD4 FITC and CD25 PE antibodies, fixed and permeabilized with the FoxP3 staining buffer set as per the manufacturer's protocol, and stained intracellularly with anti-human FoxP3 PerCP-Cy5.5 antibody (all purchased from eBioscience, San Diego, CA). Flow cytometry acquisition was performed on the FACScan (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Lymphocytes were gated on based on forward and side scatters. Treg and Teff frequencies were determined by the percentage of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^-$ cells in the lymphocyte gate, respectively (**Figure S3, SDC,** http://links.lww.com/TP/A992).

Teff Proliferation and Treg Suppressive Function Assays

$CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs were enriched from PBMCs by magnetic bead isolation using the EasySep Human $CD4^+CD25^+$T Cell Isolation Kit as per the manufacturer's protocol (StemCell Technologies, Vancouver, Canada). Sufficient enriched cells were obtained in seven DGF, 21 SGF, and nine IGF recipients to perform Teff proliferation and Treg suppressive function assays. Purities of enriched $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs were, respectively, 86%±1% (37%±2% $FoxP3^+$) and 85%±2% (3%±1% $FoxP3^+$). Teffs were labeled with 5-μm/mL CSFE (Life Technologies, Burlington, Canada) at 37°C for 5 minutes, then washed with ice-cold 10% fetal bovine serum in phosphate-buffered saline (*53*). In all assays, 4×10^4 CFSE-labeled Teffs were co-cultured with Tregs at a 1:0 or 1:1 ratio for 5 days in 200 μL of X-VIVO 15 media (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Sigma-Aldrich, Oakville, Canada) in the presence of anti-CD3/CD28-coated beads (1 bead/cell, Life Technologies). With the use of flow cytometry analysis, the unlabeled CFSE-negative Tregs were excluded. Teff proliferation (1:0 ratio) was determined by CFSE dilution (% divided function in FlowJo software). Treg suppressive function was calculated as follows: $100-(((\%\text{ divided 1:1 Teff-to-Treg ratio})/(\%\text{divided 1:0 Teff-to-Treg ratio}))\times100)$. Titration of Teff-to-Treg ratio (1:1, 1:0.5, 1:0.125) in a subset of 32 kidney transplant recipients confirmed that

the enriched $CD4^+CD25^+$ Tregs suppressed $CD4^+CD25^-$ Teff proliferation in a dose-dependent manner (**Figure S4, SDC,** http://links.lww.com/TP/A992).

Statistical Analysis

Data are expressed as mean±SEM. Analyses were performed using SPSS 20 (IBM, Armonk, NY) and considered significant at a 0.05 level. Categorical data comparisons between DGF, SGF, and IGF groups or AKI and IGF groups were made using chi-square test. Continuous data comparisons between three groups were made using one-way analysis of variance followed by Tukey's honestly significant difference post hoc analysis, whereas comparisons between two groups were made using Student's *t* test. Correlations were performed with Pearson's correlation coefficient. Multinomial logistic regression was performed to assess predictors of DGF or SGF from IGF. Receiver operating characteristic curve analysis and binary logistic regression were performed to assess the predictive accuracy of recipient, donor, and organ procurement characteristic variables for AKI. Receiver operating characteristic curve analysis was also performed to determine the optimal cutoff point of each significant variable as a marker for AKI based on the largest sums of sensitivity and specificity. Linear regression was performed to assess recipient, donor, and organ procurement characteristic variables as predictors of posttransplantation eGFR and to assess baseline recipient characteristics as predictors of pretransplantation Teff proliferation and Treg function. All significant variables in univariate analyses were considered for the multivariate analyses. We excluded ECD category from all multivariate analyses because this variable was strongly collinear with donor age (**Figure S2A, SDC**, http://links.lww.com/TP/A992) and donor age is the main determinant of ECD categorization (*31*).

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Pretransplant Recipient Circulating CD4+CD127lo/– Tumor Necrosis Factor Receptor 2+ Regulatory T Cells: A Surrogate of Regulatory T Cell–Suppressive Function and Predictor of Delayed and Slow Graft Function After Kidney Transplantation

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Background. Delayed graft function (DGF) and slow graft function (SGF) are ischemia-reperfusion–associated acute kidney injuries (AKI) that decrease long-term graft survival after kidney transplantation. Regulatory T (Treg) cells are protective in murine AKI, and their suppressive function predictive of AKI in kidney transplantation. The conventional Treg cell function coculture assay is however time-consuming and labor intensive. We sought a simpler alternative to measure Treg cell function and predict AKI. **Methods.** In this prospective observational cohort study, pretransplant recipient circulating CD4+CD25+CD127lo/– and CD4+CD127lo/– tumor necrosis factor receptor 2 (TNFR2)+ Treg cells were measured by flow cytometry in 76 deceased donor kidney transplant recipients (DGF, n = 18; SGF, n = 34; immediate graft function [IGF], n = 24). In a subset of 37 recipients, pretransplant circulating Treg cell–suppressive function was also quantified by measuring the suppression of autologous effector T-cell proliferation by Treg cell in coculture. **Results.** The TNFR2+ expression on CD4+CD127lo/– T cells correlated with Treg cell–suppressive function (r = 0.63, $P < 0.01$). In receiver operating characteristic curves, percentage and absolute number of CD4+CD127lo/–TNFR2+ Treg cell predicted DGF from non-DGF (IGF + SGF) with area under the curves of 0.75 and 0.77, respectively, and also AKI (DGF + SGF) from IGF with area under the curves of 0.76 and 0.72, respectively ($P < 0.01$). Prediction of AKI (DGF + SGF) from IGF remained significant in multivariate logistic regression accounting for cold ischemic time, donor age, previous transplant, and pretransplant dialysis modality. **Conclusions.** Pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg cell is potentially a simpler alternative to Treg cell function as a pretransplant recipient immune marker for AKI (DGF + SGF), independent from donor and organ procurement characteristics.

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Delayed graft function (DGF) and slow graft function (SGF) are a continuous spectrum of ischemia-reperfusion related acute kidney injuries (AKI) that occur in more than 20% of kidney transplant recipients.[1-3] Delayed graft function, the most severe form of AKI, is highly detrimental to kidney transplant recipients as it increases the risk for both acute and chronic rejection as well as long-term graft loss.[4-6] Although SGF is a milder form of AKI, it behaves similarly

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to DGF, and also increases the risk for acute rejection and long-term graft loss.[6,7]

Regulatory T (Treg) cells are essential in maintaining immune homeostasis in healthy individuals. A defect in their frequency or function has been implicated in autoimmune diseases, transplant rejection, and more recently AKI.[8] Murine studies demonstrated that decreasing Treg cell frequency or suppressive function before renal ischemia-reperfusion injury increased the severity of AKI.[9-11] We subsequently showed that a lower pretransplant recipient circulating Treg cell–suppressive function, measured by an in vitro assay of enriched recipient Treg cells cocultured with stimulated autologous effector T (Teff) cells, was predictive of AKI (DGF + SGF) in deceased donor kidney transplant recipients.[12] The clinical applicability of this in vitro assay as a pretransplant immune marker to predict AKI in kidney transplant recipients is however limited by the fact that it is labor-intensive, time-consuming, costly, and nonstandardized.[13] A solution to transform Treg cell–suppressive function into a clinically applicable immune marker would be to identify and quantify the most potently suppressive subset of Treg cells with phenotypic surface markers.

