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**Immune profiling and cancer post transplantation**

Hope CM *et al.* Immune profiling and cancer post transplantation

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**Abstract**

Half of all long-term (> 10 year) 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 graft. At least 30% of KTR with a history of SCC or SOC will develop a subsequent SCC or SOC lesion. Pharmacological immunosuppression is a major contributor of the increased risk of cancer for KTR, with the cancer lesions themselves further adding to systemic immunosuppression and could explain, in part, these phenomena. Immune profiling includes; measuring immunosuppressive drug levels and pharmacokinetics, enumerating leucocytes and leucocyte subsets as well as testing leucocyte function in either an antigen specific or non-specific manner. Outputs can vary from assay to assay according to methods used. In this review we define the rationale behind post-transplant immune monitoring assays and focus on assays that associate and/or have the ability to predict cancer and rejection in the KTR. We find that immune monitoring can identify those KTR of developing multiple SCC lesions and provide evidence they may benefit from pharmacological immunosuppressive drug dose reductions. In these KTR risk of rejection needs to be assessed to determine if reduction of immunosuppression will not harm the graft.

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**Key words:** Immune-profiling; Immunosuppression; Kidney; Malignancy; Transplantation

**Core tip:** Kidney Transplant Recipients (KTR) with cancer has altered leukocyte compartmentalisations and immune cell functions than KTR with no cancer. These differences can be used to determine KTR at risk of developing cancer and identify those who do not mount a reaction to their graft. Indicating there is a group of KTR that may benefit from pharmacological immunosuppressive drug dose reductions.

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**INTRODUCTION**

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](#_ENREF_1)]. Cancers in KTR have poorer prognoses for a given stage/grade than the general population, which leads to higher mortality[[5-9](#_ENREF_5)]. 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) 267 KTR (or 31%) of all KTR died with a functioning graft (ANZDATA, 2012).

Additionally, KTR have a 60 to 250- fold increased risk of developing a Non-Melanoma Skin Cancer (NMSC), which includes; squamous cell carcinoma (SCC), Basal Cell Carcinoma (BCC), Karposi’s sarcoma, Merkel cell carcinoma, and adenexal tumours[[1](#_ENREF_1),[7](#_ENREF_7),[10](#_ENREF_10)]. SCC is the most common cancer in KTR with 50% of KTR who are 15 years post transplantation developing an SCC[[11](#_ENREF_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](#_ENREF_5),[6](#_ENREF_6),[12](#_ENREF_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](#_ENREF_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](#_ENREF_14)]. 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](#_ENREF_15)]. SCC tumour characteristics that are risk factors of metastatic SCC and include: size[[16](#_ENREF_16)], depth[[16](#_ENREF_16),[17](#_ENREF_17)], thickness[[17](#_ENREF_17)], diameter[[18](#_ENREF_18)] and poor differentiation[[17](#_ENREF_17)]. Depth > 2.8 mm has a three-fold greater risk of metastasizing in KTR than the general population[[19](#_ENREF_19)].

Further evidence of tumour aggression is the invasive potential of SCC in KTR, with more perineural and lymphatic invasion that the general population[[20](#_ENREF_20)]. Metastatic incidence increases by 5%-8% with every SCC tumour accrued in KTR[[14](#_ENREF_14)]. Due to SCC lesions mainly located in UV exposed areas, e.g. the neck, face and scalp there is a possibility of invasion into subcutaneous cranial nerves in the perineural space, leading to extensive surgery and perhaps death[[21](#_ENREF_21)]. Reports observed an incident mortality of 1%-18%[[22](#_ENREF_22),[23](#_ENREF_23)]. Observational studies have showed a 37% incidence of SCC metastasizing[[18](#_ENREF_18)] which leads to the median KTR survival after diagnosis being only 2 years[[24](#_ENREF_24)]. Furthermore, it has been observed that a previous SCC is a risk factor for multiple SCC and even development of SOC[[11](#_ENREF_11),[13](#_ENREF_13),[19](#_ENREF_19)]. This is probably due to the exposure of pro-carcinogenic agents as well as the compounding effects of cancer induced, and pharmacological administrated, immunosuppression.

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.

