Immune Monitoring of Kidney Transplant Recipients with Post-transplant Malignancy

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Chris Hope

Abstract:

Half of all long-term (>10 years) Australian Kidney Transplant Recipients (KTR) will develop Squamous Cell Carcinoma (SCC) or Solid Organ Cancer (SOC), making cancer the leading cause of death with a functioning kidney graft. Immunosuppressive drugs increase the risk of cancer but prevent rejection. Finding a balance of immunosuppression may decrease cancer incidence without increasing rejection incidence. United Kingdom (UK) KTR with cancer have increased Regulatory T cells (Tregs) and decreased Natural Killer (NK) cells compared to UK KTR without cancer. However, it is not known if these immune cells and their function differ in Australian KTR with SCC or SOC. If so, then these tests will identify patients at risk of developing cancer and may benefit from reduction of immunosuppression. The presence of Donor Specific Antibodies (DSA) and a positive IFN-γ Enzyme Linked Immuno-SPOT (ELISPOT) assay associates with antibody mediated rejection and can predict cell mediated rejection episodes, respectively. It is not known if these differ in KTR with cancer vs KTR with no cancer. An immune phenotype was analysed in 116 KTR and prospectively followed for 3.5 years. The immune function of Tregs and NK cells as well as viral, mitogen and allo-responses were measured in 50/116 (43%) of these KTR.

Summary Table of Results	No Cancer	Cancer	*P*-value
Tregs cells/µl	8 (3, 19)	16 (6, 23)	0.016
NK cells/µl	107 (34, 195)	74 (43, 188)	0.980
CFSE 1:4 Treg:Eff. cell ratio, median (Range)	2 (1-7)	9 (3-15)	<0.001
CD154 1:4 Treg:Eff. cell ratio, median (Range)	13 (5-54)	36 (13-73)	0.015
PBMC (NK cell) Lysis, median (Range)	2 (0-11)	0 (0-5)	0.037
Donor Specific Antibodies (DSA)	3 (16%)	3 (10%)	0.661
Mitogen stimulation (PHA), median (Range)	1467(265-2000)	512 (51-1500)	0.002
Alloresponse (PRT), median (Range)	342 (11-1967)	151 (29-765)	0.008

KTR with cancer have different immune phenotype and function compared to KTR with no cancer. Memory B cells and CD8 γδ T cells associated with cancer development (Odds Ratio (95% C.I.); (1.03[1.00-1.06], p=0.038 and 1.01 [1.00-1.02], p=0.080, respectively). Treg numbers associate with SOC (p=0.053), predict SCC that develops (AUC=0.78), and can also predict aggressive lesions (AUC=0.86). Treg numbers are dynamic around cancer diagnosis (p=0.022) and resection (p<0.001). Australian KTR with cancer have increased non-specific Treg function (p<0.05) and decreased NK cell mediated cancer cytolysis (p=0.037), signs of a Treg induced/cancer-permissive immune system. Additionally, KTR have decreased IFN-γ release under allogeneic (p=0.008) and mitogenic stimulation (p=0.002) and similar levels of DSA (p=0.661) than KTR with no cancer.

These data indicate that KTR with cancer who have reduced allo-responses may have the potential to have alterations to their immunosuppressive drug levels. This reduction and its effects on the immune system can be monitored using the assays described in this thesis.

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Chapter 1: Introduction

1. Cancer complications post renal transplantation

Kidney Transplant Recipients (KTR) have a 3 to 12- fold increased risk of developing Non-Lymphoid or Solid Organ Cancers (SOC) when compared to the general population [1-4]. Cancers in KTR have poorer prognoses for a given stage/grade than the general population, which leads to higher mortality [5-9]. In Australia, it is observed that 20% of KTR will develop SOC within 15 years post transplantation (the median graft survival). Over a 5 year period (2007-2011) there were 267 KTR deaths from cancer or 31% of all Australian KTR deaths with a functioning graft (ANZDATA 2012 Transplantation report).

Additionally, KTR have a 60 to 250- fold increased risk of developing a Non-Melanoma Skin Cancer (NMSC) which includes; Squamous Cell Carcinoma (SCC) and Basal Cell Carcinoma (BCC), Karposi's sarcoma, Merkel cell carcinoma and adnexal tumours [1, 7, 10]. However, SCC is the most common cancer in KTR with half of all KTR who are 15 years post transplantation developing a SCC[11]. The disease progression of SCC is much more aggressive than the general population and is exemplified by the development of multiple SCC lesions and metastatic potential, phenomena that rarely occur in the immune competent[5, 6, 12].

The cumulative risk of subsequent SCC tumours is 30-32%, 60-62% and 75-80% over 1, 3 and 5 years after first tumour, respectively [13]. Compounded, this equates to approximately 10% of KTR having >5 tumours within 5 years of their first tumour, with some individual KTR reaching 40 primary SCC tumours during recipient life[14]. Hence, a single SCC lesion is a risk factor for subsequent SCC development with 60-80% of KTR with one or more tumours developing another tumour within 1-3 years[15]. Furthermore, SCC tumour characteristics are risk factors of metastatic SCC which include: size [16], depth [16, 17], thickness [17], diameter [18] and poor differentiation [17]. Indeed, depth >2.8mm has a three-fold greater risk of metastasizing in KTR than the general population[19].

Further evidence of the aggressive nature of tumours in KTR is the invasive potential of SCC in KTR, with more perineural and lymphatic invasion than the general population [20]. Additionally, metastatic incidence increases by 5-8% with every SCC tumour accrued in KTR[14] and due to SCC lesions being mainly located in UV exposed areas, e.g. the hands and face, there is a possibility of invasion into subcutaneous cranial nerves in the perineural space, leading to extensive surgery and perhaps death[21]. Reports observed an incident mortality of 1-18% [22, 23,18] exemplified when after the development of metastatic disease, the median KTR survival is only 2 years[24]. Furthermore, it has been observed that a previous SCC is not only a risk factor for multiple SCC but even development of SOC and their own incidences of metastasis and mortality[11, 13, 19].

Therefore there are various risk factors and clinical parameters that influence the development of post-transplant cancer. The next section will introduce some of these factors and the rationale behind why they are factors of risk, if they are measurable and if they can be reduced.

1.1 Immunosuppression type

There are limited and conflicting data on the use of different types of immunosuppression and the associated cancer risks. The conflict is due to immunosuppressive drugs having the ability to suppress anti-cancer immunity. The immunosuppressive types introduced in this section include; Azathioprine (AZA), Mycophenolate (MMF), Calcineurin Inhibitors (CNI), steroids and mammalian Target of Rapamycin inhibitors (mTORi). These immunosuppressants are rarely used by themselves as mono-therapies and are therefore hard to compare to one another; instead modes of action and evidence for cancer development are presented.

1.1.1 Azathioprine

Azathioprine (AZA) is catabolised to 6-mercaptopurine, which directly affects the synthesis of purines and has the ability to incorporate into DNA [25, 26]. Lymphocytes rely heavily on *de novo* purine synthesis making AZA an effective immunosuppressant. AZA was originally used as an anti-cancer therapy; however some cancers intrinsically have, or gain, purine scavenging and are, or become, resistant to AZA treatment [27]. When incorporated, the metabolite and the DNA form a complex that can block DNA repair, is photosensitive and produces reactive oxygen species (ROS) under UV exposure [25, 27]. These work synergistically to affect DNA repair which form lesions [26, 27]. One case-controlled study identified that AZA increased risk of developing SCC by 5-fold. However, in the same study Calcineurin inhibitors (CNI) and steroids were also identified as risk factors [28].

1.1.2 Mycophenolate

Mycophenolate mofetil (MMF) is a pro-drug of mycophenolic acid (MPA), which directly affects purine synthesis and is classified as an anti-proliferative drug [29]. The reaction of MPA is reversible and does not interfere with the DNA structure as AZA does [29]. One study showed a decreased photosensitivity when a cohort was randomised onto a MMF from AZA suppression regimen [30]. In another study comparing MMF to AZA usage in Organ Transplant Recipients (OTR) showed that the MMF group had a 27% adjusted risk reduction [31]. Conversely, in a 3- group, randomised control trial of 133 KTR. Group 1: 45 KTR had AZA treatment, Group 2: 44 KTR randomised to 3g daily of MMF and Group 3: 44 KTR randomised to 2g daily of MMF. There were no differences in cancer incidences between all three groups [32].

1.1.3 Calcineurin Inhibitors

Cyclosporine A (CsA) forms a complex with cyclophilin which inhibits calcineurin, hence CsA is a Calcineurin Inhibitor (CNI) [33]. Calcineurin de-phosphorylates nuclear factor of activated T cells (NFAT), which translocates to the nucleus. It is in the nucleus where NFAT activates pro-inflammatory cytokines such as IL-2 [34]. Therefore CsA indirectly affects pro-inflammatory cytokine IL-2 transcription. An isotype of cyclophilin is expressed in the mitochondria which releases apoptotic signals under oxidative stress. CsA blocks this signal transduction and allows cells to by-pass apoptosis when under oxidative stress, including ROS and UV-damage, contributing to carcinogenesis [35, 36]. Other tumorigenic side effects of CsA are direct or in-direct suppression of P53, production of TGF-β and VEGF [37-39].

When investigating this in the clinic, a retrospective analysis of 1000 KTR showed that KTR on CsA based regimens had greater cumulative incidence of tumours than those on AZA based regimens [40]. In another retrospective study any regimen with CsA had an Odd Ratio of approximately 4.5 [41]. Inversely, a CsA based mono-therapy was shown to be less carcinogenic than a MMF and Prednisone (Pred) dual-therapy[42, 43]. Another CNI, Tacrolimus (TAC), inhibits calcineurin by forming a complex with FK506-binding protein 12 (FKBP12) and outcompetes calmodulin therefore still inhibiting IL-2 transcription. Since TAC does not target cyclophilin, it avoids all interference with the mitochondria that CsA has. In a retrospective study of 609 liver transplant patients, TAC had a higher incidence rate for *de novo* cancers than CsA [44]. However in most database analyses, TAC-based immunosuppressive regimens have either no significant difference or a reduced risk of cancer incidence and/or risk over CsA-based immunosuppression regimens [45-48].

1.1.4 Corticosteroids

Corticosteroids are mainly utilised for treatment of auto-immunity, inflammatory disorders and transplantation rejection. Corticosteroids function by inhibiting transcription of IL-1, IL-2, IL-6, IFN-γ and TNF-α and transcription factors such as NF-κB [49-54]. Inhibition of these Th1 cytokines promotes a Th2 response, which provides another indirect immunosuppressive function [55]. Corticosteroids induce TGF-β and can increase the incidence of Kaposi's sarcoma cell proliferation [56, 57].

1.1.5 mammalian Target Of Rapamycin inhibitors

Both Sirolimus (SIR) and Everolimus (EVO), like TAC, bind to FKBP12. However the formed complex inhibits mammalian Target of Rapamycin (mTOR) via mTORC 1 subunit (Raptor) binding and is considered an mTOR inhibitor (mTORi). mTORi can also be classified as anti-proliferative as it induces apoptosis via p53 dependent and independent pathways. This and mTORi's ability to prevent IL-2 signalling cause it to have both anti-cancer and anti-rejection properties. Additionally, mTORi affects protein synthesis, including Vascular endothelial growth factor (VEGF) which inhibits metastatic potential in murine models [58, 59]. SIR has been used to treat patients with Renal Cell Carcinoma (RCC) and EVO has been shown to benefit patients with metastatic RCC who do not respond to mainstream treatment [60-62]. Sirolimus Conversion from CNI based regimens, is beneficial in Kaposi sarcoma and SCC involution [63-66] However it can often lead to increased adverse reactions and increases in rejection episodes if performed too early post-transplant [67, 68].

1.1.6 Anti-Thymocyte Globulin (ATG) induction therapy

Anti-Thymocyte Globulin (ATG) therapy is either horse- or rabbit- derived antibodies directed against human T cells, given as an induction therapy to transplant recipients. The T cells that reconstitute have a regulatory phenotype and return much faster than other T cells[69]. There is an association with prolonged CD4 lymphopenia and ATG as well as CD4 lymphopenia and cancer[70].

Despite the various functions of immunosuppressive types each playing a role with cancer in KTR, overall immunosuppressive load or immunosuppressive dose can also have detrimental effects and promote cancer development.

1.2 Immunosuppression dose

There is an association between immunosuppression dose and cancer incidence. KTR have 3-fold increased cancer risk compared to dialysis patients, in a retrospective registry based study[71]. Furthermore, heart transplant patients have higher levels of immunosuppression than KTR and also have corresponding increases in 5 year incidence of cancer (100% compared to 88%, respectively[14]). Additionally, KTR randomised to a low dose cyclosporine A (CsA) base regimen had reduced incidence of cancer following reduction, with the caveat that they had higher rejection rates[72].

1.3 Immunosuppression duration

Maintained immunosuppression increases the risk of cancer over time which is evident in the steady increase in KTR that accrue cancer in the years post-transplant. Australian KTR SCC incidence is 20%, 50% and 80% at 5, 15 and 30 years post transplantation respectively[11, 73]. Included in the duration of immunosuppression would be the age and aging of the KTR.

1.4 Age and Gender

Age is a risk factor of cancer development, independent of immunosuppression duration [74]. This is exemplified in a retrospective study that showed both age and male gender were risk factors [41]. When comparing KTR to the general population in an aged matched cohort of median age 39 years old, there was a 12- fold increased risk of developing non-skin cancers [4]. Age and gender can influence other parameters of cancer risk. This is particularly the case in Australia were certain, culturally male-orientated, jobs may involve higher exposure to Ultra violet (UV) radiation.

1.5 Ultra-violet radiation

It is evident that UV exposure increases the risk of skin cancer, including NMSC, by the observations recorded by clinicians of the locations of tumours. Cumulative sun exposure, including outdoor occupation, latitudinal residence and even childhood burning events all increase risk of post-transplantation cancer development [75-77]. These increases in carcinogenesis are due, in part, to the aforementioned AZA-UV interactions but mainly occur via direct UV-related mutagenesis. Due to the structure of DNA, it absorbs of UV-A (315-400nm) and UV-B light (280-315nm), in doing so the DNA itself forms cyclobutane pyrimidine dimers in two adjacent pyrimidines of the same DNA strand, which alters the structure of DNA and restricts transcription [78, 79]. A single point mutation can lead transcriptional arrest [79]. A study found that invasive SCC contained mutations of the tumour suppressor gene P53 [80]. An important conclusion from this study is that P53 mutation could have happened in childhood, as most UV exposure happens in childhood [81].

In addition to direct DNA mutagenesis, UV exposure can also have local and systemic effects on the immune system. It is thought that the local effect involves APC's, including resident keratinocytes and Langerhans cells[82, 83] whereas the systemic immunosuppression may come

from splenic cells, migrated Langerhans cells or dendritic cells. Increased expression of IL-4, IL-10, Prostaglandin E2, IL-1α and TNF-α with polarisation of immunity to a Th2 response also plays a role in systemic immunosuppression[83-85]. In combination with this, co-stimulation is effected on both APC and T cells[86]. Other cell types that are affected by UV irradiation are innate immune cells and suppressor cells[87-91]. Regulatory T cells (Tregs) that are induced by UV express lymph node homing molecule CD62L and may provide systemic immunosuppression[87, 88].

The DNA damage and immune suppression of UV can be reversed by IL-12 dependent induction of nucleotide excision repair (NER) protein[92]. Also immunity can be restored by the administration of IL-12 [93], activating APC's, increasing IFN-γ and thus balancing Th1-Th2 polarisation [93, 94].

Other clinical parameters associated with cancer risk that are also orientated by human behaviour, apart from UV exposure, are communicable diseases such as oncogenic viral infections that remain latent in the immune competent.

1.6 Viral infection

Human papillomavirus (HPV) is a group of more than 150 viruses with some types associating with anogenital, oropharyngeal and skin cancers [95, 96]. It has been speculated that HPV infection may prevent UV light-induced apoptosis [97]. Between 65 and 90% of SCC lesions from transplant recipients are positive for HPV DNA [98]. Epstein Barr virus (EBV) is associated with: sino-nasal angiocentric T-cell lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma [95]. There are data that EBV associates with mononucleosis, Burkitt lymphoma and post-transplant lymphoproliferative disorder (PTLD) in KTR [99, 100].

Chronic Cytomegalovirus virus (CMV) infection associates with graft rejection, but with malignancy however it does have indirect associations [99]. A prospective study followed 63 KTR and retrospectively included 131 KTR, with convincing data that CMV positive KTR with increased $\gamma\delta$ T cell proportions, the $V\delta2^{neg}$ sub-population in particular, had decreased cancer incidence [101]. Couzi *et. al.* furthered this observation in a case-control study of a small number (n=18) of short-term KTR (median 3 years post Tx), who developed 12 skin and 6 solid tumours over the prospective period [101].

1.7 Immune phenotyping

The association with cellular markers and cancer has been previously studied. The identification of immune cell populations and sub-populations in patient blood is called immune phenotyping. Measurement of CD4 T cells in 150 KTR revealed that KTR with skin cancer had 330 $CD4^{+}$ cells/µl of blood in comparison to KTR with no cancer who had 565 $CD4^{+}$ cells/µl (p<0.01). Additionally KTR with cancer had non-significant increases in CD8 and CD19 lymphocytes [102]. Another study involving 250 KTR over a 10 year period showed a mean of $CD4^{+}$ lymphocytes of <600 $CD4^{+}$ T cells/µl for those with cancer and >700 $CD4^{+}$ T cells/µl for those with no cancer, however there was no useful threshold found using Receiver Operator Characteristics (ROC) curve analysis [103]. Immune phenotype is more pronounced in KTR who develop non-skin cancer compared to KTR who didn't develop cancer; CD4 count: 234 cells/µl Vs. 543 cells/µl, p<0.001; CD8: 328 cells/µl Vs. 640 cells/µl p=0.1; CD19: 19 cells/µl Vs. 52 cells/µl, p<0.001 [104]. All these studies showed an association with CD4 lymphopenia and cancer. However they did not define $CD4^{+}$ subsets or other lymphocytes that may be affected by cancer.

1.7.1 Regulatory T cells (Tregs)

Immune suppressor cell existence has been debated from the early 1970's through to the mid 1990's [105-108]. The pivotal paper adoptively transferred $CD4^+CD25^+$ T cells in CD25 depleted mice, which mitigated the autoimmune diseases that manifested [108]. However, CD25 is also expressed on activated lymphocytes with only the highest proportion being suppressive *in vitro* via competitive absorption of IL-2 [108-111]. The discovery and transfection of the transcription factor *foxp3* into naïve T cells helped identify FOXP3 and its function as the master regulatory gene [112, 113] and CD127 inversed expression to FOXP3 expression has given Tregs the current phenotype $CD4^+FOXP3^+CD25^{hi}CD127^{lo}$ [110].

Tregs are required in a healthy immune system to maintain self-tolerance and immune homoeostasis during immune reactions, pregnancy and disease. Uncontrolled immune reactions and organ failure result when mutations in FOXP3 occur, as observed in the scurfy mouse models and similarly Immunodysregulation, Polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome observed in humans [114-116]. Both IPEX and X-linked Autoimmunity-Allergic Dysregulation (XLAAD) syndrome cause multi-organ failure due to mass lymphocyte proliferation of self-reactive effector cells [115]. There are many subsets of $CD4^+$ Tregs.

1.7.2 $CD4^+$ Treg subsets

The $CD4^+$ Treg in the periphery, defined by $FOXP3^+CD25^{hi}CD127^{lo}$, contain two subsets: those that originate from the thymus, known as natural Tregs (nTregs), and those that are induced in the periphery, known as induced Tregs (iTregs) [117]. The Ikaros family transcription factor, Helios, is expressed in 100% of all $CD4^+FOXP3^+$ thymocytes of mice and approximately 70% of Tregs in the periphery of both mice and humans [118]. Though the premise that Helios only defines nTreg is currently under debate, nonetheless, it may provide

evidence of *in vivo* activated Tregs [101, 119]. Other regulatory cells are CD4^{+} helpers that have suppressive function. These CD4 helper subsets are classified by the ability to secrete IL-10 (Tr1) and TGF-β (Th3), these Tregs are CD4^{+}CD25^{+} but do not express FOXP3. While these subtypes of Tregs are important the CD4^{+}CD25^{+}Foxp3^{+} Tregs are the main focus for transplantation tolerance and the main focus of this thesis. However, Tregs and their modes of actions and various roles in immunological settings are discussed below.

1.7.2.1 Treg modes of Action

Treg cell contact apoptosis requires interaction with co-stimulatory molecule Cytotoxic T cell Late Antigen-4 (CTLA-4), Fas-R/Fas-L (CD95/CD95L) Ligand and release of Perforin and Granzyme B[120-122]. Indirectly, Tregs can down-regulate B7 Co-stimulation molecules CD80/CD86 on Antigen Presenting Cells (APC, [123]). In addition, Prostaglandin E2 (PGE2) excreted by Tregs, mediates expression of indoleamine 2,3-dioxygenase (IDO) in APCs causing tryptophan starvation and leading to impaired lymphocyte proliferation [124]. Another form of suppression is the formation of localised adenosine by cleaving phosphate groups from ATP, ADP and AMP by ecto-NTPDase-1 (CD39) and ecto-5'-nucleotidase (CD73) cell surface enzymes [125]. Expression of CD39 and CD73 has been shown on murine and human Tregs [125]. Human Tregs also may work in concert with other CD73 expressing cells to elicit a regulatory response. Adenosine has been shown to act via Adenosine receptors (A1, A2a, A2b and/or A3), with A2a receptor being the dominate receptor on effector cells [126, 127]. The adenosine formed by the hydrolysis of ATP can regulate lymphocyte proliferation in autoimmune disease, transplantation and cancers [128-130]. Additionally, it has been shown that adenosine and PGE$_2$ in Tregs co-operate when regulating immune responses[129].

1.7.3 Tregs in viral infections

EBV antigen specific Tregs, mainly IL-10 secreting Tr1 and recruited nTregs, can inhibit the EPV-specific immunity permissive in tumour progression [100, 131]. Thus reduction in Tregs may be beneficial in treatment of chronic viruses. Interestingly, Treg depletion in a Herpes Simplex Virus (HSV) mouse model decreased paralysis onset, indicating that Tregs may be involved in protective immunity to HSV infection in mice at least, similarly observed in Lymphocytic Choriomenigitis virus mouse model, shown in the same study [132].

1.7.4 Tregs and Transplantation

When isolated $CD4^+CD25^-$ cells were administered to BLAB/c nu/nu mice grafted with C57BL/6 skin, there was a swifter rejection rate than administering untouched lymphocytes of the same source[108]. This indicated to the authors that there was a suppressor subpopulation within lymphocytes that limited rejection and therefore play a role in tolerance. This adoptive transfer paper has led to research directed at utilising Tregs for anti-rejection therapies. More recently there have been studies identifying/expanding Tregs that have greater antigen reactivity than polyclonal Tregs[133], however all Tregs seem to have a baseline level of allogenic suppression that is not specific to the stimulus, this non-specificity could provide the means of tumour escape, a topic for further research to be undertaken in.

In KTR, peripheral blood Tregs can differ in accordance with the situation of the patient. Two different studies on clinically tolerant, chronic rejection, stable, minimally suppressed KTR and healthy controls, showed tolerant KTR and minimally suppressed KTR had similar $CD4^+CD25^+FOXP3^+$ and $CD4^+CD25^{hi}$ cells with similar FOXP3 transcription levels when compared to the healthy controls[134, 135] and that chronically rejecting KTR had lower $CD4^+CD25^{hi}$ cells with low FOXP3 transcripts, indicating that Tregs may be protective or involved with tolerance [134, 135]. An additional study supported this in liver transplant recipients

which showed increased FOXP3 mRNA expression in $CD4^+CD25^{hi}$ T cells of tolerant patients compared to patients who had rejection episodes after cessation of immunosuppression [136]. Thus induction of Tregs for suppression of allograft cellular rejection episodes [137] and possible induction of tolerance[138] seems like an attractive substitute to immunosuppression. However, Tregs that co-express CD25 and CD39 have been denoted as a memory subtype of Treg (mTreg) and are associated with cellular rejection [139] in KTR. Additionally, increases in Tregs are also associated with cancer in the general population [140] and KTR[141].

1.7.5 Tregs in Cancer and Immune surveillance

It has been shown that the percentage of $CD4^+CD25^{high}FOXP3^+$ Tregs and Tr1 cells are increased in Head and Neck Squamous Cell Carcinoma (HNSCC) patients in comparison to healthy controls [142, 143]. Ectonucleotidase activity contributed by CD39 and CD73 is also increased on Tregs in this cohort [129]. CD39 has been shown to down-regulate IL-17 production, decreasing Th-17 cell linage. This particular Treg subtype, in the same study, has been shown to be down-regulated in autoimmune Multiple Sclerosis [128]. It has been shown that high levels of Treg in HNSCC patients from the general population associate with a poor prognosis [144-146].

Cancers and Tregs not only have commonalities between each other but they also promote each other. TGF-β and IL-10 secretions from tumours activate Th3 and Tr1 regulatory cells respectively, consequently regulating surrounding cancer cytotoxic lymphocytes [143]. Also tumour cells recruit Tregs with a series of chemokines such as C-X-C Ligand 12 (CXCL-12) and C-C motif 20 and 22 (CCL20/22) [100]. CD39 and CD73 have been shown to be expressed on Tr1 and tumour cells alike [125, 147]. Cancer progresses by the tumours' ability to secrete these soluble factors into its microenvironment. Prostaglandin E_2 (PGE_2) is a product of

Cyclooxygenase 2 (COX-2) and is involved in aiding immune escape. COX-2 is expressed on Tr1 and over-expressed on cancer cells [143, 148, 149]. In a post-transplant cancer setting, it has been shown >35 Tregs (CD4$^+$FOXP3$^+$CD25^{hi}CD127^{lo})/µl of blood in KTR with a history of SCC can predict the risk of developing a subsequent SCC lesion[141].

1.7.6 NK cells in Cancer and Immune surveillance

Carroll *et. al.* also found that Natural Killer (NK) cells, which have cytolytic ability to kill cancerous and pre-cancerous cells, are decreased in KTR with cancer. NK cells are a part of the innate immune system that identify abnormal cells and supply the signals to undergo apoptosis thus "killing" abnormal cells [150].

The identification process involves Major Histo-incompatibility Complex (MHC) class I down regulation, which some viruses and cancerous cells adopted to avoid the adaptive immune system [151]. It is an important step in enabling metastatic cells to successfully invade the host [152]. Once the cell has been identified, the NK cell only activates if there is an imbalance of CD94:NKG2A and the killer-cell immunoglobulin-like receptors (KIR) family. Once activated internal granules locate to the synapse that is created between the NK cell and target cell [150].

During the effector stage the granules are released out of the NK cell and into the synapse and onto the target cell. These proteins include perforin, granzyme A and B. It is these proteins that play their role in the killer phase of NK cells [153]. Perforin creates pores in the membrane that granzyme B can enter and activate the caspase kinase pathway and cause the target cell to undergo apoptosis [153]. This cytotoxic ability to kill cancer cells can be inhibited by Tregs but also cancer cells themselves [154, 155]. This NK-Treg interaction is a TGF-β and cell-cell contact mechanism causing down-regulation of NKG2D and induction of apoptosis[156, 157].

This leads to decreased NK cell numbers and function in the peripheral blood of cancer patients that have elevated TGF-β [158, 159]. There are two other types of NK cells: those that express CD1-d restricted T cell receptor, NK T cells (NKT) and those that lack Fc receptor CD16 and over express CD56, CD56^{bright} NK cells [160-162]. Both these cells can interact with the adaptive immune system and enhance anti-tumour ability by direct and indirect mechanism respectively [160, 162].

1.7.7 CD8 Subsets in Cancer and Immune surveillance

Another cell type with anti-tumour properties is the CD8$^+$ cytotoxic T lymphocyte (CTL). CTLs use the ability to lyse tumour cells using Fas-Fas ligand as well as perforin-IFN-γ granules similar to NK cells [163]. It has been shown that antigen specific CTL are defective in cancer patients and that removal of Tregs can restore cytolytic function [164-166]. Continuously stimulated CD4 and CD8 T cells follow a specific protein expression- and immunogenic potential- pathway to immune senescence. After T cells exit the thymus they are naïve and express both CD27 and CD28 co-stimulation molecules and home to the lymphoid organs [167, 168]. When antigen is presented they become CTL, proliferate, clear the threat, and the majority apoptose with the minority homing to lymphoid organs as central memory T cells or extra-lymphoid sites as effector memory T cells [167, 168]. Upon subsequent exposures the cells become exhausted and lose expression of co-stimulation molecules and are termed T effector memory CD45RA$^+$ or TEMRA cells [167, 169]. These cells are loosely phenotyped as CD8$^+$CD28$^-$ and shown to be regulatory in cancer patients and may associate with poor prognosis [170]. Tumours and CMV infection induce loss of CD28 [170, 171,172], perhaps as a sign of constant immune stimulation. It has been shown that Memory T cells and Natural Killer (NK) cells have anti-tumorigenic properties and that Tregs regulate both of these lymphocyte subsets [156, 173]. Thus, an excess of Tregs is associated with poor prognosis in cancer and is thought to aid cancer cells evade this immune surveillance.

1.8 Treatment options for KTR with cancer

As mentioned immunosuppression dose is a risk factor of cancer. As a form of primary cancer prevention, a reduced exposure to maintenance therapy may be beneficial. A randomised control trial comparing CNI doses showed a benefit of low dose CNI in a reduction in cancer incidence, although there was an association with increased rejection rates [72].

A form of secondary prevention can consist of converting a CNI based regimen to an mTORi based regimen[174, 175]. It has been shown that mTORi can selectively expand Tregs *in vitro* and reduce IL-2 signalling and VEGF, which has the potential to treat cancer patients [176]. Upon conversion, the amount of regulatory T cells increased in around 30% of patients and these patients still accrued cancer and thus may not benefit from mTORi conversion [177-179]. However, 30% of SRL patients do not tolerate its side effects and sudden SRL switching can cause proteinuria[180].

When KTR have a cancerous lesion, surgical resection is the recommended treatment. There are no randomised control trials investigating the effect of tumour resection and minimal evidence of benefit in KTR when reducing immunosuppression. However, treatment in the general population is associated with a decrease in Tregs and failure of Tregs to fall after tumour excision or chemotherapy may be associated to incomplete resection or relapse of disease[181, 182]. When switching or reducing immunosuppression, adequate precautions must be used. Currently there are no assays that reliably determine cancer risk although there is an immune phenotype that can predict time to next tumour in KTR with a history of SCC [141]. CNI avoidance or reduction results in increases of rejection; one way to potentially avoid these rejection episodes is to identify those KTR with cancer who have evidence of a potential alloresponse and exclude them from dose reduction. In order to reduce immunosuppression safely, both the cellular and humoral alloresponses need to be assessed.

1.8.1 Pre-treatment alloresponse measures

Assessment of alloresponses would be needed to assess risk of rejection episodes for it to be possible to reduce immunosuppression. Currently cytokines and HLA antibodies can be measured by Enzyme Linked Immuno SPOT (ELISPOT) and Luminex technologies respectively [183, 184]. Inflammatory cytokines such as IFN-γ are secreted by Th1 effector T cells and are a predictor of acute rejection and infection [185, 186]. Additionally this assay has been used to run CNI avoidance maintenance therapy with a 3-fold reduction in acute rejection as shown in literature [187]. The humoral aspect of the immune system is already routinely assessed in most transplant programmes by solid phase alloantibody detection systems [188]. HLA Donor-Specific Antibodies (DSA) are clinically relevant and observed DSA presence has informed clinicians to alter immunosuppression regimen of patients [189, 190]. However, both these techniques have not used in long-term KTR with a history of cancer.

A National Institute of Health (NIH) funded, Clinical Trials in Organ Transplant (CTOT) consortium approved, IFN-γ ELISPOT has been able to detect 6-month post-transplant acute rejection, from pre-transplant patients[191, 192]. Another CTOT entitled "CTOT09: Immune Monitoring and CNI Withdrawal in Low Risk Recipients of Kidney Transplantation" has a primary directive of using this ELISPOT in detecting those who can safely have a reduction of TAC while monitoring graft function. This indicates an assay robust enough to be used in clinical trials that has the potential to be used as a tool to guide immunosuppression regimens.

1.9 Summary, Aims and Hypotheses

KTR have increased risk of developing cancer. This is mainly due to age, gender, UV exposure, immunosuppressive drugs and immune phenotype. The impact of these associations on immune cell function has not been studied. A non-invasive method of identifying patients at risk of developing cancer may allow time for intervention therapy.

1.9.1 Aims:

1) To define if a United Kingdom- based, pre-existing immune phenotype is predictive of cancer in an Australian cohort where: UV exposures, immunosuppression regimens and SCC accrual differ.

2) To identify if this immune phenotype has the potential to identify patients at risk of developing "high risk" cancers and if the immune phenotype is dynamic around tumour resection

3) To investigate the functional ability of both Tregs and NK cells from KTR with cancer compared to KTR with no cancer

4) To measure both the humoral and cellular allo-responses of KTR and compare them to KTR with no cancer

1.9.2 Hypotheses:

1) The immune phenotype: higher numbers and proportions of Tregs and lower numbers and proportions of NK cells in KTR with cancer when compared to KTR with no cancer, will be consistent in Australian KTR.

2) This immune phenotype will associate with KTR with cancer regardless of tumour type and will be altered by cancer resection. i.e. Tregs will decrease and NK cells will increase.

3) Tregs from KTR with cancer will be more suppressive that those from KTR with no cancer and NK cells from KTR with cancer will be dysfunctional in comparison to those KTR with no cancer.

4) KTR with cancer will have no or lower allo-response when compared to KTR with no cancer.

Chapter 2: Patients, Materials and Methods

Approval was obtained from the Royal Adelaide Hospital Research Ethics Committee (protocol number 090505a) and the study was performed in accordance with Strengthening the Reporting of Observational Studies and Epidemiology (STROBE) guidelines for observational studies[193].

2.1 Patient recruitment

Kidney transplant recipients who presented to their routine clinic visits from June 2009 to August 2013 at the Central Northern Adelaide Renal and Transplantation Service (CNARTS, Royal Adelaide Hospital) who were at least 2 year post-transplant and who gave informed consent, were immune phenotyped (n=116). The cohort consists of KTR with current cancer (n=34) and KTR with a history of cancer (n=31), for a total KTR cancer cohort (n=65), also KTR without any history of cancer (n=51). Cancer categorisation was accessed through both the local hospital database as well as the Australian and New Zealand renal dialysis and transplant database (ANZDATA) as SCC is sometimes not reported in the general government database. Patient case-notes were used to identify multi-transplanted patients, and determine the type, dose and duration of immunosuppression, as well as length of transplant. For the purposes of this thesis, duration of immunosuppression is cumulative duration and includes multiple periods of time and pre-transplant exposure.

2.2 Blood Collection and sample handling

Peripheral blood by venesection was collected in Lithium-Heparin or EDTA vacuum tubes from KTR during trough levels of Calcineurin inhibitors (CNI) or mTORi, and processed within 2 hours of venesection, as previously published[141]. Blood plasma was separated by centrifugation. One of the 4ml EDTA tubes was balanced and centrifuged at 2500g for 10minutes with no brake. 100µl of plasma in 20µl aliquots were taken and stored at -80°C.

Healthy control and KTR PBMC were separated by standard Lymphoprep^{TM} (Sweden) centrifugation techniques, and re-suspended in either or Phosphate-Buffered Saline (PBS) supplemented with 2% Foetal Calf Serum (FCS) and 1mM EDTA as a cell separation media or RPMI 1640 medium (Invitrogen^{TM}, USA) supplemented with 10% FCS, 1mM L-glutamine and 1mM Penicillin/Gentamycin as a culture media. A total of 80ml of blood was collected on two separate occasions (40ml each time), the first for PBMC isolation, cryopreservation and subsequent assays, the second 40ml was used for Treg functional assays.

2.2.1 Cyropreservation

Prior to Cyropreservation the cells were centrifuged at 500g for 5 minutes and resuspended in 1ml PBS. Then, 10μl of cells was mixed with 10μl 0.4% Trypan Blue and counted on a haemocytometer. Cells were stored at 10 million per ml or 1 ml only if lower than 10 million cells were recovered. The freeze media consists of two parts; the initial media the cells were resuspended in after centrifugation was 40% FCS, 60% RPMI, the second par, 20% DMSO 80% FCS, was added drop-wise, mixed in a 50:50 ratio. The cells were then aliquoted and placed in an -1°C/min foam container and placed in a -80°C freezer overnight. The cyrovials of cells were then deposited in liquid nitrogen.

2.2.2 Thawing

A single 50ml Falcon tube was used per patient with 10ml RPMI 1640 medium (Invitrogen^{TM}, USA) supplemented with 10% human donor AB- serum (Australian Red Cross Blood Service, Adelaide, Australia) in each tube. Benzonase was added at 2μl per 10ml when defrosting to remove and DNA or RNA from dead cells. Using a pipette the frozen cells were scraped and pipetted into the 50ml Falcon tube. The cyrovial was then rinsed with the media and the Falcon tube centrifuged at 330g for 7minutes. The cells were then re-suspended in the desired media and counted as per above haemocytometer protocol.

2.3 Immune Phenotyping

Several cell phenotypes were measured in the recruited KTR. In the T cell compartment; Naïve T cells (CD45RA$^+$CD62L$^+$), T central memory (Tcm, CD45RA$^-$CD62L$^+$), T effector memory (Tem, (CD45RA$^-$CD62L$^-$) and Tem CD45RA$^+$ (TEMRA, CD45RA$^+$CD62L$^-$) were measured in both the CD4 and CD8 T cell subclasses. Additionally, CD8$^+$CD27$^-$CD28$^-$ T suppressors were measured; Naive, Tcm, Tem and TEMRA subsets were also analysed in the T suppressor subset. In the CD4 compartment; CD45RA$^+$CD38$^+$ T cells as well as CD4$^+$FOXP3$^+$CD25^{hi}CD127^{lo}Helios$^{+/-}$, CD4$^+$FOXP3$^+$CD39$^+$CD127^{lo} as well as CD4$^+$FOXP3$^+$CD39$^+$CD73$^+$ were also measured. In the B cell compartment; Naïve (CD27$^-$IgD$^+$), class-switched memory (CD27$^+$IgD$^-$) and IgG expressing B cells were measured. In the NK compartment both double positive CD56$^+$CD16$^+$ as well as CD56^{bright} were measured and finally γδ T cells, including CD4 and CD8 subclasses were measured. Collectively these make up an individual's immune phenotype.

2.3.1 Immune Phenotyping: Surface and Intra-Cellular Staining

Combinations of the following directly conjugated, anti-human antibodies were used. Antigen-Fluorochrome (Clone): CD3-FITC (HIT3a), CD3-PECy7 (UCHT1), CD3-APC (OKT3), CD3-APC-Alexa Fluor® 780 (UCHT1), CD4-PerCP5.5 (OKT4), CD8-PerCP5.5 (RPA-T8), CD16-PE (ebioCB16), CD19-APC (HIB19), CD25-APC (BC96), CD27-FITC (SK1), CD28-PE (CD28.2), CD45-PerCP (HI30), CD45RA-APC (HI100), CD62L-APC AlexaFluor® 780 (DREG-58), CD127-FITC (eBioRDR5), FOXP3-PE (PCH-101) and IgG-2b-PerCP5.5 (eBMG2b), all purchased from eBioscience, USA. Also, CD8-PE (HIT8a), CD19-FITC (SJ25C1), CD56-APC (A159), γδ TCR-PE (B1), IgD-PE (IA-6) and BD Tritest^{TM} reagent all from BD Biosciences, USA. Helios-Pacific Blue (22F6) and CD3-Pacific Blue (OKT3) were purchased from Biolegend, USA. Whole blood samples (50µl) were stained with antibodies for 30 mins in the dark at 4°C. The stained whole blood samples were

then fixed and lysed in a TQ-prep automated lysis machine (Beckman Coulter, USA), then washed with PBS and re-suspended in 50μL PBS with 100,000 (2000/μl) APC conjugated CaliBRITE Beads (BD biosciences, USA) for lymphocyte enumeration.

The $5x10^5$ PBMC were stained with antibodies for 30 mins in the dark at 4°C in 50μL of PBS. The samples were then incubated with ammonium sulphate at room temperature (RT) for 5-10 mins in the dark to lyse red blood cells. The cells were then washed in PBS and pelleted at 300 x g for 5 mins at RT. Fixation and Permeabilisation buffer (eBioscience, USA) was added and cells incubated for 2 hrs at 4°C in the dark. The PBMC were washed in Permeabilisation buffer and pelleted, blocked with rat serum for 10 mins at RT in the dark. The FOXP3 antibodies were added and incubated for 30 mins at 4°C in the dark. The PBMC were then washed in Permeabilisation buffer and re-suspended in 50μL PBS.

2.3.2 Immune Phenotyping: Flow Cytometry

All flow cytometry processing and analysis was performed by laboratory technicians blinded to the clinical status of the KTR. Total cell counts were derived by adding a known concentration and quantity of enumeration beads (APC-CaliBRITE, BD Bioscience) to the lysed and washed whole blood samples prior to analysis by flow cytometry. Leukocytes in whole blood were defined on the basis of CD45 positivity and low side-scatter[194]. Gates were determined by population clustering where possible, otherwise delineation of negative gates was established via standard fluorescence minus one (FMO) controls[140], Seen in Figure 2.3.2. FMO gating was used instead of isotype controls to determine population positivity. This is due to isotype controls sometimes over estimating background fluorescence.
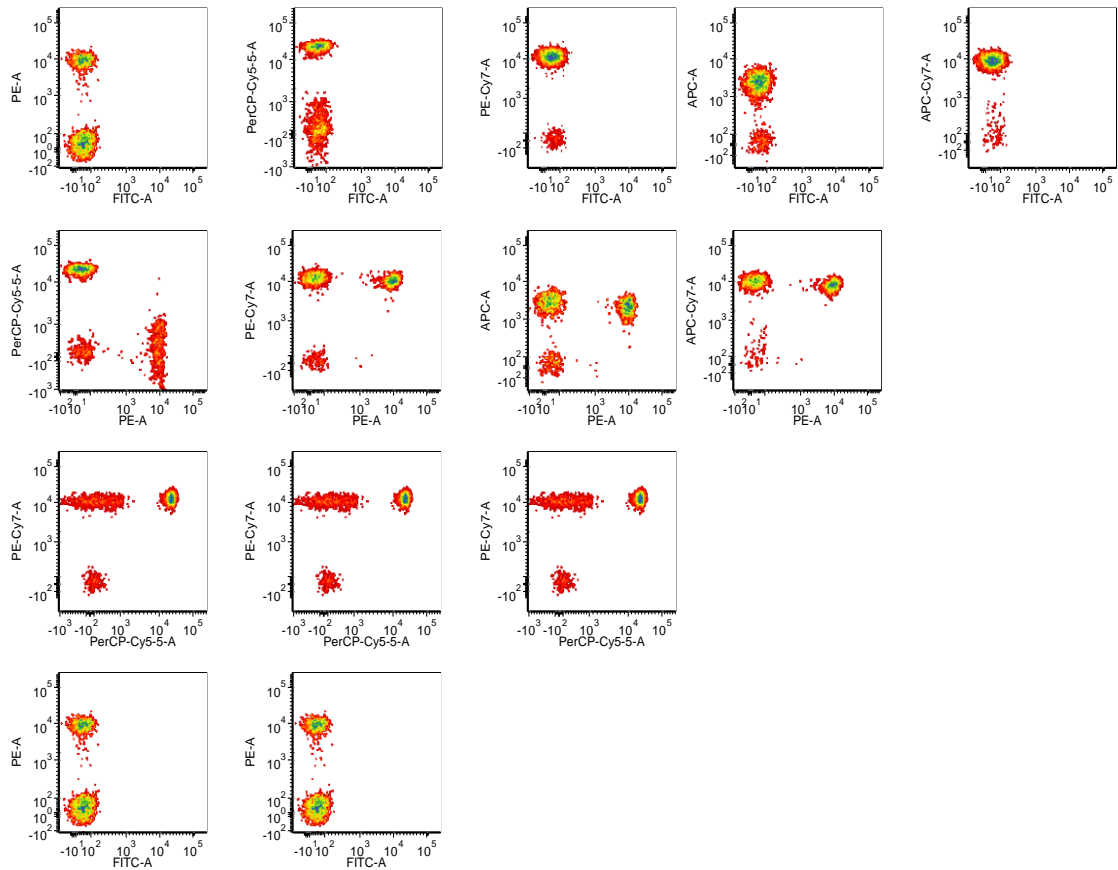
Figure 2.3.2: Example of Fluorescence Minus One (FMO) Gating: Flow cytometric density plots depicting gating FITC Fluorescence Minus One (FMO). Lymphocytes were gated on physical properties using Forward and Side Scatter profiles.

