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**TRANSPLANTATION WITH KIDNEYS
REMOVED FOR SMALL RENAL TUMOURS:
IMMUNOSUPPRESSIVE STRATEGIES AND
ROLE OF REJECTION**

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Abstract

Renal transplantation is the definitive treatment for the end-stage renal failure. Despite concerted efforts to increase the number of available organs there remains a wide gap. Kidneys with small renal cell carcinoma have been used for transplantation after ex vivo resection of tumours with excellent results. Concerns regarding the behaviour of tumour under standard immunosuppression prevent this source from being popularised. We studied tumour behaviour with standard immunosuppression and immunosuppressives with anti-proliferative properties and the effect of MHC matching on tumour behaviour. Luciferase labelled Wistar rat kidney tumour cells were injected subcutaneously into Wistar or Lewis rats to mimic well and poorly matched groups. These were divided into groups receiving Cyclosporine, Sirolimus high and Sirolimus low dose and Leflunomide. Effects of matching on tumour rejection were studied by immunosuppression withdrawal in half of the animals within each group. Tumour progression was monitored with IVIS spectrum imaging system.

When the immunosuppression was continued for the length of the study period with Cyclosporine immunosuppression, the tumour continued to grow in both strains. With high dose Sirolimus, the tumour was eradicated within 2 weeks in both Wistar and Lewis rats ($p < 0.05$). Both strains receiving low dose Sirolimus also eradicated the tumour within four weeks of treatment ($p < 0.05$). In Leflunomide group, 4/7 animals rejected the tumour within the 4 weeks of study period ($p < 0.05$).

To study the effects of rejection and matching on the tumour behaviour, the immunosuppression was stopped after 2 weeks of treatment and the animals followed for another two weeks to study these effects. After treatment withdrawal, the tumour rejection was noted which was significantly stronger in poorly matched animals than in well-matched animals ($p < 0.05$) in cyclosporine treated animals.

These results appeared to be in line with our hypothesis, that newer immunosuppressive medications with anti-neoplastic effects may be better options after transplanting kidneys after small tumour ex-vivo resection.

Acute rejection showed significant ability to lead to tumour eradication, more effectively in less well-matched animals than well-matched combinations. Thus perhaps clinically, recipients of such restored kidneys should be less well matched and immunosuppressed with agents with anti-proliferative properties. These results will need to be replicated with further studies including closely monitored clinical studies before it can be popularised at a significant new source of precious organs.

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Chapter 1

BACKGROUND / REVIEW OF LITERATURE

1.1 RENAL FAILURE

1.1.1 Background

Renal failure is one of the most common chronic medical problems with significant short and long-term morbidity and mortality. The definition of chronic kidney disease is based on the presence of kidney damage (albuminuria) or decreased kidney function (glomerular filtration rate [GFR] <60 mL/min per 1.73 m.) for 3 months or more, irrespective of clinical diagnosis (1)(2).

The biggest issue with chronic kidney disease is of the insidious onset in most of the cases and presentation with the end organ dysfunction that is irreversible, making the early diagnosis of paramount importance to halt further end organ damage.

End stage renal failure (ESRF)/Renal failure is defined as a GFR of less than 15 mL/min per 1.73 m², or the need for treatment with dialysis or transplantation(3).

Aetiology of renal failure varies considerably and includes hypertension, diabetes mellitus, glomerular disease, polycystic kidney disease, urological and congenital problems among a long list of causes. Most of the causes of renal failure can affect the transplanted kidney as well, hence the need to control the underlying disease is of paramount importance in the long run(4)(5).

Apart from the excretion of waste products, kidneys perform other very important functions including blood pressure control via renin angiotensin system, secretion of erythropoietin (85% of total body erythropoietin is secreted by interstitial cells of kidneys), acid base balance and conversion of 25 hydroxycholecalciferol (storage form of Vitamin D) to 1, 25

dihydroxycholecalciferol (active form of vitamin D).

1.1.2 Clinical features

The initial stages of chronic kidney disease are usually asymptomatic. Even during the late stages the features can be quite non-specific and in some cases leads to a late diagnosis. Peripheral oedema, uraemic symptoms, hypertension, anaemia leading to fatigue, reduced exercise capacity and in severe cases to heart failure. Other features include metabolic disturbances, uraemic encephalopathy, neuropathy, gastrointestinal, dermatological and immune dysfunctions.

1.1.3 Treatment options

CKD leads to permanent loss of renal function and treatment of the condition is thus renal replacement therapy either in the form of dialysis or renal transplantation.

1.1.3.1 *Dialysis*

Dialysis was the only form of treatment for ESRF before transplantation. It can be either by haemodialysis or by continuous ambulatory peritoneal dialysis (CAPD). The basic principle of both forms is diffusion of excess waste products across a semi permeable membrane. In haemodialysis, it is the commercially available filters across which this diffusion occurs whilst in CAPD, it's the

peritoneal cavity of the renal failure patient that acts as a diffusion membrane.

There is a need for a vascular connection via either a large bore intravenous central line or through an arteriovenous fistula to enable high flow of blood required for haemodialysis. This can lead to its own complications including line infections, thrombosis, failure of fistulas, aneurysm and upper limb steal syndrome.

Despite the effectiveness of dialysis, patients on dialysis remain unable to perform any of the endocrine functions of the kidney needing further interventions.

The third form of renal replacement therapy; hemofiltration is where blood from patients is “ultra filtrated” over the semipermeable membrane and is then replaced with fluid of appropriate biochemical composition. This form of dialysis is usually reserved for acute renal failure.

1.1.3.2 Transplantation

Transplantation is the treatment of choice these days for ESRF. This is due not only to the improvement in the quality of life but also in the long-term survival of the patients(6). With the improvements in donor management, organ preservation and transport and continuously improving immunosuppression the graft and patient survivals are improving year on year.

1.2 RENAL TRANSPLANTATION

1.2.1 History of Transplantation

The history of transplantation is long with multiple failures but persistence of committed individuals and teams lead to where we are now, where transplantation is considered the ideal choice in end stage organ failure. As opposed to other surgical fields, success of any organ transplantation required not only the perfection of surgical technique, and post-operative patient management but also was most heavily dependent on the immunosuppression of host, mastering the techniques of organ procurement and preservation as well as both the pre-operative and post-operative donor management.

The biggest technical contribution came from the work of Alexis Carrel in early nineteenth century(7). In 1954 Joseph Murray with his team was responsible of first successful human transplant between identical twins (8). This was in the back draft of multiple unsuccessful attempts of human transplants and was a major step forward. The genetic barrier to transplantation was broken in 1959 when one out of twelve patient survived long term after transplantation from non-identical twins after total body irradiation(9).

The first insight into the rejection as an immunological event was provided by the ground-breaking work of P B Medawar in 1944(10). First case of relatively successful immunosuppression was in 1960 where methotrexate and cyclophosphamide was used in a recipient of her mother's kidney which worked for 147 days(11). Use of cyclosporine as immunosuppressive for kidney

transplantation was a major step forward. Since then various refinements to existing class of immunosuppressive agents and the introduction of induction agents have brought kidney transplantation into the current modern era.

1.2.2 Transplantation and dialysis

It is now known that kidney transplantation confers significant survival benefit over the patients over long-term dialysis, but earlier studies were unable to show this benefit. Multiple reason have been cited for that observation including bias of analysis where in transplant recipients the survival was calculated from the time of transplantation as opposed to time of start of the renal replacement therapy for the dialysis patients(6)(12)(13).

Since these early reports all studies comparing the survival have shown significant improvement after transplantation across all categories(14)(15)(16). In general patients that are put on the waiting lists for transplantation are younger and healthier. Thus direct comparison between the mortality of patients on dialysis and these patients who later on have transplantation is biased towards the transplant group. But even when this bias was removed by studies with improved methodology, there was still significant survival benefit for the transplant recipients. According to a large study by Wolfe *et al.* who studied 228,552 patients on treatment for renal failure with either dialysis or transplantation the annual death rate for all patients on dialysis was 2.6 times as high as that for patients on the waiting list, and the annual death rate for patients on the waiting list was 1.7 times as high as that for transplant recipients(17)

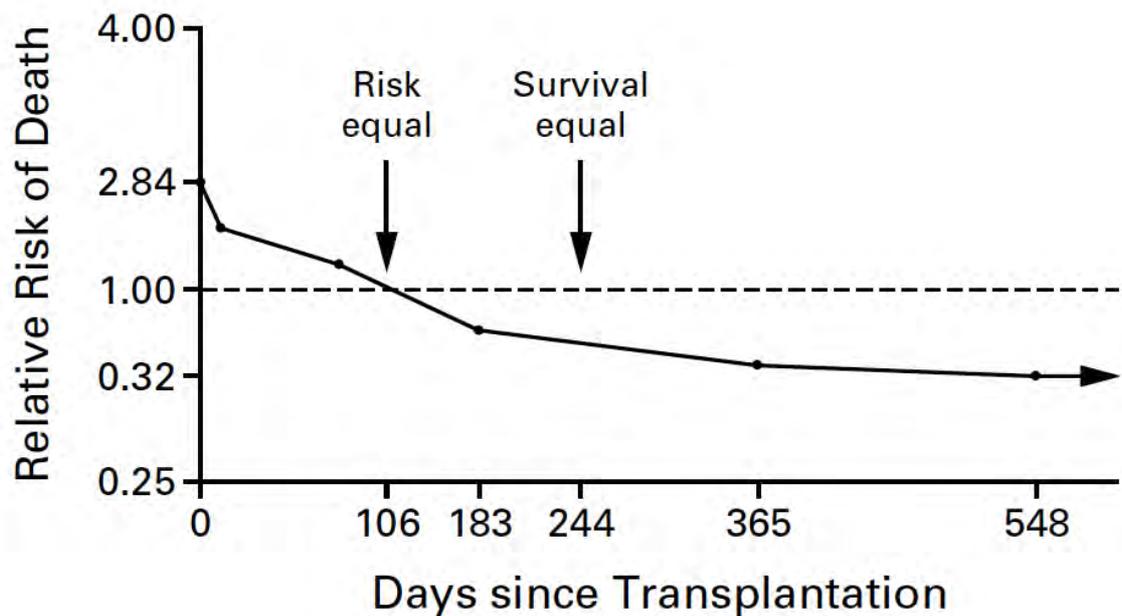


Figure 1 Comparison of mortality in all patients on dialysis. Wolfe et al.(17)

1.3 TYPES OF TRANSPLANTATION AND ORGAN AVAILABILITY

1.3.1 Types of Renal Transplants

Renal transplantation can be broadly divided into living and deceased donor transplantation.

1.3.2 Live donor transplantation

Live donation (LD) rates have increased steadily in the last decade and now accounts for around half of all the transplant procedures performed in the UK according to the latest data by NHSBT. The organs could either be from live related or live unrelated donors. There are several benefits of live donations over the deceased donations which include low rates of delayed graft function,

significantly better long term outcomes, LD being an elective procedure it gives time for optimisation of the recipient and detailed assessment and consenting of the donor. Better outcomes are the direct result of either absent or negligible cold ischaemic times and absence of agonal period and primary warm ischaemia seen in donation after cardiac death (DCD) donations. Current five-year graft and patient survivals are 91% and 96% respectively in the UK(18).

The disadvantage of LD kidney transplants affects solely to the donors in the form of operative mortality (1:3000), post-operative complications, time off work and possible psychological side effects.

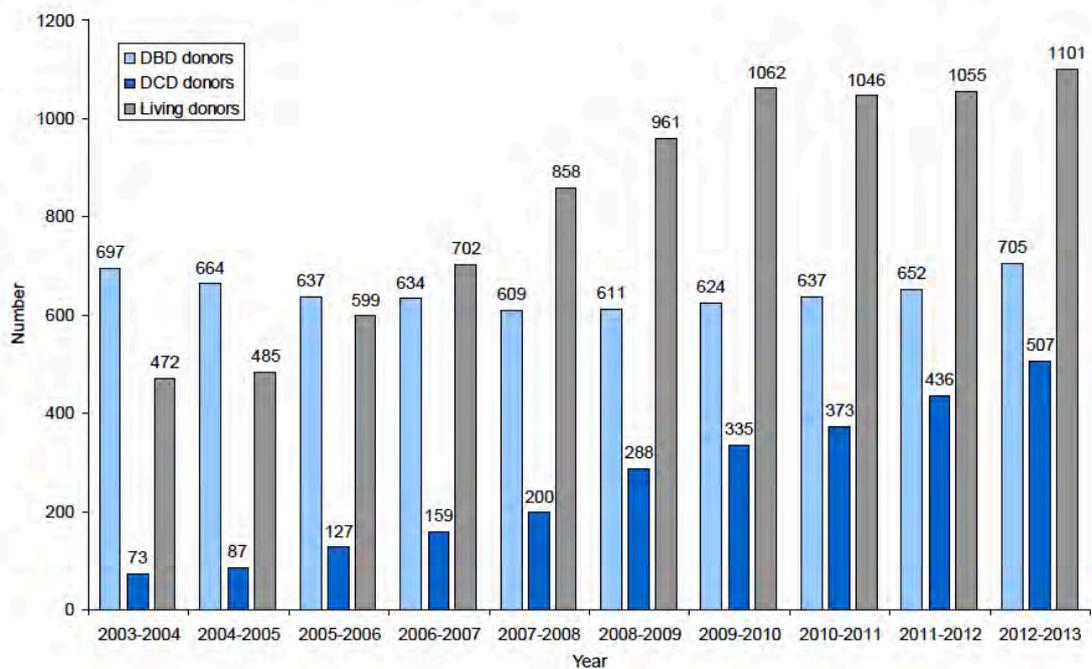


Figure 2 Transplant activities in the UK (2003-2013). Number of deceased and living donors in the UK, 2003-2013. (18)

1.3.3 Deceased Donation

1.3.3.1 *Donation after Brain Death (DBD)*

The majority of organs procured from deceased donors are still from brain dead donors. The contribution of DBD in the total deceased donor pool has reduced over the past 10 years, with around 90% of these organs being DBD in 2003-2004 to less than 60% in 2012-2013. This source was facilitated by the ethical framework established by the Harvard criteria of brain death in 1968(19), as before that time all organs had to be procured after cardiac arrest.

DBD donation occurs in a relatively controlled environment where the donor is declared brain dead after thorough brain stem testing by at least two expert clinicians after excluding reversible causes of coma. Although these organs are not subject to an agonal period and not primary warm ischaemia they are usually exposed to a cascade of damaging cytokines.

1.3.3.2 *Donation after Circulatory Death (DCD)*

Previously known as Non Heart Beating Donation (NHBD) or Donation after Cardiac Death, this form of donation has consistently increased over the past few years in the UK and now accounts for more than 40% of all deceased donations. The organs are retrieved after the donor has been declared dead based on cardio-circulatory criteria. It's not mere cardiac arrest that is defined as the point of death but there has to be a period of "no touch" before the patient can be declared dead according to dead donor rule(20). During this period, the brain would "die" thereby establishing brain death in addition to cardiac death ensuring the absence of pain and irreversibility. After establishing

death procurement can then proceed. This means that from the time of treatment withdrawal to the actual cardiac arrest the organs will be hypoperfused, then there is the period of cardiac standstill, when there is no perfusion of the organs at all which finishes when the cold perfusion starts. Warm organs with no perfusion leads collectively to organ damage. Based on how short these times can be within the permitted limits, DCD organs can be divided into Controlled and Uncontrolled.

Controlled DCD

This is further divided into Maastricht Category III and IV. (The term Maastricht criteria comes from the Consensus meeting held in Maastricht on Non Heart Beating Donation in 1994). Category III donors are the patients in ITU who are likely to die when the supportive treatment is withdrawn. The majority of DCD donors in UK are from this category. They are controlled because the treatment withdrawal only happens when the retrieval team is ready for organ procurement, thereby minimising the warm ischaemia times.

Category IV DCD donation is when patient in ITU suffers brain death but the organs are only retrieved after circulatory death.

Uncontrolled DCD

Category I DCD includes dead on arrival to Accident and Emergency department. It has to be a witnessed cardiac arrest for these patients to be considered for donation.

These patients have usually been confirmed as dead outside of the hospital and are the type of donors used by The Madrid and Barcelona units (where a doctor is present in the ambulance).

Category II DCD is where resuscitation has been attempted on the patient but it has been unsuccessful and the attempt ceases on arrival in hospital where death is declared. Only a very small number of organs are generated in the UK from these uncontrolled DCD donors as opposed to Spain, which is the world leader in donation and has a very well established Extra Corporeal Membrane Oxygenation (ECMO) programme to support these organs till they are formally retrieved.

1.3.4 Shortage of Allografts

With renal transplantation now being recognised as the treatment of choice for the renal failure patients, there is increasing demand for new organs. Despite the efforts to increase the number of organs, there still remains a wide gap between the availability of organs and potential recipients. Over the last decade there has been a steady increase in the number of donors and transplants mostly from an increasing number of DCD and live-donors. In the last few years there has been some decline in the number of patients on the waiting lists but still the gap remains wide. There are multiple reasons for this wide gap. Firstly due to transplantation being a very successful and relatively safe procedure has made way for more elderly patients who years ago would not be considered suitable or too high risk for transplantation. The ageing population also means that there are more patients with ESRF. Obesity and worsening incidence of

diabetes has also increased this number. Plus the very success of transplantation has meant that more patients will be suitable for re transplantation once the first transplant fails.

All these factors mean that there has been ever increasing pressure on the transplant waiting lists. For many years the annual number of patients added onto the waiting lists was fairly static at around 2000 but more recently this number has risen to around 3300 per year.

In the UK there are around 21,000 patients on dialysis while only 7000 are currently on the waiting list for a kidney. Although most of these 21,000 patients will have significant medical problems precluding consideration for transplantation but had there been more organs available, quite possibly more of these patients could be considered for a transplant.

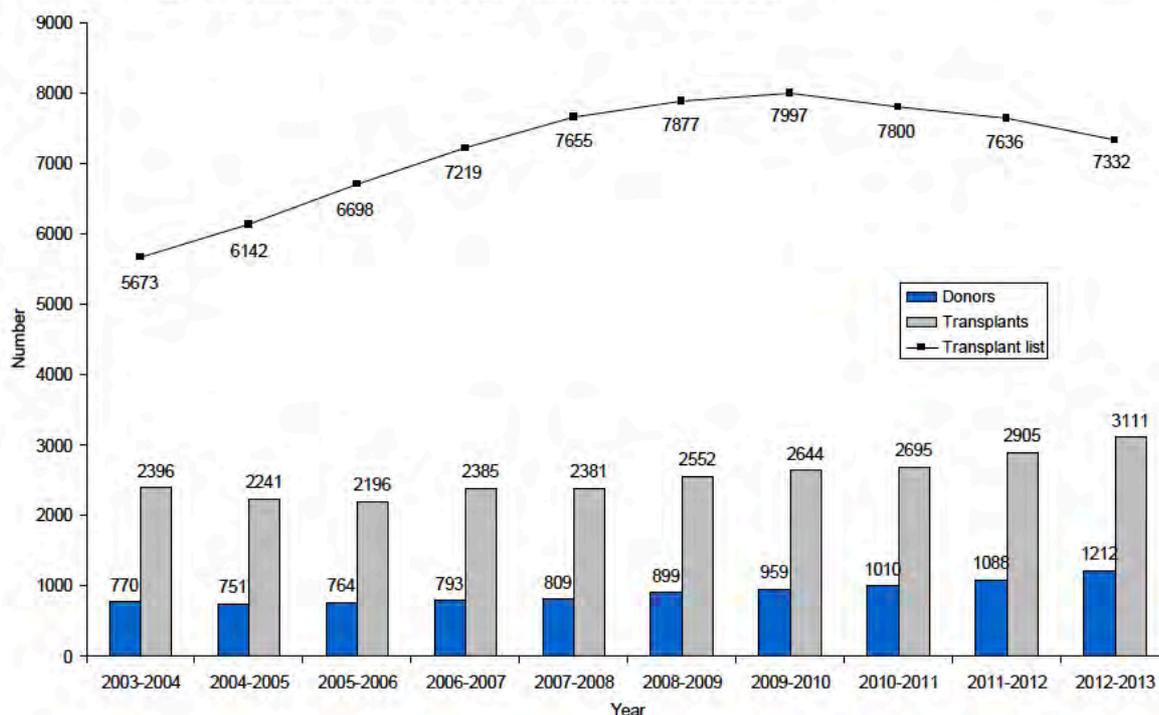


Figure 3 Discrepancy between the organs available and number of patients on transplant waiting list. Number of deceased donors and transplants in the UK, 2003-2013, and patients on the active transplant lists. (18)

1.3.5 Improving organ availability

With so much stress on the availability of organs, various strategies have been used to increase the number of organs. Two broad ways are to improve the quality and survival of transplanted organs to reduce the number of patients going back on waiting list for a re-transplant and to look for new sources of organs.

1.3.5.1 Better Immunosuppression

Immunosuppression is no way near perfect but it definitely has come a long way. These days less than 25% of patients suffer acute rejections and early graft losses has been rare(21)(22). This better early function also translated into

better long term graft survival as the patients with higher post-transplant creatinine levels had a slow, steady decline in renal function as compared to patients with better function in the initial stages(4). Death with a functioning graft is one of the leading cause of graft loss(23). This is due mostly to cardiovascular disease, infection, malignancy and diabetes(24).

Thus along with looking for new and improved immunosuppressive medications, there is a great desire to reduce cardiovascular risk factors, one of the biggest cause of death post-transplant beyond 1st year.

1.3.5.2 *New Sources*

The search for new sources of organs has been on going for a number of years now. With renal transplantation now being established as the gold standard treatment for ESRF, there is ever more increasing need to look for new sources. Over the years the type of patients accepted for donation has become less strict, both due perhaps to improved outcomes secondary to better immunosuppression and also due to increasing pressure. This lead to the Expanded Criteria Donors (ECD). These are donors over the age of 60 years or more; or over 50 years with either hypertension, raised creatinine or death by cerebrovascular(25).

Improved donor management (short cold ischaemia, better perfusate, machine perfusion and possibly ECMO etc.) in DCD donors has led to increasing number of improved organs available for transplant.

Similarly dual kidney transplant has been shown to be a viable option for marginal organs which otherwise would be discarded due to poor predicted

function post transplantation. Again when selected carefully they have shown comparable outcomes to standard criteria donors and superior results to ECD donors(26).

For live donation kidney paired donation has the potential to increase the organs available for transplantation where there are issues of ABO or HLA incompatibility, sensitization of recipients, age or graft size difference(27). There are algorithms to create matches in the donor pool by simultaneous 2 way exchanges in most cases, although there are examples of more complex exchanges in the literature.

ABO and HLA incompatible transplantation has also helped to increase the number of transplants. Ideally, however patients should be transplanted with matched ABO and compatible HLA combinations but in the absence of any available organs and potential of long wait on the waiting list the long term benefits of incompatible transplants out weight the risks(28).

Despite all these innovative new ways to increase the donor pool, there still is an acute need to look for more organs. One such source is by using the kidneys removed for small renal cell carcinomas and transplanting them after ex vivo resection(29).

1.4 PRIMARY RENAL TUMOURS

1.4.1 Incidence

Renal cell carcinoma accounts for around 2% of all cancers worldwide. The

majority of kidney cancers are RCC arising from the parenchyma of the kidney. The incidence of RCC has increased in Western countries in the last few years owing to the widespread use of US and CT scanning(30)(31).

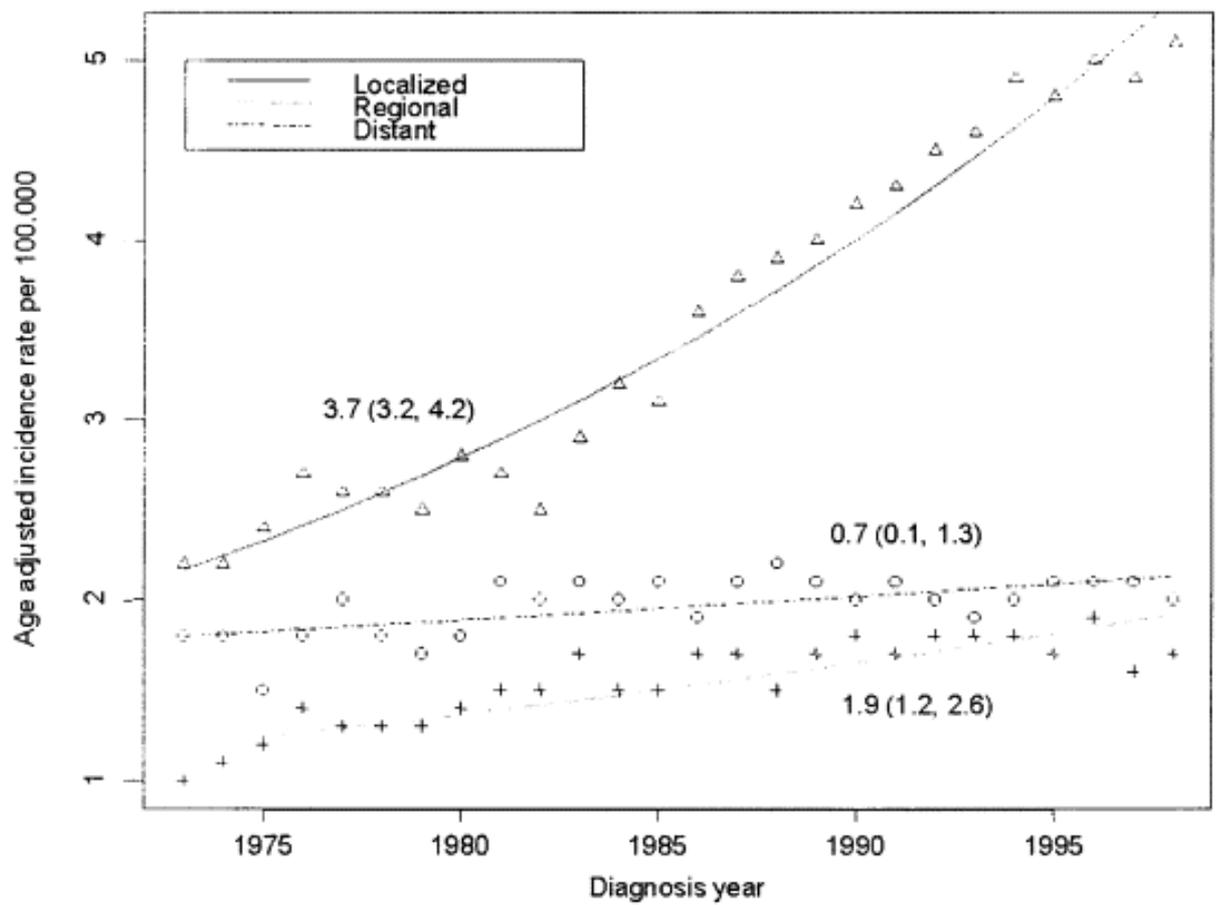


Figure 4 Incidence of RCC. Over the past decades, the incidence has gradually increased. This is due largely to increasing use of ultrasounds and CT scanning in clinical practice(32).

Most RCC's are now picked up at an early stage on investigations done for

other reasons(33). Furthermore the incidence of RCC in allografts will continue to increase as older people donate organs and graft survival is improved by better immunosuppression leading to older recipients with long time on immunosuppressive medications.

Longitudinal studies have shown that many small tumours have a slow growth pattern with low metastatic spread in tumours of <3 cm(34). This increase in the incidence is seen across all stages of the renal tumours but the biggest increase has been noticed in the small renal tumours. Autopsy studies have shown that RCC are present in 1%–20% of patients dying from unrelated cases, meaning that many of the tumours will not prove to be clinically significant in the course of patient's life. Due to these observations it's not always easy to make a management plan for some of the small renal tumours. According to Bosniak(35), a series of 43 small renal cell tumours when followed up for a up to 8 years showed variable growth. In up to half of these tumours the growth rate was less than 4mm/year and when subsequently 29 of these tumours were resected the majority came back as RCC with only a few being oncocytomas. Despite the majority of these tumours being slow growing there were cases of distant metastasis in a few cases even with these small tumours in a subsequent series. Although small, the risk of distant spread remains. Thus in majority of the cases even these tumours require surgical resection as a general rule unless there are specific contraindications.

1.4.2 Clinical features and Staging

Clinical features of renal cell carcinoma can be very non-specific. The most

common symptom is haematuria (40%) followed by flank pain and less commonly a palpable mass. Other features like malaise, fever night sweats, hypocalcaemia and weight loss are the symptoms of advanced disease and metastasis.

Renal carcinomas can also present with paraneoplastic effects due to cytokine release from the tumours (interleukin 6, erythropoietin). These can lead to hypertension, neuropathy, hypercalcaemia, raised erythrocyte sedimentation rate (ESR) and erythrocytosis.

In the majority of cases RCC presents as an incidental finding on routine radiological investigations. The other big category is where the tumour presents with distant metastasis or paraneoplastic disease. Only 5-10% of cases present with the classic triad of haematuria, flank pain and palpable mass. The staging and TNM classification is as soon in table 1.

Table 1. TNM classification and staging for renal cell carcinoma

T1a	Tumour < 4 cm in greatest dimension, limited to kidney
T1b	Tumour > 4 cm but < 7 cm in greatest dimension, limited to kidney
T2	Tumour ≥ 7 cm in greatest dimension, limited to kidney
T3	Tumour extends into major veins or invades adrenal gland or perinephric tissues, but not beyond Gerota's fascia
T3a	Tumour invades adrenal gland or perinephric tissues but not beyond Gerota's fascia
T3b	Tumour grossly extends into renal vein(s) or vena cava below diaphragm
T3c	Tumour grossly extends into vena cava above diaphragm

T4	Tumour invades beyond Gerota's fascia
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single regional lymph node
N2	Metastasis in more than one regional lymph node
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage	T stage	N stage	M stage
I	T1	N0	M0
II	T2	N0	M0
III	T1	N1	M0
	T2	N1	M0
	T3	N0	M0
IV	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
	Any T	N2	M0
	Ant T	Any N	M1

TNM classification and staging of renal cell carcinomas Ng et al. (36)

1.4.3 Treatment

Surgical treatment of renal cell carcinomas can either be with radical resection or Partial nephrectomy.

For selected small RCC cryoablation or radiofrequency ablation can be utilised as primary mode of treatment.

1.4.3.1 *Radical nephrectomy*

Total nephrectomy can now either be performed via open or laparoscopic approach. It now is regarded as an alternate standard of care for the treatment of small renal cell carcinomas due to comparable long-term outcomes with partial nephrectomy.

In classical radical nephrectomy, all the perinephric fat from the level of diaphragm is resected to include any local tumour extension, along with adrenalectomy and lymph node dissection(37). To prevent the patients from becoming adrenal insufficient, adrenal sparing radical nephrectomies are also performed. Pre-operative CT scanning is very important in planning the type of operation. It provides valuable information about the presence of local metastasis to the adrenal gland along with more significant distant disease.

The stage of the disease also heavily dictates the appropriateness of the type of operation. Tumours bigger than 7cm are best treated with radical nephrectomy, as the long-term outcomes are better. Generally patients with larger tumours (around 10cm) should have open radical surgery rather than laparoscopic(38)(39).

Anatomical position of the tumour also has important bearing, as generally hilar tumours are much more difficult to treat with partial nephrectomy. Lymph node involvement or cavoatrial extension also should generally be treated with radical surgery(40)(41).

1.4.3.2 Partial (Nephron-Sparing) Nephrectomy

Partial nephrectomy is a relatively new technique for the treatment of renal tumours and again can be performed via an open or laparoscopic approach. The following factors influence the choice of partial nephrectomy over the radical surgery

- Solitary kidney
- Small multiple tumours
- Bilateral disease
- Conditions predisposing patients to increased incidence of renal cancers (e.g. von Hippel-Lindau disease)
- Patients with either established CKD or predisposed to get CKD

Generally, most tumours less than 7 cm will be suitable for partial nephrectomy. With the long-term recurrence rates and patient survivals being comparable and

with the benefit of improved long-term eGFR than the radical nephrectomy patients, PN is now the first line treatment.

During partial nephrectomy, the rest of the kidney should be examined for any synchronous tumour as its presence might mean the need to change to a radical operation. Again with most of the partial nephrectomies, the adrenal gland is left in situ due to low incidence of adrenal metastasis excluding the upper pole tumours with direct invasion(42).

There is potential of leaving some tumour behind if the surgical margins are positive. For this reasons some centres routinely perform frozen section to confirm resection of positive margins but the evidence is not very clear on the use of frozen section as a few studies have indicated no significant difference in the recurrence free survival between the two cohorts(43)(44).

1.4.4 Current Guidelines for treatment

Due to better and wider spread use of cross sectional imaging, there has been a steady increase in the number of incidentalomas (incidentally detected tumours). This has also meant that most of the renal tumours picked up these days are smaller in size, limited to the kidney and have relatively better outcomes after appropriate treatment.

There is a huge variation of type of renal tumours and there has been a gradual shift towards treating more and more patients with less aggressive surgical option of partial nephrectomy. Partly this is because around 20% of renal tumours removed turn out to be oncocytomas with very little malignant potential at all and RN may not be the best surgical treatment.

The other big reason behind the shift towards NSS has been the recent

longitudinal and few randomized trials which have shown that the long term creatinines are better in partial nephrectomy cohort(45)(46)(47).

This effect of improved renal function is due directly to preservation of the number of functioning nephrons. Patient factors and technical aspects such as ischaemia times are also important in the final eGFR but where more renal parenchyma was resected the eGFR was negatively affected even in PN patients(48)(49).

Due to all these reasons, where technically and clinically feasible partial nephrectomy is preferred over the radical surgery.

There is higher risk of urine leak, fistulas and bleeding (50)(51)(52) in patients undergoing partial nephrectomy which may make this procedure not the best possible option for a number of patients where the primary goal may be more importantly a quick and relatively less complicated procedure than mild to moderate preservation of renal function.

1.4.4.1 Current situation of treatment

There is also evidence that despite these clear recommendations there are large number of patients who undergo RN rather than PN(53). This is due to multiple reasons, including local expertise, patients' choice, tumour anatomy and patients' comorbidities.

To further explore the current situation at a more local level we audited the practice at our hospital (Freeman hospital, Newcastle upon Tyne), which is a urology referral centre and has surgeons who regularly perform both radical and partial nephrectomies both via open and laparoscopic approaches.

Table 2. Percentages of patients undergoing radical and partial nephrectomy

Year	Total	Radical Nephrectomy	Partial Nephrectomy
2004	21	18 (86%)	3 (14%)
2005	18	12 (67%)	6 (33%)
2006	49	37 (76%)	12 (24%)
2007	42	23 (55%)	19 (45%)
2008	31	19 (62%)	12 (38%)

The above table indicates the number of small renal tumours removed at our hospital alone. The pattern of surgery has been changing with more partial nephrectomies by keyhole surgery, which obviously wouldn't be suitable for secondary procedure. However even with this there were 19 nephrectomies in 2008. All of these kidneys could have been transplanted by the Cincinnati criteria (to be explained later). These numbers are from a centre with laparoscopic urological surgery available; many units around the UK don't have this facility and therefore potentially do more radical nephrectomies for small renal cancers. If we could determine how we could treat inadvertent transfer of tumour cells in the presence of immunosuppression we could develop a safer strategy of using such kidneys and so develop an alternative source of donor organs.

1.5 POTENTIAL OF USING ORGANS FOR TRANSPLANTATION AFTER TUMOUR RESECTION

1.5.1 Literature review

1.5.1.1 *Transplantation after partial nephrectomy*

Normal practice when confronted with a tumour of kidney on procurement is to return it to the donor and not use any other organs (54). In cases of deceased donors it meant that the contralateral kidney couldn't be used as well because of the concerns of micro metastasis and bilaterality of some of the renal cell carcinomas (RCC). Penn (55), reviewing the Cincinnati transplant tumour registry (CTTR), described a total of 14 cases of ex vivo resection of small renal cell cancers detected incidentally followed by transplantation. Frozen section was employed, and where margins were clear, kidneys were used although it is not clear whether all of the tumour bearing kidneys underwent frozen section. Of the cadaveric donors, the contralateral kidneys, all of which appeared healthy, were transplanted as well. Apart from these cases of renal carcinomas, there was one case of oncocytoma within the kidney, which was transplanted after resection. Of all the cases where the tumour was adequately resected before transplantation there was no recurrence in a follow-up ranging up to 210 months. Buell *et al.* (54) presented 14 cases of transplantation after renal tumour resection from the same database as used by Penn. No recurrence has been noted up to a follow-up of 200 months. Median tumour size was 2.0 cm (range 0.5– 4.0 cm) and all were of low histological grade. They have described

two further cases since the initial data review with no recurrence and good graft function. A similar case series from Australia (56) only included elderly recipients or those with significant co morbidities and high chance of death without transplantation. Furthermore the recipients had high levels of HLA mismatching with the donors and were selected on the basis that if there was a recurrence to occur, stopping immunosuppression may help in tumour lysis by recipient's immune response. 41 patients received kidneys after ex vivo resection of tumour of which 10 were reported as benign lesion on histopathology. One patient returned to dialysis after 30 months. 4 patients died of unrelated causes. There was only one recurrence noted 9 years after transplantation out of the remaining 30 patients. Notably this tumour recurrence was at a distance from the initial resection site, this therefore might not be a tumour recurrence but another primary within a "field" change renal tissue. The patient refused any further treatment, and the lesion has grown 0.2 cm in 18 months since diagnosis. In a follow-up study on these patients this group has recently published long-term outcomes, which are significantly better than wait-listed patients on dialysis and are comparable to the live unrelated transplants (57). Mannami *et al.*(58) from Japan published a series of 42 "restored" kidneys from live donors. Eight donors with small renal cell carcinoma (<3.5 cm) underwent donor nephrectomy and ex vivo resection of the tumour followed by transplantation of the kidney. Five patients were alive, three with functioning grafts, two died with functioning grafts from unrelated causes, and one was lost to follow-up. No tumour recurrence has been noted in any of these patients. Another 8 patients had donor nephrectomies, which had benign diseases of which 5 had partial resection and kidneys were used for transplantation. Three

recipients are alive with functioning grafts, while four have gone back to dialysis (after 3,18,51,73 months). One recipient died of unrelated pathology. There have also been 6 case reports (59)(60)(61)(62)(63)(64) of live related kidney donation when a tumour was detected incidentally in or ex vivo and the kidney was transplanted after resection. No recurrence has been noted in any of these cases with a follow-up of up to more than 10 years.

1.5.1.2 Partial Nephrectomy for Tumours Diagnosed after Transplantation

Renal cell carcinoma represents around 4.6% of all the tumours in allograft recipients with only 10% of these occurring in the allograft itself (55). The other main subgroup is when a tumour was detected after transplant. Again the standard practice here has been to perform transplant nephrectomy (65) with the patient invariably returning to dialysis and normally being put on a waiting list for another transplant if feasible.

Until now, more than 50 cases of allograft renal cell tumours have been described in the literature of which at least 35 cases have had nephron sparing surgery (NSS) for their allograft tumour (66)(67)(68)(69)(70)(71)(72)(73)(74). Tumour sizes have ranged, from 0.5 to 4.0 cm although there have been two case reports of larger (6–8 cm) tumours all being successfully treated with NSS (68)(67). Postoperative follow-up is from one month to more than 10 years with one recurrence 5 years after NSS in renal allograft (75). This was in a 74-year-old recipient five years after initial transplant. A 2.4 cm RCC was incidentally

detected without any evidence of distant metastasis. It was treated with radical nephrectomy and the patient has been disease free on haemodialysis after that.

1.5.1.3 *Contralateral Transplanted Kidney with a Renal Tumour*

These kidneys again are normally not used as RCC can be bilateral especially the papillary subtype (76). Penn has described 14 cases in which the contralateral kidney was transplanted from patients with renal tumour. One patient had recurrence in the allograft, which was removed for rejection. This patient died 75 months after transplantation from a de novo cancer of one of his own kidneys. The remaining patients did not have any recurrence with a follow-up ranging from 0.5 to 153 months.

Nicol *et al.* (56) described 2 similar cases with no recurrence. Barrou *et al.* (76) has described a case of two allograft recipients from a single donor with a tubulopapillary tumour (17mm) in the right kidney; only the left kidney was utilized for transplantation. Shortly after transplantation, the recipient underwent an ultrasound (US) examination of the allograft, which did not reveal any tumour. Three months later a biopsy was done for rejection, which revealed a poorly differentiated tumour, and the patient underwent radical allograft nephrectomy. No additional chemotherapy was given apart from discontinuation of immunosuppression (prednisolone and azathioprine). Lymph nodes that had been noted to be enlarged on CT scan disappeared two month after nephrectomy. The patient underwent re transplantation two years later and was disease free and dialysis independent at 3-year follow-up. Another patient

received the heart transplant from the same donor but died from bony metastasis from the renal cell carcinoma.

1.5.1.4 Accidental Transplantation

In at least 4 cases (55)(61)(69)(77) there have been accidental transplantation of RCC mistaken as a benign pathology on procurement. Partial nephrectomy/enucleation in all these cases was performed before transplantation with adequate resection margins. Routine histopathology revealed the resected tumour to be malignant. All recipients retained the allograft because of complete excision of the tumour and were kept under close follow-up with no recurrence so far. The cases where there have been transplantation of tumour, either partially resected or unrecognized at the time of transplant have resulted in disastrous outcomes (55)(76)8.

1.5.1.5 Miscellaneous

Manammi *et al.*(58) reported a series of 8 patients who underwent nephrectomy for a distal ureteric transitional cell carcinoma (TCC). One patient had a recurrence of TCC after 15 months and was offered graft nephrectomy but opted for partial resection of ureteric tumour to prevent returning to dialysis. He died three years after partial resection from a squamous cell carcinoma of lung with liver metastasis. His TCC also had squamous metaplasia and a DNA study to determine exact origin of primary tumour could not be established because of inadequate tissue samples. The remaining patients were either alive with functioning grafts or died of unrelated causes.

1.5.1.6 Opinion of Patients and Transplant Specialists

Transplantation of kidneys with cancers is a novel idea not only among patients but also among the transplant community. To be able to exploit this potential donor pool it is of utmost importance that both the health care specialists; transplants surgeons and nephrologists and the patients (both donors and recipients) are comfortable with the idea of using such kidneys. To determine this, structured questionnaires were sent to focus group of patients on the North East renal transplant waiting list, post nephrectomy patients for small renal cancer, nephrologists and transplant surgeons in the UK.

Results are shown in Table 3 and have a generally high response rate. Those respondents that had lost their kidney, removed for tumour, had the highest consent rate and patients potentially receiving such kidney the lowest. The transplant surgeon and nephrologists had views somewhere in between. This survey was done in UK from where there have been no case reports of using organs after removal of tumour and but still the response was largely favourable. Given that since this survey there has been an increase in total number of such organs being utilized, one can extrapolate that current belief may be more favourable.

Table 3: Opinions of transplant patients and clinicians

Respondents	Response rate	Support use of kidneys
Potential recipients on waiting list	97% (113/116)	59% (67/113)
Previous nephrectomy (potential donors)	100% (15/15)	93% (14/15)
Nephrologists	58% (94/161)	78% (73/94)
Transplant surgeons	66% (43/65)	72% (31/43)

Opinion of Patients and Transplant Specialists (29)

1.5.1.7 *Summary of literature review*

One of the worries about transplantation of tumour-affected kidneys is the potential of tumour recurrence and growth in a state of potential immune inattention due the immunosuppressive therapy. Renal cell carcinoma is known to be an immunogenic tumour (78) but in the presence of immunosuppression? If there was any transplantation of tumour cells in the host, then there is a potential of continued growth in a host with a compromised immune system. Furthermore, immunosuppression in itself has been known to increase the incidence of de novo malignancy (79)(80). Because of these concerns, an immunosuppressive agent with no potential to increase de novo malignancy and better still to have antitumour activities would probably be ideal.

The incidence of RCC has increased in Western countries in the last few years owing to the widespread use of US and CT scanning (81)(33). Most RCC are now picked up at an early stage on investigations done for other reasons. Furthermore the incidence of RCC in allografts will continue to increase as older

people donate organs and graft survival is improved by better immunosuppression. Longitudinal studies have shown that many small tumours have a slow growth pattern with low metastatic spread in tumours of < 3 cm (82)(34). Autopsy studies have shown that RCC are present in 1–20% of patients dying from unrelated causes, meaning that many of the tumours will not prove to be clinically significant in the course of patient's life (83)(84).

The gold standard treatment of resectable renal cell carcinoma has been radical nephrectomy. Recent evidence has changed this practice dramatically as survival after radical nephrectomy (RN) and partial nephrectomy (PN) has shown to be comparable (85). Favourable outcomes have been observed after NSS for < 4 cm RCCs and RN has been described as “surgical overkill” (86) for these tumours.

Furthermore, local recurrence after NSS has been reported to be < 5% with recurrences mostly associated with large and multifocal tumours. A significant risk of dying in patients on dialysis particularly in older patients has been one of the driving forces to increase the number of kidney donors. Renal transplantation seems to confer a substantial survival advantage over dialysis in patients with end-stage renal failure (87). A significant number of patients accepted for dialysis are older patients, who have a mortality risk of 25%. With longer waiting times for a transplant, it is inevitable that many of the patients will die before they can receive a transplant which would have improved their quality of life and longevity(87). Furthermore 16 to 23% of suspicious lesions resected from kidneys are either benign or of low malignant potential (88)(89) and not using these kidneys with small tumours after partial nephrectomy for transplantation seems wastage of precious organs when one considers the

benefits of transplantation over dialysis.

A suspicious lesion found at multiorgan retrieval should have an excision biopsy and histological confirmation of clear margins before any of the organs can be transplanted. A malignant lesion in the kidney when unrecognized and transplanted continues to grow under the immunosuppression carries high risk of metastasis and can result in fatal outcome. If the biopsy confirms clear margins with favourable histology then these organs could be used for transplantation, as risk of recurrence is very low. The situation is more complex when it comes to using restored organs from live (related/unrelated) renal cell carcinoma patients. Major difference being that these are living cancer patients first and therefore must never be treated primarily as potential organ donors to prevent any bias in treating their primary problem which may lead to provision of less than optimal treatment and ultimately harm to these patients. This is shown by Takahara *et al.* (90) in their review of Mannami *et al.* series concerning a ureteric carcinoma patient, where adherence to standard practice for treating these tumours was not practiced with disastrous consequences. With changing trends, radical nephrectomy is now regarded as an alternate standard of care to partial nephrectomy for T1a tumours when partial nephrectomy is not technically feasible. This is due to the comparable oncological outcomes after partial nephrectomy and evidence that radical nephrectomy is an independent predictor of low GFR. A positive outcome for a recipient can never justify harm to a live donor; on the contrary, for a transplant with a live donor to be regarded as a success means that both the recipient and the donor have done well(91). Live related donors in Nicol *et al.* series were given the options of observation,

radical or partial nephrectomy without any mention of the possibility of use of organs for transplantation. Only after the patients had decided to opt for radical nephrectomy possibility of domino donation was discussed. This approach has the benefit of making sure that patients make their own decisions without any pressure from clinicians. Another important factor is to make sure that beliefs of the clinician do not affect the patient's treatment choices.

Importance of detailed informed consenting cannot be over emphasised for the recipients of such restored organs. All the relevant information especially of the origin of the organ and potential of recurrence and associated risk must be discussed fully and the patients understanding checked. Routine follow-up of the patients with annual US have been suggested to make sure any recurrence is diagnosed as early as possible. Tumours have been detected at early stage with better outcomes because of regular follow-ups. If one kidney is found to have a tumour it is important that the other kidney is closely followed up. It is easier in the live donor setting when the donor can be carefully followed up but in cadaveric donation there has to be a central database for tracking the contralateral kidney (70) which might be transplanted into a recipient in a different unit.

Immunosuppression is essential after transplant and unfortunately this has been associated with the higher incidence of cancers in recipients as opposed to the general population (80). Certain newer immunosuppressive agents have anti-tumour (92) activity and their use can, in theory not only reduce the chances of recurrence but they can also be used to treat patient should a recurrence occur.

Furthermore the Human Tissue Act 2004 (93) that covers the use of organs for

transplant in the UK allows anyone to be a donor including live related and unrelated (altruistic donor) provided there is adequate consenting. This means that donation can also occur from patients suffering from small renal cell carcinoma who have radical nephrectomy as primary treatment provided measures are taken to ensure that these patients are treated appropriately in the first place and both donor and recipients had given informed consent.

1.6 ROLE OF IMMUNOSUPPRESSION IN TRANSPLANTATION

Longevity of successful renal transplants relies most heavily on perioperative and postoperative immunosuppression. Refinements in selection of patients, surgical techniques and HLA matching all have important role in allograft survival but the biggest improvement in graft survival was noted with the introduction of cyclosporine immunosuppression.

1.6.1 Evolution of immunosuppressive agents

Since the introduction of renal transplantation into clinical practice various strategies have been utilised to prevent the rejection. This included transplantation from identical twins, whole body radiation followed by bone marrow transplantation along with renal transplantation during the very early days of transplantation. Although it was initially thought that the success of transplantation was dependent on simultaneous bone marrow and renal transplantation, later it was shown that some success could be achieved with cytoablation and corticosteroids.

After initial success, the next logical step was to look for immunosuppressive

medications to prevent rejection. The initial work was done with methotrexate and cyclophosphamide, which lead to severe bone marrow depression but moderate allograft survival (143 days) without needing bone marrow ablation. This lead to the development of the less toxic 6-mercaptopurine and later azathioprine which though variable in success but at least was not as morbid to recipients as frequently. Azathioprine inhibits DNA and RNA synthesis by interfering with the purine synthesis. This in turn blocks the lymphocyte proliferation and the production of interleukin 2 (IL-2).

Another improvement in immunosuppression came in 1960s in the form of heterologous anti-lymphocyte globulin (ALG)(94). It was used with azathioprine and improved allograft survival further but due to its heterologous nature it was used more for rescue of rejection occurring after transplant. The more 'refined' monoclonal OKT3 superseded it for a short time though most transplant specialists preferred the side effects of ATG to those of OKT3 (the first monoclonal antibody against all T cells).

During this period there were steady improvements in allograft survival but the biggest change came about with the introduction of cyclosporine in early 1980s when it was shown to significantly reduce acute rejection rates seen early soon after transplantation. This point in transplantation is often regarded as a major breakthrough as it paved the way for other solid organ transplantations. These were mainly the vital organs which though possible to transplant, their dysfunction produced death of the recipient. Such that for the transplant recipient to survive, success of the simpler non-vital graft had to be sorted first.

1.6.2 Calcineurin inhibitors (CNIs)

Cyclosporine (CsA) has been the most widely used CNI up to most of 1980s and 1990s for solid organ transplantation until FDA approved tacrolimus in 1997 which lead to it becoming the first line CNI treatment in most of the developed countries. By 2003, around 67% of new kidney transplant recipients were being discharged on Tacrolimus as a maintenance therapy(95).

Although shown to be more potent in preventing rejection than cyclosporine, Tacrolimus shares the basic mechanism of action with CsA.

1.6.2.1 *Mechanism of action*

Due to lack of precise in vitro tests of acute rejection and its effects on transplanted allografts most of the initial understanding of the mechanism of action of CsA had been based on its vitro effects on lymphocytes(96). These in vitro studies have shown that it is a potent inhibitor of generation of cytotoxic T cells as opposed to azathioprine and steroids in patients with solid organ transplantation(97).

The mechanism of action of CsA at molecular level was not described until early 1990s (98) when it was shown to be mainly as a result of CsA binding to cyclophilins in the cell cytoplasm(99). This complex then binds to Calcineurin, a calcium dependent phosphatase that normally has a pivotal role in transcription of interleukin-2 (IL-2). Thus Calcineurin inhibition by CsA leads to inhibition of IL-2 production and other lymphokines which in turn prevents further proliferation of CD4+ T cells and cytotoxic T cells from its precursors(100)(101).

CsA is also shown to exert its immunosuppressive effects by increasing the production of transforming growth factor (TGF)- β both by normal T cells and also in individuals with renal transplantation(102). This in turn inhibits IL-2 production and IL-2 stimulated up regulation of IL-2 receptors, hence affecting T cell responses(103).

1.6.2.2 *Benefits and side effects*

Even from the early days of renal transplantation CsA has shown to be more effective than azathioprine based immunosuppressive protocols in terms of long-term patient and graft survival. This was shown (fig 5) by a multicentre European trial in 1993(104)

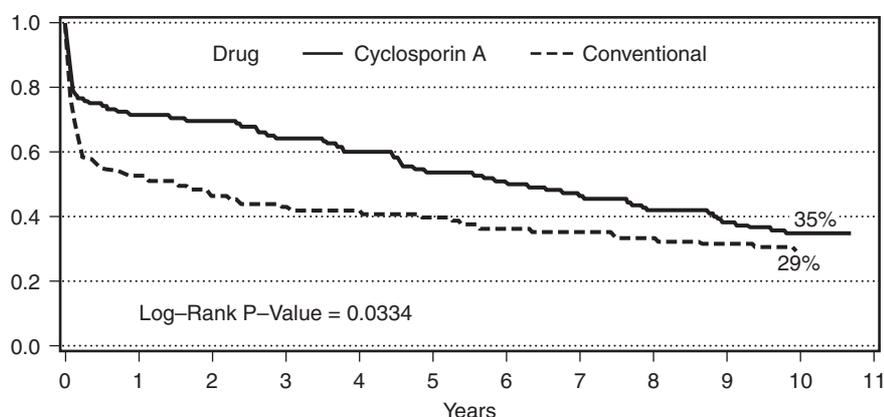


Figure 5 CsA and AzA comparison for long- term graft survival. CsA had better survival when compared to AzA (104)

Since then CsA has been used in various combinations with azathioprine, steroids, mycophenolate and induction agents with steady improvements of graft survival rates.

The groups of patients benefiting mostly were the elderly, very young, patients

with diabetes and sensitized patients as in pre cyclosporine era most of these patients were thought not suitable for transplantation.

Despite being a revolutionary drug in the field of transplantation CsA is not without its side effects. The most important side effect is of nephrotoxicity, which can be immediate, acute or chronic.

Acute nephrotoxicity is thought to be the result of reduction in blood flow and increased vascular resistance. It is quite important to differentiate this from acute rejection as the treatment course is completely opposite for these two conditions.