**IMMUNOSUPPRESSION TYPE**

There are limited and conflicting data on the use of different types on immunosuppressive drugs and the associated cancer risks. The conflict mainly due to the multiple confounding factors associated to cancer, immunosuppressive drugs in particular have the dual capacity to suppress both anti-graft and anti-cancer immunity. The immunosuppressive drug 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 in mono-therapies and are therefore hard to compare one another; instead modes of action and evidence for cancer development are presented.

**AZATHIOPRINE**

Azathioprine (AZA) is catabolised to 6-mercaptopurine, which directly affects the synthesis of purines and has the ability to incorporate into DNA[[25](#_ENREF_25),[26](#_ENREF_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](#_ENREF_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](#_ENREF_25),[27](#_ENREF_27)]. These work synergistically to affect DNA repair which form lesions[[26](#_ENREF_26),[27](#_ENREF_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](#_ENREF_28)].

**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](#_ENREF_29)]. The reaction of MPA is reversible and does not interfere with the DNA structure as AZA does[[29](#_ENREF_29)]. One study showed a decrease photosensitivity when a cohort was randomised onto a MMF from AZA suppression regimen[[30](#_ENREF_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](#_ENREF_31)]. Conversely, a 3 group randomised control trial of 133 KTR; 45 KTR randomised to AZA treatment, 44 KTR randomised to 3 g daily of MMF and 44 KTR randomised to 3 g daily of MMF with no differences in cancer incidences between all three groups[[32](#_ENREF_32)].

**CALCINEURIN INHIBITORS**

Cyclosporine A (CsA) forms a complex with cyclophilin which inhibits calcineurin, making CsA and Calcineurin Inhibitor (CNI)[[33](#_ENREF_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](#_ENREF_34)]. Therefore CsA indirectly affects pro-infalmmatory 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](#_ENREF_35),[36](#_ENREF_36)]. Other tumorigenic side effects of CsA are direct or in-direct suppression of P53, production of TGF-β and VEGF[[37-39](#_ENREF_37)].

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 an AZA based regimens[[40](#_ENREF_40)]. In another retrospective study any regimen with CsA had an Odd Ratio of approximately 4.5[[41](#_ENREF_41)]. Inversely, A CsA based mono-therapy was shown to be less carcinogenic than a MMF and Prednisone (Pred) dual-therapy[[42](#_ENREF_42),[43](#_ENREF_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. TAC does not target cyclophilin, so 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](#_ENREF_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](#_ENREF_45)].

**CORTICOSTEROIDS**

Corticosteriods are mainly utilised for treatment of auto-immunity, inflammatory disorders and transplantation rejection. Corticosteriods 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](#_ENREF_49)]. Inhibition of these Th1 cytokines promotes a Th2 response, which provides another indirect immunosuppressive function[[55](#_ENREF_55)]. Corticosteroids induce TGF-b and can increase the incidence of Karposi’s sarcoma cell proliferation[[56](#_ENREF_56),[57](#_ENREF_57)].

**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)’s *via* mTORC 1 subunit (Raptor) binding and are considered mTOR inhibitors (mTORi). mTORi can also be classified as anti-proliferatives as they induce 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 affect protein synthesis, including Vascular endothelial growth factor (VEGF) which inhibits metastatic potential in murine models[[58](#_ENREF_58),[59](#_ENREF_59)]. SIR has been used to treat patients with Renal Cell Carcinoma (RCC) and EVO has shown to benefit patients with metastatic RCC who do not response to mainstream treatment[[60-62](#_ENREF_60)]. Sirolimus Conversion from CNI based regimens, is beneficial in Kaposi sarcoma and SCC involution[[63-66](#_ENREF_63)] However it can often lead to increased adverse reactions and increases in rejection episodes if performed too early post-transplant[[67](#_ENREF_67),[68](#_ENREF_68)].

**ANTI-THYMOCYTE GLOBULIN INDUCTION THERAPY**

Anti-thymocyte globulin (ATG) is either horse- or rabbit- derived antibodies directed against human T cells, given as an induction therapy of transplant recipients. The T cells that reconstitute have a regulatory phenotype and return much faster than other T cells[[69](#_ENREF_69)]. There is an association with prolonged CD4 lymphopenia and ATG as well as CD4 lymphopenia and cancer[[70](#_ENREF_70)]. Without knowing cause and effect it is speculative to say that ATG is associated with cancer.