2.3.2.1 Immune Phenotyping: Flow Cytometry: Treg Gating with Helios

Forkhead Box Protein 3 (FOXP3) positivity was set against a negative internal control, defined by 0.1% positivity of FOXP3$^-$CD8$^+$ cells. The CD127 low (CD127^{Lo}) threshold was defined as the lower 50th centile of CD4$^+$ lymphocytes and the CD25 high (CD25^{Hi}) threshold defined as the 90th centile of the same population, as seen in Figure 2.3.2.1. At least 10,000 CD3$^+$ events were acquired, with at least 200 events in the CD4$^+$FOXP3$^+$ gate. These Tregs were then subjected onto to a population-based gating strategy for Helios positivity to define naturally occurring Tregs. Flow cytometry was performed on FACS Aria Cell Sorter or FACS Canto II Analyser (BD Biosciences, USA). Compensated data were analysed with FCS Express 4 Flow Cytometry analysis software (De Novo Software, USA).
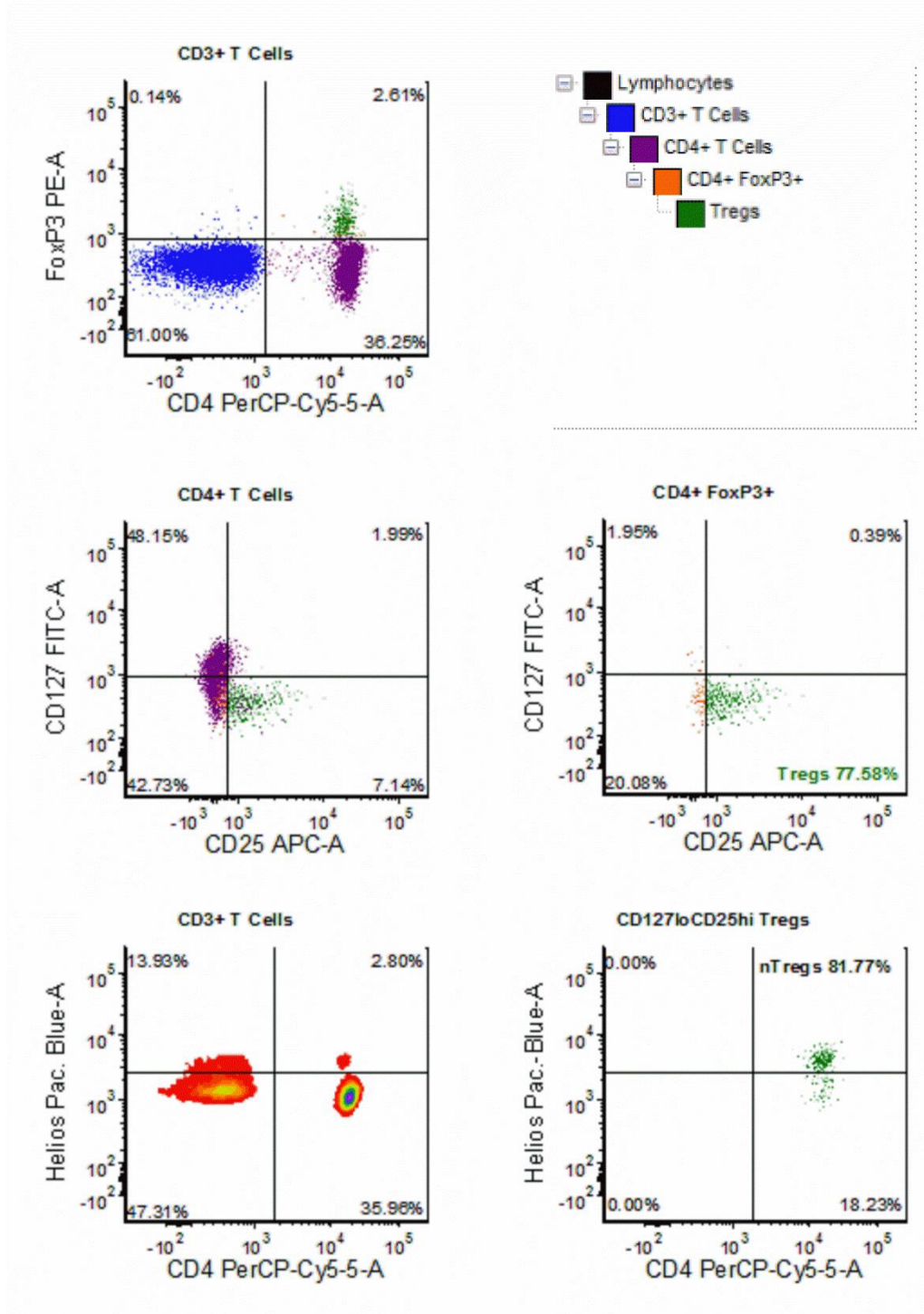
Figure 2.3.2.1: Regulatory T cell and Helios Gating Strategy for naturally occurring Tregs: Flow cytometric dot and density plots depicting gating hierarchy of gating Helios[+] regulatory T cells. Lymphocytes were gated on physical properties using Forward and Side Scatter profiles, subsequently gated by low-SCC vs. CD3 and Low-SCC vs. CD4 population gating, which enables identification of both CD3^{+} and CD3^{+}CD4^{+} T cells. These cells are then subjected to the gating strategy shown to identify Treg and nTreg populations.

2.3.3 Immune Phenotyping: Statistical Analyses

Although the immune phenotyping aims, experimental design and processing was carried out by myself, the following statistics were performed by Dr Blair Grace PHD, ANZDATA statistician from the Royal Adelaide Hospital.

Odds Ratios (OR) were generated using conditional logistic regression modelling to determine the strength of the association to cancer. This was run unadjusted and adjusted to known confounding factors such as Age, Gender and duration of immunosuppression.

The ability of immune phenotype to correctly identify those that developed cancer, within a 100 day period, was analysed using receiver operator characteristic (ROC) curves. Kaplan-Meier SCC-free survival curve and hazard ratios were developed using the trade-off value of 16 Tregs/µl as a risk stratifier.

In the 49 KTR with a history of SCC, 28 KTR developed a total of 37 SCC. Of the 37 SCC tumours that developed 22 (59%) were high risk SCC (poorly differentiated lesion, >2cm diameter with perineural invasion and invading deep structures beyond deep reticular dermis). ROC curve analysis was performed to determine the ability of Tregs to associate to this more severe SCC lesion.

Logistic regression was performed using STATA 12, as both univariate and multivariate analysis, adjusted for age, gender and duration of immunosuppression. KTR with cancer were matched for age (± 5yrs), gender and duration of immunosuppression (± 2yrs) and mTORi usage to KTR without a previous cancer. All analyses were two tailed tests. Data were analysed using Stata 12 IC (StataCorp Texas) and Prism v6.01 (GraphPad Software Inc., USA).

2.4 Treg Function

A laboratory standard Treg functional assay as well as a novel, shorter assay was utilised to determine isolated Treg bystander (non-antigen specific) function in the recruited KTR.

2.4.1 Healthy CD4$^+$CD25$^-$ effector T cell isolation

The naïve effector CD4$^+$CD25$^-$ T cells were freshly isolated on the day from a single healthy donor's PBMC, using Stem Cell Technologies (USA) separation kits in according to the manufactures procedures. An EasySep™ Human CD4$^+$ T Cell Enrichment Kit (Cat. #19052) was used, keeping the unbound fraction. This was followed with an EasySep™ Human CD25^{high} Positive Selection Kit (Cat. #18231) with the unbound CD4$^+$CD25$^-$ T effector cells being washed and resuspended in RPMI 1640 medium (Invitrogen™, USA) supplemented with 10% FCS, 1mM L-glutamine and 1mM Penicillin/Gentamycin as a culture media.

2.4.2 Healthy control and KTR CD4$^+$CD127^{lo}CD25$^+$ Treg isolation

Kidney Transplant Recipient CD4$^+$CD127^{lo}CD25$^+$ cells were freshly isolated by first using an EasySep™ Human CD4$^+$CD127^{low} T Cell Enrichment Kit (Cat. #19231), keeping the unbound T cells. Subsequently an EasySep™ Human CD25^{high} Positive Selection Kit (Cat. #18231) was used. Isolated CD4$^+$CD127^{lo}CD25$^+$ Tregs were washed and resuspended in the aforementioned culture media. These Tregs were used simultaneously in both the CFSE dilution assay as well as the CD154/CD40L Treg suppression assay.

2.4.3 CFSE dilution assay

Cell division was measured in 10µM CFSE labelled PBMC from the same single healthy donor as the CD4+ naïve T cells in the CD154/CD40L assay on the same day. To stain the PBMC, 1µl of CFDA-SE (Sigma, USA) was added to $2x10^6$ PBMC in 1ml of PBS, vortexed, incubated for 5mins in a 37°C, then placed in serum containing media, vortexed, and set aside for a further 5mins. The CFSE stained PBMCs were washed and resuspended in the aforementioned culture media. The KTR Tregs titrated down by a factor of 2 from 1:1

through to 1:32 of Treg:PBMC in 96 well U-bottomed plates. The co-culture was stimulated with CD3/CD28 beads at bead to cell ratio (1:8) over a 5 day period. Each test was run in triplicate and with negative proliferation control of PBMC alone (inadvertently, 100% suppression) and positive proliferation control (negative suppression) of PBMC with beads and no Tregs. Total proliferation was measured by quantitating the proportion of cells that expressed CFSE in-between these two controls and expressed as a percentage of the positive control. A total of 3×10^4 CD3$^+$ events were acquired on a BD FACS Canto II. Compensated data was analysed in FCS Express 4 (De Novo Software, USA). Each Treg titration from KTR with cancer was compared to KTR with no cancer using Mann-Whitney tests.

2.4.4 Suppression of CD154/CD40L expression assay

The CD154/CD40L assay was performed as previously described[195]. Healthy control and KTR Tregs were co-incubated with CD4$^+$CD25$^-$ effector T cells from the same healthy donor as the PBMC donor from the CFSE dilution assay, run on the same day. The KTR Tregs titrated down by a factor of 2 from 1:1 through to 1:32 of Treg:PBMC. The co-culture was stimulated in the presence of anti-CD3/CD28 expander beads (Invitrogen^{TM}, USA) at a bead to cell ratio (1:4) for 8-9h. After the incubation the cells were labelled with CD3-FITC, CD4-PerCP-Cy5.5 (eBioscience, USA) and CD25-PE-Cy7 (BD Bioscience, USA) antibodies. A total of 3×10^4 CD3$^+$ events were acquired on a BD FACS Canto II. The effector gate was set on unstimulated CD3$^+$CD4$^+$CD25$^-$ T cells, to exclude Tregs, and used throughout the assay. Compensated data was analysed in FCS Express 4 Flow Cytometry analysis software (De Novo Software, USA). The CD154 expression was normalised to the stimulated cells, without Tregs (100% stimulation), and data is shown as the percentage decrease of that expression. Each Treg titration from KTR with cancer was compared to KTR with no cancer using Mann-Whitney tests.

2.5 NK cell Function

Due to NK cells' anti-cancer ability and reduction in cell numbers and proportions in cancer patients, NK cell function was measured in cancer KTR. This was done in a total cytotoxicity assay and a novel measurement of NK cell interaction to the cancer cells.

2.5.1 Lactate dehydrogenase release

KTR PBMC were thawed as previously described in section 2.2.2, washed and resuspended in AIM-V BSA supplemented serum-free media (Invitrogen^{TM}, USA). The cells were then counted and resuspended at a cell concentration of $3x10^6$/ml PBMC. The PBMC were then co-cultured with $1.5x10^4$ (20:1) K562 cells or 100µL media, in triplicate, in a 96 well V-bottomed plate. The plate was spun at 240g for 4 minutes and incubated for 6 hours. After 5 hours a lysis solution was added to the maximal lysis wells. After 6 hours, the plate was spun at 240g for 4 minutes. Then, 100µl of supernatant was removed and Lactate dehydrogenase (LDH) release was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) in accordance with manufacturer's procedures. The LDH converts a colourless solution of Tetrazolium salt to Red formazan which can be quantitated by optical density (OD) readings from a photo spectrometer reading at 490nm.The reaction is uninhibited and needs to be stopped by increasing acidity (pH) with acetic acid. The OD readings are compared relative to the maximal lysis wells and expressed as a percentage of cell lysis. When comparing KTR with cancer Vs KTR with no cancer, a Mann-Whitney test was used. When comparing KTR with current cancer compared to KTR with past cancer and KTR with no cancer, a Kruskal-Wallis test was used.

2.5.2 Target Induced NK cell Lysis (TINKL)

TINKL was measured as previously published. The cells from the plate described in 2.5.1 are placed into FACS tubes and stained with 2µL of each of APC-eflour780-CD3, PE-CD16, FITC-CD19, PerCP-Cy5.5-CD45, APC-CD56 for 30mins at 4°C in the dark. The cells are washed of any unbound antibodies. At-least $3x10^{4}$ total events were acquired from the washed cells on BD FACS Canto II. Compensated data was analysed in FCS Express 4 Flow Cytometry analysis software (De Novo Software, USA). A ratio of NK cells (CD45^{+}CD3^{-}CD19^{-}CD56^{+}CD16^{+}) to CD45^{+}CD3^{+} T-cells was measured in the co-culture and compared to the background wells. The output of the assay is expressed as the percentage loss between these two ratios. A Mann-Whitney Test was used to compare KTR with cancer to KTR with no cancer and a Kruskal-Wallis Test to compare the KTR groups.

2.6 Cellular immune responses in KTR

The cellular responses in KTR or pre-transplanted dialysis patients have been shown to be predictive of outcomes in terms of graft function and rejection episodes. This section describes the methods used to determine cellular responses in the recruited KTR.

2.6.1 Anti-HLA and Anti-DSA antibody measurements (B cell responses)

The Lifecodes Tepnel Lifecodes Single Antigen 1 (LSA1) and LSA2 screen kits (Immucor, USA) were used to determine anti-HLA molecules. An MFI cut-off of 1500 units was used, as this has been defined in literature and routinely used in our diagnostic laboratory[188]. A Fishers exact test was used to compare KTR with cancer to those with no cancer.

Both these assays were performed by the Australian Red Cross Blood Service (ARCBS, Adelaide, S.A.) and data was collated and reviewed by Dr. William Hanf.

2.6.2 B-cell expansion

Healthy blood donor spenlocytes or PBMC of known haplotype were acquired from ARCBS liquid nitrogen storage and thawed as per section 2.2.2. The thawed splenocytes or PBMC were then subjected to magnetic bead isolation using an EasySep™ Human B Cell Enrichment Kit (Cat. #19054). The isolated B cells were counted and 1×10^5 viable B cells were co-incubated with a monolayer of 4×10^5/well of irradiated (43Gy) NIH-3T3-CD154 murine fibroblasts, in the RPMI 10% FCS. After 3 days of co-culture the B-cells were pooled, washed, counted then re-seeded at the starting density and expansion protocol repeated until 1×10^8 B cells had propagated. The supernatants from the last culture were tested for EBV RNA by PCR, and only those with a negative EBV test were used. Prior to cryopreservation, each cell line was tested for expression of MHC-CI and MHC-CII expression by flow cytometry. These cells were then cryogenically stored. A total of 4 B cell lines were used, shown in Table 2.1 and cover a mean(range) of 4.1 (3-5) of donor HLA antigens for KTR with cancer and 3.6 (2-6) for KTR without cancer.

2.6.3 Panel of Reactive T (PRT) cell IFN-gamma ELISPOT

When there were adequate cell numbers, the KTR responders were run in triplicate against AIM-V media, supplemented with BSA (Invitrogen™, USA), for background measurements, Cytomegalovirus Epstein Barr virus and Flu peptide pool (CEF, Mabtech, USA) for total viral responses, the 4 B cell lines in 2 different pools and Phytohemagglutinin (PHA, Sigma, USA) as a positive control. The MLR consisted of 3×10^5 thawed KTR PBMCs plated on an anti IFN-γ antibody (Endogen, USA) coated ELISPOT plate (Millipore, USA) co-incubated with the 5×10^4 B cells each (1×10^5/well) for 24h.

After which, the plates were washed 3 times with PBS and 3 times with PBSTween20. A secondary anti IFN-γ antibody (Endogen, USA) was added and incubated overnight at 4°C.

This antibody was then washed off in PBSTween20 with 1% BSA and Streptavidin-HRP was added for a 4 hour incubation at 4°C in foil. The plate was then washed again and the colouring reagent BD AEC Substrate Set (Cat.# 551951) added for 15 minutes. The reaction was stopped by running excess water over the plate and leaving it to dry overnight.

The plate was read on an AID ELISPOT reader using AID ELISPOT software (v3.1.1) from AID technologies, Germany. The wells then underwent quality control, which involved inspection of each well. A total of 6 plates were sent to Mt Sinai School of Medicine for reading on a CTL ELISPOT reader using CTL ImmunoCapture v6.4 and analysed in CTL ImmunoSpot v5.0, which trained staff then quality controlled by setting sensitivity and analysing each well. A further 5 plates were re-read at Flinders Medical Centre in the Department of Diabetes and Endocrinology using a CTL reader as well as CTL ImmunoCapture v6.4 and analysed in CTL ImmunoSpot v5.0 software. Accumulative data was analysed in Prism v6.02 using a Mann-Whitney test to compare the cancer and past cancer groups to the no cancer groups in separate analyses.

2.7 Prospective study of immunosuppression dose reductions in 12 KTR

12 KTR with a history of aggressive SCC, who were DSA negative on testing, were dose reduced on clinical grounds. Two had metastatic SCC and the remainder had developed lesions at high risk of metastasising. All 12 KTR were intolerant of mTORi conversion. The drug doses of the patients before and after reductions are listed in Table 2.7. Although the dose reductions are not statistically significant the maximal dose fell for all drugs. Additionally, 6 of the twelve KTR also stopped one or more drugs within their regimens.

Table 2.7: Drug and renal parameters after immune suppression dose reduction post diagnosis of aggressive or metastatic SCC skin.

	Before dose reduction	After dose reduction	P value
Prednisolone, mg/d (range)	5 (0-10)	5 (0-7.5)	0.202
MMF, mg/d (range)	1250 (0-2000)	1000 (0-1500)	0.135
AZA, mg/d (range)	37.5 (25-75)	25 (25-50)	0.345
Tacrolimus trough, (range)	5 (4-8)	0(0-4)	0.500
Cyclosporin trough, (range)	286 (120-680)	105 (0-349)	0.125
eGFR ml/min/1.73m^2 (range)	68 (24-89)	54 (31-89)	0.859
Creatinine µmol/l (range)	97 (70-244)	127 (78-197)	0.474

Chapter 3: Patient demographics and clinical characteristics

3.1 Introduction

When studying a heterogeneous group of patients it is important to identify all physiological, demographical and clinical characteristics that may impact the interpretation of the results. With the aim of investigating the effects cancer has on Kidney Transplant Recipients (KTR) it has been established that age, gender and immunosuppression can influence cancer acquisition and development.

This chapter introduces the cumulative cohort of KTR that were enrolled in the prospective study during June 2009 till August 2013, who form the basis of analysis in subsequent chapters.

3.1.1 Chapter aims

1) To describe the cancer status of the enrolled kidney transplant recipients and various sub-cohorts that will be subsequently analysed.

2) To identify if age, gender, induction therapy and causal nephropathy associate with cancer in the study cohort

3) To investigate if immunosuppressive drug type, duration, dose or measured serum levels associate with the cancer group.

3.1.2 Chapter hypotheses

1) Age, male gender, induction therapy will associate with cancer in our cohort.

2) Immunosuppression drug type, dose and duration will associate with cancer.

3.2 Patient selection

The total cohort consisted of 200 KTR, however there were 11 who were undergoing rejection and 33 who had presented with active infection. These patients were split into their own sub-cohort and analysed in only some of the subsequent analyses, as there was a limitation in the blood taken. They are depicted in Table 3.2.

Table 3.2.1 Entire cohort (n=200) patient demographics

	Rejection	Current cancer	Past cancer	Infection	No Cancer	*p-*value
N numbers	11	35	34	33	87	
Age*	**42 (27-68)**	**63 (38-75)**	**63 (44-78)**	**58 (37-74)**	**54 (20-77)**	**<0.001**
Male gender, *N* (%)	7 (64%)	26 (74%)	21 (62%)	18 (55%)	52 (60%)	0.520
Immunosuppression*	**1 (0-12)**	**9 (0-41)**	**11 (0-39)**	**3 (0-32)**	**3 (0-29)**	**0.014**
Drug regimen, N (%)						
Azathioprine	1 (9)	6 (17)	6 (18)	3 (9)	8 (9)	0.560
Mycophenolate	**9 (82)**	**22 (63)**	**22 (67)**	**28 (88)**	**72 (83)**	**0.044**
Calcineurin inhibitors	9 (82)	17 (49)	16 (33)	22 (67)	46 (53)	0.157
mTORi	1 (9)	9 (26)	7 (21)	1 (3)	14 (16)	0.111
Prednisolone	11 (100)	30 (86)	28 (85)	28 (88)	70 (80)	0.565
Drug dose†						
Azathioprine	**50**	**50 (13 - 75)**	**25 (25 - 50)**	**75 (25 - 75)**	**100 (75 - 100)**	**0.044**
Mycophenolate (g)	**2 (2, 2)**	**1 (1, 2)**	**1 (1, 1)**	**1 (1, 1)**	**2 (1, 2)**	**<0.001**
Cyclosporine A	**-**	**100 (100 - 150)**	**125 (100 - 150)**	**100**	**200 (100 - 200)**	**0.012**
Tacrolimus	2 (2, 3)	2 (1, 2)	2 (2, 5)	3 (2, 4)	2.5 (2, 4)	0.260
Everolimus	-	2 (1 - 3)	6 (3 - 8)	5	3 (2 - 3)	0.300
Sirolimus	-	2 (2 - 3)	2 (2 - 3)	-	2 (2 - 4)	0.786
Prednisolone	**10 (5, 10)**	**5 (5, 10)**	**5 (5, 5)**	**5 (5, 10)**	**8 (5, 10)**	**0.012**
Drug serum Levels						
Tacrolimus	7 (7, 8)	8 (5, 10)	7 (5, 7)	8 (6, 9)	7 (5, 8)	0.450
Cyclosporine A	-	59 (35, 104)	90 (43, 260)	132 (130, 134)	92 (51, 392)	0.610

*=years (range), †= median mg (range)

The remaining cohort consisted of KTR with current cancer (n=35) and KTR with a history of cancer (n=33), for a total KTR cancer cohort (n=68) and those KTR without any history of cancer (n=89), depicted in Figure 3.2.
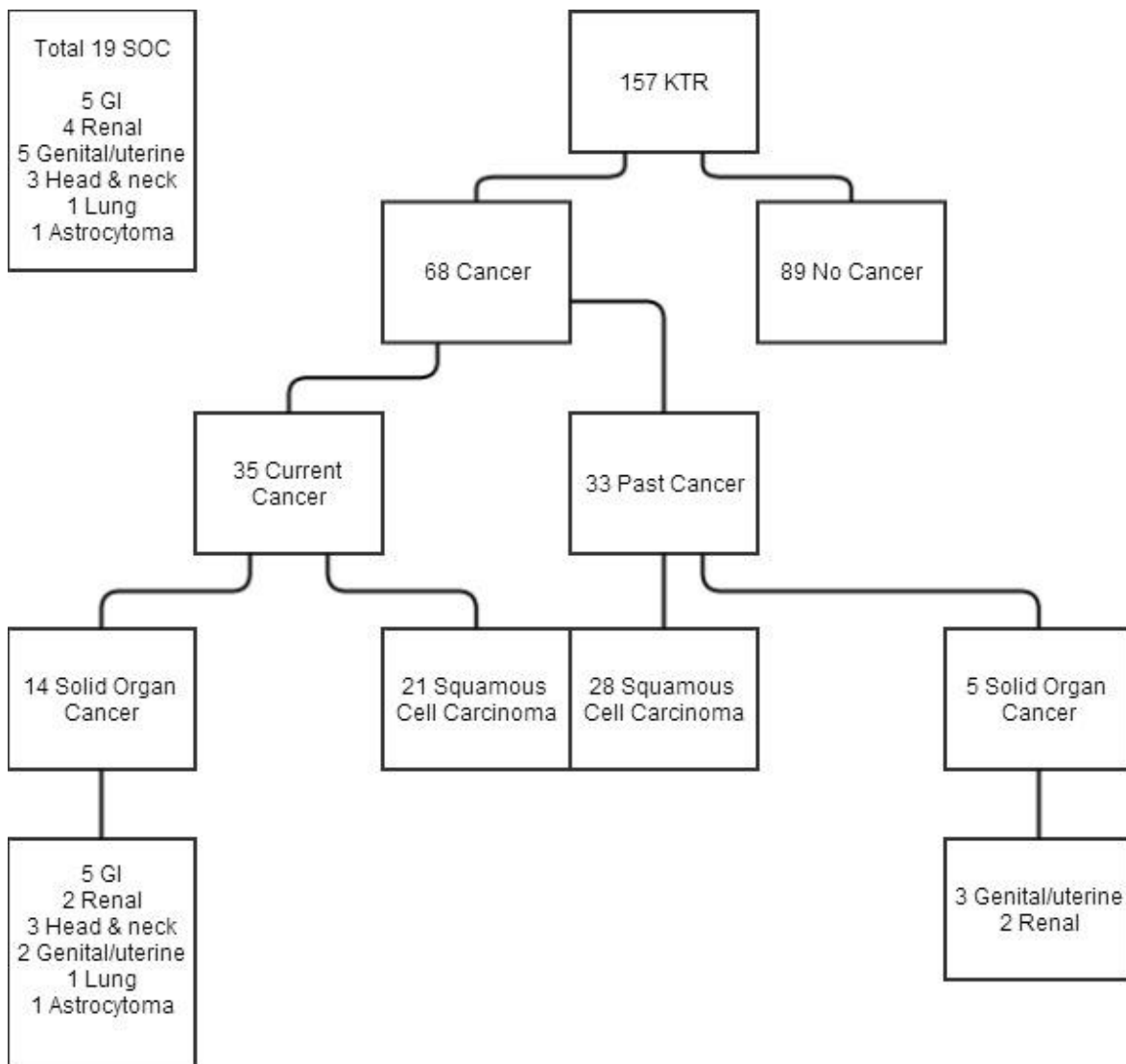
Figure 3.2: Strobe diagram of enrolled Kidney Transplant Recipient (KTR) cohort by cancer status: A total of 157 KTR enrolled, a total of 68 KTR in the cancer group consisting of 35 KTR with current cancer and 33 KTR with a past cancer. Within this group there were 19 KTR with SOC: 14 with current cancer and 5 with past cancer. There was a total of 49 KTR with SCC including both current and past cancer. The different types of SOC present in the recruited KTR are depicted in the subsequent groups and in the top left of the figure.

There were a total of 49 KTR with current or a history of SCC and 19 KTR with current of a history of SOC. Of the SOC there were; 8 KTR with Gastro-Intestinal (GI), 8 KTR with Renal, 5 KTR with genital ureteral cancer, 2 KTR with head and neck cancer, 1 KTR with lung and 1 KTR with astrocytoma.

All KTR were prospectively followed for a median time of 15 months with range (3-42). Of the KTR with SOC in situ, 14 KTR died during the course of the study. Two KTR with SCC *in situ* died of metastatic SCC. Two KTR with a history of SCC died, 1 KTR died from infection, the other from cardiovascular complications. Two KTR with no cancer at presentation developed SCC over the follow-up period and a further 2 KTR with no cancer died, 1 from infection and 1 from cardiovascular complications. Additionally from the 49 KTR with SCC there were 28 KTR who developed 37 tumours during the study. Of these 37 SCC tumours that developed, 22 (59%) were high risk SCC (poorly differentiated lesion, >2mm thickness with peri-neural invasion and invading deep structures beyond deep reticular dermis).

3.3 Patient Demographics

KTR age at transplantation was categorised into 4 groups (18-44, 45-54, 55-64 and 65+ years). Duration of immunosuppression was also categorised (0-4 (n=69), 5-9 (n=32), 10-19 (n=37) and 20+ (n=16) years). These and other categorical variables were compared using Pearson's chi-squared or Fishers exact chi-square tests as appropriate and presented in Table 3.3.1. Continuous variables between two groups were compared using Mann-Whitney test and Kruskal-Wallis rank tests and Wilcoxon rank-sum between multiple KTR sub-groups.

Table 3.3.1 Total cohort (n=157) patient demographics

	No cancer (N = 89)	Cancer (N = 68)	p-value
Age, median years (IQR)	49 (35, 58)	51 (44, 59)	0.190
Male gender, N (%)	53 (60)	46 (68)	0.300
Rejection episode(s), N (%)	19 (39)	12 (18)	0.690
Years of Immunosuppression (IQR)	**2 (1, 9)**	**10 (5, 12)**	**<0.001**
HLA mismatch	4 (2, 5)	3 (2, 5)	0.310
Causal nephropathy, N (%)			
Type 1 diabetes	6(7)	1 (1)	0.140
Type 2 diabetes	**7(8)**	**0 (0)**	**0.019**
GN	29 (33)	32 (47)	0.071
Reflux	11 (13)	3 (5)	0.097
Other	29 (35)	25 (41)	0.614

Those KTR with cancer had greater exposure to immunosuppressive therapies than KTR with no cancer. However, this cohort included patients that are within 1 year from transplant and during this first year the patient's immunosuppression is often still being adjusted. Additionally, we and others have found profound changes in immune phenotype during the first 12 months[196]. Therefore, a selection criterion of greater than 1 year post-transplant was applied to the cohort. It is nonsensical to compare KTR only 0-12 months post-transplant to KTR 10 years post-transplant.

The reduced cohort consisted of KTR with current cancer (n=34) and KTR with a history of cancer (n=31), for a total KTR cancer cohort (n=65), also KTR without any history of cancer (n=51) with demographics shown in Table 3.3.2. The 4 categories of years of immunosuppression became (1-4 (n=31), 5-9 (n=32), 10-19 (n=37) and 20+ (n=16) years) and included any pre-transplant immunosuppression. These selection criteria removed 3 KTR who developed cancer within 1 year and 38 KTR with no cancer. Additional data was collected for induction therapy in this cohort, shown in Table 3.3.2.

Table 3.3.2 Selected cohort (n=116) patient demographics

	No Cancer (*N* = 51)	Cancer (*N* = 65)	*p*-value
Age, median years (IQR)	**41 (33, 51)**	**50 (44, 58)**	**0.010**
Male gender, *N* (%)	32 (63)	44 (68)	0.580
Rejection episode(s), *N* (%)	8 (16)	12 (18)	0.810
Years of immunosuppression (Range)	7 (2, 29)	10 (2, 40)	0.098
Causal nephropathy, N (%)			
Type 1 Diabetes	5 (10)	1 (2)	0.085
Urinary Reflux	7 (14)	3 (5)	0.103
IgA Nephropathy	7 (14)	18 (28)	0.110
Drug treated nephrology	5 (10)	12 (19)	0.290
Other	27 (53)	31 (48)	0.709
Induction therapy, N (%)			
ATG	**2 (4)**	**11 (17)**	**0.037**
Anti-IL2Ra mAb	18 (35)	14 (22)	0.142
Viral Serological Status			
CMV	16 (48)	28 (68)	0.140
EBV	32 (84)	33 (80)	0.772

ATG=Anti-thymocyte Globulin, Anti-IL2Ra mAb = (anti-CD25 monoclonal antibody), Cytomegalovirus (CMV) and Epstein Barr Virus (EBV).

These results show that duration of immunosuppression was more closely relatable between groups. However KTR with cancer were older than those KTR with no cancer. There was no difference in Anti-IL2Ra mAb monoclonal antibody induction therapy. However, there was a difference in Anti-thymocyte globulin (ATG) usage. Both Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) serological status were available in 76/116 (66%) of KTR. There was no statistical difference between the CMV or EBV seropositive status of KTR with and without cancer.

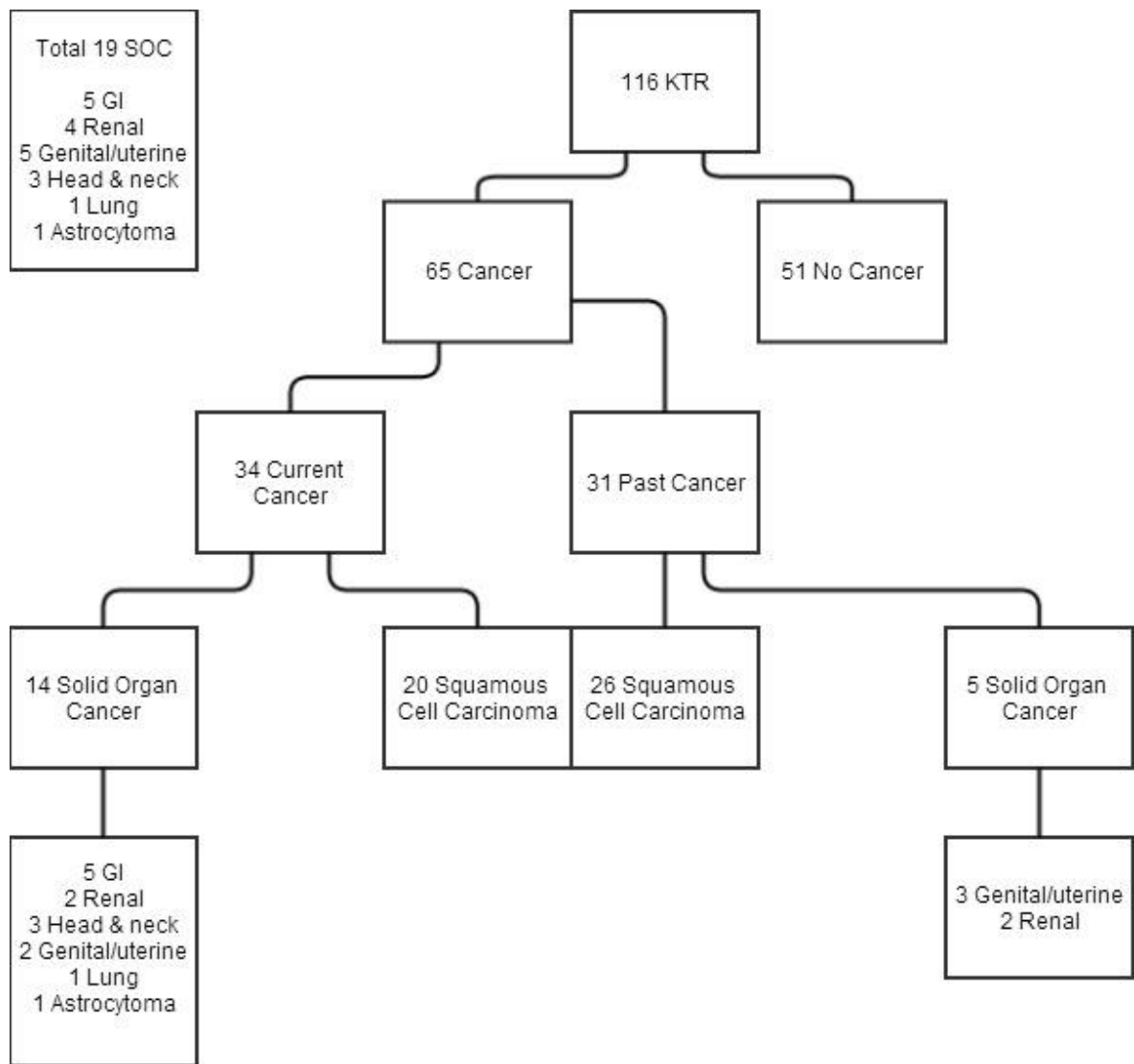**Figure 3.3 Strobe diagram of Kidney Transplant Recipient (KTR) selected cohort by cancer status:** Within the 116 KTR enrolled, a total of 65 KTR in the cancer group consisted of 34 KTR with current cancer and 31 KTR with a past cancer. Of these there were 19 KTR with SOC and 46 KTR with SCC. The different types of SOC are present in the respective categories with a total SOC breakdown in the top left of the figure.

3.4 Immunosuppression regimen dose and serum levels.

Table 3.4.1 depicts the type, dose and serum levels of the entire Cancer and Non-cancer cohort.

Table 3.4.1 Immunosuppression regimen, dose and serum levels in total cohort (n=157)

	No cancer (N = 89)	**Cancer** (N = 68)	*p*-value
Drug regimen, N (%)			
Azathioprine	8 (9)	12 (18)	0.107
Mycophenolate	**74 (83)**	**45 (66)**	**0.014**
Calcineurin inhibitors	**58 (65)**	**33 (49)**	**0.036**
mTORi	14 (16)	16 (24)	0.220
Prednisolone	73 (82)	58 (75)	0.667
Median drug dose in mg (IQR)			
Azathioprine	**100 (75, 100)**	**38 (25, 63)**	**0.004**
Mycophenolate, (g)	**1.5 (1, 2)**	**1 (1, 1)**	**<0.001**
Cyclosporine A	200 (100, 200)	100 (100, 150)	0.085
Tacrolimus	2 (2, 4)	2 (2, 3)	0.360
Everolimus	3 (2, 3)	3 (1, 5)	0.880
Sirolimus	2 (2, 4)	2 (2, 3)	0.500
Drug serum Levels, median µg/l (IQR)			
Tacrolimus	7 (5, 8)	7 (5, 10)	0.560
Cyclosporine A	92 (51, 392)	61 (43, 146)	0.370

Both Mycophenolate and Calcineurin inhibitors were used less in KTR with cancer than those KTR with no cancer. Also, both antiproliferatives Azathioprine and Mycophenolate had lower doses in the KTR with cancer than those KTR without cancer. Additionally, CNI dose was slightly lower, although not statistically significantly.

Table 3.4.2 Immunosuppression regimen, dose and serum levels in the selected KTR cohort (n=116).

	No Cancer ($N = 51$)	Cancer ($N = 65$)	p-value
Drug regimen, N (%)			
Azathioprine	6 (12)	11 (17)	0.440
Mycophenolate	41 (80)	43 (66)	0.089
Calcineurin inhibitors	30 (59)	31 (48)	0.230
mTORi	8 (16)	16 (25)	0.240
Prednisolone	35 (76)	54 (86)	0.200
Median drug dose in mg (IQR)			
Azathioprine	**100 (75, 100)**	**50 (25, 75)**	**0.024**
Mycophenolate, (g)	**1 (1, 2)**	**1 (1, 1)**	**0.008**
Cyclosporine A	200 (100, 200)	100 (100, 150)	0.150
Tacrolimus	3 (1, 5)	2 (1, 3)	0.450
Everolimus	2 (1, 2)	3 (1, 5)	0.240
Sirolimus	2 (2, 10)	2 (2, 3)	0.390
Prednisolone	5 (5, 8)	5 (5, 8)	0.900
Drug serum Levels, median µg/l (IQR)			
Tacrolimus	6.5 (5, 8)	7 (5, 10)	0.320
Cyclosporine A	92 (51, 392)	75 (57, 147)	0.620

Introduction of the selection criterion reduced the proportion of KTR with no cancer on Calcineurin inhibitors, perhaps due to early maintenance therapy mainly being a CNI based regimen. Regardless, both anti-proliferatives Azathioprine and Mycophenolate were still at lower doses in the KTR with cancer than those KTR without cancer. Both these drugs were tapered and/or ceased upon cancer diagnosis however this cannot be measured within this cohort. Age, gender and duration of immunosuppression were associated with cancer in this cohort and will be adjusted for in logistic regression modelling shown in chapter 4.

3.5 Functional study sub-cohort

Functional studies were limited by the amount of blood taken, lymphopenic patients, and access to rural or remote patients. This limited the patients' numbers to a sub-cohort of the entire recruited cohort. This cohort was used in chapter 5 and 6 to study the functional status of KTR with cancer. It consisted of 31 KTR with cancer, 17 KTR with current cancer, 14 KTR with past cancer and 19 KTR with no history of cancer. All the demographic data is shown in Table 3.5.

Table 3.5 Functional studies sub-cohort, Patient characteristics

	No cancer (N = 19)	Cancer (N = 31)	*p*-value
Age, median (IQR) years	49 (35-51)	49 (44-57)	0.242
Gender, N (%) Male	12 (63)	21 (68)	1.000
Years of immunosuppression	7 (4-24)	10 (5-12)	0.850
Rejection episodes, N (%)	4 (21)	5 (16)	0.715
Multiple transplants, N (%)	2 (11)	7 (23)	0.452
HLA mismatch, (median) range	4 (2-6)	3 (0-6)	0.704
Donor Specific Antibodies (DSA), N (%)	3 (16)	3 (10)	0.661
Non-DSA, HLA antibodies, N (%)	0 (0)	5 (16)	0.142
Drug Usage			
mTORi, N (%)	1 (5)	8 (26)	0.127
Calcineurin Inhibitors, N (%)	9 (47)	15 (48)	1.000
Azathioprine, N (%)	2 (11)	9 (29)	0.167
Mycophenolate, N (%)	16 (84)	19 (61)	0.117
Median Drug Dose (Range), mg			
mTORi	-	3 (1-8)	N/A
Cyclosporine A	200 (80-250)	125 (100-150)	0.197
Tacrolimus	2 (1-6)	3 (1-3)	0.946
Azathioprine	88 (75-100)	25 (12.5-50)	N/A
Mycophenolate	1250 (500-3500)	1000 (500-3000)	0.522

Due to numbers, both mTORi and Azathioprine drug doses could not be statistically compared. Otherwise there was no statistical difference between the two sub-groups.

3.6 Discussion

In summary, the enrolment yielded a cohort of 200 KTR. However, this included those with active infection, rejection episodes and KTR still within their first year of transplantation. The final immune phenotyping cohort consisted of 116 KTR, in which KTR with cancer were older and had increased ATG usage than those with no cancer.

KTR in our cohort were younger and had less exposure to immunosuppressive drugs than the UK study[141], but older and have had longer exposure to immunosuppressive drugs than all other post-transplant malignancy, prospective immune phenotyping studies[101-104, 197]. Our KTR with cancer was roughly 66% male, which aligns with the other post-transplant studies. Induction with ATG was prevalent throughout all studies (35% to 92%) however, only 17% of our KTR cancer cohort was treated with ATG induction therapy and none in the UK based study[141]. The other differences were in immunosuppression regimens. All other studies had >70% Calcineurin Inhibitor (CNI) use, with >70% Azathioprine use. There has only been one other immune phenotyping study involving mTORi use, which converted 13 KTR from CNI to mTORi usage[198]. Additionally, our cohort had the highest MMF usage as 66% of our cohort were administered with MMF and only 17% utilised AZA as anti-proliferative medication. These differences may affect immune phenotype and will be addressed in Chapter 4 of this thesis.

In conclusion, there were differences in age, gender and duration of immunosuppression during various iterations of analysis and found in literature, these parameters will be adjusted for in analysis in Chapter 4. However, the functional cohort which consisted of 50 KTR, (31 KTR with cancer and 19 KTR with no cancer) was already closely matched and will be used in Chapters 5 and 6 to investigate immune functions, without adjustment.

Chapter 4: Immune Phenotyping

4.1 Introduction

Immune phenotype has been previously investigated in Kidney Transplant Recipients (KTR). Literature reports that <400-600 CD4$^+$ T cells/µl of blood associates to cancer in KTR[70, 102-104]. In both studies, the majority of the cohort underwent Anti-Thymocyte Globulin (ATG) induction therapy, which is known to cause CD4 lymphopenia[70]. Both studies concluded that CD4 lymphopenia associated with cancer. One study showed that ATG impaired CD4 T cell number, but did not associate with the occurrence of cancer[103]. Furthermore, there was no CD4 cell count threshold that could accurately positively and/or negatively predict cancer using Receiver Operator Curve (ROC) analysis[103]. CD8$^+$ T cells and CD19$^+$ B cells were also investigated in the same study; there was no difference between KTR with Squamous Cell Carcinoma (SCC) when compared to KTR without SCC[104]. It was noted however, that immune phenotype was more pronounced in KTR with Solid Organ Cancer (SOC) compared to KTR with SCC: CD4 count: 234 cells/µl Vs. 543 cells/µl, p<0.001; CD8: 328 cells/µl Vs. 640 cells/µl p=0.100; CD19: 19 cells/µl Vs. 52 cells/µl, p<0.001[104].

While these studies provide some evidence that cancer may influence the peripheral immune cells, there was no investigation into sub-types of these cells, primarily because multi-parameter flow was not common place. Recently, it was reported that high numbers of CD4$^+$ Regulatory T cells (Tregs, *i.e.* CD4$^+$FOXP3$^+$CD127^{Lo}CD25^{Hi}) and low numbers of Natural Killer (NK cells, i.e. CD56$^+$CD16$^+$), in peripheral blood associated with and predicted recurrent SCC in KTR[141]. This study also showed an increase in CD8$^+$CD28$^-$. These CD8 T cells co-localise with Tregs within cancer tissue and have been shown to be suppressive from patients with cancer, and therefore abbreviated to CD8$^+$ Tsupps[170]. Furthermore, there was a decrease in CD8$^+$CD45RA$^-$CD62L$^+$ CD8 central memory T cells (CD8$^+$ Tcm), which has been shown to decrease in KTR using the corticoid steroid prednisolone, despite cancer status[141]. This indicates that immunosuppression may affect immune phenotype.

Operationally tolerant organ transplant recipients have increases in Regulatory T cells, B cells (particular naïve B cells), Vδ1 γδ T cells and decreases in CD3^{+} proportions (B:T ratio), NK cells, and Vδ2 γδ T cells within their peripheral blood[199]. Also it is already known that KTR with cancer have increases in Tregs and decreases in NK cells[141]. Therefore changes in B cells and γδ T cells that occur in these patients will be investigated.

While the United Kingdom-based study did investigate a variety of sub-populations of lymphocytes within KTR with SCC, the study did not investigate their associations to SOC. Additionally, this study did not investigate subtypes of Tregs, such as natural Tregs, which co-express the transcription factor, HELIOS. Furthermore, the study did not measure if Tregs numbers associate with predicting the severity of SCC tumour and/or if Immune Phenotype is dynamic upon cancer resection. The severity of SCC is introduced in Chapter 1: Section 1, briefly, >2.8mm depth, >2mm thickness, larger diameter and poorer differentiation are characteristics of a SCC tumour at high risk of developing in metastatic SCC. These tumours are aggressive and it would be of clinical benefit to be able to predict the severity of SCC of the tumour that is accrued. It is also important to determine if factors such as active viral infection, induction therapy and immunosuppression maintenance have an effect on immune phenotype, as there is evidence that these factors may affect immune cells, introduced in Chapter 1- Section 1.5-1.7.7). This chapter reports the results of the immune phenotyping of the cohort mentioned in Chapter 3 using methods described in Chapter 2. Immune Phenotyping was performed on fresh samples as has been shown that cryopreservation directly affects Foxp3 expression as well as many other surface markers such as CD62L and CCR5[200-204].

4.1.1 Chapter Aims

1) To determine if there are any differences in immune phenotype in Australian KTR with SCC compared to KTR with no SCC.

2) To assess if tolerance phenotype exists in long-term KTR

3) To examine if Tregs associate with Solid Organ Cancer (SOC) in KTR.

4) To investigate the effect cancer has on naturally occurring Tregs (nTreg)

5) To prospectively study if immune phenotype can predict SCC development in Australian KTR.

6) To evaluate if cancer resection alters immune phenotype of the peripheral blood.

4.1.2 Chapter Hypotheses

1) Australian KTR with cancer, both SCC and SOC, will have increased $CD4^+$ Treg and $CD8^+$ T suppressors with decreased $CD8^+$ T cm and NK cells. (*a priori*)

2) A greater number of KTR with SCC will have an immune phenotype resembling a tolerance immune phenotype. (*a priori*)

3) Differential HELIOS expression will determine if SCC in KTR induces Tregs (iTregs) or expands natural Tregs (nTregs).

4) $CD4^+$ Tregs will predict cancer onset (*a priori*)

5) $CD4^+$ Treg number will associate with severity of the cancer developed

6) $CD4^+$ Tregs will associate with cancer tissue presence.