Chronic CsA nephrotoxicity is also a big problem in the long term and is thought to be a result of increased circulating levels of TGF- β .

Apart from major nephrotoxic side effects, CsA can cause hypertrichosis, hyperkalemia, hypomagnesemia, hyperuricaemia, glucose intolerance, reversible hepatotoxicity, neurological disturbances and minor gastrointestinal upsets.

1.6.2.3 *Cancer risk*

One of the other most serious adverse effects of CsA is development of neoplasia. As with other immunosuppressive medicines there is an increase in the incidence of lymphomas(105) and skin cancers post solid organ transplantation. The normal tumour scavenging ability of the competent immune system is disabled leading to more aggressive and invasive tumours.

The effects of CsA on tumour development are studied in detail and it is believed that the overall level of immunosuppression is also important as well as the specific agents used. It has been shown by Hojo that, independent of its

immunosuppressive effect, CsA can lead to morphological changes including increased cell motility and anchorage independent invasive growth leading to increased number of metastasis in a mouse model. These effects were attributed to the raised TGF- β levels associated with CsA treatment as these were counteracted by anti TGF- β monoclonal antibodies(106). Tumour angiogenesis by vascular endothelial growth factor dependent mechanism can also account for the increased incidence of tumours with CsA treatment (107) as opposed to Rapamycin.

CsA also increased IL-6 production in Epstein Barr virus infected cells which leads to increased proliferation of B cells and possibly be the cause of lymphoproliferative disorders(108).

1.7 NEWER IMMUNOSUPPRESSIVES WITH ANTI-NEOPLASTIC EFFECTS

The search for an ideal immunosuppressive agent did not stop after CsA showed initial promising results as it had some major side effects in the long run especially chronic allograft nephrotoxicity blunting the initial advantage over contemporary immunosuppressives and also the increased incidence of cancer. Sirolimus, everolimus and Leflunomide are a few agents, which have both the immunosuppressive properties and also are known for anti-neoplastic effects.

1.7.1 Mammalian Target of Rapamycin (mTOR) inhibitors

Two of the most common mTOR inhibitors are Sirolimus and Everolimus. Most of the animal and clinical studies for mTORs have been done with Sirolimus. These agents are very closely related to each other although in future there

may emerge some important clinical differences.

1.7.1.1 *Mechanism of action*

Sirolimus is a fermentation product of the microorganism *Streptomyces hygroscopicus*. It is a potent inhibitor of T and B cells as a response to antigen stimulation. For the activation of lymphocytes there has to be interaction of interleukins and IL receptors as well as co-stimulatory molecules (CD28), which then leads to activation of TORC1 complexes. Sirolimus binds to FK binding protein 12(FKBP 12) and this complex blocks the activation of TORC1 complex leading to cell cycle arrest in late G1 phase(109).

It also has a direct inhibitory effect on the apoptosis of dendritic cells which have a pivotal role in the transplant immunology(110). It also inhibits cytokine and growth factor stimulated proliferation of the fibroblasts, tumour cells and smooth muscle cells(111)(112)(113).

1.7.1.2 *Role in transplantation*

There have been several studies looking at the role of sirolimus either as a primary immunosuppressive agent or as a replacement of cyclosporine. The Sirolimus European Renal Transplant Study Group, which looked at first cadaveric renal transplant recipients receiving either CsA based or sirolimus based primary immunosuppression showed very similar graft and patient survivals as well as episodes of acute rejections(114). Similar results were noted when azathioprine used in the above-mentioned study was replaced with mycophenolate mofetil (MMF). The acute rejection rates and patient and graft survivals were similar with different side effect profile(115). An RCT performed

in 2006 compared sirolimus MMF and prednisolone with tacrolimus based immunosuppression and again showed these two to be comparable although the acute rejection rate was higher in sirolimus based regime but it did not reach statistical significance(116). Two large trials from USA showed significantly improved acute rejection rates and graft survivals with CNI based immunosuppression when compared to sirolimus based treatments(117)(118). The Rapamune US study group showed reduced acute rejection rates with sirolimus when compared with azathioprine in CsA and steroid based regimes but the 12-month creatinine was lower in sirolimus group(119).

Sirolimus has also been used as sole agents in the maintenance phase of immunosuppression after transplantation. One of the first big trials with more than 400 patients from Manchester showed better creatinine clearance in the sirolimus arm when it was used as a maintenance immunosuppressive after the withdrawal of CsA at 3 months post transplant(120). An RCT from Cambridge suggested that the impaired graft function possibly due to CNI nephrotoxicity could be reversed with sustained improvement at 2 years post transplant by conversion to sirolimus at three months. This along with other similar studies point to potential benefits of sirolimus as a maintenance agent in terms of graft function and to potentially reduce the incidence of chronic allograft nephropathy(121).

1.7.1.3 *Side effects*

It was the result of large studies where sirolimus was used alone rather than in combination with CsA that shed light on potential side effects of sirolimus. Most of the side effects are the result of the immunosuppressive and anti proliferative

effects although some remain to be clearly explained so far.

The risk of infection is not much different from other immunosuppressive agents. This effect is most probably due to its immunosuppressive properties. Hyperlipidaemia can be a problematic side effect as most of the renal transplant patients are arteriopathies as well and are already at a high risk of heart disease. More than half of the patients on sirolimus treatment develop increased levels of triglycerides and cholesterol, which may also have implication with chronic allograft rejection(122).

Among one of the most serious side effects is pneumonitis. Symptoms can vary from fever, fatigue cough to pulmonary failure.

From a surgical point of view, delayed wound healing is an important side effect. This may lead to skin and soft tissue infections or poor wound healing internally at the anastomosis sites e.g. ureteric anastomosis. These effects have been linked to its effects on fibroblasts and neovascularization. Developments of lymphocoeles are also more common in patients on sirolimus treatment.

Other side effects include skin rashes sometimes needing dose reduction or even withdrawal of treatment, anaemia, thrombosis, aphthous ulcers, proteinuria among other minor side effects.

1.7.1.4 *Effects on neoplasia*

mTOR inhibitors belong to the relatively new group of immunosuppressive medications, which have some anti tumour potential as well. This effect can be very useful as all the patients on immunosuppression are at an increased risk of

developing malignancies and if a good immunosuppressive agent can also be able to have potent anti tumour properties then it can solve this major issue.

Both in vitro and in vivo studies have shown that sirolimus has both these properties. Although mTOR inhibitors have potent immunosuppressive effects, which in itself are risk factors for tumour cells to evade one's immune system, but it's anti tumour effects are significantly potent as well.

There are several studies which suggest that with sirolimus based immunosuppression the over all rates of de novo malignancies are significantly less than CsAs. A large retrospective analysis was performed on post transplant malignancies in more than 33 thousand deceased donors from 264 centres in USA. This study looked at both the skin and non-skin solid organ malignancies rates and showed that the rate of malignancies with sirolimus/everolimus and mTOR plus CNI combination was 0.6% as compared to CNI alone which was 1.81%(92).

Efficacy of mTOR inhibitors has also been shown in cases of advanced renal cell carcinoma. A large double blind trial randomised 272 patients into an Everolimus group and 138 in a placebo arm for the treatment of advanced renal cell carcinoma. There was progression of disease in 37% of patients in the treatment arm as opposed to 65% in the placebo arm (p 0.0001) with a median disease free survival of 4.0 versus 1.9 months(123).

Similar encouraging results were noted when Everolimus was used for the patients with advanced pancreatic neuroendocrine tumours. 207 patients in the treatment were compared with 203 patients in placebo arm and were found have significantly better progression free survival of 11 months as compared to

4.6 months in the placebo arm ($p < 0.001$). 34% of patients were still alive at 18 months as compared to 9% with placebo(124).

mTOR inhibitors were found to be useful in hepatocellular carcinomas and in advanced breast carcinomas as well(125)(126).

mTORs have been shown to be effective for the post transplant lymphoproliferative disorders as well. A case of complete remission of disseminated PTLD has been reported in literature after conversion to sirolimus(127).

With an increasing cohort of patients on immunosuppression post transplant there is an increasing incidence of Kaposi's sarcoma. mTOR inhibitors have been found useful for their treatment as well. A case series of 25 patients with cutaneous Kaposi's sarcoma when converted from cyclosporine to sirolimus showed complete biopsy proven remission within 6 months of treatment(128).

These effects of mTOR inhibitors are very encouraging and over the past 10 years there has been mounting evidence on their efficacy. Although by no means these are the perfect solutions but a medication to be immunosuppressive and anti neoplastic in transplant setting is very useful feature that we wished to explore.

1.7.2 Leflunomide

Leflunomide has also been used for immunosuppression after solid organ transplantation with variable success. It has been the analogues of the metabolites of Leflunomide that have shown most promise, as the half-life of the drug is more manageable. The metabolites of leflunomide are called

malononitrilamides (MNAs) and FK778 is the most common and well-studied agent in organ transplantation.

Currently leflunomide is used approved as a disease-modifying agent for rheumatoid arthritis, although there are several possible applications of this drug.

1.7.2.1 Mechanism of action

Leflunomide exerts its immunosuppressive and anti-inflammatory effects through multiple pathways. The most important mechanism of action is the inhibition of pyrimidine synthesis. Activated lymphocytes depend exclusively on the de novo synthesis of the uridine monophosphate, as they are unable to use the pyrimidine salvage pathway. Leflunomide inhibits the mitochondrial enzyme, dihydroorotate dehydrogenase which is responsible for the production of pyrimidines(129)(130), leading to the inability of lymphocytes to synthesise RNA and DNA.

The other main mode of action of leflunomide is the inhibition of tyrosine phosphorylation. With the substitution of uridine in vitro, the inhibition of dihydroorotate dehydrogenase pathway occurs with no effect on tyrosine phosphorylation pointing to a separate mechanism of action(131).

1.7.2.2 Role in transplantation

Leflunomide is currently only licenced for the use in rheumatoid arthritis patients. But in past it has been used for solid organ transplantation.

A phase II multicentre study looked at the primary end point of acute rejection in renal transplant recipients. The study divided patients into 3 groups; high dose

leflunomide, low dose leflunomide and placebo, with all groups receiving tacrolimus and corticosteroids. A total of 149 patients were randomised into these groups. There was no significant difference between the graft survival, but the acute rejection rates were lower in leflunomide group (26.5%, 25.9%) as opposed to placebo (39.1%)(132).

There has also been evidence in animal studies that leflunomide can reduce the chronic rejection in allografts(133).

1.7.2.3 *Side effects*

Most of the insight into the side effects of leflunomide has come from the studies where it was used for the treatment of arthritis. A phase II trial of 358 patients divided into leflunomide, sulphasalazine and placebo, looked at the efficacy and safety profile of leflunomide for arthritis patients has shown that the most common side effects were diarrhoea (17%), nausea (10%), alopecia (8%), and rash (10%). There were transient abnormalities in the liver function of the leflunomide group of patients(134). There have also been reports of anaemia, microangiopathy, oesophagitis and electrolyte disturbances(135)(136).

Leflunomide has shown to have some anti proliferative effects along with immunosuppression and hence is a suitable agent for the inclusion in our study(137)(138)(139)(140).

1.8 **TRANSPLANT IMMUNOLOGY**

Much of the improvement in the outcomes of the allografts after transplantation has been due to the development of better immunosuppressive agents, and it

has been the improving understanding of the immunology of transplantation that has paved the way for better immunosuppressive agents.

Immunological response after transplantation is a dynamic multifaceted process with a wide range of activation and stimulatory mechanisms involved.

Immunological response to the transplantation can be divided into various stages; pre transplantation and post transplantation or by the type of immune system – innate or adaptive.

1.8.1 Innate immune response to transplantation

Even before the organs are transplanted there is plenty of trauma or tissue injury leading to activation of innate immune response against the transplanted tissue.

In cases of donation after circulatory arrest, there is organ injury due to first by warm ischaemia and then by variable periods of cold ischaemia followed by reperfusion injury. In case of donation after brain dead (DBD) donors the overwhelming release of cytokines as direct result of neuroendocrine responses due to brain death also leads to activation of complement and coagulation cascades even before the organs are transplanted.

Up regulation of cell surface adhesion molecules including P selectins and integrins along with the milieu of cytokines helps in the migration of immune mediator cells. There are suggestions that the more the initial injury to the organ, the worse the immunological outcomes(141) probably as a result of the induction of proinflammatory cytokines including interleukin 1 (IL-1), IL-6 and tumour necrosis factor (TNF- α)(142).

The complement system normally is activated as a response to non specific

infections but it can also be stimulated as a result of hypoxia and tissue injury (143)(144) which is a hallmark of organ transplantation as a result of procurement process.

1.8.2 Adaptive alloimmunity

Adaptive immunity or the specific or acquired immunity is more specific and directed than the innate immunity. Hence it requires the effector cells to be stimulated and then migrate to the site of interest, which is allograft in transplantation. The whole process is an intricate combination of antigen presentation, allorecognition and then activation, migration and targeting of the allograft by the effectors cells.

These stages are of paramount importance and are discussed.

1.8.2.1 Antigen recognition and presentation

The immune system distinguishes self from non-self antigens to prevent auto immunity. The histocompatibility antigens are unique to every individual of even the same species and thus are the targets of the immune system in clinical transplantation. The most important and well characterised is the Major Histocompatibility Complex (MHC) antigens but more recently the role of minor histocompatibility antigens have been recognised as well.

Genes in the MHC locus are divided into class I, II and III but class I and II are thought to exert the most influence on the allograft rejection.

Class I MHC proteins are expressed on almost all nucleated cells and generally activate the CD8⁺ T cells and normally present proteins synthesised intracellularly. Class II MHC complexes are expressed on B-lymphocytes,

dendritic cells and some endothelial cells. These complexes are generally responsible for activation of CD4+ cells and generally process extracellular peptides although this is not absolute as there can be some cross presentation(145).

There are three ways the antigens are presented. In the direct antigen presentation the donor derived MCH proteins as well as other allogeneic proteins will be presented to recipient T cells by the donor antigen presenting cells. Even with the elimination of leukocytes for the allograft, which is essential for the direct pathway of antigen presentation, the rejection can still occur. The second type of antigen presentation is via the indirect pathway where the antigens derived from the donors are processed and presented by the recipient antigen presenting cells to the recipients T cells leading to downstream activation of cytokines leading to rejection response(146).

In the 'semi direct' pathway the donor APCs transfer cellular membrane proteins and MHCs to the recipient APCs. These chimeric APCs then stimulate both CD4 + and CD8+ T cells.

1.8.2.2 Role of co stimulation

Once the antigens are presented to the T cells receptors (TCR), their fate is largely dependent on the type of co stimulation at the time of antigen presentation. These cells depending on the co stimulatory signal can start proliferating, become anergic or develop into memory cells.

It has been shown that where there is absence of co stimulatory signals through a lack of stimulatory cytokines, T cells can become anergic, which can be reversed by replacing the missing cytokines(147). There are a large number of

co-stimulatory signals but probably the most studied one is CTLA 4, which inhibits T cell activation.

Broadly speaking co stimulatory interactions are either from CD28-B7 family or tumour necrosis factor receptor (TNFR) / tumour necrosis factor (TNF) family.

CD28 is present on the T cell surface and it interacts with CD80 and CD86.

Interaction of CD28 with CD80/86 promotes activation of T cells while CTLA 4 interaction with CD80/86 inhibits T cell activation.

CD40 ligand (CD154) is expressed on the surface of activated T cells. This interacts with CD40 and is needed for the activation of B cells and dendritic cells. These interactions lead to production of different cytokines, which activate various effector cells.

1.8.2.3 T cell differentiation and the role of regulatory cells

After the initiation of immune response by antigen presentation to TCR, naive T cells differentiate into helper T cells. These cells mostly are CD4+ and once activated also express CD154. Generally Th1 differentiation is promoted by IL-2, Th2 by IL-4, Th17 by IL-6 and T_{reg} by TGF- β .

Th1 cells have been shown to be responsible for acute rejection associated with transplantation(148)(149) while Th2 and T_{reg} cells are implicated in the negative feedback mechanisms leading to protection from alloimmunity(150). This has lead to a vast interest in their role to prevent allograft rejection.

CD4+ CD25+ regulatory cells are the most well studied group of regulatory cells. Earlier experiments have suggested that these regulatory cells are needed to prevent autoimmunity. After thymectomy of an adult rat and split

dose gamma irradiation the rats developed autoimmune diseases. This has been shown to be reversed by transfer of CD4⁺ CD25⁺ T_{reg} cells(151)(152). More recently their role in prevention of allograft rejection has been studied. These T cell subsets were isolated in the long term surviving cardiac (153)(154) and pancreatic(155) allografts pointing towards their role in the development of tolerance.

1.8.2.4 *Humoral mechanism of rejection*

Two main mechanism of tissue damage by antibodies are either through the activation of complement or antibody dependent cellular cytotoxicity. If a patient is transplanted a kidney who already has preformed antibodies against the MHC of the transplanted organ, it will lead to hyper-acute rejection. This type of rejection is also seen if the organ is transplanted across the ABO blood group without implementing the immunosuppressive protocols of ABO incompatible transplantation (antibody removal). These scenarios of hyper acute rejection are quite rare these days due to the refinements in tissue typing and cross matching. Development of these antibodies could be because of multiple previous blood transfusions where the recipient gets sensitized by the HLA antigens of the WBCs in the blood, previous pregnancies where it's the exposure to the paternal antigens which leads to the development of an antibody response or previous transplant or via cross reactivity of various infective agents' antigens.

The humoral arm of adaptive immune system also plays an important role in the long term as well. Donor HLA specific antibodies (DSA) can develop during the episodes of acute rejections. Presence of DSA is an adverse sign for the

allograft both for the short and the long term as its quite difficult to treat and can show resistance to treatment with antilymphocytes and steroids regimes(156). Positivity of C4d staining (157) on renal biopsy is an indicator of the antibody mediated rejection and necessitates a more intensive therapeutic rescue regimen.

1.8.3 Role of Natural Killer (NK) cells

Natural killer cells are an important part of innate immune response. Their main role has been against the cancer cells and virus infected cells as a first line of defence. Although most of the NK cells are present in peripheral blood, lymph nodes and bone marrow, they can be recruited to the sites of inflammation by various cytokines(158). A lack of MHC class I expression either completely or even partially can lead to recognition by NK cells and leads to the lysis of these cells. Many of the cancer cells lack normal MHC expression thus making them susceptible to attack by NK cells.

NK cells also play active role in adaptive immunity by their interaction with the dendritic cells that can be positively or negatively influenced by them. Some T cells also express NK cell receptors which influences their interaction with the other cells of adaptive immune system(159).

The response from NK cells is much quicker than T and B cells as it's due mostly to the preformed secretory granules containing effectors with properties to induce apoptosis. There also is some evidence of memory with NK cells as the second response to the same antigens is even quicker than the initial stimulus(160).

1.9 TUMOUR IMMUNOLOGY

1.9.1 Immunological response to tumours

As tumour cells are also usually self, the majority of the antigens associated with them do not incite an immune response due to the tolerance process. Still there are few antigens that are recognised by either the innate immune system or adaptive immune response of the host. The antigens could be originating from tumour / viral genes, they could be the result of mutation in normally occurring genes or they could be the result of overexpression of normal genes or foetal antigens(161). All these processes make these tumour antigens susceptible to recognition by MHC and therefore attack by the T cells.

Broadly, tumour antigens can be divided into two categories.

1.9.1.1 *Tumour specific antigens*

These antigens are produced from the tumour cells and may be the result of several mutations. These antigens are thus new and so can lead to induction of cell-mediated immune response. The majority of these antigens are the result of chemical or viral exposure. The T cells then eliminate the cells expressing these antigens thus either leading to complete destruction of the tumour load or selecting the cells, which either express these antigens at very low level or don't express them at all. Thus making them invisible to the immune system.

Virally induced tumour antigens can be similar among different tumours induced by the same virus and injecting hosts with the cells from one type of tumour can protect them from the other tumours caused by the same virus. This principle is

used for the production of vaccine for cervical cancer where human papilloma virus is present in 80% of invasive tumours(162).

1.9.1.2 Tumour associated antigens

These antigens are not specific to the tumour and are either produced at some stage of development or are produced at very low levels and tumour cells alter their expression positively. Alpha foetoprotein (AFP) is normally expressed during the embryonic stages of development and is only present in very small amounts in non-pregnant adults. Its levels are raised to many folds in liver cancers and it forms an important prognostic indicator. Similarly carcinoembryonic antigen (CEA) is overexpressed by a proportion of colonic cancers. In some types of breast cancers HER2 antigens are overexpressed which is not an embryonic antigen.

1.9.2 Immune response to tumour development

There are several pathways that regulate the human cells and prevent tumour development. Just because of the mere numbers of cells present in humans and multiple stimuli from carcinogens, without these protective mechanisms life would be impossible. Nucleotide excision repair (NER) pathway is one of the several well characterised ways by which unregulated growth is prevented(163). There are other simpler mechanisms where disruption of extracellular-matrix association can lead to apoptosis. If the tumour cells evade these basic defence mechanisms then there is the specific protection by the immune system.

The immune system can help achieve this by either protecting against tumour inducing viruses and reducing pre tumour inflammatory response or by

specifically attacking the tumour/tumour associated antigens.

Immune system can both protect and indirectly promote the tumour growth contrary to the initial belief, a model called immune-editing. Initial studies have shown the importance of T, NK T cells and NK cells in their role of tumour destruction as in their absence animals are more prone to develop several cancers. But more recent studies have shown that cancers developed in animal models with deficient immune system are much more immunogenic than the animals with intact immunity. This observation points towards these tumours being naturally selected to be less immunogenic and thus more able to evade host response. Perhaps this is the final step required for any tumour to evade the host immune system as otherwise hosts with normal immune systems should destroy all the tumour load(164).

Vesely *et al.* describe this process of immune-editing in three stages(165) Fig 6. The first phase is elimination where cancer cells are attacked by the immune system and are destroyed. The second phase of equilibrium is where there is a balance between the growth of tumour cells and destruction and the final stage is escape where least immunogenic tumours evade the immune system and undergoes the phase of exponential growth(165).

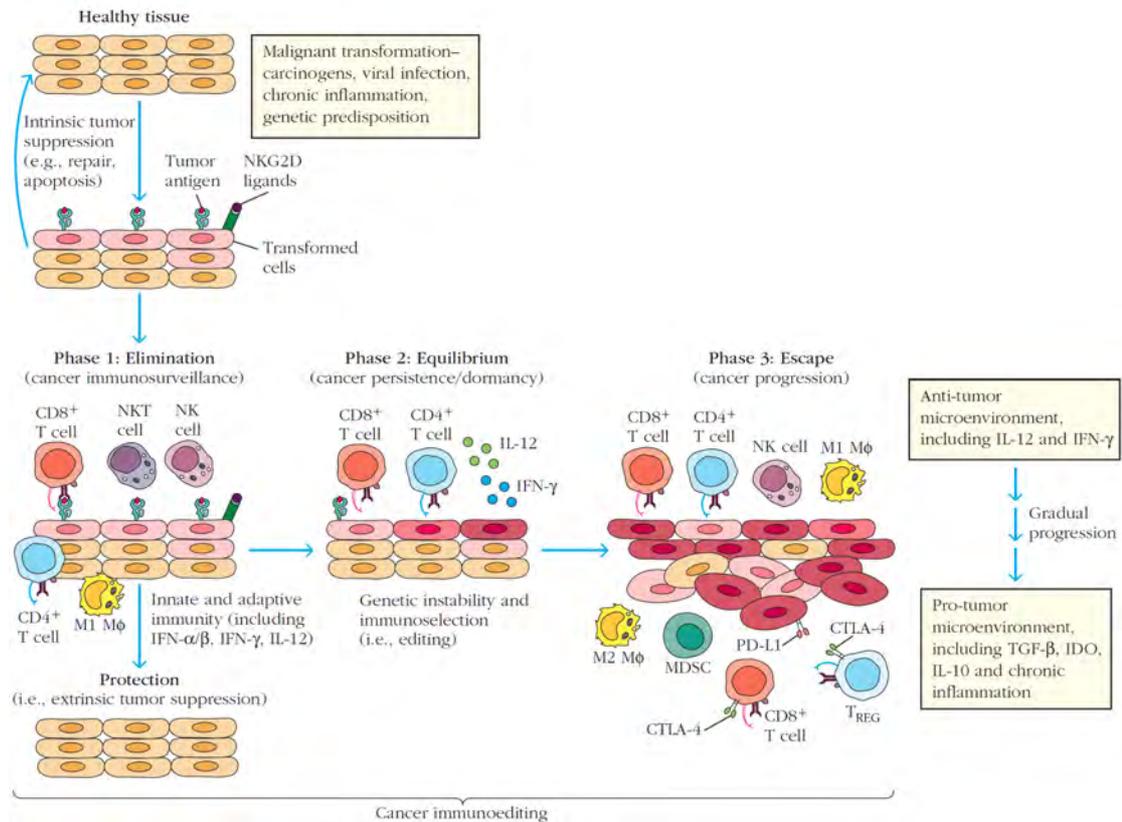


Figure 6 Immune-editing. Three stages of Elimination of cancer cells (first stage), Equilibrium between growth of tumour cells and destruction (second stage) and Escape associated with exponential growth (third stage). (165)

Key immunological players of cancer eradication are natural killer (NK) cells and certain macrophages from the innate immune system and T_{H1} and cytotoxic T cells of adaptive immune system as well as antibody response to tumours by B cells. Conversely, T_{H2} and T_{reg} cells' prominence in the tumour milieu is an adverse prognostic sign. Again there are several pathways through which the tumour cells evade the natural defence of the hosts including reduced MHC expression or selection of such tumour cells which are less immunogenic, up-regulation of anti-apoptotic mediators and lack of co-stimulatory signals which are essential to mount an immune response and in its absence can lead to tolerance of the tumour.

1.10 SUMMARY OF LITERATURE REVIEW

The use of organs affected by small renal tumours have been attempted in past. Most of the initial reports of the use of these organs have been retrospective studies. One of the very first reports of the use of organs affected with small renal cell carcinomas was from the Cincinnati group where Penn(55) looking at Cincinnati transplant tumour registry (CTTR), described a total of 14 cases of ex vivo resection of small renal cell cancers detected incidentally followed by transplantation. There were no recurrences from these cases and the other organs were transplanted from these donors without any adverse effects. Similar results were reported by Buell(54) again with no recurrence. Later on Nicol (56) presented a quite impressive case series from Australia where these kidneys with small renal tumours were transplanted after ex-vivo resection after careful selection of recipients. Their group chose elderly patients or patients with comorbidities and high chance of death without transplantation. All the recipients had full informed consent and were given the choice of rejecting these organs. Of the 41 kidneys transplanted after ex vivo resection, 10 had benign pathology and 31 had a malignant tumour. There was only one case of recurrence that was away from the site of resection. Similar case series were reported from Japan(58) with no recurrence. All these and several case series(29) point towards the unexpectedly promising results after transplanting these organs after tumour resection.

Similarly there are more than 50 cases of allograft renal tumours, which have been successfully treated by partial nephrectomy rather than graft nephrectomy.

Chapter 2

EXPERIMENTAL DESIGN

2.1 Research hypothesis

The main aims of our study were

- To assess the role of standard immunosuppressive agents on transplanted tumour growth and compare with immunosuppressive agents with anti-neoplastic properties.
- To assess the role of matching on tumour growth
- To assess the role of rejection in eliminating transplanted tumour load.

There are more than 7000 new cases of renal cell carcinomas diagnosed in the UK every year and more than 50% of these are T1a tumours (less than 4 cm unilateral tumours). The current standard of treatment for these tumours is partial nephrectomy and radical nephrectomy is now regarded as an alternate standard. Despite this there still are a large number of patients undergoing radical nephrectomy for these small tumours due to various reasons. There is a potential of these organs to be restored by ex vivo resection of the tumours and then be transplanted. The current literature, although quite limited, is very encouraging. Although the long term incidence of recurrence and patient survival are comparable between patients undergoing partial nephrectomy and radical nephrectomy(85) and this is one of the main reason of partial nephrectomy now being regarded as a standard procedure for these tumours in a urological setting , the situation after transplantation is more complicated.

Transplant recipient are on life long immunosuppression and as explained earlier, host immune system is an important barrier to tumour development and growth. In the event of any inadvertent tumour transplantation in these patients

the results could be devastating.

The main aim of this research project was to look at the role of different immunosuppressive agents on tumour development and growth in a transplant setting to identify the immunosuppressive agents, which can be most suited to situations where these restored organs are transplanted.

The hypothesis was that under the influence of regular immunosuppression any transplanted tumour load will continue to grow. Also perhaps there will be an ideal form of immunosuppression that could be used for these restored organs. Certain immunosuppressive agents with anti neoplastic properties (Sirolimus and Leflunomide) could be used in these situations and the anti neoplastic properties utilised to rid of any inadvertently transplanted tumour from the host.

The other observation from a pure immunological point of view is of the role of matching in transplantation. The better-matched organs could incite less of an immune response against them and vice versa. As the tumours are also derived from the donors, they share most of the antigens with them apart from the tumour specific or tumour derived antigens. In cases of tumour transplantation along with these restored organs or tumour recurrence in these kidneys matching can possibly play an important role. Our hypothesis was that the tumour derived from more mismatched donors will incite a more robust rejection response thereby producing a better clearing of any tumour load. This situation can be used as a helpful aid to other treatments when a recurrence is diagnosed in these organs. A graft nephrectomy will be required for most of these situations followed by withdrawal of all immunosuppression. In this scenario, without any immunosuppression the hosts immune system will be better equipped to reject any left over tumour load e.g.; in local lymph nodes.

Barrou *et al.* (76) has described a case of two allograft recipients from a single donor with tubulopapillary tumour (17mm) in the right kidney; only the left kidney was utilized for transplantation. Shortly after transplantation, the recipient underwent an ultrasound (US) examination of the allograft, which did not reveal any tumour. 3 months later a biopsy was done for rejection, which revealed a poorly differentiated tumour, and the patient underwent radical allograft nephrectomy. No additional chemotherapy was given apart from discontinuation of immunosuppression (prednisolone and azathioprine). Lymph nodes that had been noted to be enlarged on CT scan disappeared two month after nephrectomy. The patient underwent re transplantation two years later and was disease free and dialysis independent at 3-year follow-up. This example suggests that hosts immune system can be used for rejection of any left over tumour and by transplanting organs with less well matching perhaps this rejection of tumours will be even stronger.

The immunosuppressive agents used for our project were Cyclosporine, Sirolimus and Leflunomide. This choice of immunosuppression gave us the opportunity to assess the tumour behaviour under “normal” immunosuppression without any anti neoplastic effects (cyclosporine) and newer agents with some antiproliferative properties.

The tumour was transplanted into two different strains of rats to mimic well-matched and poorly matched groups. The role of matching and rejection of tumour is compared in these two groups after withdrawal of any immunosuppression.

To enable real time in vivo monitoring, a highly sensitive IVIS spectrum imaging system was used for analysis. At the end of the study period further analysis by flow cytometry was performed to assess immunological response to the tumour in the transplant setting.

Our project can be outlined as below

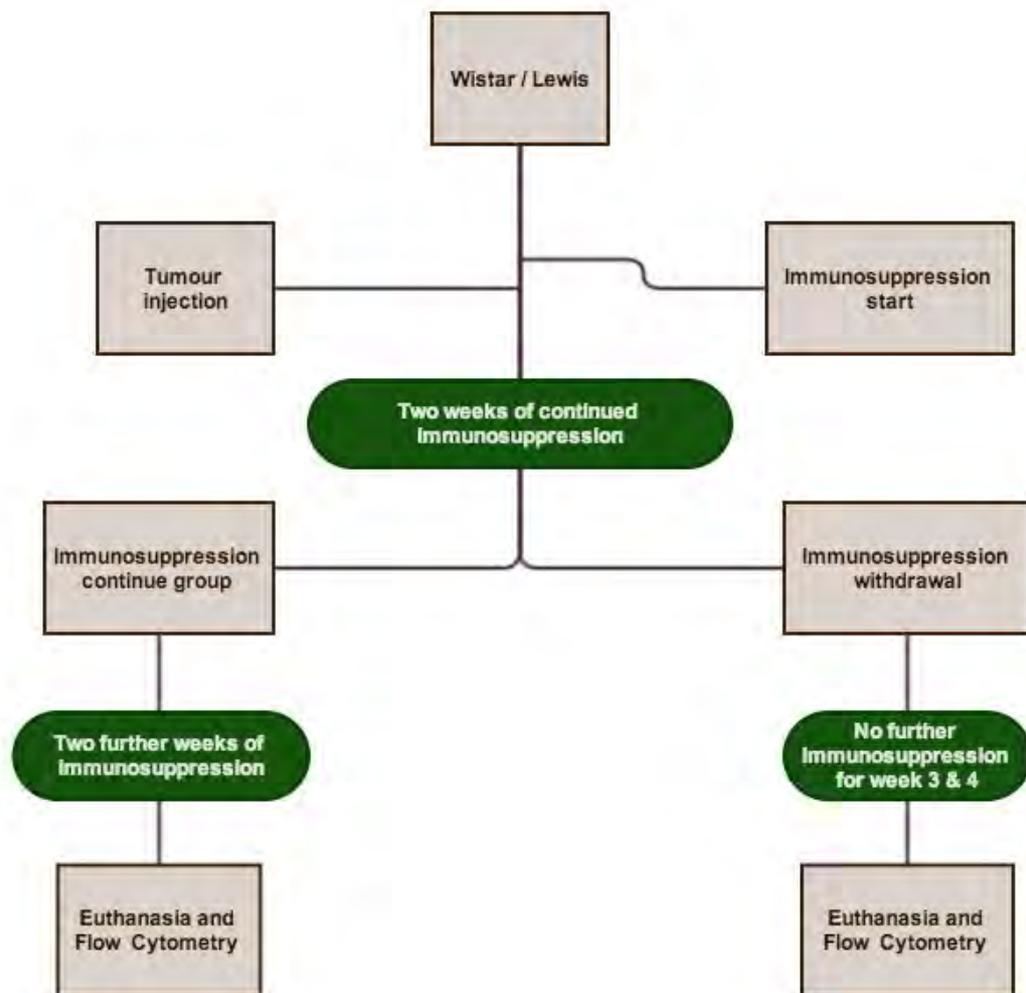


Figure 7 Flow sheet showing project outline.

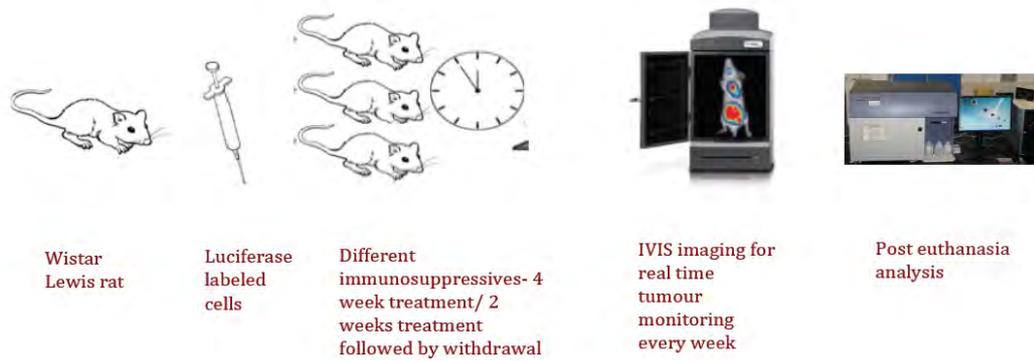


Figure 8 Schematic diagram outlining the project methodology.

2.2 Subcutaneous injection of renal tumour

The purpose was to induce a tumour by injecting a known number of tumour cells under the surface of the skin. Such a model has been used previously (166). The normal immunosuppression used in this situation was cyclosporine. After inoculation of 1.2×10^7 tumour cells the animals were monitored for four weeks and then euthanased to measure their tumour size and then they underwent a post mortem to determine whether or not there has been distant spread. After tumour injection 5 initial groups were developed: one with no immunosuppression (control), then four groups one receiving the standard immunosuppressive drug cyclosporine which was administered for 4 weeks or the test immunosuppressive anti-proliferative drugs: again administered for 4 weeks. The animals receiving immunosuppression were further divided into treatment continue group where immunosuppression was continued for 4 weeks and treatment withdrawal group where immunosuppression was stopped after two weeks; to assess the role of rejection with no immunosuppression.

We used two different rat strains; Wistar and Lewis. The cell line was from Wistar origin and was developed after exposure to an oral carcinogen and were shown to be stable(167). Well-matched groups were mimicked when these tumour cells were injected into Wistar animals and poorly matched when these tumour cells (of Wistar origin) were injected into Lewis rats.

2.3 Monitoring the tumour

To enable in vivo visualisation and objective estimation of tumour load as a measure of tumour behaviour in our model we transfected the cell line with Luciferase and Green Florescent Protein (GFP). Stably transfected cells were then selected for injection in the subcutaneous tissue and the animals were scanned in IVIS Spectrum imaging system to monitor the tumour growth. Towards the end of the study period of 4 weeks the animals were euthanased by schedule 1 of Home Office protocol and a formal post mortem was performed to assess any distant metastasis.

2.4 Choice of Immunosuppressive agent

We studied the effects of three immunosuppressive agents on the transplanted tumour behaviour.

2.4.1 Cyclosporine

This, along with tacrolimus is the most commonly used immunosuppressive agent used in clinical transplantation. CsA revolutionised the immunosuppression by significantly reducing the rates of acute rejection in 1990s compared to previous agents. Although from the mid of 2000s, tacrolimus has taken over as the most commonly used primary immunosuppressive agent, they both are Calcineurin inhibitors and share mechanism of action and side effects. One of the important side effects of CsA is the increased incidence of tumour development as is with most of the immunosuppressive agents. These effects are thought to be due to increased cell mobility and increased anchorage independent growth and also due to increased angiogenesis(106)(107).

2.4.2 Sirolimus

The profile of Sirolimus fits very well to the research hypothesis of an immunosuppressive medication that has antineoplastic properties as well. Although the rates of acute rejection may be slightly better with CNIs when compared to Sirolimus, its potency as antineoplastic agent has been tested for different tumours. Sirolimus behaved as a one of the two test agents in the study to exploit these anti tumour effects. We used two different doses of Sirolimus; the high dose (2.0mg/kg/day) as used in many animal studies for urothelial tumours (168) and low dose (0.5mg/kg/day) normally used to study the immunosuppressive effects(169)(170).

2.4.3 Leflunomide

The other immunosuppressive we used for our research was Leflunomide. Leflunomide exerts its immunosuppressive and anti-inflammatory effects through multiple pathways. The most important mechanism of action is the inhibition of pyrimidine synthesis. Activated lymphocytes depend exclusively on the de novo synthesis of the uridine monophosphate, as they are unable to use the pyrimidine salvage pathway. Leflunomide inhibits the mitochondrial enzyme, dihydroorotate dehydrogenase which is responsible for the production of pyrimidines(129)(130), leading to the inability of lymphocytes to synthesise RNA and DNA.

Leflunomide is currently only licenced for the use in rheumatoid arthritis patients. But in past it has been used for solid organ transplantation.

It has shown improved outcomes when used with CNIs and steroids (132) and also has shown reduced acute rejection rates in animal models (133).

Various studies have shown anti tumour role of these agents(171)(138)(172)(139), which makes leflunomide suitable to assess our hypothesis.

2.5 Grant and licence

The work carried out in this project was appropriately licenced. The project was licenced through Home Office animal licence (Licence no. PPL 60/4042) for a total of five years. The animal work on our project lasted from June 2010-Feb 2012. I held personal licence granted by Home office after successfully completing modules 1-4 of animal handling, kept at all times in the animal department of Newcastle University.

The grant was provided by Northern Counties Kidney Research Fund (NCKRF).

The Initial grant was of £3000 for the pilot work and the subsequent grant was for £ 19,270.54 (Grant reference BH111133).

Chapter 3

DEVELOPMENT OF STABLY TRANSFECTED RAT TUMOUR CELL LINE

3.1 INTRODUCTION

Transfection of the cell line with luciferase and Green fluorescent protein was one of the very important steps of our experiments. Despite the rapid growth (24 hours), without any transfection with luciferase and GFP, all the assessment of the tumour growth under experimental conditions would have been very subjective with high chances of bias and human error. Using the objective method of measuring the tumour load with IVIS Spectrum Imaging system as described in more detail later prevented this.

Injecting native cells into rats means that tumours can only be detected once palpable and the rats would have required sacrificing. Transfecting the cells with a reporter gene allows the real-time monitoring at subclinical levels and the rats can be kept alive throughout the study period.

Plus, without knowing the effects of the different immunosuppressive agents on tumour growth, it would have been impossible to determine any subtle changes. If there was not a big difference in the effectiveness of one immunosuppressive over the other then these changes would have gone unnoticed.

Apart from refining the results and removing the bias to a great deal from these experiments, transfection of our cell line also meant that the animals had to undergo the experiments for shorter period. Because of the very high sensitivity of the IVIS imaging system we needed a lot less tumour load to be able to study the effects of immunosuppressive agents and the role of matching on them. This was a major refinement, leading to lot less animal stress during these experiments.

3.2 FLUORESCENCE AND BIOLUMINESCENCE

Bioluminescence results in the release of photons, which can be picked up by photon sensitive equipment. The photon emission is secondary to the generation of an excited high-energy state of the electrons in the molecules, which are inherently unstable. When the molecules come back to their resting state, this leads to energy production in the form of photons emission.

There are some basic differences between bioluminescence and fluorescence. For any sort of fluorescence there has to be an external source of energy, in the form of light that is used to excite the molecules from its resting state while in bioluminescence this is a chemical process. Usually the amount of light emitted by the fluorescent assays is much higher than the bioluminescent assays, as there is an external source of energy, so the amount of energy delivered can be increased to a great deal. But this does not translate to the better overall sensitivity of these assays. This is due to the fact that any estimation of photons must be calculated by subtracting the background "noise" or background signals. As there is an external source of energy, usually the background signal is also very bright in cases of fluorescence, hence reducing the overall sensitivity of the assay.

The photons needed to excite the fluorochromes can also interfere with the results of the assays. But in biological samples, the tissues may have their own inherent fluorescence that can interfere with the final results.

These issues are much less of a problem in cases of bioluminescence as there is no external light source required. This means that inherent

fluorescence will also not be interfering with the analysis. Due to these, there is a very small background signal making any light measurement far more sensitive towards the experimental assay.

Thus bioluminescence is preferred over the fluorescent processes although fluorescence has its important uses as well, especially in light microscopy and flow cytometric analysis where bioluminescence cannot be used or has very limited role.

The most commonly used bioluminescent enzyme is Firefly Luciferase (others are Renilla luciferase and Aequorin).

3.2.1 Firefly Luciferase

This is a naturally occurring enzyme found in firefly and in bacterial species such as *Vibrio* sp. and results in bioluminescence in these organisms. The chemical process leading to light (photon) emission has the following steps.

Conversion of Luciferin (substrate of Luciferase) to high-energy state Oxyluciferin, which is an unstable molecule. This is an active process needing an input of energy in the form of ATP and requires oxygen.

This high energy state of oxyluciferin is unstable and thus gets converted to the stable low energy state of oxyluciferin, releasing the energy absorbed in the initial reaction as photons.

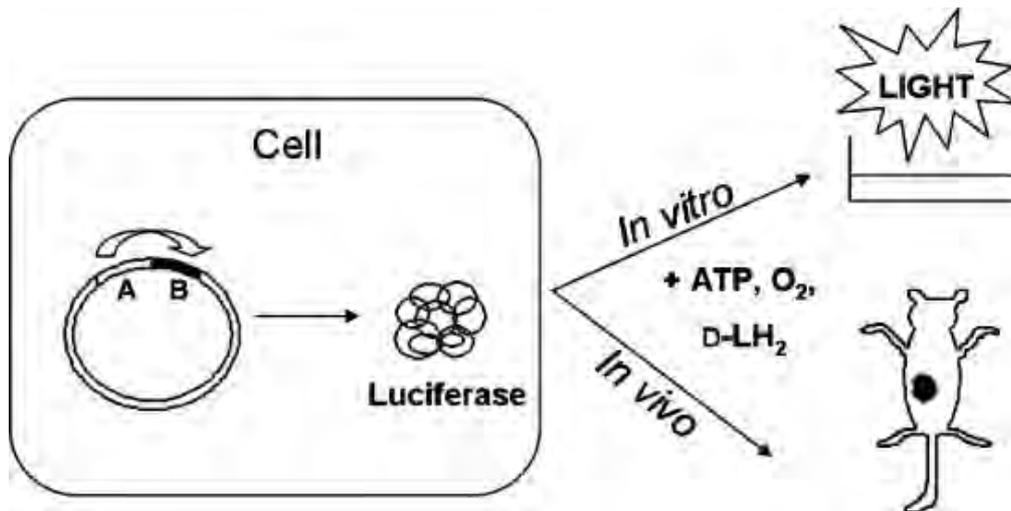


Figure 9 Schematic of mechanism of bioluminescence (173). Cells are initially transfected with Luciferase enzyme. Once transfected, these cells have the ability to emit light in the form of photons in the presence of O₂, ATP and luciferase.

3.2.2 Green Florescent Protein (GFP)

This is another naturally occurring protein that has excellent florescent properties and has been used as a reporter extensively. The enhanced form of GFP (eGFP) is preferred now because it offers higher intensity emissions after blue light excitation(174).

The other benefit of GFP has been the ease of determining the transfection process with florescent microscopy. It is useful for flowcytometry as opposed to the bioluminescent proteins.

3.3 IVIS SPECTRUM IMAGING SYSTEM

This is an imaging modality specifically designed to image small animals for the detection of florescent and/or bioluminescent signal with very high sensitivity. A schematic diagram of the imaging system is shown in the figure below.

This can be divided into three distinct parts;

Imaging Chamber

This contains a sound proof, light tight imaging chamber with heated stage to keep the anaesthetised animals' body temperature regulated. It has integrated gas anaesthesia and an Oxygen delivery mechanism.

The stage is motor controlled to adjust the lens position for the best possible image capture.

CCD Camera

A highly sensitive back illuminated CCD camera, with 13.5-micron pixels. The camera is thermoelectrically cooled to -90°C to reduce the background noise.

Lenses

There is 6-inch diameter optics with focal length varying from $f/1$ - $f/8$. It also homes an emission filter.

The basic purpose of this arrangement is

- To be able to capture the faintest possible light signals with minimal detectable radiance of $70 \text{ photons/sec/sr/cm}^2$
- To reduce the background noise, which is achieved by keeping the animals anaesthetised so that there is no movement and maximum amount of information is gathered, by means of super cooling of the CCD camera and by the use of complex software algorithms to calculate the actual signals and subtracting the background "noise".

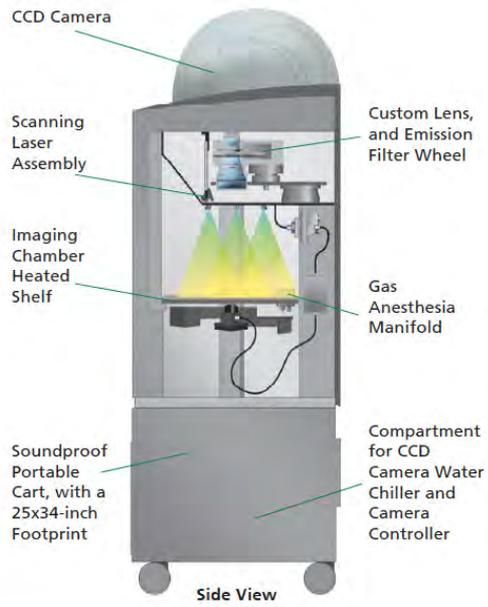


Figure 10 Schematic diagram of IVIS Spectrum taken from the IVIS manual.

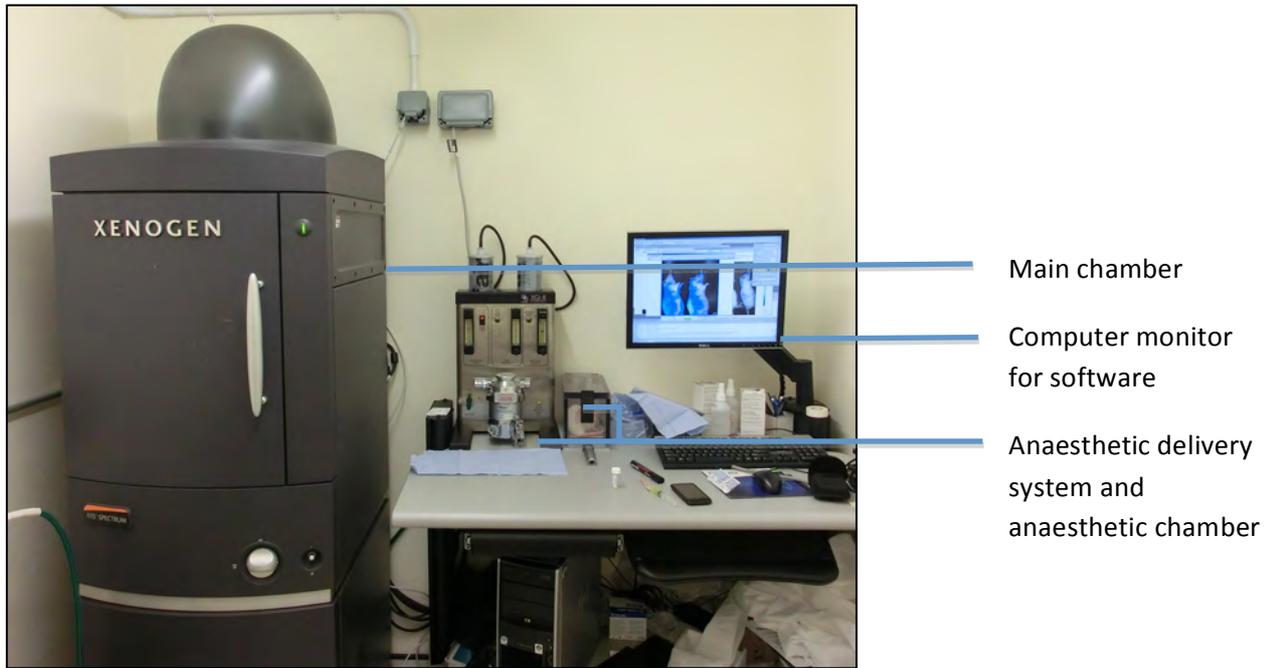


Figure 11 IVIS spectrum imaging system. Used for our experiments in Newcastle University. Main chamber homes the anaesthetised animals. Anaesthetic delivery system mixes oxygen with anaesthetic gases both to induction chamber and main chamber. Computer software is used to analyse the acquired data.

Principle of IVIS spectrum

Florescence

Emitted light from the excitation filter wheel feeds through a fibre optic bundle to illuminate the specimen from either the top, in epi-illumination (reflectance) mode, or from underneath the stage. If the specimen has fluorochrome it will absorb the light of excitation wave length and emit its own light of certain wave length depending upon the fluorochrome. This light is then picked up by the super cooled CCD cameras through an array of filters to create the image. The software in the attached CPU has the ability to subtract the background illumination from the final signal received, generating the true reading of fluorescence.

Bioluminescence

The specimen was placed in the light proof dark chamber and luciferin was added to the sample to start the reaction with luciferase. Exposure times can be either automatic or it can be set to capture the images depending on their light emission. Again the photons produced are picked up by the CCD camera. Again for the accuracy of the measurement of light emission, the background noise is subtracted from the original image.

To enable the real time monitoring of the rat kidney tumour cells, these were transfected with luciferase. This is described along with the basic cell culture below.

3.4 MATERIALS AND METHODS

3.4.1 Cell culture

The cell line used throughout the experiments was BP36B, a rat renal tumour cell line. Basic cell culture techniques were practiced and perfected on A549 (human lung carcinoma) (175) before embarking on the transfection of the BP36B cell line.

The rat tumour cell line, BP36b, was acquired under a standard MTA from the Cell Bank at the RIKEN BioResource Centre (Ibaraki, Japan). These cells were developed by Tokuzen *et al.* (167) by exposing the Wistar male rats to

N-ethyl-N-hydroxyethylnitrosamine (EHEN), a known carcinogen, and by feeding EHEN to 6 weeks old male Wistar rats for 3 weeks, 14 rats showed the presence of the tumour. The cells were isolated and cultured in RPMI 1640 with 10% FCS and antibiotics in the humidified incubator with 5% CO₂ at 37°C.

Tokuzen *et al.* developed three rat renal tumour cell lines (BP13, BP30 and BP36B). All of these cell lines had epithelial appearance in monolayers and were moderately differentiated basophilic tumours of proximal tubular origin.

The cell line chosen for our experiments was BP36B as the doubling time of this cell line was 17 hours as compared to 29 and 21 hours respectively for the other tumour cell lines. Also this cell line was most successful in inducing tumour in a xenotransplant model after pre-treatment of the nude mice with anti-asialo GM1 antibody (an NK cell depleting antibody)(176).

This cell line was reported very stable even after 100 passages. The cell characteristics were tested in the cell lines for up to 3 years from the receipt and the doubling times and the cell line characteristics have remained stable throughout this period. These cells were then split into 6 flasks initially and later on **1:8 splitting ratio** was adopted once the cell line was stabilised in our lab.



Figure 12 Light microscopic appearance of BP36B rat renal tumour cell line (10X). These cells grew in monolayer and remained epithelial in appearance. The doubling times were consistent with the reported times by the cell bank.