The Treg cells are CD4+ T cells most commonly identified by their expression of the transcription factor forkhead box P3 and the surface markers CD25 and CD127.[8] The use of these markers in the clinical setting is however problematic. Because forkhead box P3 is an intracellular protein, the fixation and permeabilization step required before its staining lengthens the Treg cell quantification process by flow cytometry and does not allow its use for Treg cell isolation and potential cellular therapy.[14,15] Although CD25 is a surface marker, only the CD4+ T cells with the highest CD25 expression are suppressive Treg cells in humans, and the flow cytometry gating strategy to identify these CD4+CD25^{hi} cells is variable.[15,16] Similarly, the downregulation of CD127 on CD4+ T cells was shown to be an equivalent surface marker alternative to CD25^{hi} for the identification of suppressive Treg cells, but cannot exclude recently activated Teff cells.[8,15]

In the context of an inflammatory environment, such as AKI after kidney transplantation, tumor necrosis factor receptor 2 (TNFR2) is another recently discovered surface marker that is particularly interesting for the identification of the most potently suppressive subset of Treg cells. The TNFR2 is a cytokine receptor mostly restricted to lymphocytes and endothelial cells.[17] It is preferentially expressed on Treg cells as compared to Teff cells and mediates many of the biological functions of TNF-α.[18] TNF-α blockade has indeed been shown to both dampen and exacerbate autoimmune diseases.[17] Although TNF-α is traditionally thought to be upregulated and proinflammatory in AKI, it could also have an immunoregulatory role via its interaction with kidney-infiltrating Treg cells.[19] Recent murine and human studies showed that TNF-α signaling through TNFR2+ Treg cells increased their survival, proliferation, and suppressive function.[18]

Based on previous literature, we therefore hypothesized that TNFR2 expression on circulating Treg cells could serve as a surrogate phenotypic surface marker of pretransplant Treg cell–suppressive function in patients awaiting a kidney transplantation. Moreover, based on our previous finding that pretransplant recipient Treg cell–suppressive function predicted AKI (DGF + SGF) after kidney transplantation, we hypothesized that pretransplant recipient TNFR2+ Treg cells could predict those who will suffer from AKI (DGF + SGF).

MATERIALS AND METHODS

Our prospective observational cohort study was approved by the McGill University Health Centre Research Ethics Board, registered on ClinicalTrials.gov (NCT01232816), and conducted in adherence with the declarations of Helsinki and Istanbul.

Pretransplant Recipient Circulating CD4+CD127lo/–TNFR2+ and CD4+CD25+CD127lo/– Treg Cell Frequencies

Peripheral blood was drawn in heparin-coated tubes in the operating theater, before induction immunosuppression and skin incision. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare, Piscataway, NJ). The PBMCs were surface stained with antihuman CD4 fluorescein isothiocyanate (clone OKT4; eBioscience, San Diego, CA), CD127 PE-Cy5 (clone eBioRDR5; eBioscience), and CD25 PE (clone BC96; eBioscience) or TNFR2 PE (clone TR75-89; BD Biosciences) for 30 minutes in the dark at 4°C, then washed with phosphate-buffered saline (Wisent, St-Bruno, Canada). Flow cytometry acquisition was performed on the FACScan (BD Biosciences), and data analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Forward and side scatters were used to gate on lymphocytes. The Treg cell frequency was determined by the percentage of CD4+CD25+CD127lo/– or CD4+CD127lo/–TNFR2+ cells in the lymphocyte gate. Gating strategies for CD4, CD127, CD25, and TNFR2 were based on single-color compensation. Using the same peripheral blood sample, lymphocyte count was obtained from our clinical hematology laboratory and absolute Treg cell counts were calculated using the following formula: % cells in lymphocyte gate × lymphocyte count.

Pretransplant Recipient Treg Cell–Suppressive Function Assay

In a subset of 37 kidney transplant recipients, pretransplant Treg cell–suppressive function was quantified as previously described.[12] Briefly, CD4+CD25+ Treg cells and CD4+CD25–Teff cells were enriched from PBMCs by magnetic bead isolation (EasySep Human CD4+CD25+ T Cell Isolation Kit; StemCell Technologies, Vancouver, Canada). Purities of enriched CD + CD25+ Treg cells and CD4+CD25- Teff cells were 86 ± 1% and 85 ± 2% respectively. The Teff cells were labeled with 5 μm/mL carboxyfluorescein succinimidyl ester (Life Technologies, Burlington, Canada) at 37°C for 5 minutes, then washed with ice-cold 10% fetal bovine serum in phosphate-buffered saline. In all assays, 4×10^4 CFSE-labeled Teff cells were cocultured with Treg cells at a 1:0 or 1:1 ratio for 5 days in 200 μL of X-VIVO 15 media (Lonza, Basel, Switzerland) supplemented with 10% human AB serum (Sigma-Aldrich, Oakville, Canada) in the presence of anti-CD3/CD28–coated beads (1 bead/cell; Life Technologies). Unlabeled CFSE-Teff cells were excluded by flow cytometry analysis gating. The Teff cell proliferation was measured by CFSE dilution (% divided function, FlowJo software). The Treg cell–suppressive function was then calculated using the following formula: $100 - \frac{\%\ \text{divided 1:1 Teff:Treg ratio}}{\%\ \text{divided 1:0 Teff:Treg ratio}} \times 100$.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using SPSS 20 (IBM, Armonk, NY) and considered significant if P value is 0.05 or less. Categorical data were compared using χ^2 test. Continuous data between 3 groups were compared using 1-way analysis of variance followed by Tukey honestly significant difference post hoc analysis, whereas continuous data between the 2 groups was compared using Student t test. Correlations were made with Pearson correlation coefficient. Linear regression was performed to assess recipient baseline characteristics as predictors of pretransplant circulating CD4+CD127lo/–TNFR2+ Treg cells. Logistic regression and receiver operating characteristic (ROC) curve were performed to assess recipient, donor, and organ procurement variables as predictors of DGF from non-DGF (immediate graft function [IGF] + SGF) or AKI (DGF + SGF) from IGF. The ROC curve was also used to determine a range of cutoff values for each significant variable that predicted DGF or AKI (DGF + SGF), as well as an optimal cutoff value based on the largest sums of sensitivity and specificity. Multivariate logistic regression was used to assess the independent predictive ability of CD4+CD127lo/–TNFR2+ Treg cell for DGF and AKI (DGF + SGF) in models containing variables that were significantly different between our outcomes of interest with the exception of expanded criteria donor (ECD) category as this variable was strongly collinear with donor age, and donor age is the main determinant of ECD categorization.[20] The multivariate logistic regression models were internally validated by generating 95% confidence intervals for the odds ratios using the bootstrap technique with 1000 replicates.

RESULTS

Patient Characteristics

Seventy-six consecutive consenting adult deceased-donor kidney transplant recipients were enrolled and prospectively divided into (1) DGF (n = 18), (2) SGF (n = 34), and (3) IGF (n = 24) groups. The IGF and SGF groups were combined into a non-DGF group (n = 58). The DGF and SGF groups were also combined into an AKI group (n = 52) because these 2 groups are a continuous spectrum of renal ischemia-reperfusion injury.[3] The DGF recipients were defined as those requiring dialysis within 7 days after transplantation. Recipients not requiring dialysis after transplantation were defined as having SGF if their 24-hour serum creatinine decreased by less than 20%, and IGF if it decreased by more than 20%.[12,21] Initiation of posttransplant dialysis was decided independently by the treating physicians.