When statistically assessing all *a priori* hypotheses, the results will be considered statistically significant if the significance is $p < 0.05$, for all other hypotheses, a significance of $p < 0.01$ will be needed, due to testing multiple parameters.

4.2 Chapter Methods

4.2.1 Antibody Panel

For a research based assay to become more clinically relevant it needs to be non-invasive, efficient, rapid, reproducible, cost effective and validated in multiple populations. To address these issues the amount: of physical handling, blood needed for reproducible staining and antibodies was reduced.

Recent developments into Treg subtypes indicate a possibility that CD39 and CD73 Tregs may also be increased, thus two separate Treg assay samples were created. Investigation into Natural Killer cell markers with the addition of APC-beads allowed enumeration of T, B and NK cells. Cell markers CD27, IgD and IgG were used to determine B cell nativity. An additional tube was created to investigate γδ T cells and CD4/CD8 subtypes. A commercially available Tritest® dye was also used to enumerate T and B cell numbers. The cell markers and associated fluorochromes are summarised in Table 4.2.1.

Table 4.2.1	FITC	PE	PerCP-Cy5.5	PeCy7	APC	APC-eFlour780
CD8 Subsets	CD27	CD28	CD8	CD3	CD45RA	CD62L
CD4 Memory	CD27	CD38	CD4	CD3	CD45RA	CD62L
CD4 Treg I	CD127	FOXP3	CD4	CD39	CD73	CD3
CD4 Treg II	CD127	FOXP3	CD4	CD3	CD25	CD62L
NK Cell	CD19	CD16	CD45		CD56 + Beads	CD3
B Cell	CD27	IgD	IgG	CD3	CD19	
γδ T Cell	CD8	γδ	CD4	CD3		
Tritest ®	CD3	CD19	CD45		Beads	

This panel enables identification of the following cells. $CD8^+$: $CD27^-$, $CD28^-$, $CD27^-CD28^-$, naïve, Tcm, Tem and TEMRA with the addition of $CD27^-CD28^-$: naïve, Tcm, Tem and TEMRA. The $CD4^+$ sub-populations included: naïve, Tcm, Tem, TEMRA and $CD45RA^+CD38^+$. Two subsets of NKT cells: $CD16^+$ T cells and $CD56^+$ T cells were measured and 4 different NK cell types: $CD16^+$ and $CD56^+$ single positive, $CD56^{++}$ NK "brights" and $CD56^+CD16^+$ NK cells. The selection of antibodies in the B cell tube enabled measurement of naïve B cells, class-switched memory B cells as well as any IgD or IgG expressing B cells. There were 4 distinct $CD3^+\gamma\delta$ T subpopulations analysed; $CD4^+CD8^-$ (CD4 single positive), $CD4^+CD8^+$ (double positive), $CD4^-CD8^+$ (CD8 single positive), and $CD4^-CD8^-$ (double negative). Out of the 7 tubes approximately 35 different cell types were analysed. To build this panel the antibodies were titrated and added to the panel a single antibody at a time, sequentially.

4.2.2 Antibody Titrations

To titrate multiple antibodies in a multi-colour panel, single antibodies need to be run alone and together within the panel. This was performed with every addition of a new antibody to assess antibody interactions. When HELIOS was added to the Treg tubes within the panel, it was added on the Pacific Blue (excitation of 400-450nm) channel and the titration of CD3-Pacific blue will be used an example. The Pacific Blue antibody did not leak into the other 6 channels in the compensation tube and there was no effect on CD3% positivity in the Fluorescence Minus One (FMO). As it can be seen the antibody stained the cells using only 1µL of stock antibody.
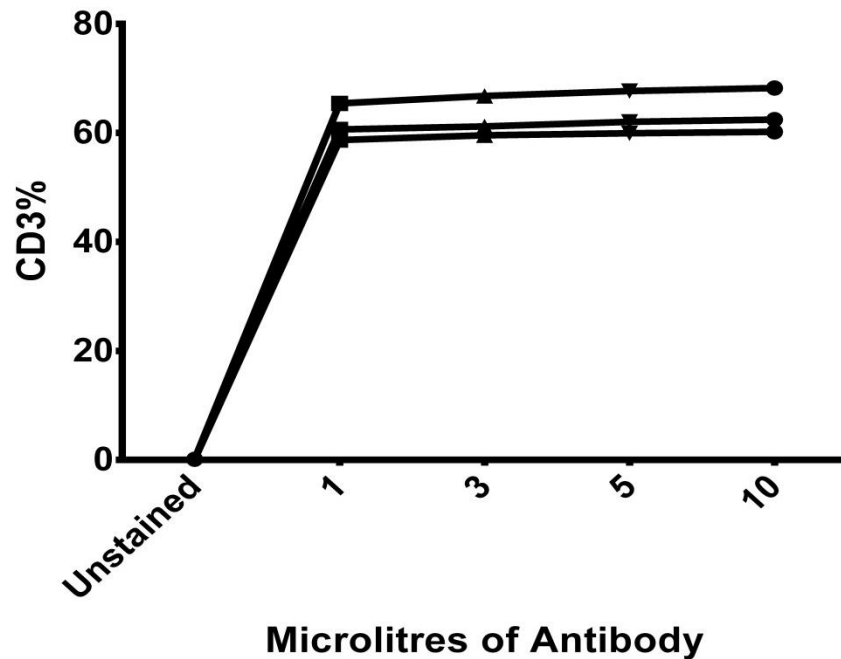

Figure 4.2.2: Pacific Blue antibody titration: A Line graph depicting the proportion of CD3 positive T cells in Peripheral Blood Mononuclear Cells (PBMC) from 3 individuals stained with 1,3,5 and 10µL of Pacific Blue conjugated CD3 antibody, analysed with FACS.

All antibodies were titrated and used in the panel shown in Table 4.2.1 as per protocol mentioned in Chapter 2: Section 2.3.

4.2.3 Titration of whole blood

Two aliquots: 50 and 100 µl of whole blood from 7 KTR were stained and several primary gates were analysed. Decreased volume of blood did not affect the cell surface molecules and was comparable, seen in Figure 4.2.2. The greatest variation was from γδ T cells (gd T cells) with a p-value = 0.110.
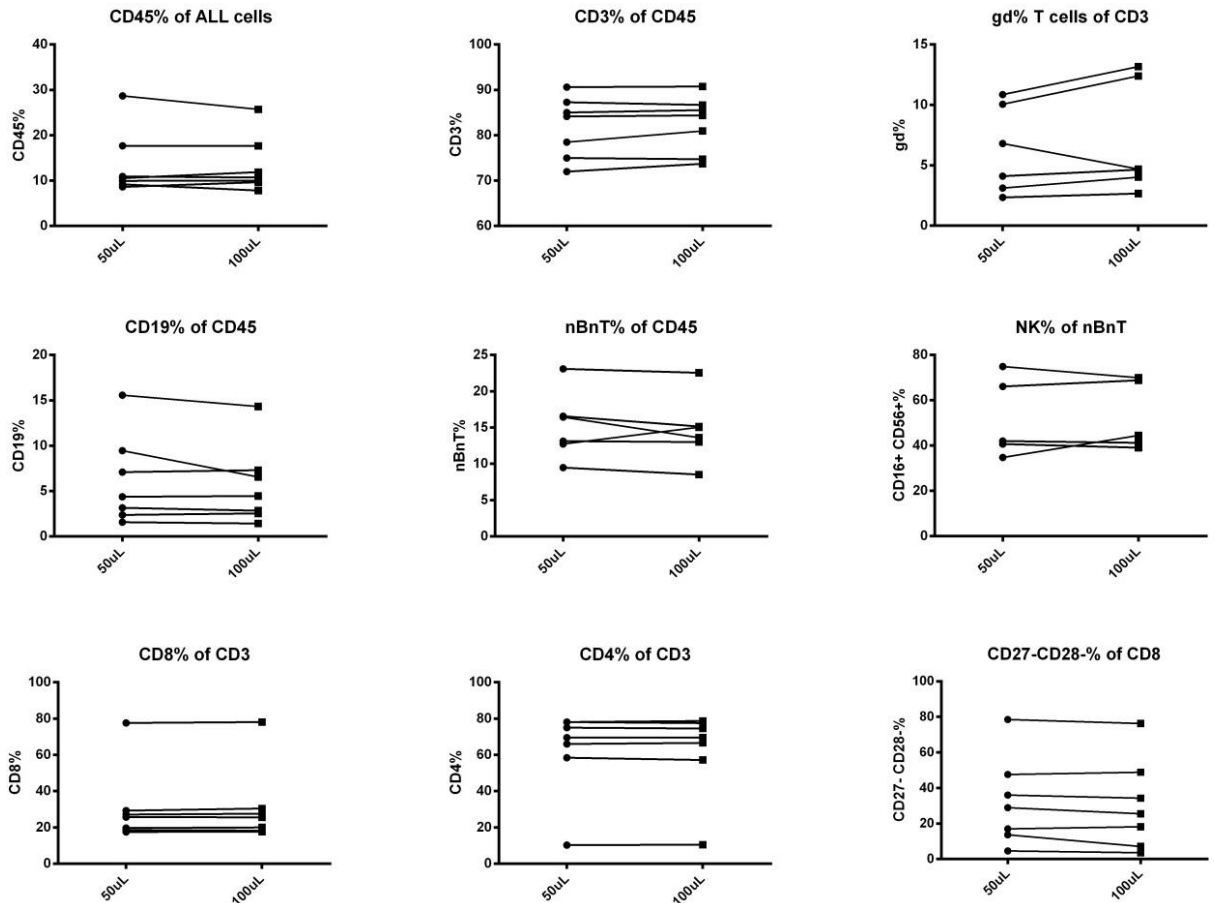

Figure 4.2.3: Staining of 50 and 100µl whole blood: A series of line graphs depicting proportions of various cell types using FACS antibodies in 50µL and 100µL of peripheral whole blood from KTR (n=7) with p-value median (range) = 0.330 (0.110-1.00) using Wilcoxon Paired tests. The greatest variation was from γδ T cells (gd T cells) with a p-value = 0.110.

Therefore, 50µl was sufficient to measure the proportions of cells, reducing the amount of blood needed to run an immune phenotype.

4.2.4 Regulatory T cell staining optimisation

The current gold standard of identifying Tregs includes staining for the FOXP3 regulatory gene transcription factor. As FOXP3 is an intracellular transcription factor that requires permeabilisation for the antibody to bind. The permeabilisation technique required optimisation and techniques which need to be consistent throughout the study. This section shows the investigation into the Treg staining.

4.2.5 FOXP3 antibody titration

To reduce the amount of antibody used, a assay was run on 7 KTR PBMCs. The antibody was satined at 2.5 and 5µl volumes. Below is a graphical representation of the FOXP3$^+$% of CD4 T cells. It shows that there is no difference between using 2.5 or 5µL of antibody. This was compared using a paired T-test with a p-value >0.999. The greatest variation was an 0.8% increase in FOXP3 positivity as shown in Figure 4.2.5.
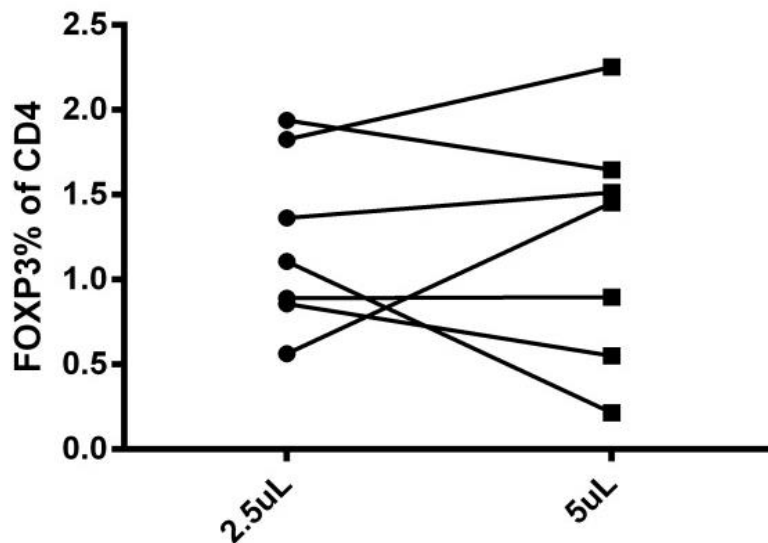

Figure 4.2.5: FOXP3 antibody titration: A line graph showing the proportion of positive FOXP3 cells from CD4+ FACS events. 7 Kidney Transplant Recipient PBMC stained with 2.5 or 5 µl of anti-FOXP3 were compared. There was no difference between using 2.5 or 5µl of FOXP3 antibody, using paired T test, p>0.999 with the greatest variation = 0.8%.

4.2.6 FOXP3 Whole blood staining

It is acknowledged, but unpublished, that when staining FOXP3 in Whole blood the signal to noise ratio is much greater than when staining PBMC, creating decreased levels of FOXP3$^+$ CD4 T cells. In 2010, eBioscience released a Whole blood FOXP3$^+$ staining kit (Cat # 88-8996) which was used, as per protocol, to stain 23 KTR. The results were compared to PBMC on the same sample to determine if there is a decrease in the amount of FOXP3$^+$ cells when using lysed Whole blood in comparison to ficoll separated PBMC, seen in Figure 4.2.6.
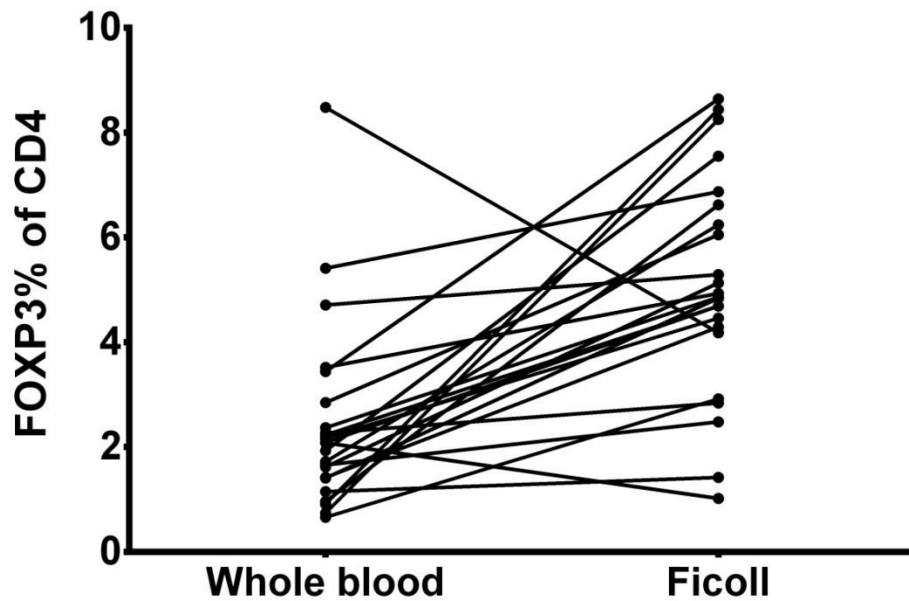

Figure 4.2.6: FOXP3 staining in ficolled PBMC compared to lysed whole blood: A line graph depicting the comparison of the proportion of fluorescently labelled FOXP3 in CD4 positive FACS events from lysed Whole blood and ficolled PBMC, in 23 KTR. Mean FOXP3% for ficoll is 5.1% Vs. 2.4% for whole blood, p<0.001 using paired Wilcoxon Two-tailed test.

There was a two-fold decrease in the mean FOXP3 expression between using Ficoll separated PBMC to Whole blood. Therefore, PBMC was used to determine CD4$^+$FOXP3$^+$CD25^{hi}CD127^{lo} Tregs as outlined in Chapter 2: Section 2.3 and reported in Section 4.3 of this chapter.

4.2.7 FOXP3 clone validation

In 2007 a study showed that T cell receptor activation of CD4 naïve cells in the presence of TGF-β induced FOXP3. The paper, and running commentaries, suggested that anti-FOXP3 antibody clone PCH-101 has non-specific binding properties under specific conditions[205]. An assay was used to determine if PCH-101 was increasing the Treg signal via non-specific binding. The clone PCH-101 was compared to clone 259D/C7. The assay utilised 5 samples and the proportion of FOXP3+ CD4 T cells was similar for both clones. This indicates that PCH-101 is appropriate for use in the freshly isolated unstimulated blood for immune phenotyping (Figure 4.2.7). The greatest shift was 0.4%.
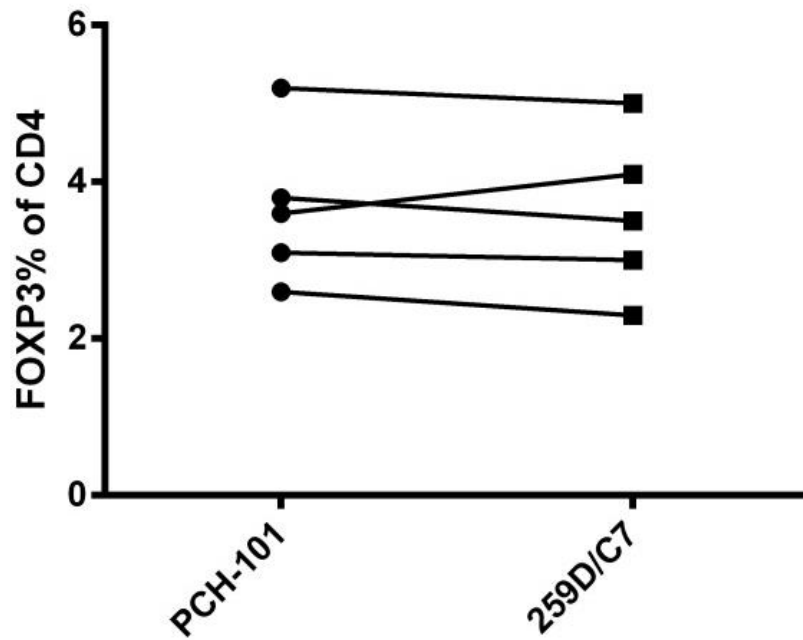

Figure 4.2.7: FOXP3 clone comparison: A line graph of FOXP3% of CD4 events from FACS analysis of 5 PBMC samples stained with PCH-101 and 258D/C7 clones. There is no difference in clones p>0.999, using a paired Wilcoxon Two-tailed test.

4.3 Results

4.3.1 Utilisation of HELIOS as natural Treg (nTreg) marker

The expression of FOXP3 was measured in the presence and absence of HELIOS and vice versa to determine any differences that may occur with the addition of a new antibody (n=3). There were no antibody interactions and therefore both HELIOS and FOXP3 can be used simultaneously (data not shown). To investigate the interactions cancer may have on Tregs, the Ikaros family transcription factor, HELIOS, was used as a marker of thymic-derived or naturally occurring Tregs (nTreg), rather than peripherally derived or induced Tregs (iTregs). The expression of HELIOS was measured in the Tregs of 51 KTR and 6 Healthy controls, the results are shown in Figure 4.3.1.
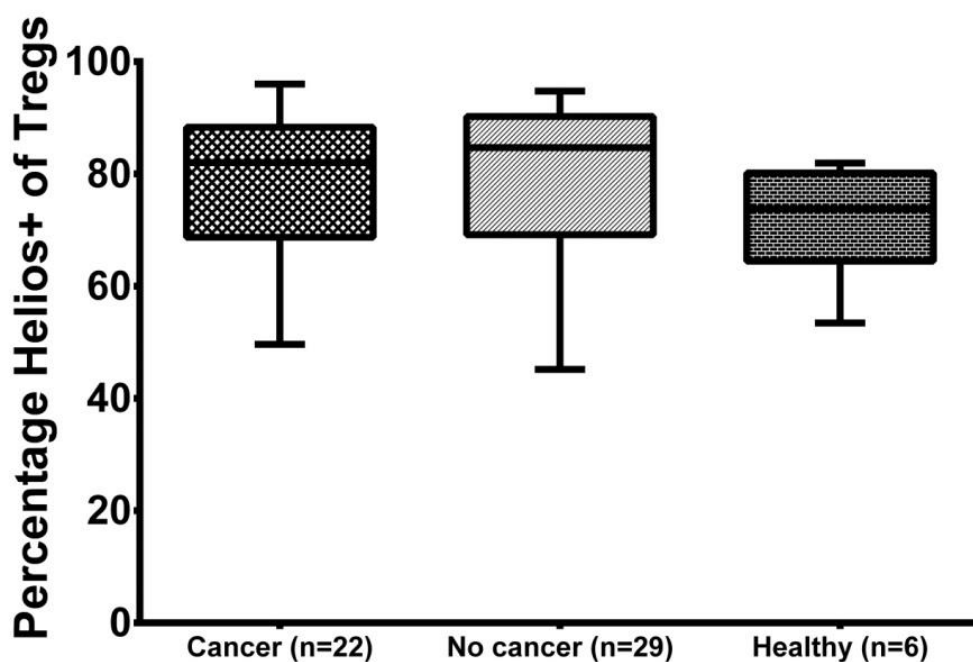

Figure 4.3.1: Helios co-expression in gated Tregs of Kidney Transplant Recipients with cancer and with no cancer: A box and whisker plot depicting the percentage co-expression of the Ikaros transcription factor, Helios, in Regulatory T cells in Kidney Transplant Recipients (KTR) with cancer Vs. KTR with no cancer Vs. Healthy controls with median% (range); 82 (50-96), 85 (45-95) and 74 (53-82) respectively.

Although there were, not statistically different, increased proportions in KTR (82-85%) to that observed (74%) and reported in the general population (70%) there were no differences between those with cancer and those without[118].

4.3.2 Immune Phenotype and association to cancer in KTR

This section will investigate one of the primary aims of this thesis: To determine if there are any differences in immune phenotype in Australian KTR; with SCC or with SOC, when compared to those KTR with no cancer. Approximately 37 different cell types, many with both proportion and enumeration, were investigated. However, there were *a priori* hypotheses that included investigation into; $CD8^+$ T suppressors ($CD8^+CD27^-CD28^-$), $CD8^+$ central memory T cells ($CD8^+CD45RA^+CD62L^-$), $CD4^+$ T cells, $CD4^+$ regulatory T cells ($CD4^+FOXP3^+CD127^{lo}CD25^{hi}$), and NK cells ($CD3^-CD19^-CD16^+CD56^+$). These cells were investigated in two different ways. Firstly, to investigate the presence of cancer in KTR, current cancer was compared to KTR who never had cancer and secondly, KTR with a history of cancer was compared to KTR who never had cancer. As mentioned when statistically assessing all *a priori* hypotheses, the results will be considered statistically significant if the significance is $p<0.05$, for all other hypotheses, a significance of $p<0.01$ will be needed, due to testing multiple cell types. Table 4.3.2.1 shows the investigated immune phenotype of these three groups. This is raw phenotype data that has not been adjusted for age, gender and duration of immunosuppression.

Table 4.3.2.1: Median (Range) of immune phenotype values comparing between kidney transplant recipients groups by Kruskal-Wallis non-parametric testing

	Current cancer N = 35	Past cancer N = 33	No cancer N = 89	p-value
CD45 Count	1740 (1140, 2390)	1400 (740, 2080)	1820 (1160, 2990)	0.086
CD3 of CD45	76 (67, 85)	79 (69, 86)	78 (66, 82)	0.540
CD3 Count	1280 (890, 1870)	1050 (570, 1830)	1340 (860, 2340)	0.170
CD8 of CD3	35 (24, 45)	35 (27, 45)	31 (25, 41)	0.650
CD8 Count	430 (230, 560)	440 (170, 640)	380 (260, 660)	0.840
naive T cells of CD8	17 (7, 25)	20 (13, 33)	23 (12, 44)	0.075
TCM cells of CD8	6 (3, 11)	6 (3, 13)	6 (2, 12)	0.970
$CD8^+CD27^-CD28^-$%	47 (22, 66)	45 (17, 58)	35 (15, 57)	0.290
$CD8^+CD27^-CD28^-$ Count	190 (70, 345)	222 (30, 370)	120 (35, 300)	0.390
CD4 of CD3	60 (45, 67)	53 (46, 62)	63 (49, 71)	0.120
CD4 Count	790 (430, 1040)	510 (290, 860)	800 (450, 1430)	0.062
$CD4^+CD45RA^+CD38^+$%	16 (9, 26)	15 (9, 27)	21 (13, 34)	0.070
$CD4^+FOXP3^+$%	**3 (2, 5)**	**4 (3, 6)**	**2 (2, 4)**	**<0.001**
$CD4^+FOXP3^+$ Count	29 (15, 35)	22 (14, 36)	19 (10, 37)	0.390
CD19 of CD45	2 (1, 7)	3 (2, 4)	4 (2, 6)	0.190
CD19 Count	49 (17, 100)	40 (17, 75)	59 (27, 140)	0.160
$CD4^+$ of γδ T cells	**36 (27, 52)**	**24 (12, 37)**	**39 (22, 55)**	**0.033**
$CD8^+$ of γδ T cells	**33 (21, 53)**	**36 (23, 53)**	**24 (16, 38)**	**0.027**

The proportion of FOXP3 was greatest in those KTR with a history of cancer and increased in those KTR with current cancer compared to those KTR with no cancer (p<0.001). Although not statistically significant, $CD4^+$ single positive γδ T cells were decreased (p=0.033) in KTR with past cancer and $CD8^+$ single positivity γδ T cells were increased (p=0.027) in KTR with current and past cancer compared to those KTR with no cancer.

To investigate the association immune phenotype has with cancer KTR, current cancer and past cancer were grouped together (KTR cancer, n=65) and compared to those KTR with no cancer (n=51). Non-parametric Mann-Whitney two-tailed tests were utilised to determine differences between the two groups. The results presented in Table 4.3.2.2 are unadjusted for age, gender and duration of immunosuppression.

Table 4.3.2.2: Median (IQR) of immune phenotype values compared between kidney transplant recipients with cancer and with no cancer by Mann-Whitney two-tailed tests.

	Cancer (n=65)	No Cancer (n=51)	p-value
CD45 lymphocytes/µl	1540 (1100, 2350)	1680 (1140, 2900)	0.380
Treg Parameters			
FOXP3% of CD4+ T cells	*4 (3, 5)*	*2 (2, 4)*	*<0.001*
Treg% of CD4+ T cells	*2 (2, 3)*	*1 (1, 2)*	*<0.001*
Tregs cells/µl	*16 (6, 23)*	*8 (3, 19)*	*0.016*
B cell Parameters			
B cell% of CD45 cells	3 (1, 5)	4 (2, 6)	0.084
B cells/µl	41 (17, 91)	56 (28, 116)	0.190
Naïve B cell% of B cells	41 (22, 72)	55 (37, 74)	0.067
Naïve B cells/µl	**13 (5, 50)**	**26 (13, 55)**	**0.028**
Memory B cell% of B cells	17 (9, 32)	14 (6, 21)	0.065
Memory B cells/µl	7 (3, 14)	6 (2, 14)	0.680
γδ T cell Parameters			
TCRγδ% of T cells	8 (4, 14)	6 (4, 9)	0.110
CD8% of TCRγδ T cells	36 (21, 53)	30 (17, 41)	0.087
NK cell Parameters			
CD3⁻CD19⁻% of CD45 cells	18 (11, 27)	18 (13, 25)	0.710
NK cells/µl	74 (43, 188)	107 (34, 195)	0.980
CD56⁺⁺% of CD3⁻CD19⁻ cells	2 (1, 4)	2 (1, 3)	0.310

Tregs = Regulatory T cells, TCRγδ = T- cell Receptor γδ subunit positive cells, NK = Natural Killer cells

When comparing KTR groups, the KTR with cancer had higher Treg parameters including proportions and counts, however there were no differences in NK cell parameters between the two groups shown in Table 4.3.2.2. Additionally, Naïve B cell counts are lower in KTR with cancer when compared to KTR without cancer (median. 13 naïve B cells/µl Vs. 26 naïve B cells/µl, respectively, p=0.028).

To investigate the strength of the association immune phenotype has with cancer Odds Ratios (OR) were developed with logistic regression modelling. The odds ratios were run unadjusted

in univariate logistic regression models and adjusted for age, gender and duration of immunosuppression in multivariate modelling (Table 4.3.2.3).

Table 4.3.2.3: Odds Ratios for the association immune phenotype has with cancer using logistic regression modelling in selected cohort (n=116).

	Unadjusted			Adjusted		
	OR	95% C.I.	p-value	OR	95% C.I.	p-value
Treg Parameters						
FOXP3% of CD4 T cells	**1.26**	**[1.04-1.53]**	**0.019**	**1.31**	**[1.03-1.66]**	**0.028**
Tregs% of CD4 T cells	**1.61**	**[1.17-2.20]**	**0.003**	**1.61**	**[1.13-2.29]**	**0.008**
Tregs/µl	**1.02**	**[0.99-1.05]**	**0.127**	**1.03**	**[1.00-1.06]**	**0.090**
B cell Parameters						
B cell% of CD45 Lymphocytes	0.97	[0.89-1.05]	0.483	0.98	[0.89-1.08]	0.653
B cells/µl	0.99	[0.99-1.00]	0.358	1	[0.99-1.00]	0.659
Naïve B cell% of B cells	**0.99**	**[0.97-1.00]**	**0.058**	**0.98**	**[0.97-1.00]**	**0.082**
Naïve B cells/µl	0.99	[0.99-1.00]	0.577	1	[0.99-1.00]	0.883
Memory B cell% of B cells	**1.03**	**[1.00-1.06]**	**0.038**	**1.04**	**[1.00-1.07]**	**0.025**
Memory B cells/µl	0.99	[0.96-1.01]	0.228	0.99	[0.97-1.02]	0.568
γδ T cell Parameters						
TCRγδ% of T cells	**1.07**	**[1.00-1.14]**	**0.043**	1.03	[0.97-1.12]	0.310
CD8% of TCRγδ T cells	**1.02**	**[1.00-1.04]**	**0.055**	1.01	[0.99-1.04]	0.341
CD8+TCRγδ T cells/µl	**1.01**	**[1.00-1.02]**	**0.080**	1	[0.99-1.02]	0.293
NK cell Parameters						
CD3⁻CD19⁻% of CD45 lymphocytes	0.99	[0.96-1.02]	0.697	0.99	[0.95-1.03]	0.587
NK cells/µl	1.00	[1.00-1.00]	0.879	1.00	[1.00-1.00]	0.846
CD56⁺⁺% of CD3⁻CD19⁻ lymphocytes	1.11	[0.91-1.35]	0.314	1.12	[0.89-1.39]	0.338

Tregs = Regulatory T cells, TCRγδ = T- cell Receptor γδ subunit positive cells, NK = Natural Killer cells

The Treg parameter with the strongest association was the proportion of $CD4^+$ Tregs ($CD3^+CD4^+FOXP3^+CD127^{lo}CD25^{hi}$% of CD4 T cells, p=0.003) which stayed consistently strong in multivariate analysis (p=0.008, Table 4.4.3). Natural Killer cells did not associate with cancer in either model. Memory B cells showed association with cancer (p=0.038) and remained consistent in multivariate analysis (p=0.025). All γδ T cell parameters had increased odds ratios, but the strength and statistical significance was lost in multivariate analysis.

Further investigation of Tregs and the presence of cancer, both squamous cell carcinoma (SCC) and Solid Organ Cancer (SOC), has on KTR, was undertaken. Absolute numbers of $CD4^+FOXP3^+CD127^{lo}CD25^{hi}$ Tregs were measured and the results are shown in Figure 4.3.2.
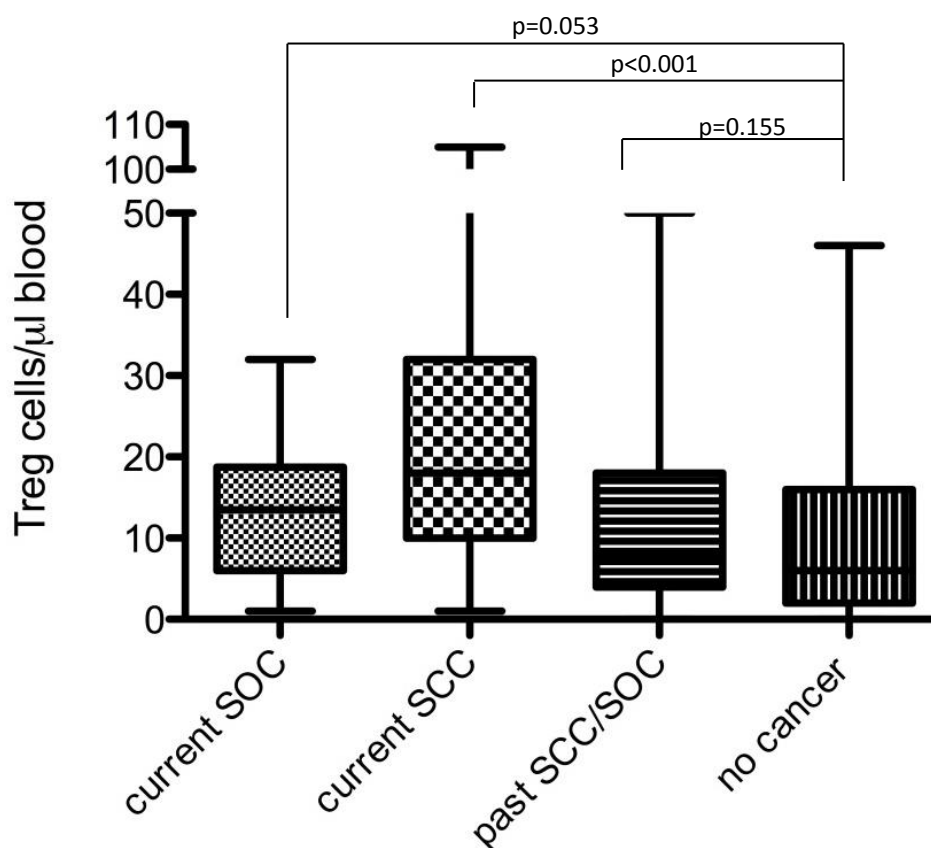

Figure 4.3.2: Absolute numbers of peripherally circulating Regulatory T Cells (Tregs, i.e. CD4+FOXP3$^+$CD127^{lo}CD25^{hi}) in kidney transplant recipients with various categories of cancer: Absolute numbers of $CD4^+FOXP3^+CD127^{lo}CD25^{hi}$ T cell (Tregs) in the peripheral blood of KTR determined by flow cytometry: current solid organ cancer (SOC, n=14); Current squamous cell carcinoma (SCC, n=21); past SOC/SCC (n=33) represents KTR who did not have a current cancer in situ; no cancer represents those KTR who did not have a history of SCC or SOC (n=89). Current SOC vs. no cancer (p=0.053) Current SCC vs. no cancer (p<0.001) Past SCC/SOC compared to no cancer (p=0.155) using Mann Whitney two tailed test.

KTR with current SOC and current SCC had greater numbers of peripheral blood Tregs than KTR with no cancer (p=0.053 and p<0.001, respectively). KTR with past cancer had similar levels to KTR with no cancer (p=0.155). The next section of this chapter shows the impact that various parameters have on Treg and other cell types.

4.3.3 Viral and immunosuppression drug related associations to immune phenotype

With any heterogeneous cohort, investigation into potential effects of measurable variables should be considered. In this section the impact of viral infections, induction therapy and immunosuppression regimens were investigated.

4.3.3.1 Infection and immune phenotype

Cytomegalovirus (CMV) has been shown to increase $CD8^+CD28^-$ T suppressors[206]. It has also been shown that KTR with CMV positivity and increased $\gamma\delta$ T cells have less cancer[101]. These two cell populations along with Tregs were analysed for association to CMV seropositivity as determined by routine diagnostic PCR performed by SA pathology, Adelaide, Australia. Additionally, Tregs were also analysed to determine any association to EBV infection and shown in Figure 4.3.3.1 to Figure 4.3.3.4.
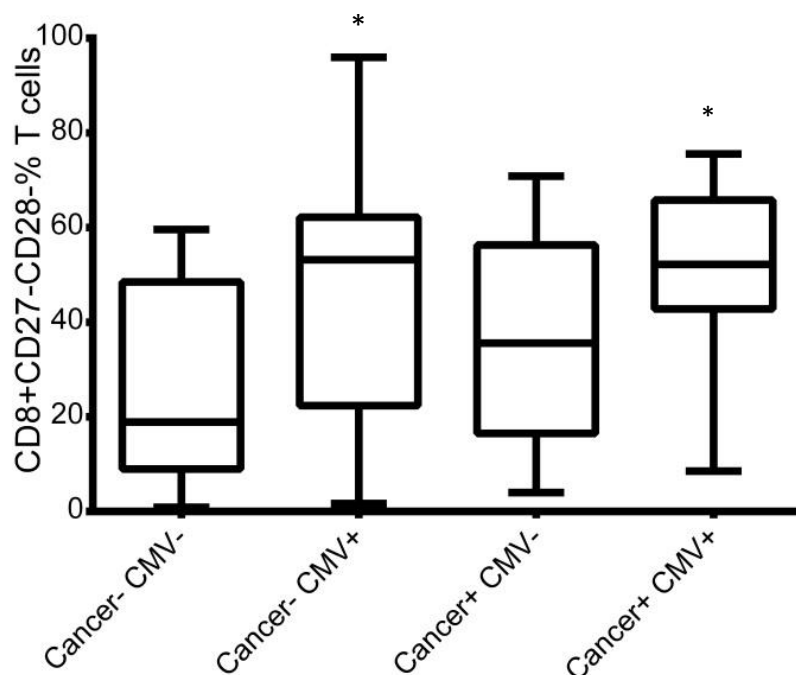

Figure 4.3.3.1: $CD8^+CD27^-CD28^-$ proportion in KTR with and without Cancer and CMV status: Proportions of $CD8^+CD27^-CD28^-$ T cells in KTR with cancer and with CMV (n=31) or without (n=15) and KTR with no cancer with CMV (n=23) or without (n=17). CMV positivity increased CD8 T suppressors in both KTR with cancer (p=0.016) and KTR with no cancer (p=0.010) using Mann-Whitney Two-Tailed Tests.
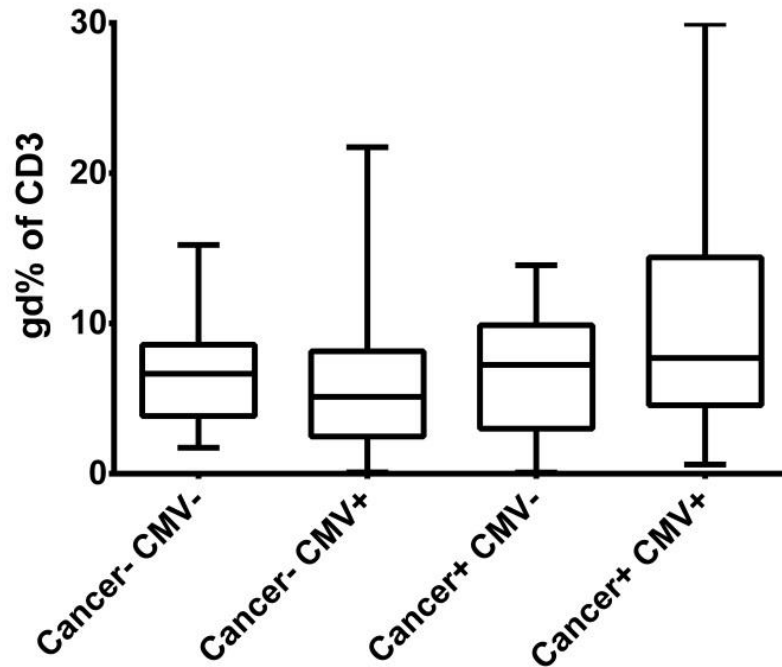
Figure 4.3.3.2: Proportion of γδ T cells (gd T cells) in KTR with and without Cancer and CMV status: Proportions of γδ T cells of CD3+ T cells in KTR with cancer and with CMV (n=31) or without (n=15) and KTR with no cancer with CMV (n=23) or without (n=17). CMV positivity did not change γδ T cells in both KTR with cancer (p=0.308) and KTR with no cancer (p=0.385) using Mann-Whitney Two-Tailed Tests.
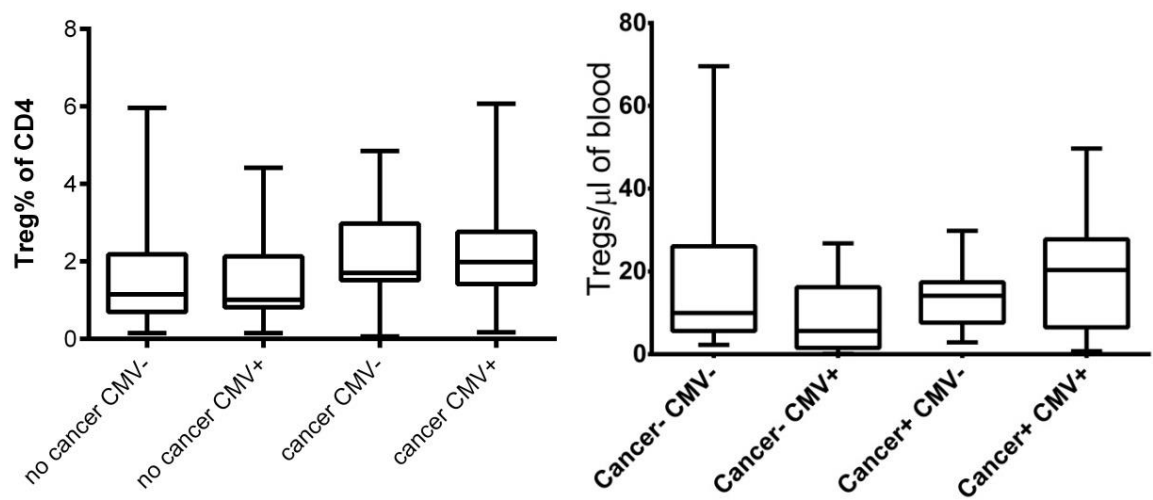
Figure 4.3.3.3: Proportion and numbers of Regulatory T cells in KTR with and without Cancer and CMV status: Proportions and numbers of regulatory T cells (Tregs) in KTR with cancer and with CMV (n=31) or without (n=15) and KTR with no cancer with CMV (n=23) or without (n=17). CMV positivity did not change either proportion of Tregs in both KTR with cancer (p=0.086) and KTR with no cancer (p=0.780) or Treg number in KTR with cancer (0.124) and KTR with no cancer (0.32) using Mann-Whitney Two-Tailed Tests.
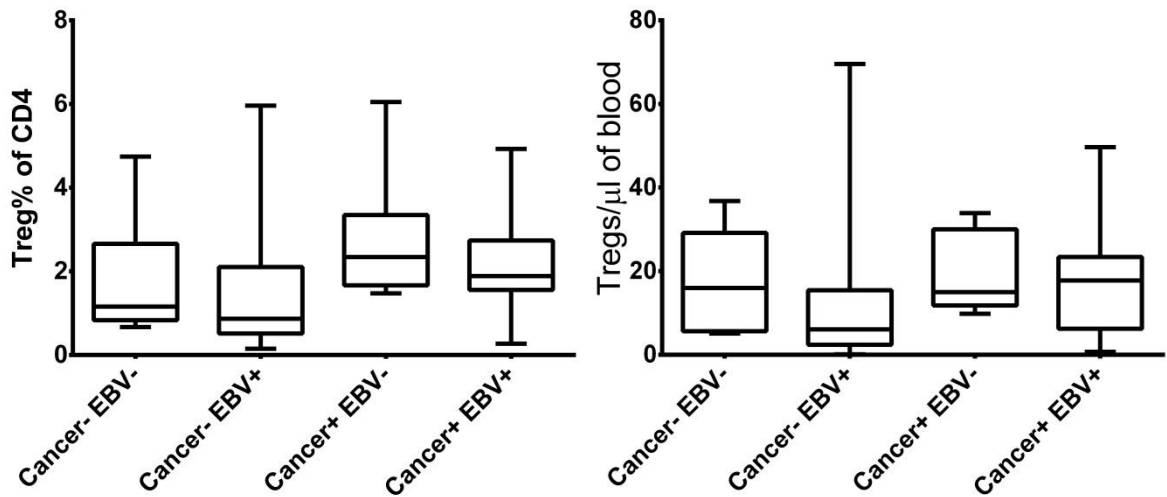
Figure 4.3.3.4: Proportion and numbers of Regulatory T cells in KTR with and without Cancer and EBV seropositivity: Proportions and numbers of regulatory T cells (Tregs) in KTR with cancer and with EBV (n=29) or without (n=8) and KTR with no cancer with EBV (n=30) or without (n=6). EBV positivity did not change either proportion of Tregs in both KTR with cancer (p=0.373) and KTR with no cancer (p=0.321) or Treg numbers in both KTR with cancer (p=0.435) and KTR with no cancer (p=0.135) using Mann-Whitney Two-Tailed Tests.

Although there was no difference of CMV seropositivity between KTR groups (Table 3.3.2). CD8 T suppressors were increased regardless cancer status (Figure 4.3.3.1) whereas γδ T cells did not change (Figure 4.3.3.2). Both CMV (Figure 4.3.3.3) and EBV (Figure 4.3.3.4) positivity did not affect Treg proportion or number.

4.3.4 Induction therapy and immune phenotype

Anti-Thymocyte Globulin (ATG) has been shown to affect CD4^{+} Treg proportions, both ATG and anti-IL2Ra (IL-2) were analysed to determine associations to CD4 T cells and Treg numbers. The 116 KTR were analysed, 12 KTR had ATG induction, 32 KTR had anti-IL-2Ra (IL-2) leaving 72 KTR with no induction (nil) as depicted in Figure 4.3.4.
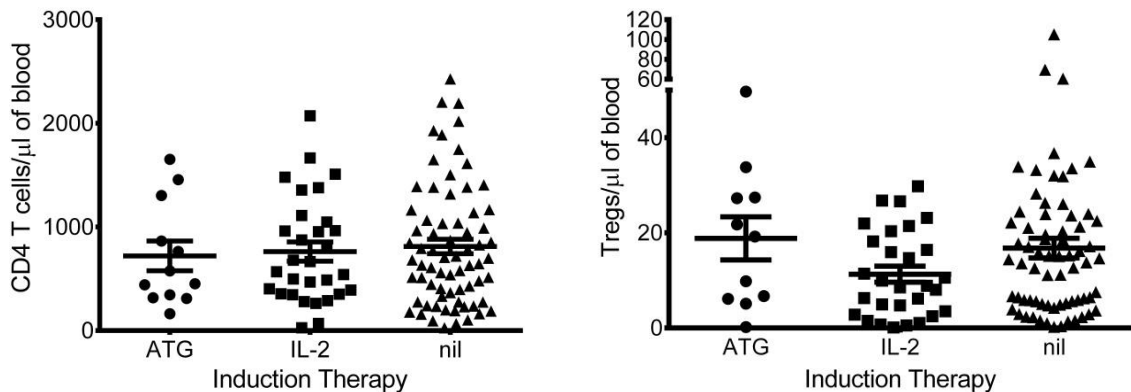

Figure 4.3.4: The effect induction therapy has on numbers of CD4 and Regulatory T cells in KTR: A total of 12 KTR had ATG induction, 32 had anti-IL2Ra induction and 72 KTR with neither induction therapy. There was no difference between groups using Kruskal-Wallis testing and upon further analysis, although the median Treg number for ATG induction was 19 Tregs/µl and 14 Tregs/µl for those with no induction, this was not significant using Mann-Whitney two-tailed tests (p=0.439).

