Role of TNFR2-expressing regulatory T cells in recovery from acute renal ischemic injury

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December 2014

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master in Science in Experimental Surgery.

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Acknowledgements

This work was only possible due to the invaluable guidance, support, and encouragement provided by my supervisor, Dr. Steven Paraskevas. I would like to sincerely thank him for generously taking me on as an undergraduate student and supporting me through two summers and my Master's degree. He has been a dedicated mentor not only in research, but also in the clinical aspects of medicine.

Dr. Minh-Tri Nguyen has been an invaluable guide in my research, teaching me how to construct a project and follow through with experiments. He guided me as an undergraduate student and taught me all the techniques necessary for the completion of my project. I would like to express my gratitude for the time he took to pass on his knowledge, and for his dedication and patience as a teacher. Without him, I would not have had a project.

I would also like to thank Craig Hasilo for his support and guidance in my experimental work and in the writing of my thesis. He has been a great resource for discussing all things lab-related and non-lab-related.

Shuqing Liu has provided technical guidance and has helped me complete experiments that ran late into the night.

I would like to express my infinite gratitude to my parents, Mark and Hana, for supporting me throughout my studies and for their unwavering belief in me.

And last but not least, a huge thank you to Armita, Anna, and Scott for being wonderful friends throughout my Master's.

Thank you!

Abstract

Background: Ischemia reperfusion injury is the main cause of delayed graft function in kidney transplantation (DGF). DGF, part of a spectrum of post-transplant acute kidney injury (AKI), is highly detrimental to short- and long-term graft outcomes by increasing the risk for acute and chronic rejection. Regulatory T cells (Tregs) are conventionally identified by their expression of the surface markers CD4 and CD25 and the master transcription factor forkhead box P3 (FoxP3). Tregs are critical in immune tolerance of the graft and are able to attenuate murine AKI. The most highly suppressive subset has been shown to express tumour necrosis factor receptor type 2 (TNFR2). In this study, we evaluated whether pretransplantation Treg suppressive function predicts AKI and investigated the role of TNFR2 in AKI.

Methods: From 2010-2013, we enrolled 87 recipients of a deceased donor kidney transplant as part of an ongoing study. Of these, 52 experienced AKI (DGF or slow graft function (SGF)) and 24 had immediate graft function (IGF). Pretransplantation peripheral blood CD4+CD25^{hi}FoxP3+ and CD4+TNFR2+CD127- Treg frequency was quantified using flow cytometry. A Treg microsuppression assay was used to determine Treg functional activity. To investigate the role of TNFR2 in AKI, we also developed a murine model of unilateral autologous renal ischemia-reperfusion. All murine experiments were conducted with 7-12 week old C57BL/6 males and transgenics were developed on this background. TNFR2-/- x FoxP3-RFP+ mice were bred. Rag1 -/- recipients were adoptively transferred with either TNFR2 -/- Tregs or wild type Tregs and wild type effector T cells (Teffs).

Results: In human transplant recipients, pretransplantation Treg suppressive function and percentage of CD4+CD120b+CD127- Tregs was decreased in AKI recipients. CD120b on recipient peripheral blood Tregs correlated positively with the Treg suppression assay (r=0.38, *P*=0.02). Univariate and multivariate analyses accounting for donor age, cold ischemic time, and dialysis modality showed that pretransplantation CD4+CD120b+CD127- Treg percentage predicts AKI (odds ratio, 0.56; *P*=0.01; odds ratio, 0.57; *P*=0.04).

Conclusion: Our results suggest that people with higher Treg CD120b expression may be at lower risk of AKI following kidney transplantation.

Résumé

Informations de base: L'ischémie-reperfusion est la cause principale du retard de la reprise de fonction rénale, qui est une forme d'insuffisance rénale aiguë. Le retard de la reprise de fonction rénale est nuisible à la survie du greffe à court et à long terme en augmentant le risque de rejet aigu et chronique. Les cellules T régulatrices (Treg) ont classiquement été identifiés par l'expression des marqueurs de surface CD4 et CD25 et le facteur de transcription maître forkhead box P3 (FoxP3). Les Tregs jouent un rôle critique dans la tolérance immunitaire de la greffe et peuvent atténuer l'insuffisance rénale aiguë murine. Le sous-ensemble le plus fortement suppressive exprime le recépteur pour le facteur de nécrose tumorale type 2 (TNFR2). Nous avons évalué si la fonction suppressive des Tregs peut prévoir l'insuffisance rénale aiguë et nous avons étudié le rôle du TNFR2 dans l'insuffisance rénale aiguë.

Méthodes: Les bénéficiaires de donneur de greffe de rein décédés (n = 76) ont été divisés en groupes d'insuffisance rénale aiguë (n = 52) et de fonction immediate de la greffe (n = 24). Les Tregs CD4+ CD4+CD25^{hi}FoxP3+ et CD4+CD120b+CD127- pré-transplantation ont été quantifié par la cytométrie en flux. Des souris TNFR2-/- x FoxP3-RFP+ ont été développé et testé et les Tregs et Teffs ont été injecté dans des souris Rag1-/- pour tester un modèle d'insuffisance ischémique rénale aiguë.

Résultats: La fonction suppressive des Tregs prégreffe et le pourcentage de Tregs CD4+CD120b+CD127- a été diminué chez les receveurs avec l'insuffisance ischémique rénale aiguë. Il y a une corrélation positive entre le CD120b sur les Tregs et l'essai de suppression des Treg (r = 0,38, p = 0,02). Les analyses univariée et multivariée qui comprennent l'âge du donneur, le temps d'ischémie froide, et la modalité de dialyse ont

montré que le pourcentage de Tregs CD4+CD120b+CD127- prégreffe prédit l'insuffisance ischémique rénale aiguë (rapport de côtes, 0,56; *P*=0,01; rapport de côtes, 0,57; *P*=0,04).

Conclusion: Nos résultats suggèrent que le pourcentage de Tregs CD4+CD120b+CD127- prégreffe peut être utilisé pour prédire les receuveurs qui développeront l'insuffisance rénale aiguë.

Abbreviations

AKI: acute kidney injury

APC: antigen-presenting cell

ATN: acute tubular necrosis

AUC: area under the curve

CAPD: continuous ambulatory peritoneal dialysis

CCR: chemokine receptor (beta)

CIT: cold ischemic time

CK: contralateral kidney

CTLA4: cytotoxic T lymphocyte antigen 4

CXCR: chemokine receptor (alpha)

CD4: cluster of differentiation 4

DGF: delayed graft function

FoxP3: forkhead box P3

G-CSF: granulocyte-colony stimulating factor

GITR: glucocorticoid-induced TNF-receptor related protein

HD: hemodialysis

ICAM-1: intercellular adhesion molecule 1

IFN: interferon

IGF: immediate graft function

IK: ischemic kidney

IL: interleukin

IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IRI: ischemia-reperfusion injury

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

MUHC: McGill University Health Centre

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor κB

NK: natural killer cells

PGE2: prostaglandin E2

RFP: red fluorescent protein

ROC: receiver operating characteristic

RVH: Royal Victoria Hospital

SGF: slow graft function

TCR: T cell receptor

Teffs: effector T cells

Tfh: follicular helper T cells

Tregs: regulatory T cells

TGF: tumour growth factor

Th: helper T cells

TNF: tumour necrosis factor

TNFR2: tumour necrosis factor receptor type 2

TNFRSF: TNF receptor superfamily

Contributions

Under Dr. Steven Paraskevas' and Dr. Minh-Tri Nguyen's supervision, I have learned specific lab techniques and skills through both the delayed graft function study and the mouse study.

In the human clinical study, I recruited 34 patients (out of a total of 87 patients) between September 2012 and December 2013. My role involved explaining the study to patients and obtaining consent, collecting blood samples, performing Treg and Teff cell purification, and evaluating Treg suppressive function by flow cytometry analysis.

The mouse study allowed me to acquire knowledge of cell culture, cell isolation, adoptive transfer, flow cytometry, FACS sorting, mouse handling, mouse breeding, and mouse surgery (including the unilateral and bilateral renal ischemia reperfusion models). Over the course of a year, I developed and tested a line of TNFR2-/- x FoxP3-RFP+ mice. I bred four generations of mice to obtain male mice homozygous for both TNFR2 knock-out and FoxP3-RFP knock-in genes. Breeding involved planning which mice to cross, monitoring mice from birth through growth, genotyping F3 and F4 generations, and identifying mice for use in experiments. I isolated splenocytes and performed adoptive transfer of Treg and Teff cell ratios into Rag1-/- mice. I developed proficiency in a surgical procedure modeling murine ischemic injury that required bilateral clamping of the renal artery.

Introduction and Literature Review

- **Renal Transplantation and the Alloimmune Response**
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Renal Transplantation and the Alloimmune Response

Kidney transplantation is now considered the standard of care for treating patients who suffer from chronic kidney disease and who are in end stage renal failure (Merion, Ashby et al. 2005). Renal transplantation may be performed using kidneys from deceased or living donors. There has been a slight overall increasing trend towards living-donor transplantation, but deceased-donor transplantation is still predominant (Merion, Ashby et al. 2005).

The world's first successful kidney transplant was between identical twins, and was performed in Boston in1954. The recipient, Richard Herrick, lived for eight more years (Murray, Merrill et al. 1958). Dr. Joseph Murray headed the transplant team, and went on to win the Nobel Prize in Medicine in 1990 for his work in the field of transplantation. Montreal's Royal Victoria Hospital (RVH) was home to Canada's (and the Commonwealth's) first successful kidney transplant between identical twins in 1958, and the recipient, Moira Johnson, went on to live for another 29 years (Murray, Merrill et al. 1958). These first kidney transplants were done without need for immunosuppressive medication, but set a precedent for further development of renal allograft transplantation (Starzl, Rosenthal et al. 1983). Later on, in 1963, Canada's first kidney transplant between unrelated individuals also took place at the RVH, the kidney being from a deceased donor. The first powerful immunosuppressive agent, azathioprine, was debuted in 1963 and was routinely used in kidney transplantation throughout the 1960s (Starzl, Rosenthal et al. 1983). Since then, immunosuppressive regimens have greatly evolved as has the understanding of the complexities of the immune system.

The immune response to the transplanted tissue is the primary obstacle in organ transplantation. The concept of allograft rejection has evolved beyond its early classification into hyperacute, acute, and chronic processes, with the realization that all types share certain cellular and molecular pathways (Schwartz, Nankivell et al. 2010, Lakkis 2012). Hyperacute rejection is evident within minutes to hours after transplantation, and involves the deposition of pre-formed donor specific antibodies (Schwartz, Nankivell et al. 2010, Lakkis 2012). Acute rejection appears days to weeks later, but can occur at any time after a transplant (Schwartz, Nankivell et al. 2010, Lakkis 2012). It is often characterized by mononuclear cellular infiltrates of the graft, although antibody mediated processes may be involved simultaneously (Schwartz, Nankivell et al. 2010, Lakkis 2012). What was traditionally referred to as chronic rejection is now recognized to represent the end stage of a subclinical, low-grade immune response, often involving chronic antibody deposition, occurring over months to years (Halloran 2004, Schwartz, Nankivell et al. 2010, Lakkis 2012). These processes are accentuated by tissue remodeling, collagen and extracellular matrix deposition leading to fibrosis, and epithelial-mesenchymal transition in tubular cells. Vascular changes are characteristic of chronic rejection, leading to impaired perfusion which ultimately results in ischemia and organ atrophy. Although acute rejection can respond to immunosuppressive therapy, chronic rejection typically is not improved via increased immunosuppression (Tilney, Schmid et al. 1994, Halloran 2004, Schwartz, Nankivell et al. 2010).

While there have been improvements in short-term allograft survival, long-term graft survival has not seen significant developments (Meier‐Kriesche, Schold et al. 2004, Meier‐Kriesche, Schold et al. 2004). Not all renal allografts have the same

survival time, and certain factors, such as the onset of delayed graft function (DGF), and ensuing inflammation, may predispose a transplanted organ to decreased life expectancy (Perico, Cattaneo et al. 2004, Yarlagadda, Coca et al. 2008, Yarlagadda, Coca et al. 2009, Schwartz, Nankivell et al. 2010).

Delayed Graft Function

Renal function post-transplantation can be classified as immediate graft function (IGF), slow graft function (SGF), and delayed graft function (DGF), thus constituting the spectrum of ischemia reperfusion injury. There is no formal definition of DGF, but by convention, it has generally been defined as the need for dialysis following a renal transplant (Perico, Cattaneo et al. 2004, Hassanain, Tchervenkov et al. 2009). SGF, on the other hand, is also not formally recognized, but considered to be a state of milder injury in which dialysis is not needed, but serum creatinine does not decline (Humar, A, Clinical Transplantation, 2002).