Recipient, donor, and organ procurement characteristics were collected prospectively (Tables 1 and **Table S1, SDC**, http://links.lww.com/TP/B196). Donor age and cold ischemic time (CIT) were significantly higher in DGF and SGF recipients. The use of ECDs and the presence of a previous transplant were also significantly more frequent in DGF and SGF recipients. The use of pretransplant dialysis was significantly different between AKI (DGF + SGF) and IGF, but not DGF and non-DGF (IGF + SGF) recipients. Induction and maintenance immunosuppressive regimen were similar between groups (details in Table 1 and **Table S1, SDC**, http://links.lww.com/TP/B196). None of the recipients, especially those with an autoimmune etiology of end-stage renal disease, were on immunosuppressive therapy within 6 months preceding their transplant. As expected, estimated glomerular filtration rate was significantly lower in DGF and SGF recipients up to 6 months after transplantation (Table 1 and **Table S1, SDC**, http://links.lww.com/TP/B196).

Expression of TNFR2 on CD4+CD127lo/– T Cells is a Surrogate Phenotypic Surface Marker of Treg Cell–Suppressive Function

Based on previous literature, we first sought to verify whether expression of the surface marker TNFR2 is a surrogate marker of Treg cell–suppressive function in a subset of 37 deceased donor adult kidney transplant recipients. Expression of TNFR2 on CD4+CD127lo/– T cells positively correlated with Treg cell–suppressive function (r = 0.63, $P < 0.01$; Figure 1). Identification of Treg cell using the CD4+CD127lo/– TNFR2+ markers positively correlated with the CD4+CD25+CD127lo/– markers (r = 0.40, $P < 0.01$), and yielded a larger cell population (4.39 ± 0.30% or 59.95 × 10^6/L instead of 2.54% ± 0.12% or 34.31 × 10^6/L, $P < 0.01$).

Pretransplant Recipient Circulating CD4+CD127lo/–TNFR2+ Treg Cells Were Lower in DGF and SGF Recipients

We then examined whether the percentage and absolute number of pretransplant circulating CD4+, CD4+CD127lo/–, CD4+CD25+CD127lo/–, and CD4+CD127lo/–TNFR2+ T cells were different between DGF, SGF, and IGF recipients. Only the percentage and absolute number of CD4+CD127lo/–TNFR2+ Treg cells were significantly lower in DGF (3.08 ± 0.59%, 39.70 ± 10.45 × 10^6/L) and SGF (3.95 ± 0.35%, 54.83 ± 5.34 × 10^6/L) in comparison to IGF (6.05 ± 0.57%, 82.72 ± 10.99 × 10^6/L) recipients ($P < 0.05$; Figures 2 and 3, and **Figure S1, SDC**, http://links.lww.com/TP/B196). The same results were observed when comparing DGF with non-DGF or AKI to IGF recipients ($P = 0.02$; Figures 2 and 3, and **Figure S1, SDC**, http://links.lww.com/TP/B196).

Recipient Baseline Characteristics Were Not Predictive of Pretransplant Recipient Circulating CD4+CD127lo/–TNFR2+ Treg Cells

Certain recipient baseline characteristics, such as age,[22,23] sex,[24] autoimmune etiology of end-stage renal disease,[25-28] body mass index,[29,30] vitamin D supplementation,[31] statin therapy,[32] blood transfusion history,[33] sensitization,[34] and dialysis modality/duration[35] were shown to have immunomodulatory potential. We therefore investigated whether the aforementioned variables influenced the percentage or absolute number of pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg cells. In our cohort of recipients, none of the recipient baseline characteristics was predictive of CD4+CD127lo/–TNFR2+ Treg cells (**Table S2, SDC**, http://links.lww.com/TP/B196).

Prediction of DGF or AKI Based on Pretransplant Recipient Circulating CD4+CD127lo/–TNFR2+ Treg cells

Univariate Analyses

Pretransplant Recipient Circulating CD4+CD127lo/–TNFR2+ Treg Cells Predicted DGF

Because pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg cells were significantly decreased in DGF

TABLE 1.

Recipient, donor, and organ procurement characteristics

	Non-DGF (IGF + SGF)	DGF	P[a]	IGF	AKI (SGF + DGF)	P[b]
Recipient						
n	58	18		24	52	
Age, y	57 ± 2	57 ± 3	0.85	57 ± 3	57 ± 2	0.95
Male	42	15	0.35	15	42	0.09
African American race	6	4	0.19	3	7	0.91
Diagnosis						
SLE	1	0	0.73	0	1	0.77
GN	14	7		5	16	
DM2	16	4		6	14	
HTN	2	1		1	2	
Other	25	6		12	19	
BMI, kg/m^2	28 ± 1	29 ± 1	0.54	27 ± 1	28 ± 1	0.27
PRA > 50%	8	2	0.66	5	5	0.14
PRA class I, %	13 ± 3	5 ± 5	0.24	16 ± 5	9 ± 3	0.22
PRA class II, %	8 ± 3	5 ± 5	0.61	10 ± 6	6 ± 3	0.54
Previous transplant	3	5	<0.01	1	7	0.22
Blood transfusion history	15	4	0.82	5	14	0.40
HLA mismatches	3.5 ± 0.1	3.9 ± 0.3	0.27	3.6 ± 0.3	3.6 ± 0.2	0.86
Pretransplant vitamin D	19	6	0.83	10	15	0.42
Pretransplant statin	29	8	0.68	13	24	0.52
Pretransplant dialysis						
None	5	1	0.34	4	2	<0.01
PD	11	1		7	5	
HD	40	15		12	43	
Time on dialysis, y	3.8 ± 0.4	5.0 ± 1.0	0.19	4 ± 1	4 ± 1	0.81
Pretransplant eGFR, mL/min per 1.73 m^2	11 ± 1	9 ± 1	0.11	10 ± 1	11 ± 1	0.63
Immunosuppression regimen						
ATG Tac, MMF, steroid[c]	27	5	0.29	10	22	0.33
Alemtuzumab Tac, MMF[d]	30	13		13	30	
Daclizumab Tac, MMF, steroid[c]	1	0		1	0	
Posttransplant outcomes						
Day 1 eGFR, mL/min per 1.73 m^2	13 ± 1	9 ± 1	0.01	14 ± 1	11 ± 1	0.09
Day 7 eGFR, mL/min per 1.73 m^2	37 ± 3	14 ± 2	<0.01	51 ± 5	23 ± 2	<0.01
Day 14 eGFR, mL/min per 1.73 m^2	47 ± 2	23 ± 3	<0.01	58 ± 3	32 ± 2	<0.01
Day 30 eGFR, mL/min per 1.73 m^2	48 ± 2	31 ± 3	<0.01	58 ± 4	37 ± 2	<0.01
Day 90 eGFR, mL/min per 1.73 m^2	52 ± 2	38 ± 5	<0.01	63 ± 3	41 ± 2	<0.01
Day 180 eGFR, mL/min per 1.73 m^2	53 ± 3	41 ± 6	0.04	65 ± 4	44 ± 3	<0.01
AR within 180 d	7	6	0.04	3	9	0.59
Donor						
Age, y	49 ± 2	58 ± 3	0.01	38 ± 4	57 ± 2	<0.01
ECD	23	11	0.11	4	30	<0.01
DBD	57	17	0.38	24	50	0.33
DCD	1	1		0	2	
Terminal eGFR, mL/min per 1.73 m^2	121 ± 8	117 ± 11	0.79	118 ± 10	120 ± 9	0.86
Procurement						
CIT, h	15 ± 1	20 ± 2	<0.01	14 ± 1	17 ± 1	0.05
Machine perfusion	49	14	0.61	21	42	0.67

[a] *P* value for comparisons between DGF and non-DGF groups.
[b] *P* value for comparisons between AKI and IGF groups.
[c] Maintenance immunosuppression starting on day 1 with tacrolimus (through levels, 4-8 ng/mL), MMF, and corticosteroid tapering protocol.
[d] Maintenance immunosuppression starting on day 1 with tacrolimus (through levels, 4-8 ng/mL) and MMF.
AR, acute rejection; ATG, antithymocyte globulin; BMI, body mass index; DBD, donor after brain death; DCD, donor after cardiac death; DM2, type 2 diabetes mellitus; eGFR, estimated GFR; GN, glomerulonephritis; HD, hemodialysis; HTN, hypertension; MMF, mycophenolate mofetil; PD, peritoneal dialysis; PRA, panel-reactive antibody; SLE, systemic lupus erythomatosus; Tac, tacrolimus.