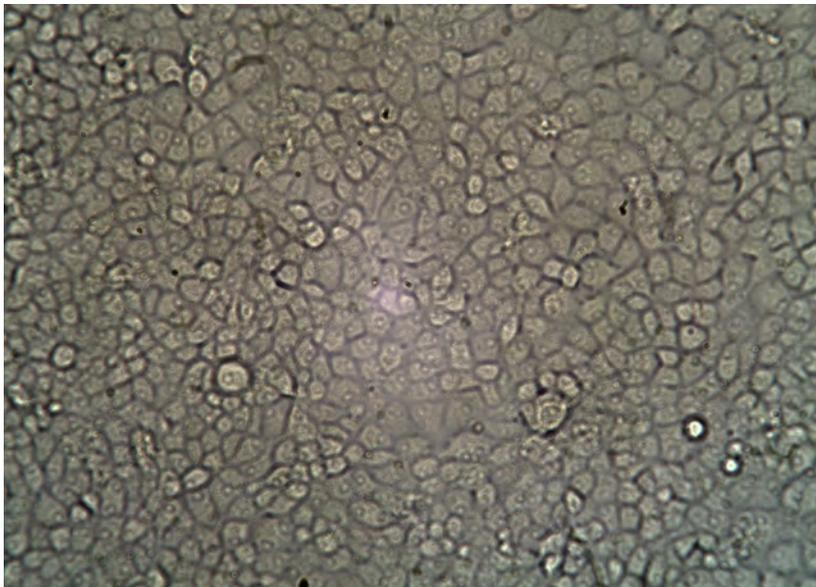


Figure 13 Light microscopic appearance of BP36B rat renal tumour cell line (40X). These cells grew in monolayer and remained epithelial in appearance. The doubling times were consistent with the reported times by the cell bank.

The cell culture medium (Roswell Park Memorial Institute RPMI-1640) was purchased from the Biosera® Labtech International Ltd. and was stored at 2-8°C. L-Glutamine (Sigma-Aldrich Company Ltd) was added at a concentration of 0.3g/l. along with heat inactivated FBS (Sigma-Aldrich Company Ltd) to a concentration of 10% to prepared final enriched medium for cell culture usage.

To avoid microbial contamination Penicillin and Streptomycin (1,000 units of Penicillin and 0.1 mg if streptomycin/ml) (Sigma-Aldrich Company Ltd) were used routinely for all the cell cultures as per protocol of the cell culture lab. When required Amphotericin B was also added to a final concentration of 2.5mg/lit to prevent fungal infection.

Our cells were cultured in multi-well plates and flasks with porous caps in an open incubator system. The average temperature of the incubator was kept at 37°C with a CO₂ concentration of 5% in a humidified environment. For buffering purposes, the RPMI 1640 used for our cell cultures contained 2gm/lit of sodium bicarbonate.

3.4.2 Thawing of cryopreserved cell line

The cells were kept in liquid nitrogen and were carefully removed. These eppendorfs were then quickly placed in pre-heated water baths at 37°C. This reduces the damage to the cells that can accompany slow thawing of the cryopreserved cells. The aim was to thaw the cells in less than a minute.

The eppendorf was then transferred to the hood and wiped with 70% alcohol. The cells along with the contained medium were then transferred to a centrifuge tube containing the RPMI1640 that again was warmed at 37°C and cells

centrifuged at 200g for 5 minutes to form a clear pellet.

The centrifuge tube was then brought back in the hood and the excess medium decanted carefully to avoid disturbing the cell pellet at the base at this stage.

Then the cells were re-suspended in the RPMI 1640 medium (supplemented with L-Glutamine, 10% FBS and antibiotics). Care was taken to re-suspend cells gently by repeated pipetting of the medium.

Next the cells were transferred into two 75cm² flasks containing approximately 30 mls of culture medium to fully immerse the cells.

These flasks were then closed with porous caps and inspected under light microscope before being incubated at 37°C in humidified CO₂ incubator.

A record book was kept updated in the lab for any changes to the number of cryopreserved cells.

To ensure a constant growth rate the cells were subcultured at a confluence of approximately 70-90%. Typically this resulted in passaging the cells two to three times a week.

Cells were passaged by removing the media and washing with Dulbecco's Phosphate Buffered Saline (DPBS) gently to remove any left over culture media. Pre-warmed trypsin was added to cover the cell layer all over; typically between 3-5 mls for 75cm² flasks. The flask was gently rocked and left for 1-2 minutes to allow the cells to detach from the flask.

After this 5-10 mls of culture media was added to inactive the trypsin and prevent cell damage. The cells were then pelleted by centrifugation at 400 g for 5 minutes and re-suspended in fresh culture media prior to distribution into new flasks and place back in the incubator.

3.4.3 Cryopreservation

The cells undergo both karyotypic and phenotypic changes if they are subcultured for long time. To prevent this and keep them as original to their initial properties it is important to cryopreserve them. We aimed to cryopreserve our cell line as soon as there was a surplus.

We adopted following protocol for freezing the cells

The cells were cryopreserved at a high concentration. As there is cell damage during the thawing process, it is important to start with a higher concentration of healthy cells for cryopreservation. We normally used at least on T75 flask for this purpose that roughly contains 7.5×10^6 cells.

After trypsinization of the cells they were re-suspended in the growth medium to inactivate the trypsin followed by centrifugation for 5 minutes.

The supernatant was decanted and the cells were suspended in 1 ml of freezing medium. The freezing medium was prepared before the start of the freezing process and included 70% RPMI, 20% FBS and 10% Dimethyl Sulfoxide (DMSO).

These suspended cells were then transferred into the pre-labelled Eppendorf tube. This was then placed in the polycarbonate container for gradual freezing. This contained isopropyl alcohol which helps in lowering of the temperature of the cells 1-2°C per minute. This is important as it prevents cell damage by either too rapid or too slow cooling.

The cells were left in the -80°C freezer overnight and then next day were transferred into the liquid nitrogen in a dedicated Dewar.

Labelling of the cells included type and passage of cells and date of freezing. This was recorded in the nitrogen lab book as well.

3.5 RESULTS

3.5.1 Puromycin kill curve

For the selection of the transfected cells, puromycin was used. The adequate dosing was calculated by undertaking the kill curve.

BP36B cells were grown in 24 well tissue culture plate in 0.5 ml of culture medium.

These cells were cultured till they reach around 70% confluence to get the best results. This was followed by adding rising concentration of puromycin into the wells. We used the following concentrations; 1mg/ml, 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml, 10mg/ml, 12mg/ml, 15mg/ml, 20mg/ml. This was done in duplicates.

In the final well the cells were cultured in normal growth medium without any puromycin and they acted as controls. The culture medium was replaced every 2 days for 10 days and the wells were examined for any signs of visual toxicity e.g.; separation of the cells for the adherent surface of the multi-well plate.

The cells with 10mg/ml of puromycin were completely destroyed within 1 week while with 6 and 8 mg/ml concentrations there were still some cells left in the medium beyond 7 days; hence 10mg/ml was used as the final concentration in the experiments to select the stably transfected cells.

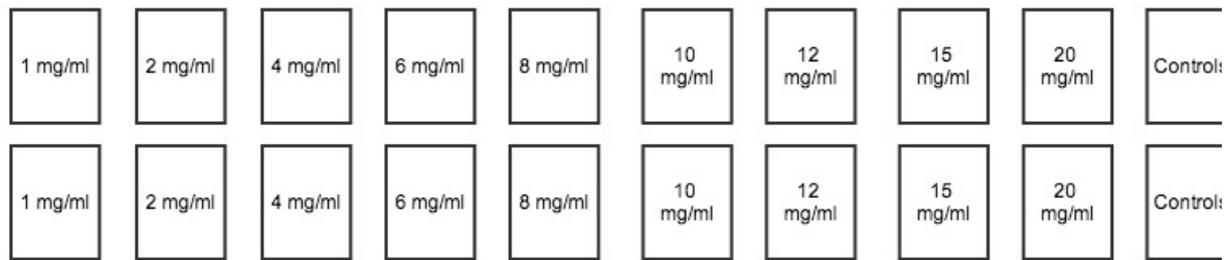


Figure 14 Selection antibiotic. Schematic diagram of the concentration of Puromycin in the multi-well plate to determine the kill curve. The boxes represent the wells with the concentration.

3.5.1 Transfection

Transfection is the process of introducing nucleic acids into cells by non-viral methods. Transduction is the process whereby foreign DNA is introduced into another cell via a viral vector. These are common methods to introduce a foreign gene into host cells. We used viral vector to transduce our cell line. We tried transfection with two different vectors as with the first set of experiments, the cell line failed to transfect.

3.5.1 Failed transfection

The initial attempt of transduction was done with Firefly luciferase lentiviral particles acquired from GeneCopoeia™ Rockville, USA. In this vector the luciferase and Puromycin genes were under the CMV promoter.



Figure 15 Lentiviral vector. These were used for transduction of tumour cells initially. The tumour cells successfully formed monoclonies but were not bioluminescent. Both luciferase and Puromycin genes was under CMV promoter. Figure from literature provided by vendor.

These viral particles were delivered on dry ice from local distributor from France.

Transduction was performed according to the instructions by the manufacturer.

Prior to transduction the cells were grown in the cell culture to make sure they were not infected and there was no change in the growth pattern.

At day 1, 5×10^4 cells were plated in a well in 12 well-plate. These were grown in the standard culture medium with the necessary antibiotics for 24 hours at 37°C with 5% CO₂ overnight.

On day 2 for each well, We prepared 0.5 ml of virus suspension diluted in complete medium with Polybrene at a final concentration of 5–8 µg/ml to increase the permeability. Following concentrations of the letivirus (0.1µl, 5µl, 10µl, 50µl and 100µl) to determine the most appropriate concentration for best transfection results.

The cells were infected by removing the old culture medium and replacing it with 0.5 ml of diluted viral supernatant. The lentivirus and the medium were gently mixed by rotation. Vortex was avoided as per instruction by the manufacturer.

For one well, We added 0.5ml of complete DMEM with Polybrene. This well behaved as control. The plate was placed for 2 hours at 4-8°C and then transferred to 37°C incubator with 5% CO₂ and incubated overnight. On day 3 the cells were split and medium was replaced and on day 4 the culture medium was replaced not containing any polybrene and incubated for another 48 hours.

From day 6 the selection process was started with Puromycin at a dose of 10ug/ml as calculated by the kill curve. The old medium was replaced with fresh complete medium containing the puromycin every 3–4 days until drug-resistant colonies became visible. This time in our experiment was around 7-9 days.

Intriguingly, the antibiotic selection was very clear with stable monoclonal formation upon usage of puromycin but We were not able to elicit any luciferase expression. This was later found to be due most probably due to mycoplasma infection.

The expression of luciferase was tested by using IVIS spectrum imaging system but no bioluminescent signals were detected.

These experiments were repeated twice with similar results. The manufacturer was consulted and the cells were attempted a re-transfection with a new batch of the same lentivirus without any success.

To increase the chances of any subtle expression of luciferase, Weeven undertook the ultrasonic lysis of these cells but were still unable to detect any successful transfection.

3.5.2 Successful transfection

Following the initial failed attempts at transfecting the cells, Wechanged the vector.

The other change was that this time the viral vector also carried the gene for eGFP as well as Luciferase. The vector used was LVP 020 from GenTarget Inc San Diego, USA. These came in dry ice; with a total volume of 200uL containing 1×10^7 IFU/ml. Luciferase was expressed under a tetracycline includible suCMV promoter. These were able to express high levels of luciferase without any induction. CMV promoter is one of the strongest promoters when compared to other RNA polymerase II promoters and hence it was advantageous that the most important gene (luciferase) was under its promotion.



Figure 16 Lentiviral construct. Luciferin was expressed under suCMV promoter. This lentiviral construct also expressed GFP. The puromycin and GFP genes were under the influence of Rsv promoter. The tumour cells once transduced were successfully expressing luciferase and GFP. Lentiviral construct from the GenTarget Inc San Diego, USA information sheet.

The GFP and Puromycin were both under the Rous sarcoma virus (Rsv) promoter(177).

The protocol from the manufacturer for transduction was followed in principle.

The cells were seeded as previously in a 24 well plates in the RPMI 1640 medium with antibiotics at a rough seeding of 5×10^4 cells in 0.5ml of medium.

The cells were around 40% confluent at the time of transduction.

On day 1, the old culture medium was replaced with the fresh medium. The lentiviral particles were thawed to the room temperature. Three different concentrations of the lentiviral particles were added to the cell lines (5uL, 10uL, 25ul and 50uL). These meant that the multiplicities of infection MOI were 1, 2, 5 and 10 respectively. These cells were incubated in the CO₂ humidified incubator at 37°C for 72 hours.

The culture medium was then changed with the one containing puromycin. The optimal dose of puromycin was calculated prior to these experiments as below. The optimal dose of Puromycin was found to be 10ug/ml. The cells were grown in this medium under standard conditions and the medium changed every 2-3 days.

There were different rates of growth of cells with differing MOIs. The cells with MOI of 5 were the fastest to grow and there were 2-3 monoclonies in the multiwell plate. Cells infected with MOI of 2 were the second fastest to form monoclonies while the cells with MOI of 1 were very slow to grow and it took them 3 weeks before any appreciable monoclonity formation was noticed.

Once the cells were growing to near confluence in the monoclonies they were trypsinised and transferred initially to petri-dishes and then to 25cm² flasks followed by 75cm² flask.

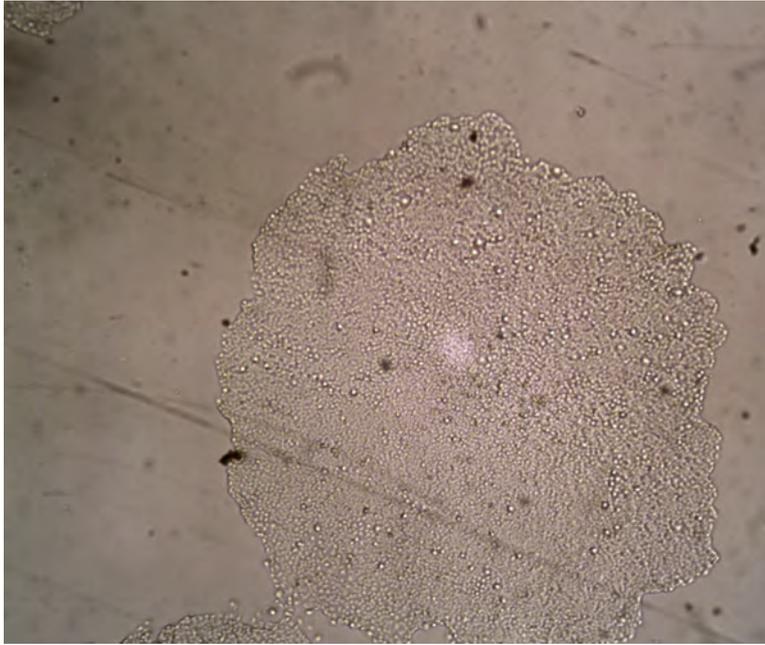


Figure 17 Visible monocolonies. Cells transfected with a MOI of 5.0. Magnification of 10X.



Figure 18 Slow growing monocolonies. Very slow growth rate and not very clear monocolonies formed by the cells infected with MOI of 1.0. Magnification 10X.

3.6 IN VITRO CONFIRMATION OF TRANSFECTION

For the confirmation of successful transduction we used three methods.

3.6.1 Fluorescence microscopy

This was the easiest method of determining successful transduction. The fact that the cells were being selected with puromycin was already an indirect indication that they would be expressing GFP as well, as both of these genes were under the same promoter sequence in the viral vector.

The cells were tested under the fluorescent microscope to assess transduction. There were two controls for this. First were the BP36B cells with no transduction.

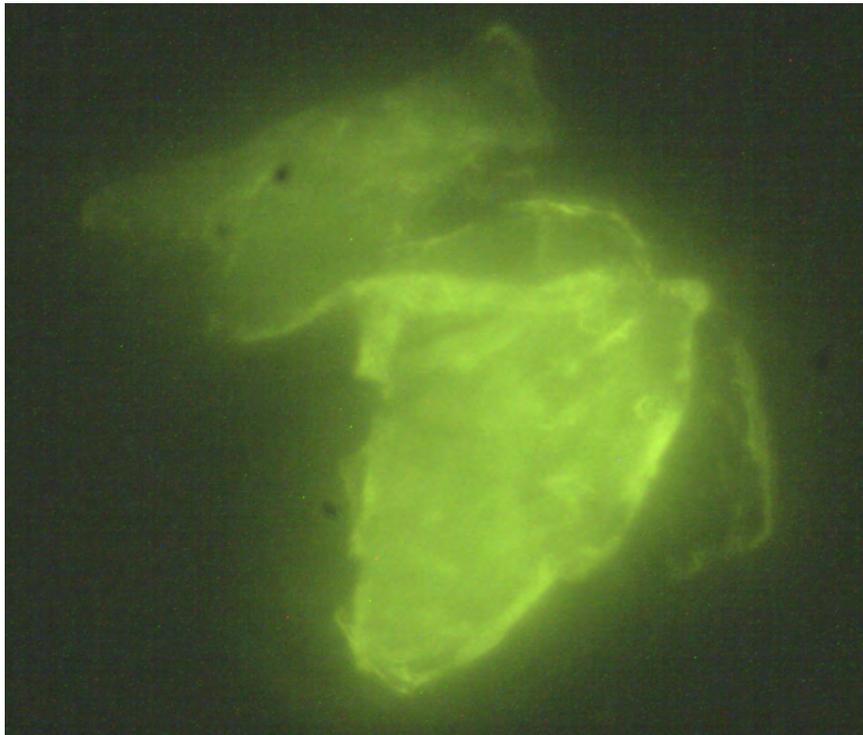


Figure 19 Fluorescent microscopy. Clumps of BP36B cells as seen with fluorescent microscopy. To get brighter signals, the cells were trypsinised and concentrated before being examined under fluorescent microscope.

The other control was BP36B cell line with Florescene diacetate (FDA). This is normally used for cell viability testing. The control cells did not show any significant florescence. The cells with FDA produced easily visible staining under microscopy. Then our transfected cells with MOI of 2 and 5 were examined under the same settings and the cells in clumps revealed good florescent signal.

3.6.2 Luminometer for luciferase

The expression of luciferase as a marker of successful transfection was elicited by light emitted after the lysis of the transfected cells followed by the exposure to luciferin. We used premade Bright-Glo™ Luciferase Assay System from Promega Corporation Wisconsin, USA.

The cells were trypsinised and suspended in the culture medium and equal amount of Bright-Glo reagent was added in the Eppendorf tubes. Both the cells and the reagent were at room temperature to aid cell lysis.

This was left for 2 minutes then these were transferred to the Luminometer. After 10 seconds of exposure, the cells gave following readings.

Table 4: Bioluminescent signals from luminometer. Cells with different MOIs were used for the measurements. Maximum signals were seen with the cells transfected with MOI of 2.5.

Control	MOI 2.5	MOI 5.0	MOI 5.0
14.7/10sec	>9999/10sec	1487/10sec	3980/10sec

3.6.3 IVIS spectrum imaging

The cells were tested in the IVIS spectrum imaging system as well, for conformation of transduction. We again used Bright-Glo™ Luciferase Assay System to lyse the cells and expose them to the luciferin. The Eppendorf tubes were then transferred to the IVIS and images were taken at the standard exposure time of 30 seconds for controls, cells with MOI of 2.5 and the ones with MOI of 5.0 after waiting for 2 minutes to allow for the lysis of the cells.

The cells were initially examined for the expression of luciferase by the bioluminescence and then for GFP for fluorescence.

The photon emission was brightest with cells transduced with MOI of 2.5 when compared to the cells with MOI of 5.0. This was in keeping with the luminometer readings undertaken previously. The control cells did not exhibit any bioluminescence.

Thursday, February 24, 2011, 17:18:51
 Living Image Version: 4.1.0.11858 (Oct 22 2010)
 Camera: 150847N4728, Andor

Group: TEst
 Experiment: test
 Comment1: BP368 rat idney tumor cell_luciferase -eppendr
 Comment2:

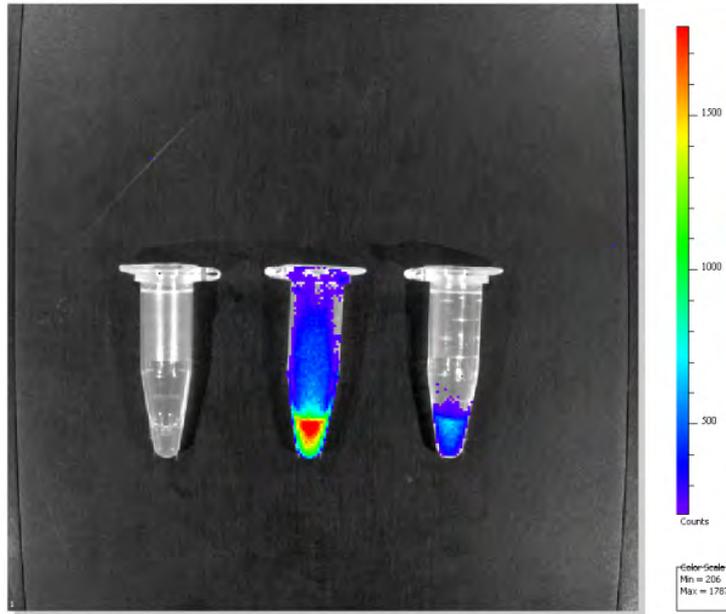


Figure 20 IVIS Spectrum image. Bioluminescence from luciferase transduced cells. The control cells (Left) did not show any signals, the brightest glowing cells (middle) were transduced with MOI of 2.5 and cells with MOI of 5.0 (right) were also positive for the expression of luciferase but were less bright.

Similar results were noticed when the florescence was tested for these cells. Again the cells infected with MOI of 25 were the brightest and there was no signal from the control cells.

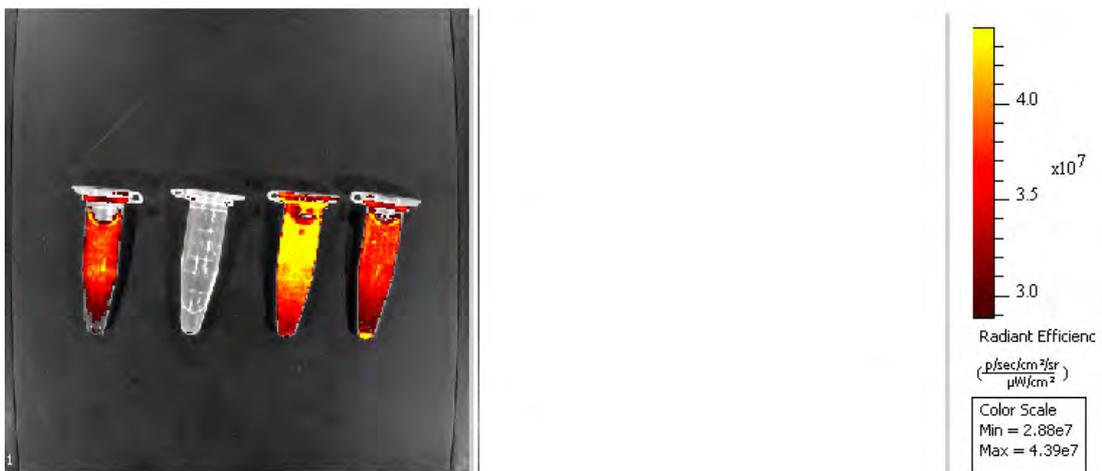


Figure 21 IVIS image for florescence. MOI 5.0, Controls, MOI 2.5 and MOI 5.0 from left to right

Chapter 4

IN VIVO ANALYSIS OF RENAL TUMOURS

4.1 IN-VIVO ANALYSIS FOR PROOF OF CONCEPT

After making sure that the cell line was successfully transfected and we were able to reliably track the tumour growth in real time, the next stage was to check the behaviour of tumour in vivo before assessing different immunosuppressive medicines.

4.1.1 Aims

Before embarking on the live animal work it was important to make sure that the luminescent signals would be picked up when these cells are injected in the animals. For this purpose we used recently culled animals. This was important, as the cells would be injected in the subcutaneous tissue as compared to the transparent and thin Eppendorf tubes which could easily transmit the emitted light from these cells, in other words this was to check the penetrance of the emitted light.

The other issue that could affect these animal studies was the auto fluorescence of the animals. This is more of a problem for GFP signals but not an issue for luciferase, as it does not exist in rats naturally.

The third important issue was the ability of detecting signals from any deep-seated metastasis of the tumour tissue should it happen during the study period.

4.1.2 Methods

The injection of cells and the subsequent detection of fluorescence were as follows

The animals were humanely culled by schedule 1 of the home office protocol.

As these were dead animals, it was important to inject the cells in the subcutaneous tissue along with the substrate, luciferin. Hence, we again used the Bright-Glo system from Promega as described earlier to inject these animals.

The fur was shaved on the ventral aspect of the animal and a subcutaneous injection of cells mixed with Bright-Glo was done in the right flank.

A deep injection was done on the left flank near the spleen and on the right side deep in the groin region.

The animal was then transferred in the IVIS machine and image was taken with automatic exposure.

Then the carcass was turned dorsum up and again was injected with the cell and reagent mixture in the mediastinum. Again the animal was transferred into the dark chamber and image acquisition performed.

4.1.3 Results

With subcutaneous injection in the right flank, there was a very bright signal after 30 seconds of exposure.

There was a good signal from the deep injection in the left flank and the right groin region. This signal was less intense than the more superficially placed cells in the right flank. As both the injections were done at the same time and the photon intensity of the right sided injection was very high compared to the other injection, the minimum units or signal pick up were quite high. This was

not of any clinical significance but an important lesson was learnt here was that if there was one very intense signal at one area and a weaker signal from an adjacent area on the same frame then the weaker signal may not be picked up in that image.

To reduce any false negative results due to this phenomenon, whenever there was an intense signal and a chance of distant spread, we used to cover the brighter area to look for weaker signals.

The deep injection in the mediastinum also produced good signals after an exposure time of 60 seconds.

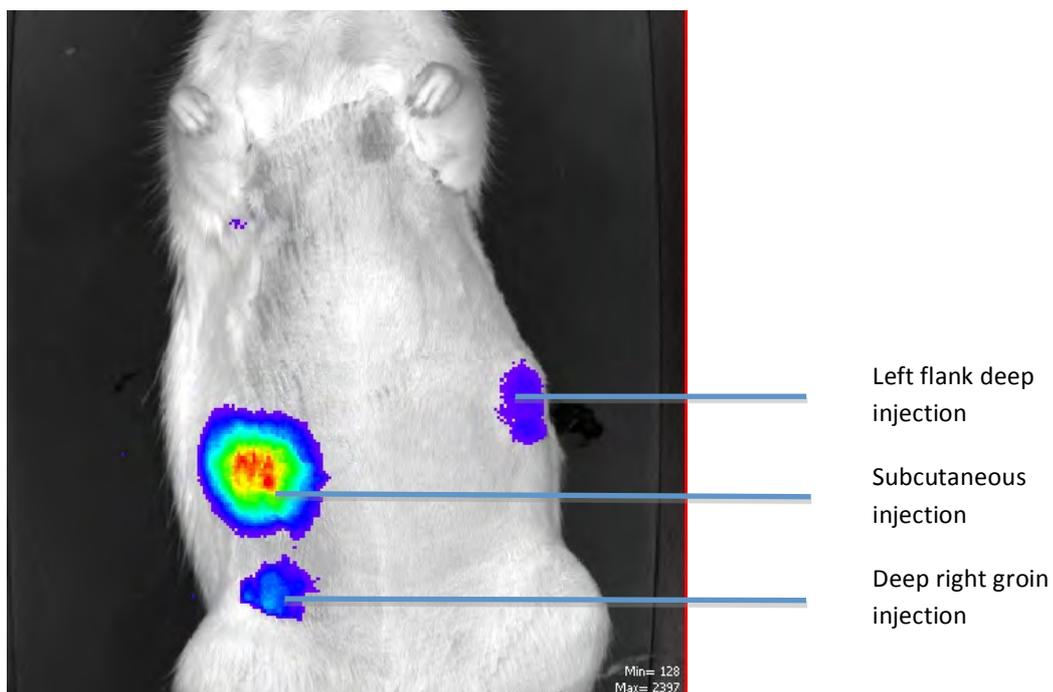


Figure 22 Ventral view of bioluminescent signals from dead rat. Both the transfected cells and luciferin (Promega Bright Glo system) were injected to see if subcutaneous injection will lead to signals pick up by the IVIS spectrum imaging system.

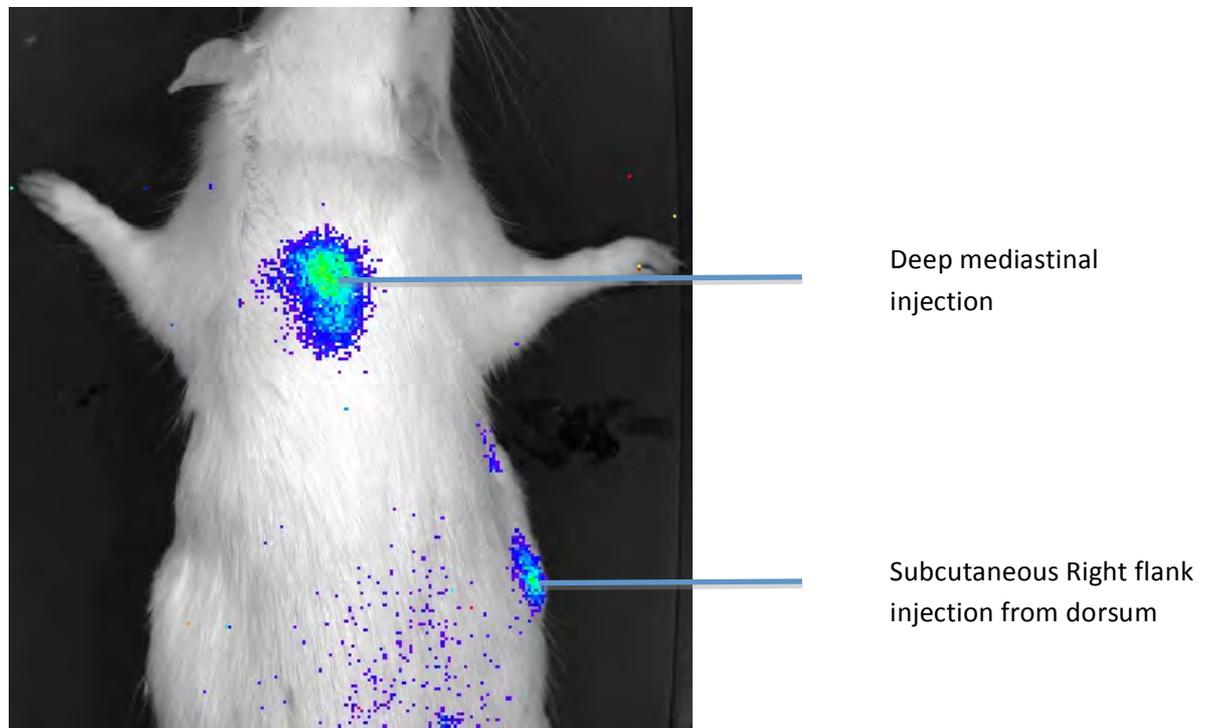


Figure 23 Dorsal view of bioluminescent signals from dead rat. Both the transfected cells and luciferin (Promega Bright Glo system) were injected into the mediastinum to see if IVIS spectrum imaging system will pick up deep seated signals in cases of metastasis.

As these analyses were only to see if the bioluminescence was picked up in rats with our model, no background noise was calculated and there were no radiance calculations.

4.2 PILOT ANIMALS: IN-VIVO ANALYSIS

4.2.1 Aims

After the proof of the concept, firstly that the cells were successfully expressing luciferase and later that we would be able to detect these signals through these

rats after subcutaneous injection, we wanted to experiment on small number of animals to make sure that all the above still held in the living animal scenario.

4.2.2 Methods

Pilot study on live animals looked at followings

- Tumour growth in well matched (Wistar) and poorly matched (Lewis) rats with no immunosuppression
- Tumour growth in the presence of Cyclosporine
- Role of rejection by stopping immunosuppression after 2 weeks.

4.2.2.1 Cell preparation

The cells even when fully trypsinised did not separate from each other completely and remained in clumps. This made it difficult to determine the exact number of cells by cell counters, flowcytometry or by haemocytometers.

To standardize the numbers of cells injected into the animals we used two 75cm² flasks at approximately 80% confluence for injection. This gave a tumour load of around 1.2×10^7 cells per animal at the time of subcutaneous injection which is similar to the literature figures of between 10^6 - 10^8 cells in small animals(178)(179).

After the cells were trypsinised they were transferred into the 20ml tube containing complete medium and were transferred to the animal facility in

Newcastle University. There the cells were spun and the medium discarded and cells washed gently with DPBS twice before being suspended in 100uL of DPBS for the injection.

4.2.2.2 *Animal housing conditions*

All the rats were housed in the animal facility of Newcastle University to very high standards. The facility is run by senior veterinary and technical professionals and is regularly monitored by the home office staff to ensure animal well-being is not compromised at any stage.

The rats were bought from Charles River® Margate United Kingdom. These animals were housed for a week for acclimatisation before any intervention was performed. The starting weight of the rats was between 70-80gm as they grow quite quick; especially Wistar rats and dosing of luciferin can become an issue with bigger animals. weonly used male Wistar and Lewis rats for these experiments to avoid any hormonal fluctuations during the study period.

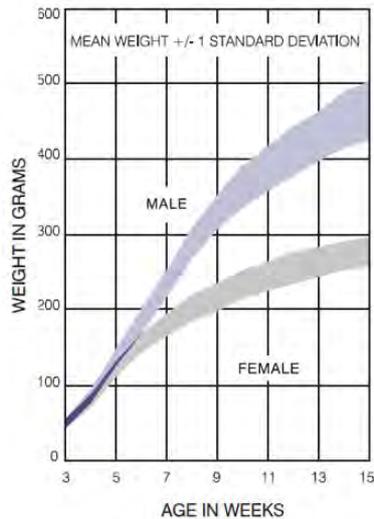


Figure 24 Wistar Rats weights (Charles River)

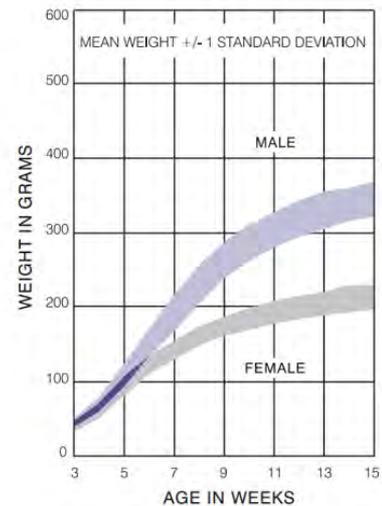


Figure 25 Lewis Rats weights

Difference in weight gain between the two species

All the rats were kept in clean quite uncluttered rooms. There was a separate procedure room to prevent any distress to the other rats. The light dark cycle was 14 hours/ 10 hours. The rooms temperature was kept between 20-24°C at all times.

The bedding was wooden shaving, which was kept dry and changed regularly. Tap water was supplied without any restrictions and animals were fed pelleted chow.

4.2.2.3 Tumour cell injection

The animals were anaesthetised as per protocol described later. Right flank of the animals were shaved in preparation of the injection.

A 25 G needle was used for the subcutaneous injection of the tumour cells. Before any injection of the cells, a small proportion of these cells were checked in the IVIS machine by adding Bright-Glo to make sure they were still expressing the luciferase.

The flank skin was pinched and after gentle mixing of the cells in the syringe they were injected in the subcutaneous plane.

A single syringe was used for injecting one animal. we avoided vigorous shaking to minimise the trauma to the cells, although a gentle flick before the injection was done to make sure they were adequately suspended in the medium.

4.2.2.4 Intraperitoneal Luciferin injection

To enable the animals to bio-luminesce, substrate of luciferase, luciferin was injected into the rats. The easiest way of injecting these animals with multiple luciferin injections was through intraperitoneal injections as repeated intravenous administration through tail vein can lead to thrombosis and prevent further luciferin delivery.

The preferred site was the left lower abdominal quadrant with a 21-gauge needle.

The dose of luciferin was calculated for all the animals before starting the experiments based on their weights.

The animals were manually restrained with the head pointing down to get the bowel out of the way of injection and then the luciferin was injected with a needle just penetrating the abdominal wall.

The luciferin was bought from Gold biotechnology® Missouri USA. This was D-Luciferin, potassium salt that was water-soluble. The luciferin was dissolved in PBS to make the stock solution with a concentration of 80mg/ml. This was achieved by dissolving 1 gm of GoldBio Luciferin in 12.5mls of PBS. The recommended dose of luciferin was 150mg/kg body weight of the rat and the optimal concentration for IP injection was 15mg/ml. For a 100gm rat this came to 1ml of the solution to get the recommended concentration of 150mg/kg.

weused the following formula to calculate the dose

$$\text{Dose of Luciferin} = 0.15 \times \text{Weight of rat in grams}$$

4.2.2.5 *Luciferin kinetic curve*

At the beginning of every new batch of animals the kinetic curve of luciferin was calculated. Intraperitoneal (IP) injection of luciferin has to be absorbed through the peritoneal membrane and then it gets into the blood stream, followed by the delivery to the subcutaneous right flank region where the transfected cells were transplanted. This process can vary a lot depending on multiple variables

including the size of the animal, accuracy of the injection, temperature of the injected luciferin and the general well being of the animal as well as the conscious status of the rat.

To minimise any variability we tried to keep these variables to as minimum as possible and also calculated the kinetic curve of luciferin. It gave a rough estimation of the peak absorption of the drug in that particular rat which could be generalised for similar animals.

Kinetic curve was calculated by multiple exposures to the IVIS camera after IP injection (Fig 26). Five minutes after the injection the animals were first scanned and every two minutes for a total of 25 minutes then onwards. The brightest reading occurred when there was a maximum concentration of luciferin available at the target tissue and that time was the optimal for further imaging. For our experiments this time was 15 minutes after the luciferin injection. To reduce any bias even further, we always took two readings when imaging the animals on either side of the 15 minutes and the higher reading was used for the analysis.

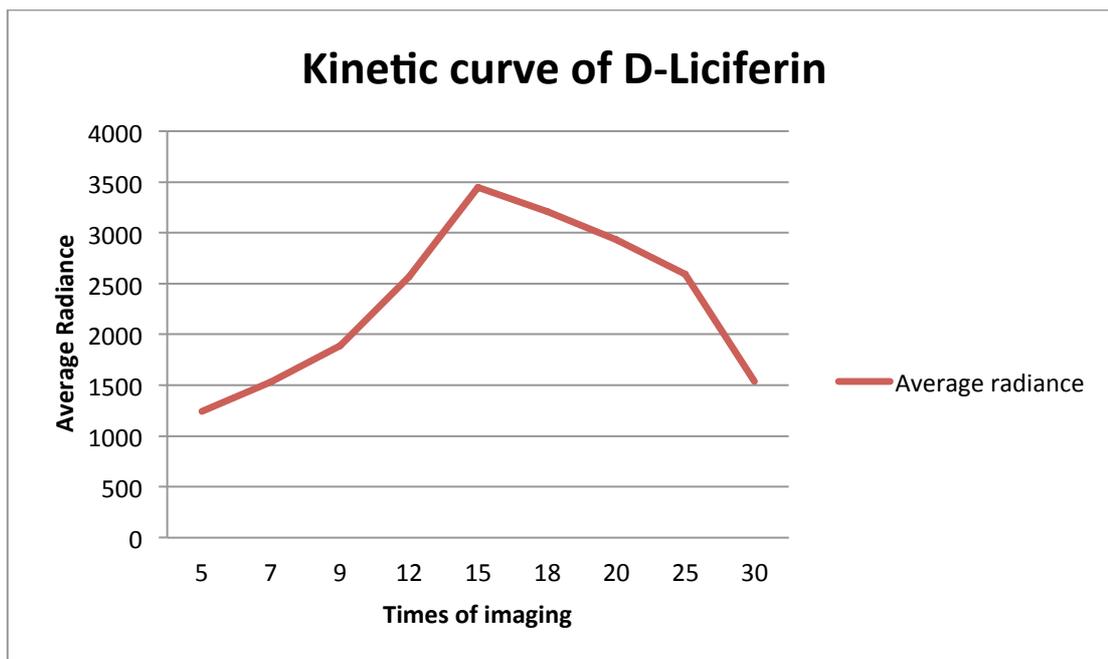


Figure 26 Kinetic curve of Luciferin. Calculated for Lewis rat in the Pilot study. The radiance was calculated by subtracting the background signals from the signals of Region of Interest (ROI).

4.2.2.6 *Anaesthetic protocol*

The animals were induced in the anaesthetic induction chamber of either the IVIS spectrum machine or the separate induction chambers in the procedure room.

Before the experiments we made sure that the vaporiser containing Isoflorane and the Oxygen tank were full. Firstly the Oxygen was turned on at a flowmeter rate of 1 litre/minute and the rat was placed in the induction chamber and the lid secured. The stopcock to the induction chamber was then opened with the vapour dial at 3% for isoflurane. Normally it took the animals 2-3 minutes before they fell unconscious. At this stage the stopcock was turned for the imaging chamber of the IVIS and the animals were placed there with their noses

positioned into the nose cones. At this point the concentration of the isoflurane was between 1-2% depending on the size of the rats.

Once the animals were there and they were stably anaesthetised, the imaging was commenced.

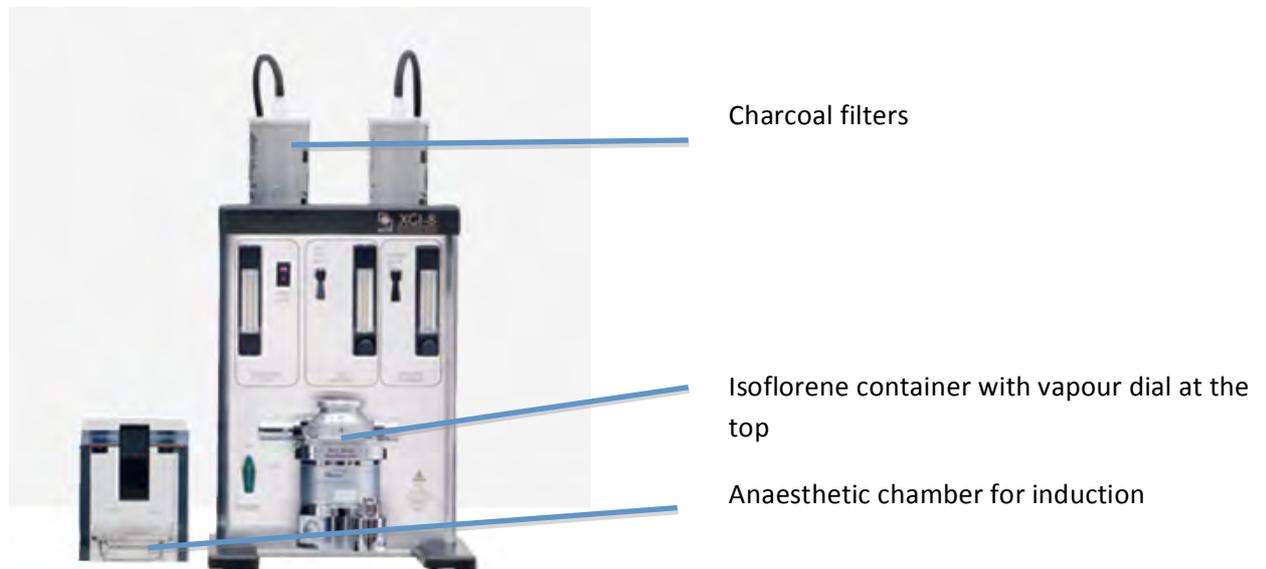


Figure 27 Anaesthetic chamber and isoflurane delivery system of IVIS spectrum.



Figure 28 Anaesthetic manifold. Nose cones for maintenance of anaesthesia in the imaging chamber

4.2.2.7 *Experimental protocol*

The cells were trypsinised, washed, prepared and injected according to the above-mentioned protocols. After the injection of the cells, the animals were

also injected with luciferin and then scanned in the IVIS spectrum. The animals were scanned under the automatic exposure if there were positive signals and in the absence of any signals they were exposed for the maximum exposure of 5 minutes to detect any faint signals.

After the first scan the animals were recovered in the cages under direct observation until mobile.

After the initial scan, the animals were scanned once every week for a total of 4 weeks. After the end of the study period of 4 weeks these animals were culled by schedule 1 as per home office protocol by cervical dislocation and a post mortem performed to detect any gross metastasis. Harvesting of spleen and enlarged lymph nodes was performed at this stage for flow cytometric analysis.

4.2.3 Results

4.2.3.1 Tumour growth in well matched (Wistar) and poorly matched (Lewis) rats with no immunosuppression

4.2.3.1.1 Well-matched group

Wistar rats were injected with 1.2×10^7 BP36b cells in the right flank and the animals were scanned on the day of injection and then every week for 4 weeks. Generally there was a steady decline in the number of cells as depicted by reducing luminescence on IVIS images. By week 1 most of the cells were “rejected” and by the 2nd week there was no residual luminescence at all (fig 29). This demonstrates that in the absence of any immunosuppression there

was a trend to eliminate the tumour load. As it was the Wistar cell line injection into Wistar rats, this group was a well-matched combination. Despite the cell line and the animals of being of similar strain, they were not identical as Wistar rats are out bred. Due to the small number of animals, no statistical analysis was performed at this stage.

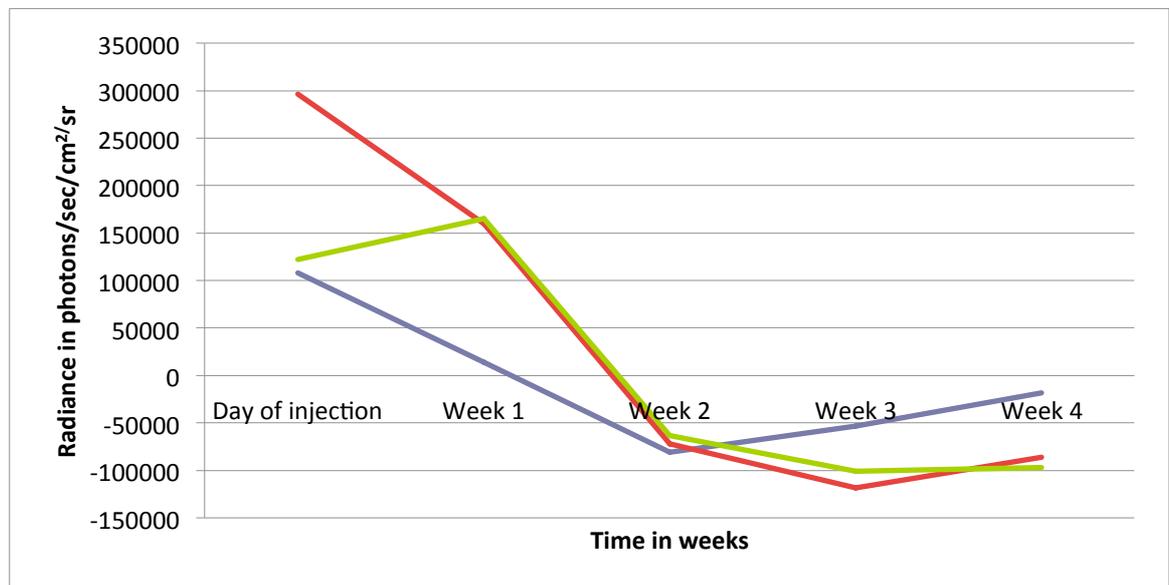


Figure 29 Tumour behaviour in the well-matched group with no immunosuppression. The entire tumour load was cleared within two weeks. The three lines represent three Wistar rats in this group.

4.2.3.1.2 *Poorly matched group*

Two Lewis rats were injected with 1.2×10^7 cells into the right flank. The animals were scanned on day 0 and then once every week. Tumour present at day 0 of injection disappeared at 1st week IVIS scan as opposed to the Well-matched Wistar group above, where tumour took two weeks to clear from the

immuno-competent animals. This was in line with the hypothesis that the tumour would be rejected quicker in a poorly matched animal.

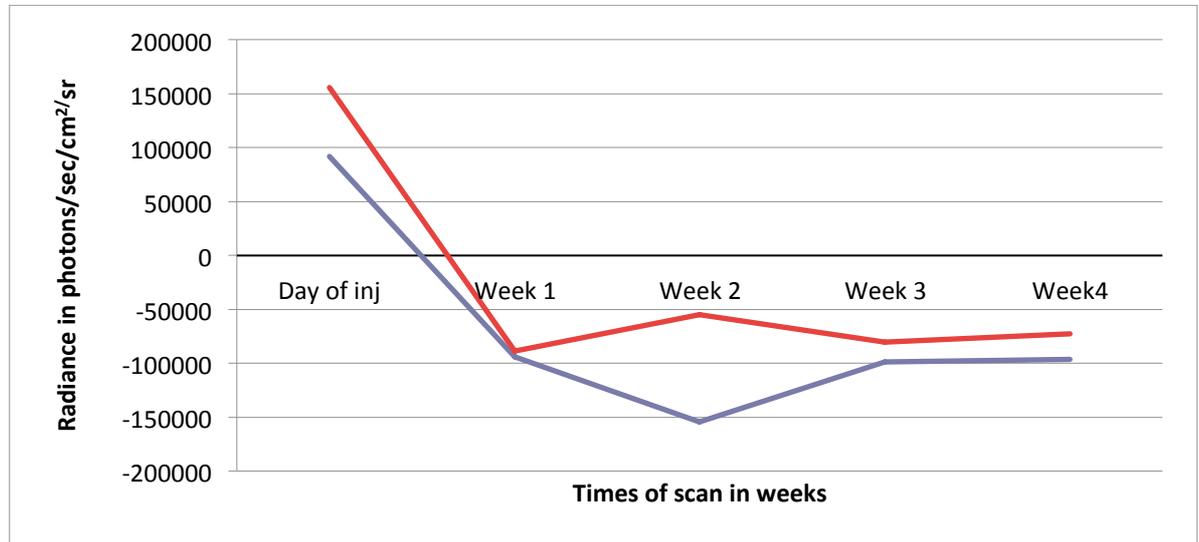


Figure 30 Tumour behaviour in the poorly matched group with no immunosuppression. The entire tumour load was cleared within first week. The two lines represent the Lewis rats in this group.

4.2.3.2 *Tumour growth in the presence of Cyclosporine in Lewis rats*

Two Lewis rats were injected into the right flank with 1.2×10^7 cells under the cyclosporine immunosuppression which was continued for four weeks. The dose of cyclosporine was selected to be 25mg/kg/day as per the commonest dose in literature for immunosuppression purposes(180)(181)(182). Animals were scanned every week in the IVIS spectrum. In the presence of cyclosporine the tumour continued to grow for the duration of the study with rapid exponential growth towards the later half.

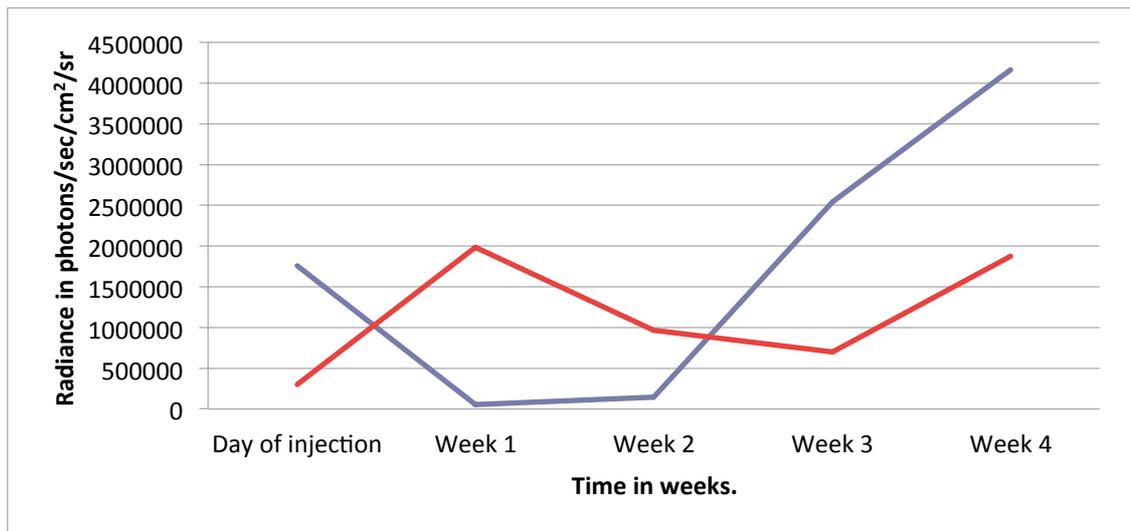


Figure 31 Tumour behaviour in the poorly matched group with CsA immunosuppression. The tumour continued to grow with time during the study period. The two lines represent the Lewis rats in this group.

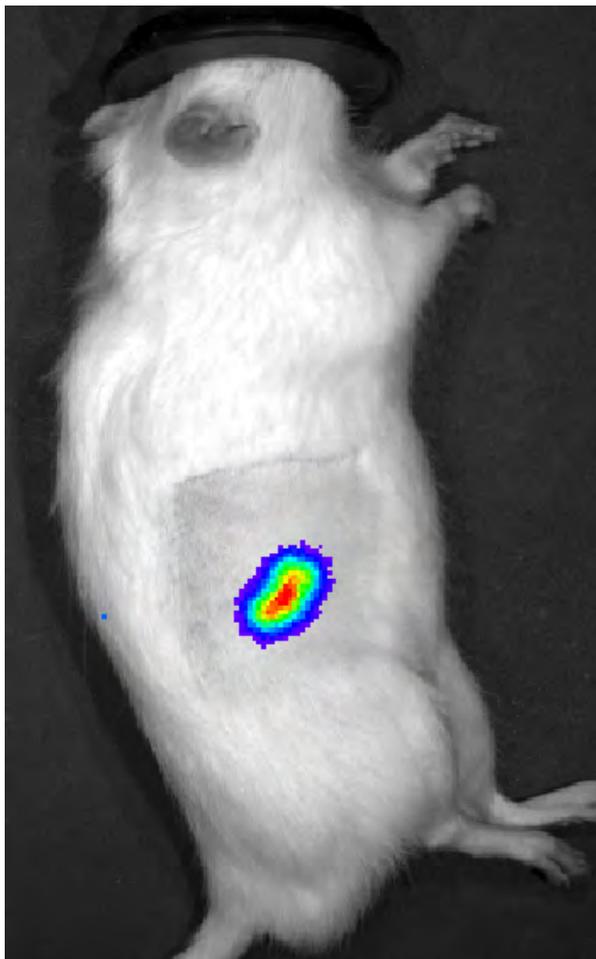


Figure 32 Lewis rat with cyclosporine immunosuppression. The tumour injected into the right flank has continued to grow.

