The utilisation of biomarkers of a reduced immune response to predict squamous cell carcinoma in long-term renal transplant recipients

Matthew Bottomley

Green-Templeton College



And Nuffield Department of Surgical Sciences

University of Oxford

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Matthew Bottomley, Green-Templeton College

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Cutaneous squamous cell carcinoma (SCC) is the most common malignancy in renal transplant recipients (RTR) with an incidence up to 250 times that of the general population. SCC is frequently recurrent and more aggressive than in non-transplant cohorts. Immunological and clinical measures have been developed to stratify RTR by future SCC risk but their comparative performance in long-term RTR has not been assessed.

We hypothesised that biomarkers of two potential mechanisms underlying a reduced immune response may predict SCC development. These biomarkers comprised 'signatures' of immune regulation developed in RTR who have successfully ceased immunosuppression without development of graft dysfunction ('operational tolerance') and immunophenotypic changes associated with immunosenescence. Immunosenescence leads to a reduction of immune responses with increasing age.

A prospective cohort study was undertaken of 117 long-term RTR, half with a history of previous SCC. RTR were stratified by phenotype based on a majority ('CD57hi') or minority ('CD57lo') of peripheral blood $CD8^+$ T cells expressing CD57. CD57hi RTR were 2.4 times more likely to develop SCC during follow-up, independent of age or history of previous SCC. This was more predictive than previously developed clinical and immunological measures. CD57hi RTR exhibited other functional and phenotypic changes associated with immunosenescence.

Signatures of operational tolerance did not predict SCC risk but were heavily influenced by the effect upon circulating B cells due to immunosuppressive regime, particularly azathioprine and calcineurin inhibition. Further evaluation indicated that azathioprine use was associated with the presence of donor-specific antibodies.

This study indicates that biomarkers of 'operational tolerance' are confounded by immunosuppression use and may not be suitable for clinical application in their current format. Biomarkers of immunosenescence, such as the CD57hi phenotype proposed in this study, may identify RTR at increased risk of subsequent SCC who may benefit from pre-emptive immunosuppression reduction.

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Abbreviations and glossary

βPV	Beta papilloma virus (human papilloma virus of the beta genus)
Allogeneic transplant	Transplant between genetically distinct organisms
Allograft	Tissue transplanted from donor genetically distinct (allogeneic) to recipient
AMANN	Alpha-1,2-mannosidase
ANOVA test	Analysis of variance test
APC	Antigen presenting cell(s)
ATG	Anti-thymocyte globulin (also known as 'thymoglobulin' [trade name]) – a polyclonal T cell depleting agent
BAFF	B-cell activating factor
Basiliximab	A non-depleting anti-interleukin 2 receptor (CD25) monoclonal antibody
BCC	Basal cell carcinoma
BFA	Brefeldin A
BMI	Body mass index
Breg	B cells that display suppressive function *ex vivo*
CD	Cluster of differentiation
CMV	Cytomegalovirus
mCMV	Murine cytomegalovirus
CnI	Calcineurin inhibitor
CVD	Cardiovascular disease
DBD	Donation after brain death
DCD	Donation after cardiac death
DMBA	7,12-dimethylbenz[α]anthracene
DMSO	Dimethyl sulfoxide
DSA	Donor-specific antibody/antibodies
DTH	Delayed-type hypersensitivity
DWFG	Death with a functioning graph
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
FACS buffer	PBS supplemented with 2% FCS and 0.1% sodium azide
FCS	Foetal calf serum
FOXP3	Forkhead box protein P3 (also known as 'scurfin')
gdT cell	Gamma-delta T cell ($CD3^+$gamma-delta-TCR^+)
GUSB	Beta-glucuronidase
GWAS	Genome-wide association study
HPV	Human papilloma virus
HS^{AT+}	Antithrombin-binding heparan sulfate
IE	Immediate-early; proteins produced early in the CMV viral infection cycle that

	do not require *de novo* synthesis of protein or virome integration
IFN	Interferon
IFTA	Interstitial fibrosis and tubular atrophy (previously referred to as 'chronic allograft nephropathy')
IL	Interleukin
IQR	Interquartile range
IRP	Immune risk profile (also called 'immune risk phenotype' by some authors)
Isograft	Tissue transplanted from donor genetically identical (syngeneic) to recipient
ITN consortium	"Immune Tolerance Network" consortium (based in the US)
KLRG-1	Killer cell lectin-like receptor G1
LCMV	Lymphocytic choriomeningitis virus
MB	Matthew Bottomley
MDRD	Modified Diet in Renal Disease
MFI	Mean fluorescence intensity
MMF	Mycophenolate mofetil
NK cell	Natural killer cell ($CD3^-CD56^+$)
NMSC	Non-melanoma skin cancer
NODAT	New-onset diabetes after transplantation
Ns	Not significant
OpTS	Operational tolerance 'signature'
OTC	Oxford Transplant Centre
OTR	Organ transplant recipient(s)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Programmed cell death protein
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
PTLD	Post-transplant lymphoproliferative disease
pTreg	Peripherally-induced Treg. Treg that arise from conversion of $CD4^+CD25^-$ T cells in the periphery.
qPCR	Quantitative polymerase chain reaction
R10	1640-RPMI enriched with 10% FCS, 2mM L-glutamine and 100U/mL penicillin and streptomycin (also referred to as 'complete media')
REC	Research Ethics Committee
RISET consortium	"Reprogramming the Immune System for the Establishment of Tolerance" consortium (based in EU and Israel)
RT	Room temperature
RT-PCR	Real time-polymerase chain reaction (also can refer to reverse transcription PCR) – to avoid confusion in this study "qPCR" (quantitative PCR) is used.
RTR	Renal transplant recipient(s)
RTR_{No}	Renal transplant recipient taking part in the study without a history of previous SCC at enrolment

RTR_{SCC}	Renal transplant recipient taking part in the study with a history of previous SCC at enrolment
SCC	Cutaneous squamous cell carcinoma
SEM	Standard error of the mean
SIR	Standardised incidence rate. The rate of a condition or disease compared to another population (usually the incidence within the age- and gender-matched general population in the context of transplantation)
Syngeneic transplant	Transplant between genetically identical organisms (e.g. identical twins)
TCm	Central memory T-cell
Tem	Effector memory T-cell
TEmRA	Effector memory T-cell expressing CD45RA
TGF	Transforming growth factor
Tim	T-cell immunoglobulin and mucin domain
TNF	Tumour necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate
Treg	Regulatory T-cell ($CD3^{+}CD4^{+}CD69^{-}CD25^{hi}CD127^{lo}FoxP3^{+}$ for the purposes of this thesis though other phenotypes have been defined)
TSDR	Regulatory T cell (Treg) specific demethylated region
tTreg	Thymically-derived Treg. Treg that arise naturally during development during T cell maturation.
UVR	Ultraviolet (UV) radiation
Xenograft	Tissue transplanted between species

Throughout this thesis, genes are italicised, whilst proteins are not (e.g. '*FOXP3*' refers to the gene; 'FOXP3' refers to the protein).

Statistical denotation

ns	Not significant
*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Chapter 1: Introduction

1.1 Long-term graft and patient survival as a barrier to improving outcomes

Over the last thirty years improvements in surgical technique and the prevention and management of acute rejection have led to remarkable improvements in short-term kidney allograft survival. One-year cadaveric graft survival in the early eighties was approximately 80%, with around half of all recipients experiencing an episode of acute rejection [1, 2]. In contrast, one-year graft survival in 2010 irrespective of donor origin was in excess of 95%, with less than 15% of recipients experiencing an episode of acute rejection [3-5].

These improvements mean transplantation has become the gold standard treatment for end-stage renal failure (ESRF). Renal transplant recipients (RTR) consistently demonstrate improved survival and a better quality of life compared to those who remain on dialysis [6-8]. Additionally, it is more cost-effective to receive a transplant than to remain on dialysis [9].

As short-term (less than one year post-transplant) survival improved, it was presumed that this would be reflected in long-term (greater than one year post-transplant) graft survival. However, a number of studies have highlighted that the rate of graft loss ("graft attrition") beyond one year post-transplant is relatively unchanged over the last twenty years [5, 10-13]. The data for the UK over the last decade is shown in Figure 1.1.

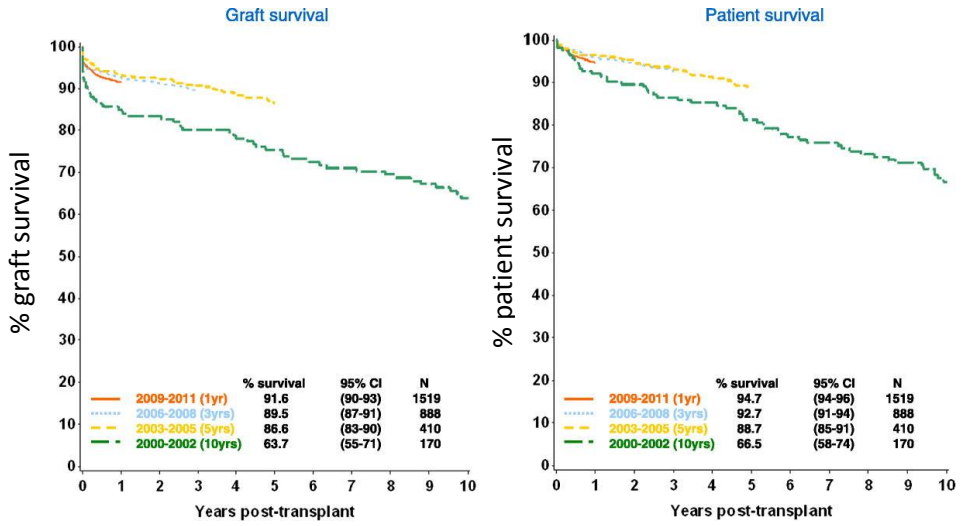


Figure 1.1: Graft & patient attrition in donation after cardiac death (DCD) renal transplantation by year of transplant in the UK. *The improvement in graft outcomes is seen in the first six months post-transplant: after this the rate of (non-death censored) graft attrition and patient survival is unchanged. DCD data was chosen as it illustrates the point most clearly but the same trend is seen for donation after brain death (DBD) and living donation. Data from NHS Blood and Transplant renal transplant registry [14].*

The latest registry data from the US suggest that whilst the likelihood of a return to dialysis by ten years post-transplant may be falling, the incidence of death with a functioning graft (DWFG) has increased and now accounts for approximately half of all graft loss after one year post-transplant [5]. This means a major barrier to long-term graft survival is actually patient survival as improvements in long-term graft survival have been offset by increased mortality post-transplant (Figure 1.2).

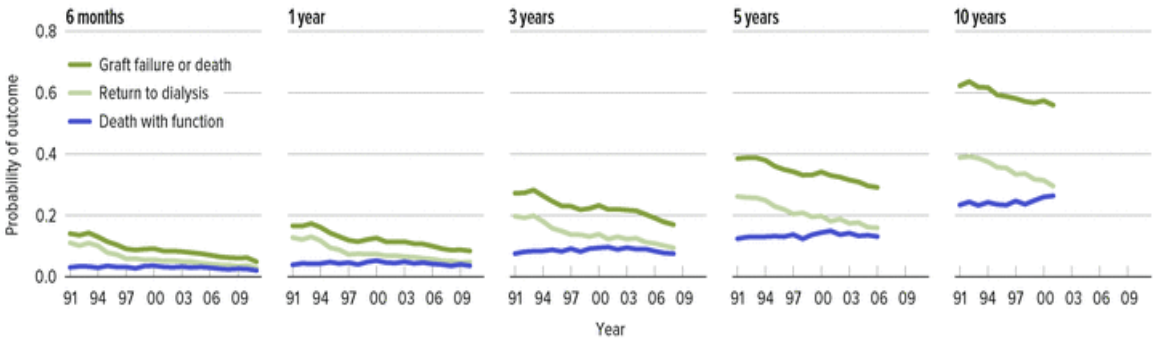


Figure 1.2: Likelihood of graft failure or DWFG after transplant in the US by year of transplant. *Whilst the likelihood of graft failure is falling in long-term RTR, the likelihood of DWFG is climbing. Reproduced from the Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients (OPTN/SRTR) annual report for 2011 [5].*

This increase in DWFG is due partly to the increasing average age of recipients compared to ten years ago [5, 14, 15]. Improved perioperative medical and surgical care combined with the accumulating evidence of superiority of transplantation over other modalities of renal replacement therapy means that the risk-benefit ratio increasingly favours the use of transplantation in recipients who previously would have been considered too high risk due to other comorbidities. Thus the increased incidence of DWFG is partially a reflection of transplantation in older, more infirm patients: whether outcomes in the remaining 'optimal' transplants have improved remains contentious [16, 17]. However, it is generally accepted that long-term transplant outcomes are influenced by factors distinct from those affecting short-term outcomes.

1.2 Death with a functioning graft

The chief causes of DWFG in long-term RTR are cardiovascular disease (CVD) and malignancy [11, 18, 19]. CVD is more prevalent in RTR due to adverse metabolic factors associated with uraemia and chronic inflammation pre-transplant, as well as the impact of immunosuppressive agents post-transplant [20]. For example tacrolimus, a calcineurin inhibitor, is associated with 'new onset diabetes after transplant' (NODAT); corticosteroids (subsequently referred to as 'steroids') are associated with hypertension and dyslipidaemia; and mammalian target of rapamycin (mTOR) inhibitors such as sirolimus are associated with proteinuria and dyslipidaemia. However, aggressive primary and secondary prevention of CVD in RTR, along with increased awareness of the atypical presentation of CVD in this population, have led to reductions in mortality attributable to this [18]. In contrast to both the above, malignancy is increasingly frequent and in Australia and New Zealand has now overtaken CVD to become the leading cause of DWFG since 2002 [18, 21]. Malignancy, predominantly *de novo* post-transplant [22, 23], is rapidly becoming one of the key barriers to long-term RTR survival.

1.3 Malignancy after organ transplantation

Contemporary registry studies suggest the incidence of malignancy in RTR is at least three times that of the general population [24-28] but the true incidence is likely to be greater than this as cutaneous malignancy is poorly reported to modern transplant registries (see below). The overrepresentation of malignancy in transplant recipients is not universal; certain malignancies such as breast and prostate do not appear to be more common in transplant populations. In contrast, malignancies with a viral aetiology such as Kaposi's sarcoma (human herpesvirus 8) and lymphoma (Epstein-Barr virus) are significantly overrepresented.

Not only is malignancy more common in transplant recipients, outcomes are also poorer. Interrogation of the Cincinatti International Tumour Registry suggests that cancer is often more advanced at time of diagnosis and is associated with poorer survival compared to non-transplanted cohorts, irrespective of stage [29].

1.4 Post-transplant non-melanomatous skin cancer

The most common site for malignancy after transplant is the skin and is predominantly non-melanomatous skin cancer (NMSC). NMSC consists commonly of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), although rare tumours such as Merkel cell and sebaceous carcinomata are also included. Whereas the relative incidence of BCC and SCC in the general population is approximately 4:1, this ratio is reversed in transplant recipients [30-36]. This means that SCC is by far the most common malignancy in RTR with up to a 250-fold increased risk compared to the general population [33, 34, 37-40]. Extrapolating from studies in the general population, the development of NMSC may identify RTR at increased risk of subsequent malignancy in other solid organs [41-44].

The incidence of SCC over time following transplant varies according to a number of factors. The strongest of these factors is cumulative UV exposure; accordingly Australasia suffers the highest rate of post-transplant NMSC in the world with an estimated 20 year cumulative SCC

incidence of 50-80% [31, 32, 45-47]. In Northern Europe and the UK the incidence at twenty years is around 20-30% based predominantly on single centre reports [33, 35, 37, 48]. One retrospective study in the UK estimated thirty year prevalence to be as high as 65% [34]. Duration of immunosuppression plays a key role also; a prospective study in the UK estimated the annual incidence of SCC in a white RTR cohort at 1.3% in the first five years, 1.4% between five and ten years, and 5.3% after ten years post-transplant [49]. The median time from renal transplant to first SCC is around 8-10 years [30, 31, 34, 50, 51].

The true excess incidence of NMSC in transplant populations is likely to be at least 30% greater than registry data suggest [46, 52-55]. Many national and international registries report only the first incidence of each type of malignancy and those removed in a primary or private healthcare setting, particularly BCC, may not be reported [54]. Furthermore, this discrepancy is not homogenous but for unclear reasons appears to be greater in males and with increasing age [54]. This must be considered when interpreting registry-based data and has led to some registry studies, particularly those in Australasia, opting not to include NMSC when undertaking research on risk factors for malignancy in transplant populations.

In addition to increased incidence, post-transplant SCC is considerably more aggressive than in the general population. In organ transplant recipients (OTR) SCC occurs at least a decade earlier in life than would otherwise be expected [56, 57], with around 30-50% developing a further SCC within twelve months of a first, in contrast to less than 10% within five years in the general population [34, 35, 58-61]. Half of OTR with SCC will develop more than one SCC per year during follow-up [34]. The risk of metastasis in OTR is up to 8%, double that of the general population [60, 62-64]. The median survival from diagnosis of metastasis in OTR is less than three years [65].

SCC from OTR differ histologically from those in the general population, with an increased propensity to develop a spindle cell morphology and a decreased inflammatory infiltrate [56].

Further analysis of this infiltrate has consistently demonstrated a reduction in the peritumoural $CD8^+/FOXP3^+$ ratio compared to non-OTR [66-68]. In malignancies in non-transplant populations a decreased $CD8^+/FOXP3^+$ ratio within the tumour has been associated with a poorer prognosis [69-71].

NMSC represents a significant post-transplant burden. The accumulation of multiple NMSC is a major cause of morbidity amongst RTR. As well as an emotional and physical burden on the patient there is also an economic implication for the healthcare provider as current guidelines recommend regular annual dermatological screening for all RTR [72]. 3% of Australian RTR with NMSC undergo plastic surgical procedures requiring inpatient stays per year [32].

There are currently no objective clinical methods to stratify risk of SCC in order to guide the frequency of screening: a number of clinical risk scores have been proposed having been developed in distinct populations, but none have been validated in independent cohorts [32, 34, 73, 74].

The factors involved in SCC development in RTR are complex and interacting. These will be considered in turn.

1.5 Clinical factors contributing to post-transplant SCC risk

Three chief clinical factors that contribute to SCC risk are UV radiation exposure [73-76]; duration, intensity and type of immunosuppression [25, 33, 47, 66, 74, 77-82]; and age at transplant [30, 32-34, 40, 73, 74].

1.5.1 UV radiation exposure and skin type

The contribution of UV radiation to SCC risk is evidenced by the increased disease incidence in both transplant and non-transplant populations in countries with closer proximity to the equator [75]. SCC most commonly develops on UV-exposed areas of the body such as the

arms and craniofacial region [33, 76, 79]. Both the risk and tumour burden of SCC is increased in RTR reporting previous UV exposure [73, 74, 76, 79].

UV radiation, particularly UVB, is mutagenic through formation of thymidine dimers and reactive oxygen species [83, 84]. In particular, inactivating mutations in the tumour suppressor gene p53 have been implicated [85, 86] with some evidence of a role for activating mutations in oncogenes such as Ras [87-90]. p53 mutations are detected in a high proportion of actinic keratoses and SCC and associate with resistance to stress-induced apoptosis in keratinocytes [91].

UV radiation has a suppressive effect on the adaptive immune system occurring through a number of mechanisms. The relevance of this is seen in a tumour transplant model where chronic UV radiation-induced SCC, which are rejected by healthy mice, are accepted by mice pre-treated with either immunosuppressive drugs or UV radiation [92]. UV radiation depletes the epidermis of mature Langerhans cells (cutaneous antigen presenting cells [APC]) by inducing DNA damage which leads to their migration to regional lymph nodes [93, 94]. Antigen presentation by these damaged cells favours the generation of tolerant and suppressive T cell populations [95]. UV radiation also leads to infiltration and expansion of immature Langerhans cells, IL-10 producing $CD11b^+$ macrophage populations and $CD4^+CD25^+FOXP3^+$ regulatory T cells within the epidermis [96-100]. Various populations of UV-induced regulatory T cells have been identified within this system depending on the conditions used but the best characterised resemble the 'prototypic' $CD4^+CD25^+FOXP3^+$ cell [101-103].

The tolerising effect of UV radiation, if given at significant doses, can be systemic. In mice and humans application of a hapten to skin leads to sensitisation and a hypersensitivity response upon re-challenge. Contact hypersensitivity upon repeat exposure to a hapten is inhibited if the sensitisation site is pre-treated with UV radiation due to the mechanisms outlined above. This effect is not seen if an alternate hapten is applied, indicating the effect is antigen-specific

[104-106]. However, this hyporesponsiveness upon re-challenge also occurs in remote areas of skin that were not exposed to UV radiation and can also be induced in healthy, unirradiated mice by adoptive transfer of splenocytes from UV irradiated mice [92, 104, 107].

The effect of UV radiation upon remote immune responses has led to the suggestion that SCC development correlates with UV-mediated suppression of cell-mediated immunity in humans [105, 106, 108]. Previous work in long-term RTR has demonstrated that those with a history of SCC have a greater proportion and number of circulating $CD4^+CD25^+FOXP3^+$ Treg compared to RTR without a history of SCC. It is unknown whether these are induced by UV radiation due to a lack of specific markers for UV-induced Treg [66, 109].

The immunosuppressive and mutagenic effect of UV radiation is not absolute and synergises with the phenotype of the individual, in particular skin type, but this is also reflected in hair and eye colour. Non-brown eyes, blonde or ginger hair and paler skin have been reproducibly associated with increased skin cancer risk in various transplant and non-transplant cohorts [74, 79, 110-114]. This may be due to reduced constitutive melanin production and altered distribution of melanocytes in paler skin as well as a diminished induction of melanin upon UV exposure [115]. Melanin absorbs UV radiation and UV-generated free radicals, preventing penetration to the rapidly dividing cells in the basal layer of the epidermis [116]. This may also reduce the susceptibility to UV-mediated immunosuppression [105].

1.5.2 Pharmacological immunosuppression

Maintenance pharmacological immunosuppression is required to prevent rejection of an allograft. However, not only does immunosuppression prevent an alloresponse, it also impairs immune surveillance and anti-tumour responses [117-119] as well as having direct effects to promote malignant transformation.

Since the publication of the results from the ELITE-Symphony study, the UK has predominantly used mycophenolate mofetil (MMF) and tacrolimus or sirolimus as long-term maintenance

therapy after renal transplant [4, 72]; prior to this a combination of azathioprine, ciclosporin and/or prednisolone was used. The duration of immunosuppression correlates with immunosuppression risk as discussed but also the intensity of immunosuppression. A retrospective registry study found that maintenance therapy with ciclosporin, prednisolone and azathioprine was associated with a three-fold increased risk of SCC compared to prednisolone and azathioprine only [40], confirming the results of earlier single-centre reports [120, 121]. Heart transplant recipients who receive greater maintenance doses of immunosuppression develop a greater number of SCC at an earlier stage post-transplant and are more likely to develop metastasis compared to RTR [40, 77, 122, 123].

Only a single prospective study has assessed the effect of immunosuppression reduction on subsequent development of malignancy: in a study of 231 RTR, 8 of 116 randomised to receive a reduced dose of ciclosporin developed SCC compared to 15 of 115 who received 'normal' dose ciclosporin during the first five years after transplant [124]. However, the rate of acute rejection was significantly greater in the reduced dose arm (9 episodes vs 1).

In animal models and *in vitro* cell culture, tacrolimus, ciclosporin, azathioprine and steroids have all been demonstrated to have potentially mutagenic effects directly upon premalignant and malignant cells.

Tacrolimus and ciclosporin, collectively referred to as 'calcineurin inhibitors', promote the development and progression of tumours in animal models by increasing synthesis of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) [125-127] as well as suppressing UV radiation induced apoptosis and DNA repair in the skin [128, 129].

Azathioprine is incorporated into DNA where it enables greater UV energy absorption, creates mutagenic reactive oxygen species and inhibits DNA repair [130-132]. Steroids inhibit the

function of NF-κB [133] which may synergise with UV-induced Ras mutations to lead to epidermal neoplasia [89, 90].

All these agents have been associated with increased risk of SCC in patients receiving them as monotherapy for other indications such as asthma or autoimmunity [134-137]. In contrast, sirolimus, a mammalian target of rapamycin (mTOR) inhibitor, is generally considered anti-neoplastic with a number of effects including reduction of the effect of VEGF and sensitisation of tumour cells to DNA damage-induced apoptosis [127, 138].

Despite the above data, clinical evidence for increased SCC risk with any single class of agent in the context of organ transplantation is conflicting and no single class of immunosuppressive drug has been consistently demonstrated to increase the incidence of malignancy [25, 47, 51, 66, 77-82]. This suggests that the overall burden of immunosuppression contributes significantly more to SCC risk than any single agent and that the mutagenic properties of some agents may be antagonistic when used in combination. The exception to this is sirolimus which was associated with reduced malignancy risk in a recent meta-analysis of 21 randomised trials, although at the expense of increased mortality [139]. However, two randomised trials of sirolimus in reducing recurrence of SCC yielded disappointing results; sirolimus was poorly tolerated and appeared to provide sustained benefit only to those with a history of a single SCC at recruitment, suggesting early initiation was required to prevent or slow SCC recurrence [140, 141].

The study by Dantal *et al.* suggests that many RTR are maintained on greater doses of immunosuppression than is necessary for stable graft function [124]. 'Over-immunosuppression' is associated with increased SCC risk, however, there is still a subset that requires larger doses to adequately suppress immunity against the allograft.

Whilst primary prevention of SCC is clearly the desired goal, there is currently no method of prospectively identifying those who are 'over-immunosuppressed' to guide pre-emptive immunosuppression reduction. As a consequence immunosuppression reduction is often cautiously undertaken after the occurrence of malignancy or recurrent NMSC.

Unfortunately, there is minimal evidence to suggest post-SCC immunosuppression reduction reduces accrual of disease. Evidence is based on case series only, where improvement is only seen in about half [142-144]. The impact may be limited as it is likely to take place after many of the widespread pre-malignant changes in the skin (referred to as 'field cancerisation') have already taken place.

1.5.3 Increasing age

Increasing age at transplantation has been consistently demonstrated to be a risk factor for subsequent SCC development [30, 32-34, 40, 51, 73, 74]. This is considered to be a result of cumulative exposure to risk factors and accumulation of intracellular changes required for SCC development.

There is some evidence to suggest the immune system undergoes remodelling, becoming less effective as a person ages. This has been described as "immunosenescence" in the adaptive immune system and will be discussed in greater depth later in this chapter.

1.5.4 Other contributing factors

Smoking: Smoking is associated with increased SCC risk in both transplant and non-transplant populations [32, 47, 75, 76, 110, 145-147]. Mechanistically, smoking is linked with increased oxidative stress which is associated with SCC development in RTR [132]. Smoking also leads to exposure to an array of carcinogens contained within tobacco, including arsenic and polycyclic aromatic hydrocarbons, which have been demonstrated to contribute to skin cancer risk [75, 148-151].

Genetics: Whilst confounding issues such as recall bias and shared exposure to environmental risk factors should be considered, family history of SCC appears to be associated with increased risk of the same in the general population [152]. It seems likely that some mutations in various tumour suppressor and oncogenes are inherited in a germline fashion; interestingly germline mutations of p53 do not typically lead to SCC [153].

Xeroderma pigmentosum is an inherited defect in UV-induced DNA repair which typically presents with cutaneous malignancy [84]. This suggests that polymorphisms and mutations within this pathway may predispose to SCC development [154]. A number of single nucleotide polymorphisms (SNP), including those relating both to phenotypic traits and DNA repair pathways, have been associated with increased risk of NMSC [51, 155-157]. Earlier studies suggested an association between certain HLA alleles and SCC after transplant but these have not generally been replicated [158-160].

Human papillomavirus (HPV) infection: The most overrepresented malignancies in transplant recipients are associated with oncogenic viruses [24, 26, 28]. This suggests that there also may be a viral aetiology in SCC. HPV of the beta genus (beta papilloma virus, βPV [161]) is strongly linked to the development of SCC in the genetic condition epidermodysplasia verruciformis, where recurrent and severe βPV infection and markedly increased viral loads are associated with development of aggressive SCC in early adult life [162]. This has led to the suggestion of a similar link to SCC in both the general population and in RTR, however this remains controversial.

βPV DNA from the natural reservoir, the hair follicle, can be isolated from 80-90% of non-transplanted participants and 80-98% of OTR [163]. HPV infection occurs in childhood and DNA can be isolated from healthy neonates as young as ten days old [164]. There are at least 25 different genotypes of βPV and the majority of hair follicles are colonised with multiple and overlapping strains; this co-infection is more marked in OTR [163]. There is evidence to

suggest that UV radiation may synergise with HPV infection to induce SCC by impairing DNA repair mechanisms [165-167].

Total and persisting βPV load may be more important for SCC risk rather than the specific viral genotype [168]. Whilst βPV DNA is found more frequently within SCC compared to normal skin and more frequently within SCC from OTR compared to non-OTR [169], the viral load (quantified by HPV copies per cell) appears to be greatest in pre-malignant actinic keratosis [170]. Transgenic models with the E2 protein of the HPV-8 genome present under the control of the keratin promoter have demonstrated that homozygous mice develop cutaneous SCC at an increased rate compared to heterozygotes [171]. Gene expression studies suggest βPV is not active within established SCC [172, 173]. This suggests that HPV may be involved at an early stage of malignant transformation but once malignancy is established βPV infection is dispensable. In immunosuppressed populations, decreased immune surveillance or viral clearance may permit ongoing viral replication and thus increased likelihood of malignant transformation.

Uraemia and dialysis: Registry studies have suggested that both increasing uraemia and the commencement of dialysis may be associated with a slight increase in risk of malignancy both within the genitourinary tract and elsewhere in the body [26, 174-177]. Uraemia may impair immune responses to infection and malignancy directly [175, 178] and may contribute through other mechanisms such as chronic inflammation and oxidative stress [179, 180].

1.6 Quantifying immunosuppressive effect in transplant recipients

Whilst the aetiology of SCC in RTR is complex, it is clear that immunosuppression plays a significant role through a number of mechanisms and is therefore a major contributor to long-term morbidity and mortality. However, reduction of immunosuppression after the

development of cutaneous malignancy may be too late, whilst inappropriate reduction may lead to rejection.

One of the issues is that the dosing of immunosuppression in RTR is imprecise. Azathioprine, prednisolone and mycophenolate are dosed by weight initially before being adjusted based on clinical judgement or fixed protocols. Tacrolimus, ciclosporin and sirolimus are dosed based on trough serum levels. These trough levels are hindered by significant intra- and interpatient variability and correlate poorly with cumulative dose and physiological effect [181, 182]. This has been demonstrated most clearly with ciclosporin. Inhibition of calcineurin prevents activation of the transcription factor nuclear factor of activated T cells (NFAT) which leads to a reduction in interleukin-2 (IL-2), interferon gamma (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) expression in lymphocytes. In a study of 133 RTR, despite similar trough levels of ciclosporin, those who went on to develop malignancy during follow-up had 50% greater reduction in two-hour post-ciclosporin IL-2/IFN-γ/GM-CSF gene expression in peripheral blood compared to those who did not [183]. A similar problem has been recently demonstrated with tacrolimus in liver transplant recipients: mean serum concentration correlates poorly with both mean intracellular concentration and the mean extent of calcineurin inhibition after seven days of therapy [184].

At a population level serum drug levels are a continuum and so 'optimal' trough levels (or the 'therapeutic window') represent a compromise between unacceptably high rates of rejection and the development of drug toxicity [182, 185]. This risk-benefit balance is not homogenous and will vary not only between individuals but potentially over time.

An aim of this study, therefore, was to assess potential markers which may indicate an altered risk-benefit balance, whereby the risk of rejection is lower than average and the risk of malignancy is greater than average. In a cohort such as this, pre-emptive reduction of immunosuppression would be desirable.

At present no validated markers exist to direct the management of optimal immunosuppressive dose or regimen. The immune system is likely to be the source of any potential marker, given its key role in the clearance of tumours and in graft rejection. Using peripheral blood mononuclear cells (PBMC) allows easy access for repeated sampling and so is the method of choice for immune phenotyping.

1.7 Immunological contributors to SCC: using the immune phenotype to stratify SCC risk

Previous work has demonstrated that alterations in circulating lymphocytes in RTR may be associated with increased risk of future malignancy. These populations have been demonstrated to contribute to the risk of malignancy in other settings and so may play a mechanistic role.

1.7.1 $CD4^+$ T cells

The association between a reduced $CD4^+$ T cell count and malignancy is established in the setting of HIV [186]. However, in transplantation this is less clear. Studies have suggested that a lower $CD4^+$ T cell count after transplant may associated with increased subsequent cancer risk [187, 188]. However, this predictive value was relatively weak and other studies have failed to replicate these findings [189, 190].

Part of the inconsistency described above may be due to the heterogeneity of malignancies studied and the poor resolution of $CD4^+$ populations. Within the $CD4^+$ compartment there are both effectors and regulatory populations. Whilst a number of different regulatory T cell populations have been identified *ex vivo* and in animal models, the canonical Treg population in humans possesses the phenotype $CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ [191-194]. Treg are found within the tumour bed of malignancies and their frequency is often positively correlated with a poorer prognosis [71, 195-197].

Upon activation, Treg are able to inhibit effector responses through a number of direct and indirect mechanisms. Many of these act upon effector cells preventing cytokine secretion and proliferation, but also upon antigen presenting cells where they reduce the quality of antigen presentation or alter the balance of costimulatory signalling towards inhibition of effector activation. These include the secretion of inhibitory cytokines such as IL-10 and TGF-β [198-200]; cell surface signalling such as through CTLA-4 [201] and induction of apoptosis in effector cells using Fas ligand and cytolytic mediators such as perforin and granzyme [202]. Treg may also degrade or sequester mediators required for a full effector response such as tryptophan, by metabolism by indoleamine-2,3-dioxygenase-1 (IDO); adenosine-triphosphate by degradation to adenosine monophosphate and adenosine by CD39 and CD73 respectively; and IL-2 by binding and uptake through the IL-2 receptor [203-205]. This is summarised in the setting of malignancy in Figure 1.3.

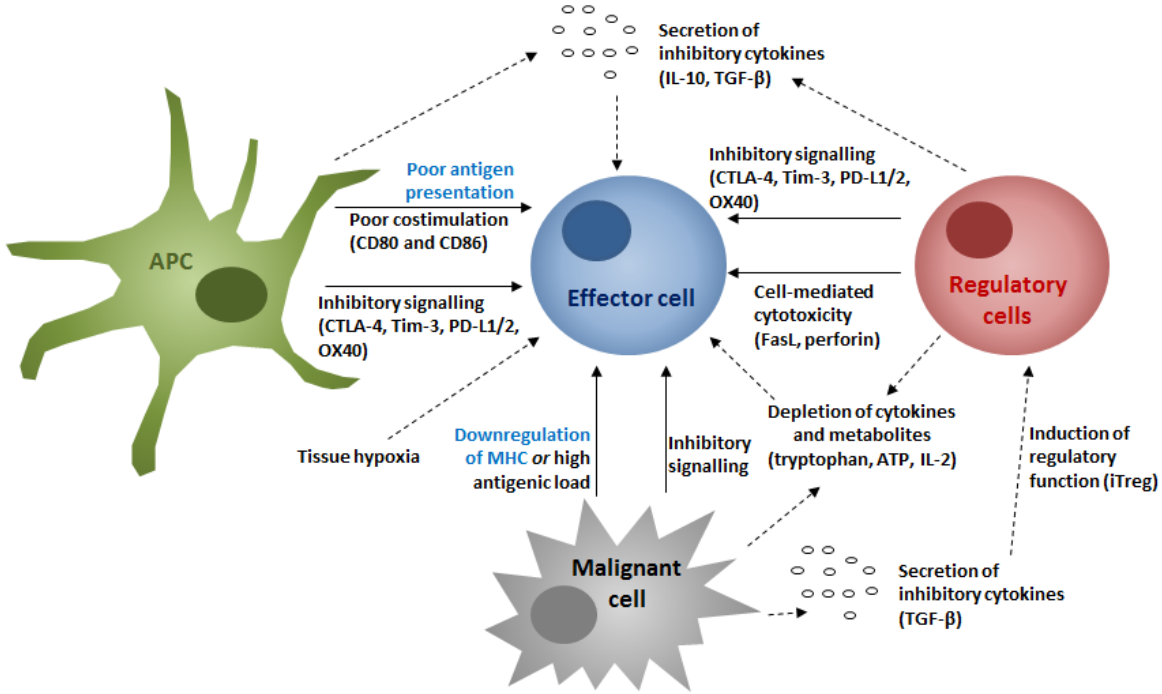


Figure 1.3: Mechanisms contributing to regulation of tumour clearance by effector cells. *APC, antigen presenting cell. In this context, an APC may be a macrophage, dendritic cell (or Langerhans cell) or B cell. Regulatory cells may represent various populations, including Treg, $CD8^+$ T cells and B cell regulatory populations, and myeloid derived suppressor cells (MDSC). Effector cell relates predominantly to $CD8^+$ T cells, but may also include natural killer (NK), natural killer T (NKT), gamma-delta T and cytotoxic $CD4^+$ T cells. The above mechanisms contribute to effector, particularly $CD8^+$, cell dysfunction and lead to a failure of tumour clearance. Those mechanisms highlighted in blue represent mechanisms of immune escape for the tumour. Examples of relevant mediators for each pathway are provided in brackets. Solid arrows represent mechanisms directed against specific cells through cell contact, whilst dashed arrows represent non-specific mechanisms through remodelling of the tumour microenvironment. Adapted from [206].*

Treg occur 'naturally', arising from the thymus (tTreg) during T cell development [207], as well as being induced in the periphery (pTreg) from $CD4^+CD25^-FOXP3^-$ populations in the periphery in response to activation in the presence of transforming growth factor beta (TGF-β) and IL-2 [208, 209]. Notably, TGF-β is produced by tumour cells including SCC, and this may induce conversion to pTreg in infiltrating T cells [210, 211]. As well as inducing pTreg, TGF-β is able to block IL-2-induced T cell proliferation and inhibit Th1 and Th2 responses [209, 212, 213].

Markers such as Helios and Neuropilin-1 have been postulated to distinguish between pTreg and tTreg [214-216], however subsequent studies have disputed this with evidence that these

markers may be upregulated in all T cells with activation [217-221]. To date, no widely accepted marker exists to discriminate these two populations. Therefore in this thesis 'Treg' refers to Treg of the $CD25^{hi}CD127^{lo}FOXP3^{+}$ phenotype and does not distinguish between pTreg and tTreg unless specified otherwise.

Two studies have previously found that RTR with a history of SCC have a higher number and proportion of Treg; furthermore a high number of circulating Treg was predictive of future SCC development with a surge in number seen around the time of SCC development [66, 109]. Further characterisation of these suggested that the majority of circulating Treg in RTR express Helios, but this did not differ between those with and without a history of cancer; the authors concluded that this suggested either Treg were recently activated or predominantly tTreg [109]. Because activated T cells may transiently express *FOXP3* [208], the extent of demethylation of a region within the *FOXP3* promoter has been proposed as a more specific marker of 'true' tTreg than *FOXP3* expression alone [222]. Further analysis of one of these cohorts confirmed that the proportion of $CD4^{+}$ T cells with a fully demethylated "Treg specific demethylated region" (TSDR) was greater in RTR with a history of SCC [223].

1.7.2 $CD8^{+}$ T cells

$CD8^{+}$ T cells are essential for elimination of tumours in animal models by production of effector cytokines such as IFN-γ and through cell-mediated cytolysis [202, 224, 225]. Despite the antigenicity of tumours and intact chemokine-mediated $CD8^{+}$ infiltration, tumour-infiltrating $CD8^{+}$ T cells often fail to clear tumours due to a number of mechanisms which lead to anergy, suppression and exhaustion. These are summarised in Figure 1.3.

Whilst no study has directly demonstrated impaired $CD8^{+}$ T cell function in the setting of SCC in RTR, indirect evidence arises from previous studies of immune phenotype.

Carroll *et al.* found a reduced number of central memory T cells (TCm) was predictive of SCC development [66]; these cells are required for a prolonged anti-tumour response in animal

models [224]. In liver transplantation, <40% CD28-expressing $CD8^+$ cells was independently associated with increased risk of malignancy in the first decade after liver transplant; cutaneous malignancy was relatively infrequent in this study [226]. $CD8^+CD28^-$ cells, which are frequently $CD57^+$, are considered to be poorly proliferative ("senescent"), terminally differentiated effectors [227]; a subset of these may demonstrate regulatory function in malignancy [228, 229]. In the setting of established malignancy in non-RTR, increased number of $CD8^+CD28^-$ or $CD57^+$ cells are associated with a poorer prognosis [230-232].

1.7.3 $CD19^+$ B cells

B cells undertake effector functions through the production of immunoglobulin (antibodies) as well as through antigen presentation with co-stimulation and production of effector cytokines, which enables polarisation of T cell responses [233-235]. Recently, populations of B cells with regulatory properties *ex vivo* have been described in both animals and humans [236-240]. These have been implicated in the development of operational tolerance in RTR and will be discussed further below.

Little work has been undertaken regarding the potential role of B cells in the development of SCC in RTR. Hope *et al.* found an increased proportion, but not absolute number, of memory B cells was independently associated with an increased risk of subsequent SCC [109]. In animal models of chemical and HPV-driven SCC, knock-out of B cells leads to a failure of carcinogenesis [150, 241]. In other experimental models of malignancy, B cells have been found to have both pro- and anti-tumour effects [242-246].

1.7.4 Other populations

NK cells arise from the innate immune system and recognise tumour cells through killer cell receptors, which recognise markers upregulated with stress, as well as virally or malignant transformation-induced downregulation of 'self' molecules such as MHC Class I [247]. Gamma-delta T cells are a population of predominantly $CD3^+CD4^-CD8^-$ and $CD3^+CD4^-CD8^+$ T cells bearing an atypical T cell receptor (TCR) containing a gamma and a delta chain that

recognises cells that present unprocessed, non-peptide phosphorylated compounds [248]. These compounds are generated naturally under conditions of stress and are found within microbial structures. There is a degree of functional overlap within these two populations, in that both express receptors such as NKG2D, which recognise stress markers such as MICA/B, overexpressed on malignantly transformed cells. Both these cells are able to secrete effector cytokines and engage in cell-mediated killing, akin to $CD8^+$ T cells.

In RTR, there is also evidence to suggest that both NK cells and gamma-delta T cells may assist in the prediction of post-transplant SCC. Carroll *et al.* found a decrease in NK cells, associated with an increase in Treg number, was predictive of future SCC [66]. An increased number of gamma-delta cells, driven by CMV seropositivity, was previously found to protect against malignancy development in the first decade after renal transplant [249]. An increased number of gamma-delta T cells may be predictive of SCC development [109]; this predictive value was lost on adjustment for age and duration of immunosuppression.

Changes in immune phenotype that have been previously suggested to be predictive of SCC development are summarised in Table 1.1.

Cell population	Description	Reference
$CD4^+$ T cells	RTR who develop "skin cancer" post-transplant have ↓ number of $CD4^+$ T cells (univariate analysis).	[188]
$CD8^+$ T cells	↓proportion of $CD28^-$ $CD8^+$ T cells and ↓number and proportion of $CD8^+$ TCm predictive of recurrent SCC development (multivariate analysis).	[66]
Gamma-delta T cells	↑proportion amongst T cells predictive of recurrent SCC development after sampling (univariate analysis).	[109]
Treg	↑number predictive of recurrent SCC development within 100 days of sampling (multivariate analysis).	[109]
Treg and NK cells	RTR with ↑Treg (35/µl blood) and ↓NK cell number (<100/µl blood) at increased risk of recurrent SCC development after sampling (multivariate analysis)	[66]
$CD19^+$ B cells	↑proportion of memory B cells predictive of recurrent SCC development within 100 days of sampling (univariate analysis).	[109]

Table 1.1: Changes in immune phenotype previously associated with SCC development in RTR.

1.8 Mechanisms behind increased SCC risk

Whilst it is apparent that alterations in immune phenotype are potentially useful to identify RTR at increased risk of SCC, the mechanisms behind these changes are unclear. Furthermore, no study has compared the performance of clinical risk factors with immunological factors in the prediction of SCC.

The most consistent evidence for a link with SCC development exists for regulatory (particularly Treg) populations and alterations in effector populations. Therefore this project will focus predominantly on the contribution of both of these to the development of SCC.

1.8.1 Increased immune regulation in RTR with SCC

In a number of experimental models of transplantation, donor antigen exposure under the cover of monoclonal antibodies to block T cell-signalling leads to the induction of Treg, which are able to delay or prevent allograft rejection [250-252]. Conversely, depletion of these Treg using an anti-CD25 antibody results in accelerated rejection of an allograft [253, 254]. Induced Treg can be transferred to a naïve host, where they inhibit allograft rejection in the new host

without further tolerance induction: an effect termed 'infectious tolerance' [255, 256]. This lack of immunological response to the allograft is associated with intact rejection of a third party graft, indicating antigen specificity of the acquired hyporesponsiveness. This can be referred to as *prope* tolerance, as first described experimentally by Billingham *et al.* [257]. These results suggest that Treg may play a key role in inducing tolerance in transplantation in animal models.

In RTR who develop SCC, there is an immune milieu supporting the development of increased Treg number and the development of tolerance to tumour antigens. It can be postulated that in a subset of patients an increased number of regulatory cells may develop specific for other antigens; namely antigens from the allograft, potentially leading to tolerance.

We therefore hypothesised that RTR who develop SCC may be most likely to have an 'allograft tolerising' immunological environment, utilising some of the mechanisms described in Figure 1.3. This may have a cumulative effect with pharmacological immunosuppression, leading to 'over immunosuppression'.

In order to assess whether immune regulation may be relevant in this cohort, it was important to define a measure of decreased immune reactivity to the graft.

'Operational tolerance'

The best clinical example of decreased immune reactivity to a graft is provided by a small number of RTR worldwide who have ceased pharmacological immunosuppression for a prolonged period and have maintained stable allograft function, with apparent intact response to other potential immunological insults. Whilst the exact definition is debated, this can be broadly defined as 'operational tolerance' [258]. This is often prefaced with 'spontaneous' to highlight that this has not been deliberately induced.

In a study setting, 'spontaneous operational tolerance' is usually defined as the cessation of pharmacological immunosuppression with maintenance of stable graft function without

significant proteinuria for a period of time, usually taken to be greater than at least six months or one year. The specifications of 'lack of proteinuria' and 'stable graft function' are controversial, given that many of these operationally tolerant RTR ceased immunosuppression after many years of nephrotoxic therapy and in some following earlier episodes of acute rejection [259, 260]. Defining an arbitrary cut-off for proteinuria and serum creatinine may exclude RTR who otherwise have genuine operational tolerance to a previously damaged graft. A number of studies do not specify a threshold for proteinuria and rather than an absolute cut-off for serum creatinine simply define 'stable' as minimal variance (e.g. less than 30%) in serum creatinine over the last year [261].

The word 'operational' is used to distinguish this phenomenon from *prope* tolerance. This is because *prope* tolerance cannot be demonstrated in this cohort, as it is difficult to prove an absence of an ongoing immune response to the allograft without serial histology, which is not practical or ethical, or tests of lymphocyte reactivity against the allograft. Immune infiltration has been demonstrated in the grafts of a number of operationally tolerant RTR at similar levels to both non-rejecting and rejecting biopsies, indicating that histology alone may not be informative [259, 262]. Furthermore, an intact alloresponse to third party grafts cannot be directly tested. The prevalence of operational tolerance within stable RTR is unknown, but is presumed to be rare [263-266].

The case histories of these cohorts do not implicate a 'unifying' clinical event to suggest a trigger for the spontaneous development of operational tolerance, although it appears to occur mostly in older RTR with relatively longstanding grafts [259, 260]. Many operationally tolerant RTR have received HLA mismatched allografts and exhibited the capacity to mount an alloresponse earlier in their transplant course through episodes of acute rejection. Immunosuppression is stopped for a variety of reasons including non-compliance, malignancy

or due to nephrotoxicity of immunosuppression, particularly in the era where immunosuppressive options were limited.

There is poor understanding behind the mechanisms responsible for both the development and maintenance of operational tolerance, but it is likely that this cohort represents a heterogeneous group where differing degrees and modes of hyporesponsiveness exist. Whilst operationally tolerant RTR do not appear more susceptible to infection than their immunosuppressed counterparts, the cohort heterogeneity is highlighted by their humoral and cell-mediated response to seasonal flu vaccination [267]. Some operationally tolerant RTR display a markedly reduced response akin to pharmacologically immunosuppressed RTR, suggestive of a non-specific reduction in immune function, whilst others display a response similar to healthy controls which is suggestive of hyporesponsiveness specifically against the allograft. Further highlighting this heterogeneity and lack of understanding, around 30% of 'operationally tolerant' RTR, who are immunologically and clinically indistinguishable, display a subsequent decline in renal function which is presumed to be a delayed immunological response and a loss of 'operationally tolerant' status [259, 260]. This may occur over a decade after ceasing pharmacological immunosuppression.

Attempts have been made to identify unifying features in the small number of RTR that demonstrate 'operational tolerance'. This in itself is a problem, as the number of RTR demonstrating retrospective operational tolerance worldwide numbers less than 100, meaning that many studies are on small groups of these, amounting to little more than case series. This means studies have found different findings, often inconsistently replicated elsewhere. These studies are summarised in Table 1.2.

First author	Year	OT cohort size	Comparator groups	Key findings in operationally tolerant RTR	Reference
VanBuskirk	2000	4	STA, AR	Weak donor-reactive DTH response in OT. Mediated by IL-10 or TGF-β	[268]
Brouard	2005	5	MIS, STA, CR, HC	Altered TCR Vβ usage, similar to MinSt. ↑*CD25* and ↓*IL2* expression vs CR. No difference in *IFNγ* or *IL10* expression.	[269]
Baetan	2006	6	STA, CR, HC	↑ no. of central memory $CD8^+$ T cells & ↓ no. of $CD8^+CD28^-$ cells vs CR. No difference in proliferative capacity.	[270]
Louis	2006	8	MIS, STA, CR, HC	↑B cell numbers vs CR/STA. ↑$CD4^+CD25^{hi}$ numbers vs CR. No difference in cytokine production or proliferation	[271]
Ballet	2006	5	STA, HC	Heterogeneous humoral and cell-mediated response to seasonal flu vaccination in OT	[267]
Brouard	2007	17	MIS, AR, CR, HC	49 genes differentially expressed on microarray, many related to TGF-β. Three clusters of genes, relating to reduced immune activation and signal transduction, and reduced costimulation, and immune quiescence.	[264]
Braudeau	2007	7	STA, CR, HC	↑$CD4^+CD25^{hi}FOXP3^+$ numbers vs CR, similar to HC. Similar suppression of $CD4^+CD25^-$ proliferation.	[272]
Braud / Sivozhelezov	2008	8	CR, HC	223 genes differentially expressed on microarray analysis. 12 clusters of genes, including immune and defence responses, and response to biotic and external stimuli.	[273, 274]
Braudeau	2008	8	STA, CAR, CRF, HC	↓*MyD88* and *TLR4* gene expression vs CR. ↓$TLR4^+$ monocytes vs CR.	[275]
Moares-Viera	2010	5	STA, MIS, CR, HC	↑$CD4^+CD25^{hi}FOXP3^+$ number vs CR, but similar to HC. ↓phosphorylation of STAT6 in monocytes vs all groups. ↑*IL4Rα* gene expression vs MIS	[276]
Pallier	2010	12	STA, CR, HC	↑number and proportion of B cells vs STA & CR, but similar to HC. ↑transitional and memory B cells vs STA and CR, but similar to HC. ↑expression of costimulatory markers in memory B cells of OT vs STA & CR, and in some cases HC. ↑expression of inhibitory B cell transcripts and B cell survival factors vs CR, STA and in some cases HC. No inhibition in B cell activation vs other groups	[277]
Sagoo*	2010	11	HC, CR, SCn, SnCn, MIS	↑proportion of B and NK cells vs HC, CR, SCn& SnCn. ↑B/T cell ratio vs all groups except MIS. Distinct gene expression profile in OT (see Table 1.2). ↓recently activated $CD4^+$ T cells vs all groups except SCn. No difference in $CD4^+CD25^{hi}$. ↓direct pathway donor responsiveness in OT. ↑*FOXP3*/*AMANN* ratio vs CR., ↑transitional:memory B cell ratio vs CR.	[278]
Newell*	2010	25	STA, HC	22 of 30 genes with ↑expression vs STA B cell specific. ↑expression of *CD20* in urine vs HC and STA. ↑expression of *FOXP3*, *Perforin* and *CD3* in urine vs HC (similar to STA). ↑number of total, naive and transitional B cells vs STA. ↑number of memory B cells vs HC. Identification of three gene signature to discriminate OT and STA (Table 1.2)	[279]
Lozano	2011	12 (+12 liver)	STA, CR, HC, LNT	Minimal overlap in altered gene expression between liver and kidney OT. Altered genes in liver OT clustered around NK cells; in kidney OT clustered around B cells. 24 of 35 common genes differentially expressed by kidney OT in this paper, the Newell cohort and the Sagoo cohort vs SCn found in B cells	[280]

First author	Year	OT cohort size	Comparator groups	Key findings in operationally tolerant RTR	Reference
Danger	2012	11	STA, CR	↓*PARVG* gene expression vs STA & CR. 2 SNPs for this gene, accounting for 20% of variability.	[281]
Danger	2012	15	STA	↑expression of miR-142-3p in B cells vs STA. Increased *TGF-β* gene expression in B cells vs STA.	[282]
Moraes-Vieira	2012	5	MIS, STA, CR, HC	↑*TGF-β* expression in PBMC vs MIS & STA. ↑*GATA3* (Th2 transcription factor) expression in whole blood and $CD4^+$ T cells vs all other groups. No difference in *FOXP3* or *IFN-γ* expression between groups.	[283]
Silva	2012	5	STA, CR, HC	Similar B cell number vs HC (HC have more B cells than CR). ↑transitional B cell proportion vs STA & CR. Overrepresentation of 2 CDR3 lengths in OT in BCR diversity analysis. ↑downstream signalling after CD40 stimulation in transitional B cells vs CR.	[284]
Becker	2012	4	STA, IR, BC	Looked at kidney biopsies by immunohistochemistry. No difference in cellular infiltrate number vs other groups. ↑$FOXP3^+$ proportion of infiltrating cells vs other groups. ↓expression of PI3K and c-rel (downstream mediators of NF-κB signalling) vs other groups.	[262]
Haynes	2012	11	IT, MIS, STA, CR	Reduced donor HLA-specific indirect pathway inflammatory response using transvivo DTH model vs MIS, STA & CR. Inflammatory response restored in 80% of OT by blocking TGF-β but not IL-10. ↑linked suppression by allospecific Treg vs CR, but not significant when compared to MIS & STA. ↑naïve B cells vs mono & CR, but no effect upon above with depletion.	[285]
Chesneau	2014	9	STA, HC	↑proportion transitional and naïve B cells and ↓plasmablasts vs STA. Intact *in vitro* maturation and differentiation into plasma cells in OT. ↓expression of *IRF4* and *PDRM1* in B cell (implicated in terminal differentiation of plasma cells) from OT, with ↑apoptosis. ↑IL-10 production by activated OT B cells	[286]
Roedder	2014	69	STA, CR, HC	Identified 21 genes that differentiate OT from STA & CR. Genes enriched in pathways relating to apoptosis; TNF-α, IL-4 and IL-6 signalling; and B cell activation. Enriched in B cells (7 genes), NK cells (6 genes) and dendritic cells (7 genes). Identified three-gene signature to discriminate OT vs STA& CR.	[265]
Braza	2015	13	ST, CR, HC	↑% of $CD4^+$ T cells with fully demethylated TSDR vs STA & CR. ↑proportion of $CD45RA^-$ $CD25^{hi}CD127^{lo}FOXP3^{hi}$ (memory) mTreg within lymphocytes vs all other groups. ↑suppressive capacity of mTreg vs STA. ↑expression of GITR and CD39 in mTreg vs STA & HC.	[287]

Table 1.2: Summary of the main immunological findings from studies on operationally tolerant RTR. **indicates the studies which produced signatures of tolerance which will be assessed during this project. 'OT' operationally tolerant RT; 'CR' chronic rejecting RTR; 'STA' RTR with stable graft function; 'HC' healthy controls; 'MIS', RTR on minimal immunosuppression (often steroid monotherapy); 'AR' RTR with acute rejection; 'CAR' RTR with deteriorating renal function and features of antibody mediated rejection on biopsy; 'CRF' Non-RTR with 'non-immune' chronic kidney disease; 'SCn' stable RTR taking calcineurin inhibitor; 'SnCn' stable RTR not taking calcineurin inhibitor; 'LNT' Liver transplant recipients who failed immunosuppression weaning; 'IR' RTR demonstrating interstitial rejection on biopsy; 'BC' RTR displaying borderline changes on biopsy; 'IT' RTR with a syngeneic transplant (from an identical twin). All findings are in peripheral blood unless stated otherwise.*

Potential mechanisms maintaining 'operational tolerance'

Despite the heterogeneity of reports on operationally tolerant RTR, consistent themes can be identified.

Although there is evidence for the involvement of Treg in experimental models of induced tolerance, the role of Treg in spontaneous operational tolerance is unclear. In general there is an association between normalised levels of Treg and good graft function, as seen when operationally tolerant and stable RTR are compared to chronic rejectors [271, 272, 276, 287]. However, a difference in number or proportion of Treg in circulating blood has not been consistently reported in operationally tolerant compared to stable RTR, although a recent study suggested an increase in circulating memory Treg with operational tolerance [287].

A series of biopsies from operationally tolerant RTR suggested increased frequency of $FOXP3^+$ cells amongst infiltrating lymphocytes compared to other groups [262]. Whilst it is possible that these were activated T cells, they were associated with a reduction in NF-κB signalling throughout the graft. This may indicate that Treg number in peripheral blood is poorly representative of number elsewhere, such as in the draining lymph nodes or the graft itself. However, in another study urinary *FOXP3* transcripts were not significantly elevated in operationally RTR compared to RTR with stable graft function [279].

A role for TGF-β has been consistently demonstrated in RTR who exhibit operational tolerance, with evidence of increased production and downstream signalling in B cells and whole blood [282, 283], or evidence of a role in suppression of indirect pathways of antigen presentation [268, 285].

Finally, operational tolerance appears to be associated with alterations in circulating B cells, in particular an overrepresentation of immature (transitional) and naïve B cells [271, 277-280]. B cells also demonstrate alterations in function, such as evidence of increased inhibitory signalling or failure to appropriately mature [277, 282, 286]. B-cells were traditionally considered pathogenic in transplantation due to their presence in acutely rejecting allografts and potential to produce donor

specific antibodies (DSA) [288]. Moreover, alloantibodies have been demonstrated to be the cause of hyperacute rejection and may contribute to chronic rejection [289].

There is accumulating evidence that a number of B cell populations with the ability to regulate immune responses exist. First described in animals, these were later identified in humans through *ex vivo* study, in the setting of autoimmunity [236, 237, 240]. Populations of circulating and peripheral B cells with suppressive function *ex vivo* (termed 'Breg' for simplicity) have also been found in healthy individuals [290], as well as in the setting of parasite infection [291], allergy [292], transplantation [293] and cancer [238, 294]. A specific marker for Breg has not been elucidated, but suppressive B cells have been reported as enriched within various populations including: $CD19^{+}CD24^{hi}CD38^{hi}$, representing a subset of transitional B cells cycling from the bone marrow to the spleen for final maturation into naïve B cells; $CD19^{+}CD24^{hi}CD27^{+}$, representing a subset of memory B cells; and $CD19^{+}CD25^{+}CD71^{hi}CD73^{lo}$, present throughout mature and immature B cell populations [236, 237, 292].

In the absence of a specific marker, the secretion of IL-10 has been taken to identify putative Breg, although it is increasingly evident that mechanisms of suppression may be both IL-10- dependent and independent. Mechanisms identified in human Breg include secretion of cytokines such as IL-10 [236, 290, 291, 293]; cell contact-mediated signalling [236, 290]; inhibition of downstream TCR signalling [238]; and induction and maintenance of Treg [290, 295]. Breg may play a role in prolonging allograft survival. B-cell depletion from non-sensitised RTR greatly increased the incidence of acute T-cell-mediated rejection in a randomised trial, leading to early suspension of the study [296]. A decrease in IL-10 secreting $CD24^{hi}CD38^{hi}$ B cells able to suppress Th1 cytokine expression was noted in RTR who developed acute rejection compared to those who did not [293].

Taking all the above together, it has been extrapolated that Breg may play a role in the maintenance of operational tolerance although a clear mechanism for this has not been identified as yet. In one mouse model of tolerance induction, induction of TGF-β-secreting $Tim\text{-}1^{+}$ B cells is specifically required to induce allospecific iTreg and prolong cardiac allograft survival [297].

Interestingly, the mechanism of tolerance in renal transplantation may differ from that of liver transplantation, where operational tolerance is relatively more frequent [280]. In liver transplantation, alterations in gamma-delta and NK cell populations appear to associate with operational tolerance.

'Linked suppression': a mechanism for malignancy development with tolerance

The phenomenon of 'linked suppression' may be relevant in operational tolerance. Linked suppression describes the phenomenon where activated regulatory populations specific for antigen presented by host APC are able to suppress T cell responses to unrelated antigens presented by the same APC.

First described in transgenic mouse models of transplantation [256], this has been demonstrated indirectly using human PBMC and HLA antigen in the setting of operational tolerance in *trans vivo* models [268, 285]. Briefly, injection of donor HLA antigen and PBMC from non-tolerant RTR into the footpad of an immunodeficient mouse leads to a delayed-type hypersensitivity (DTH) response due to cell-mediated alloreactivity. This is not seen using PBMC from operationally tolerant RTR. Injection of a third party recall antigen, such as tetanus toxoid, leads to a DTH response with PBMC from both. However, if donor HLA antigen and third party antigen are injected together with operationally tolerant PBMC, no response is seen. This phenomenon appears to be B cell independent [285].

It is possible that APC that present tumour antigen for which regulatory populations have developed also present alloantigen in lymphoid tissue. This could lead to suppression of this alloresponse and the development of regulatory mechanisms to this new antigen. This would require co-localisation of both tumour and alloantigen but is conceptually feasible given the propensity of malignancy to disseminate to distant sites and provides an immunological basis for the hypothesis that overrepresentation of subclinical operational tolerance may be seen in RTR who develop SCC.

Identifying a 'signature' of operational tolerance

Two studies published back-to-back by European and American consortia of transplant immunology institutes independently identified 'signatures' of operational tolerance using blood samples (Table 1.3). These were created by comparing the immune phenotype of the peripheral blood from operationally tolerant RTR to the immune phenotype of healthy controls, RTR exhibiting stable graft function under a variety of immunosuppressive regimens and RTR demonstrating a chronic deterioration in graft function.