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.

**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](#_ENREF_71)]. Furthermore, heart transplant patients have higher levels of immunosuppression than KTR and also have corresponding increases in cancer (100% compared to 88% 5 year incidence, respectively[[14](#_ENREF_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](#_ENREF_72)].

**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](#_ENREF_11),[73](#_ENREF_73)]. Included in the duration of immunosuppression would be the age and aging of the KTR.

**AGE AND GENDER**

Age is a risk factor of cancer development, independent of immunosuppression duration[[74](#_ENREF_74)]. This is exemplified in a retrospective study that showed both Age and male gender were risk factors[[41](#_ENREF_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](#_ENREF_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.

**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](#_ENREF_75)]. These increases in carcinogenesis are in part to the aforementioned AZA-UV interactions but mainly via direct UV-related mutagenesis. Due to the structure of DNA, it absorbs of UV-A (315-400 nm) and UV-B light (280-315 nm), in doing so the DNA itself forms cyclobutane pyrimidine dimers in two adjacent pyrimidines of the same DNS strand, which alters the structure of DNA and restricts transcription[[78](#_ENREF_78),[79](#_ENREF_79)]. A single point mutation can lead transcriptional arrest[[79](#_ENREF_79)]. A study found that invasive SCC contained mutations of the tumour suppressor gene P53[[80](#_ENREF_80)]. An important conclusion from this study is that P53 mutation could have happened in childhood, as most UV exposure happens in childhood[[81](#_ENREF_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](#_ENREF_82),[83](#_ENREF_83)]. Whereas the systemic immunosuppression may come from splenic cells, migrated Langerhans cells, dendritic cells. Increased expression of IL-4, IL-10, Prostogalndin E2, IL-1α and TNF-α with polarisation of immunity to a Th2 response also plays a role in systemic immunosuppression[[83-85](#_ENREF_83)]. In combination with this, co-stimulation is effected on both APC and T cells[[86](#_ENREF_86)]. Other cell types that are affected by UV irradiation are innate immune cells and suppressor cells[[87-91](#_ENREF_87)]. Regulatory T cells (Tregs) that are induced by UV express lymph node homing molecule CD62L and may provide systemic immunosuppression[[87](#_ENREF_87),[88](#_ENREF_88)].

The DNA damage and immune suppression of UV can be reversed by IL-12 dependent induction of nucleotide excision repair (NER) protein[[92](#_ENREF_92)]. Also immunity can be restored by the administration of IL-12[[93](#_ENREF_93)], activating APC’s, increasing IFN-γ and thus balancing Th1-Th2 polarisation[[93](#_ENREF_93), [94](#_ENREF_94)].

Other clinical parameters are 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.

**VIRAL INFECTION**

Human papillomavirus (HPV) is a group of more than 150 viruses with some types associating with anogenital, oropharyngeal and skin cancers[[95](#_ENREF_95),[96](#_ENREF_96)]. It has been speculated that HPV infection may prevent UV light-induced apoptosis[[97](#_ENREF_97)]. Between 65% and 90% of SCC lesions from transplant recipients are positive for HPV DNA[[98](#_ENREF_98)].

Epstein Barr virus (EBV) is associated with: sino-nasal angiocentric T-cell lymphoma, Hodgkin lymphoma and nasopharyngeal carcinoma[[95](#_ENREF_95)]. There are data that EBV associates with mononucleosis, Burkitt lymphoma and post-transplant lymphoproliferative disorder (PTLD) in KTR[[99](#_ENREF_99),[100](#_ENREF_100)].

Chronic Cytomegalovirus virus (CMV) infection can cause graft rejection, but with malignancy however it does have indirect associations with cancer[[99](#_ENREF_99)]. A prospective study followed 63 KTR and retrospectively included 131 KTR, with convincing data that CMV positive KTR with increased γδ T cell proportions, the Vδ2neg sub-population in particular, had decreased cancer incidence[[101](#_ENREF_101)]. This case-control study compared 18 short-term KTR (median 3 years post Tx), who developed 12 skin and 6 solid tumours over the prospective period and compared to 45 KTR who did not develop cancer. The skin nor solid organ tumour types were not disclosed.