Although ATG had increased Treg numbers compared to those with no induction therapy (19 Vs. 14, respectively) this was not significant (p=0.439) using Mann-Whitney two-tailed tests.

4.3.5 Immunosuppression regimen and immune phenotype

Although KTR immunosuppression regimens mainly consist of two or more immunosuppressive drugs, they are most often based with either calcineurin inhibitors (CNI) or mammalian target or rapamycin inhibitors (mTORi). These two are often administered in conjugation with anti-proliferatives, either Azathiroprine (AZA) or Mycophenolate (MMF). Due to a limitation of patient number, CNIs with mTORi's were paired as well as AZA with MMF. Figure 4.3.5.1 and Figure 4.3.5.2 are both a series of box plots depicting the effect these drugs have on the important cell types.
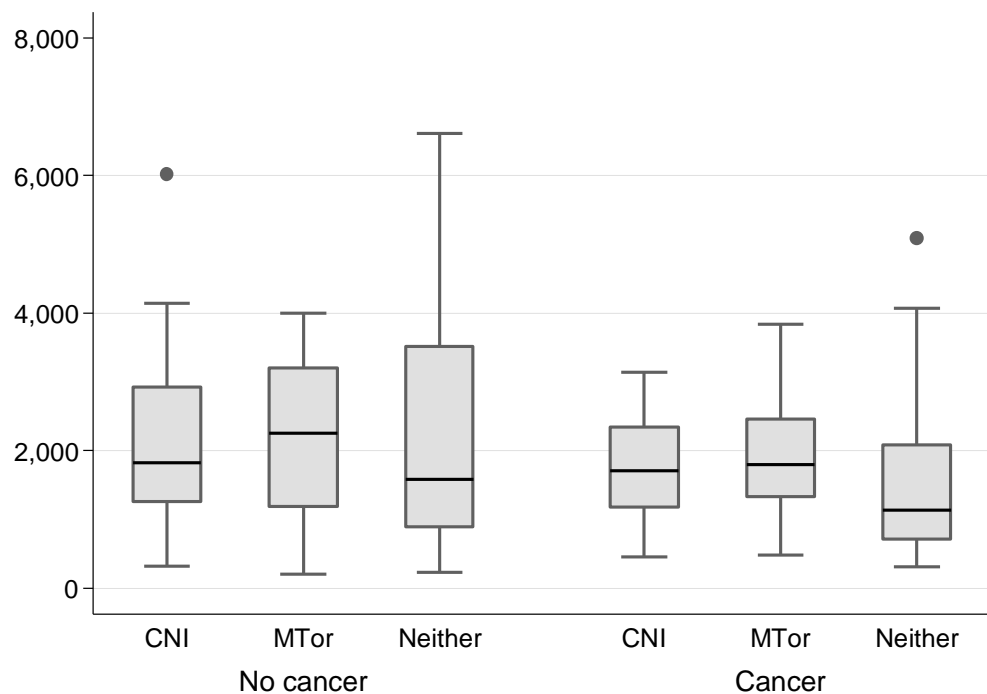

Figure 4.3.5.1: Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) drug interactions to CD45 count: The immune phenotypes of Kidney Transplant Recipients (KTR) on Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) based regimens were compared in KTR with cancer and KTR with no cancer. CD45 lymphocytes, CD45 lymphocyte counts were similar across the groups.
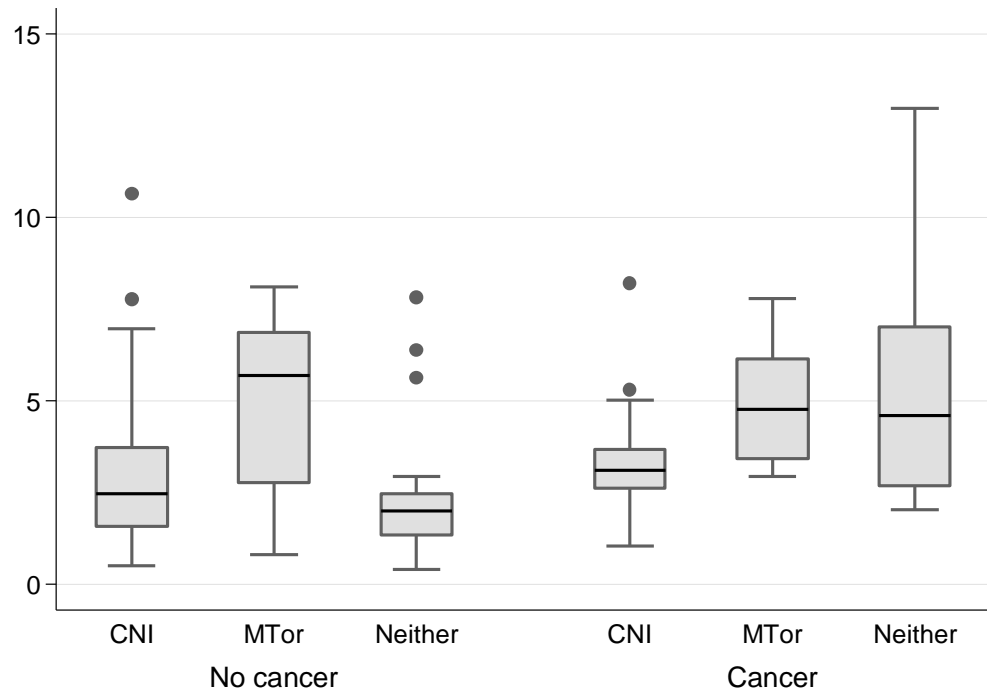
Figure 4.3.5.2: Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) drug interactions to proportion of FOXP3^{+} CD4 T cells: The immune phenotypes of Kidney Transplant Recipients (KTR) on Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) based regimens were compared in KTR with cancer and KTR with no cancer. There were increases in FOXP3 portions between cancer and no cancer. There were also increases in the mTORi groups compared to CNI and KTR on neither mTORi nor CNI.
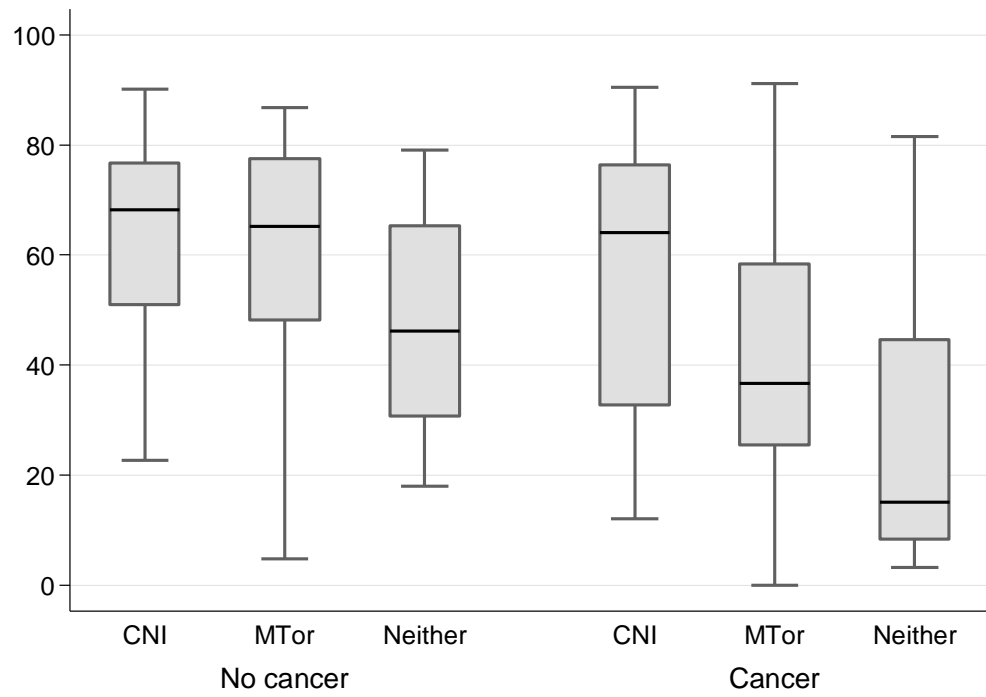
Figure 4.3.5.3: Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) drug interactions on naïve B cell proportion: The immune phenotypes of Kidney Transplant Recipients (KTR) on Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) based regimens were compared in KTR with cancer and KTR with no cancer. KTR on CNI had similar naïve B cell proportions despite cancer status. However, mTORi-based regimens or KTR on neither regimen had decreased numbers and proportions of naïve B cells in those KTR with cancer.
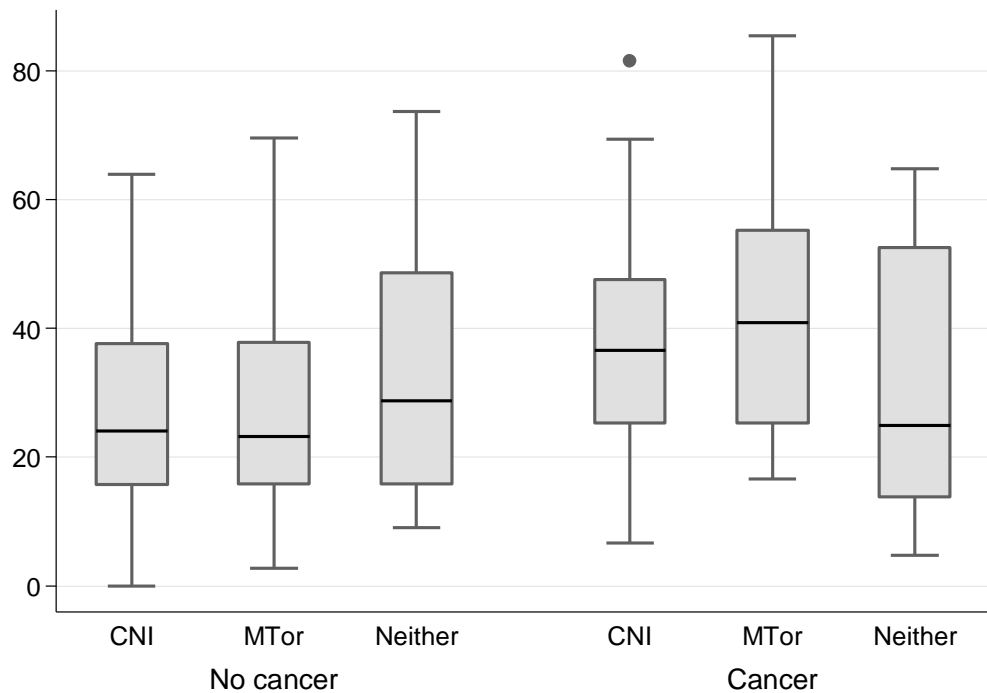
Figure 4.3.5.4: Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) drug interactions CD8 γδ T cells (gd T cells): The immune phenotypes of Kidney Transplant Recipients (KTR) on Calcineurin inhibitors (CNI) and mammalian Target of Rapamycin (mTORi) based regimens were compared in KTR with cancer and KTR with no cancer. CD8+ single positive γδ CD3^{+} T cells were higher in KTR with cancer but unaffected by drug regimen.

Despite there being no difference in CD45 lymphocyte count between both immunosuppression and cancer status there was an increase in FOXP3 proportion and numbers as well as Treg numbers in those KTR treated with mTORi drug-based regimens. However, it can also be seen that all Treg parameters were increased in KTR with cancer despite drug regimen. Those KTR receiving CNI had similar naïve B cell proportions and numbers despite cancer status. However, mTORi-based regimens or KTR on neither regimen had decreased numbers and proportions of naïve B cells compared to those KTR with cancer. CD8+ single positive γδ CD3+ T cells were higher in KTR with cancer but unaffected by drug regimen. NK cells were not different between all groups.
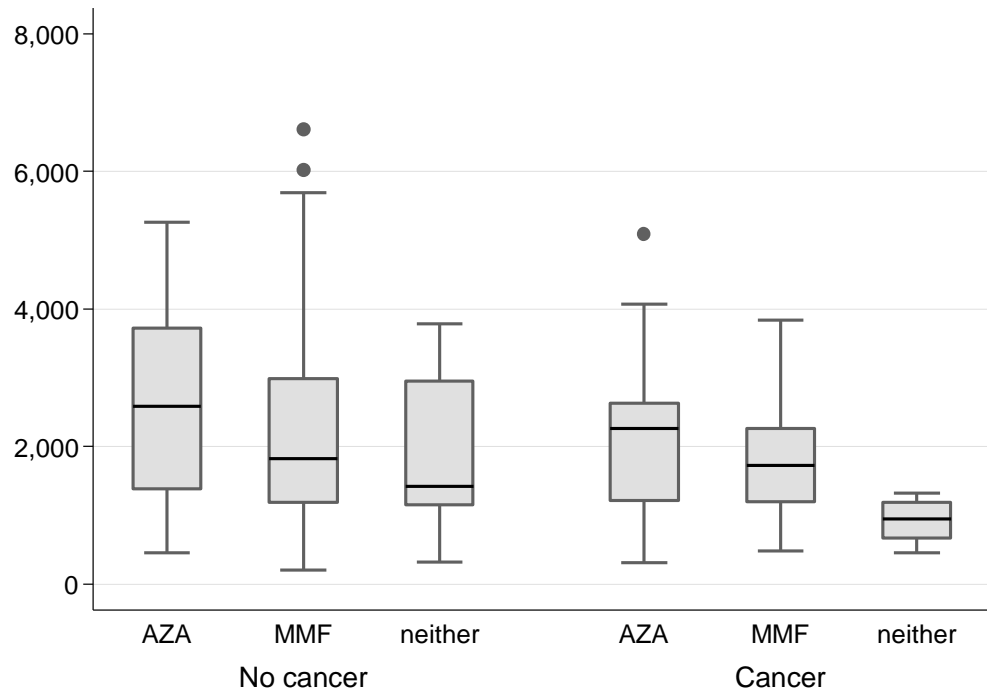
Figure 4.3.5.5: Azathioprine (AZA) and Mycophenolate (MMF) drug interactions to CD45 count: The immune phenotypes of Kidney Transplant Recipients (KTR) on Azathioprine (AZA) and Mycophenolate (MMF) based regimens were compared in KTR with cancer and KTR with no cancer. CD45 lymphocyte counts are similar between anti-proliferative drug use and cancer status, however those KTR not treated with anti-proliferatives in the absence of cancer had greater numbers of lymphocytes compared to KTR on neither anti-proliferatives that also have cancer. Those receiving neither AZA nor MMF may be on higher levels of prednisolone doses and therefore may have lymphopenia.

Figure 4.3.5.6: Azathioprine (AZA) and Mycophenolate (MMF) drug interactions to proportion of FOXP3+ CD4 T cells: The immune phenotypes of Kidney Transplant Recipients (KTR) on Azathioprine (AZA) and Mycophenolate (MMF) based regimens were compared in KTR with cancer and KTR with no cancer. There were increases in FOXP3 proportion of CD4 T cells between cancer and no cancer. This is more evident in those KTR who are on AZA treatments no anti-proliferatives than those on MMF treatments.

Figure 4.3.5.7: Azathioprine (AZA) and Mycophenolate (MMF) drug interactions to FOXP3$^+$ T cell count: The immune phenotypes of Kidney Transplant Recipients (KTR) on Azathioprine (AZA) and Mycophenolate (MMF) based regimens compared in KTR with cancer and KTR with no cancer. There were increases in FOXP3 numbers between cancer and no cancer. There were also increases in Treg parameters of the AZA groups compared to MMF and KTR on neither. This was more evident in those KTR who are on AZA and also have cancer.
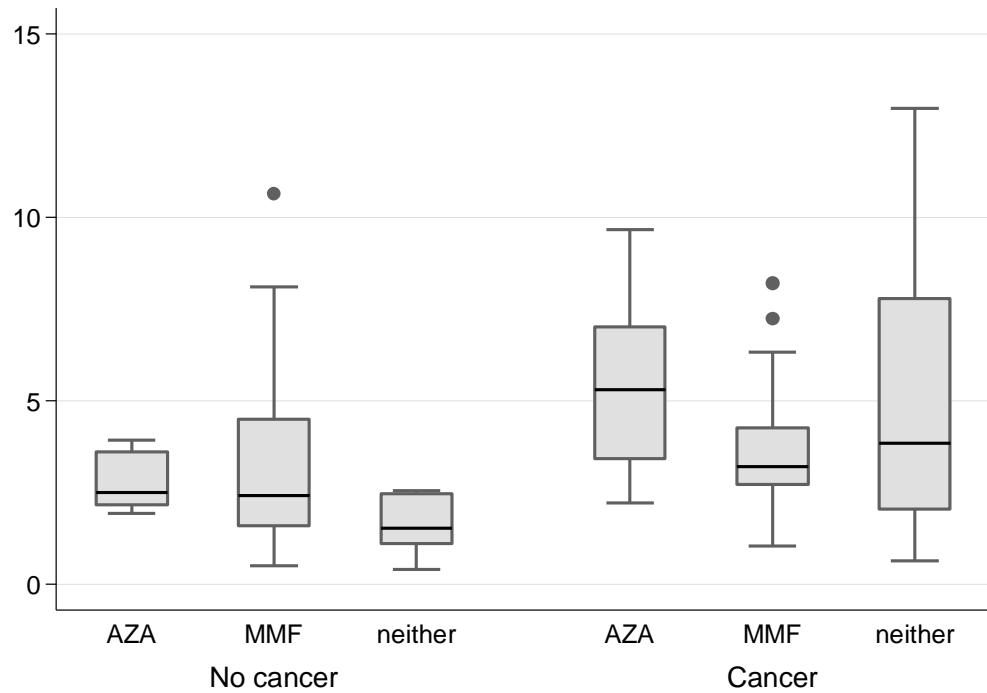
Figure 4.3.5.8: Azathioprine (AZA) and Mycophenolate (MMF) drug interactions to Treg count: The immune phenotypes of Kidney Transplant Recipients (KTR) on Azathioprine (AZA) and Mycophenolate (MMF) based regimens were compared in KTR with cancer and KTR with no cancer. There were increases in Treg numbers between cancer and no cancer. There were also increases in Treg parameters of the AZA groups compared to MMF and KTR on neither. This was more evident in those KTR who are on AZA and who also have cancer.
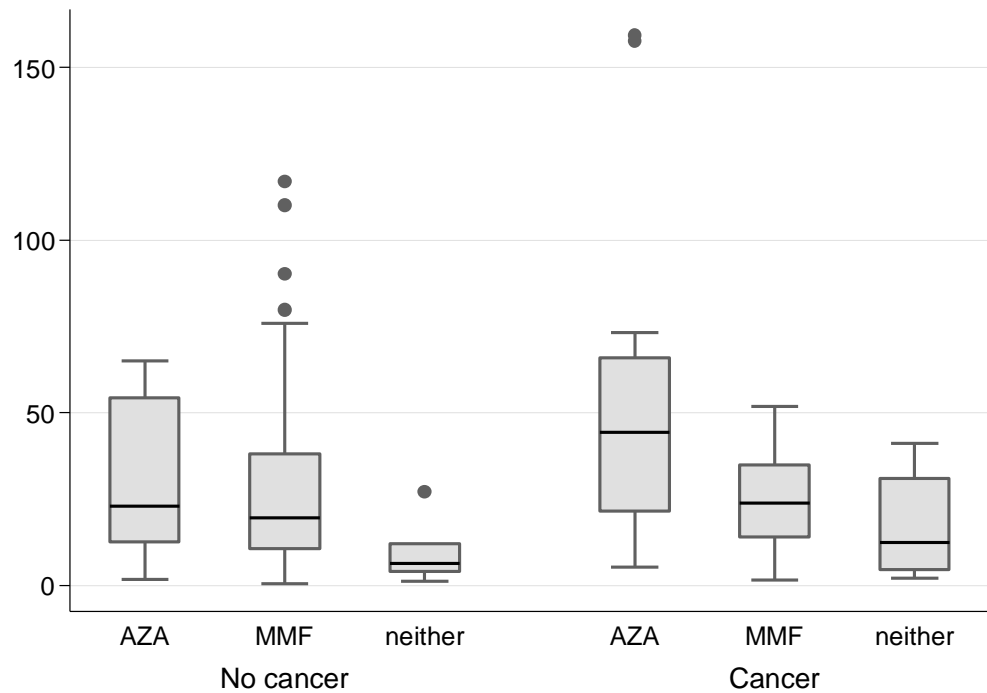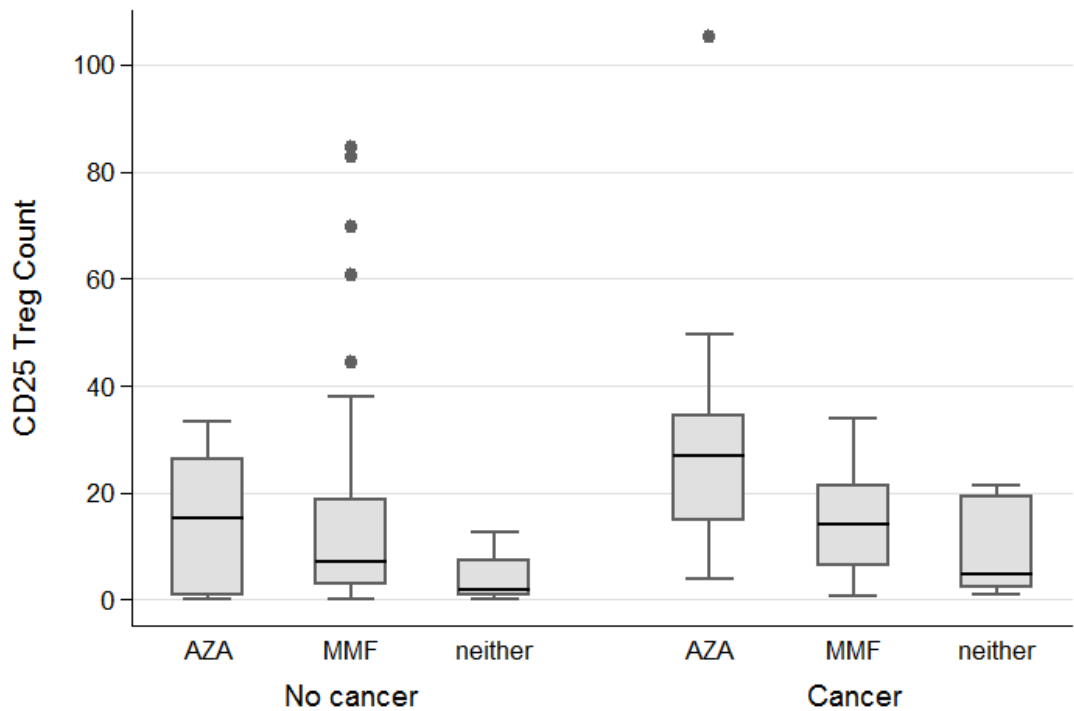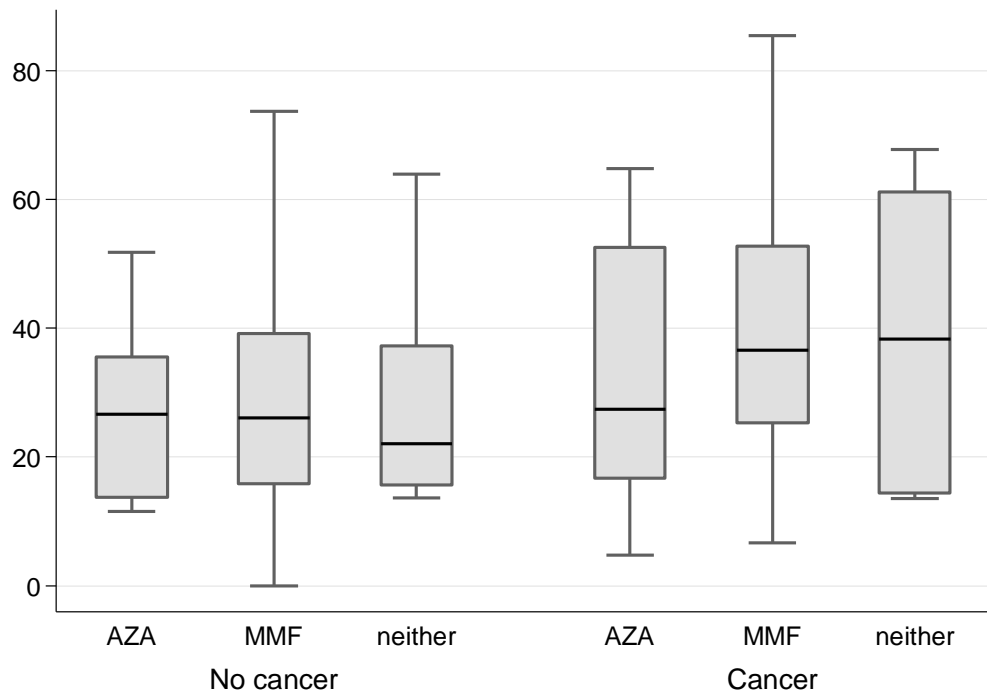
Figure 4.3.5.9: Azathioprine (AZA) and Mycophenolate (MMF) drug interactions to CD8$^+$ gd T cells: The immune phenotypes of Kidney Transplant Recipients (KTR) on Azathioprine (AZA) and Mycophenolate (MMF) based regimens were compared in KTR with cancer and KTR with no cancer. CD8$^+$ single positive γδ CD3$^+$ T cells were slightly higher in KTR with cancer but unaffected by drug regimen.

It can be seen that those KTR on neither anti-proliferative and have cancer had lower total lymphocyte numbers when compared to those KTR with no cancer (Figure 4.3.5.5). However as they were not receiving anti-proliferatives they may be on higher levels of prednisolone and therefore may suffer from lymphopenia. It can also been seen that all Treg parameters were greater in those KTR with cancer despite immunosuppression regimen. Interestingly, those KTR with cancer and on AZA had greater Tregs than those KTR with cancer on MMF or neither drug regimen. CD8$^+$ single positive γδ CD3$^+$ T cells were slightly higher in KTR with cancer but unaffected were by drug regimen. NK cells were slightly lower in those KTR with cancer on AZA-based regimen compared to KTR on AZA-based regimens with no cancer.

In summary, CD4$^+$ FOXP3$^+$ proportion and numbers, naïve B cell and CD3$^+$CD8$^+$γδT cells were altered by cancer status. However, it was observed that mTORi and AZA treated KTR also had increases in Treg parameters. Additionally, KTR on mTORi or not being treated with either mTORi or CNI (neither) had decreased naïve B cells within the KTR group with cancer, showing evidence that immunosuppression can alter some of the cancer immune phenotype parameters. Furthermore, CD3$^+$CD8$^+$γδT cells were increased in KTR with cancer regardless of immunosuppression regimen and similar between immunosuppression groups.

Sections 4.3.2-4.3.5 provides data of a peripheral blood immune phenotype that associates to cancer in KTR (Section 4.3.2) despite viral seropositivity (Section 4.3.3), induction therapy (Section 4.3.4) and immunosuppression maintenance (Section 4.3.5). The next sections will provide evidence of the clinical utility of this phenotype, including using it as a tool to predict cancer development.

4.3.6 Immune phenotype ability to predict cancer

KTR with current and past SCC were grouped together to form a group of KTR that have higher risk of developing cancer within the 3 year follow-up to make the 49 KTR described in Chapter 3: Section 3.2. To prospectively study the predictability that immune phenotype has for determining risk of cancer in KTR, Receiver operator characteristic (ROC) curves were employed in these 49 KTR. The accuracy and sensitivity/specificity of each cell type's ability to predict cancer onset in KTR with previous SCC (n=49), within 100 days of being phenotyped, was measured (Figure 4.6.1).

Figure 4.3.6.1: Regulatory T cell (Treg) Receiver Operator Characteristic (ROC) curves for the development of Squamous Cell Carcinoma in Kidney Transplant Recipients: A ROC curve for the detection of SCC development in KTR within 100 days of measurement. The regulatory T cells (Tregs) ROC with the Area Under the Curve (AUC)=0.78, specificity=68% and sensitivity=71% at a trade-off value of 16 Tregs/μl.

A sensitivity/specificity trade-off value of >16 Tregs/μL has a sensitivity of 66% and a specificity of 78%, Figure 4.3.6.1. No other cell type or combinations thereof gave increased predictability.

Figure 4.3.6.2: Memory B cell Receiver Operator Characteristic (ROC) curves for the development of Squamous Cell Carcinoma in Kidney Transplant Recipients: A ROC curve for the detection of SCC development in KTR within 100 days of measurement. Memory B cells curve with AUC=0.68 along with a specificity=85% and sensitivity=56%, trade-off of 3 memory B cells/μl.

Figure 4.3.6.3: CD8 γδ T cell Receiver Operator Characteristic (ROC) curves for the development of Squamous Cell Carcinoma in Kidney Transplant Recipients: A ROC curve for the detection of SCC development in KTR within 100 days of measurement. CD8 γδ T cell curve with AUC=0.64 with specificity=55% and sensitivity=69% at a trade-off value of 27 CD8 γδ T cell/μl.
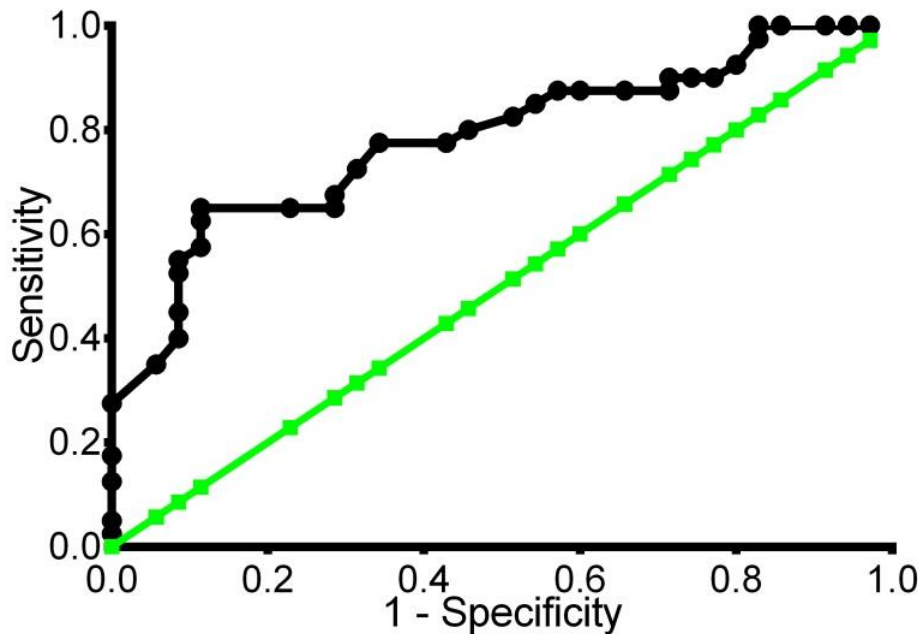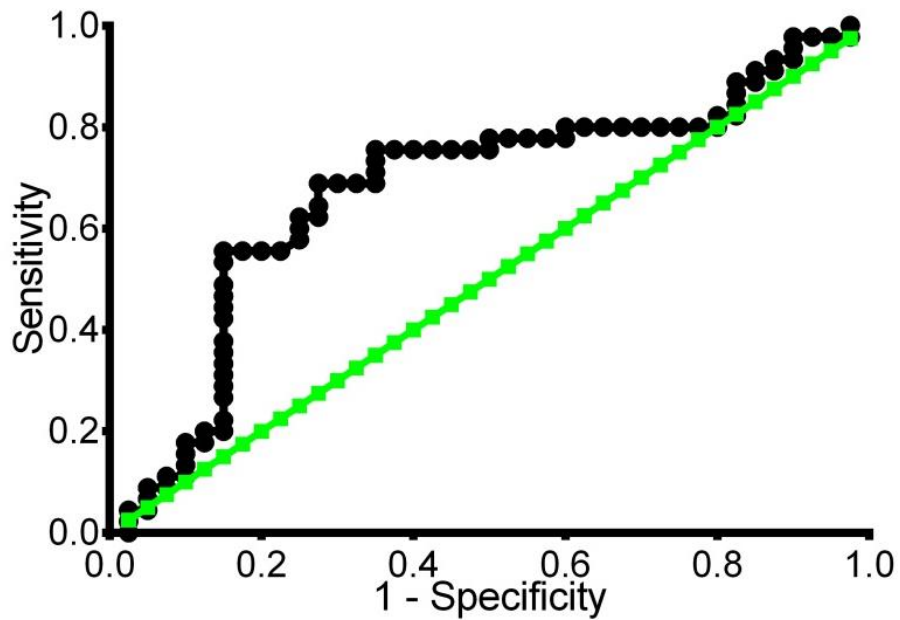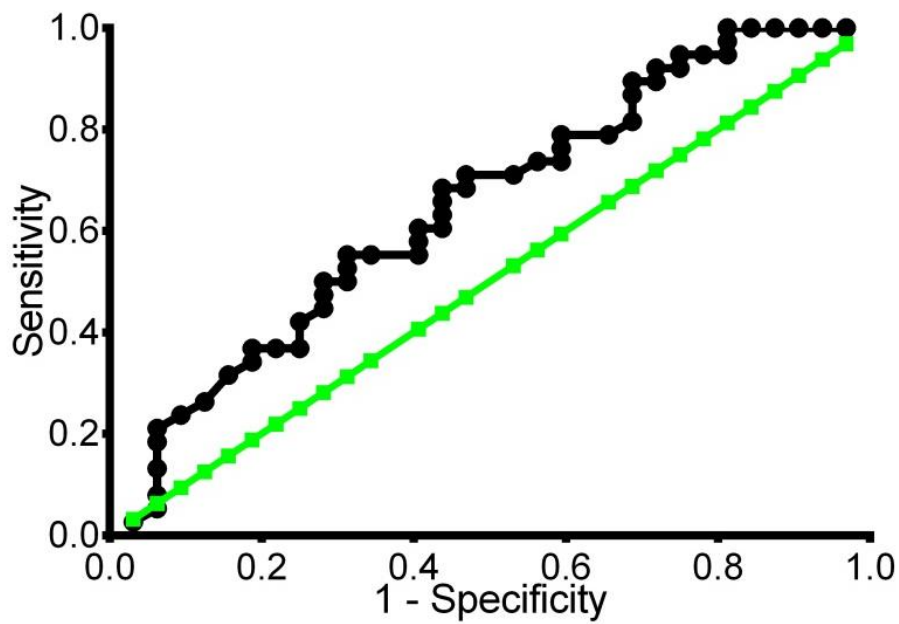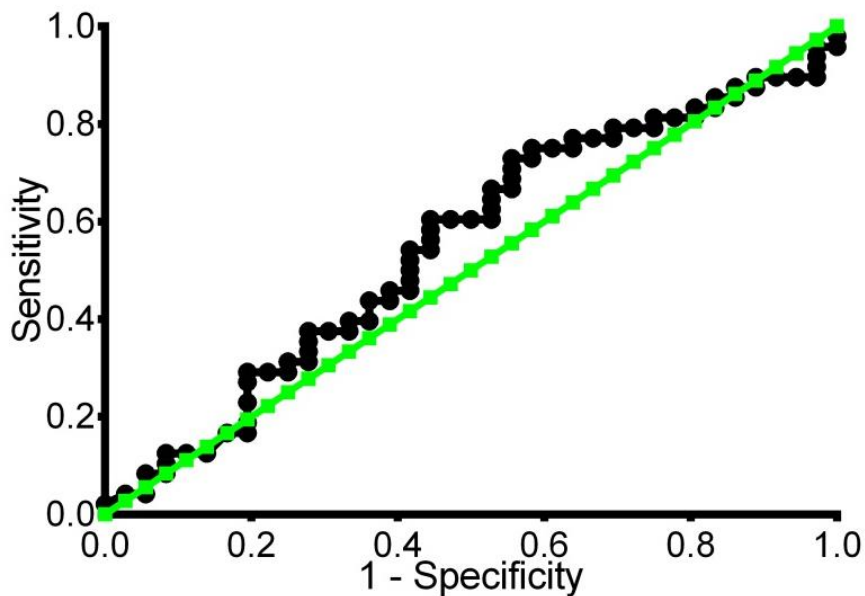
Figure 4.3.6.4: Natural Killer (NK) Receiver Operator Characteristic (ROC) curves for the development of Squamous Cell Carcinoma in Kidney Transplant Recipients: A ROC curve for the detection of SCC development in KTR within 100 days of measurement. NK cell ROC with AUC=0.55, a specificity = 38% and sensitivity = 72% at a trade-off value of 64 NK cells/µl.

In summary, Regulatory T cell number had greater accuracy (AUC = 0.78) than the other cell types. The accuracy of the other cell types in descending order; Memory B cell number (AUC = 0.68), CD8$^+$γδ T cell number (AUC = 0.64) and Natural Killer cell number (AUC = 0.55) did not provide any benefit as single parameters or when used with Treg number (data not shown). Further investigation found a sensitivity/specificity trade-off of 16 Tregs/µl. To determine if those KTR with a history of SCC were above the 16 Tregs/µl threshold accumulated SCC at a higher ratio compared to those KTR with a history of SCC below this threshold, a Kaplan-Meier SCC-free survival curve and hazard ratios (HR) were developed. This was performed using the trade-off value of 16 Tregs/µl as a risk stratifier. The results are shown in Figure 4.3.6.5.
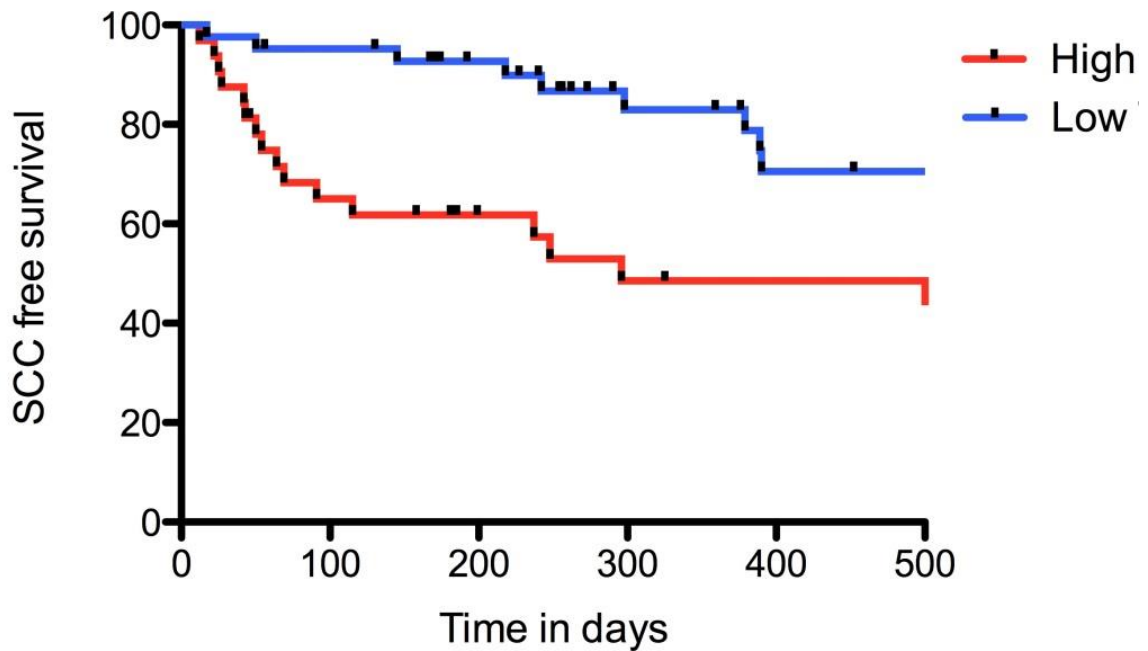
Figure 4.3.6.5: Percentage SCC free-survival of dichotomised data: High levels (>16 Cells/µL) of CD4$^+$FOXP3$^+$CD127^{Lo}CD25^{Hi} Regulatory T Cells (red line) indicate less SCC-free survival compared to low levels (<16 Cells/µL, blue line). KTR with high levels of Tregs have a hazard ratio of 3.4 with 95% confidence intervals of 1.6–7.5 and a log-ranked chi-squared p-value of <0.01.

Figure 4.3.6.5 shows that KTR with a history of cancer and >16 Tregs/µl had a HR = 3.4 (95%CI of 1.6 to 7.5) chi squared p<0.01 when compared to KTR with a history of cancer with <16 Tregs/µl, thus providing evidence that Tregs (>16 Tregs/µl) themselves have the power to predict those at a 3.4-fold increased hazard of developing SCC. However, this data cannot predict severity of accumulated SCC tumours and does not seem to diverge or dichotomise the data after 100 days of immune phenotyping.

To increase the clinical application, a ROC curve was used to determine if Tregs alone could predict the development of high risk SCC (i.e. poorly differentiated lesion, >2mm thickness, perineural invasion and invading deep structures beyond the deep reticular dermis layer) in KTR with previous SCC (n=49), within 100 days, shown in Figure 4.3.6.6.

Area under ROC curve = 0.8590
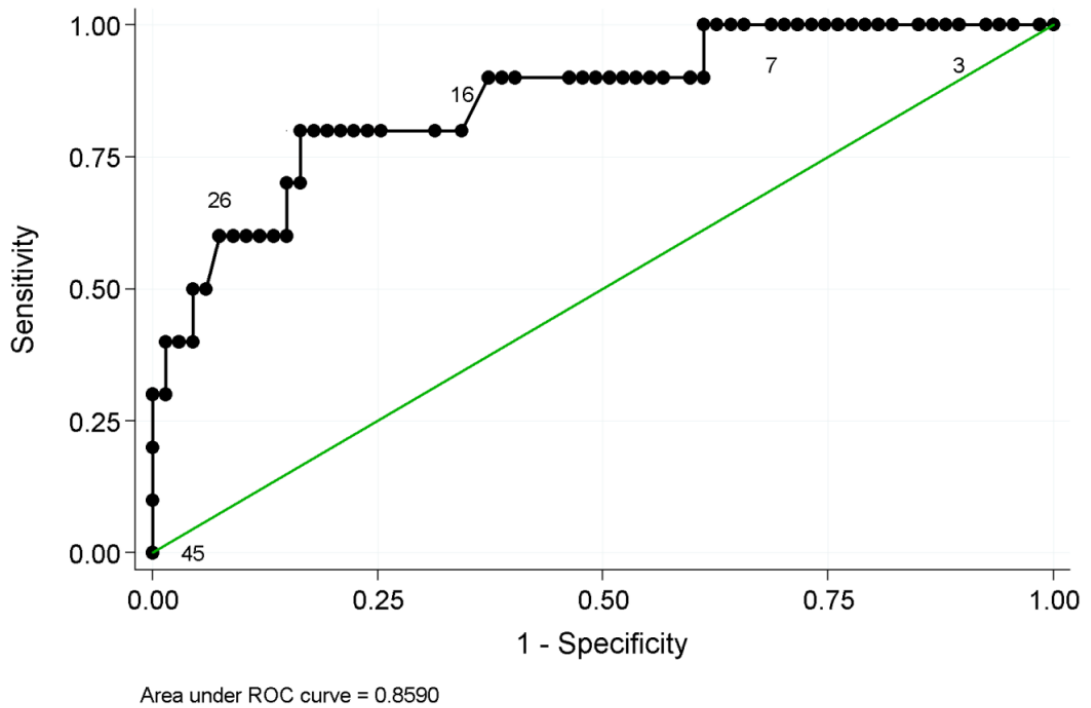
Figure 4.3.6.6: Regulatory T cell (Treg) numbers as a predictor of high risk squamous cell carcinoma of the skin (SCC) in kidney transplant recipients (KTR): A receiver operator characteristic (ROC) curve for developing a high risk squamous cell carcinoma of the skin after kidney transplant with a previous SCC (n=49) within 100 days of $CD4^+FOXP3^+CD127^{lo}CD25^{hi}$ Treg enumeration in peripheral blood, by flow cytometry. The optimal sensitivity-specificity trade-off is at 19 Tregs/μl with Area under the ROC curve of 0.86 and sensitivity of 80% and specificity of 78%.

The AUC was 0.86 and when using the sensitivity/specificity trade-off of 19 Tregs/μl this test had a sensitivity of 80% and a specificity of 78% (Figure 4.3.6.6). This assay now provides greater prognostic power as the greater the number of Tregs the more severe the SCC that becomes clinically apparent with-in 100 days. This does not answer if the Tregs dynamically change due to cancer presence, the next section investigates this.

4.3.7 Cancer presence and immune phenotype

There were 9 Kidney Transplant Recipients (KTR) who developed cancer that were also immune phenotyped prior to and at diagnosis of cancer, during the course of the study. Studying the Treg levels in these patients enables investigation into dynamic changes of Treg numbers upon clinical presentation and diagnosis of the tumour, the results are shown in Figure 4.3.7.1.
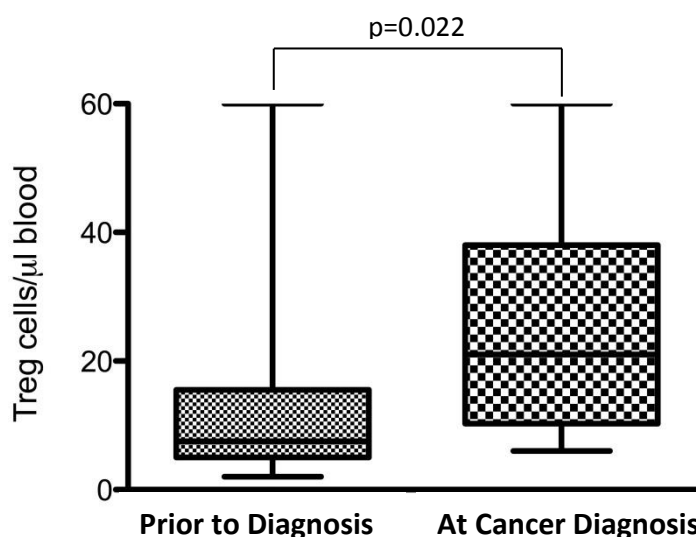
Figure 4.3.7.1: Changes in regulatory T cell number in kidney transplant recipients: A boxplot (median and range) of changes in enumerated Regulatory T cell numbers in kidney transplant recipients' peripheral blood. Enumerated prior to cancer diagnosis and at cancer diagnosis (n=9, p=0.022).