DGF is a significant complication of kidney transplantation, and has been noted to occur in 5-50% of deceased donor kidney transplants (Sellers, Gallichio et al. 2000, Perico, Cattaneo et al. 2004, Daly, Power et al. 2005). It is considered a form of acute kidney injury (AKI), and causes complications in post-transplant management, increases morbidity, prolongs patient hospitalization and increases health care costs (Perico, Cattaneo et al. 2004, Daly, Power et al. 2005, Yarlagadda, Coca et al. 2008, Yarlagadda, Coca et al. 2009). Furthermore, DGF increases the risk of acute and chronic graft rejection, as well as the risk of chronic allograft nephropathy and premature graft loss (Yarlagadda, Coca et al. 2008, Yarlagadda, Coca et al. 2009). SGF is a milder form of

AKI and does not have a requirement for dialysis. Although there have been significant advances in the field of transplantation since 1950, there have been no significant therapeutic advances with respect to the prevention or treatment of DGF (Yarlagadda, Coca et al. 2008, Siedlecki, Irish et al. 2011). Three major categories of factors are associated with DGF: donor, recipient, and transplant procedure (Perico, Cattaneo et al. 2004, Daly, Power et al. 2005). Major risk factors associated with DGF include donor age, cold ischemic time, and recipient hypotension (Siedlecki, Irish et al. 2011). Some risk factors which have been identified for DGF are principally those related to either the health of the kidney or the transplant procedure itself. These include higher donor age, longer cold ischemia time and intraoperative hypotension. To date, the association with specific recipient factors has been weak, including such variables as obesity, diabetes and African-American race. Well-defined risk factors in the recipient have thus far been unknown.

Ischemia Reperfusion Injury

The major cause of DGF is ischemia reperfusion injury (IRI) (Siedlecki, Irish et al. 2011). There are, however, many variables, such as serum electrolyte levels, fluid balance and urine output, which determine the need for dialysis, and consequently the decision to utilize it may be somewhat subjective (Perico, Cattaneo et al. 2004, Daly, Power et al. 2005). In a patient with DGF, the transplanted kidney is not completely functional, and the damage in the kidney induces the tubular cells to undergo turnover and regeneration (Siedlecki, Irish et al. 2011). Cells undergo both apoptosis and necrosis, with the latter producing an array of inflammatory mediators that chemotactically attract

more cells to the site of injury (Tilney, Schmid et al. 1994, Siedlecki, Irish et al. 2011). This innate immune response increases antigen release and presentation to cells of the acquired immune response. This increases the risk of a subsequent acute allograft rejection. Decreasing the incidence of DGF is crucial in the effort to increase kidney graft survival (Siedlecki, Irish et al. 2011). Although there is currently no way to physically quantify DGF, its clinical significance lies in the possible prediction of reduced long-term survival of the graft (Daly, Power et al. 2005, Hassanain, Tchervenkov et al. 2009, Yarlagadda, Coca et al. 2009).

CD4+ T Cells

The human immune response has conceptually been divided into two principal classes. The innate immune response is immediate yet non-specific, while the adaptive response is developed and antigen-specific. The adaptive immune system retains information in immunological memory, and responds quickly and specifically upon subsequent exposure to the same antigen. Furthermore, a core feature of these two systems is that they both involve humoral and cell-mediated components. Humoral responses are mediated by macromolecules such as complement proteins, secreted antibodies, and antimicrobial peptides. Cell-mediated responses are mediated by the activation of phagocytes, cytotoxic T cells, and cytokine release. Cytotoxic T cells directly cause target cell apoptosis, whereas helper T cells are able to recruit multiple cell types in order to protect against various pathogens (Tilney, Schmid et al. 1994, Schwartz, Nankivell et al. 2010).

White blood cells include cells of both myeloid and lymphoid lineages; those derived from the latter being further categorized as natural killer (NK) cells, B lymphocytes, and T lymphocytes. T cells are essential in cell-mediated adaptive immunity, maturing in the thymus into different subsets, each with a distinct role. T helper cells have a glycoprotein, called cluster of differentiation 4 (CD4) found on their surface (Tilney, Schmid et al. 1994). CD4 is also found on the surface of monocytes, macrophages, and dendritic cells and is essential for the proper functioning of the adaptive immune system (Tilney, Schmid et al. 1994, Park, Li et al. 2005). Depletion of CD4+ T cells prior to an organ transplant is important in avoiding acute rejection, but leaves the body susceptible to infection (Jones, Ha et al. 2002, Wood and Sakaguchi 2003).

Antigen-presenting cells (APCs) present antigen via major histocompatibility complex (MHC) II to T helper (Th) cells. Through the release of various specific cytokines at the site of inflammation, naïve CD4+ Th cells process the signal from the APC and are guided to divide and differentiate into one of several subtypes, specifically Th1, Th2, Th3, Th17, Th9, follicular helper T cells (Tfh), or regulatory T cells (Tregs) (Afzali, Lombardi et al. 2007, Gutcher and Becher 2007). Effector T cells (Teffs) include Th1, Th2, Th3, Th17, Th9 subtypes. Each subtype produces different cytokines to direct different immune responses (Afzali, Lombardi et al. 2007, Gutcher and Becher 2007). In humans, there is no distinct Th17 lineage, although T effector (Teff) cells secreting interleukin-17 (IL-17) have been identified (Afzali, Lombardi et al. 2007, Koenen, Smeets et al. 2008). Each subset of Th cells produces a signature cytokine and is characterized by a specific transcription factor (Koenen, Smeets et al. 2008). Th17 is

characterized by *RORγt*, and Tregs are characterized by forkhead box P3 (FoxP3) (Solez, Axelsen et al. 1993, Afzali, Lombardi et al. 2007).

The Balance of Tregs and Teffs

Tregs have been shown to be anti-inflammatory, and their role appears to be to modulate or terminate an immune response. For this reason, it is thought that they can potentially extend the life of the allograft (Afzali, Lombardi et al. 2007). Evidence in knockout mice and humans with the rare IPEX disorder indicates that normal function of Tregs includes not only suppression of Teff function, but the normal termination of the Teff response to foreign antigens. Therefore, precisely controlled balance between Tregs and Teffs is essential to proper functioning of the immune response. Recent evidence in mice has shown that naive Th cells can be induced to differentiate into either Teff or Treg cells, thus favouring either the pro-inflammatory or the anti-inflammatory pathways (Loverre, Divella et al. 2011). While the differentiation into either lineage is mutually exclusive in mice, there is no evidence that this phenomenon also occurs in humans (Weaver, Harrington et al. 2006, Afzali, Lombardi et al. 2007). Skewing naive CD4+ Th cells towards a pro-inflammatory lineage, namely Teff cells, and away from an anti-inflammatory pathway like Treg cells, may be central to allograft rejection. Consequently, the promotion of Tregs and the suppression of Teff cells may improve allograft survival (Afzali, Lombardi et al. 2007).

Treg Cells

While their role had been hypothesized many years earlier, the existence of the Treg phenotype was demonstrated in 1995, when it was discovered that mouse Tregs constitutively express CD25 (Sakaguchi, Sakaguchi et al. 1995). Based on this finding, human Tregs were first defined as CD4+CD25+ T cells (Sakaguchi, Sakaguchi et al. 1995). It was subsequently found that FoxP3-expressing Tregs show downregulated levels of the IL-7 receptor CD127 (Liu, Putnam et al. 2006). CD4+ T cells expressing little or no CD25 have similar levels of CD127. Liu et al demonstrated that using the combination of surface markers CD4, CD25, and CD127 to identify Treg cells allowed for the identification of a highly purified subset of Tregs and contained a larger number of cells compared to Tregs isolated with other surface markers (Liu, Putnam et al. 2006). Furthermore, this subset of cells demonstrated high suppressive capacity (Liu, Putnam et al. 2006). Treg cells were identified in this study by their CD4+CD25+CD127- status.

Tregs are produced in the thymus in response to self-antigens and mediate tolerance in the periphery (Sakaguchi, Sakaguchi et al. 1995, Ohkura, Kitagawa et al. 2013). Treg cells with both a Treg phenotype and suppressive function used to be restricted to a subset of Tregs identified by the surface markers CD4 and CD25(hi). Today, the accepted identification strategy for Tregs consists of the surface markers CD4 and CD25 (the IL-2 receptor α chain), and the intracellular transcription factor FoxP3 (Ohkura, Kitagawa et al. 2013).

IL-2 is crucial to Treg survival and function, and the loss of either IL-2 or FoxP3 results in deficiency in Treg numbers or lethal early-onset inflammatory disease (Buckley 2004, Malek and Bayer 2004, Chatila 2005). In the presence of antigen, the cytokines IL-2 and TGF-β can induce naïve T cells to become Tregs (Chen, Jin et al. 2003, Zheng,

Wang et al. 2007). Tregs suppress cells of both the innate and adaptive immune systems, either directly via cell-cell contact or indirectly via soluble mediators (Wood and Sakaguchi 2003). Activated T cells can be directly suppressed by Fas ligand-mediated and granzyme B signaling pathways (Gondek, Lu et al. 2005, Baatar, Olkhanud et al. 2007). They can be indirectly suppressed by cytokine deprivation that leads to apoptosis, or by uptake of IL-2 (Pandiyan, Zheng et al. 2007). Tregs also secrete soluble factors including IL-10, TGF-β, and IL-35 (Shevach 2009). They constitutively express the adhesion molecules integrin α_E, CD62L, CD44, and various selectin ligands, as well as the chemokine receptors CCR2, CCR4, CCR5, CCR6, CCR7, and CXCR3 (Huehn, Siegmund et al. 2004). These molecules allow Tregs to reach the site of inflammation (Ring, Schäfer et al. 2006, Maganto-García, Bu et al. 2011).

Tregs are an essential part of the immune system that guards against excessive damage to tissues from ongoing inflammation, including the documented effects of ischemia-reperfusion in a wide variety of tissues. There are two populations of Tregs, those produced in the thymus (natural Tregs) and those produced in the periphery (inducible Tregs) (Lan, Ansari et al. 2005).

Treg Cells and Acute Kidney Injury

Tregs are known to home to sites of inflammation, and this is true of course, in all post-ischemic tissues, including the kidney. In the context of kidney injury, deficiency of Tregs has been shown to result in enhanced renal inflammation and tissue injury, acute tubular necrosis (ATN), and loss of renal function (Kinsey, Sharma et al. 2013). Tregs have been shown to traffic to the site of acute injury within the kidney to promote repair

after ischemic injury (Gandolfo, Jang et al. 2009). The adoptive transfer of Tregs from a naïve wild-type mouse into another naïve wild-type mouse prior to ischemic injury was able to protect against renal injury and dysfunction (Kinsey, Huang et al. 2010). Furthermore, the mice that received Tregs showed prolonged survival (Kinsey, Huang et al. 2010). Kinsey et al. showed that adoptive transfer of clinically relevant doses of Tregs prior to ischemic injury in a mouse model was protective against renal injury (Kinsey, Huang et al. 2010). Adoptive transfer of Tregs 24 hours after acute injury resulted in increased number of Tregs trafficking to the site of renal injury (Kinsey, Huang et al. 2010). It also resulted in decreased Teff production of IFN-γ and TNF-α within the kidney (Kinsey, Huang et al. 2010). The repair phase of renal ischemic injury was characterized by increased trafficking of Tregs to the site of inflammation and promotion of healing (Kinsey, Huang et al. 2010). Often, AKI is only discovered about 24 hours after renal injury has already occurred, thus validating the clinical relevance of this study (Gandolfo, Jang et al. 2009). This work suggests that therapeutic adoptive transfer of Tregs in humans may be possible as a means of protection against acute renal injury.

Regulatory T Cells and Rejection

The role of Tregs is to suppress the activation, proliferation, and effector functions of Teff cells. In vitro, Treg cells (CD4+ CD25+FoxP3+) can inhibit production and release of cytokines by CD4+ CD25- T cells, as well as down-regulate the responses of CD8+ T cells, NK cells, and CD4+ cells (Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009, Li and Turka 2010). Tregs exert their suppressive function both in vitro and in vivo on CD4+ and CD8+ T cells, NK cells, B cells, and APCs. A recent

study in vivo showed that Treg cells have the potential to prevent allograft rejection in mice (Presser, Sester et al. 2009). The ability of CD4+CD25+FoxP3+ cells to control immune responses makes them important in the maintenance of allograft tolerance.

Long-term graft survival has not seen a significant improvement, despite recent drug research and development. Currently, research is being conducted on Tregs, since they suppress alloreactive effector T cells, although their mechanism of action is still not completely understood (Afzali, Lombardi et al. 2007). Tregs can also suppress other types of immune cells, such as B cells, monocytes, and dendritic cells. They express an antigen-specific receptor, and when they interact with their particular ligand, they migrate to the site of inflammation. The balance between Tregs and Teffs is thought to be crucial to the development or rejection or to allograft tolerance (Afzali, Lombardi et al. 2007, Li and Turka 2010, Waldmann 2010, Zhao, Qiu et al. 2010).

Activated Tregs can suppress non-antigen-specific donor effector cells, a mechanism termed "bystander suppression" (Maloy and Powrie 2001, Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009). It has been suggested that Tregs exhibit specificity by suppressing cells they encounter at the site of inflammation (Afzali, Lombardi et al. 2007). Also, tolerant and healthy individuals appear to have higher levels of Tregs and FoxP3 mRNA as opposed to patients with chronic rejection, who show much lower levels of Tregs and FoxP3 mRNA (Muthukumar, Dadhania et al. 2005).

TGF-β promotes Tregs, while TGF-β together with IL-6 skew towards Th17 cells (Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009, Loverre, Divella et al. 2011). The blockade of cytokines involved in Th17 development, especially IL-6, could

potentially lessen the immune reaction involved in allograft rejection (Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009).