4.2.3.3 *Role of rejection by stopping immunosuppression after 2 weeks.*

Two Lewis rats were injected with 1.2×10^7 cells into the right flank under the immunosuppression of cyclosporine. To study the effects of rejection the treatment was continued for two weeks and then stopped. Imaging of these animals was carried out until 4 weeks after the original injection. Under the immunosuppression the tumour kept on growing while after stopping the immunosuppression the tumour disappeared within 2 weeks highlighting the possible role of rejection(fig33).

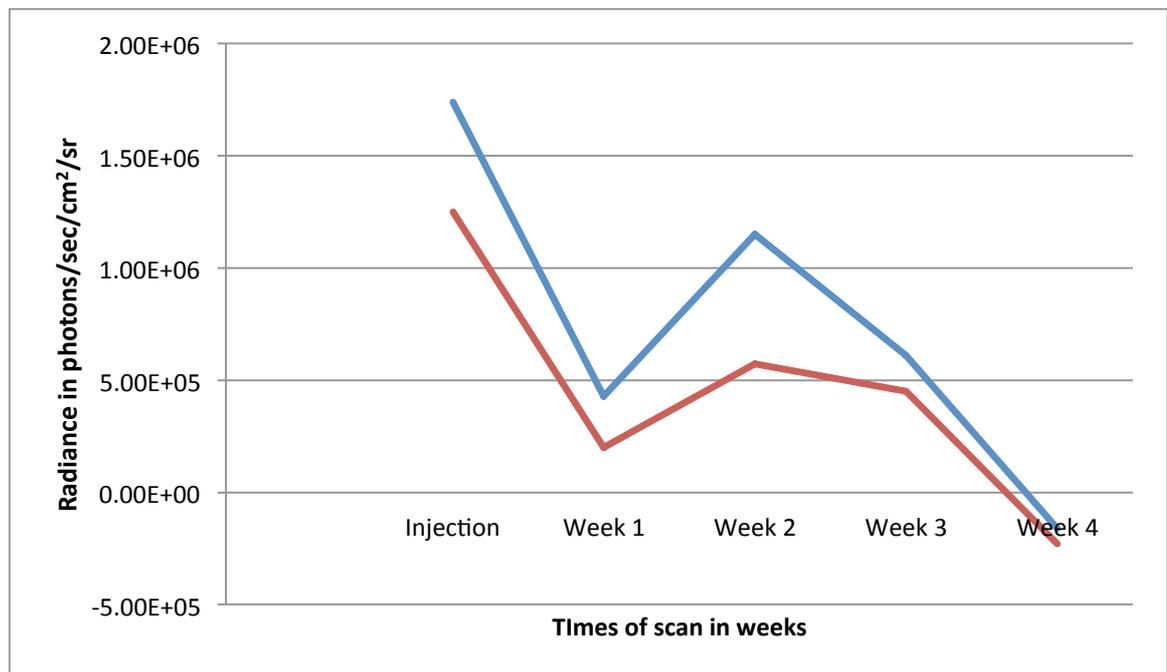


Figure 33 Tumour behaviour in the poorly matched group with CsA immunosuppression stopping after two weeks. The tumour continued to grow with time during the initial half while after stopping the treatment the entire tumour load was cleared. The two lines represent the Lewis rats in this group.

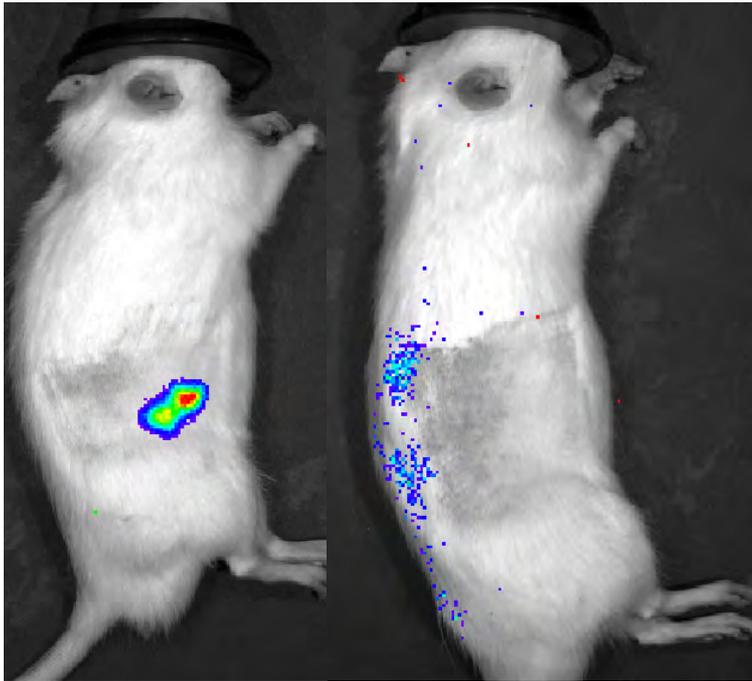


Figure 34 Effects of treatment withdrawal. Example of a rat at Week 2 (left)(with immunosuppression) and week 4 (right) (after stopping the immunosuppression for 2 weeks). The coloured area over the fir in the right image is the “noise” of the IVIS imaging due to long exposure times to detect even very low radiance from the region of interest.

The basic purpose of these experiments was to make sure that the proposed intervention would be possible before embarking on the large-scale experiments. With these preliminary experiments we detected the trend of tumour cells being rejected as any other allogeneic transplanted tissue.

With the initial success of these experiments, we started the experiments on the proposed groups.

4.3 CONTROL GROUP TO STUDY BEHAVIOUR OF TUMOUR WITHOUT IMMUNOSUPPRESSION IN A WELL-MATCHED COMBINATION

4.3.1 Background

The tumour cells were derived from the Wistar rats and when they were transplanted into the Wistar animals, they behaved as a well-matched combination. As the Wistar rats were out bred animals, despite being of the same strain, there were some differences between the animals' MHC and the cell line. This arrangement mimicked the human situation very closely, where even in very good matches between the donor and the recipients there are still some differences in the HLA loci. Thus this was therefore a good model to study the effects of tumour transplantation.

4.3.2 Aim

The main aim was to test animals without immunosuppression and so monitor the growth of the tumour in non-immunocompromised situations, as these animals then behaved as the controls for the study period.

These experiments also gave insight into the role of matching on the transplanted tumour behaviour.

4.3.3 Tumour injection and analysis

There were a total of 6 animals in this group. All were Wistar male rats, which were acclimatised for one week before the tumour injection in the right flank

under anaesthesia. Before the injection of the cells they were tested and confirmed for bioluminescence.

The animals were scanned on the day of injection, and once a week there after as per above-mentioned protocol. As these animals were not under the influence of any immunosuppressive medication, the scanning of these animals was stopped once there were no further bioluminescent signals detected, meaning that the entire tumour load had been rejected.

In this group we observed that there was a reduced signal to the initial radiance on the 1st week's scan and by the second week all the animals had rejected the tumour cells completely.

After the last scan these animals were culled. A careful post mortem failed to detect any gross tumour growth either at the site of initial injection or any evidence of enlarged lymph nodes.

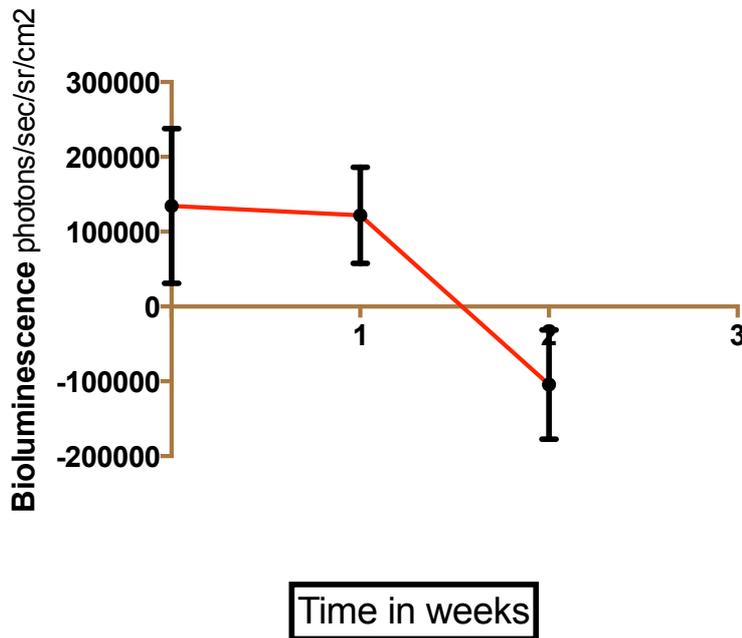


Figure 35 Role of acute rejection. Rejection of all of the injected tumour cells in the absence of any immunosuppression in well-matched Wistar animals within two weeks.

For the data analysis, after the animals were scanned in the IVIS spectrum, the regions of interests (ROI) were designated manually. A region of interest (ROI) is a user-specified area in an optical image, which the software uses to create, and computes objective values of the photon emission which is direct representation of the number of healthy luciferase expressing cells. This data was then used for statistical analysis. we used two types of ROI; *measurement ROI* which measured the signal intensity in an area of the image with positive signals or initial injection (in case of no signals) and an *average background ROI* that measured the average signal intensity in a user-specified area of the image that was considered background.

The image data output was in photons and emission of photons from the region of interest was calculated as *radiance*, which was displayed in photons/sec/cm²/sr.

While the counts are a relative measure of the photons incident on the CCD camera, the photons are absolute physical units that measure the photon emission from the subject.

The radiance unit of photons/sec/cm²/sr is the number of photons per second that leave a square centimetre of tissue and radiate into a solid angle of one steradian (sr). Measurements in units of radiance automatically take into account camera settings (for example, integration time, binning, f/stop, and field of view). As a result, images of the same subject acquired during the same session have the same signal amplitude regardless of the camera settings because the radiance on the animal surface does not change. The advantage of working with image data in the photons mode is that camera settings can be changed during an experiment without having to adjust the images or the measured ROI data.

4.4 CONTROL GROUP TO STUDY BEHAVIOUR OF TUMOUR WITHOUT IMMUNOSUPPRESSION IN A POORLY MATCHED COMBINATION

4.4.1 Background and aims

Lewis rats are an inbred strain and are hence syngeneic. These animals are immunologically compatible in cases of transplantation between the individuals. But there are significant differences between Lewis and Wistar strains, meaning when these animals were injected with the tumour cells of Wistar origin, this group behaved as a poorly matched combination in comparison to the above group of Wistar animals.

Again the aim was to test animals without immunosuppression in order to monitor the growth of the tumour in non-immunocompromised situations, as these animals then behaved as the controls for the study period for Lewis strain.

These experiments also gave insight in to the role of matching on the transplanted tumour behaviour.

4.4.2 Tumour injection and analysis

There were a total of 6 animals in this group. All were Lewis male rats, which were acclimatised for one week before the tumour injection in the right flank under anaesthesia. Before the injection, the cells were tested for bioluminescence.

The animals were scanned on the day of injection, and once a week there after as per above-mentioned protocols. As these animals were also not under the influence of any immunosuppressive medication the scanning of these animals was stopped once there were no further bioluminescent signals detected.

In this group again we noticed the rejection of transplanted tumour cells but this was much faster when compared to Wistar control rats. All the animals rejected the entire tumour load within the 1st week of injection of tumour cells. To confirm further, these animals were again scanned for the 2nd week and again did not reveal any residual tumour load.

After the last scan these animals were culled and a careful post mortem conducted. This examination failed to detect any gross tumour growth either at the site of initial injection nor were there any enlarged adjacent lymph nodes.

These results were in line with our hypothesis that the response to the transplanted tumour could be dependent on the degree of matching between the host and the donor. This finding can have important bearing when it comes

to transplantation with restored kidneys after tumour resection.

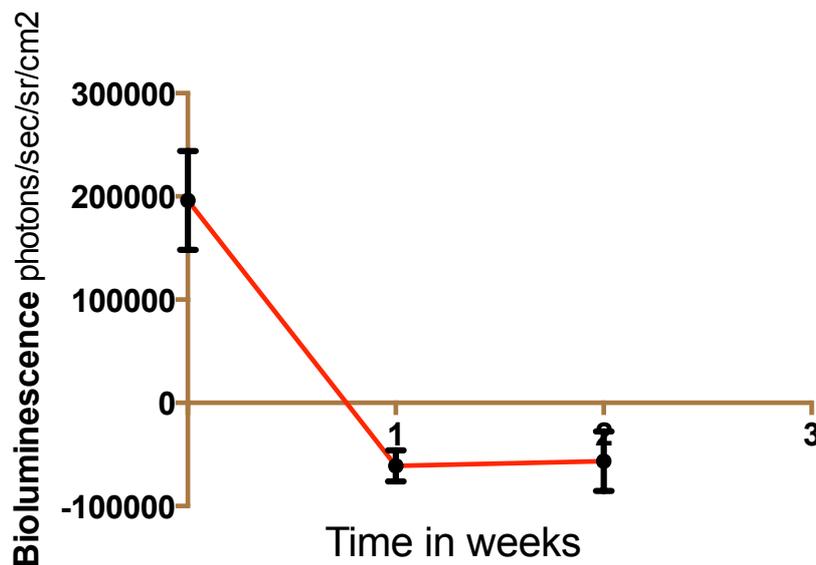


Figure 36 Role of acute rejection. Rejection of all of the injected tumour cells in the absence of any immunosuppression in poorly-matched Lewis animals within just one week.

4.4.3 Comparison

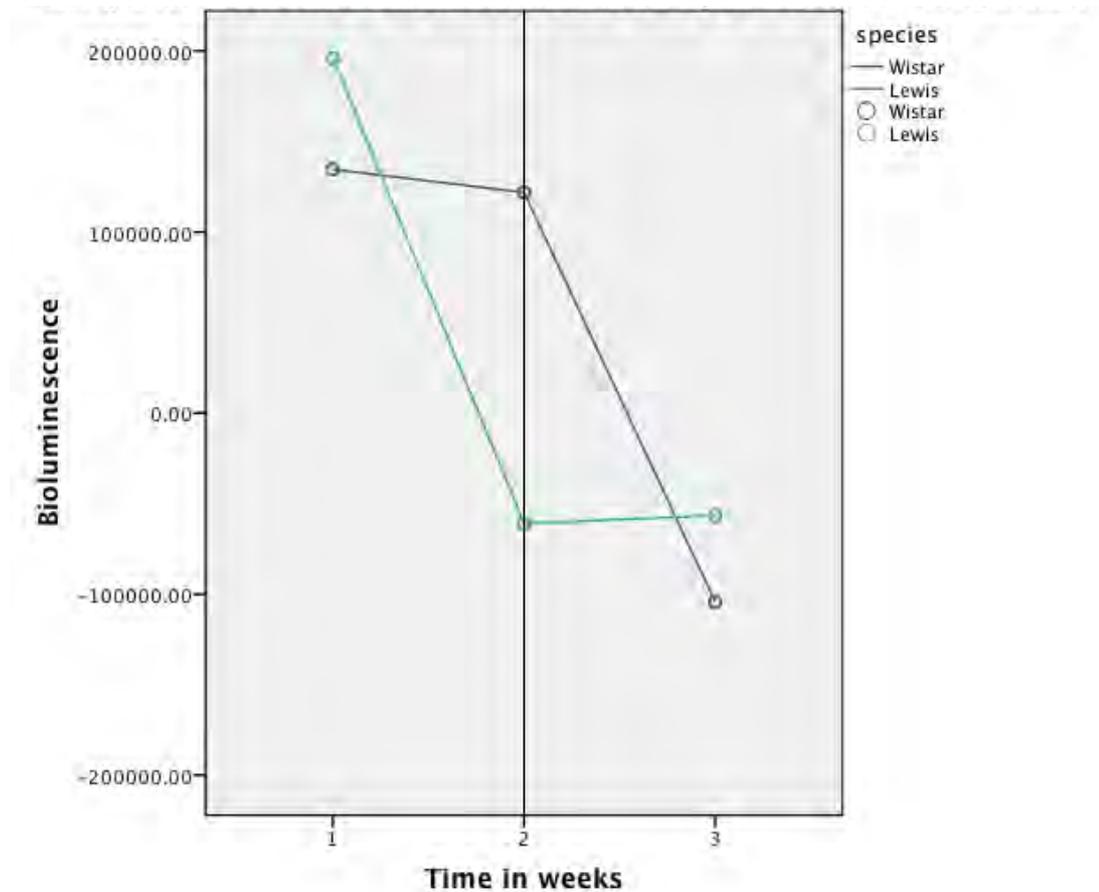


Figure 37 Direct comparison between Wistar and Lewis rats. Complete rejection of transplanted tumour cells; stronger in poorly matched Lewis animals

When both these groups were compared, there was a statistically significant difference between the two groups in terms of tumour rejection ($p < 0.05$). This is in line with our hypothesis, that with increasing mismatch between the donor cells (Wistar origin) and hosts, there will be stronger rejection response.

4.5 CYCLOSPORINE GROUP

Next we looked at the effects of cyclosporine on the transplanted tumour cells. This was really important because in clinical scenarios if there is inadvertent tumour transmission along with the restored kidneys (after T1a tumour resection followed by transplantation), the patients would not be immuno-competent as they would most likely be on at least one or two immunosuppressive medications. This, we know, takes away one of the body's first lines of defence against de novo and transplanted tumours(165) hence making these patients at much higher risk of unchecked tumour growth.

To check our hypothesis of the effects of immunosuppression on tumour growth, and role of matching and rejection on the tumour load elimination we divided these animals into two groups for each strain; ones with continued immunosuppression for 4 weeks of study period and one with two weeks of immunosuppression and two week after withdrawal to study rejection.

4.5.1 With continued immunosuppression

In this group of rats, the treatment with cyclosporine was continued for the entire study period of four weeks to study its effects on transplanted tumour cells.

Cyclosporine was given at a dose of 25mg/kg/day. The cyclosporine was started 2 days before the injection of the rats with transfected tumour cells to make sure there was adequate level of cyclosporine in circulation at the time of the injection. The cyclosporine was administered by oral gavage. After initial few gavages the animals were more acclimatised and tolerated the procedure

without much resistance. There were no incidences of gavage failure or any trauma associated with it.

The dosing was done once everyday in the mornings without fail. The oral solution was used and the formulation was Neoral® oral solution (Novartis Pharmaceuticals Corporation New Jersey, USA).

To further look at the effects of matching on tumour dynamics this treatment 'continued' group was further divided into well-matched and poorly matched animals.

4.5.1.1 Well-matched and poorly matched combination

There were a total of four Wistar rats in this group. The area of injection of tumour cells was shaved at the time of injection and was kept shaved to reduce any background interference with the signals and also to enable us to identify the area of initial injection site. This helped in marking the Regions of Interests (ROIs) when analysing the results. With continued immunosuppression we began to palpate the tumours at the site of initial injection, which continued to grow till the end of the study period.



Figure 38 Wistar rat with right flank injection. Three weeks after the injection of tumour cells. Very high signal intensity from the tumour in the right flank subcutaneous tissue. This tumour was palpable at this stage and grew even further till the end of study period.

Based on the bioluminescent signals there was initially a steady growth of the tumour till third week of the continued immunosuppression followed by exponential growth leading to formation of palpable tumour masses in the right flank. This perhaps was due to neo-angiogenesis as was evident on gross post mortem examination of these rats.



Figure 39 Dissection of the right flank. The skin is dissected off the ventral abdominal wall. The tumour clearly visible with evidence of increased vascularity around the tumour

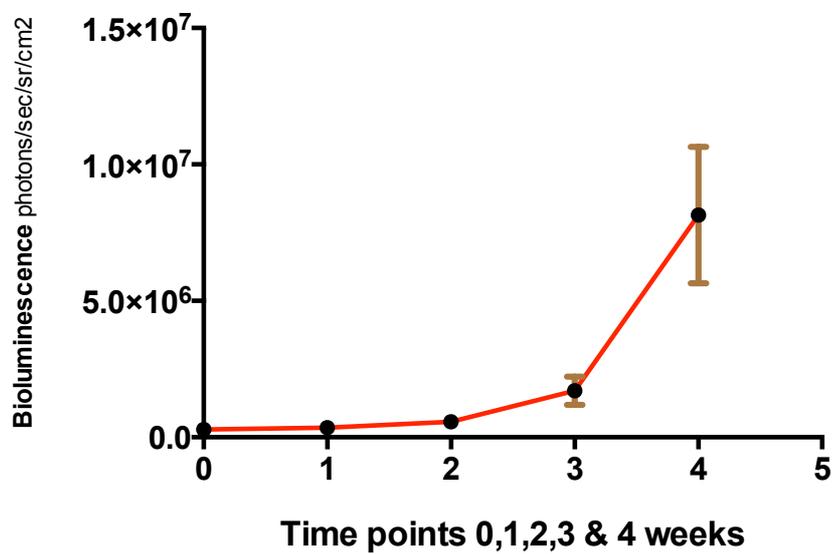


Figure 40 CsA treatment continue group. Rapid growth of transplanted tumour cells towards the end of study period represented by bright bioluminescent signals.

Similarly there were six Lewis rats in the other group. Same protocol of tumour injection and monitoring was adhered to for these animals as well.

Again, by the end of the study period there were palpable tumours in the right flank.

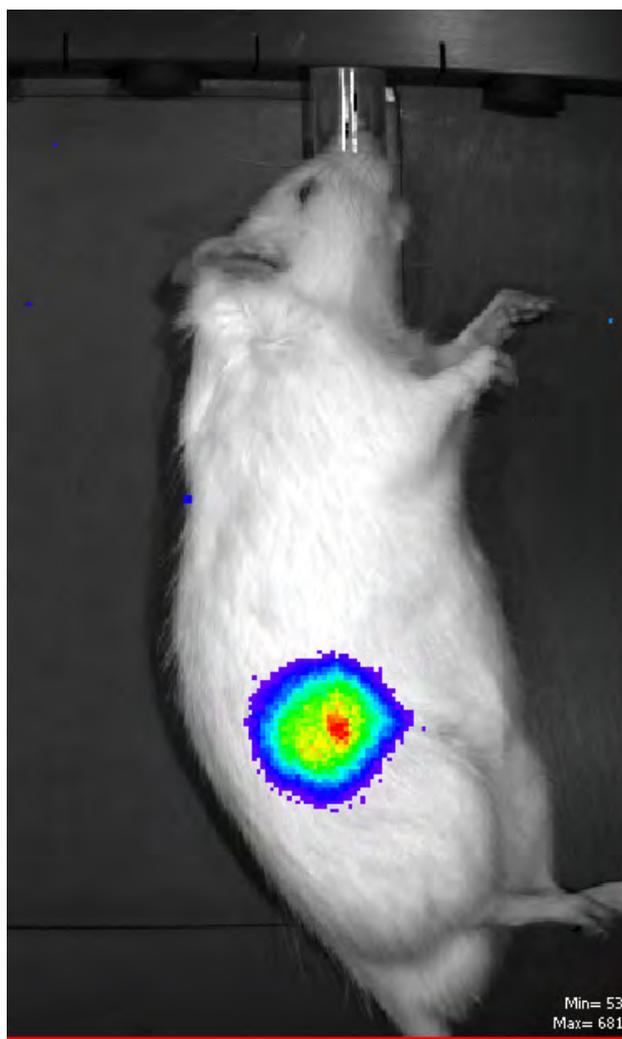


Figure 41 Lewis rat with right flank injection. Four weeks after the injection of tumour cells. Very high signal intensity from the tumour in the right flank subcutaneous tissue. This tumour was palpable at this stage.

Despite the fact that these animals were poorly matched to the injected tumour cells, under the influence of cyclosporine there was uninterrupted growth in these rats as well.

With continued cyclosporine immunosuppression, there was both gross and indirect (bioluminescent) evidence of continued tumour growth in both strains.

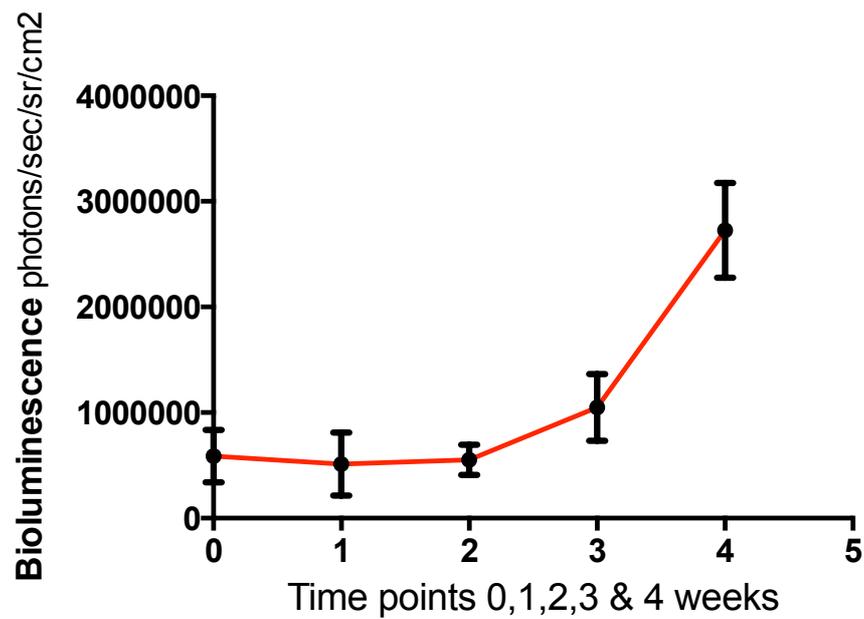


Figure 42 CsA treatment continue group. Rapid growth of transplanted tumour cells towards the end of study period represented by bright bioluminescent signals.

4.5.2 With treatment withdrawal

In this protocol the rats were given the cyclosporine for two weeks. To assess the role of rejection on the tumour cells we experimented with the treatment withdrawal protocol. The animals were scanned like the previous protocols. But after two weeks of treatment, the cyclosporine was stopped but animals were kept alive for another 2 weeks without any treatment. These were scanned as normal to monitor the tumour growth.

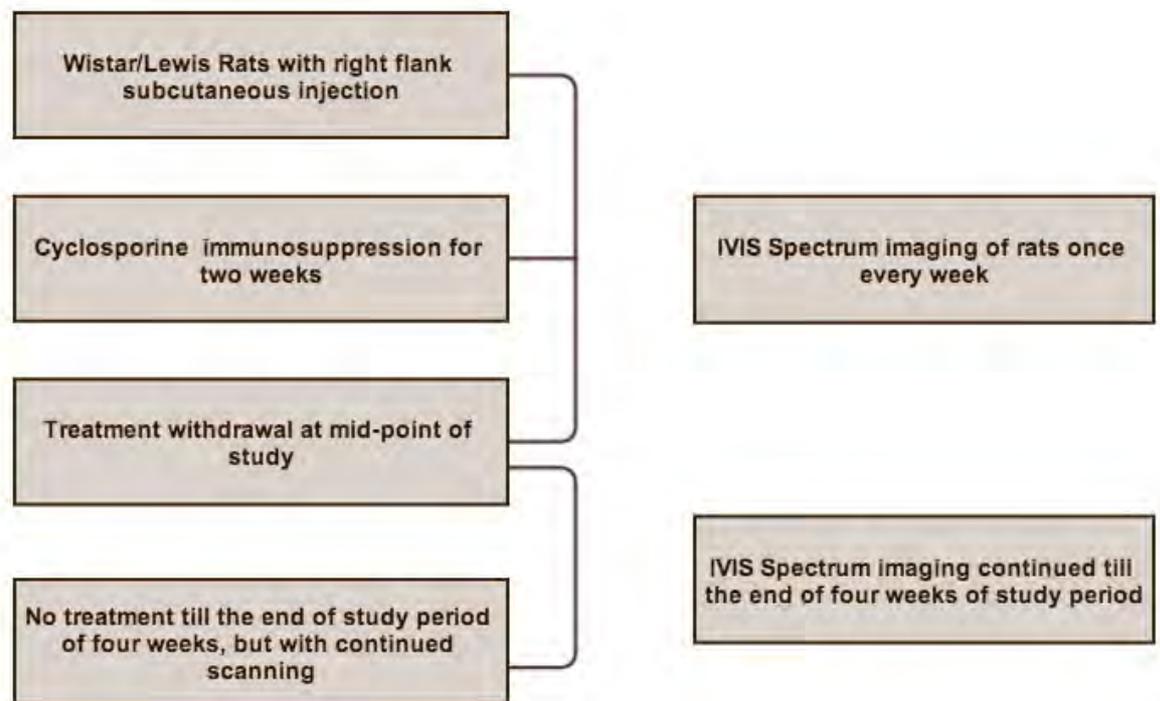


Figure 43 Flow diagram of protocol to study role of rejection and matching. After two weeks of continued immunosuppression the treatment is withdrawn to study the effects of acute rejection.

4.5.2.1 *Well-matched and poorly matched combination*

In this group, the rats were given standard 25mg/kg/day of cyclosporine via oral gavage as previously for two weeks. The animals were scanned once every

week. At the two-week mark, the cyclosporine was stopped and rats were continued to be scanned once every week for another week till the end of study period. At the end of a total of four weeks, all the animals were euthanased and post mortem performed as usual.

There were a total of eight animals in this group. There were strong bioluminescent signals from these rats till the immunosuppression was stopped. After that the bioluminescence started to reduce and eventually disappeared in majority of the animals. At the end of the study period, there were still four Wistar rats with positive tumour load.

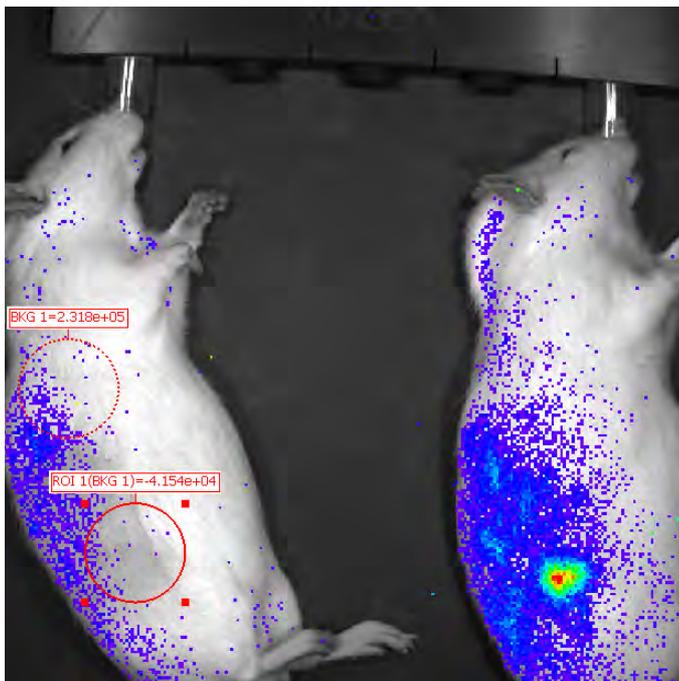


Figure 44 CsA treatment withdrawal group. Wistar rats at 4 weeks post tumour cells injection. This image was taken after 300 seconds of exposure (maximum) to detect any left over signals from the transfected tumour cells. The rat on the left side has rejected the entire tumour load while there was still positive signals from the Wistar on the right of the image.

By the end of the study, half of the animals have rejected the tumour fully while the rest still had considerable load of the tumour cells. This was in contrast to the animals

in the well matched treatment continued group where the tumour continued to grow in all the animals.

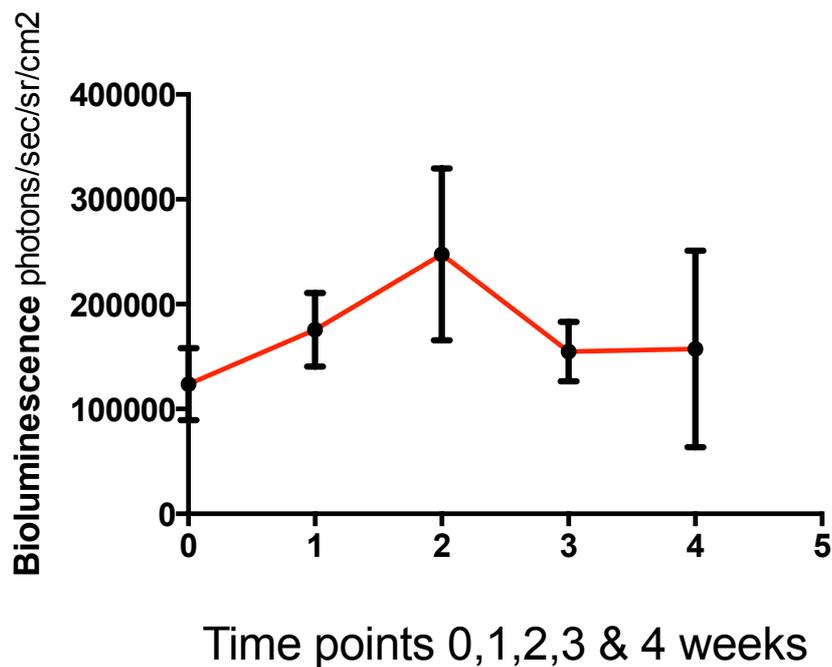


Figure 45 CsA treatment withdrawal in Wistar rats. Continued growth of the transplanted tumour cells under the influence of CsA immunosuppression till two weeks. This was followed by reduction in bioluminescent signals due to withdrawal of immunosuppression.

Again with the Lewis rats the treatment with cyclosporine was continued for two weeks, at which point the treatment was stopped and once weekly scanning was continued.

There were a total of six Lewis rats in this group. Again under the cyclosporine immunosuppression the tumour continued to grow but after stopping the immunosuppression, by the end of the study period there was no detectable bioluminescence from any of the animals.

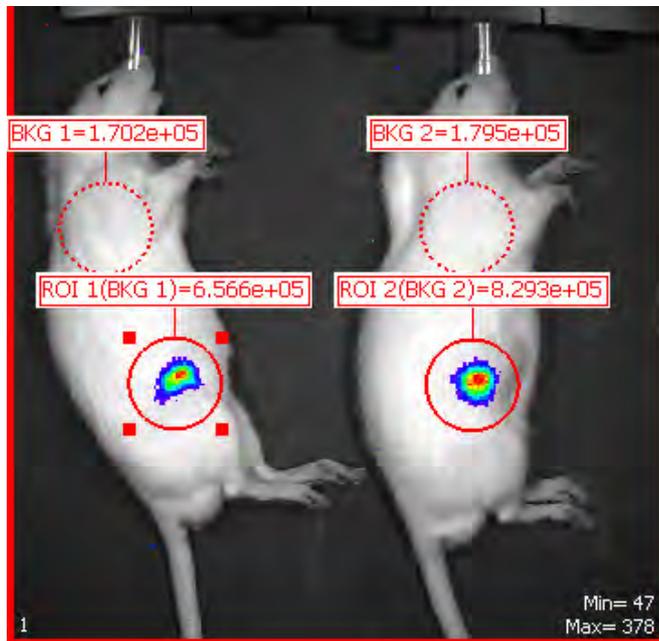


Figure 46 CsA poorly matched rats. Lewis rats at two weeks of cyclosporine immunosuppression. Bright signals were achieved from all the animals tested.



Figure 47 CsA Poorly matched rats with treatment withdrawal. Lewis animals at four weeks after the initial injection. This scan was done two weeks after stopping the immunosuppression and no signals were detected

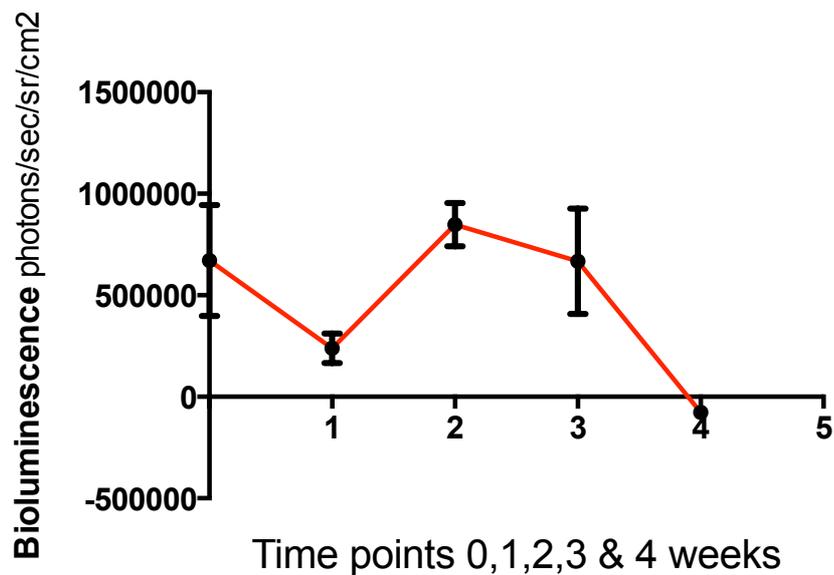


Figure 48 CsA Poorly matched rats with treatment withdrawal. Continued growth of the transplanted tumour cells under the influence of CsA immunosuppression till two weeks. This was followed by complete disappearance of bioluminescent signals after the withdrawal of immunosuppression.

4.5.3 Comparison

When the results of the two strains (Wistar and Lewis) were compared head to head, we found that under the influence of continued cyclosporine immunosuppression the tumour continued to grow in both strains. The growth was exponential towards the end of the study period, most likely due to neoangiogenesis.

The growth of tumour was significantly more in Wistar group of animals as compared to Lewis at the end of 4 weeks ($p<0.05$).

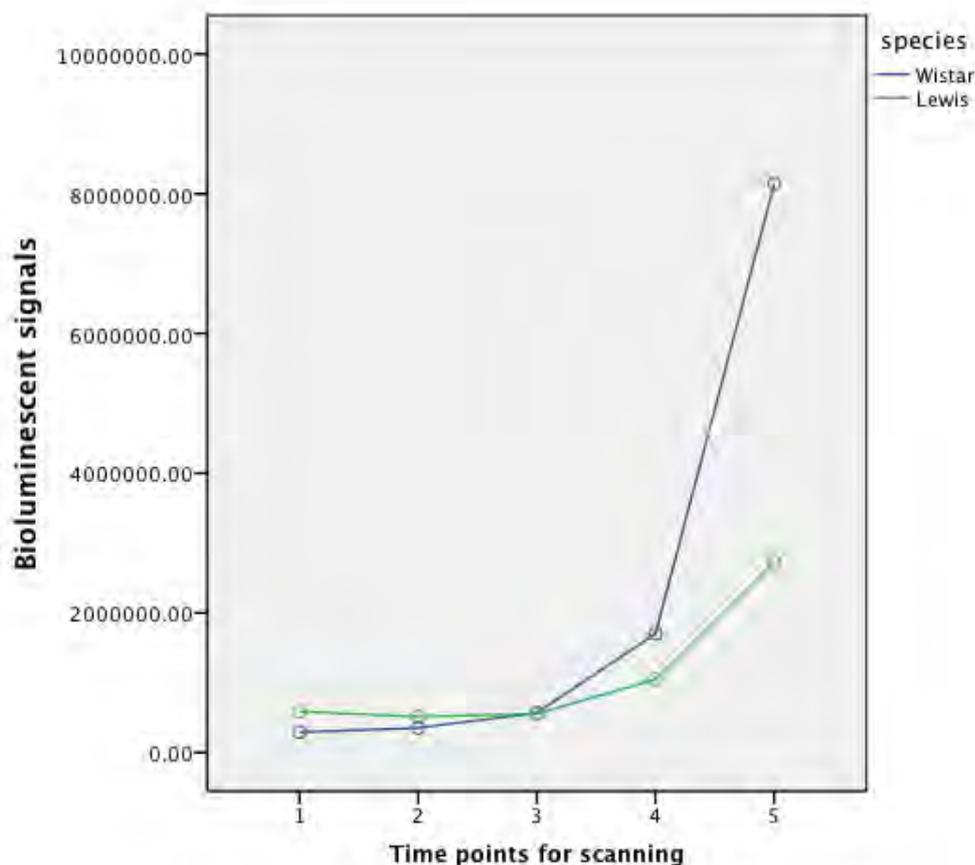


Figure 49 Direct comparison of Wistar and Lewis rats with continued CsA immunosuppression. The growth of tumour was significantly stronger among well-matched Wistar animals. The scanning schedule in weeks is shown at the X-axis.

When comparison was made between the two strains, representing well matched and poorly matched combinations, **after withdrawal of treatment** the poorly matched animals appeared significantly well equipped to reject any tumour load than well matched Wistar animals. The most likely explanation of this effect is the level of MHC matching, mounting stronger acute rejection response in the less well-matched animals. Again on direct comparison the results were statistically significant ($p < 0.05$).

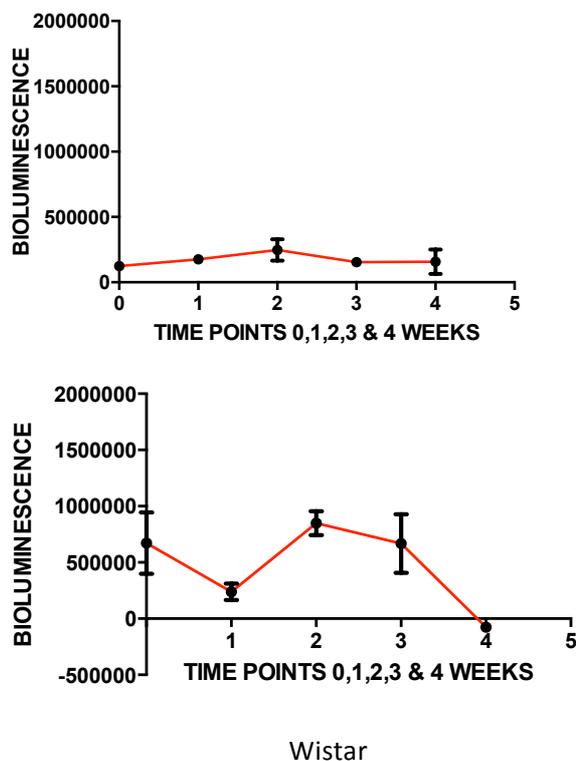


Figure 50 Direct comparison of Wistar and Lewis rats in withdrawal of CsA immunosuppression. Lewis rats were significantly more effective in rejecting tumours when compared with the well-matched Wistar rats.

4.6 SIROLIMUS TREATMENT GROUP

After finishing the control group of cyclosporine immunosuppression, we looked at the effect of Sirolimus immunosuppression on the transplanted tumour behaviour.

4.6.1 Background

The reason for selecting Sirolimus was two fold. Firstly, Sirolimus has shown promising results due to its anti neoplastic properties. Secondly, long-term treatment with cyclosporine can lead to chronic allograft nephropathy and preservation of a smaller (resected) kidney with a non-nephrotoxic drug could be preferable.

The most important reason for using an mTOR inhibitor was the possibility of tumour regression in an immunosuppressed host.

Sirolimus is a fermentation product of microorganism *Streptomyces hygroscopicus*. It is a potent inhibitor of T and B cells as a response to antigen stimulation. For the activation of lymphocytes there has to be interaction of interleukins and IL receptors as well as co-stimulatory molecules (CD28), which then leads to activation of TORC1 complexes. Sirolimus binds to FK binding protein 12(FKBP 12) and this complex blocks the activation of TORC1 complex leading to cell cycle arrest in late G1 phase(109).

It also has a direct inhibitory effect on the apoptosis of dendritic cells which have a pivotal role in the transplant immunology(110). It also inhibits cytokine and growth factor stimulated proliferation of the fibroblasts, tumour cells and smooth muscle cells(111)(112)(113).

Sirolimus has also been used as a sole agent in the maintenance phase of immunosuppression after transplantation. One of the first big trials with more than 400 patients from Manchester showed better creatinine clearance in the sirolimus arm when it was used as a maintenance immunosuppression after the withdrawal of CsA at 3 months post transplant(120). An RCT from Cambridge suggested that the impaired graft function possibly due to CNI nephrotoxicity could be reversed with sustained improvement at 2 years post transplant by conversion to sirolimus at three months. This along with other similar studies point to potential benefits of sirolimus as a maintenance agent in terms of graft function and to potentially reduce the incidence of chronic allograft nephropathy(121).

There are several studies, which suggest that with sirolimus based immunosuppression the over all rates of de novo malignancies are significantly less than CsAs. There was a large retrospective analysis performed on post transplant malignancies in more than 33 thousand deceased donors from 264 centres in USA. This study looked at both the skin and non-skin solid organ malignancies rates and showed that the rate of malignancies with sirolimus/everolimus plus CNI combination was 0.6% as compared to CNI, alone which was 1.81% (92).

Efficacy of mTOR inhibitors has also been showed in cases of advanced renal cell carcinoma. A large double blind trail randomised 272 patients into everolimus group and 138 in placebo arm for the treatment of advanced renal cell carcinoma. There was progression of disease in 37% of patients in the treatment arm as opposed to 65% in the placebo arm ($p \leq 0.0001$) with a median disease free survival of 4.0 versus 1.9 months(123).

Similar encouraging results were noted when everolimus was used for the patients with advanced pancreatic neuroendocrine tumours. 207 patients in the treatment were compared with 203 patients in placebo arm and were found to have significantly better progression free survival of 11 months as compared to 4.6 months in the placebo arm ($p < 0.001$). 34% of patients were still alive at 18 months as compared to 9% with placebo(124).

mTOR inhibitors were found to be useful in hepatocellular carcinomas and in advanced breast carcinomas as well(125)(126).

mTORs have been shown to be effective for the post transplant lymphoproliferative disorders as well. A case of complete remission of disseminated PTLD has been reported in literature after conversion to sirolimus(127).

With increasing cohort of patients on immunosuppression post transplant there is an increasing incidence of Kaposi's sarcoma. mTOR inhibitors have been found useful for their treatment as well. A case series of 25 patients with cutaneous Kaposi's sarcoma when converted from cyclosporine to sirolimus showed complete biopsy proven remission within 6 months of treatment(128).

These effects of mTOR inhibitors are very encouraging and over the past 10 years there has been mounting evidence on their efficacy. Although by no means these are the perfect solutions but a medication to be immunosuppressive and anti neoplastic in transplant setting is very useful feature that we tried to explore.

4.6.2 Dose

The dose used in animal studies varies between the types of intended intervention. For its immunosuppressive and inhibitory effects on vascular intimal hyperplasia the dose range is reported to be between 0.3-0.5mg/kg/day orally. The studies where both cyclosporine and sirolimus have been used together the target dose was lower to achieve the therapeutic levels due to synergism in the pharmacokinetics(183)(184)(185).

Where Rapamycin was used for the treatment of urothelial carcinomas, the most common dose used was 2mg/kg/day(168)(186).

For our experiments, we used two different doses of sirolimus. The lower dose we used was 0.5mg/kg/day while the high dose tested was 2mg/kg/day.

4.6.3 Sirolimus high dose

4.6.3.1 *With continued immunosuppression*

Again this group was divided into well-matched and poorly matched animals. There were 6 animals in each group tested.

4.6.3.1.1 *Well-matched and poorly matched combination*

1.2×10^7 cells were injected into the Wistar and Lewis rats in the right flank as per protocol under general anaesthesia. Sirolimus was started a day before the tumour injection and was continued for a total of four weeks of treatment via oral gavage. There were 6 Wistar rats in this group and the behaviour of tumour

was studied by once weekly IVIS spectrum scanning under general anaesthesia.

With sirolimus dosing, all the Wistar animals were free of tumour load at the third scanning (two weeks from the day of subcutaneous injection).

This effect was stronger in the high dose group (2mg/kg/day) when compared to low dose group (0.5mg/kg/day).

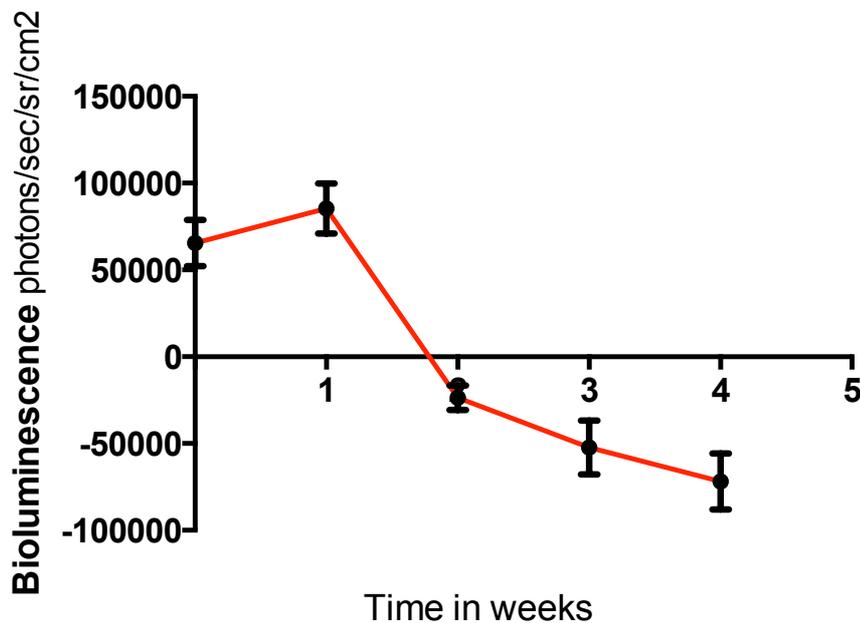


Figure 51 Well-matched (Wistar) animals with continued sirolimus immunosuppression. All tumour load was cleared by the week two of the study period.

With high dose of sirolimus all the six **Lewis rats** cleared the tumour load as evident by lack of any IVIS bioluminescent signals by week two of study period.

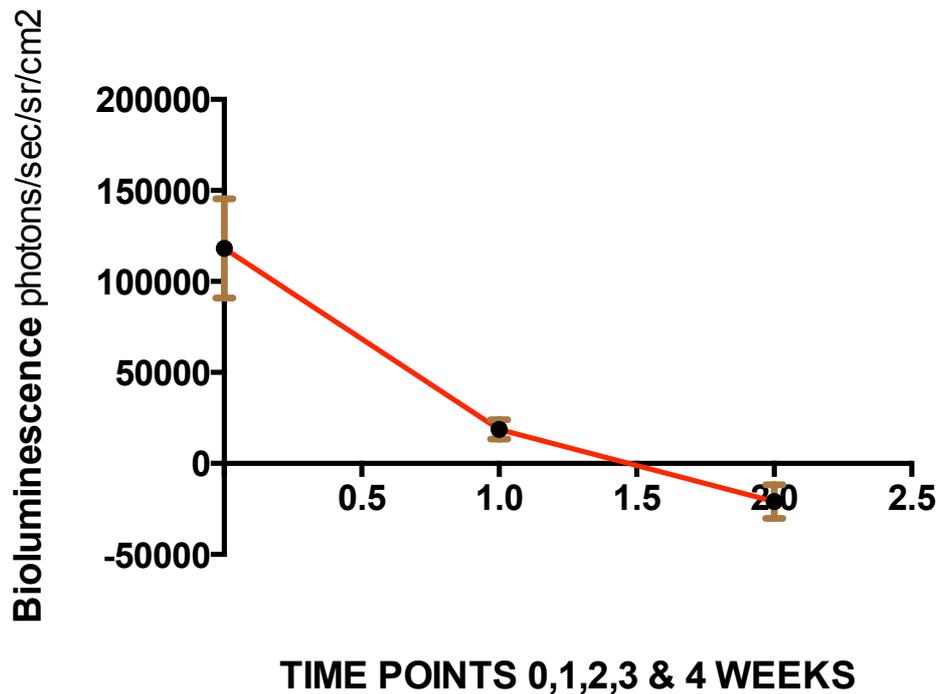


Figure 52 Sirolimus high dose with continuous immunosuppression in poorly matched animals. Again all animals rejected the tumour cells with first two weeks of study period. The scanning was done once every week (Week 0, 1,2,3,4)

4.6.3.2 *With treatment withdrawal*

To study the role of acute rejection in tumour elimination these animals were given sirolimus immunosuppression for initial two weeks from tumour injection and then the immunosuppression was withdrawn till the end of the study period. Tumour behaviour was monitored with once weekly IVIS imaging as previously. This group was further divided into two subgroups based on matching.

4.6.3.2.1 *Well-matched and poorly matched combination*

There were 6 rats in each of these high dose groups.

All the animals had rejected the tumour cells by the week 2 of IVIS spectrum scanning. All the animals were scanned till the completion of the study period of four weeks even if there was no detectable signal while the study was still underway. This helped not only in confirming the initial results but also ruled out absence of signals due to technical problems.

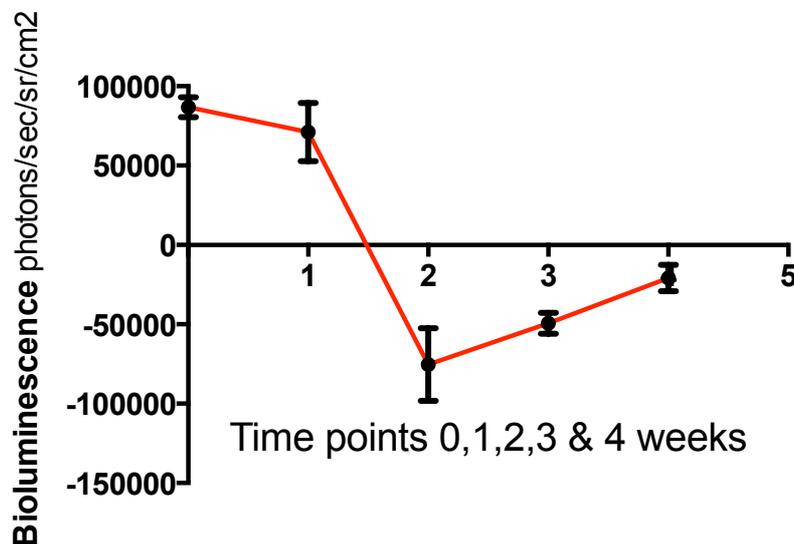


Figure 53 Well-matched Wistar treatment withdrawal. Animals again rejected the tumour load with the first two weeks of high dose sirolimus. There was no reappearance of tumour cells after treatment withdrawal at any point of study period.

There were six animals in poorly matched group as well but three animals died during scanning. This was completely unexpected and happened during the 1st scanning episode for these animals. All the other variables were similar to any other scanning protocol after tumour injection. These animals were acclimatised

for a week before any experimentation, were kept in same conditions to the rest of the animals, the anaesthetic protocol was similar as well. Other animals studied and scanned that day did not have any adverse effects. The post mortem examination of these animals did not reveal any thing unusual either.