The European signature, identified by the "Reprogramming the Immune System for the Establishment of Tolerance" (RISET) consortium, was a multiparameter signature comprising altered peripheral blood lymphocyte populations (quantified by flow cytometry), altered patterns of gene expression (quantified by real-time quantitative PCR) and donor hyporesponsiveness (assessed using direct pathway interferon-gamma EliSPOT) [278].

Variable	Direction of change in operational tolerance	Method of detection	Further information
RISET signature			
$CD19^+$:$CD3^+$ ratio	Increased	Flow cytometry	The ratio of B- and T- cells
$\%CD4^+$ $CD25^{int}$	Decreased	Flow cytometry	The proportion of $CD4^+$ T cells expressing CD25 at an intermediate level; these are taken to be 'recently activated' T cells
***FOXP3*/*AMANN* ratio**	Increased	qPCR	The ratio of *FOXP3* and alpha-1,2-mannosidase (*AMANN*) gene expression in whole blood
Expression of panel of ten genes:		Whole blood qPCR (relative quantification)	
TCL1A	Increased		T cell leukaemia/lymphoma 1A
CD79B	Increased		CD79 molecule beta
***MS4A1* (*CD20*)**	Increased		CD20
FCRL1	Increased		Fc receptor-like 1
FCRL2	Increased		Fc receptor-like 2
HS3ST1	Increased		Heparan sulfate 3-*O*-sulfotransferase-1
TLR5	Decreased		Toll-like receptor 5
SLC8A1	Decreased		Solute carrier family 8, member 1
PNOC	Increased		Prepronociceptin
SH2D1B	Increased		SH2 domain-containing 1B
Donor-specific direct pathway hyporesponsiveness	Decreased	IFN-γ ELISPOT	The ratio of spots formed after recipient PBMC incubation with CD2-depleted donor or third party PBMC
ITN signature			
Expression of panel of three genes:		Whole blood qPCR (absolute quantification)	
IGKV1-D	Increased		Immunoglobulin kappa variable region 1D
IGKV4-1	Increased		Immunoglobulin kappa variable region 4-1
IGLL1	Increased		Immunoglobulin lambda-Like Polypeptide 1

Table 1.3: The variables comprising the 'signatures' of operational tolerance from the RISET and ITN consortia. *All variables are derived from PBMC or whole blood.*

In contrast, the American signature identified by the "Immune Tolerance Network" (ITN) consortium, utilised the expression of three genes in peripheral blood [279]. Both consortia cross-validated their signature using samples from the other. Both signatures were found to have a sensitivity and specificity of greater than 80% for identifying operational tolerance; both therefore identified a subset of stable RTR on immunosuppression who appeared to demonstrate the signature.

We chose to analyse these signatures as, at the commencement of this project, they represented the largest cohorts of operationally tolerant RTR analysed to date and the only signatures which had been externally validated in a second cohort. We hypothesised that these signatures would be overrepresented in RTR who develop SCC and would be associated with alterations in the B cell compartment.

1.8.2 Diminished effector responses

The second *a priori* hypothesis in this project was that alterations in effector responses may lead to an increased risk of SCC development. Previous studies have identified that potential changes in NK cells, gamma-delta T cells and $CD8^+$ cells may identify RTR at increased risk of SCC.

One striking feature about SCC risk is that it correlates positively with age at transplant and with duration of immunosuppression, which is also a measure of increasing age. Ageing is associated with an increased risk of cancer and infection after transplant and in the general population, with evidence of impaired vaccination responses [298-301]. Non-sensitised elderly RTR demonstrate reduced rates of rejection compared to young RTR [302]. This phenomenon is not universal as impaired vaccination response is not uniform and a subset of elderly transplant participants will still experience acute rejection. The decreased ability to respond to vaccination appears to be linked to the process of 'immunosenescence' [303, 304].

This led us to hypothesise that the process of 'immunosenescence' may play a role in the development of SCC in RTR.

Immunosenescence

Immunosenescence refers to the remodelling of the immune system associated with increasing age. This process leads to a reduction in the strength of adaptive immune responses. The innate immune system undergoes a related process, termed 'inflamm-ageing', in which it becomes chronically activated at a low level [305]. Immunosenescence is a conserved process amongst mammals [306-308]. Given the ubiquitous nature of this process despite a marked difference in

length of life across the species, immunosenescence has been previously described as a function of age compared to life expectancy rather than chronological time elapsed since birth [305].

Biomarkers of immunosenescence

An accumulation of $CD8^+$ T cells with ageing was first described in depth in a healthy octogenarian cohort [309]. Clustered changes in adaptive immunity, particularly an increase in the proportion of $CD8^+$ T cells with reduction in $CD4^+$ T- and $CD19^+$ B-cells; reduction in lymphocyte proliferation after mitogenic stimulation; and reduction in IL-2 production, were associated with increased mortality in the group displaying these attributes [310-312]. In these studies cause of death was not reported.

Expansions of $CD8^+$ T cells associated with poor prognosis were later identified as a relative decrease in naïve $CD8^+$ T cells offset by an increase in oligoclonal $CD8^+CD28^-CD57^+$ terminally differentiated effector (TEMRA) cells [313-317]. This is thought to occur due to thymic involution leading to a loss of naïve T cell output with homeostatic expansion of effector memory T cells. Changes in other compartments are also seen, such as the accumulation of expansions of terminally differentiated NK cells and $CD27^-IgD^-$ 'senescent' B cells [318-320]. This is associated with an increase in serum levels of inflammatory cytokines such as IL-6, tumour necrosis factor alpha (TNF-α) and IL-1β [305]. Thus the process of immunosenescence affects all aspects of immunity.

These changes are akin to those seen in disease states marked by chronic antigenic stimulation, including HIV [227, 321, 322]; malignancy [323-325]; chronic viral infection [326, 327]; autoimmunity [328, 329]; tuberculosis [330]; and bone marrow and solid organ transplantation [326, 327, 331-334]. This led to the hypothesis that it is cumulative antigenic load, rather than ageing *per se*, which leads to immunosenescence [335].

The increase in $CD8^+$ and decrease in $CD4^+$ T cells, poor proliferation and the expansion of $CD8^+CD28^-$ cells, along with CMV seropositivity, have been defined as an "immune risk profile" (IRP). The IRP was associated with increased risk of mortality and was suggested to be readily identifiable simply by using the CD4/CD8 ratio, with a ratio of less than one being associated with the other features [311, 314, 336].

Even at the extreme of age, development of the IRP is not inevitable in the presence of CMV, but instead may identify 'pathological ageing' due to the interplay of many factors. A study in centenarian survivors indicated a complete lack of the IRP in this cohort despite 70% of the cohort being CMV seropositive throughout the six-year follow-up period [337]. The CD4/CD8 ratio may be under a degree of genetic control as well as showing gender-specific differences [338-340]. The IRP does not appear to be influenced by morbidity or frailty, indicating this is not simply a biomarker of other, clinically quantifiable factors [341, 342]. It is still unclear whether a 'genetically low' CD4/CD8 ratio in earlier life is prognostically relevant, although it may associate with impaired vaccination response [338].

In the absence of other immunological markers to quantify ageing, the IRP has become the 'standard' for defining immunosenescence [343] and has been validated in predicting poorer outcomes in other 'potentially immunosenescent' cohorts, including further studies of the elderly and in the setting of HIV [344-346].

Immunosenescence in transplantation

One prospective and one retrospective study have suggested that changes seen in immunosenescence may identify RTR at decreased risk of acute rejection post-transplant [347, 348]. In liver transplantation, an increased proportion of $CD8^+CD28^-$ cells, as seen in immunosenescence, was associated with increased risk of cancer post-transplant [226]. Carroll *et al.* found that an increased proportion of $CD8^+CD28^-$ cells were associated with increased risk of subsequent SCC in long-term RTR [66]. These studies did not assess whether these markers occurred in the context of other changes seen in immunosenescence.

It should be noted that the IRP was developed and validated in cohorts only including participants that "were not on a drug regimen known to affect the immune system" [311]. Clearly transplant patients are taking 'immune altering' drugs, as well as experiencing chronic exposure to a defined set of alloantigens from the transplant; it is not clear whether the IRP will remain valid in this cohort. The prognostic relevance of immunosenescence has not been prospectively explored in a

long-term transplant cohort, despite previous demonstration of the immune changes seen in ageing.

1.9 Biomarkers of a reduced immune response

In this study the potential contribution of clinical and immunological factors to the development of SCC in long-term RTR will be explored. It is clear that the excess incidence of SCC within OTR is a combination of factors. Traditional factors, such as UV exposure, mostly accumulate prior to transplant. Immunological factors relate not only to the cumulative dose of immunosuppression but also the immune response in preventing the development of malignancy. SCC could therefore act as a surrogate endpoint for a reduced immune response, if the other factors were accounted for.

In this project, we will explore the role of two potential mechanisms of reduced immune responses which may be present in RTR who develop SCC: operational tolerance and immunosenescence. Both of these mechanisms have been associated with alterations in immune phenotype in circulating blood, which could be used to establish their presence or absence.

An immune phenotype that is reliably associated with a clinical outcome could be considered a 'biomarker': that is "[a] characteristic that can be measured objectively and evaluated as an indicator of a normal biological process, a pathogenic process, or a pharmacological response to a therapeutic intervention" [349]. If these phenotypes were present in RTR who develop SCC and provided predictive value beyond clinical factors that also predispose to cutaneous malignancy development, it could be argued that these immune phenotypes are acting as 'biomarkers' of a reduced immune response. This may represent an immune system which may be adequately suppressed by lower doses of pharmacological immunosuppression compared to RTR not displaying the biomarker.

1.10 Global aims and hypotheses

1.10.1 Aims

- To establish the value of markers of immunosenescence and operational tolerance in the clinical prediction of SCC, including comparison with pre-existing clinical risk factors and clinical scores;

- To assess the prevalence of two previously identified 'signatures' of operational tolerance in long-term, stable RTR, and validate these two signatures against each other.

- To identify immunological mechanisms that may contribute to increased SCC risk in some RTR;

1.10.2 Hypotheses

- RTR who develop SCC will have greater prevalence of the two signatures of operational tolerance compared to RTR who do not develop SCC;

- The PBMC of RTR who develop SCC will display more features of immunosenescence compared to RTR who remain SCC-free;

- The above immunological markers will provide predictive value for SCC development independent of previously identified clinical risk factors and therefore provide additional prognostic information.

Chapter 2: Methods and materials

Methods and materials will be addressed in order of appearance in Chapters 3-5. Where a technique is used in multiple chapters, the methodology will be described in the chapter where first used.

2.1 Chapter 3

2.1.1 Clinical Methods

Study design

The study was designed as a prospective, longitudinal study. Renal transplant recipients (RTR) generally remain under long-term follow-up at a single unit, barring relocation, which provides a stable population that can be monitored over a prolonged period without significant loss to follow-up. The case-control design was used as the frequency of the tolerance signature in the general RTR population is unknown, but is presumed at this stage to be relatively rare. The study was reported in accordance with "Strengthening the Reporting of Observational Studies in Epidemiology" (STROBE) guidelines [350].

The study was registered with the National Institute for Health Research Clinical Research Network (NIHR CRN, study reference 10308). Funding was provided by the Wellcome Trust through a clinical research training fellowship grant (reference number: 0987447/Z/12/Z) and the Oxford Hospitals Research Services Committee grant scheme (reference number: 1088).

Ethical considerations

Sampling coincided with clinic visits to the Oxford Transplant Centre (OTC) or satellite unit in order to minimise inconvenience for participants and maximise recruitment. Study samples were taken at the time of routine clinical sampling, which occurs on all follow-up visits.

Due to the effects of age and immunosuppression, RTR may have a highly variable peripheral blood lymphocyte count. Previous studies in transplant recipients at the OTC suggested that a total of

approximately 24ml of blood, a physiologically negligible quantity, is sufficient to allow phenotypic analysis as well as storage of sufficient PBMC for further evaluation.

In order to allow patients a reasonable period of time to consider whether they wished to participate in the study without unduly delaying the clinic, participants were given advance notice of the study, along with contact details for the investigation team so that any questions could be addressed. Participant information sheets and a covering letter were posted a mean of 26 days prior to the clinic appointment.

The study was reviewed by an NHS Research Ethics Committee (REC) who gave a favourable opinion for the ethical conduct of the study. The study was reviewed through the proportionate review system, which expedites ethical review by allocating low-risk studies to the first available committee meeting, irrespective of location in the UK. All ethical applications and subsequent amendments were reviewed by the West of Scotland REC (reference 12/WS/0288, Appendix ii.A and B).

Participants

'Cases' were defined as RTR with stable graft function with a previous history of histologically-confirmed invasive cutaneous squamous cell carcinoma (SCC), referred to as RTR_{SCC}. An *a priori* decision was made to exclude RTR with SCC prior to (n=0) or within one year of transplantation (n=1) to ensure the cohort reflected post-transplant SCC only.

'Controls' were matched by age, sex and cumulative duration of immunosuppression (including pre-transplant immunosuppression where relevant) and are referred to as RTR_{No}. Table 2.1 details the inclusion and exclusion criteria used for recruitment. To be eligible for inclusion, a potential participant had to satisfy all inclusion criteria.

Inclusion Criteria	Exclusion Criteria
Male and female RTR aged greater than 18 years old.	Unable to provide informed consent to participate
A stable, functioning renal transplant (defined as serum creatinine increased ≤30% above baseline value in preceding 12 weeks).	Previous invasive malignancy during the last five years (except cutaneous basal cell carcinoma)*
Have provided informed consent to participate	Evidence of systemic infection at time of recruitment (see below)
	Transplant recipient of any organ other than kidney previously, or recipient of a syngeneic renal allograft
	Previous evidence of human immunodeficiency virus (HIV) infection
	Less than one year post-transplant
	Squamous cell carcinoma diagnosed prior to or within one year of transplant
	Known genetic tendency towards cutaneous malignancy (e.g. Muir-Torre syndrome)

Table 2.1: Recruitment inclusion and exclusion criteria. **or cutaneous squamous cell carcinoma for RTR_{SCC}.*

Systemic infection was defined as clinical evidence of systemic infection either at the time of recruitment or within the previous week. In the event of this occurring (n=3), enrolment was deferred.

Sample size

The paucity of data due to the novelty of the signatures of operational tolerance complicated the development of a power calculation to guide sampling size. At commencement of this project, no previous studies had estimated the prevalence of these signatures in stable RTR. Whilst operational tolerance is presumed to be a rare phenomenon in renal transplantation, previous studies have estimated the prevalence of operational tolerance using other measures to be approximately 5-10% [263, 264, 266]. This has been further supported by work published during the study [265]. In the original study describing the development of the RISET signature of operational tolerance, it was noted during the validation step that 5 of 34 RTR (15%) with stable graft function using calcineurin inhibitors would be predicted to display operational tolerance [278]. We used these figures to estimate the prevalence of operational tolerance amongst the RTR population.

Sample sizes were calculated to cover a range of possible estimates of prevalence. With 200 patients (100 in each group), there was an 80% power to detect a difference with p=0.05 in

prevalence between 8% and 17%, assuming a prevalence of the signatures of operational tolerance in RTR patients without SCC between 0% and 6%. This is depicted by the shaded cells in Table 2.2 of sample size in each group for varying difference in prevalence between groups.

		Prevalence of tolerance 'signature' in RTR without history of SCC (RTR_{No})						
		0%	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**
Prevalence of tolerance 'signature' in RTR with history of SCC (RTR_{SCC})	**7%**	108	137	166	194	222	248	275
	8%	93	116	138	159	180	200	220
	9%	83	100	117	134	150	166	181
	10%	74	88	102	115	128	141	153
	11%	67	78	89	100	111	121	131
	12%	61	70	80	89	97	106	114
	13%	56	64	72	79	87	94	101
	15%	51	58	65	71	78	84	90
	16%	48	54	59	65	70	76	81
	17%	44	50	54	59	64	69	73

Table 2.2: Sample size calculated using SAS 9.2 to compare two independent proportions using Pearson's chi-square test at a power of 80% and a p-value 0.05. *Values calculated by C Ciria, Centre for Statistics in Medicine, University of Oxford, and represent number per arm.*

Based on a previous study undertaken at this centre, it was predicted that 27% of participants within 10 years post-transplant; 43% within 15 years post-transplant; and 61% greater than 20 years post-transplant would have a history of skin cancer, of which approximately half would represent SCC [30]. As Caucasian RTR represent the vast majority of those who develop SCC, estimation of the size of the available cohort with SCC was limited to this group. At commencement of the study there were approximately 750 living Caucasian patients with a functioning kidney-only transplant under follow-up at OTC, of which 260 were greater than 10 years post-transplant. It was estimated that the OTC would be following up approximately 98 RTR with a history of SCC (Table 2.3). Thus the intention was to recruit all eligible RTR with a history of SCC followed up at the OTC.

Time since transplant	Number in database	Expected NMSC incidence	Expected n with NMSC	Expected n with SCC
<5 years	335	9%	30	15
5-10 years	155	27%	41	20
10-20 years	174	43%	74	37
>20 years	86	61%	52	26
Total			**197**	**98**

Table 2.3: Total estimated number of RTR with a history of SCC under follow-up at the OTC. *Data is based on a previous study undertaken at this centre [30]*

Patient identification and recruitment

Patients were recruited during the period March 2013 – November 2014. Oxford University Hospitals NHS Trust uses an electronic appointments system, allowing advance identification of potentially eligible patients.

Patients were screened prior to their clinic attendance. Screening consisted of using the Trust's renal database to confirm age, gender, date of transplantation and (in conjunction with Trust's pathology database) to confirm stable renal function and any previous diagnoses of malignancy (including cutaneous). Following this initial screening, if patients were deemed potentially eligible, they were sent an invitation letter and patient information sheet (Appendix ii.C).

During clinic, potential participants were approached by the investigator (MB) and invited to discuss the study further in private. If the patient was willing to participate, written consent was provided and the participant completed a proforma providing information on phenotype (such as natural hair and eye colour); occupation; UV radiation exposure (including history of sunburns; number of holidays where the patient sunbathed or spent significant amounts of time in the sun; and previous ultraviolet sunbed use); and family history of malignancy (Appendix ii.E-F). The participant and investigator together characterised the skin type of the participant as defined by the Fitzpatrick classification [351].

Clinical data were collected from the Trust renal and transplant databases and patient transplant and general medical notes. Information relating to HLA type was not recorded locally for 4 donor-

recipient pairs and 5 donors: this information was kindly provided by the NHS Blood and Transplant (NHSBT) service.

Information provided by participants at enrolment was used to calculate chronic UV exposure as defined by Harwood *et al.* [34]. Chronic UV exposure was defined as employment in an outdoor occupation for greater than five years; continuous residence in a sunny climate for greater than six months; or more than 11 holidays to a sunny climate where the participant sunbathed. For the purposes of this study, a smoker was classified as a person who reported a smoking history of greater than one pack-year. A 'pack-year' was defined as the equivalent of smoking twenty cigarettes per day for a one year period (therefore a person who smoked ten cigarettes a day for two years would be considered to have a one pack-year smoking history) [352].

Clinical risk scores for NMSC development

Three previously published risk scores developed for use in transplant cohorts to predict NMSC or SCC risk were assessed. These were calculated as:

- **Harden risk score** [73] as calculated by the sum of**:**
    - 1.26 if male gender (0 if female);
    - 0.1 multiplied by age at transplantation;
    - 1.87 if green eyes (0 otherwise)

    A score of $\geq$8.25 was considered to identify participant at markedly increased risk of SCC within 10 years of transplantation.
- **Harwood risk score** [34] as determined by:

Risk score	*Phenotype*
1	Fitzgerald skin type 5-6
2	Fitzgerald skin type 1-4, <35 years old at first transplant
3	Fitzgerald skin type 1-4, 35-44 years old at first transplant, <5 lifetime sunburns
4	As per risk score 3 **but** >5 lifetime sunburns **or** 45-54 years old at first transplant
5	Skin type 1-4 and greater than 55 years old at first transplant

- **Urwin risk score** [74] as calculated by the sum of:
    - 2 points if age >50 at first transplant;

- 2 points if average daily lifetime exposure to sunlight >1 hour;
- 2 points if >30 years in a tropical climate;
- 3 points if the participant had an SCC prior to transplant;
- 2 points if the participant had another NMSC prior to transplant;
- 1 point for any history of childhood sunburn (taken to be <16 years old);
- 1 point for Fitzgerald skin type 1.

Venous sampling

Venous blood was provided by the participant at the same time as routine clinical sampling. Blood was stored on ice and processed within 4 hours of venepuncture. Where possible, blood was taken at trough levels of immunosuppression. Estimated glomerular filtration rate (eGFR) was calculated from serum creatinine using the four-variable Modified Diet in Renal Disease (MDRD) equation, which has been demonstrated to be the most accurate estimating equation in renal transplant recipients with reduced eGFR [353].

Serum taken at enrolment was tested for titres of immunoglobulin G (IgG) to CMV by indirect chemiluminescence immunoassay (Liaison, Diasorin, Saluggia, Italy) by S. Wareing (Oxford University Hospitals Virology Department). A titre of greater than 14IU/mL was considered positive. The coefficient of variation for the assay has been reported to be <8%, specificity 99.65%, sensitivity 99.88%, and repeatability >98% [354]. The upper limit of detection was 180IU/mL; if the titre was above this serum was diluted 1 in 6 with phosphate-buffered saline (PBS) and repeated. A randomly selected subset of CMV seropositive participants was tested for CMV viral load by PCR of whole blood during follow-up sampling during the study; the lower limit of detection was 164 copies per millilitre of blood.

Study outcomes

The primary outcome of the study was the time from enrolment to the first diagnosis of cutaneous SCC during follow-up. Secondary outcomes were: total number of SCC during follow-up; time between SCC events; and occurrence of metastatic SCC. All SCC were diagnosed histologically; the date of biopsy/excision was taken as the date of the event. For the purposes of recurrence,

multiple SCC excised on the same day did not count as recurrence in itself (i.e. this counted as one event and not multiple events). Excision from the same site within one year, where either scar tissue was described in the histological report, there was incomplete excision reported initially, or was clinically described as recurrence, were not treated as a separate event. Where there was diagnostic uncertainty then the reporting histologist's opinion was taken as final. If the reporting histologist was unable to offer a favoured diagnosis, a non-diagnosis of SCC was presumed. Study outcomes were identified through the Trust's pathology database; where patients received dermatological screening in other NHS Trusts, study outcomes were identified through discussion with the patient's GP and the treating hospital's histopathology department where appropriate.

The relationship and overlap between SCC and keratoacanthoma (KA) is unclear and subject to much debate [355, 356]. Despite the similar clinical management of both in transplant recipients, there is some evidence to suggest that the immune response to KA may differ from that of SCC. KA demonstrate greater inflammatory infiltrate compared to SCC and normal skin, with the greatest density of granzyme B^+ and $CD8^+$ T cells seen in regressing KA [357]. In order to maximise the homogeneity of the study outcome and immune responses that may be relevant, throughout the study we treated KA and SCC as distinct entities. Thus, development of KA during follow-up was not counted as an event.

Participants without an outcome event were censored at the time of their latest follow-up data, graft loss or death, whichever occurred first. When a participant was censored at time of graft loss for survival and sampling purposes, observation continued for subsequent SCC development.

Ethical approval was granted for follow-up of these participants for five years from date of recruitment; this is ongoing.

Recruitment of other study groups

During the study, an ethical amendment was made to enable recruitment of an additional cohort of participants. These were patients with a history of cutaneous SCC but with no history of continuous systemic immunosuppression (for a cumulative total of more than one month) or transplantation.

Other inclusion and exclusion criteria were applied as for the main cohort. These participants did not enter into longitudinal sampling but provided a single venous blood sample at time of recruitment. Further information is provided in Section 2.3.

2.1.2 PBMC isolation and flow cytometry

Peripheral blood mononuclear cell (PBMC) isolation and preparation

Venous sampling was undertaken at recruitment, then at the first routine follow-up appointment after 6 months from recruitment, then 12 months after recruitment.

Ethylenediaminetetraacetic acid (EDTA)-preserved whole blood (BD Biosciences, Oxford, UK) was diluted 1:1 with PBS. PBMC were isolated using density gradient centrifugation ('Lymphocyte Separation Medium LSM 1077', PAA, UK) at 2000rpm for 20 minutes at room temperature.

Isolated PBMC were washed in PBS before contaminating erythrocytes were lysed using lysis buffer ('Pharm Lyse', BD Biosciences). PBMC quantification was undertaken manually using a Neubauer chamber haemocytometer. An aliquot of PBMC was phenotyped by flow cytometry (see below). Remaining PBMC were resuspended in 'freezing medium' (a 1:1 mix of RPMI-1640 media (Sigma-Aldrich, UK) and heat-inactivated (30 mins at 56^{O}c) fetal calf serum (FCS, Gibco, Life Technologies, UK) with 10% dimethyl sulphoxide [DMSO, Sigma-Aldrich, UK]) and slow frozen to -80^{O}c at 1^{O}c/minute using a cell cooling block (Biocision, California, US) for 24 hours before transfer to liquid nitrogen for long-term storage.

Serum samples (BD Biosciences) were spun at 1700g for 5 mins at 4^{O}c prior to removal of the sera. Sera was divided into aliquots and stored at -80^{O}c.

PBMC staining for flow cytometry

Aliquots of 4 x 10^5 PBMC were stained for 45 minutes at 4^{O}c with a pre-mixed cocktail of surface-staining antibodies or isotype controls. Stock antibodies were diluted to optimal concentration (see below) in FACS buffer (2% heat-inactivated foetal calf serum (FCS) and 0.1% sodium azide in PBS).

Intracellular staining was performed after surface staining, following fixation and permeabilisation in the appropriate solution (FOXP3 Fixation/Permeabilisation Kit, eBioscience, UK or

Cytofix/Cytoperm kit, BD Biosciences, UK) according to manufacturer instructions. Cells were subsequently stained with antibodies or appropriate isotype controls. 2% mouse serum was used to prevent non-specific binding.

Data acquisition was performed using a Beckman Coulter Navios system (Beckman Coulter, UK) for analysis of freshly isolated PBMC and using a BD Canto II (BD Biosciences) for all analysis of thawed PBMC.

Data were analysed using Kaluza 1.2 software (Beckman Coulter, UK) and FlowJo version X.0.7 (Tree Star Inc, OR, US). Major subsets were delineated using the gating strategies illustrated in Figure 2.1; other subsets are illustrated in the relevant section in Chapters 3-5. Dead cells and non-lymphocytes were excluded by forward and side scatter gating.

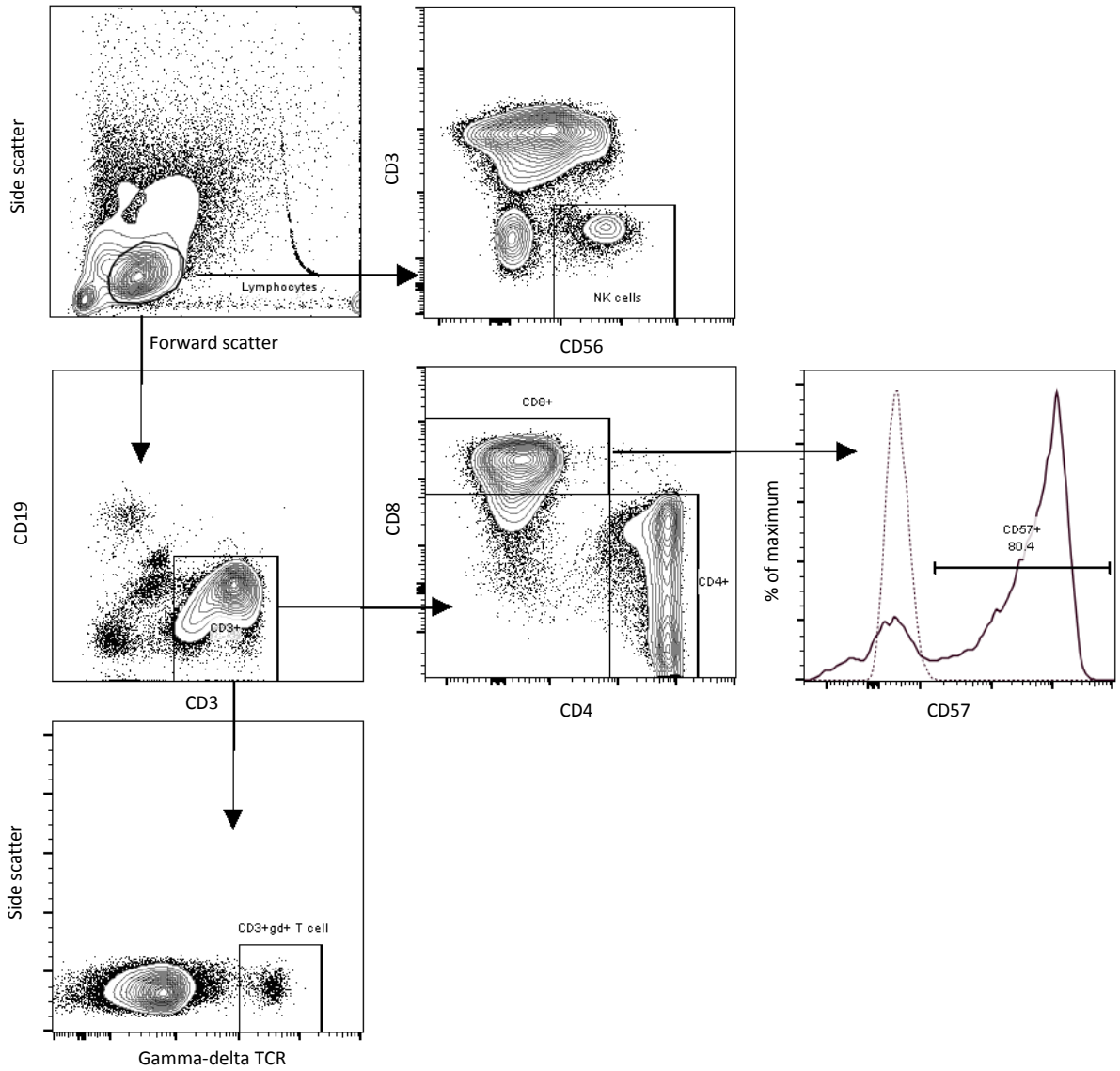


Figure 2.1: Representative gating strategy for delineation of major lymphocyte subsets. *Gating strategy for $CD3^{-}CD56^{+}$ NK cells, $CD3^{+}$gamma-delta^{+}, $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells. CD57 positivity on $CD8^{+}$ and $CD4^{+}$ T cells was established using an isotype control (dotted line).*

Results are expressed as a percentage of the lymphocyte gate or as an absolute cell number per microlitre (µl) of blood unless specified otherwise. This was calculated by applying the percentage of the relevant population within the lymphocyte gate on flow cytometry to the lymphocyte count acquired on clinical bloods taken at the same clinic visit.

Antibodies

The antibodies (and clones) used in the fresh PBMC phenotyping panel were: CD3-eFluor 450 (UCHT1), gamma-delta TCR-FITC (B1.1), CD28-APC- eFluor 780 (CD28.2), CD57-PE (NK1.1), CD38-APC (HIT2), CD20-APC-eFluor 780 (2H7) from eBioscience, Hatfield, UK; CD4-ECD (SFCI12T4D11) and

CD19-Krome Orange (J3-119) from Beckman Coulter, Wycombe, UK; CD8-PerCP-Cy5.5 (SK1), CD16-PE-Cy7 (3G8), CD56-APC (B159), CD27-FITC (M-T271), CD127-PE (HIL-7R-M21), CD25-PE-Cy7 (M-A251), CD69-Alexa Fluor 700 (FN50), CCR7-PE-Cy7 (3D12), CD24-PE (ML5), Granzyme-B-Alexa Fluor 700 (GB11), IgM-PE-Cy5.5 (G20-127) and IgD-PE-Cy7 (IA6-2) from BD Biosciences; CD45RA-APC (MEM-56) from Invitrogen (Life Technologies, Paisley, UK); FOXP3-Alexa Fluor 647 (259D) from Biolegend, London, UK. Where appropriate, isotype controls were used for gating.

2.1.3 Flow cytometry preparatory work

Antibody titration

All antibodies chosen for the panel were titrated in order to identify the optimal concentration to give strong positive staining without a significant increase in background staining. Each antibody was tested at a range of concentrations (Figure 2.2).

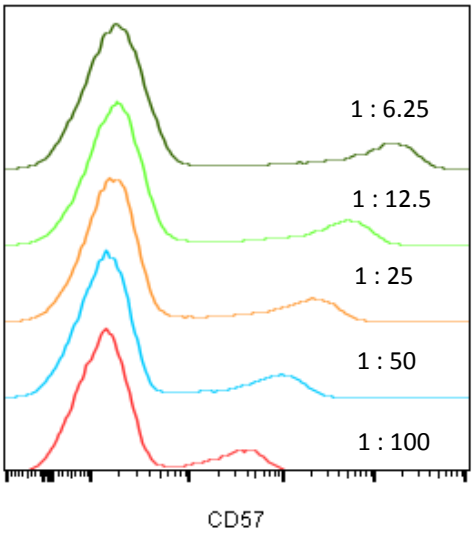


Figure 2.2: Representative staining of whole PBMC for CD57-PE with serial dilution. *The dilution refers to concentration of 'neat' antibody (as provided by the manufacturer) diluted in FACS buffer.*

In order to objectively assess the optimal concentration of antibody, staining was quantified using the 'stain index' [358], as calculated by:

$$Stain\ index = \frac{(MFI_{pos} - MFI_{neg})}{2 \times SD_{neg}}$$

Where 'MFI' is the median fluorescence intensity of the positive or negative population and 'SD' is the standard deviation. Plotted on a graph, this allows the optimal concentration of antibody to be

established. The optimal concentration was in the saturation region, where a doubling of antibody concentration no longer yielded a proportional increase in stain index (Figure 2.3).

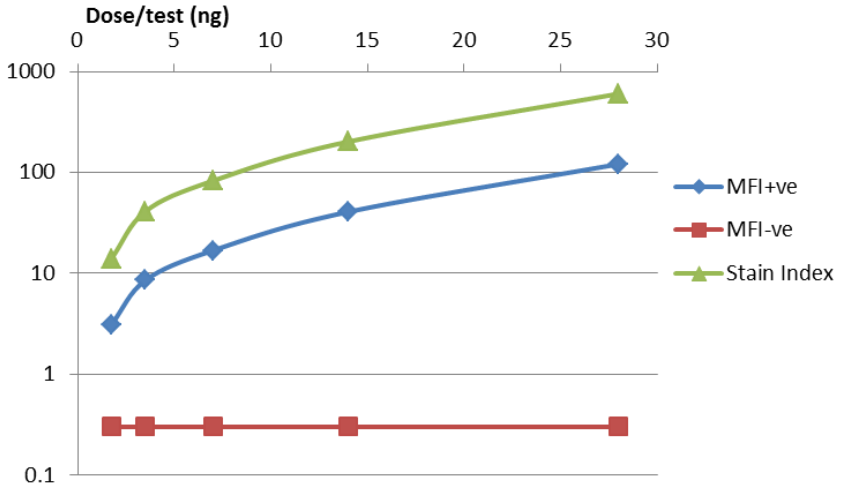


Figure 2.3: A representative graph of the stain index against antibody dose per sample. *This graph illustrates the change in stain index for CD57-PE.*

Panel validation

External validation

The study described in this thesis was undertaken on a different flow cytometer (Navios system, Beckman Coulter) to previous clinical studies reported by this laboratory. Furthermore, because this flow cytometer provided 10-colour capabilities (compared to 8-colour in previous studies [BD Canto II, BD Biosciences]) a new panel of antibodies was developed. To provide external validation for staining and analysis, PBMC from four healthy volunteers were isolated and stained using both this panel and an antibody panel from the previously validated and published clinical study on RTR with SCC (Figure 2.4 and Table 2.3) [223]. To minimise any potential bias each sample was analysed blind to the result from the other panel. The results table was generated following analysis of all samples.

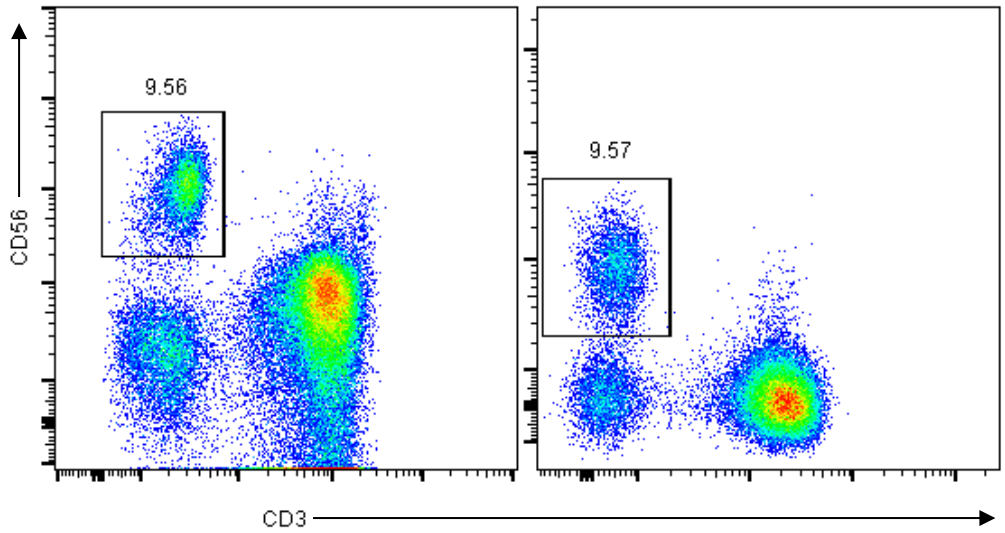


Figure 2.4: An example of staining of CD3+CD56- (NK) cells from the study panel (left) and validation panel (right). *These plots represent data acquired using different antibody clones and captured using Navios (left) and BD Canto II (right) systems.*

Concordance between the two panels was generally good; key populations are summarised in Table 2.4.

		Mean proportion of lymphocytes (%)	
Cell population	**n**	**Study Panel**	**Validation Panel**
$CD3^+$	4	79.4%	81.0%
$CD4^+$	4	51.7%	53.3%
$CD3^-CD56^+$	4	9.6%	10.0%
$CD8^+$	4	22.4%	22.6%
$CD4^+CD25^{hi}CD127^{lo}FOXP3^+$	3	1.6%	1.5%
$CD3^-CD19^+$	3	1.8%	2.4%

Table 2.4: Inter-assay variability compared to a second, previously validated, panel. *The mean proportion, expressed as a percentage of the lymphocyte gate, are provided for a selection of lymphocyte populations. 'n' refers to number of samples stained with both panels.*

Internal validation

To assess the intra-assay reproducibility of results, PBMC from a healthy donor were isolated then stained in triplicate then acquired and analysed separately as before (Table 2.5).

Cell population	Mean proportion	Standard deviation	Coefficient of Variation
$CD3^+$	77.4%	1.3%	1.7%
$CD4^+$	40%	0.6%	1.5%
$CD8^+$	28.5%	1.3%	4.6%
$CD3^-CD56^+$	9.3%	0.7%	7.5%
$CD19^+$	4.4%	0.7%	15.9%
$CD4^+FOXP3^+$	7.0%	0.6%	8.6%
$CD4^+CD25^{hi}CD127^{lo}FOXP3^+$	5.8%	0.5%	8.6%
$CD19^+CD24^{hi}CD38^{hi}$	0.33%	0.03%	9.1%

Table 2.5: Intra-assay replicability of staining. *The mean proportion, standard deviation and coefficient of variation (calculated as standard deviation divided by the mean, expressed as a percentage) of selected cell populations isolated then stained in triplicate.*

The coefficients of variation reported here are similar to those reported elsewhere with testing of a flow cytometry panel used in an international collaborative study (The ONE Study), with the largest coefficient of variation seen in $CD19^+$ cells amongst the major subsets during intra-assay testing [359]. The variability of Treg staining is also consistent with that reported during the validation of a previous study in a similar cohort [360].

2.2 Chapter 4

2.2.1 Recruitment of a 'training set' to define cut-offs for the tolerance signature variables

The original RISET and ITN studies did not expressly set 'cut-offs' for operational tolerance for each variable within the signatures. Instead, both referred to direction of change for each parameter e.g. an increase in CD19/CD3 ratio. In order to create 'normal values' for each of the variables within both tolerance signatures, it was necessary to use a 'training set'. For widespread use of the tolerance signature, a large cohort would be needed to establish normal values; this cohort was merely intended to serve as a pilot to generate values for this study.

There were a number of options for this:

1. Utilise a subset of the participants in the study, chosen at random. This would ensure that the characteristics and sampling of the main cohort would be exactly representative of the

main cohort. However, this would reduce the number of participants within the main cohort for analysis.

2. Utilise stored samples previously collected from earlier studies in similar populations, avoiding participants recruited into this study [66, 223]. This would have the advantage of speed, as the samples were already collected, but would have the disadvantage of having not necessarily been processed identically to study participants. In particular the method of mRNA extraction would differ. Perhaps most importantly, this would also have the disadvantage of creating a selection bias for the training set, as these participants were generally otherwise ineligible for the study due to reasons often relevant to the study outcome such as subsequent development of malignancy or graft loss.
3. Recruit a new cohort matched for variables potentially relevant to the tolerance signature.

Rather than utilise participants already in the study, which would reduce the number of participants who could be analysed subsequently in the 'test set', it was decided to recruit an additional cohort.

This was guided by preliminary analysis of two variables from the RISET tolerance signature, the CD19:CD3 ratio and proportion of $CD4^+$ T cells expressing $CD25^{int}$, which were tested for correlation with major clinical covariates. A non-significant, weak correlation was seen with age at enrolment and calcineurin inhibitor use, and a stronger correlation with azathioprine use (Table 2.6). Given the data presented in Chapter 3 demonstrating that CMV is associated with alterations in the immune phenotype, RTR in the 'training set' were also matched for CMV serostatus.

Variable	B:T cell ratio	p	Proportion of $CD4^+$ T cells expressing $CD25^{int}$	p
Age at enrolment	-0.17	0.06	0.07	0.45
Age at first transplant	-0.11	0.25	-0.11	0.1
Total duration of immunosuppression	-0.05	0.62	-0.02	0.87
Total number of HLA mismatches	-0.07	0.48	-0.02	0.85
Gender	0.08	0.39	0.05	0.59
Renal function (eGFR)	0.03	0.79	0.14	0.13
Immunosuppression:				
Azathioprine	-0.34	<0.001	0.09	0.36
Calcineurin inhibitor	0.18	0.06	0.04	0.70
Steroids	-0.004	0.97	0.02	0.87

Table 2.6: Bivariate correlation of major clinical variables with the ratio of the percentage of $CD19^+$ to $CD3^+$ lymphocytes within the lymphocyte gate and proportion of $CD4^+$ T cells expressing $CD25^{int}$. *Correlations are provided as an r value, using Pearson's test, and was performed on the main cohort (n=117).*

This training cohort was thus matched by age at enrolment, azathioprine and calcineurin inhibitor use and CMV serostatus. It was not practical to completely match all immunosuppression, so azathioprine and calcineurin inhibitor use were matched, as the major two immunosuppressants in use throughout the main cohort. The lack of correlation with duration of immunosuppression, confirmed on final analysis (Chapter 4), enabled the recruitment of participants who had otherwise been considered 'too low risk' for SCC due to duration of immunosuppression. An empirical decision was made not to recruit any patient who had received their transplant less than four years previously to allow stabilisation of any immune phenotype after transplant.

The training set was recruited to be 20% of the size of the main cohort as it was felt this would be large enough to provide cut-off values without too much influence by outliers. Participants were recruited according to the same inclusion and exclusion criteria as the main cohort and participant samples were processed identically.

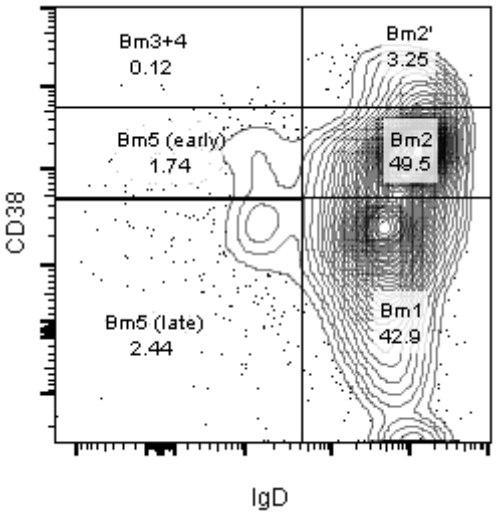
2.2.2 Flow cytometry for the signatures of tolerance

Flow cytometry was performed on freshly isolated PBMC using the same isolation and staining method as Chapter 3.

B cell gating strategy

Further B cell phenotyping was performed on $CD19^{+}CD20^{+}$ populations, as demonstrated in Figure 7 and Chapter 4. Two different classifications were used to characterise B cells. The CD27-IgD classification allows resolution of naïve and antibody isotype switched and unswitched memory populations [361], but cannot distinguish $CD19^{+}CD24^{hi}CD38^{hi}$ transitional from naïve (as both are $CD27^{-}IgD^{+}$) B cells. To distinguish transitional B cells, $CD24^{hi}CD38^{hi}$ gating was used (gating strategy illustrated in Chapter 4); naïve B cells were calculated as $CD27^{-}IgD^{+}$ cells minus those that were $CD24^{hi}CD38^{hi}$.

The Freiberg classification, originally designed for lymphoid tissue but subsequently validated in peripheral blood, allows resolution of B cell populations using CD38 and IgD expression [362]. The Freiberg classification suffers from the limitation that unswitched memory cells (which are also $CD38^{-}IgD^{+}$) can overlap with Bm1 'naïve' B cell populations (Figure 2.5). To avoid this problem, analyses of B cell populations were performed using both of these classifications in parallel.



Population	Contains
Bm1	Naïve B cells, unswitched memory
Bm2	Naïve B cells
Bm2'	Transitional B cells
Bm3+4	Plasmablasts
Bm5	Memory B cells

Figure 2.5: B cell populations distinguished using the CD38-IgD classification. *Plot shows singlet $CD19^{+}CD20^{+}$ lymphocytes in a healthy volunteer.*

Distinguishing activated $CD4^{+}$ T cells and Treg

The original RISET tolerance signature utilised proportion of 'recently activated' $CD4^{+}$ T cells, which were defined as $CD4^{+}CD25^{intermediate}$ ($CD4^{+}CD25^{int}$) [278]. These were $CD4^{+}$ T cells gated as $CD25^{+}$ but not $CD25^{hi}$, which are enriched for Treg [193, 363-365]. Since the development of this gating strategy, additional phenotypic markers have been identified to assist in the enumeration of Treg, in particular low surface expression of the α-chain of the IL-7 receptor (CD127) [192, 194] and the

activation marker CD69 [366], and high intracellular expression of FOXP3 [191, 193]. We therefore sought to utilise these markers to avoid accidental inclusion of Treg within the 'recently activated T cell' population.

Figure 2.6 demonstrates the original method of identifying recently activated ($CD4^+CD25^{int}$) T cells. However, this has the problem of excluding $CD127^{hi}FOXP3^{lo}$ cells. Therefore we adopted a different strategy. $CD4^+CD25^+$ T cells [229, 363] were gated on CD127 expression also. Those that were $CD25^{hi}$ (the cut-off being determined by the point at which $CD127^{hi}$ and $CD127^{lo}$ populations become distinguishable) were gated for FOXP3 expression, and those that were $CD127^{lo}FOXP3^+$ and $CD69^-$ were classified as Treg. These were subtracted from the $CD4^+CD25^+$ population to leave a "$CD4^+CD25^+$ effector" population. We also utilised $CD4^+CD25^{int}$ gating, as originally referenced, for comparison. The threshold for FOXP3-positivity on the CD127-FOXP3 plot was then applied to other populations, such as $CD8^+$ T cells.

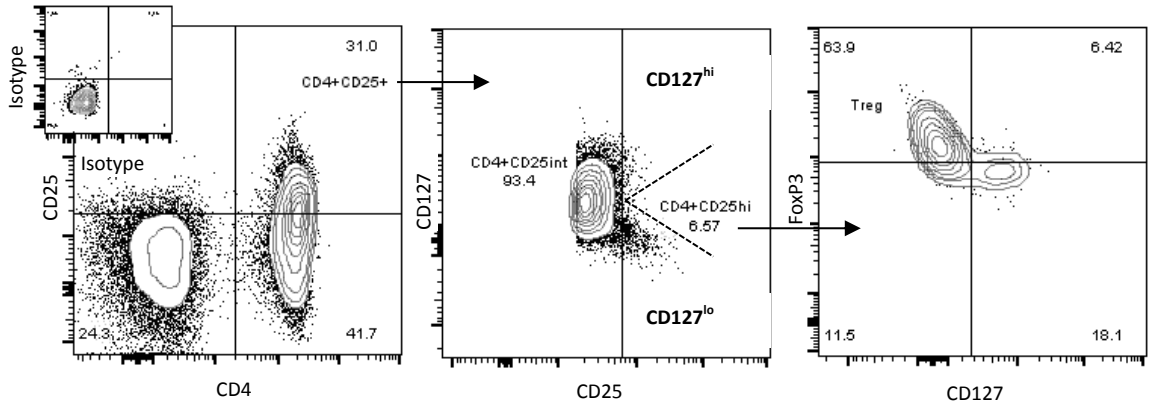


Figure 2.6: The gating strategy for distinguishing $CD4^+CD25^{hi}$ effectors and Treg. *Singlet $CD3^+$ lymphocytes were gated on CD4 and CD25; $CD4^+CD25^+$ were divided into $CD25^{int}$ and $CD25^{hi}$ using CD127. $CD25^{hi}$ were then subdivided using CD127 and FOXP3 expression. The dotted lines on the middle plot indicate how the 'CD25^{int}' and 'CD25^{hi}' populations were gated using CD127, by gating on the point at which $CD127^{lo}$ and $CD127^{hi}$ populations became identifiable. Use of CD69 to further discriminate Treg did not significantly change these results.*

2.2.3 RNA extraction and Quantitative (q-) Real Time PCR

Whole blood RNA extraction

3ml of venous blood was taken into vacuum-filled blood tubes containing RNA preservation solutions ("Tempus" RNA preservation tubes, Life Technologies, Paisley, UK). These tubes were frozen at -80^{O}c until extraction of RNA.

Total RNA was extracted from whole blood using the magnetic bead method ("MagMAX", Life Technologies), as per manufacturer's instructions. Briefly, thawed RNA-stabilised blood was diluted with PBS then centrifuged at 5000g for 15 minutes at 4^{O}c. The pellet was resuspended and treated with a pre-digestion wash before further centrifugation. The crude RNA pellet was then treated with protease and DNAse to remove contaminants, before addition of nucleic acid-binding magnetic beads for RNA capture, with use of a magnetic stand to allow removal of supernatant. Following a series of wash steps, RNA was eluted and quantified by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, Loughborough, UK). All samples yielded RNA with a 260/280nm optical density ratio of greater than 2.00. RNA was stored at -80^{O}c prior to reverse transcription. Gel electrophoresis was undertaken on a subset of samples (n=10) that yielded the greatest concentration of RNA. No DNA contamination or RNA degradation was seen.

Reverse transcription

Complementary DNA (cDNA) was generated using recombinant moloney murine leukemia virus reverse transcriptase and random hexamers (Life Technologies) using a starting quantity of 1µg of total RNA. RNA was heated to 65^{O}c for 10 mins then chilled to 4^{O}c for 5 minutes prior to reverse transcription, in order to dissociate secondary structures. For first-strand generation, the PCR conditions were 25^{O}c for 10 mins, 48^{O}c for 30 mins and 95^{O}c for 5 mins Prior to commencement of the study, all gene expression assays were tested for linear amplification using serial dilution, confirming no inhibition of reverse transcription, with all demonstrating an amplification efficiency of 85-110% (Figure 2.7).

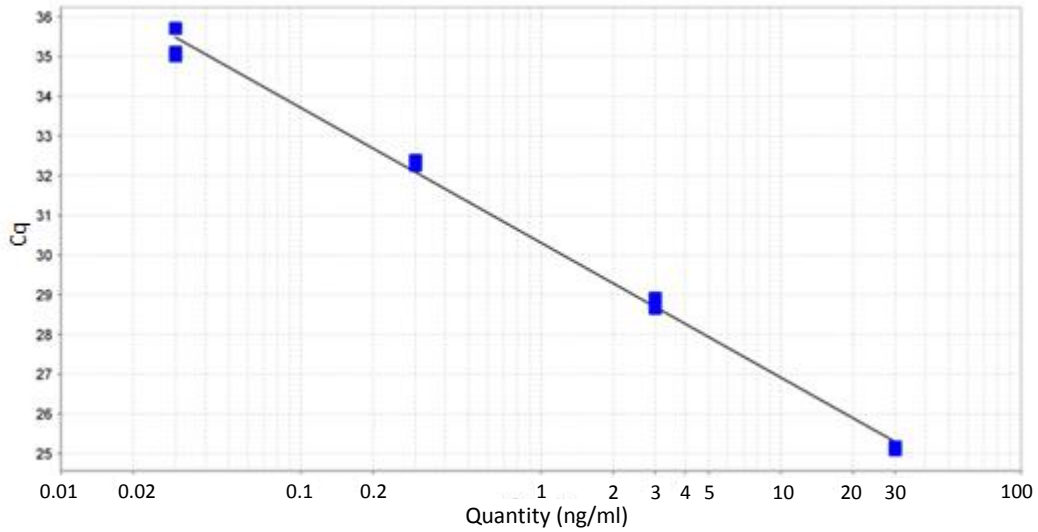


Figure 2.7: A representative standard curve obtained using serial dilution of cDNA to confirm no inhibition of qPCR, with linear amplification. *The assay illustrated here (*GUSB*) yielded an efficiency of 97% (r^2 0.996). Quantity refers to starting quantity of cDNA in nanograms (ng).*

Quantitative/Real Time-PCR

Gene expression was normalised to beta-glucuronidase (*GUSB*) as this gene demonstrated greatest consistency during evaluation of a panel of sixteen candidate reference genes across a variety of individuals from different arms of the study and healthy controls (Table 2.6). *HPRT* was used as a second reference gene as this was the reference gene utilised during the original developing of the RISET tolerance signature [278]. Quantitative/Real-time PCR (qPCR) was undertaken using 30ng cDNA using either inventoried assays or using custom primers and probes, as detailed in Table 2.7.

Target	Target description	n	Mean Cq	Cq SD	Cq COV
18S	18S ribosomal RNA	7	13.7	0.60	4.4%
ACTB	Beta actin	8	19.4	0.60	3.1%
B2M	Beta-2-microglobulin	8	21.6	1.05	4.8%
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	8	22.8	0.71	3.1%
GUSB	Beta-glucuronidase	8	27.5	0.52	1.9%
HMBS	Hydroxymethylbilane synthase	7	31.5	0.54	1.7%
HPRT1	Hypoxanthine phosphoribosyltransferase 1	8	29.6	1.03	3.5%
IPO8	Importin 8	8	29.9	0.63	2.1%
PGK1	Phosphoglycerate kinase 1	6	26.2	0.93	3.5%
POLR2A	RNA polymerase II	8	27.4	0.62	2.2%
PPIA	peptidylprolyl isomerase A (cyclophilin A)	8	23.9	0.70	2.9%
RPLP0	ribosomal protein, large, P0	8	24.0	0.90	3.7%
TBP	TATA-binding protein	8	30.0	1.07	3.6%
TFRC	Transferrin receptor	8	30.5	0.57	1.9%
UBC	Ubiquitin C	8	23.9	0.68	2.9%
YWHAZ	Protein Kinase C Inhibitor Protein	8	29.3	0.85	2.9%

Table 2.7: Results from reference gene stability assessment. *Where less than 8 genes are assessed, there was a large (>0.5 Cq) variation between duplicates for one sample; this sample was excluded from analysis. 'SD', Standard deviation. ' COV', coefficient of variation. Cq values differ significantly from those in Table 2.8 as this evaluation was performed using a lower starting quantity of cDNA.*

The initial ITN signature used absolute mRNA quantification [279]. However, subsequent work has identified relative quantification is also able to distinguish tolerant RTR and RTR under immunosuppression ([367] and D. Phippard, ITN consortium, personal communication). For ITN assays (*IGLL1*, *IGKV4-1* and *IGKV1D-13*) *18S* and *GAPDH* were utilised as reference genes in addition to *GUSB* and *HPRT*, as these genes were utilised as reference genes by the ITN consortium (D. Phippard). The correlation (r value) between results obtained by normalisation to *GUSB* and the mean of *18S*/*GAPDH* was 0.97 for *IGKV1D-13* and 0.96 for *IGKV4-1* during the study.

To validate and confirm the choice of reference gene, the variability of Cq values of *GUSB*, *HPRT*, *18S* and *GAPDH* was retrospectively compared across all samples (Table 2.8). This confirmed the choice of *GUSB* as the reference gene, with a coefficient of variation at least 30% less than the other candidate reference genes. Cq values across all four genes did not differ when stratified by immunosuppressive regimen or age at enrolment.

Target	Mean Cq	Cq SD	Cq COV
18S	10.6	0.41	3.9%
GAPDH	21.6	0.92	4.3%
HPRT	30.3	1.12	3.7%
GUSB	25.7	0.66	2.6%

Table 2.8: Retrospective confirmatory validation of reference gene stability across the whole RTR cohort. *Values are given as untouched mean Cq values, with 30ng of starting cDNA in all reactions. 'SD', Standard deviation. ' COV', coefficient of variation.*

Samples were analysed in duplicate using 384-well plates on the Quantstudio 7 platform (Life Technologies) and repeated if there was >0.5 Cq standard deviation between replicates. In order to ensure inter-plate consistency a control sample was run on all plates. This was a single pool of RNA extracted as above from a healthy volunteer who expressed all genes at detectable levels. RNA from the sample was reverse transcribed and amplified in parallel with the other samples for that plate. The plate was disregarded if the control sample demonstrated >0.75 ΔCq difference from the averaged ΔCq value for this sample across all plates (no plates were excluded on this criterion). A no template control was run for all assays. qPCR conditions were 50^{O}c for 2 minutes, then 95^{O}c for 10 minutes, then 45 cycles of 95^{O}c for 15 seconds and 60^{O}c for 1 minute. The details of assays used are listed in Table 2.9.

Assay Target	Sequence or assay ID	Supplier
FOXP3	5'- AAG TGG CCC GGA TGT GAG A -3' (FWD) 5'- CAT TGT GCC CTG CCC TTC T -3' (REV) 6FAM- ACT TCC TCA AGC ACT GCC AGG CGG –TAMRA (Probe)	Sigma (probe) Eurogentec (primers)
AMANN	5'- CGG CCA GAA GTT ATG GAG ACT TAC -3' (FWD) 5'- CTT AGG CCT GAA TAG CCT CCA TTC -3' (REV) 6FAM- TGG GAA GCC GTA GAG GCC TTG GAA A –TAMRA (Probe)	Sigma (probe) Eurogentec (primers)
TLR5	Hs00152845_m1	Life Technologies
PNOC	Hs00173823_m1	Life Technologies
SH2D1B	Hs01592483_m1	Life Technologies
SLC8A1	Hs00253432_m1	Life Technologies
HS3ST1	Hs01099196_m1	Life Technologies
FCRL1	Hs00364705_m1	Life Technologies
FCRL2	Hs00229156_m1	Life Technologies
MS4A1	Hs00544818_m1	Life Technologies
TCL1A	Hs00172040_m1	Life Technologies
CD79B	Hs00236881_m1	Life Technologies
IL10	Hs00961622_m1	Life Technologies
HPRT	5'- AGT CTG GCT TAT ATC CAA CAC TTC G -3' (FWD) 5'- GAC TTT GCT TTC CTT GGT CAG G -3' (REV) 6FAM- TTT CAC CAG CAA GCT TGC GAC CTT GA –TAMRA (Probe)	Sigma (probe) Eurogentec (primers)
GUSB	Hs99999908_m1	Life Technologies
18S	5' GCC CGA AGC GTT TAC TTT GA – 3' (FWD) 5' TCC ATT ATT CCT AGC TGC GGT ATC – 3' (REV) 6FAM- AAA GCA GGC CCG AGC CGC C –TAMRA (Probe)	Life Technologies
GAPDH	NM_002046.3	Life Technologies
IGLL1	Hs00252263_m1	Life Technologies
IGKV4-1	AIN1E62	Life Technologies
IGKV1D-13	5' GGG CTT CTG CTG CTC TGG 3' (FWD) 5' TGG AGA CTG GGT CAA CTG GAT 3' (REV) 6FAM- CCA GGT GCC AGA TGT G –NFQ (Probe)	Life Technologies

Table 2.9: List of primers and probes used for gene expression analysis

Gene expression was normalised to *GUSB* unless specified otherwise, using the $2^{-\Delta Cq}$ method [368-370]. This value was used to generate the *FOXP3*/*AMANN* ratio. qPCR results were reported using the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines [371].

2.2.4 Detection of anti-HLA antibodies

As the tolerance signatures pertain predominantly to alterations in B cell populations, we assessed B cell antibody production by detection of anti-HLA and donor specific antibodies.

Anti-HLA antibodies were detected using the Luminex bead assay (One Lambda Inc., California, US), according to the manufacturer's instructions and the protocol used for clinical samples by the

Transplant Immunology laboratory, Churchill Hospital, Oxford (developed by M. Chen). Briefly, 10µl of serum was cultured for 30 minutes with beads conjugated to HLA epitopes. After washing, beads were then incubated with anti-human IgG bound to phytoerythrin (PE) for 30 minutes. After a further wash step, samples were analysed using a Luminex 100 IS fluorescence detector system (Qiagen, Manchester, UK).

All samples were screened for anti-HLA antibodies using mixed HLA Class I and Class II screening beads. Samples with a marginal or positive result suggestive of anti-donor specificity were further tested. Samples with a marginal result were further assessed using Class I or Class II PRA beads, which are coated with a mix of multiple Class I or Class II HLA epitopes, allowing further elucidation of HLA specificity. For those with a positive result where definite anti-donor reactivity could not be confirmed using screening or PRA beads, sera were tested against Class I or Class II single antigen flow beads. Each bead is coated in a single glycoepitope and sets of 96 (Class I) or 76 (Class II) different beads are used to confirm donor-specificity of anti-HLA antibodies. Any sample with a high negative control binding was re-tested following 'Adsorbout bead' (One Lambda Inc.) incubation to reduce non-specific binding. All serum samples requiring single antigen bead testing were pre-treated with 0.3% EDTA to inactivate complement.

The mean fluorescence intensity (MFI) on each individual sample bead was normalised to a negative control bead within each sample and a negative control sample provided by the manufacturer (LSNC, One Lambda Inc.) using the equation: $[MFI_{\text{sample beads}} - MFI_{\text{sample negative control beads}}] - [MFI_{\text{negative control sample beads}} - MFI_{\text{negative control beads}}]$ [372]. This value is used to identify marginal and positive results. All bead mixes contain a positive and negative control bead and all sample batches were run with a positive and negative control sample, containing a high titre of mixed anti-HLA antibodies, and no anti-HLA antibodies, respectively. For single antigen flow beads, the cut-off for positivity was an MFI of 500 and represents the threshold used for clinical samples at the Oxford Transplant Centre. Analysis was performed by MB with assistance and confirmation of positive

results by M. Chen and Professor S. Fuggle (Oxford University Hospitals Transplant Immunology Laboratory).