recipients, we investigated whether they could predict if a recipient will have DGF or not after transplantation. In logistic regression analysis, each percentage increase in CD4+CD127lo/−TNFR2+ Treg cell decreased the odds of having DGF by 30% ($P = 0.02$), whereas each 1×10^6/L increase decreased the odds by 2% ($P = 0.03$; Table 2). Predictive accuracy for DGF was assessed in ROC curve analysis, in which area under the curves (AUCs) of 0.75 and 0.77 were obtained for CD4+CD127lo/−TNFR2+ Treg cell percentage and absolute number, respectively ($P < 0.01$; Figure 4A). The performance of various cutoff

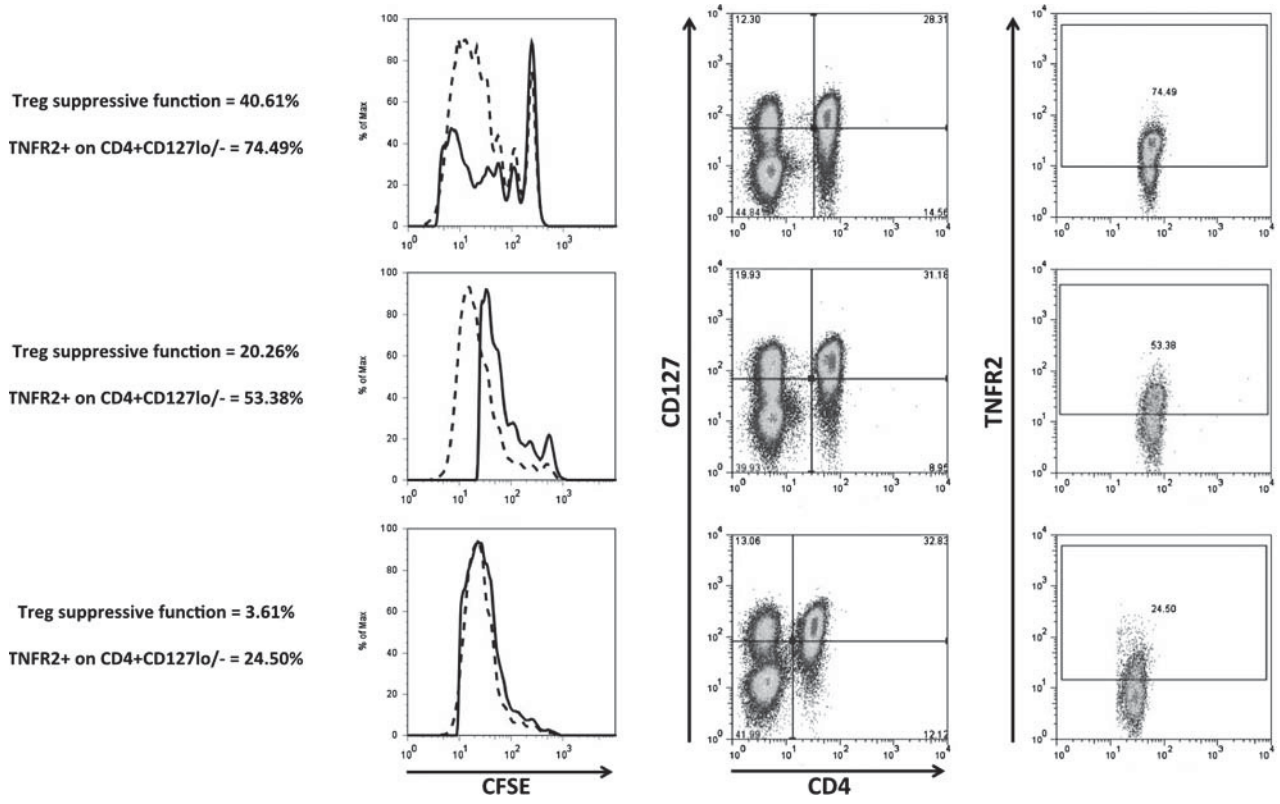

FIGURE 1. Pretransplant recipient circulating Treg cell–suppressive function correlates with TNFR2+ expression on CD4+CD127lo/– T cells. Representative examples of circulating Treg cell–suppressive function measured by the suppression of stimulated autologous CFSE-labeled Teff cell by Treg in coculture and the corresponding TNFR2 expression on CD4+CD127lo/– T cells are shown for 3 recipients before transplantation.

values for the previous 2 variables with regard to sensitivity, specificity, and predictive value is presented in Table 3. Identifying Treg cells with CD4+CD127lo/– or CD4+CD25+CD127lo/– was not predictive of DGF (**Figure S2, SDC**, http://links.lww.com/TP/B196). Cold ischemic time was the only other significant variable in the prediction of DGF (AUC = 0.75, $P < 0.01$), whereas donor age and previous transplant were not (Figure 4B).

Pretransplant Recipient Circulating CD4+CD127lo/−TNFR2+ Treg Cells Predicted AKI

Because DGF and SGF represent a continuous spectrum of renal ischemia-reperfusion injury,[3] we also examined whether pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg cells predicted posttransplant AKI (combined DGF and SGF group). In logistic regression analysis, each percentage increase in CD4+CD127lo/–TNFR2+ Treg cell decreased the odds of having AKI by 31% ($P < 0.01$), whereas each 1×10^6/L increase decreased the odds by 2% ($P < 0.01$; Table 4). Predictive accuracy for AKI was assessed in ROC curve analysis, in which AUCs of 0.76 and 0.72 were obtained for CD4+CD127lo/–TNFR2+ Treg cell percentage and absolute number, respectively ($P < 0.01$; Figure 5A). The performance of various cutoff values for the previous 2 variables with regards to sensitivity, specificity, and predictive value is presented in Table 3. Identifying Treg cells with CD4+CD127lo/– or CD4+CD25+CD127lo/– was not predictive of AKI (**Figure S3, SDC**, http://links.lww.com/TP/B196). Other significant variables in the prediction of AKI included donor age (AUC = 0.81, $P < 0.01$), ECD category (AUC = 0.71, $P < 0.01$), and pretransplant dialysis modality (AUC = 0.67, $P = 0.02$; Figure 5B).

Multivariate Analyses

For our multivariate analyses, we considered all variables that were significantly different between our outcomes of interest. Because a strong correlation existed between ECD category and donor age (**Table S3, SDC**, http://links.lww.com/TP/B196), we excluded ECD category and retained donor age in our multivariate analyses because donor age is the main determinant of ECD categorization.[20] No or weak correlations existed among the other aforementioned variables included in our multivariate analyses (**Table S3, SDC**, http://links.lww.com/TP/B196).

Pretransplant Recipient Circulating CD4+CD127lo/−TNFR2+ Treg Cells Were Not Independently Associated With DGF

In multivariate logistic regression analysis adjusting for the effects of CIT, donor age, and previous transplant, CD4+CD127lo/–TNFR2+ Treg cell percentage and absolute number did not remain significant variables in predicting which recipients will have DGF or not (Table 2).