The tumour load was eliminated fully by week three scanning.

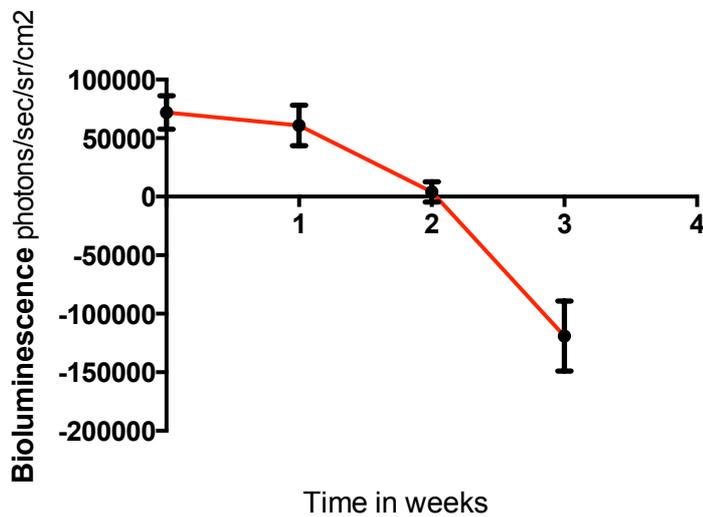


Figure 54 Poorly matched treatment withdrawal. In these three Lewis animals, there was some residual signals by the week two scanning. At week three there were no signals left again proving the effectiveness of treatment withdrawal.

4.6.4 Sirolimus low dose

4.6.4.1 *With continued treatment*

Similarly this group was divided into two to include both Wistar and Lewis strains.

4.6.4.1.1 *Well-matched and poorly matched combination*

This group consisted of 6 Wistar and 6 Lewis rats receiving 0.5mg/kg/day of sirolimus. By week 3, all the animals had rejected the entire tumour load. This was consistent with the earlier finding with the high dose group, apart from a slightly longer time taken for tumour cells elimination.

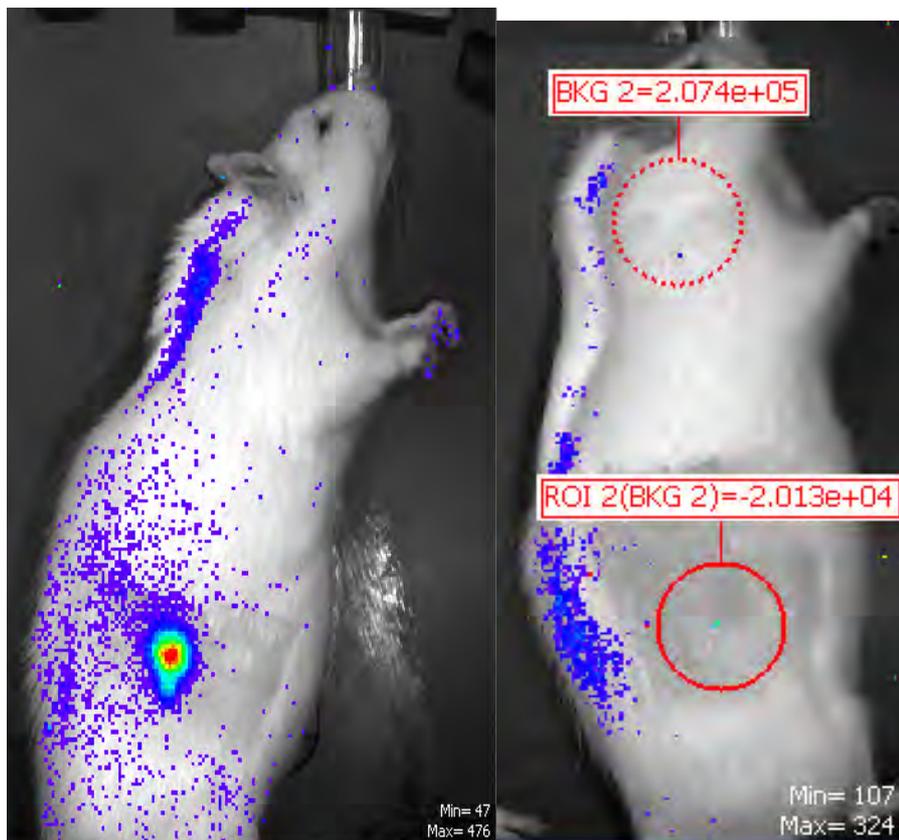


Figure 55 Low dose sirolimus treatment. Week 1 and week 4 IVIS images of the Wistar rat with low dose sirolimus treatment. There is complete elimination of tumour load during the study period.

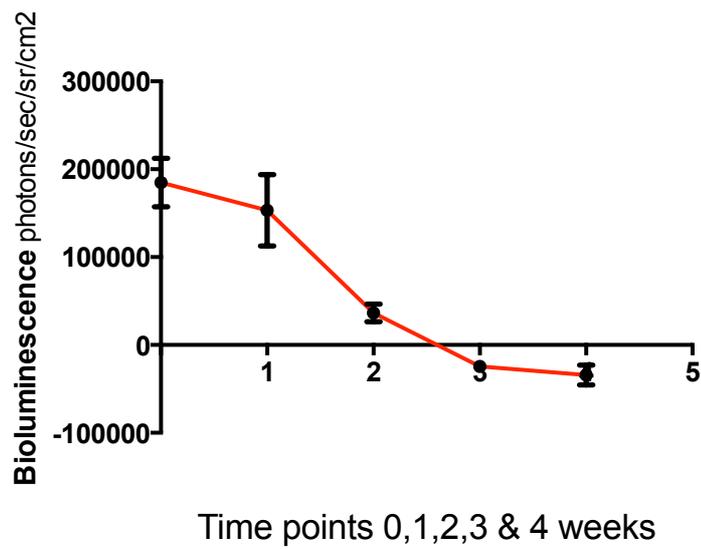


Figure 56 Low dose sirolimus treatment. Well matched Wistar animals with continued immunosuppression with low dose Sirolimus, again was successful in clearing all the tumour load by week three of scanning.

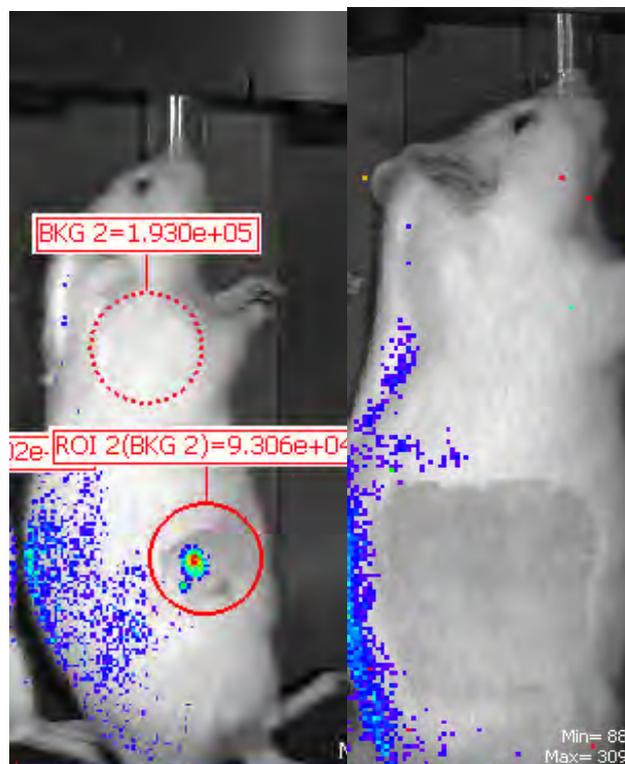


Figure 57 Poorly matched – Low dose sirolimus. Week 1 and week 4 IVIS images of the Lewis rat with low dose sirolimus treatment. There is complete elimination of tumour load during the study period.

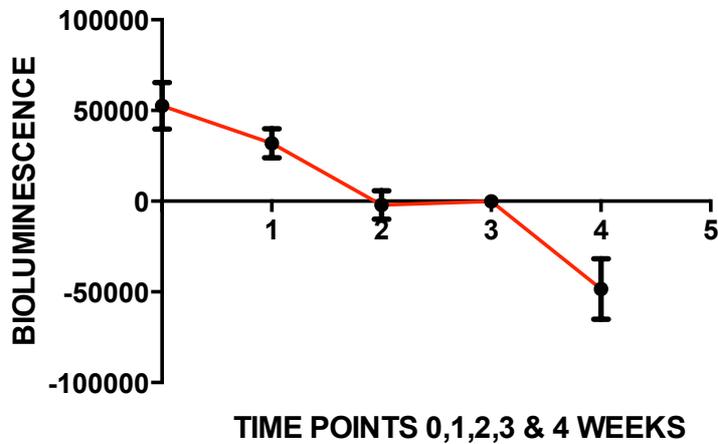


Figure 58 Low dose sirolimus-Lewis rats. Poorly-matched Lewis animals with continued immunosuppression with low dose Sirolimus, again was successful in clearing all the tumour load by week four of scanning.

4.6.4.3 *Treatment withdrawal*

This group was divided into Wistar and Lewis animals as well.

4.6.4.3.1 *Well-matched and poorly matched combination*

This group had 6 Wistar rats receiving low dose sirolimus treatment for two weeks followed by discontinuation. Again all the animals rejected the tumour load at the end of the study period of 4 weeks. By the time the treatment was withdrawn, the tumour load was already reducing. This was in contrast to the cyclosporine group.

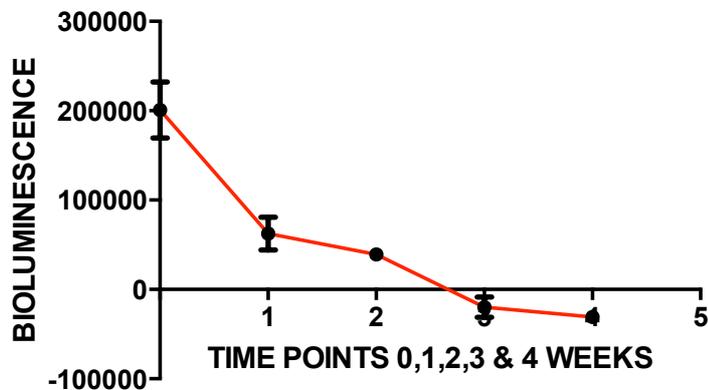


Figure 59 Well-matched rats with low dose sirolimus. The tumour load was reducing which continued upon treatment withdrawal and by the end of study period there were no bioluminescent signals left.

4.6.4.4 *Poorly matched treatment withdrawal*

This group had 6 Lewis rats receiving low dose sirolimus treatment for two weeks followed by discontinuation. In this group, all the animals rejected the tumour load by week 3 of the study period. Again this effect was in accordance to the previous observations that the rejection of the tumour is significantly stronger in the less well-matched group of rats. Due to the very strong primary effect of sirolimus itself, there was no significant difference between these groups on direct comparison. This observation was most likely due the fact that at the time of treatment withdrawal, the tumour load is already so small that we fail to elicit any significant differences between these groups.

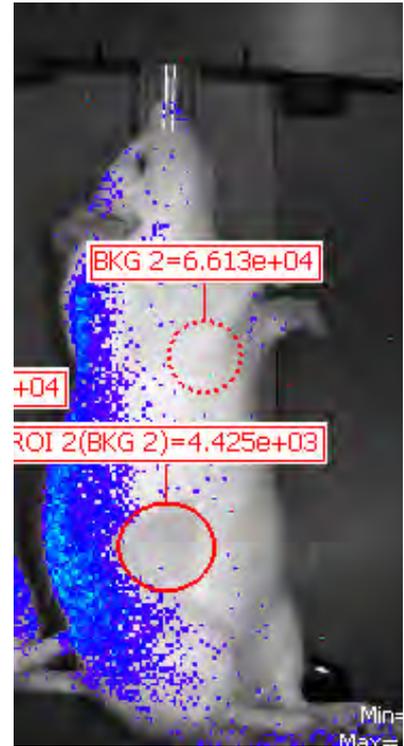
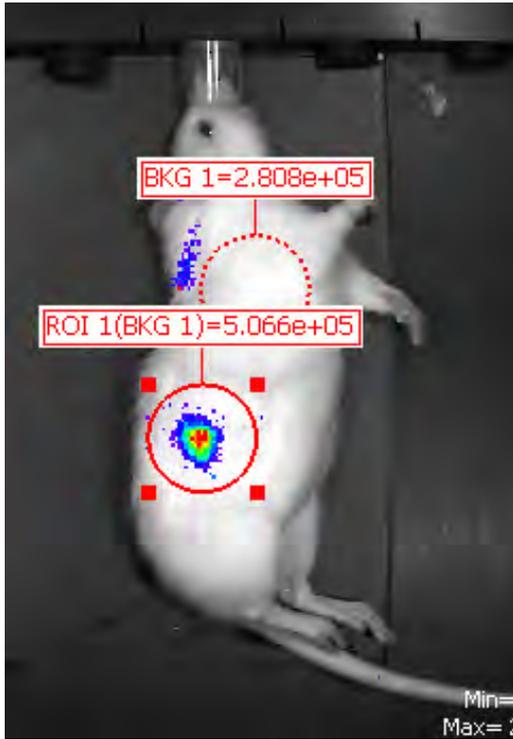


Figure 60 Lewis rats-Treatment withdrawal. Week 0 and week 4 IVIS images of the Lewis rat with low dose sirolimus treatment withdrawal. There is complete elimination of tumour load during the study period.

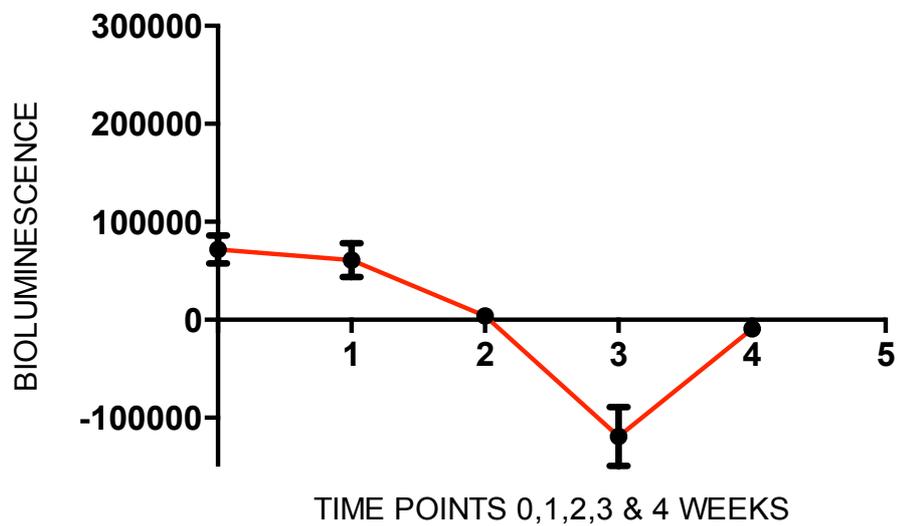


Figure 61 Lewis animals with low dose sirolimus treatment withdrawal. The tumour load was reducing which continued upon treatment withdrawal and by the end of week three there were no bioluminescent signals left.

4.7 LEFLUNOMIDE

4.7.1 Background and dosing

Leflunomide has both immunosuppressive and anti-inflammatory effects. The main effect is exerted by its inhibition of pyrimidine synthesis pathway. This prevents the availability of pyrimidine to the lymphocytes, which depend solely on the de novo synthesis of the pyrimidines as they lack the pyrimidine salvage pathway.

Although currently not used as a standard immunosuppressive in solid organ transplantation but it has been used previously with promising results comparable to the cyclosporine immunosuppression. Various studies have shown anti tumour role of these agents(171)(138)(172)(139)(140), which makes leflunomide suitable to assess our hypothesis.

The dose of leflunomide used for our experiments was 20mg/kg/day. This was based on the various dose ranges used in literature for rats in transplantation models. The doses range from 5mg/kg/day to 35 mg/kg/day(187)(188)(133).

The powdered form of leflunomide was purchased from Stratech Scientific Ltd. Suffolk, United Kingdom. The powdered form is not soluble in water and hence was dissolved in DMSO according to the literature and manufacturer's instructions(189)(190)(191). 54mg leflunomide was dissolved in 1 ml of filtered DMSO to make up stock solution that was then diluted in drinking water to make up final concentrations for animal use.

we only studied the effects of leflunomide in Wistar rats (well matched group). This was due to the use of some animals at the beginning of the pilot study for the proof of the concept.

4.7.2 With continued immunosuppression in well matched group

There were 7 Wistar animals in this group. The tumour cells were injected in the right flank and animals were gavaged with oral solution of leflunomide at the previously agreed dose of 20mg/kg/day. The rats were scanned in IVIS spectrum once weekly.

Three out of 7 Wistar rats in this group rejected the tumour by the end of the study period. The remaining number of animals showed a trend towards reducing tumour load as well, but were unable to eliminate the bioluminescent signals completely during the course of study period.

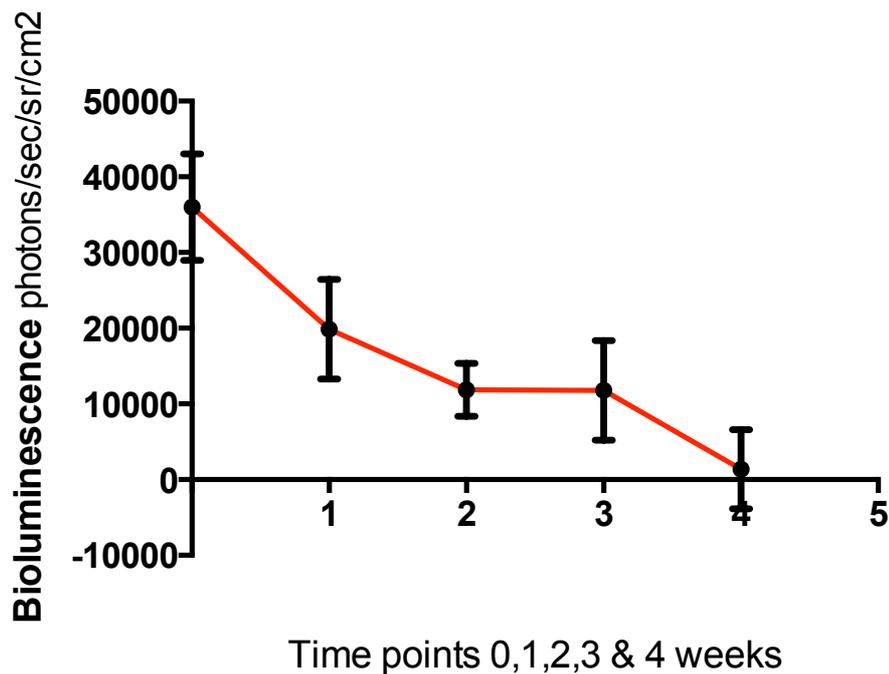


Figure 62 Well-matched Wistar animals in the leflunomide treatment continue arm. There was a steady decline in the bioluminescent signals from these animals from tumour injection site. Despite weakening signals not all the animals rejected complete tumour load by the end of the study period.

4.7.3 With treatment withdrawal in well matched animals

Again 7 Wistar rats were studied in this group. Leflunomide was stopped after two weeks of continuous treatment and animals were monitored for tumour behaviour. By the end of the study period, four out of the 7 animals studied in this group had rejected the tumour and showed no bioluminescence. The rest of the animals although did have positive signals but the intensity of the signal was significantly lower than at the beginning of the study.

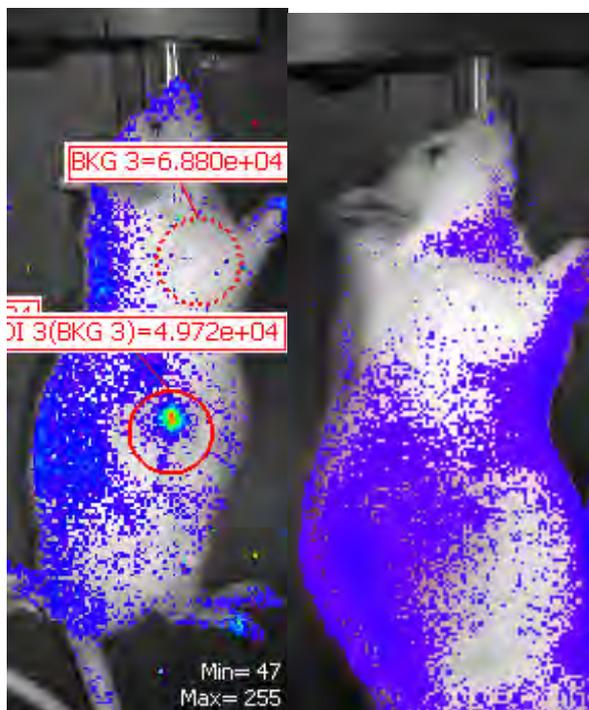


Figure 63 Leflunomide treated Wistar animals-treatment withdrawal. Complete elimination of the tumour load from the Wistar rats. There is considerable background “noise” which is due to long exposure times to detect any small residual tumour bioluminescence.

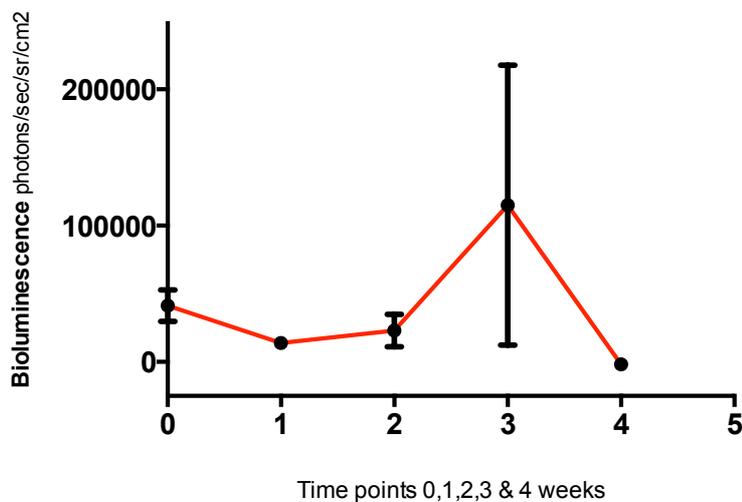


Figure 64 Treatment withdrawal after two weeks in Leflunomide treated animals. By the end of study period 4/7 animals have rejected the tumour load and rest were showing reducing tumour load.

4.8 COMPARATIVE ANALYSIS

The focuses of the comparative analyses were to look at the role of matching and acute rejection on the tumour elimination. The second part was focused at looking at the role of different immunosuppressive agents on the behaviour of transplanted tumour cells.

4.8.1 Role of matching on tumour rejection

As mentioned earlier when there was no immunosuppression given, the poorly matched animals were far more effective in rejecting the tumour load as opposed to the well-matched Wistar animals. This difference between groups was significant ($p < 0.05$). The tumour elimination was much more rapid in the Lewis animals as below.

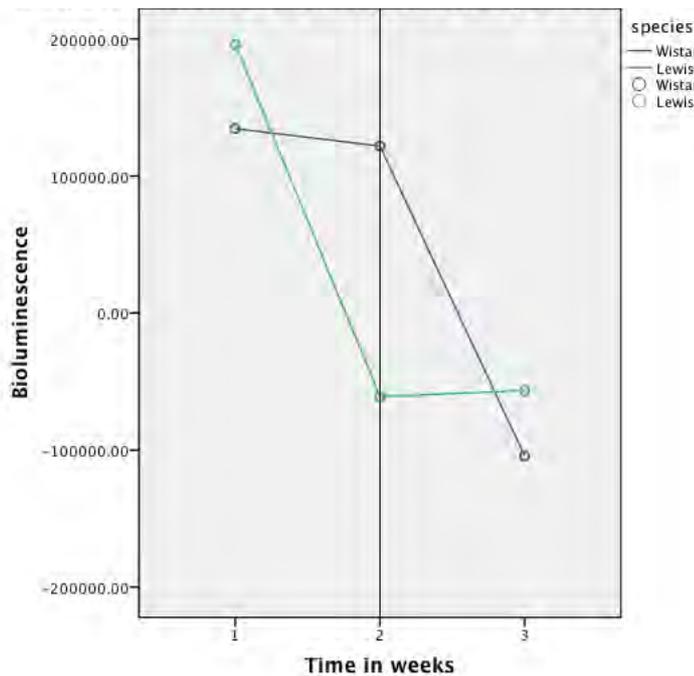


Figure 65 Direct comparison between Wistar and Lewis rats after tumour cells injection. Complete rejection of transplanted tumour cells; stronger in poorly matched Lewis animals

Similar effect was evident in the treatment groups. Although to some extent it could be appreciated among all treatments studied but this effect was most pronounced in the cyclosporine group. This was due to the observation that the tumour growth continues under this immunosuppression and at the time of withdrawal of treatment there was still a significant tumour load.

Two different sets of observations proved our hypothesis of a positive role of acute rejection in tumour destruction. Firstly, reduction of tumour load after treatment withdrawal among the same group of animals, and secondly comparing the effects of withdrawal of treatment across well matched and poorly matched strains.

4.8.2 Rejection within similar strain

The best example of role of rejection within a strain was in cyclosporine group as mentioned above. For Wistar rats, when the treatment was withdrawn, half the animals rejected the tumour load entirely as opposed to the continued growth of the tumour in the treatment continue group (fig 66 and fig112).

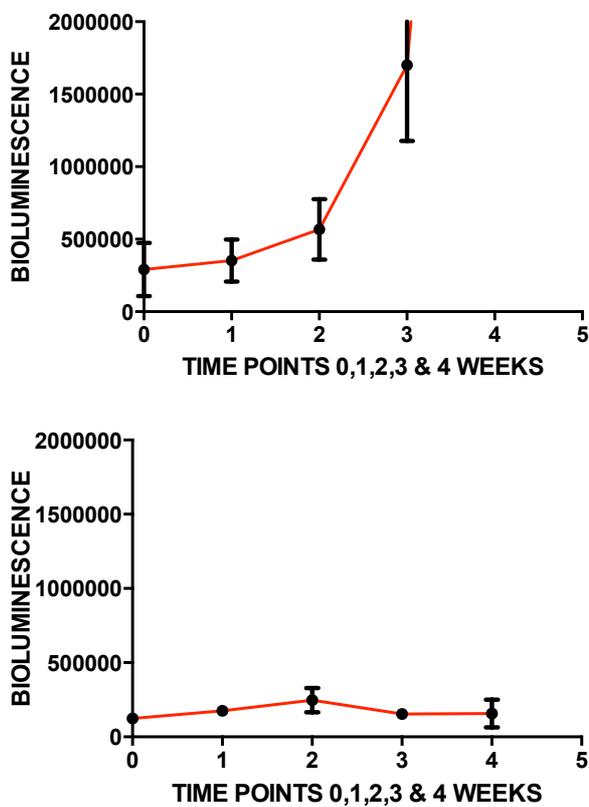


Figure 66 Comparison between treatment continue/withdrawal groups in CsA treatment-Wistar animals. As opposed to continued treatment, there was significant reduction in tumour load after treatment withdrawal. For comparison purposes the bioluminescent ranges are kept similar on Y axis hence the final reading in CsA group is out of the scale.

The tumour load was significantly reduced at the end of the study period after treatment withdrawal ($p < 0.05$ Mann Whitney U test). These statistical

differences were noted from week 3 and 4, before that the differences in the bioluminescence were not significant affirming the role of acute rejection after the treatment withdrawal.

Similar effects were seen in the Lewis rats before and after treatment withdrawal (fig 67 and fig 113).

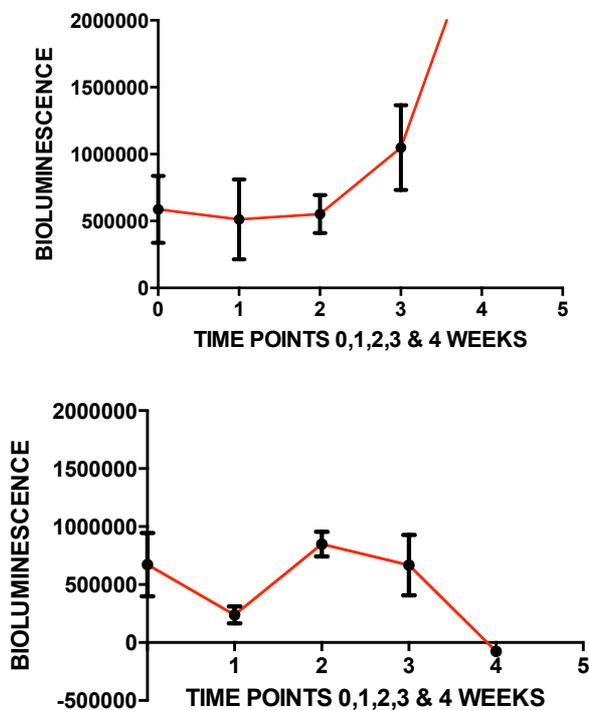


Figure 67 Comparison between treatment continue/withdrawal groups in CsA treatment-Lewis animals. As opposed to continued treatment, there was significant reduction in tumour load after treatment withdrawal. For comparison purposes the bioluminescent ranges are kept similar on Y axis hence the final reading in CsA group is out of the scale. By the end of study in treatment withdrawal group, all the animals have cleared the tumour load fully.

Again there were no differences between the two groups when compared up to week three of the treatment but by week 4 there was significant difference in signal emissions ($p < 0.05$).

4.8.3 Rejection between strains

Again this effect was best shown in the cyclosporine group. When Wistar and Lewis rats were compared against each other after treatment withdrawal, poorly matched Lewis animals were significantly ($p < 0.05$) well equipped in rejecting the tumour cells. This effect was likely due the wider MHC differences in these animals to the injected tumours cells from Wistar rats as all the animals in the Lewis group rejected the tumour load after treatment withdrawal while only half were able to clear the tumour load by the end of the study period (fig 68 and fig 114).

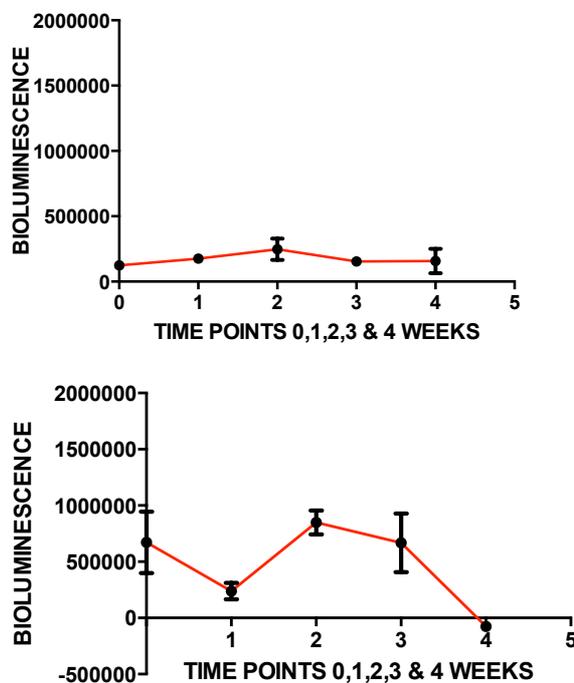


Figure 68 Direct comparison: treatment withdrawal Wistar v Lewis. Although well matched animals showed significant tumour rejection after treatment withdrawal, the poorly matched Lewis animals were most effective

in eliminating complete tumour load. These results were clear in CsA group, as there was significant tumour load at the time of withdrawal of treatment.

Although there were brighter bioluminescent signals in the Lewis animals at the time of injection (time 0), by the end of the study period despite having more tumour load to start with, these rats were better in rejecting the tumour load ($p < 0.05$ at week 4).

As mentioned earlier due to effectiveness of sirolimus in tumour elimination even at low dose, we did not see any difference between treatment withdrawal and continuation groups and between the well-matched and poorly matched groups (fig 69 and fig 115).

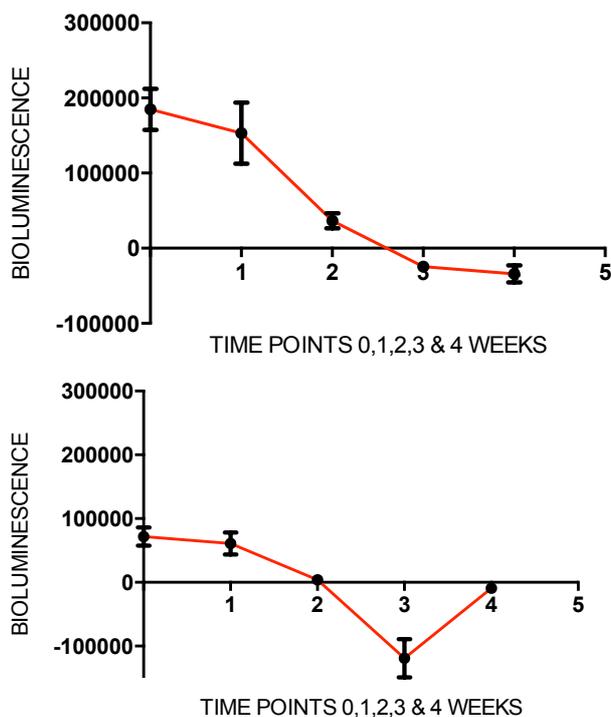


Figure 69 Sirolimus treatment low dose. There was no difference between well-matched and poorly matched strains. This was most likely due to effectiveness of Sirolimus as an anti neoplastic agent, leaving both animal strains with very little tumour load at the time of treatment withdrawal.

4.9 ROLE OF IMMUNOSUPPRESSION

The direct comparisons between the immunosuppressive groups pointed towards our hypothesis that newer immunosuppressive medication will be better in rejecting the transplanted tumours.

4.9.1 Cyclosporine v High dose Sirolimus

With high dose sirolimus treatment both Wistar and Lewis rats eliminated the entire tumour load within the study period as opposed to the cyclosporine treated animals where transplanted tumour continued to grow. These results were statistically significant ($p < 0.05$). (fig70 and fig 116).

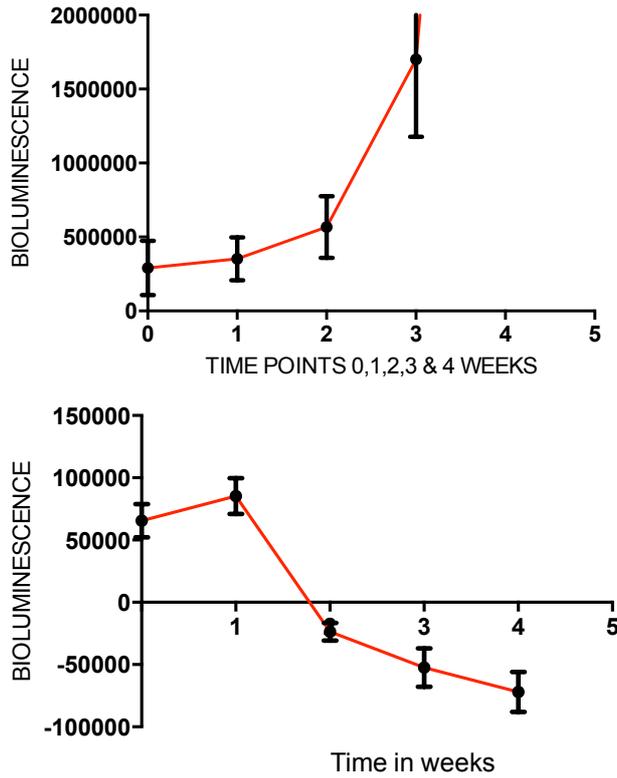


Figure 70 Direct comparison between Wistar rats on CsA (Left) or Sirolimus high dose (right) immunosuppression. As opposed to steady increase in tumour load with CsA, there was complete elimination of bioluminescent signals with Sirolimus.

Similar results were noted with Lewis rats (fig 71 and fig 117).

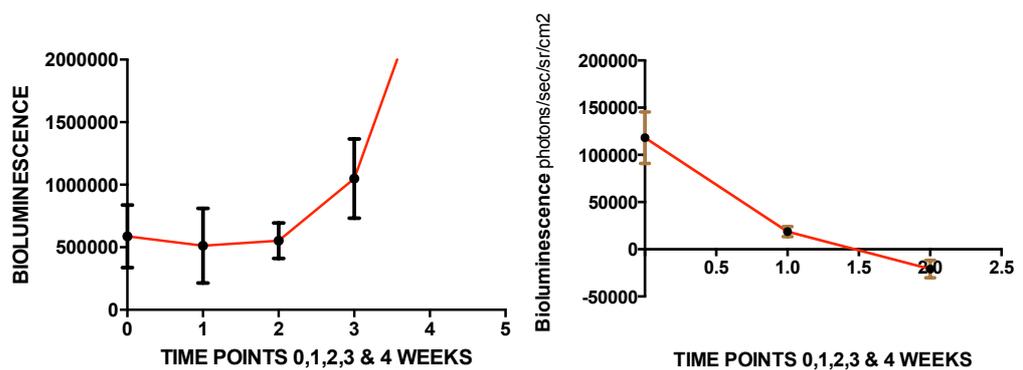


Figure 71 Direct comparison between Lewis rats on CsA or Sirolimus (high dose) immunosuppression. As opposed to steady increase in tumour load with CsA, there was complete elimination of bioluminescent signals with Sirolimus.

4.9.2 Cyclosporine v Low dose Sirolimus

Both Wistar and Lewis rats eliminated the entire tumour load within the study period as opposed to the cyclosporine treated animals where transplanted tumour continued to grow. These results were statistically significant as well ($p < 0.05$). These animals treated with low dose took slightly longer than the high dose sirolimus group but still were able to entirely clear the tumour load within the 4 weeks of the study period (fig 72 and fig 118).

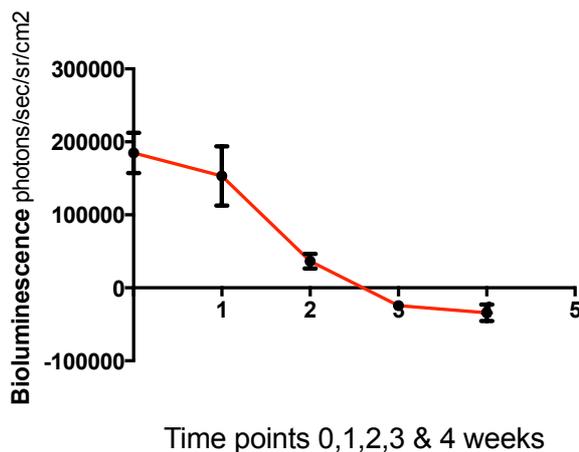
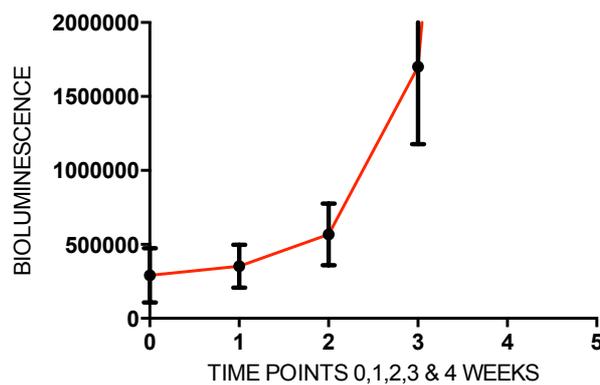


Figure 72 Direct comparison between Wistar rats on CsA or Sirolimus (low dose) immunosuppression. As opposed to steady increase in tumour load with CsA, there was complete elimination of bioluminescent signals with Sirolimus.

Results with Lewis (poorly matched) animals were similar with statistically significant improved results (fig 73 and fig 119).

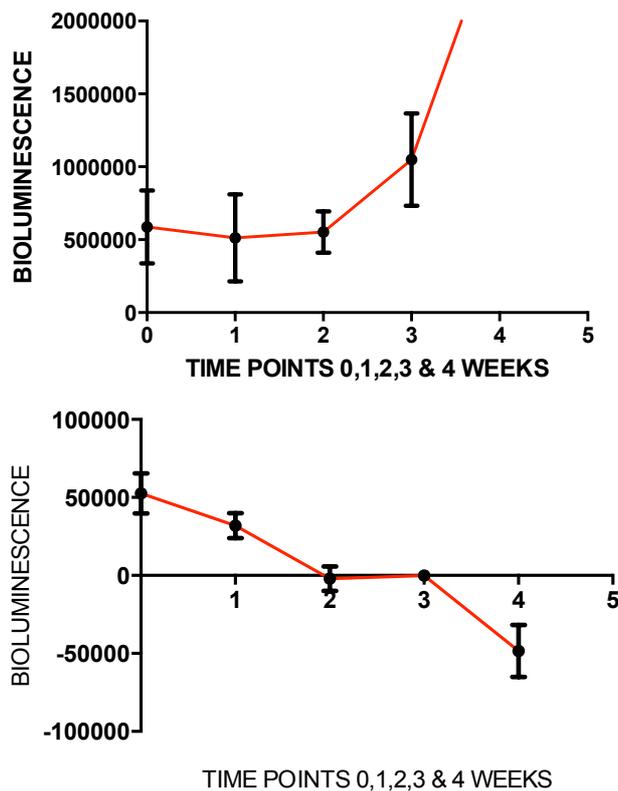


Figure 73 Direct comparison between Lewis rats on CsA and Sirolimus (low dose) immunosuppression. As opposed to steady increase in tumour load with CsA, there was complete elimination of bioluminescent signals with Sirolimus.

4.9.3 Cyclosporine v Leflunomide

Despite the lack of leflunomide treated animals' complete elimination of tumour, there were 3 animals by the end of the study period that had managed to clear the tumour load (fig 74 and fin 120). This was in contrast to the continued tumour growth in the cyclosporine group.

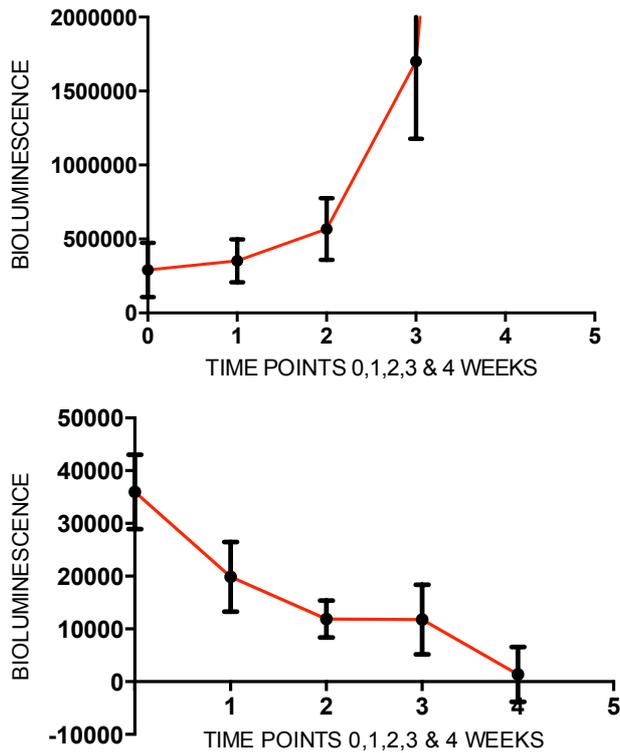


Figure 74 Direct comparison between Wistar rats on CsA or Leflunomide immunosuppression. As opposed to steady increase in tumour load with CsA, there was gradual reduction of bioluminescent signals with Leflunomide.

4.10 POST EUTHANASIA ANALYSIS

At the end of the study period of four weeks, all the animals were euthanased by Home Office Schedule 1 of cervical dislocation. During every IVIS imaging session, the right flank was palpated for evidence of gross tumour growth as well. This examination was repeated on the euthanased animal after dissection of the subcutaneous tissue of initial tumour injection (fig).



Figure 75 Palpable tumour in the right flank at the site of injection 4 weeks prior. On right, comparison with a standard 21 gauge needle. This was a Lewis rat with continued Cyclosporine immunosuppression for 4 weeks.



Figure 76 Dissection of the right flank skin off the ventral abdominal wall. The tumour clearly visible with evidence of increased vascularity around the tumour.

The dissection of the injection area was performed whether or not there was any detectable positive signal. After this, a midline laparotomy was performed to assess any gross evidence of distant metastasis. The inguinal and para-aortic regions were carefully dissected to detect any grossly enlarged lymph nodes. Only in 5 animals with very bright bioluminescent signals there were detectable inguinal lymph nodes. Due to lack of lymph node yield, the spleen was used for the flowcytometric analysis for the sake of reproducibility.



Figure 77 Dissection and isolation of subcutaneous tumour in a Wistar rat with Cyclosporine immunosuppression.

4.11 DISCUSSION

The need for increasing the organs for transplantation is very real. Any increase, whether by increasing the standard pools of live and deceased donors or looking for new sources will help the cause. Whatever the source, it has to be able to provide safe allografts. Restored kidneys sourced from patients with small renal tumours have the potential to increase the donor pool significantly

as shown earlier but there is no clear evidence that these kidneys can be safe. After partial nephrectomy, in patients with T1a tumours the oncological outcomes and long-term survival is comparable with patients having radical nephrectomy, hence the shift towards partial nephrectomy as the preferred method of treatment in the majority of patients(43)(44). Although this finding was the background of the reported cases of deliberate transplantation of these restored organs, but there remains a few important clinical and ethical questions. First of all, what would happen if these organs were transplanted and the recipient given immunosuppression, would that increase the chances of recurrence and metastasis? What should be the best possible immunosuppression in order to prevent any recurrence? What would be the best strategy with regards to immunosuppression if there was a recurrence, should the immunosuppression be continued, stopped or replaced? Can we use the body's natural immune response to fight any foreign tumour transplanted along with the allograft and would the tumour be significantly immunogenic for this strategy to work? There have been no experiments to answer these questions and hence the literature is completely lacking in this regards.

We set up our experiments specifically to answer these particular questions.

4.11.1 Role of rejection

Acute rejection, exercised by the hosts' immune system is one of the barriers to transplantation. Although it provides its own challenges for transplantation, it has an important role in preventing the tumour growth.

Even before they are transplanted, the allografts undergo trauma of transplantation; harvesting, warm and cold ischaemic damage and ischaemia reperfusion injury. This “primes” these organs for the maximal immune response from the adaptive immune system (192).

While the initial responses are non-specific and mediated by complement system, Natural killer cells and macrophages, the adaptive immune response is very specific to the donor antigens. MHC is the glycoproteins that are at the core of adaptive immunity. These are one of the most polymorphic proteins. MHC class I proteins are present on the cell surface of all nucleated cells and present intracellular antigens to CD8+ T cells. MHC class II proteins are present on the surface of antigen presenting cells and B cells. They present exogenous antigens to CD4+ T cells. Both subclasses of MHC play an important role in the allograft rejection as the graft survival can be prolonged when the transplanted donor tissue is lacking in either one or both of them(193)(194)(195)(196)(197)(198). For transplantation, greater the antigenic difference between donor and the recipient, the more is the propensity for rejection and vice versa. Hence as a general rule, it is preferable to transplant organs across minor MHC differences.

Immune system also plays a paramount role in preventing tumour development. Cancer cells, in order to propagate have to evade the individual's own immune system. This is due to the fact that the tumours either express tumour specific antigens or tumour associated antigens. These are non-self and are readily recognised by the host immune system. For the tumours to grow, they have to evade the immune system. This process of “immune editing” selects for the “escape mutants” that are less immunogenic and hence can propagate in the

presence of a competent immune system. Reduced MHC expression and poor costimulatory signals by the tumour cells are a few of the mechanism for such immune editing (199)(200)(201).

Based on their ability to incite an immune response the tumour cells can be classified into immunogenic (regressors) which are rejected in the naïve syngenic animals, intermediate immunogenic (progressors) which require prior immunisation for rejection and non immunogenic tumours(202). More immunogenic the tumour, the better equipped the immune system will be to cause rejection. Renal cell carcinoma is an immunogenic tumour (203) as is evident by the tumour infiltration of the T cells (204). Hence our experiments to assess the role of rejection in different experimental conditions are quite relevant.

To assess the role of rejection we divided the experimental groups into well-matched Wistar animals and poorly matched Lewis animals. As the tumour cells were of Wistar origin, there were far less differences in the MHC proteins when these tumour cells were transplanted into the Wistar animals as compared to the Lewis rats. Furthermore, as the Wistar rats were outbred and not syngenic despite the donor cells of being same strain they were not identical to them. This recreates the human transplant scenario very closely, where even the most closely matched transplants (excluding identical twins) have some minor allogeneity(205).

When the tumour cells were transplanted without any immunosuppression there was an aggressive response against the tumour cells by both the groups. This is in line with the hypothesis that the immunogenic renal cell tumours will incite

an immune response that will lead to their rejection in the presence of a competent immune system.

This immune response was significantly stronger in the poorly matched Lewis animals as compared to the well-matched Wistar rats. This effect is again due to wider MHC differences in the poorly matched animals as highlighted above.

These effects were also seen quite clearly in the cyclosporine immunosuppression arm of experiments. The tumour cells continued to grow in both the arms but when the immunosuppression was stopped in the treatment withdrawal arms of both well-matched and poorly matched groups there was a steady decline in the tumour load. This points towards the efficacy of acute rejection once the immunosuppression is stopped. Of interest here was the finding that this rejection was again found to be significantly more effective in the poorly matched Lewis animals due to wider MHC differences between the donor and recipient tissues.

These findings have important clinical bearing. In cases of transplantation with restored kidneys after ex vivo resection of tumours, if there was any recurrence of tumour then acute rejection could be used to clear the recipients of any tumour load after the graft nephrectomy. In these situations, perhaps the allografts with less well MHC matching will prove better at rejecting any residual tumour load.

4.11.2 Effects of cyclosporine immunosuppression

Cyclosporine immunosuppression has revolutionised the field of transplantations since its introduction in 1980s. It has greatly increased the graft

survival and is now the most commonly used immunosuppressive medication along with Tacrolimus for renal transplantation. It exerts its effects by reducing the production of Interleukin-2 (IL-2) and IL-2 receptor expression, thereby reducing T cell expression.

Renal transplant confers significant quality of life and survival benefits for patients on dialysis but the risk of developing cancer after transplant also increases. The cumulative risk adjusted risk of cancer increases in direct proportion to the time since transplantation. In adults aged less than 35 years this risk after 10 years significantly higher than the general population while 55 years and older patients this risk increases even further(206).

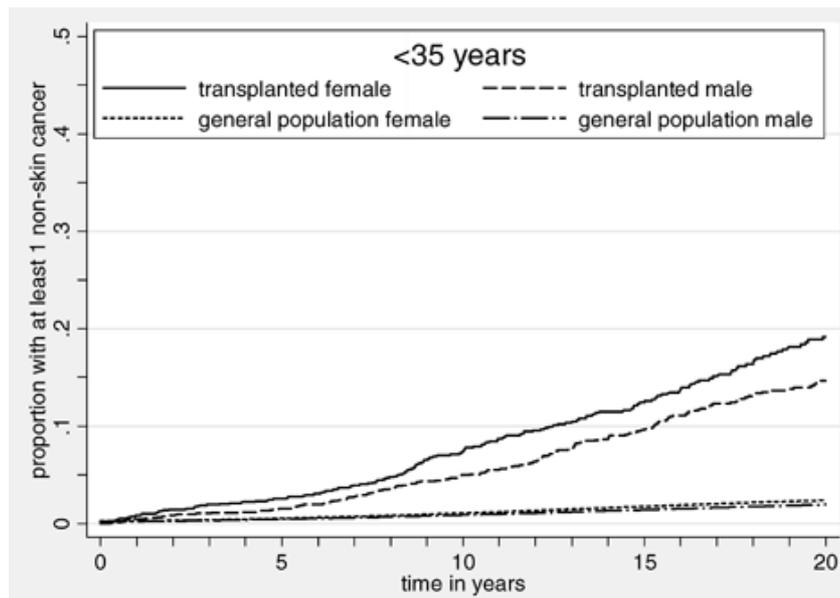


Figure 78 Cumulative risk of cancer. Cumulative risk of cancer (excluding non-melanocytic skin and lip cancer) in kidney transplant recipients by age at transplantation(206)

Cancer rates described in literature normally underestimate the real picture due to relatively short follow-ups and more focus on patients transplanted recently. A large US study looking at the incidence of cancer after renal transplantation

only reported cancers in the first three years after transplantation. Prevalence studies show a different picture, with an incidence of 34-50% if transplant recipients were followed for 20 years or more(207)(208)(209).

Although the risk of cancer increases with increasing age and being on dialysis also increases incidence of cancers but the significantly higher incidence of cancer over and above the general population is believed to be due to the effects of immunosuppressive medications. An impaired immune response due to strong immunosuppression is one of the most important mechanisms. Other potential mechanisms include recurrent infections with oncogenic viruses.

Then there is direct neoplastic effect of the cyclosporine immunosuppression. This is due to aberrant production of cytokines regulating tumour growth, metastasis and angiogenesis(210).

All these factors make it of paramount importance that any restored kidney transplanted must be free of any gross cancer as cancer growth may be uninterrupted in immunosuppressed hosts. Also tailoring of immunosuppression to agents with antineoplastic properties may lead to better outcomes when transplanting these organs.

Our experiments on both Wistar and Lewis rats showed these effects of cyclosporine immunosuppression. When the immunosuppression with CsA was continued in either strains there was on-going growth of the tumour cells despite, in both the strains.

The growth was exponential towards the end of the study period due most probably to neoangiogenesis.

These findings have important clinical implications. If there was an inadvertent tumour transfer or de novo renal tumour development then under the influence of continued CsA immunosuppression the tumour cells are likely to grow uninterrupted. Hence not only it would be important to very closely monitor these implants but also change the immunosuppression if possible.

4.11.3 Immunosuppression with antineoplastic agents

Calcineurin nephrotoxicity was one of the main side effects of CNIs. mTOR inhibitors are devoid of this side effect and hence promised a great deal for renal transplant patients. Despite initial expectations these agents have not been able to replace CNIs for long-term immunosuppression due to their own side effects profile. But there is growing body of evidence that these can be good alternatives to CNIs after an initial period of with calcineurin inhibitors.