**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](#_ENREF_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 Receive Operator Curve (ROC) analysis[[103](#_ENREF_103)]. Additionally, 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](#_ENREF_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](#_ENREF_104)]. All these studies showed an association with CD4 lymphopenia and cancer, however the majority of the cohorts underwent Anti-Thymocyte Globulin (ATG) induction therapy. However they did not define CD4+ subsets or other lymphocytes that may be affected by cancer.

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+CD127LoCD25Hi) and low numbers of Natural Killer (NK cells, *i.e.,* CD56+CD16+), in peripheral blood associated with and predicted recurrent SCC in KTR[[105](#_ENREF_105)]. 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[[106](#_ENREF_106)]. 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[[105](#_ENREF_105)]. This indicates that immunosuppression may affect immune phenotype and warrants investigation.

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, Vδ2 γδ T cells within their peripheral blood[[107](#_ENREF_107)]. Transplant patients have increased Regulatory T cells, B cells (memory B cells), CD8+ γδ T cells and CD8+ CD27-CD28- T cells and decreases in CD4 counts, NK cells and CD8+ Tcm[[105](#_ENREF_105),[108](#_ENREF_108)].

**REGULATORY T CELLS (TREGS)**

Immune suppressor cell existence has been debated from the early 1970’s through to the mid 1990’s[[109-112](#_ENREF_109)]. The pivotal paper adoptively transferred CD4+CD25+ T cells in CD25 depleted mice, which mitigated the autoimmune diseases that manifested[[112](#_ENREF_112)]. However, CD25 is also expressed on activated lymphocytes with only the highest proportion being suppressive *in vitro* via competitive absorption of IL-2[[112-115](#_ENREF_112)]. The discovery and transfection of the transcription factor *foxp3* into naïve T cells helped identify FOXP3 and its function as the master regulatory gene[[116](#_ENREF_116),[117](#_ENREF_117)] and CD127 inversed expression to FOXP3 expression has given Tregs the current phenotype CD4+FOXP3+CD25hiCD127lo [[114](#_ENREF_114)].

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[[118-120](#_ENREF_118)]. Both IPEX and X-linked Autoimmunity-Allergic Dysregulation (XLAAD) syndrome cause multi-organ failure due to mass lymphocyte proliferation of self-reactive effector cells [[119](#_ENREF_119)].

**CD4+ TREG SUBSETS**

The CD4+ Treg in the periphery, defined by FOXP3+CD25hiCD127lo, 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)[[121](#_ENREF_121)]. 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[[122](#_ENREF_122)]. Though the premise that Helios only defines nTreg is currently under debate, nonetheless, it may provide evidence of *in vivo* activated Tregs[[101](#_ENREF_101),[123](#_ENREF_123)]. Despite the debate it seems that KTR with cancer have similar Helios expression than KTR without cancer[[108](#_ENREF_108)].

**TREG MODES OF ACTION**

Treg apoptosis induction requires cell contact with co-stimulatory molecule Cytotoxic T cell Late Antigen-4 (CTLA-4), Fas/Fas ligand interaction and release of Perforin and Granzyme B[[124-126](#_ENREF_124)]. Indirectly, Tregs can down-regulate B7 Co-stimulation molecules CD80/CD86 on Antigen Presenting Cells (APC[[127](#_ENREF_127)]). 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[[128](#_ENREF_128)]. 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[[129](#_ENREF_129)]. Expression of CD39 and CD73 has been shown on murine and human Tregs[[129](#_ENREF_129)]. 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[[130](#_ENREF_130),[131](#_ENREF_131)]. The adenosine formed by the hydrolysis of ATP can regulate lymphocyte proliferation in autoimmune disease, transplantation and cancers[[132-134](#_ENREF_132)]. Additionally, it has been shown that adenosine and PGE2 in Tregs co-operate when regulating immune responses[[133](#_ENREF_133)]. Other regulatory cells are CD4+ helpers that have suppressive function are classified by the ability to secret of IL-10 (Tr1) and TGF-β (Th3) which they are also induced by, respectively.