Th17 Cells and IL-17

Although IL-17 is a ubiquitous cytokine produced by various cell types, its main source is CD4+ memory T cells (Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009, Li and Turka 2010). IL-17 has pro-inflammatory properties, and is therefore heavily associated with inflammatory and autoimmune diseases in both mice and humans (Afzali, Lombardi et al. 2007, De Serres, Sayegh et al. 2009, Li and Turka 2010). IL-17 is a pleiotropic cytokine that acts through several mechanisms to increase inflammation (Afzali, Lombardi et al. 2007). IL-17 induces IL-6, IL-8, and the C3 opsonin (Fossiez, Djossou et al. 1996, Van Kooten, Boonstra et al. 1998). It stimulates production of nitric oxide, a vasodilator; IL-6, another pro-inflammatory cytokine; and prostaglandin E2 (PGE2) (Yao, Fanslow et al. 1995, Attur, Patel et al. 1997). IL-17 can also act synergistically with other pro-inflammatory cytokines such as IFN-ɣ and TNF-α (Albanesi, Cavani et al. 1999). This ultimately results in increased inflammation caused by increased gene expression. The up-regulation in gene expression leads to an increase in other inflammatory mediators, such as IL-8, which mediates chemotaxis, and granulocyte-colony stimulating factor (G-CSF), a hematopoietic growth factor which promotes growth and development of myeloid cells at the site of inflammation (Fossiez, Djossou et al. 1996, Starnes, Broxmeyer et al. 2002). IL-17 also promotes T cell activation by causing other cytokines to induce the production of co-stimulatory molecules such as ICAM-1 (Yao, Painter et al. 1995). In addition, IL-17 is a co-

stimulator for T cells, and it also regulates proliferation, maturation, and chemotaxis of neutrophils (Zhang, Zhang et al. 2008).

FoxP3

Approximately 5-10% of CD4+ T cells also express CD25 (Chen, Subleski et al. 2010). Between 1 and 3% of CD4+ T cells express FoxP3 and are considered to be Tregs with suppressive function (Chen, Subleski et al. 2010). In 2003, FoxP3 was shown to be the master regulator of mouse Treg cell development and function, and has since been confirmed as a specific marker of Treg cells in humans (Fontenot, Gavin et al. 2003). FoxP3 is a nuclear transcription factor and the master regulator controlling the development and function of Treg cells. It is found on the p arm of the X chromosome (Zheng and Rudensky 2007). FoxP3 suppresses key T cell cytokine genes, such as those encoding IL-2, IL-4, and IFN-γ, thus preventing Tregs from producing these cytokines (Sakaguchi, Sakaguchi et al. 1995, Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003). It also suppresses the response elements linked to nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT) (Bettelli, Dastrange et al. 2005).

The central role of FoxP3 in Treg suppressive capacity was first shown in scurfy mice (Brunkow, Jeffery et al. 2001, Khattri, Cox et al. 2003). Scurfy mice have a spontaneous mutation in the FoxP3 gene, and this genetic anomaly results in lymphoproliferative disease which causes death after approximately 4 weeks (Brunkow, Jeffery et al. 2001). The role of FoxP3 was subsequently shown in patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett, Christie et al. 2001). IPEX is very similar to the diseases observed in mice

following CD4+CD25+ Treg cells depletion (Bennett, Christie et al. 2001). Immunopathologies resulting from FoxP3 deficiency are due to unchecked activation and proliferation of Teff cells (Sakaguchi, Yamaguchi et al. 2008, Sakaguchi, Miyara et al. 2010). When FoxP3 is ectopically expressed in peripheral CD4+CD25- T cells, these cells gain the suppressive function associated with Treg cells (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Khattri, Cox et al. 2003). They also gain the ability to express the Treg signature molecules CD25, cytotoxic T lymphocyte antigen 4 (CTLA4), and glucocorticoid-induced TNF-receptor related protein (GITR) (Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Khattri, Cox et al. 2003). Furthermore, the expression of these Treg signature molecules correlates with FoxP3 expression in human CD4+ T cells (Yagi, Nomura et al. 2004).

Since FoxP3 is intracellular, it is not a good marker for the detection of Treg suppressive function. In naïve mice, CD4+FoxP3+ Treg cells can be identified by the expression of CD25. The population of human Tregs, however, is heterogeneous, both with respect to phenotype and function (Sakaguchi, Sakaguchi et al. 1995). In humans, FoxP3 expression is proportional to CD25 expression in FoxP3+ Treg cells (Baecher-Allan, Brown et al. 2001, Sakaguchi, Miyara et al. 2010). Only 1-2% of cells that have the highest expression of CD25 have functional suppressive capacity (Baecher-Allan, Brown et al. 2001, Sakaguchi, Miyara et al. 2010). Cells with intermediate expression of CD25 tend to have low levels of FoxP3 and are considered naïve T cells (Sakaguchi, Miyara et al. 2010). However, it should be noted that to date there is no clear distinction between high, intermediate, and low levels of CD25 expression. Furthermore, under

inflammatory conditions, activated T effector cells may express CD25 and be misidentified as Tregs (Sakaguchi, Miyara et al. 2010).

Treg Suppressive Mechanisms

The molecular mechanisms by which Tregs are functionally suppressive are poorly understood. As mentioned previously, Tregs are able to modulate the cytokine microenvironment, disrupt metabolic activity of the target cell, and alter the ability of dendritic cells to activate other immune cells.

TNF

Tumour necrosis factor (TNF) is a proinflammatory cytokine which regulates immune cells (Wakefield, James et al. 1991, Aggarwal 2003). It is mainly produced by activated macrophages but can also be produced by CD4+ lymphocytes and NK cells (Wakefield, James et al. 1991). TNF interacts with two receptors, TNF receptor type 1 (TNFR1; CD120a) and TNF receptor type 2 (TNFR2; CD120b) (Wajant, Pfizenmaier et al. 2003). While TNFR1 is ubiquitously expressed, TNFR2 is primarily expressed on lymphocytes (Wajant, Pfizenmaier et al. 2003). TNFR1 mediates the pro-apoptotic and cytotoxic effects of TNF, and can be activated by both membrane-bound and soluble trimers of TNF (Locksley, Killeen et al. 2001, Aggarwal 2003). Upon ligand binding to the receptor, TNFR1 receptors form a trimer and mediate the TNF signal via an intracellular death domain, TNFR1-associated death domain (TRADD) (Naismith and Sprang 1998, Wajant, Pfizenmaier et al. 2003). This signal activates NF-κB and

mitogen-activated protein kinase (MAPK), and induces death signaling (Perkins 2000, Wajant, Pfizenmaier et al. 2003).

TNFR2 is primarily involved in cellular activation, proliferation, and survival pathways, and lacks a death domain (Grell, Douni et al. 1995). In contrast to TNFR1, TNFR2 transduces negative feedback in inflammatory environments in response to TNF binding (Chen, Bäumel et al. 2007). In wild type mice, 30-40% of Tregs in the periphery express TNFR2, whereas only 10% of Teff cells express TNFR2 (Chen, Subleski et al. 2010, Chen and Oppenheim 2011). In addition, there are fewer TNFR2 receptors per cell on Teff cells than on Treg cells (Chen and Oppenheim 2011). TNFR2-expressing CD25+ or FoxP3+ cells form the most potently suppressive subset of Tregs, whereas TNFR2- Tregs in wild type mice, regardless of whether they express CD25 or FoxP3, show minimal or no suppressive capacity (Chen, Subleski et al. 2008). In response to inflammatory stimuli in vivo, Tregs lacking TNFR2 are unable to control the immune response whereas TNFR2+ Tregs proliferate (Chen, Subleski et al. 2008). It has been demonstrated that even within the CD4+CD25-FoxP3+ subset, TNFR2+ Tregs have potent suppressive capacity (Chen, Subleski et al. 2008).

In humans, thymically produced Tregs, or natural Tregs, constitutively express TNFR2 whereas thymically produced Teffs do not express TNFR2 (Annunziato, Cosmi et al. 2002, Chen, Bäumel et al. 2007). FoxP3+ cells in the periphery have higher levels of TNFR2, independent of the level of CD25 expression (Chen, Bäumel et al. 2007). Approximately 70% of FoxP3+ Tregs expressed TNFR2 compared to 20% of FoxP3- Teffs (Chen and Oppenheim 2011, Chen and Oppenheim 2011).

In conditions of ongoing inflammation, such as in malaria, TNFR2 expression is

up-regulated and can indicate a subset of more highly suppressive Tregs (Chen, Subleski et al. 2010, Chen and Oppenheim 2011). In patients with rheumatoid arthritis, TNFR2 is up-regulated on Tregs within the synovial fluid (Chen and Oppenheim 2011). This finding has been interpreted to mean that these Tregs have enhanced suppressive capacity (Chen and Oppenheim 2011).

It was recently shown that almost all human CD4+CD25^{high}FoxP3+ cells (97.4-100%), as well as a large proportion of CD4+CD25^{low}FoxP3+ cells (approximately 34%), express TNFR2 (Chen, Subleski et al. 2010). The population of CD4+CD25$^{high/low}$TNFR2+ cells includes a substantially higher amount of FoxP3+ cells than the CD4+CD25+ subset (Chen, Subleski et al. 2010). Cells identified as CD4+CD25+TNFR2+ exhibited Treg suppressive capacity (Chen, Subleski et al. 2010, Chen and Oppenheim 2011). They expressed high levels of FoxP3 and, at a ratio of 1:1, were able to suppress proliferation of CD4+CD25-TNFR2- Teff cells by greater than 60% (Chen, Subleski et al. 2010). The same study showed that at a ratio of 1:1 Treg to CD4+ Teff cells, CD4+CD25+TNFR2+ cells almost completely suppressed IFN-γ production (Chen, Subleski et al. 2010).

The mechanism by which CD4+CD25+TNFR2+ Tregs mediate suppression is unknown. CTLA-4 is found in high levels on both human and mouse CD4+CD25+TNFR2+ cells, and has been shown to have a role in the suppressive capacity of Tregs (Wing, Onishi et al. 2008, Chen and Oppenheim 2011).

When stimulated with IL-2 and anti-CD3, both human and mouse Tregs shed large quantities of soluble TNFR2 (sTNFR2) in vitro (Chen, Subleski et al. 2010). CD4+CD25+ Treg cells are anergic to TCR stimulation and are capable of secreting anti-

inflammatory cytokines such as IL-10 and TGF-β (Tang, Boden et al. 2004, von Boehmer 2005, van Mierlo, Scherer et al. 2008). The adoptive transfer of Tregs decreases levels of acute phase proteins (van Mierlo, Scherer et al. 2008). TNF is one of the most potent activators of the acute phase reaction and acts via IL-6 to promote the release of several acute phase proteins from the liver (van Mierlo, Scherer et al. 2008). CD4+CD25+ Tregs isolated from wild-type mice reduce IL-6 production, whereas CD4+CD25+ Tregs isolated from TNFR2-/- mice do not have this effect (Epstein, Gabay et al. 1999, van Mierlo, Scherer et al. 2008). This data shows that TNFR2+ Tregs can block TNF and reduce inflammation by shedding sTNFR2. TNF transiently silences the suppressive capacity of Treg cells, which is restored several days after their activation (van Mierlo, Scherer et al. 2008).

TNF has shown unexpected immunosuppressive effects (Chen, Bäumel et al. 2007). For example, mice deficient in TNF have more severe inflammation in models of chronic inflammation (Cope, Liblau et al. 1997, Zganiacz, Santosuosso et al. 2004, Zakharova and Ziegler 2005). It was proposed that the pro-inflammatory effects of TNF derive from the uncoupling of Teffs from Treg-mediated suppression (Chen, Bäumel et al. 2007). Prolonged exposure to TNF, however, has been shown to be protective in some autoimmune states (Cope, Liblau et al. 1997, Cope 1998, Zakharova and Ziegler 2005, Chen, Bäumel et al. 2007). Short-term exposure of Teff and Treg co-cultures to TNF results in Teff proliferation, whereas longer exposure to TNF restores Treg-mediated suppression of Teff cells (Chen, Subleski et al. 2010). TNF synergizes with IL-2 to enhance activation of Tregs (Chen, Bäumel et al. 2007).

The precise mechanism of action of TNFR2-expressing Treg suppression is unknown and remains to be understood. It is thought that TNFR2 acts as a co-stimulator for antigen-driven T cell responses (Aggarwal 2003). TNF together with IL-2 promotes proliferation of both mouse and human FoxP3+ Tregs, suggesting a role for TNFR2 in the expansion of Treg cells (Chen, Bäumel et al. 2007). Teff cells react quicker to TNF than do Treg cells, allowing inflammation to occur. At the peak of Teff infiltration, Tregs are activated and mediate suppression of Teff cytokine production and proliferation (Chen, Bäumel et al. 2007). TNF therefore has a dual role in inflammation; it can act as a pro-inflammatory agent to promote removal of antigens and it can act as an anti-inflammatory agent to suppress the immune response. Interestingly, natural Tregs seem to require TNF signaling to mediate TNFR2-expressing Treg suppression, while inducible Tregs may use a different mechanism of suppression (Housley, Adams et al. 2011).

Both human and mouse activated Tregs produce high levels of soluble TNFR2 which has been shown to contribute to the suppressive capacity of TNFR2-expressing Tregs. In addition, in both mice and humans, the interaction of TNF with TNFR2 increases FoxP3 expression.

Other members of the TNF receptor superfamily (TNFRSF) are preferentially expressed by either resting or activated Tregs. These molecules include GITR, 4-1BB, and OX40 (Chen and Oppenheim 2011). Some of these molecules are also expressed by activated Teff cells, and thus, a more specific marker of Tregs, especially functional Tregs, is necessary.