2.2.5 ELISA for serum immunoglobulin

As well as testing for antigen-specific immunoglobulin production, the total concentration of IgG and IgM were quantified in sera from RTR.

Serum immunologlobulin concentration was determined by ELISA (Total Human IgM and IgG Ready-SET-Go kits, eBioscience), according to the manufacturer's instructions. Thawed and centrifuged sera were diluted (1:10,000 for IgM and 1:500,000 for IgG) and incubated for two hours in 96-well flat bottom plates (Nunc MaxiSorp, Thermo Scientific) pre-coated with capture antibody. After washing, plates were incubated for one hour at room temperature with horseradish peroxidase (HRP) conjugated detection antibody, before further washing and addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution for 15 minutes. All incubations were performed at room temperature and (with the exception of the final incubation) on a rocker. The substrate reaction was stopped with 2N sulphuric acid, and the plate read immediately at a wavelength of 450nm using a microplate reader (eMax system, Molecular Devices, Wokingham, UK). All testing was performed in duplicate.

2.3 Chapter 5

2.3.1 Thawing of PBMC and flow cytometry

PBMC were thawed by immersion in a 37^{O}c waterbath. When mostly thawed, 4µg DNAse I (Roche, Burgess Hill, UK) was added then 5mL of RPMI over a 2 minute period. Cells were washed twice prior to preparation for further analysis. Staining of PBMC was performed as in Chapter 3, but events were acquired on a BD CantoII (BD Biosciences). Analysis was performed as before, gating on live CD3^{+} lymphocytes.

For further phenotyping and functional assays, additional antibodies (clone) used were: PD-1-FITC (EH12.2H7), CD19-FITC (HIB19), CD45RA-FITC (HI100), Tim-3-PE (F38-2E2) and CD57-Pacific Blue (HNK-1) from Biolegend; CD3-ECD (UCHT1) from Beckman Coulter; KLRG-1-APC (REA-261) from

Miltenyi Biotec (Bisley, UK); and CD28-PE (CD28.2) and CD8-APC-Cy7 (SK1) from BD Biosciences. 7AAD (eBioscience) was used to discriminate live/dead cells in all assays using thawed PBMC.

2.3.2 $CD8^+$ T cell cytotoxicity assay

The cytotoxicity assay was used to assess the degranulation of lysosomes by circulating $CD8^+$ T cells and is a modified version of that described by Yap *et al.* using IL-2 rather than anti-CD28 for costimulatory signalling [373]. Preliminary work was undertaken to establish the optimal concentration of anti-CD3 and IL-2. This revealed a plateau of CD107a staining at the dose used subsequently for the assay.

Wells of a 96-well flat bottom plate were coated with 2µg/mL anti-CD3 antibody (OKT3, Biolegend, UK) overnight at 4^Oc. After washing twice with PBS, wells were loaded with 0.5 x 10^6 PBMC in complete medium (RPMI supplemented with 10% FCS [Gibco], L-glutamine and penicillin-streptomycin) supplemented with 125U/mL IL-2 (R&D Systems) and incubated for 6 hours at 37^Oc 5% CO_2. CD107a (Lysosome Associated Membrane Protein [LAMP]-1)-APC-Cy7 (Clone H4A3, Biolegend, UK; 2.5µL/well) was added at the beginning of the culture and monensin (1x, eBioscience) was added after 2 hours. An unstimulated control was used, which was incubated for 6 hours in an uncoated well in the presence of complete medium and CD107a-APC-Cy7 only. At the end of the incubation period cells were stained for surface markers. Gating for $CD107a^+$ cells was performed using the unstimulated control.

The binding of the conjugated antibody to CD107a during the incubation period is a measure of the turnover of CD107a at the cell surface. This enables quantification of the degree of lysosomal fusion with the cell membrane and therefore the extent of lysosomal degranulation with polyclonal TCR stimulation.

2.3.3 T cell stimulation for intracellular cytokine staining

In order to assess the production of cytokines by T cells, PBMC were thawed as above and plated at 0.5 x 10^6 per well in a 96-well flat-bottom plate. Cells were activated for 6 hours at 37^Oc 5% CO_2 using phorbol-12-myristate-13-acetate (PMA, 50ng/mL, Sigma-Aldrich) and ionomycin (1µg/mL,

Sigma-Aldrich) with brefeldin A (1x, BD Biosciences) in complete medium. Unstimulated cells, incubated with brefeldin A and complete media only, were used as negative controls and for gating. Cells were stained for surface markers as before and permeabilised prior to intracellular cytokine staining using the Cytofix/Cytoperm staining kit (BD Biosciences). Antibodies used for cytokine staining were: IL-2-FITC (MQ1-17H12, eBioscience), TNF-α-FITC (Mab11, eBioscience) and IFN-γ-eFluor 450 (4S.B3, eBioscience).

2.3.4 Recruitment of non-transplant participants

Participants with a history of SCC but no history of continuous systemic immunosuppression were recruited from the Outpatient Department at the Oxford Dermatology Unit, Churchill Hospital. Potentially eligible participants were identified by the treating consultant prior to clinic and participation was discussed with the patient during their appointment. If amenable, the patient was referred to MB for formal consent and phlebotomy at the clinic. Clinical blood count and renal function was assessed at enrolment. Additional exclusion criteria for this cohort were: renal impairment requiring dialysis, a history of previous solid organ transplantation, and a history of previous systemic immunosuppression of cumulative duration of greater than one month. All participants had a history of 'recent' SCC (within the preceding 750 days). Whilst formal matching was not undertaken at this stage, potential recruits who were 'younger' were approached preferentially to facilitate ease of subsequent matching to the main cohort.

2.3.5 CMV pentamer staining

To assess CMV-specificity of circulating $CD8^+$ T cells, 5µL of phycoerythrin (PE)-bound recombinant Pro5 MHC Class I pentamers complexed to the NLVPMVATV $pp65_{495\text{-}503}$ peptide (ProImmune, Oxford, UK) were incubated with 0.75 x 10^6 thawed PBMC for 10 minutes at RT. Optimal dosing of pentamer was established by titration on a test sample prior to staining of study samples.

After washing, PBMC were counterstained for 30 mins at 4^Oc in the dark, with fixation for 30 minutes with 'fixation buffer' (PBS with 2% formaldehyde and 1% FCS) before data acquisition (BD CantoII).

Single live $CD19^-$ cells were gated for analysis; $CD19^+$ cells were gated out as these can bind non-specifically to pentamers. To ensure specificity of staining, two control samples were stained during preliminary testing. One was a HLA-$A*0201^+$ RTR who was CMV seronegative; the second was a CMV seropositive HLA-A3/68 (i.e. HLA-$A*0201^-$) RTR. No pentamer staining was seen with either.

2.3.6 Immunohistochemistry

Excised SCC from study participants during the study period or the preceding year were chosen in order to assess correlation with enrolment peripheral blood phenotype.

SCC histology on clinical specimens was first reviewed by a consultant dermatopathologist (E. Ieremiah, Oxford University Hospitals Pathology Department) to confirm the diagnosis prior to further staining. Immunohistochemistry was performed on samples by P. Allen (Oxford University Hospitals Pathology Department). 3-4µm thick sections were cut on coated super frost slides. Adjacent sections were single stained for FOXP3 or double-stained for CD8 and CD57.

FOXP3 epitope retrieval was performed on the Bond Max staining machine with Bond Epitope Retrieval Solution 2 (EDTA-based buffer at pH9.0) at 100^Oc for 20 minutes. Detection of the FOXP3 primary antibody was performed using the 'Bond Polymer Refine Detection System' (Leica Biosystems). Stained sections were enhanced with 1% copper sulphate followed by a light haematoxylin counterstain. Sections were then dehydrated, cleared and mounted in DPX.

Double staining utilised 3,3'-diaminobenzidine (DAB, brown) for CD57 and alkaline phosphatase (red) for CD8 staining. Epitope retrieval for CD57/CD8 was performed with Bond Epitope Retrieval Solution 1 (Citrate-based buffer at pH 6.0) at 100^Oc for 20 minutes. CD57 detection was performed using 'Bond Polymer Refine Detection System' as for FOXP3 with CD8 detection performed using 'Bond Polymer Refine Red Detection System' (Leica Biosystems). Double-stained sections were counterstained with haematoxylin then mounted with aqueous mountant.

The sections were stained on the Leica Bond Max staining platform (Leica Biosystems, UK). The FOXP3 primary antibody (clone 236A/E7, Abcam, UK) was used at 1:100 dilution. The CD57 (clone

NK-1, Leica Biosystems) and CD8 primary antibodies (clone 4B11, Leica Biosystems) were in ready-to-use format and so were used undiluted.

Cells were counted and summated over 10 high power fields (HPF), equating to a total surface area of 2mm^2. These were then adjusted to give a value per mm^2. Cells were counted by E. Ieremiah (Oxford University Hospitals Pathology Department). Seven samples were reviewed and cells counted independently by a second consultant histopathologist (O. Espinosa, Oxford University Hospitals Pathology Department) to assess inter-observer variability. The coefficient of variation in intratumoural FOXP3$^+$ cell count was 8% and for CD8$^+$CD57$^+$ cell count was 6%. The peritumoural coefficients were larger, reflecting the larger surface area that could be examined, leading to more variation in counts. The coefficient of variation for FOXP3$^+$ counts in the peritumoural region was 13%, and for CD8$^+$CD57$^+$ 30%. This large coefficient of variation for peritumoural CD8$^+$CD57$^+$ cells is misleading, as peritumoural CD8$^+$CD57$^+$ counts were low and the mean absolute difference in counts between observers was 1 cell.

2.4 Statistical analyses

All analyses were performed on Graphpad Prism for Windows 5.03 (Graphpad, San Diego, CA) or IBM SPSS Statistics for Windows 20.0 (IBM Corp., New York, NY). Continuous variables are shown as median (interquartile range) unless specified otherwise, except hazard ratios which are reported as hazard ratio (95% confidence interval). Categorical variables are reported as number (percentage of group).

Comparison of continuous variables between groups was performed using the non-parametric Mann-Whitney (two groups) or Kruskal-Wallis (multiple groups) test or the parametric Student's t-test (two groups) or Analysis of Variance (ANOVA, multiple groups). For categorical variables the chi-squared test or Fisher's exact test were used. Where the Kruskal-Wallis test was significant, a subsequent post-hoc Dunn test was applied and these are the results shown on figures, unless indicated otherwise. All statistical tests were performed using a two-tailed test.

Correlations were performed using Pearson's test for parametrically distributed data and Spearman's test for non-parametrically distributed data. Survival analyses were performed using Cox regression, as described in the relevant section. Regression was performed using the 'standard' *en bloc* entry method, where all predictors are entered into the model simultaneously.

The relationship between cohort size and the number of covariates in a multivariate analysis is controversial [374]. A formal *a priori* calculation could not be performed as there were a number of variables of interest in this study. Green suggests as a 'rule of thumb' that the minimum sample size for multivariate regression should be "104 + n" (where 'n' is the number of independent variables being examined) for testing individual predictors with a medium effect size [374, 375]. A medium effect size is one that is likely to be clinically relevant and so this is an acceptable approximator for sample size. Thus the maximum number of covariates used in any regression analysis with multiple variables was guided by this.

Linear regression for interaction of immunosuppression with the signatures of tolerance was performed using normally-transformed variables where appropriate.

Odds ratios were calculated by logistic regression using variables significant on univariate analysis. All variables were checked to ensure the assumption of proportional hazards was not violated for Cox analysis: for continuous variables using a residual plot, whilst for categorical variables a log-log plot was used. The normality of data was assessed using the Shapiro-Wilk test and confirmed visually by Q-Q plots.

ROC curves were generated and tested using a non-parametric assumption of distribution. Throughout the study a p-value of less than 0.05 was considered significant, unless indicated otherwise. In order to prevent Type II (false positive) errors due to multiple testing, where appropriate a Bonferroni correction was applied, the adjusted threshold for significance is indicated where used.

Notation for statistical significance is given for each figure, but in general ‘ns’ denotes ‘non-significant’, *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Error bars on graphs indicate mean ± SEM unless indicated otherwise.

Chapter 3: Participant recruitment, phenotype and prediction of cutaneous squamous cell carcinoma development

3.1 Introduction

A number of reproducible clinical risk phenotypes exist for SCC development in renal transplant recipients including UV exposure, increasing duration of immunosuppression (which is also a function of increasing age) and increasing age at first transplant [31-34, 37, 46, 74, 75, 79, 110, 142, 376]. These risk factors were established across whole transplant cohorts where, particularly with more historical studies, long-term RTR are underrepresented. Low risk groups for SCC such as the young, those with a very short duration of transplant and those with Fitzpatrick skin type 5 may also be enrolled in these studies. Risk scores have been developed to aid the identification of RTR at increased risk of SCC within the general transplant population [32, 34, 73, 74]. These risk scores have been developed in various cohorts where a sizeable majority are in the first decade after transplant and have not been validated in a cohort of patients who are globally high risk and who may have had previous SCC.

Previous work suggests that the circulating immune phenotype of RTR is altered in those with a history of SCC and may be able to predict the future development of SCC. Increased number of Treg, gamma-delta T cells, $CD8^{+}CD28^{-}$ cells and reductions in NK cell numbers and $CD4^{+}$ cells after lymphocyte depleting induction have all been associated with subsequent SCC development [66, 109, 188].

As discussed in Chapter 1, we hypothesised that two potential mechanisms underlying a reduced immune response may contribute to SCC development: immune regulation (identified using previously defined 'signatures' of operational tolerance) and immunosenescence. Operational tolerance and alterations in B cell compartments will be discussed further in Chapter 4.

For an immune phenotype to be clinically useful, it should ideally provide prognostic information on top of that provided by clinical factors. However, no study has compared the performance of clinical and immunological factors in predicting SCC development in long-term RTR. This chapter will address the questions raised here; namely:

1. Can pre-existing risk scores identify those at highest risk of SCC in long-term RTR?
2. Are markers of immunosenescence predictive of SCC development?
3. Do immunological or clinical predictors of SCC risk perform better in this cohort?

3.2 Recruitment

117 RTR were recruited in total to the study: the process of identification and recruitment is summarised in Figure 3.1.

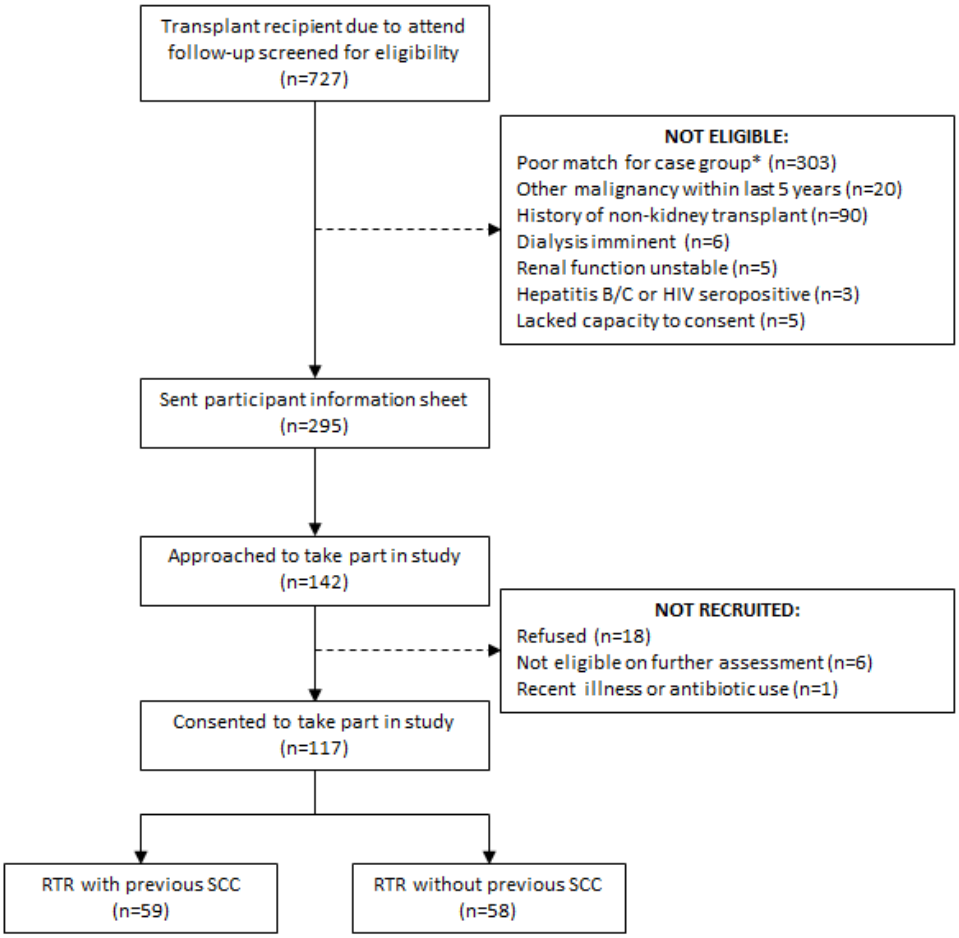


Figure 3.1: Flow diagram detailing participant identification and recruitment to the main arms of the study. **poor match reasons include: age less than 40 (n=35); less than five years post-transplant (n=234); not skin type 1-4 (n=32); and syngeneic graft (n=1).*

Whilst the initial protocol design as discussed in Chapter 2 was to recruit 200 RTR, recruitment was halted early for two main reasons. The first was that the vast majority of RTR with a history of SCC had been recruited at this point: a number of RTR with a history of SCC were excluded from the study due to previous malignancy within the last 5 years (n=10), or due to the imminent failure of their transplant, unstable renal function or a recent re-transplant (n=4). Two potential participants declined to take part. The second reason was that interim analysis at this point had demonstrated the marked effect of azathioprine upon B cell populations, discussed in more detail in Chapter 4. Given the widespread use of azathioprine in this cohort it was felt that the prevalence of the tolerance signature was likely to be much lower than expected; even with a cohort size of 200, the study was likely to be underpowered to detect a difference in SCC development associated with possession of the signature.

3.3 Clinical phenotype

The clinical phenotype of participants is summarised in Table 3.1. As expected given the lag period between transplantation and SCC development, the majority of RTR recruited had a median duration of immunosuppression of over twenty years. All but four participants were Caucasian. Around two-thirds were male, and the majority had received a period of dialysis prior to transplantation. Most participants did not receive induction therapy. Despite a trend towards an increased proportion having received treatment for rejection (59% vs 41%, p=0.054), RTR with a history of SCC (hereafter referred to as RTR_{SCC}) displayed similar renal function to those without a history of SCC (RTR_{No}). RTR_{SCC} were older than RTR_{No} (66 (58 – 74) vs 61 (55 – 67), p=0.03). Whilst it was initially intended to match groups by age, sex and duration of immunosuppression, due to the increased prevalence of SCC in older RTR, age could not be fully matched.

Variable	All (n=117)	RTR_{SCC} (n=59)	RTR_{No} (n=58)	p
Caucasian[£]	113 (97)	58 (98)	55 (95)	0.36
Male	80 (68)	41 (71)	39 (67)	0.21
Age at enrolment (years)	63 (56 – 71)	66 (58 - 74)	61 (55 - 67)	0.03
Body mass index (kg/m^2)	25.5 (22.7 – 28.7)	25.2 (21.7 – 28.2)	26.2 (23.2 – 29.7)	0.09
Age at first transplant (years)	42 (32 – 51)	43 (31 – 52)	40 (32 – 49)	0.30
Dialysis before transplant	88/112 (79)	44/56 (79)	44/56 (79)	1.0
Duration of dialysis (months)	13 (7 – 24)	15 (8 – 35)	13 (7 – 22)	0.53
Total duration of immunosuppression (months)[#]	262 (207 – 326)	283 (208 – 353)	249 (203 – 312)	0.17
Received more than one transplant	23 (20)	13 (22)	10 (17)	0.56
Induction therapy:				0.53
None	99 (85)	51 (86)	48 (83)	
IL-2 receptor antagonist	11 (9)	5 (9)	6 (10)	
Anti-thymocyte globulin (ATG)	7 (6)	3 (5)	4 (7)	
Previously treated for rejection	54/108 (50)	32/54 (59)	22/54 (41)	0.054
Number of times treated	2 (1 – 3)	2 (1 – 3)	2 (1 – 3)	0.42
Total HLA-ABDR mismatches:	2 (1 – 4)	2 (2 – 4)	2 (1 – 4)	0.71
Serum creatinine (mmol/L)	122 (102 – 164)	117 (90 – 168)	126 (106 – 161)	0.27
eGFR ($mL/min/1.73m^2$)	45.7 (34.0 – 59.2)	50.9 (35.5 – 64.0)	44.2 (34.0 – 57.5)	0.19
CMV IgG seropositive at enrolment	76 (65)	40 (68)	36 (62)	0.52

Table 3.1: Clinical phenotype of study participants at enrolment. *Continuous variables are reported as median (interquartile range) whilst categorical variables are reported as number (percentage of column). Where there are missing data, a denominator of number of known values in that column is given.* [£]*3 participants were mixed Indian-Caucasian (no SCC), whilst one was Afro-Caribbean (prev SCC).* [#]*including pre-transplant immunosuppression (n=10 [4 RTR_{SCC}] received immunosuppression prior to transplant). Variables are compared between arms (RTR_{SCC} and RTR_{No}) using Mann-Whitney test for continuous variables and chi-squared or Fisher's exact test for categorical variables.*

3.4 Immunosuppression at enrolment

RTR_{SCC} and RTR_{No} did not differ in terms of immunosuppressive regimen, dosage or trough levels at time of enrolment (Table 3.2).

Variable	RTR_{SCC} (n=59)	RTR_{No} (n=58)	p
Immunosuppression:			
Calcineurin inhibitor	49 (83)	47 (81)	0.78
Azathioprine	46 (78)	38 (66)	0.14
MMF	4 (7)	9 (16)	0.15
Prednisolone	24 (41)	22 (38)	0.76
Sirolimus	4 (7)	2 (3)	0.35
Daily dosage (mg/kg)*:			
Ciclosporin	1.90 (1.60 – 2.90)	2.05 (1.58 – 2.55)	0.81
Tacrolimus	0.06 (0.06 – 0.10)	0.06 (0.04 – 0.08)	0.25
Azathioprine	0.85 (0.55 – 1.13)	0.80 (0.58 – 1.20)	0.85
MMF	11.9 (7.9 – 15.1)	14.6 (13.0 – 19.0)	0.28
Prednisolone	0.07 (0.06 – 0.08)	0.06 (0.05 – 0.07)	0.12
Trough level (ng/mL)^:			
Ciclosporin	76 (54 – 104)	83 (59 – 100)	0.80
Tacrolimus	7.4 (6.3 – 9.4)	6.5 (5.7 – 7.8)	0.32
Number of immunosuppressive agents&:			
1	3 (5)	8 (14)	
2	45 (76)	40 (69)	0.27
3	11 (19)	10 (17)	

Table 3.2: Immunosuppressive regimen of participants at enrolment. **Daily dosage is reported as a median (IQR) across RTR receiving that medication. ^if below limit of detection, was given value 1 unit below this (i.e. if <30ng/mL, given arbitrary value of 29ng/mL). &refers to the total number of immunosuppressive agents taken (i.e. prednisolone and ciclosporin would be counted as two). Difference assessed by chi-squared test. Variables are compared between arms (RTR_{SCC} and RTR_{No}) using Mann-Whitney test for continuous variables and chi-squared or Fisher's exact test for categorical variables. 'MMF', mycophenolate mofetil.*

3.5 Other clinical risk factors for malignancy development

A number of previously identified clinical risk factors for malignancy were assessed in both groups. RTR_{SCC} were more likely to report previous malignancy in a first-degree relative (parents or siblings) and trended towards a paler skin type (Table 3.3).

Three clinical risk scores previously proposed for use in the prediction of NMSC or SCC development in renal transplant recipients were compared between RTR_{SCC} and RTR_{No} [34, 73, 74]. Whilst all three trended towards a greater value in the RTR_{SCC} group, only the Urwin risk score was found to be significantly different. The Harden risk score utilised a threshold score of greater than or equal to 8.25 to identify RTR at high risk of NMSC during the first ten years post-transplant: only two RTR, both RTR_{SCC}, were in this high-risk group.

Variable	RTR_{SCC} (n=59)	RTR_{No} (n=58)	P
Smoker (>1 pack-year history)	20 (34)	23 (40)	0.52
Previous non-NMSC malignancy	5 (9)	11 (19)	0.10
History of malignancy in parents/siblings	31 (53)	20 (35)[&]	0.04
Chronic UV exposure [34]	38 (64)	32 (55)	0.31
Fitzpatrick skin type:			
Type I-II	27 (47)	17 (29)	0.07
Type III-IV	30 (51)	41 (71)	
Type V-VI	1 (2)	0	
Non-brown eye colour	44 (75)	37 (65)	0.26
Harden risk score [73]	5.26 (4.2 – 6.16)	4.96 (4.35 – 5.82)	0.43
Score $\geq$8.25	2 (3)	0	0.50
Urwin risk score [74]	2 (1 – 3)	1.5 (0 – 2)	0.008
Harwood risk score [34]	4 (2 – 4)	3 (2 – 4)	0.10

Table 3.3: Prevalence of other risk factors for NMSC in study participants. *[&]2 participants were adopted and thus unable to provide a family history. Difference between categorical variables assessed by chi-squared or Fisher's exact test as appropriate, and between continuous variables by Mann-Whitney test. The calculation of the risk scores is explained further in Chapter 2: Methods.*

3.6 Enrolment immune phenotype

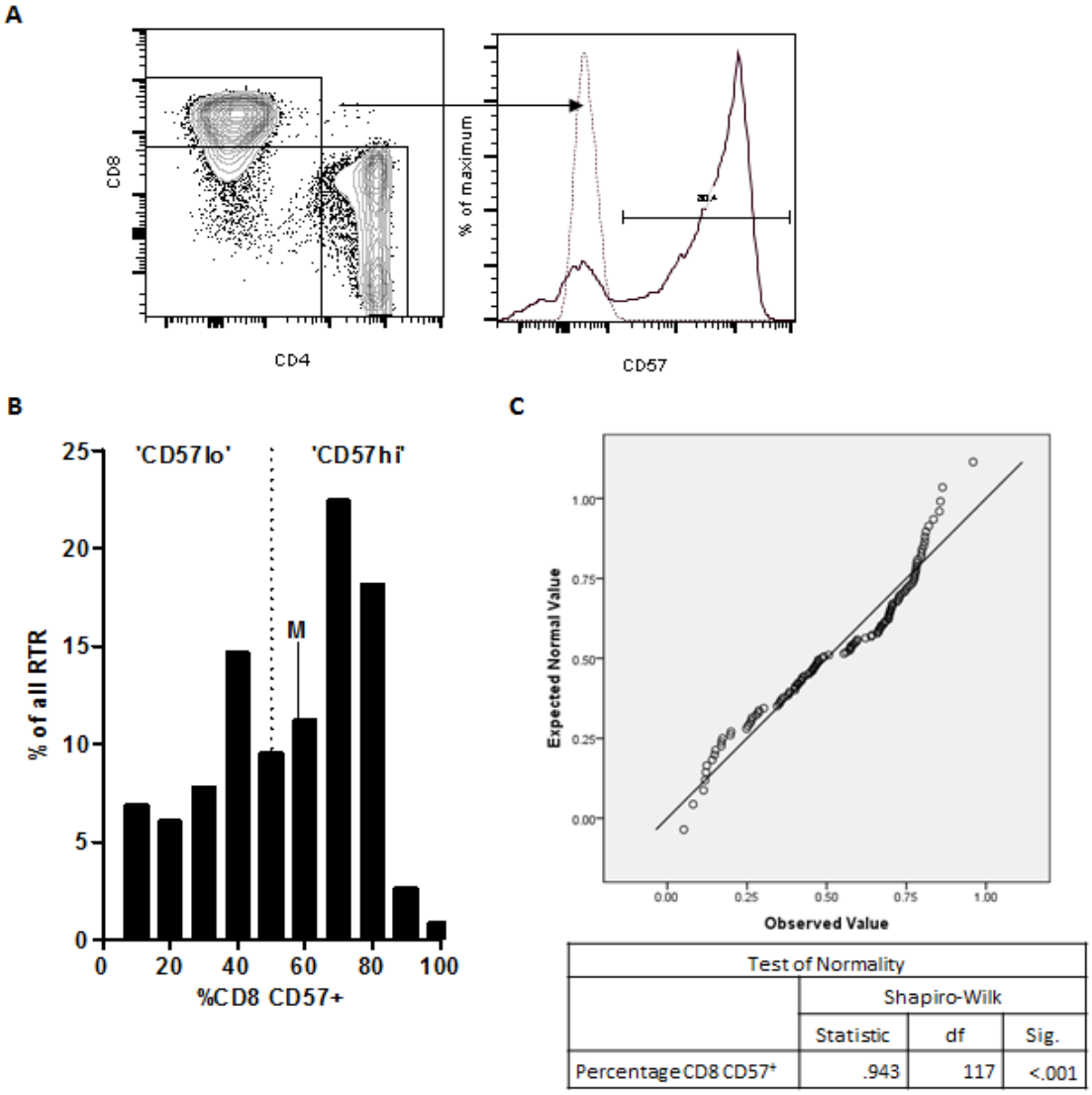
RTR were immune phenotyped at enrolment by flow cytometry using fresh, isolated PBMC. There was a trend towards a decreased number of $CD3^+$ T cells in RTR_{SCC}, but this did not represent a loss of one particular subtype as the proportion of $CD4^+$, $CD8^+$ and gamma-delta cells was unaltered compared to RTR_{No} (Table 3.4). There was a significant increase in the baseline percentage but not absolute number of Treg in RTR_{SCC}.

Amongst the markers of immunosenescence, there was no difference at enrolment in the mean proportion of $CD8^+$ T cells expressing CD57 nor in the CD4/CD8 ratio; both in terms of absolute value and the proportion of RTR with a ratio less than one; a value previous associated with increased risk of mortality in the elderly [309, 310, 315, 377]. No difference was seen in the proportion of T cells within major $CD8^+$ compartments.

Variable	RTR_{SCC}	RTR_{No}	p
Lymphocyte count	1135 (863 – 1658)	1360 (1040 – 1728)	0.15
Monocyte count	500 (410 – 635)	475 (413 – 600)	0.58
Total ($CD3^+$) T cell count	945 (649 – 1339)	1157 (785 – 1467)	0.25
Gamma-delta T cell count	33 (14 – 79)	38 (13 – 97)	0.77
Gamma-delta T cell percentage	2.6 (1.5 – 5.5)	3.0 (1.2 – 6.7)	0.83
$CD4^+$ T cell count	567 (431 – 700)	666 (474 – 836)	0.13
$CD4^+$ T cell percentage	48 (39 – 61)	49 (40 – 60)	0.79
Treg ($CD4^+CD69^-CD25^{hi}CD127^{lo}FOXP3^+$) cell count	15 (10 – 23)	13 (9 – 18)	0.27
Treg percentage (of $CD4^+$ population)	2.8 (1.9 – 3.6)	2.3 (1.5 – 2.9)	0.02
$CD57^+$ percentage (of $CD4^+$ population)	11 (7 – 28)	11 (6 – 18)	0.35
$CD8^+$ T cell count	275 (132 – 410)	284 (179 – 510)	0.20
CD4/CD8 ratio	2.1 (1.3 – 4.3)	2.1 (1.2 – 3.7)	0.57
$CD8^+$ T cell percentage	22 (15 – 31)	24 (16 – 32)	0.61
$CD8^+CD57^+$ T cell count	162 (48 – 282)	146 (77 – 310)	0.65
Naïve $CD8^+$ T cell (percentage of $CD8^+$)	11 (4 – 33)	15 (5 – 34)	0.96
$CD8^+$ TEm (percentage of $CD8^+$)	24 (14 – 39)	22 (12 – 43)	0.90
$CD8^+$ TEMRA (percentage of $CD8^+$)	54 (29 – 73)	42 (20 – 74)	0.40
$CD8^+$ TCm (percentage of $CD8^+$)	1 (0.4 – 4)	1 (0.3 – 3)	0.50
$CD57^+$ percentage (of $CD8^+$ population)	67 (39 – 76)	53 (36 – 70)	0.18
>50% of $CD8^+$ T cells $CD57^+$ ('CD57hi' phenotype)	36 (61)	29 (50)	0.21
$CD4^+/CD8^+$ ratio	2.10 (1.17 – 3.72)	2.12 (1.34 – 4.28)	0.57
$CD4^+/CD8^+$ ratio <1	9 (15)	11 (19)	0.59
NK cell ($CD3^-CD56^+$) count	30 (16 – 109)	43 (13 – 121)	0.97
NK cell percentage	3.5 (1.4 – 9.7)	3.1 (1.0 – 9.1)	0.70
$CD19^+$ B cell count	19 (8 – 44)	19 (12 – 40)	0.45
$CD19^+$ B cell percentage	1.7 (0.9 – 3.7)	1.8 (0.9 – 2.9)	0.38

Table 3.4: Immune phenotype of study participants at enrolment. *Continuous variables are reported as median (interquartile range), whilst categorical variables are reported as number (percentage of group). Counts are reported as cells per microlitre of blood, whilst percentages are reported as a fraction of the lymphocyte population, unless specified otherwise. Groups were compared by Mann-Whitney test (continuous variables) or chi-squared test (categorical variables). For two participants a contemporaneous clinical lymphocyte count was not available and so absolute cell numbers could not be calculated.*

The percentage of CD57-expressing $CD8^+$ T cells showed a bimodal distribution across the cohort. The cohort mean and nadir of the two peaks approximated to 50% and so participants were dichotomised into 'CD57hi', where the majority ($\geq$50.0%) of $CD8^+$ T cells expressed CD57, and 'CD57lo', where the minority (<50.0%) expressed CD57 (Figure 3.2).



Test of Normality			
	Shapiro-Wilk		
	Statistic	df	Sig.
Percentage CD8 CD57+	.943	117	<.001

Figure 3.2: The distribution of percentage of CD8$^+$ T cells expressing CD57 is bimodal across the whole cohort, with a population mean and nadir at approximately 50%. *A) Representative staining and gating strategy for CD8 and CD57. B) Histogram of distribution of percentage of CD57-expressing CD8$^+$ T cells amongst study participants; the solid line labelled 'M' represents the median for the cohort (58%, mean 54%). The dashed line indicates the 50% cut-off. C) Q-Q plot demonstrating the bimodal distribution of the histogram in B, along with Shapiro-Wilk test indicating significant deviation from normality.*

Taking sections 3.2-3.5 together, these results indicate that the cohort recruited is relatively heterogeneous in terms of clinical risk factors for SCC. The only clinical marker of note which differed between those with and without a history of SCC is age at enrolment, which will be adjusted for in subsequent testing. In keeping with previous studies, RTR_{SCC} had a greater proportion of Treg within the CD4$^+$ population.

3.7 Effect of immunosuppression upon NK phenotype

As has been previously reported [117, 378], NK cell numbers were significantly reduced in those receiving azathioprine. A further analysis of NK cell subsets was undertaken to see whether one particular population was affected by azathioprine. The reduction was seen across both $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ populations, but was particularly marked in the $CD56^{dim}CD16^{+}$ population, leading to a significant reduction in the $CD56^{dim}$:$CD56^{bright}$ ratio (Figure 3.3). $CD56^{dim}CD16^{+}$ NK cells are thought to represent 'mature' NK cells that develop full cytolytic function upon activation, are capable of antibody-dependent cell-mediated cytotoxicity (ADCC) and are poorly proliferative [379-381]. $CD56^{bright}CD16^{-}$ NK cells are considered their precursors and exhibit enhanced secretion of cytokines such as IFN-γ and TNF-α with proliferation in response to IL-2, but poorer cytolytic function [379-382]. In keeping with this, we found the expression of granzyme B and the activating receptor NKG2D (CD314) to be significantly greater in CD56dim NK cells compared to CD56bright (data not shown).

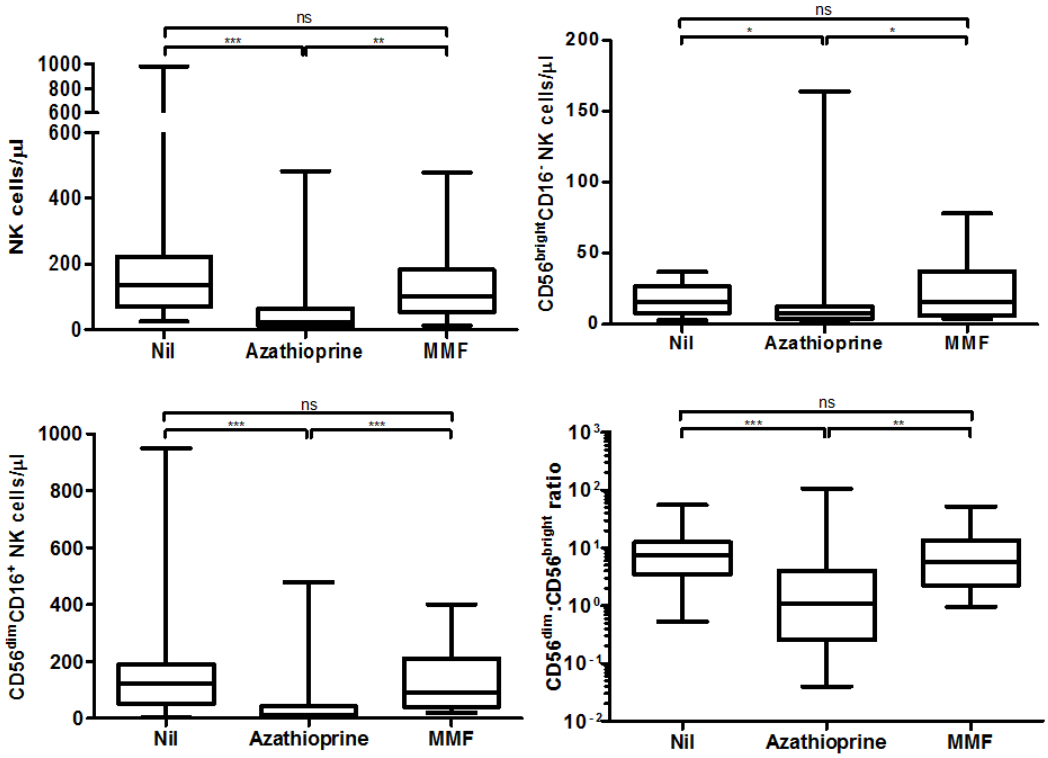


Figure 3.3: Azathioprine use is associated with a reduction in the number of circulating cytolytic, mature NK cells. *Total circulating NK cells numbers, numbers of $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ cells, and ratio of these in RTR on no antimetabolite; MMF; and azathioprine. 'ns' not significant, *p<0.05, **p<0.01, ***p<0.001 using Kruskal-Wallis with Dunn's post-hoc tests. p value is given for Kruskal-Wallis test where no lines are shown. Box and whisker plots indicate (from top to bottom) maximum value, 75th, 50th and 25th centiles and minimum value, respectively.*

Azathioprine did not have an effect upon total circulating monocytes or T cell numbers; in particular there was no reduction in the number of total or naïve CD4 and CD8 T cells (data not shown).

In keeping with previous reports, we found calcineurin inhibition was associated with a trend towards a reduced number (median [IQR] 18 (10 – 36) vs 13 (9 – 18), p=0.2) but particularly proportion of Treg within $CD4^+$ T cells (median [IQR] 3.0 (1.8 – 4.3)% vs 2.4 (1.5 – 3.0)%, p=0.06).

3.8 SCC development

Study participants were followed up for a median (IQR) of 630 (531 – 723) days with no difference between RTR_{SCC} and RTR_{No} in follow-up duration. This represents a total of 70435 'at risk' days. During follow-up two RTR lost their grafts and returned to dialysis and seven RTR died (Table 3.5). There was no difference in the proportion of DWFG between those with and without a history of SCC at enrolment. Whilst this study was not designed to detect differences in mortality, it should be noted that of the six deaths during follow-up, five of the participants demonstrated a CD57hi phenotype.

Cause of loss to follow-up	RTR_{SCC}	RTR_{No}
All death with functioning graft:	4	2
Malignancy	1	1
Cardiovascular disease	2	1
Infection	1	0
Graft loss	0	2
Other loss to follow-up	1	0
Total	5	4

Table 3.5: Causes of non-event censoring or loss to follow-up. *Variables are reported as number of cases. One RTR_{SCC} died of pulmonary infection but with widespread pulmonary metastases of cutaneous SCC origin.*

32 participants developed a total of 66 SCC during follow-up. Eight RTR_{No} developed a first presentation of SCC. On univariate analysis, RTR_{SCC} were over three and a half times more likely to develop SCC during follow-up (Table 3.6 and Figure 3.4). 15 RTR developed multiple SCC during follow-up, of which three were RTR_{No}.

3.9 Prediction of SCC development during study

The ability of clinical and immunological factors to predict the development of SCC was studied next (Table 3.6). On univariate analysis both age at enrolment and first transplant were predictive of SCC, as well as history of previous SCC prior to the study. Notably, previously identified risk factors for SCC were not predictive in this cohort, including smoking, chronic UV exposure and duration of immunosuppression. No individual immunosuppressive agent was predictive of SCC development; MMF and sirolimus were not analysed due to infrequency of use.

Variable	Univariate	
	HR (95% CI)	**p**
Age at enrolment (years)	1.08 (1.04 – 1.12)	<0.001
Male gender	1.44 (0.64 – 3.20)	0.38
Age at first transplant (years)	1.04 (1.01 – 1.07)	0.005
Received dialysis pre-transplant	2.09 (0.73 – 5.96)	0.17
Duration of dialysis pre-transplant (months)	1.01 (1.00 – 1.01)	0.09
Total immunosuppression duration (months)	1.00 (1.00 – 1.00)	0.76
Number of HLA mismatches	1.06 (0.85 – 1.33)	0.60
eGFR at enrolment (ml/min/1.73m^2)	1.00 (0.98 – 1.02)	0.64
CMV seropositive	2.00 (0.86 – 4.62)	0.11
Positive smoking history	1.28 (0.63 – 2.58)	0.50
History of previous SCC	3.67 (1.65 – 8.19)	0.001
History of previous non-NMSC malignancy	1.13 (0.43 – 2.92)	0.81
Malignancy in first-degree relative	0.63 (0.31 – 1.32)	0.22
History of chronic UV exposure	1.89 (0.88 – 4.10)	0.11
Increasing Fitzpatrick skin type	0.75 (0.48 – 1.16)	0.19
Non-brown eye colour	1.28 (0.57 – 2.84)	0.55
Number of immunosuppressive agents taken at enrolment	1.44 (0.77 – 2.72)	0.26
Use of azathioprine at enrolment	2.25 (0.87 – 5.85)	0.10
Use of ciclosporin at enrolment	3.79 (0.90 – 15.9)	0.07
Use of prednisolone at enrolment	0.64 (0.30 – 1.35)	0.24

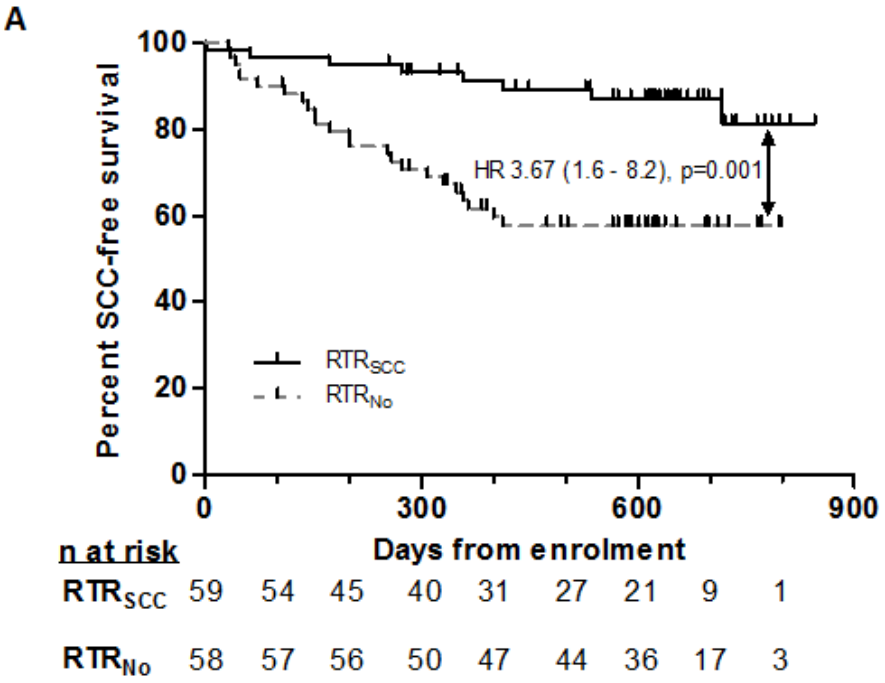
Table 3.6: Univariate predictive value of clinical factors for earlier SCC development during follow-up. *Variables are expressed as a hazard ratio, per unit increase for continuous variables (units in parentheses where appropriate). A Bonferonni correction was applied for multiple testing: the threshold for significance was set at $p \leq 0.003$.*

Immunologically, a high proportion of CD57-expressing $CD8^+$ T cells were predictive of SCC development on univariate analysis (Table 3.7 and Figure 3.4). Number of Treg were not predictive of SCC development; this remained the case when the analysis was repeated using only those with a history of SCC at enrolment (HR (95%CI): 0.99 (0.95 – 1.02), p=0.42). Despite a greater proportion of Treg within $CD4^+$ T cells in the RTR_{SCC} cohort, this was not predictive of further SCC development:

again, this was also the case when only RTR_{SCC} were analysed (HR (95% CI): 0.97 (0.72 – 1.31), p=0.83).

Variable	HR (95% CI)	p
Number of lymphocytes (cell/µl)	1.00 (1.00 – 1.00)	0.87
Number of $CD3^+$ cells (cell/µl)	1.00 (1.00 – 1.00)	0.65
Number of $CD4^+$ T cells (cell/µl)	1.00 (1.00 – 1.00)	0.44
Number of $CD8^+$ T cells (cell/µl)	1.00 (1.00 – 1.00)	0.70
Number of NK cells (cell/µl)	1.00 (1.00-1.00)	0.55
Number of Treg (cell/µl)	0.98 (0.95 – 1.02)	0.36
Percent of $CD4^+$ Treg	0.92 (0.67 – 1.27)	0.61
Number of gamma-delta T cells (cell/µl)	1.004 (1.00 – 1.01)	0.03
Number of $CD57^+$ $CD8^+$ T cells (cell/µl)	1.001 (1.00 – 1.00)	0.32
>50% $CD57^+$ $CD8^+$ T cells (CD57hi phenotype)	3.60 (1.55 – 8.34)	0.003
CD4:CD8 ratio	0.99 (0.88 – 1.12)	0.87
CD4:CD8 ratio <1	1.34 (0.55 – 3.27)	0.52

Table 3.7: Univariate predictive value of immunological factors for earlier SCC development during follow-up. *Variables are expressed as a hazard ratio, per unit increase for continuous variables (units in parentheses where appropriate). A Bonferonni correction was applied for multiple testing: the threshold for significance was set at $p \leq 0.004$.*



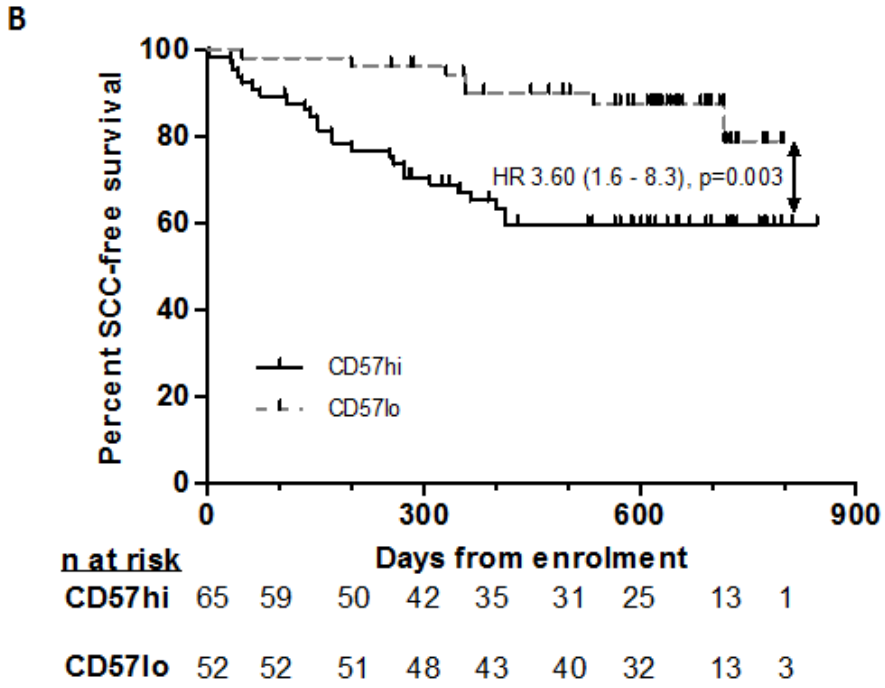


Figure 3.4: SCC development occurs earlier during follow-up in those with a history of previous SCC and in CD57hi RTR. *Hazard ratio represents univariate Cox regression analysis, with RTR stratified by history of previous SCC (A) and CD57 phenotype (B).*

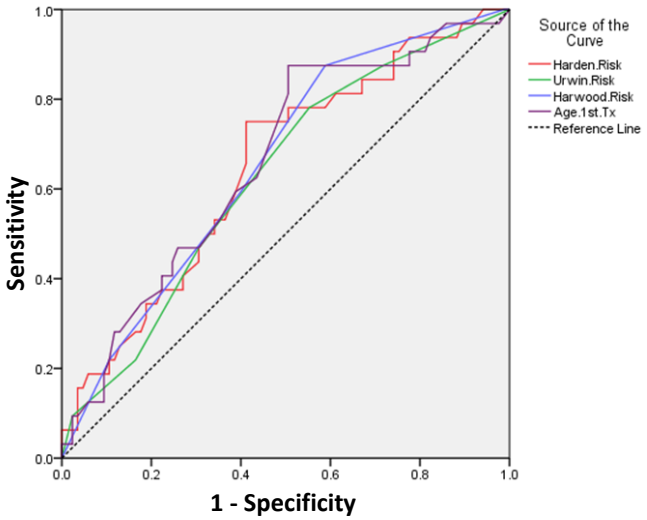
The multivariate model included age at enrolment, history of pre-study SCC, and CD57hi phenotype as covariates (Table 3.8). Age at first transplant was also significant; however age at enrolment and age at first transplant were strongly correlated ($r=0.74$, $p<0.001$) and so to prevent the risk of these variables competing in the multivariate analysis, the model was constructed using each variable

independently. Replacing age at enrolment with age at transplantation in the model did not significantly change the value of the results, though age at first transplant lost significance as a predictive variable.

Variable	HR (95% CI)	p
Age at enrolment (years)	1.05 (1.01 – 1.09)	0.007
History of previous SCC	2.88 (1.27 – 6.50)	0.01
>50% $CD57^+$ $CD8^+$ T cells	2.43 (1.01 – 5.87)	0.048
Age at first transplant (years)	1.03 (1.00 – 1.06)	0.06
History of previous SCC	3.34 (1.49 – 7.56)	0.003
>50% $CD57^+$ $CD8^+$ T cells	2.80 (1.18 – 6.62)	0.02

Table 3.8: Multivariate analysis for prediction of earlier SCC during follow-up. *For multivariate modelling >50% $CD57^+$ $CD8^+$ T cells was adjusted for age at enrolment and history of previous SCC. The second model replaced age at enrolment with age at first transplant.*

All three risk scores offered predictive value for the development of SCC during the study, although this was not the case when only RTR with a first occurrence of SCC were analysed (Supplemental Data Table i.1). When ROC curves were constructed to assess the predictive value of the scores, the area under the curve (c-statistic) indicated that they offered no additional predictive value beyond age at transplant (Figure 3.5).



Variable	c-statistic (95% CI)	p
Age at first transplant (increasing)	0.66 (0.55 - 0.77)	0.008
Harden risk score (increasing)	0.65 (0.52 - 0.74)	0.01
Urwin risk score (increasing)	0.63 (0.55 - 0.76)	0.03
Harwood risk score (increasing)	0.66 (0.55 – 0.77)	0.009

Figure 3.5: Previously published clinical risk scores do not offer additional predictive value above that provided by age at first transplant in predicting SCC development in long-term RTR. *ROC curves were tested for significant deviation from the reference line, presuming a non-parametric distribution.*

This was confirmed by recalculating the Harden and Urwin risk scores without the age at transplant component: neither of the scores continued to offer predictive value (Table 3.9).

Variable	Univariate		Without age component	
	HR (95% CI)	p	HR (95% CI)	p
Harden risk score	1.37 (1.09 – 1.74)	0.008	1.12 (0.72 – 1.75)	0.61
Urwin risk score	1.36 (1.07 – 1.71)	0.01	1.26 (0.94 – 1.69)	0.12
Harwood risk score	1.60 (1.15 -2.23)	0.005	N/A	N/A

Table 3.9: Predictive value of clinical risk scores for SCC development during study. *Variables are expressed as a hazard ratio per unit increase in score, calculated by Cox modelling. The univariate analysis was then repeated using the risk score without the age component. It was not possible to do this for the Harwood score due to the nature of the score.*

The results presented in this section suggest that stratifying long-term, high-risk RTR on the basis of CD57 expression upon $CD8^+$ T cells enables identification of a subset at increased risk of SCC development after sampling. This is independent of age at enrolment, age at transplantation and history of previous SCC. In contrast previously developed clinical risk scores offer limited ability to predict SCC development other than the predictive value offered by age at transplant.

3.10 Stability of CD57 expression

For clinical utility as a biomarker of increased SCC risk, it is important to assess the stability of CD57 expression with time and SCC development to evaluate how frequently the marker would need to be tested in a clinical setting. 104 participants were phenotyped again at least six months (median [IQR] of 238 [196 – 252] days) and 86 participants one year (385 [364-441] days) after initial phenotyping. Analysis was undertaken blinded to the original phenotyping result.

The median (IQR) coefficient of variation from baseline was 4.8% (2.0 – 10.1%), corresponding to a median absolute standard deviation of 2.2% (1.1 – 5.0%) at the first repeat sampling. The median coefficient of variation at the second repeat sampling was 5.4% (1.9 – 9.8%), corresponding to a median absolute standard deviation of 2.0% (0.8 - 4.9%). These values did not differ in those who developed SCC between sampling (Figure 3.6). The slope of the best fit lines of enrolment percentage against first sampling (0.96 [0.91-1.01]) and second sampling (0.91 [0.84-0.98]) percentage were close to one, indicating a strong correlation with the enrolment phenotype over a one year period.

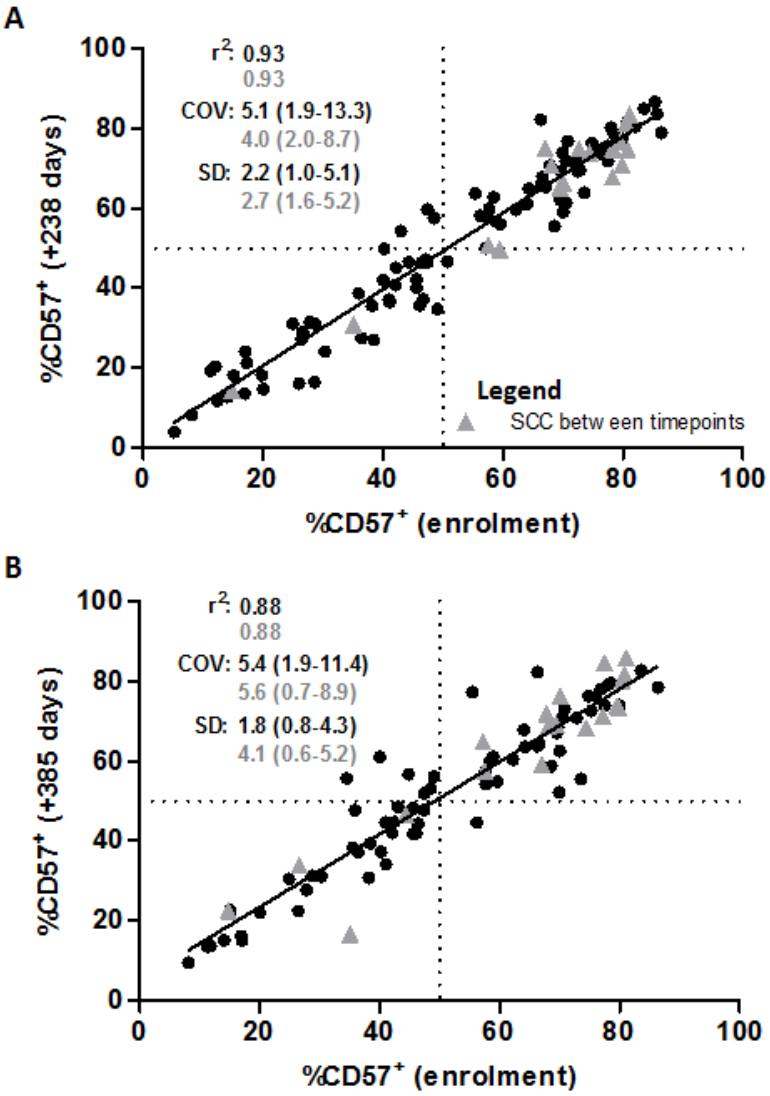


Figure 3.6: CD57 expression upon CD8$^+$ T cells is stable with time and SCC development. *Correlation between enrolment CD57 expression upon CD8$^+$ T cells and expression at two subsequent timepoints. A) First timepoint, a median of 238 days after enrolment; B) Second timepoint, a median of 385 days after enrolment. The coefficient of determination (r^2), coefficient of variation (COV) and standard deviation (SD) are given for those who remained SCC-free (black) and those who developed SCC between time points (grey). COV and SD are reported as median (IQR). Correlations are calculated using Pearson's test.*

These results illustrate that CD57 expression upon CD8$^+$ T cells is stable with time and show no alteration in stability with SCC development.

3.11 Determinants of CD57 phenotype

Given the predictive value of CD57 phenotype in predicting disease behaviour it was important to assess whether any clinical factors which may also be relevant in SCC development may have impacted upon this. A logistic regression was therefore undertaken using CD57 phenotype as the

dependent variable, testing clinical covariates for predictive value (Table 3.10). Only increasing age at enrolment and CMV seropositivity were predictive of CD57 phenotype. Importantly, factors previously found to be predictive of SCC recurrence risk, such as Fitzpatrick skin type and chronic UV exposure, did not differ between those who were CD57hi and those who were CD57lo.

Variable	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age at enrolment	1.09 (1.04-1.14)	<0.001	1.07 (1.02-1.13)	0.005
Age at first transplant	1.06 (1.02-1.10)	0.001		
Male gender	1.38 (0.63-3.04)	0.60		
Current or previous smoker	0.56 (0.26-1.20)	0.14		
Duration of immunosuppression	1.00 (0.99-1.00)	0.35		
Number of transplants	0.69 (0.32-1.49)	0.34		
Total number of HLA-ABDR mismatches	1.10 (0.86-1.39)	0.45		
Number of HLA-AB mismatches	1.07 (0.80-1.43)	0.65		
CMV IgG seropositivity	14.4 (5.48-37.8)	<0.001	12.5 (4.60-33.7)	<0.001
Post-transplant CMV seroconversion*	3.43 (0.70-16.8)	0.13		
T-cell depleting induction therapy	0.58 (0.12-2.72)	0.49		
History of previous SCC	1.57 (0.75-3.26)	0.23		
Chronic UV exposure	1.36 (0.64-2.85)	0.42		
Fitzpatrick skin type (I-II, III-IV, V-VI)	1.51 (0.72-3.15)	0.27		
Serum creatinine	1.00 (0.99-1.00)	0.45		
eGFR	1.00 (0.98-1.02)	0.73		
On calcineurin inhibitor at enrolment	1.48 (0.57-3.80)	0.42		
Trough level (ciclosporin)	0.99 (0.98-1.01)	0.27		
On azathioprine at enrolment	1.26 (0.56-2.82)	0.58		
On MMF at enrolment	0.46 (0.14-1.50)	0.20		
On steroids at enrolment	0.60 (0.28-1.26)	0.18		
Previous treatment for rejection	1.00 (0.47-2.14)	1.00		
Pre-transplant dialysis	0.69 (0.27-1.74)	0.43		
Duration of pre-transplant dialysis	1.00 (0.99-1.01)	0.45		
Previous non-NMSC malignancy	0.58 (0.20-1.67)	0.31		

Table 3.10: Clinical predictors of CD57hi phenotype. *Continuous variables are expressed as an odds ratio per unit (as defined in Table 3.6) increase. The multivariate (bivariate) regression model included those variables significant on univariate analysis. *compared to pre-transplant seroconversion (only in those RTR who were seropositive at enrolment). Age at transplant was predictive on univariate analysis but correlated strongly with age at enrolment; to prevent possible competition between these the stronger of the two was included in the multivariate model. Significance was set at $p \leq 0.002$ for univariate testing, using a Bonferonni correction.*

In support of the association between CMV seropositivity and CD57 expression, we found a significant positive correlation between CMV IgG titre and percentage of CD57-expressing $CD8^+$ T cells in CMV positive RTR (r^2 0.17, $p<0.001$). This phenotype may thus be driven by subclinical CMV viraemia, which may occur in a subset of transplant recipients in the absence of antiviral prophylaxis

[383, 384]. PCR on peripheral blood was undertaken to assess CMV viral load in a subset of stable CMV positive participants (n=29): CMV viral load was below the limit of detection in all but one (this sample was taken during the participant's final hospital admission, during which the participant subsequently died).

Given the effect of immunosuppression on other aspects of the immune phenotype, the effect of calcineurin inhibitors, steroids and antimetabolites upon CD57 expression was assessed. Steroid use at enrolment was associated with a significantly reduced proportion of CD57-expressing $CD8^+$ T-cells (median [IQR] 66% [40 – 77%] vs 48% [26 – 68%], p=0.01). However, the proportion of participants taking steroids did not differ between RTR_{SCC} and RTR_{No} or CD57hi and CD57lo groups, nor did the mean dose. When stratified by CMV serostatus, a significant reduction in CD57 expression was seen only in CMV seropositive RTR (71% [59 – 79%] vs 59% [45 – 71%], p=0.008) despite no difference in age at sampling (data not shown). A similar trend was seen in seronegative RTR, though this did not reach significance (40% [25 – 47%] vs 25% [13 – 42%], p=0.12)

3.12 Prediction of SCC recurrence

Whilst previous analyses suggest the CD57hi phenotype predicts earlier occurrence of SCC after sampling, the date of sampling is an arbitrary time point and has no clinical meaning. A more clinically relevant read-out would the time of occurrence of (next) SCC from a fixed time point such as time of previous SCC.