Pretransplant Recipient Circulating CD4+CD127lo/−TNFR2+ Treg Cells Predicted AKI

In multivariate logistic regression analysis, CD4+CD127lo/–TNFR2+ Treg cell percentage and absolute number remained significant predictors of AKI when adjusting for the effects of CIT, donor age, previous transplant, and pretransplant dialysis modality, as demonstrated by odds ratios of 0.72

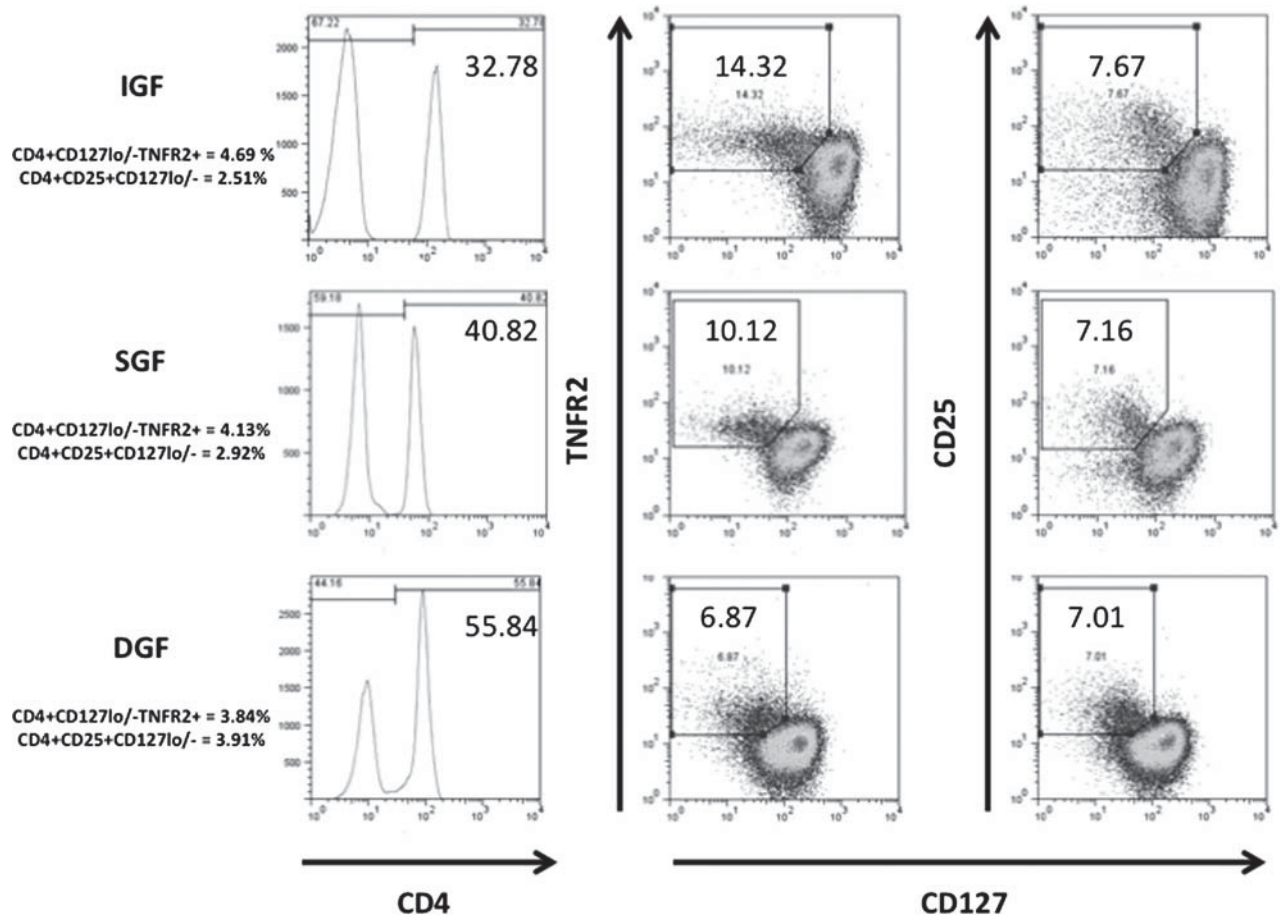

FIGURE 2. Pretransplant recipient circulating CD4+CD127lo/−TNFR2+ Treg cells were lower in DGF and SGF in comparison to IGF recipients. Representative CD4+CD127lo/−TNFR2+ and CD4+CD25+CD127lo/− flow cytometry analyses for a DGF, SGF, and IGF recipient are shown.

(P = 0.02) and 0.98 (P = 0.02), respectively (Table 4). This analysis was internally validated by generating 95% confidence intervals for the odds ratios with the bootstrap resampling technique using 1000 replicates (**Table S4, SDC**, http://links.lww.com/TP/B196).

CD4+CD127lo/−TNFR2+ Treg Cell-Based Logistic Regression Model to Predict AKI

Because pretransplant dialysis modality and donor age were also significant variables in the prediction of AKI in multivariate logistic regression and are known risk factors for AKI,[20,36,37] we incorporated these variables with pretransplant recipient circulating CD4+CD127lo/−TNFR2+ Treg cells to create a logistic regression model that could be used before transplantation to guide organ allocation by estimating the probability of a recipient to develop AKI after transplantation (probability of AKI = $\frac{1}{1+e^{-z}}$ with z = −4.28 + (−0.34 × CD4+CD127lo/−TNFR2+ Treg %) + (0.09 × donor age) + (0.70 × peritoneal dialysis [0/1]) + (2.96 × hemodialysis [0/1]). We chose CD4+CD127lo/−TNFR2+ Treg cell percentage instead of absolute number in the model because it had the highest AUC of the two in ROC curve analysis. This logistic regression model improved the prediction accuracy for AKI on ROC curve analysis with an AUC of 0.90 (P < 0.01) in comparison to using CD4+CD127lo/−TNFR2+ Treg cell percentage, donor age, or dialysis modality alone (Figures 5 and 6). Its prediction accuracy, however, did not differ significantly from a logistic regression model using only the clinically available variables donor age and pretransplant dialysis modality (AUC = 0.88; Figure 6). Nevertheless, at the optimal cutoff probability value based on the best sums of sensitivity and specificity, the model including CD4+CD127lo/−TNFR2+ Treg cell had a better specificity and positive predictive value (PPV) while maintaining a similar sensitivity and negative predictive value (Table 5).

DISCUSSION

We previously found that pretransplant recipient Treg cell–suppressive function was predictive of AKI (DGF + SGF) after kidney transplantation. Because of its labor-intensive and time-consuming nature, the conventional assay to measure Treg cell–suppressive function by suppression of autologous Teff cell proliferation by Treg cell in coculture is however impractical for widespread clinical use as an immune marker for AKI (DGF + SGF). This is especially true in deceased donor kidney transplantation, which often occurs in a semi-urgent fashion and during off-hours. We found that the measurement of circulating recipient CD4+CD127lo/−TNFR2+ Treg cells before transplantation could be a suitable rapid and simple alternative to the conventional Treg cell–suppressive function assay as an immune marker for AKI (DGF + SGF).