Use of mTOR inhibitors as denovo immunosuppressive agents was studied by ORION and Symphony studies. ORION study had 469 patients who were divided into three groups. Group 1 had sirolimus and tacrolimus with gradual withdrawal of tacrolimus after week 13. Group two was given MMF and sirolimus. This group was discontinued early due to high rates of acute rejections. Group 3 was given tacrolimus and MMF. There were no significant differences in the primary end points of eGFR and graft loss among the groups but the rates of biopsy proven acute rejections were significantly higher in the sirolimus groups(211). The Symphony study – another randomised control trial, in its one year results reported better results in terms of acute rejection, eGFR and graft failure with tacrolimus, MMF and steroid based regime than three other regimes including one containing sirolimus. A further two years follow up

of the Symphony study showed continued benefit with Tacrolimus arm of the study with those patients maintaining eGFR and low rates of acute rejections. Although the total number of patients who remained on sirolimus by the end of the follow up had numerically high eGFR but due to higher incidence of acute rejections and side effects a significant patients crossed over to the tacrolimus arm and these results would not bear any clinical relevance(118,212).

Sirolimus has been used with the combination of CNI's. The rates of acute rejection episodes in the combination has been shown to be less than the other treatment arms(213,214). Although the rates of acute rejection were low, but the side effects including lymphocoele and the wound infections were higher. Also the one-year renal function was also worse off in the combination regime(215). Due to this, in the 2009 Kidney Disease: Improving Global Outcomes (KIDIGO) guidelines it was recommended against to use both CNI and sirolimus in combination(216).

The other strategy of using mTOR inhibitors is to employ them as maintenance therapy as a replacement of CNI. One of the first large studies was published in 2003, which randomised 430 patients into either receiving sirolimus, CsA and steroids or have CNI withdrawal after 3 months. The eGFRs and graft survival rates at 36 months were significantly better in the CNI withdrawal group, with better compliance rates as well(217). Due to chronic allograft nephropathy associated with CNIs, withdrawal of these agents after an initial period post transplant was shown to be beneficial(121). The timing of withdrawal of CNI is varied in different studies but to see the best results it is important to withdraw them before the irreversible changes of allograft nephropathy set in. The ZEUS study, a large multicentre RCT comparing CsA, MMF and prednisolone versus

everolimus, MMF and prednisolone showed significantly better results in terms of eGFR in the everolimus arm. Initially the acute rejection rates were higher but over the whole study period they were comparable as well(218).

There was a lot of excitement about mTOR inhibitors in the beginning as potentially the agents that would be able to completely replace CNIs and prevent the problems associated with chronic allograft nephropathy but due to their own side effect profile they have been unable to replace CNIs completely. But as maintenance therapy after an initial period of 3-5 months of CNIs they have been found superior as described above. Along with these benefits, when the antineoplastic effects of these agents are taken into account they have very promising prospects, if not in all then definitely in special circumstances.

Antineoplastic effects of mTOR inhibitors are well documented. These are not only shown to be helpful in skin cancers including melanomas but also in post-transplant lymphoproliferative disorders and Kaposi sarcomas. There is gathering evidence for their effectiveness for metastatic renal cell carcinoma, HER 2 positive breast cancers, neuroendocrine and pancreatic tumours(128,219–224).

The CONVERT trial, which had 830 patients, randomised to conversion to sirolimus or continuation with CsA immunosuppression has shown that the incidence of non melanoma skin cancer was significantly lower in sirolimus group (1.2 v 4.3 p<0.001). The rate of other malignancies was lower again in the sirolimus group but failed to reach statistical significance (p 0.058)(225). Similar results were noted when patients with one cutaneous cancer were randomly assigned to either continue on cyclosporine immunosuppression or

convert to sirolimus. In the conversion arm, the survival free from cutaneous squamous cell cancer was significantly longer than in CsA arm(226).

Knoll and colleagues conducted a systematic review of 21 randomised control trials recently. This review looked at the data of 5876 transplant recipients and showed that there was 56% reduction in the incidence of non melanoma skin cancer and 40% reduction for the rest of malignancies with sirolimus switch. Patients receiving de novo sirolimus did not show any improvement in the cancer occurrence. These results in themselves are very promising but the conversion to sirolimus came at a cost of increased risk of non-cancer related deaths. Most likely causes postulated by the authors were increased cardiovascular risks and infections. These effects were more pronounced when higher doses of sirolimus were used. Increased risk of rejection could be due to over immunosuppression in the sirolimus group due to known higher risks of acute rejections requiring steroid pulsing(227).

Despite the reduction in the incidence of cancer, increase in the non-cancer deaths in sirolimus group seems quite discouraging. But when this was looked at in the context of what happened to the patients who could not get an organ for transplantation then the situation was much more promising. The transplant waiting list is a “very dangerous” place to be, as the average risk of dying while being wait listed is between 6- 10% per year(228–230). This is also depended on the age of the patients listed, with worsening survival with advancing age. Patients becoming unfit and inactive on the waiting list complicates the situation as the risk of dying increases even further. According to an analysis about 30% deaths on waiting list occur in patients who had inactive status(231).

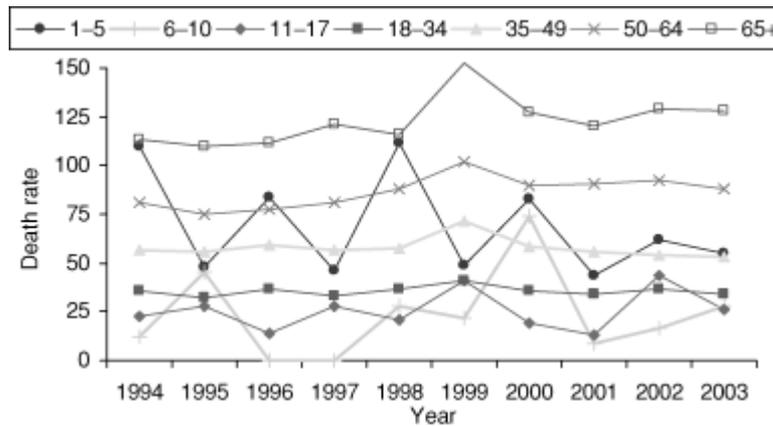


Figure 79 Death rate on the kidney waiting list by age group, 1994-2003 (229)

Thus an elderly patient who has to wait for longer (blood group O and B) the risk of dying may be more than 50% over a period of five years(229,232).

In our experiments, when sirolimus was used as immunosuppressive agent all animals were able to reject tumour load within the study period. Although this effect was stronger in the higher dose sirolimus animals, the low dose group was also significantly better in rejecting the tumour load when compared to both CsA and leflunomide groups.

Because the effect of sirolimus was found to be very strong, we was unable to elicit any difference between the treatment continue and withdrawal groups as by the time of withdrawal of immunosuppression the tumour load was already very little.

4.11.4 Conclusions

In summary, we have shown with our experiments that we can use acute rejection, level of matching and manipulation of immunosuppression to our benefit. In the light of the above mentioned findings and extrapolating them to the clinical situation, we suggest that kidneys removed for small renal cell carcinoma can be used for transplantation in a select group of patients. Perhaps the best approach while using these organs will be to do vigilant wide local excision with US guidance if needed, to make sure there is no residual macroscopic tumour left in the restored organ. After transplantation, continue on CNI based immunosuppression for a period of 3-5 months followed by conversion to prevent common risks associated with mTOR inhibitors in the immediate post operative period. To use acute rejection for any tumour elimination, the donor recipient combination should perhaps be less than ideal HLA match.

Of course patient selection and informed consenting when using these organs will be more important than any other ordinary kidney transplantation.

High risk, elderly patients with unacceptably high risk of dying while waiting for a kidney will benefit the most from these relatively higher risk organs. Both the donor and recipient consent will need to be very thorough and clear. Potential recipients must know that these organs have less number of nephrons to start with due to partial resection. The risk of tumour recurrence and potential risk of relatively aggressive nature must be clearly indicated. Other side effects associated with transplanting these kidneys will include risk associated with mTOR inhibitors and risks of bleeding, urine leak and lymphocoeles due to partial resection.

On the other hand the benefits, both in terms of quality of life and survival must also be highlighted and the decision must be left for the patients to make to avoid any clinician bias.

Chapter 5

FLOW CYTOMETRIC ANALYSIS

5.1 AIMS

Flow cytometric analysis was performed to assess the effect of tumour transplantation, different immunosuppression and matching with respect to the distribution of subclasses of T cells and Natural Killer population in our experimental model.

5.2 BACKGROUND AND PRINCIPLES

Flow cytometry is the technique of measuring the characteristics of small particles as they flow in the fluid medium. The most important properties normally measured are the number, size, shape, granularity and, by labelling with fluorescent markers, the specific properties of the particles. The particles are passed through a laser beam suspended in a fluid medium. These particles cause dispersion of the light, which is then picked up by appropriately positioned photomultiplier tubes. These are then converted to electrical signals which are interpreted by the on board computer.

The three main parts of the flow cytometer are the fluidics, optics and electronics.

Fluidics acts are the medium through which the particles, usually cells are transported. The cells of sizes ranging between 0.2- 150 micrometres are suitable for flow cytometric analysis. This requires the cells to be isolated from tissues before they can be analysed e.g. spleen. For the results to be precise ideally only one cell should pass through the light beam at one time. The arrangement of the flow chamber is such that it makes it possible for the cells to be passed in a single file on low flow settings.

When the light strikes these cells it diverges into different directions. The extent of this depends on the physical properties of the cells. There are two different type of scattering that is detected by flow cytometer; forward scattered light (FSC) and side scattered light (SSC).

FSC is detected just off the original laser beam path and gives the indication of the size of the cells. SSC is usually detected at around 90° angle and gives the indication about the shape and granularity of the cells. Combined together these measurements can be used to differentiate between different white blood cells for instance.

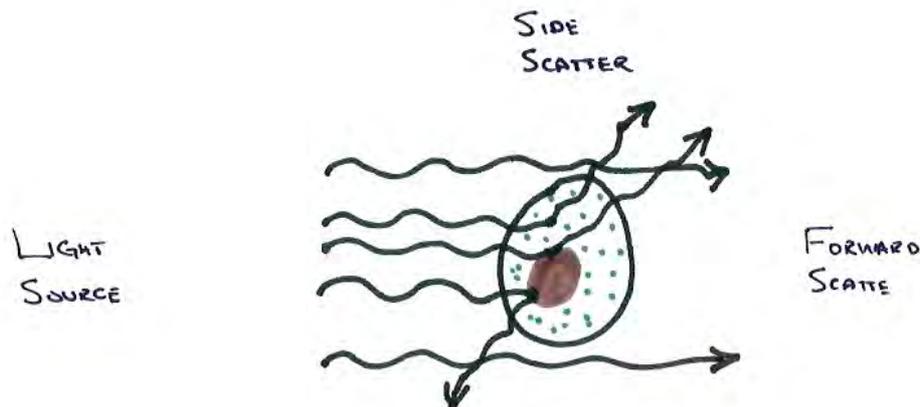


Figure 80 Forward and side scatter of incident beam of light as a basic principle of flow cytometry.

The granulocytes being the biggest and most granular scatter the light most and appear farthest in both the FSC and SSC. Monocytes are large in size but are smoother hence appear to disperse the beam most for FSC. Lymphocytes are

the smallest and also non granular and hence are found towards the left of the SSC/FSC plot as shown.

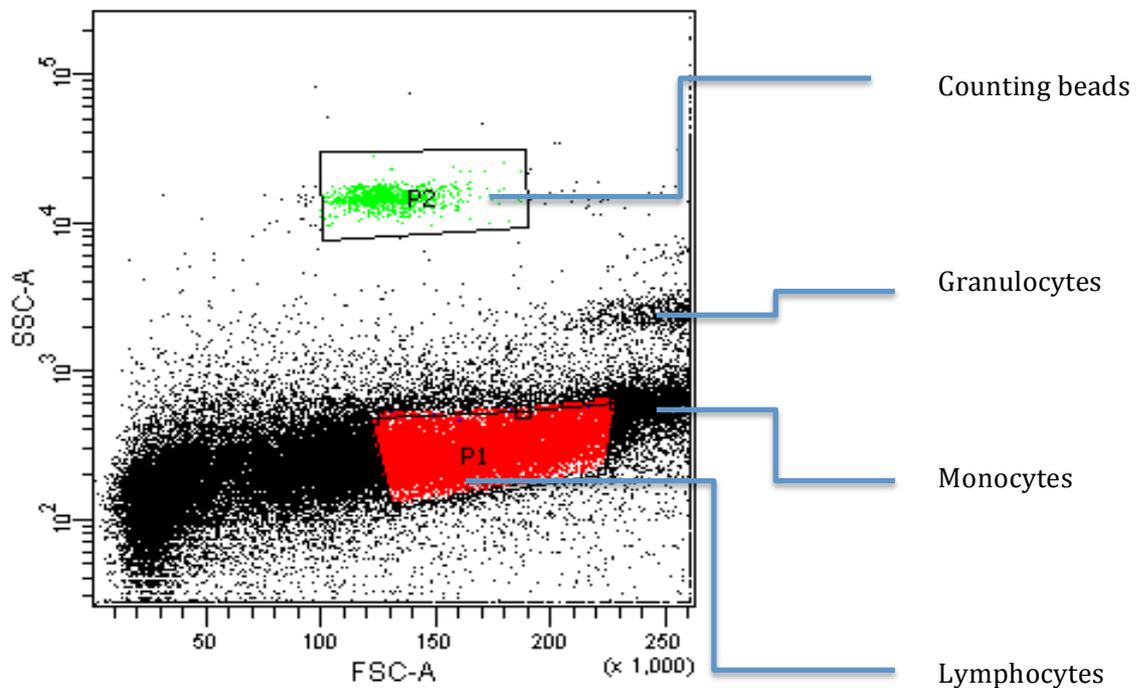


Figure 81 Forward and side scatter. An example of forward and side scatter of cells separated from a Wistar rat's splenic tissue after euthanasia. The bigger and more irregular the cell shape the more FSC and SCC they cause.

A more targeted use of flow cytometry is to incubate the cells with antibodies conjugated with a fluorochrome that will bind to cell specific antigens. These fluorochromes are excited when hit by monochromatic light moving electrons out from their normal orbit to an unstable high-energy state. Upon their return to baseline they release energy in the form of photons of light, which invariably is of higher wavelength and is different from the excitation wavelength. These FSC, SSC and fluorescence signals are channelled by a set of filters and

mirrors to specific sensors. These sensors for the fluorescent light are called photo multiplier tubes (PMTs).

These PMTs then convert the photon energy to electrical signals, which are then “read” by the attached computer to produce output data.

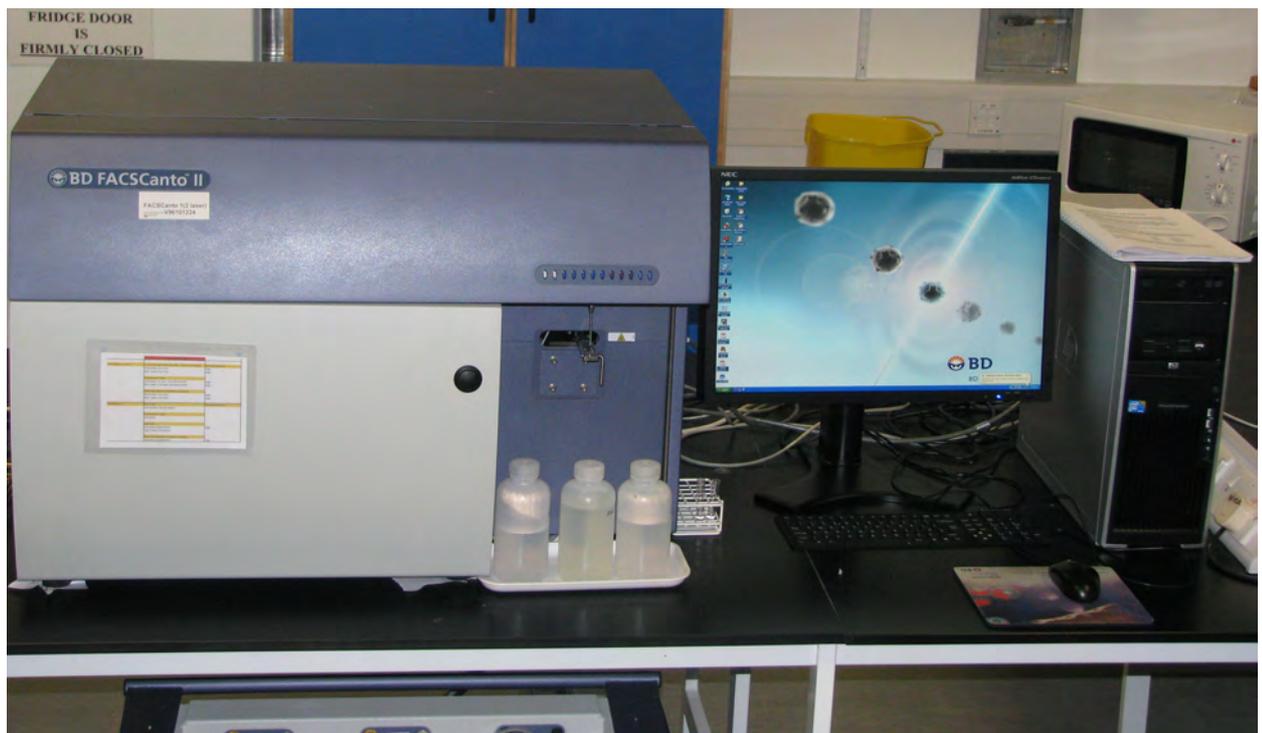


Figure 82 BD FACSCanto II Flow cytometer. Main flow cytometer is on the left of the picture and the data is analysed by the attached computer towards the right of the picture.

5.3 CELL MARKERS IN TRANSPLANTATION AND CANCER

The most important lymphocytes in the context of transplantation and tumour immunology are CD4+, CT8+ T cells and Natural killer cells. Thus these cell subsets were studied in our experimental animals. One of the earlier studies showing the role of CD4+ and CD8+ cells in allo-rejection, was from Cobbold *et*

al.(233). CD4+ and CD8+ cells were depleted separately and then together and the rate of skin graft rejection was studied. This study showed that the survival of skin grafts was similar among controls and the animals with removal of just CD8+ T cells. When CD4+ cells were depleted there was a modest increase in the survival of skin allografts while a long-term graft survival was achieved when both CD4+ and CD8+ cells were depleted. This study pointed towards the crucial role that both these cell types play and also highlight their interplay with each other for graft rejection.

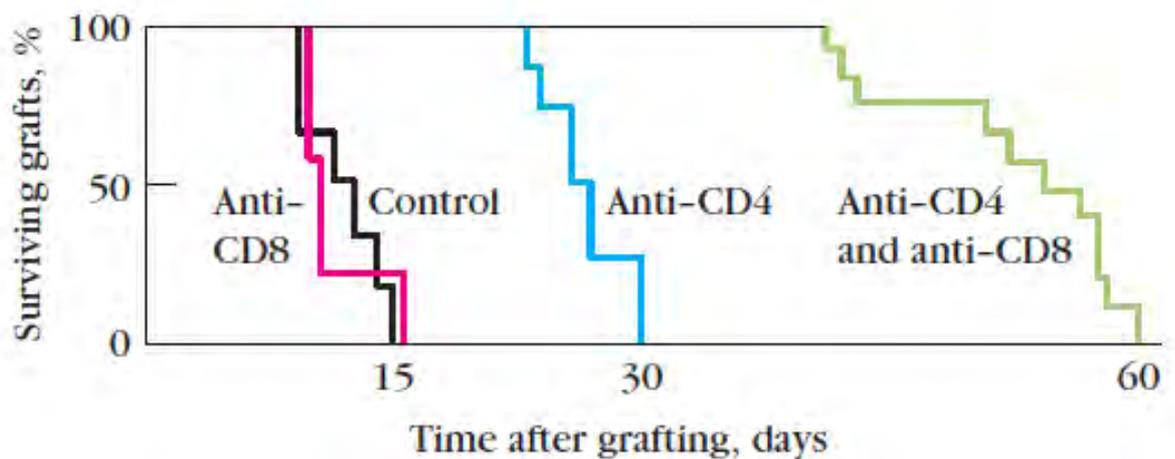


Figure 83 Role of CD4+ and CD8+ T cells in skin allograft rejection. Depletion of CD8+ cells did not have much effect on allograft rejection (similar to controls). With CD4+ cells depletion there was only a modest increase in the allograft survival which when both these cell populations were depleted then there was significant increase in the survival of these allografts. This study highlighted the importance of both these cells in rejection. (reproduced from reference 233)

5.3.1 CD4+ T cells

These cells have pivotal role in transplant immunology. Once the T cells are activated in the presence of co-stimulatory signals they change into effector

cells. The CD4+ “helper” T cells upon activation produce a number of cytokines that help exert immunological attack on the non-self alloantigens. These can both be “normal” alloantigens from the transplanted kidney or it could be tumour antigens. The role of the CD4+ cells as the initiator of the graft rejection has been widely demonstrated by various groups(234)(235)(236)(237). Initiation of immune response is of paramount importance in body’s natural defence against pathogens as well as the transplanted or de novo tumour cells.

5.3.1.1 *Activation status and significance*

Majority of the CD4+ cells are naïve under resting conditions. For these cells to mount any immune response against transplanted cell they have to be activated. There are several markers that get expressed on the activated T cells. These include CD25, CD69 and CD154 among others(238)(239)(240).

5.3.2 **CD8+ cells**

Cytotoxic T lymphocytes (CTLs) (CD8+) have the most profound effects against tumour development and progression. These cells are dependent on the production of cytokines mainly produced by the Th1 cells eliciting a coordinated defence mechanism against tumours. These cells are also the biggest mediators of acute rejection. CTLs are normally present in low levels in non rejecting allografts while in rejecting organs their number is significantly higher(241)(242) and rejection can be delayed by depletion of these lymphocytes(243).

5.3.3 Natural killer cells

These cells are part of the innate immune system. These are one of the first defence mechanisms of the body against foreign and tumour antigens. Although initially thought to be not important effector cells in solid organ transplantation(244), more recent studies have shown their role in small animal model(245). Although these cells alone may be insufficient to cause full blown acute rejection themselves, but by interaction with other cells of innate and adaptive immune system they have an important role to play.

Their role against tumours is clearer both in small animals and humans. Mice deficient of NK cells have higher incidence of lymphomas and sarcomas, as is the case in humans(246)(247)(248)(249).

The two ways by which NK cells are thought to play a anti neoplastic role is by recognising “**missing self**” – where tumour cells and virus infected cells under express the MHC class I proteins in order to evade the immune system(250)(251). This mechanism is of particular benefit to the host as one of the properties of the tumour cells is to not express MHC class I molecules in an attempt to evade the attack by CTLs. These tumour cells can escape cytotoxic T cells but are recognised by NK cells via “missing self” mechanism and eliminated. The other mechanism by which NK cell exercise their defence is by the identifying “**induced-self**” antigens. These are the antigens which are either not expressed at all on normal healthy cells or are expressed at very low levels but as a result of malignancy, infection or trauma are up-regulated (252)(253)(254)(159). When these induced-self antigens are expressed in significant amount they are recognised by the NK cells, which eliminate them.

In short NK cells are an important player in the tumour cells destruction.

5.3.4 Regulatory T cells

In the past 10-15 years there has been growing interest at the role of these subsets of T lymphocytes. From a phenotypic point of view these cells are identified as CD4⁺ CD25⁺ FoxP3⁺ cells(255). Liu *et al.* has described the down- regulation of CD127 as a potentially important marker of T_{reg} cells(256), while TNFR2 has also been implicated as an alternative marker for these cells(257). Despite some evidence that there is a subpopulation of CD4⁺CD25⁻ FoxP3⁺ T_{reg} that is positive for TNFR2 (258), the vast majority of studies use CD25 and FoxP3 positivity as a marker for identifying these cells.

The role of T_{regs} as immunoregulatory cells has now been firmly established although there is still a lot about these cells that remains unanswered. They have been shown to keep in check the autoimmune and inflammatory processes both in humans and in rodents(259)(260).

Experiments in humanised mice have shown their ability to counter graft versus host disease. In these mice, when human lymphocytes were transplanted, severe GVHD ensues but co-transfer of T_{regs} prevents this life threatening phenomenon(261). Their protective role has also been shown for the solid organ transplantation by the Oxford group(262).

Tumour associated antigens expressed by the precancerous cells are recognised by the immune system thus preventing a large number of malignancies. It is only when these initial defences are evaded that the transition from pre-cancer to cancer occurs in at least some cases. With T_{reg}

cells keeping immune system “in check”, it was hypothesised(263) that this could lead to tumour cells evading host defences. There has been evidence to suggest that this hypothesis may be correct as large number of T_{reg} cells are isolated from established tumours and draining lymph nodes, thereby pointing towards their potential role(264). Similarly depletion of T_{reg} cells resulted in better antineoplastic responses. Marabelle *et al.* showed that there were large numbers of T_{reg} cells expressing OX40 and CTLA-4 as well as FoxP3. Presence of these T_{regs} at the vicinity of the tumour was a poor prognostic factor for the mice. When anti OX40 and anti CTLA-4 antibodies were injected to target these T_{reg} cells directly, these mice achieved a systemic clearance of tumour load(265). Similar results have been reported by either depleting the tumours with T_{regs} or by injecting them with T_{reg} depleted CD4+ T cells(266)(267)(268)(269)(270)(271)(272).

5.4 MATERIALS AND METHODS

5.4.1 Choice of cell markers

We selected CD25 as our T cell activation marker. This is expressed by both CD4+ and CD8+ T cells as well as by the Treg cells. By using this as one of the markers we were able to differentiate the populations of Treg (CD 4+ CD25+ FoxP3+) as well as activated CD4+ T cells (CD 4+ CD25+ FoxP3-). Effector CD8+ T cells also express CD25 as a marker of activation and was identified as CD 8+ CD25+. CD161 (also called NK receptor protein 1) was used for the

detection of the NK cells. These are present on all NK cells and very small proportion of T cells(273,274).

5.4.2 Antibodies

The cell surface antigens targeted to identify the above-mentioned cell populations included CD4, CD8, CD25 and CD161. The cells were stained according to the protocols provided by the distributors. Antibodies to CD4, CD8, CD25, CD161 and FoxP3 were anti-rat and acquired from eBioscience Ltd. (Hatfield, United Kingdom), while CD161 was bought from BioLegend (London, United Kingdom). The following table shows the dilution used for flow cytometric analysis and the conjugated fluorochromes.

Antibodies	CD4	CD8	CD25	CD161	FoxP3
Dilutions	1/400	1/100	1/100	1/200	1/50
Fluorochromes	FITC	FITC	PE	FITC	PE-Cy 5

Concentration of the antibodies titers used for staining different T lymphocytes as well as the fluorochromes for the detection in flow cytometric analysis.

5.4.3 Isolation of Cells for Flow Cytometry

Only a very small number of rats had positively detectable lymph nodes. Hence the FACS analysis was performed by harvesting the spleens at post mortem examination.

The splenic tissue was cut into small pieces in a petri dish. With a 5ml syringe plunger against a cell strainer the tissue was teased into a single cell suspension. Any gross tissue was then removed carefully. Cells were suspended in 10 ml of PBS and strained again before being centrifuged at 400g for 5 minutes. The supernatant was discarded and to remove red blood cells were re-suspended in 5 ml RBC lysis buffer (eBioscience) and incubated for 5 minutes before adding 15mls of PBS and centrifuging for 5 minutes before resuspending in PBS. For most of the analysis a single lysis cycle was sufficient to achieve good results but occasionally the process required repeating.

5.4.4 Staining the Surface Antigens

The cells suspended in PBS were placed in aliquots of 50 μ L and antibodies added at the correct dilution (see table) before incubation at 4°C for 30 minutes. After the incubation the cells were washed with 2 mls PBS followed by spinning. The supernatant was discarded and cells re-suspended in 2 ml of PBS, washed and spun again followed by re suspension in 0.5 ml of PBS prior to flow cytometric analysis.

5.4.5 Staining for FoxP3

Intracellular staining for FoxP3 was performed after staining the cell surface antigens. Cells were pulse centrifuged and resuspended in 1ml of fixation/permeabilization working solution (eBioscience) followed by incubation

at 4°C for 30 minutes. Then 2 ml of PBS was added and the sample was spun at 400g for 5 minutes and supernatant was discarded. The pellet was re-suspended in 100uL of permeabilization buffer and FoxP3 antibody was added at a dilution of 1/50 and the sample incubated for 30 minutes at room temperature. This was followed by addition of 2 ml of PBS and centrifugation. The supernatant was discarded and the above step repeated. Finally the sample was re-suspended in 0.5 ml of PBS prior to flow analysis.

5.4.6 Flow cytometric analysis, gating and compensation

After the staining of both cell surface and intracellular antigens, the cells were analysed in the flowcytometer. Both the lymphocyte population and the counting beads were gated in order to count the absolute and relative number of cells analysed. These gated lymphocytes were then further analysed based on the different fluorochromes used. Fig 84 gives an example of our analysed data. Additional dot plots are shown in chapter 10 fig 121- 125.

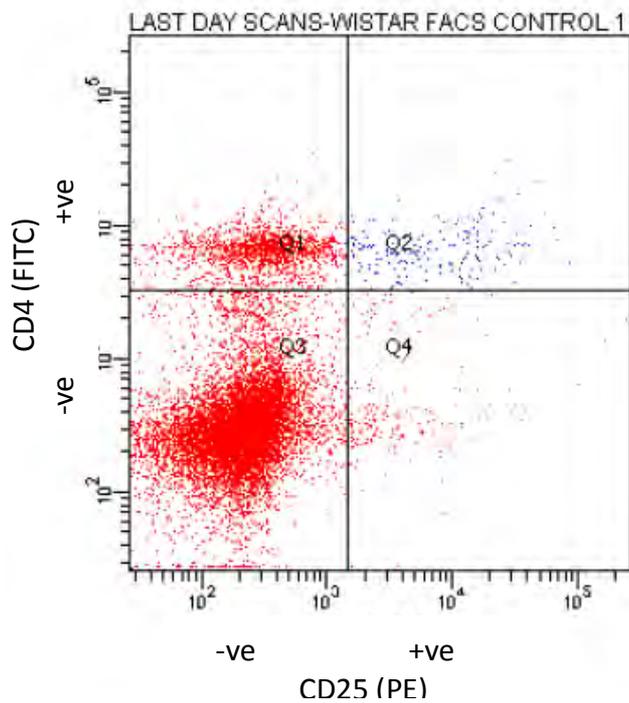
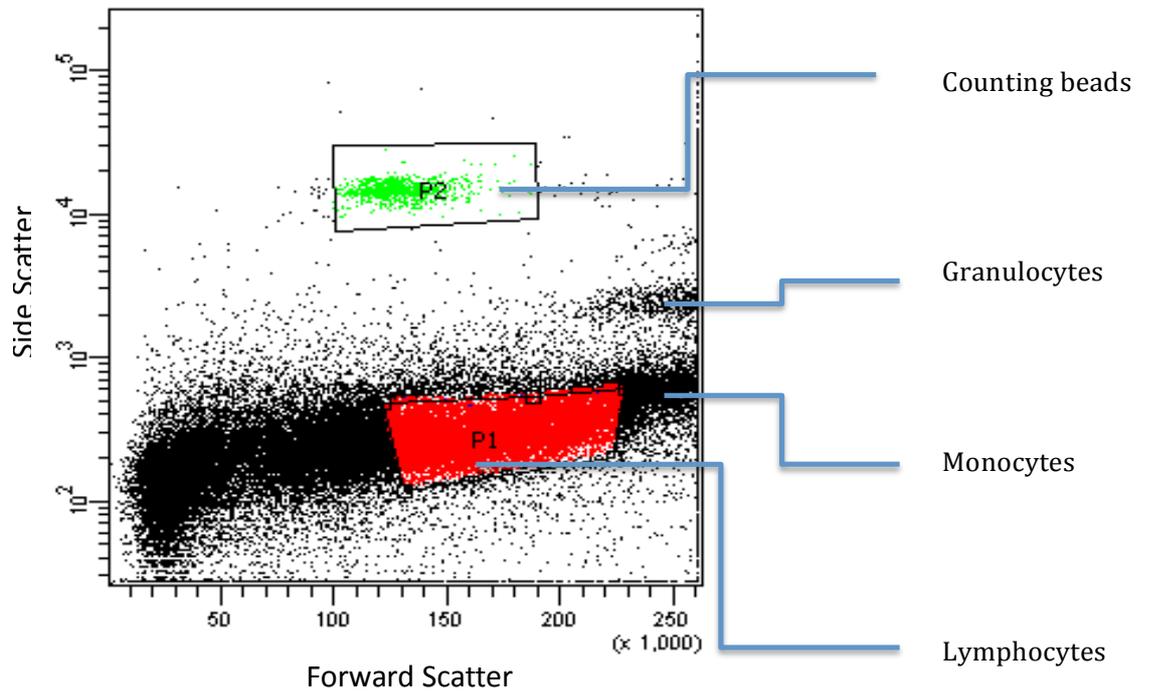


Figure 84 Representative flow data showing the method of analysis. Splenic cells were isolated and stained to detect antigens. The cells were first analysed by forward scatter and side scatter to identify lymphocytes. The lymphocyte population was gated (P1) and the gated population was then analysed by fluorescence. In the representative example shown the lymphocytes were analysed to determine the % of CD4+, CD25+ cells

For compensation settings, the unstained cells and the individual antibodies for CD4, CD8, CD161, Fox P3 (fixed and unfixed) were used for our experiments. Unstained tubes were used first to ensure that the populations of cells were in the right place on the dot plot. This was done using the FSC and SSC to screen the cells of interest. These cells of interest were then gated P1. These tubes are then run through the FACS machine and the data recorded as compensation control tubes.

The positive population was gated for each tube followed by adjusting the P1 gate and then it was “applied to all compensation” controls. The P2 gate is adjusted to fit the positive populations.

This process was repeated for each stained tube to create a compensation matrix for the fluorochrome used (as above). These compensations were then named and saved for all our future experiments.

5.5 STATISTICAL ANALYSIS

The flowcytometric data was analysed using the GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. Due to wide variations in the data points, non parametric analysis using Kruskal-Wallis test followed by post hoc analysis by Dunn’s multiple comparison testing were performed. The results with p values of <0.05 were considered as statistically significant.

5.6 RESULTS

5.6.1 Control comparison

Before analysing the effect of tumour and different immunosuppressive agents on the distribution of different T cells subsets, we looked at the control Wistar and Lewis rats. These were normal animals with *no tumour injection or immunosuppression*.

5.6.1.1 CD4+ cells

These were the percentage of CD4 + cells as a fraction of the total lymphocyte events. Interestingly, there were considerably more CD4+ cells in the Wistar control animals than Lewis animals.

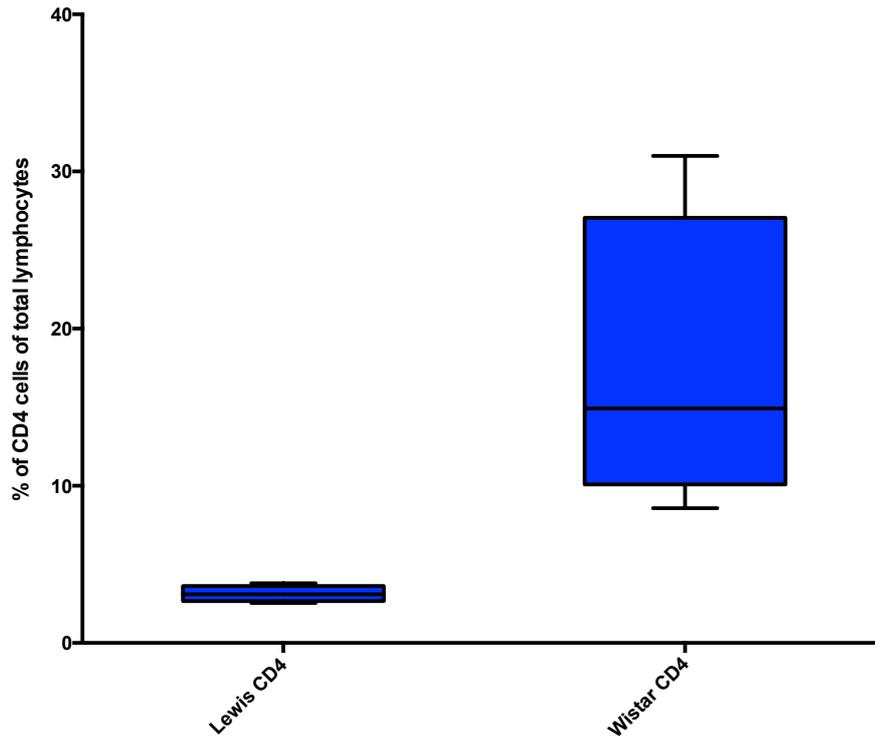


Figure 85 Comparison of CD4+ cells. The difference between the CD4 cells distribution between Lewis and Wistar rats. The difference was statistically significant ($p=0.028$)

The observed difference was statistically significant ($p=0.028$).

5.6.1.2 Activated CD 4 cells

Although the proportion of CD4 cells was significantly higher in Wistar than the Lewis animals, the percentage of activated (CD4+ CD25+) cells was significantly more in Lewis rats ($p=0.0283$). The significance of these results was unclear and we were unable to see any clear explanation in the literature.

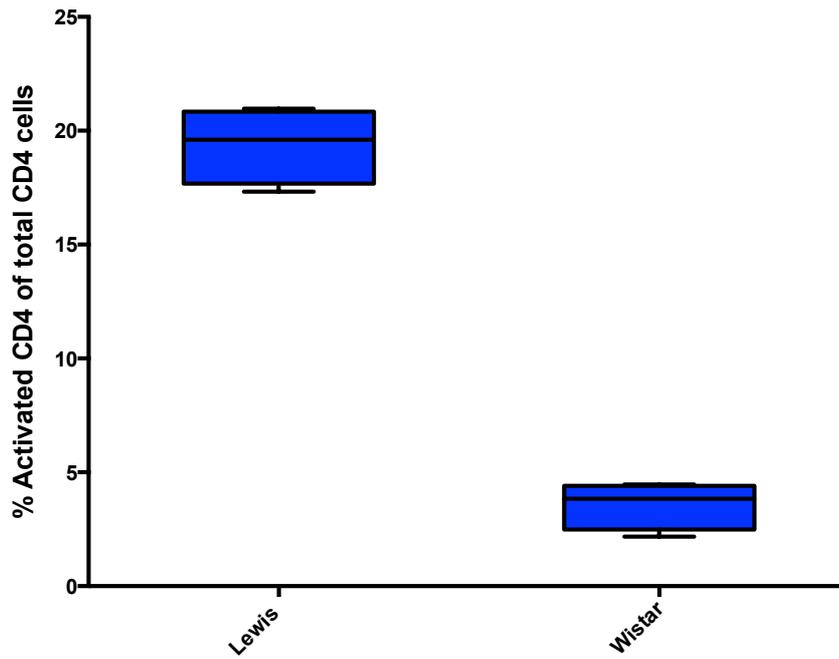


Figure 86 Comparison of activated CD4+ cells. Percentage of activated (CD4+ CD25+) cells between Lewis and Wistar rats. P=0.028

5.6.1.3 *Regulatory T cells (T_{reg})*

Similar to the higher percentage of activated CD4+ cells in Lewis rats, the percentage of T_{reg} cells as a fraction of the total CD4+ cells was higher in the Lewis rats. These results were statistically significant when compared to Wistar rats (**p=0.0286**).

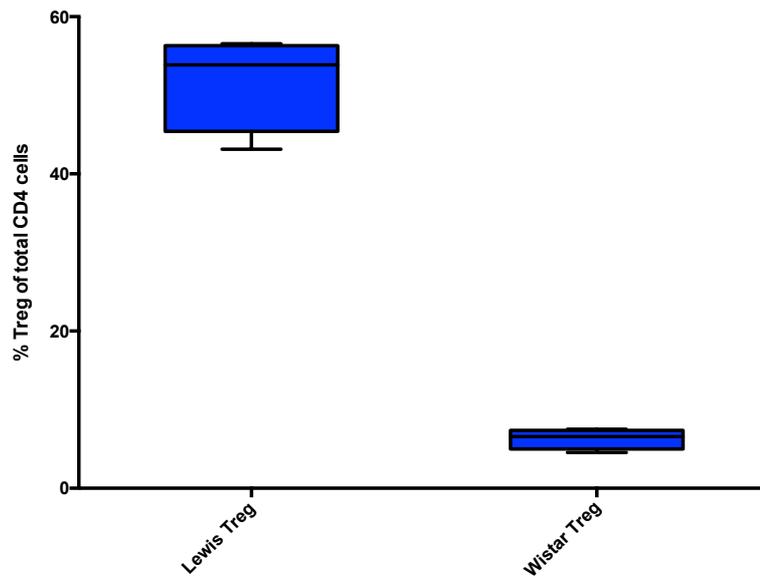


Figure 87 Comparison of Treg cells. Treg formed bigger proportion of CD 4+ cells in the Lewis rats than the Wistar strain.

5.6.1.4 *CD 8+ cells*

CD 8+ cells were between 5-15% of total lymphocytes in Lewis rats and between 3-8 % in the Wistar rats but the differences were neither marked nor reached statistical significance ($p=0.114$).

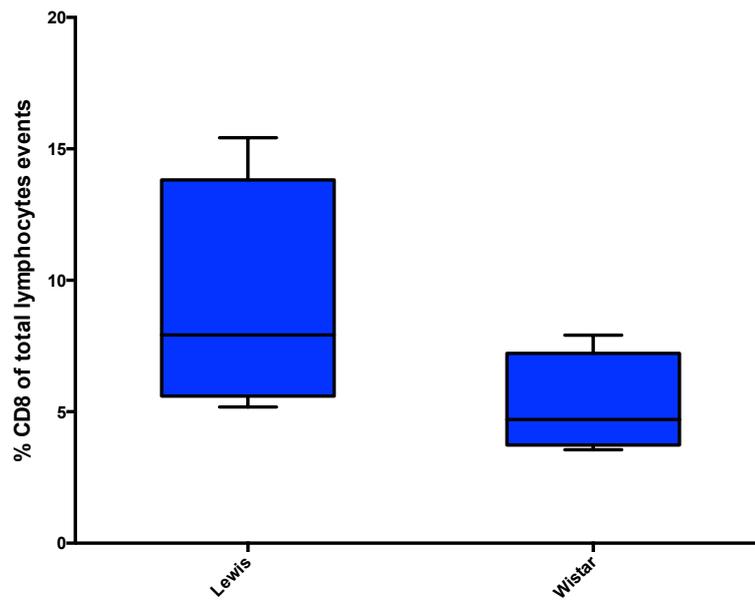


Figure 88 Comparison of CD8+ T cells among Wistar and Lewis rats.

5.6.1.5 Activated CD8 cells

The proportion of activated CD8⁺ T cells was very small in both the groups. These were the cells expressing both CD8 and CD25 cell surface markers. In Lewis rats the percentage of activated CD8 cells was between 0.1-0.3% while it was 0.08%-0.18% in Wistar rats. Again there was no statistical difference between these two strains (**p=0.342**).

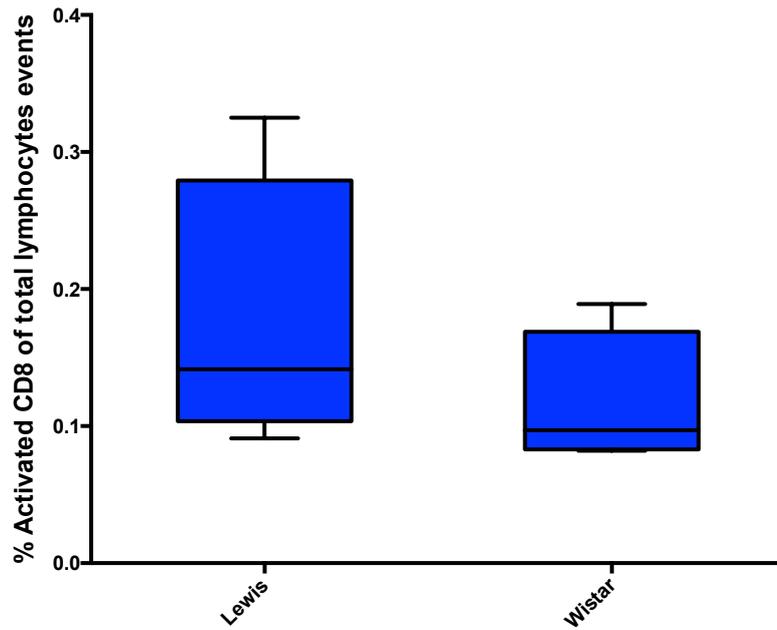


Figure 89 Activated CD8 cells in Wistar and Lewis groups.

we also compared the percentage of activated CD 8 cells of the total CD8 + cell population. Again there was no significant difference ($p=0.485$).

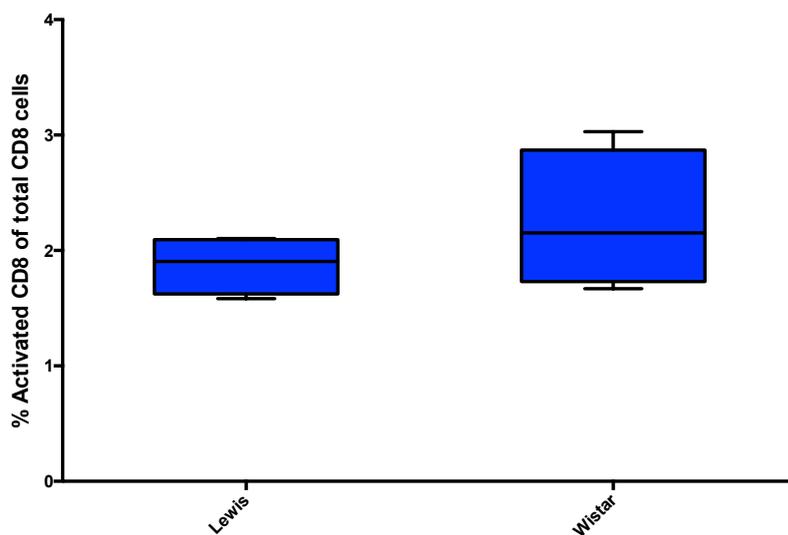


Figure 90 Percentage activated CD8 of total CD8 cells

5.6.1.6 *Natural killer cells*

The Natural killer cell proportion varied from 3-5% for the Lewis rats and for the Wistar rats it was between 1-4% with no statistically significant difference among these strains ($p=0.485$).

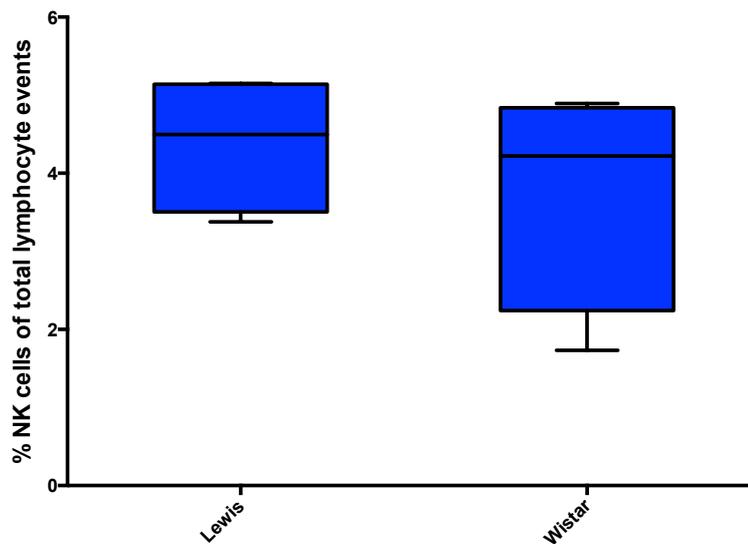


Figure 91 Comparison of NK cells. Natural killer cells distribution in Wistar and Lewis rats was not significantly different

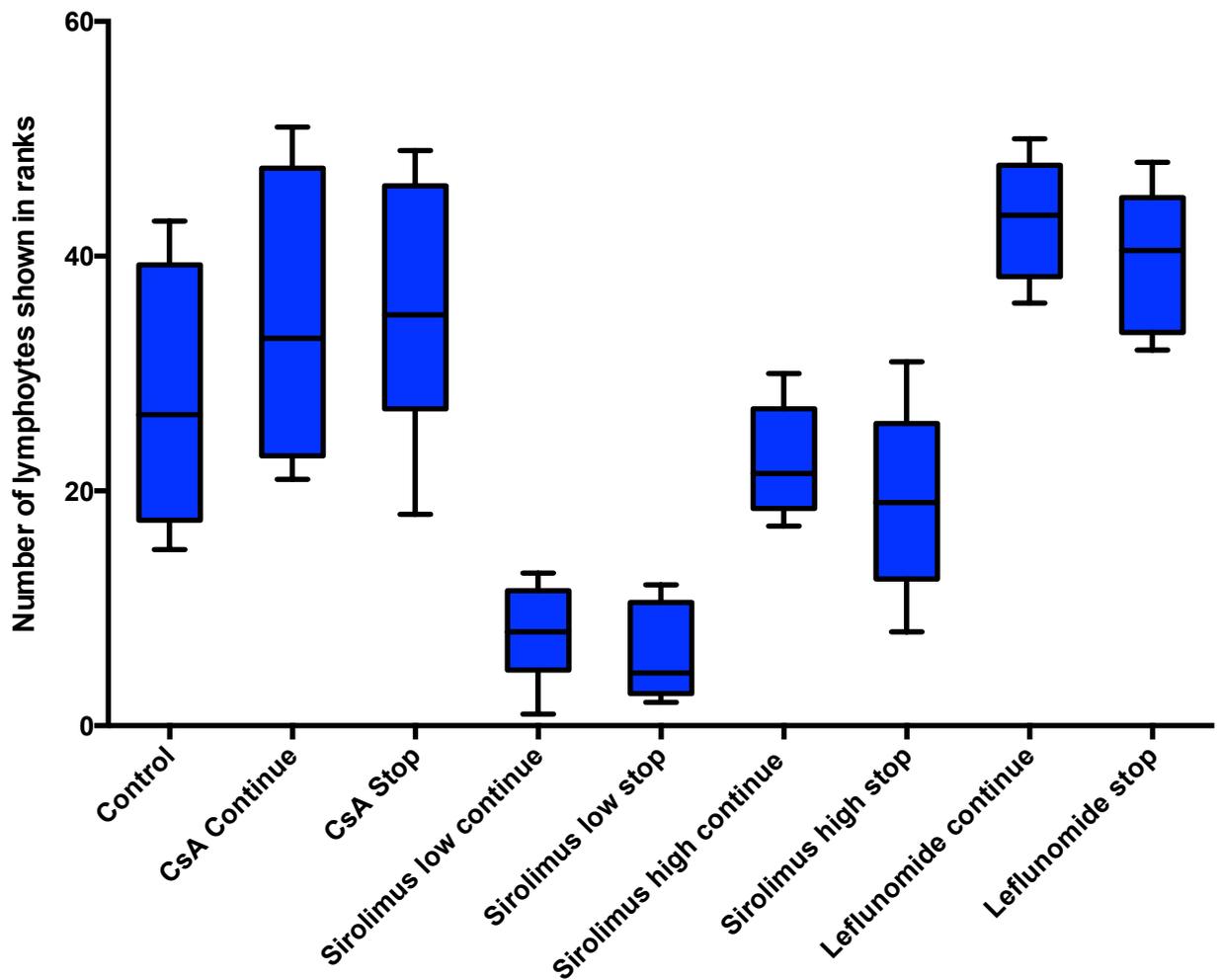
5.6.2 Cell population comparison between treatment groups- Wistar group

The cell population and their activated proportions were then analysed among different treatment groups. These included animals with tumour injection and cyclosporine, sirolimus or leflunomide immunosuppression for treatment continue and treatment withdrawal groups for the Wistar animals; and tumour injection only and tumour injection with cyclosporine or sirolimus immunosuppression for treatment continue and treatment withdrawal groups for Lewis animals.

5.6.2.1 *CD4+ cells*

There were wide variations for the CD4+ cell population among different groups but these cells were the most common. For the control and cyclosporine groups it varied from 8-70% of the total lymphocyte population studied. These cells were greatly suppressed in the sirolimus treated animals (0.3-15%). For leflunomide, again their percentage varied from 19-55%.

Despite these wide variations, the difference in the percentages between CsA stop and sirolimus low dose; and between sirolimus low dose and leflunomide groups were statistically significant. All treatment groups and the controls were compared for CD4+ cells among each other. The comparisons with the significant differences are highlighted in the table below the graph.



CsA Stop vs. Sirolimus low continue	CsA Stop vs. Sirolimus low stop	Sirolimus low continue vs. Leflunomide continue	Sirolimus low continue vs. Leflunomide stop	Sirolimus low stop vs. Leflunomide continue	Sirolimus low stop vs. Leflunomide stop
0.0346	0.0153	0.0014	0.0069	0.0005	0.0029

Figure 92 Comparison of proportions of CD4+ cells. Comparison of proportions of CD4+ cells under different immunosuppressive medications. The number of lymphocytes are shown on the Y axis as ranks as the non parametric test used for these comparison was Kurskal-Wallis test. The table below it shows the statistically significant results on post hoc paired analysis with Dunn's test.

5.6.2.2 *Activated CD4 cells*

These were the cells expressing both CD4 and CD25 cell surface receptors only. The proportions of activated CD4 cells ranged from 0.06-12% among various groups studied. The only single statistically significant result was between sirolimus low dose and high dose treatment continuation groups (**p <0.0103**).