It was hypothesised that the CD57hi phenotype would identify RTR at risk of developing earlier recurrence of SCC. In order to maximise the size of this cohort for analysis, given the data previously showing stability of the CD57 marker over a one year period, all RTR with SCC during the study or in the preceding year prior to enrolment were selected for analysis, using the first SCC during this time period as 'time zero'. This created a cohort of 42 RTR with a history of recent SCC which was an insufficient number for multivariate analysis.

CD57hi phenotype identified RTR who developed SCC recurrence at a significantly earlier time point (Figure 3.7). By 600 days post-SCC, only a third of CD57hi RTR remained free of recurrence, in contrast to two-thirds of CD57lo RTR. Age at first transplant and enrolment, duration of immunosuppression and all three clinical risk scores were not predictive of earlier SCC recurrence on univariate analysis. As CD57 phenotype is heavily influenced by CMV seropositivity we assessed the predictive value of this in identifying earlier SCC recurrence: this also predicted earlier SCC recurrence on univariate analysis (HR: 3.8 (95%CI: 1.1 – 13.2), p=0.03). During follow-up, two RTR developed metastatic SCC: both of these were CD57hi and CMV seropositive. During the 'at risk' period, seven (16%) had reduction of immunosuppression; four (28%) of CD57lo RTR and three (11%) CD57hi RTR. Of these, all three CD57hi and one of four CD57lo developed further SCC, suggesting this did not have a significant impact upon the findings in Figure 3.7.

Analysis of those with a first presentation of SCC during the study or previous year (n=13) revealed half of the CD57hi cohort developed a further SCC within 200 days, whereas all CD57lo remained recurrence-free throughout follow-up (Figure 3.7). This indicates a positive predictive value of 50% and a negative predictive value of 100% for second SCC development during follow-up.

These results suggest that a high proportion of CD57-expressing $CD8^+$ T cells, driven to an extent by latent CMV infection, can be used to predict earlier recurrence of SCC in high-risk RTR.

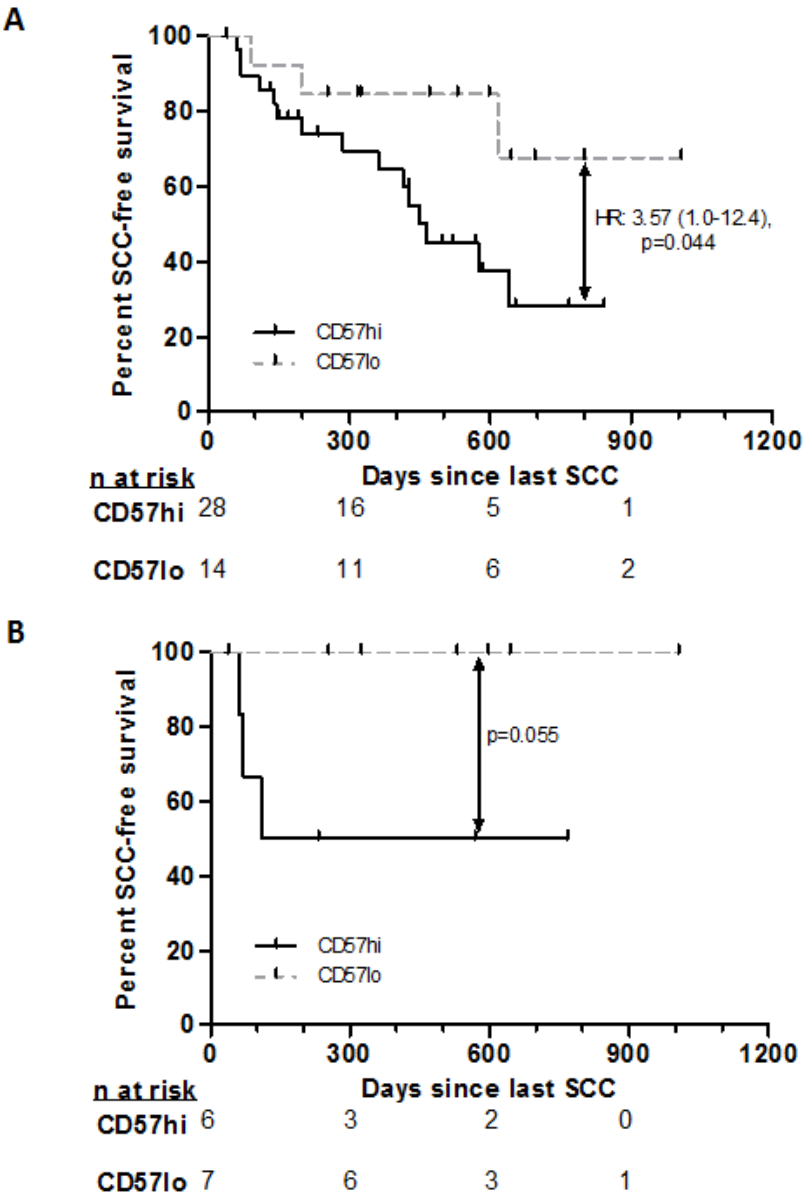


Figure 3.7: CD57hi phenotype identifies RTR who develop earlier recurrence of SCC. *A) Time from previous SCC to next SCC in RTR who developed SCC during the study or preceding year. B) Time from first SCC to second SCC in RTR only with a first presentation of SCC during the study or preceding year. Hazard ratios using univariate Cox regression are presented for A; the p value for B represents the result of log-rank testing.*

3.13 Summary of key findings

- 117 RTR were recruited. RTR_{SCC} and RTR_{No} were matched for sex and duration of immunosuppression. RTR_{SCC} and RTR_{No} displayed similar clinical phenotypes. RTR_{SCC} were significantly older at enrolment, but not at time of first transplant;
- A number of previously identified risk factors for SCC development, including chronic UV exposure, did not differ between RTR_{SCC} and RTR_{No}: only one of three published risk scores examined was significantly increased in those with a history of SCC;
- RTR_{SCC} had an increased proportion of circulating Treg within the $CD4^+$ T cell compartment, but this did not translate to an increase in absolute number;
- Age at enrolment and first transplant, a history of previous SCC, and >50% CD57-expressing $CD8^+$ T cells predicted SCC development during follow-up;
- CD57 phenotype was stable over a one year period and with SCC development, although was influenced by CMV serostatus, use of steroids and age at enrolment. Other potential risk factors for SCC did not differ between those who were CD57hi and CD57lo;
- CD57hi phenotype and CMV seropositivity predicted earlier recurrence of SCC in those who developed SCC during the study or preceding year.

3.14 Discussion

Whilst numerous risk scores have been published to identify RTR at increased risk of SCC amongst the general transplant population, little work has been undertaken to establish measures specifically within selected cohorts at increased risk of SCC. While UV exposure is a key risk factor for SCC development, two other major factors are cumulative duration of immunosuppression and increasing age at transplant. There is an expanding cohort of older, long-term transplant recipients as a result of increased access to transplantation for those with comorbidities and increased age [5, 385]. This cohort can be expected to further increase in size over coming years. This means the future burden of SCC within transplant populations is also likely to grow.

In this chapter, the performance of previously identified clinical and immunological predictors of SCC was compared, along with potential markers of immunosenescence. These results indicate that CD57hi phenotype may be stronger predictor of SCC development and recurrence than other previously identified risk factors in long-term RTR. This phenotype is associated strongly, but not exclusively, with CMV seropositivity.

3.14.1 Phenotype at enrolment

Clinical phenotype

Recruitment for this study focussed upon RTR who would be at 'high-risk' for SCC development during follow-up. This was achieved by recruiting a cohort of RTR who had developed SCC previously and a matched cohort who had not developed SCC. Whilst the groups were matched specifically for duration of immunosuppression and gender, an important difference between the two groups was age at enrolment. This difference arose from the nature of recruitment, where both groups were recruited simultaneously with matching employed later in the process. At this stage it became clear that there were insufficient older RTR with long-standing transplants without a history of SCC that were eligible to take part. The difference was permitted to remain in order to maximise the study cohort size, as opposed to retrospective exclusion of patients in order to improve matching. Thus adjustment for age at enrolment was made on subsequent analyses.

RTR_{SCC} and RTR_{No} were relatively well matched in many other respects at baseline. A greater proportion of RTR_{SCC} had received treatment for rejection previously despite no difference in number of HLA mismatches. The link between rejection and previous cutaneous malignancy may relate to the alteration in immunosuppression at time of rejection, or temporary use of more powerful immunosuppressants such as high-dose steroids and anti-thymocyte globulin (ATG, polyclonal antibodies which primarily deplete T cells) in resistant rejection [386, 387]. It was decided to count any treatment for rejection rather than only histologically-confirmed rejection to account for use of high-dose steroids or other therapies that may be started then subsequently stopped with a biopsy that is non-diagnostic of rejection. Rejection may be associated with an increase in maintenance immunosuppression, which could lead to protumoural effects, as discussed in Chapter 1. Despite the increased prevalence of earlier treatment for rejection amongst RTR_{SCC}, renal function at enrolment was similar in the two groups.

Few RTR received induction therapy at the time of transplant, reflecting clinical practice when the majority received their transplant. Given the widespread use of induction therapy in current practice, the relationship between induction therapy and malignancy is likely to become increasingly relevant, although this may take a number of years to become apparent given the lag period between transplant and many malignancies. There is evidence for increased malignancy risk with lymphocyte depleting agents such as anti-thymocyte globulin [189, 388-391]; however increased risk with non-depleting agents such as basiliximab (an IL-2 receptor antagonist) has not been clearly demonstrated [389, 391, 392]. A previous study suggested RTR with low $CD4^+$ T cell counts after immune reconstitution, of which about 60% received ATG, were at increased risk of cutaneous malignancy development over a ten year period [187].

There was no difference in maintenance immunosuppressive regimen at enrolment, both in terms of agents used and dosage. Cumulative immunosuppressive dose was not calculated for participants as this information was not always accurately documented or was missing altogether, particularly for RTR who were transplanted elsewhere. Whilst total duration of immunosuppression

has been consistently shown to be a risk factor for SCC development, studies have not demonstrated a clear link between the cumulative dose of any individual agent and SCC risk [66, 393, 394].

The net effect of immunosuppression with multiple drugs, best quantified clinically by total duration, may be significantly more important in determining SCC risk than absolute cumulative dose of any drug. Whilst the overall intensity of immunosuppression is likely to be important, as evidenced by an early reduction in immunosuppression dose leading to a reduction in SCC burden [124], evidence that any one agent is particularly associated with malignancy is conflicting. Interpretation of the literature is limited by variable study design and overlapping immunosuppressive regimes, small cohort size and incomplete matching in many studies [40, 47, 79-81, 388, 395, 396]. The most convincing evidence pertinent to the study presented here comes from long-term follow-up of the 'Australian Multicentre Trial of Ciclosporin Withdrawal' cohort (n=489), where the incidence of both skin and non-skin cancer at twenty years post-transplant did not differ between those who were randomised to receive dual therapy with azathioprine and prednisolone and those who received long-term ciclosporin monotherapy [47].

RTR in this study were taking a relatively homogenous immunosuppressive regime with a combination of steroids, ciclosporin and/or azathioprine in the majority. Surprisingly few RTR were taking sirolimus, particularly in the RTR_{SCC} group. This may reflect the poor tolerability of sirolimus previously reported or a move away from sirolimus use given the equivocal findings of two randomised studies in which sustained benefit from a switch to sirolimus after SCC development was only seen in those with a history of a single lesion [140, 141].

In contrast to previous work attempting to identify immunological predictors of SCC development in long-term RTR cohorts [66, 109, 188], this study also took into account risk factors for SCC development related to sun exposure.

There was a non-significant trend towards paler skin in those with a previous history of SCC. This is in keeping with previous studies, where paler skin is a risk factor for SCC development [74, 79, 110-

113]. Surprisingly, self-reported chronic UV exposure did not differ between the two groups. The lack of increased UV exposure in those with a history of SCC may represent inaccurate reporting although efforts were taken to minimise this by use of open questions and by breaking down lifetime sun exposure into periods (childhood, pre-transplant and post-transplant). An alternative explanation is that, given the long lag period since transplant and the relatively long duration of immunosuppression, in long-term RTR UV exposure may be less relevant.

Two of three previously developed clinical risk scores did not differ between the two groups. The third score, the Urwin score, did differ. Potentially up to 40% of the Urwin score is made up by a number of factors indicative of UV exposure, whereas the Harden score does not incorporate this, whilst the Harwood score utilises cumulative sunburn episodes only to distinguish between a score of 3 and 4. The Urwin score may therefore differ as a result of more accurate characterisation of UV exposure. The final explanation is that the lack of discrimination may represent a survival bias, where those with the most sun exposure developed the most aggressive malignancies and so did not survive to be recruited to this study; however, this would be expected to affect the Urwin score more than the others given the increased weight given to sun exposure-related factors, which clearly was not the case. The lack of difference between groups may also represent the selected nature of this cohort, in contrast to the populations used to develop the measures which were unselected. This will be discussed further later.

RTR_{SCC} were more likely to report a history of any malignancy in a first-degree relative than RTR_{No}. These malignancies were variable in origin and only a small number reported cutaneous malignancy in parents or siblings. This may represent recall bias or shared environmental exposure to carcinogens [397, 398], but may also represent an inherited genetic component of malignancy development as discussed in Chapter 1. Whilst a number of studies have suggested increased risk of SCC if there is a family history of the same in the general population [152, 399-403], only one corrected for inherited phenotypic and environmental risk factors [152]. One study found an association between parental melanoma and hepatic malignancy and SCC, whilst another found an

association with SCC and genitourinary malignancy. However, both were interrogations of the same national registry and did not adjust for risk factors for malignancy [399, 401].

Immunological phenotype

In contrast to previous work, we found that RTR with a history of SCC at enrolment did not have an increase in Treg number, though there was an increase in the proportion within the $CD4^+$ gate. This is in agreement with the findings by Sherston *et al.*, who re-tested survivors of the original work by Carroll *et al.* [66, 223]. This may also reflect increased use of calcineurin inhibitors in this study compared to previous studies, with a greater proportion of RTR using these and a greater median daily dose. Calcineurin inhibition has been previously associated with a reversible reduction in number and proportion of Treg in peripheral blood and an inhibition of *FOXP3* expression with relative preservation of the epigenetic changes in the Treg-specific demethylated region (TSDR) required for *FOXP3* transcription [117, 223, 404-407]. We too saw a trend towards lower number and proportion of Treg within $CD4^+$ T cells with use of calcineurin inhibitors.

The univariate effect of steroids on $CD8^+CD57^+$ cells was unexpected. There was no other clinical factor that differed between those taking steroids and not taking steroids, including mean age and proportion who were CMV seropositive that might otherwise explain this (data not shown). This phenomenon has been described previously in steroid-treated polymyalgia rheumatica [408], but this is the first description in the setting of transplantation. It is hypothesised that, given the association of immunosenescence with chronic inflammation (discussed further in Chapter 5), the use of steroids reduces this inflammation and slows the accumulation of these cells, though why this preferentially occurs with steroids and not other immunosuppression is unclear at this stage. The effect of slowing the accumulation of these cells and potentially 'protecting' from SCC may be counteracted by the additional immunosuppressive effect. Despite this effect, the proportion of RTR in the CD57hi and lo groups taking steroids did not differ and thus is unlikely to impact upon the finding regarding CD57hi phenotype and SCC risk.

3.14.2 Azathioprine and NK cells

The effect of azathioprine upon circulating NK cell number has been previously described in transplantation, inflammatory bowel disease and rheumatoid arthritis [117, 378, 409]. Generally these studies have found a preferential loss of the $CD56^{dim}CD16^{+}$ population in those taking azathioprine, in agreement with the data presented here [378, 410]. Azathioprine has also been demonstrated to impact negatively upon NK cell activation, leading to reduced cytotoxicity and cytokine secretion [378, 410-412]. A previous study in inflammatory bowel disease found the reduction in circulating NK cell numbers with azathioprine therapy was also seen locally in the colon, indicating that the loss of circulating NK cells may reflect changes in peripheral tissue [378]. This is important as the majority of NK cells are not in blood but are located in lymphoid tissue [413]. Whilst the spleen contains mostly circulating $CD56^{dim}CD16^{+}$ NK cells, lymph nodes contain predominantly $CD56^{bright}CD16^{-}$ NK cells, where they undergo activation-induced maturation in response to antigenic stimulation and cytokines from antigen presenting cells [381, 414, 415]. Animal models demonstrate that these NK cells play a critical role early in the immune response through the generation of cytokines including interferon gamma, which induces polarisation towards a Th1 response against viruses, tumours and intracellular bacterial pathogens [416-419].

The relevance of a reduction in the NK cell-mediated response to various malignancies, though notably not SCC, has been demonstrated in animal models utilising antibody-mediated NK cell depletion, and NK receptor germline and conditional knockout [418, 420-422]. The effect of azathioprine upon the NK cell-mediated immune priming process is likely to be detrimental and needs to be explored further, particularly in animal models of cutaneous malignancy [150, 241, 423]. This may represent an important mechanism of initiating an effective immune response during the early phase of malignancy development.

Given the data above and the NK cell sparing effects of MMF, this may provide a rationale for pre-emptive conversion from azathioprine to MMF in a subset of RTR who are at high risk of SCC; for

example CD57hi RTR, where the adaptive immune response may be already impaired. The mechanistic role of azathioprine in lymphocyte depletion will be discussed further in Chapter 4.

3.14.3 Prognostic value of clinical and immunological variables for SCC development

Whilst immune phenotype of circulating lymphocytes has been used to identify increased SCC risk previously [66, 109, 188], this is the first study to compare the predictive value of immunological variables against clinical markers. It was found that a high proportion of CD57-expressing $CD8^+$ T cells are associated with an increased risk of SCC development during follow-up, with a decreased interval between successive SCC in those with recurrent disease. This phenotype is itself associated with increasing age and CMV seropositivity, but CMV seropositivity did not predict earlier SCC occurrence after sampling and the CD57hi phenotype remained predictive after adjustment for both age at transplant and enrolment. Clinical aspects relating to the association of CMV with malignancy will be dealt with in this Chapter, whilst molecular and immunological factors will be addressed in Chapter 5.

UV exposure, clinical risk scores, and SCC in long-term RTR

Whilst risk factors for *de novo* SCC in RTR have been well characterised, studies on risk factors for further SCC that do not represent incomplete removal or metastasis are more sparse. Euvrard *et al.* retrospectively analysed 188 RTR with previous post-transplant SCC and found that eye colour, duration of immunosuppression and sun exposure were predictive of further disease [58]. This reflects similar findings elsewhere [34]. In contrast, the study presented here found duration of immunosuppression was not predictive of earlier SCC development: this likely relates to the selected nature of the cohort, where all were chosen for a long duration of immunosuppression. Eye colour is unlikely to be directly predictive of SCC development, but is likely a more reliably quantified manifestation of overall phenotype, including hair colour and (most relevantly) skin type [75]. Sun exposure in that study was characterised in a different manner to our study and was reported as low, medium or high, whereas this study used more strictly defined cut-offs for chronic

UV exposure and defined it as a binary variable [34]. A retrospective Australian study found UV exposure to be predictive of SCC development and numbers in a relatively young cohort (median age 40.2 years) in the first decade post-transplant [75]. These are in contrast to our findings; reasons for this are discussed below.

Three previously developed risk scores based on clinical phenotype [34, 73, 74] were assessed for performance in this cohort: all three were predictive of SCC development after sampling. A fourth clinical risk score has been previous published, however this was not included as this incorporates previous actinic keratosis as a covariate [32]. This may not have definitive histological evidence in all cases in our cohort, for example where a clinical diagnosis is made, and so this covariate could not be objectively applied. All three analysed scores utilise age at transplant but offered no predictive value beyond this. Furthermore, on univariate analysis no score was predictive of earlier SCC recurrence. Whilst all three scores were developed in populations distinct from the one in this study, they represent the only objective clinical stratification methods developed in transplant populations. All three of the scores have not been validated in an independent cohort and this study provided the opportunity to do so. All three scores demonstrated poor performance in a globally high-risk population, with no additional predictive value beyond that of increasing age. Why might this be? The Harden score was developed in a northern British population relatively early post-transplant and was designed to identify RTR at risk of developing NMSC in the first ten years post-transplant [73]; the population studied here is much later post-transplant. The Harwood score was also developed in a cohort predominantly in the first decade post-transplant and was devised in a multi-ethnic population in a large urban British cohort, where a quarter of the cohort was skin type V or VI [34]. The participants in this project, predominantly Caucasian, would therefore all score highly on this basis. Finally, the Urwin score was developed in an Australian cohort; this partly explains the increased weight given to UV-related factors [74]. The relative contribution of UV radiation to the development of SCC is likely to be much greater in a cohort from a subtropical country as evidenced by the earlier onset of SCC. The finding that these scores perform poorly in this study may highlight a potential limitation of them: the scores may identify

RTR at increased risk of SCC amongst an unselected transplant population (comprising RTR of all ages and races) but do not offer sufficient precision to identify selected RTR at greatest risk of SCC. In a clinical setting, these risk scores could be used to 'triage' RTR at increased risk of SCC, based on clinical phenotype, who could then be further stratified by CD57 phenotype.

A key feature in the cohort studied here was the long duration of time since transplant; a feature less prevalent in previous studies of clinical phenotype. As discussed previously, it is possible that those who develop the most aggressive SCC due to high UV exposure are 'selected against' for enrolment into this study, due to their death or ineligibility for another reason. This is undoubtedly a contributor but actually quantifying the extent of this is difficult; whilst SCC is highly prevalent in transplant populations the actual mortality from this is relatively low, though still higher than the general population, as less than 10% metastasise [62, 65]. Studies in European and North American cohorts have estimated SCC-specific mortality to be up to 10% [62, 63, 424]. A study in a more UV exposed cohort in New Zealand found case-mortality due to metastatic SCC to be 15% at ten years after diagnosis of first SCC [45]. It would be expected that those with the most UV exposure would be those with the most recurrent disease and would be most likely to develop metastasis [34].

In the analysis of those with SCC during the study or preceding year, chronic UV exposure was not predictive of earlier SCC recurrence. Furthermore, although limited by small numbers, chronic UV exposure and all three risk scores were not predictive of first SCC development in those who had no history of SCC at enrolment. This suggests a second explanation for the poor performance of the risk scores; the aetiology of SCC in this population is different to those who develop SCC earlier post-transplant, with immunosuppression and by extension alterations in the immune system playing a much more significant role.

Using immune phenotype to predict SCC development and recurrence

CD57hi phenotype was found to be predictive of SCC development after sampling and was predictive of earlier SCC recurrence on univariate analysis. This marker was stable over a one year period without being significantly affected by SCC development. The stability of the marker is both

a strength and a weakness: the stability means that testing would only need to be intermittent in a clinical setting, providing long-term prognostic value. However, the lack of change with SCC development means that it could not be used to predict imminent onset of disease.

Treg proportion and number were not predictive of SCC development, in contrast to previous reports [66, 109]. This may also relate to the different outcome examined in this study. It was felt that a marker to independently identify RTR at increased risk of the development of any SCC over a long period of time would be the most clinically useful measure. The prognostic information previously described with increased Treg number has generally been of a short term nature, has required additional risk stratification using pre-existing risk factors (such as number of previous SCC), or was only predictive of high-risk SCC, a feature which requires histological examination. Treg predictive value has only been previously assessed from sampling, an arbitrary timepoint with no clinical meaning. Thus, the CD57hi phenotype appears to a stronger performer in this respect.

The lack of overrepresentation of CD57hi phenotype at enrolment in RTR_{SCC} is interesting. This may represent a survival bias, if those with the most aggressive disease were in the CD57hi group. This chapter demonstrates a number of reasons why the CD57hi phenotype may be associated with reasons why a patient may not be eligible for the study, such as increased mortality or development of other malignancy.

Previous work in European cohorts has estimated the risk of SCC recurrence within a year of first SCC to be around 30-50% [34, 35, 58, 59]; this is higher than the recurrence rate in RTR with a first SCC in this study (24%), though obviously this is limited by the small numbers and may reflect the diminished influence of UV radiation. Importantly, all those with recurrence had a CD57hi phenotype. This finding needs to be confirmed in a larger cohort of RTR with first manifestation of SCC, as this may provide a method to identify RTR who would benefit from immunosuppression reduction or switch to sirolimus prior to the development of further malignancy.

An association between the accumulation of $CD57^+$ or $CD28^-$ $CD8^+$ T cells and malignancy has been reported previously in the setting of transplantation. A decreased proportion of $CD8^+CD28^+$ cells

were found to be predictive of malignancy development in the first ten years post-liver transplant; however CMV serostatus was not assessed and cutaneous malignancy was uncommon and poorly defined [226]. In established malignancy in the general population, an increased proportion of circulating CD57-expressing $CD8^+$ T cells has been associated with a poorer prognosis in gastric cancer [425], poorer response to interferon alpha therapy in melanoma [231] and a better response to therapy, but poorer prognosis otherwise, in renal cell carcinoma [232]. Interestingly in these studies the cut-offs were much lower than in this chapter (18%, 23% and 30% respectively) and highlights the increased proportion of these cells frequently seen with chronic antigenic stimulation, such as in transplantation. In bladder cancer survival after intra-vesical IL-2 therapy was better in those with a lower absolute number of circulating $CD8^+CD57^+$ cells [230]. The finding that an increased proportion of CD57-expressing $CD8^+$ T cells is predictive of SCC development is also in keeping with the findings by Carroll *et al.*, who found that an increasing proportion of $CD8^+CD28^-$ cells was predictive of SCC development [66]. However in that study the predictive power of this was lost with adjustment for age and the relationship to CMV serostatus was not explored. Furthermore, the authors looked only at time to next SCC from sampling and not time from previous SCC.

Clinical aspects of CMV and malignancy

CMV is a double-stranded DNA beta-herpes virus (human herpes virus 5) and the largest member of its family [426]. It is transmitted easily between humans, being passed through saliva, breastfeeding, sexual contact, the faecal-oral route and during childbirth [426, 427]. Primary infection is frequently asymptomatic in healthy individuals, with the virus rapidly establishing latency, although in the neonatal period congenital infection can be more catastrophic [428]. Latency is a "reversibly quiescent state in which viral genomes are maintained, but viral gene expression is highly restricted and no virus is produced" [340].

The main reservoir *in vivo* during latency is cells of the myeloid lineage including dendritic cells, megakaryocytes and macrophages, as well as their $CD34^+$ haematopoietic precursors within bone marrow [429, 430]; whether CMV is able to maintain latency in other types of cell is subject to

debate [431]. In the presence of an intact immune system viral replication is controlled, however with immune deficiency or immune suppression (such as in organ transplantation) viral reactivation can occur, leading to infection and inflammation in a number of organs including the lung, GI tract, the allograft, and the central nervous system, including the eye [432]. Thus sites of infection may differ from the sites where latency is maintained. Prophylaxis against CMV reactivation is often commenced during the early post-transplant period in those who demonstrate pre-existing seropositivity or those who receive a seropositive organ; this is usually of a three or six month duration, during the period of greatest immunosuppression [432, 433].

Evidence of an IgG humoral response to CMV, indicative of latent infection, can be found in a large proportion of the general population and rises with age; in general CMV seropositivity can be detected in around 60-80% of the general population [315, 434]. This prevalence is in keeping with the results in this chapter.

There is a strong association between CMV seropositivity and changes seen in the immune system with age, including the CD57hi phenotype. This may explain the finding that both CD57hi phenotype and CMV seropositivity were predictive of earlier SCC recurrence. It is important to note, however, that CD57hi phenotype is not limited to those who are CMV seropositive i.e. it is not simply a marker of CMV seropositivity. This is confirmed by the predictive value of CD57hi phenotype but not CMV seropositivity for SCC development during follow-up. The alterations in immune phenotype associated with CMV will be discussed further in Chapter 5. As up to 5% of renal transplant recipients may demonstrate asymptomatic CMV viraemia [384], plasma was tested for CMV viral load on a subset of participants during follow-up, and found to be absent in almost all (the one exception being a clinical sample performed when the participant was hospitalised and near end of life). This suggests that the CMV in long-term RTR is predominantly latent or below the limit of detection, although cross-sectional testing as in this chapter may miss intermittent reactivation or reactivation in peripheral tissues that may not be detectable in peripheral blood [383, 384, 435].

The association between CMV and increased propensity to malignancy after renal transplant has been previously suggested, although results are conflicting. In a single centre study of 455 RTR, CMV positive RTR were at increased risk of malignancy development in the first twelve years post-transplant. CMV seropositivity was found to associate with an increased number of $CD8^+CD57^+$ cells compared to those who were CMV seronegative [327]. However the function of these cells was not explored in depth, with the authors simply referring to them as 'exhausted' due to their inability to produce IL-2. Follow-up was during the first decade post-transplant and as a consequence cutaneous malignancy was relatively underrepresented and poorly defined. A smaller study in RTR (n=100) found the opposite result and suggested that CMV is protective of malignancy development in the first decade post-transplant due to an expansion of protective gamma-delta T cells [249]. An expansion of gamma-delta T cells was seen in the results reported in this chapter; this did not appear to be protective against SCC, in keeping with the work by Hope *et al.* [109]. It may be that the initial protective expansion of gamma-delta cells is later outweighed by progressive impairment in other T cell populations [340], or may relate to the contribution of specific gamma-delta subsets [249, 436]. Whether CMV reactivation plays a role in accelerating this process is unclear; the former study suggested that the proportion of CD57-expressing $CD8^+$ cells was highest in RTR who had demonstrated CMV replication despite viral prophylaxis after transplant [327].

A recently published British study attempted to address the conflicting results above through analysis of the UK Transplant Registry (n=13,215 RTR). Both one and ten year survival in CMV seronegative OTR was significantly better than those who were exposed to CMV (either through pre-existing seropositivity or receipt of a seropositive organ) across all organs, however there was no difference in the risk of developing a first malignancy between seropositive and seronegative OTR and the increased mortality appeared to predominantly relate to cardiovascular disease [437]. However, all OTR were censored at ten years post-transplant, 35% of OTR had unknown CMV status, only the first malignancy was recorded and NMSC was not included as an outcome, which limits the interpretation of this study with regards to the results presented in this chapter. There are no previous reports linking cutaneous malignancy and CMV seropositivity. It may be

hypothesised that the reason for this is that the role of other environmental factors is dominant at earlier time points, however with increasing duration of a reduced immune response after transplant other factors, such as the influence of CMV and the CD57hi phenotype, may come to the fore.

3.14.4 Conclusion

In this chapter it has been demonstrated that an immune phenotype comprising a majority of $CD8^+$ T cells expressing CD57, termed 'CD57hi', is a predictor of earlier SCC development during study follow-up and may also be associated with earlier recurrence of disease. A combination of CMV infection and increasing age appear to drive this phenotype in the majority of participants.

The association of the CD57hi phenotype and CMV with cutaneous malignancy are novel findings. It is unclear how this may relate to increased SCC risk. There is conflicting evidence that CD57 expression may represent a marker of impaired T cell function [311, 327, 438]. Alternatively, the expression of CD57 may represent a biomarker for another process; for example, the accumulation of terminally differentiated $CD8^+CD57^+$ cells has been reported with age and immunosenescence. This has not been previously explored in the setting of post-transplant cutaneous malignancy.

The association between CD57hi phenotype and alterations in lymphocyte phenotype and function, which may explain the increased propensity for SCC development, will be explored further in Chapter 5.

Chapter 4: Markers of operational tolerance and B-cell populations in long-term renal transplant recipients

4.1 Introduction

As discussed in Chapter 1, we hypothesised that two potential mechanisms may underlie the development of SCC in some RTR: immunosenescence and immunoregulation with bystander suppression due to the development of tolerance to the allograft. In Chapter 3, markers of immunosenescence were assessed and compared to other immunological and clinical predictors of SCC development. A 'CD57hi' immunophenotype was found to be more predictive of SCC development and recurrence than other immunological markers and was independently predictive irrespective of clinical risk factors. In this chapter, we explore the potential contribution of tolerance to the development of SCC.

A difficulty with exploring the development of tolerance in human transplantation has been a lack of reliable biomarkers of tolerance to the allograft. It is not ethical to blindly stop immunosuppression in otherwise stable RTR to demonstrate a lack of immune response to the graft. This has led to a search for biomarkers that may guide immunosuppression minimisation without risk of graft dysfunction.

One population that has been of particular interest is the small worldwide cohort of RTR that have stopped maintenance pharmacological immunosuppression and have retained stable graft function subsequently. Whilst it is not possible to conclusively demonstrate a lack of immune response to the allograft, this cohort is considered to demonstrate 'operational tolerance' [260, 263, 264, 278, 279, 439].

Whilst a number of small studies have demonstrated various phenotypic changes in this cohort, two large collaborative studies compared the immune phenotype of operationally tolerant RTR with RTR

in various clinical states, who remain on maintenance immunosuppression [278, 279]. Both these studies were able to elucidate phenotypic changes associated with operational tolerance, termed 'signatures' of operational tolerance.

Neither of these signatures have been tested for prevalence in an independent cohort of stable RTR who remain on immunosuppression. To investigate this further, we hypothesised that the 'signatures' would be most likely to be found in RTR who exhibit stable long-term graft function but particularly in those who develop SCC, due to the synergistic effect of a tolerogenic immune milieu and pharmacological immunosuppression leading to effective 'over'-immunosuppression.

One 'signature', defined by the RISET consortium and referred to here as the 'RISET tolerance signature', consists of a cross-platform signature utilising flow cytometry, gene expression (qPCR) and donor responsiveness as assessed using direct pathway interferon-gamma EliSPOT [278]. The second signature, defined by the Immune Tolerance Network (ITN) and referred to here as the 'ITN tolerance signature', utilised the expression of three genes in peripheral blood [279]. Both 'signatures' largely relate to B cell-related variables, suggesting that operational tolerance may be associated with marked alterations in B cell populations and B cell gene expression in peripheral blood [277].

In this chapter, we aim to answer the questions:

1. What is the prevalence of the RISET and ITN tolerance signatures amongst long-term RTR with stable graft function?
2. Are these signatures overrepresented in those who develop SCC?
3. Do clinical factors influence the prevalence of these signatures?
4. Are these signatures associated with clinical outcomes?
5. Do the ITN and RISET signatures demonstrate concordance in those identified as 'tolerant'?

4.2 B cell phenotype at enrolment

The phenotype of B cell populations at enrolment are summarised in Table 4.1. Both the absolute number and percentage of B cells within circulating transitional, memory and effector (plasmablast) populations did not differ between RTR_{No} and RTR_{SCC}. There was a trend towards a reduced number of naïve B cells in RTR_{SCC} although there was marked variation in numbers, with significant overlap between the two groups. Two B cell populations have been associated with regulatory properties, namely $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ B cells: neither of these differed in percentage or number between groups.

Variable	RTR_{SCC}	RTR_{No}	p
Number $CD19^{+}CD27^{-}$ naïve B cells (cells/10µl)	39 (11 – 132)	71 (25 – 142)	0.10
Number of $CD19^{+}CD27^{+}$ memory B cells (cells/10µl)	74 (31 – 143)	76 (41 – 158)	0.43
Number of $CD19^{+}CD38^{hi}IgM^{-}IgD^{-}$ plasmablasts (cells/10µl)	5 (3 – 10)	4 (3 – 12)	0.85
Number of $CD24^{hi}CD38^{hi}$ transitional B cells (cells/10µl)	2 (1 – 6)	2 (1 – 5)	0.39
Number of $CD24^{hi}CD27^{+}$ B cells (cells/10µl)	53 (26 – 112)	55 (25 – 138)	0.42
Percentage naïve B cells	31.8% (15.3 – 54.5)	43.9% (18.4 – 59.6)	0.27
Percentage memory B cells	52.3% (35.5 – 70.5)	41.9% (29.2 – 67.8)	0.19
Percentage plasmablasts	3.1% (1.4 – 4.6)	2.6% (1.2 – 3.5)	0.20
Percentage transitional B cells	1.3% (0.8 – 2.7)	1.2% (0.7 – 2.9)	0.85
Percentage $CD24^{hi}CD27^{+}$ B cells	36.9% (20.5 – 56.5)	31.8% (17.6 – 54.3)	0.41
Ratio of switched to unswitched memory B cells	1.56 (0.88 – 3.31)	1.27 (0.79 – 2.25)	0.29

Table 4.1: B cell phenotype at enrolment. *Continuous variables are reported as median (interquartile range), whilst categorical variables are reported as number (percentage of group). Counts are reported as cells per microlitre of blood unless specified otherwise, whilst percentages are reported as a fraction of the $CD19^{+}$ B cell population. Groups were compared by Mann-Whitney test (continuous variables) or chi-squared test (categorical variables). For two participants a contemporaneous clinical lymphocyte count was not available and so absolute cell numbers could not be calculated.*

4.3 Pilot 'training set' recruitment

The original papers describing the tolerance signatures did not specify absolute threshold values for distinguishing operational tolerance but merely direction of change (e.g. the increase in a variable was associated with operational tolerance). In order to establish the prevalence and concordance of both signatures it was necessary to establish cut-offs for each variable: it was hypothesised that RTR displaying an immune phenotype outside of this 'norm' would thus be more likely to demonstrate operational tolerance. We therefore recruited a 'training set' to establish reference values for each variable within both tolerance signatures.

Preliminary work identified that the training set should be matched by age at enrolment; CMV serostatus; and ciclosporin and azathioprine use (Chapter 2). The clinical phenotype of the training set compared to the main cohort is summarised in Table 4.2. The cohorts were generally well matched though induction therapy was more common in the training set. Previous treatment for rejection was less frequent in the training set. The proportion of RTR within the training set taking each immunosuppressive agent was broadly similar, although the daily dosage of azathioprine was slightly greater and the daily dose of tacrolimus lower in the training set.

Variable	Main cohort	Training set
n	117	24
Caucasian[£]	113 (97)	24 (100)
Male	80 (68)	16 (67)
Age at enrolment (years)	63 (56 – 71)	63 (60 – 73)
Age at first transplant (years)	42 (32 – 51)	56 (49 – 60)
Total duration of immunosuppression (months)	262 (207 – 326)	126 (83 – 175)
Induction therapy:		
None	99 (85)	10 (42)
Anti-CD25	11 (9)	11 (46)
Thymoglobulin	7 (6)	1 (4)
Alemtuzumab	0	2 (8)
Previously treated for rejection	54 (50)	7 (29)
Number of HLA-ABDR mismatches	2 (1 – 4)	3 (2 – 4)
Serum creatinine (mmol/L)	122 (102 – 164)	137 (120 – 173)
eGFR (mL/min/1.73m^2)	45.7 (34.0 – 59.2)	44.2 (34.1 – 50.8)
CMV IgG seropositive at enrolment	76 (65)	15 (63)
Immunosuppression:		
Calcineurin inhibitor	96 (82)	20 (83)
Azathioprine	84 (72)	17 (71)
MMF	13 (11)	5 (21)
Prednisolone	46 (39)	5 (21)
Daily dosage (mg/kg)[*]:		
Ciclosporin	2.0 (1.6 – 2.7)	2.3 (1.3 – 3.0)
Tacrolimus	0.06 (0.05 – 0.09)	0.04 (0.02 – 0.06)
Azathioprine	0.8 (0.6 – 1.2)	1.2 (0.9 – 1.4)
MMF	13.6 (11.6 – 16.8)	20.3 (10.6 – 23.6)
Prednisolone	0.07 (0.06 – 0.08)	0.06 (0.06 – 0.07)

Table 4.2: Clinical phenotype of the training set. *The main cohort characteristics are provided for comparison. Median (interquartile range) is provided for continuous variables, and number (percentage) for categorical variables.*

Samples provided by the training set were used to create 'normal' values for the tolerance signature covariates. The mean value for the training set was used as the 'normal' value. In any potential trial of immune-phenotype guided weaning of immunosuppression, a false-positive for operational tolerance would be more deleterious to an otherwise stable patient than a false-negative result as inappropriate immunosuppression reduction could potentially lead to graft rejection. Therefore an *a priori* decision was made to tighten the threshold for operational tolerance for all variables by requiring a greater than 0.5 standard deviation (SD) difference from the normal value (Table 4.3). Whilst 0.5 SD was an arbitrary value, it ensured that only those participants who had a definite phenotype in the direction of operational tolerance for that variable would be included, rather than those with a borderline phenotype.

Variable	Direction of change for tolerance	Normal (mean) value	Operational tolerance threshold
CD19:CD3 ratio	Greater than	0.052	0.080
%CD4 $CD25^{int}$	Less than	26.1%	19.2%
%CD4 $CD25^{+}$ effector	*Less than*	*36.2%*	*28.4%*
FOXP3/AMANN ratio	Greater than	0.77	1.09
TCL1A expression	Greater than	0.025	0.041
TLR5 expression	Less than	0.33	0.24
SH2D1B expression	Greater than	0.15	0.22
SLC8A1 expression	Less than	0.30	0.25
MS4A1 expression	Greater than	0.51	0.75
FCRL1 expression	Greater than	0.042	0.059
FCRL2 expression	Greater than	0.014	0.021
CD79B expression	Greater than	0.25	0.32
HS3ST1 expression	Greater than	0.0027	0.0037
PNOC expression	Greater than	0.0042	0.0055
IGKV4-1 expression	Greater than	1.20	1.89
IGKV1D-13 expression	Greater than	5.23	7.20

Table 4.3: The cut-offs for 'operational tolerance' developed using the training set. *For each variable, the mean value is increased or decreased by 0.5 standard deviations as appropriate to create the cut-off threshold. %CD4 $CD25^{+}$ effector is italicised to indicate it was not part of the original tolerance signature, but is provided for comparison.*

4.4 Tolerance signature phenotype at enrolment

Using the cut-offs defined in Section 4.3 a total of six participants (5% of the cohort) demonstrated a phenotype in keeping with operational tolerance using the ITN tolerance signature (Table 4.4). There was no difference in the number of RTR with and without a history of SCC who displayed this phenotype and no difference in the level of gene expression between groups.

We did not analyse the donor-specific hyporesponsiveness aspect of the RISET signature as donor PBMC or splenocytes were not available for the vast majority of participants: this was predominantly due to most donors being cadaveric at time of transplant (n=96, 82% of cohort), or the donors having subsequently died, been lost to follow-up, or living in a location too remote from Oxford to enable sampling (n=14, 12% of cohort). This would leave too few participant-donor pairs to analyse in a meaningful fashion.

Of the remaining aspects of the RISET signature which were examined, no participant displayed a phenotype in keeping with tolerance. Across the RTR_{SCC} and RTR_{No} cohorts as a whole there was no difference in the distribution of any variables between groups, nor a difference in the number of aspects of the tolerance signature which were present in participants in each group. As discussed in Chapter 2, the original tolerance signature utilised the percentage of $CD4^+$ T cells that were $CD25^{int}$ as a measure of percentage of recently activated $CD4^+$ T cells. Replacing this with the alternative measure percentage of $CD4^+$ T cells that were $CD25^+$ but not Treg (i.e. percentage of $CD25^+$ effector) did not change this result. The gene panel was particularly poorly represented amongst participants, with no participant displaying the gene panel phenotype of tolerance in its entirety. Surprisingly despite the increased proportion of Treg at enrolment amongst RTR_{SCC} there was no difference in *FOXP3*/*AMANN* ratio compared to RTR_{No}. The proportion of recently activated T cells, calculated using either method, correlated negatively with *AMANN* expression but did not correlate with *FOXP3* expression (r=-0.23 p=0.01 and r=0.03 p=0.78 respectively, for the $CD25^{int}$ method).

There appeared to be minimal overlap between the two signatures, with no correlation between the number of variables present in the RISET signature and the number of variables present in the ITN signature (r^2 0.004, p=0.52). The CD19:CD3 ratio did not correlate with either *IGKV4-1* (r^2 0.02, p=0.10) or *IGKV1D-13* (r^2 0.001, p=0.74) expression.

	Variable	Median (IQR) values			p	No. with 'tolerant' phenotype		p
		Whole cohort	RTR_{SCC}	RTR_{No}		RTR_{SCC} (n=59)	RTR_{No} (n=58)	
RISET	**CD19:CD3 ratio**	0.021 (0.011 – 0.046)	0.021 (0.012 – 0.051)	0.021 (0.010 – 0.039)	0.88	4 (7)	3 (5)	1.00
	%CD4 $CD25^{int}$	14.4% (9.0 -21.1)	14.6% (9.1 – 21.1)	14.3% (8.8 – 21.3)	0.62	36 (61)	42 (72)	0.19
	%CD4 $CD25^{+}$ effector	*21.6% (14.8% - 31.5%)*	*23.9% (14.4 – 33.9)*	*21.2% (15.6 – 30.9)*	*0.84*	*39 (66)*	*39 (67)*	*0.90*
	***FOXP3/AMANN* ratio**	0.77 (0.47 – 1.05)	0.77 (0.48 – 1.26)	0.77 (0.47 – 1.03)	0.62	16 (27)	9 (16)	0.13
	***TCL1A* expression**	0.01 (0.004 – 0.019)	0.009 (0.004 – 0.022)	0.010 (0.005 – 0.018)	0.67	8 (14)	7 (12)	0.81
	***TLR5* expression**	0.22 (0.16 – 0.32)	0.23 (0.16 – 0.33)	0.22 (0.17 – 0.31)	0.93	32 (54)	34 (59)	0.63
	***SH2D1B* expression**	0.081 (0.048 – 0.15)	0.083 (0.053 – 0.15)	0.077 (0.043 – 0.14)	0.57	8 (14)	9 (16)	0.76
	***SLC8A1* expression**	0.22 (0.17 – 0.26)	0.22 (0.18 – 0.28)	0.21 (0.17 – 0.24)	0.33	39 (66)	46 (79)	0.11
	***MS4A1* expression**	0.24 (0.14 – 0.41)	0.22 (0.15 – 0.40)	0.26 (0.14 – 0.45)	0.43	6 (10)	7 (12)	0.74
	***FCRL1* expression**	0.017 (0.010 – 0.029)	0.015 (0.009 – 0.032)	0.018 (0.012 – 0.028)	0.39	1 (2)	4 (7)	0.21
	***FCRL2* expression**	0.005 (0.003 – 0.009)	0.005 (0.004 – 0.010)	0.005 (0.003 – 0.008)	0.90	7 (12)	5 (9)	0.56
	***CD79B* expression**	0.20 (0.16 – 0.30)	0.19 (0.15 – 0.29)	0.21 (0.16 – 0.32)	0.37	12 (20)	14 (24)	0.62
	***HS3ST1* expression**	0.002 (0.001 – 0.003)	0.002 (0.001 – 0.003)	0.002 (0.001 – 0.003)	0.86	8 (14)	6 (10)	0.59
	***PNOC* expression**	0.002 (0.002 – 0.003)	0.002 (0.002 – 0.004)	0.002 (0.002 – 0.003)	0.25	4 (7)	3 (5)	1.00
				Total displaying all aspects of gene panel:		0	0	-
				Total displaying all aspects of RISET operational tolerance phenotype:		0	0	
				Total displaying three aspects of the RISET operational tolerance phenotype:		1 (2)	0	
				Total displaying two aspects of the RISET operational tolerance phenotype:		9 (15)	9 (16)	0.79
				Total displaying one aspect of the RISET operational tolerance phenotype:		35 (59)	36 (62)	
				Total displaying no aspect of the RISET operational tolerance phenotype:		14 (24)	13 (22)	
ITN	***IGKV4-1* expression**	0.47 (0.24 – 1.02)	0.51 (0.30 – 1.14)	0.42 (0.24 – 0.91)	0.22	5 (9)	3 (5)	0.72
	***IGKV1D-13* expression**	1.94 (0.98 – 3.98)	2.18 (1.02 – 4.39)	1.86 (0.87 – 3.50)	0.32	8 (14)	5 (9)	0.40
				Total displaying both aspects of ITN operational tolerance phenotype:		4 (7)	2 (3)	
				Total displaying one aspect of the ITN operational tolerance phenotype:		5 (9)	4 (7)	0.67
				Total displaying no aspect of the ITN operational tolerance phenotype:		50 (85)	52 (90)	

Table 4.4: Operational tolerance phenotype at enrolment. *Values are provided for the cohort as a whole, then for each group, as a median (IQR). The number (percentage of column) of participants within each group displaying the tolerance phenotype is also provided. Significance testing was performed using Mann-Witney (median) or chi-squared test (number). For each the total number displaying all aspects of each tolerance signature is provided. %CD4 CD25 effector is not included in the overall tolerance signature data prevalence, but is provided for comparison*

4.5 B cell phenotype and immunosuppression

The number of participants displaying the operational tolerance phenotype was lower than expected, particularly with regards to the RISET tolerance signature. One of the most striking differences was between the prevalence of the individual covariates that were B cell related (mean prevalence of 12% across all variables) and those that were not B cell related (mean prevalence of 43%, Figure 4.1). This suggested that there may be an effect by another variable upon the B cell aspect of the tolerance signature, which did not have such an effect upon the non-B cell aspects, leading to a 'skewing' of aspects of the signature in some individuals. Given the effect of azathioprine upon NK cells, we hypothesised that immunosuppression may be the source of this effect.

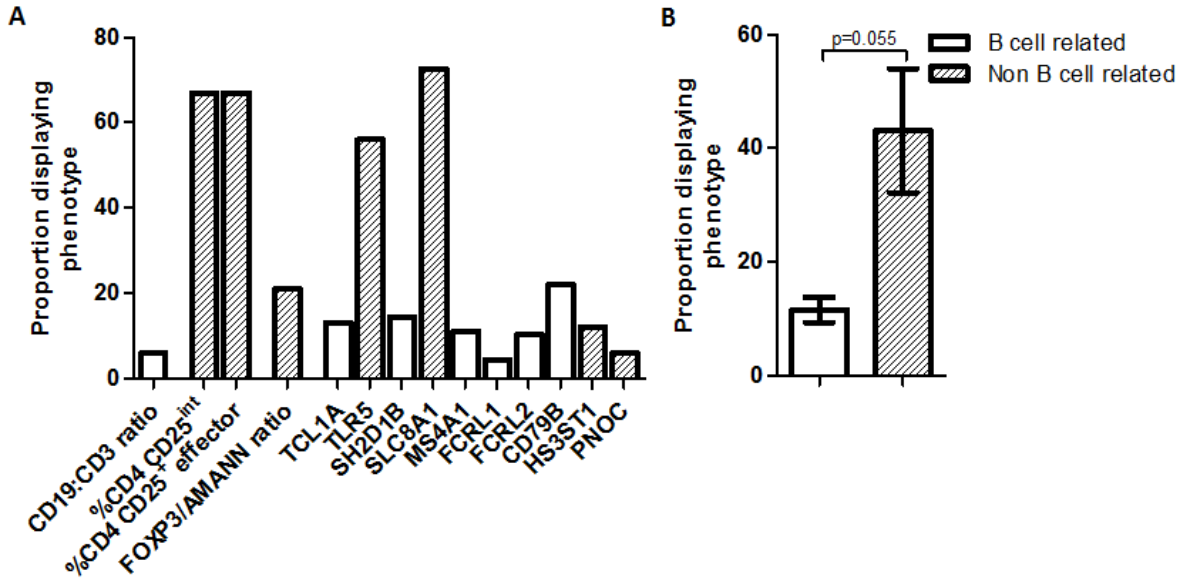


Figure 4.1: Overall prevalence of each aspect of the RISET tolerance signature. *A) Individual aspects of the RISET tolerance signature: clear bars indicate variables relating to B cells, whilst shaded bars indicate variables not related to B cells. B) Prevalence of variables grouped by whether B cell related or non-B cell related, according to Sagoo* et al. *[278]. On further exploration of the literature, PNOC is also strongly associated with B cells [440]. In B, bars indicate the mean prevalence whilst error bars indicate SEM, whilst the p value is calculated by Mann-Witney test.*

Stratifying RTR by antimetabolite use, RTR on azathioprine at enrolment had a trend towards lower circulating B cell numbers, with significantly fewer circulating $CD19^{+}CD24^{hi}CD38^{hi}$ transitional and total $CD19^{+}CD27^{-}IgD^{+}$ naïve B cells (Figure 4.2). There was preservation of the $CD19^{+}CD20^{+}CD27^{+}$ memory compartment, with no alteration of the ratio of $CD27^{+}IgD^{-}$ (isotype switched) to $CD27^{+}IgD^{+}$

(isotype unswitched) memory B cells and no alteration in the number of circulating $CD38^{hi}IgM^{-}IgD^{-}$ plasmablasts (data not shown). As was seen with NK cells in Chapter 3, MMF did not have this effect. When the analysis was repeated using the alternative CD38-IgD classification, the number of Bm2' ($CD38^{hi}IgD^{+}$, equivalent to transitional) and Bm1/2 ($CD38^{lo}IgD^{+}$, equivalent to naïve and unswitched memory) cells were similarly reduced with azathioprine therapy with sparing of the Bm3+4 ($CD38^{hi}IgD^{-}$, plasmablast) and Bm5 ($CD38^{+/-}IgD^{-}$, switched memory, data not shown). When the cohort was stratified by steroid or calcineurin inhibitor use, no association with reduced B cell number was seen.

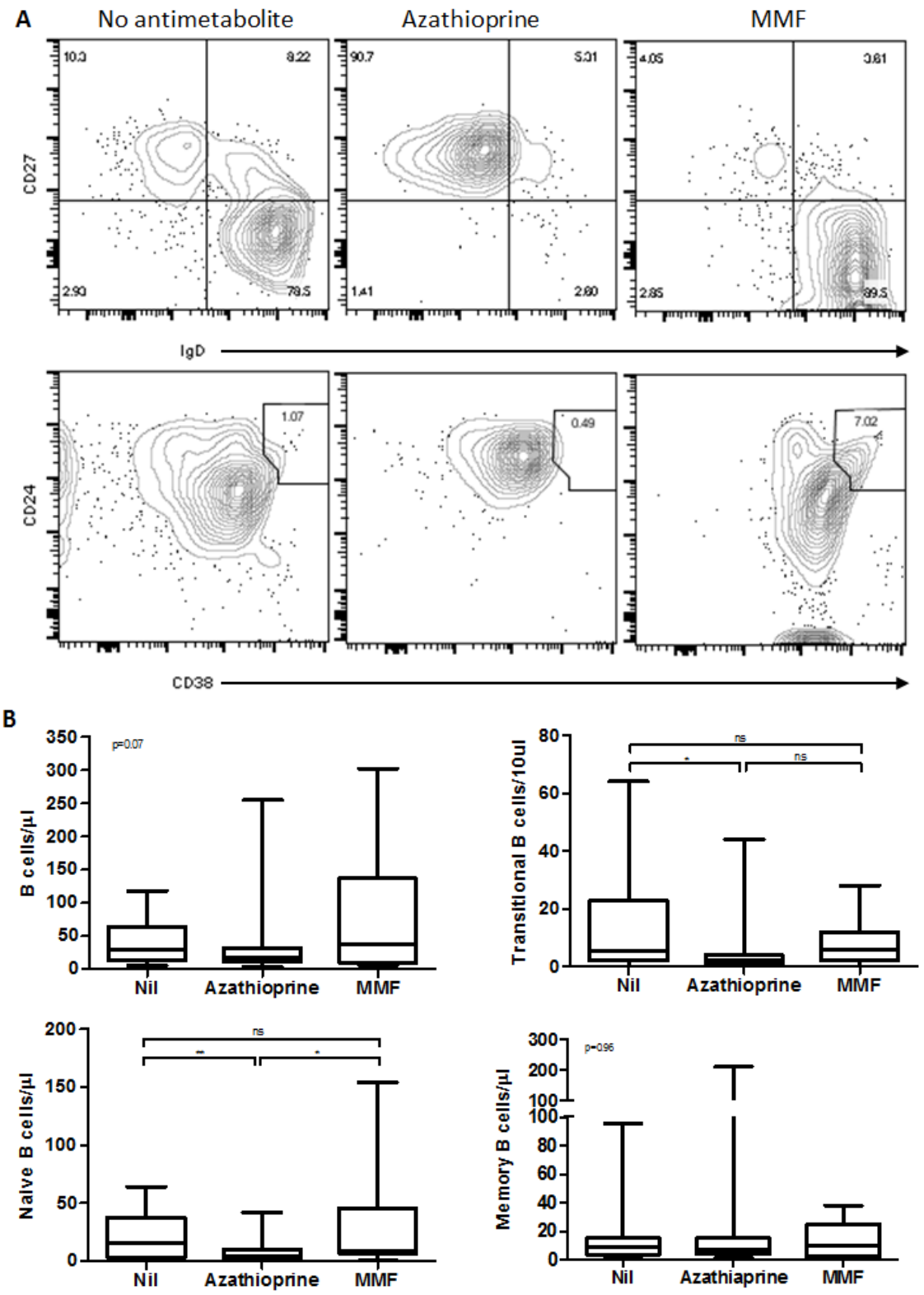


Figure 4.2: Azathioprine, but not MMF, use is associated with a reduction in the number of circulating immature and naïve B cells. *A) Representative staining of B cell populations in RTR on no antimetabolite; azathioprine; and mycophenolate mofetil (MMF). B) Circulating B cell numbers in RTR taking no antimetabolite, azathioprine and MMF. 'ns' not significant, *p<0.05, **p<0.01. p value is given for Kruskal-Wallis test where no lines are shown. Box and whisker plots indicate (from top to bottom) maximum value, 75th, 50th and 25th centiles and minimum value, respectively.*

The effect upon naïve and transitional B cell populations appeared to be dose dependent, with a weak negative correlation with daily azathioprine dose seen in both (Figure 4.3).

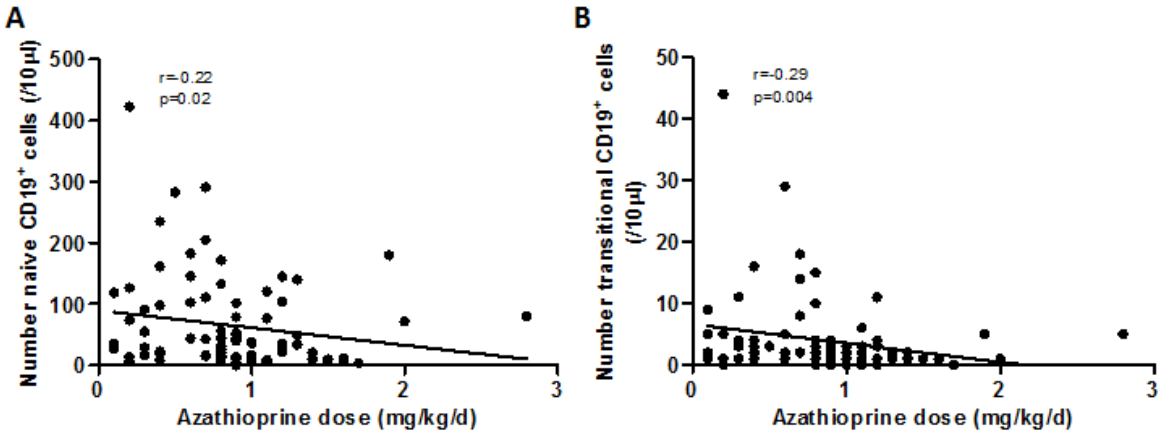


Figure 4.3: The reduction of circulating total naïve and transitional B cell numbers is dose dependent. *A) Correlation between naïve B cell number and daily azathioprine dose. B) Correlation between transitional B cell number and daily azathioprine dose. Azathioprine dose given as mg/kg/day. Correlations tested using two-sided Spearman's test, as all three variables were non-parametrically distributed. Only RTR taking azathioprine at enrolment were included (n=82). Both cell populations are given per 10µl blood.*

Analysis of these factors is complicated by polypharmacy: over 90% of RTR were taking multiple immunosuppressive agents and thus some of these effects may be due to the overlapping effect of different agents. In order to overcome this, a linear regression analysis was undertaken to assess the effect of each immunosuppressant individually on the B cell compartment. Time (in months) since last transplant was also included to account for changing trends in immunosuppressive regime over time and any potential effect of cumulative immunosuppressive dose. For example, those taking MMF are more likely to have received their transplant at a later date than those taking azathioprine and so will have a shorter cumulative duration of immunosuppression. These results are shown in Table 4.5 and illustrate that whilst azathioprine therapy is associated with decreased antigen-naïve and transitional B cell numbers, leading to an overall reduction in B cell number, calcineurin inhibition is associated with alterations within the memory compartment, with increased numbers of plasmablasts and an increased proportion of class switched memory B cells. The absolute number of both unswitched and switched memory B cells was unaltered by any immunosuppressive regime, although there was a trend towards an increase number of switched memory B cells with calcineurin inhibition (data not shown).

	CnI	MMF	Aza	Steroids	Sirolimus
Number of $CD19^+$ cells	↔	↔	↓	↔	↔
Proportion of lymphocytes $CD19^+$	↔	↔	↔*	↔	↔
Number of naïve B cells	↔	↔	↓	↔*	↔
Number of transitional B cells	↔*	↔	↓	↔	↔
Number of memory B cells	↔	↔	↔	↔	↔
Number of plasmablasts	↑	↔	↔	↔	↔
Ratio of switched to unswitched memory B cells	↑	↔	↔	↔	↔

Table 4.5: The effect of individual immunosuppressant agents upon the B cell compartment. *Arrows indicate direction of change in those taking the agent. Results are generated using linear regression on log-transformed variables, using each agent, age at enrolment and time since last transplant as covariates. Significance was adjusted for multiple testing using Bonferonni correction and taken to be $\underline{\leq}0.007$, though covariates with $p<0.05$ and $p\underline{\leq}0.01$ are indicated in brackets with * and ** respectively. 'CnI', calcineurin inhibitor; 'MMF', mycophenolate mofetil; 'Aza', azathioprine. The actual values are shown in Supplementary Data Table i.2.*

The data presented in Section 4.5 suggests that immunosuppression has a marked effect upon the B cell compartment in peripheral blood, with azathioprine particularly affecting antigen-inexperienced, pre-germinal centre B cell populations, and calcineurin inhibitors impacting upon antigen-experienced, post-germinal centre populations.

4.6 Effect of immunosuppression upon markers of operational tolerance

The findings in Section 4.5 raised the question of whether immunosuppression may have an effect upon the signatures of operational tolerance. We hypothesised that, given its effect upon circulating B cell populations, azathioprine and calcineurin inhibition would particularly have an effect upon the B cell related aspects of the signatures.

The CD19:CD3 ratio and all genes which are related to B cells were found to be reduced in RTR taking azathioprine on univariate analysis (Table 4.6). Those variables which did not relate to B cells, such as the proportion of recently activated T cells, were not affected.