**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](#_ENREF_100),[135](#_ENREF_135)]. 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 have an early role in protective immunity to HSV infection, similarly observed in Lymphocytic Choriomenigitis virus mouse model, shown in the same study[[136](#_ENREF_136)].

**TREGS AND TRANSPLANTATION**

In regards to transplantation, when isolated CD4+CD25- cells are administered to BLABc nu/nu mice grafted with C57BL/6 skin there is a swifter rejection rate than administering untouched lymphocytes of the same source[[112](#_ENREF_112)]. This indicates CD4+CD25- T cellsubpopulation has greater cytotoxicity when absent from CD4+CD25+ T cells and that CD4+CD25+ T cells are possible inducers of tolerance.

In KTR, 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+CD25hi cells with similar FOXP3 transcription levels when compared to the healthy controls[[137](#_ENREF_137),[138](#_ENREF_138)] and that chronically rejecting KTR had lower CD4+CD25hi cells with low FOXP3 transcripts, indicating that Tregs may be protective or involved with tolerance[[137](#_ENREF_137),[138](#_ENREF_138)]. An additional study supported this in liver transplant recipients which showed increased FOXP3 mRNA expression in CD4+CD25hi T cells of tolerant patients compared to patients who had rejection episodes after cessation of immunosuppression[[139](#_ENREF_139)]. Thus induction of Tregs for suppression of allograft cellular rejection episodes[[140](#_ENREF_140)] and possible induction of tolerance[[141](#_ENREF_141)] seem 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 episodes[[142](#_ENREF_142)] in KTR. Increases in Tregs are also associated with cancer in the general population[[143](#_ENREF_143)] and KTR[[105](#_ENREF_105)].

**TREGS IN CANCER AND IMMUNE SURVEILLANCE**

It has been shown that the percentage of CD4+CD25highFOXP3+ Tregs and Tr1 cells are increased in Head and Neck Squamous Cell Carcinoma (HNSCC) patients in comparison to healthy controls[[144](#_ENREF_144),[145](#_ENREF_145)]. Ectonucleotidase activity contributed by CD39 and CD73 is also increased on Tregs in this cohort[[133](#_ENREF_133)]. 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[[132](#_ENREF_132)]. It has been shown that high levels of Treg in HNSCC patients from the general population associate with a poor prognosis[[146-148](#_ENREF_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[[145](#_ENREF_145)]. 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](#_ENREF_100)]. CD39 and CD73 have been shown to be expressed on Tr1 and tumour cells alike[[129](#_ENREF_129),[149](#_ENREF_149)]. Cancer progresses by the tumours’ ability to secrete these soluble factors into its microenvironment. Prostoglandin E2 (PGE2) 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[[145](#_ENREF_145),[150](#_ENREF_150),[151](#_ENREF_151)]*.*

In a post-transplant cancer setting, it has been shown that Tregs (CD4+FOXP3+CD25hiCD127lo) in blood from KTR with a history of SCC can predict the risk of developing a subsequent SCC lesion[[105](#_ENREF_105)]. Another study has shown that Tregs alone can predict cancer onset and associate to the severity of the cancer developed[[108](#_ENREF_108)]. In this same study Hope *et al*[[108](#_ENREF_108)] shows prospectively that Tregs increase in KTR when the cancer becomes apparent and then decreases post-resection of tumour tissue.

**NK CELLS IN CANCER AND IMMUNE SURVEILLANCE**

Carroll *et al*[[152](#_ENREF_152)] revealed 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. 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[[153](#_ENREF_153)]. It is an important step in metastatic cells to successfully invade the host[[154](#_ENREF_154)]. 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[[152](#_ENREF_152)]. 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[[155](#_ENREF_155)]. 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[[155](#_ENREF_155)]. This cytotoxic ability to kill cancer cells can be inhibited by Tregs but also cancer cells themselves[[156](#_ENREF_156),[157](#_ENREF_157)]. This NK-Treg interaction is a TGF-β and cell-cell contact mechanism of down-regulation NKG2D and induction of apoptosis, respectively[[158](#_ENREF_158),[159](#_ENREF_159)]. This leads to decreased NK cell numbers and function in the peripheral blood of cancer patients that have elevated TGF-β[[160](#_ENREF_160),[161](#_ENREF_161)]. 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, CD56bright NK cells [[162-164](#_ENREF_162)]. Both these cells can interact with the adaptive immune system and enhance anti-tumour ability by direct and indirect mechanism respectively[[162](#_ENREF_162),[164](#_ENREF_164)].