Purpose of Study

Recently, TNFR2 has been shown to identify a highly suppressive subset of CD4+CD25+FoxP3+ Treg which suppresses the activation of Teffs by neutralizing TNF either by membrane TNFR2-TNF interaction or by shedding of soluble TNFR2 (sTNFR2). Preliminary data from our lab also showed a reduction in TNFR2 expression on the Tregs of kidney transplant recipients suffering from AKI. Whether TNFR2 expression on Tregs is critical for reducing AKI is however unknown. We hypothesized that TNFR2 expression on Tregs is essential in Treg-mediated protection from AKI.

Our lab designed and conducted an observational study in deceased donor renal transplant recipients to investigate whether pretransplantation recipient Tregs and their suppressive function predicts AKI and short-term outcomes post-transplantation. Dr. Minh-Tri Nguyen established a protocol for evaluating Treg suppressive function in kidney transplant patients. In the first phase of the trial, we recruited 53 patients into this study and recently published the data (Nguyen, Fryml et al. 2014).

As a follow-up to the human clinical study, we designed a project to evaluate the frequency and percentage positivity of TNFR2 on Tregs. We found a correlation between a high percentage of TNFR2 expression and protection from AKI.

We also used a murine model to test whether TNFR2 is important in Treg-mediated suppression of the immune response. The objectives were to 1) develop and test a mouse line that is both TNFR2-/- and FoxP3-RFP+; and 2) use a mouse model of renal ischemia reperfusion injury to quantify infiltration of TNFR2-/- x FoxP3-RFP+ Treg cells into ischemic kidneys.

Materials and Methods:

- **DGF Study**
    - **Patients**
    - **Human Peripheral Blood Mononuclear Cell Isolation**
    - **PBMC Stimulation**
    - **Flow Cytometry Acquisition and Analysis**

- **Mouse Model**
    - **Mice**
    - **CD4+ T Cell Isolation from Mouse Splenocytes**
    - **Adoptive Transfer and Mouse Model of Renal Ischemic Injury**

DGF Study

Patients

Adult patients undergoing a first deceased donor kidney transplant were approached upon admission for the transplant and consented to this study through the Multi-Organ Transplant Program at the MUHC. The protocol to obtain human blood samples from patients was approved by the McGill University Health Centre Research Ethics Board and registered on ClinicalTrials.gov (NCT01232816). A sample of 40 ml of freshly drawn blood was obtained from each patient at day 0 (prior to transplantation), day 180, and day 365. During the second phase of the trial, we recruited a further 34 patients, to bring the total recruitment in this study to 87.

Human Peripheral Blood Mononuclear Cell Isolation

The blood was collected in heparin-coated tubes and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. Blood was diluted in a 1:1 volume with phosphate buffered saline (PBS) pH 7.2 and carefully layered over 14 ml Lymphocyte Separation Medium (Wisent, QC, CAN). The sample was centrifuged at 1200 rpm for 20 minutes at 20 °C. The buffy layer containing PBMCs was transferred to another tube using a pipette and washed twice in PBS at 300 rpm for 10 minutes. Cells were resuspended in PBS counted using Trypan Blue. Cells were split into 2 fractions, for staining for Th17 or Treg cells.

PBMC Stimulation of Th17 Cells

The Th17 fraction of cells was cultured in X-Vivo 15 Media (Lonza, NJ, USA) supplemented with 10% human AB serum (Sigma, ON, CAN) stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ ml) (Sigma, ON, CAN), ionomycin (1 ug/ ml) (Sigma, ON, CAN), and monensin (Ebioscience, CA, USA) in X-Vivo culture medium (Sigma-Aldrich, ON, CAN) in a 6-well plate for 5 hours at 37 °C in a sterile incubator. After 5 hours, these cells were washed twice in PBS and divided up equally into 3 FACs tubes. Three samples were stained for surface markers as follows: 1) unstained; 2) FITC anti-CD4, 3) FITC anti-CD4 (Ebioscience, CA, USA).

Surface and Intracellular Staining for Treg and Th17 Cells

Cells were equally distributed in 5 ml polystyrene round-bottom tubes (Becton Dickinson, NJ, USA) for immunolabeling (approximately 1 x 10^6 PBMCs in 100 ul of PBS). Surface staining was performed prior to intracellular labeling, as some antibodies which bind surface markers may not recognize fixed antigens.

The Treg fraction was kept on ice in the 4 °C refrigerator in PBS/ 2% FBS (Wisent, QC, CAN) for 5 hours and then divided up equally into 7 FACS tubes. Seven samples were stained as follows: 1) unstained; 2) FITC anti-CD4; 3) PE anti-CD27; 4) PECy5 anti-CD127; 5) FITC anti-CD4, PE anti-CD25; 6) FITC anti-CD4, PE anti-CD120b, PECy5 anti-CD127; 7) FITC anti-CD4, PE anti-CD25, PECy5 anti-CD127.

After staining, both fractions of cells were incubated in the dark for 30 minutes at 4 °C. Cells were washed twice in PBS and incubated overnight at 4 °C in fixation/ permeabilization buffer (Ebioscience). Cells were then washed twice in permeabilization buffer (Ebioscience) and stained for intracellular markers using PE anti-IL-17 (Th17

fraction) and PerCP anti-FoxP3 (Treg fraction). Cells were washed twice with permeabilization buffer and resuspended in PBS for immediate analysis by flow cytometry.

Flow Cytometry Acquisition and Analysis

Treg and Teff cell frequencies were measured by flow cytometry acquisition of peripheral blood mononuclear cells stained with surface and intracellular markers. We used side scatter and forward scatter plots to gate on the lymphocyte population within each of our samples. Treg cells were identified as CD4+CD25+FoxP3+ cells and Teff cells were identified as CD4+CD25- cells found within the lymphocyte gate. Each step of the procedure is explained in the methods described below.

Samples were acquired using a FACSCaliber flow cytometer (Becton Dickinson, CA, USA) and analyzed with Cellquest software. Per sample, approximately 1 x 10^5 cells were acquired.

Mouse Model

Examining the Role of TNFR2 on Murine Tregs in the Response to Unilateral Renal Ischemic Injury

Male TNFR2-/- mice on a C57BL/6 background were generously provided by Dr. Pnina Brodt's lab. Mice underwent unilateral renal clamping for 30 minutes in order to induce a murine model of IRI. After 30 minutes the clamp was removed and blood flow was restored. N=3 mice were sacrificed at day 1 and day 7 after IRI. Lymphocytes were

isolated from the contralateral kidney, ischemic kidney, and spleen. Kidneys and spleens were removed and placed separately into Hank's Balanced Salt Solution (HBSS) without calcium (Wisent, CA, USA).

Kidneys were cut up with a scalpel and digested with 2ul/ ml Collagenase D (Roche, QC, CAN) in HBSS with calcium (Wisent, CA, USA) for 45 minutes at 37 °C. Spleens were combined and pulverized with the ends of 10 ml syringes (BD Biosciences, ON, CAN). Both kidney and spleen cells were then washed twice in HBSS without calcium at 300 rpm for 10 minutes at 20 °C. Lymphocytes were isolated using gradient density centrifugation. Splenocytes were carefully layered over 4 ml of Histopaque (Sigma, ON, CAN) and centrifuged at 800 rpm for 15 minutes at 20 °C. The buffy coat containing lymphocytes was pipetted up and washed twice in HBSS without calcium at 400 rpm for 5 minutes at 4 °C. Cells were then counted using trypan blue.

Generation of TNFR2-/- x FoxP3-RFP+ Mice

We began planning the generation of the TNFR2-/- x FoxP3-RFP+ mice starting in October 2012, and began breeding mice in December of 2012. C57BL/6 males bearing the TNFR2 -/- mutation were crossed with C57BL/6 females bearing the reporter gene FoxP3-RFP (The Jackson Laboratory, Maine, USA) and subsequent generations inbred to obtain mice homozygous for both transgenes. The gene encoding TNFR2 is located on mouse chromosome 6 and the gene encoding FoxP3 is found on the p arm of the X chromosome. Due to these genetics, male TNFR2-/- mice were bred with female FoxP3-RFP mice. Male TNFR2-/- mice on a C57BL/6 background were generously provided by Dr. Pnina Brodt's lab. They were crossed to female FoxP3-RFP knock-in on a C57BL/6

background. The F3 generation was genotyped to identify mice homozygous for TNFR2-/- and FoxP3-RFP. A 0.5 cm length of the tail tip was cut off and DNA was extracted with proteinase K (Invitrogen, ON, CAN). PCR was performed using from primers prepared according to the genotyping protocol (The Jackson Labs, stock number 008374) provided by the supplier. An electrophoresis gel was run using a 1.5% agarose gel in order to identify mice homozygous for both TNFR2 knockout and FoxP3-RFP knock-in.

CD4+ T Cell Isolation from Mouse Splenocytes

Spleens from homozygous male mice from the F3 and F4 generations were removed and placed in Hank's Balanced Salt Solution (HBSS) without calcium (Wisent, CA, USA). Spleens were combined and pulverized with the ends of 10 ml syringes (BD Biosciences, ON, CAN). Cells were washed twice in HBSS without calcium at 300 rpm for 10 minutes at 20 °C. Lymphocytes were isolated using gradient density centrifugation. Splenocytes were carefully layered over 4 ml of Histopaque (Sigma, ON, CAN) and centrifuged at 800 rpm for 15 minutes at 20 °C. The buffy coat containing lymphocytes was pipetted up and washed twice in HBSS without calcium at 400 rpm for 5 minutes at 4 °C. Cells were then counted using trypan blue.

Cells were resuspended in PBS and CD4+ T cells were isolated by negative selection using the EasySep™ Mouse CD4+ T Cell Enrichment Kit (Stemcell Technologies, BC, CAN).

CD4+ T cells were stained for CD4 using FITC anti-CD4 (Ebioscience, CA, USA) to confirm CD4 status. Cells were incubated for in the dark for 30 minutes at 4 °C and

then washed twice in PBS. Cells were sorted using the FACSAria cell sorter (BD Biosciences, ON, CAN).

CD4+FoxP3-RFP live cells from the sort were frozen in 500 ul of 100% fetal bovine serum (FBS) (Wisent, ON, CAN) and 500 ul of FBS supplemented with 20% dimethyl sulfoxide (DMSO) (Sigma, ON, CAN). Cells were stored in -80 °C.

Adoptive Transfer and Mouse Model of Renal Ischemic Injury

Rag1-/- mice were purchased and bred according to animal care regulations. Male mice underwent adoptive transfer and were injected with 50 000 Tregs and 200 000 Teffs, such that a ratio of 1:4 Tregs: Teffs was used. These numbers were based on a study conducted by Kinsey et al. in which the researchers calculated clinically relevant doses of Tregs (Kinsey, Huang et al. 2012, Kinsey, Sharma et al. 2013).

AKI was induced in mice by bilateral clamping of the renal vessels for 30 minutes with atraumatic vascular clamps (100g closing pressure) via midline laparotomy under isoflurane anesthesia. Experience in our laboratory and in the literature suggests 30 minutes of IRI inflicts a reproducible sub-lethal renal injury.

Mice were sacrificed 7 days after ischemic injury. The right and left kidneys from each mouse were combined and digested with collagenase D (2 mg/ mL) (Roche, USA) in HBSS with calcium (Wisent, CA, USA) for 45 minutes. Cells were washed twice in HBSS without calcium (Wisent, CA, USA) at 300 rpm for 10 minutes at 20 °C. After washing, kidney-infiltrating lymphocytes were isolated by density gradient centrifugation, and the frequency of TNFR2+CD4+CD25+FoxP3+ Tregs quantified by flow cytometry analysis.

Results

- **DGF Human Study**

- **IRI Mouse Study**

Delayed Graft Function Study

Patient Characteristics

Adult deceased donor kidney transplant recipients (n=87) were enrolled into the study. Recipients were divided into two groups: 1) IGF (n=24), defined as recipients with a decrease in 24 hour serum creatinine of 20% or greater, and 2) AKI (including both DGF and SGF patients) (n=52), defined as recipients either requiring dialysis within 7 days post-transplantation, or without requiring dialysis within 7 days post-transplantation but with a decrease in 24 hour serum creatinine of less than 20%. Reduced serum creatinine levels indicate reduced kidney function. DGF and SGF groups were combined into the AKI group because they are on a continuous spectrum of ischemia reperfusion injury. The decision to start dialysis post-transplantation was made independently by the treating physicians. Recipient, donor, and organ procurement information was collected prospectively (Table 1). Patients in IGF and AKI groups received similar immunosuppression regimens (Table 1).

Although we recruited 87 patients, we have used 53 patients in our analysis. As the study requires time points be taken over the course of a year, we currently only have complete lymphocyte data sets for 53 patients. As can be seen from the cohort diagram (Diagram 1), 11 patients were excluded from the study because of incomplete patient data. There were 2 groups in this study, IGF (n=24) and AKI (n=52). From the IGF group, 8 patients were excluded because of incomplete lymphocyte data. From the AKI group, 15 patients were excluded because of incomplete lymphocyte data. We were

therefore left with 16 patients in the IGF study group and 37 patients in the AKI study group.

Mouse Study

Examining the Role of TNFR2 on Murine Tregs in the Response to Unilateral Renal Ischemic Injury

The data show that TNFR2-/- IK has significantly less CD4+IL17A+ Th17 infiltration than the wild-type IK at day 1 but not at day 7 (Table 3, Figures 3-4, Table 4). A graduate student performed further analysis and these analyses are presented in the discussion section.