Treg numbers increased from a point in time prior to cancer compared to another time point at the diagnosis of cancer (p=0.022, Figure 4.3.7.1). This indicates that Tregs have a dynamic upward shift from a point in time until the cancer is diagnosed.

To investigate the impact presence or absence of cancer has on peripheral blood immune phenotype, 23 of the 35 (66%) KTR with current cancer were re-immune phenotyped after cancer resection, data shown in Figure 4.3.7.2 (Panel A). To measure immune phenotype stability, over the same time period there were 18 KTR who were immune phenotyped that did not have cancer and who did not develop cancer, that were re-immune phenotyped, the data are shown in Figure 4.3.7.1 (Panel B).

Panel A: KTR with resected cancer
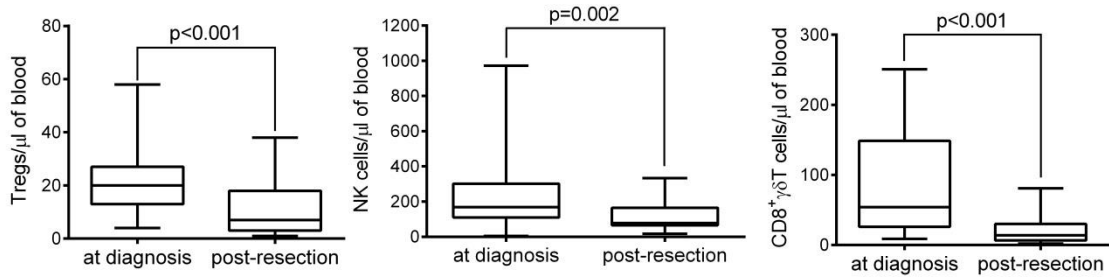
Panel B: KTR with no cancer, observed over two time points
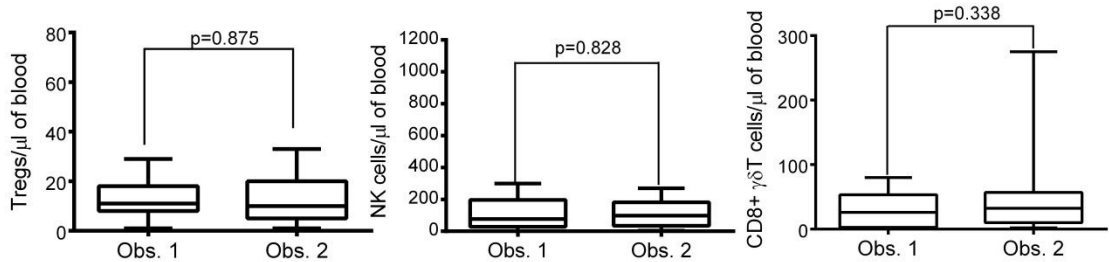
Figure 4.3.7.2: Immune phenotypes of Kidney Transplant Recipients with or without cancer over two time points: Boxplots (median and range) changes in cells type with resection of SCC or SOC (Panel A, n=23) or over two observations of stable KTR (Panel B, n=18). Left hand boxplot represents cell value at the time of cancer diagnosis or the first observation (Obs. 1) and right hand side represents the value after resection or at the second observation (Obs. 2). Prospective follow-up between time points for cancer patients were median (IQR) of 6 (1.5-14) months compared to stable KTR median (IQR) of 12 (3-16) months.

After SCC or SOC resection (n=23); Treg, NK cell and $\gamma\delta$ T cell number fell significantly (all $p<0.01$, Figure 4.7.2), in comparison to KTR with no SCC development (n=19) where immune phenotype was stable with prospective follow-up (Figure 4.7.2). To control for surgical resection, KTR with resected Basal Cell Carcinoma (BCC) were also investigated as seen in Figure 4.3.7.3.
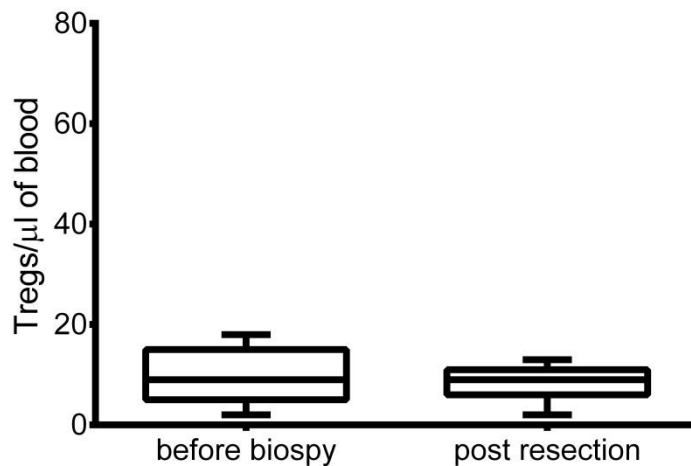
Figure 4.3.7.3: Lesion Resection of post-biospy proven BCC tumours.

Boxplot of enumerated regulatory T cell number of Kidney Transplant Recipients (n=12) that had suspect lesions removed that were subsequently re-classified as Basal Cell Carcinoma (BCC) tumours.

Those KTR (n=12) with suspected malignant lesions, who later had histologically classified BCC also had low levels of Treg (<19 Tregs/μL) and after resection their Tregs remained static (Figure 4.3.7.3).

4.4 Discussion

Previous studies have investigated if immune cells associate with cancer in KTR[103, 104, 205]. In these studies the majority of KTR investigated underwent ATG induction therapy and pronounced CD4 lymphopenia was observed. The conclusions from both studies indicated that CD4 lymphopenia associated with cancer but did not determine if the CD4 lymphopenia was ATG- or *de novo* cancer induced. Additionally, there was no CD4 cell threshold that could accurately predict cancer. In this thesis, more KTR with cancer used ATG than KTR with no cancer and only 17% of KTR underwent ATG induction. When investigating ATG usage and cancer status effect on CD4 count, there was no difference in CD4 count between all cancer groups (p=0.062) as seen in Table 4.3.4.1. The 3 studies showed that CD4 counts <400-600 CD4 cells/μl had an association to cancer. Our medians were of 790, 510 and 800cells/μl, for KTR with current, past and no cancer, respectively. This data indicates that CD4 lymphopenia is associated with ATG induction therapy rather than cancer status in KTR.

When the same studies compared CD8^{+} T cells and CD19^{+} B cells in KTR with SOC to KTR with SCC, there were greater differences in KTR with SOC than SCC[104]. The study reported here did not find any differences in CD8 counts in KTR with current, past or no cancer having 430, 440 and 380cells/µl, respectively. There was also no difference in overall CD19^{+} B cell counts, 49, 40 and 59cells/µl, respectively. Our numbers are similar to other studies; 543 Vs. 650 CD4 cells/µl, 640 Vs. 435 CD8 cells/µl, 52 Vs. 41 CD19 cells/µl when comparing KTR with cancer between a reported study to this study, all data shown in Table 4.3.4.1.

A more recently performed study investigated the use of immune phenotype to predict SCC development in KTR at risk of multiple SCC[141]. This study provided evidence that sub-types of immune cells associate to SCC and can predict SCC development, namely, increases in CD4^{+} Regulatory T cells and CD8^{+} T suppressors with decreases in CD8^{+} T central memory, and Natural Killer cells. This study, reported here, showed an increase in Tregs but not CD8 T suppressors, when comparing KTR with cancer against those KTR with no cancer: median value of 16 Vs. 8 Tregs/µl and 206 Vs 120 CD8 T suppressors /µl, respectively. Furthermore, this study showed no difference in CD8^{+} central memory T cells and Natural Killer cells, when comparing KTR with cancer against KTR with no cancer. Therefore, this data, in part, validates previous studies which showed that high regulatory T cells (Tregs) and low Natural Killer (NK) cells associate with SCC in KTR based in the United Kingdom (UK)[141]. The UK cohort was older, had longer exposure to immunosuppression and was primarily treated with Calcineurin inhibitors (CNI), Azathioprine (AZA) and steroid free based immunosuppression regimens. What this study also showed was a difference in immune phenotype with those that had Cytomegalovirus (CMV) and prednisolone usage, warranting investigation into viral seropositivity and immunosuppression usage.

CMV was shown to increase CD8 T suppressor cells which also have been shown to be increased in cancer patients. Additional data indicated that CMV positivity increases γδ T

cells[101]. The same study showed that CMV seropositivity provides protection from cancer development. Our study showed no difference in CMV infection between those with cancer and those without (68% vs 48%, Chi-Squared p=0.140, Chapter 3: Section 3.3: Table 3.3.2), additionally we showed KTR with cancer have increased $CD8^+CD4^-$ γδ T cell numbers = 33 vs 24 cells/µl, respectively. The data in this chapter showed an increase in CD8 T suppressors in CMV seropositive KTR (Figure 4.5.1.1) but no difference in γδ T cell numbers and CMV seropositive KTR (Figure 4.5.1.2). Additionally, neither CMV nor EBV positivity affected Treg, γδ T cell or B cell proportion or numbers. The only association to immune phenotype we found was that CMV seropositive KTR had increased the $CD8^+CD27^-CD28^-$ parent population (p<0.02) which we also observed in the UK study and has been previously reported[206]. The contradictory data between CMV infection, cancer status and γδ T cells, indicates further investigation is needed to dissect the mechanism behind the observations.

A new finding in this current study showed that SOC and SCC are associated with high number and proportion of CD8 single positive γδ T cells (i.e. $CD8^+CD4^-$ $CD3^+$ γδ T cells). The function of γδ T cells are, when compared to αβ T cells, poorly understood but are involved in mucosal, anti-viral and anti-cancer immunity [207, 208]. In addition, a high proportion of γδ T cells is associated with operational tolerance in liver transplant recipients [209]. Early data suggested that $CD8^+$γδ T cells have been shown to be cytotoxic compared to $CD4^+$ counterparts[210]. In animal models of mucosal immunity, Tregs impair $CD4^+$γδ T cell function [211]. In renal cell cancer high number of γδ T cells portent good prognosis [212]. Conversely, γδ T cells and the $Vδ2^{neg}$γδ T cell sub-population have been shown to be reduced in KTR with cancer, especially in CMV positive KTR [101]. The KTR in our cohort with SOC (n=14) who survived (n=6) had a higher number of total γδ T cells (i.e. $CD3^+$γδ T cells) compared to those who died of their malignancy (p=0.110, data not shown), they also had less aggressive disease at presentation. However, we found no association of CMV status on $CD3^+$γδ T cells or cancer status. We showed that KTR with cancer have a non-statistically increased proportion

of γδ T cells in the CD3 portion of lymphocytes, whereas Couzi. *et. al.* showed γδ T cell proportion of lymphocytes are decreased in lymphocyte gate[101]. This signifies a different population of γδ T cells are being studied between groups. Additionally, the adjusted logistic regression models (Table 4.3.2.3) and cancer resection data (Figure 4.3.7.2 Panel A) show γδ T cells associate with age, gender and/or duration of immunosuppression and may be influenced by cancer tissue presence. Additionally, there was a difference in age, duration and type of immunosuppression between studies and therefore may account for these differences. However, given we have shown γδ T cells numbers are dynamic around cancer resection it may be that the differences between studies reflects the timing of sample collection.

Investigation into NK cells showed that there wasn't any association between low NK cells and SCC development. It has been shown that AZA preferentially depletes NK cell number and impairs function[213][214]. Therefore the predominance of Mycophenolate (MMF) as opposed to AZA in the drug regimen of the current cohort may explain this difference in immune phenotype than in other previously published papers[102-104]. NK cell number in Australian KTR, irrespective of cancer status, was higher than their comparators in the UK. However NK cell numbers decrease with age[161][215] and the UK cohort were older than the Australian cohort. Furthermore, when correcting for age and gender there was no association with use of AZA on NK cell parameters in our cohort (Unadjusted p= 0.797, Adjusted for AZA use p= 0.224).

When investigating B cells, cancer patients had lower overall B cells, with a compartmental shift from naïve to memory. It is known that naïve B cells decrease and memory B cells increased with age[216] and could be a sign of B cell immune senescence. Indeed, operationally tolerant KTR not taking any immune suppression have increased memory B cell subtypes, compared to stable KTR on CNI based regimens [217][218]. Although the confounders of age, gender and duration of immunosuppression were taken into account, complex drug regimens

could not. However CNI usage, dosage and levels were the same between groups. Also Tregs themselves have direct and indirect abilities to suppress IgG production by giving apoptotic signals to B cells in vitro and in vivo [219]. This data suggests that we may be seeing an effect of a greater number of Tregs in an aging population. Additionally, this could explain why we observe that cancer is affecting the B cell compartmentalisation by adjusting the proportions of the cells but does not affect total numbers. B cell numbers did not change after cancer resection (data not shown). A recently described B cell sub-type with the regulatory ability by forming ATP catabolites utilising the CD39 and CD73 ectonucleotidase pathway [220] and may play a role in cancer. We measured this novel regulatory B cell (Breg, $CD19^+CD21^+CD39^+CD73^-$) in a limited cohort consisting of 35 KTR (23 KTR with cancer and 12 KTR with no cancer). There are higher proportions of Bregs in KTR with cancer but this did not reach significance (p=0.18). Additionally, the adenosine formation by high levels of CD73 expression is vital in IgG/A class-switching [118] and could be a reason to why we do not see large amounts of IgG^+ B cells in our cohort and inversely see IgD^+ accumulation.

High Treg, $CD4^-CD8^+\gamma\delta$ T cells and memory B cell proportions predicted SCC development in KTR with a previous SCC. However Treg number was the best predictor and there was no additive benefit of adding $CD4^-CD8^+\gamma\delta$ T cells as in the most part they co-segregated with Treg. Such co-segregation has been found within tumour tissue [221]. When comparing any SCC development vs. high risk SCC development the AUC of the ROC increased. This suggests high Treg number has a strong association with cancer progression and is more helpful in predicting aggressive SCC development. Tregs alone can stratify risk in these KTR and may provide us a means to identify patients at risk of developing cancer for increased dermatological review, preventative treatment, and possible immunosuppression dosage manipulations.

The current study shows that absolute numbers of Tregs were higher in those with current SOC or SCC compared to KTR with no current cancer. These data are consistent with the literature showing increased Treg numbers portend poor prognosis in non-immunosuppressed populations with cancer [222]. Additional markers, such as HELIOS, may identify Treg induced by tumour tissue or Treg with enhanced suppressive ability [119, 223]. We found that a median of 84% of Tregs expressed HELIOS (Figure 4.3.1). Although this is higher than reported for healthy controls (70%) there was no difference between KTR groups[224]. Therefore using an objective Treg gating strategy, the majority of Treg in this study are naturally occurring Treg (nTreg) rather than induced Treg (iTreg) depicted in Chapter 2: Figure 2.3.2.1. Though the premise that Helios only defines nTreg is currently under debate [119, 223] nonetheless, it may provide evidence of in vivo activated Tregs.

Two studies have shown that ATG induction reduced CD4 T cell numbers by approximately half while increasing Treg proportion by 2-fold, within the first 6-12 months post-transplant [69] [70] indicating a rebound of Treg, rather than an induction [225]. In our study investigating immune phenotype more than 10 years after ATG use, CD4 T cell and Treg parameters were not statistically different between ATG users and non-users: CD4 T cell median cell count (452cells/µl Vs. 682cells/µl, p=0.534); Treg numbers (25 Tregs/µl Vs. 16 Tregs/µl, p=0.223).

Finally we assessed whether immune phenotype changes with presence or resection of tumour tissue. These data do not encompass the entire current cancer groups as not all KTR were able to be followed at both the time of cancer diagnosis and at follow-up. Primarily this relates to those KTR who were diagnosed with late stage disease and died within 30 days of diagnosis. We have data of 9 KTR who were immune phenotyped prior to cancer development and at cancer diagnosis; it was observed that Treg numbers do significantly increase when tumours become clinically apparent (Figure 4.3.7.1).

After SOC/SCC resection the numbers of Treg, NK cell and γδ T cell fell significantly. This was compared to the immune phenotype in those KTR who did not develop any type of cancer, which is stable over time. In those KTR where resection was performed for clinical suspicion of SCC but histology characterised benign lesions (n=12), the Treg numbers were <19 cell/μL (Figure 4.3.7.3). Furthermore, the Tregs did not change after BCC resection (Figure 4.3.7.3), indicating that a malignant SCC or SOC in particular is informing the peripheral blood and that general skin surgery does not affect Treg levels.

With regards to other factors that may alter immune phenotype, mTORi are shown to increase Treg numbers regardless of cancer status (Figure 4.3.5.2) and those on CNI with cancer have less Treg numbers than those on mTORi or not on either (Figure 4.3.5.2). These data align with literature, were Tregs are seen to increase in patients that have been converted from CNI- to mTORi-based regimens. It also seems that AZA treated KTR have increased Tregs when compared to those not on AZA, however these patients (n=17) would be older and may be on mTORi that could be effecting the results.

In conclusion, this is the first time that immune phenotype has been shown to associate with the development of SOC in KTR. Secondly, immune phenotype in KTR with cancer is dynamic and resection of SOC or SCC results in decreases of Tregs, NK cells and CD4-CD8+γδ T cells in peripheral blood. Larger studies will be required to determine whether failure of these cell types to fall after cancer resection will predict recurrence. We have shown that Treg monitoring can predict the development of high risk SCC and allow us to identify KTR who may benefit from increased dermatological review, preventative treatment and possible immunosuppression dosage manipulations. The latter will only be possible if there is empirical evidence to suggest it can be done without impairing graft function. This will be investigated in subsequent chapters of this thesis.

Chapter 5: Immune Cell Function

5.1 Introduction

Kidney Transplant Recipients (KTR) with cancer have been shown in Chapter 4 and in previous studies to have a different immune phenotype to KTR without cancer[101-104, 141]. KTR with cancer have increased numbers and proportions of Regulatory T cells (Tregs) and decreased numbers and proportions of Natural Killer (NK) cells[141, 226]. However, the immune system's effectiveness cannot be gauged by cell numbers and proportions alone; this chapter investigates the immune function of KTR with cancer.

It has been shown that Tregs isolated from tumour tissue and the peripheral blood of cancer patients have higher suppressive function than Tregs from the blood of normal donors[143, 227, 228]. Importantly, the stage and grade of Head and Neck Squamous Cell Carcinoma (HNSCC) are associated with greater numbers and greater suppression capacity of the Tregs on a cell-per-cell basis than healthy controls[229] and, as such, also associate with poor cancer prognosis[228].

Chapter 1 introduced how Calcineurin Inhibitors (CNI) regimens are associated with reduced numbers and proportions of Tregs and how mammalian Target of Rapamycin inhibitors (mTORi) maintain these Treg parameters[196, 230]. Furthermore, Chapter 4: Section 4.3.5 showed that Tregs numbers and proportions are increased by mTORi usage in KTR with no cancer and CNI usage decreases Tregs in KTR with cancer. A proposed mechanism is CNI's ability to reduce Nuclear Factor of Activated T cells (NFAT), decreasing production of IL-2 which is vital for function and homeostasis, in mice[231]. Molecular interactions between NFAT and FOXP3 show that NFAT acts as a molecular switch between immune stimulator and immune regulator, thus down regulation decreases FOXP3 expression and FOXP3's ability to form these regulatory complexes[230, 232]. Additionally, FOXP3 mRNA transcription was decreased in CNI treated PBMC compared to Rapamycin in an allo-stimulated mixed

lymphocyte reaction[233]. There is also an inverse correlation to CNI level and Treg function[234]. Differing results may be due to blood handling; blood from KTR on CNI had decreased FOXP3 expression >2 hours of venesection and this may affect Treg function. Therefore, Tregs need to be isolated within this time to accurately determine Treg function.

Tregs promote cancer survival whereas NK cells have anti-cancer abilities. The function or dysfunction of NK cells plays an important role in the apoptosis of pre-cancer and cancerous cells. Patients with genetically (MCM4 or GATA2 mutations) related NK cell deficiencies in either number or function, have increased risk of infections, in particular: Herpes viruses, Human Papilloma Virus (HPV), Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) (reviewed elsewhere [235]).

NK cells are large granular lymphocytes that lack the CD3 T cell complex. They function by identifying and spontaneously causing apoptosis in cancerous and infected cells without prior antigen presentation [150, 236]. The identification process requires abnormal cells to display stress signals such as down-regulation of "self" surface proteins: Major Histo-incompatibility Complex (MHC) class I and regulatory Killer cell immunoglobulin-like receptors (KIR) [152, 153, 237]. The down regulation of MHC-I, reduces the effectiveness of cytotoxic CD8+ T cells and adaptive immune responses but makes the cells more sensitive to NK and innate immune responses[238]. Once an NK cell identifies this down-regulation, it binds and activates, expressing a type II transmembrane glycoprotein CD69 and other surface markers of activation[239]. Internal granules locate to the immune synapse that is created between the NK cell and the target cell and the effector molecules (perforin, Tumour Necrosis Factor-α (TNF-α), granzymes and interferons) are released into the synapse and onto the target cell. Upon degranulation, Lysosome-Associated Membrane Protein 1 (LAMP-1, CD107a) is exposed on the surface of the NK cell[153]. The released perforin creates pores in the target cell membrane through which granzyme B can enter the target cell and initiate apoptosis via the caspase

kinase pathway. Therefore there are several ways to measure NK cell activity including: CD69 up-regulation in the activation stage, CD107a in the effector stage, release of cytokines (perforin, granzyme B, interferon γ) in the killing stage, and total cytolysis of the target cells.

Cancer cells have greater metabolic demands than normal cells[240], utilising glycolysis and lactate pathways, via Lactate Dehydrogenase (LDH), causing an 18-fold increase in glucose utilisation, even under aerobic conditions[241]. This LDH can be measured as a cytotoxic assay (first described in 1988[242]). Additionally, in *in vitro* assays, NK cells undergo apoptosis when they are exhausted from their last kill. Recently, it has been shown that the loss of NK cells from an *in vitro* assay with a set number of NK cells, can relate to the amount of target cells killed. This loss has been termed "target induced NK cell loss" (TINKL). These two assays have been chosen for clinical application. LDH is a single platform, self-contained, non-radioactive, sensitive assay that can be used in any laboratory. TINKL is a flow-based assay that can be readily implemented in clinical flow laboratories.

It is widely accepted that NK cell function is decreased in cancer patients however it is not reported if KTR with cancer have further reduced NK cell function. The effect immunosuppression has on NK cells have been investigated both *in vitro* and *in vivo*[243, 244]. Immunosuppressive drugs: AZA, MMF, CNI, and prednisolone all have individual effects. These effects depend on the how the NK cells are stimulated and how NK function is measured. One particular study showed only a decrease in NK function in short-term KTR compared to healthy controls, which as not observed in long term KTR[243]. Both IFN-γ and CD107a expression have been shown to decrease when NK cells were co-cultured in the presence of clinically relevant concentrations of a variety of immunosuppressive drugs[244]. This chapter focuses on both Treg and NK cell function from the patients outlined in Chapter 3: Section 3.5 using the methodologies from Chapter 2: Section 2.5.

5.1.1 Chapter Aims:

1) To utilise Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay in PBMC, a laboratory standard, to show differences in Treg suppressive function of KTR.

2) To determine utilisation of CD154/CD40L suppression of expression assay, a short duration assay, for Tregs from KTR and compare to CFSE results.

3) To investigate any differences in NK cytotoxic function in KTR with current cancer compared to those with a past cancer or those with no cancer.

5.1.2 Chapter Hypotheses:

1) The CFSE dilution assay will determine that Tregs from KTR with cancer have greater suppressive function that Tregs from KTR with no cancer.

2) The CD154 suppression of expression assay will correlate to CFSE dilution suppression assay and provide a quicker, more cost effective alternative to CFSE.

3) The NK function of KTR with cancer or a history of cancer will be dysfunctional compared to those KTR with no history of cancer.

5.2 Chapter Methods:

All regulatory T cell techniques were performed on fresh samples as cryopreservation has been shown to decrease function in expanded Tregs[245] and cytokine profiles[200, 246, 247].

5.2.1 Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay

CFSE is an amine dye that is drawn into cells and forms fluorescent protein that conjugates upon deacetylation. The cells are stimulated and upon doubling, reduce fluorescence 2-fold as the amine concentration is halved with the daughter cell. Therefore, a dilution is a measurement of the cells that have proliferated. With the addition of Tregs, this dilution is decreased and it is this proportional decrease compared to the (unsuppressed) positive control that is measured. With the aim of determining overall Treg function, a bead coated with anti-

CD3 and co-stimulatory anti-CD28 antibodies was used to pan-stimulate the reacting T cells. This will enable a broad-spectrum or bystander regulatory response. The CFSE dilution was measured as total proliferation as seen in Figure 5.2.1.

A) No stimulation control
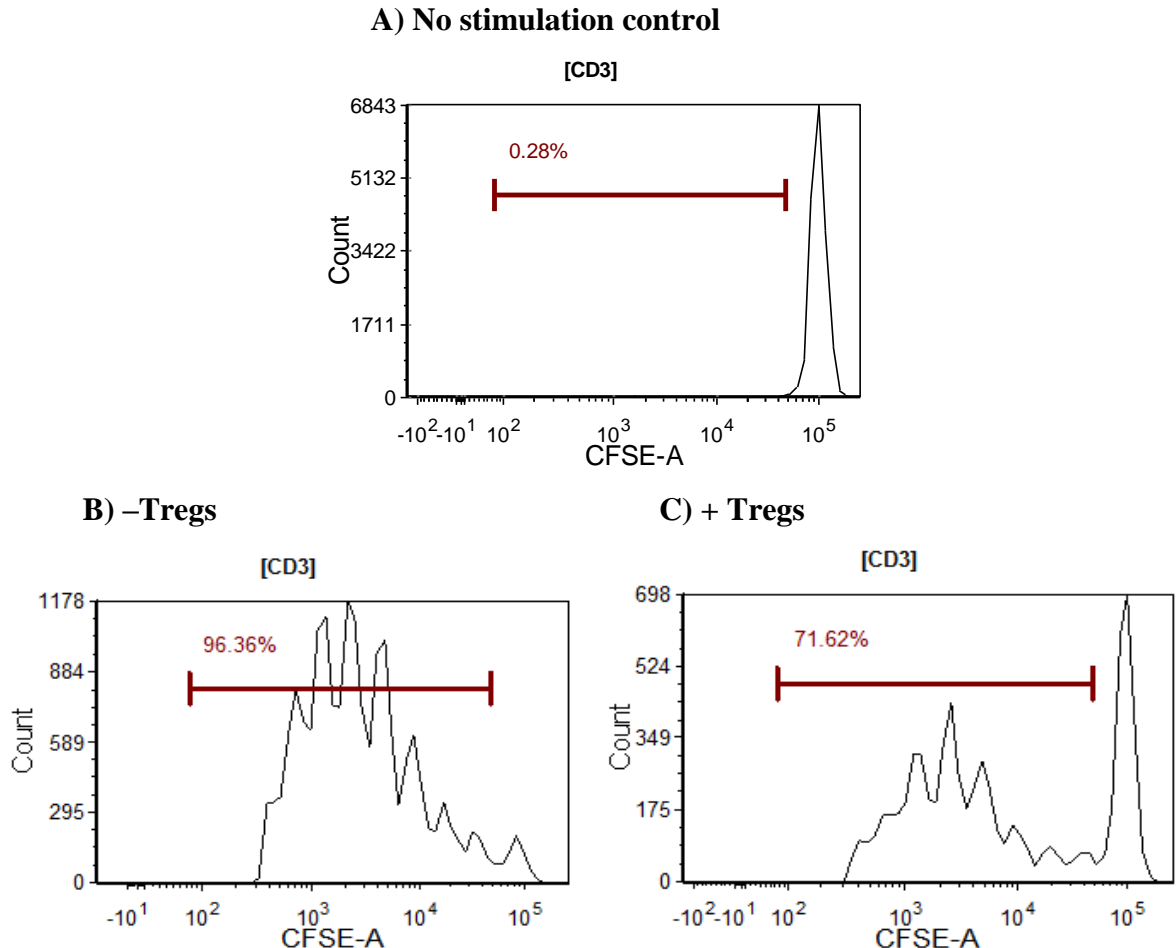
B) –Tregs

C) + Tregs

Figure 5.2.1: CFSE dilution pattern with Tregs at 1:4 ratio: Histogram plots of CFSE dilution from CD3$^+$ FACS events from CFSE stained healthy indivdual PBMC stimulated by CD3/CD28 beads. A) unstimulated PBMC, B) no regulatory T cells (Tregs) and C) Kidney Transplant Recipients (KTR) tregs added at Treg:responder cell ratio 1:4.

The suppression of CFSE expression is measured as;

[(CFSE% of graph B - CFSE% of graph C) / CFSE% of graph B] x 100

The results were expressed as a percentage, with percentages below 0% classed as 0%.

5.2.1.1 Optimising bead to responder cell Ratio

When stimulating PBMC it is important to induce the greatest range of proliferation with the lowest concentration of beads. The stimulation needs to be strong enough to be able to delineate any differences between regulatory ability but not too strong and not able to be suppressed. Previously, a bead to cell ratio of 1:4 was used for the CD154 suppression of expression assay[195, 248]. As this is an 8 hour assay and the CFSE is a 5 day assay, it was determined that the highest bead concentration to use would be 1:8 and a titration of 2-foldwould be investigated, depicted in Figure 5.2.1.1.
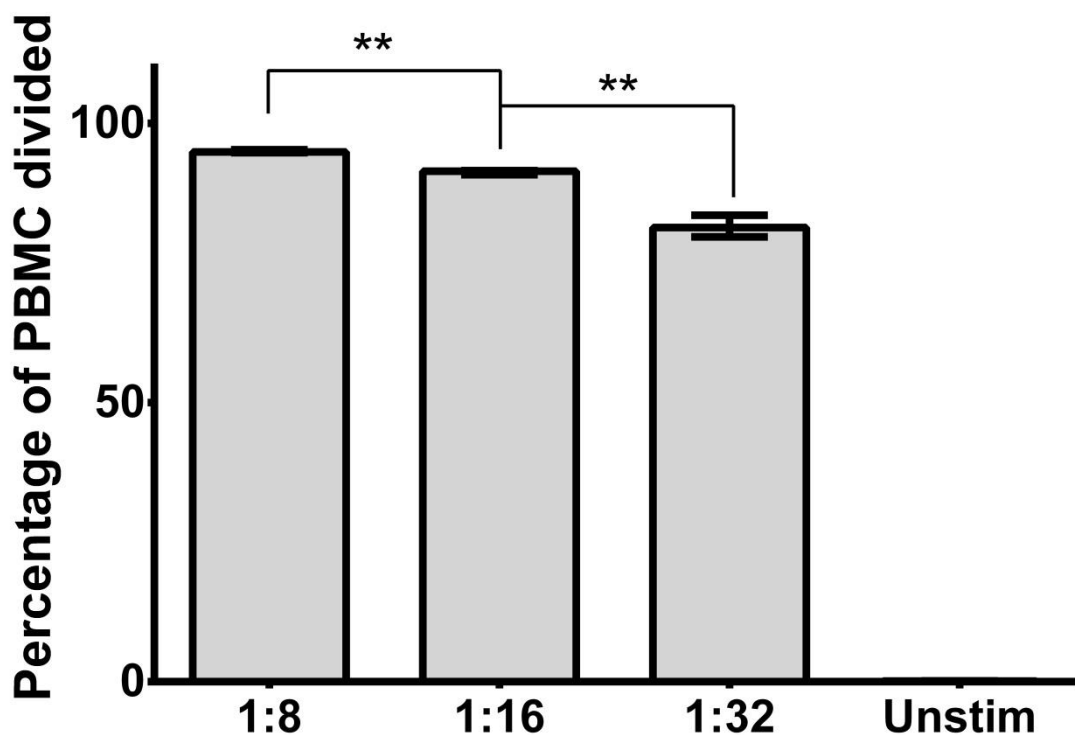
Figure 5.2.1.1: Optimising assay stimulation: A bar graph depicting median (range) of 4 experiments performed on 1 healthy individual's PBMC. The titration of beads gave a titrated stimulation of 95%, 91% 81% and 0%, respectively, p=<0.001 using a paired parametric ANOVA.

The individual used in this experiment remained consistent as the responder for the rest of this chapter. The bead:cell ratio of 1:8 (half that recommended) was chosen as 1:16 had a statistically reduced level of stimulation and 1:32 had further reduced proliferation.

5.2.1.2 Optimising regulatory T cells (Treg) to responder cell ratio

To determine optimal Treg ratio, induced Tregs (iTregs) from naïve T cells where used. The responding PBMC were stimulated with 1µg/ml of CD3/CD28 antibodies for a 5 day period. Naïve CD4+ T cells were isolated from 3 healthy donor buffy coat PBMCs using Naive CD4+ T cell Isolation Kit II for human cells. Naïve CD4$^+$ T cells were stimulated with monocyte derived Dendritic cells (mDCs), IL-2 and TGF-β for 5 days. These mDC are matured from CD14 negative selected monoyctes and placed in T25 culture flasks. With the addition of GM-CSF and IL-4 for a period of 4 days followed by LPS for another day, the monocytes mature to form mDCs. Once the iTregs were induced they were placed in the aforementioned CFSE assay and run at Treg to cell ratios 1:2, 1:4, 1:8, 1:16 and 1:32, depicted in Figure 5.2.1.2.
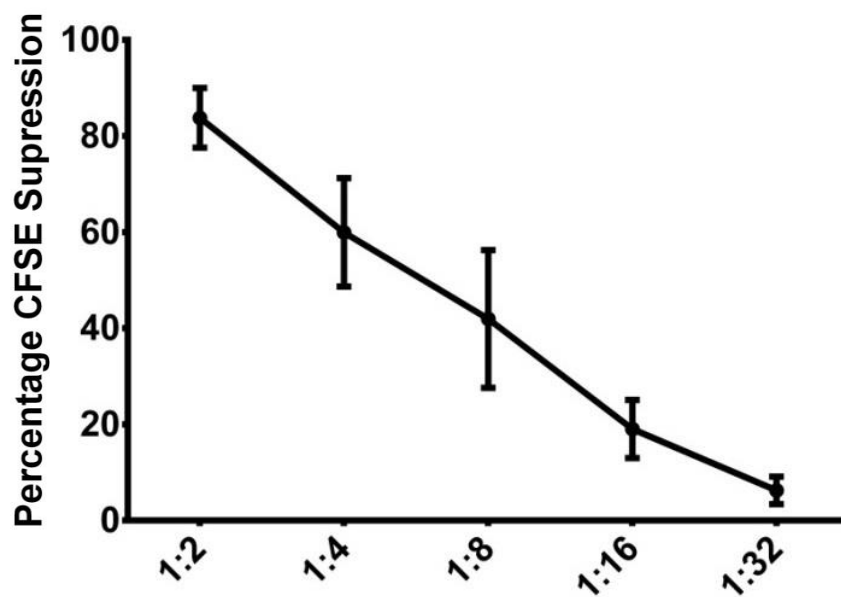
Figure 5.2.1.2 Induced Treg suppression titration: A line graph depicting the titration of induced regulatory T cells (iTreg, n=3) to responder cell ratios ranging from 1:2 down to 1:32. The data shows the mean percentage suppression of CFSE dilution at each ratio with standard deviation.

In summary, The Treg: Responder ratio of 1:8 gave the largest difference. However the median value was 42% suppression and, with the hypothesis of Tregs in KTR with cancer having greater suppression, the ratio 1:4 was chosen for experiments with KTR Tregs.

5.2.2 CD154 suppression of expression assay

Upon T cell receptor stimulation, costimulation molecule CD154 (CD40L) is expressed on the cell surface. CD154/CD40L binds to CD40 on APC's (mainly B cells) and provide co-stimulation to proliferate. It has been shown that CD154/CD40L suppression of expression is a surrogate marker for cell proliferation[195, 248]. Additionally, there is no expression of CD154 on resting naïve CD4 T cells. This is due to the transient expression of CD154 which has a peak expression at 6-8 hours after activation[248]. All magnetically isolated CD4$^+$CD25$^-$ naïve T cells had greater than 95% purity before use in the assay, as measured with flow cytometery (data not shown). All isolated cells did not express CD154 at baseline level, represented in Figure 5.2.2.1 (A).

5.2.2.1 Gating of CD154 in the CD154 suppression of expression assay

As mentioned, CD154/CD40L is not expressed on resting cells and this can be seen in the top plot of Figure 5.2.2.1. However, on stimuation there was an increase in CD154 expression to 41%, Figure 5.2.2.1 (B). With the addition of KTR Tregs, a reduction in CD154 expression was measured, a representative plot is depicted in Figure 5.2.2.1 (C).

A) No stimulation control
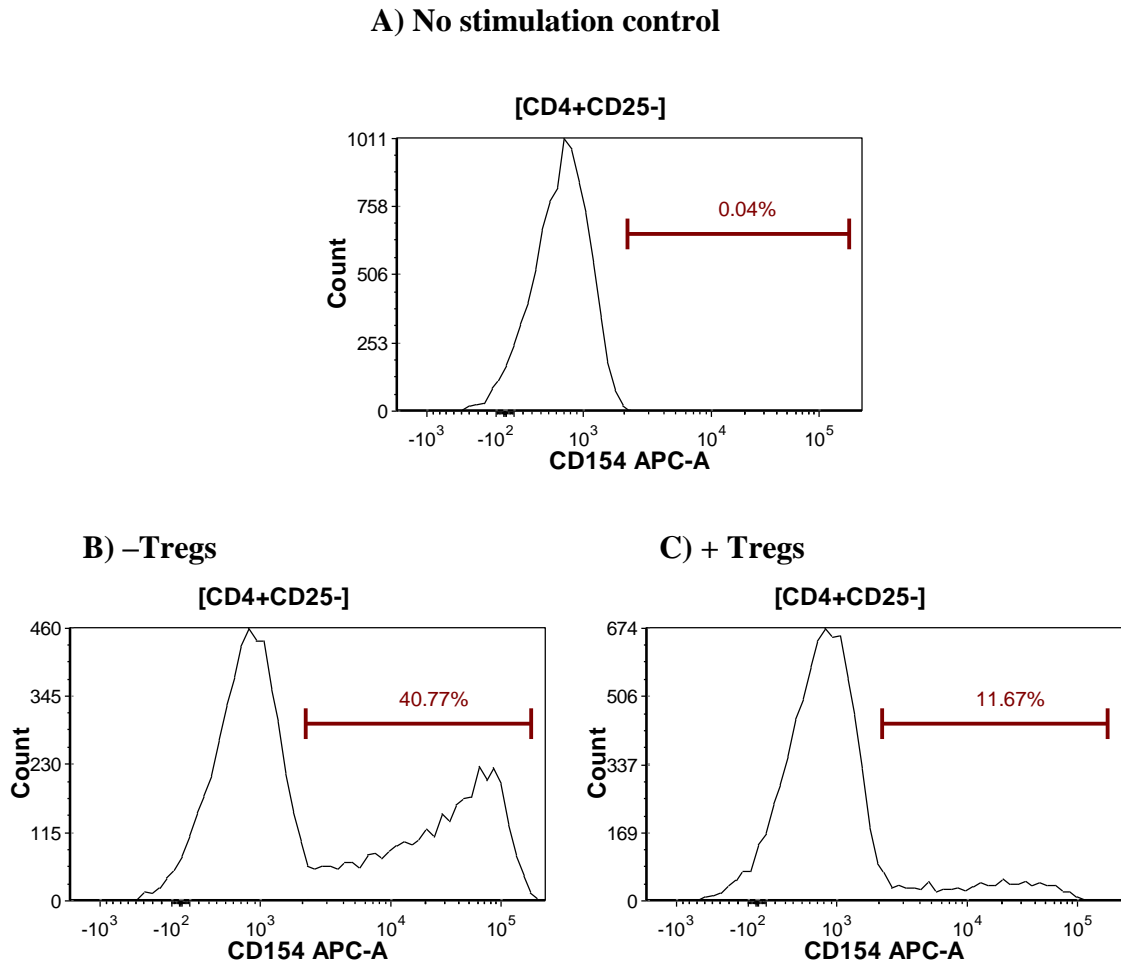
B) –Tregs

C) + Tregs

Figure 5.2.2.1: CD154 suppression of expression with naturally occurring regulatory T cells (nTregs) at 1Treg:4 responder naïve CD4$^+$ T cells: Histogram plots of CD154 suppression of expression in CD4$^+$CD25$^-$ FACS events from healthy indivdual isolated CD4$^+$CD25$^-$ naïve T cells. The top plot is unstimulated PBMC, the bottom two plots are stimulated by CD3/CD28 beads. The bottom left plot is with no regulatory T cells (Tregs) and the bottom right plot is with KTR tregs added at Treg:responder cell ratio 1:4.

The suppression of CD154 expression is measured as;

[(CD154% of graph B - CD154% of graph C) / CD154% of graph B] x 100

The results were expressed as a percentage, with percentages below 0% classed as 0%.

5.2.3 Lactate Dehydrogenase (LDH) release assay

NK cell killing can be measured by using a cancer cell line K562 which lacks MHC-class I on its cell surface. Technically the gold standard of NK cell function, is a chromium release assay. This assay involves ^{52}Cr radioactive isotope labelling of the K562. An alternative method is measurement of Lactated dehydrogenase (LDH) released from the lysed K562 cells. The LDH converts a colourless solution of Tetrazolium salt to Red Formazan which can be quantitated by optical density (OD) readings from a photospectrometer at 490nm. The reaction is uninhibited and needs to be stopped by decreasing pH with acetic acid.

Cytotoxicity is measured by the OD$_{490}$ of:

[(PBMC+K562 – PBMC alone – K562 Alone) / (K562 Max Lysis – K562 Alone)] x 100

The results were expressed as a percentage, with percentages below 0% classed as 0%.

5.2.3.1 The effect of cryopreservation on Lactate Dehydrogenase (LDH) release analysis

Cryopreservation may potentially impact cell function, and if intended to be used for cell storage, then it is important to measure if cryopreservation impacts NK cell function. Both healthy control and KTR PBMC were cryopreserved as per protocol outlined in chapter 2: Section 2.2.1. Measurements of LDH release were taken from 4 healthy controls and 8 KTR from fresh PBMC and compared post-cryopreserved PBMC.
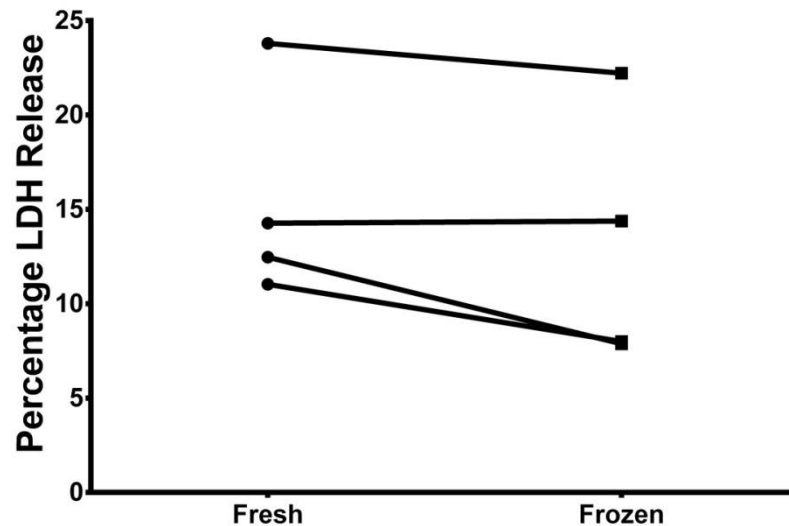
5.2.3.1.1 Lactate Dehydrogenase (LDH) release measured in freshly isolated and thawed peripheral blood mononuclear cells (PBMC) from Healthy Controls: A line plot of paired LDH released from PBMC isolated from 4 Healthy Controls run fresh and again after thawing. The LDH values were similar despite cryopreservation, p-value=0.250, using a paired non-parametric Wilcoxon test.

It can be seen in Figure 5.2.3.1.1 that healthy control PBMC lysis of K562 is not affected by cryopreservation. This test was run again in KTR to determine if there are any detrimental effects on KTR PBMC after cryopreservation and the results can be seen in Figure 5.2.3.1.2
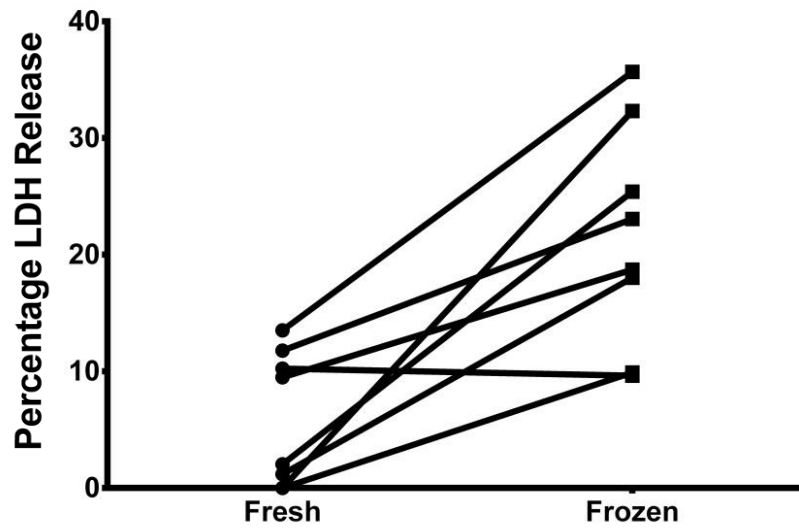
5.2.3.1.2 Lactate Dehydrogenase (LDH) release measured in freshly isolated and thawed peripheral blood mononuclear cells (PBMC) from Kidney Transplant Recipients: A line plot of LDH release from a co-culture of K562 with PBMC from 8 Kidney Transplant Recipients (KTR), run fresh and again after thawing. The LDH values were increased by cryopreservation, p-value=0.156, using a paired non-parametric Wilcoxon test.

It can be seen that PBMC have increased K562 cell lysis after cryopreservation.