**CD8 SUBSETS IN CANCER AND IMMUNE SURVEILLANCE**

Another cell type with anti-tumour properties is CD8+ cytotoxic T lymphocytes (CTL). CD8+ cytotoxic T cells or lymphocytes (CTL) are in the effector arm of the adaptive immune system. CTLs use the ability to lyse tumour cells using Fas-Fas ligand as well as perforin-IFN-γ granules similar to NK cells[[165](#_ENREF_165)]. It has been shown that antigen specific CTL are defective in cancer patients and that removal of Tregs can restore cytolytic function[[166-168](#_ENREF_166)].

CD4 and CD8 T cells follow an immunogenic pathway to immune senescence. T cells exiting the thymus are naïve since they express both CD27 and CD28 co-stimulation molecules and home to the lymphoid organs[[169](#_ENREF_169),[170](#_ENREF_170)]. When antigen is presented they become CTL, clear the threat, and the majority apoptose with the minority homing to lymphoid organs as central memory T cells or extra-lympoid sites as effector memory T cells[[169](#_ENREF_169),[170](#_ENREF_170)]. Upon subsequent exposures the cells become exhausted and lose expression of co-stimulation molecules and are termed T effector memory CD45RA+ or TemRA cells[[169](#_ENREF_169),[171](#_ENREF_171)]. These cells are loosely phenotyped as CD8+CD28- and shown to be regulatory in cancer patients and may associate with poor prognosis[[106](#_ENREF_106)]. Tumours themselves may induce this loss of CD28[[106](#_ENREF_106),[172](#_ENREF_172)] and they are also expanded in patients with CMV infection[[173](#_ENREF_173)]. It has been shown that Memory T cells and Nautral Killer (NK) cells have anti-tumorigenic properties and that Tregs regulate both of these lymphocyte subsets[[158](#_ENREF_158),[174](#_ENREF_174)]. Thus, an excess of Tregs is associated with poor prognosis in cancer and is thought to aid cancer cells evade this immune surveillance.

**IMMUNE CELL FUNCTIONS**

Kidney Transplant Recipients (KTR) with cancer have increased numbers and proportions of Regulatory T cells (Tregs) and decreased numbers and proportions of Natural Killer (NK) cells[[105](#_ENREF_105),[108](#_ENREF_108)]. 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 KTR with cancer have higher suppressive function than Tregs from the blood of normal donors[[145](#_ENREF_145),[175](#_ENREF_175),[176](#_ENREF_176)]‎. 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[[177](#_ENREF_177)]‎ and, as such, also associate with poor cancer prognosis in the general population[[176](#_ENREF_176)].

In the Transplant population it is known that 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[[178](#_ENREF_178),[179](#_ENREF_179)]. Furthermore, 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[[180](#_ENREF_180)]. 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[[178](#_ENREF_178),[181](#_ENREF_181)]. Additionally, FOXP3 mRNA transcription was decreased in CNI treated PBMC compared to Rapamycin in an allo-stimulated mixed lymphocyte reaction[[182](#_ENREF_182)]. There is also an inverse correlation to CNI level and Treg function[[183](#_ENREF_183)].

 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[[184](#_ENREF_184)]).

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[[152](#_ENREF_152),[185](#_ENREF_185)]. 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)[[154](#_ENREF_154),[155](#_ENREF_155),[186](#_ENREF_186)]. 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[[187](#_ENREF_187)]. 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[[188](#_ENREF_188)]. 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[[155](#_ENREF_155)]. 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[[189](#_ENREF_189)], utilising glycolysis and lactate pathways, via Lactate Dehydrogenase (LDH), causing an 18-fold increase in glucose utilisation, even under aerobic conditions[[190](#_ENREF_190)]. This LDH can be measured as a cytotoxic assay (first described in 1988[[191](#_ENREF_191)]). 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*[[192](#_ENREF_192),[193](#_ENREF_193)]. 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[[192](#_ENREF_192)]. 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[[193](#_ENREF_193)].