TNFR2-/- x FoxP3-RFP Mouse Model

The TNFR2-/- x FoxP3-RFP mouse was generated by breeding 2 TNFR2-/- males with 3 FoxP3-RFP+ females. The TNFR2-/- x FoxP3-RFP mouse used in this study was genotyped (Figure 5A and Figure 5B). The TNFR2-/- x FoxP3-RFP mouse is a viable mouse model. The gene encoding TNFR2 is located on mouse chromosome 6 and the gene encoding FoxP3 is found on the p arm of the X chromosome. Based on genotyping, F3 mice that were homozygous for TNFR2-/- and either hemizygous (males) or homozygous (females) for FoxP3-RFP were bred to produce F4 offspring. Genotyping showed that TNFR2-/- x FoxP3-RFP males of the F4 generation were homozygous for TNFR2-/- and hemizygous for FoxP3-RFP.

Adoptive Transfer of Rag1-/- Mice

Rag1-/- mice underwent successful adoptive transfer. A total of 11 Rag1-/- mice (8 for the 3 day time point and 3 for the 7 day time point) were adoptively transferred with 250 000 lymphocytes in a ratio of 1:4 Tregs: Teffs. The mice did not present with any visible health problems prior, during, and shortly after adoptive transfer, or in the 24 hours following adoptive transfer prior to IRI.

Mouse Model of Renal Ischemic Injury

F4 mice that underwent bilateral ischemic clamping did not all survive. At the 3 day time point, only 3 out of 5 seven week old TNFR2-/- x FoxP3-RFP+ mice survived. The experiment was repeated and two out of 3 seven week old TNFR2-/- x FoxP3-RFP+ mice survived. At the 7 day time point, 1 out of 3 seven week old TNFR2-/- x FoxP3-RFP+ mice survived.

FlowJo analysis of the data showed that the unstained C57BL/6 lymphocytes isolated from the spleen (Figure 6) were distributed on the scatter plot as expected. Compensation controls for CD4 (efluor 450/ Pacific Blue) and IL-17 (FITC/ Alexafluor 488) and all samples yielded unexpected results. Cell populations in all samples were very different from the unstained C57BL/6 lymphocytes isolated from the spleen. The forward scatter and side scatter show very different profiles from the expected distribution of lymphocytes (Figures 6-9).

Discussion

- **DGF Human Study**

- **Mouse Study**

DGF Human Study

Our study has shown a novel association between pretransplantation peripheral blood recipient Treg suppressive function and AKI in deceased donor kidney transplant recipients.

Significant differences in both cold ischemic time (CIT) and donor age were observed between IGF and AKI patients. A significant difference was also observed in the percentage of CD4+TNFR2+CD127- Tregs between IGF and AKI patients (Table 2).

Notably, previous research in our lab identified donor age as a significant predictor of patients who are likely to develop AKI after transplantation in unvariate analysis (Nguyen, Fryml et al. 2014). Multivariate logistic regression taking into account CD4+ TNFR2+CD127- Treg percentage, CIT, and dialysis modality also identified donor age as a significant predictor of patients who are likely to develop AKI after renal transplantation (Nguyen, Fryml et al. 2014).

Previous research carried out in our lab showed that importantly, Treg function was able to predict AKI and was able to significantly distinguish AKI from IGF recipients in univariate analysis. When accounting for CD4+ TNFR2+CD127- Treg percentage, donor age, and dialysis modality in multivariate logistic regression, CIT no longer significantly predicts AKI (Nguyen, Fryml et al. 2014). Machine perfusion of deceased donor grafts decreases the association between CIT and AKI development, and may explain the lack of significance when taking into account Treg function (Ciancio, Gaynor et al. 2010).

Neither CIT nor donor age directly link the recipient's outcome to a recipient variable. CD4+ TNFR2+CD127- Treg percentage, however, is the only variable identified so far that depends entirely on the recipient. This exciting new finding highlights the importance of CD4+ TNFR2+CD127- Treg percentage as a significant predictor of AKI. CD4+ TNFR2+CD127- Treg percentage has clinical relevance as a potential marker in identifying AKI recipients. Furthermore, this marker may be useful in better matching donor organs to recipients.

In order to determine whether AKI and IGF outcome study groups were similar with respect to Teff and Treg frequencies, flow cytometry was performed on both study groups and published in a paper by our group (Nguyen, Fryml et al. 2014).

No significant differences were observed between CD4+CD25- Teff frequencies, CD4+CD25+FoxP3+ Treg frequencies, FoxP3 expression on CD4+CD25+Tregs, and Treg-to-Teff ratio in AKI and IGF recipients (Nguyen, Fryml et al. 2014).
In a paper previously published by our group, none of the recipient baseline characteristics with immunomodulatory potential, including age, sex, body mass index, autoimmune disease diagnosis, vitamin D supplementation, statin therapy, previous blood transfusion, sensitization, and dialysis modality or duration were predictive of pretransplantation Teff proliferation or Treg function (Nguyen, Fryml et al. 2014). Variability in the purity of enriched CD4+CD25- Teffs and CD4+CD25+ Tregs did not correlate with proliferation or suppressive function, respectively (Nguyen, Fryml et al. 2014). Variability in FoxP3 expression percentage in CD4+CD25+ Tregs enriched from different recipients did not correlate with suppressive function (Nguyen, Fryml et al.

2014). Overall, the significant finding is that there is lower pretransplantation Treg suppressive function in AKI recipients.

We examined whether recipient peripheral TNFR2% on CD4+TNFR2- Tregs correlates with Treg suppressive function. Dr. Minh-Tri Nguyen performed statistical analysis and created a graph to demonstrate correlation. These results were published in an abstract and showed a moderate positive correlation between recipient peripheral TNFR2% on CD4+TNFR2- Tregs and Treg suppressive function. I have included the figure to show that my data collection has supported further analysis by our group (Figure 1) (Minh-Tri Nguyen MD 2013).

Recipients with AKI showed lower CD4+ TNFR2+CD127- Treg percentage compared to recipients with IGF, which was not related to recipient characteristics with immunomodulatory potential. The findings suggest that recipient peripheral blood CD4+ TNFR2+CD127- Treg percentage, measured prior to transplantation, can potentially be used as a novel independent marker for risk of AKI.

Previous markers for AKI have been studied in donor urine machine perfusion fluid, and early posttransplantation recipient urine samples (Hall, Yarlagadda et al. 2010, Hollmen, Kyllönen et al. 2010, Moers, Varnav et al. 2010, Hollmen, Kyllonen et al. 2011, Hall, Doshi et al. 2012). However, measuring pretransplantation recipient peripheral blood CD4+ TNFR2+CD127- Treg percentage can allow prediction of AKI prior to renal transplantation and the onset of ischemic injury within the graft. In future, it may be feasible to quantify the percentage of recipient CD4+TNFR2+CD127- Tregs using flow cytometric analysis. Quantification has tremendous potential to be useful in

the identification of kidney recipients at risk for AKI, and thus can help in the donor allocation process.

Inflammation is a central event in AKI, and peripheral Tregs are known to traffic to areas of inflammation to mediate the Teff response and reduce inflammation. In murine models of ischemic AKI, Tregs have been shown to traffic to the kidney, reduce infiltration of innate immune cells, and inhibit the production of proinflammatory cytokines by Teff cells (Gandolfo, Jang et al. 2009, Kinsey, Sharma et al. 2009). The protective effect of Tregs depends on suppressive molecules, such as CTLA-4, or secreted soluble factors such as IL-10 and adenosine (Kinsey, Sharma et al. 2009, Kinsey, Huang et al. 2012, Lai, Yong et al. 2012). Tregs deficient in CTLA-4 or in the ability to produce IL-10 or adenosine were shown to lose their suppressive function in a murine model of ischemic AKI. Kidney transplant recipients with lower CD4+TNFR2+CD127- Treg percentage may therefore be more prone to developing AKI after transplantation. It is possible, then, that therapies targeted to enhance recipient CD4+TNFR2+CD127- Treg percentage may reduce the risk of AKI posttransplantation.

We showed that TNFR2 percentage on CD4+CD127- Tregs correlates positively with Treg suppressive function. The formal Treg suppressive function assay has several limitations with respect to clinical application in deceased donor kidney transplantation because it is time consuming, labour intensive, requires a large amount of recipient blood to isolate a small population or Tregs (less than 10% of total CD4+ T lymphocytes in healthy individuals), and is not standardized as a technique. Since expression of TNFR2 on CD4+CD25+FoxP3+ Tregs has been shown to identify the most highly suppressive subset of Tregs, we studied TNFR2 as a potential independent novel recipient-based

peripheral blood immune marker for AKI when measured prior to transplantation (Chen, Subleski et al. 2008, Chen, Subleski et al. 2010). Our results indicate that TNFR2 percentage on CD4+CD127- Tregs can be used as a marker of Treg suppressive function. I have included a ROC curve generated by Dr. Minh-Tri Nguyen supported by data I collected (Figure 2). It should be noted that the ROC value of 0.68 implies that caution should be taken when using pretransplantation recipient peripheral blood CD4+TNFR2+CD127- Treg percentage alone as a predictor of recipients who will develop AKI after transplantation (Figure 2). By itself, the variable may not have the desired discriminatory power. Since clinical utility is considered above 0.75, we weren't far and therefore we could have a test that works if perhaps we had a larger sample size.

The results of this study are limited by a small sample size. However, larger database studies identified similar risk factors for AKI, including CIT and donor age (Ojo, Wolfe et al. 1997, Humar, Ramcharan et al. 2002). While both of these factors are related to the donor organ, Treg suppressive function and the use of TNFR2 as a novel marker are characteristic of the recipient. TNFR2 may be associated with higher suppressive function in this heterogeneous cell population. Since we already showed that function correlates with AKI, then it is logical to extrapolate that TNFR2 expression could be a more easily assessed measure of it.

We next investigated the role of TNFR2 in the recovery of acute renal ischemic injury in a murine model. We wanted a murine model that would allow us to easily identify FoxP3+ Tregs, and therefore chose to tag FoxP3 with red fluorescent protein to allow sorting by FACS. We wanted to study the effect of reconstituting ratios of Teff cells and FoxP3+ Treg cells, both TNFR2+/+ and TNFR2-/-, in immunocompromised

mice (Rag1-/-) to better understand the role of TNFR2 in Treg-mediated suppression. Rag1-/- mice were chosen to allow adoptive transfer studies and thereby eliminate the effect of the TNFR2KO mutation in other tissues and cell types.

Mouse Study

CD4+IL17A+ Infiltration in Wild-Type and TNFR2-/- Mice

The results we obtained suggest that there is less CD4+IL17A+ Th17 cell infiltration in TNFR2-/- IK compared to wild-type IK, at day 1 but not at day 7. Further analysis by Dr. Minh-Tri Nguyen found that there are some significant differences with respect to CD4+IL17A+ Th17 cell infiltration. In particular, a Kruskal-Wallis test followed by a Mann-Whitney non-parametric test detected a significant difference in the comparison between wild-type CK, wild-type IK, and TNFR2-/- IK infiltrating CD4+IL17A+ Th17 cells. We expected that TNFR2-/- IK would have fewer CD4+IL17A+ Th17 infiltrating cells. TNFR2-/- mice lack the TNFR2 receptor on Tregs, and since this receptor is responsible for mediating the effects of TNF on Treg cells, their suppressive response is dampened down. Furthermore, the lack of TNFR2 will prevent Tregs that normally express TNFR2 from homing to sites of TNF production. Th17 cells also respond to TNF, and it is thought that they respond faster to TNF than do Tregs because of higher sensitivity (Chen and Oppenheim 2011). Lack of TNFR2 results in lower suppressive potential on the part of Treg cells (Chen, Subleski et al. 2008). In TNFR2-/- mice, TNF acts on Th17 cells as it does in wild-type mice. It is possible that over time, there is less Treg suppression and therefore more infiltrating CD4+IL17A+ Th17, thus explaining greater infiltration in the wild-type kidney at day 7 as compared to

day 1. Since Th17 cells also respond to TNF, they are likely to be negatively affected by the TNFR2 KO mutation. Further experiments need to be carried out as this experiment was limited by small sample size (n=3 at each time point).

Development and Testing of TNFR2-/- x FoxP3-RFP Mouse Line

We successfully developed and tested a line of TNFR2-/- x FoxP3-RFP mice. The transgenic mice are indistinguishable from wild-type littermates, and are viable and fertile. These mice were usable in experiments, and provided a good murine model for the investigation of recovery from acute ischemic renal injury.

Mouse Model of Renal Ischemic Injury

Lymphocytes are 6-30 um in size, and are generally clearly identifiable on a flow cytometry scatter plot. The scatter plots from each sample do not show the expected lymphocyte distribution. Instead, the spread of lymphocytes over the scatter plots (Figures 6-9) suggests that the populations of Treg and Teff cells that were adoptively transferred into Rag1-/- mice did not retain their characteristic size and shape. Cell populations in all samples were very different from the unstained C57BL/6 lymphocytes isolated from the spleen, and appear to be dying or already dead (Figures 6-9). Cell populations start at the origin and spread out in a fan shape with no distinct divisions between dead cells, lymphocytes, and neutrophils. The data is thus unquantifiable.