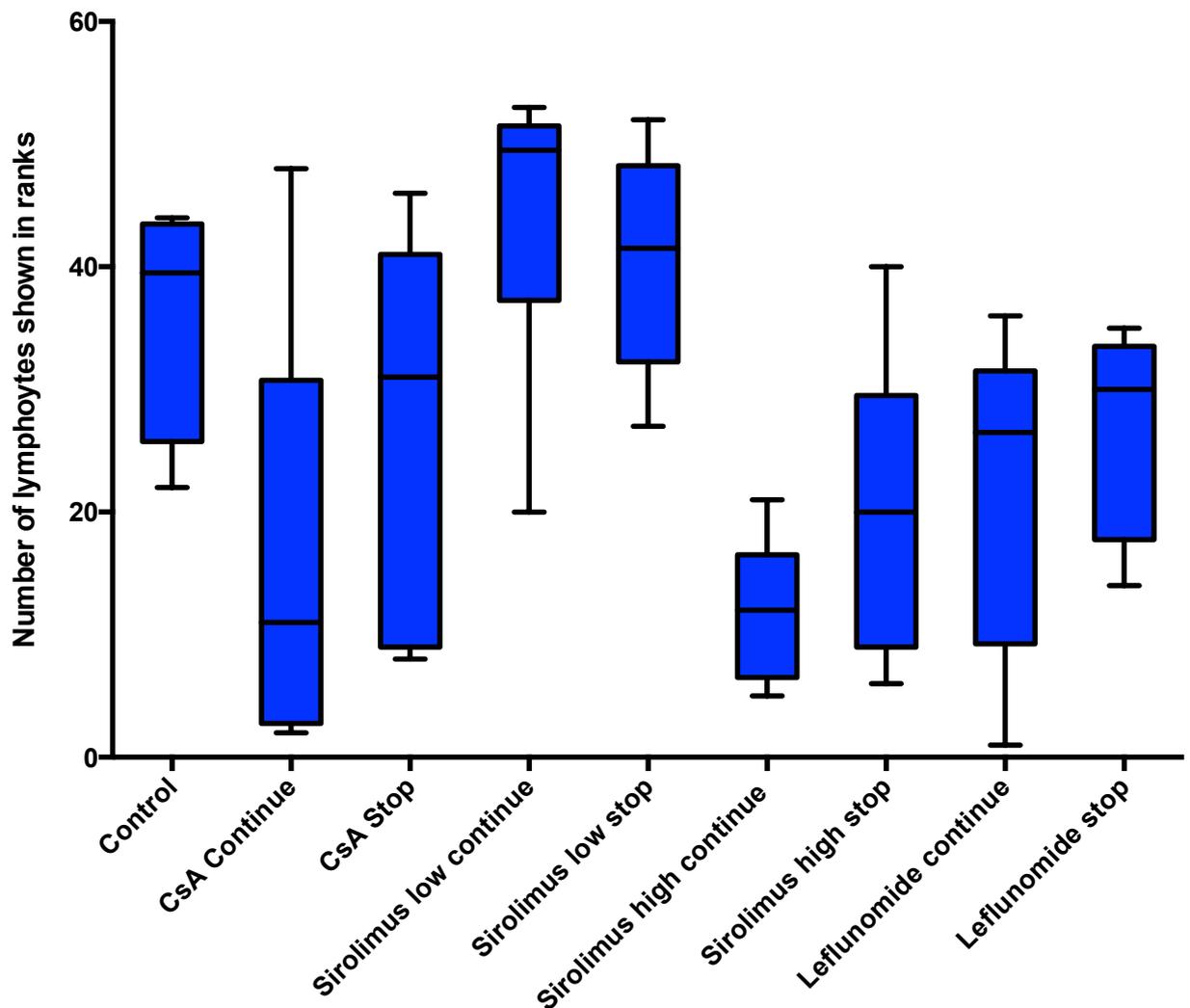


Figure 93 Comparison of proportions of activated CD4+ cells. Comparison of proportions of activated CD4+ cells under different immunosuppressive medications. The only one statistically significant paired comparison was between sirolimus low and high dose treatment continue groups ($p < 0.0103$).

5.6.2.3 *Regulatory T cells*

These are the CD4, CD25 and FoxP3 expressing cells. Generally the animals treated with Sirolimus low dose had higher proportions of T_{reg} cells population although the differences between the groups were too small to be significant.

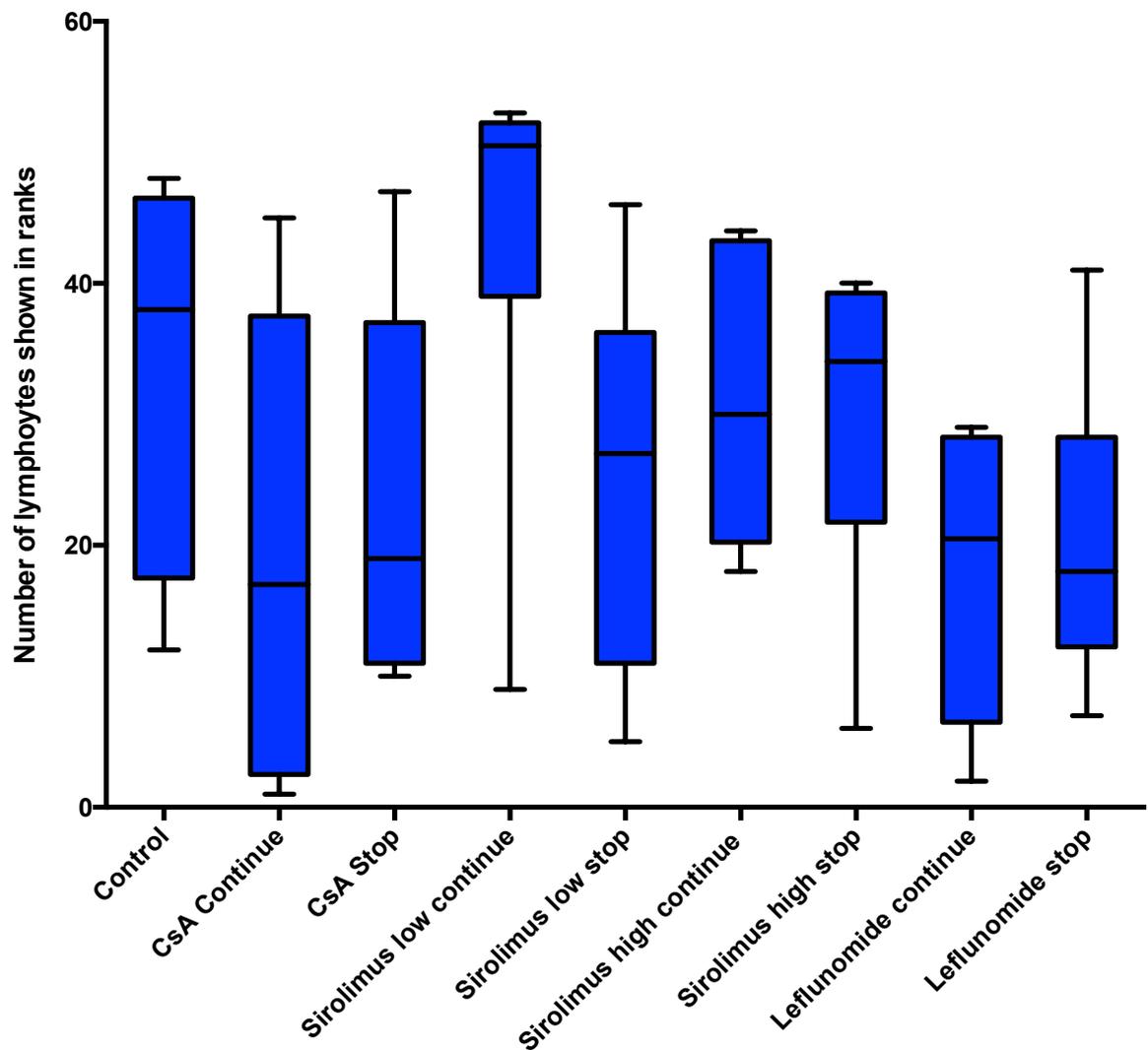


Figure 94 Treg comparisons. Comparison of proportions of Treg as a proportion of total CD4+ cells under different immunosuppressive medications

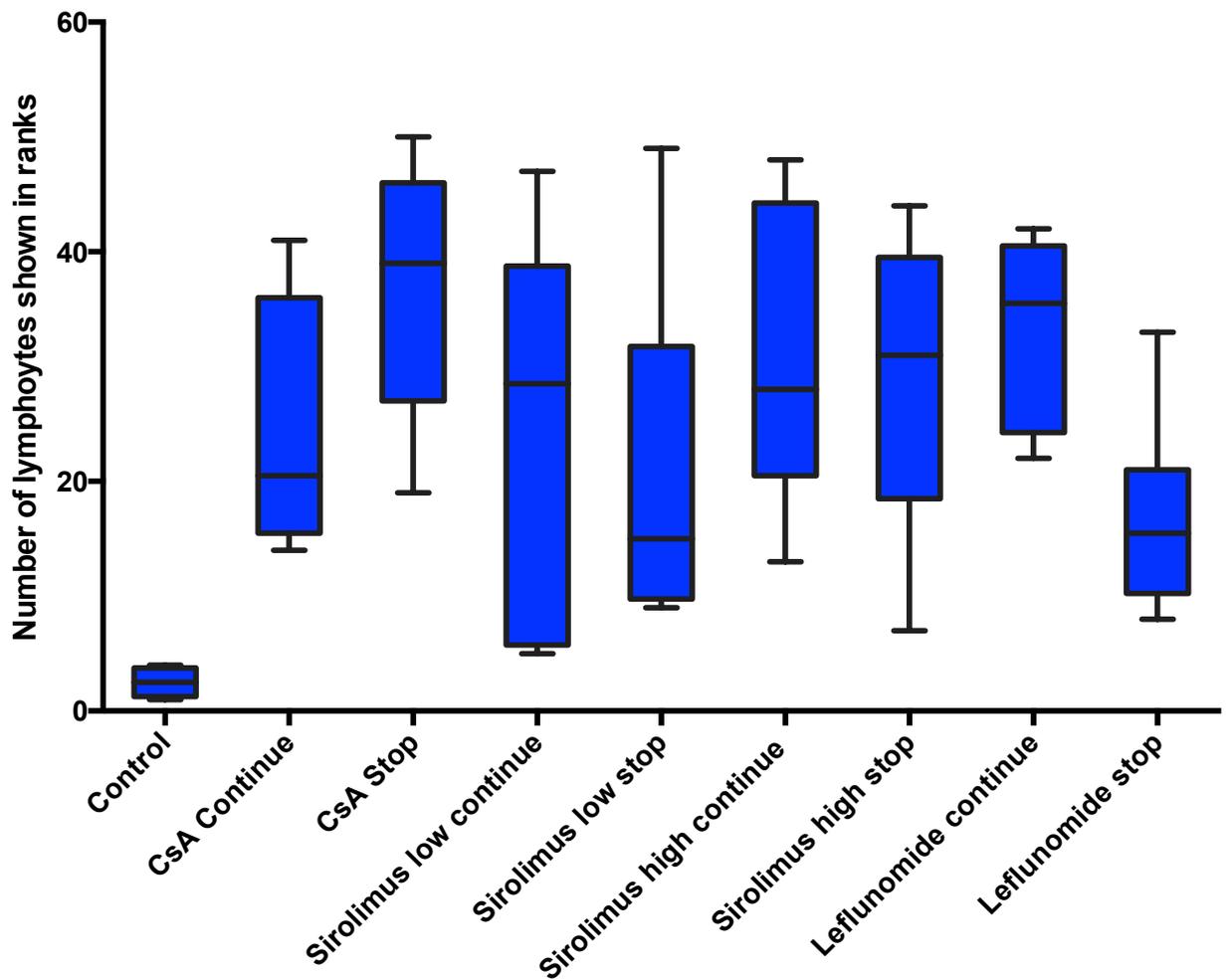
5.6.2.4 CD8 + cells

welooked at the total number of CD8 cells and activated CD8 (CD8+CD25+) as a proportion of total lymphocytes events as well as the number of activated CD8 cells out of the total CD8 population.

Control animals had very small CD8 population ranging from 0.08-0.19% of the total lymphocytes. This was in contrast to the animals with tumour injection and

immunosuppression, where the mean value varied from 5-9%. These results are in line with the fact that CD8+ cells are one of the main effector cells when it comes to rejection and tumour destruction.

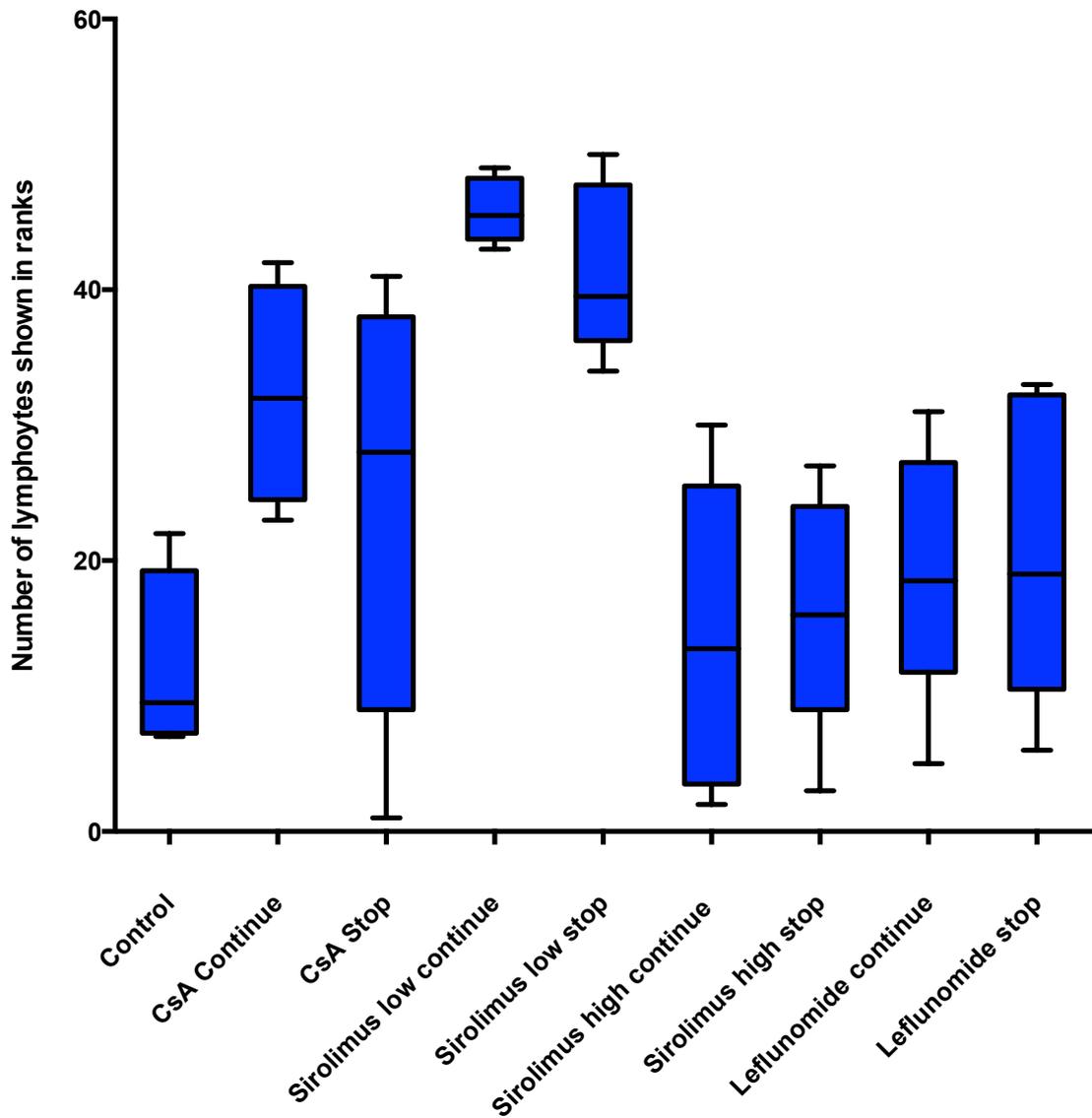
All treatment groups and the controls were compared for CD8+ cells among each other. The comparisons with the significant differences are highlighted in the table below the graph



Treatment	Control vs. CsA Stop	Control vs. Leflunomide continue
Significance	0.0042	0.0378

Figure 95 CD8+ cell comparisons. Comparison of proportions of CD8+ cells of total lymphocytes under different immunosuppressive medications. The CD8+ cells were in abundance as compared to the control animals with no tumour injection.

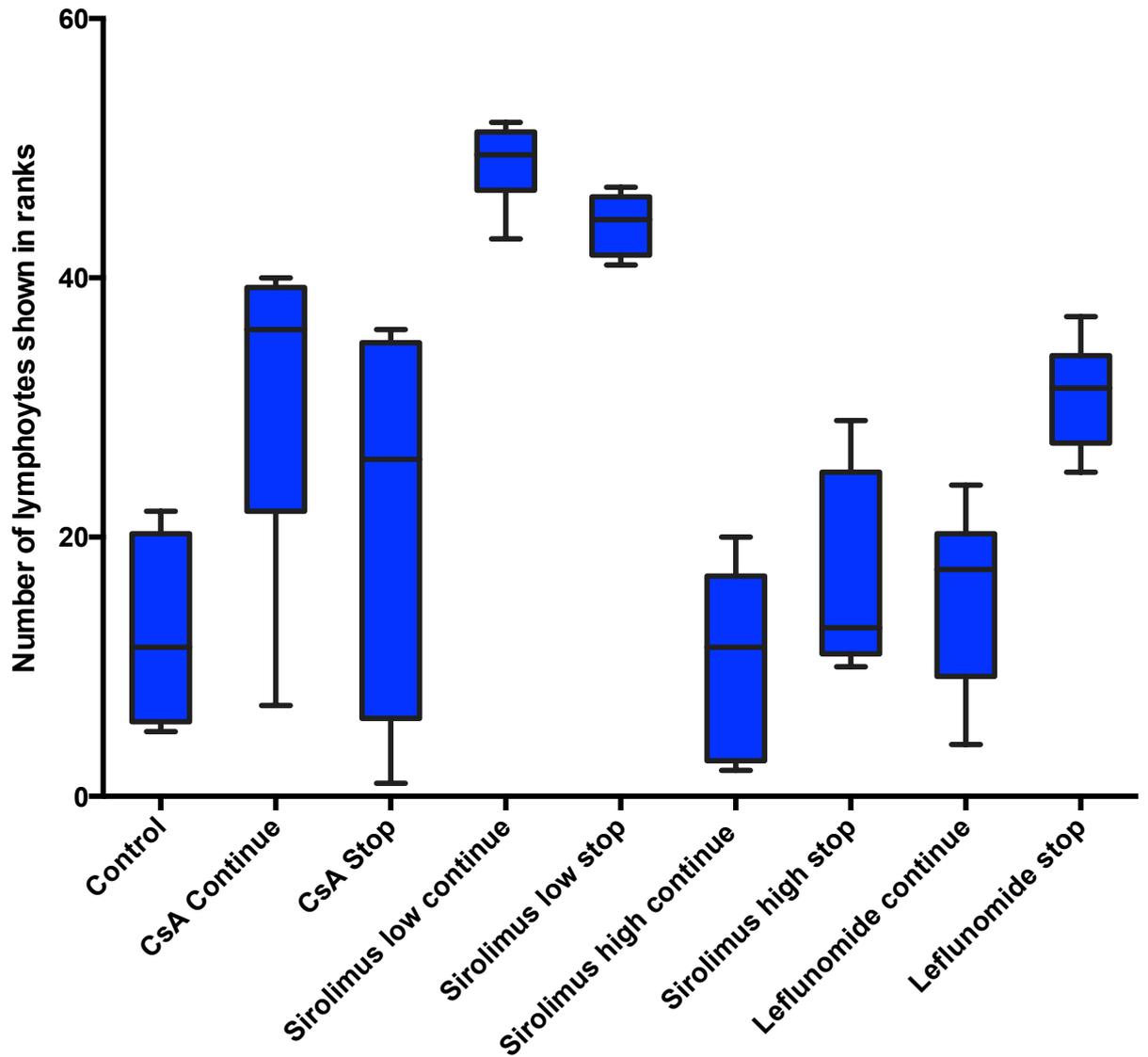
When the activated CD8 population was analysed, generally there were more of these cells in the animals with tumour injection, although the significant differences were only seen between control and Sirolimus low dose continue groups and sirolimus group versus leflunomide treatment continue group.



Treatments	Control vs. Sirolimus low continue	Sirolimus low continue vs. Sirolimus high continue	Sirolimus low continue vs. Sirolimus high stop	Sirolimus low continue vs. Leflunomide continue
Significance	0.0117	0.0071	0.0308	0.0481

Figure 96 Activated CD8 T cell comparisons. Comparison of proportions of activated CD8+ cells of total lymphocytes under different immunosuppressive medications. The cells populations with significant differences in numbers are shown in brown colour. The activated CD8+ cells were generally in higher number as compared to the control animals with no tumour injection.

Next we analysed the number of activated CD8+ cells as a proportion of total CD8+ cells. Sirolimus low dose group again had the most number of activated CD8+ cells among all groups and the results were significant when compared to controls, sirolimus high and leflunomide treatment continue groups.



Treatment	Control vs. Sirolimus low continue	Control vs. Sirolimus low stop	Sirolimus low continue vs. Sirolimus high continue	Sirolimus low continue vs. Sirolimus high stop	Sirolimus low continue vs. Leflunomide continue	Sirolimus low stop vs. Sirolimus high continue	Sirolimus low stop vs. Leflunomide continue
Significance	0.0073	0.0435	0.0005	0.0188	0.0050	0.0046	0.03

Figure 97 Activated CD8 comparisons. Comparison of proportions of activated CD8+ cells of total CD8+ under different immunosuppressive medications. The cells populations with significant differences in numbers are shown in brown colour. The numbers of activated CD8+ cells were highest among the sirolimus low dose group.

5.6.2.5 *Natural killer cells*

There were no significant differences between the populations of NK cell among various immunosuppressives studied. The means ranged from 3-14%.

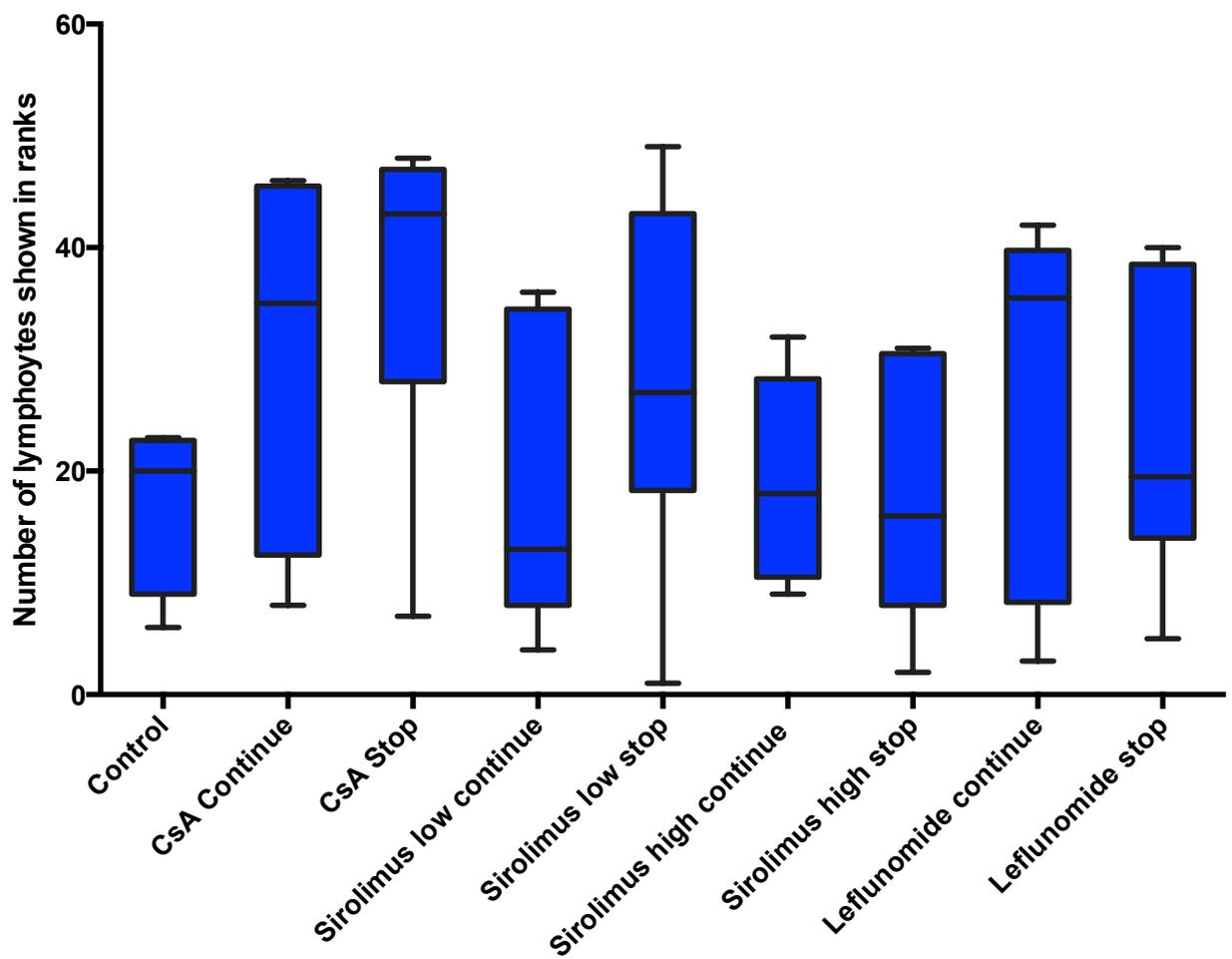
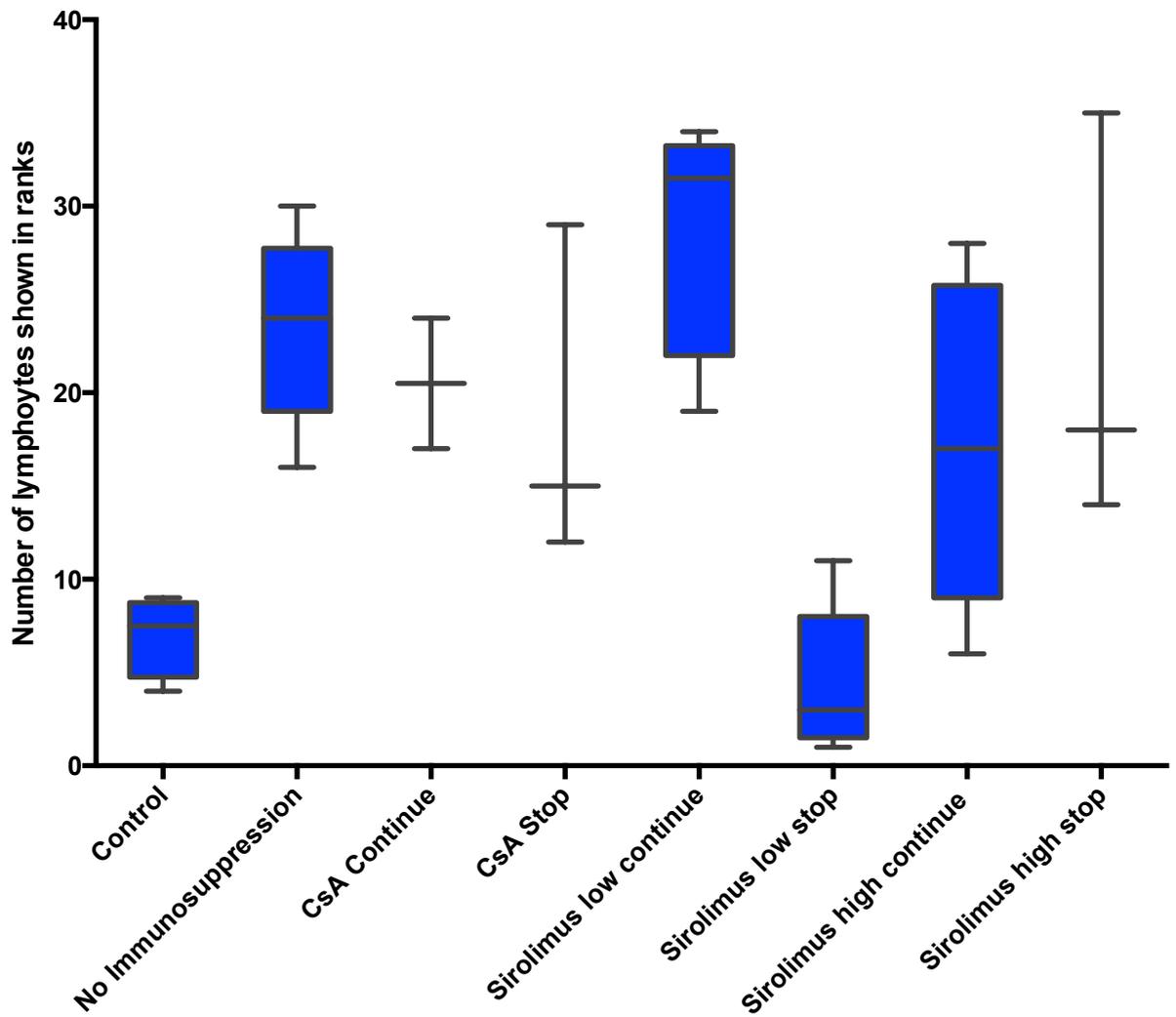


Figure 98 NK cells comparisons. Natural Killer cells distribution among different experimental groups. The numbers were variable under different conditions with generally more NK cells when the tumour was injected but none of these reached statistical significance.

5.6.3 Cell population comparison among treatment groups - Lewis animals

5.6.3.1 *CD4+ cells*

In Lewis animals, the proportion of CD4+ cells of the total lymphocyte was lower than the Wistar animals, with a mean of 3.7%. There were wide variations between the groups with the sirolimus low dose continue group having the most numbers of CD4 population (mean 28.6%). This was significantly higher than that of the control animals.



Treatment	Control vs. Sirolimus low continue	Sirolimus low continue vs. Sirolimus low stop
Significance	0.0295	0.0026

Figure 99 CD4+ comparisons. Comparison of proportions of CD4+ cells of all lymphocytes under different immunosuppressive medications.

5.6.3.2 Activated CD4 cells

These are the lymphocytes expressing both CD4 and CD25 cell surface receptors. Again the biggest and the only statistically significant difference was measured between the controls and sirolimus low dose treatment continue groups ($p < 0.0003$). The mean of activated CD4 cells for control was 19.38% while for the sirolimus group it was 1.03%.

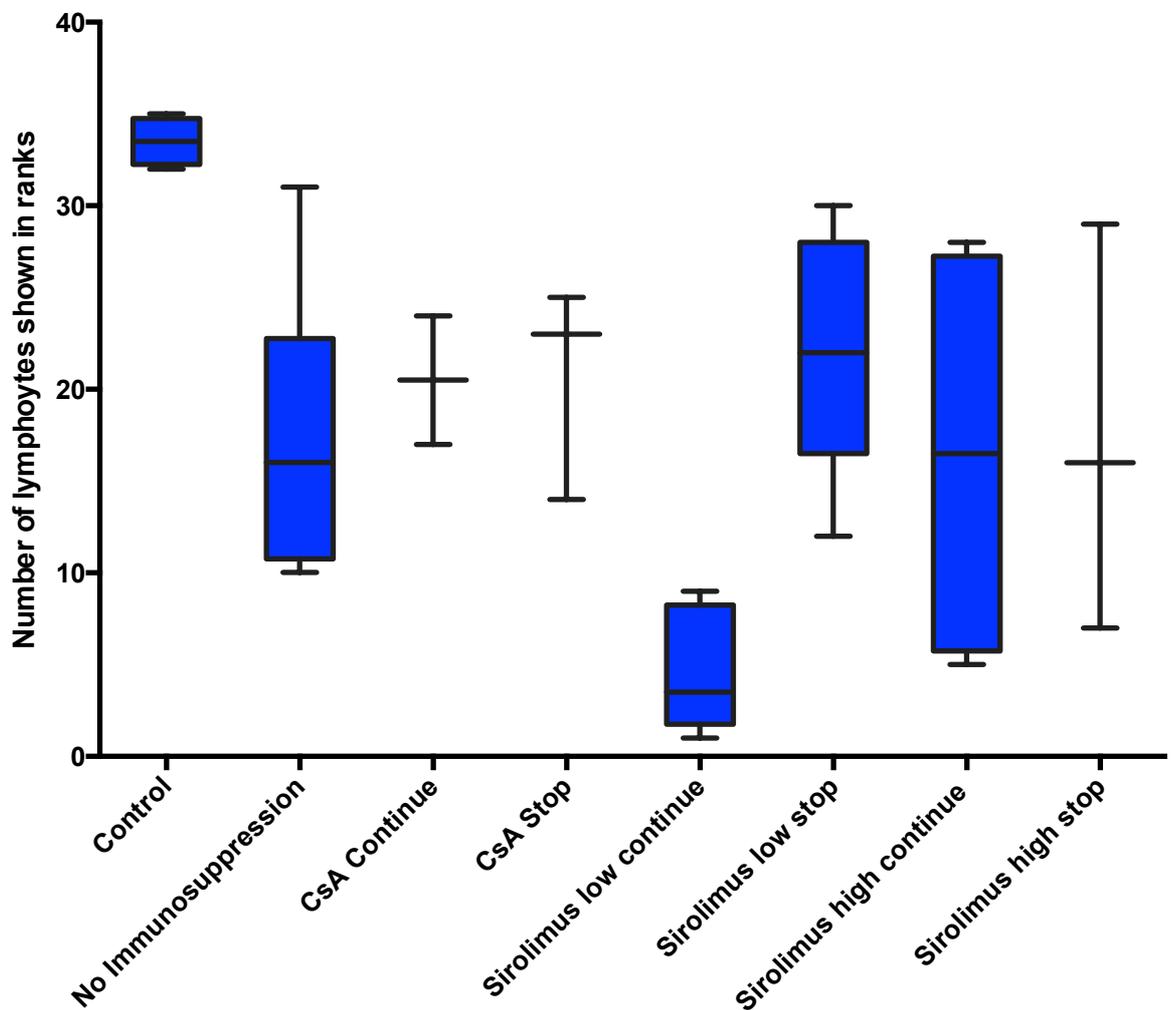
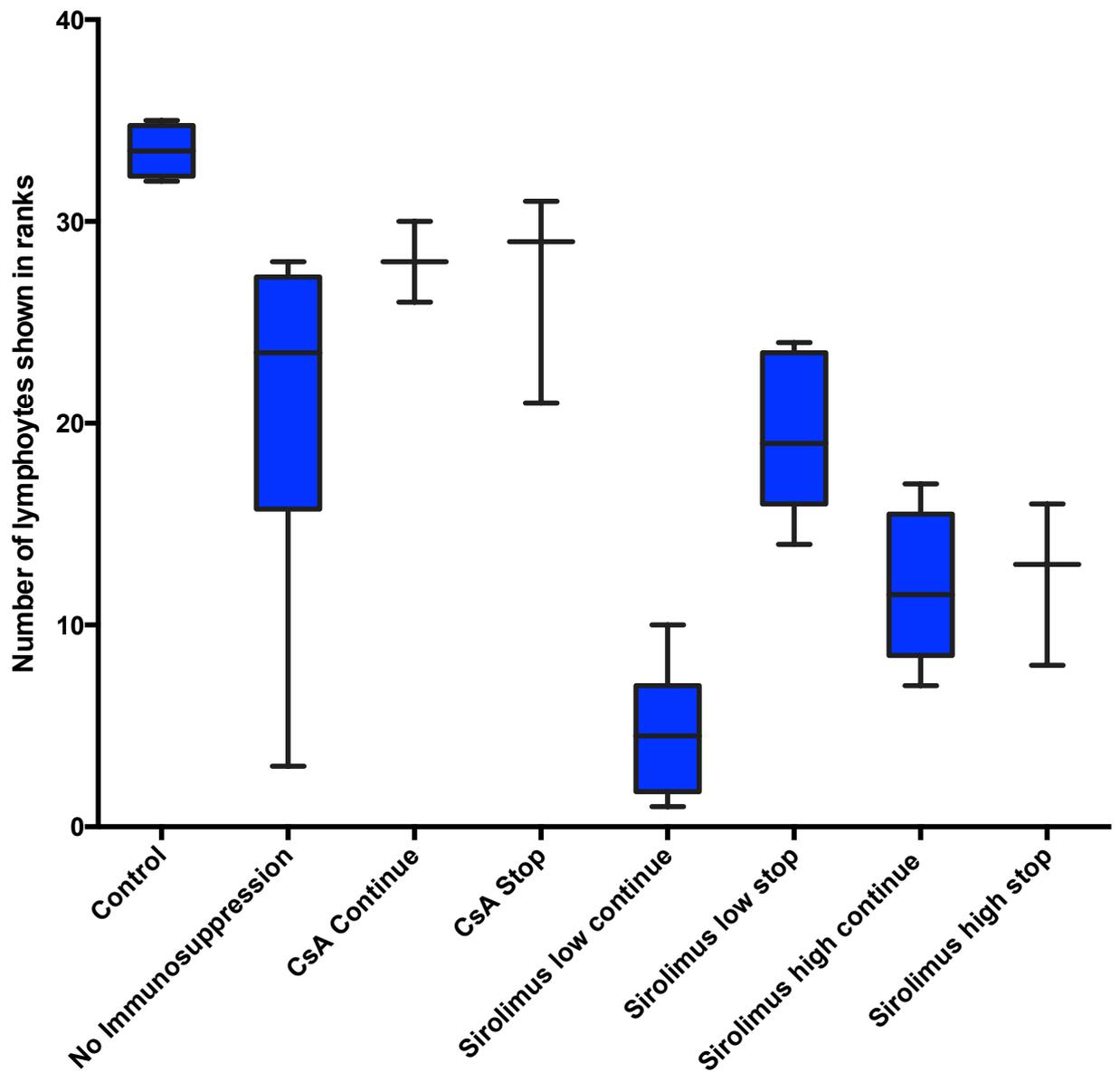


Figure 100 Activated CD4 comparisons. Comparison of proportions of activated CD4+ cells of all lymphocytes under different immunosuppressive medications. The only one significant difference was between the control and sirolimus low dose continue groups ($p < 0.0003$).

5.6.3.3 *Regulatory T cells*

The control animals with no immunosuppression had a mean of 51% T_{regs} of the total number of CD 4 positive cells. There was a reduction in this proportion in the rats with immunosuppression and those with just the tumour injection. The biggest difference that was between the controls and the sirolimus treated animals in the continuation arm.



Treatment	Control vs. Sirolimus low continue	Control vs. Sirolimus high continue
Significance	0.0004	0.0295

Figure 101 Treg comparisons. Comparison of proportions of Treg cells of the CD4+ population under different immunosuppressive medications. The cells populations with significant differences in numbers are shown in brown colour

5.6.3.4 CD 8+ cells

Again we looked at the total number of CD8 cells and activated CD8 (CD8+CD25+) as a proportion of total lymphocytes events as well as the number of activated CD8 cells out of the total CD8 population.

The proportion of CD8 cells was quite evenly distributed throughout the groups and there were no significant differences between them. The range of the percentage of CD8+ cells of the total lymphocytes was between 3-19 %.

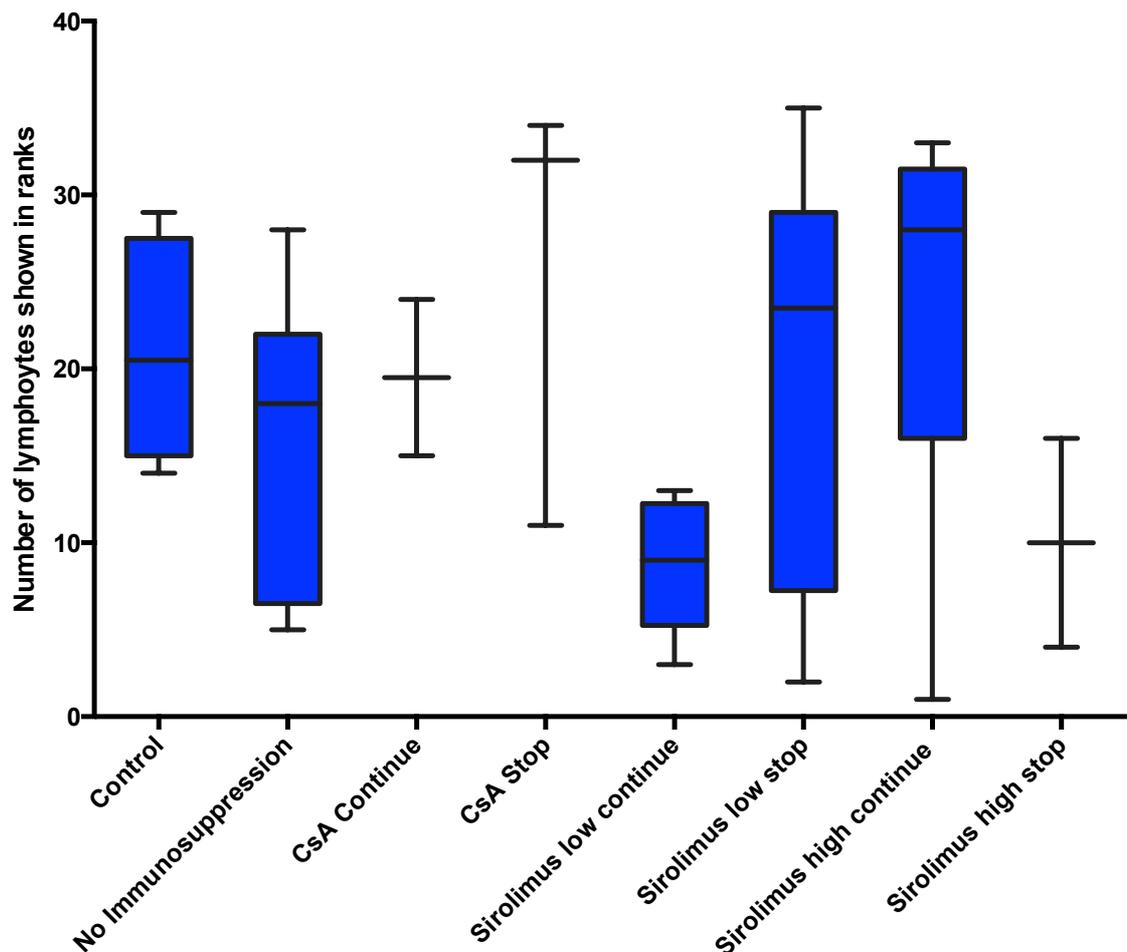


Figure 102 CD8 comparison. Comparison of proportions of CD8+ cells of all lymphocytes under different immunosuppressive medications.

The number of activated CD8+ T cells of the total population was a small fraction ranging between 0.3-2.9%. The sirolimus low dose stop group had the largest number of activated T cells of total lymphocytes ($p < 0.0385$).

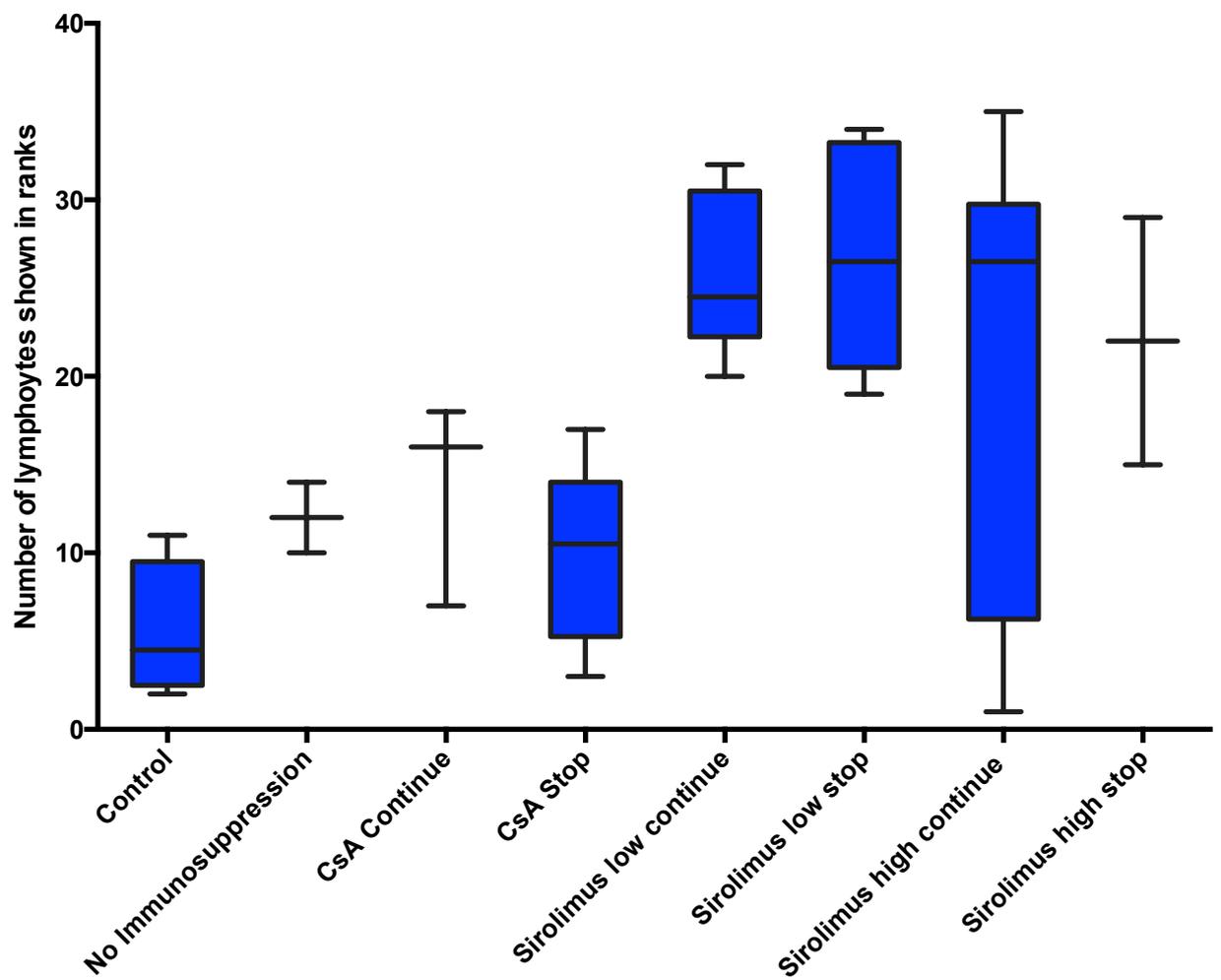
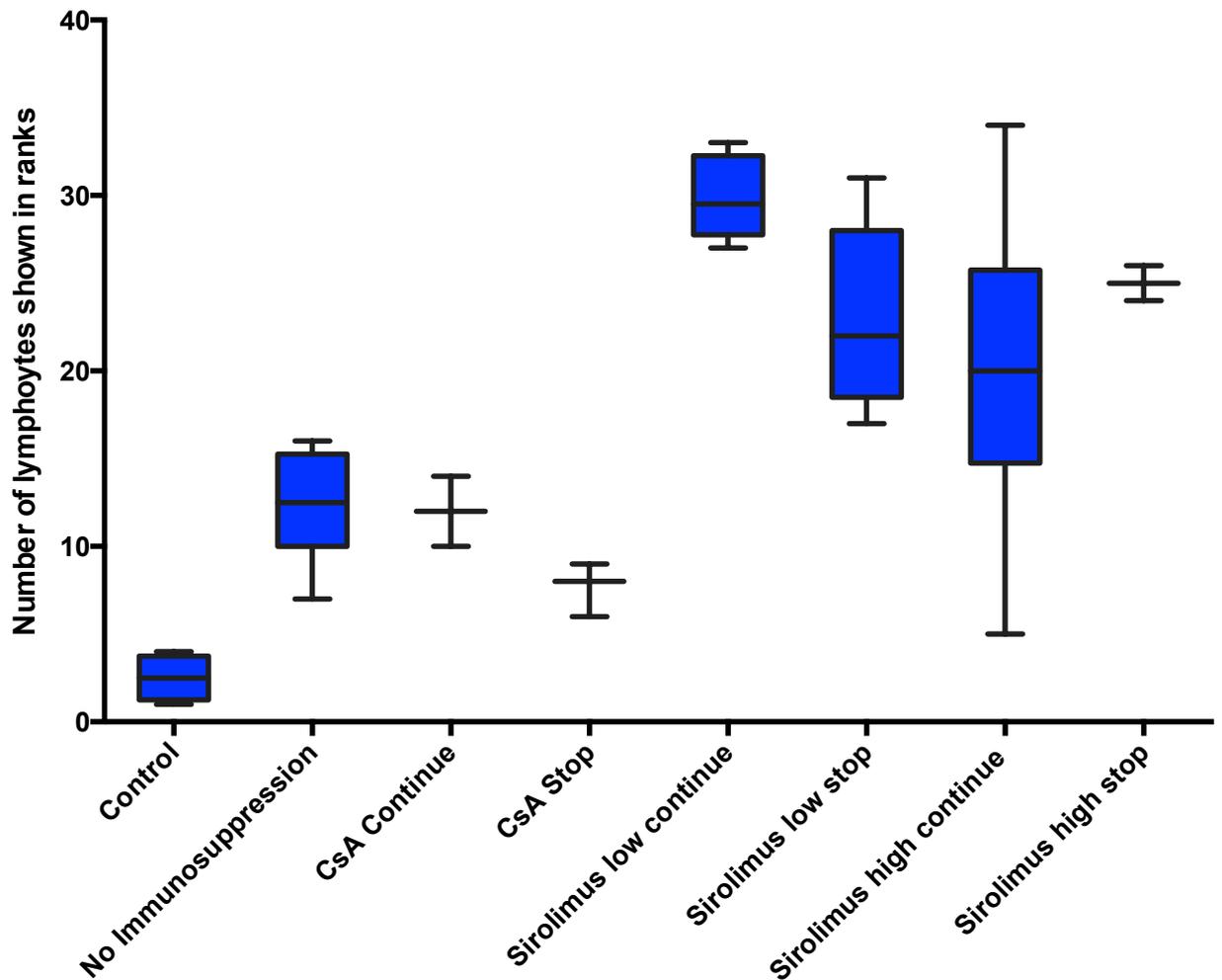


Figure 103 Activated CD8 comparisons. Comparison of proportions of activated CD8+ cells of all lymphocytes under different immunosuppressive medications. The cells populations with significant differences in numbers are shown in brown colour. Again, like Wistar animals, the highest numbers of these activated cells were seen in sirolimus low dose groups.

When we compared the proportion of the activated CD8+ cells of total CD8+ cells, we found wide variation among the groups. Again the biggest and highly significant difference was between the controls and the sirolimus low dose continue group (mean was 1.8% for controls and 45% for the sirolimus group).



Treatment	Control vs. Sirolimus low continue	CsA Stop vs. Sirolimus low continue
Significance	0.0006	0.0460

Figure 104 Activated CD8 comparisons. Comparison of proportions of activated CD8+ cells of all lymphocytes under different immunosuppressive medications. The cells populations with significant differences in numbers are shown in brown colour.

5.6.3.5 *Natural Killer cells*

There was no difference between the NK cells between the groups with the mean percentage ranging from 4.3-16%. There were wide variations even within the groups as well.

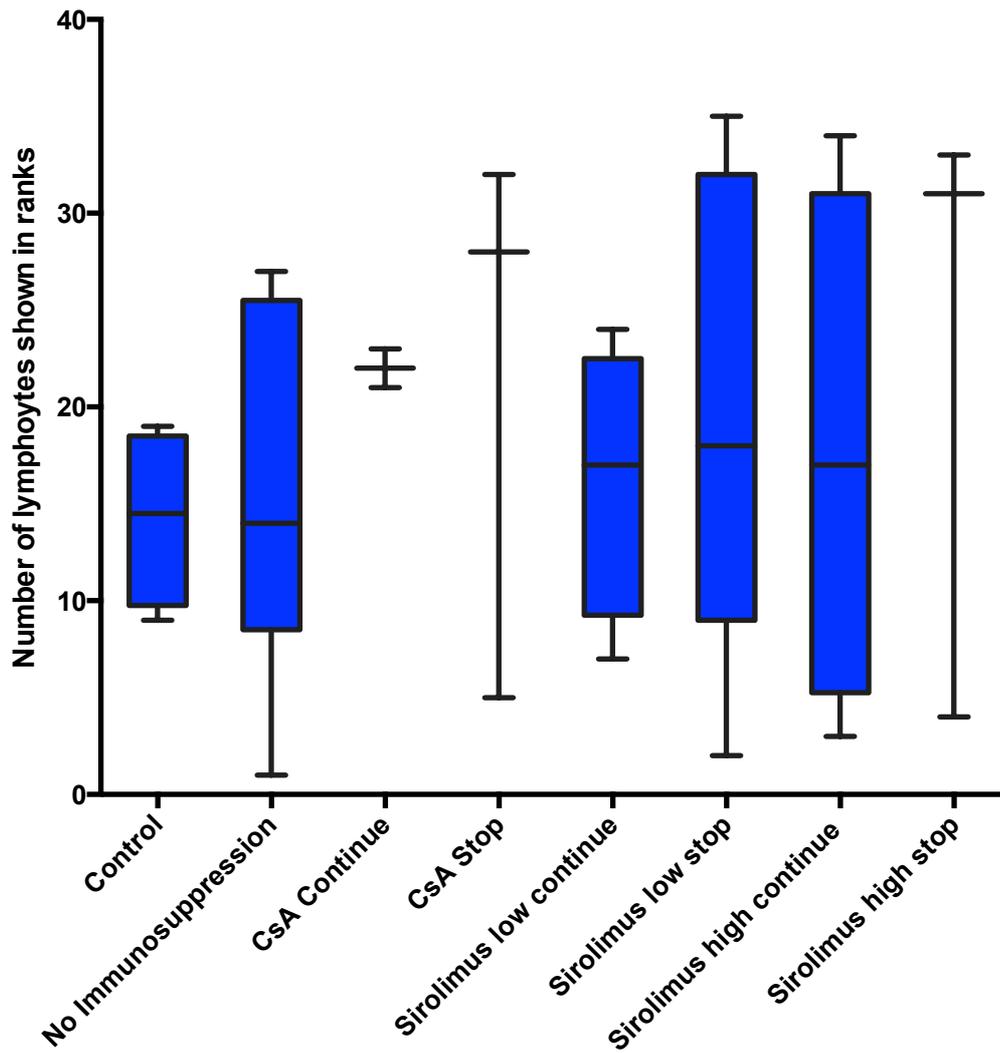


Figure 105 NK cells comparisons. Comparison of proportions of NK cells of all lymphocytes under different immunosuppressive medications.