**TREATMENT OPTIONS FOR KTR WITH CANCER**

The aforementioned assays give clinicians the ability to objectively identify patients that may develop pre-metastatic cancer with relatively high specificity and specificity. 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](#_ENREF_72)], thus investigating the benefit of reduced immunosuppression as primary cancer prevention. However, those with reduced CNI had increases in rejection episodes[[72](#_ENREF_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[[194](#_ENREF_194),[195](#_ENREF_195)]. There was a benefit, however not all conversions were successful (30%) and an additional 30% did not tolerate the mTORi side effects[[14](#_ENREF_14),[196](#_ENREF_196),[197](#_ENREF_197)]. 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[[198-200](#_ENREF_198)]. Monitoring HLA molecules and specific Donor Specific Antibodies (DSA) routinely has decreased antibody mediated rejection episodes dramatically[[201](#_ENREF_201),[202](#_ENREF_202)]. Interferon-γ ELISPOT has been used to predict 6-month graft function and rejection episodes[[200](#_ENREF_200)]. Additionally it has been used pre-transplant to categorise patients into CNI or mTORi maintenance therapy[[203](#_ENREF_203)]. 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)”[[204](#_ENREF_204)]. 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[[205](#_ENREF_205),[206](#_ENREF_206)]. 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[[205](#_ENREF_205),[206](#_ENREF_206)]. KTR with unresolved BK pathogenesis also had a non-significant decrease in EBV peptide and PHA mitogenic IFN-γ ELISPOT responses[[205](#_ENREF_205)], indicating over-immunosuppression[[207](#_ENREF_207)]. This may share a link with development of malignancy as they are both considered manifestations of over-immunosuppression.

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 trail comparing CNI doses showed a benefit of low dose CNI in a reduction in cancer incidence, although there was an association with increase rejection rates[[72](#_ENREF_72)].

Alternatively, secondary prevention consists of CNI conversion to an mTORi based regime which has been done[[194](#_ENREF_194),[195](#_ENREF_195)]. It has been shown that mTORi can selectively expand Tregs *in vitro* and reduce IL-2 signalling, which has the potential to treat cancer patients[[208](#_ENREF_208)]. 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[196,197,[209](#_ENREF_209)]. However, 30% of SRL patients do not tolerate its side effects and sudden SRL switching can cause poteinuria[[210](#_ENREF_212)].

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. Failure of Tregs to fall after tumour excision, chemo or immunotherapy is due to incomplete resection or predicted relapse of disease[[211](#_ENREF_213),[212](#_ENREF_214)].

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[[105](#_ENREF_105)]. 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.

**PRE-TREATMENT ALLORESPONSE MEASURES**

Assessment of allo-responses 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[[198](#_ENREF_198),[213](#_ENREF_215)]. Inflammatory cytokines such as IFN-γ are secreted by Th1 effector T cells and are a predictor of acute rejection and infection[[200](#_ENREF_200),[204](#_ENREF_204)]. A National Institute of Health (NIH) funded Clinical Trials in Organ Transplant (CTOT) consortium approved ELISPOT has been able to detect 6-month post-transplant acute rejection in pre-transplant patients[[214](#_ENREF_216),[215](#_ENREF_217)]. Additionally a similar assay has been used to run CNI avoidance maintenance therapy with a 3-fold reduction in acute rejection as shown in literature[[203](#_ENREF_218)]. The humoral aspect of the immune system is already routinely assessed in most transplant programmes by solid phase alloantibody detection systems[[202](#_ENREF_202)]. HLA Donor-Specific Antibodies (DSA) are clinically relevant and observed DSA presence has informed clinicians to alter immunosuppression regime of patients[[199](#_ENREF_199),[201](#_ENREF_201)]. However both these techniques have not been measured in long-term kidney transplant recipients with a history of cancer.

**CONCLUDING REMARKS**

Long-term immunosuppression increases the risk of cancer development. The dose of immunosuppression can be increased by closely monitoring graft function and survival. In this review we present that there are several emerging immune monitoring tools that are available to potentially help reduce immunosuppression. Future studies may be undertaken to determine if these assays can help identify those at risk of cancer development and if reduction of immunosuppression is of benefit.

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