5.2.3.3 Effect of anticoagulant used on Lactate Dehydrogenase (LDH) Release

There is evidence that anticoagulants effect the activation of lymphocytes and it is very important to determine if the anticoagulant may impact the cell function of the given assay[249, 250]. Lithium Heparin (LiHep) and Ethylenediaminetetraacetic acid (EDTA) tubes are the two main anticoagulants used in our blood collection centre. EDTA is a metal ion chelating agent that may affect calcium dependent activation such as CD69 expression[251]. With this knowledge we tested to see if EDTA would affect NK cytotoxic function. Figure 5.2.3.3
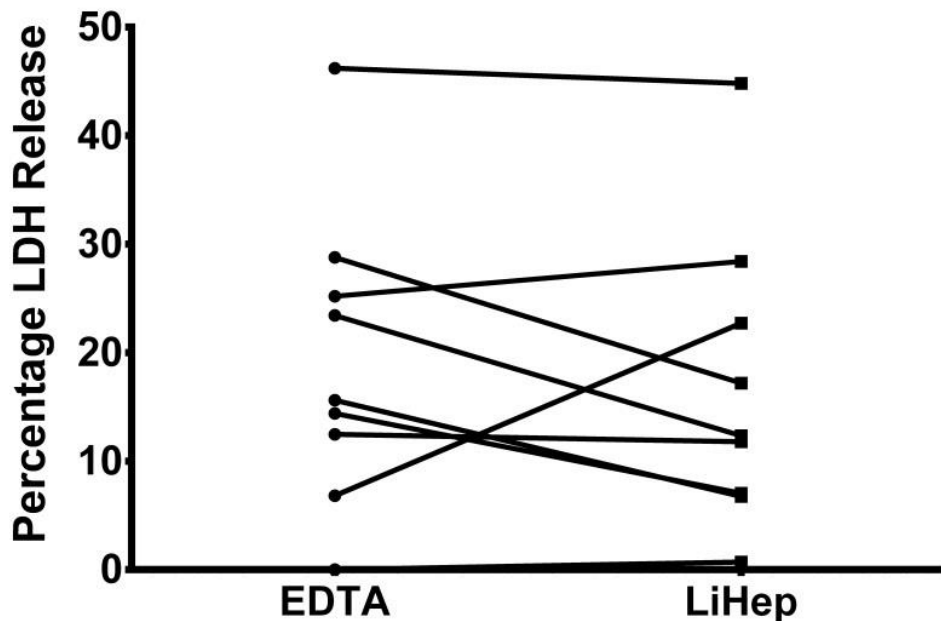
Figure 5.2.3.3: The effect of anti-coagulant on lactate dehydrogenase (LDH) release: A line graph depicting LDH release from a co-culture of K562 and PBMC from 3 Kidney Transplant Recipients (KTR) run from PBMC's isolated from EDTA and LiHep anti-coagulated blood. The results are similar, p=0.426 using a paired non-parametric Wilcoxon U-test.

This indicates that there was no difference in NK cytotoxicity using EDTA or LiHep as the anticoagulant at any of the three measured PBMC:K562 ratios of 20:1, 10:1 and 5:1.

5.2.3.4 Co-Culture incubation time

According to the manufacturer's procedures a 4hr incubation is used to measure LDH release. However, the TINKL protocol requires a 6hr incubation period. Because these assays were to be used in tandem, measurement of LDH release from both 4hrs and 6hrs was investigated. Using 4 healthy control PBMC, it can be seen there is no difference using a 4 or 6 hour incubation period, p=0.625, Figure 5.2.3.4.
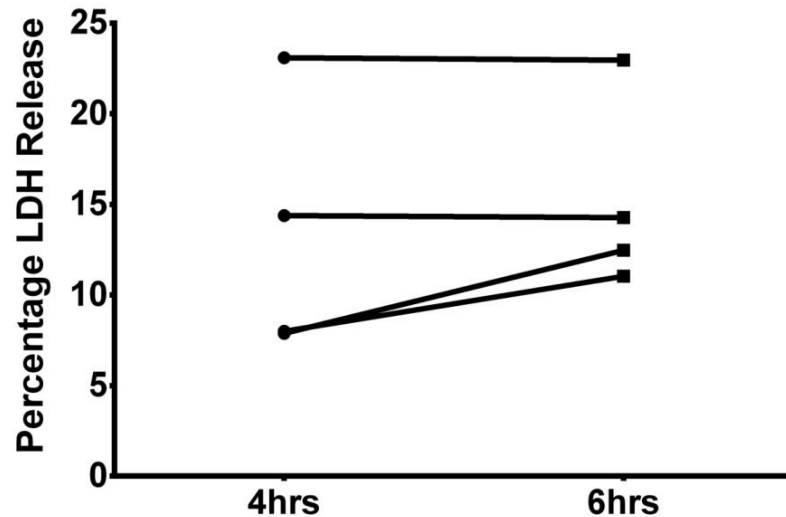
Figure 5.2.3.4: Lactate dehydrogenase release (LDH) release measured after 4 or 6 hour incubation: A dot plot of LDH release from a co-culture of K562 with 4 healthy control Peripheral Blood Mononuclear Cells (PBMC) over a 4 and 6 hour time period. Results were paired and analysed using a non-parametric Wilcoxon U-test. There is no difference in LDH release at 4 or 6 hrs, p=0.625.

The previous experiments established that anticoagulant, cryopreservation and incubation time did not affect NK function. However it is known that KTR have decreased NK cell numbers. Thus, before investigation into the impact cancer has on NK cell function in KTR, the correct ratio of effector to target cells was investigated.

5.2.3.5 Peripheral blood mononuclear cell (PBMC) to K562 Target cell ratio

With literature evidence of decreased NK function under immunosuppression[243, 244], and with the hypothesis that NK cell function will be decreased in KTR with cancer, the ratio of PBMC:K562 target cells was run double the manufactures' recommendation and titrated down 2 additional ratios in 5 healthy controls and 3 KTR as seen in Figure 5.2.3.5.
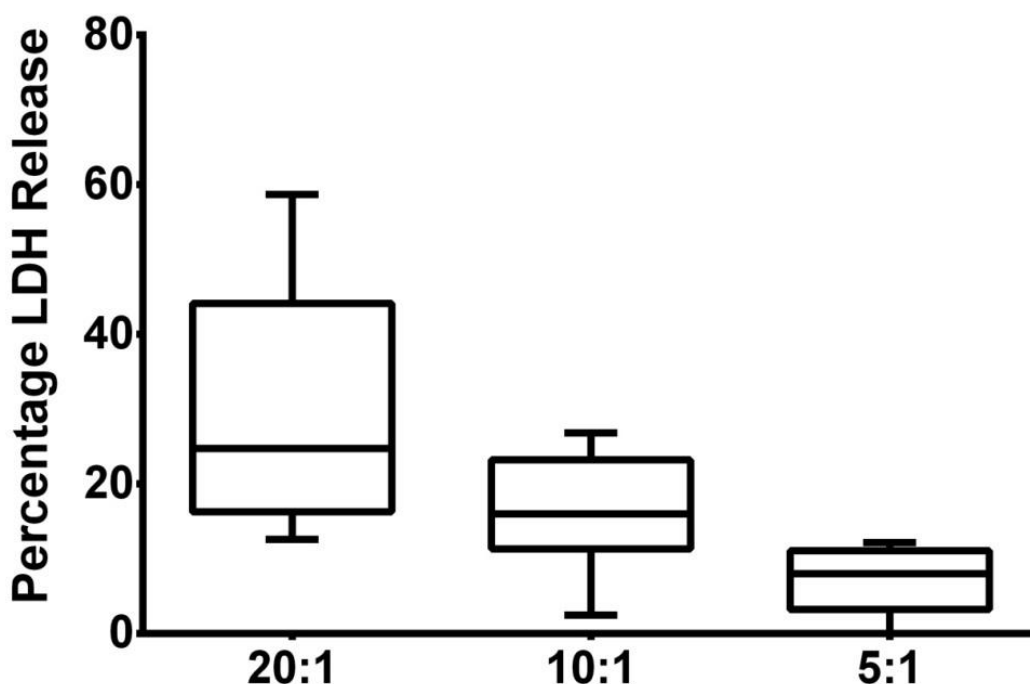
Figure 5.2.3.5: Lactate dehydrogenase release (LDH) measured at three different ratios of Peripheral Blood Mononuclear Cell (PBMC): K562 target cells: A box and whisker plot of the median (range) LDH release of 5 healthy controls and 3 Kidney Transplant Recipients (KTR) from a PBMC/K562 co-culture assays. The PBMC were titrated at PBMC: K562 target cell ratios of 20:1, 10:1 and 5:1. Median values between 10:1 compared to 20:1 was 17% Vs 25% and difference in range was 24% Vs. 46% when comparing 10:1 Vs. 20:1.

In summary, the LDH assay can be run using freshly isolated or cryopreserved PBMC from EDTA or Li-Hep anticoagulated tubes. Additionally, the LDH assay can be run for an incubation period of 4 or 6 hours, at a PBMC:K562 ratio of 20:1, in healthy controls. Even though the LDH assay is affected by cryopreservation in KTR, LDH release is increased. This could be due to a variety of mechanisms including; loss of immunosuppressant effects; loss of a suppressive cell population; and/or cell mediators during the freeze thaw process.

5.2.4 Target Induce NK cell Loss (TINKL) Assay

In vitro co-cultures of NK cells and NK sensitive target cells (K562), lead to K562 lysis. Upon release from killing, the NK cell can undergo apoptosis itself. This apoptosis and loss of NK cells numbers associates to the amount of target cells killed as measured by ^{51}Cr release. This has been termed "target induced NK cell loss" or TINKL [252]. However, it is not known if immunosuppression affects TINKL.

5.2.4.1 The effect of cryopreservation on TINKL analysis

The original TINKL assay is described for use in fresh, overnight rested PBMC for handling and time constraint purposes. This section investigated the use of cryopreservation for batch processing and clinical study purposes. The optimisation is mainly driven by the ability to simultaneously investigate the LDH release from the same PBMC:K562 co-cultures as TINKL analysis. Furthermore, cryopreservation enables the use of the same cells in other assays outlined in Chapter 6. Healthy control PBMC were used to determine the effects of cryopreservation and the results are depicted in Figure 5.2.4.1.1.
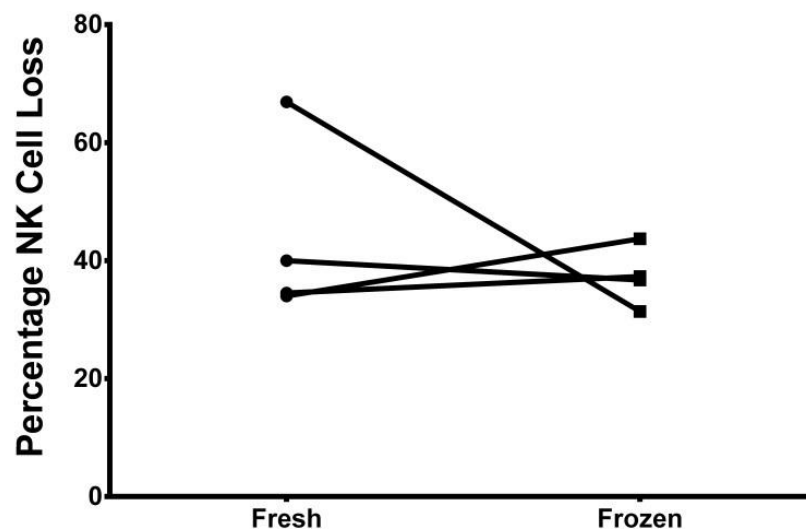
Figure 5.2.4.1.1: Target Induced NK Cell Loss (TINKL) measured in fresh and frozen samples from Healthy Controls: A Line graph depicting TINKL from co-cultures of PBMCs from 4 Healthy Controls and K562s. The samples were run fresh and again after cryopreservation. There was no difference when using fresh or frozen PBMC, p=0.875 using a non-parametric paired Wilcoxon test.

Figure 5.7.3.2.1 shows that there was no effect of cryopreservation of healthy control PBMC, with 1 out of 4 samples having a noticeable non-significant decrease. This test was run again on KTR PBMC.
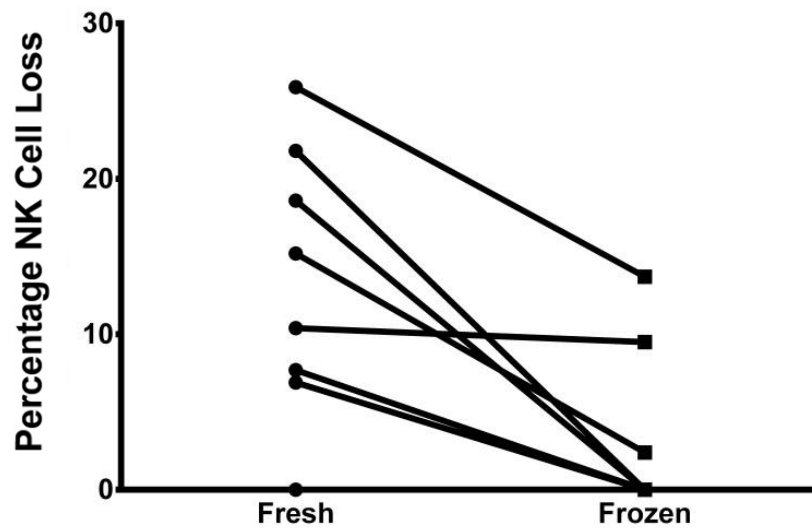
Figure 5.2.4.1.2: Target Induced NK cell Loss (TINKL) measured in freshly isolated and thawed peripheral blood mononuclear cells (PBMC) from Kidney Transplant Recipients: A line plot of LDH release from a co-culture of K562 with PBMC from 8 Kidney Transplant Recipients (KTR), run fresh and again after thawing. The TINKL values were affected by cryopreservation, p-value= 0.0156, using a paired non-parametric Wilcoxon test.

In summary, TINKL can be used post-thawing process in healthy controls, however, there was a reduction in TINKL values after cryopreservation in KTR and therefore TINKL assays should not be performed in frozen material.

5.3 Results

5.3.1 CFSE dilution assay of nTreg function in Kidney Transplant Recipients (KTR)

To define Treg suppressive differences in KTR, 16 KTR, 8 with cancer and 8 with no cancer, were assessed. The characteristics of the 16 KTR are shown in Table 5.3.1. Isolated nTregs were run in the optimised assay as previous mentioned in Chapter 2: Section 2.4. The CFSE results are expressed as a percentage reduction of the maximally proliferated cells (Figure 5.3.1.1) and the cumulative results are shown in Figure 5.3.1.2. The patients used in the following analyses were a sub-cohort from Chapter 3: Section 3.5 that are matched, as shown in Table 3.5. The 16 KTR characterisitics used in the Treg suppression assays are outlined in Table 5.3.1.

Table 5.3.1 Cohort of KTR run in Treg Funcitonal Assays

	Cancer	No cancer	p-value
Number, *N*	8	8	-
Age in years (Range)	67 (52-80)	53 (34-66)	0.009
Male gender, *N* (%)	8 (100)	3 (37.5)	0.026
Years of Immunosuppression (Range)	11 (3-22)	3 (0-11)	0.016

Due to random sampling of the 50 KTR; age, gender and duration of immunosuppression are significantly different between the two groups.
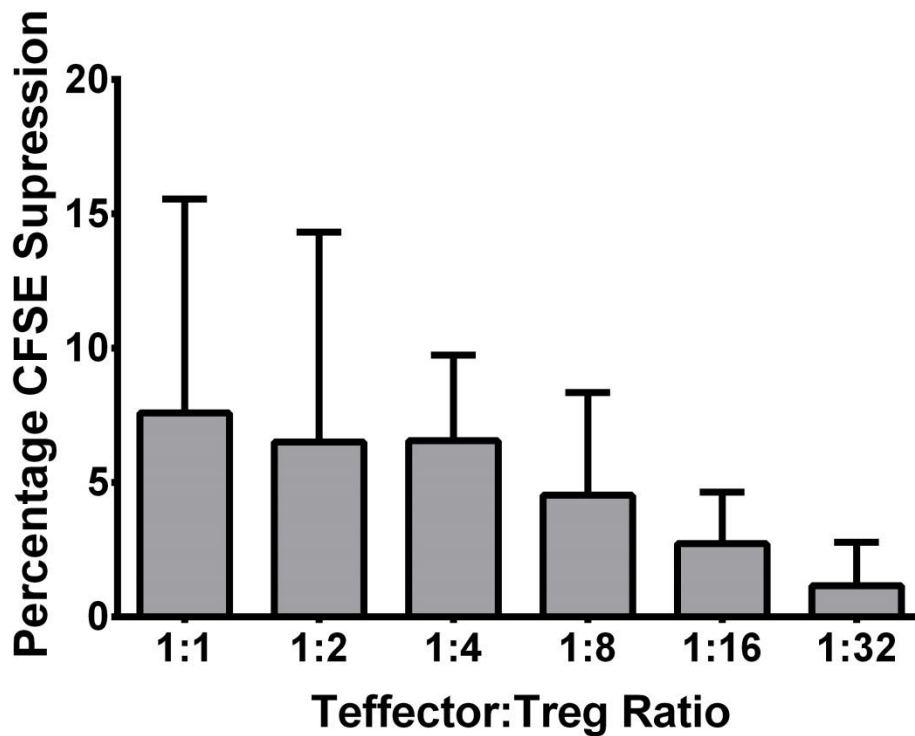
Figure 5.3.1.1: Titrated peripheral blood naturally occurring regulatory T cell (nTreg) suppression of CFSE dilution: A bar graph depicting precentage Treg suppression of CFSE dilution from 13 KTR. Samples were run in triplicate at titrating ratios, and the graph shows the median with Inter-Quartile Ranges (IQR) of the tripicate means.

CFSE dilution assay with nTregs and bead stimulus yeilds a lower level of suppression than using iTregs with anitbody stimulus, however it is a plausible assay to use with isolated nTregs from KTR. These results were then split into those KTR with cancer and those KTR with no history of cancer, depicted in Figure 5.3.1.2.
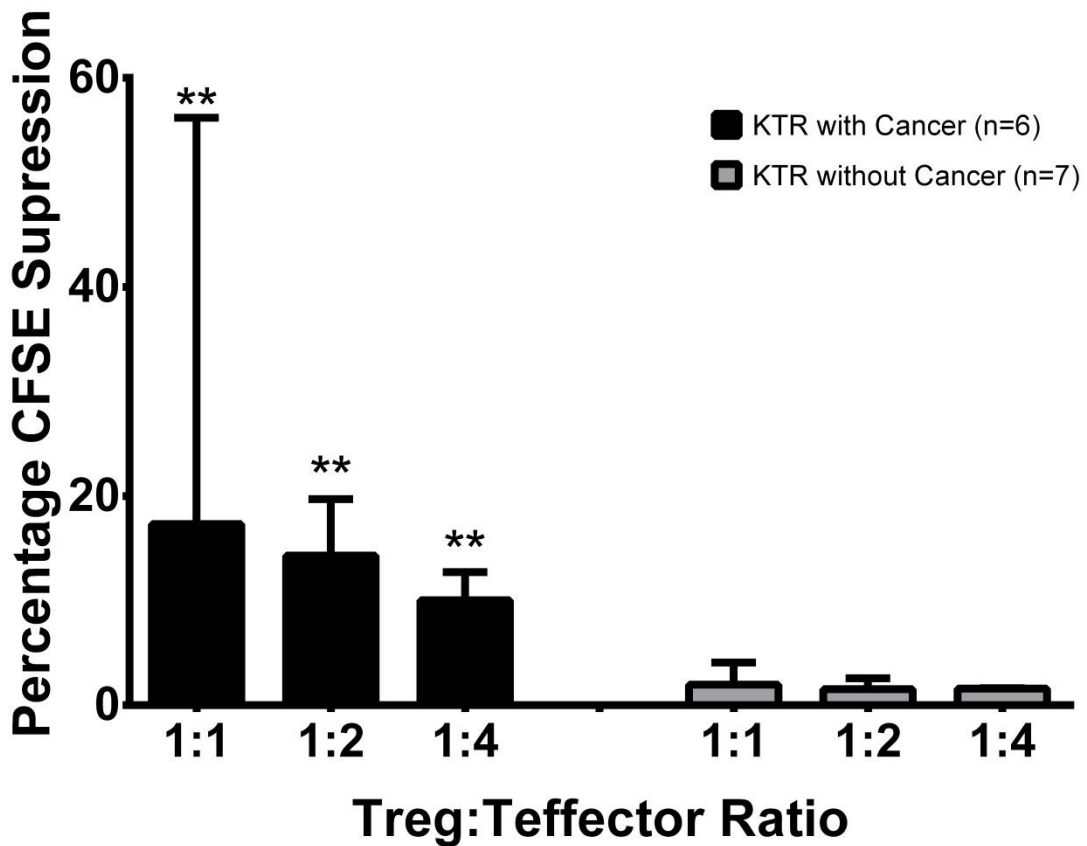
Figure 5.3.1.2: Isolated peripheral blood regulatory T cell (nTreg) suppression of CFSE dilution: A column graph depicting the median with inter-quartile range (IQR) of KTR with (black)/without (grey) cancer Tregs suppression of CD3/CD28 bead stimulated CFSE dilution at ratios 1:1, 1:2 and 1:4. With median% (Range) for; 1:1; 18 (4-83) Vs. 2 (2-12) $p<0.01$, 1:2; 14 (8-29) Vs. 2 (1-7) $p<0.01$ and 1:4; 9 (3-15) Vs. 2 (1-7), $p<0.01$, respectively using a Kraskal-Wallis non-parameteric test.

When comparing KTR with cancer to those KTR with no cancer there was an increase in nTreg suppression abilty over all titrations, $p<0.05$, using a Kraskal-Wallis non-parameteric test as seen in Figure 5.3.1.2. It can be seen that this data may have been conflicitng the titration data in Figure 5.3.1.1.

5.3.2 CD154 suppression of expression of natural Treg (nTreg) function in Kidney Transplant Recipients (KTR)

To validate the CD154 suppression of expression assay in KTR, 16 KTR and 3 healthy donor nTregs where run as per protocol in Chapter 2: Section 2.4. The titration of Treg suppression is depicted in Figure 5.3.2.
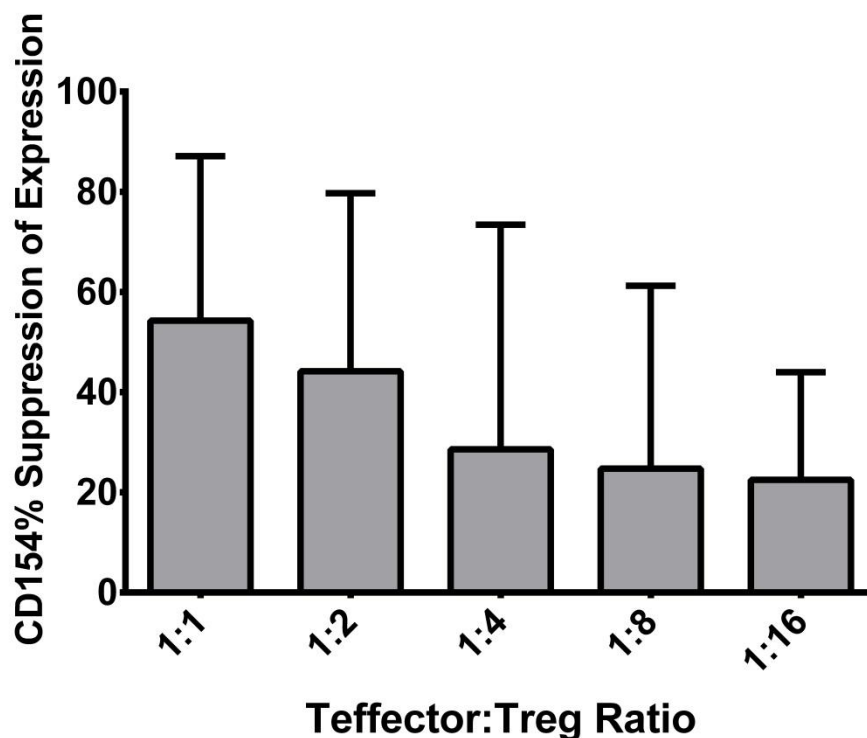
Figure 5.3.2.1: Titrated peripheral blood regulatory T cell (Treg) suppression of CD154 expression on naïve CD4 T cells: A bar graph of the titrated CD154 suppression of expression response from isolated Treg samples in 16 KTR. Samples were run in triplicate. The graph shows the median of the triplicate mean values, with range.

Titrations 1:1, 1:2 and 1:4 give the greatest differences in Treg function and were used to determine differences in Treg function from KTR with cancer and those KTR with no cancer. The patients used in the following analysis were from Chapter 3: Section 3.5 and were matched as shown in Table 3.5, with no statistically differences between measured variables, including; age, gender, and dose, type and duration of immunosuppression. The cumulative results were then grouped into KTR with cancer and those without cancer and are shown in Figure 5.3.2.1.
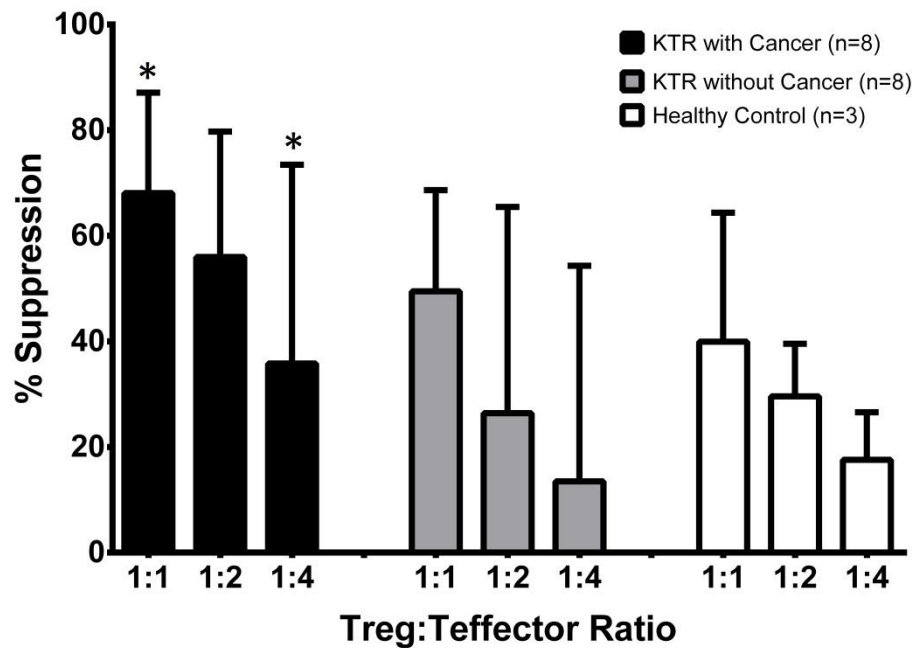
Figure 5.3.2.2: Isolated peripheral blood regulatory T cell (Treg) suppression of CD154 expression on naïve CD T cells: A column graph showing the accumulative median and range of Treg suppression, as measure of CD154 suppression of expression, from KTR with cancer (n=8), without cancer (n=8) and healthy control Tregs (n=3) at titrating Treg:Teff ratios, 1:1, 1:2 and 1:4. With median% (Range) for; 1:1; 68 (46-87) Vs. 49 (36-69) p= 0.038, 1:2; 56 (16-80) Vs. 26 (16-65) p= 0.083 and 1:4; 36 (13-73) Vs. 13 (5-54), p= 0.015, respectively, using a Mann-Whitney non-parameteric tests for each ratio.

Isolated Tregs from 8 KTR with cancer showed greater suppression of CD154 expression on healthy naïve CD4 T cells than 8 KTR with no cancer (median%(range); 1:1, 68 (46-87) Vs. 49 (36-69) p= 0.038, 1:2 56 (16-80) Vs. 26 (17-65) p= 0.083, 1:4 36 (13-73) Vs. 13 (5-54) p= 0.015, respectively, using a Mann-Whitney non-parameteric test for each ratio). Additionally, KTR with cancer had an statistically non-significant increased function compared with healthy controls: 1:1 median%(range) 68 (46-87) Vs. 40 (33-64) p= 0.117, 1:2 56 (16-80) Vs. 30 (14-40) p= 0.085, 1:4 36 (13-73) Vs. 18 (16-27) p= 0.085, respectively, using a Mann-Whitney non-parameteric test for each ratio as shown in Figure 5.3.2.2. Additionally, there was no difference between KTR with no cancer and healthy controls, with p-values =0.568, 0.667 and 0.497 for each ratio resepectively.

5.3.3 Correlations of CD154 to CFSE suppression assays

The titrations of the functional assay were tested, comparing 1:1, 1:2 and 1:4 in a correlation matrix. The CD154 assay had a mean correlation co-efficient of 0.95 compared to CFSE which was 0.78. When comparing assays the correlation co-efficient was 0.58, the highest correlation being the ratio of 1:4 with a coefficient of 0.84.

5.3.3.1 Correlations of Treg suppression assays to Age and duration of immunosuppression

Due to sampling of the 50 KTR cohort, there were differences in age, gender and duration of immunosuppression between KTR with cancer and KTR with no cancer (Table 5.3.1). To investigate if these paratemeters are affecting Treg suppression, correlation analyses were performed. Age did not relate to Treg function (r^2= 0.197, p= 0.086 for the CFSE assay and r^2= 0.046, p= 0.425 for the CD154 assay). Duration of immunosuppression did not affect Treg suppression in the CFSE assay (r^2= 0.246, p= 0.062) but did for the CD154 assay (r^2= 0.458, p= 0.007). All values determined by spearman non-parameteric tests.

5.3.4 Cancer and LDH release in KTR

The optimised technique was run on batched material for LDH release assay as described in Chapter 2 performed at a 20:1 PBMC: K562 ratio. A total of 32 KTR were analysed, 15 KTR with no cancer, 7 KTR with history of cancer and 10 KTR with current cancer, as seen in Figure 5.3.4.

Table 5.3.4 KTR run in the LDH release assay

	Current	Past	No cancer	p-value
Number, *N*	10	7	15	-
Age in years (Range)	62 (51-74)	60 (54-70)	59 (45-74)	0.613
Male gender, *N* (%)	8 (80%)	5 (71%)	8 (53)	0.863
Years of Immunosuppression (Range)	13 (0-33)	10 (4-18)	7 (0-23)	0.126
Tregs/µl (Range)	16 (5-34)	17 (6-27)	6 (0-67)	0.317
memory B cells/µl (Range)	12 (1-42)	7 (0-64)	17 (4-36)	0.510
CD8+ γδ T cells/µl (Range)	**88 (5-415)**	**157 (36-293)**	**28 (0-120)**	**0.011**
NK cells/µL (Range)	186 (1-1009)	161 (108-648)	83 (15-227)	0.465
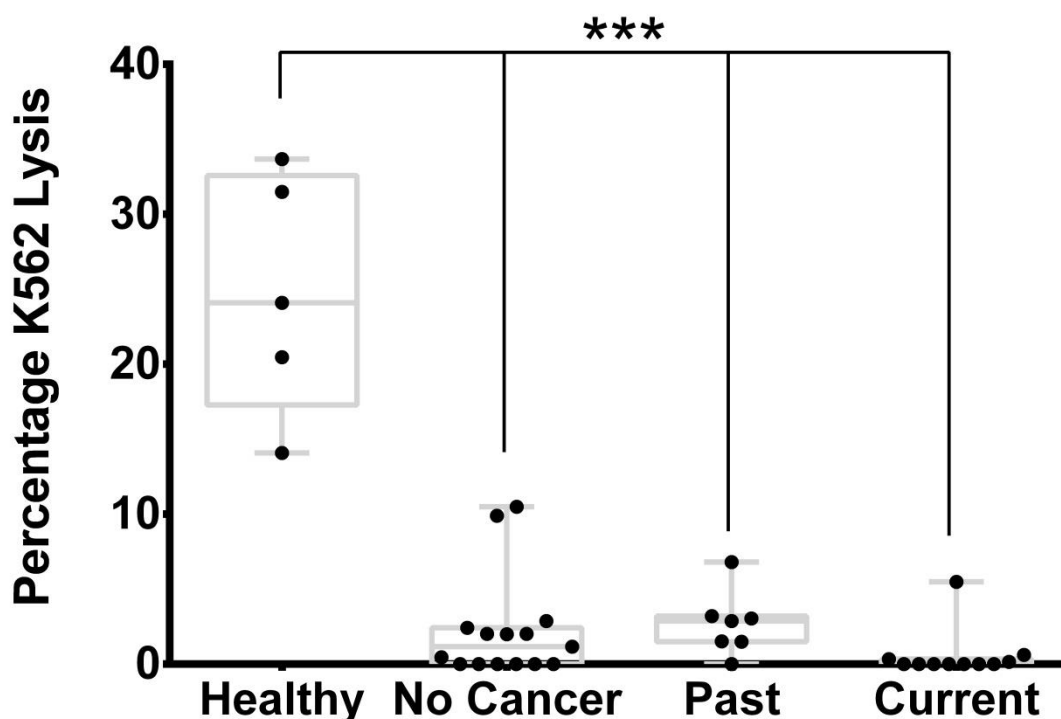
Figure 5.3.4.1: Lactate Dehydrogenase (LDH) release from Kidney Transplant Recipients (KTR) and Healthy Controls: A box and whisker plot depicting Lactate Dehydrogenase (LDH) release from healthy controls (n=5), KTR with no cancer (n=15), KTR who had cancer in the past (n=7) and KTR with current cancer (n=10). Both KTR with current cancer and those with no cancer have significantly lower LDH release than healthy controls using Kruskal-Wallis test (p<0.001).

KTR with no cancer and KTR with current cancer had statistically less LDH release than healthy controls (p<0.001), whereas those KTR with past cancer did not, as seen in Figure 5.8.2.1. When comparing KTR to healthy controls, KTR have decreased ability to lyse K562.
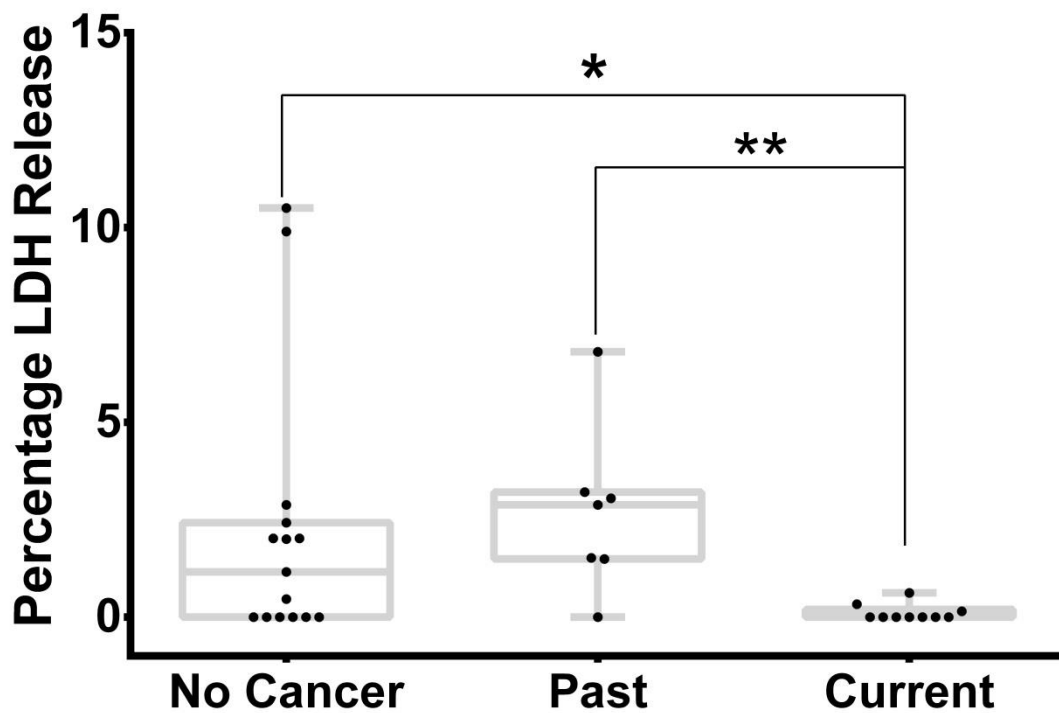
Figure 5.3.4.2: Lactate Dehydrogenase (LDH) release in Kidney Transplant Recipients (KTR): A box and whisker plot depicting the accumulative data of Lactate Dehydrogenase (LDH) release from KTR with no cancer (n=15), KTR who had cancer in the past (n=7), and KTR with current cancer (n=10). KTR with current cancer have less LDH release than those KTR with a past cancer (p= 0.002) and no cancer (p= 0.037) using Mann-Whitney Tests. KTR with Past cancer had similar LDH release to those with no cancer (p= 0.209).

When comparing KTR groups, KTR with current cancer had lower LDH release than those KTR with no cancer, p= 0.037 and KTR with past cancer (p= 0.002). Additionally KTR with past cancer had similar LDH release to KTR with no cancer (p= 0.209).

5.3.4.3 Isolated CD56+ NK cell lysis ability

To identify if there are cells or mediators in the PBMC that are restricting NK cells' ability to lyse K562, the ability of isolated NK cells to lyse K562 target cells was measured in a limited number of patients. When using a ratio of 20:1 PBMC:K562, the ratios of NK:K562 were run at a median (range) of 0.3 (0.01-3.12). This was based on the known number of NK in the patient's PBMC. Therefore a titration of NK cells was run from 10:1 to 0.04. The results are shown in Figure 5.3.4.3.
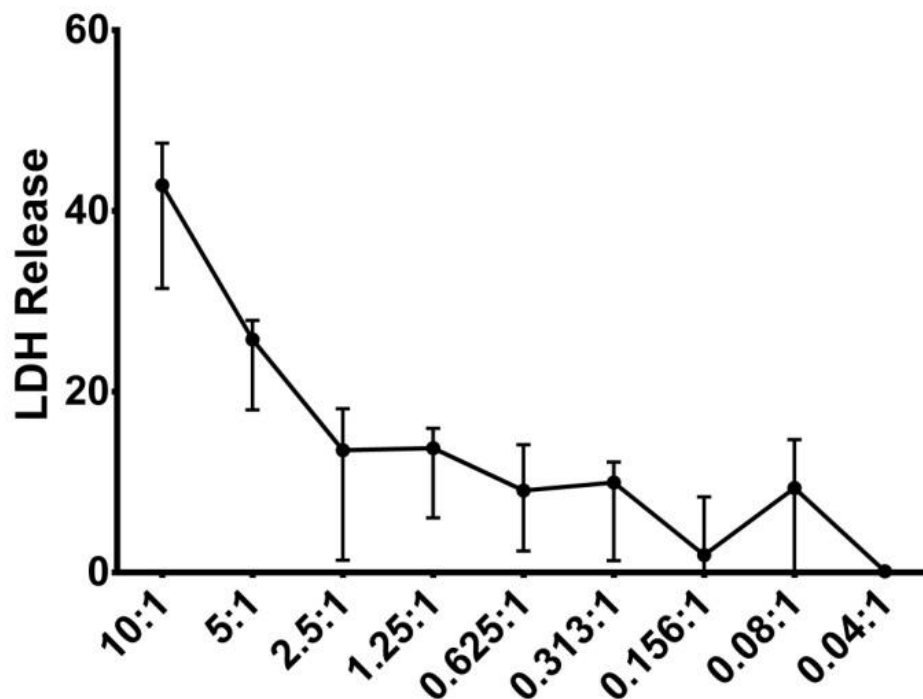
Figure 5.3.4.3.1: Lactate Dehydrogenase (LDH) release from Isolated Natural Killer cells from Kidney Transplant Recipients (KTR) with cancer: Isolated NK cells from 7 KTR were run at ratios 10:1 to 0.04:1 in 2-fold dilutions in LDH release assay.

The isolated NK cell ratios were then compared to results from PBMC run at 20:1 at the approximate NK:K562 ratios as defined by the known number of NK in the PBMC, Figure 5.9.1. The median (Range) of NK:Target ratio in the PBMC used in the assay was 0.9 (0.21-1.95), similar to that of the previous experiments (Figure 5.3.4.1 and 5.3.4.2).
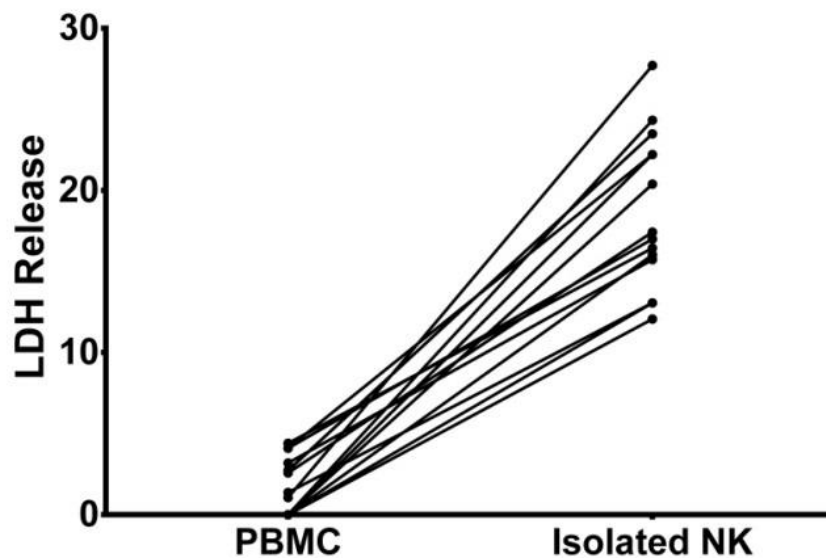
Figure 5.3.4.3.2: Lactate Dehydrogenase (LDH) release from Peripheral Blood Mononuclear Cells (PBMC) and Isolated Natural Killer cells from Kidney Transplant Recipients (KTR) with cancer: 5 KTR PBMC and Isolated NK cells run in triplicate at similar NK:Target ratios. This comparison shows that NK cells were being restricted by cells within the PBMC, $p<0.001$ using a non-parametric paired Wilcoxon test. Additionally, testing the means of the triplicates was significantly different ($p=0.008$) using a non-parametric Mann-Whitney test.

In summary, KTR PBMC have decreased ability to lyse K562 compared to Healthy Controls and this is exacerbated in KTR with cancer. When NK cells are isolated from the PBMC of KTR with cancer they follow a standard titration pattern. When comparing isolated NK cytolysis at a similar ratio as present in PBMC, there is an increase in LDH release and therefore K562 lysis (Figure 5.3.4.3.2).

5.4 Discussion

Both the Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution suppression assay and the CD154/CD40L suppression of expression assay show that Tregs isolated from KTR with cancer had greater function on a cell-to-cell basis than Tregs from KTR with no cancer. CFSE results correlated to CD154/CD40L suppression of expression in KTR with a spearman correlation coefficient of 0.84 at a Treg to effector T cell ratio of 1:4. This enabled use of CD154/CD40L suppression of expression assay in KTR, which is a more clinically applicable assay due to its shorter time frame. There were greater disparities between Tregs from KTR with cancer than KTR with no cancer in the CFSE assay (5 days) when compared to the CD154/CD40L assay (8 hours). The KTR with cancer assessed in these Treg suppression assays were also older, and had greater duration of immunosuppression but we know these two factors are significant in the development of cancer. When assessing the effect of age or duration of immunosuppression however there was no correlation suggesting the independent factor is cancer rather than age or duration of immunosuppression.

The Treg suppression of CD154 expression may have clinical implications, as CD154 expression on CD4 helper cells is required for: maturation of APCs[253], effector cell activation[254, 255], maturation [256], differentiation [256, 257], and expansion [254]. It has been found that epidermal Langerhans cells' allo-stimulatory properties are increased with CD154/CD40L activation[258]. CD154 suppression on CD4 T helper cells plays a role in tolerance induction[257, 259, 260]. Inversely CD154 induction on CD4 T helpers increases CD8+ Cytotoxic T Lymphocyte (CTL) anti-tumour abilities[261], primarily in response to CD154/CD40L upregulated co-stimulatory molecules[261] e.g. Interferon-gamma (IFN-γ[254]) and Interleukin-12 (IL-12[261]). Furthermore, sustained expression of CD154 and recall expression of CD154 is dependent on autocrine IL-2[260, 262] and IL-15[262]. This indicates a reduction, or removal, of Tregs will increase immunological responses, which has been shown in CD8 CTL responses during influenza infection[263].

KTR with cancer have increased proportions and numbers of CD4$^+$ Tregs that are capable of suppressing PBMC proliferation and CD154 expression on naïve CD4 T cells compared to KTR with no cancer. This reduction of CD154 may reduce allogenic and tumorgenic immunity by reducing IFN-γ, IL-12, IL-2 and IL-15 release. Reductions in allogenic immunity by measure of IFN-γ will be investigated in Chapter 6.

As NK cells were decreased in KTR from the UK, we focussed on NK function and phenotype in this current study. NK cell activity was measured in KTR with cancer by the Lactate Dehydrogenase (LDH) release assay outlined in Chapter 2: Section 2.5. KTR PBMC had decreased ability to lyse K562 compared to healthy controls and this was exacerbated in KTR with cancer. This is despite there being an almost 2-fold amount of NK cells added to the assay (NK cells/µl (range) 186 (1-1009) Vs. 83 (15-227), KTR with cancer Vs. KTR with no cancer, respectively). Although there was no significant difference between Treg, memory B cell and NK cells, there were more CD8$^+$ γδ T cells in the PBMC from KTR with current cancer than KTR with no cancer (p=0.011, Table 5.3.4). Cytolysis of cancer cells by γδ T cells has been shown previously[264]. Although these cells were not identified as CD8$^+$ γδ T cells and they were not targeting K562, there is potential for γδ T cells to have anti-tumour properties. There was an increase in CD8$^+$ γδ T cells in the PBMC of both KTR with past and current cancer when compared to KTR with no cancer, however there only an observed increase in cytolytic function between KTR with past cancer compared to KTR with current cancer, indicating that KTR with cancer may in fact have defective NK and γδ T cells. This is further exemplified as KTR with past cancer had similar NK and Treg numbers.

The function of NK cells was restored when isolated from PBMC and run at similar NK:K562 ratios to that of the PBMC. Previous data from Chapter 4: Section 4.3.2 shows one of the major differences in the PBMC from differing KTR groups is the number and proportion of Tregs. It is known Treg can affect NK function[154, 155] and therefore Tregs may play a role in

NK dysfunction in KTR with cancer. This hypothesis is explored in the future directions section of this thesis in Chapter 7: Section 7.2.