Variable	Azathiaprine (n=84)	No azathioprine (n=33)	p
%CD19:%CD3 ratio	0.02 (0.009 – 0.03)	0.04 (0.02 – 0.07)	<0.001
%CD4 $CD25^{int}$	14.3 (9.05 – 21.6)	14.1 (9.13 – 19.4)	0.57
%CD4 $CD25^{+}$ effector	21.5 (13.8 – 33.8)	21.6 (16.5 – 29.4)	0.89
FOXP3/*AMANN* ratio	0.77 (0.47 – 1.05)	0.72 (0.46 – 1.18)	1.00
TCL1A*	0.008 (0.004 – 0.16)	0.18 (0.007 – 0.05)	0.001
TLR5	0.22 (0.16 – 0.30)	0.25 (0.16 – 0.42)	0.50
SH2D1B*	0.06 (0.04 – 0.10)	0.21 (0.14 – 0.29)	<0.001
HS3ST1	0.002 (0.001 – 0.002)	0.002 (0.001 – 0.003)	0.56
MS4A1*	0.22 (0.14 – 0.37)	0.39 (0.18 – 0.66)	0.02
SLC8A1	0.22 (0.17 – 0.26)	0.20 (0.17 – 0.28)	0.93
FCRL1*	0.01 (0.01 – 0.02)	0.03 (0.01 – 0.04)	<0.001
CD79B*	0.19 (0.15 – 0.26)	0.28 (0.18 – 0.37)	0.008
PNOC	0.002 (0.002 – 0.003)	0.002 (0.002 – 0.004)	0.69
FCRL2*	0.005 (0.003 – 0.008)	0.007 (0.003 – 0.01)	0.08

Table 4.6: Azathiaprine impacts upon the B cell-related variables within the RISET tolerance signature of operational tolerance. **indicates B cell related variables. Significance tested using Mann-Witney test. The difference between "%CD4 $CD25^{int}$" and "%CD4 $CD25^{+}$ effector" are discussed in Chapter 2.*

The effect of other immunosuppression upon aspects of the RISET tolerance signature was examined next. Steroid therapy was associated with a significant elevation in *SLC8A1* and *TLR5* and reduction in *HS3ST1* gene expression on univariate analysis. Conversely, calcineurin inhibitors appeared to be associated with a reduction in *SLC8A1* and *TLR5* expression. MMF use was not associated with any change in these variables.

Despite the data from Chapter 3 suggesting a reduction in the proportion of Treg in circulating blood with calcineurin inhibition, there was no effect upon the *FOXP3*/*AMANN* ratio. This is because a significant reduction in *FOXP3* expression was offset by a significant reduction in *AMANN* expression, leading to no alteration in the ratio upon univariate analysis (Figure 4.4).

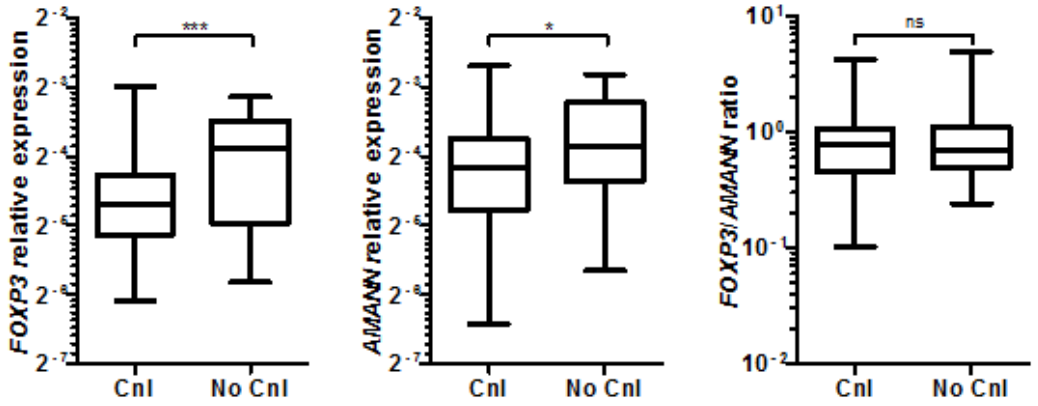


Figure 4.4: Calcineurin inhibition reduces both* FOXP3 *and* AMANN *relative expression, leading to no alteration in* FOXP3/AMANN *ratio. *Expression is relative to* GUSB*. 'ns', not significant, *p<0.05, ***p<0.001. 'CnI', calcineurin inhibitor. Box and whisker plots indicate (from top to bottom) maximum value, 75th, 50th and 25th centiles and minimum value, respectively.*

Variables in both tolerance signatures were examined by linear regression, correcting for other immunosuppression and age at enrolment, as in Section 4.5. This analysis revealed that immunosuppression has a significant impact upon multiple parts of the RISET tolerance signature (Table 4.7). As with the univariate analysis, azathioprine was associated with a reduction, or a trend towards a significant reduction, in all B cell-related variables with the exception of *FCRL2*. Also of note, the CD19/CD3 ratio, *MS4A1* and *FCRL1* expression demonstrated a trend towards a negative correlation with increasing enrolment age. The analysis confirmed that steroids were associated with reduced *H3ST1* and increased *TLR5* expression, with a trend towards an association with increased expression of *SLC8A1*. No immunosuppressant had a significant association with alterations in the *FOXP3*/*AMANN* ratio. Only three variables in the RISET signature, the percentage of recently activated $CD4^+$ T cells, the *FOXP3*/*AMANN* ratio, and *FCRL2* expression, appeared to be unaffected by immunosuppression.

	CnI	MMF	Aza	Steroids	Sirolimus
CD19/CD3 ratio	↔	↔	↔**	↔	↔
%$CD4^+$ $CD25^{int}$	↔	↔	↔	↔	↔
%$CD4^+$ $CD25^+$(effector)	↔**	↔	↔	↔	↔
FOXP3/*AMANN* ratio	↔	↔	↔	↔	↔
TCL1A expression	↔	↔	↓	↔*	↔*
TLR5 expression	↔	↔	↔	↑	↔
SH2D1B expression	↔	↔	↓	↔	↔
HS3ST1 expression	↔	↔	↔	↓	↔
MS4A1 expression	↔	↔	↔*	↔	↔**
SLC8A1 expression	↔	↔	↔	↔**	↔
FCRL1 expression	↔	↔	↔*	↔	↔
CD79B expression	↔	↔	↔*	↔	↔
PNOC expression	↔	↔	↔	↔	↔*
FCRL2 expression	↔	↔	↔	↔	↔

Table 4.7: The effect of individual immunosuppression upon the RISET tolerance signature. *Arrows indicate direction of change in variable value in those taking the agent, compared to those not taking the agent. Results are generated as for Table 4.5 using linear regression on log-transformed variable. Significance was adjusted for multiple testing using Bonferonni correction and taken to be ≤ 0.004, but covariates with $p<0.05$ and $p\leq 0.01$ are indicated in brackets with * and ** respectively. 'CnI', calcineurin inhibitor; 'MMF', mycophenolate mofetil; 'Aza', azathioprine. Actual values are shown in Supplementary Data Table i.3.*

The above analysis was repeated with the ITN tolerance signature. We similarly hypothesised that, as the markers also relate to B cell function and immunoglobulin expression, the expression of these would be reduced in those taking azathioprine and increased in those taking calcineurin inhibitors. In keeping with previous reports [367], one of the genes (*IGLL1*) amplified poorly in many samples and so a two-gene signature was examined (*IGKV1D*-13 and *IGKV4-1*). Surprisingly, azathioprine did not have an effect upon the expression of these genes, but calcineurin inhibition did (Table 4.8).

	CnI	MMF	Aza	Steroids	Sirolimus
IGKV4-1 expression	↑	↓	↔	↔	↓
IGKV1D-13 expression	↑	↔	↔	↔	↓

Table 4.8: The effect of immunosuppression upon the ITN tolerance signature. *Results were generated as for Table 4.7. Significance was taken as $p<0.05$. Actual values are shown in Supplementary Data Table i.4.*

The results presented in this section suggest that all three major immunosuppressive agents used in long-term RTR significantly impact upon the immune phenotype, leading to marked changes in the expression of both the RISET and ITN tolerance signatures. This means that any tests of association between SCC and the tolerance signatures will require adjustment for immunosuppressive regime.

4.7 B cell effector function and immunosuppression

One of the key components of B cell effector function *in vivo* is maturation into short-lived plasmablasts and long-lived plasma cells, with production of antibody. Preformed or post-transplant (*de novo*) development of antibodies with a specificity for donor HLA alleles, or "donor specific antibodies" (DSA) are associated with an increased risk of acute antibody-mediated rejection (AMR) [441-444]. RTR who develop DSA may also have poorer long-term graft survival [372, 445-447], with chronic antibody-mediated damage contributing to the development of transplant glomerulopathy. This leads to an irreversible decline in renal function and ultimately interstitial fibrosis and tubular atrophy (IFTA) [448, 449]. There is no consensus regarding treatment of AMR with a paucity of randomised controlled trials: many strategies to eliminate DSA do not yield sustained reductions in DSA titre or fail to demonstrate an appreciable benefit on clinical outcome [450-456]. AMR, particularly when associated with transplant vasculitis, may have a poorer outcome than acute cellular rejection with up to 20% of RTR losing their grafts within one year [442, 443].

Given the changes in B cell phenotype seen with azathioprine and calcineurin inhibition, we hypothesised immunosuppression and the signatures of tolerance may be associated with alterations in the humoral response to the allograft. All RTR were assessed for the presence of donor and non-donor specific anti-HLA Class I and II IgG antibodies using the solid phase Luminex assay. For 3 donor-recipient pairs the donor HLA Class II alleles were unknown and so these were only assessed using HLA Class I data. Antibodies to HLA-A, HLA-B, HLA-Bw, HLA-DR and HLA-DQ were assessed.

22 RTR, representing 19% of the cohort, possessed DSA. 4 RTR demonstrated Class I only DSA, 15 Class II only, and 3 both Class I and Class II DSA. 39 (33%) of RTR demonstrated non-donor specific anti-HLA antibodies (nDSA): 11 Class I, 19 Class II and 9 both Class I and Class II. 12 (55%) RTR with DSA also demonstrated nDSA, representing a significantly greater proportion than RTR without DSA (n=27 [28%], p=0.02). One participant demonstrated a weak positive antibody to Class II, and was included as a positive result. For 17 RTR with DSA which were tested with single antigen beads, the median MFI on the strongest positive bead was 16000 (IQR: 2500 – 22000).

RTR demonstrating DSA exhibited a significantly lower eGFR compared to RTR without DSA (median [IQR] eGFR 33 [29 – 55] vs 48 [38 – 62]ml/min/1.73m^2, p=0.01). There was no difference in renal function between RTR with nDSA and those without (data not shown). A history of SCC at enrolment and CD57 phenotype were not associated with increased prevalence of DSA. DSA seropositivity was not associated with any significant difference in peripheral blood B cell numbers or subset proportions on univariate analysis (data not shown).

There was no difference in the expression of any of the RISET or ITN tolerance signature markers between those who were DSA seropositive and seronegative except *TCL1A*, expressed at a lower level in those who were positive for DSA (data not shown). There was no association between possession of the ITN signature and DSA seropositivity. During follow-up, one RTR with Class I DSA at enrolment subsequently developed rapid graft dysfunction leading to graft loss, whilst another with Class I and II nDSA and stable impaired graft function (serum creatinine 461µmol/l) at enrolment subsequently developed further graft dysfunction, leading to graft loss.

Previous studies have looked at risk factors for *de novo* DSA development, but have frequently reported univariate risk factors or have compared immunosuppressive regimes rather than individual agents. To test the interaction between immunosuppression and the presence of DSA, a logistic regression method was employed. Univariate risk factors for the development of *de novo* DSA that have been previously described are: graft from a living donor [457] and from a deceased donor [458]; age at transplant (both younger and older age at transplant have been identified as

risk factors) [444, 445, 458-460]; increasing HLA mismatch [444, 445, 457-459, 461, 462]; increasing duration of immunosuppression [460, 463]; previous acute rejection [444, 445, 459, 464]; and non-adherence to immunosuppression [445].

An exploratory model was constructed to assess as many of the previously identified risk factors as possible (Table 4.9). It was not feasible to include non-adherence, but total HLA mismatch, additional previous transplants, time since last transplant, donor source, treatment for previous suspected rejection, age at transplant and use of all individual immunosuppressive agents were included in the model. As DSA have been frequently described in the context of chronic graft decline [445, 465], renal function (as measured by eGFR) was also included as a covariate.

Using this model azathioprine use was associated with the presence of DSA independent of other risk factors. As previously reported there was an association between declining eGFR and the presence of DSA, independent of other covariates. Notably, when this analysis was repeated for nDSA, no association with azathioprine was seen, although a significant association with acute rejection was present (data not shown).

Variable	Univariate		Multivariate	
	OR (95% CI)	**p**	**OR (95% CI)**	**p**
Graft from deceased donor	0.68 (0.18 – 2.53)	0.56		
Recipient of more than one transplant	1.26 (0.41 – 3.86)	0.69		
Increasing HLA AB-DR mismatch of last transplant	1.30 (0.95 – 1.76)	0.10	1.24 (0.86 – 1.79)	0.24
Increasing age at last transplant (years)	0.97 (0.93 – 1.01)	0.12	0.97 (0.92 – 1.02)	0.21
Previous treatment for acute rejection	2.80 (0.99 – 7.96)	0.053	2.27 (0.73 – 7.01)	0.16
Increasing renal function (eGFR)	0.98 (0.95 – 1.01)	0.10	0.96 (0.92 – 0.99)	0.02
Increasing time since last transplant (months)	1.004 (1.00 – 1.01)	0.18	0.97 (0.92 – 1.02)	0.21
Immunosuppression at enrolment:				
On calcineurin inhibitor	0.69 (0.22 – 2.14)	0.52		
On azathioprine	2.92 (0.80 – 10.6)	0.10	4.54 (1.02 – 20.2)	0.047
On MMF	0.33 (0.04 – 2.68)	0.30		
On steroids	2.15 (0.84 – 5.50)	0.11	2.21 (0.71 – 6.89)	0.17
On sirolimus	0.86 (0.10 – 7.73)	0.89		

Table 4.9: Multivariate analysis of factors associated with the presence of DSA. *All variates tested by univariate analysis with a significance of p<0.20 were included in the multivariate model.*

These results led to a discrepancy between the flow cytometry data, suggesting increased humoral responses in those treated with calcineurin inhibitors, and the lack of association with IgG DSA. This led us to hypothesise that RTR on calcineurin inhibition are able to generate primary IgM humoral

responses, but fail to generate long lasting IgG memory responses, which require T cell help and germinal centre formation [466, 467]. In order to assess this, quantification of total serum IgG and IgM was assessed by ELISA and analysed using the same model as previously, utilising each individual immunosuppressant, age at enrolment and time since last transplant as covariates.

The results demonstrate that total serum IgM, indicative of early, relatively T cell-independent responses, are increased in those receiving calcineurin inhibitors, independent of age, time since last transplant, and other immunosuppression (Table 4.10). However, this is not associated with an increase in total serum IgG, indicative of long-lasting memory formation and plasma cell response. Adding CD57 phenotype as a covariate indicated that this was not associated with alterations in serum IgG and IgM levels (data not shown).

	CnI	MMF	Aza	Steroids	Sirolimus
Total serum IgM	↑	↔	↔	↔	↔
Total serum IgG	↔	↔	↔	↔	↔

Table 4.10: The effect of immunosuppression upon total serum antibody levels. *Results were generated as for Table 4.5, using log-transformed total antibody concentrations. Significance was taken as $p<0.05$. Actual values are given in the Supplementary Data Table i.5.*

The results in Section 4.7 indicate that whilst calcineurin inhibition and azathioprine use are associated with alterations in the circulating B cell compartment, only azathioprine is associated with the development of humoral responses against the allograft. Calcineurin inhibition was associated with an increase in serum IgM levels, suggesting that the increased proportion of plasmablasts seen on immunophenotyping are most likely short-lived, early response plasmablasts, the formation of which is not inhibited by calcineurin inhibition. However, this does not translate to an increase in IgG antibodies and the formation of DSA as the T cell help required for sustained humoral immunity and class switch is lacking.

4.8 Tolerance signatures and the prediction of SCC development

The final aspect of the tolerance signatures explored was their ability to predict SCC development during follow-up with the hypothesis that possession of a greater number of variables from either

tolerance signature would be a predictor of SCC development. As discussed in Chapter 3, this was an exploratory analysis only, due to lack of power.

Whilst possession of either tolerance signature was not associated with increased risk of SCC during follow-up, there was a strong association between possessing the CD19:CD3 ratio aspect of the tolerance signature and earlier development of SCC (Table 4.11). No other single variable or any individual gene in the gene panel was predictive of earlier SCC development.

Variable	Univariate		Adjusting for immunosuppression		Adjusting for IS, age and SCC history	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
			RISET variables			
Increasing number of variables present	1.31 (0.76 – 2.26)	0.34	1.49 (0.86 – 2.59)	0.16		
Elevated CD19:CD3 ratio	3.86 (1.48 – 10.1)	0.006	3.63 (1.36 – 9.71)	0.01	4.20 (1.53 – 11.6)	0.005
Reduced %CD4 $CD25^{int}$	0.77 (0.38 – 1.58)	0.48				
Reduced %CD4 $CD25^{+}$ effector	1.11 (0.53 – 2.36)	0.78				
Elevated *FOXP3/AMANN* ratio	1.38 (0.62 – 3.07)	0.44				
Increasing number of aspects of gene panel present	1.04 (0.88 – 1.23)	0.63	1.06 (0.88 – 1.27)	0.54		
			ITN variables			
Displaying whole signature	2.91 (0.88 – 9.61)	0.08	2.06 (0.61 – 6.91)	0.24		
Increasing number of variables present	1.31 (0.69 – 2.52)	0.41	1.14 (0.60 – 2.19)	0.69		
Elevated *IGKV4-1* expression	1.74 (0.53 – 5.71)	0.36	1.40 (0.42 – 4.66)	0.58		
Elevated *IGKV1D-13* expression	1.33 (0.47 – 3.79)	0.60	1.11 (0.38 – 3.22)	0.85		

Table 4.11: Predictive value of tolerance signature factors for SCC development during follow-up. *Each variable was assessed for presence using the cut-offs defined in Table 4.3. Variables are expressed as a hazard ratio, using those who did not display the phenotype as the reference value. All variables were then adjusted for immunosuppression (as for Table 4.7) where it was shown to have an effect in tables 4.7 and 4.8, then subsequently corrected for age at enrolment and history of SCC (as described in Chapter 3) if significance remained. Hazard ratios were calculated by Cox regression. Values for individual genes within the gene panel are provided in the Supplemental Information Appendix Table i.6.*

In order to further explore what other underlying changes in immune phenotype may be associated with an increased CD19:CD3 ratio, RTR with and without 'tolerant range' CD19:CD3 ratio were compared. RTR taking azathioprine at enrolment were not included in this analysis to remove the confounding effect of this immunosuppressant.

RTR with an elevated CD19:CD3 ratio had a trend towards an increase in the number of B cells without a change in the number of T cells. This was predominantly due to an expansion of naive B cells, though there was a trend towards an increased number of all B cell populations (Table 4.12).

There was no alteration in the total number of $CD3^+$ T cells, nor any alteration in major T cell subsets. Notably, those possessing an elevated CD19:CD3 ratio did not have an increase in number of Treg, NK cells or a decrease in the number of recently activated T cells in circulating blood and no overrepresentation of CD57hi RTR was present within this population (data not shown).

Variable	Elevated CD19:CD3 ratio	'Normal' CD19:CD3 ratio	p
Number of $CD3^+$ T cells (cells/µl)	930 (494 – 1519)	1094 (528 – 1371)	0.98
Number of $CD19^+$ B cells (cells/µl)	855 (473 – 2512)	300 (120 – 630)	0.02
Number naïve B cells (cells/10µl)	566 (352 – 1328)	98 (39 – 395)	0.01
Number of memory B cells (cells/10µl)	145 (51 – 754)	86 (30 – 153)	0.32
Number of plasmablasts (cells/10µl)	10 (4 – 25)	5 (2 – 12)	0.32
Number of $CD24^{hi}CD38^{hi}$ transitional B cells (cells/10µl)	24 (14 – 45)	4 (2 – 10)	0.01
Number of $CD24^{hi}CD27^+$ B cells (cells/10µl)	114 (39 – 633)	56 (21 – 138)	0.41
Percentage naïve B cells	76% (62 – 87%)	58% (28 – 73%)	0.048
Percentage memory B cells	20% (9 – 33%)	31% (19 – 51%)	0.18
Percentage plasmablasts	1.1% (0.9 – 1.4%)	2.1% (1.1 – 3.3%)	0.08
Percentage transitional B cells	4.8% (1.4 – 6.0%)	1.9% (0.8 – 4.4%)	0.30
Percentage $CD24^{hi}CD27^+$ B cells	16% (6.6 – 27%)	21% (13 – 40%)	0.35
Ratio of switched to unswitched memory B cells	1.3 (1.0 – 1.6)	1.3 (1.0 – 2.8)	0.58

Table 4.12: Alterations in immune phenotype associated with elevated CD19:CD3 ratio, in those not taking azathioprine (n=33). *'Percentage' refers to the percentage of $CD19^+$ B cells. All results are median (IQR). Significance tested using Mann-Witney U test. Significance was set at $p \leq 0.004$ using a Bonferroni correction.*

Analysing clinical phenotype stratified by normal or elevated CD19:CD3 ratio, there was no difference in age at recruitment or first transplant, the number with a history of previous SCC at enrolment, total duration of immunosuppression or renal function (eGFR), nor a difference in the proportion who were CMV seropositive or had been treated for rejection. There was no difference in those using calcineurin inhibitors or steroids at enrolment between groups. This was not altered when the analysis was repeated using only those not on azathioprine.

Despite an increased CD19:CD3 ratio being predictive of SCC development, the absolute number of total $CD19^+$ B cells in peripheral blood was not predictive of SCC development after adjustment for azathioprine use, nor was any individual B cell population (data not shown).

Studies have previously demonstrated that B cells with regulatory properties are found in the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ compartments, and that these cells frequently express IL-10 [236, 237, 240]. Attempts to stimulate both B cells enriched by cell sorting and unsorted PBMC *in vitro* using various durations of incubation with combinations of PMA, ionomycin, LPS and CpG as reported previously [237, 278, 279] to assess for IL-10 production were unsuccessful due to the difficulty in gating positivity accurately. Various methods were attempted, including fluorescence minus one (FMO), isotype controls and unstimulated cells, but none provided an adequate negative control to enable the $IL\text{-}10^+$ gate to be set reproducibly and accurately. Instead, whole blood *IL10* gene expression was assessed. No difference was seen between those in the elevated CD19:CD3 cohort and those who were not, both when the whole cohort was analysed and when only those not taking azathioprine were analysed (data not shown).

These results suggest that an increased ratio of $CD19^+$ B cells to $CD3^+$ T cells is predictive of future development of SCC. However, this is not associated with other alterations in the immune phenotype that have been previously described in RTR displaying operational tolerance. An elevated CD19:CD3 ratio is associated with an increased proportion of naïve B cells in RTR not taking azathioprine.

4.9 Summary of key findings

- There was no difference in B cell or ITN/RISET tolerance signature-related phenotype between those with and without a history of SCC at enrolment;
- Azathioprine has a marked effect upon B cell populations with a marked reduction in the number of circulating naïve and transitional B cells, whilst calcineurin inhibition increased the number of plasmablasts with an increased proportion of switched memory B cells;
- Steroids, calcineurin inhibitor and azathioprine therapy have a marked effect upon various aspects of both the RISET and ITN 'signatures' of operational tolerance;
- Independent of potential confounders, azathioprine use is associated with the presence of DSA;
- The ITN and RISET tolerance signatures demonstrate minimal overlap in this cohort with the RISET signature in particular displaying low prevalence;
- While the tolerance signatures *per se* do not predict SCC development, the ratio of B:T cells is independently predictive of SCC development during follow-up;
- An elevated B:T cell ratio is associated with a general increase in B cell number with an increased proportion of naïve B cell populations alteration in the proportion of B cell or other lymphocyte subsets.

4.10 Discussion

4.10.1 Azathioprine and circulating B cell populations

In this chapter the association between B cell phenotype, two previously identified 'signatures' of operational tolerance and SCC development were explored. A history of previous SCC did not initially appear to be associated with alterations in the B cell compartment. Further analysis revealed that azathioprine had a profound effect upon circulating B cells, with a reduction particularly in antigen-inexperienced (naïve and transitional) B cell populations independent of other immunosuppression.

The lack of a difference in B cell populations between RTR_{SCC} and RTR_{No} at enrolment contrasts with the findings by Hope *et al.*, who reported RTR with a history of any post-transplant malignancy had reduced numbers of naïve circulating B cells and a trend towards a greater proportion of memory B cells [109]. This same study found an increased proportion of memory B cells to be predictive of malignancy development within 100 days. However, no assessment of the role of immunosuppression was made by the authors: of note, RTR with a history of malignancy were more likely to be taking azathioprine and at a greater dose, though these did not reach significance. The findings presented in this chapter suggest that there is a dose-dependent effect of azathioprine upon B cells leading to an overrepresentation of memory B cells within the $CD19^{+}$ population. When this is considered, the data presented by Hope *et al.* may be in agreement with that presented here, with their findings representative of increased azathioprine use and dosage.

The finding that azathioprine therapy is associated with reduced B cell numbers in humans is a relatively novel one. This is surprising given its long duration of use in clinical practice. A study analysing renal transplant recipients for the presence of the ITN tolerance signature looked at eight RTR with long-term transplants and found that azathioprine use was associated with reduced numbers of total and naïve B cells [367]. Administration of azathioprine to mice also results in a reduction in B cell populations [468]. In a study of non-transplant patients with systemic lupus erythematosus (SLE, an antibody-mediated autoimmune condition) azathioprine, but not MMF,

therapy was found to be associated with a reduction in circulating naïve and transitional B cell numbers [469]. The results reported in this chapter validate these findings in the largest transplant cohort to date. In accordance with the findings presented in this chapter, Moreso *et al.* noted that increasing duration of ciclosporin therapy (up to ten years) was associated with increased expression of the ITN signature, but that this was not seen in RTR treated with azathioprine for over 30 years [367]. The authors hypothesised that this may be due to a selection bias in those on azathioprine who have survived to extremely long-term. However, the data presented in this chapter would refute this, as all participants in this study have long-standing allografts and the change in B cell phenotype was seen only in those taking azathioprine.

Why should azathioprine particularly affect B cell populations? Given the similar effect upon NK cells and the notable impact upon the youngest B cell populations, this would suggest that azathioprine is exerting its effect in the bone marrow upon the lymphoblast population. There appears to be sparing of myeloblasts based upon the lack of alteration of monocyte numbers with azathioprine use. The myelosuppressive effects of azathioprine at toxic doses are well known, but a preferential effect upon B cells even at therapeutic doses has not been clearly demonstrated. An *in vitro* study exploring the effect of immunosuppressive agents upon T and B cell lymphoblast lines found that the azathioprine concentration required to induce cytostasis was similar in B and T cells (14.4μM) but the concentration required to induce cytotoxicity was about one-third lower in B cell compared to T cell lymphoblasts (17.8μM vs 26 μM) [470]. It should be noted that these concentrations are significantly greater than that seen in the blood of uraemic patients and RTR following administration, as azathioprine is rapidly metabolised to 6-mercaptopurine (6-MP) *in vivo* [471-473]. 6-MP had an effect at a much lower concentration but the concentration needed for cytostatic and cytotoxic effect did not appear to differ significantly between T and B cell lymphoblasts. This does not rule out an effect from the accumulation of the downstream metabolites of 6-MP upon lymphoblast proliferation; 6-thioguanine nucleotides in particularly have been associated with antiproliferative effects in leucocytes but a preferential effect upon T or B lymphoblasts has not been explored [474-476].

An alternative explanation may be that azathioprine acts upon all lymphoblast lineages equally but due to the altered pathway for maturation, the effect upon phenotype is more prominent in B and NK cells. Studies in mice suggest that only minute quantities, potentially as few as 10 per day, of T cell precursors are required to traffic from the bone marrow to the thymus to support ongoing T cell output and that these are detectable in blood in very low numbers [477-480]. As the thymus cannot support long-term precursor survival this would imply that T cell numbers should be exquisitely sensitive to a loss of bone marrow precursor output. However, with increasing age and thymic involution, output of T cells falls with maintenance of T cell numbers in the periphery relying upon peripheral clonal expansion of T cells [308, 481, 482], meaning that a reduction in thymic output may not be detectable clinically. In support of this, there was no difference in numbers of naïve $CD4^+$ and $CD8^+$ T cells between RTR taking azathioprine and those not taking azathioprine (Chapter 3). In contrast, readily detectable numbers of young B cells and NK cells traffic straight from the bone marrow to the circulating blood and lymphoid organs with minimal peripheral expansion except following antigenic stimulation.

It needs to be considered why MMF does not have the same effect given both inhibit purine metabolism. Azathioprine is rapidly metabolised to form thioguanine nucleotides. These are purine analogues which predominantly act to inhibit purine, RNA and DNA synthesis and prevent chromosomal replication leading to cell cycle arrest [473, 483], though azathioprine may have other mechanisms of immunosuppressive action, including modulation of CD28 signalling [484]. In contrast, MMF is the prodrug of mycophenolic acid which acts to reversibly inhibit inosine monophosphate dehydrogenase (IMPDH) leading to depletion of the intracellular guanine nucleotide pool [485-488]. Both these mechanisms are particularly potent in lymphocytes, which lack mechanisms to 'salvage' nucleotides from breakdown products. MMF is a stronger inhibitor of the type II isoform of IMPDH, which is expressed in activated, proliferating B and T cells, than type I IMPDH, constitutively expressed in resting lymphocytes. Despite this, total IMPDH activity is relatively low in normal, non-malignant rat bone marrow compared to the thymus and spleen [489]. This may explain the difference between MMF and azathioprine, where MMF may have minimal

action on immature lymphoblasts in the bone marrow, in contrast to azathioprine, which is a non-selective inhibitor of purine metabolism [485, 486].

4.10.2 The impact of immunosuppression upon the 'signatures' of operational tolerance

In order to understand the impact of immunosuppression on the signatures of operational tolerance and, by proxy, potential mechanisms, one needs to explore the individual roles of the aspects of the signatures.

The RISET signature of operational tolerance can be subdivided into three areas [278]. The first is a measure of T cell activation and regulation provided by the percentage of recently activated $CD4^+$ T cells and the *FOXP3*/*AMANN* ratio. This is a function of *FOXP3* expression which is most highly expressed by Treg, and *AMANN* expression which codes for alpha-1,2-mannosidase which mediates N-glycosylation and has been suggested to be expressed in memory T cells [490]. Thus the ratio has been suggested to effectively represent the regulatory to effector T cell balance [491]. In this chapter, no correlation between *FOXP3*/*AMANN* ratio and the proportion of memory T cells was seen and *AMANN* expression negatively correlated with the proportion of recently activated $CD4^+$ T cells. Whilst the aforementioned paper suggested that the ratio of *FOXP3* to *AMANN* expression is representative of a regulatory-effector balance, the findings in this study would be more in keeping with the earlier findings of Sawitzki *et al.*, who reported that alpha-1,2-mannosidase is highly expressed within the kidney and heart allografts of mice and rats undergoing successful tolerance induction protocols and that expression in PBMC and graft infiltrating leucocytes is downregulated prior to rejection. Studies of alpha-mannosidase inhibition *in vitro* in human and murine T cells suggest this enzyme has an inhibitory role in effector T cells, particularly naïve cells [490, 492]. Alpha-mannosidase may behave differently in different T cell populations, with upregulation of alpha-1,2-mannosidase in Treg and downregulation in effector $CD4^+$ T cell populations upon activation in a signal-strength dependent manner [490, 492, 493]. Chemical inhibition of alpha-1,2-mannosidase prior to adoptive transfer of Treg demonstrated a key role for this enzyme in Treg migration, preventing their trafficking to secondary lymphoid tissue [493]. This led to a failure to

suppress rejection of a murine skin allograft. Inhibition of this enzyme in effector T cell populations also leads to accelerated allograft rejection [490]. Taken together, the data presented here along with previous findings would suggest that an increased *FOXP3*/*AMANN* ratio may not be indicative of increasing Treg predominance over memory as previously suggested [491]. With reduction of $CD4^+$ T cell activation, which may be due to increased immunoregulation, *AMANN* expression climbs. This causes the *FOXP3*/*AMANN* ratio to fall rather than increase.

The second aspect of the RISET signature relates to B cells and comprises the ratio of the proportion of $CD19^+$ and $CD3^+$ T cells within the lymphocyte population, and the expression of a number of genes. These are *MS4A1* (CD20); *FCRL1* (Fc receptor-like 1), which codes for a transmembrane protein which positively regulates BCR signalling, particularly expressed on naive B cells [494, 495]; *FCRL2* (Fc receptor-like 2), coding for a transmembrane protein related to *FCRL1*, which inhibits B-cell receptor (BCR) signalling and is expressed predominantly on memory B cells [494, 496, 497]; *PNOC* (prepronociceptin), coding for a precursor of the neuropeptide nociceptin, particularly expressed in activated B cells [440]; *TCL1A* (T cell leukaemia/lymphoma 1A), coding for a transcriptional regulator expressed mainly in naïve B cells [498, 499]; and *CD79B,* coding for half of a heterodimeric component of the BCR and expressed on all B cells [500-502]. The changes in expression of these genes mirror the loss of B cell populations with azathioprine. *FCRL2,* which is predominantly expressed upon memory B cells, was not associated with alterations with azathioprine use and this is likely to be because azathioprine was not associated with a reduction in memory B cell numbers in Table 4.5.

The final area of the tolerance signature relates to innate immunity. *TLR5* and *SLC8A1* are constitutively expressed in monocytes [491, 503, 504]: *TLR5* encodes the Toll-like receptor 5, which is a pattern recognition receptor that detects bacterial flagellin [505, 506], whilst *SLC8A1* (solute carrier family 8 member 1) codes for a sodium-calcium exchanger (NCX) in the endoplasmic reticulum that facilitates TNF-α production [504]. In particular monocytes express NCX1 and NCX3 [504]. *HS3ST1* (coding for heparan sulphate-3-O-sulfotransferase-1) is the rate limiting enzyme for

the production of antithrombin-binding heparan sulphate (HS^{AT+}) from heparan sulphate proteoglycans (HSPG) [507]. HS^{AT+} has traditionally been considered a natural anticoagulant. HS^{AT+} is predominantly expressed by endothelial cells, where it is bound to the luminal surface. Expression of *HS3ST1* by leucocytes has not been explored but is presumed to be predominantly upon monocytes (see below). *SH2D1B* (SH2 domain-containing protein 1B) encodes the intracellular adaptor protein EAT-2 (Ewing's sarcoma associated transcript-2) which is expressed predominantly in NK cells in humans, and mediates NK cell activation, with a stimulatory and suppressive role reported in different settings [508-511]. The reduction in NK cell number with azathioprine explains the reduction in expression of this gene in peripheral blood with azathioprine therapy.

A study of bone marrow transplant recipients looked at expression of the RISET tolerance signature and found that *TLR5* expression was reduced in bone marrow transplant recipients who received Treg therapy and did not develop graft versus host disease (GVHD), compared to those who receive conventional therapy and developed GVHD, and healthy controls [491]. The opposite picture was seen for *HS3ST1* expression and no difference was observed for *SH2D1B*. The authors hypothesised that this pattern of gene expression may therefore reflect a reduced inflammatory environment in the recipients of Treg therapy [491]. In the aforementioned study no bone marrow recipient received steroid therapy. In animal models, stimulation through TLR-2, TLR-3, TLR-4 and TLR-9 have been shown to prevent the induction of tolerance to skin and cardiac allografts; TLR-4 signalling acting via $CD8^+$ T cells and TLR-9 in a $CD4^+$ dependent fashion, though these were seen in different models limiting direct comparison [512, 513]. In liver transplantation, circulating monocytes from recipients with acute rejection displayed elevated levels of TLR-2 and TLR-4 compared to those with stable graft function [514]. Due to the variable behaviour of members of the TLR family it is difficult to generalise between Toll-like receptors, but taken together these studies might suggest that reduced *TLR5* expression would favour tolerance. However, there is emerging evidence to suggest that TLR-5 may have a modulatory role. TLR-5 is expressed at a higher level upon human Treg compared to $CD4^+CD25^-$ effectors, and signalling through this enhances *FOXP3* expression and Treg suppressive function *in vitro* [515]. In a murine skin transplant model, administration of

recombinant flagellin led to prolongation of allograft survival, with an increase in Treg number within the draining lymph nodes and spleens of treated mice during administration [516]. Furthermore, Treg expressed greater levels of cytokines such as TGF-β1, TLR-5 and IL-10, and were more suppressive *ex vivo*. In our study there was no correlation between *TLR5* expression and number or percentage of Treg or activated T cells (data not shown).

In this chapter we found that steroid use was associated with increased whole blood expression of *TLR5*. These results are in agreement with those from a study of the murine liver, with marked upregulation of TLR-5, -7 and -9 expression upon Kupffer cells, which are also a part of the monocyte-macrophage-DC axis, within the murine liver in response to dexamethasone treatment [517]. Dexamethasone pre-treatment of Kupffer cells led to a marked reduction in TLR-5-mediated *IL6* expression and upregulation of *IL10* expression, although this did not translate to increased IL-10 secretion, without an apparent effect upon downstream NF-κB signalling. Thus steroid-induced upregulation of TLR-5 may be associated with downstream dysregulation of TLR-5 signalling through the MAPK pathway [518], leading to reduced cell activation upon ligation. An alternative explanation, given the positive association described between flagellin load and TLR-5 expression [516], is that steroid therapy increases bacterial load within the gut, leading to increased flagellin translocation and subsequent upregulation of *TLR5* expression. However it seems unlikely that steroids alone would have this effect and not any other immunosuppressant.

The role of *HS3ST1* as a mediator of anticoagulation has been challenged in recent years. Surprisingly, knockout of this gene from mice does not lead to a procoagulant state despite marked reduction in HS^{AT+} levels, with similar levels of fibrin in blood compared to wild type littermates, even upon procoagulant challenge [519]. $HS3ST1^{-/-}$ mice exhibit an exaggerated response to LPS challenge compared to $HS3ST1^{+/+}$ littermates with increased mortality [520]. This difference is inflated in the presence of exogenous antithrombin as $HS3ST1^{+/+}$ but not $HS3ST1^{-/-}$ mice are rescued. This suggests HS^{AT+} may potentiate antithrombin anti-inflammatory signalling, a finding supported by work *in vitro*, suggesting a contributory role to regulation of NFκB signalling in monocytes [521].

In clinical and animal studies antithrombin treatment in severe sepsis is not associated with a benefit in those receiving concomitant heparin, which would be expected to competitively inhibit antithrombin binding to HS^{AT+} [522, 523]. These studies provide indirect evidence to suggest that HS^{AT+} and antithrombin may modulate TLR signalling, potentially through NFκB. *HS3ST1* expression has been documented in tissues other than the endothelium and the role of heparan sulphate proteoglycans is vast and varied with significant overlap: ligands include various cytokines and growth factors, including fibroblast growth factor and MIP-1α [524-527]. *HS3ST1* expression upon leucocytes particularly has not been explored though the data above, taken together, would suggest expression is likely in monocytes and potentially also neutrophils.

In this chapter, steroid usage was associated with upregulation of *SLC8A1* and downregulation of *HS3ST1* expression. This would initially appear to suggest a proinflammatory effect of steroids upon monocytes. The difficulty with understanding the potential effect of upregulated NCX expression in monocyte and lymphocyte function in response to steroid therapy is that the exchanger has a bidirectional function [504]. In the resting state, NCX1 removes Ca^{2+} from the cytoplasm and would act to suppress activation and TNF-α secretion; however in response to some stimuli the exchanger reverses direction and contributes to activation-induced calcium influx into the cytoplasm. Most work on NCX1 has been performed in cardiac muscle, where sodium-calcium exchange is particularly relevant. Sirolimus and ciclosporin 'lock' NCX into the 'forward' calcium efflux state or prevent trafficking to the cell surface in non-lymphoid cells without affecting expression, leading to suppression of activation [528-530]. A study in neonatal rat cardiac myocytes found dexamethasone upregulated *SLC8A1* expression in a dose-dependent fashion and was associated with an increase in intracellular sodium, in keeping with a similar role for steroids [531]. The importance of this gene in transplantation has not been evaluated, but in the original RISET tolerance signature paper expression of *SLC8A1* was highest in chronic rejectors then stable transplant recipients, although the expression was highly variable [278].

The upregulation of *HS3ST1* with the development of operational tolerance may be indicative of modulation of TLR signalling. Its downregulation with steroids may suggest a pro-inflammatory effect though it is feasible that downregulation of HS^{AT+} may have an effect on other members of the TLR family; in particular it may synergise with the upregulation of TLR5 to enhance regulatory signalling [520]. Like *SLC8A1*, *HS3ST1* expression has not been assessed in the setting of transplantation, but in the original RISET study there was no difference in expression between RTR with stable graft function and those with chronic rejection, though expression was markedly reduced compared to those not on immunosuppression in keeping with the putative role for steroids suggested in this chapter [278].

Donor hyporesponsiveness, assessed using the direct pathway of allorecognition, comprises the final aspect of this part of the RISET signature. This was not assessed due to the difficulty in obtaining donor PBMC or splenocytes due to the significant period of time since transplantation: future prospective studies should focus on the association between donor hyporesponsiveness and the other aspects of the signature pertaining to T cell function.

Whilst the RISET tolerance signature was associated with significant alterations with azathioprine and steroid usage, the ITN tolerance signature was influenced more by calcineurin inhibitors. The original comparator population for the development of the ITN signature was a population of RTR taking calcineurin inhibitors or sirolimus [279]. As has been described in previous work, the amplification of *IGLL1* was found to be unreliable and so this was not utilised [367]. Previous data has suggested that using relative expression of the remaining two gene signatures, *IGKV1D-13* and *IGKV4-1*, is sufficient to distinguish tolerant and non-tolerant RTR in a small cohort [367]. In this same paper it was also noted that RTR taking calcineurin inhibitors for a significant period of time were generally more likely to develop a phenotype in keeping with operational tolerance but that this phenomenon was not seen in azathioprine treated long-term RTR. This is in keeping with the results presented in this chapter. *IGKV1D-13* and *IGKV4-1* were expressed at comparable levels in healthy controls and tolerant RTR, but were suppressed in RTR receiving "standard

immunosuppression" (consisting of triple therapy of calcineurin or mTOR inhibition, an antiproliferative, and prednisolone) during the original development of the tolerance signature [279]. This may mean that 'increased' expression of these genes with calcineurin inhibition is actually the reverse; that calcineurin inhibition is associated with a failure to suppress expression of these genes in peripheral blood (see Section 4.10.6 for further discussion).

Newell *et al.* found the ITN tolerance signature associated with alterations in B cell populations, namely an increase in transitional and naïve B cells, and hypothesised that the upregulation of these genes in operational tolerance may be due to peripheral editing of the BCR receptor [279]. Both *IGKV4-1* and *IGKV1D-13* code for proteins that contribute to immunoglobulin structure. During the maturation process of each B cell, rearrangement of selected genes from within each of the variable (V), diversity (D) and joining (J) regions leads to the assembly of a specific immunoglobulin complex, which determines the subsequent antigen specificity [532, 533]. *IGKV1* and *IGKV4* are gene families within the variable region of kappa light chains: upregulation of expression of these would be expected to be seen in response to activation-induced maturation of B cells and antibody secretion [532, 534]. The results presented in this chapter indicate that calcineurin inhibition is associated with increased numbers of antigen-experienced B cells and plasmablasts, whilst both *IGKV1D-13* and *IGKV4-1* expression correlated with the absolute number and percentage of plasmablasts in peripheral blood (data not shown). Thus receptor editing may not be relevant as proposed by Newell *et al.*, but may simply reflect the increasing presence of plasmablasts in circulating blood. Might this be relevant in operational tolerance? Recently a population of $CD19^{+}CD138^{hi}$ 'regulatory plasma cells' have been described in mice, acting through secretion of IL-10 and IL-35 [535]. However these have not yet been described in humans. Furthermore, it is presumed these would be located in germinal centres and so would not be directly quantified by the ITN tolerance signature in peripheral blood.However, it is feasible that the signature may be a biomarker for their presence.

Teasing out the role of steroids in the RISET tolerance signature and the role of calcineurin inhibitors in the ITN tolerance signature illustrates the difficulty of interpreting the interaction of immunosuppression with both signatures. It is difficult to discriminate the impact of immunosuppression, which may lead to downstream pro- and anti-inflammatory effects and may suggest an alternate route for their immunosuppressive effect which is unrelated to operational tolerance. Alternatively, these changes may suggest that they support or antagonise the development of operational tolerance (Figure 4.5). Further work regarding the effect of immunosuppression upon these pathways is needed, which may elucidate novel mechanisms by which they exert their immunosuppressive effect.

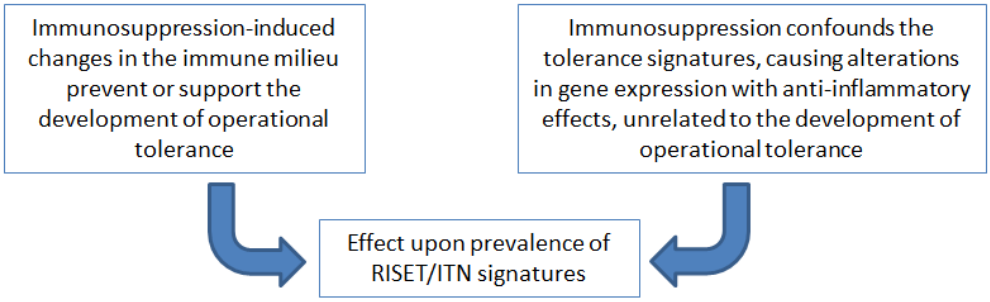


Figure 4.5: The potential interaction between immunosuppression and the tolerance signatures.

4.10.3 Prevalence of the tolerance signatures in long-term RTR

This is the first study to look at the use of variables described within the ITN and RISET tolerance signatures to predict a clinical outcome, namely the development of SCC. We found that the signatures as a whole had no predictive value, even when adjusted for immunosuppression use. Whilst the apparent prevalence of subclinical operational tolerance using the ITN signature was approximately 5%, the apparent prevalence using the RISET signature was zero. In the original development of the tolerance signatures, the prevalence of the signatures in stable RTR receiving conventional dual therapy was 15% and 25% [278, 279]; however, these were only small test cohorts. In larger studies the prevalence of operational tolerance has been estimated at around 5% in stable RTR receiving conventional dual therapy immunosuppression on the basis of possession of previously suggested signatures of operational tolerance [263, 264], although in reality there is no way currently to estimate true prevalence without immunosuppression cessation and these may

simply represent 'false positives'. Zoller *et al.* surveyed 149 American renal transplant units regarding their experiences of cessation of immunosuppression [266]. 48 individuals who had stopped immunosuppression were identified from a total RTR cohort of over 6000. 11 retained stable graft function for at least one year, and five retained stable function for at least three years. That one signature should identify a similar proportion of potentially tolerant RTR to these estimates and one substantially less is a cause for concern for potential future clinical use. Tolerance is unlikely to represent a binary phenomenon, but is likely to represent a spectrum of increasing hyporesponsiveness to an allograft, eventually crossing a threshold where immunosuppression is no longer necessary [285]; a potential explanation for the difference in prevalence is that the cut-offs used for these two signatures may identify different stages in this process. The poor correlation between signatures has been recently recognised elsewhere. In the absence of further cohorts of operationally tolerant RTR, Baron *et al.* recently published a meta-analysis of all previous studies that have elucidated genetic signatures of operational tolerance [536]. This study highlighted that almost no genes were commonly distributed across the studies, and the few that were did not possess discriminative value for identifying tolerance.

A potential explanation of the poor predictive value of the signatures would be an incorrect definition of 'tolerance' used in this study. In the absence of a reference tolerant group to confirm a threshold to distinguish tolerance and non-tolerance, a training set provided reference values which were then 'tightened' to create arbitrary thresholds for potential operational tolerance. The population used to generate these cut-offs were stable transplant recipients a median of ten years post-transplant. It is feasible that some of this population may exhibit sub-clinical tolerance and may already display the tolerance signature, which would mean the bar would be set artificially high by adding an additional 0.5 standard deviations. However it seems unlikely that sufficient numbers of this training set would display this phenotype to significantly affect the threshold, given the presumed infrequency of operational tolerance amongst stable transplant recipients. Secondly, despite similar matching for the confounding effect of calcineurin inhibition, the prevalence of the ITN signature was similar to previous estimates of operational tolerance frequency; this may relate

to the use of only two genes but suggests that the threshold is not inappropriately high. We did not analyse the donor hyporesponsiveness previously described in the RISET cohort, but this would not increase the apparent frequency of operational tolerance in the absence of the other variables.

A more likely explanation for the low prevalence is the effect of immunosuppression, as previously discussed. However, the effect of immunosuppression also raises concerns about the robustness of the signatures themselves. Both signatures were developed by comparison of operationally tolerant RTR with RTR remaining on immunosuppression [278, 279]. It is possible that, rather than representing signatures of operational tolerance, they actually represent signatures of immunosuppression. This is a possibility that was raised by Newell *et al.* at the time of signature development. A potential limitation of the study presented here is the overlap between immunosuppressive therapies: almost all participants are taking more than one immunosuppressant. This would be expected to reduce the apparent effect of any individual immunosuppressant: the fact that so many remained significant after correction for multiple testing highlights the dramatic effect of immunosuppression on some variables.

4.10.4 B cells and operational tolerance

Over the last fifteen years, evidence has emerged to support a role for populations of B cells in suppressing immune responses. These were demonstrated initially in the autoimmune setting in mice [537-543], although subsequent work has identified populations in humans; these were initially demonstrated to be enriched in the $CD24^{hi}CD38^{hi}$ transitional and $CD24^{hi}CD27^{+}$ memory B cell compartments in the settings of autoimmunity [236, 237, 240] and allergy [292]. As the mechanism of development and maintenance of operational tolerance is unclear, following the publication of the ITN and RISET signatures attempts to dissect this mechanism has focused primarily on B cells, given the overrepresentation of B cell-related markers within them.

Initial studies assessed the phenotype of B cells from operationally tolerant RTR, suggesting that this state was associated with an increased number of circulating B cells [277-279, 286], which produce increased levels of IL-10 [279, 286] compared to immunosuppressed RTR. B cells

expressed BANK-1 (an inhibitor of B cell activation) and a ratio of Fc receptors that would favour inhibition, with increased expression of the B cell survival factor B cell activating factor (BAFF) [277]. Silva *et al.*, in agreement with other studies [271, 277, 279, 286], found that peripheral B cells in operationally tolerant RTR were similar to that seen in healthy controls and that transitional and naïve B cells were markedly reduced in RTR taking immunosuppression. This led to the conclusion that part of the 'state' of operational tolerance related to preservation of the B cell compartment [284]. This conclusion should now be interpreted with caution as a substantial proportion of the RTR taking immunosuppression were taking azathioprine but it is interesting given the finding in this chapter than an increased proportion of B cells in RTR on immunosuppression predicts SCC onset (discussed further in Section 4.10.7). A study by Danger *et al.* noted stably increased expression of miR-142-3p in the B cells of operationally tolerant RTR compared to RTR on immunosuppression, at levels comparable to that of healthy controls [282]. Further work demonstrated that this was not simply due to an absence of immunosuppression but suggested that the upregulation of this may be a result of increased TGF-β1 expression and signalling [282]. Moraes-Vieira *et al.* provided independent support for these findings with the report that TGF-β and TGF-βR were stably expressed at higher levels in the peripheral blood in operationally tolerant RTR compared to those on immunosuppression [283]. Furthermore, GATA-3 was expressed at higher levels in RTR displaying operational tolerance, suggesting a Th2-skewed immune milieu may exist in operational tolerance.

Later studies have focused on the function of B cells in operational tolerance. Chesneau *et al.* found B cells from operationally tolerant RTR proliferated and produced IgG and IgM at similar levels as immunosuppressed RTR and healthy volunteers, but produced greater levels of IL-10. Plasma cells displayed alterations in gene expression and were more likely to undergo apoptosis in healthy volunteers and operationally tolerant RTR [286]. Whilst the authors assessed the potential confounding role of ciclosporin, other immunosuppression (importantly azathioprine) was not assessed. Further work by the same authors suggested that operationally tolerant RTR have a greater number of granzyme B-expressing memory B cells, which are able to inhibit T cell

proliferation by inducing apoptosis in a contact dependent, IL-10 independent, IL-21-mediated manner [544]. This phenomenon has been otherwise described in the setting of insufficient CD40 signalling [545, 546].

A limitation of these studies is that a precise marker to identify B cells with regulatory properties is still awaited. The main marker used to identify cells with regulatory function to date, based on *in vitro* work and indirect evidence from autoimmunity [547, 548], is the expression of IL-10 upon *in vitro* stimulation. No one B cell population identified by phenotypic markers is highly enriched for this behaviour. Mechanisms are unlikely to solely relate to the expression of IL-10 *in vivo*: for example evidence exists for cell contact mediated inhibition of $CD4^+$ responses [541, 543]. Furthermore, as the specificity and function of B cells that potentiate operational tolerance are unknown, studies have used methods of polyclonal stimulation which may not accurately reflect the *in vivo* response of these cells and may mask the effect of the true 'active' drivers of operational tolerance through non-specific activation. Nonetheless, when looking at the body of research in cohorts demonstrating operational tolerance, the results do consistently suggest alterations in the B cell compartment are associated with this clinical state.

Whilst previous results suggest that B cells are likely to play a role in mediating operational tolerance, the findings in this chapter indicate that the 'signatures' used to identify those who are operationally tolerant are altered by immunosuppressive therapy in variable ways. This hampers the clinical applicability of these tolerance signatures as they stand, and suggests the need to identify novel signatures which are unaffected by the immunosuppressive regimen. Haynes *et al.* tested a method of quantifying the indirect pathway of allorecognition in a 'spectrum' of RTR, ranging from syngeneic grafts between monozygotic twins to chronic rejectors, but including stable recipients on immunosuppression and a cohort of operationally tolerant RTR [285]. The response to alloantigen correlated with predicted alloreactivity, with no response in syngeneic grafts and a minimal response in operationally tolerant RTR and a progressively increasing response in the other groups. However when TGF-β1 was blocked a greater response was noted in the operationally

tolerant RTR, suggesting a role for TGF-β1-mediated regulation of the indirect pathway response. Furthermore, depletion of B cells did not impact upon this. The same groups were assessed for the probability of tolerance using the RISET signature and poor correlation with alloreactivity was seen. This suggests that the RISET signature does not accurately reflect other mechanisms that may be relevant in the setting of operational tolerance and agrees with our findings that correlation is poor between different measures.

Future studies of operationally tolerant cohorts will need to choose immunosuppressed RTR controls carefully to avoid an immunosuppression effect; in particular RTR taking azathioprine should be avoided. Whether the depletion of B cells in those taking azathioprine is reversible upon cessation is unclear, though experience with NK cells suggests it is [117]. Until this is clearer, use of control RTR with a history of azathioprine use is undesirable. A mechanistic explanation for why these specific genes in both signatures were significantly altered in the setting of operational tolerance has been lacking. The data presented in this chapter may provide an explanation for this.

It is likely that certain immunosuppressants will favour operational tolerance whilst others will not. An alternative interpretation of the data presented in this chapter regarding immunosuppression and operational tolerance is that azathioprine 'redirects' the immune system away from operational tolerance, with markers of operational tolerance appropriately absent in those taking azathioprine due to the increased predisposition towards a humoral response (Figure 4.6).

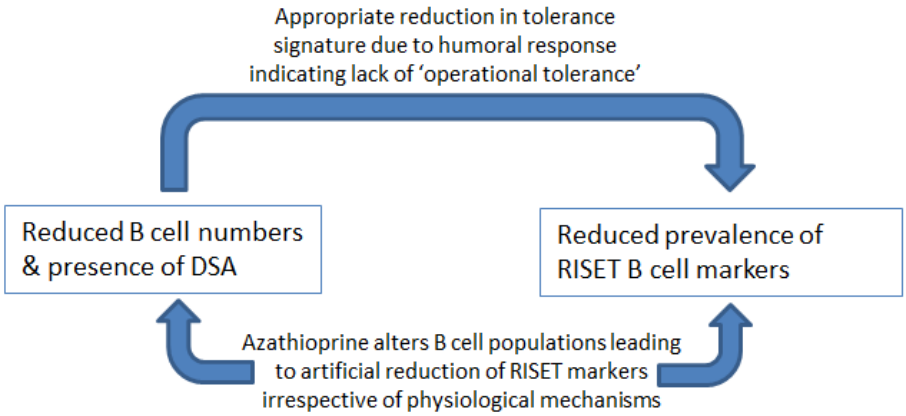


Figure 4.6: The interaction between azathioprine and the RISET tolerance signature. *The figure illustrates two alternate interpretations of the data presented in this chapter.*

Untangling the effect of immunosuppression upon biomarkers of tolerance represents a significant challenge in future research in this area. Discriminating between 'false negatives' and 'false positives' due to immunosuppression and true changes in the immune milieu induced by immunosuppression represent a significant hurdle to future research in this area. The 'next generation' of signatures of tolerance are beginning to emerge and it remains to be seen whether these will suffer from the same problem [536].

4.10.5 Prevalence of donor specific antibodies in long-term RTR

The prevalence of nearly 20% of RTR possessing DSA and 30% with nDSA is in keeping with other studies, although generally these have looked at populations much earlier post-transplant and variation is introduced through the threshold and method used for classifying DSA [445, 458, 459, 462, 463, 465, 549]. Longitudinal studies suggest that the prevalence of DSA increases with time post-transplant with the greatest rise in incidence in the first year, then a subsequent annual rate of *de novo* DSA development estimated at 2-5% [445, 458, 550, 551]. This is the first report of DSA prevalence in a cohort over twenty years post-transplant and the finding that prevalence is similar to that reported previously, despite the increased duration of time post-transplant, can be interpreted in a number of ways. Firstly, it is possible that the increased prevalence seen over time in previous studies plateaus at a point where the development of *de novo* DSA is counterbalanced by the attrition rate on graft survival due to chronic antibody damage. A second interpretation is that, given the long-term survival of RTR in this study, the nature or specificity of the DSA is such that there is less pathogenicity towards the graft. However, it should be noted that DSA seropositivity was associated with poorer renal function in our cohort; in a cross-sectional analysis causality cannot be determined but may indicate that these DSA are indeed detrimental to graft survival.

Late AMR, associated with *de novo* DSA, has been associated with a poorer prognosis and may be more resistant to therapy [552, 553]. Historical samples were not analysed in this chapter but given the time since transplant it is presumed that the majority of DSA in this study would be *de novo*

post-transplant. In keeping with previous findings, the majority of DSA in this study were due to HLA Class II-directed antibodies [444, 445, 457, 459, 549]. Some studies have reported a poorer outcome in the presence of Class II or C1q-fixing antibodies [462, 552, 554-556], whilst others report no difference [443, 445, 463, 557]. During the follow-up period of the study (nearly two years), only a single RTR with DSA (representing 5% of the DSA-positive subset) lost their graft. This is substantially less than the 64% one year survival after positive DSA testing estimated by Terasaki *et al.* in a prospective study of unselected American RTR [447], though is akin to that found by Lachmann *et al.* in a larger cohort of RTR with DSA with retained graft function [465]. Longer prospective follow-up of the cohort may provide further insight into the relative pathogenicity of the detected DSA compared to previous reports.

4.10.6 Donor specific antibodies, the tolerance signatures and immunosuppression

Surprisingly, given the findings above regarding switched memory B cells and plasmablast number, it was azathioprine rather than calcineurin inhibition that was associated with the presence of donor specific antibodies after adjustment for previously reported risk factors. This may reflect the poor correlation between antibody levels and short-lived circulating plasmablast and memory B cell numbers that has been previously reported [558], as the main source of IgG DSA is from long-lived antibody-secreting plasma cells in the bone marrow [534, 559].

Why might ciclosporin be associated with upregulation of genes relating to immunoglobulin production, but not associated with DSA formation? The explanation may be that calcineurin inhibitors mediate their effects indirectly upon B cells. In clinical studies, ciclosporin continuation is associated with a lower rate of DSA formation than switching to everolimus [457]. Ciclosporin has been demonstrated *in vitro* not necessarily to inhibit B cells directly [560, 561], but to have an inhibitory effect on the T cell help necessary for long-term, high-affinity antibody formation and through reduction of the production of cytokines necessary for germinal centre formation and B cell differentiation, such as IL-2 and IL-21 [466, 467, 560, 562, 563]. Work in human B cells stimulated by various methods including TLR, polyclonal BCR, IL-21 and CD40-mediated signalling has

demonstrated that IgG and IgM production by B cells can be downregulated in the presence of high-dose steroids and escalating doses of mycophenolic acid (the active component of MMF) and everolimus but not ciclosporin [564, 565]. This would be in keeping with the finding presented in this chapter that total serum IgM concentration is elevated with calcineurin inhibition. However, the data here suggests that this is not necessarily the only mechanism as the proportion of class-switched memory B cells was not reduced in the presence of calcineurin inhibitors, which would be expected if there was an inhibition of T cell help. Another possibility may be that circulating switched memory cells seen in our cohort are specific for antigens encountered prior to commencement of immunosuppression and so represent a pre-formed memory (secondary) response. A lasting alloresponse may be lacking due to the inhibition of primary responses and an absence of T cell help. This would explain the upregulation of *IGKV1D-13* and *IGKV4-1* and increased serum IgM levels, but a lack of IgG DSA and no increase in total IgG concentration in peripheral blood. In contrast, azathioprine may not inhibit T cell help in the germinal centre, potentially allowing formation of a memory response. Early studies, though often using higher doses of immunosuppression than seen in this study, provide a direct comparison of ciclosporin and azathioprine in the generation of humoral responses to influenza vaccination in transplant recipients; a process which requires T cell help. The authors found that humoral responses were impaired in RTR taking ciclosporin with low dose steroids with the response in those taking azathioprine and steroids comparable to healthy controls [566], which would support our hypothesis. Studies of primary and recall responses have suggested that azathioprine is associated with inhibition of primary antibody responses, but less so secondary responses [567, 568].