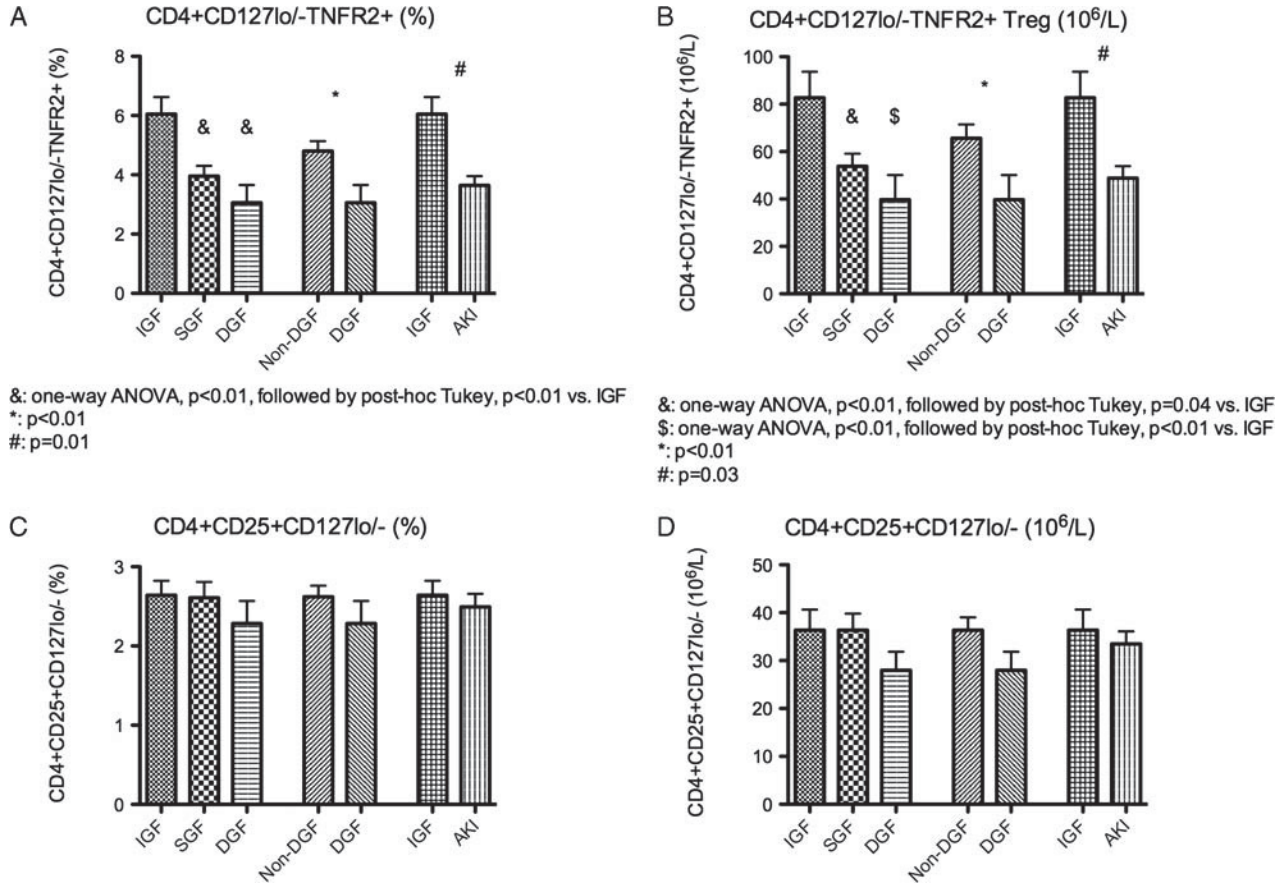

FIGURE 3. Pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg (A) percentage and (B) absolute number, but not CD4+CD25+ CD127lo/– Treg cell (C) percentage and (D) absolute number, were significantly lower in DGF and SGF in comparison to IGF, significantly lower in DGF in comparison to non-DGF (IGF + SGF), and significantly lower in AKI (DGF + SGF) in comparison to IGF recipients.

Although Treg cells are currently conventionally identified with the surface markers, CD4, CD25, and CD127, this combination failed to predict DGF or AKI (DGF + SGF) in our study. On the other hand, the combination of CD4, CD127, and TNFR2, which partially correlated with CD4+ CD25+CD127lo/–, was able to predict DGF and AKI (DGF + SGF). This reflects the known heterogeneity of Treg cells,[38] and perhaps both populations identify Treg cells with distinct functional capacities in AKI. Previous murine and healthy subjects literature reported that TNFR2 identifies a highly suppressive subset of Treg cells, independent of CD25. The TNFR2+ T cells were shown to contain a substantial portion of CD25– cells, whereas CD25+ T cells that were TNFR2– had minimal to no suppressive activity.[18,39-41] Our results also suggest that upregulation of TNFR2 on CD4+CD127lo/– T cells could identify highly suppressive Treg cells in uremic patients awaiting a deceased donor kidney transplant, as it correlated with the conventional Treg cell–suppressive function assay.

In an inflammatory environment, such as AKI, where TNF-α is upregulated,[19] the presence of TNFR2 on Treg cells could be essential for their maximal suppressive function via several mechanisms. First, TNF-α binding to TNFR2+ Treg cells serves as a negative feedback loop to prevent excessive effector immune responses by stimulating Treg cell activation/expansion.[39] Second, interaction between TNF-α and TNFR2 + Treg cells also increases their resistance to oxidative stress by upregulation of the antioxidant thioredoxin-1, thereby

TABLE 2.

Logistic regression analysis to predict DGF

Univariate Analysis			
Variables	**OR**	**95% CI**	***P***
CD4+CD127lo/–TNFR2+ Treg, %	0.70	0.52-0.95	0.02
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.96-0.99	0.03
CIT, h	1.15	1.04-1.26	<0.01
Donor age, y	1.04	0.99-1.08	0.06
Previous transplant	7.05	1.49-33.35	0.01
Multivariate analysis with CD4+CD127lo/–TNFR2+, %			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg, %	0.73	0.51-1.05	0.09
CIT, h	1.14	1.03-1.27	0.02
Donor age, y	1.01	0.97-1.05	0.58
Previous transplant	8.40	1.20-58.88	0.03
Multivariate analysis with CD4+CD127lo/–TNFR2+, 10^6/L			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg (10^6/L)	0.98	0.96-1.01	0.10
CIT, h	1.16	1.04-1.29	<0.01
Donor age, y	1.01	0.97-1.06	0.60
Previous transplant	7.16	1.11-46.25	0.04

95% CI, 95% confidence interval; OR, odds ratio.