The most plausible reason for the distribution of lymphocytes observed in our data is that the cells were no longer viable and were either dying or already dead. The cells were originally isolated and sorted from the spleens of TNFR2-/- x FoxP3-RFP

mice. The duration of isolation was approximately 10 hours, and sorting lasted approximately 5-6 hours. Cells were kept in culture medium throughout the isolation procedure up until sorting. They were resuspended in PBS for sorting to prevent interference of the phenol red within the media with the signal emitted by RFP+ cells. Cells were subsequently frozen and banked until enough TNFR2-/- x FoxP3-RFP+ cells were accumulated to carry out the experiment. Previous results from our lab indicate that approximately 70-80% of cells that are frozen and then thawed are viable. The thawing procedure is stressful to cells and is likely responsible for some cell death as well as change in cell morphology and function.

We used Rag1-/- mice for adoptive transfer, which do not produce mature B or T cells. There was no problem with the tail-vein injection itself. Adoptive transfer into Rag1-/- mice was used to reconstitute the population of T cells within these immunocompromised mice, and to be able to detect infiltration of Teff and Treg cells into the kidney after ischemia reperfusion injury. Bilateral IRI is traumatic for the mice, as is the prolonged period of maintenance anaesthesia (approximately 40 minutes). These factors may be responsible for the death of the mice.

This experiment was limited by the number of Tregs we were able to isolate and bank, which in turn was limited by the number of male TNFR2-/- x FoxP3-RFP mice we had in stock. This experiment needs to first be optimized and then repeated, but I did not have the time within my Master's. Treg and Teff cells that are isolated from TNFR2-/- x FoxP3-RFP mice should be used fresh and directly adoptively transferred to maximize cell viability. Freezing and thawing cells should be avoided to prevent excessive cell injury and death.

A less invasive way to perform bilateral clamping is through the dorsal side. The kidneys can be clamped outside the body and pushed back in to prevent dehydration. Using this technique may also minimize trauma to the mouse and prolong survival post-IRI.

This study needs to be modified, optimized, and repeated with a larger number of mice in order to establish significance of any potential results. It will be interesting to determine whether TNFR2 is indeed responsible for the suppressive effects of TNFR2-expressing Tregs. If the results show that TNFR2 contributes to the suppression of natural Tregs, then further studies may look at the mechanism of TNFR2-mediated suppression to determine whether Tregs shed TNFR2 to suppress Teffs. The results from this study may elucidate a new mechanism for in vivo Treg-mediated protection from AKI via TNFR2, and may provide the basis for the clinical development of novel TNFR2/ Treg-targeted therapeutic strategies to prevent or dampen AKI in kidney transplant recipients.

Diagrams, Tables, and Figures

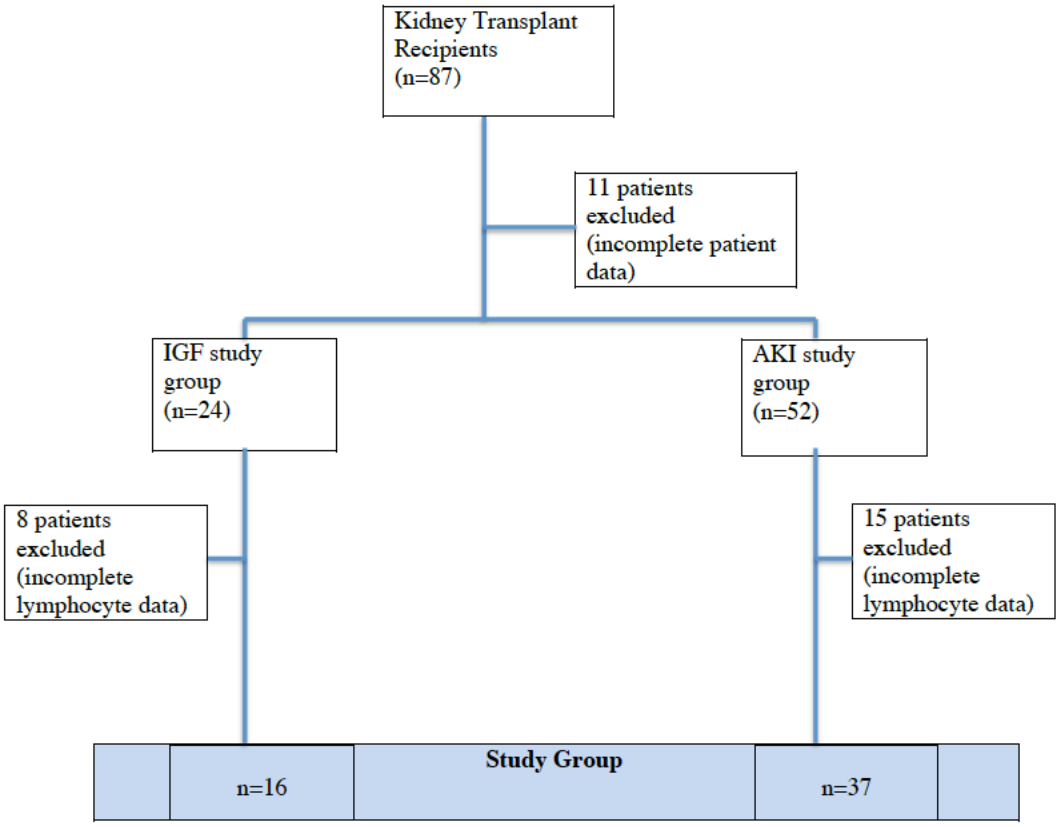


Diagram 1. Patient categorization based on study group. This cohort diagram shows the patients we recruited (n=87) and the groups they were divided into. Eleven patients were initially excluded because of incomplete patient data. There were 2 groups in this study,

IGF (n=24) and AKI (n=52). From the IGF group, 8 patients were excluded because of incomplete lymphocyte data. From the AKI group, 15 patients were excluded because of incomplete lymphocyte data. We were therefore left with 16 patients in the IGF study group and 37 patients in the AKI study group.

		IGF	AKI	*P*
Recipient Characteristics				
n		24	52	
Age, yr		57.46±3.05	57.25±1.52	0.46
African American		3	7	0.91
Diagnosis				
	Autoimmune	0	1	0.49
	DM	6	14	0.86
	GN	5	16	0.37
	HTN	1	2	0.95
	Other	12	19	0.27
Weight, kg		72.63±3.19	78.83±2.55	0.99
BMI, kg/m2		26.88±0.89	28.29±0.76	0.36
PRA>50%		5	5	0.14
PRA class I		15.67±5.33	8.55±2.98	0.09
PRA class II		9.86±5.50	6.27±2.99	0.25
Previous Tx		1	7	0.22
Previous Blood Transfusion		5	14	0.4
HLA mismatch		3.58±0.28	3.63±0.16	0.21
Pre-Tx Dialysis				
	CAPD	7	5	0.01
	HD	12	43	0.01
Time on dialysis, yr		3.98±0.59	4.19±0.50	0.51
CD4+TNFR2+CD127-, %		2.74±0.32	1.87±0.14	0.03
TNFR2+, %		79.03±3.51	73.46±2.16	0.41
Pre-Transplant Creatinine*		72.63±6.03	71.53±4.39	0.95
Immunosuppression Regimen				
	ATG	10	22	0.96
	Campath (Alemtuzumab)	13	30	0.77
	Simulect, Basiliximab	1	0	0.14
Vitamin D		10	15	0.42
Statin		13	24	0.52
Donor Characteristics				
Age, yr		37.83±3.57	57.35±1.82	<0.01
DBD		24	50	0.33
DCD		0	2	0.33
Procurement Information				
	CIT, hr	13.75±1.25	16.94±0.89	0.5

*Creatinine was measured prior to transplant, on the day of transplant.

AKI, acute kidney injury; ATG, antithymocyte globulin; ATN, acute tubular necrosis; BMI, body mass index; CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after cardiac death; DM2, diabetes mellitus type 2; GN, glomerulonephritis; HLA, human leukocyte antigen; HTN, hypertension; IGF, immediate graft function; PRA, panel reactive antibody; Tx, transplant.

Table 1. Clinical information regarding recipient, donor, and organ procurement characteristics. Pre-transplant dialysis (both CAPD and HD), CD4+TNFR2+CD127- %, and donor age are all significant, with respective *P* values of 0.01, 0.01, 0.03, and <0.01.

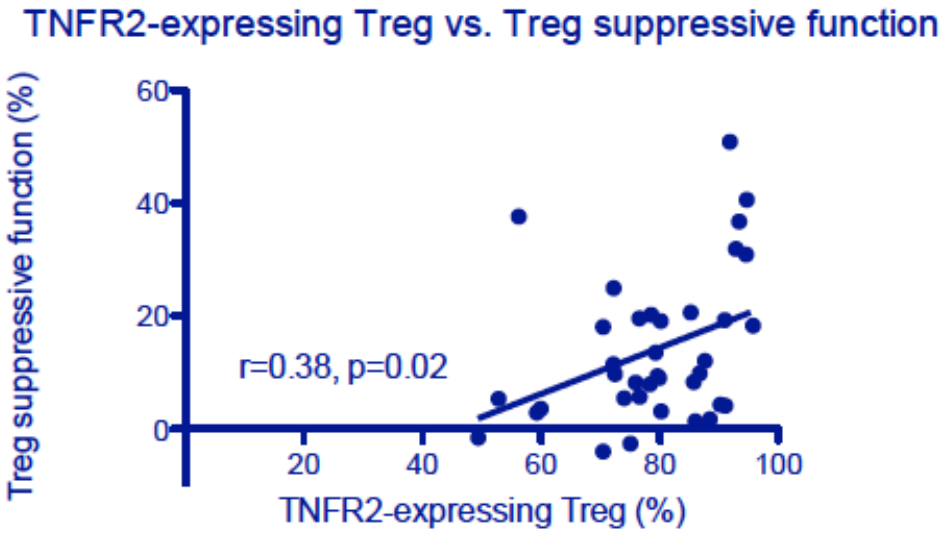
Table 2. Percentage of CD4+TNFR2+CD127-Tregs and CD4+CD25- Teffs in patients prior to transplant. This table shows the percentage of CD4+TNFR2+CD127- Tregs and percentage of CD4+CD25- Teffs identified from peripheral mononuclear blood cells from 40ml of each patient's blood sample. These numbers are the raw data that was collected based on flow cytometry and subsequent FlowJo analysis. Further analysis was performed on this data by a graduate student in our lab, and these analyses are presented and explained in the discussion.

	CD4+TNFR2+CD127- %	CD4+CD25- %
P1	2.221128	25.415
P2	1.173405	16.43
P3	2.012604	20.695
P4	0.905625	9.575
P5	1.801503	26.865
P6	1.87824	17.08
P7	3.90678	13.79
P8	1.556945	23.19
P9	1.559709	7.135
P10	2.007972	13.875
P11	1.420706	39.335
P12	1.444902	20.535
P13	0.875185	19.655
P14	0.808914	24.485
P15	0.893345	32.425
P16	0.62706	23.535
P17	0.72872	19.945
P18	0.72789	41.025
P19	4.280892	32.045
P20	1.630096	28.355
P21	4.931776	28.05
P22	0.85525	37.06
P23	2.719352	22.72
P24	3.808533	23.44
P25	2.117577	14.42
P26	1.93397	21.46
P27	1.840992	35.57
P28	2.558106	22.435
P29	1.489268	31.465
P30		
P31	1.153509	29.04
P32	1.363786	34.29
P33	1.02157	15.12

P34		
P35	4.615992	26.54
P36	3.217536	21.035
P37	1.305832	31.62
P38	2.058516	34.195
P39	1.85033	38.225
P40	2.174844	11.395
P41		
P42	2.217039	20.22
P43		
P44	2.612916	37.765
P45	2.146298	27.67
P46	2.366161	29.82
P47	1.889132	37.13
P48	1.06711	31.72
P49	0.558333	43.975
P50	2.142124	35.49
P51	0.728276	34.685
P52	1.0633	27.745
P53	2.469061	44.695
P54	1.1692	34.085
P55	0.866592	18.43
P56	2.59063	44.325
P57	7.504848	32.45
P58	3.838934	29.72
P59	1.452728	35.355
P60		
P61	1.772652	21.245
P62	0.72633	5.71
P63	1.664096	20.405
P64	4.327557	30.365
P65	0.41262	14.645
P66	4.55927	36.46
P67	1.016512	13.345
P68	1.673784	34.535
P69	2.345745	28.09
P70		
P71		
P72		
P73	0.573776	4.49
P74	3.106722	33.94
P75	4.24964	46.705
P76	2.17143	29.88

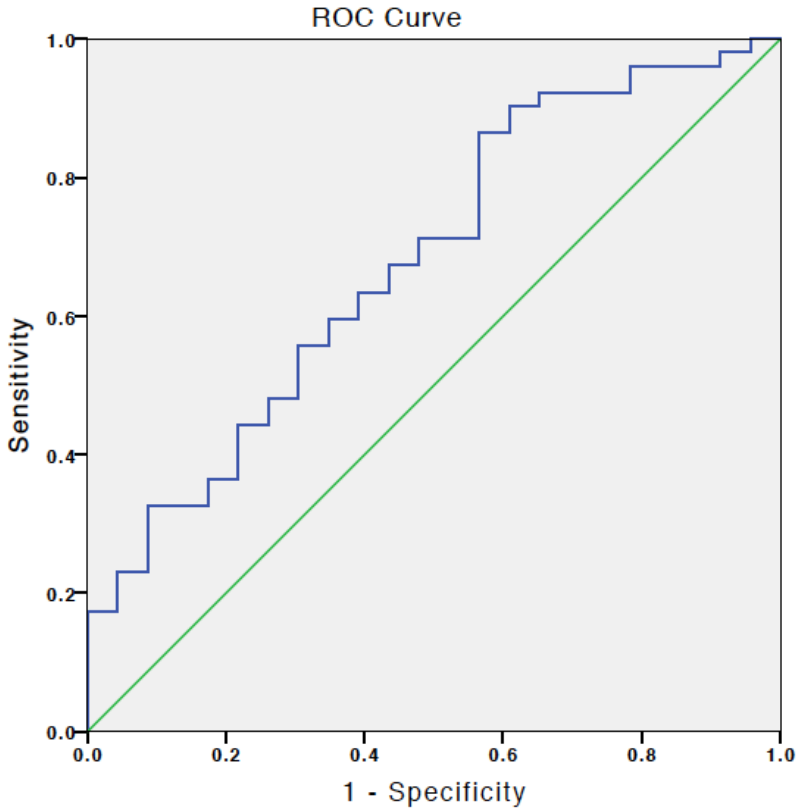
P77	2.901314	28.72
P78	2.45603	39.235
P79		
P80	2.54184	46.31
P81	0.747285	42.045
P82		
P83	1.74666	32.445
P84	2.016064	28.96
P85		
P86	2.984501	41.425
P87		
P88	1.94472	49.22
P89	2.272732	42.83
P90	2.850787	19.765
P91	3.0951	42.045

Figure 1. Correlation between TNFR2-expressing Tregs and Treg suppressive function. This graph shows that pre-transplant recipient peripheral blood TNFR2 expression on Tregs correlates positively with the formal Treg suppressive function assay. N=37 patients were used to generate this correlation, which is significant at *P*=0.02. This graph was generated by a graduate student using the data I collected, and is included to show the usefulness of the data collected.