Immunosuppression regimen of KTR alters the NK cell repertoire and function. KTR on CNI had reduced CD16 expression and degranulation and NK activation and KTR on mTORi had reduced IFN-γ release in NK/K562 co-cultures[265]. These phenotypic and functional differences make NK cells from KTR physiologically different, and maybe more dysfunctional than healthy controls. A caveat when using PBMC as effector cells is the difference in NK cell numbers, KTR have been shown to have less NK cells[266], and could be contributing to the difference in K562 lysis that is observed. A studying measuring K562 lysis with ^{52}Cr showed higher levels of cytolysis, median (range) of 6% (2-18%) in KTR with CMV infection compared to our value of 0.5% (0-11%), however they used a PBMC:K562 ratio of 100:1 compared to 20:1, the KTR did not have cancer and were in the early transplant period[267].

Another result is that Target Induced NK cell Loss (TINKL) cannot be accurately determined on frozen material. These observations indicate that thawed NK cells from KTR, but not healthy controls, can kill multiple K562 cells thus increasing LDH release but decrease the NK cell loss. It is known that IL-2 increases NK cell function and that immunosuppression specifically targets IL-2[243 244].

In conclusion, KTR with cancer have increased Treg function and decreased NK cell function. Additionally, KTR Treg function can be measured by suppression of CFSE dilution and also a novel, faster assay (8 hours) by measuring the suppression of the activation marker CD154, as these assay correlate. The TINKL assay cannot be used post cryopreservation, however the LDH assay can. LDH release is decreased in KTR PBMC, however once NK cells are removed from PBMC, NK cell function is restored to that of PBMC from normal controls.

Chapter 6: Measurement of Alloresponses

6.1 Introduction

The previous sections of this thesis showed that Kidney Transplant Recipients (KTR) with cancer have higher levels of more suppressive Regulatory T cells (Treg, Section 4.4-4.7, 5.3 and 5.5) and similar levels of low functioning Natural Killer (NK) cells (Section 4.4-4.7, 5.7 and 5.8) than KTR with no cancer. Furthermore, Tregs themselves can predict cancer in KTR with a history of cancer and decrease upon cancer resection. These assays give clinicians the ability to objectively identify patients that may develop pre-metastatic cancer with relatively high specificity and specificity (78% and 80%, respectively). However they do not inform clinicians if KTR will benefit from cancer prevention therapy.

A randomised control trial randomised pre-transplant KTR to a standard level Calcineurin inhibitor (CNI) regimen and a CNI sparing regimen[72], thus investigating the benefit of reduced immunosuppression as primary cancer prevention. However, those with reduced CNI had increases in rejection episodes[72]. Other studies investigated converting CNI based regimens to mammalian Target of Rapamycin inhibitor (mTORi) based regimens as secondary prevention therapy, as mTORi are used as anti-cancer therapies[174, 175]. There was a benefit, however not all conversions were successful (30%) and an additional 30% did not tolerate the mTORi side effects[14, 198, 268]. Furthermore, immune phenotype has revealed that those who maintain high levels of Tregs after mTORi conversion (>20 Tregs/µL) do not benefit from conversion and may benefit from immunosuppressive drug reduction. To perform immunosuppressive drug reduction as secondary cancer prevention, risk of graft rejection will need to be measurable.

Pre-transplant anti-Human Leukocyte Antigen (HLA) and Interferon-γ ELISPOT associate post-transplant with antibody and cellular mediated rejection episodes[183, 185, 190]. Monitoring HLA molecules and specific Donor Specific Antibodies (DSA) routinely has decreased

antibody mediated rejection episodes dramatically[188, 189]. Interferon-γ ELISPOT has been used to predict 6-month graft function and rejection episodes[185]. Additionally it has been used pre-transplant to categorise patients into CNI or mTORi maintenance therapy[269]. These studies are limited in clinical application as donor specific cells were used to stimulate the mixed lymphocyte reactions, requiring use of precious or non-existent deceased donor material. This restricts the utility of ELISPOT to live recipient/donor pairs. An IFN-γ ELISPOT assay has been developed that utilises a variety of unrelated HLA disparate material to measure total allo-response and is termed "Panel of Reactive T cells (PRT)"[186]. This assay has been shown to have potential to determine post-transplant risk of rejection when measured pre-transplant. However there are no current studies utilising IFN-γ post-transplant as a form of rejection prediction in long-term KTR.

The IFN-γ ELISPOT may be extended to guide immunosuppression reductions[270, 271]. There are a few studies utilising a viral peptide stimulated IFN-γ ELISPOT to discriminate KTR who may benefit from reduced immunosuppressive drugs as a form of treatment[270, 271]. KTR with unresolved BK pathogenesis also had a non-significant decrease in EBV peptide and PHA mitogenic IFN-γ ELISPOT responses[270], indicating over-immunosuppression[272]. This may share a link with development of malignancy as they are both considered manifestations of over-immunosuppression.

This Chapter focuses on HLA antibody detection, IFN-γ release from allogenic stimulation, viral peptides and mitogen antigens.

6.1.1 Chapter Aims

1. To determine if there are differences in the presence and levels of anti-HLA and DSA in KTR with cancer compared to KTR without cancer.

2. To investigate the use of the Clinical Trials in Organ Transplant (CTOT) IFN-γ ELISPOT in Australian KTR to determine the IFN-γ release of Allo-stimulated cells.

3. To extend the CTOT IFN-γ ELSPOT to measure both viral peptide and mitogen stimulation to determine effector immune function of KTR

6.1.2 Chapter Hypotheses

With the premise that KTR with current cancer are over-immunosuppressed;

1. KTR with cancer will have no or reduced HLA or DSA antibodies within their peripheral blood.

2. KTR with current cancer will have reduced or no ability to produce IFN-γ under an allogeneic stimulus compared to those KTR with or without history of cancer.

3. KTR with current cancer will produce less IFN-γ under viral peptide and mitogen stimulation.

6.2 Chapter Methods

6.2.1 Panel of Reactive Antibodies (PRA) solid phase assay

Screening antibodies in KTR serum has been implemented as routine immune monitoring of organ transplant patients across Australia and various institutions across the world. An MFI cut-off of 1500 units was used, as this has been defined in literature and used in our diagnostic laboratory[188]. Both these assays were performed by the Australian Red Cross Blood Service (ARCBS, Adelaide, S.A.) and data was collated and reviewed by Dr. William Hanf.

6.2.2 Panel of Reactive T cells (PRT) stimulated Interferon-γ Enzyme Linked Immuno-SPOT (ELISPOT) assay.

The panel of reactive T cell (PRT) IFN-γ ELISPOT was implemented to measure total allo-responses from the cohort of KTR (n=50) outlined in Chapter 3: Section 3.5, with demographic data presented in Table 3.5. KTR PBMC were stimulated with B cells propagated from donor material stored by the Australian Red Cross Blood Service (ARCBS), Adelaide. The HLA types of the donated material are shown in Table 6.2.2.1.

Table 6.2.2.1 HLA types of donor B cells used in PRT panel

B cell sample	HLA - A	HLA - B	HLA - DR
1	2,2	8,44	1,15
2	2,31	18,62	4,11
3	1,2	8,44	3,7
4	3,11	7,35	3,4

The HLA molecules expressed by the B cells are similar to approximately 90% of the Caucasian population, with the most frequent HLA haplotypes of Caucasians being: A1, A2, A3, B8, B7, B44, DR3, DR2, and DR4.

The propagated B cells were analysed for HLA-CI and HLA-CII expression with expression shown in Table 6.2.2.2.

Table 6.2.2.2 Proportion of CD19$^+$ B cells and expression of MHC-CI and MHC-CII

B cell sample	CD19%	MHC-CI	MHC-CII
1	86.19	99.59	99.9
2	98.1	98.79	99.28
3	98.66	99.71	93.44
4	98.79	98.67	97.71

These B cells cover approximately 4 out of the 6 donor HLA molecules or 66% of all HLA molecules from the patient cohort outlined in Chapter 3 Section 5, depicted in Figure 6.2.2.1.
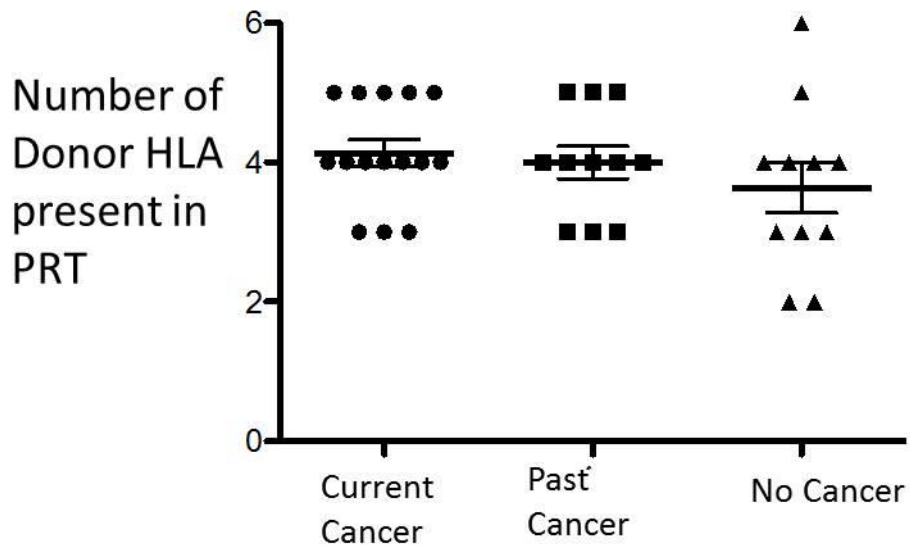
Figure 6.2.2.1: HLA of B cell lines that cover Donors Specific HLA of KTR with Current, Past and No cancer: A plot showing median and interquartile range of B cell HLA molecules covering donor specific HLA. All KTR have approximately 4 out of 6 HLA matches to the B cell lines used in the Panel of Reactive T cells (PRT).

In summary, B cells from healthy donors were expanded as per Chapter 2: Section 2.6.2. These B cells cover 90% of the Caucasian population and 66% of all donor HLA molecules in this study. The coverage is similar between groups with KTR with approximately 4 out of 6 HLA donor molecules being represented in the PRT. These B cells were used in all subsequent Panel of Reactive T cell (PRT) experiments.

6.2.2.3 Cross referencing the Enzyme Linked Immuno-SPOT (ELISPOT) assay

One of the ELISPOT techniques' limitations to wide-spread use is cross-laboratory validation, which now has been addressed[273]. I underwent a 3 month traineeship at Mount Sinai Medical School to learn the optimised, National Institute of Health (NIH) accredited, Clinical Trials in Organ Transplantation (CTOT) IFN-γ ELISPOT technique[273]. Once returned, investigation was undertaken into standardising and cross-validating two different aspects of reading and analysing ELISPOT results. There are two steps to test reproducibility: image capturing and

image processing. Imagine processing consists of the settings for the software for counting the spots within the picture and the errors between the different software.

6.2.2.3.1 Cross referencing laboratories

IFN-γ ELISPOT was performed according to the CTOT Standard Operating Procedure (S.O.P) outlined in Chapter 2: Section 2.6.3, using B cells propagated as per CTOT protocol outlined in Chapter 2: Section 2.6.2 and characterised in Section 6.2.2 of this chapter. The plates were captured and processed at the Centre of Clinical and Experimental Transplantation (CCET) in Adelaide (Adel) on an AID branded ELISPOT reader. The plates were then sent to the CTOT laboratory in the US where they were re-captured and re-analysed by the CTOT staff members on a CTL brand ELISPOT reader, depicted in Figure 6.2.2.3.1.1.
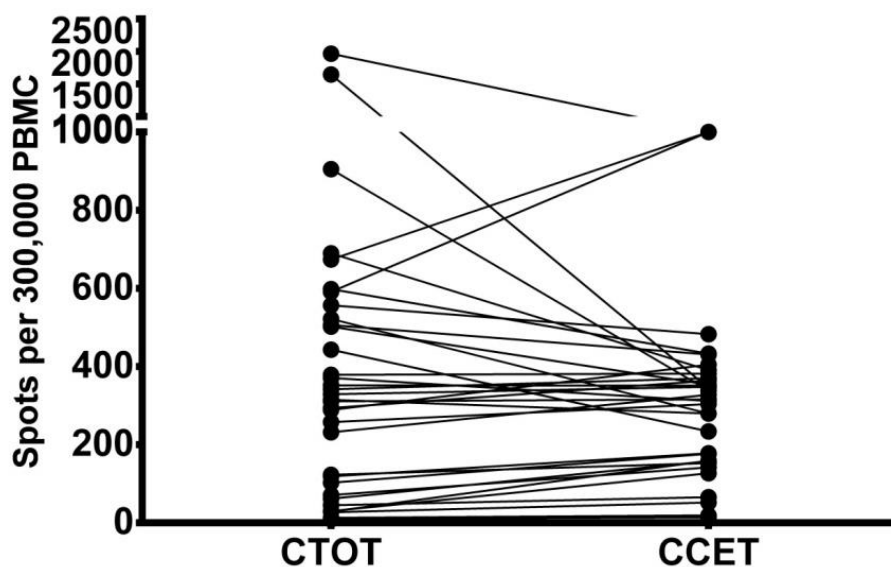
6.2.2.3.1.1 Cross referencing Enzyme Linked Immuno-SPOT (ELISPOT) across laboratories: A line graph depicting changes in spots from the Centre of Clinical and Experimental Transplantation (CCET) to the Clinical Trial in Organ Transplantation (CTOT) laboratory. Although there is no statistical change between paired (0.913) or median values (p=0.868), the range of the CTOT spans greater values than CCET (9 - 1967 compared to 14 - 1000, respectively).

Despite the absence of difference between the paired values and the median values from the different laboratories, the range was decreased when read on the AID branded machine at CCET. Instrument error between institutions was investigated in the next section. A CTL

reader (similar to that in the US) was accessed at Flinders Medical Centre (FMC), Adelaide, Australia, where extra plates were re-read and the results were compared to the AID reader at CCET. The results are depicted in Figure 6.2.2.3.1.2.
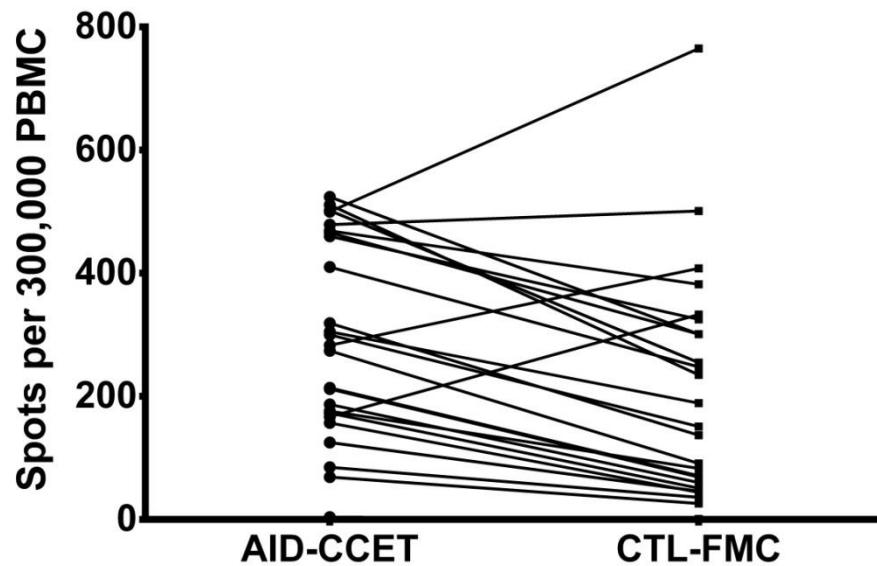
6.2.2.3.1.2 Cross referencing Enzyme Linked Immuno-SPOT (ELISPOT) across Instruments: A line graph depicting changes in spots from the Centre of Clinical and Experimental Transplantation (AID-CCET) to the Flinders medical centre (CTL-FMC). The ranges of the CTL-FMC = 1 - 765 in comparison to the AID-ADEL = 4 - 524.

6.2.2.3.2 Instrument error between institutions

A total of 138 wells were analysed; 33 background, 33 PHA wells and 72 test wells. The plates were read and analysed on a CTL machine in the CTOT laboratory in the US. The photos were saved in a format that the Adelaide based AID machine could read. The results from both readings would enable measurement of the error from the counting software. The physical plates were then re-photographed and re-counted on the AID machine. Total error of both the image capture and the software counts was calculated, with any additional error from the image capture. The mean of the total error was 26%, with the greatest error coming from the background and PHA wells. When analysing tests results alone, the error was 14%. However, the error from background wells alone was 40% and this would affect final results, as background wells are subtracted from test wells.

6.2.2.4 Correlation of results between institutions

Results from 32 KTR analysed on the AID machine in Adelaide, at the CCET were compared to the CTL machine in the CTOT laboratory in New York. The results are shown in Figure 6.2.2.4.1.
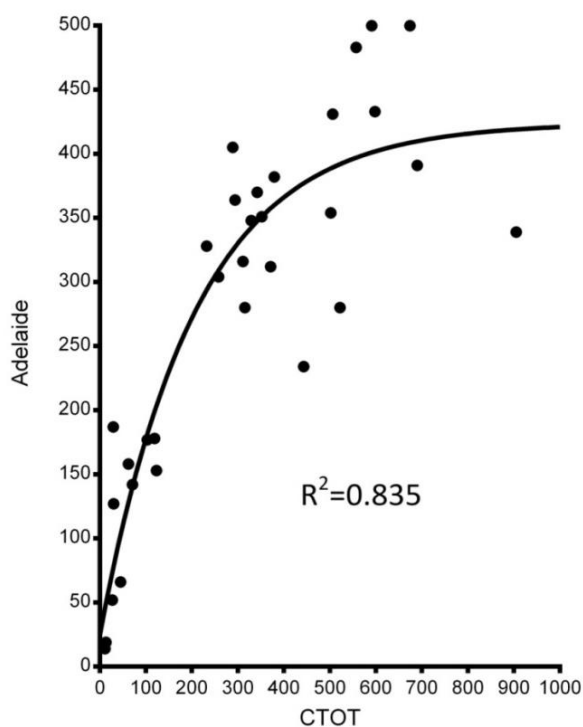
Figure 6.2.2.4.1 Correlation of results between Adelaide (AID-CCET) and New York (CTL-CTOT): A dot plot of 32 KTR PRT IFN-γ ELISPOT pairs run at the CCET laboratory on an AID branded machine Vs. the CTOT laboratory on a CTL branded machine. A non-linear correlation curve was used for correlation analysis resulting in a R^2 value = 0.835.
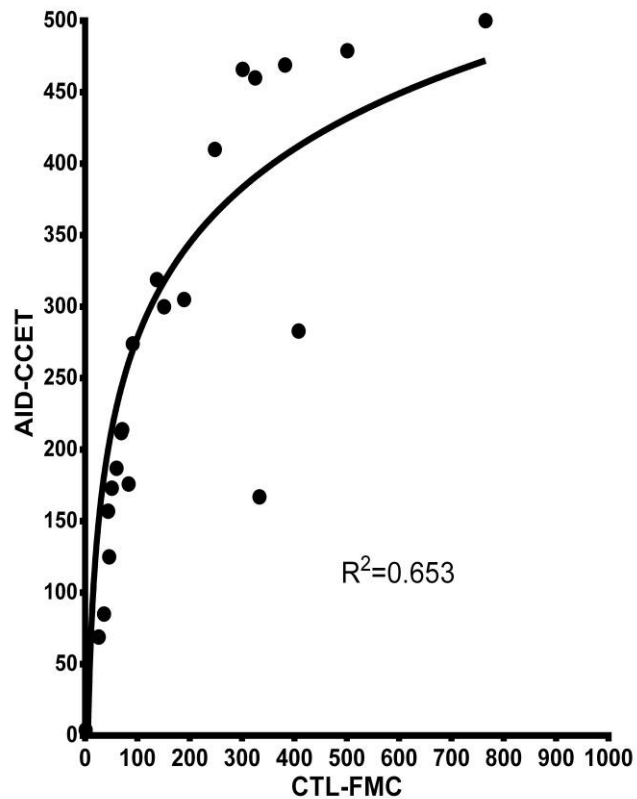
Figure 6.2.2.4.2 Correlation of results between Adelaide (AID-CCET) and Flinders Medical Centre (CTL-FMC): A dot plot of 25 KTR PRT IFN-γ ELISPOT pairs run at the CCET laboratory on an AID branded machine Vs. the CTOT laboratory on a CTL branded machine. A non-linear correlation curve was used for correlation analysis resulting in a R^2 value = 0.653.

In summary, when comparing AID to CTL results there is no direct statistical difference and there is a strong correlation in results, but CTL have greater reading range. The results in the following chapters have been read on CTL machines from CTOT and from Flinders Medical Centre, Adelaide, Australia. Unfortunately, the results from FMC cannot be compared to CTOT as they were different KTR pairs.

6.3 Results

The patients that were investigated with the above techniques outlined in Table 6.3.

Table 6.3 Patient Characteristics of KTR in subsequent studies.

	Cancer (C)	Current (Cu)	Past (P)	No Cancer (NC)	p-value	p-value
Numbers, *N*	29	17	12	18	CvNC	CuvPvNC
Age in year (Range)	61 (51-74)	62 (51-74)	60 (54-70)	58 (51-76)	0.176	0.342
Male Gender, *N* (%)	21 (72%)	12 (71%)	9 (75%)	11 (61%)	0.826	0.869
Duration in years (Range)	12 (0-33)	13 (0-33)	10 (4-18)	9 (0-29)	0.543	0.422
Tregs/µL	**24 (1-105)**	**28 (1-105)**	**19 (3-50)**	**10 (0-26)**	**0.043**	**0.039**
Memory B cells/µL	12 (1-64)	10 (1-42)	15 (1-64)	17 (1-79)	0.616	0.409
CD8$^+$ γδ T cells/µL	89 (0-868)	136 (0-868)	53 (6-195)	31 (0-120)	0.738	0.598
NK cells/µL	192 (1-1009)	136 (1-294)	277 (23-1009)	99 (15-227)	0.430	0.328

This cohort was used two different ways firstly in two groups KTR Cancer Vs. KTR with No cancer (CvNC) and KTR with current cancer Vs. KTR with past cancer Vs. KTR with No cancer (CuvPvNC). The Age, duration of immunosuppression and cell numbers were analysed with Mann-Whitney non-parametric tests for CvNC and Kruskal-Wallis tests were used for CuvPvNC. Gender was analysed with Chi-Square tests. As it can be seen there are no significant differences between groups, with the exception of Treg numbers.

6.3.1 Anti-Human Leukocyte Antigen (HLA) and Anti-Donor Specific Antibodies (DSA) in Kidney Transplant Recipients (KTR)

To determine overall humoral immune responses, measurement of anti-HLA and DSA were implemented in the cohort outlined in Chapter 3: Section 3.5 and characteristics shown in Table 3.5. The 50 KTR plasma samples were collected, labelled, stored and prepared by myself, however the performance and analysis of the Luminex assay was done by Dr. William Hanf at the Australian Red Cross Blood Service, Adelaide, South Australia, Australia.
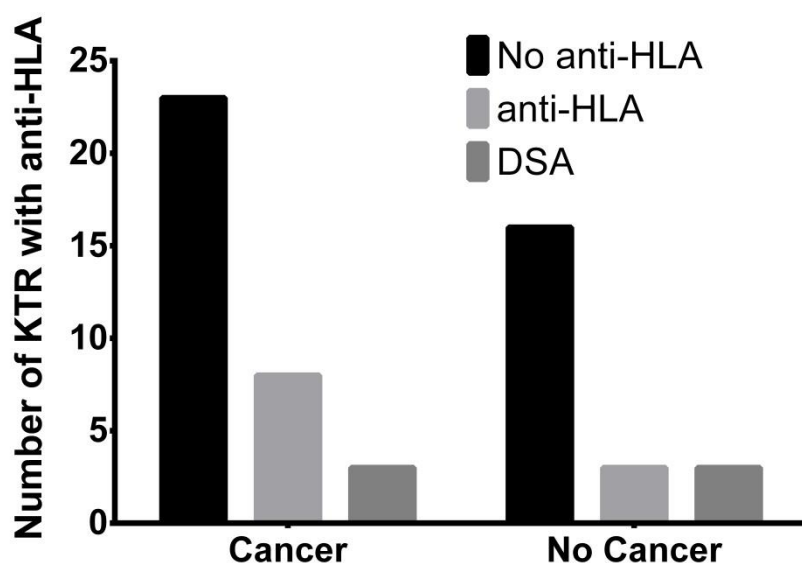
Figure 6.3.1: Human Leukocyte Antigen (HLA) antibodies and Donor Specific Antibody (DSA) levels in 50 Kidney Transplant Recipients with (n=31) and without cancer (n=19): A bar graph depiciting levels of HLA antibodies and DSA in peripheral blood plasma of KTR. There is no difference in HLA andtibodies (p=0.142) or DSA (p=0.661) in KTR with cancer compared to KTR with no cancer, using Fisher's exact tests.

KTR with cancer (n=31) had similar HLA antibody MFI levels (MFI >1500) to those KTR with no cancer (n=19, 8 (26%) Vs. 3 (16%), respectively, p=0.500). Additionally, KTR with cancer had the similar amount of DSA antibodies and a non-statistically different amount of anti-HLA and non-DSA, shown in Figure 6.2.

6.3.2 Panel of Reactive T-cells (PRT) Interferon-gamma (IFN- γ) response in Kidney Transplant Recipients (KTR) as measured by Enzyme Linked ImmunoSPOT (ELISPOT).

This section will address one of the major aims of this thesis, which is to measure cellular allo-responses of KTR with cancer and compare them to KTR with no cancer. This assay was set up as mentioned in Chapter 2. A total of 53 KTR and 7 Healthy controls were run in the assay. There was 1 KTR who was removed from analysis as they were currently in a rejection episode. Additionally, 2 Healthy controls were dropped due to low or no response in positive control wells. Furthermore, 6 KTR with infections were not used for subsequent analyses.
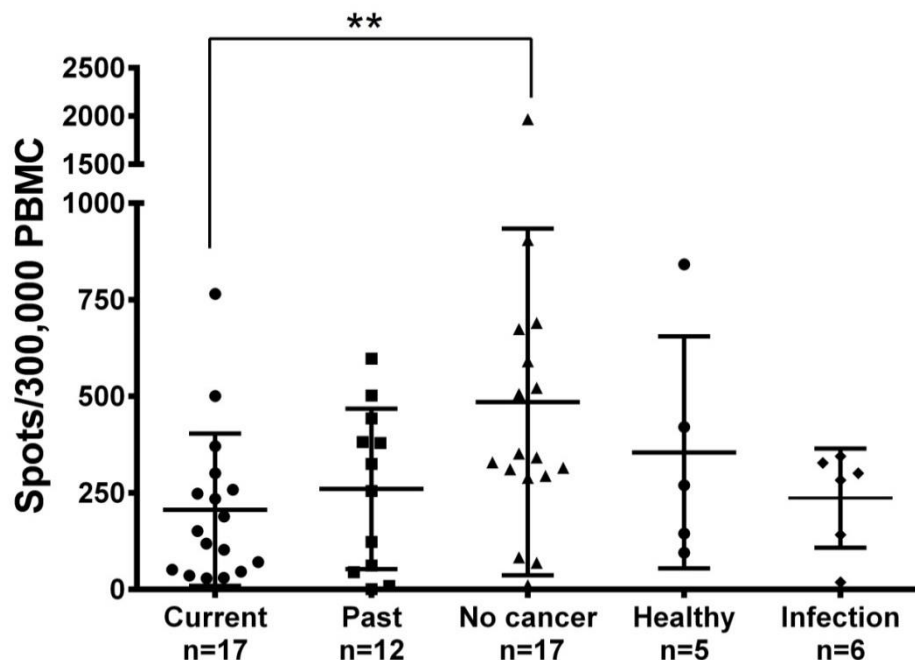
Figure 6.3.2.1 Panel of Reactive T cells (PRT) Interferon-gamma (IFN-γ) release in Kidney Transplant Recipients (KTR) as measured by Enzyme Linked ImmunoSPOT (ELISPOT): IFN-γ was measured in 52 KTR and 5 Healthy donors. KTR with cancer (n=17) had lower (p=0.008) and KTR with past cancer (n=12) had similar (p=0.138) IFN-γ responses than KTR with no cancer (n=17), using Mann-Whitney tests.

KTR with cancer had less allo-response than those KTR with no cancer with a median spots per 300,000 PBMC (range) of 151 (29-765) vs. 342 (11-1967), p=0.008 and a median (range) of 290 (1-598) for KTR with past cancer.
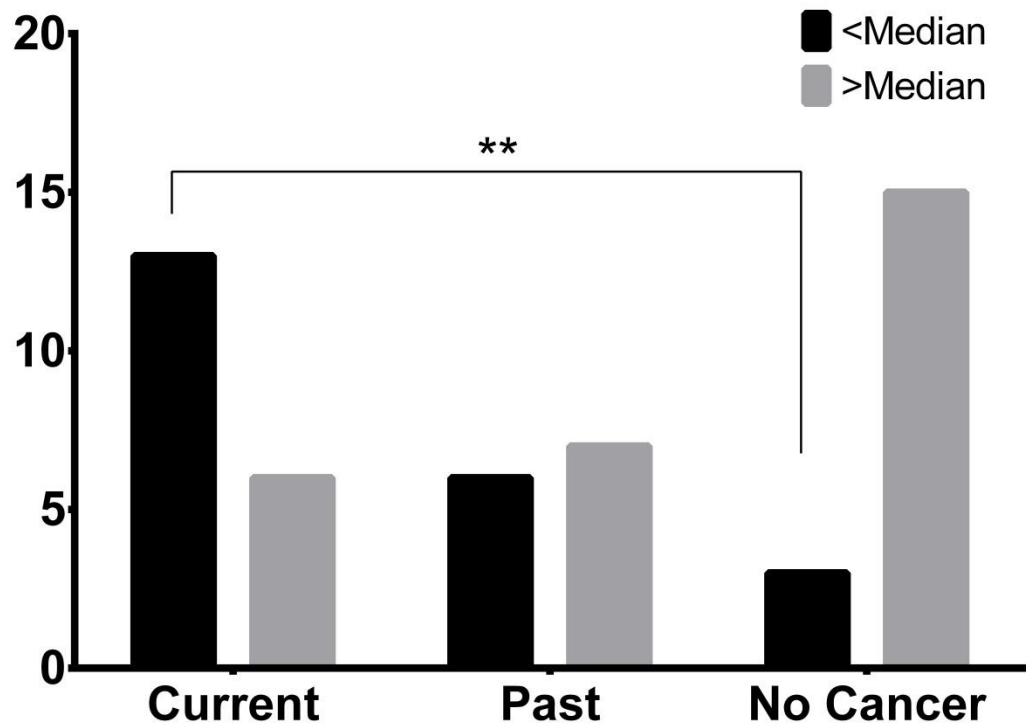
Figure 6.3.2.2 Dichotomised Panel of Reactive T cells (PRT) Interferon-gamma (IFN-γ) Enzyme Linked ImmunoSPOT (ELISPOT) data by total median value of 292 spots per 300,000 PBMC: A bar graph showing the PRT IFN-γ ELISPOT of 50 KTR dichotomised using a median value cut-off of 292 spots per 300,000 PBMC. There are a greater proportion of KTR with current cancer (n=17) that have <292 spots than KTR with no cancer (n=17), p=0.007, using a Chi-squared test. There is no difference between past (n=12) and no cancer or past and current cancer (p=0.203 and p=0.452, respectively), using Chi-squared tests.

Using a median cut-off of 292 spots per 300,000, the majority of KTR with cancer, half of those KTR with past cancer, and minority of those KTR with no cancer, are below. These patients who are below this threshold are potential candidates to take part in a dose reduction trial as described later in Chapter 7: Section 7.2.

6.3.3 Viral and mitogen stimulated Interferon-γ release

This section covers the third and final aim of this chapter. Viral peptide and mitogen stimulation were measured by IFN-γ release, as a secondary measure of immune function and capacity to clear viral infections.
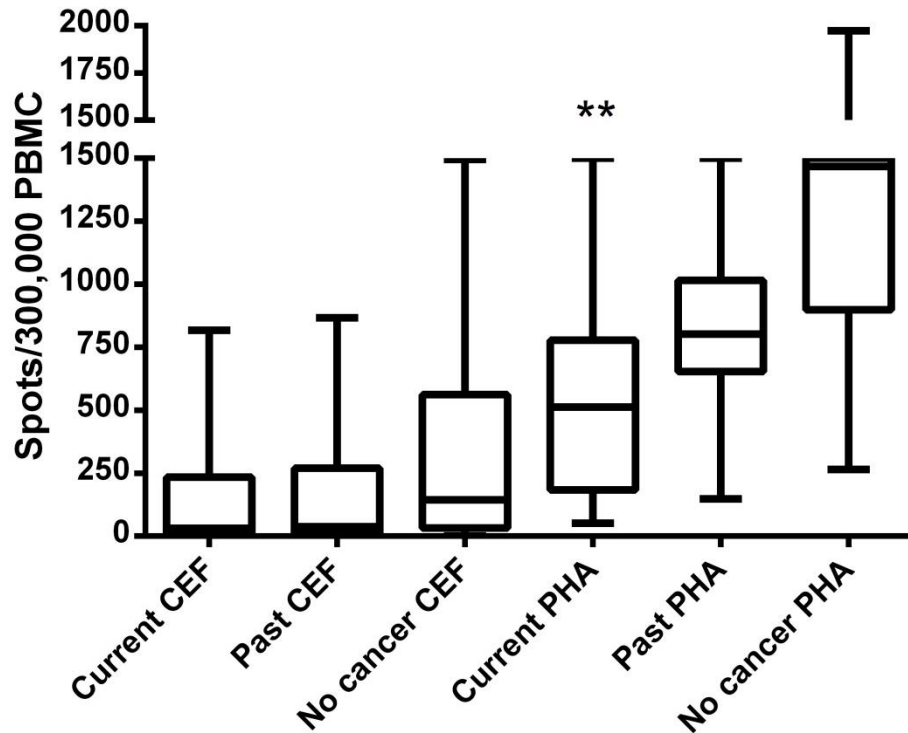
Figure 6.3.3 Viral and mitogen stimulated Interferon-gamma (IFN-γ) release as measured by Enzyme Linked ImmunoSPOT (ELISPOT): A boxplot depicting Interferon-γ ELIPSOT responses to alloantigen, to viral antigens (CEF CMV, EBV and Flu) and to maximal stimulation with PHA in 46 KTR. Those KTR with cancer (n=17) have less mitogen response when compared to KTR with no cancer (n=17), Kruskal-Wallis p-value = 0.019.

KTR with cancer have differential IFN-γ release to alloantigen, CEF (CMV, EBV and FLU) viral peptides and to maximal stimulation mitogen (PHA) when compared to KTR with no cancer. The CEF responses are not significantly lower, p=0.240. However, Mitogen responses are lower in KTR with cancer in comparison to those KTR without cancer, p=0.003 using a Mann-Whitney non-parametric Test.

6.4 Discussion

KTR with cancer had similar levels of anti-Human Leukocyte Antigen (HLA), Donor Specific Antibodies (DSA) and lower viral, mitogen and allo-stimulated interferon-gamma (IFN-γ) release than KTR with no cancer.

Anti-HLA and DSA are humoral responses directed towards the kidney graft, which spearhead antibody mediated rejection episodes[188]. There were 3/31 (9.7%) KTR with cancer and DSA (>1500 MFI) and 8/31 (26%) with anti-HLA antibodies (>1500 MFI) KTR with cancer that were at potential risk of antibody mediated rejection[188], especially if reduction of immunosuppression was considered a treatment option for the cancer.

Measurement of IFN-γ via ELISPOT was performed in these patients. A panel of B cells that covered the most frequent Caucasian HLA molecules as well as the majority (66%) of HLA haplotypes of the donors to the KTR was utilised. The PRT IFN-γ release data was captured and processed on different machines. The CTOT laboratory, in the US, has an optimised protocol for the capturing and processing for the PRT ELISPOT. The ELISPOT results showed that there was a strong correlation and no statistical difference between centres, but there was a discrepancy in the number of spots the machine is capable of quantifying. A CTL machine at Flinders Medical Centre (FMC) was accessed and the results also showed a strong correlation and increases in range when compared to the AID branded machine. This indicates that results between machines are comparable but if high level readings are needed then a CTL machine is required.

When comparing KTR from Table 6.3 and Table 3.5 (In Chapter 3: Section 3.5) it can be seen that these patients are matched in age, gender and type, dose and duration of immunosuppression. There were no observable differences in memory B cells, CD8$^+$ γδ T cells in this sub-cohort of patients. However, NK cells were similar and Tregs were increased

in KTR with cancer and KTR with current cancer when compared to KTR with no cancer (p=0.043 and p=0.039, respectively). There was no association of Treg numbers or proportions to IFN-γ release (p=0.208 and p=0.103, respectively). It may be low patient numbers that make the analyses too under powered to define whether Treg numbers associate with PRT.

KTR with infection and current cancer have lower PRT levels than KTR with past and no cancer. This is further exemplified when using a median cut off of 291 spots per 300,000 PBMC. A majority of KTR with cancer have less than this median compared to equal numbers of KTR with past cancer and a minority of KTR with no cancer.

Due to the relatively small number of KTR investigated, the effect of drug dose on PRT in those with current and past cancer was unable to be defined. Longitudinal data is needed to determine whether changes in immunosuppression affect PRT in this population of long-term KTR. One hypothesis is that the reason those with past cancer have higher PRT is that their immunosuppression has been more aggressively reduced compared to current cancer. Follow up of KTR from first malignancy and immunosuppressive drug dose reduction, may show an increase in PRT.

Further investigation into viral and mitogen stimulated PBMC from KTR with cancer revealed similar levels of viral peptide stimulation but decreased mitogen stimulation, exemplifying a restricted immune system. BK viral peptide stimulated IFN-γ ELISPOT can guide immunosuppression changes during BK infection and donor-specific ELISPOT has been used to determine immune tapering and mTORi conversions in the first 12 months pre-transplantation. However, the use of IFN-γ ELISPOT to taper long-term KTR has yet to be performed.

Chapter 7: Concluding remarks and future directions

7.1 Key findings

7.1.1 Patient Cohort Characteristics

The KTR characteristics were similar to previous published KTR cohorts. The main differences were age, duration and type of immunosuppression. There was also a higher burden of SCC skin than previous studies and this is the first report of immune phenotype in KTR with SOC.

7.1.2 Immune Phenotype

Unlike clinical variables, high numbers of peripheral blood Regulatory T cells (Tregs) can predict onset of a poor prognosis of cancer, within 100 days of immune phenotyping. Tregs have a dynamic association with cancer: i.e. increase upon cancer diagnosis, they are increased in KTR with cancer, and decrease after cancer resection.

7.1.3 Immune Cell function

KTR with cancer had increased Treg suppressive capacity on a cell-to-cell basis compared to KTR with no cancer. Suppression of CD154/CD40L expression provides a surrogate marker of Treg suppression of proliferation in an immunosuppressed cohort.

KTR with cancer had decreased total cell lysis compared to KTR with no cancer. Additionally, removal of NK cells from PBMC reconstituted NK cell function.

7.1.4 Allo-responses

KTR with cancer had lower allo-response in the Panel of Reactive T cell (PRT) Interferon-gamma (IFN-γ) release Enzyme-Linked ImmunoSPOT (ELIPSOT) assay when compared to KTR with no cancer. KTR with cancer had less mitogen stimulated IFN-γ release than KTR with no cancer.

7.2 Future directions

There were 33 KTR recruited with infection whose immune phenotype was not reported in this thesis. This was mainly due to the heterogeneity of the active infection which therefore did not provide any specific, beneficial information. As infection can be considered as over-immunosuppression, it shares commonality to cancer. However, infection often occurs in the early transplant period, whereas cancer is occurs in long-term KTR. An investigation into the level of Tregs in KTR with infection may identify a sub-group that, since infection, have increased risks of cancer. Chapter 6: Section 6.3.2 shows that, albeit only 6 (18%) of these, KTR with infection that were run in a PRT IFN-γ ELISPOT had similar levels to that of KTR with cancer. This provides some evidence that KTR with infection may also benefit from reduction in immunosuppression, or at least investigation of benefit from immunosuppression. A larger prospective study of immune phenotype may reveal that chronic infection leads to long-term increases in Regulatory T cells (Tregs), decreases in allo-responsiveness and possible increased risk of cancer development.

While it is interesting that Treg, $CD8^+$ γδ, and NK numbers decreased upon cancer resection, a prospective follow up is needed to determine whether failure of these cell types to fall in numbers may predict cancer recurrence. This information would be significant to help the decision to perform pre-emptive anti-cancer therapy *e.g.* increase cancer surveillance.

Natural Killer (NK) cell function is decreased in KTR with cancer when analysing PBMC. However, when NK cells are removed from PBMC their function is increased. One difference in PBMC between KTR with cancer and KTR with no cancer is Treg proportion and number, thus our hypothesis is that Tregs are reducing NK cell function. We can test this in two different experiments. The first assay could be run on isolated NK cells with addition of isolated Tregs at titrating ratios to determine if the LDH released titrates. The second assay would be removal of Tregs from PBMC and running the resulting cells (Treg depleted

PBMC) in comparison with PBMC; the difference will determine the amount of PBMC mediated lysis that Tregs are restricting. The addition of titrated Tregs will determine if the effect can also be titrated. Furthermore, investigation into the cancer lysing function of $CD8^+$ $\gamma\delta$ T cells may show a protective effect from cancer but may also reveal they are defective in KTR with cancer.

The IFN-γ release assay can be used to determine cellular rejection episodes 6 months post-transplant in pre-transplant patients. The allo-stimulated IFN-γ ELISPOT release assay had a median level of 291 spots per 300,000 PBMC for all KTR, with a majority of KTR with cancer producing less than this. Therefore, KTR with cancer indeed have less allo-response than KTR with no cancer and may have immunosuppression reduced safely.

In this cohort, 10 of the 23 KTR (43.5%) had immunosuppression dose reductions after cancer diagnosis, which resulted in a non-significant reduction in the median level of the dose. Half of the patients stopped or reduced CNIs. A prospective study is needed to determine if there is an IFN-γ ELISPOT cut-off that can predict, with reasonable accuracy, those KTR who will have a rejection episode post immunosuppression reduction. ELISPOTs would be run for a baseline and after any clinical intervention, be it cancer resection or immunosuppression reduction. Those lower than the revealed cut-off will then be used as criteria for KTR enrolment in a randomised control trial to determine benefit of dose reduction in regards to reduced cancer incidence with minimal rejection episodes.

Those KTR with past cancer, had lower levels of Regulatory T cells, higher levels of NK cell function and variable PRT levels compared to KTR with cancer, indicating that intervention led to adequate immunosuppressive drug reductions reconstituting their immune system function.

7.3 Conclusions

Australian Kidney Transplant Recipients with cancer have: higher proportions and numbers of more suppressive Regulatory T cells, similar proportions and numbers of, less functional NK cells, similar Donor Specific Antibody levels, and lower mitogenic and allogenic responses than Australian KTR with no cancer. Tregs associate with Solid Organ Cancer, predict Squamous Cell Carcinoma (SCC) and high risk SCC, and they are dynamic around cancer diagnosis and resection. This data indicates a sub-population of KTR that may be over immunosuppressed and may benefit from reducing immunosuppressive drugs, a benefit that can be measured by the assays outlined in this thesis.

References:

1.   Dantal J, Pohanka E. Malignancies in renal transplantation: an unmet medical need. *Nephrol Dial Transplant* 20071.   Dantal J, Pohanka E. Malignancies in renal transplantation: an unmet medical need. *Nephrol Dial Transplant* 2007; **22 Suppl 1:** i4-10.

2.   Kasiske BL, Snyder JJ, Gilbertson DT*, et al.* Cancer after kidney transplantation in the United States. *Am J Transplant* 2004; **4:** 905-913.

3.   Miao Y, Everly JJ, Gross TG*, et al.* De novo cancers arising in organ transplant recipients are associated with adverse outcomes compared with the general population. *Transplantation* 2009; **87:** 1347-1359.

4.   Apel H, Walschburger-Zorn K, Haberle L*, et al.* De novo malignancies in renal transplant recipients: experience at a single center with 1882 transplant patients over 39 yr. *Clin Transplant* 2013; **27:** E30-36.

5.   Karagas MR, Stukel TA, Greenberg ER*, et al.* Risk of subsequent basal cell carcinoma and squamous cell carcinoma of the skin among patients with prior skin cancer. Skin Cancer Prevention Study Group. *Jama* 1992; **267:** 3305-3310.

6.   Barksdale SK, O'Connor N, Barnhill R. Prognostic factors for cutaneous squamous cell and basal cell carcinoma. Determinants of risk of recurrence, metastasis, and development of subsequent skin cancers. *Surg Oncol Clin N Am* 1997; **6:** 625-638.

7.   Hartevelt MM, Bavinck JN, Kootte AM*, et al.* Incidence of skin cancer after renal transplantation in The Netherlands. *Transplantation* 1990; **49:** 506-509.

8.   Wisgerhof HC, van der Geest LG, de Fijter JW*, et al.* Incidence of cancer in kidney-transplant recipients: a long-term cohort study in a single center. *Cancer Epidemiol* 2011; **35:** 105-111.

9.   Martinez JC, Otley CC, Stasko T*, et al.* Defining the clinical course of metastatic skin cancer in organ transplant recipients: a multicenter collaborative study. *Arch Dermatol* 2003; **139:** 301-306.

10.   Penn I. Skin disorders in organ transplant recipients. External anogenital lesions. *Arch Dermatol* 1997; **133:** 221-223.

11.   Carroll RP, Ramsay HM, Fryer AA*, et al.* Incidence and prediction of nonmelanoma skin cancer post-renal transplantation: a prospective study in Queensland, Australia. *Am J Kidney Dis* 2003; **41:** 676-683.

12.   Cantwell MM, Murray LJ, Catney D*, et al.* Second primary cancers in patients with skin cancer: a population-based study in Northern Ireland. *Br J Cancer* 2009; **100:** 174-177.

13.   Wisgerhof HC, Edelbroek JR, de Fijter JW*, et al.* Subsequent squamous- and basal-cell carcinomas in kidney-transplant recipients after the first skin cancer: cumulative incidence and risk factors. *Transplantation* 2010; **89:** 1231-1238.