An association between azathioprine and DSA has been reported previously, although this is usually as a result of univariate analysis or comparison of whole immunosuppressive regimen rather than individual agents [460, 550]. Gathering evidence for the 'pro-DSA' role of azathioprine is complicated by the fact that MMF has been demonstrated *in vitro* and *in vivo* to prevent antibody formation, particularly in primary antibody responses [488, 565, 569]. As MMF is used in place of azathioprine, this may artificially inflate the association of azathioprine and DSA in univariate

analyses. Van der Mast *et al.* suggested an increased rate of DSA in RTR converted from ciclosporin to azathioprine compared to those who were switched to MMF, but interpretation is limited by study size and lack of control group [570]. In a controlled trial, Raftery *et al.* found that ciclosporin but not azathioprine monotherapy prevented the formation of anti-HLA antibodies in response to mismatched blood transfusion in previously unsensitised non-transplanted participants with ESRF [571]. However, the dose of ciclosporin used (15mg/kg) was much higher than that used in current clinical practice as evidenced by the high (100%) rate of side effects in the ciclosporin arm. A clinical study compared RTR who received MMF after transplant with RTR who never received MMF (of which half had received azathioprine) and found DSA prevalence to be over double in those who did not receive MMF [572]. A large cross-sectional study found azathioprine monotherapy was associated with increased risk of anti-HLA antibodies, though this was not seen on correction for pre-transplant sensitisation, gender and other immunosuppression [573]. RTR taking ciclosporin and tacrolimus were less likely to have HLA antibodies on multivariate analysis. However this study did not distinguish between donor and non-donor specific antibodies and did not correct for renal function or previous rejection. A series of small studies inconsistently suggested a reduction in the rate of progressive graft dysfunction with a switch from azathioprine to MMF, but these did not assess humoral responses and are confounded by the effect of the reduced calcineurin inhibitor dose which was instigated at the same time in some studies [574-576]. Conversion from azathioprine (half were also taking ciclosporin) to tacrolimus and MMF, with continuation of steroids, was found to 'rescue' chronic humoral rejection with a reduction in DSA titres in a small case series [577], but direct comparison of azathioprine and MMF in a randomised trial demonstrated no benefit in outcomes at two years post-transplant, though this time point may be too early to see the effects of chronic antibody-mediated rejection due to *de novo* DSA [578]. A study in SLE demonstrated that patients taking azathioprine monotherapy had comparable serum IgG levels to untreated controls, whilst patients taking MMF had a marked reduction in both circulating plasmablasts and total serum IgG [469]. This effect was not seen in the results presented in this chapter, although polypharmacy and different antigenic stimuli may cloud this.

The mechanism behind why azathioprine is associated with incomplete suppression of a humoral alloresponse is unclear. In light of the data presented in this chapter, the increased prevalence of humoral responses may relate to the imbalance of naïve and memory B cell populations, where a lack of naïve B cells and potentially suppressive transitional B cells allows expansion of memory B cells in the B cell niche. This may encourage secondary memory antibody responses, which are less well controlled by immunosuppression, to predominate [567]. An azathioprine-induced reduction in total B cells may lead to an increase in soluble BAFF concentration through homeostatic feedback mechanisms: elevated levels of soluble BAFF in peripheral blood have been previously associated with subsequent development of DSA in RTR [579].

Future work should focus on alterations in B cell populations in tertiary lymphoid organs and bone marrow in models of humoral rejection in the presence of azathioprine. For example, B cells are required for the maintenance of splenic lymphoid structures in mice [580]. As such, immunosuppression may have profound effects on this and may lead to dysregulation of B cell maturation and antibody secretion. This may prove informative not only in the field of transplantation, but also in other fields where azathioprine is in regular use, such as autoimmunity and inflammatory bowel disease.

In this study, all comparisons have been performed within the RTR population. Therefore it is unclear whether immunosuppression-induced alterations in B cell phenotype reflect a change towards or away from that seen in non-immunosuppressed populations. Further work needs to be undertaken comparing this cohort to a matched non-immunosuppressed cohort to clarify this. For the purpose of analysis 'calcineurin inhibitors' were taken as a single group. Ciclosporin and tacrolimus both inhibit calcineurin but via different mechanisms. Ciclosporin forms a complex with ciclophylin, which inhibits the enzymatic function of calcineurin which prevents nuclear factor of activated T cells (NFAT) translocation to the nucleus. In contrast, tacrolimus forms a complex with FK506-binding protein, which then inhibits calcineurin [581]. In both cases this leads to a failure to express IL-2 following TCR stimulation. However, unlike ciclosporin tacrolimus may also act via

other pathways and therefore may be more potent in inhibiting IL-2 production [582]. Thus the effect upon B cells and germinal centre reactions may differ between the two. In a univariate analysis of phenotype, Lachmann *et al.* found that RTR taking ciclosporin based therapy were more likely to have DSA than those taking tacrolimus, though this did not correlate for confounders such as time since transplant [465]. Other work has suggested that both ciclosporin and tacrolimus are protective against IgG DSA formation [573]. Our study did not assess for the presence of IgM DSA – these cannot be discounted and are likely to occur, in those taking calcineurin inhibitors. However, the clinical significance of these is unclear, with suggestion that the presence of isolated IgM DSA, in the absence of IgG class switching, is not associated with an increased rate of allograft loss and may even be beneficial to graft survival [583-585].

4.10.7 B cells in the pathogenesis of SCC

After adjustment for potential confounders, an increasing ratio of B cells to T cells was found to be predictive of earlier SCC during follow-up. The mechanism by which an elevated proportion of $CD19^+$ B cells within the lymphocyte gate, without a reduction in $CD3^+$ T cell number, should predispose a transplant recipient to SCC is unclear, although this is associated with trends towards an increased proportion of naïve B cells and an increased number of $CD19^+CD24^{hi}CD38^{hi}$ transitional B cells which have been demonstrated *in vitro* to have immunoregulatory effects [236, 237, 290, 292, 295]. Unfortunately it was not possible to optimise the assay to assess for IL-10 production, this being considered a 'hallmark' of B cells with regulatory properties: however, whole blood *IL10* expression was not increased in those with the highest CD19:CD3 ratio. Whilst this is a suboptimal method to assess IL-10 production, as these cells are resting and it is an assay of whole blood as opposed to specific populations, previous work has suggested that resting production of IL-10 by *ex vivo* circulating B cells in operationally tolerant RTR may be greater [279].

The association between an increasing $CD19^+$ to $CD3^+$ lymphocyte ratio and the development of SCC is a novel finding and was not associated with alterations in any T- or NK cell subsets in peripheral blood. A role for B cells in the development of SCC has been previously suggested by Hope *et al.*

[109], though as discussed this was limited by the confounding influence of azathioprine, which was corrected for in this study. One explanation for the lack of association with changes in specific B cell populations in peripheral blood may be that the increased proportion is a biomarker for alterations in B cell populations elsewhere in the body, such as the spleen.

A mechanistic role for B cells in cutaneous malignancy development has been previously described, in which the B cells have mediated their effects in distant lymphoid tissue. In a murine model of chemically-induced cutaneous SCC, splenic and lymph node B cells producing IL-10 inhibited $CD8^+$ T cell responses, leading to papilloma and malignancy development [150]. Similarly, in a murine model of cutaneous SCC where a subset of HPV-16 genes are expressed constitutively under the keratin promotor, dysplastic skin is characterised by deposits of IgG and IgM produced by distant B cell populations, which promotes malignancy through Fc receptor gamma-mediated chronic inflammation and macrophage reprogramming [241, 423]. Knockout of lymphocytes ablates this whilst adoptive transfer of B cells restores dysplasia development [241]. Subsequent work in this model demonstrated that depletion of B cells using a monoclonal anti-CD20 antibody led to reduced angiogenesis and keratinocyte hyperproliferation; administration of this in established SCC did not in itself lead to regression though B cell depletion did improve the response to chemotherapy [586]. A recent review by Tan and Coussens highlighted that in humans, increased levels of circulating immune complexes and tumour antigen antibody titres are not necessarily associated with an effective immune response against malignancy [587]; instead they may correlate with disease stage and are often associated with a poorer prognosis in many cancers including bladder, breast, head and neck, and genitourinary malignancies [588-591]. In the setting of breast cancer, Domschke *et al.* reported that the presence of tumour-reactive T cells in the bone marrow at time of tumour resection were associated with reduced cancer-related mortality risk; however, in patients who demonstrated the presence of tumour antigen antibodies there was a trend towards increased mortality risk: only 20% of those demonstrating tumour-reactive T cell response had tumour antigen associated antibodies, in contrast to 70% of those without a tumour-reactive T cell response [592].

B cells also infiltrate malignancies directly in the clinical setting [593-600]. Animal studies and *in vitro* studies of B cells from cancer patients demonstrate that their presence may lead to downregulation or inappropriate polarisation of T cell responses through cell-cell contact and local cytokine production [150, 238, 601-603], induction of Treg [601, 604, 605], modulation of dendritic cell or $CD4^+$ induction of $CD8^+$ responses [245, 606], and secretion of soluble factors that encourage tumour survival [243]. On the other hand B cells may contribute positively to promote an adaptive response through antigen presentation and costimulation directly or through cytokine secretion [596, 607], local formation of antibodies (which, as they are produced locally at greater concentrations may be more effective) [244], or direct cytotoxic activity against tumors [608]. It has been proposed that these conflicting results depend on the model used, with the activation state of B cells playing a key role [609]. The picture is no clearer in humans, where in some cases markers of B cell and plasma cell infiltration have been associated with a better prognosis [595-599], whilst in others a poorer prognosis [594, 600, 610].

It is likely that the net contribution of B cells *in vivo* to the immune response to malignancy also depends on the clinical setting, such as stage and type of malignancy, and is complicated by the factor that increased B cell infiltration often correlates with other factors, such as increased infiltration by other lymphocytic populations and more advanced disease [599, 600]; these factors are inconsistently accounted for in studies. In a small clinical trial in metastatic colon cancer, $CD20^+$ B cell depletion using rituximab was associated with a reduction in both circulating B cells and IgM immune complexes, and half of those who completed treatment showed a transient regression in metastases [242]. However, the use of the same therapy in a small study in renal cell carcinoma demonstrated no benefit [611].

In the setting of cutaneous SCC little work regarding B cell infiltration has been undertaken, though a immunohistochemical study, without clinical correlation, of SCC from RTR and non-RTR found B cells made up a variable minority of infiltrating lymphocytes, and that numbers were not increased in RTR compared to non-RTR. However, there was a significant increase in infiltrating $CD138^+$

plasma cells in RTR, suggesting tertiary lymphoid structures and antibody production may be more common in RTR than non-RTR [612]; this may be a contributor to the increased aggression of SCC seen after organ transplantation. However, as discussed above, interpretation of immunohistochemistry alone is difficult given the multifaceted and often distant role of B cells in malignancy and requires correlation with clinical outcomes. This is difficult with SCC in RTR where the tumours are usually completely excised and the next occurrence of disease frequently represents a separate event, rather than metastasis or recurrence.

Taken together, previous work in animals combined with the results presented in this chapter suggest a pathogenic role for B cells particularly in the early development of SCC, through promotion of chronic inflammation. Given the above finding regarding azathioprine and depletion of B cell populations, this might be hypothesised to be beneficial for SCC development. However, the intact humoral response to allograft in the presence of azathioprine as an immunosuppressant implies that this agent, in contrast to other commonly used immunosuppressive agents, may allow a humoral response to take place against dysplastic skin facilitating the chronic inflammation that precedes malignancy development in animal models.

4.10.8 Conclusion

This chapter has demonstrated that the 'signatures' of operational tolerance are affected significantly by the immunosuppressive regimen in use, limiting their clinical applicability at present. Future signatures of operational tolerance ideally would need to be unaffected by immunosuppression, although this presumes that certain immunosuppressive agents do not predispose RTR towards or away from operational tolerance. Discriminating a confounding and a true effect of immunosuppression in the development and maintenance of operational tolerance represents a substantial challenge in future biomarker research.

Azathioprine in particular depletes naïve and transitional B cell populations, but does not appear to alter mature B cell populations or humoral responses, as evidenced by circulating phenotype and immunoglobulin production: indeed, in contrast to other immunosuppressive agents azathioprine is

associated with the development of graft-directed antibodies, and it is possible that this relates to dysregulation of the B cell compartment and alteration of germinal centre reactions. In contrast, calcineurin inhibitors appear to prevent class switching and the development of long lasting IgG responses, though may not impact upon IgM responses.

Correcting for the effect of immunosuppression, the data presented here show an association between an increasing proportion of B cells and the development of SCC. Whilst the effect of azathioprine means the CD19:CD3 ratio is unlikely to be clinically informative without adjustment, data from animal models and other malignancies provide indirect demonstration of the detrimental role of B cells in cutaneous carcinogenesis. This should be the focus of further mechanistic work in this area.

Chapter 5: $CD8^+$ T cell dysfunction and SCC development

5.1 Introduction

An initial aim of the study presented in this thesis was to assess whether underlying 'natural' reductions in the immune response may be predictive of the development of SCC in long-term renal transplant recipients.

The results in Chapters 3 and 4 indicate that: a) an increasing proportion of CD57-expressing $CD8^+$ T cells have predictive value for earlier SCC during follow-up and a more aggressive disease course with earlier recurrence; and b) the 'signatures' of operational tolerance signatures do not predict SCC development *per se* though this is with the caveat that they are heavily influenced by immunosuppression. An increasing ratio of T to B cells was found to be predictive of SCC development, however this required adjustment for azathioprine use. This means that this parameter would be difficult to interpret in a clinical setting.

A further aim of the study was to attempt to elucidate the mechanisms that may underlie the development of SCC, both in the general population and in long-term RTR. $CD8^+$ T cells are a key population required for elimination of UV and chemically induced cutaneous SCC in mice [151, 613]. The role for $CD8^+$ T cells in preventing SCC in humans is supported indirectly by previously published immunohistochemical and tumour 'crawl out' data suggesting that there may be decreased $CD8^+$ infiltration into the SCC of RTR compared to non-RTR [66-68]. This may partly explain the increased aggression and recurrence of SCC seen in RTR. We decided to focus on the mechanisms which might associate increasing CD57 expression in peripheral blood with SCC development; in particular a role for $CD8^+$ T cell dysfunction in these patients.

$CD8^+CD57^+$ cells have been described as dysfunctional previously, although the evidence for this is conflicting. Studies have found impaired cytokine production in CMV-specific $CD8^+CD28^-$ or $CD8^+CD57^+$ T cells [312, 614-617], whilst another suggested impaired cytotoxicity in bone marrow

transplant recipients with a high proportion of these cells [618]. Impaired production of IL-2 has also been reported in these cells in both healthy populations and long-term RTR [332, 619]. Other studies have suggested $CD8^+CD28^-$ and $CD8^+CD57^+$ T cells show normal or even greater effector function [316, 438, 620-623]. These comparisons are often made with $CD8^+CD28^+$ and $CD8^+CD57^-$ populations from the same host. Changes in the $CD8^+$ compartment with age and immunosenescence can be accompanied by alterations in the $CD4^+$ compartment [322, 624], which may affect overall CD4/8 T cell function and are not assessed by studies of one cell population.

Another potentially important variable which has been minimally explored is the role of immunosuppression in immunosenescence. A recent study found indirect evidence of intact cytotoxic function and cytokine secretion in terminally differentiated cells in a cohort of RTR but did not explore $CD8^+$ subsets specifically, and did not have a non-immunosuppressed comparator group [373]. Another study found a reduction in total IL-2 and IFN-γ secretion by $CD3^+$ T cells in RTR compared to non-RTR irrespective of age and CMV serostatus, but did not look at the relative contribution of T cell subsets [625]. It is possible that CD57-expressing cells are differentially affected by immunosuppression compared to CD57-negative cells.

In the setting of chronic antigenic stimulation, particularly with higher antigenic loads, $CD8^+$ dysfunction may occur in the form of immune exhaustion [626-635]. In long-term RTR, where chronic antigenic stimulation is likely to be ongoing, there may be overlap with these factors: this has not been explored in long-term RTR. One study suggested the increased risk of malignancy in the first decade after transplant with CMV seropositivity was due to 'immune exhaustion', but analysis was limited to demonstrating a lack of IL-2 production by $CD8^+CD57^+$ cells [327].

Whilst studies have examined intratumoural infiltration and gene expression in SCC in RTR and non-RTR [66-68, 636] and other have looked at the prognostic value of circulating immune phenotype [66, 109, 188], no study has assessed these two in parallel in the same participants. This will be evaluated in CD57lo and CD57hi RTR in the data reported here.

This chapter explores the issues above, aiming to answer the questions:

1. Is the CD57hi phenotype associated with other immune phenotype changes?
2. Is the CD57hi phenotype associated with functional changes in the $CD8^+$ compartment?
3. Does immunosuppression affect $CD8^+CD57^+$ cells to a greater extent?
4. Does the CD57hi phenotype associate with altered lymphocyte infiltration into the SCC?

5.2 Lymphocyte compartment changes with CD57hi phenotype

First, other changes within the lymphocyte compartment that correlated with CD57hi phenotype were examined.

Greater expression of CD57 upon $CD8^+$ T cells was associated with increasing differentiation of both the $CD4^+$ and $CD8^+$ compartments. The proportion of CD57-expressing $CD8^+$ T cells demonstrated a negative correlation with the proportion of naïve and central memory (TCm) $CD8^+$ T cells and a positive correlation with terminally differentiated effector memory (TEMRA) $CD8^+$ T cells (Figure 5.1A). This correlation was weaker, but also held true with regard to the $CD4^+$ compartment (Figure 5.1B), with a significant and strong correlation between CD57 expression in the $CD8^+$ and $CD4^+$ compartments (r^2 0.65, $p<0.001$).

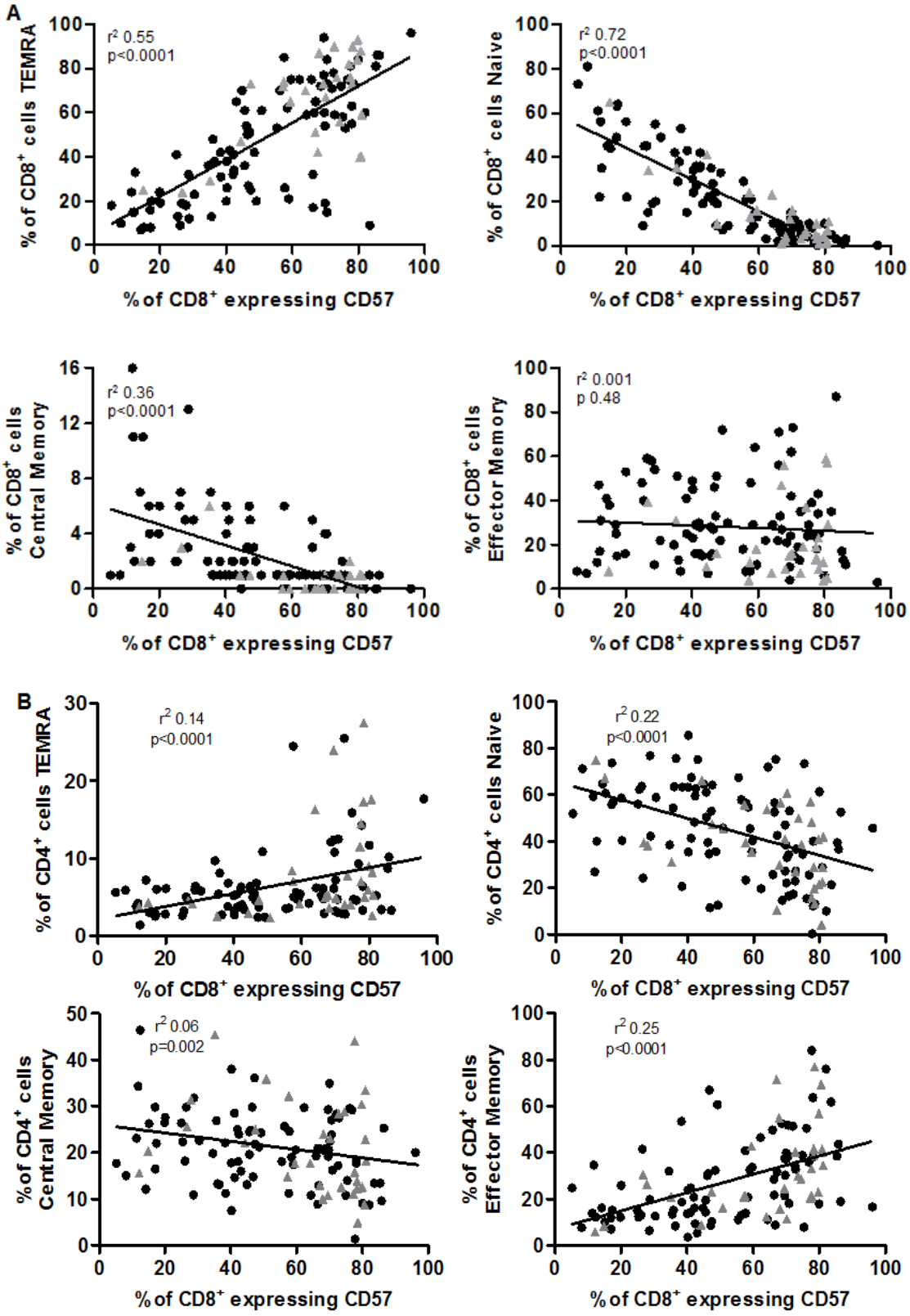


Figure 5.1: Increasing CD57 expression upon CD8⁺ T cells correlates with increasing differentiation of the circulating CD8⁺ (A) and CD4⁺ (B) compartments. *Black circles indicate RTR without SCC during follow-up whilst grey triangles indicate RTR who developed SCC during follow-up.*

It was found in Chapter 3 that CMV seropositivity was strongly associated with an increased proportion of CD57-expressing $CD8^+$ T cells ('CD57hi' phenotype). RTR were therefore stratified by CMV serostatus. In keeping with the previous result CD57-expressing cells were predominantly found in the effector compartment (Figure 5.2). CMV seropositivity was associated with both increased differentiation of the $CD8^+$ T cell compartment and an increase in CD57-expressing $CD8^+$ T cells, particularly within the TEMRA compartment.

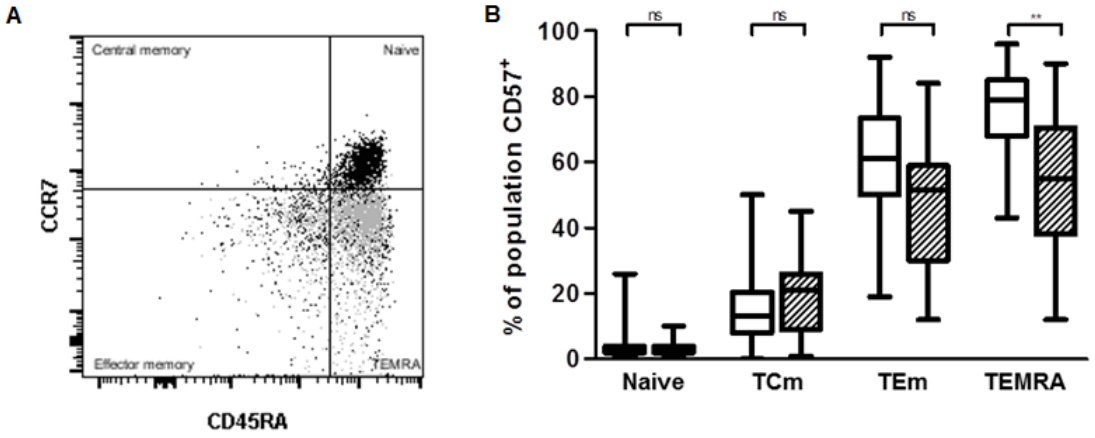


Figure 5.2: CD57 expression is predominantly within the CD8+ effector compartment and is associated with CMV seropositivity*. A) Representative gating indicating the location of all $CD8^+$ T cells (black) and $CD8^+CD57^+$ T cells (grey) within CD8 subsets. B) Frequency of CD57-expressing cells amongst CD8 populations (stratified by CMV seropositivity: non-shaded bars indicate seropositive RTR, total n=117). 'ns', not significant; ** p<0.01 using Mann-Witney test. Box and whisker plots indicate (from top to bottom) maximum value, 75^{th}, 50^{th} and 25^{th} centiles and minimum value, respectively.*

There was a moderate positive correlation between the $CD4^+/CD8^+$ ratio and the proportion of $CD8^+$ T cells expressing CD57 (r^2 0.21, p<0.001). Wikby *et al.* described increased risk of two-year mortality in a Swedish longitudinal study in elderly participants displaying a circulating CD4/CD8 ratio of less than 1 [309-311]; 20 participants, representing 17% of the total cohort, demonstrated a ratio <1 at enrolment; all but two of these displayed a CD57hi phenotype. This meant that 28% of the CD57hi phenotype cohort, but only 4% of the CD57lo cohort, displayed a CD4/CD8 ratio of less than 1 (p<0.001) (Table 5.1).

	$CD4^+/CD8^+$ ratio <1	$CD4^+/CD8^+$ ratio ≥1
CD57hi	18 (28)	47 (72)
CD57lo	2 (4)	50 (96)
Total	*20 (17)*	*97 (83)*

Table 5.1: 2x2 table indicating number (percentage) of RTR with CD57hi and CD57lo phenotype who also express the immune risk profile (CD4/CD8 ratio <1). *Number in brackets indicates percentage of CD57hi or CD57lo or total cohort. The difference in proportions was significant (p<0.001) by chi-squared test.*

The CD57hi phenotype was not associated with an increase in Treg number, *FOXP3* expression or *IL10* expression in peripheral blood across the whole RTR cohort. In fact, the CD57hi phenotype was associated with lower numbers of Treg and *FOXP3* expression (Figure 5.3). Analysing only CMV seropositive RTR indicated a weak but significant negative correlation between CMV IgG titre and both the percentage (r^2 0.08, p=0.02) and number (r^2 0.07, p=0.03) of Treg. An exploratory multivariate analysis was undertaken to further assess this. The reduction in Treg number in CD57hi RTR remained significant upon adjustment for age at enrolment, calcineurin inhibitor use and history of previous SCC or SCC during follow-up. $CD8^+CD57^+$ cells expressed FOXP3 at a lower level than $CD8^+CD57^-$ cells when quantified by percentage of expressing cells and mean fluorescence intensity, with FOXP3 expression being seen in only a small percentage of circulating $CD8^+$ T cells. There was no difference in total number or percentage of $CD8^+FOXP3^+$ cells between CD57hi and CD57lo RTR.

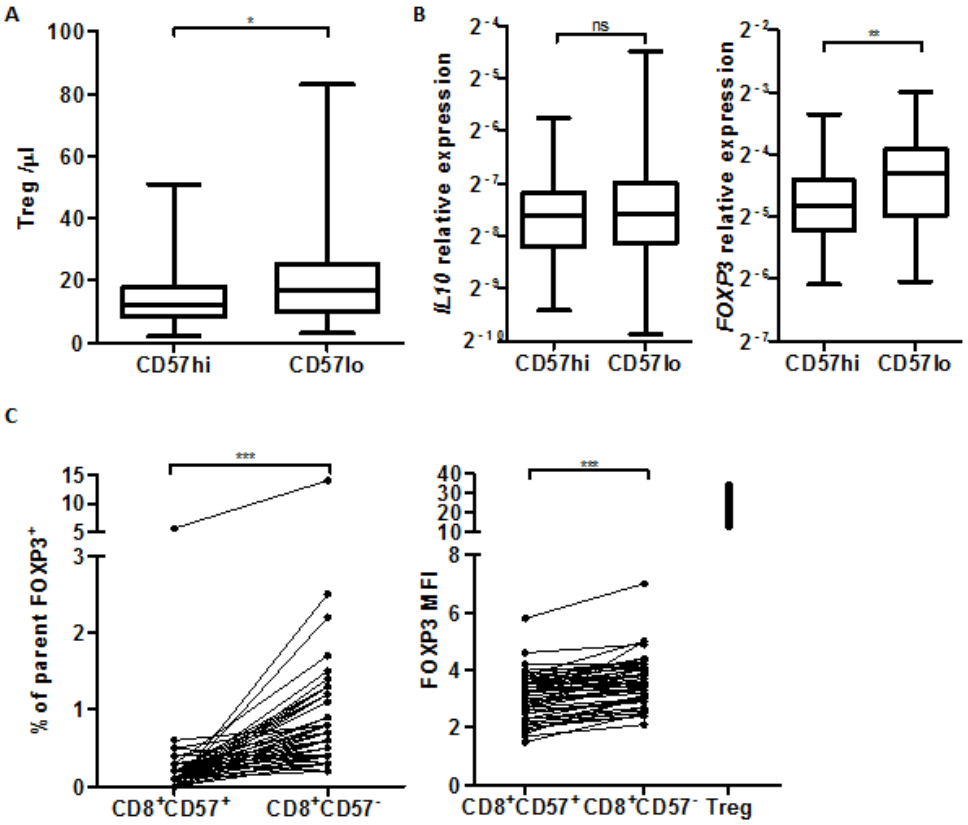


Figure 5.3: CD57hi phenotype is not associated with an increase in Treg,* FOXP3 *expression or* IL10 *expression in peripheral blood and $CD8^+CD57^+$ cells do not express FOXP3. *A) Number of Treg in freshly isolated PBMC B) whole blood* FOXP3 *and* IL10 *expression, normalised to* GUSB*, stratified by CD57 phenotype (n=117). C) Percentage of FOXP3-expressing and mean fluorescence intensity (MFI) of FOXP3 staining on freshly isolated $CD8^+$ cells stratified by CD57 phenotype (n=55). MFI of FOXP3 on Treg is provided for comparison. 'n' differs between A and B as the flow cytometry staining panel to enable this analysis was added after study commencement. 'ns' not significant, *p<0.05, **p<0.01, ***p<0.001 using Mann-Whitney test (A and B) or Wilcoxon matched-pairs rank test (C). Box and whisker plots indicate (from top to bottom) maximum value, 75th, 50th and 25th centiles and minimum value, respectively.*

5.3 CD57 as a phenotypic marker of terminal differentiation and cellular senescence

The results in Section 5.2 indicated that increasing CD57 expression correlated with differentiation of the $CD8^+$ and $CD4^+$ compartment. Alternative phenotypic markers of increasing differentiation and senescence of $CD8^+$ T cells are downregulation of CD27 and CD28 and upregulation of 'killer-cell lectin-like receptor G1' (KLRG-1) [227, 637-639].

As expected, the CD57hi phenotype was associated with increased downregulation of CD28 and CD27 (Figure 5.4A). When the expression of CD27 upon $CD8^+$ T cells was explored, a significantly greater proportion of $CD8^+CD57^+$ T cells lacked CD27 expression in CD57hi RTR, compared to CD57lo

(Figure 5.4B). The same was seen for CD28 but not KLRG-1 expression (data not shown). The phenotype of CD57-expressing $CD8^+$ T cells compared to that of CD57-negative $CD8^+$ T cells was explored in a subset of RTR. CD57-positivity on $CD8^+$ T cells was associated with co-expression of KLRG-1 and a lack of CD28 expression (Figure 5.4C and D). The total proportion of $CD8^+$ T cells expressing KLRG-1 did not differ between CD57hi and CD57lo, though there was a trend towards a greater proportion in CD57hi (CD57hi mean [IQR] 72.0% [60.6 – 83.4] vs CD57lo mean [IQR] 61.4% [48.0 – 74.9], p=0.18).

Taken together, the results from Sections 5.2 and 5.3 indicate that the expression of CD57 within the $CD8^+$ T cell compartment is a summary measure of the accumulation of phenotypically senescent $CD4^+$ and $CD8^+$ T cells, with increasing differentiation of both compartments. Not only is there a greater proportion of $CD57^+$ $CD8^+$ T cells in CD57hi RTR, but also at an individual cellular basis they are at a later stage of differentiation.

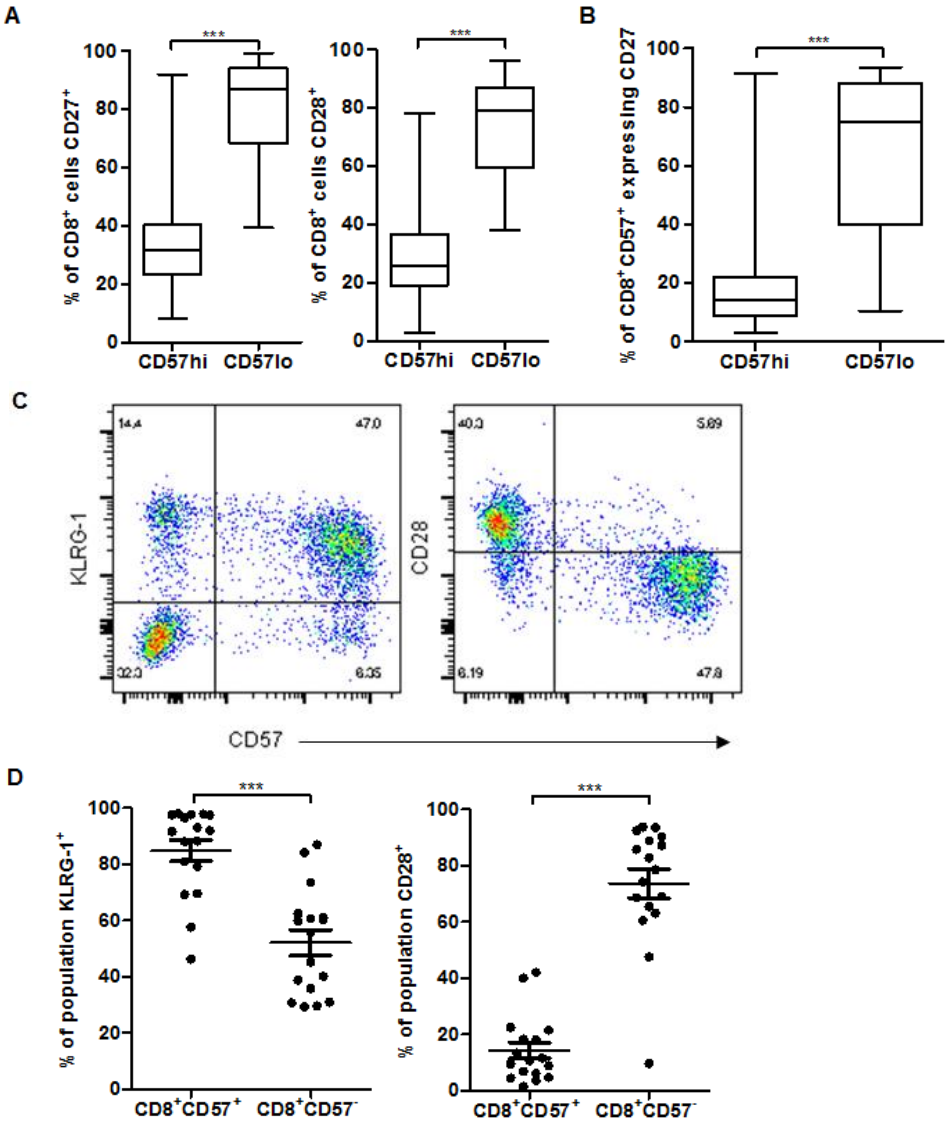


Figure 5.4: CD8⁺CD57⁺ cells display other phenotypic markers of cellular senescence. *A) Percentage of CD8⁺CD27⁺ and CD8⁺CD28⁺ cells within CD8 gate, stratified by CD57 phenotype (n=116). B) Expression of CD27 within CD8⁺CD57⁺ gate stratified by CD57 phenotype (n=116) C) Representative staining of KLRG-1 and CD28 on a subset of RTR, gated on live CD3⁺CD8⁺ lymphocytes (n=19 RTR). D) KLRG-1 and CD28 expression amongst CD8⁺ T cell populations.. ***, p<0.001 using Student's t-test. Box and whisker plots indicate (from top to bottom) maximum value, 75th, 50th and 25th centiles and minimum value, respectively.*

5.4 Phenotypic markers for CD8⁺ T cell dysfunction

Other mechanisms of T cell dysfunction have also been described in the setting of cancer; predominantly exhaustion but also anergy [633-635, 640-643]. Due to the difficulty in distinguishing anergy we opted to focus on exhaustion [644]. Constitutive programmed death-1 (PD-1, CD279) and T-cell immunoglobulin and mucin domain 3 (Tim-3, CD366) upregulation has

been described on exhausted $CD8^+$ T cells particularly in the context of chronic antigenic stimulation, such as persistent viral infection and malignancy [628-631, 633, 634, 640-643, 645, 646]. No study has looked at the co-expression of these markers in the peripheral blood of RTR with respect to $CD8^+$ dysfunction. Thawed PBMC from a subset of RTR, matched for age and gender, were assessed for PD-1 and Tim-3 expression in $CD8^+$ T cells. We hypothesised that increasing CD57 expression might synergise with $CD8^+$ T cell exhaustion.

There was no increase in expression of either PD-1 or Tim-3 on CD57-expressing $CD8^+$ T cells compared to those that were CD57-negative (Figure 5.5A and B). Expression of Tim-3 and PD-1 in peripheral blood tended to be mutually exclusive, with co-expression consistently present on less than 3% of total circulating $CD8^+$ T cells (Figure 5.5A and C). CD57hi RTR did not express increased levels of either PD-1 or Tim-3 or both in circulating $CD8^+$ T cells (Figure 5.5C).

The proportion of $PD\text{-}1^+Tim\text{-}3^+$ $CD8^+$ T cells demonstrated a positive correlation with age at enrolment (r^2 0.26, p=0.03) but no correlation with duration of immunosuppression. The proportion of single-positive cells did not correlate with either variable. The proportion of double-positive cells did not differ between those with and without SCC at baseline, and those who developed SCC during follow-up (data not shown).

These results suggest that there is no phenotypic evidence of greater exhaustion in circulating $CD8^+$ T cells from CD57hi RTR. Only a small minority of circulating $CD8^+$ T cells display phenotype markers of exhaustion in stable long-term RTR, which increases with age. It is not possible to rule out exhaustion definitively using cell surface markers alone; therefore $CD8^+$ T cell function was also assessed.

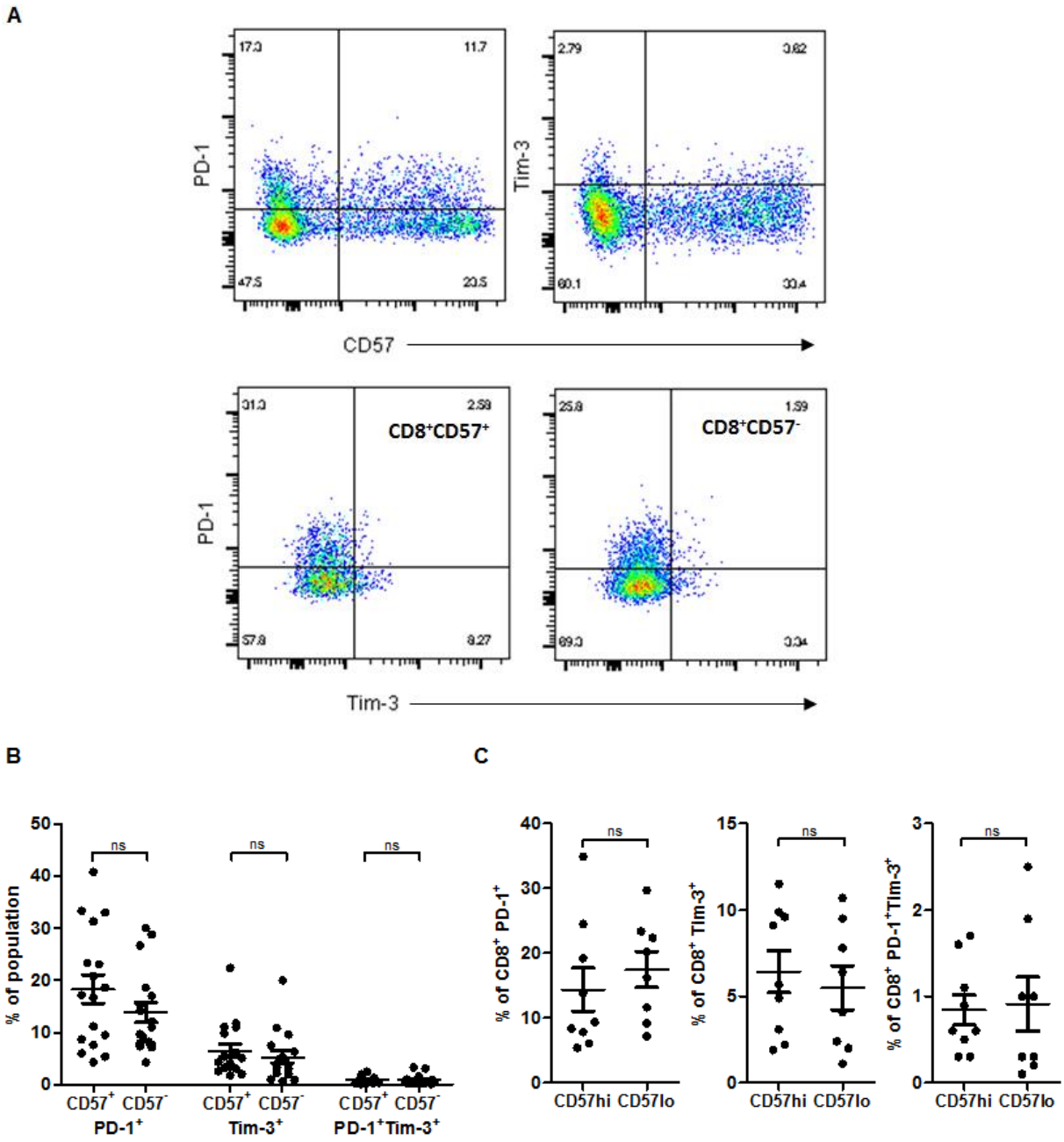


Figure 5.5: Circulating CD8$^+$CD57$^+$ cells do not express markers of exhaustion at greater levels than CD57$^-$ cells. *A) Representative staining of Tim-3 and PD-1. B) Tim-3, PD-1 and co-expression of both amongst CD57$^+$ and CD57$^-$ CD8$^+$ T cell populations (n=19 RTR) C) Total expression of PD-1 and Tim-3 and co-expression of both stratified by CD57 phenotype. Gating for PD-1 and Tim-3 were set using isotype controls. 'ns', not significant by Student's t-test. For B and C, mean and standard error of the mean (SEM) for each group are also indicated.*

5.5 CD8$^+$ T cell cytotoxicity

The ability of circulating CD8$^+$ T cells to exhibit cytotoxic activity was evaluated first. In the absence of a known tumour antigen to stimulate tumour-reactive CD8$^+$ T cells, this was undertaken using

polyclonal stimulation with plate-bound CD3 and soluble IL-2. IL-2 co-stimulation was used in preference to CD28 signalling, as a lack of CD28 upon most $CD57^+$ cells would confound any potential interpretation of an impaired response.

This assay demonstrated firstly that $CD8^+CD57^+$ T cells are capable of being activated to the same extent as $CD8^+CD57^-$ T cells following anti-CD3 stimulation in the presence of IL-2, as evidenced by upregulation of CD69 (Figure 5.6).

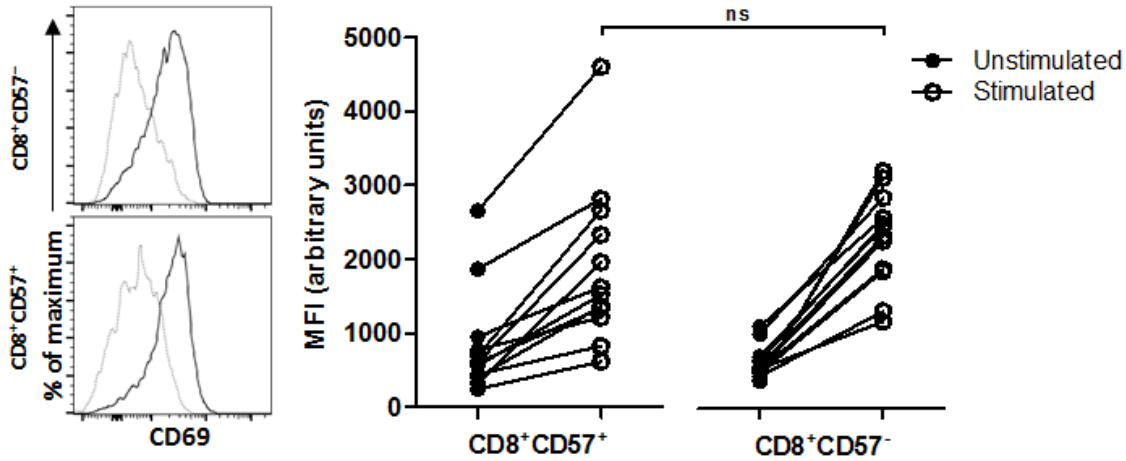


Figure 5.6: $CD8^+CD57^+$ cells are activated to an equal degree by polyclonal anti-CD3/IL-2 stimulation. *Thawed PBMC were stimulated for 6 hours with 2µg/mL plate-bound anti-CD3 and 125IU/mL IL-2. Representative staining shown with (black line) and without stimulation (grey line) on left, with grouped results on right. 'ns', not significant using Student's paired t-test.*

Unstimulated $CD8^+CD57^+$ T cells expressed high levels of granzyme B *ex vivo* (Figure 5.3A). The turnover of cell surface lysosomal-associated membrane protein-1 (LAMP-1, CD107a) during a 6 hour stimulation period (using anti-CD3 and IL-2 stimulation), assessed using fluorescence intensity and the percentage of $CD107a^+$ T cells at the end of the culture, did not differ between the $CD8^+CD57^+$ and $CD8^+CD57^-$ compartments (Figure 5.6B and C). When re-stratified by SCC at enrolment and during follow-up, no difference was seen.

Overall lysosomal degranulation in response to polyclonal stimulation was greater in CD57hi compared to CD57lo RTR (Figure 5.6D). These results indicate that both TCR-mediated activation and cytotoxicity are not specifically impaired in $CD57^+$ cells, and that CD57hi RTR do not display a deficit in degranulation of soluble cytolytic mediators in the circulating $CD8^+$ compartment.

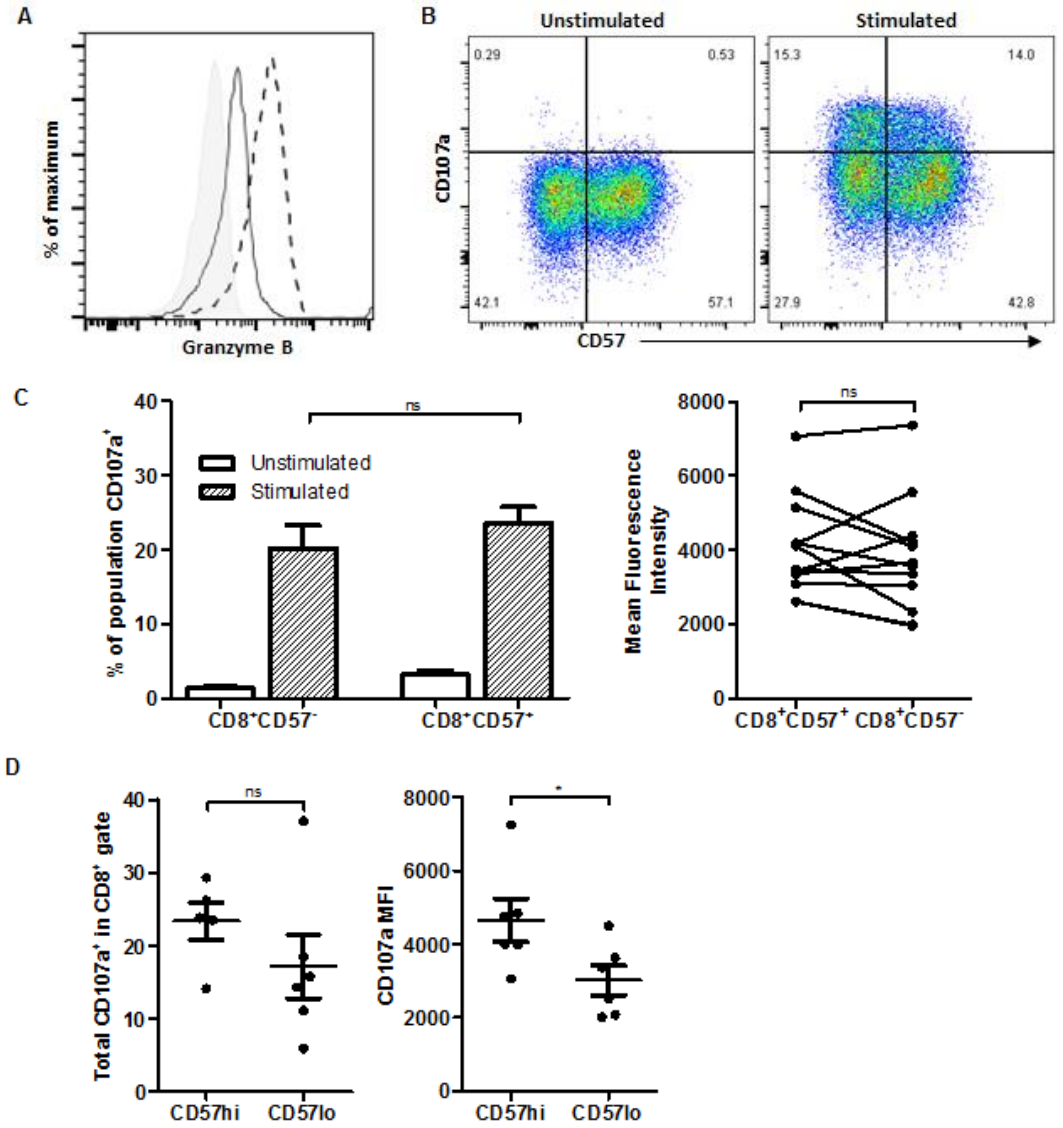


Figure 5.7: CD8$^+$CD57$^+$ cells express high levels of granzyme B and are not impaired in degranulation of lysozymes upon polyclonal stimulation. *A) Representative granzyme B staining in an isotype control (gray filled plot), freshly isolated CD8$^+$CD57$^-$ (black solid line), and CD8$^+$CD57$^+$ (black dashed line) cells. B) Representative staining of CD107a (LAMP-1) by CD8$^+$ cells with and without stimulation. Gated on live CD8$^+$ cells. Stimulation was for 6 hours with plate-bound anti-CD3 and IL-2 (as per Figure 5.6) with monensin added after 2 hours. CD107a-APC-Cy7 was present in the culture medium and so binding represents turnover at the cell surface. C) Percentage of cells expressing CD107a within live CD8$^+$CD57$^+$ and CD8$^+$CD57$^-$ gates, and mean fluorescence intensity of CD107a in those gates after stimulation (n=12 RTR). 'ns', not significant. D) Total percentage and MFI of CD8$^+$CD107a$^+$ upon stimulation stratified by CD57 phenotype (n=6 RTR per arm, matched by age and sex). 'ns', not significant *, p<0.05 by t-test. RTR matched by age and sex and taking calcineurin inhibitors at sampling were used for this analysis. For C (left graph) and D, mean and SEM for each group are indicated.*

5.6 Cytokine secretion by $CD8^+$ T cells

Secretion of effector cytokines was assessed next. We focused on TNF-α, IL-2 and IFN-γ as there is the greatest evidence of impaired production of these molecules with $CD8^+$ dysfunction. To rule out any effect due to variation in immunosuppressive regime, only RTR taking calcineurin inhibitors were used. Other immunosuppression in the subset at time of sampling was azathioprine in 55%, MMF in 27% and steroids in 36%; stratification by use of these did not demonstrate any significant differences in total cytokine production (data not shown).

Upon polyclonal stimulation, there was a trend towards a greater proportion of $CD57^+$ $CD8^+$ T cells secreting IFN-γ, and a significantly greater proportion of $CD57^+$ cells secreting TNF-α (Figure 5.8). Few $CD57^+$ T cells secreted IL-2. The same phenomenon was seen in the $CD4^+$ compartment.

Given that CD57 is predominantly expressed in effector memory T cells, it is possible that the lack of IL-2 production is common to all effector memory and is therefore a reflection of this. To assess for this, IL-2 secretion by T cell compartment was assessed. In all naïve and memory compartments, IL-2 production was diminished in $CD57^+$ T cells compared to $CD57^-$ (Supplementary Data Figure i.1).

It was hypothesised that CD57hi RTR would, therefore, have a deficit in total $CD4^+$ and $CD8^+$ T cell IL-2 production compared to CD57lo. Surprisingly, there was no difference in the total proportion of $CD4^+$ and $CD8^+$ T cells producing IL-2 comparing CD57hi and CD57lo RTR (Figure 5.8). Whilst there was a trend towards a significant reduction in the proportion of IL-2 producing $CD8^+$ T cells in CD57hi ($p=0.09$), there was no difference in the $CD4^+$ compartment (Supplementary Data Figure i.2). No difference in TNF-α production was noted, whilst IFN-γ production was greater in CD57hi. When re-stratified for those with a history of SCC at enrolment, or who developed SCC during follow-up, no difference was seen in the production of all three cytokines.

In the setting of chronic viral infection, a progressive reduction in polyfunctionality has been described in exhausted $CD8^+$ T cells [647]. The ability to secrete both or either TNF-α and IFN-γ was assessed in $CD8^+$ T cells from CD57hi and CD57lo RTR (n=3 per arm); there was no difference in the proportion secreting both, one or neither or these cytokines (Supplementary Data Figure i.3).

The results of sections 5.5 and 5.6 together indicate that $CD8^+CD57^+$ cells behave as functional effector T cells upon polyclonal stimulation.

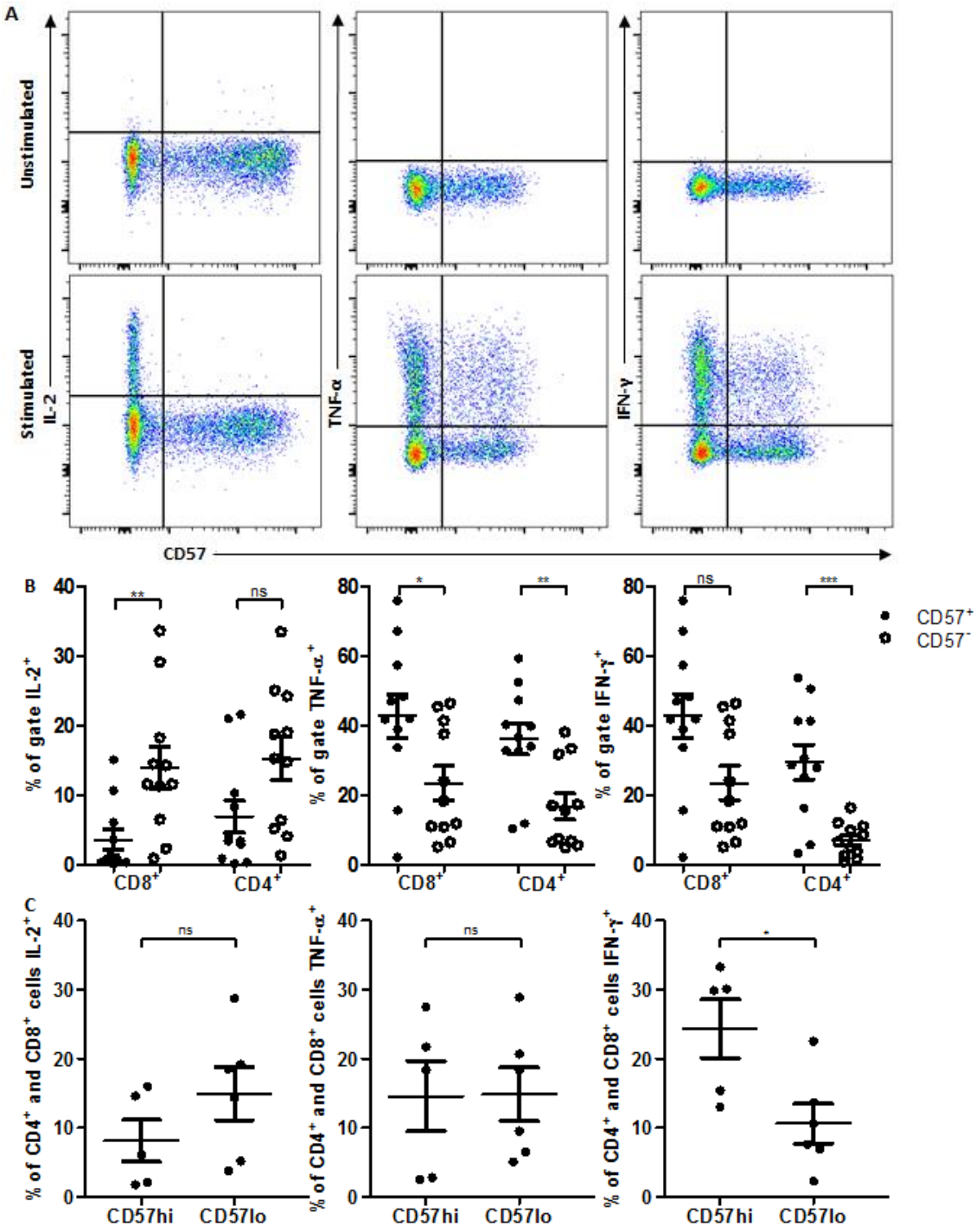


Figure 5.8: CD57hi RTR do not demonstrate an impairment in production of effector cytokines compared to CD57lo. *A) Representative staining of IL-2, TNF-α and IFN-γ gated on live $CD8^+$ cells, after six hours stimulation with PMA and ionomycin in the presence of brefeldin A. B) Production of the three cytokines by $CD57^+$ and $CD57^-$ cells in the CD4 and CD8 gates (n=12). C) Total production of the three cytokines stratified by CD57 phenotype (n=6 per arm). RTR matched by age and sex and taking calcineurin inhibitors were used for this analysis. 'ns', not significant, *p<0.05, **p<0.01, ***p<0.001 using Student's t-test (paired testing was used in B). In B and C mean and SEM are indicated for each group.*

5.7 The effect of immunosuppression on $CD8^+$ function

In order to assess whether $CD8^+CD57^+$ cells may be differentially affected by immunosuppression, a comparative cohort of non-transplant recipients without a history of continuous systemic immunosuppression and with a history of recent SCC were recruited (n=20). The non-RTR cohort as a whole was older than the RTR cohort (median [IQR] age: 80 [71 – 84] vs 63 (56 – 71]), reflecting the earlier onset of SCC in transplant recipients. 10 (50%) of the non-RTR cohort were CMV seropositive. One participant in the non-RTR group developed further SCC during non-RTR follow-up (median follow-up: 224 days [IQR: 189 – 260]), though 2 RTR had SCC excised on the day of sampling.

Firstly, a subset were matched by age, sex and CMV serostatus to individual RTR_{SCC} from the main cohort, to assess for differences in the proportion of CD57-expressing $CD8^+$ T cells (n=12 per arm). There was no difference in the proportion of circulating $CD8^+$ T cells expressing CD57 in CMV seropositive RTR, although there was a significant increase in CMV seronegative RTR. There was no difference in the number of circulating total $CD4^+$ and $CD8^+$ T cells between the groups and indicates that the increased aggression of SCC seen in CD57hi/CMV seropositive RTR is not simply due to an increased number or proportion of $CD8^+CD57^+$ cells.

Next, the function of $CD8^+$ and $CD4^+$ T cells were compared. Again, only RTR taking a calcineurin inhibitor at sampling were used. It was not possible to match RTR and non-RTR by age for this analysis.

There was no difference in cytotoxic function between RTR and non-RTR (Figure 5.9B). Both $CD4^+$ and $CD8^+$ T cells from RTR displayed a marked reduction in total IL-2 and TNF-α production compared to non-RTR (Figures 5.9 C-D). Production of these cytokines by $CD4^+$ and $CD8^+$ T cells in RTR was abrogated due to a reduction in the $CD57^-$ compartment, with preserved cytokine production by $CD57^+$ cells (Supplementary Data Figure i.4). IFN-γ production was preserved (Figure 5.9E).

Based on the correlation between CMV IgG titre and proportion of CD57-expression discussed in Chapter 3, it was hypothesised that RTR developing SCC have experienced significantly more subclinical CMV reactivation. To assess this, CMV IgG titres in the above matched RTR and non-RTR were compared: despite a comparable proportion of CD57-expressing $CD8^+$ T cells, CMV titres were significantly greater in RTR compared to non-RTR (Figure 5.9F).

These results suggest that the impairment in cytokine secretion with immunosuppression is limited predominantly to the $CD57^-$ compartment, with relative preservation of the $CD57^+$ compartment's ability to secrete cytokines. Lysosomal degranulation is not impaired with immunosuppression. The finding that the CMV IgG response was much greater in CD57hi RTR compared to CD57hi non-RTR suggests that CMV-specific responses may be overrepresented within the lymphocyte compartment in CD57hi RTR.

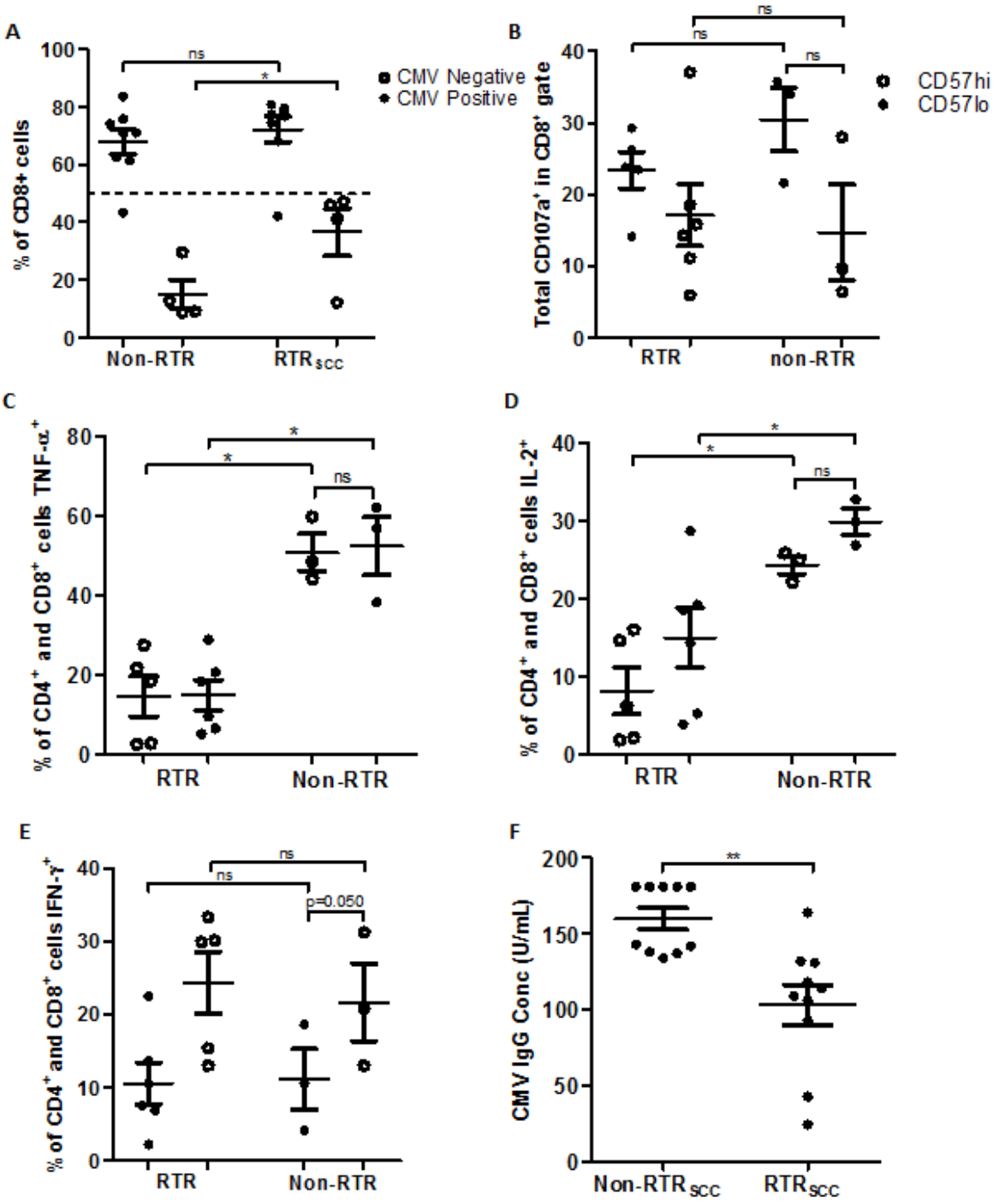


Figure 5.9: RTR do not display a greater proportion of CD57-expressing CD8$^+$ cells compared to matched non-RTR with a history of SCC, but demonstrate a greater humoral response to CMV. CD8$^+$ T cells from RTR display reduced production of IL-2 and TNF-α but not IFN-γ compared to non-RTR. *A) Proportion of CD57-expressing CD8$^+$ T cells in RTR and non-RTR, stratified by CMV serostatus. B) Comparison of percentage of CD107a$^+$ CD8$^+$ T cells and (C-E) total CD4$^+$ and CD8$^+$ cytokine production in RTR and non-RTR. F) Anti-CMV IgG titres in CMV seropositive RTR and non-RTR. Undiluted sera results are shown for CMV titres, where sera with a titre greater than 180IU/mL were given the nominal value of 181IU/mL. 'ns', not significant, *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA with Bonferonni correction (A-E) or Student's t-test (F). Results are for 11 RTR and 6 non-RTR. Mean and SEM are indicated for each group on each graph.*

5.8 CMV-specificity within the CD8+ compartment and SCC development

It has been suggested that the TCR repertoire is reduced with immunosenescence and increasing differentiation of the $CD8^+$ compartment [373, 648]. We therefore hypothesised that the association between CD57 phenotype and SCC development may relate specifically to changes in the TCR repertoire.