	Contralateral Kidney	Ischemic Kidney	Spleen	

Figure 2. Pretransplantation recipient peripheral blood CD4+TNFR2+CD127- Treg percentage as predictor of AKI. (N=53, AUC, 0.68; CI, 0.55-0.81; *P*<0.05). The ROC value of 0.68 is poor and implies that pretransplantation recipient peripheral blood CD4+TNFR2+CD127- Treg percentage is a poor predictor of recipients who will develop AKI after transplantation. This ROC curve was generated by a graduate student using the data I collected, and is included to show the usefulness of the data collected.



AUC= 0.68 (0.55-0.81), *P*<0.05

		CD4+ Gate		CD4+ Gate		CD4+ Gate
	CD4+IL17a+ (%)	CD25+FoxP3+ (%)	CD4+IL17a+ (%)	CD25+FoxP3+ (%)	CD4+IL17a+ (%)	CD25+FoxP3+ (%)
Day 1						
M11	1.58	1.87	1.63	1.54	2.88	9.02
M12	1.17	5.46	2.51	11.9	7.31	12.1
M13	1.58	3.8	3.08	6.17	11.4	9.33
N	3	3	3	3	3	3
Mean	1.443333333	3.71	2.406666667	6.536666667	7.196666667	10.15
Std Deviation	0.23671361	1.796691404	0.73050211	5.189723821	4.261130523	1.695847871
Day 7						
M71	0.266		4.86	7.34	7.53	9.01
M72	2.7	5.39	4.56	4.79	4.96	6.54
M73	4.12	6.5	4.2	5.11	3.9	5.85
N	3	2	3	3	3	3
Mean	2.362	5.945	6.81	8.62	8.195	10.7
Std Deviation	1.949105436	0.784888527	0.330454233	1.389112426	1.866610118	1.661455185

Table 3. CD4+CD25+FoxP3+ Treg and CD4+IL-17a+ Teff percentages in the contralateral kidney, ischemic kidney, and spleen in a TNFR2-/- murine model of IRI. These mice underwent unilateral renal clamping, which was used as a model of murine IRI. N=3 in each group, the groups being the time points day 1 and day 7. Data are presented as percentages. Lymphocytes were isolated from the contralateral (control) kidney, ischemic kidney, and the spleen. Lymphocytes were stained to determine percentages of infiltrating Treg and Teff cells. Tregs were stained for CD4, CD25, and FoxP3 markers. Teffs were stained for CD4 and IL-17a. During analysis with FlowJo software, Tregs were gated on CD4+ cells. From the CD4+ population, cells were gated on CD25 and FoxP3 status. This table quantifies the percentages of infiltrating CD4+CD25+FoxP3+ Tregs and CD4+IL-17a Teffs.

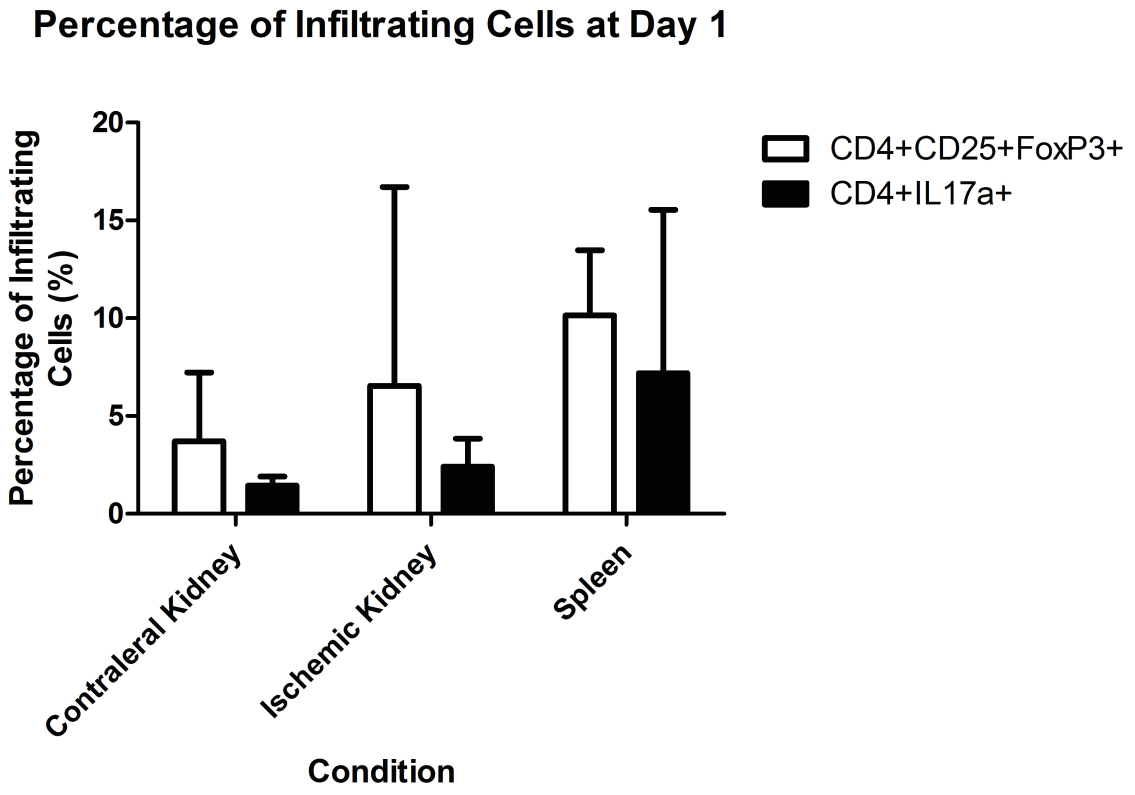


Figure 3. This figure is a graphical representation of the data presented in Table 3. N=3 TNFR2-/- mice in each group. It shows the percentages of infiltrating CD4+CD25+FoxP3+ Tregs and CD4+IL-17a Teffs at day 1 post-IRI.

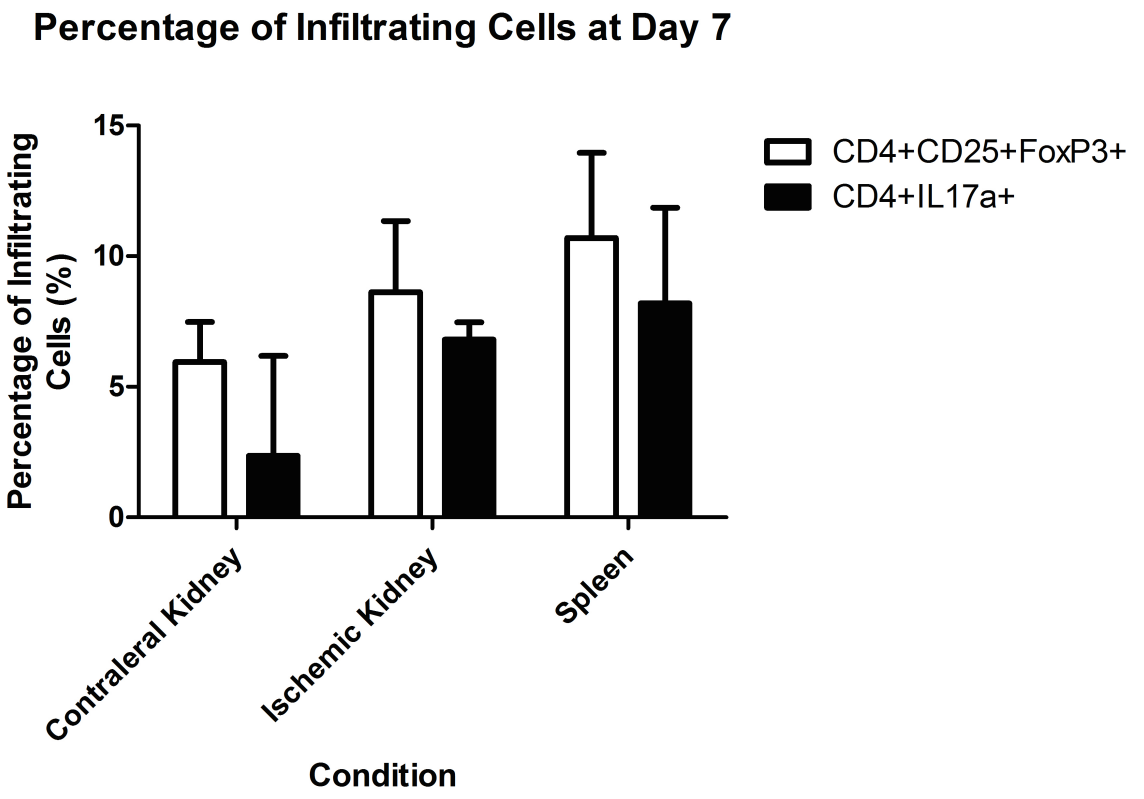


Figure 4. This figure is a graphical representation of the data presented in Table 3. N=3 TNFR2-/- mice in each group. It shows the percentages of infiltrating CD4+CD25+FoxP3+ Tregs and CD4+IL-17a Teffs at day 1 post-IRI.

Table 4. Percentage of infiltrating CD4+IL17A+ Th17 cells into the kidney at days 1 and 7 after IRI in wild-type C57BL/6 and TNFR2-/- mice. N=4 wild-type and n=3 TNFR2-/- mice underwent unilateral renal IRI for 30 minutes. Mice were sacrificed at days 1 and 7 after IRI. Lymphocytes were isolated from the contralateral kidney (CK) and the ischemic kidney (IK) by density gradient centrifugation and were identified using flow cytometry and staining for CD4 and IL-17a. Data are presented as percentages of infiltrating CD4+IL17A+ Th17 cells from the population of lymphocytes within the kidney.