14.   Euvrard S, Kanitakis J, Decullier E*, et al.* Subsequent skin cancers in kidney and heart transplant recipients after the first squamous cell carcinoma. *Transplantation* 2006; **81:** 1093-1100.

15. Lindelof B, Sigurgeirsson B, Gabel H, *et al.* Incidence of skin cancer in 5356 patients following organ transplantation. *Br J Dermatol* 2000; **143:** 513-519.

16. Johnson TM, Rowe DE, Nelson BR, *et al.* Squamous cell carcinoma of the skin (excluding lip and oral mucosa). *J Am Acad Dermatol* 1992; **26:** 467-484.

17. Rowe DE, Carroll RJ, Day CL, Jr. Prognostic factors for local recurrence, metastasis, and survival rates in squamous cell carcinoma of the skin, ear, and lip. Implications for treatment modality selection. *J Am Acad Dermatol* 1992; **26:** 976-990.

18. Peat B, Insull P, Ayers R. Risk stratification for metastasis from cutaneous squamous cell carcinoma of the head and neck. *ANZ J Surg* 2012; **82:** 230-233.

19. Brantsch KD, Meisner C, Schonfisch B, *et al.* Analysis of risk factors determining prognosis of cutaneous squamous-cell carcinoma: a prospective study. *Lancet Oncol* 2008; **9:** 713-720.

20. Lott DG, Manz R, Koch C, *et al.* Aggressive behavior of nonmelanotic skin cancers in solid organ transplant recipients. *Transplantation* 2010; **90:** 683-687.

21. Streams BN, Eaton JS, Zelac DE. Perineural spread of squamous cell carcinoma involving the spinal accessory nerve in an immunocompromised organ transplant recipient. *Dermatol Surg* 2005; **31:** 599-601.

22. Buell JF, Hanaway MJ, Thomas M, *et al.* Skin cancer following transplantation: the Israel Penn International Transplant Tumor Registry experience. *Transplant Proc* 2005; **37:** 962-963.

23. Mackenzie KA, Wells JE, Lynn KL, *et al.* First and subsequent nonmelanoma skin cancers: incidence and predictors in a population of New Zealand renal transplant recipients. *Nephrol Dial Transplant* 2010; **25:** 300-306.

24. Moloney FJ, Kelly PO, Kay EW, *et al.* Maintenance versus reduction of immunosuppression in renal transplant recipients with aggressive squamous cell carcinoma. *Dermatol Surg* 2004; **30:** 674-678.

25. O'Donovan P, Perrett CM, Zhang X, *et al.* Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* 2005; **309:** 1871-1874.

26. Zhang X, Jeffs G, Ren X, *et al.* Novel DNA lesions generated by the interaction between therapeutic thiopurines and UVA light. *DNA Repair (Amst)* 2007; **6:** 344-354.

27. Gueranger Q, Kia A, Frith D, *et al.* Crosslinking of DNA repair and replication proteins to DNA in cells treated with 6-thioguanine and UVA. *Nucleic Acids Res* 2011; **39:** 5057-5066.

28. Ingvar A, Smedby KE, Lindelof B, *et al.* Immunosuppressive treatment after solid organ transplantation and risk of post-transplant cutaneous squamous cell carcinoma. *Nephrol Dial Transplant* 2010; **25:** 2764-2771.

29. Allison AC, Eugui EM. Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection. *Transplantation* 2005; **80:** S181-190.

30. Hofbauer GF, Attard NR, Harwood CA, *et al.* Reversal of UVA skin photosensitivity and DNA damage in kidney transplant recipients by replacing azathioprine. *Am J Transplant* 2012; **12:** 218-225.

31. O'Neill JO, Edwards LB, Taylor DO. Mycophenolate mofetil and risk of developing malignancy after orthotopic heart transplantation: analysis of the transplant registry of the International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 2006; **25:** 1186-1191.

32. Clayton PA, McDonald SP, Chapman JR*, et al.* Mycophenolate versus azathioprine for kidney transplantation: a 15-year follow-up of a randomized trial. *Transplantation* 2012; **94:** 152-158.

33. Walsh CT, Zydowsky LD, McKeon FD. Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. *J Biol Chem* 1992; **267:** 13115-13118.

34. Stepkowski SM. Molecular targets for existing and novel immunosuppressive drugs. *Expert Rev Mol Med* 2000; **2:** 1-23.

35. Lemasters JJ, Nieminen AL, Qian T*, et al.* The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* 1998; **1366:** 177-196.

36. Zamzami N, Larochette N, Kroemer G. Mitochondrial permeability transition in apoptosis and necrosis. *Cell Death Differ* 2005; **12 Suppl 2:** 1478-1480.

37. Hojo M, Morimoto T, Maluccio M*, et al.* Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature* 1999; **397:** 530-534.

38. Guba M, von Breitenbuch P, Steinbauer M*, et al.* Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 2002; **8:** 128-135.

39. Wu X, Nguyen BC, Dziunycz P*, et al.* Opposing roles for calcineurin and ATF3 in squamous skin cancer. *Nature* 2010; **465:** 368-372.

40. McGeown MG, Douglas JF, Middleton D. One thousand renal transplants at Belfast City Hospital: post-graft neoplasia 1968-1999, comparing azathioprine only with cyclosporin-based regimes in a single centre. *Clin Transpl* 2000**:** 193-202.

41. Marcen R, Pascual J, Tato AM*, et al.* Influence of immunosuppression on the prevalence of cancer after kidney transplantation. *Transplant Proc* 2003; **35:** 1714-1716.

42. Abou Ayache R, Thierry A, Bridoux F*, et al.* Long-term maintenance of calcineurin inhibitor monotherapy reduces the risk for squamous cell carcinomas after kidney transplantation compared with bi- or tritherapy. *Transplant Proc* 2007; **39:** 2592-2594.

43. Giese T, Sommerer C, Zeier M*, et al.* Monitoring immunosuppression with measures of NFAT decreases cancer incidence. *Clin Immunol* 2009; **132:** 305-311.

44. Wimmer CD, Angele MK, Schwarz B*, et al.* Impact of cyclosporine versus tacrolimus on the incidence of de novo malignancy following liver transplantation: a single center experience with 609 patients. *Transpl Int* 2013; **26:** 999-1006.

45. Opelz G, Dohler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. *Am J Transplant* 2004; **4:** 222-230.

46.   Mayer AD, Dmitrewski J, Squifflet JP*, et al.* Multicenter randomized trial comparing tacrolimus (FK506) and cyclosporine in the prevention of renal allograft rejection: a report of the European Tacrolimus Multicenter Renal Study Group. *Transplantation* 1997; **64:** 436-443.

47.   Pirsch JD, Miller J, Deierhoi MH*, et al.* A comparison of tacrolimus (FK506) and cyclosporine for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Group. *Transplantation* 1997; **63:** 977-983.

48.   Wiesner RH. A long-term comparison of tacrolimus (FK506) versus cyclosporine in liver transplantation: a report of the United States FK506 Study Group. *Transplantation* 1998; **66:** 493-499.

49.   Kleinert H, Euchenhofer C, Ihrig-Biedert I*, et al.* Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-kappa B. *Mol Pharmacol* 1996; **49:** 15-21.

50.   Lee SW, Tsou AP, Chan H*, et al.* Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. *Proc Natl Acad Sci U S A* 1988; **85:** 1204-1208.

51.   Almawi WY, Hess DA, Rieder MJ. Multiplicity of glucocorticoid action in inhibiting allograft rejection. *Cell Transplant* 1998; **7:** 511-523.

52.   Zanker B, Walz G, Wieder KJ*, et al.* Evidence that glucocorticosteroids block expression of the human interleukin-6 gene by accessory cells. *Transplantation* 1990; **49:** 183-185.

53.   Arya SK, Wong-Staal F, Gallo RC. Dexamethasone-mediated inhibition of human T cell growth factor and gamma-interferon messenger RNA. *J Immunol* 1984; **133:** 273-276.

54.   Vacca A, Felli MP, Farina AR*, et al.* Glucocorticoid receptor-mediated suppression of the interleukin 2 gene expression through impairment of the cooperativity between nuclear factor of activated T cells and AP-1 enhancer elements. *J Exp Med* 1992; **175:** 637-646.

55.   McFarland HF. Complexities in the treatment of autoimmune disease. *Science* 1996; **274:** 2037-2038.

56.   Cai J, Zheng T, Lotz M*, et al.* Glucocorticoids induce Kaposi's sarcoma cell proliferation through the regulation of transforming growth factor-beta. *Blood* 1997; **89:** 1491-1500.

57.   Trattner A, Hodak E, David M*, et al.* The appearance of Kaposi sarcoma during corticosteroid therapy. *Cancer* 1993; **72:** 1779-1783.

58.   Bansbach CC, Wancio D, Sehgal SN. Sirolimus (rapamycin) inhibits mitogen-induced stimulation of protein synthesis in primary lymphocytes. *Inflamm Res* 1995; **44 Suppl 2:** S179-180.

59.   Luan FL, Ding R, Sharma VK*, et al.* Rapamycin is an effective inhibitor of human renal cancer metastasis. *Kidney Int* 2003; **63:** 917-926.

60.   Hudes GR. Targeting mTOR in renal cell carcinoma. *Cancer* 2009; **115:** 2313-2320.

61.   Calvo E, Escudier B, Motzer RJ*, et al.* Everolimus in metastatic renal cell carcinoma: Subgroup analysis of patients with 1 or 2 previous vascular endothelial growth factor receptor-tyrosine

kinase inhibitor therapies enrolled in the phase III RECORD-1 study. *Eur J Cancer* 2012; **48:** 333-339.

62.   van den Eertwegh AJ, Karakiewicz P, Bavbek S*, et al.* Safety of everolimus by treatment duration in patients with advanced renal cell cancer in an expanded access program. *Urology* 2013; **81:** 143-149.

63.   Stallone G, Schena A, Infante B*, et al.* Sirolimus for Kaposi's sarcoma in renal-transplant recipients. *N Engl J Med* 2005; **352:** 1317-1323.

64.   Alberu J, Pascoe MD, Campistol JM*, et al.* Lower malignancy rates in renal allograft recipients converted to sirolimus-based, calcineurin inhibitor-free immunotherapy: 24-month results from the CONVERT trial. *Transplantation* 2011; **92:** 303-310.

65.   Campistol JM, Eris J, Oberbauer R*, et al.* Sirolimus therapy after early cyclosporine withdrawal reduces the risk for cancer in adult renal transplantation. *J Am Soc Nephrol* 2006; **17:** 581-589.

66.   Mathew T, Kreis H, Friend P. Two-year incidence of malignancy in sirolimus-treated renal transplant recipients: results from five multicenter studies. *Clin Transplant* 2004; **18:** 446-449.

67.   Cibrik D, Silva HT, Jr., Vathsala A*, et al.* Randomized trial of everolimus-facilitated calcineurin inhibitor minimization over 24 months in renal transplantation. *Transplantation* 2013; **95:** 933-942.

68.   Mjornstedt L, Sorensen SS, von Zur Muhlen B*, et al.* Improved renal function after early conversion from a calcineurin inhibitor to everolimus: a randomized trial in kidney transplantation. *Am J Transplant* 2012; **12:** 2744-2753.

69.   Gurkan S, Luan Y, Dhillon N*, et al.* Immune reconstitution following rabbit antithymocyte globulin. *Am J Transplant* 2010; **10:** 2132-2141.

70.   Ducloux D, Bamoulid J, Courivaud C*, et al.* Thymic function, anti-thymocytes globulins, and cancer after renal transplantation. *Transpl Immunol* 2011; **25:** 56-60.

71.   Vajdic CM, McDonald SP, McCredie MR*, et al.* Cancer incidence before and after kidney transplantation. *Jama* 2006; **296:** 2823-2831.

72.   Dantal J, Hourmant M, Cantarovich D*, et al.* Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. *Lancet* 1998; **351:** 623-628.

73.   Ramsay HM, Fryer AA, Hawley CM*, et al.* Non-melanoma skin cancer risk in the Queensland renal transplant population. *Br J Dermatol* 2002; **147:** 950-956.

74.   Kessler M, Jay N, Molle R*, et al.* Excess risk of cancer in renal transplant patients. *Transpl Int* 2006; **19:** 908-914.

75.   Ramsay HM, Fryer AA, Reece S*, et al.* Clinical risk factors associated with nonmelanoma skin cancer in renal transplant recipients. *Am J Kidney Dis* 2000; **36:** 167-176.

76. Urwin HR, Jones PW, Harden PN*, et al.* Predicting risk of nonmelanoma skin cancer and premalignant skin lesions in renal transplant recipients. *Transplantation* 2009; **87:** 1667-1671.

77. Ramsay HM, Fryer AA, Hawley CM*, et al.* Factors associated with nonmelanoma skin cancer following renal transplantation in Queensland, Australia. *J Am Acad Dermatol* 2003; **49:** 397-406.

78. Kim JK, Patel D, Choi BS. Contrasting structural impacts induced by cis-syn cyclobutane dimer and (6-4) adduct in DNA duplex decamers: implication in mutagenesis and repair activity. *Photochem Photobiol* 1995; **62:** 44-50.

79. Donahue BA, Yin S, Taylor JS*, et al.* Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. *Proc Natl Acad Sci U S A* 1994; **91:** 8502-8506.

80. Brash DE, Rudolph JA, Simon JA*, et al.* A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci U S A* 1991; **88:** 10124-10128.

81. Marks R, Jolley D, Lectsas S*, et al.* The role of childhood exposure to sunlight in the development of solar keratoses and non-melanocytic skin cancer. *Med J Aust* 1990; **152:** 62-66.

82. el-Ghorr AA, Norval M. A monoclonal antibody to cis-urocanic acid prevents the ultraviolet-induced changes in Langerhans cells and delayed hypersensitivity responses in mice, although not preventing dendritic cell accumulation in lymph nodes draining the site of irradiation and contact hypersensitivity responses. *J Invest Dermatol* 1995; **105:** 264-268.

83. Rivas JM, Ullrich SE. The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J Leukoc Biol* 1994; **56:** 769-775.

84. Chung HT, Burnham DK, Robertson B*, et al.* Involvement of prostaglandins in the immune alterations caused by the exposure of mice to ultraviolet radiation. *J Immunol* 1986; **137:** 2478-2484.

85. Gurish MF, Lynch DH, Daynes RA. Changes in antigen-presenting cell function in the spleen and lymph nodes of ultraviolet-irradiated mice. *Transplantation* 1982; **33:** 280-284.

86. Ullrich SE. Modulation of immunity by ultraviolet radiation: key effects on antigen presentation. *J Invest Dermatol* 1995; **105:** 30S-36S.

87. Schwarz A, Maeda A, Wild MK*, et al.* Ultraviolet radiation-induced regulatory T cells not only inhibit the induction but can suppress the effector phase of contact hypersensitivity. *J Immunol* 2004; **172:** 1036-1043.

88. Schwarz A, Navid F, Sparwasser T*, et al.* In vivo reprogramming of UV radiation-induced regulatory T-cell migration to inhibit the elicitation of contact hypersensitivity. *J Allergy Clin Immunol* 2011; **128:** 826-833.

89. Simon JC, Hara H, Denfeld RW*, et al.* UVB-irradiated dendritic cells induce nonproliferating, regulatory type T cells. *Skin Pharmacol Appl Skin Physiol* 2002; **15:** 330-334.

90. Schwarz A, Beissert S, Grosse-Heitmeyer K*, et al.* Evidence for functional relevance of CTLA-4 in ultraviolet-radiation-induced tolerance. *J Immunol* 2000; **165:** 1824-1831.

91.   Moodycliffe AM, Nghiem D, Clydesdale G*, et al.* Immune suppression and skin cancer development: regulation by NKT cells. *Nat Immunol* 2000; **1:** 521-525.

92.   Schwarz A, Maeda A, Kernebeck K*, et al.* Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. *J Exp Med* 2005; **201:** 173-179.

93.   Ando O, Suemoto Y, Kurimoto M*, et al.* Deficient Th1-type immune responses via impaired CD28 signaling in ultraviolet B-induced systemic immunosuppression and the restorative effect of IL-12. *J Dermatol Sci* 2000; **24:** 190-202.

94.   Suemoto Y, Ando O, Kurimoto M*, et al.* IL-12 promotes the accessory cell function of epidermal Langerhans cells. *J Dermatol Sci* 1998; **18:** 98-108.

95.   Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006; **118:** 3030-3044.

96.   Bouwes Bavinck JN, Feltkamp M, Struijk L*, et al.* Human papillomavirus infection and skin cancer risk in organ transplant recipients. *J Investig Dermatol Symp Proc* 2001; **6:** 207-211.

97.   Storey A, Thomas M, Kalita A*, et al.* Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* 1998; **393:** 229-234.

98.   Harwood CA, Surentheran T, McGregor JM*, et al.* Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. *J Med Virol* 2000; **61:** 289-297.

99.   van Zanten J, de Leij L, Prop J*, et al.* Human cytomegalovirus: a viral complication in transplantation. *Clin Transplant* 1998; **12:** 145-158.

100.   Baumforth KR, Birgersdotter A, Reynolds GM*, et al.* Expression of the Epstein-Barr virus-encoded Epstein-Barr virus nuclear antigen 1 in Hodgkin's lymphoma cells mediates Up-regulation of CCL20 and the migration of regulatory T cells. *Am J Pathol* 2008; **173:** 195-204.

101.   Couzi L, Levaillant Y, Jamai A*, et al.* Cytomegalovirus-induced gammadelta T cells associate with reduced cancer risk after kidney transplantation. *J Am Soc Nephrol* 2010; **21:** 181-188.

102.   Ducloux D, Carron PL, Rebibou JM*, et al.* CD4 lymphocytopenia as a risk factor for skin cancers in renal transplant recipients. *Transplantation* 1998; **65:** 1270-1272.

103.   Thibaudin D, Alamartine E, Mariat C*, et al.* Long-term kinetic of T-lymphocyte subsets in kidney-transplant recipients: influence of anti-T-cell antibodies and association with posttransplant malignancies. *Transplantation* 2005; **80:** 1514-1517.

104.   Ducloux D, Carron PL, Motte G*, et al.* Lymphocyte subsets and assessment of cancer risk in renal transplant recipients. *Transpl Int* 2002; **15:** 393-396.

105.   Gershon RK, Cohen P, Hencin R*, et al.* Suppressor T cells. *J Immunol* 1972; **108:** 586-590.

106.   Kronenberg M, Steinmetz M, Kobori J*, et al.* RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. *Proc Natl Acad Sci U S A* 1983; **80:** 5704-5708.

107.   Green DR, Webb DR. Saying the 'S' word in public. *Immunol Today* 1993; **14:** 523-525.

108. Sakaguchi S, Sakaguchi N, Asano M, *et al.* Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995; **155:** 1151-1164.

109. Baecher-Allan C, Brown JA, Freeman GJ, *et al.* CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 2001; **167:** 1245-1253.

110. Liu W, Putnam AL, Xu-Yu Z, *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006; **203:** 1701-1711.

111. O'Garra A, Vieira P. Regulatory T cells and mechanisms of immune system control. *Nat Med* 2004; **10:** 801-805.

112. Zhang L, Zhao Y. The regulation of Foxp3 expression in regulatory CD4(+)CD25(+)T cells: multiple pathways on the road. *J Cell Physiol* 2007; **211:** 590-597.

113. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299:** 1057-1061.

114. Gambineri E, Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr Opin Rheumatol* 2003; **15:** 430-435.

115. Chatila TA, Blaeser F, Ho N, *et al.* JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* 2000; **106:** R75-81.

116. Wildin RS, Ramsdell F, Peake J, *et al.* X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 2001; **27:** 18-20.

117. Valmori D, Merlo A, Souleimanian NE, *et al.* A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest* 2005; **115:** 1953-1962.

118. Schena F, Volpi S, Faliti CE, *et al.* Dependence of immunoglobulin class switch recombination in B cells on vesicular release of ATP and CD73 ectonucleotidase activity. *Cell Rep* 2013; **3:** 1824-1831.

119. Zabransky DJ, Nirschl CJ, Durham NM, *et al.* Phenotypic and functional properties of Helios+ regulatory T cells. *PLoS One* 2012; **7:** e34547.

120. Huang CT, Workman CJ, Flies D, *et al.* Role of LAG-3 in regulatory T cells. *Immunity* 2004; **21:** 503-513.

121. Mantel PY, Ouaked N, Ruckert B, *et al.* Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 2006; **176:** 3593-3602.

122. Sakaguchi S, Setoguchi R, Yagi H, *et al.* Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in self-tolerance and autoimmune disease. *Curr Top Microbiol Immunol* 2006; **305:** 51-66.

123. Cederbom L, Hall H, Ivars F. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 2000; **30:** 1538-1543.

124. Jung ID, Jeong YI, Lee CM*, et al.* COX-2 and PGE2 signaling is essential for the regulation of IDO expression by curcumin in murine bone marrow-derived dendritic cells. *Int Immunopharmacol* 2010; **10:** 760-768.

125. Mandapathil M, Hilldorfer B, Szczepanski MJ*, et al.* Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. *J Biol Chem* 2010; **285:** 7176-7186.

126. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 2001; **414:** 916-920.

127. Raskovalova T, Huang X, Sitkovsky M*, et al.* Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. *J Immunol* 2005; **175:** 4383-4391.

128. Fletcher JM, Lonergan R, Costelloe L*, et al.* CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* 2009; **183:** 7602-7610.

129. Mandapathil M, Szczepanski MJ, Szajnik M*, et al.* Adenosine and prostaglandin E2 cooperate in the suppression of immune responses mediated by adaptive regulatory T cells. *J Biol Chem* 2010; **285:** 27571-27580.

130. Sitkovsky M, Lukashev D, Deaglio S*, et al.* Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. *Br J Pharmacol* 2008; **153 Suppl 1:** S457-464.

131. Marshall NA, Vickers MA, Barker RN. Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. *J Immunol* 2003; **170:** 6183-6189.

132. Lund JM, Hsing L, Pham TT*, et al.* Coordination of early protective immunity to viral infection by regulatory T cells. *Science* 2008; **320:** 1220-1224.

133. Schoenbrunn A, Frentsch M, Kohler S*, et al.* A converse 4-1BB and CD40 ligand expression pattern delineates activated regulatory T cells (Treg) and conventional T cells enabling direct isolation of alloantigen-reactive natural Foxp3+ Treg. *J Immunol* 2012; **189:** 5985-5994.

134. Louis S, Braudeau C, Giral M*, et al.* Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation* 2006; **81:** 398-407.

135. Moraes-Vieira PM, Silva HM, Takenaka MC*, et al.* Differential monocyte STAT6 activation and CD4(+)CD25(+)Foxp3(+) T cells in kidney operational tolerance transplanted individuals. *Hum Immunol* 2010; **71:** 442-450.

136. Pons JA, Revilla-Nuin B, Baroja-Mazo A*, et al.* FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal. *Transplantation* 2008; **86:** 1370-1378.

137. Baan CC, Velthuis JH, van Gurp EA*, et al.* Functional CD25(bright+) alloresponsive T cells in fully immunosuppressed renal allograft recipients. *Clin Transplant* 2007; **21:** 63-71.

138. Yoshizawa A, Ito A, Li Y*, et al.* The roles of CD25+CD4+ regulatory T cells in operational tolerance after living donor liver transplantation. *Transplant Proc* 2005; **37:** 37-39.

139. Dwyer KM, Hanidziar D, Putheti P, *et al.* Expression of CD39 by human peripheral blood CD4+ CD25+ T cells denotes a regulatory memory phenotype. *Am J Transplant* 2010; **10:** 2410-2420.

140. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* 2001; **45:** 194-205.

141. Carroll RP, Segundo DS, Hollowood K, *et al.* Immune phenotype predicts risk for posttransplantation squamous cell carcinoma. *J Am Soc Nephrol* 2010; **21:** 713-722.

142. Strauss L, Bergmann C, Gooding W, *et al.* The frequency and suppressor function of CD4+CD25highFoxp3+ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2007; **13:** 6301-6311.

143. Bergmann C, Strauss L, Zeidler R, *et al.* Expansion of human T regulatory type 1 cells in the microenvironment of cyclooxygenase 2 overexpressing head and neck squamous cell carcinoma. *Cancer Res* 2007; **67:** 8865-8873.

144. Bates GJ, Fox SB, Han C, *et al.* Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006; **24:** 5373-5380.

145. Ling KL, Pratap SE, Bates GJ, *et al.* Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. *Cancer Immun* 2007; **7:** 7.

146. Fox SB, Launchbury R, Bates GJ, *et al.* The number of regulatory T cells in prostate cancer is associated with the androgen receptor and hypoxia-inducible factor (HIF)-2alpha but not HIF-1alpha. *Prostate* 2007; **67:** 623-629.

147. Stagg J, Divisekera U, McLaughlin N, *et al.* Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A* 2010; **107:** 1547-1552.

148. Denkert C, Winzer KJ, Hauptmann S. Prognostic impact of cyclooxygenase-2 in breast cancer. *Clin Breast Cancer* 2004; **4:** 428-433.

149. Mrena J, Wiksten JP, Thiel A, *et al.* Cyclooxygenase-2 is an independent prognostic factor in gastric cancer and its expression is regulated by the messenger RNA stability factor HuR. *Clin Cancer Res* 2005; **11:** 7362-7368.

150. Biron CA, Nguyen KB, Pien GC, *et al.* Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999; **17:** 189-220.

151. Karre K, Ljunggren HG, Piontek G, *et al.* Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 1986; **319:** 675-678.

152. Algarra I, Cabrera T, Garrido F. The HLA crossroad in tumor immunology. *Hum Immunol* 2000; **61:** 65-73.

153. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2002; **2:** 735-747.

154. Wolf AM, Wolf D, Steurer M*, et al.* Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* 2003; **9:** 606-612.

155. Smyth MJ, Teng MW, Swann J*, et al.* CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* 2006; **176:** 1582-1587.

156. Trzonkowski P, Szmit E, Mysliwska J*, et al.* CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. *Clin Immunol* 2004; **112:** 258-267.

157. Friese MA, Wischhusen J, Wick W*, et al.* RNA interference targeting transforming growth factor-beta enhances NKG2D-mediated antiglioma immune response, inhibits glioma cell migration and invasiveness, and abrogates tumorigenicity in vivo. *Cancer Res* 2004; **64:** 7596-7603.

158. Yoon SJ, Heo DS, Kang SH*, et al.* Natural killer cell activity depression in peripheral blood and ascites from gastric cancer patients with high TGF-beta 1 expression. *Anticancer Res* 1998; **18:** 1591-1596.

159. Lee JC, Lee KM, Kim DW*, et al.* Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol* 2004; **172:** 7335-7340.

160. Terabe M, Swann J, Ambrosino E*, et al.* A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* 2005; **202:** 1627-1633.

161. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22:** 633-640.

162. Cooper MA, Fehniger TA, Turner SC*, et al.* Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* 2001; **97:** 3146-3151.

163. Qin Z, Schwartzkopff J, Pradera F*, et al.* A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res* 2003; **63:** 4095-4100.

164. Colombo MP, Piconese S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. *Nat Rev Cancer* 2007; **7:** 880-887.

165. Lee PP, Yee C, Savage PA*, et al.* Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* 1999; **5:** 677-685.

166. Sutmuller RP, van Duivenvoorde LM, van Elsas A*, et al.* Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* 2001; **194:** 823-832.

167. Campbell JJ, Bowman EP, Murphy K*, et al.* 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. *J Cell Biol* 1998; **141:** 1053-1059.

168. Forster R, Schubel A, Breitfeld D*, et al.* CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999; **99:** 23-33.

169.   Gupta S, Su H, Bi R, *et al.* Life and death of lymphocytes: a role in immunesenescence. *Immun Ageing* 2005; **2:** 12.

170.   Filaci G, Fenoglio D, Fravega M, *et al.* CD8+ CD28- T regulatory lymphocytes inhibiting T cell proliferative and cytotoxic functions infiltrate human cancers. *J Immunol* 2007; **179:** 4323-4334.

171.   Tsukishiro T, Donnenberg AD, Whiteside TL. Rapid turnover of the CD8(+)CD28(-) T-cell subset of effector cells in the circulation of patients with head and neck cancer. *Cancer Immunol Immunother* 2003; **52:** 599-607.

172.   Gamadia LE, Rentenaar RJ, Baars PA, *et al.* Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers. *Blood* 2001; **98:** 754-761.

173.   Valmori D, Scheibenbogen C, Dutoit V, *et al.* Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(-) effector subset exerting ex vivo tumor-specific cytolytic activity. *Cancer Res* 2002; **62:** 1743-1750.

174.   Sanchez-Fructuoso A, Conesa J, Perez Flores I, *et al.* Conversion to sirolimus in renal transplant patients with tumors. *Transplant Proc* 2006; **38:** 2451-2452.

175.   Yelken B, Caliskan Y, Ozkan O, *et al.* Conversion to sirolimus in renal transplant recipients: a single-center experience. *Artif Organs* 2010; **34:** E230-237.

176.   Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 2005; **105:** 4743-4748.

177.   Carroll RP, Hester J, Wood KJ, *et al.* Conversion to sirolimus in kidney transplant recipients with squamous cell cancer and changes in immune phenotype. *Nephrol Dial Transplant* 2012.

178.   Euvrard S, Morelon E, Rostaing L, *et al.* Sirolimus and secondary skin-cancer prevention in kidney transplantation. *N Engl J Med* 2012; **367:** 329-339.

179.   Hoogendijk-van den Akker JM, Harden PN, Hoitsma AJ, *et al.* Two-Year Randomized Controlled Prospective Trial Converting Treatment of Stable Renal Transplant Recipients With Cutaneous Invasive Squamous Cell Carcinomas to Sirolimus. *J Clin Oncol* 2013.

180.   van den Akker JM, Wetzels JF, Hoitsma AJ. Proteinuria following conversion from azathioprine to sirolimus in renal transplant recipients. *Kidney Int* 2006; **70:** 1355-1357.

181.   Kono K, Kawaida H, Takahashi A, *et al.* CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. *Cancer Immunol Immunother* 2006; **55:** 1064-1071.

182.   Perez SA, Karamouzis MV, Skarlos DV, *et al.* CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. *Clin Cancer Res* 2007; **13:** 2714-2721.

183.   Terasaki PI, Ozawa M. Predicting kidney graft failure by HLA antibodies: a prospective trial. *Am J Transplant* 2004; **4:** 438-443.

184.   Zhang W, Caspell R, Karulin AY, *et al.* ELISPOT assays provide reproducible results among different laboratories for T-cell immune monitoring--even in hands of ELISPOT-inexperienced investigators. *J Immunotoxicol* 2009; **6:** 227-234.

185. Heeger PS, Greenspan NS, Kuhlenschmidt S, *et al.* Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 1999; **163:** 2267-2275.

186. Poggio ED, Clemente M, Hricik DE, *et al.* Panel of reactive T cells as a measurement of primed cellular alloimmunity in kidney transplant candidates. *J Am Soc Nephrol* 2006; **17:** 564-572.

187. Bestard O, Cruzado JM, Lucia M, *et al.* Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. *Kidney Int* 2013.

188. Eng HS, Bennett G, Tsiopelas E, *et al.* Anti-HLA donor-specific antibodies detected in positive B-cell crossmatches by Luminex predict late graft loss. *Am J Transplant* 2008; **8:** 2335-2342.

189. Amico P, Honger G, Mayr M, *et al.* Clinical relevance of pretransplant donor-specific HLA antibodies detected by single-antigen flow-beads. *Transplantation* 2009; **87:** 1681-1688.

190. Riethmuller S, Ferrari-Lacraz S, Muller MK, *et al.* Donor-specific antibody levels and three generations of crossmatches to predict antibody-mediated rejection in kidney transplantation. *Transplantation* 2010; **90:** 160-167.

191. Hricik DE, Rodriguez V, Riley J, *et al.* Enzyme linked immunosorbent spot (ELISPOT) assay for interferon-gamma independently predicts renal function in kidney transplant recipients. *Am J Transplant* 2003; **3:** 878-884.

192. Gebauer BS, Hricik DE, Atallah A, *et al.* Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. *Am J Transplant* 2002; **2:** 857-866.

193. Vandenbroucke JP, von Elm E, Altman DG, *et al.* Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *Annals of internal medicine* 2007; **147:** W163-194.

194. Nicholson JK, Hubbard M, Jones BM. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. *Cytometry* 1996; **26:** 16-21.

195. Hill D, Eastaff-Leung N, Bresatz-Atkins S, *et al.* Inhibition of activation induced CD154 on CD4+ CD25- cells: a valid surrogate for human Treg suppressor function. *Immunol Cell Biol* 2012; **90:** 812-821.

196. Segundo DS, Ruiz JC, Izquierdo M, *et al.* Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation* 2006; **82:** 550-557.

197. Ducloux D, Carron PL, Racadot E, *et al.* CD4 lymphocytopenia in long-term renal transplant recipients. *Transplant Proc* 1998; **30:** 2859-2860.

198. Carroll RP, Hester J, Wood KJ, *et al.* Conversion to sirolimus in kidney transplant recipients with squamous cell cancer and changes in immune phenotype. *Nephrol Dial Transplant* 2013; **28:** 462-465.

199. Puig-Pey I, Bohne F, Benitez C, *et al.* Characterization of gammadelta T cell subsets in organ transplantation. *Transpl Int* 2010; **23:** 1045-1055.

200. Costantini A, Mancini S, Giuliodoro S, *et al.* Effects of cryopreservation on lymphocyte immunophenotype and function. *J Immunol Methods* 2003; **278:** 145-155.

201. Weinberg A, Song LY, Wilkening C, *et al.* Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol* 2009; **16:** 1176-1186.

202. Elkord E. Frequency of human T regulatory cells in peripheral blood is significantly reduced by cryopreservation. *J Immunol Methods* 2009; **347:** 87-90.

203. Sattui S, de la Flor C, Sanchez C, *et al.* Cryopreservation modulates the detection of regulatory T cell markers. *Cytometry B Clin Cytom* 2012; **82:** 54-58.

204. Seale AC, de Jong BC, Zaidi I, *et al.* Effects of cryopreservation on CD4+ CD25+ T cells of HIV-1 infected individuals. *J Clin Lab Anal* 2008; **22:** 153-158.

205. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* 2007; **110:** 2983-2990.

206. Courivaud C, Bamoulid J, Gaugler B, *et al.* Cytomegalovirus exposure, immune exhaustion and cancer occurrence in renal transplant recipients. *Transpl Int* 2012; **25:** 948-955.

207. Rahimpour A, Mattarollo SR, Yong M, *et al.* gammadelta T cells augment rejection of skin grafts by enhancing cross-priming of CD8 T cells to skin-derived antigen. *J Invest Dermatol* 2012; **132:** 1656-1664.

208. Braza MS, Klein B. Anti-tumour immunotherapy with Vgamma9Vdelta2 T lymphocytes: from the bench to the bedside. *Br J Haematol* 2013; **160:** 123-132.

209. Martinez-Llordella M, Puig-Pey I, Orlando G, *et al.* Multiparameter immune profiling of operational tolerance in liver transplantation. *Am J Transplant* 2007; **7:** 309-319.

210. Morita CT, Verma S, Aparicio P, *et al.* Functionally distinct subsets of human gamma/delta T cells. *Eur J Immunol* 1991; **21:** 2999-3007.

211. Yurchenko E, Levings MK, Piccirillo CA. CD4+ Foxp3+ regulatory T cells suppress gammadelta T-cell effector functions in a model of T-cell-induced mucosal inflammation. *Eur J Immunol* 2011; **41:** 3455-3466.

212. Kobayashi H, Tanaka Y, Nakazawa H, *et al.* A new indicator of favorable prognosis in locally advanced renal cell carcinomas: gamma delta T-cells in peripheral blood. *Anticancer Res* 2011; **31:** 1027-1031.

213. Alamartine E, Sabido O, Berthoux F. In-vitro effects of cyclosporin A, FK506, 6-mercaptopurine, and prednisolone on lymphokine-activated killer cells. *Nephrol Dial Transplant* 1994; **9:** 1456-1461.

214. Vacher-Coponat H, Brunet C, Moal V, *et al.* Tacrolimus/mycophenolate mofetil improved natural killer lymphocyte reconstitution one year after kidney transplant by reference to cyclosporine/azathioprine. *Transplantation* 2006; **82:** 558-566.

215.	Chidrawar SM, Khan N, Chan YL*, et al.* Ageing is associated with a decline in peripheral blood CD56bright NK cells. *Immun Ageing* 2006; **3:** 10.

216.	Colonna-Romano G, Aquino A, Bulati M*, et al.* Memory B cell subpopulations in the aged. *Rejuvenation Res* 2006; **9:** 149-152.

217.	Newell KA, Asare A, Kirk AD*, et al.* Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest* 2010; **120:** 1836-1847.

218.	Pallier A, Hillion S, Danger R*, et al.* Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. *Kidney Int* 2010; **78:** 503-513.

219.	Lim HW, Hillsamer P, Banham AH*, et al.* Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol* 2005; **175:** 4180-4183.

220.	Saze Z, Schuler PJ, Hong CS*, et al.* Adenosine production by human B cells and B cell-mediated suppression of activated T cells. *Blood* 2013; **122:** 9-18.

221.	Ma C, Zhang Q, Ye J*, et al.* Tumor-infiltrating gammadelta T lymphocytes predict clinical outcome in human breast cancer. *J Immunol* 2012; **189:** 5029-5036.

222.	Miller AM, Lundberg K, Ozenci V*, et al.* CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *J Immunol* 2006; **177:** 7398-7405.

223.	Himmel ME, MacDonald KG, Garcia RV*, et al.* Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *J Immunol* 2013; **190:** 2001-2008.

224.	Thornton AM, Korty PE, Tran DQ*, et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 2010; **184:** 3433-3441.

225.	Sewgobind VD, Kho MM, van der Laan LJ*, et al.* The effect of rabbit anti-thymocyte globulin induction therapy on regulatory T cells in kidney transplant patients. *Nephrol Dial Transplant* 2009; **24:** 1635-1644.

226.	Hope CM, Grace BS, Pilkington KR*, et al.* The immune phenotype may relate to cancer development in kidney transplant recipients. *Kidney Int* 2014.

227.	Strauss L, Bergmann C, Szczepanski M*, et al.* A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* 2007; **13:** 4345-4354.

228.	Mandapathil M, Szczepanski MJ, Szajnik M*, et al.* Increased ectonucleotidase expression and activity in regulatory T cells of patients with head and neck cancer. *Clin Cancer Res* 2009; **15:** 6348-6357.

229.	Bergmann C, Strauss L, Wang Y*, et al.* T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease. *Clin Cancer Res* 2008; **14:** 3706-3715.

230. Akimova T, Kamath BM, Goebel JW, *et al.* Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant* 2012; **12:** 3449-3461.

231. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, *et al.* Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 2002; **196:** 851-857.

232. Wu Y, Borde M, Heissmeyer V, *et al.* FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 2006; **126:** 375-387.

233. Baan CC, van der Mast BJ, Klepper M, *et al.* Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* 2005; **80:** 110-117.

234. San Segundo D, Fabrega E, Lopez-Hoyos M, *et al.* Reduced numbers of blood natural regulatory T cells in stable liver transplant recipients with high levels of calcineurin inhibitors. *Transplant Proc* 2007; **39:** 2290-2292.

235. Musaro A, McCullagh KJ, Naya FJ, *et al.* IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* 1999; **400:** 581-585.

236. Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989; **47:** 187-376.

237. Karre K, Ljunggren HG, Piontek G, *et al.* Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. 1986. *J Immunol* 2005; **174:** 6566-6569.

238. O'Leary JG, Goodarzi M, Drayton DL, *et al.* T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* 2006; **7:** 507-516.

239. Lanier LL, Buck DW, Rhodes L, *et al.* Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen. *J Exp Med* 1988; **167:** 1572-1585.

240. Shim H, Chun YS, Lewis BC, *et al.* A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc Natl Acad Sci U S A* 1998; **95:** 1511-1516.

241. Greiner EF, Guppy M, Brand K. Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J Biol Chem* 1994; **269:** 31484-31490.

242. Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 1988; **115:** 61-69.

243. Morteau O, Blundell S, Chakera A, *et al.* Renal transplant immunosuppression impairs natural killer cell function in vitro and in vivo. *PLoS One* 2010; **5:** e13294.

244. Meehan AC, Mifsud NA, Nguyen TH, *et al.* Impact of commonly used transplant immunosuppressive drugs on human NK cell function is dependent upon stimulation condition. *PLoS One* 2013; **8:** e60144.

245. Peters JH, Preijers FW, Woestenenk R, *et al.* Clinical grade Treg: GMP isolation, improvement of purity by CD127 Depletion, Treg expansion, and Treg cryopreservation. *PLoS One* 2008; **3:** e3161.

246. Kvarnstrom M, Jenmalm MC, Ekerfelt C. Effect of cryopreservation on expression of Th1 and Th2 cytokines in blood mononuclear cells from patients with different cytokine profiles, analysed with three common assays: an overall decrease of interleukin-4. *Cryobiology* 2004; **49:** 157-168.

247. Brooks-Worrell B, Tree T, Mannering SI, *et al.* Comparison of cryopreservation methods on T-cell responses to islet and control antigens from type 1 diabetic patients and controls. *Diabetes Metab Res Rev* 2011; **27:** 737-745.

248. Ruitenberg JJ, Boyce C, Hingorani R, *et al.* Rapid assessment of in vitro expanded human regulatory T cell function. *J Immunol Methods* 2011; **372:** 95-106.

249. Brunialti MK, Kallas EG, Freudenberg M, *et al.* Influence of EDTA and heparin on lipopolysaccharide binding and cell activation, evaluated at single-cell level in whole blood. *Cytometry* 2002; **50:** 14-18.

250. Freer G, Matteucci D, Mazzetti P, *et al.* Generation of feline dendritic cells derived from peripheral blood monocytes for in vivo use. *Clin Diagn Lab Immunol* 2005; **12:** 1202-1208.

251. Bezouska K, Nepovim A, Horvath O, *et al.* CD 69 antigen of human lymphocytes is a calcium-dependent carbohydrate-binding protein. *Biochem Biophys Res Commun* 1995; **208:** 68-74.

252. Warren HS, Wu F, Horn PL, *et al.* Peripheral blood natural killer (NK) cell function in healthy adults assessed using the target-induced NK loss (TINKL) assay. *J Immunol Methods* 2013; **392:** 68-70.

253. Caux C, Massacrier C, Vanbervliet B, *et al.* Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* 1994; **180:** 1263-1272.

254. Grewal IS, Xu J, Flavell RA. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* 1995; **378:** 617-620.

255. Yang Y, Wilson JM. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science* 1996; **273:** 1862-1864.

256. van Essen D, Kikutani H, Gray D. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature* 1995; **378:** 620-623.

257. Hollander GA, Castigli E, Kulbacki R, *et al.* Induction of alloantigen-specific tolerance by B cells from CD40-deficient mice. *Proc Natl Acad Sci U S A* 1996; **93:** 4994-4998.

258. Peguet-Navarro J, Dalbiez-Gauthier C, Rattis FM, *et al.* Functional expression of CD40 antigen on human epidermal Langerhans cells. *J Immunol* 1995; **155:** 4241-4247.

259. Filatenkov AA, Jacovetty EL, Fischer UB, *et al.* CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J Immunol* 2005; **174:** 6909-6917.

260. Buhlmann JE, Gonzalez M, Ginther B*, et al.* Cutting edge: sustained expansion of CD8+ T cells requires CD154 expression by Th cells in acute graft versus host disease. *J Immunol* 1999; **162:** 4373-4376.

261. Zitvogel L, Mayordomo JI, Tjandrawan T*, et al.* Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* 1996; **183:** 87-97.

262. Skov S, Bonyhadi M, Odum N*, et al.* IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. *J Immunol* 2000; **164:** 3500-3505.

263. Ballesteros-Tato A, Leon B, Lund FE*, et al.* CD4+ T helper cells use CD154-CD40 interactions to counteract T reg cell-mediated suppression of CD8+ T cell responses to influenza. *J Exp Med* 2013; **210:** 1591-1601.

264. Lertworapreecha M, Patumraj S, Niruthisard S*, et al.* Cytotoxic function of gamma delta (gamma/delta) T cells against pamidronate-treated cervical cancer cells. *Indian J Exp Biol* 2013; **51:** 597-605.

265. Neudoerfl C, Mueller BJ, Blume C*, et al.* The Peripheral NK Cell Repertoire after Kidney Transplantation is Modulated by Different Immunosuppressive Drugs. *Front Immunol* 2013; **4:** 46.

266. Hutchinson P, Chadban SJ, Atkins RC*, et al.* Laboratory assessment of immune function in renal transplant patients. *Nephrol Dial Transplant* 2003; **18:** 983-989.

267. Venema H, van den Berg AP, van Zanten C*, et al.* Natural killer cell responses in renal transplant patients with cytomegalovirus infection. *J Med Virol* 1994; **42:** 188-192.

268. Hoogendijk-van den Akker JM, Harden PN, Hoitsma AJ*, et al.* Two-year randomized controlled prospective trial converting treatment of stable renal transplant recipients with cutaneous invasive squamous cell carcinomas to sirolimus. *J Clin Oncol* 2013; **31:** 1317-1323.

269. Bestard O, Cruzado JM, Lucia M*, et al.* Prospective assessment of antidonor cellular alloreactivity is a tool for guidance of immunosuppression in kidney transplantation. *Kidney Int* 2013; **84:** 1226-1236.

270. Chakera A, Bennett S, Lawrence S*, et al.* Antigen-specific T cell responses to BK polyomavirus antigens identify functional anti-viral immunity and may help to guide immunosuppression following renal transplantation. *Clin Exp Immunol* 2011; **165:** 401-409.

271. Binggeli S, Egli A, Schaub S*, et al.* Polyomavirus BK-specific cellular immune response to VP1 and large T-antigen in kidney transplant recipients. *Am J Transplant* 2007; **7:** 1131-1139.

272. Schaub S, Hirsch HH, Dickenmann M*, et al.* Reducing immunosuppression preserves allograft function in presumptive and definitive polyomavirus-associated nephropathy. *Am J Transplant* 2010; **10:** 2615-2623.

273. Ashoor I, Najafian N, Korin Y*, et al.* Standardization and cross validation of alloreactive IFNγ ELISPOT assays within the clinical trials in organ transplantation consortium. *Am J Transplant* 2013; **13:** 1871-1879.

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Appendix: Published Paper

Hope, C.M., Grace, B.S., Pilkington, K.R., Coates, P.T., Bergmann, I.P. & Carroll, R.P. (2014) The immune phenotype may relate to cancer development in kidney transplant recipients. *Kidney International, v. 86, pp. 175-183*