As no tumour antigen has been identified for SCC, it was not possible to assess for the frequency of SCC-specific $CD8^+$ T cells in peripheral blood directly. Instead, indirect evidence of clonal expansion was sought by evaluating the proportion of CMV-specific T cells within the $CD8^+$ T cell compartment [312, 649]. The HLA-A*0201-restricted peptide NLVPMVATV ($pp65_{495-503}$) from the CMV lower matrix protein pp65 was used, as this is a major target of the cellular response to CMV, forming an immunodominant epitope [650, 651]; it has been previously estimated that 70-90% of all CMV-reactive $CD8^+$ T cells may be specific for pp65 [650].

Pentamer binding T cells were strongly concentrated in the TEMRA subset across all participants, with a trend towards a greater proportion of $CD8^+CD57^+$ cells binding pentamer than $CD8^+CD57^-$ (Figure 5.10B and C). There was no difference in the total proportion of $CD8^+$ cells binding $pp65_{495-503}$, when stratified by CD57 phenotype (Figure 5.10D), though the proportion was highly variable amongst RTR. No correlation was seen between the proportion of CD57-expressing $CD8^+$ T cells; duration of immunosuppression; anti-CMV IgG titre; and the proportion of $pp65_{495-503}$ binding $CD8^+$ T cells (data not shown). There was a moderate positive correlation between age and the proportion of tetramer binding cells (r^2 0.25, p=0.046). The most striking finding was that CMV seropositive RTR who developed SCC during follow-up, irrespective of their CD57 status, had the greatest proportion of circulating $pp65_{495-503}$ specific $CD8^+$ T cells, with a mean proportion over twice that of those who did not develop SCC during follow-up, despite no overall difference in age at sampling (Figure 5.10C). There were insufficient CMV seropositive HLA-A*0201$^+$ non-RTR for meaningful analysis of pentamer binding (n=3), though there was no obvious trend in the three individuals analysed.

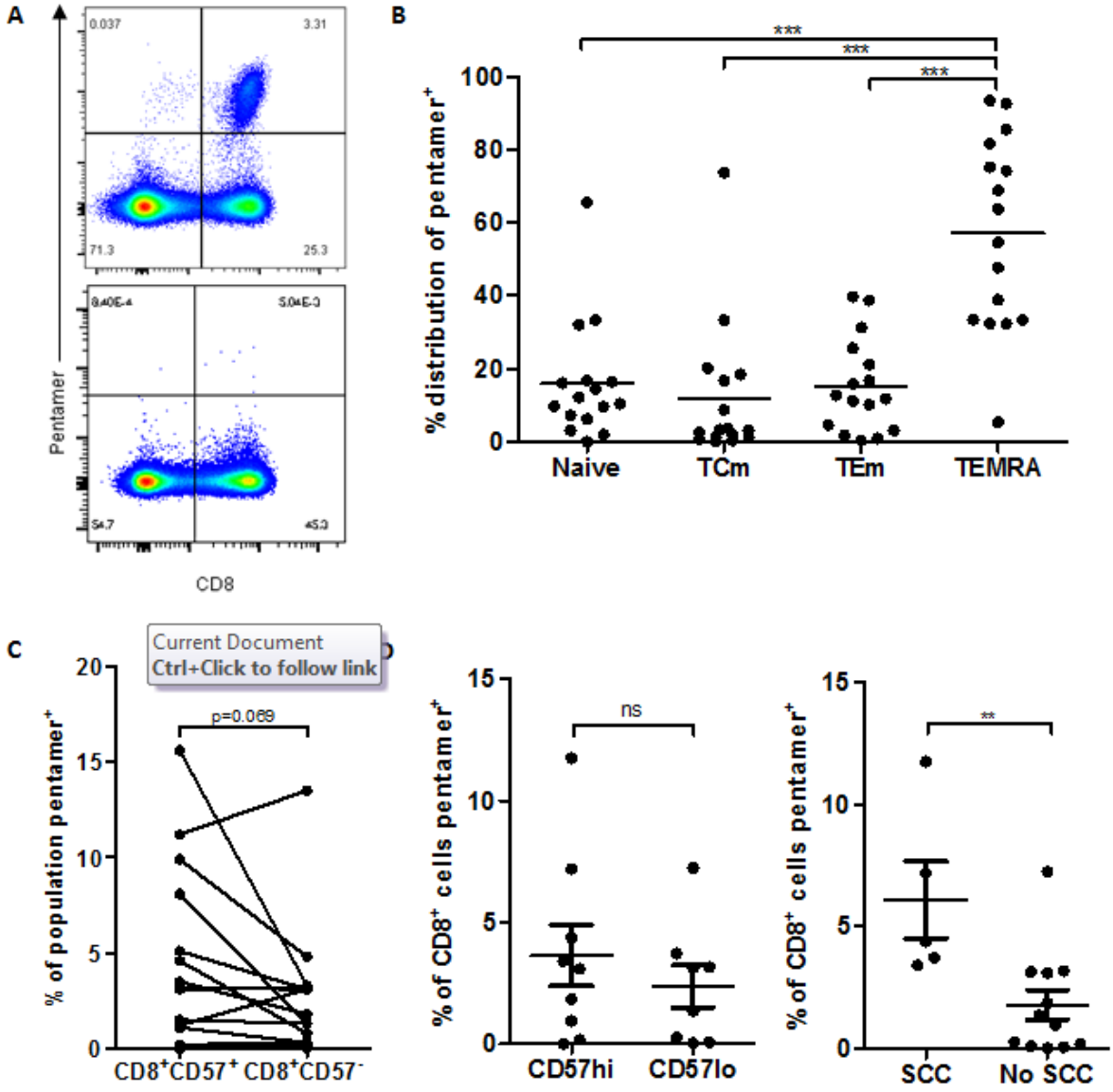


Figure 5.10: pp65-specific T cells are concentrated in the TEMRA compartment. Seropositive CD57hi RTR do not have a greater proportion of pp65-specific T cells, but RTR that develop SCC during follow-up have a significantly greater proportion of pp65-specific T cells. *A) Representative pentamer staining. Plots are gated on singlet, live CD19$^-$ lymphocytes. The plots indicate two RTR with a high (top) and low (bottom) proportion of pentamer-binding CD8$^+$ cells. B) Distribution of pentamer$^+$ cells within each CD8$^+$ population (n=17 CMV seropositive HLA-A*0201$^+$ RTR). C) Percentage of pp65$_{495\text{-}503}$ specific CD8$^+$ T cells within the CD57$^+$ and CD57$^-$ gates. D) Total proportion of pp65$_{495\text{-}503}$–specific CD8+ T cells stratified by CD57 phenotype and SCC development during follow-up. For this analysis CMV seropositive, HLA-A*0201$^+$ RTR were used. 'ns', not significant, **p<0.01 using Student's t-test (for B a paired test was used). Group mean and SEM are indicated on each graph.*

The results presented in this section indicate that there is an accumulation of CMV-specific CD8$^+$ clones with age. However, CMV-seropositive RTR who develop SCC during follow-up have the greatest number of pp65$_{495\text{-}503}$ specific CD8$^+$ T cells, despite no difference in age. This would be

compatible with the hypothesis that oligoclonal expansion of the TEMRA population due to CMV reactivation may lead to a skewed or reduced TCR repertoire.

5.9 Tumour infiltration

The characterisation of $CD57^+$ cells has been limited to peripheral blood thus far. We looked at tumour infiltration by $CD8^+$ and $FOXP3^+$ cells stratified by CD57 phenotype. We hypothesised that the CD57hi phenotype would be associated with reduced infiltration by $CD8^+$ cells. The reduced number of $FOXP3^+$ cells in peripheral blood may be due to the presence in peripheral tissue and so we hypothesised that the number of tumour infiltrating $FOXP3^+$ cells would be greater in CD57hi RTR. This would lead to a reduction in the tumour infiltrating CD8/FOXP3 ratio in CD57hi RTR. We hypothesised that this would be accompanied by an increase in the proportion of CD57-expressing cells.

Immunohistochemistry upon excised SCC from RTR who developed SCC during the study or preceding year was performed to compare the infiltration of SCC by $CD8^+$, $CD8^+CD57^+$ and $FOXP3^+$ T cells from CD57hi and CD57lo RTR. Cells were double stained for CD8 and CD57 or stained for FOXP3 to enable quantification of 'Treg' ($FOXP3^+$) and $CD8^+$ populations. SCC from 16 RTR and 11 non-RTR were analysed (Figure 5.11 and Table 5.1); CD57hi RTR were older than CD57lo RTR. There was no difference between tumour depth and tumour differentiation between RTR groups, though there was a trend towards poorer differentiation in CD57hi non-RTR.

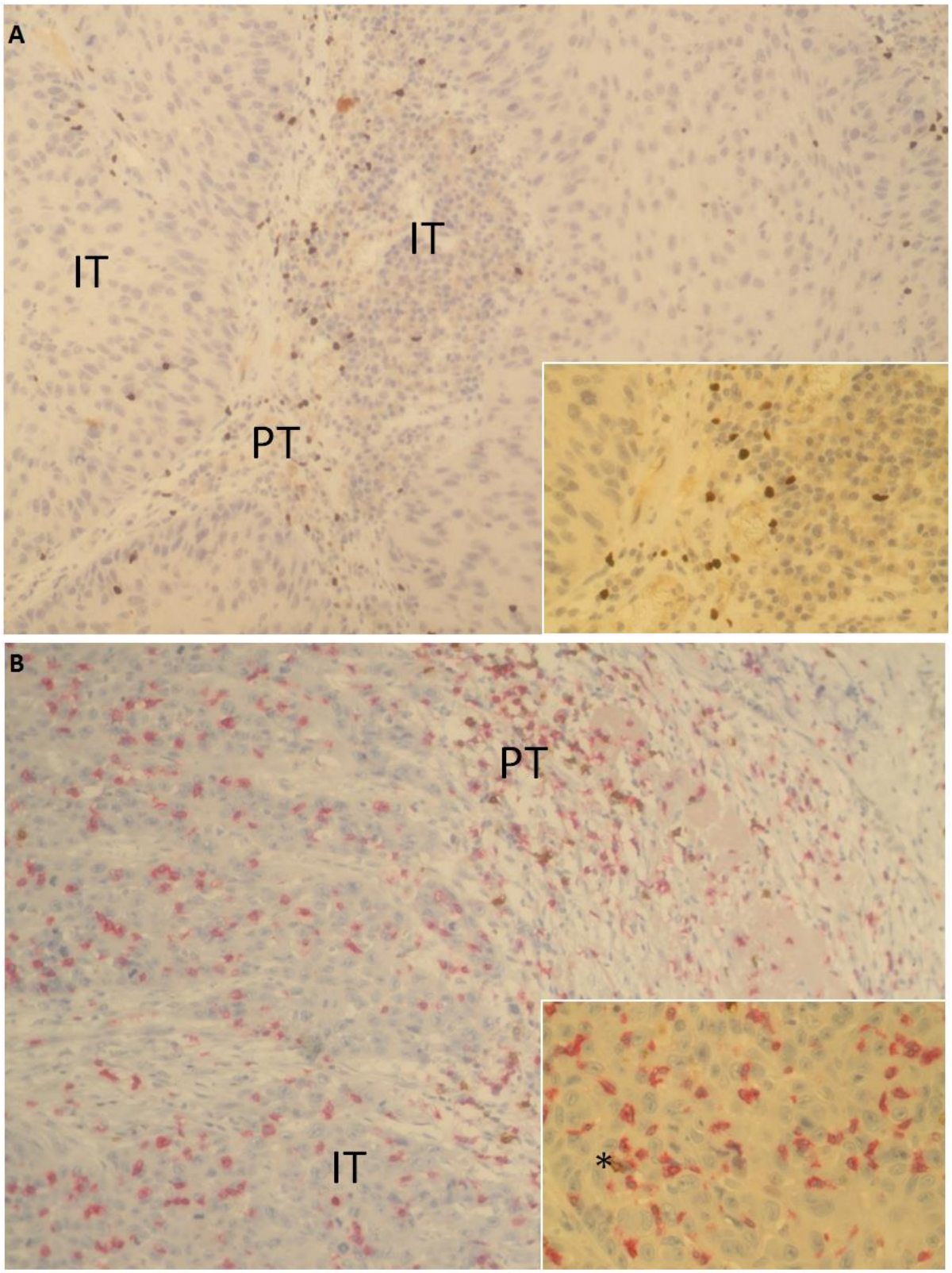


Figure 5.11: Representative immunohistochemistry of SCC staining. *A) FOXP3 staining B) CD8 (pink) and CD57 (brown) staining. Both are medium power magnification, with an example of a high power field inset. Slides are counterstained with haematoxylin. * indicates a $CD8^{+}CD57^{+}$ double-stained cell. 'PT' peritumour, 'IT' intratumour.*

Variable	RTR		Non-RTR	
	CD57hi (n=10)	CD57lo (n=6)	CD57hi (n=6)	CD57lo (n=5)
Age at resection	73 (65 – 76)**	57 (53 – 64)**	81 (73 – 84)	81 (67 – 84)
Max. depth (mm)	2.9 (1.5 – 5.4)	3.8 (2.3 – 5.0)	3.5 (2.1 – 4.8)	2.6 (1.7 – 5.9)
Differentiation:				
Well	5 (50)	2 (33)	1 (17)	5 (100)
Moderate or Poor	5 (50)	4 (67)	5 (83)	0
No. of $CD8^+$ cells:				
Total	75 (23 – 105)	139 (40 – 337)	285 (93 – 394)	364 (199 – 567)
Intratumoural	7 (3 – 9)	19 (1 – 40)	37 (20 – 111)	12 (6 – 213)
Peritumoural	68 (33 – 102)	120 (39 – 297)	236 (70 – 307)	245 (148 – 454)
$\%CD8^+$ that are $CD57^+$				
Total	6.5 (3.0 – 13.7)	1.1 (0.6 – 6.5)	2.6 (1.3 – 3.6)*	0.3 (0.1 – 1.2)*
Peritumoural	6.0 (3.2 – 11.9)	1.0 (0.0 – 6.7)	2.6 (1.8 – 4.1)*	0.4 (0.2 – 1.2)*
No. of $FOXP3^+$ cells				
Total	119 (90 – 178)	230 (143 – 370)	262 (147 – 304)	248 (160 – 334)
Intratumoural	22 (4 – 38)	19 (16 – 26)	42 (28 – 85)	24 (17 – 89)
Peritumoural	105 (75 – 145)	210 (110 – 355)	215 (119 – 228)	190 (99 – 307)
CD8/FOXP3 ratio				
Total	0.52 (0.42 – 0.97)	0.64 (0.12 – 1.58)	1.08 (0.64 – 1.28)	1.66 (1.00 – 1.87)
Intratumoural	0.46 (0.22 – 0.86)	0.75 (0.04 – 2.33)	1.25 (0.67 – 2.00)	0.44 (0.24 – 3.81)
Peritumoural	0.54 (0.46 – 1.13)	0.60 (0.15 – 1.66)	1.07 (0.58 – 1.39)	1.29 (1.07 – 1.85)

Table 5.2: immune infiltration into SCC from CD57hi and CD57lo RTR and non RTR. *Continuous variables are reported median (IQR), whilst categorical variables are reported number (percentage of group). Absolute numbers are reported per mm^2. There were insufficient $CD8^+CD57^+$ cells within many tumours to calculate a percentage of $CD8^+$ cells, and so 'intratumoural' $\%CD57^+$ is not reported. *p<0.05, **p<0.01 using Mann-Witney test.*

There were no significant differences between CD57hi and CD57lo RTR in the parameters examined due to the marked variation in infiltration by cell populations between samples. Of note there was a trend towards an increase in the proportion of total and peritumoural $CD8^+CD57^+$ cells in CD57hi cohorts compared to CD57lo. In keeping with previous studies [66-68], RTR demonstrated a lower CD8/FOXP3 ratio compared to non-RTR. The total infiltration of both $CD8^+$ cells and $FOXP3^+$ cells was markedly lower in CD57hi cohorts, suggesting an overall reduction in inflammatory infiltrate.

5.10 Summary of key findings

- CD57hi phenotype was associated with changes previously described in the context of immunosenescence, particular in those who were CMV seropositive;
- Co-expression of Tim-3 and PD-1 is rare in $CD8^+$ T cells in the peripheral blood of long-term RTR and does not associate with CD57hi phenotype;
- Circulating $CD8^+$ T cells from CD57hi RTR do not demonstrate an impairment in cytotoxicity, as assessed by granzyme B expression and turnover of cell surface CD107a, upon polyclonal stimulation;
- Overall production of the effector cytokines IL-2, TNFα and IFNγ is not impaired in CD57hi compared to CD57lo RTR, though $CD57^+$ T cells do not produce IL-2;
- CD57hi phenotype is not associated with increased Treg number or *FOXP3* expression;
- Immunosuppression does not impact significantly upon $CD4^+CD57^+$ and $CD8^+CD57^+$ T cell function in contrast to $CD57^-$ populations;
- The humoral IgG response to CMV is significantly greater in RTR compared to matched non-RTR;
- CMV seropositive CD57hi RTR do not demonstrate an overrepresentation of $pp65_{495\text{-}504}$ specific $CD8^+$ T cells compared to CD57lo RTR, but RTR developing SCC during follow-up demonstrate the highest proportion of pp65-specific $CD8^+$ T cells;
- $CD8^+CD57^+$ cells are found within and around SCC. SCC from CD57hi RTR and non-RTR trend towards a reduction in the total CD8/FOXP3 ratio, compared to CD57lo participants.

5.11 Discussion

In this chapter, the association between CD57hi phenotype and the more aggressive SCC course seen in Chapter 3 was explored further. The key finding is that the CD57hi phenotype and associated immunosenescence is a distinct phenomenon without features of T cell exhaustion in peripheral blood.

5.11.1 Immunosenescence and the CD57hi phenotype

Defining immunosenescence

The results presented in this chapter demonstrate that the CD57hi phenotype is associated with other phenotypic changes previously described in ageing associated with an increased mortality risk, including reduction of the CD4/CD8 ratio and an increase in the proportion of terminally differentiated $CD8^+$ cells with a reduction in the proportion of naïve and central memory T cell.

Defining pathological ageing objectively has proved a challenge. The body undergoes a number of alterations with age of which changes in the immune system are only one aspect [652]. However, this process of immune remodelling appears to be more marked in some individuals. The process of 'immune ageing' that is associated with increased mortality defines immunosenescence and identifies it as a clinically relevant phenomenon [311, 317, 377, 653, 654].

The CD57hi phenotype as a biomarker for immunosenescence

The IRP threshold for immunosenescence, namely a CD4/CD8 ratio of <1, is a pragmatic one which divides ageing cohorts into those with poorer and better outcomes. In this respect, the 50% cut-off for proportion of CD57-expressing $CD8^+$ T cells is also a pragmatic cut-off for immunosenescence. It forms a natural division as this corresponds to the nadir and the mean across the population. It is possible that the association between SCC and CD57 expression is a continuum rather than a dichotomy; larger studies are needed to confirm whether more levels of stratification are appropriate.

The CD57hi phenotype is associated with other changes described previously in immunosenescence; namely the expansion of the $CD8^+$ compartment, leading to reduction of the CD4/CD8 ratio. The CD57hi phenotype was associated strongly, but not exclusively, with CMV

seropositivity whereas in this cohort all RTR possessing the IRP (n=20) were seropositive. Conversely not all CMV seropositive RTR are CD57hi and other factors are likely to play a role in the development of immunosenescence. Evidence for this was seen in Section 5.7, where CMV seronegative RTR had a greater proportion of CD57-expressing $CD8^+$ T cells than CMV seronegative non-RTR. End-stage renal failure has been associated with immune phenotype changes seen in immunosenescence and this may be an important contributing factor [655-657]. A numbers of SNP have been identified that appears to be associated with the accumulation of $CD4^+CD28^-$ cells in CMV seropositive individuals. Notably many were located close to the genes encoding for MHC Class II alleles [340]. This builds on earlier work suggesting that the accumulation of $CD8^+CD28^-$ cells may be influenced by SNP in the genomic loci in the HLA-DRA and HLA-DO regions on chromosome 6 and suggests that the development of immunosenescence is partially influenced by genetically-mediated alterations in antigen presentation [658]. Thus both genetic predisposition and uraemia may be two factors that play a role in the development of immunosenescence alongside CMV infection.

Comparison of the CD57hi phenotype and the IRP

There was not a perfect concordance between a CD4/CD8 ratio <1 and the CD57hi phenotype; indeed this cut-off equated to a proportion of $CD57^+$ $CD8^+$ T cells of about 80%. Notably a CD4/CD8 ratio of <1 was not predictive of earlier SCC development nor was it predictive of mortality in this study, being present in only 2 of 6 who died during follow-up. In contrast, the CD57hi phenotype was present in all but one RTR who died during follow-up. The small number of deaths during the follow-up period precludes more in depth analysis.

This lack of predictive value of the CD4/CD8 ratio might be explained by the younger age of the cohort in this study. However, an extension of the original studies looked at the IRP in a younger cohort and found that it began to provide prognostic information from about 60 onwards, although a subset of younger participants also displayed this phenotype [377]. Other studies have demonstrated impaired immune responses become detectable in the mid-sixties [654, 659]. Therefore this is unlikely to be the explanation. The CD57 marker presented here may instead

represent an 'earlier' marker of immunosenescence or may be more suited to an immunosuppressed cohort. Notably, previous studies of the IRP have excluded participants taking medication that may affect the immune system [309-311, 337, 377, 660]. A previous study in liver transplantation found a similar cut-off of less than 40% $CD8^+CD28^+$ cells was predictive of increased risk of malignancy in the first decade post-transplant [226]. This was not further explored in the context of immunosenescence but may indicate this is a prognostic tool that could be useful in other transplantation settings. Previous studies have suggested a decreased risk of acute rejection in RTR displaying features of immunosenescence [347, 348, 661]. On the other hand, a recent study noted that there was an increased rate of chronic graft dysfunction in immunosenescent RTR, though this did not reach significance and the cause of graft failure was not explored in depth [373].

The common feature in all these studies, taken with the results presented in this study, is that the altered outcome can also be explained by an increased physiological effect of pharmacological immunosuppression. This may provide indirect evidence that immunosenescence in the context of organ transplantation synergises with pharmacological immunosuppression.

T cell differentiation and CD57hi phenotype

The CD57hi phenotype was associated with the loss of circulating naïve and TCm cells with an accumulation of $CD8^+$ TEMRA cells. In addition, CD57hi RTR demonstrated an expansion of $CD57^+CD28^-CD27^-KLRG\text{-}1^+$ cells. This means that through two different methods of quantifying $CD8^+$ T cell differentiation it was confirmed that the CD57hi phenotype is associated with a more differentiated T cell compartment. In the setting of melanoma, tumour infiltrating $CD57^+$ cells have been found to undergo incomplete differentiation, with failure to downregulate CD27 and CD28, and a lack of cytotoxic activity [662]. There was no evidence for this phenotype in circulating $CD8^+CD57^+$ cells in RTR in our study.

In health, naïve $CD8^+CCR7^+CD62L^+CD45RA^+$ T cells are activated in response to TCR ligation and appropriate co-stimulation [663]. There is rapid clonal expansion into an effector population that produces cytokines such as TNF-α and IFN-γ, along with cell-mediated cytotoxic activities through

secretion of mediators such as perforin and granzyme and ligation of death receptors. This is followed by apoptosis of the majority with resolution of the original immunological insult. A subset will form memory cells, subsets of which can be distinguished by phenotypic and functional markers [664]. TEm circulate through nonlymphoid tissue and demonstrate rapid cytolytic action upon activation, but are generally poorly proliferative, in contrast to TCm that circulate through lymph nodes and demonstrate greater cytokine secretion with a capacity to self-renew [665-669]. TCm, as opposed to TEm, have been shown to provide superior protective immunity against bacteria and viruses in some models [667, 670], but not others [671, 672]: findings that may depend on the site and pathogen in question. A recent study in a cohort of deceased organ donors across a wide range of ages has suggested that the frequency of circulating $CD4^+$ and $CD8^+$ central memory cells is representative of the frequency at other sites, including the spleen, most of the GI tract, and inguinal, lung and mesenteric lymph nodes [673]. The variability for naïve and TEMRA cells was much greater but correlated with the spleen but not lymph nodes for $CD8^+$ TEMRA cells, with the converse true for $CD4^+$ and particularly $CD8^+$ naïve cells. In contrast, looking at CMV seropositive individuals only, another study found that the loss of TCm in peripheral blood was not mirrored in lymph nodes [669].

What might a loss of central memory $CD8^+$ T cells mean for both alloimmunity and antitumour immunity? $CD8^+$ central memory cells generated in response to pre-transplant viral infections may be the chief mediators in skin allograft rejection [674]. Transfer of tumour-reactive naïve or central memory $CD8^+$ T cells leads to more efficient B16 melanoma rejection than transfer of effector or late effector memory T cells, suggesting they may have a key role in propagating tumour clearance *in vivo* [224, 675]. This is likely due to their ability to self-renew thereby maintaining the immune response against larger or metastatic tumours. However, a subset of central memory $CD8^+$ T cells have also been implicated in allograft tolerance in murine models of lung and islet transplantation [676, 677]. Increasing differentiation of the $CD8^+$ T cell compartment and greater proportion of $CD28^-$ cells has been associated with a decreased risk of renal allograft rejection [347, 348]. In liver transplantation these parameters were associated with increased risk of rejection following

immunosuppression withdrawal [678], although this was after lymphocyte depletion at induction, which means the repopulating $CD8^+$ T cells may have been relatively enriched for allospecific effector cells.

Taken together, the results in this chapter and Chapter 3 indicate that the 'CD57hi' phenotype is a marker of increased immunosenescence, which appears to portend a poorer prognosis with regards to the development of SCC and may indicate increased mortality risk also. The threshold for this is distinct to the IRP that has been developed and validated in the elderly and in HIV, and appears to have superior predictive value. The effect of increasing differentiation in the $CD8^+$ T cell compartment may be clinically beneficial for allograft responses but detrimental for tumour immunity. This may synergise with immunosuppression and manifest in SCC development.

5.11.2 The role of CMV

An important, but unanswered, question that remains is why CMV should be directly or indirectly associated with cutaneous malignancy and immunosenescence.

CMV and immunosenescence

Within one year of primary CMV infection stable expansions of CMV specific $CD4^+$ and $CD8^+$ cells can be detected in peripheral blood. It is estimated that up to 10% of the circulating T cell population may be specific for CMV and this may be even higher in the elderly [679-682]. This accumulation is of predominantly effector memory T cells with specificity for a limited number of viral epitopes [617, 683]. This phenomenon is termed 'memory inflation' and much of our understanding of this comes from work in murine CMV (mCMV) [684]. It is hypothesised that lifelong recurrent antigenic challenge leads to the changes seen in immunosenescence: CMV may significantly contribute to this with expansion particularly of the $CD8^+$ memory compartment required to maintain CMV latency [685, 686].

The expansion of CMV-specific T cells has been hypothesised to compromise immunity to other pathogens. In CMV seronegative individuals the T cell response to EBV is more marked than that seen in those who are CMV seropositive [687]. CMV seropositive individuals may demonstrate a

reduced response to influenza vaccination [688]. On the other hand, there are some animal data to suggest that latent beta- and gamma-herpesvirus infection may have a cross-protective effect upon infection by other pathogens, through promotion of innate responses [689, 690]. A recent study has indicated a similar phenomenon may occur in humans [340]. Increased levels of IFN-γ and IL-13, as well as an enhanced $CD8^+$ response to IL-6, were found in young CMV seropositive participants, with a better response to annual influenza vaccination over a three year period compared to seronegative participants. This effect was lost with age and was not seen with EBV seropositivity. The authors suggested that CMV may promote strong immune responses in earlier life, but in later life this net benefit is lost and the immune system becomes impaired [340]. Thus the early benefit becomes outweighed by the net reduction in adaptive immune function [691].

The incomplete overlap between CMV and immunosenescence combined with the variable effect of CMV infection with age of the host may explain why some studies have demonstrated an association between CMV seropositivity and increased risk of malignancy post-transplant [327] and others have not [249, 437]. The CD57hi phenotype may thus provide a method of identifying those RTR where CMV infection has become pathogenic rather than beneficial. In 'normal' un-immunosuppressed populations this may occur in later life, but in the immunosuppressed this transition may occur earlier and to a greater extent, due to increased viral load and immunosuppression (Figure 5.12).

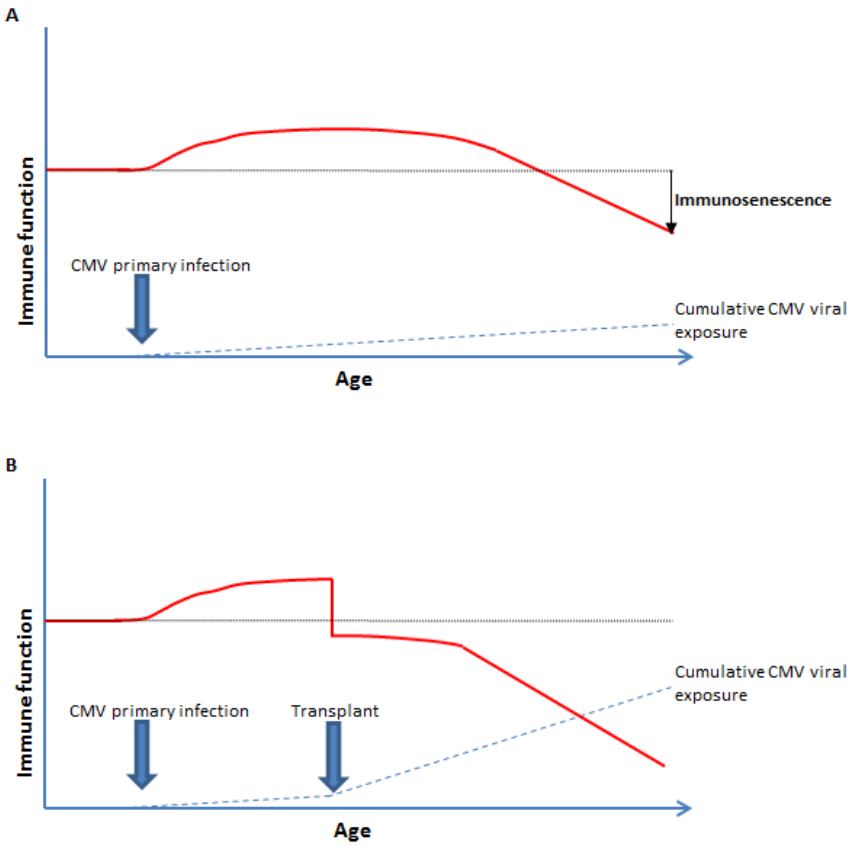


Figure 5.12: CMV may initially be protective and encourage immune responses, but later is associated with impaired immune response: a model for altered immunosenescence in transplantation. *A) The process in a normal, non-immunosuppressed person. Primary CMV infection is associated with an increase in the immune response, which in later life declines below normal (immunosenescence). B) The process in a person receiving a transplant. The improved immune response is blunted by immunosuppression, and increased viral reactivation leads to accelerated decline in the immune function, with a synergistic effect from immunosenescence and immunosuppression.*

CMV reactivation

Memory inflation is thought to occur through vigorous $CD8^+$ responses to repeated viral reactivation. There is evidence that subclinical CMV reactivation may occur both in the elderly and in transplant recipients [384, 435].

Many complications associated with ageing, such as dementia, atherosclerosis and type 2 diabetes have long been known to occur in the milieu of a chronic low-grade inflammatory state, including increased levels of IL-1β, IL-6, C-reactive protein (CRP) and TNF-α [692-694]. This is perhaps better described by the term used by Franceschi to describe the pro-inflammatory changes seen in the innate immune system with immunosenescence: so-called 'inflamm-ageing' [305, 695].

Inflammation, particular involving TNF-α, may induce CMV reactivation [696-701]. The critical step for this is activation of NF-κB, which leads to immediate-early (IE) gene expression due to the presence of consensus sequences within the IE promoter region [698, 699, 702]. Thus the chronic inflammatory state seen in immunosenescence may cyclically drive CMV reactivation. The role of TNF-α may be twofold as it may also play a role in promoting differentiation and induction of downregulation of CD28 upon $CD8^+$ T cells [619].

Corticosteroids inhibit NF-κB signalling and may explain the association between steroids and the reduction in CD57 expression within the $CD8^+$ T cell compartment [133]. However, calcineurin inhibitors affect NF-κB signalling also, without an effect upon the CD57 phenotype [703]. This suggests the aetiology may be more complex and may reflect a balance between NF-κB inhibition and inhibition of specific anti-viral immune responses. For example, in mouse models, depletion of NK cells in combination with $CD8^+$ T cells reveals a key role in maintaining latency and preventing mCMV reactivation, compared to depletion of $CD8^+$ T cells alone [704]. Depletion of NK cells by azathioprine may thus have an impact upon CMV reactivation.

The markedly increased humoral response to CMV in otherwise matched RTR compared to non-RTR suggests that a greater CMV antigenic stimulus is occurring with immunosuppression. However, the evidence for humoral immunity being a marker of increased CMV exposure in humans is indirect. Humoral immunity to CMV has been previously found to correlate with increasing phenotypic changes seen in immunosenescence, as was reported here [705-707]. Phenotypic changes are more marked in transplant recipients who have demonstrated post-transplant viral replication [327]. In mice, the dose of the original mCMV inoculum correlates positively with the magnitude of subsequent memory inflation [708]. Finally, increasing humoral immunity to CMV correlates inversely with the TCR repertoire [709]. Thus an increased antigenic load can be linked to more advanced features of immunosenescence which correlates with increased humoral immunity and decreasing TCR diversity. Suessmuth *et al.* recently looked at the TCR repertoire of haematopoietic stem cell transplant recipients who developed post-transplant CMV reactivation with expansions of

$CD8^+$ T cells in the reconstituting T cell populations and found that this was associated with 'holes' in the TCR repertoire as compared to a reference group of healthy controls [326].

Applied to the results in this study, this means that the proportion of $CD57^+$ cells may not be increased with immunosenescence in populations under immunosuppression compared to those without, but the extent of TCR repertoire restriction may be greater.

In the canonical stepwise model of tumour development and immunoediting, the development of a tumour passes through distinct stages, akin to evolutionary pressure on a population. Initially 'tumour elimination' occurs, where the majority of transformed cells are eliminated by an effective $CD8^+$ response. Later 'tumour equilibrium' develops where there is a turnover of transformed cells to replace those that are eliminated, eventually leading to selection of surviving cells which are able to inhibit or evade the immune response. This is referred to as 'sculpting'. Finally, there is 'tumour escape', where transformed cells are able to escape from immune control and accumulate to form a tumour with an immunosuppressive environment [710]. A significant reduction in TCR repertoire would be expected to expedite the second and third step of this model, where mutation and selection of tumour cells leads to earlier expansion of populations for which there is minimal repertoire coverage.

Detection of CMV-specific T cell expansions

The increased humoral response did not correlate to an increased proportion of CMV-specific $CD8^+$ T cell clones. The lack of correlation between the IRP, CMV IgG and expansions of pp65-specific clones has been reported previously [312, 709, 711]. Though the $pp65_{495\text{-}503}$ peptide was chosen for its immunodominant properties [650, 651], there is evidence that other peptides, particularly IE-1, may become immunodominant at later time points. With increasing duration of CMV latency there is an expansion of IE-1-specific T cell clones, whereas pp65-specific lines may remain stable [712]. IE-1 is expressed early in the viral replication cycle and can be expressed during viral persistence, whilst pp65 is a late protein which is expressed only with replication. This means with time more antigen exposure to IE-1 than pp65 may occur, leading to expansion of IE-1-specific clones.

Advanced immunosenescence may be paradoxically associated with a reduction in the number of clonal expansions. This is thought to be due to selective expansion of a small number of clones, associated with contraction of the remaining clonal populations [312]. Pentamers may therefore not provide optimal resolution to assess clonal expansions. Future assessments of TCR repertoire restriction in this cohort may be better performed using spectratyping or cloning and sequencing [713, 714].

Despite the above limitations pp65-specific T cells were expanded with age, in accordance with previous findings [615, 648, 709]. These were most expanded in RTR who developed SCC during follow-up. This may have three interpretations: firstly, even with the poor resolution discussed above oligoclonality is most profound in those who go on to develop SCC. Alternatively this may indicate a clonal expansion representing an ongoing immune response to active CMV replication in peripheral tissues. Finally, it has been suggested that the proportion of pp65-specific $CD8^+$ T cells, but not CMV IgG titre, may reflect the presence of CMV DNA within circulating monocytes [711, 715], in which case this expansion in those who develop SCC may suggest the greatest latent CMV burden, with poorest viral control.

CD57hi phenotype appears to be associated with an increased burden of CMV reactivation, with suggestion that those with the greatest burden of CMV are most at risk of developing SCC during follow-up. This may provide indirect evidence of functional over-immunosuppression with reactivation of CMV and consequent effects upon SCC risk, or may suggest increased CMV-driven immunosenescent changes and clonal expansion within the T cell compartment.

CMV and mutagenesis

A final possibility to explain the association between CMV and SCC development is that CMV may be directly carcinogenic.

Low levels of CMV gene expression have been consistently identified in a subset of a variety of malignancies, including colonic cancer [716], cervical cancer [717], and glioma [718]. CMV gene expression has also been detected in a subset of cutaneous SCC in non-transplant cohorts [719,

720]. Interpretation of these data are difficult given the myeloid CMV reservoir and evidence for reactivation within inflammatory environments. This may mean CMV reactivation is an epiphenomenon of neoplasia-associated inflammation [721]. However, some studies provide more convincing evidence that the protein expression arises from transformed cells rather than infiltrating leucocytes [716, 718].

In vitro, CMV proteins have been demonstrated to have transformative properties. For example, mtrII is able to bind to the tumour suppressor protein p53 and inhibit its activity *in vitro* in human cell lines [722]. Products of the immediate-early genes (IE-1 and IE-2) are potentially responsible for a number of potential causes of transformation, including upregulation of the oncogenes *c-myc*, *c-fos* and *c-jun* and disruption of various cell-cycle checkpoints [723-725]. On the other hand there is also data to suggest that CMV may inhibit cellular proliferation and cell cycle progression in human fibroblasts [726, 727]. Interaction of CMV with other viruses may change its behaviour: IE-1 and IE-2 are able to cooperate with adenovirus protein E1A to mediate malignant transformation in baby rat kidney cells in a 'hit and run' manner, where transient expression is sufficient to induce subsequent oncogenesis [728]. Similar cooperation is seen in human fibroblasts, where TNF-α mediated apoptosis is inhibited [729].

CMV can infect human keratinocytes [730], though whether CMV is able to maintain latency in keratinocytes is unknown. The potential interaction with other viruses is particularly interesting given the postulated role of HPV infection in SCC development. A potential interaction between other strains of HPV and CMV has been previously suggested, where a Taiwanese study found primary cervical tumours positive for both HPV-16 and CMV DNA were associated with increased incidence of lymph node metastasis, compared to those positive for HPV only [717].

In summary, demonstration of a direct mutagenic effect in keratinocytes by CMV is lacking, but indirect evidence suggests it is plausible. Given the findings of this project, this is an area deserving of further study.

5.11.3 Exhaustion and cellular senescence

Evidence for forms of $CD8^+$ cellular dysfunction which have been described extensively in the setting of malignancy and chronic viral infection were sought in PBMC from RTR; these were exhaustion and cellular (replicative) senescence [731]. Firstly, these will be briefly reviewed.

T cell exhaustion

Exhaustion of $CD8^+$ T cells was first described in mouse models of chronic viral carriage. This is characterised by accumulation of dysfunctional virus-specific $CD8^+$ T cells that demonstrated markers of activation but exhibit poor proliferative function and poor cytokine secreting ability, with high levels of inhibitory receptors such as lymphocyte activation gene protein 3 (LAG-3), PD-1, cytotoxic lymphocyte antigen-4 (CTLA-4) and Tim-3 [631, 632, 634, 640, 641, 732, 733]. This occurs predominantly in response to chronic, high levels of antigenic load though other mechanisms, such as inhibitory signalling, contribute [626, 627, 734]. There is a progressive loss of function, with progressive loss of IL-2 then TNF-α production, with loss of IFN-γ and granzyme B secretion in advanced exhaustion [647]. Similar phenotypic and functional disturbances were later described in cancer [629-631, 633-635, 640, 642, 643, 735]. Exhausted cells demonstrate dysregulation of T-bet and Eomesodermin (Eomes), two members of the t-box transcription family required for cytolytic function and memory formation [736-739]. The upregulation of inhibitory receptors such as PD-1 and CTLA-4 has led to attempts to manipulate these pathways for clinical benefit [740, 741].

Cellular senescence

Cellular senescence is an immunological description distinct from that of immunosenescence, which refers to the changes seen in the immune system as a whole with ageing. However, there is considerable overlap between the two, as cellular senescence is most commonly found in $CD28^-$ $CD27^-CD57^+$ cells which are expanded in immunosenescence [707].

Cellular senescence is a state encountered in T cells which have undergone multiple rounds of antigen-driven proliferation. With advancing differentiation, CD27 then CD28 are downregulated. Downregulation of CD28 coincides with upregulation of CD57 [707, 742]. KLRG-1 is increasingly expressed during this process [617, 638, 743]. Senescent T cells remain metabolically active.

CD57, also known as HNK-1, is a terminally sulphated carbohydrate epitope found on a variety of glycoproteins within the central nervous system and also upon lymphocytes: predominantly NK cells and $CD8^+$ T cells [744]. Its exact role in immunity is unknown, though it is predominantly expressed upon lymphocytes at an advanced stage of differentiation [227]. The CD57 epitope has been found upon cell adhesion proteins such as neural cell adhesion molecule (NCAM) in the nervous system and laminin-1, and is a ligand for L- and P-selectin [745-747]. These indicate it may have a structural or lymphocyte trafficking role. However, it has also been identified as a ligand for IL-6 and is present within the IL-6 receptor on resting lymphocytes, where it may be required for the early stages of IL-6-mediated signalling [748]. It is not clear why it is upregulated during advanced differentiation and cellular senescence.

$CD27^-CD28^-CD57^+$ cells are predominantly $CCR7^-CD45RA^+$, although this pattern may invert upon antigenic stimulation [749, 750]. These cells display the shortest telomeres amongst T cells [639], are prone to activation-induced cell death [227], and may not proliferate [227], though this last feature has been disputed [438]. These cells demonstrate high levels of cytolytic activity upon stimulation, and in some settings, notably CMV, express increased levels of both T-bet and Eomes, unlike exhausted cells [373, 738, 739, 751, 752].

Overlap between immunosenescence and cellular mechanisms of $CD8^+$ dysfunction

No difference in PD-1 or Tim-3 expression by $CD8^+$ T cells was observed when stratified by CD57 phenotype. This is the first study to look at the expression of both molecules together in the peripheral blood of long-term RTR and this was only seen in a small minority (<3%) of $CD8^+$ T cells.

The relative infrequency of $CD8^+PD\text{-}1^+Tim\text{-}3^+$ cells is in keeping with other studies in non-transplant cohorts [630, 646, 735, 753]. One possibility is that circulating populations do not reflect dysfunctional changes within peripheral sites. Evidence from Hepatitis C infection and in atherosclerosis would suggest that is not true [630, 646]. In colorectal cancer, the proportion of circulating double-positive dysfunctional $CD8^+$ T cells were increased in patients with cancer compared to healthy controls [754]. This was reflected within the tumour, with a significantly

greater proportion of tumour-infiltrating double-positive $CD8^+$ T cells compared to matched blood. Notably, in healthy controls there was no difference in the proportion of double-positive cells between non-cancerous colon and blood.

We confirmed our phenotypic findings in functional analyses. These demonstrated no impairment in polyclonal $CD8^+$ cytotoxic function or $CD8^+/CD4^+$ cytokine secretion in CD57hi RTR compared to CD57lo. There was no loss of polyfunctionality for secretion of IFN-γ and TNF-α in $CD57^+$ cells or in CD57hi RTR. These results suggest these are *bona fide*, functional effector $CD8^+$ T cells. This is in keeping with comparative studies of cellular senescence and exhaustion in non-transplant cohorts, where senescent effector populations arising from CMV infection are phenotypically and functionally distinct from exhausted populations arising through HIV or EBV [628, 630, 735, 755].

Recent data has emerged to suggest that cellular senescence may be induced within tumour infiltrating lymphocytes by the tumour, Treg, or by gamma-delta T cells with regulatory function [756-758]. This may contribute to cellular senescence locally in SCC, but is unlikely to be a major factor in peripheral blood given that some RTR with no history of malignancy and who did not develop malignancy during the study also had expansions of these cells at a similar level to those who did.

There are other features of cellular senescence that were not assessed in this study. Whilst it is unlikely T cells can divide indefinitely (the so-called 'Hayflick limit' [759]), the use of the above markers to identify populations with an inability to proliferate has been increasingly disputed. There is accumulating evidence that 'terminally differentiated' $CD8^+$ T cells are able to proliferate in response to anti-CD3 antibody stimulation [227, 321, 347, 438]. Chong *et al.* confirmed proliferation of $CD8^+CD57^+$ cells in a number of ways including using proliferation dye and radio-isotope uptake. They suggested that variations in methodology may explain previous negative findings, providing evidence that some proliferation dyes and culture media conditions were able to inhibit proliferation [438]. Another key factor is also likely to be subset selection, with $CD57^+CD27^+$ cells or $CD57^+CD28^+$, a rare subset, retaining proliferative capacity, whereas $CD57^+CD27^-$ and

$CD57^+CD28^-$ do not [227, 749]. Given this, it would be presumed that proliferation is most impaired in CD57hi RTR, where a greater proportion of $CD57^+$ cells are $CD27^-$ and $CD28^-$. Telomere length was not assessed: studies in humans using adoptively transferred $CD8^+$ T cells as immunotherapy have demonstrated that the longevity of the anti-tumour response is greatest for those clones with the longest telomere length [760, 761].

These results indicate that in RTR there is minimal overlap between immunosenescence and exhaustion in circulating $CD8^+$ cells; rather the features seen are in keeping with those of cellular senescence. An impaired $CD8^+$ response due to cellular senescence may not only be relevant to anti-tumour responses, but may be relevant to clearance of other viruses; in particular HPV [762]. The link between HPV and SCC is controversial, but there is increasing evidence that the viral load may be more important than the specific genotype [168, 171]. Studies of SCC and precursors have illustrated that the highest load of HPV occurs prior to the development of SCC, during the pre-malignant actinic keratosis phase [170]. In the presence of impaired $CD8^+$ responses, particularly if NK cells are also impaired through azathioprine use, then it is likely that viral load will increase. This may contribute towards the increased propensity to SCC with CD57hi phenotype.

5.11.4 Regulatory T cells and immunosenescence

In contrast to previous studies [66, 109], this project demonstrated minimal prognostic value of circulating Treg number or proportion for SCC development. In Chapter 3, this was partly attributed to differences in the immunosuppressive regime and the primary outcome between previous studies and this one.

In this chapter, a second possible explanation for this was provided: CD57hi RTR have fewer circulating Treg. CMV seropositive RTR demonstrate a negative correlation between CMV humoral response and the number and proportion of Treg. This was confirmed by a second method, with lower expression of *FOXP3* within whole blood in CD57hi RTR. The reduced number of Treg remained significant even with adjustment for confounders such as calcineurin inhibitor use and age. Studies in the general population have suggested minimal change in number of Treg with

ageing. There are conflicting results regarding changes in the frequency of Treg with age: results have varied depending on the strategy to identify Treg [194, 763-766]. No study has stratified by CMV status. A study in the general population found a reduced number of Treg in CMV seropositive participants early after myocardial infarction, though the cohorts were not matched for age [767].

In haematopoietic stem cell transplant recipients, CMV seropositivity is associated with a decrease in reconstituting circulating Treg [768]; thus this may represent another manifestation of the postulated increased CMV reactivation seen in CD57hi RTR. This may parallel the same phenomenon seen with TCm: despite a reduction in circulating TCm with CMV seropositivity, TCm-like cells are still found in lymph nodes [669]. Whilst Treg were defined as $CD127^{lo}FOXP3^{+}$ in this study to distinguish them from effector T cell populations, recent studies have suggested that FOXP3 may be downregulated in inflamed peripheral tissue in otherwise *bona fide* Treg with fully demethylated TDSR and suppressive function, albeit reduced [769]. IL-6 is a key cytokine in inducing FOXP3 degradation by the ubiquitin pathway by inhibition of the protective deubiquitinase USP7 [770]; serum levels of this cytokine are markedly increased in elderly RTR, particularly those demonstrating other features of immunosenescence [348]. Thus Treg may downregulate FOXP3 expression during chronic inflammation and immunosenescence, which would reduce their apparent number upon phenotyping.

Chronic inflammation may be an early event in SCC development. Evidence comes from animal models, where SCC is invariably induced through a method of propagating chronic inflammation and from humans where malignant transformation into SCC occurs in chronic inflamed wounds such as ulcers [241, 613, 771, 772]. Immunosenescence thus may contribute to SCC induction through the maintenance of a chronic inflammatory environment. Whether circulating Treg function is impaired with immunosenescence has not been explored and will be a focus of further work.

Sherston *et al.* compared Treg number with the proportion of $CD4^{+}$ T cells with a fully demethylated TSDR in long-term RTR with a history of SCC and found the proportion of fully demethylated $CD4^{+}$ to be almost twice the number of Treg identified by phenotypic FACS analysis [223]. Whilst there was

undoubtedly an effect of calcineurin inhibition, as highlighted by the authors, even in the non-calcineurin inhibitor arm there was still a marked variation in the ratio of the proportion of 'Treg' to fully-demethylated TSDR. Repeating this analysis with CD57hi and CD57lo RTR may reveal a greater proportion of fully-demethylated $CD4^+$ T cells that were not detectable by FACS in CD57hi RTR.

Irrespective of underlying mechanism, the finding that number and proportion of Treg fall with immunosenescence in RTR has important implications. This may explain why increased number of circulating Treg were not predictive of SCC in this study and also raises the wider question whether *FOXP3* and Treg-based prognostic markers developed in younger or non-immunosenescent populations would be useful in elderly, immunosenescent populations.

It is possible other populations of regulatory cells may become more relevant with immunosenescence. In various settings including malignancy, *ex vivo* $CD8^+$ cell populations have been found to have immunosuppressive properties, including those displaying $CD8^+CD28^-$ and $CD8^+CD57^+$ phenotype [228, 756, 773-779]. Increased numbers of suppressive $CD8^+CD25^+FOXP3^+$ cells have been described with ageing [780], though we found no difference in $CD8^+FOXP3^+$ number between CD57hi and CD57lo RTR in this study. Whilst $CD8^+CD57^+$ cells did not express FOXP3, immunosuppressive properties cannot be excluded. Following treatment with alemtuzumab, *in vitro* treated and *ex vivo* $CD8^+CD28^-$ cells from RTR may have a suppressive effect upon PBMC proliferation but not IFN-γ production, and only at a relatively high ratio of $CD8^+CD28^-$ to responders: the authors concluded this was likely to be due to competition for "immune space" [333]. Carroll *et al.* found RTR with a history of SCC were more likely to demonstrate reduced PBMC proliferation in response to PHA stimulation and this correlated with $CD8^+CD28^-$ number [66]. Whether this is due to suppression of remaining PBMC or simply hyporesponsiveness to PHA by these cells was not further explored. A study in RTR found *ex vivo* effector-phenotype TEMRA cells to have no suppressive effect upon proliferation *in vitro*, despite increased production of IL-10; however the ratio of 'suppressors' to responders was not as high as that used in the study above [347]. These findings suggest that there may be a subset of terminally differentiated cells

demonstrating a low level of suppressive function in the setting of this study, but the *in vivo* relevance of these remains unclear.

5.11.5 Immunosuppression and CD8+ populations

We hypothesised that immunosuppression may have a greater effect upon $CD8^+CD57^+$ cells, leading to an increased 'effect' of immunosuppression upon the CD8 population in CD57hi RTR. This was not the case and in fact the opposite was seen. Whilst the role of individual immunosuppressive agents was not examined, all RTR were taking a calcineurin inhibitor. When compared to matched non-RTR, we found that $CD8^+CD57^+$ cells retain their cytokine producing and cytolytic capabilities despite immunosuppression, in contrast to $CD8^+CD57^-$ cells. This suggests that once a $CD8^+$ T cell reaches the late stage of differentiation, it appears to be relatively untouched by immunosuppression directly.

Pre-existing alloreactive memory is well recognised in transplantation and is considered a hurdle in the development of tolerance strategies [781]. The lack of effect of immunosuppression upon the immediate effector function of clonally expanded $CD57^+CD28^-$ cells has not been previously reported. A previous study in mice found that cytotoxic function and cytokine secretion in effector memory cells was impaired by calcineurin inhibitor therapy but mice do not develop $CD57^+CD28^-$ or TEMRA populations and so the effect upon these cells specifically could not be assessed [782]. Hebib *et al.* found that IFN-γ and TNF-α production was similar in $CD8^+CD57^+$ compared to $CD8^+CD57^-$ cells from RTR using both PMA and ionomycin and anti-CD3 stimulation, but did not directly compare secretion with non-RTR [332].

The effect upon T cells is likely to be dose-dependent, and as such it should be recognised that the dose of ciclosporin and tacrolimus in long-term RTR is relatively low, and greater doses, used earlier post-transplant, may have more of an effect upon this population.

5.11.6 Tumour infiltration by CD8+ and FOXP3+ cells

Finally, we assessed the difference in tumour infiltration between CD57hi and CD57lo RTR. We looked at tumours from RTR and non-RTR that developed recent SCC, so that immune phenotype

was likely to be relatively representative of that at the time of tumour excision. The wide variation in tumour infiltrate and relatively small number of cases meant that no variables were significantly different, however a number of trends can be seen. The difference in age between CD57hi and CD57lo RTR was unavoidable and must be borne in mind as a potential confounding factor, however most trends in CD57hi and lo RTR were also seen in non-RTR, who were well matched for age.

The most marked trend seen was in the infiltration of $CD8^+$ cells. This was reduced in RTR compared to non-RTR, with relative preservation of $FOXP3^+$ infiltration. This led to a reduction in the CD8/FOXP3 ratio compared to non-RTR, as has been previously described [66, 67, 155]. In both CD57hi cohorts there was a marked reduction in total and peritumoural $CD8^+$ infiltration compared to CD57lo participants. In RTR this was also associated with a reduction in infiltrating $FOXP3^+$ cells, leading to minimal effect upon the ratio. However, in CD57hi non-RTR there was a decrease in the total and peritumoural CD8/FOXP3 ratio compared to CD57lo non-RTR. This was associated with poorer differentiation in the tumours from CD57hi non-RTR, despite no difference in tumour depth or age at excision.

In a cross-sectional analysis one must not assume causation, but it is tempting to speculate that a reduced $CD8^+$ infiltrate in CD57hi individuals leads to a more aggressive, poorly differentiated tumour at time of excision. However the converse may be true and poor differentiation may be associated with reduced $CD8^+$ infiltrate. A trend towards this was seen in one study of cutaneous SCC in non-RTR, though was not replicated in another [210, 357]. A reduction in CD8/FOXP3 ratio is associated with a poor prognosis in the setting of a number of other malignancies, including ovarian, hepatic and colorectal cancer, and is in keeping with the increased aggression seen with SCC in RTR compared to non-RTR [69-71]. Longitudinal follow-up in a larger cohort may allow further insight into the prognostic value of this marker in non-RTR with SCC.

The reduction in both $CD8^+$ and $FOXP3^+$ infiltration in CD57hi RTR compared to CD57lo RTR is interesting. The reduction in $CD8^+$ infiltration, albeit non-significant, may suggest reduced $CD8^+$ responses to SCC, which would be in keeping with the hypothesis of a lower proportion of $CD8^+$ T

cells specific for tumour antigens. There may be altered chemokine signalling with immunosenescence to drive T cell infiltration into these tumours; or it may be due to alterations in trafficking as a result of increased differentiation. The overall reduction in tumour-associated Treg in CD57hi RTR reflects that seen in peripheral blood and suggests that Treg are not preferentially located in peripheral tissues. The fate of Treg in immunosenescent RTR remains unclear.

In both CD57hi cohorts the proportion of infiltrating $CD8^+$ cells expressing CD57 was increased, reflecting the trend seen in peripheral blood. Furthermore, the proportion of these cells was greater in CD57hi RTR compared to CD57hi non-RTR. Whilst $CD8^+CD28^-$ cells have been described within solid organ tumours previously [228, 323], tumour-infiltrating $CD57^+$ cells have been less well characterised. An increasing proportion of circulating CD57-expressing $CD8^+$ cells is associated with poorer prognosis in a number of malignancies [230-232, 425], but these studies have not compared tumour infiltration with circulating numbers: this is the first study to report this correlation. Increasing CD57 expression in infiltrating lymphocytes has been associated with better outcomes in colorectal cancer [783, 784]. However in both of these studies CD57-expressing cells were not further phenotypically assessed and so may represent enhanced NK cell infiltration. Presuming that a significant proportion of $CD8^+CD57^+$ cells are CMV-specific, this may add weight to the hypothesis that CMV reactivation is taking place in the tumour.

It is possible that FOXP3 is being expressed by activated T cells, or by $CD8^+$ regulatory cells. A previous project in this laboratory assessed this in the setting of SCC in RTR, and found that co-expression of FOXP3 and CD8 was seen in a small proportion of $CD8^+$ T cells and was only detectable in a subset of tumours. Furthermore, CD4, CD69 and FOXP3 co-staining, which would be expected to identify activated effector $CD4^+$ T cells, was present in a low proportion of $CD4^+$ T cells (approximately 10% of $CD4^+$ T cells, compared to $CD69^-FOXP3^+$ pattern in 70%) [360]. So whilst FOXP3 expression upon other T cell populations cannot be excluded, this is unlikely to be a significant contributor to the number of $FOXP3^+$ cells.

5.11.7 Conclusion

In this chapter, functional and phenotypic changes within the lymphocyte compartment with CD57hi phenotype have been further explored. The phenotype is associated with accumulation of senescent $CD8^+$ T cells, with loss of naïve T cell populations. This phenomenon has been previously described in the setting of ageing and HIV and associates with increased mortality. We provide results of the first assessment of the performance of the IRP within a transplant cohort. We show that it did not appear to provide prognostic value in this cohort of transplant recipients, unlike the CD57hi phenotype.

The $CD57^+$ cells that accumulate produce effector cytokines and display cytotoxic function upon stimulation, despite immunosuppression. This distinguishes them from exhausted $CD8^+$ cells, which are not present in significant proportions in the circulating blood in this cohort. We have provided indirect evidence to suggest that the CD57hi phenotype is associated with remodelling of the immune system with expansion of oligoclonal populations and a presumed restriction in TCR repertoire as has been described elsewhere. This may facilitate tumour antigen escape and may manifest in a reduced lymphocytic infiltrate into SCC, with a marked reduction in the number of $CD8^+$ cells infiltrating SCC from CD57hi RTR. The increased proportion of $CD8^+CD57^+$ cells in peripheral blood is reflected in tumours.

Although this process is driven to a degree by CMV, other factors appear to influence this process, as the CD57hi phenotype is not simply a biomarker of CMV seropositivity. The recurrent association between CMV and SCC in this project should stimulate further efforts to establish the nature of the link between these two.

Chapter 6: Conclusion

In this final chapter, the implications and limitations of this study will be discussed along with potential directions for further research.

6.1 Implications of main study findings

Reducing incidence of late graft loss

One of the global aims of this study was to improve understanding of the mechanisms behind the development of SCC in long-term RTR. Interestingly, our findings may also have important implications for other causes of late graft loss.

DWFG is caused predominantly by infection, malignancy and CVD in long-term RTR. The results of this study support those of earlier work that suggested that CMV may be associated with an increased incidence of malignancy in the first decade post-transplant [327].

Whilst this study focused on SCC, other malignancies did occur during follow-up in this project. These were relatively uncommon and were heterogeneous in origin (1 melanoma, 1 ovarian cancer, 1 lung cancer, 2 post-transplant lymphoproliferative disease, 1 presumed colonic cancer which was not histologically confirmed before death). Two of these malignancies were in CD57hi RTR. With longer follow-up, a greater number of events may confirm an increased propensity to other malignancy in immunosenescent RTR.

Infection is another major cause of DWFG, and whether immunosenescent RTR are more predisposed to infection is an important question: extrapolation from data in the general population would suggest this is the case but this has not yet been explored in a transplant setting.