Lane #: 1 2 3 4 5 6 7 8 9 10 11 12

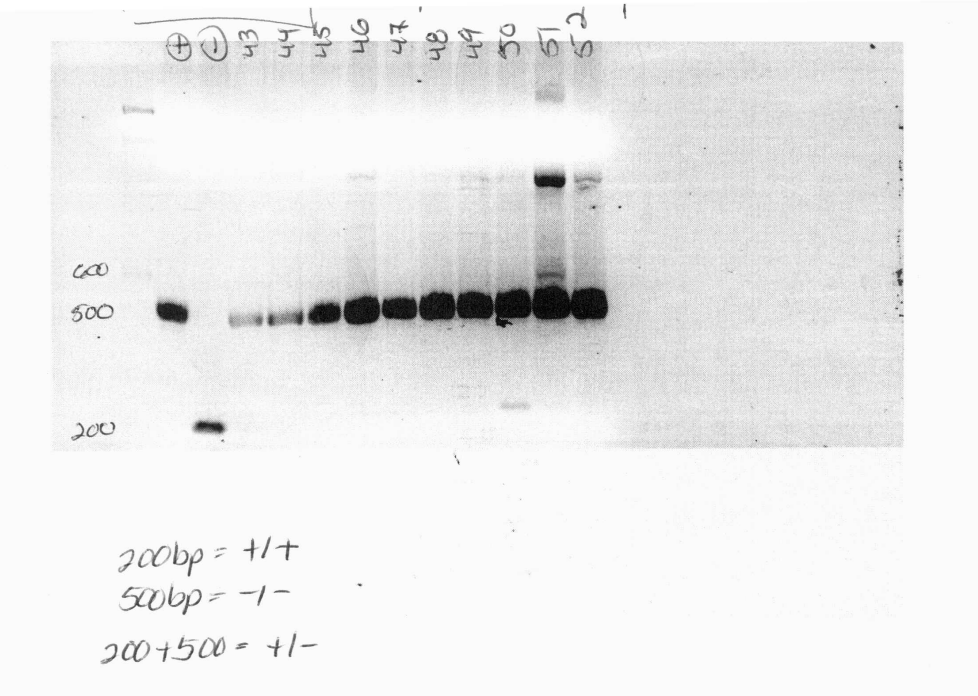
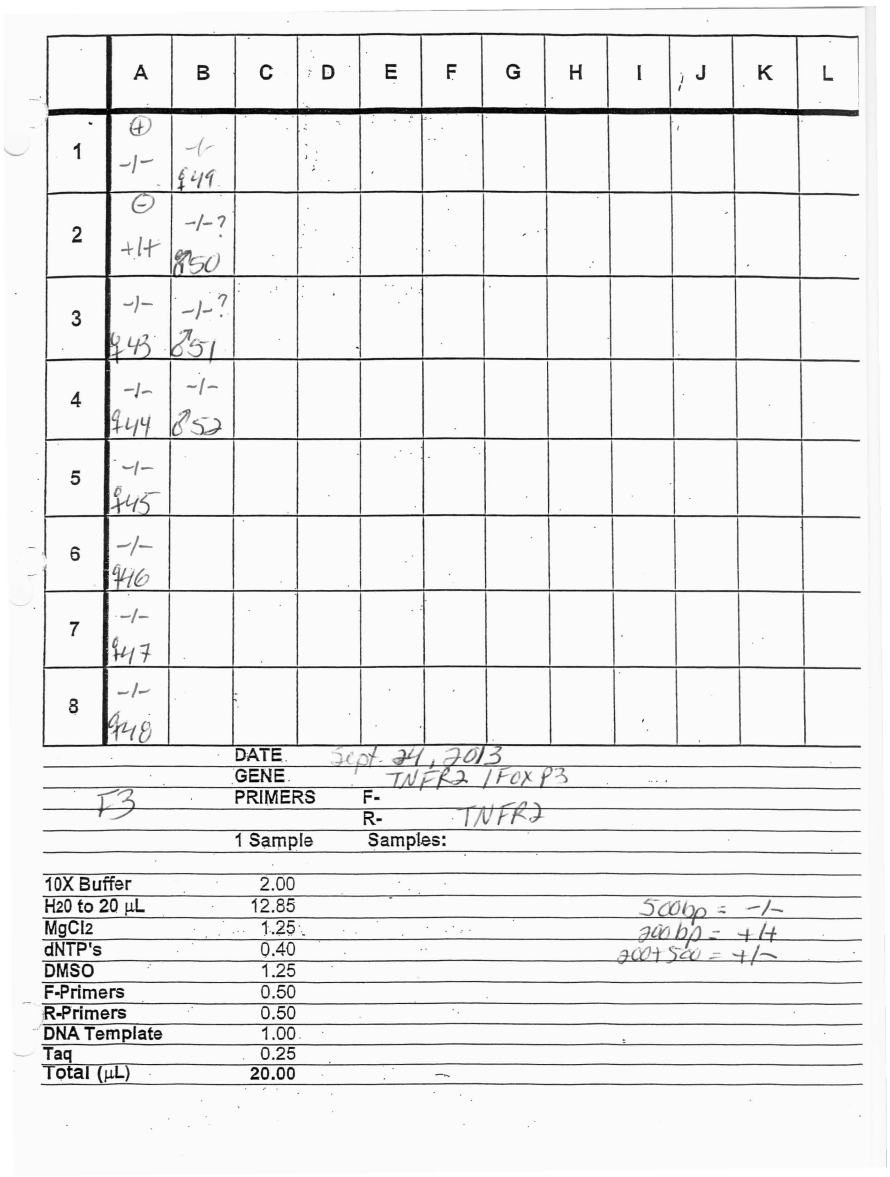


Figure 5A. Male TNFR2-/- mice were crossed with female FoxP3-RFP+ mice on a C57BL/6 background to generate homozygous TNFR2-/- x FoxP3-RFP+ mice. Mice in the F3 generation were genotyped to identify homozygous mice. We cut 0.5 cm off the tail, isolated DNA, ran PCR, and ran a gel to identify homozygous mice. These homozygous TNFR2-/- x FoxP3-RFP+ mice were then bred together to generate F4 mice. Male F4 mice were used in our experiments to isolate Tregs and Teffs for adoptive transfer into Rag1-/- mice. This figure shows the gel we obtained from F3 mice using TNFR2 primers. A single band 200 bp indicates TNFR2+/+ status. A single band at 500 bp indicates TNFR2-/- status. A band at 200 bp and a band at 500 bp indicates TNFR2+/- status. Lane 1 is the positive control, and has a band at 500 bp. Lane 2 is the negative control, and has a band at 200 bp. Lanes 3 through 9 and lane 12 have bands at 500 bp and are TNFR2-/-. Lanes 10 and 11 have a band at 500 bp and a larger band higher up. These 2 mice were excluded from further breeding.



	A	B	C	D	E	F	G	H	I	J	K	L
1	⊕ −/−	−/− ♀49										
2	⊖ +/+	−/−? ♀50										
3	−/− ♀43	−/−? ♂51										
4	−/− ♀44	−/− ♂52										
5	−/− ♀45											
6	−/− ♀46											
7	−/− ♀47											
8	−/− ♀48											

DATE Sept 24, 2013
GENE TNFR2 / FOXP3
F3 PRIMERS F-
R- TNFR2
1 Sample Samples:

10X Buffer	2.00
H20 to 20 µL	12.85
MgCl2	1.25
dNTP's	0.40
DMSO	1.25
F-Primers	0.50
R-Primers	0.50
DNA Template	1.00
Taq	0.25
Total (µL)	20.00

500bp = −/−
200 bp = +/+
200+500 = +/−

Figure 5B. This figure supplements Figure 5A and tabulates the results from the genotyping gel we ran on the F3 generation of TNFR2-/-x FoxP3-RFP+ mice.

TNFR2-/- x FoxP3-RFP+ Mouse Model

The following figures (Figures 6-9) show the flow cytometry data that was collected from Rag1-/- mice. Rag1-/- mice were adoptively transferred with a ratio of 1:4 Tregs to Teffs from TNFR2-/- x FoxP3-RFP+ mice. These mice then underwent bilateral clamping of the renal artery at days 3 and 7 after adoptive transfer to induce a murine model of IRI. Tregs and Teffs were subsequently identified by flow cytometric analysis of CD4+CD25+FoxP3+ Tregs and CD4+IL-17a+ Teffs. Scatter plots of CD4+CD25+FoxP3+ Tregs and CD4+IL-17a+ Teffs are presented in Figures 6-9.

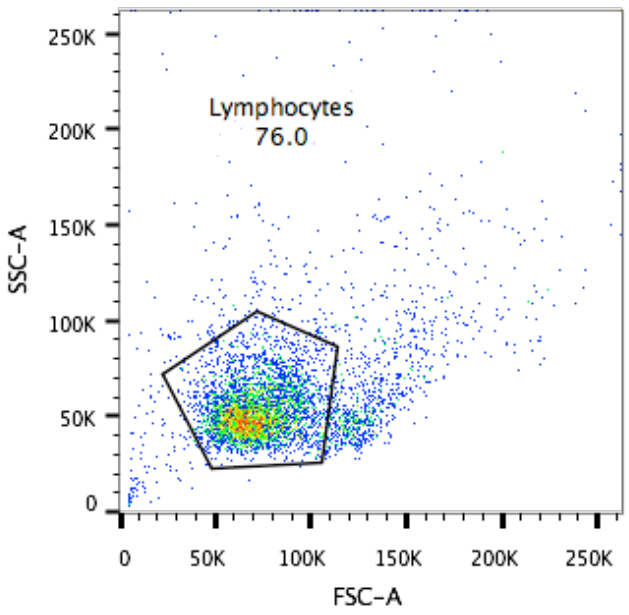


Figure 6: This scatter plot shows results from the compensation control, which consists of unstained C57BL/6 lymphocytes isolated from the spleen. Of the entire population of cells in the scatter plot, 76% are identified as lymphocytes based on side scatter (SSC) and forward scatter (FSC).

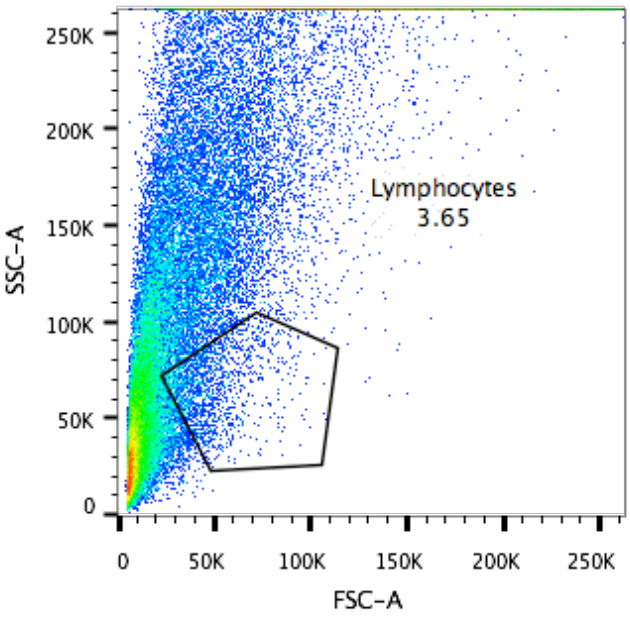


Figure 7: Lymphocytes isolated from the kidney of a Rag1-/- mouse adoptively transferred with TNFR2-/- x FoxP3-RFP Tregs: Teffs in a ratio of 1:4. Mouse underwent bilateral IRI surgery for 30 minutes and was sacrificed 3 days after surgery. PBMC isolation was performed and cells were stained for CD4. The gate represents the area on the graph where lymphocytes are expected to be found. 3.65% of the population is identified as lymphocytes based on SSC and FSC.

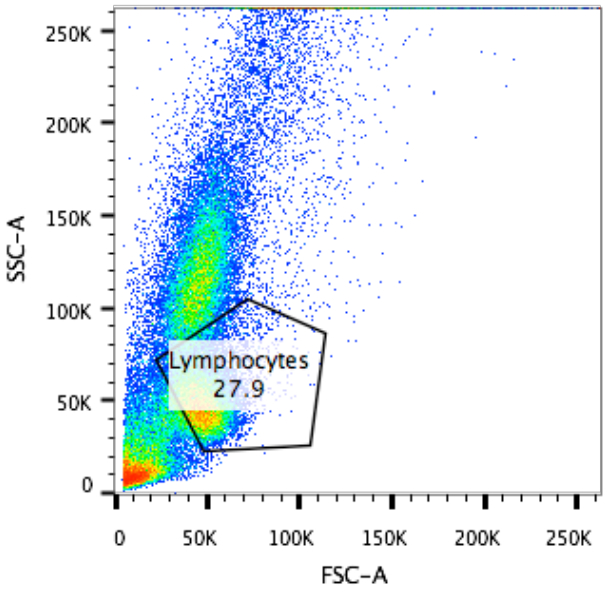


Figure 8: Lymphocytes isolated from the spleen of a Rag1-/- mouse adoptively transferred with TNFR2-/- x FoxP3-RFP Tregs: Teffs in a ratio of 1:4. Mouse underwent bilateral IRI surgery for 30 minutes and was sacrificed 3 days after surgery. PBMC isolation was performed and cells were stained for CD4. The gate represents the area on the graph where lymphocytes are expected to be found. 27.9% of the population is identified as lymphocytes based on SSC and FSC.

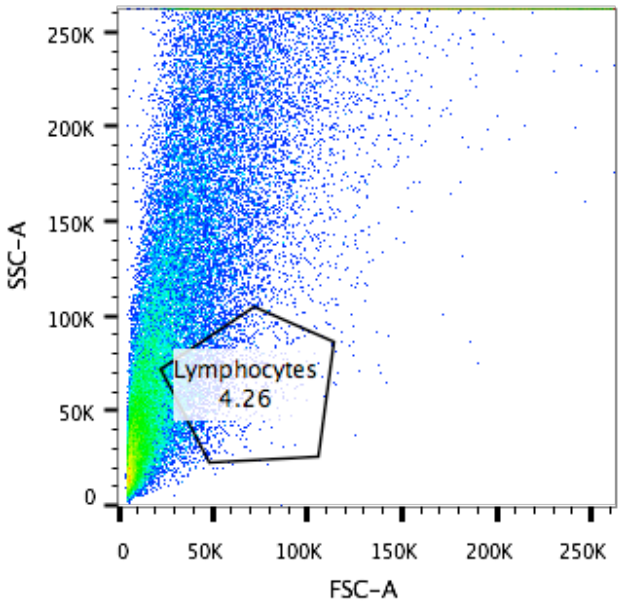


Figure 9: Lymphocytes isolated from the kidney of a Rag1-/- mouse adoptively transferred with TNFR2-/- x FoxP3-RFP Tregs: Teffs in a ratio of 1:4. Mouse underwent bilateral IRI surgery for 30 minutes and was sacrificed 3 days after surgery. PBMC isolation was performed and cells were stained for CD4 and IL-17. The gate represents the area on the graph where lymphocytes are expected to be found. 4.26% of the population is identified as lymphocytes based on SSC and FSC.

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