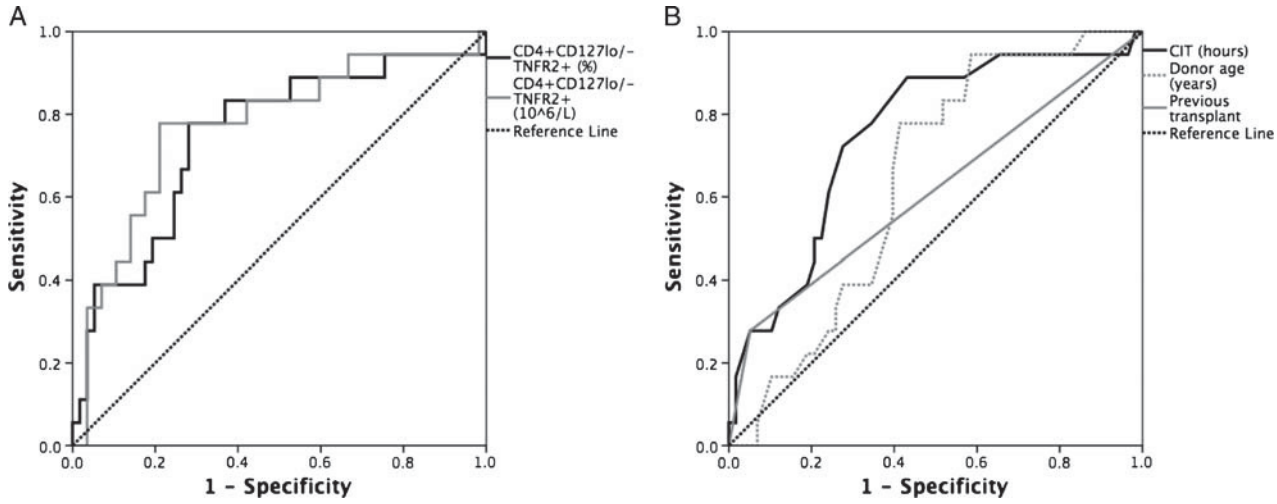

FIGURE 4. Predictors of DGF in ROC curve analysis. A, Pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg percentage (AUC, 0.75; 95% CI, 0.61-0.88, $P < 0.01$; optimal cutoff value, 3.32%; sensitivity, 77.8%; specificity, 71.9%; PPV, 46.7%; NPV, 91.1%) and absolute number (AUC, 0.77; 95% CI, 0.64-0.90; $P < 0.01$; optimal cutoff value, 37.13×10^6/L; sensitivity, 77.8%; specificity, 78.9%; PPV, 53.8%; NPV, 91.8%) predicted DGF in ROC curve analysis. B, Cold ischemic time (AUC, 0.75; 95% CI, 0.63-0.88; $P < 0.01$; optimal cutoff value, 14.5 hours; sensitivity, 88.9%; specificity, 56.9%; PPV, 39.0%; NPV, 94.3%) predicted DGF in ROC curve analysis, whereas donor age (AUC, 0.65; 95% CI, 0.52-0.77; $P = 0.07$) and previous transplant (AUC, 0.61; 95% CI, 0.45-0.78; $P = 0.15$) did not. 95% CI, 95% confidence interval; NPV, negative predictive value.

increasing their survival in an inflammatory environment.[42] Third, Treg cells can shed soluble TNFR2, which then acts as a decoy to decrease the availability of TNF-α to exert its proinflammatory activities.[43] Circulating TNFR2+ Treg cells could therefore be homing to the ischemia-reperfusion injured transplanted kidney and subsequently decrease damage by directly suppressing effector immune activity or by inhibiting the proinflammatory functions of TNF-α.

Similarly to the conventional pretransplant Treg cell–suppressive function assay, the pretransplant measurement of the most potently suppressive subset of circulating CD4+CD127lo/–TNFR2+ Treg cells was independently predictive of AKI (DGF + SGF), but with a lower accuracy, specificity, and PPV.[12] The addition of CD4+CD127lo/–TNFR2+ Treg cell to known clinical risk factors for AKI to form a predictive model improved the specificity and PPV, but did not improve the accuracy compared to using a model without CD4+CD127lo/–TNFR2+ Treg cell. Although clinical variables, such as donor age, are undoubtedly strong predictors of AKI, this could also be attributed to the fact that we limited our identification of the most potently suppressive subset of Treg cells to only 3 surface markers. This was done with the intentional purpose of optimizing cost-effectiveness, rapidity, and simplicity. The measurement of circulating CD4+CD127lo/–TNFR2+ Treg cell, from blood draw to flow cytometry analysis, can be done within 2 hours using a basic flow cytometer that is widely available for dedicated use in pretransplant immune monitoring and does not require complex fluorescence compensation. With the wider availability of multicolor flow cytometers and automatic compensation, identification of the most potent subset of Treg cells with additional described markers, such as CD45RA,[44] CTLA-4,[45] HLA-DR,[46] ICOS,[47] and CD62L[48] could increase the correlation between phenotypically identified potent Treg cells and

TABLE 3.

Sensitivity, specificity, and predictive value for predicting DGF or AKI using CD4+CD127lo/–TNFR2+ Treg

	Cutoff Value	Sens, %	Spec, %	PPV, %	NPV, %
Prediction of DGF					
CD4+CD127lo/–TNFR2+ Treg, %	1.49	27.8	96.5	71.4	80.9
	3.32[a]	77.8	71.9	46.7	91.1
	6.19	94.4	24.6	28.3	93.3
CD4+CD127lo/–TNFR2+, 10^6/L	19.26	33.3	96.5	75.0	82.1
	37.13[a]	77.8	78.9	53.8	91.8
	72.65	94.4	33.3	30.9	95.0
Prediction of AKI					
CD4+CD127lo/–TNFR2+ Treg, %	2.76	36.5	95.7	95.2	40.7
	4.27[a]	71.2	69.6	84.1	51.6
	6.79	94.2	39.1	77.8	75.0
CD4+CD127lo/–TNFR2+, 10^6/L	31.67[a]	38.5	95.7	95.2	40.7
	43.26	55.8	78.3	85.3	43.9
	110.35	94.2	26.1	74.2	66.7

[a] Optimal cutoff value based on the best sums of sensitivity and specificity.
NPV, negative predictive value; Sens, sensitivity; Spec, specificity.

TABLE 4.

Logistic regression analysis to predict AKI

Univariate Analysis			
Variables	**OR**	**95% CI**	***P***
CD4+CD127lo/–TNFR2+ Treg, %	0.69	0.55-0.86	<0.01
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.97-0.99	<0.01
CIT, h	1.09	1.01-1.18	0.05
Donor age, y	1.08	1.04-1.12	<0.01
ECD	6.82	2.04-22.78	<0.01
Previous transplant	3.58	0.42-30.86	0.25
Pretransplant dialysis			
None	1.00		
PD	1.43	0.18-11.09	0.73
HD	7.17	1.17-43.97	0.03
Multivariate analysis with CD4+CD127lo/–TNFR2+ (%)			
Variables	OR	95% CI	*P*
CD4+CD127lo/–TNFR2+ Treg (%)	0.72	0.55-0.94	0.02
CIT, h	1.08	0.94-1.23	0.27
Donor age, y	1.09	1.04-1.15	<0.01
Previous transplant	2.67	0.14-49.48	0.51
Pretransplant dialysis			
None	1.00		
PD	2.16	0.15-31.19	0.57
HD	22.54	1.67-303.43	0.02
Multivariate analysis with CD4+CD127lo/–TNFR2+, 10^6/L			
Variables			
CD4+CD127lo/–TNFR2+ Treg, 10^6/L	0.98	0.97-0.99	0.02
CIT, h	1.09	0.95-1.23	0.22
Donor age, y	1.10	1.04-1.17	<0.01
Previous transplant	1.80	0.09-36.24	0.70
Pretransplant dialysis			
None	1.00		
PD	1.69	0.12-23.98	0.70
HD	20.14	1.40-289.36	0.03

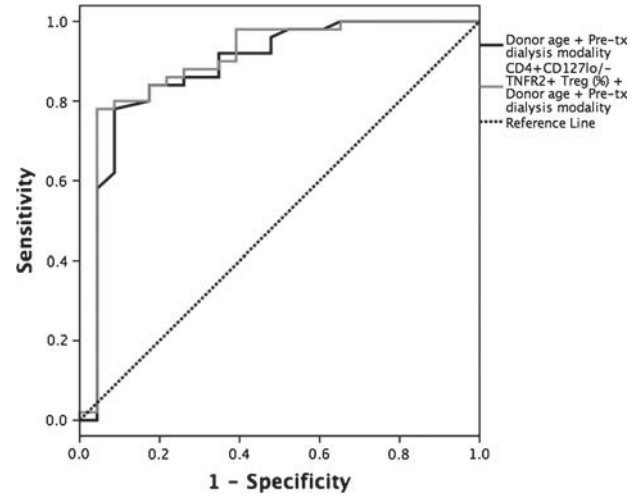

FIGURE 6. Prediction of AKI in ROC curve analysis using logistic regression models based on clinical variables alone (AUC, 0.88; 95% CI, 0.79-0.98; $P < 0.01$) or in combination with CD4+CD127lo/–TNFR2+ Treg cell percentage (AUC, 0.90; 95% CI, 0.81-0.99; $P < 0.01$).

the conventional Treg cell–suppressive function assay without compromising cost-effectiveness, and potentially improve the predictive value for AKI.