The leading cause of DWFG is CVD. This was not an *a priori* hypothesis in this study and so baseline cardiovascular risk factors were not assessed. However, previous studies have found an increase in $CD8^+CD57^+$ and $CD4^+CD28^-$ cells, which correlate strongly with $CD8^+CD57^+$ cells, may be predictive of cardiovascular mortality in non-transplant patients with pre-existing CVD [785, 786]. An association

between CMV antigenaemia and CVD has been previously observed in the first year after renal transplant [787]. Notably, the National Health and Nutrition Examination Survey (NHANES) III found that CMV seropositivity was a predictor of cardiovascular mortality in the general population, but the significance of this was narrowly lost after adjustment for confounders (HR 1.19 (95% CI: 0.95–1.49) [788]. This may support the concept discussed earlier that CMV alone is not a poor prognostic factor but is a function of the immune response to CMV, potentially leading to an immunosenescent phenotype. The findings in this project agree with this hypothesis, with an increased rate of mortality unrelated to malignancy in the CD57hi cohort. This study adds to the increasing body of evidence that CMV may be a direct or indirect contributory factor to the bulk of morbidity and mortality in long-term RTR and provide a potential method to stratify RTR by the extent of the CMV-associated immunological alterations. Whether the CD57hi phenotype is predictive of cardiovascular mortality is an important topic for further study.

Up to half of long-term graft loss is due to graft failure [5]. The exact causes of "chronic allograft nephropathy", or "interstitial fibrosis and tubular atrophy" [789], are contentious and complex but chronic humoral alloimmunity is a contributing factor in a proportion of cases [790, 791]. The finding that nearly 20% of this cohort possesses DSA suggests that even in the very long term this may continue to contribute to graft loss. In this study, azathioprine use was associated with possessing DSA at enrolment. Whilst HLA alleles are by no means the only target of antibodies directed against the graft, these are particularly associated with a poor prognosis [792]. Why azathioprine should be associated with DSA formation is unclear, but further study in animal models may elucidate this.

A return to dialysis is linked to increased rates of CVD, infection and an increased incidence of depression compared to patients who were never transplanted [793, 794]. Many long-term RTR will be sensitised or have accumulated comorbidities which will make them unsuitable for further transplantation. For those who are eligible for re-transplantation graft failure means a return to the transplant waiting list, further increasing the disparity between the waiting list and available organs.

Preserving long-term allografts is therefore an important priority in transplant research. The findings of this study may lead to clinical studies investigating immunosuppression modulation away from azathioprine in long-term RTR: this may yield a reduction in the incidence of late graft loss due to chronic humoral responses against the allograft.

Immunosenescence in the development of SCC

Another global aim of this study was to test two potential ways of identifying RTR who may be at increased risk of developing subsequent cutaneous malignancy. The reasoning for this is that early reduction in immunosuppression has been shown to reduce the risk of future SCC, though with the risk of rejection in a subset of patients [124]. A method to identify those who may benefit from immunosuppression reduction with lesser risk of rejection would be valuable.

The results in this study illustrate that the 'CD57hi' phenotype may provide a way of stratifying the risk of future SCC. For the first time, we compared the performance of immunological phenotype to clinical phenotype for SCC risk prediction. The CD57hi phenotype was a stronger predictor than previous 'accepted' risk factors such as UV exposure and duration of immunosuppression. This is partly a result of cohort selection, but the same phenomenon was seen even in those with a first occurrence of SCC during the study. A potential explanation could be poor elucidation and accurate accounting of chronic UV exposure, but three previously published clinical risk scores were also employed to assist in quantifying this. These measures have not been validated outside of the cohorts they were developed in and their poor performance in an independent cohort of long-term RTR is also a major finding of the study.

The CD57hi phenotype characterised in this study may have a number of clinical applications. Firstly, it may enable stratification of dermatological surveillance. For example a transplant recipient with a CD57hi phenotype may be followed up more regularly in clinic, whilst RTR with a CD57lo phenotype may be seen less frequently. However, it should be noted that more intensive dermatological follow-up has not been demonstrated to improve the detection rate of SCC in RTR, and great emphasis is placed on RTR to self-examine. Even in this respect the marker may be useful

if it enabled RTR at greatest risk of SCC to be better informed and educated: this would provide a strong motivator for self-examination. Transplant recipients as a cohort are poorly compliant with sun protection measures and this marker may enable focused interventions in high risk groups [795-797].

Secondly, current guidelines suggest that RTR with recurrent SCC are considered for a reduction in immunosuppression [72, 455]. However, the evidence for late immunosuppression reduction altering SCC accrual is poor. Identification of RTR at highest risk of future SCC by CD57hi phenotype may enable pre-emptive reduction or switch in immunosuppression; either after first SCC or even prior to the development of SCC.

There is little consensus of how immunosuppression should be adjusted after SCC, though many clinicians reduce or stop azathioprine. The findings in this study that azathioprine is associated both with a reduction in NK cell number and the development of DSA supports the reduction or cessation of azathioprine. Taken together, these data should prompt further research into whether a switch from azathioprine to MMF in CD57hi RTR may reduce both the risk of SCC and of DSA formation and late graft dysfunction. In the presence of a dysfunctional CD8 compartment this may partially restore anti-tumour immunity.

It costs approximately £7000 per year to care for a kidney transplant recipient [798]: a major ongoing cost is that of immunosuppression and dermatological surveillance. A reduction in the intensity of either of these interventions even in a subset of RTR may result in a substantial saving for the health provider.

A final use of this marker is in clinical research. Previous studies of immunosuppression adjustment have shown disappointing outcomes upon SCC recurrence, but have found greater benefit of intervention in those with a low burden of disease at recruitment [140, 141]. In controlled trials of RTR it is difficult to recruit a sizeable cohort with sufficient power to detect an endpoint which may only occur in a small proportion of patients; this is partly why the previously referenced trials focused upon SCC recurrence. This marker may enable recruitment of a high-risk population with

sufficient power to detect whether changes in immunosuppression prevent development of future cutaneous malignancy.

An important aspect of CD57 stratification is that it is likely to be easily replicable. Whilst a full phenotypic assessment of circulating leucocytes requires a number of markers and a multi-laser flow cytometer, the CD57hi marker can be assessed using only two channels (CD8 and CD57). The $CD57^+$ population is easily identifiable on this plot and provides a similar result to that provided by 'full' phenotyping (Figure 6.1). In multicentre studies this marker would be easy to standardise between centres.

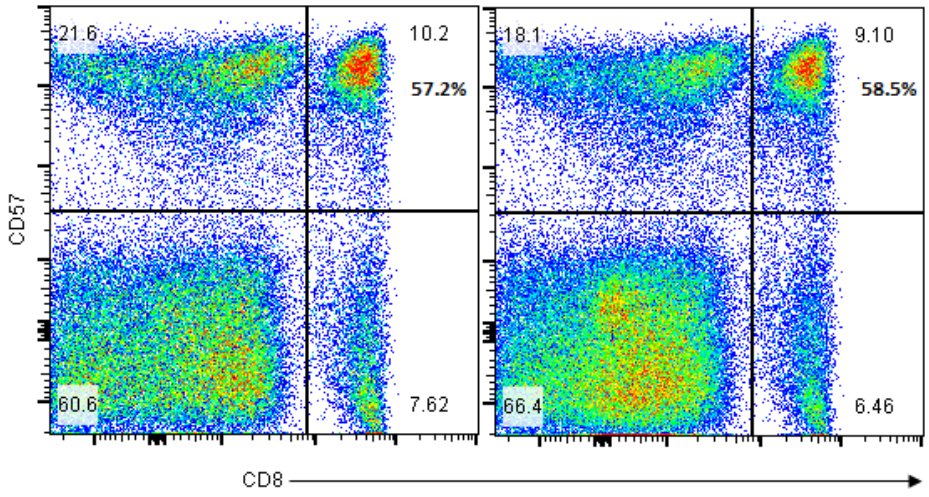


Figure 6.1: Gating on CD8 and CD57 alone allows elucidation of CD57 expression on $CD8^+$ T cells. *The same sample was gated only on lymphocytes using forward and side scatter (left) or was not gated prior to plotting (right). The percentages represent the proportion of $CD8^+$ cells expressing CD57 in each sample. The percentage of CD57-expressing $CD8^+$ T cells using the study gating strategy, performed blinded, was 57.2%.*

A key question that arises from this and other studies is: can immunosenescence be reversed or prevented? There is minimal evidence to suggest so at present. The differentiation of $CD8^+$ cells is partly under the control of the mTOR (mammalian target of rapamycin) pathway; *in vitro* studies and animal models suggest that mTOR but not calcineurin inhibitors may prevent the differentiation of effector cells, promoting differentiation of central memory T cells, and so may slow differentiation and clonal expansion of the T cell compartment [782, 799, 800]. However, in the clinical setting rapamycin therapy was associated with an *increased* accumulation of CMV-specific effector cells after transplant [801]. Whilst this may be protective against CMV reactivation, it may

also accelerate immunosenescence. Further investigation of the influence of different immunosuppressive drugs on immunosenescence in the context of RTR is clearly warranted.

An alternative approach may be to try to reduce CMV reactivation and studies are currently assessing the use of antiviral therapy upon the $CD8^+$ and $CD4^+$ compartment. However, the effects over the short-term appear to be small and an effect upon clinical endpoints has not been demonstrated [802, 803]. Some CMV-exposed RTR currently receive antiviral prophylaxis for up to six months post-transplant; there may be an argument to extend this if an effect upon immunosenescence is demonstrated. CMV vaccination may represent a long-term solution and is presently undergoing clinical trials, but this approach will not be of benefit to the current and near-future cohort of transplant recipients as is it only being tested in the perinatal period at present [804].

In adult rodents and primates a 25% reduction in calorific intake improves the production, maintenance and function of naïve T cells with increasing age [805, 806]. If this were employed in conjunction with other measures such as antiviral therapy, this may slow the development of some features of immunosenescence. However, this would likely have to be a lifelong therapy and this lifestyle change may not be acceptable to many RTR.

'Signatures' of operational tolerance and immunosuppression

The final aim of this study was to assess the prevalence of two previously defined 'signatures' of operational tolerance in long-term RTR with the hypothesis that this may be overrepresented in RTR who develop SCC. The finding that both signatures are affected by immunosuppression is important and expands upon recently published work [367]. Whilst subclinical operational tolerance may occur in a subset of long-term RTR, our findings demonstrate that current markers may not be refined sufficiently to identify these patients. The marked effect of immunosuppression makes it difficult to determine which of the current markers are significant without further understanding of the mechanisms behind the alterations in the immune phenotype. Using azathioprine as an example, the data presented here demonstrate that it had a marked effect in

'moving' the circulating immune phenotype away from the RISET tolerance signature. This may reflect an appropriate move, given that azathioprine may be associated with the development of DSA and as such patients are unlikely to be tolerant. However, azathioprine did not have an effect upon the ITN tolerance signature, which is concerning and suggests the two signatures are not measuring the same effect.

The results of this study cast sufficient doubt about the robustness of the signatures as they stand that any proposals to use these to identify RTR who may benefit from immunosuppression reduction will require much further validation. Studies are currently attempting to find novel markers associated with operational tolerance which are not affected by immunosuppressive regimen (M. Hernandez-Fuentes, Kings College London, personal communication).

6.2 Immunosenescence as a mechanism of operational tolerance

The implication of identifying a marker of increased SCC risk would be that this marker might be used to define RTR who would benefit from immunosuppression reduction. Might reduction in immunosuppression eventually result in cessation in some of these RTR without risk of rejection?

In this project, operational tolerance and immunosenescence have been treated as two separate phenomena. An overlap was not clearly demonstrated in this project using the current published RISET and ITN 'signatures' of operational tolerance, though many alternative explanations for this have been discussed. It is possible that there is overlap between immunosenescence and operational tolerance, though it should be remarked that immunosenescence is unlikely to represent 'true' tolerance. The data from the general population suggest that the reduction in immune function in immunosenescent RTR would not be donor-specific but is likely to be global. However, it is feasible that in some RTR demonstrating advanced immunosenescence the clonal expansion and TCR repertoire reduction of $CD4^+$ and $CD8^+$ T cells is so profound that the remaining donor-reactive populations form only a small minority. Alternatively, as discussed in Chapter 5, there is some evidence of an as-yet-undetermined subset of $CD8^+CD28^-$ cells possessing non-antigen-specific immunosuppressive properties; potential expansion of these in immunosenescence

may associate with increasing immunoregulation, though this may also be simply due to competition for cytokines and chemokines.

Given that CMV has been demonstrated to be closely associated with the development of immunosenescence, this could mean that CMV is both an enemy and an ally in transplantation. CMV is associated with deleterious effects at the time of transplant, with reactivation leading to various end-organ complications, including direct cytopathic effects upon the graft [432, 433]. However, once the perioperative period has passed the later immunosenescence-driving effects of CMV may then enable the reduction of immunosuppression. In those individuals who already display elements of immunosenescence at time of transplant, there may be reduced risk of acute rejection [347, 348]. A caveat of this hypothesis is that viral-specific clones can demonstrate cross-reactivity with HLA alleles and can therefore be drivers of 'heterologous immunity' [807-809] meaning that immunosenescence would not inevitably associate with operational tolerance. Designing an experimental approach to investigate this further could provide useful additional insights.

The overall effect of immunosenescence may be dependent upon the extent of cross-reactivity within clonal expansions and the remaining T cell populations. RTR in this study have maintained stable graft function for a median duration of over twenty years and therefore pre-existing memory populations could be hypothesised to display low cross-reactivity with alloantigen. We had hoped to analyse this possibility but this was not possible due to a paucity of donor material. However it is feasible that a combination of stratification by CD57 (to identify immunosenescence) and tests of donor reactivity, such as IFN-γ ELISPOT, could be combined to identify those with non-alloreactive clonal expansions, who may safely reduce immunosuppression. If these analyses were performed prior to transplant, this potentially could enable identification of RTR who would be able to undergo 'less intensive immunosuppression' from the time of transplant onwards.

As studies of immunosenescence have previously occurred in non-transplant populations, the additional presence of an allograft is difficult to interpret. Whether cessation of

immunosuppression would change the balance of clonal expansion and enable expansion of previously non-dominant alloreactive clones is unclear, but this may provide an explanation for why RTR with 'operational tolerance' may develop a delayed response to the graft many years after stopping immunosuppression [259, 260].

Previous studies in operationally tolerant RTR have minimally explored the potential role of immunosenescence. One study identified that reduced vaccination responses were seen in a proportion of operationally tolerant RTR but did not provide further details to enable evaluation of co-existing immunosenescence; however three of the five operationally tolerant RTR investigated were aged older than 60 [267]. A greater proportion of CD28-negative $CD8^+$ T cells and a lower number of TCm were found in chronic rejecters compared to age-matched operationally tolerant RTR: however this study did not match for CMV status and so this finding cannot be interpreted [270].

Immunosenescence is associated with the opposite effect upon many previously identified molecules of immune regulation. For example, Treg have been demonstrated to be associated with allograft tolerance in animal models but the same data are lacking in humans, with a minimal apparent role for Treg in the peripheral blood signatures of operational tolerance. The results in this project may provide an explanation if those developing immunosenescence demonstrate lower levels of Treg and elevated levels of traditional effector molecules such as perforin and granzyme B. On cohort analysis, those developing donor hyporesponsiveness by a regulatory route (i.e. increased number of Treg and immunoregulation) would be offset by those developing hyporesponsiveness due to immunosenescence, with decreased number of Treg and an increasingly pro-inflammatory milieu. Future studies may need to account for underlying immunosenescence in assessing these markers and this may mean that a 'unified' signature that is utilisable in all RTR (as the 'signatures' of operational tolerance were intended to be) may need to validate and utilise markers which are not affected by these two states.

In summary, an overlap between immunosenescence and operational tolerance cannot be excluded. This has not been explored to date but the identification of a marker to stratify immunosenescence may enable future research in this area.

6.3 Potential limitations of this study

In any cohort study, a potential limitation is that the cohort studied is not representative of the greater population. The size of this study (n=117) represents approximately 23% of the cohort of white RTR greater than five years, 40% of those greater than 10 years post-transplant, and over 65% of those greater than 15 years post-transplant under follow-up at OTC. We estimate the cohort also includes over 90% of RTR with a history of SCC. It is likely that the cohort is therefore large enough to make assumptions about similar cohorts in temperate climates. The final size of the study is also comparable to previous studies in this field [66, 109]. The age of this cohort is likely to be representative also, with the size of long-term RTR cohorts of this age likely to increase in the future, due to increasing age of recipients at transplantation, greater numbers of transplants taking place per year and general long-term survival of allografts.

The immunosuppressive regimen used by the majority of participants in this study is an 'older' one in that ciclosporin and azathioprine are generally used more than tacrolimus and MMF. Whilst the effect of newer agents upon the findings of this study cannot be quantified, those RTR who are at highest risk of SCC (i.e. long-term, older RTR) are likely to be taking these agents. Over 95% of the cohort was Caucasian and so the applicability of these findings to non-Caucasian populations will need to be validated in future.

In any prospective study it is important to be certain that all outcome events are captured. In this respect transplant recipients are perfectly suited for longitudinal studies as they return regularly for follow-up. Many RTR upon moving away to a new area continue to return to the original transplant unit for continuity, as demonstrated by the small number of participants (n=1) who were lost to follow-up in our study. In order to ensure that no events were missed a number of methods were undertaken in parallel. Local pathology databases were monitored for any new histology results

during follow-up. For any patient where it was unclear what dermatology follow-up arrangements were in place, the GP provided details of any appointments or histology based on correspondence with the treating dermatologist. Finally, to ensure no additional events were missed, at any study follow-up sample the participant was asked whether they had seen a dermatologist during the last year and whether they had any samples taken. The robustness of data collection was confirmed in that no participant reported an SCC at follow-up that the investigating team was not already aware of.

One potential limitation of this study is the cohort size. The tolerance signature was present in a much lower proportion of participants than expected: this meant the study was subsequently underpowered to identify predictive value of the 'signatures' for SCC development. However, as discussed in Chapter 5 the signatures themselves are confounded by other clinical factors: this means that for clinical purposes the signatures are not useful and any predictive value is far outweighed by the contribution of immunosenescence, which appears to play a much larger role.

In RTR who are at increased risk of death due to other causes, such as CVD, it is important to be aware of the potential effect of competing risk. For example, if there is unbalanced death in one arm of a study due to CVD, those RTR who have died are no longer at risk of developing SCC and this would potentially reduce the number of events in that arm. This could potentially be an issue given that the CD57hi phenotype may identify RTR at increased risk of death during follow-up. However, this would therefore act to *reduce* the apparent association between CD57hi phenotype and SCC, rather than inflate it. Of the seven deaths, three occurred after an SCC. Therefore whilst these participants were 'censored' for the time to recurrence analysis, the *a priori* analysis of predictive value for time to first SCC would not be affected. So potentially only four participants could demonstrate a competing risk effect which is unlikely to significantly impact upon the result.

In an exploratory study there is the need for a balance between exploration of novel hypotheses, and the effect of multiple testing. Minimising the risk of a type I error (falsely discounting a positive finding) needs to be offset by the risk of type II errors (accepting a falsely positive result). In order

to balance this, adjustments for multiple tests have been made where possible throughout the thesis. However, the optimal method to ensure that a novel finding is 'real' is to validate it independently. To this end, ongoing studies in other centres have expressed interest in validating the prognostic benefit of stratification by CD57. It is reassuring that increasing CD57 expression has been indirectly identified by a previous study as a predictor of malignancy risk in RTR [327].

6.4 Future directions for study

The follow-up period in this study represents a relatively short duration at present. Ethical approval was originally granted for five year follow-up of this cohort and this will provide more information regarding the long-term prognostic value of the CD57hi phenotype. This will also provide the opportunity to study the stability of this marker over a much longer period.

As discussed above, it is important to validate the findings of this study in a second cohort and to assess its performance in other populations. Ideally this study would recruit RTR after diagnosis of first SCC with a study outcome of time to second SCC, stratified by CD57 phenotype. It is also unclear whether this marker may be prognostically informative in non-immunosuppressed populations who develop SCC, as up to 30% may develop a second SCC within five years; a similar prospective study may elucidate this [810].

An important cohort for further assessment of this marker is RTR who are younger than those studied here. Whilst data using other biomarkers of immunosenescence suggest that alterations in immune phenotype may begin to become prognostically important from about the age of 60 onwards, some studies have suggested that reduced responses to vaccines are seen in younger populations [304]. As post-transplant SCC occurs at an earlier age than SCC in the general population, this may enable very early identification of those at highest risk, potentially decades prior to developing SCC.

The next step in Oxford will be to validate retrospectively whether CMV is associated with earlier onset of first SCC, as this cohort was relatively underrepresented in this study. A retrospective

cohort study of white RTR transplanted over the last 20 years at the OTC who remain under follow-up in Oxford or an Oxford satellite unit (approximately 800, based on a preliminary database search) may provide confirmation that CMV seropositivity at transplant is specifically associated with the onset of earlier SCC, without the limiting factor of survival bias. Presuming the prognostic value of this marker was validated, the next step would be to consider a prospective trial of immunosuppression reduction in this cohort.

Further work should be undertaken to elucidate the mechanisms underpinning the findings identified in this study. The effect of immunosenescence upon allogeneic responses has not been explored due to the lack of donor material. Another study, ongoing within the Wood group, has biobanked donor PBMC at time of transplant with follow-up of recipients now approaching five years post-transplant. Through a combination of assays, including ELISPOT and mixed lymphocyte reaction, it would be informative to assess the presence or absence of alloreactive responses in matched CD57hi and CD57lo RTR.

In order to fully understand the effect of immunosenescence upon SCC development, it is likely that animal models will need to be utilised. Models of memory inflation using mCMV are well characterised using the BALB/c and B6 mouse strains and draw many parallels with the effect seen in humans [684, 685]. The B6 mouse is also the strain used for both UV radiation and DMBA/TPA models of SCC and so these models could therefore be combined to assess the effect of memory inflation upon SCC development [151, 241, 613].

It was not possible to obtain freshly excised tumour tissue in order to perform functional assays directly in this study, due to the clinical necessity of keeping the excised material intact. However, future work may be able to utilise pre-cancerous tissue such as Bowen's disease (SCC in situ). In particular, quantification of HPV viral load within actinic keratoses from CD57hi and CD57lo RTR may add weight to the potential contribution of this virus to SCC risk with immunosenescence.

The findings of this study indicate the need to identify novel signatures of tolerance that are unaffected by immunosuppression, for clinical use. Without further mechanistic understanding of

the development and maintenance of operational tolerance, it is difficult to see how these can be developed when the confounding and 'true' effects of immunosuppression upon biomarkers cannot be distinguished.

6.5 Concluding remarks

The CD57hi phenotype provides a method of identifying RTR at increased risk of SCC, possibly providing greater prognostic accuracy than previously identified clinical markers in long-term RTR. Previously identified signatures of operational tolerance have limited clinical applicability due to the effect of immunosuppression and do not appear to be predictive of SCC development.

The CD57hi phenotype is partially, but not definitively, driven by CMV infection and leads to clonal expansion of effector T cell populations with indirect evidence of a reduction in the $CD8^+$ TCR repertoire. These are changes previously described in immunosenescence and has been associated with increased mortality in the elderly and in HIV positive cohorts.

The increasing age of RTR at transplant means that immunosenescence is likely to become an increasingly relevant phenomenon in transplantation. With validation, this marker may provide a method to stratify RTR by risk of long-term complications partially arising from pharmacological immunosuppression and may enable identification of RTR who would benefit from pre-emptive immunosuppression reduction.

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Appendices

i. Supplemental Data

Chapter 3

Variable	Univariate analysis	
	HR (95% CI)	p
Harden risk score	1.32 (0.83 – 2.10)	0.24
Urwin risk score	0.87 (0.49 – 1.52)	0.62
Harwood risk score	1.73 (0.90 – 3.31)	0.10

Table i.1: Predictive value of clinical risk scores for SCC development in RTR_{No} only. *Variables are expressed as a hazard ratio per unit increase in score, calculated by Cox modelling.*

	CnI	MMF	Aza	Steroids	Sirolimus
Number of $CD19^+$ cells	0.14 (-0.11 – 0.40, 0.27)	-0.01 (-0.32 – 0.29, 0.92)	**-0.33 (-0.50 – -0.09, 0.005)**	-0.09 (-0.24 – 0.09, 0.38)	-0.19 (-0.72 – 0.02, 0.06)
Proportion of lymphocytes $CD19^+$	0.19 (-0.05 – 0.42, 0.12)	0.06 (-0.22 – 0.35, 0.64)	-0.25 (-0.39 - -0.01, 0.04)	0.00 (-0.15 – 0.15, 0.99)	-0.15 (-0.59 – 0.10, 0.16)
Number of naïve B cells	0.12 (-0.16 – 0.54, 0.29)	-0.01 (-0.43 – 0.41, 0.96)	**-0.44 (-0.86 – -0.29,<0.001)**	-0.22 (-0.49 – -0.04, 0.02)	-0.11 (-0.81 – 0.21, 0.25)
Number of transitional B cells	0.26 (0.00 – 0.62, 0.05)	0.00 (-0.37 – 0.35, 0.97)	**-0.42 (-0.68 – -0.19, 0.001)**	-0.14 (-0.33 – 0.06, 0.18)	0.09 (-0.27 – 0.68, 0.40)
Number of memory B cells	0.07 (-0.23 – 0.40, 0.59)	-0.10 (-0.51 – 0.24, 0.47)	-0.09 (-0.34 - 0.16, 0.48)	0.03 (-0.17 – 0.23, 0.76)	-0.19 (-0.84 – 0.07, 0.10)
Number of plasmablasts	**0.42 (0.18 – 0.69, 0.001)**	-0.04 (-0.35 – 0.26, 0.77)	-0.08 (-0.27 – 0.14, 0.51)	-0.11 (-0.25 – 0.07, 0.28)	-0.01 (-0.39 – 0.35, 0.92)
Ratio of switched to unswitched memory B cells	**0.37 (0.15 – 0.74, 0.004)**	0.24 (-0.01 – 0.70, 0.06)	0.08 (-0.16 – 0.32, 0.52)	0.05 (-0.15 – 0.23, 0.66)	0.17 (-0.09 - 0.77, 0.12)

Table i.2: The effect of individual immunosuppressant agents upon the B cell compartment. *Results are generated using linear regression on log-transformed variables, using each agent, age at enrolment and time since last transplant as covariates and are presented as beta (95% CI, p value), with 7 degrees of freedom. Significance was adjusted for multiple testing using Bonferonni correction and taken to be $\leq$0.007. 'CnI', calcineurin inhibitor; 'MMF', mycophenolate mofetil; 'Aza', azathioprine. Statistically significant variables are shown in bold.*

	CnI	MMF	Aza	Steroids	Sirolimus
CD19/CD3 ratio	0.18 (-0.06 – 0.43, 0.13)	0.03 (-0.25 – 0.33, 0.79)	-0.31 (-0.47 – 0.08, 0.007)	0.03 (-0.13 – 0.18, 0.79)	-0.12 (-0.57 – 0.13, 0.22)
%$CD4^+$ $CD25^{int}$	0.21 (-0.04 – 0.33, 0.12)	0.09 (-0.15 – 0.29, 0.52)	0.12 (-0.08 – 0.22, 0.37)	0.06 (-0.09 – 0.15, 0.61)	0.15 (-0.09 – 0.44, 0.19)
%$CD4^+$ $CD25^+$(effector)	0.37 (0.07 – 0.39, 0.006)	0.13 (-0.10 – 0.29, 0.32)	0.13 (-0.07 – 0.20, 0.31)	0.10 (-0.06 – 0.15, 0.36)	0.18 (-0.04 – 0.44, 0.10)
FOXP3/*AMANN* ratio	-0.02 (-0.21 – 0.19, 0.91)	-0.09 (-0.32 – 0.15, 0.47)	-0.14 (-0.25 – 0.07, 0.27)	-0.06 (-0.16 – 0.09, 0.60)	-0.03 (-0.33 – 0.24, 0.77)
TCL1A expression	0.06 (-0.22 – 0.38, 0.60)	-0.06 (-0.44 – 0.27, 0.63)	**-0.41 (-0.70 – -0.22, <0.001)**	-0.22 (-0.42 – -0.04, 0.02)	-0.23 (-0.95 – -0.09, 0.02)
TLR5 expression	-0.07 (-0.17 – 0.09, 0.56)	0.15 (-0.05 – 0.26, 0.18)	0.10 (-0.06 – 0.15, 0.37)	**0.44 (0.11 – 0.27, <0.001)**	0.10 (-0.09 – 0.29, 0.30)
SH2D1B expression	-0.11 (-0.29 – 0.09, 0.29)	-0.09 (-0.33 – 0.13, 0.38)	**-0.70 (-0.72 – -0.41, <0.001)**	0.01 (-0.11 – 0.13, 0.89)	0.04 (-0.21 – 0.35, 0.60)
HS3ST1 expression	-0.08 (-0.24 – 0.13, 0.54)	0.04 (-0.19 – 0.25, 0.78)	-0.09 (-0.20 – 0.09, 0.46)	**-0.36 (-0.32 – -0.09, 0.001)**	-0.12 (-0.41 – 0.12, 0.27)
MS4A1 expression	0.05 (-0.17 – 0.25, 0.69)	-0.01 (-0.26 – 0.23, 0.91)	-0.30 (-0.39 – -0.06, 0.01)	-0.16 (-0.24 – 0.03, 0.12)	-0.28 (-0.72 – -0.12, 0.007)
SLC8A1 expression	-0.13 (-0.14 – 0.05, 0.32)	-0.04 (-0.13 – 0.09, 0.73)	0.03 (-0.06 – 0.08, 0.81)	0.28 (0.02 – 0.14, 0.006)	0.11 (-0.06 – 0.20, 0.31)
FCRL1 expression	0.19 (-0.04 – 0.35, 0.12)	0.08 (-0.16 – 0.31, 0.53)	-0.28 (-0.35 – -0.04, 0.02)	-0.006 (-0.13 – 0.12, 0.95)	-0.02 (-0.31 – 0.26, 0.87)
CD79B expression	0.06 (-0.10 – 0.17, 0.64)	-0.04 (-0.19 – 0.13, 0.72)	-0.29 (-0.25 – -0.03, 0.01)	-0.18 (-0.17 – 0.01, 0.07)	-0.18 (-0.37 – 0.02, 0.08)
PNOC expression	-0.07 (-0.20 – 0.12, 0.61)	-0.06 (-0.23 – 0.14, 0.63)	-0.07 (-0.16 – 0.09, 0.60)	-0.09 (-0.14 – 0.06, 0.41)	-0.25 (-0.49 – -0.04, 0.02)
FCRL2 expression	0.16 (-0.09 – 0.40, 0.22)	0.03 (-0.26 – 0.33, 0.84)	-0.16 (-0.33 – 0.07, 0.21)	-0.05 (-0.20 – 0.12, 0.63)	-0.06 (-0.46 – 0.26, 0.58)

Table i.3: The effect of individual immunosuppression upon the RISET tolerance signature. *Results are generated as for Table i.2 using linear regression on log-transformed variable. Significance was adjusted for multiple testing using Bonferonni correction and taken to be ≤0.004. 'CnI', calcineurin inhibitor; 'MMF', mycophenolate mofetil; 'Aza', azathioprine. The actual values are shown in Supplementary Data Table i.3. Statistically significant variables are shown in bold.*

	CnI	MMF	Aza	Steroids	Sirolimus
IGKV4-1 expression	**0.34 (0.15 – 0.68, 0.002)**	**-0.22 (-0.64 – -0.01, 0.04)**	-0.02 (-0.23 – 0.20, 0.87)	-0.07 (-0.24 – 0.11, 0.45)	**-0.24 (-0.88 – -0.11, 0.01)**
IGKV1D-13 expression	**0.40 (-0.21 – 0.74, 0.001)**	-0.20 (-0.60 – 0.03, 0.07)	-0.03 (-0.24 – 0.19, 0.80)	-0.02 (-0.19 – 0.15, 0.85)	**-0.20 (-0.80 – -0.03, 0.03)**

Table i.4: The effect of immunosuppression upon the ITN tolerance signature. *Results were generated as for Table i.3. Significance was taken as p<0.05. Statistically significant variables are shown in bold.*

	CnI	MMF	Aza	Steroids	Sirolimus
Total serum IgM	**0.31 (0.06 – 0.52, 0.01)**	-0.01 (-0.29 – 0.26, 0.92)	0.02 (-0.17 – 0.20, 0.87)	-0.16 (-0.27 – 0.03, 0.13)	0.06 (-0.25 – 0.43, 0.60)
Total serum IgG	0.13 (-0.08 – 0.26, 0.30)	-0.08 (-0.27 – 0.14, 0.54)	-0.01 (-0.14 – 0.14, 0.54)	-0.17 (-0.20 – 0.02, 0.10)	-0.04 (-0.30 – 0.20, 0.72)

Table i.5: The effect of immunosuppression upon total serum antibody levels. *Results were generated as for Table 4.9, using log-transformed total antibody concentrations. Significance was taken as p<0.05. Statistically significant variables are highlighted in bold.*

Variable	Univariate		Adjusting for IS		Adjusting for IS, age and SCC history	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**	**HR (95% CI)**	**p**
Displaying *TCL1A* expression variable*	1.57 (0.68 – 3.64)	0.29	2.16 (0.90 – 5.19)	0.08	2.45 (0.99 – 6.03)	0.051
Displaying *TLR5* expression variable[&]	0.91 (0.43 – 1.93)	0.81	0.73 (0.33 – 1.64)	0.45		
Displaying *SH2D1B* expression variable*	0.77 (0.32 – 1.87)	0.56	1.40 (0.48 – 4.09)	0.54		
Displaying *SLC8A1* expression variable	0.89 (0.40 – 1.97)	0.77				
Displaying *MS4A1* expression variable	1.26 (0.55 – 2.92)	0.58				
Displaying *FCRL1* expression variable*	0.82 (0.25 – 2.69)	0.74	1.04 (0.31 – 3.51)	0.95		
Displaying *FCRL2* expression variable	0.87 (0.56 – 1.36)	0.54				
Displaying *CD79B* expression variable*	0.93 (0.44 – 1.96)	0.84	1.08 (0.50 – 2.32)	0.84		
Displaying *H3ST1* expression variable[&]	1.25 (0.54 – 2.88)	0.61	1.13 (0.48 – 2.66)	0.77		
Displaying *PNOC* expression variable	0.89 (0.27 – 2.92)	0.84				

Table i.6: Predictive value of individual genes within the RISET gene panel for SCC development during follow-up. *Variables are expressed as a hazard ratio, with not displaying the relevant phenotype taken as the reference value. All variables were then adjusted for immunosuppression (steroid ([&]) or azathioprine ([*])), then subsequently corrected for age at enrolment and history of SCC (as described in Chapter 3).*

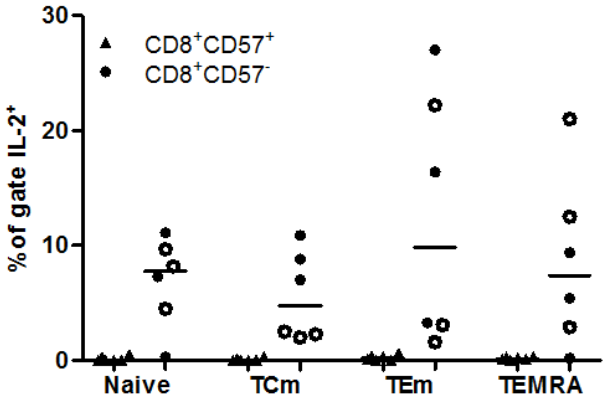


Figure i.1: The reduction in IL-2 expression with CD57-positivity is seen in all CD8+ T cell compartments and is not a defect specifically of CCR7- CD8+ T cells. *Open circles indicate CD57hi RTR, whilst closed circles indicate CD57lo RTR. **p<0.01 by Student's t-test.*

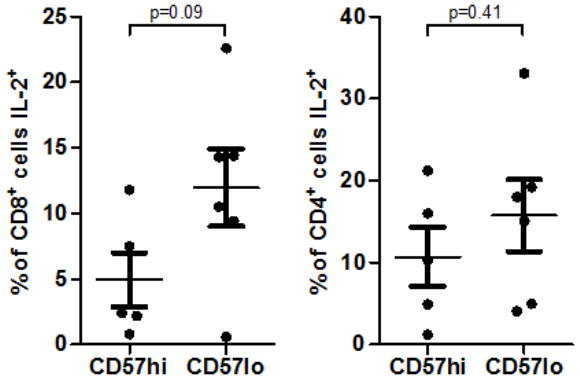


Figure i.2: IL-2 production in CD4+ and CD8+ T cells is not significantly reduced in CD57hi compared to CD57 lo RTR. *Groups are compared using Student's t-test.*

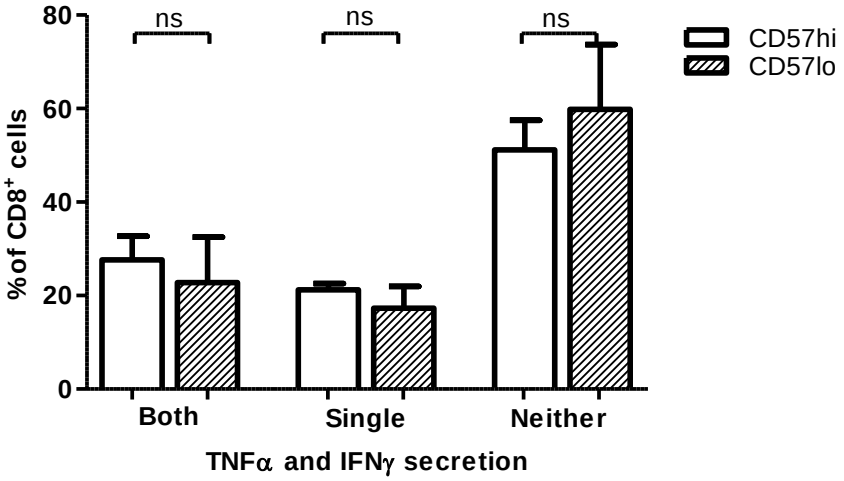


Figure i.3: There is no deficit in the ability of CD8+ T cells from CD57hi RTR to secrete more than one cytokine, compared to CD57lo RTR. *Groups compared by Student's t-test.*

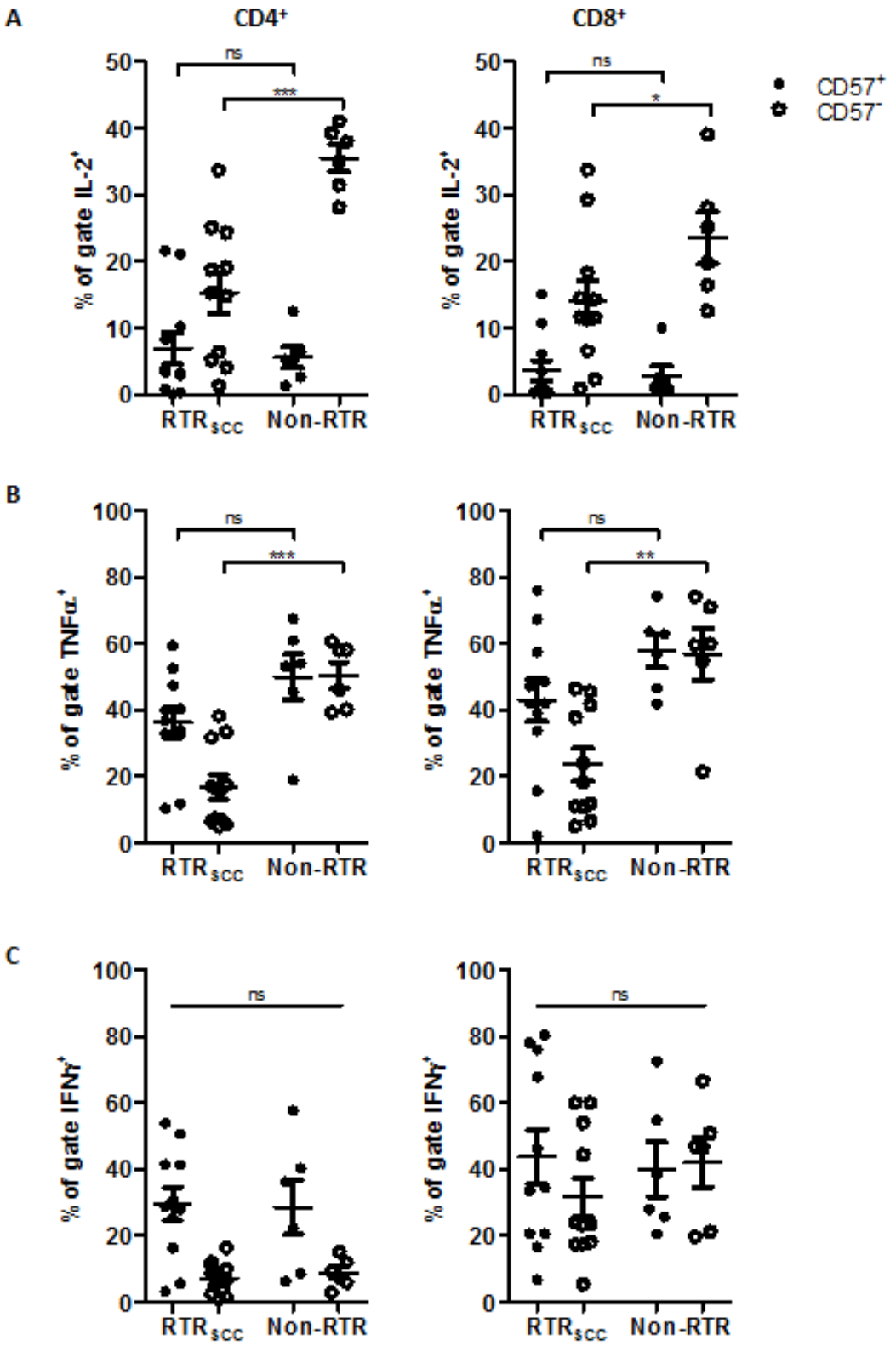


Figure i.4: Immunosuppression impacts upon cytokine secretion by the CD57⁻ compartment in both CD4⁺ and CD8⁺ T cells. There is relative preservation of the CD57⁺ compartment and IFNγ secretion. *A) IL-2 secretion B) TNFα secretion C) IFNγ secretion by CD4+ (left) and CD8+ (right) T cells. Closed circles indicate CD57⁺ populations, open circles CD57⁻. 'ns', not significant, *p<0.03, **p<0.01, ***P<0.001 using ANOVA with post-hoc Bonferonni correction for multiple tests (p≤0.03 was taken as the threshold for significance).*

ii. Study documentation

A: Notification of Research Ethics Committee favourable opinion

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 5
Ground Floor - Tennent Building
Western Infirmary
38 Church Street
Glasgow
G11 6NT

Date 08 November 2012

Direct line 0141 211 2102
Fax 0141 211 1847
E-mail sharon.macgregor@ggc.scot.nhs.uk

Dr Paul Harden
Consultant Nephrologist
Oxford Radcliffe Hospitals NHS Trust
Oxford Kidney Unit
Churchill Hospital
Headington
Oxford
OX3 7LE

Dear Dr Harden

Study title: **An investigation into the role of B-cells and the tolerance signature in renal transplant recipients who develop cutaneous squamous cell carcinoma**
IRAS project number: **112813**
REC reference: **12/WS/0288**

The Proportionate Review Sub-committee of the West of Scotland REC 5 reviewed the above application on 07 November 2012.

Ethical opinion

The Committee were satisfied with the recruitment process, information and consent, anonymity, statistics and that the risks to participants were minimal. The patient burden appears to be small and there are no material ethical issues.

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Covering Letter		30 October 2012
Investigator CV		30 October 2012
Letter of invitation to participant	2.0	17 October 2012
Other: Funder's letter		17 July 2012
Other: Student CV: Bottomley		30 October 2012
Other: Supervisor's CV: Wood		30 October 2012
Participant Consent Form	2.0	18 October 2012
Participant Information Sheet	2.0	17 October 2012
Protocol	1.2	17 October 2012
REC application		01 November 2012
Referees or other scientific critique report		12 July 2012

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/WS/0288	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

SMacgregor

for
Dr Gregory Ofili
Chair

Enclosures:	List of names and professions of members who took part in the review
	"After ethical review – guidance for researchers"
Copy to:	Dr M Bottomley, Oxford University Hospitals NHS Trust Ms Heather House, Oxford University Hospitals NHS Trust

B: Notification of NHS Trust approval

Oxford University Hospitals NHS
NHS Trust

HH/AA/OD/10308

Dr Paul Harden
Consultant Nephrologist
Oxford Kidney Unit
Churchill Hospital
Headington, Oxford
OX3 7LE

From the R & D Lead
OUH Research & Development
Joint Research Office
Block 60, Churchill Hospital
Old Road, Headington
Oxford OX3 7LE

Tel: (01865) (5)72970
Fax: (01865) (5)72242
oliver.dove@ouh.nhs.uk

Friday 18th January 2013

Dear Dr Harden,

Re: Role of the tolerance signature and B cells in RTR with cutaneous SCC
NIHR CSP Reference: 112813
Research and Development Reference: 10308
Research Ethics Committee Reference: 12/WS/0288

Confirmation of Trust Management Approval

On behalf of the Oxford University Hospitals NHS Trust, I am pleased to confirm Trust Management Approval and Indemnity for the above research on the basis described in the application, protocol and other supporting documents.

Conditions of Approval

Your attention is drawn to the attached conditions of approval. Breach of these conditions may result in Trust Management Approval being revoked.

Ethics Correspondence

In order to facilitate good communications and avoid unnecessary delays please copy all correspondence with the Research Ethics Committee (REC) to R&D, providing copies of all relevant documents.

Research Sponsorship

The Oxford University Hospitals NHS Trust accepts the role of Research Sponsor for this study in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004, and amendments 2006 and 2008 and the requirements of Department of Health Research Governance Guidelines.

Site Specific Assessment
This Trust Management Approval letter also incorporates site specific assessment for the Oxford University Hospitals NHS Trust site.

Recruitment date
Please inform OUH R&D when the first participant has been recruited to the study in Oxford by sending the date to the following email address
ResearchRecruitment@ouh.nhs.uk
Provision of this information is a condition of your NHS permission and required to ensure the trust meets its national reporting requirement.

Approved Documents

Document Type	Version	Date
Protocol	1.2	17th October 2012
Letter of Invitation to Participant	2.0	17th October 2012
Participant Consent Form	2.0	18th October 2012
Participant Information Sheet	2.0	17th October 2012
Referees or other scientific critique report		12th July 2012
Funder's Letter		17th July 2012
Investigator C.V.	Paul Harden	30th October 2012
Investigator C.V. (student)	Matthew Bottomley	30th October 2012
Investigator C.V. (academic supervisor)	Kathryn Wood	30th October 2012
R&D Form		
SSI Form		

I wish you every success with the study.

Yours sincerely,



Ms Heather House
Research and Development Lead

Copy to:	OUH Study Finance	study.finance@nhs.net

C: Patient invitation letter and information sheet (transplant recipient)

Oxford University Hospitals NHS
NHS Trust

Please address all inquiries to:
Oxford Transplant Centre
Churchill Hospital
Roosevelt Drive
Oxford
OX3 7LJ

Telephone: +44 (0) 1865 222 508
Email: matthew.bottomley@nds.ox.ac.uk

Sunday, 02 August 2015

[redacted]

Dear [redacted]

We are writing to you to ask you to consider participating in a research study currently being run in collaboration between the Oxford Transplant Centre and the University of Oxford. We are looking at patients who have received a kidney transplant in order to better understand why you may have a risk of developing a skin cancer (squamous cell carcinoma). We are recruiting two groups of participants:

- those who have received a kidney transplant and subsequently developed cutaneous squamous cell carcinoma;
- those who have received a kidney transplant and have not developed cutaneous squamous cell carcinoma;

This is an important topic as skin cancer is much more common in patients who have received a kidney transplant compared to people who have not, and may cause much anxiety and additional hospital visits for patients. We suspect that the immune system plays an important role in determining whether patients (and in particular transplant recipients) will develop cancer or not. With your help, we hope to better understand the role of the immune system in the development of cancer.

We have attached an information sheet which will provide you with further details about the study and would ask that you take the time to read the leaflet before considering whether you would be willing to participate.

In the next few weeks, during your next transplant clinic visit, we will approach you to ask whether you would be willing to take part in this study. If you have decided not to take part, this will in no way impact upon the medical care you receive.

Thank you for taking the time to read the attached information; if you have any questions or concerns regarding the study, please do not hesitate to contact us on the above details.

Yours sincerely,

Dr Matthew Bottomley
Clinical Research Fellow

Dr Paul Harden
Consultant Transplant Physician

Enc: Patient information sheet

Oxford University Hospitals NHS
NHS Trust

Please address all inquiries to:
Oxford Transplant Centre
Churchill Hospital
Roosevelt Drive
Oxford
OX3 7LJ

Telephone: +44 (0) 1865 222 508
Email: matthew.bottomley@nda.ox.ac.uk

"An investigation into the role of regulatory B-cells and the tolerance signature in renal transplant recipients who develop cutaneous squamous cell carcinoma"

PATIENT INFORMATION SHEET

You are invited to take part in a research study organised by the Oxford Transplant Centre in collaboration with researchers into the immune system in transplantation in the University of Oxford. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you would like to talk to someone about taking part in medical research, the Oxford University Hospitals "Patient Advice and Liaison Service" (PALS) is a confidential service that provides impartial advice to patients and their families. Their website is http://www.ouh.nhs.uk/patient-guide/pals.aspx (telephone 01865 221473).

Thank you for reading this.

What is the purpose of the study?
Patients who receive a kidney transplant are at a higher risk of skin cancer than people who haven't had a transplant. One type of skin cancer in particular (termed 'squamous cell carcinoma') is responsible for a significant proportion of this and the frequency of it seems to increase with time after a transplant. We think one of the causes of this is the drugs that patients take following a transplant to prevent rejection. However, some patients may develop skin cancer once; a few may develop it on multiple occasions; and others may not develop it at all. We are interested in why this is. We think this may be due to variations in the immune system between patients.

Amongst other things, we are interested in particular in the role of two factors in the immune system:

1. A type of white cell termed a 'regulatory B-cell'. These cells are naturally present in the body in humans. They have been shown to help prevent autoimmune disease

(where the immune system attacks the body) and rejection of transplants. It has been suggested that they may be involved in the development of cancer.
2. A series of blood markers in the blood that represent a number of aspects of the immune system (termed a 'signature'). This signature looks at different types of white blood cells and how they function. This signature may be relevant in how the immune system reacts to the transplant.

We think that patients who develop this type of skin cancer may have alterations in these two factors. We also want to assess whether these factors are different in patients who go on to develop skin cancer in the future.

Why have I been chosen?
You have been invited to take part EITHER because you have been diagnosed with a skin cancer after receiving a kidney transplant in the past OR you have not developed a skin cancer after your transplant.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. You will be treated the same whether you choose to take part or not, or if you choose to leave the study later.

What will happen to me if I take part?
The study will take up to five years and will take place at the Oxford Transplant Centre at the Churchill Hospital. We will take an extra 50ml (about 3 tablespoonsful) of blood when you have your routine blood tests at your next visit to transplant clinic.

Subsequently, during routine check-ups following your transplant we will take a further 50ml of blood with your blood tests that you would have anyway as part of your clinical care. At your clinic appointments you often have to give a sample of urine to help the doctors monitor how your transplant is working – we will take some of the leftover urine also for analysis.

These extra blood tests and urine samples will be taken at six months after the first tests, then yearly, for up to five years. You will not need to come to Oxford any more times than you would as part of your standard care.

Your blood and urine will be taken to the University of Oxford, where tests will be performed on it to assess how your immune system works.

If you have had skin cancer, the cancerous tissue that you have had cut out (or will have cut out) will normally be looked under the microscope by experts (pathologists) to assess it further. We will look at the parts of the removed tissue that are not needed by them. We will look at the immune system and how it is responding to the cancer. This will not impact upon the tests that the pathologists need to do as part of your routine care.

We will look in your patient records for information relating to your transplant, such as how long you have had the transplant, what drugs we have given you to prevent rejection, and so on. We will then continue to monitor your treatment for up to five years, looking for any

problems that may develop with your transplant. We will compare this to the other group that we are looking at, to see if there is a difference.

What do I have to do?
Other than attending your follow-up appointments and providing us with extra blood and urine, you will need to do nothing different (except for what is written in the next paragraph and underlined).

You should continue taking all your medications as prescribed by the doctor as normal. However, it is important that you do not take your ciclosporin or tacrolimus or any immunosuppressive medications on the morning of the clinic visit until after you have had your blood taken so that the levels of the drug can be measured.

What are the possible disadvantages and risks of taking part?
There are no additional risks to taking part. If you take part in this study you will have to give an additional amount of blood at certain time-points (discussed above). This would be done at the same time as blood tests you would normally have, so you will not need to give blood twice.

What are the possible benefits of taking part?
Whilst you are unlikely to benefit directly, future patients in your position may benefit. The results of the study may provide further understanding of how the immune system works following kidney transplantation. This may one day allow us to tailor the anti-rejection tablets that patients have to take following a transplant. This may help to reduce the risk of skin cancer developing. Understanding how skin cancer develops may allow us to research new, more effective, treatments for it.

What happens when the research study stops?
Your blood will be frozen and stored in a secure University of Oxford storage facility in 'anonymised' form (i.e. you will not be identifiable from the samples) for use in future research. You can request for samples not to be stored beyond the end of this study if you prefer. In this case, samples will be destroyed when the study ends. This will be an option on the consent form.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of this research will be kept strictly confidential. Your samples will be labelled with a number only, which will be maintained throughout the study. You will not be able to be identified from this. All samples will be stored, identified and referred by the number only. Information from your medical notes will also have this number, so you cannot be identified.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study.

What will happen to the results of the research study?
Oxford University and the Oxford University Hospitals NHS Trust may have an interest in using the genetic and biological information found from the testing or analysis of your blood for the development of new products that might help the diagnosis, treatment and prevention of disease.

Results will be published in scientific journals and will be discussed in medical conferences. You will NOT be able to be identified in any report/publication. We should be able to tell you the outcome of the study in non-medical terms if you are interested; please contact Dr Paul Harden at the Oxford Transplant Centre if you wish to hear about the study outcome at a future clinic appointment: we should be able to give you an outline of the study results in about five years' time. At the end of the study we will publish a summary of the research in the Transplant Unit newsletter, though if important findings are made earlier we would publish those sooner.

Who is organising and funding the research?
This research is organised by the Oxford Transplant Centre in collaboration with the Transplant Research Immunology Group at the University of Oxford. The doctors coordinating the research in Oxford are Dr. Paul Harden (consultant transplant physician), and Dr Matthew Bottomley (clinical research fellow). The study is funded by the Wellcome Trust (www.wellcome.ac.uk).

Who has reviewed the study?
The study has been reviewed by the funding panel at the Wellcome Trust and doctors and researchers in the Oxford Transplant Unit and University of Oxford. The study was given a favourable ethical opinion for conduct by the Local Research Ethics Committee.

Contact for Further Information
If you require any further information please contact Matthew Bottomley who is the main researcher in Oxford running this study.

Thank you for reading this information sheet and for considering participating in this research. This leaflet is for you to keep.

Matthew Bottomley (Clinical Research Fellow) and Dr Paul Harden (Consultant Transplant Physician), Oxford Transplant Centre and Transplant Research Immunology Group

Tel: +44 (0) 1865 222508

E-mail: matthew.bottomley@nds.ox.ac.uk

D: Patient information sheet (non-transplant recipient)

Oxford University Hospitals NHS
NHS Trust

Please address all inquiries to:
Oxford Transplant Centre
Churchill Hospital
Roosevelt Drive
Oxford
OX3 7LJ

Telephone: +44 (0) 1865 222 568
Mobile: +44 (0) 7772 489 605
Email: matthew.bottomley@ndcn.ox.ac.uk

"An investigation into the role of regulatory B-cells and the tolerance signature in renal transplant recipients who develop cutaneous squamous cell carcinoma"

PATIENT INFORMATION SHEET – GROUP 4

You are invited to take part in a research study organised by the Oxford Transplant Centre and the Dermatology Unit at the Churchill Hospital in collaboration with researchers into the immune system in transplantation in the University of Oxford. Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you would like to talk to someone about taking part in medical research, the Oxford University Hospitals "Patient Advice and Liaison Service" (PALS) is a confidential service that provides impartial advice to patients and their families. Their website is http://www.ouh.nhs.uk/patient-guide/pals.aspx (telephone 01865 221473).

Thank you for reading this.

What is the purpose of the study?
Patients who receive a kidney transplant are at a higher risk of skin cancer than people who haven't had a transplant. One type of skin cancer in particular (termed 'squamous cell carcinoma') is responsible for a significant proportion of this and the frequency of it seems to increase with time after a transplant.

We have previously demonstrated that kidney transplant recipients who develop this type of skin cancer may have alterations in parts of their immune system. We would like to now see whether these changes are also present in patients who have a history of this skin cancer, but have never had a transplant.

Why have I been chosen?
You have been invited because you have been diagnosed with a skin cancer ('squamous cell carcinoma') in the past, and you have not had an organ transplant or received drugs to suppress the immune system.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. You will be treated the same whether you choose to take part or not, or if you choose to leave the study later.

What will happen to me if I take part?
We will take an extra 50ml (about 3 tablespoonsful) of blood during your clinic appointment, and ask you to provide a urine sample and complete a questionnaire. Your blood and urine will be taken to the University of Oxford, where tests will be performed on it to assess how your immune system works.

The cancerous tissue that you have had cut out (or will have cut out) will normally be looked under the microscope by experts (pathologists) to assess it further. We will look at the parts of the removed tissue that are not needed by them. We will look at the immune system and how it is responding to the cancer. This will not impact upon the tests that the pathologists need to do as part of your routine care.

We will look in your patient records for information such as any previous cancers you may have had, and how they were treated.

Your involvement in the study will come to an end at this point.

What do I have to do?
You will need to do nothing different. You should continue taking all your medications as prescribed by the doctor as normal.

What are the possible disadvantages and risks of taking part?
There are no additional risks to taking part. Taking part in the study should only take 10 minutes. The amount of blood we are taking is only a very small percentage of the blood in your body (less than 1%), and most people tolerate this very well. There may be some slight bruising after providing the blood sample, but this will settle down naturally.

What are the possible benefits of taking part?
Whilst you are unlikely to benefit directly, future patients in your position may benefit. The results of the study may provide further understanding of how the immune system works following kidney transplantation and in the response to skin cancer. Understanding how skin cancer develops may allow us to research new, more effective, treatments for it.

What happens when the research study stops?
Your blood will be frozen and stored in a secure University of Oxford storage facility in 'anonymised' form (i.e. you will not be identifiable from the samples) for use in future research. You can request for samples not to be stored beyond the end of this study if you prefer. In this case, samples will be destroyed when the study ends. This will be an option on the consent form.

What if something goes wrong?
If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you

may have grounds for a legal action but you may have to pay for it. If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of this research will be kept strictly confidential. Your samples will be labelled with a number only. You will not be able to be identified from this. All samples will be stored, identified and referred by the number only. Information from your medical notes will also have this number, so you cannot be identified.

What will happen to the results of the research study?
Oxford University and the Oxford University Hospitals NHS Trust may have an interest in using the genetic and biological information found from the testing or analysis of your blood for the development of new products that might help the diagnosis, treatment and prevention of disease.

Results will be published in scientific journals and will be discussed at medical conferences. You will NOT be able to be identified in any report/publication. We should be able to tell you the outcome of the study in non-medical terms if you are interested; please contact Dr Reed (consultant dermatologist) at the Dermatology Unit if you wish to hear about the study outcome at a future clinic appointment; this may take a number of years before the results become fully available.

Who is organising and funding the research?
This research is organised by the Oxford Transplant Centre and Oxford Dermatology Unit in collaboration with the Transplant Research Immunology Group at the University of Oxford. The doctors coordinating the research in Oxford are Dr Paul Harden (consultant transplant physician), Dr John Reed (consultant dermatologist) and Dr Matthew Bottomley (clinical research fellow). The study is funded by the Wellcome Trust (www.wellcome.ac.uk).

Who has reviewed the study?
The study has been reviewed by the funding panel at the Wellcome Trust and doctors and researchers in the Oxford Transplant Unit and University of Oxford. The study was given a favourable ethical opinion for conduct by the Local Research Ethics Committee.

Contact for Further Information
If you require any further information please contact Matthew Bottomley who is the main researcher in Oxford running this study.

Thank you for reading this information sheet and for considering participating in this research. This leaflet is for you to keep.

Matthew Bottomley (Clinical Research Fellow) and Dr Paul Harden (Consultant Transplant Physician), Oxford Transplant Centre and Transplant Research Immunology Group

Tel: +44 (0) 1865 222508

E-mail: matthew.bottomley@nds.ox.ac.uk

E: Informed Consent Form

Oxford University Hospitals NHS
NHS Trust

Please address all inquiries to:
Oxford Transplant Centre
Churchill Hospital
Roosevelt Drive
Oxford
OX3 7LJ

Telephone: +44 (0) 1865 222 508
Email: matthew.bottomley@nds.ox.ac.uk

Version 2.1 (08/02/13)

Patient Identification Number for this trial

CONSENT FORM

Title of Project: "Investigation into the role of regulatory B-cells and the tolerance signature in renal transplant recipients who develop cutaneous squamous cell carcinoma"

Name of Researchers: Dr Matthew Bottomley and Dr Paul Harden

Please initial box

1. I confirm that I have read and understand the information sheet dated …….. (version ……..) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by members of the research group other than the principal investigator and by regulatory authorities or sponsor or individuals from the NHS trust where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree that samples I provide may be stored in anonymised form for future research. I consider this sample a gift to OUH Trust and the University of Oxford and I understand that I will not gain any direct benefit from it. Yes ☐ No ☐
5. I agree to take part in the above study ☐

Name of Patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

F: Recruitment proforma

Baseline SCRIPT Study Proforma **Participant no……..**

CONFIRM NO ANTIBIOTICS / ANTIVIRALS IN LAST TWO WEEK ☐

Name of Patient: …… D.O.B: ……/……/…… Sex: M / F

Height: ……Cm Fitzpatrick Skin Class: …… (see chart)

Weight: …… Kg/Lb

Hair colour (age 15)? …… Eye Colour? ……

Number of Painful sunburns:

Childhood (up to 16 years old): ……

Adult (pre-transplant): ……

Adult (post-transplant): ……

Number of holidays to a sunny climate, where you sunbathed:

Childhood: ……

Adult (pre-transplant): ……

Adult (post-transplant) ……

Occupation or hobby that resulted in time spent outside: **YES / NO**

Profession(s)/Hobby: …… Duration (years) …… Hours outside (ave/day): ……

Profession(s)/Hobby: …… Duration (years) …… Hours outside (ave/day): ……

Profession(s)/Hobby: …… Duration (years) …… Hours outside (ave/day): ……

Smoking history: **YES / NO** Pipe / Cigar / Cigarette / Other ……

How many years: ……

Average quantity/day: ……

Sunbed use: **YES / NO**

Age first used: ……

Average visits/month:…… Years of use: ……

Lived abroad for over six months: **YES / NO**

Country: …… Duration: ……

Country: …… Duration: ……

Country: …… Duration: ……

Country: …… Duration: ……

Any cancer in your parents/siblings, or in you, ***including before your transplant***: **YES / NO**

Relative …… Cancer: …… Age:……

Relative …… Cancer: …… Age:……

Relative …… Cancer: …… Age:……

Version 1.1
8 March 2013

iii. Publications arising from this project