CD4+CD127lo/–TNFR2+ Treg cell, however, was not significantly different between DGF and SGF recipients, and could not independently predict DGF from non-DGF (IGF/SGF) recipients. Although DGF is considered the most severe form of ischemia-reperfusion injury, evidence suggests that the contribution of SGF to immunological outcomes and graft survival is more similar to DGF than IGF.[3,7,49] Moreover, the classification of graft function into DGF or SGF is based on a subjective decision to dialyze a recipient within the first week after transplantation. It is therefore possible that for 2 recipients with the same severity of ischemia-reperfusion injury, one was classified as DGF because the

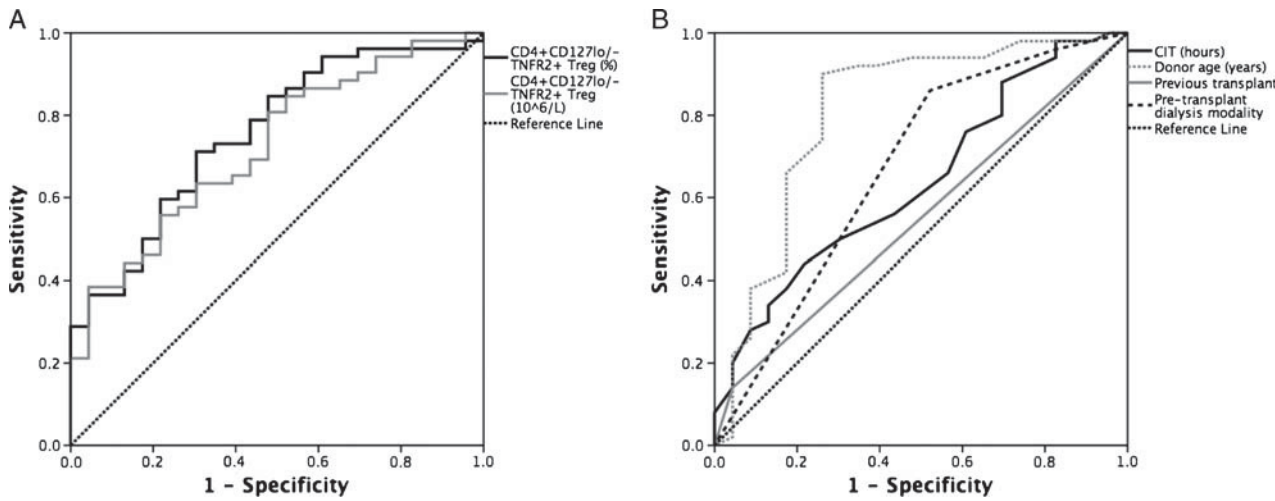

FIGURE 5. Predictors of AKI in ROC curve analysis. A, Pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg percentage (AUC, 0.76; 95% CI, 0.64-0.87; $P < 0.01$; optimal cutoff value, 4.27%; sensitivity, 71.2%; specificity, 69.6%; PPV, 84.1%; NPV, 51.6%) and absolute number (AUC, 0.72; 95% CI, 0.60-0.84; $P < 0.01$; optimal cutoff value, 31.67 × 10^6/L; sensitivity, 38.5%; specificity, 95.7%; PPV, 95.2%; NPV, 40.7%) predicted AKI in ROC curve analysis. B, Donor age (AUC, 0.81; 95% CI, 0.69-0.93; $P < 0.01$; optimal cutoff value, 46.5 years; sensitivity, 88.5%; specificity, 75.0%; PPV, 88.4%; NPV, 75.0%), expanded criteria donor category (AUC, 0.71; 95% CI, 0.58-0.83; $P < 0.01$; sensitivity, 57.7%; specificity, 83.3%; PPV, 88.2%; NPV, 45.5%), and pretransplant dialysis modality (AUC, 0.67; 95% CI, 0.53-0.81; $P = 0.02$; sensitivity, 86.0%; specificity, 47.8%; PPV, 78.2%; NPV, 61.1%) predicted AKI in ROC curve analysis, whereas cold ischemic time (AUC, 0.63; 95% CI, 0.49-0.76; $P = 0.08$) and previous transplant (AUC, 0.55; 95% CI, 0.41-0.68; $P = 0.52$) did not.

TABLE 5.

Sensitivity, specificity, and predictive value for predicting AKI using logistic regression models

Models	AUC (95% CI), *P*	Optimal Cutoff Value	Sens	Spec	PPV	NPV
Donor age + pretransplant dialysis modality	0.88 (0.79-0.98), <0.01	0.74	80.0	82.6	90.9	65.5
CD4+CD127lo/–TNFR2+ Treg (%) + donor age + pretransplant dialysis modality	0.90 (0.81-0.99), <0.01	0.76	78.0	95.7	97.5	66.7

clinical decision was to initiate dialysis but graft function was about to recover, whereas the other was classified as SGF because dialysis requirement was imminent but graft function recovered in time to avoid dialysis.

Our study is limited by the fact that it was conducted in a single institution with a small sample size. This could explain why CIT, donor age, and previous transplant were not predictors of both DGF and AKI in multivariate analyses. With regards to pretransplant dialysis modality, it was only predictive of AKI but not DGF. Recipients undergoing a preemptive transplant rarely develop DGF,[50] but could have a very limited residual native kidney function still making them susceptible to developing SGF. Although the 24% rate of DGF in our cohort was on par with the literature, we had a higher rate of SGF at 45%.[49] This could be related to the fact that almost 50% of our grafts are from ECDs. Because we recruited consecutive consenting patients in a prospective manner before transplantation at a time when their graft outcome was unknown, it is also possible that, by chance, more patients volunteering to participate in our study developed SGF. This volunteer effect could influence our results. Because of the urgent nature of deceased donor kidney transplantation, we also did not test the stability of circulating CD4+CD127lo/–TNFR2+ Treg cells by serial measures in the pretransplant setting. We acknowledge that this would be an important goal of a separate study.

Despite the limitations of our study, our results nevertheless indicate that circulating CD4+CD127lo/–TNFR2+ Treg cell is a potential novel immune marker for AKI. This is in fact the second study supporting the concept that a decreased pretransplant recipient circulating Treg cell–suppressive function is linked to AKI. External validation of circulating CD4+CD127lo/–TNFR2+ Treg cell as a predictor of AKI could eventually guide organ allocation and therapeutic interventions aimed at individual specific targets of Treg cell. For example, pretransplant infusion of maximally suppressive Treg cells isolated based on CD127 and TNFR2 expression could potentially decrease ischemia-reperfusion injury and decrease the risk for AKI. The TNFR2+ Treg cells could also be expanded ex vivo or in vivo with specific TNFR2 agonists.[51,52] Another therapeutic avenue would be to simulate soluble TNFR2 shedding by Treg cells with etanercept (fusion protein composed of the extracellular domain of TNFR2 and the hinge and Fc domains of human IgG1), which has been shown to dampen renal ischemia-reperfusion injury in mice.[53] This is in contrast with strong clinical predictors of AKI, such as donor age, which cannot be manipulated especially in the current context of organ demand and supply mismatch.

In conclusion, our findings suggest that pretransplant recipient circulating CD4+CD127lo/–TNFR2+ Treg cell is a potential alternative to the conventional Treg cell–suppressive function assay as an immune marker for AKI (DGF + SGF), independent of donor and organ procurement characteristics. Measuring pretransplant circulating CD4+CD127lo/–TNFR2+ Treg cells could therefore allow identification of recipients at risk for AKI before transplantation, and consequently guide organ allocation and AKI-targeted immunotherapies to specific measures of CD4+CD127lo/–TNFR2+ Treg cells.

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