5.7 DISCUSSION

Development of tumour is a complex and a multistage process in animals and humans. These factors could be intrinsic (genetic mutations, oncogenes) and extrinsic (radiation and carcinogenic exposure and certain viral infections). Whatever the mechanism, the tumours have tumour specific and tumour associated antigens that make them unique. These antigens are recognised by the immune system, which help in keeping these mutant/tumours cells under control. The interaction of the immune system with tumours was shown quite eloquently by Macfarlane Burnet. The syngeneic rats were injected with one type of irradiated tumour cells and divided into two groups. Then competent non-irradiated tumour cells of same type were injected in the first group of rats and another type in the other set. The “immunised” animals with same tumour type rejected the competent tumour cell load while the other immunised group showed tumour propagation(275). This work was one of the earliest proofs of the close interaction between the immune system and the tumour cells.

We now know that almost all the effectors of immune system are actively involved in the tumour immunity. Macrophages and dendritic cells have a specific role in antigen presentation. CD8+ cell mediated cytotoxicity plays perhaps the most important role of them all. Then there are CD4+ cells and their various subsets and natural killer cells. Tumour cells are believed either to induce immune system thereby making them exposed to immune destruction or evade the immune system with various mechanisms. It is this interplay between

these two mechanisms that determines the fate of most of the tumours(276). More immunogenic tumour are easily attacked by the immune system and carry a better prognosis while the immune resistant variant are more difficult to control. The resistant variants have various mechanisms that help them evade immune system including; selective overgrowth of antigen negative variants of tumour cells, down-regulation of expression of MHC antigens and then selective selection of these cell lines. Other relatively newly discovered mechanisms include lack of costimulation that is required for continued propagation of the cytotoxic T cells and important role of regulatory T cells.

There is a lot of information that we still don't know fully about the role of immune system in tumour development and growth. But specifically the CD4+, CD8+, NK and T_{reg} cells play important roles that are being recognised more and more.

Literature regarding the role of cytotoxic CD8+ T cell as having anti-tumour properties is very clear. The role of CD4+ cells is becoming clearer in the recent years as well. They are known for a long time to be required for the priming, initial activation and expansion of CD8+ cells(277)(278)(279). As the direct effectors of anti-tumour effects are CD8+ cells, CD4+ cells play a pivotal role by making sure there are enough activated, primed and continuous supply of these CTLs at the tumour microenvironment. One of the main reasons of incomplete or ineffective tumour regression despite initial CTL response is the lack of CD4+ T cell help(280)(281). Most tumour cells express MHC class I molecules that are recognised by CTLs but there is now emerging evidence that for some class II expressing tumour cells, CD4+ cells behave as effectors as they are not recognised by the CTLs(282)(283).

Apart from the helper subset of CD4⁺ cells, there are regulatory T cells (CD4⁺ CD25⁺ FoxP3⁺). These cells were first characterised as mediators of self-tolerance by Sakaguchi *et al.*(284). Along with development of tolerance and prevention of autoimmunity both in humans and animals, these cells are capable of dampening the tumour cytolytic responses by CD8⁺ cells. These cells are seen in increased numbers in the tumour microenvironment and their presence in most of the tumours is generally regarded as a negative prognostic sign(285–292). Movement of these cells are regulated either by direct chemokine driven mechanisms, local tissue expansion or by conversion of FoxP3⁻ to FoxP3⁺ cells(293,294). Whatever the mechanism of infiltration, in majority of the established tumours, the higher the proportion of T_{reg} cells at the tumour microenvironment or circulation the worse is the prognosis. Furthermore, T_{reg} cells not only are important in tumour microenvironment but also in prevention of autoimmunity in normal subjects and may have a potential role in development of allograft tolerance in transplant subjects. In purely transplant setting, higher numbers of T_{reg} cells is a good prognostic feature but the situation becomes a little complicated when these transplant recipients develop cancer.

Role of different immunosuppressive agents have been widely studied on the immune phenotyping of the transplant recipients. Generally, CNIs in transplant setting either cause reduction in the number of T_{reg} cells or inhibits their function. Gao *et al.* showed that whereas mTOR inhibitors were able to promote de novo generation of alloantigen specific T_{reg} cells, CNIs completely inhibited this process(295). Several other studies have shown similar effects of CNIs on T_{reg} cells(296–298). In contrast to the CNIs, mTOR inhibitors have generally

shown a more positive role on the population of T_{regs} , hence the potential promise for help in tolerance. They were first shown to expand T_{regs} in vitro by Battaglia *et al.*(299). These in vitro effects were seen both in humans and mice. Similar results were noticed in in-vivo studies where both the function and numbers of T_{regs} increased by mTORs inhibitors(300,301). In the context of transplantation without any tumour growth, these effects are desirable for potential tolerance but in cases of tumour development, high percentage of T_{reg} population circulating or in the tumour microenvironment is a worse prognostic sign. This interplay between tumour development and T_{reg} cells is a two way process with both having the potential to promote each other's(302).

Another potentially very important observation was made by Hope *et al.* on cancer related immune phenotype in kidney transplant recipients. They showed that although the number of T_{regs} was higher in the KTR with cancers as is shown by the previously mentioned studies, there was a decline in the number of T_{reg} population after tumour resection(303). This observation could partially be due to removal of the positive feedback mechanism of tumour microenvironment on the T_{reg} population or due to inevitable reduction of immunosuppression after the diagnosis of tumour in most KTRs(304).

In our analysis, the T lymphocyte counts showed wide variations between the groups. It will be difficult and may be artificial to completely infer cause and effect relationship on the tumour rejection based on this analysis but there are some very interesting trends.

Firstly, there were marked differences in the distribution of T cells between the two strains. Although the number of CD 4+ cells was significantly higher in

Wistar rats than the Lewis but the proportion of the activated CD4 cells was higher in Lewis rats in comparison. Similarly the T_{reg} cells were more abundant in Lewis rats. With CD4 cells having more indirect role in activating the immune system and T_{reg} having a “controlling” effect of the immune response these finding could be important in tumour control. The rest of the cell populations (CD8, activated CD8 and NK cells) were similar between both these strains.

Due to the above-mentioned differences, it was meaningful to compare the effect of different immunosuppressives on various cell populations in each strain separately.

For the Wistar rats, CD 4+ cell population was lower in the sirolimus low dose group as compared to the controls and the CsA group. But the activated CD4+ cells were highest in the Sirolimus low dose group. There were no apparent differences for T_{reg} population.

The results from activated CD8 cells were more consistent. All of the animals had higher proportions of CD8+ cells as compared to the controls. Similar results were seen when specifically the activated CD8+ cells' percentage of the total CD8+ population was studied. The highest number of CD8+ cells were seen in the animals treated with low dose of sirolimus. These cells are normally one of the first effective responses for destroying the tumour cells. The preservation of these cells in higher number in the sirolimus treated group may point towards the ability of sirolimus treated animals to destroy the tumour cells more effectively than the CsA immunosuppression. Interestingly, in the high dose sirolimus animas groups these cells were not in much numbers and

perhaps it points towards the excessive immunosuppression as a much higher immunosuppressive dose was used in this group.

For the Lewis animals, the T_{reg} population was the lowest in the sirolimus low dose group and activated CD8+ cells population was the highest. These results again, may explain the reason for the better response against the tumour cells in the sirolimus arm of animals.

The effects on CD8+ cells were most consistent in our flowcytometric data and also in line with the clinical observation of better tumour clearance in these animals.

Normally immunosuppression with sirolimus has shown an increase in the number of T_{reg} cells, but in our model the population of T_{reg} reduced along with an increase in the number of CTLs. Although this result may seem against most of the literature but one important difference in our model was that it was not simply a transplant model but the transplanted cells were malignant as well. As shown by Hope *et al.*(303), when the SCC were excised in KTRs, the T_{reg} cells population also went down. The reason we might be seeing decline in the number of T_{reg} cells might be due to the strong anti-neoplastic effects leading to reducing tumour load and thereby eliminating the stimulus to keep the numbers high of T_{reg} cells by the tumour microenvironment. Not a lot is known as to why the T_{reg} numbers reduce after tumour excision and further research is needed in this area.

For the natural killer cells we did not find any meaningful differences for any of the treatment regime.

There are a few limitations with our flow cytometric analysis. First of all, the results of the CsA may not be the true reflection of the actual lymphocyte count at the end of the study period. This is because of technical failure of the FACS machine at the beginning of the experiments and loss of a few samples from the Wistar animals with no immunosuppression. This also meant that by the time we were able to confidently do the flow cytometric analysis, the CsA treated animals had already finished four weeks of the experiments for a few weeks. The ideal time to look at the effects of tumour and immunosuppression with flow cytometry is immediately after the completion of four weeks of treatment according to the study protocol. Due to above mentioned reason CsA treated animals had a few weeks, without any treatment before they were euthanased for the flow cytometric analysis. This time might have caused the reversion of the lymphocyte count to baseline, which will not be the true reflection of the exact influence of tumour on the lymphocyte count. The results of the sirolimus and leflunomide are more reliable from this respect.

The other possible limitation of the flow analysis could be the small number of animals in each arm. Some of the trends seen in our experiments as described above might have become clinically significant if we had big enough numbers. This may especially be true as the bioluminescent differences described in the previous chapter were far more drastic among different treatment arms and subtle but important changes in the various subpopulations of lymphocytes might need significantly bigger number to increase the power of our analysis.

Finally, the ideal place to look for the immune effectors would have been the tumour microenvironment. This as we know has the most reflective population of lymphocytes. Such an analysis would be easily possible for the animals were

the tumour growth was good and by the end of experiment there were either palpable tumour or at least bioluminescent signals to enable accurate selection of tissue containing tumour microenvironment. But as all of the sirolimus treated and most leflunomide treated animals completely eliminated the tumour load such analysis could have become very subjective with lot of variations. Second best place to look for the lymphocytes would have been the regional lymph nodes but again they were only detectable in a very small proportion of rats. Hence to keep the analysis more objective and to minimise variability We chose the splenic tissue as the source of these effectors of immune system.

Chapter 6

CLINICAL CASES

6.1 CLINICAL CASES

Previously mentioned case series/reports already pointed towards the feasibility of transplanting kidneys after ex vivo resection of tumours. With these results there was an indication to perhaps the best matching and immunosuppressive strategy.

Based on the above-mentioned observations, we undertook transplantation of such restored organs in a controlled environment in Freeman Hospital, Newcastle upon Tyne.

The clinical cases are divided into the benign and malignant.

6.1.1 Transplantation with kidney removed for benign pathology

The indications of nephrectomy due to benign causes are few. Most common causes include intractable haematuria, loin pain, refractory proteinuria to maximum medical therapy and iatrogenic kidney injury. Our case is presented below(305)

6.1.1.1 *Case report*

A 59-year-old male underwent a resection of a large retroperitoneal 'tumour', which involved the left colon for a pre-operatively diagnosed sarcoma. Final histology confirmed "Benign Fibromatosis" which is associated with an optimistic prognosis. The patients subsequently underwent a reversal of colostomy and intraoperatively sustained an iatrogenic injury to the left mid ureteric injury, which presented on the 10th post-operative day as loin pain and

sepsis. An initial nephrostomy and drainage of urinoma was performed followed by an unsuccessful attempt at antegrade and retrograde stent insertion. A defect measuring >4 cm was identified in the mid-ureter. A renogram confirmed a differential function of 41% on the left side and the patient's glomerular filtration rate (GFR) was calculated at 68-ml/min/1.73 m².

The patient was counselled regarding the options of either nephrectomy followed by auto-transplantation (AT) or nephrectomy alone. The risks of both these options were discussed with patient in detail. The patient was not keen to pursue any reconstructive surgery and opted for a nephrectomy. We then discussed the possibility of 'Altruistic domino donation' with the patient. Permission for using the left kidney for potential transplantation was approved by UK Transplant (UKT) under the category of "domino donation" according to the Human Tissue Act(93) .

A 70-year-old recipient on thrice weekly haemodialysis for 6-years was identified. The patient had ESRF secondary to hypertension and had concomitant ischemic heart disease. His mismatch was 1:1:1. This recipient had waited for 6 years for a cadaveric renal transplant and had no prospect of a live donor transplant. It would have been unlikely for him to receive a cadaveric transplant during his lifetime based on his performance status and co-morbidities making him completely dialysis dependent for life. Both donor and recipient were scheduled for surgery on the same day and did not meet each other. An open nephrectomy was performed in view of the patient's previous extensive open surgery and adhesions via a loin incision and the kidney flushed with cold preservation solution. The kidney was prepared for transplantation after a thorough examination by the transplant team (Figs. 1 and 2).

The donor organ was implanted into the right iliac fossa and the ureter was successfully anastomosed into the bladder over a stent. Post-operative recovery was unremarkable for both the donor and the recipient. Donor was discharged on 2nd post-operative day with creatinine of 1.25 mg/dl. Recipient had immediate graft function and creatinine on discharge was 1.53 mg/dl. The donor and the recipient have subsequently communicated anonymously with each other by letter and are doing well at 8-month follow-up.



Figure 106 Left kidney after back benching. Artery, vein and ureter demonstrated.



Figure 107 Left kidney after reperfusion. Ureter being anastomosed over the JJ stent.

6.1.1.2 Discussion

Kidneys removed for small renal tumours have been successfully transplanted after ex vivo tumour resection with extremely low recurrence rates (55)(29). Kidneys removed for benign aetiologies are however not associated with any risk of tumour transmission. Transplantation with renal grafts from benign aetiologies can be potentially linked to the established concept of altruistic donation. With the “donor” being treated for their primary pathology with nephrectomy, such donations are regarded as Altruistic domino donation in the United Kingdom (UK) under Human Tissue Act (HTA).

In current clinical practice the commonest indications for simple nephrectomy (SN) include intractable loin pain, renal artery aneurysm (58), ureteric injury (306) and refractory nephrotic syndrome (58). Auto transplantation (AT) can be

an option for these patients (excluding nephrotic syndrome) but on occasions this may not be the appropriate treatment in clinical situations such as extensive previous surgery, increasing risk of unsuccessful ureteric repair leading to post-operative complications, medical co-morbidities or patient choice. Counselling of patients undergoing SN and recipients of these kidneys is extremely important. The recipient should be fully aware of the origin and quality of the organ. This technique has the advantage of favourable ischaemic times. Using this approach we successfully used the renal graft from our donor for transplantation. In normal clinical circumstances the graft would have ended up as a nephrectomy specimen and this recipient would have been continued to be on thrice weekly haemodialysis. Our approach was psychologically very rewarding for the donor who viewed this as a positive outcome from a surgical complication. Furthermore these organs should be transplanted locally as they may pose specific technical problems (short vessels, ureter, etc.) and transplanting them into local recipients may minimise the discard rates and potential risk of complications, a concept endorsed by NHS Blood and Transplant (NHSBT). We aim to highlight the importance of this new concept to urologists in the UK and worldwide as this could be a very important source of precious renal allografts worldwide.

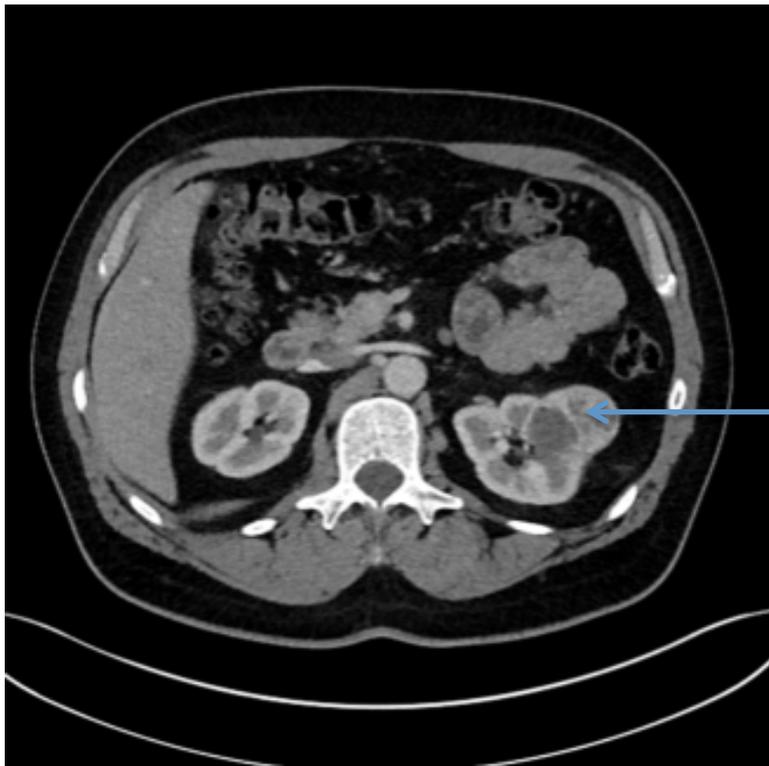
After the successful transplantation of this restored kidney and in the light of the available literature as mentioned earlier and promising results from our experiments we carefully selected suitable kidneys for transplantation after ex-vivo resection of malignant tumours.

6.1.2 Transplantation with kidney removed for malignant pathology

For transplantation of these restored organs we selected our recipients carefully, the information given to the patients was robust making sure that they understood the quality of the organs and the possibility of potential complications.

The patients selected were elderly and high-risk from the transplant waiting list. These patients would not tolerate delayed graft function from normal cadaveric kidneys and were more likely to die from cardiovascular complication while on waiting list than the risks associated transplantation with these organs.

Three patients with small renal cell carcinoma were identified. These patients were independently assessed by the urology teams and a decision to undergo radical nephrectomy was made totally independent of the possibility of transplantation. Once this decision was made these patients were approached for the possibility of donating these organs after ex vivo excision to which they readily agreed. The possibility of using the organs for transplantation had no bearing on the original decision of the type of cancer surgery. All these patients underwent staging CTs and baseline investigations.



Left renal tumour
identified on CT
abdomen

Figure 108 CT scan of a donor with the Left exophytic renal tumour.

Recipients were aged between 69-78 years with mean age of 72 years and had multiple comorbidities. They were explained in detail about the risks and benefits of such transplantation in detail before any decision to perform the surgery. We devised an information leaflet (see below), which was used as an additional tool to help these patients in understanding the procedure.

Information sheet for patients receiving tumour excised kidneys

Source of organ

This organ is being donated by a patient suffering from a small tumour in this kidney. In most cases such tumours could be treated by removing only the tumour affected part safely but this kidney will be removed completely either because of the patient's wishes or for the technical reasons.

Tumour excision (resection)

The affected part of the kidney will be removed before the kidney is transplanted. Though the tumour will appear to be all removed there will be a small chance that there will still be some tumour cells remaining within the kidney.

Risk of recurrence

If the patient had kept this kidney rather than donating it, the risk of tumour recurrence with local removal of the tumour is less than 5%. However the use of immunosuppression which will be necessary to prevent you rejecting this kidney brings an unknown to the risk of recurrence of the tumour. Current medical literature on the use of such organs after tumour removal has shown very low recurrence rates.

Surveillance after transplantation

The transplanted kidney will be scanned every six months to make sure it is healthy. Should there be any evidence of a tumour development within the transplant the immunosuppression will be reduced or stopped and the kidney can be removed. Regular surveillance will increase the chances of picking any tumour growth at early stages thus reducing risk to your health. Despite the surveillance there remains the possibility that a tumour may develop within the transplanted kidney and potentially spread elsewhere in the body before it is identified. In the circumstance treatment may be possible but the condition would not be curable.

Quality of organ

Part of the kidney affected by the tumour has been removed, thereby leaving less than the full kidney for transplantation. Current evidence from literature suggests that despite this, these kidneys work as well as live unrelated transplants.

Because this kidney is from a live donor, the organ is of better quality and has not suffered from long periods of cold storage without its blood supply (which is normal for organs from deceased donors).

Further information

Please feel free to ask any questions regarding this information sheet or any other queries not answered here from your consultant or registrar.

Prof David Talbot, Mr David Rix, Mr Muhammad Khurram

6.1.2.1 *Technique*

After the nephrectomy, the tumours were excised under direct vision +/- ultrasound guidance by an experienced urologist. The calyces were over sewn with 5/0 PDS and Surgicell and Tachosil was sewn into the defects as well. The implantation was as standard with particular attention at haemostasis.

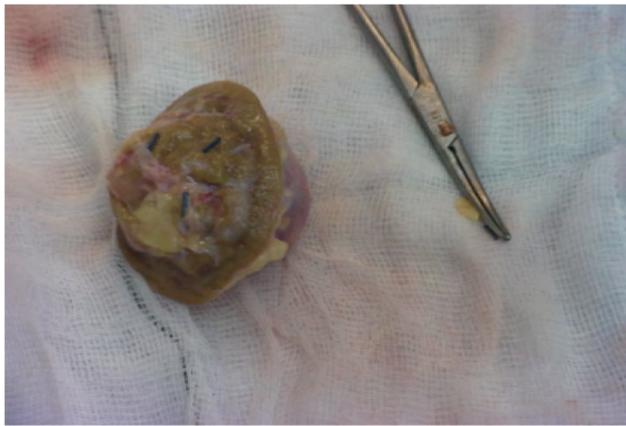


Figure 109 Excised tumour

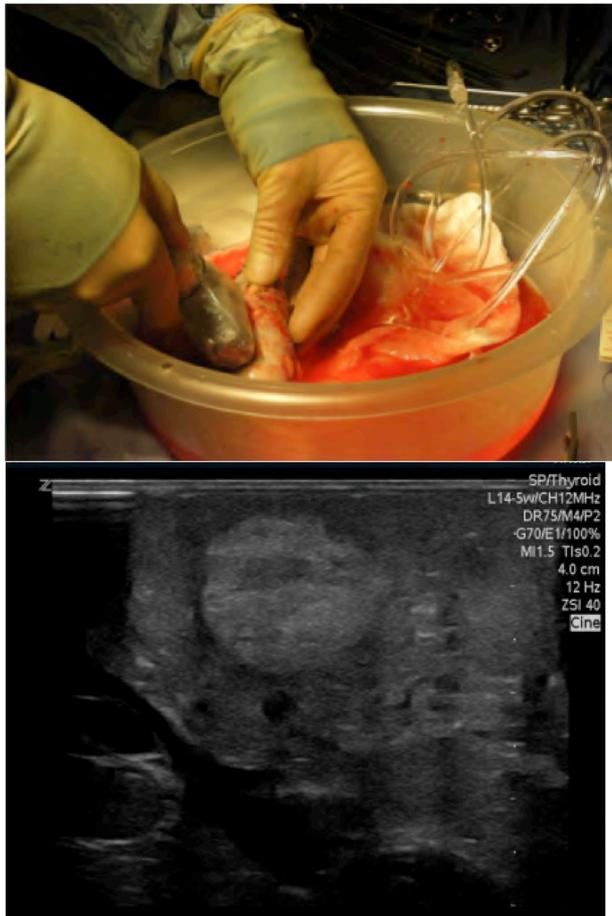


Figure 110 Back benching and USS to assess the completion of resection



Figure 111 Kidney following repair and at reperfusion

The mean age of the three recipients was 72 years. One was taken off the deceased donor transplant list while other two were high risk as well and were unlikely to receive a kidney allograft from the deceased donor pool. The histology was clear cell in two while chromophobe carcinoma in the other with clear resection margins. One of the recipients had early post-operative renal vein thrombosis requiring graft nephrectomy at day 10. While the other two were dialysis independent with 18 month serum creatinine of 133 and 205 $\mu\text{mol/L}$. One of the recipients had a urine leak, which was treated conservatively.

Both these patients were converted to sirolimus immunosuppression after the initial period on tacrolimus. The reason for not starting sirolimus earlier was the higher incidence of wound complications associated with it.

There has been no evidence of tumour recurrence in these patients on the strict follow up. Both these patients remain off dialysis with good renal function, while in the absence of these kidneys they would have most likely had been still on dialysis.

Chapter 7

FINAL DISCUSSION

7.1 OVERVIEW

Kidney transplantation still remains the best treatment for end stage renal failure; which is one of the most common chronic diseases. With the aging population and the increasing incidence of diabetes and obesity, the incidence of chronic renal failure is increasing as well, putting more pressure on the already stretched waiting lists for transplants. This situation is further compounded by the very success of transplantation, as it is becoming a much safer procedure meaning more elderly dialysis patients are now being considered for transplantation than ever before. This disparity has necessitated the need to look for new sources of organs for transplantation.

In the past decade, the gold standard treatment for small renal tumours has changed from radical to partial nephrectomy. This is due to comparable oncological outcomes(85) and better long term eGFRs(45)(46). Despite this, a large number of patients still undergo radical nephrectomy for T1a tumours. These kidneys have been used in small limited series for transplantation after ex-vivo resection of the tumours. One of the first series looking at these transplants with restored organs was from Cincinnati, where Penn(55) reported on their data retrospectively. A perspective series by Nicol *et al.* (56) had 31 cases of transplantation with kidneys with renal cell carcinomas after ex vivo resection. Since then there has been another 24 transplants again from Australia with no evidence of recurrence and good allograft function(307). Apart from these case series there have been multiple case reports with good results. So far there are close to 100 cases reported in the literature of such transplants from restored organs.

Despite the fact that there has been only one potential recurrence(56), the risk of malignancy is real as these patients are immunosuppressed. There is no single postoperative immunosuppressive strategy evident from literature. The immunosuppression was not modified in a majority, while CsA / tacrolimus was substituted with sirolimus after 3 months. This was not due to any direct experiments showing its efficacy in preventing recurrence or tumour destruction in such restored organs, but due to its previously known antiproliferative properties.

The main aim of our work was to test different immunosuppressive agents to answer this question directly and find the best agent for immunosuppression for these organs. There have been no direct experiments to look at the effects of different immunosuppressive agents on tumour growth in vivo in a transplant model.

7.2 SUMMARY OF FINDINGS

7.2.1 Overview

An experimental model of rat tumour transplantation was developed. One of the most important reasons for using the rodent model was the fact that there was already a large body of literature on transplantation, immunosuppression and tumour behaviour in these animals. These animals are big enough to consider kidney transplantation as part of future work of my project, yet small enough to

study enough numbers to give sufficiently powered analysis for the multiple groups.

The rat kidney tumour cells were transfected with luciferase to enable real time in vivo monitoring of the tumour growth. Once successful transfection was confirmed by in vitro and pilot experiments, two different strains of rats (Wistar and Lewis) were selected for per protocol experiment. Two different strains of rats behaved as well matched and poorly match transplants groups after injection of tumour cells. These differences were important as they helped us study the effects of matching and role of rejection on transplanted tumour cells.

Any success with transplanting restored organs in immunosuppressed hosts will rely very heavily on the choice of immunosuppression. We looked at the CNIs as the standard immunosuppression (majority of the patients these days are on Tacrolimus- a calcineurin inhibitor). This was compared directly with sirolimus and leflunomide as the agents having anti neoplastic properties as well as immunosuppression.

7.2.2 Role of matching and rejection

The immune system plays a central role in the success and failure of the transplant. One of the biggest barriers in human organ transplant was to suppress immune system fully to enable longevity of the transplanted organs. This was achieved years ago and immunosuppression has come a long way from the days of total body irradiation and cyclophosphamide, but still remains a rapidly evolving science. Where on one hand the immune system is a big hurdle for the successful transplantation, on the other hand it plays a pivotal role in

preventing tumour development and progression. In transplant recipients a fine balance between these two mechanisms is very important for the patients as a whole.

As already discussed, the organs are “primed” to trigger a strong immune response after the trauma of retrieval(141). On transplantation of these organs, the first line of attack is from the non-specific innate immune cells – NK cells, macrophages and complement system. The adaptive immune response is stronger, more important and lasting in damaging these organs after the initial attack from the innate system. These cells recognise both MHC class I and II molecules and both of them play a complementary role in transplant rejection(193–195,197). The more the differences between the donor and the recipient, the stronger the immune response and stronger the rejection. Hence generally we aim for less mismatching for better long-term outcomes.

Tumour cells are also immunogenic to a varying extent. For tumours to develop and flourish, one of the prerequisites is to evade the host immune response. If the tumour associated and tumour specific antigens are recognised by the innate and adaptive immune system, the tumour cells expressing them are more likely to be destroyed. Tumours have various pathways of “immunoediting” to prevent elimination by strong immune response(199,200). Renal cell carcinoma cells are classified as regressors as they are strongly immunogenic(202,203) and hence can be suitable targets by the immune system.

This interplay between the immune system, allograft and transplanted tumour cells was the basis of our hypothesis of using immune system to clear the

tumour load should a recurrence occurs in transplanted organs after ex vivo resection of tumours.

7.2.2.1 Rejection in controls with no immunosuppression

First of all, the tumour cells were injected into both Wistar and Lewis animals without any immunosuppression. Although such a scenario is not likely to be replicated in human transplant setting, these groups gave a lot of insight into the role of rejection. In the absence of any immunosuppression both the well-matched and poorly-matched animals rejected the tumour load. This rejection was very effective and strong when compared to the later experiments on the animals receiving immunosuppression. Again when both these groups were compared with each other the rejection was significantly stronger in the Lewis (poorly-matched) rats than the well-matched Wistars. These difference were in line with the hypothesis that more the mismatch on MHC loci, the stronger the immunological response. All the Wistar rats rejected all tumour cells with two weeks of study period while Lewis rats only took one week to achieve this.

7.2.2.2 Rejection in immunosuppression groups

Rejection was found to play an important role in the rats receiving CsA immunosuppression. When the immunosuppression was stopped after two weeks of continued treatment, the transplanted tumour cells were exposed to the host immune response. As opposed to the behaviour of these transplanted tumours under CsA immunosuppression, where these cells continued to flourish, when the treatment was withdrawn there was a sharp reduction in the bioluminescent signals in both the well-matched and poorly-matched groups.

Theoretically this was a direct proof of our hypothesis again. When the immunosuppression was withdrawn the host immune response was quite effective in causing the destruction of the foreign tumour cells.

These effects of rejection were not seen in the other immunosuppression groups. The reason was effective elimination of the tumour load to a great extent (or completely) by the anti-neoplastic properties of these newer agents (sirolimus and leflunomide).

7.2.2.3 *Role of matching in rejection of tumours*

The role of donor recipient matching was best seen in the CsA group again, both in the treatment continue and treatment withdrawal groups. In the treatment continue group under the influence of CsA, towards the end of the study period there was exponential growth of the transplanted tumour cells. This growth although very strong in both the strains, was significantly more in the Wistar animals towards the end of the study period. With no statistical differences in the initial tumour load this could be explained due to wider MHC differences in Lewis rats to the injected tumour cells.

Similarly, after the withdrawal of the immunosuppression, half of the Wistar animals were able to reject the tumour cells. This was in contrast to all the animals completely eliminating the tumour load in Lewis rats. With all the other experimental variables being same, this effect is due to poor matching.

Considering these finding, from a clinical point of view, when restored kidneys after ex vivo resection of tumour are transplanted we can potentially employ

hosts immune system in cases of any future recurrence of tumour. Unlike the normal situation of trying to look for the best possible MHC match between the donor and recipient, in these transplants we might want to transplant across less than ideal tissue match. Thus, if there were any tumour recurrence or de novo tumour development, then withdrawal of immunosuppression would be helpful in the elimination of any possible distant (lymph node) spread via recipient's immune system.

7.2.3 Role of immunosuppression

There have been significant advances in the past decade in the treatment of renal tumours. These days the proffered treatment for the T1a (<4cm) tumours is partial nephrectomy. This is because of similar oncological outcomes(308–310) and preservation of the renal function by preserving the nephron mass. In urological patients partial nephrectomy can be the treatment of choice but the stakes will be very high if the kidneys removed for small renal cell tumours were to be transplanted after ex vivo resection in hosts that are immunosuppressed. Hence, establishing the role of immunosuppression in a tumour transplant model was our other main objective.

With standard CsA immunosuppression We found that the tumour has continued to grow and such growth was very rapid towards the end of study period most probably due to neo-angiogenesis. This effect was seen in both well-matched and poorly matched groups. This is in line with the current available evidence on the effects of standard immunosuppression on cancer development. Not only does any immunosuppression takes out the protection provided by the immune system but also there is evidence that CsA can lead directly to cancer

development due to aberrant production of cytokines regulating tumour growth, metastasis and angiogenesis(210).

The effects of standard immunosuppression by CsA were directly compared with the newer antineoplastic medication. Sirolimus is licenced for use in renal transplantation and has antineoplastic effects. We tested a low and a high dose of this agent. With both doses, in both strains (Wistar and Lewis) there were complete elimination of cancer cells within the study period. The effects were slightly stronger with higher dose but still very equally effective in the low dose regimens.

Similar effects, although relatively less dramatic were seen with leflunomide. These animals showed continued reduction in tumour load and by the end of study period more than half of the animals had rejected their tumour load fully, in contrast to the CsA immunosuppression where there was an exponential increase in the tumour growth.

These results point favourably towards sirolimus as quite possibly the drug of choice after transplantation with these restored organs.

7.2.4 Flow cytometry

The flow cytometry was performed to look at the direct effect of tumour cells transplantation and role of different immunosuppressive agents at immune effectors level. We looked at non-specific effectors of immune system that have a direct role in tumour recognition and elimination-Natural killer (NK) cells(246–249). These cells recognise the tumour cells or virus infected cells which under express the MHC class I molecules (missing self)(250,251). The other cells

looked at were the effectors from the adaptive immune system. These cells are important both in a transplant situation and also have an important role in cases of tumour development. CD4⁺ helper T cells exert their role by producing number of cytokines that initiate graft rejection by stimulating more direct effectors(234–236). CD8⁺ T cells are normally found in small numbers in non-rejecting graft but their number increase in cases of acute rejection and also in the tumour microenvironment, highlighting their significance. Lastly, We looked at T_{reg} cells. There is a lot of literature pointing towards their role in tolerance and tumour behaviour.

Before analysing the response of immune system We looked at the control animals with no tumour or immunosuppression to establish a baseline. The analysis of both Wistar and Lewis strains revealed that there were marked differences in the distribution of T cells among them. The absolute number of CD4 cells was significantly higher in Wistar rats but the number of activated CD4 cells was more in the Lewis rats. Although, the reason for this is not very clear it might explain more powerful rejection by the Lewis rats. Similarly the T_{reg} cells were more abundant in Lewis rats. With CD4 cells having a more indirect role in activating the immune system and T_{reg} having a “controlling” effect of the immune response these finding could be important in tumour control. The rest of the cell populations (CD8, activated CD8 and NK cells) were similar between both these strains.

In the immunosuppressed animals, there were subtle differences in the cell populations among different agents as well. For Wistar rats, the number of CD4⁺ cells were the lowest in sirolimus low dose treatment arm as compared to the CsA group while the activated CD4 cells population was the highest in the

sirolimus low dose group. For these animals there were no apparent differences in the populations of NK and T_{reg} cells.

Activated CD8⁺ results were more consistent. All the animals had higher proportion of CD8 cells when compared to the controls. This will be due to their primary role in the acute rejection and tumour immunology. Similar results were seen when specifically the activated CD8⁺ cells' percentage of the total CD8⁺ population was studied. The highest number of CD8⁺ cells were seen in the animals treated with low dose of sirolimus. These cells are normally one of the first effective responses for destroying the tumour cells. The preservation of these cells in higher numbers in the sirolimus treated group may point towards the ability of sirolimus treated animals to destroy the tumour cells more effectively than the CsA immunosuppression. Interestingly, in the high dose sirolimus animals groups these cells had reduced numbers and perhaps this points towards the excessive immunosuppression, as a much higher immunosuppressive dose was used in this group.

For the Lewis animals, the T_{reg} population was the lowest in the sirolimus low dose group and activated CD8⁺ cells population was the highest. These results again, may explain the reason for the better response against the tumour cells in the sirolimus arm of animals.

The effects on CD8⁺ cells were most consistent in our flowcytometric data and also in line with the clinical observation of better tumour clearance in these animals.

Normally immunosuppression with sirolimus has shown an increase in the number of T_{reg} cells, but in our model the population of T_{reg} reduced along with

an increase in the number of CTLs. Although this result may seem against most of the literature but one important difference in our model was that it was not simply a transplant model but the transplanted cells were malignant as well. As shown by Hope *et al.* (303), when the SCC were excised in KTRs, the T_{reg} cells population also went down. The reason we might be seeing a decline in the number of T_{reg} cells might be due to the strong anti-neoplastic effects leading to reducing tumour load and thereby eliminating the stimulus to keep the numbers high of T_{reg} cells by the tumour microenvironment. Not a lot is known as to why the T_{reg} numbers reduce after tumour excision and further research is needed in this area.

7.3 Clinical implications

Chronic renal failure is one of the most common chronic diseases and for the majority of the patients renal transplant is the optimal treatment. Not only does it improve the quality of life but it also has survival benefits(15–17). Unfortunately, the prevalence of disease is such that we still do not have enough organs available for transplantation. This is despite recent increase in the numbers of available organs, via the use of more and more marginal organs, increasing DCD donation and a general increase in live donors. Thus there is still a need to look at new sources to increase the number of available organs for transplantation.

Use of kidneys for transplantation after *ex vivo* resection of small renal cell tumours may not seem practical but there have been a few cases where this type of transplantation has either happened deliberately or inadvertently. The first report was by Penn(55) in 1995 from Cincinnati where he looked at their

database for types of transplantation and reported 14 such transplants with no recurrence. Longer follow up by Buell *et al.* (54) also did not report any tumour recurrence in 2005. Subsequently, Nicol *et al.* reported a series where a total of 41 such organs were transplanted after *ex vivo* resection. Of these 10 were benign tumours and the rest were malignant. They also reported excellent results with only one recurrence 9 years after the original transplant. Notably this recurrence was away from the site of original resection. All these reports, although very encouraging, were done at a small and well controlled environment. There was no mention of any specific immunosuppressive strategies post operatively.

With around 5000 RCC diagnosed every year in the UK and the majority of them being T1a tumours, potentially there is a large source that can be tapped for precious organs. Although, partial nephrectomy is now considered the treatment of choice for these small cancers, there is clear evidence both nationally and also from our local regional urology referral centre (section 1.4.4.1) that a large proportion of patients with small renal tumours still undergo radical nephrectomy(53). This means these radically resected kidneys can potentially be transplanted in selected patients. The biggest hurdle of course is the fact that the transplant recipients are immunosuppressed thus high risk of developing recurrence with standard immunosuppression. Our study was aimed at looking at the role of using newer agents to better tailor the immunosuppression after such transplantations.

We have shown quite clearly in the animal model that sirolimus was best at destroying the transplanted tumour load. This effect was independent of the level of matching between the donor and the recipients. Sirolimus is already

licensed for use in transplant and there is evidence to support its use beyond the initial few months in special situations if not in all cases(121,225–227) in order to reduce CNI induced nephrotoxicity or due to sirolimus' role in preventing skin cancers. Thus, clinically if these kidneys are transplanted then the patients should be electively converted to sirolimus immunosuppression.

The other finding from our study was the role that host's immune system played in rejecting the tumour load once the immunosuppression was stopped. Clinically, this can be potentially important in rare situations of recurrence after transplantation of such organs. In cases of recurrence, the patients can undergo transplant nephrectomy and simply stopping the immunosuppression can be enough to stop any microscopic spread of the donor-derived tumour. For this to happen effectively, extrapolating from our experiments, the matching between the donors and recipients should be less than ideal. This will make any donor derived malignancy more immunogenic and thus prone to be acutely rejected.

With initial encouraging results from the literature and using sirolimus with a degree of mismatch between the donor and recipient, transplanting these organs can become a very real possibility at a larger scale.

Of course the most important factors to consider before any of these organs can be transplanted are patient selection and consenting.

Although it might seem a big ethical dilemma to offer an organ removed for cancer from one patient and then offer it for transplantation to another. But when we consider that some of the elderly and high risk patients on the waiting list have very little chance of getting an organ for transplantation and the risk of dying while waiting is quite high, the situation becomes quite clear. According to

many reports the risk of dying while being wait listed is between 6-10% per year(228–230). This is also dependent on the age of the patients listed, with worsening survival with an advancing age. Patients becoming unfit and inactive on the waiting list complicates the situation as the risk of dying increases even further. According to an analysis about 30% of deaths on waiting list occur in patients who had inactive status(231). Thus an elderly patient who has to wait for longer (blood group O and B) the risk of dying can be 50% over five years(232). This is far more than any risk associated with transplanting these restored organs. Thus elderly high-risk patients who would otherwise be unlikely to get a transplant from the normal waiting list will benefit the most from such restored organs.

It is of paramount importance to consent both the donors and recipients without any bias. The decision to do partial or radical nephrectomy should be purely clinical and must be made in discussion with the patient after discussing the pros and cons of each procedure. Potential of transplanting the organ after ex vivo resection should have no bearing on what type of treatment is offered to the RCC patients. If for some reason either clinically or due to patient's choice the radical nephrectomy is being performed only then should the "donors" be asked for their consent using these organs for transplantation.

For recipients as well the consenting process must be very vigorous and it must be ensured by the senior clinicians that the recipients fully understand the source of these organs, the small nephron mass to start with and the potential for recurrence and metastasis of tumour. Only when everyone is fully satisfied can these transplants go ahead.

After transplantation, perhaps these patients would need routine US monitoring of the allografts to pick up any early signs of recurrence, all in order to reduce any potential morbidity associated with a recurrence.

7.4 Limitations to the study

Perhaps the central limitation to our study would be the use of rat kidney tumour cells in a rodent transplant model. To extrapolate the results from animal studies onto human scenario, always has its inherent risks of not being a true representation of real life situation. But with a large body of work done on rats and mice both on immunology, transplantation and tumour behaviour this was the closest model to test our hypothesis.

With sirolimus immunosuppression in the treatment withdrawal arm, the effect on tumours was so strong that there were hardly any tumour cells left by the time we stopped immunosuppression to look at the role of rejection. Perhaps a higher initial tumour load would have meant that there were still significant number of cells present at the time of withdrawal to study the effects of rejection better. But this would mean that we would have to increase the tumour load across all arms of the study period for the results to be comparable. Plus there was no way of us predicting such a strong response with sirolimus before the start of experiments.

With flow cytometry, the results of the CsA may not be the true reflection of the actual lymphocyte count at the end of the study period. This is because of technical failure of the FACS machine at the beginning of the experiments and loss of a few samples from the Wistar animals with no immunosuppression. This also meant that by the time we were able to confidently do the flow

cytometric analysis, the CsA treated animals had already finished four weeks of the experiments for a few weeks. This time might have caused the reversion of the lymphocyte count to baseline, which would not be the true reflection of the exact influence of tumour on the lymphocyte count. The results of the sirolimus and leflunomide are more reliable from this respect.

The other possible limitation of the flow analysis could be the small number of animals in each arm. There were very clear differences in bioluminescent signals under different treatment conditions but only subtle changes in the various subpopulations of lymphocytes. Perhaps with bigger numbers these differences would be more pronounced. This however would have come at the cost of using significantly larger number of rats with no guarantee that there will be any clear answer.

Finally, the ideal place to look for the immune effectors would have been the tumour microenvironment or regional lymph nodes. The reason for choosing splenic tissue was to ensure consistency as not all the animals were left with any residual tumour and regional lymph nodes were only found in a very small proportion of the subjects. Hence to keep the analysis more objective and minimise variability We chose the splenic tissue as the source to study the immune system.

7.5 Future work

The main role of this project was to prove the concept of using acute rejection to eliminate the tumour load and the role of level of matching on the strength of

rejection. The other aim was to determine the best immunosuppressive medication in tumour transplant scenario.

The future work can take this project forward by injection the tumours into the renal subscapular regions followed by kidney transplantation. This will be followed by using Sirolimus as the agent with antineoplastic properties at low dose (the best immunosuppressive agent with most effective dose based on our work). The biggest benefit of this project will be not only to look at the effect of sirolimus on the tumour but it will also determine the acute rejection episodes in the renal parenchyma as it is a known weakness of sirolimus. This will mimic the transplantation situation more closely as opposed to the subcutaneous injection.

A further step proposed is to use nude or humanised mice with human kidney tumour cells. This will resemble the human transplant scenario even further.

APPENDICES

Renal Transplantation with Kidneys Affected by Tumours

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Review Article

Renal Transplantation with Kidneys Affected by Tumours

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Renal transplantation confers improvement in quality of life and survival when compared to patients on dialysis. There is a universal shortage of organs, and efforts have been made to overcome this shortage by exploring new sources. One such area is the use of kidneys containing small tumours after resection of the neoplasm. This paper looks at the current evidence in the literature and reviews the feasibility of utilizing such a source.

1. Introduction

Renal transplantation is the optimal mode of treatment for the patients with end stage renal failure. One of the major problems for transplantation is the discrepancy between the donor and recipient numbers with far less donor than recipients. As a consequence, patients with renal failure have to wait for a long time before they can be offered an allograft. This situation is especially worse in some countries like Japan, with small cadaver programme where the average waiting time is 16 years [1].

A significant number of patients die from the complications of chronic renal insufficiency on long-term dialysis before they get a transplant. This situation is more important especially in cases where chronic kidney disease has led to other medical problems and patient either die of the complications or become too unwell for a transplant [2]. Various measures including the use of marginal donors and use of kidneys from Maastricht category II non-heart-beating donors (NHBD) [2] have been utilized to increase the donor pool along with measures to improve and prolong graft function and survival. In addition, increasingly elderly donors are used, therefore increasing the risk of renal malignancy.

One potential area, first described by Penn [3] has been to transplant kidneys after ex vivo resection of small tumours.

This was a very radical idea, because firstly, there has been evidence of transmission of donor-derived malignancy into recipient from the very early days of transplantation [4]. Secondly as a general rule, organs from donors with malignancies have not been used for the same fear with some exceptions such as central nervous system tumours [5]. Surprisingly-outcomes of the patients described in Penn's series were not as bad as could have been anticipated.

The contemporary experience with partial nephrectomy and its success for the treatment of small renal cell cancers has led to extrapolation of similar technique for the management of allograft malignancy [6] albeit sporadically.

The purpose of this paper is to summarise the current evidence with regards to the utilization of kidneys with tumours for transplant and the use of conservative surgery for allografts where possible.

2. Material and Methods/Review Criteria

Pubmed, medline, EMBASE and CINHALL were linked searched for "renal tumour/tumor," "kidney tumour/tumor," "allograft tumour/tumor," "nephron sparing surgery," "partial nephrectomy," and "transplant" to identify potentially relevant articles. Articles concerning the use of kidneys after resection of renal tumour for transplant and partial

nephrectomy of allograft for renal tumours were selected. References of the selected article were also searched to identify further articles of interest.

3. Results

From the above-mentioned criteria of the literature search the following different types of case reports/case series were identified which are discussed separately.

3.1. Use of Kidneys after Resection of Tumours. Normal practice when confronted with a tumour of kidney on procurement is to return it to the donor and not use any other organs [7]. In cases of deceased donors it meant that the contralateral kidney cannot be used as well because of the concerns of micro metastasis and bilaterality of some of the renal cell carcinomas (RCC).

Penn [3], reviewing the Cincinnati transplant tumour registry (CTTR), described a total of 14 cases of ex vivo resection of small renal cell cancers detected incidentally followed by transplantation. Frozen section was employed, and where margins were clear, kidneys were used although it is not clear whether all of the tumour bearing kidneys underwent frozen section. Of the cadaveric donors, the contralateral kidneys, all of which appeared healthy, were transplanted as well. Apart from these cases of renal carcinomas, there was one case of oncocytoma within the kidney which was transplanted after resection. Of all the cases where the tumour was adequately resected before transplantation there was no recurrence in a followup ranging up to 210 months.

Buell et al. [7] presented 14 cases of transplantation after renal tumour resection from the same database as used by Penn. No recurrence has been noted up to a followup of 200 months. Median tumour size was 2.0 cm (range 0.5–4.0 cm) and all were of low histological grade. They have described two further cases since the initial data review with no recurrence and good graft function.

A similar case series from Australia [8] only included elderly recipients or those with significant co morbidities and high chance of death without transplantation. Furthermore the recipients had high levels of HLA mismatching with the donors and were selected on the basis that if there was a recurrence to occur, stopping immunosuppression may help in tumour lysis by recipient's immune response. 41 patients received kidneys after ex vivo resection of tumour of which 10 were reported as benign lesion on histopathology. One patient returned to dialysis after 30 months. 4 patients died of unrelated causes. There was only one recurrence noted 9 years after transplantation out of the remaining 30 patients. Notably this tumour recurrence was at a distance from the initial resection site, this therefore may not be a tumour recurrence but another primary within a "field" change renal tissue. The patient refused any further treatment, and the lesion has grown 0.2 cm in 18 months since diagnosis. In a followup study on these patients this group has recently published long-term outcomes which are significantly better

than wait-listed patients on dialysis and are comparable to the live unrelated transplants [9].

Mannami et al. [10] from Japan published a series of 42 "restored" kidneys from live donors. Eight donors with small renal cell carcinoma (<3.5 cm) underwent donor nephrectomy and ex vivo resection of the tumour followed by transplantation of the kidney. Five patients were alive, three with functioning grafts, two died with functioning grafts from unrelated causes, and one was lost to followup. No tumour recurrence has been noted in any of these patients. Another 8 patients had donor nephrectomies which had benign diseases of which 5 had partial resection and kidney used for transplantation. Three recipients are alive with functioning grafts, while four have gone back to dialysis (after 3,18,51,73 months). One recipient died of unrelated pathology.

There have also been 6 case reports [11–16] of live related kidney donation when a tumour was detected incidentally in or ex vivo and the kidney was transplanted after resection. No recurrence has been noted in any of these cases with a followup of up to more than 10 years.

3.2. Partial Nephrectomy for Tumours Diagnosed after Transplantation. Renal cell carcinoma represents around 4.6% of all the tumours in allograft recipients with only 10% of these occurring in the allograft itself [3].

The other main subgroup is when a tumour was detected after transplant. Again the standard practice here has been to perform transplant nephrectomy [17] with the patient invariably returning to dialysis and normally being put on a waiting list for another transplant if feasible.

Until now, more than 50 cases of allograft renal cell tumours have been described in the literature of which at least 35 cases have had nephron sparing surgery (NSS) for their allograft tumour [6, 11, 18–36]. Tumour sizes have range, from 0.5 to 4.0 cm although there have been two case reports of larger (6–8 cm) tumours all being successfully treated with NSS [18, 19]. Postoperative followup is from one month to more than 10 years with one recurrence 5 years after NSS in renal allograft [37]. This was in a 74 year old recipient five years after initial transplant. A 2.4 cm RCC was incidentally detected without any evidence of distant metastasis. It was treated with radical nephrectomy and patient has been disease free on hemodialysis.

3.3. Contralateral Transplanted Kidney with a Renal Tumour in Cadaveric Donor. These kidneys again are normally not used as RCC can be bilateral especially the papillary subtype [38]. Penn [3] has described 14 cases in which the contralateral kidney was transplanted from patients with renal tumour. One patient had recurrence in the allograft which was removed for rejection. This patient died 75 months after transplantation from a de novo cancer of one of his own kidneys. The remaining patients did not have any recurrence with a followup ranging from 0.5 to 153 months.

Nicol et al. [8] described 2 similar cases with no recurrence. Barrou et al. [38] has described a case of two allograft recipients from a single donor with tubulopapillary

tumour (17 mm) in the right kidney; only the left kidney was utilized for transplantation. Shortly after transplantation, the recipient underwent an ultrasound (US) examination of the allograft which did not reveal any tumour. 3 months later a biopsy was done for rejection which revealed a poorly differentiated tumour and the patient underwent radical allograft nephrectomy. No additional chemotherapy was given apart from discontinuation of immunosuppression (prednisolone and azathioprine). Lymph nodes that had been noted to be enlarged on CT scan disappeared two month after nephrectomy. The patient underwent re transplantation two years later and was disease free and dialysis independent at 3 year followup. Another patient received the heart transplant from the same donor but died from bony metastasis from the renal cell carcinoma.

3.4. Accidental Transplantation. In at least 4 cases [3, 13, 20, 39] there have been accidental transplantation of RCC mistaken as a benign pathology on procurement. Partial nephrectomy/enucleation in all these cases was performed before transplantation with adequate resection margins. Routine histopathology revealed the resected tumour to be malignant. All recipients retained the allograft because of complete excision of the tumour and were kept under close follow-up with no recurrence so far.

The cases where there have been transplantation of tumour, either partially resected or unrecognized at the time of transplant have resulted in disastrous outcomes [3, 38].

3.5. Miscellaneous. Manammi et al. [10] reported a series of 8 patients who underwent nephrectomy for a distal ureteric transitional cell carcinoma (TCC). One patient had a recurrence of TCC after 15 months and was offered graft nephrectomy but opted for partial resection of ureteric tumour to prevent returning to dialysis. He died three years after partial resection from a squamous cell carcinoma of lung with liver metastasis. His TCC also had squamous metaplasia and a DNA study to determine exact origin of primary tumour could not be established because of inadequate tissue samples. The remaining patients were either alive with functioning grafts or died of unrelated causes.

3.6. Opinion of Patients and Transplant Specialists. Transplantation of kidneys with cancers is a novel idea not only among patients but also among the transplant community. To be able to exploit this potential donor pool it is of utmost importance that both the health care specialists; transplants surgeons and nephrologists and the patients both donors and recipients are comfortable with the idea of using such kidneys. To determine this, structured questionnaires were sent to focus group of patients on the North East renal transplant waiting list, postnephrectomy patients for small renal cancer, nephrologists and transplant surgeons in the UK [40].

Results are shown in Table 1 and have a generally high response rate. Those respondents that had lost their kidney, removed for tumour, had the highest consent rate and

TABLE 1

Respondents	Response rate	Support use of kidneys
Potential recipients on waiting list	97% (113/116)	59% (67/113)
Previous nephrectomy (potential donors)	100% (15/15)	93% (14/15)
Nephrologists	58% (94/161)	78% (73/94)
Transplant surgeons	66% (43/65)	72% (31/43)

patients potentially receiving such kidney the lowest. The transplant surgeon and nephrologists had views somewhere in between.

This survey was done in UK from where there have been no case reports of using organs after removal of tumour and but still the response was largely favourable. Given that since this survey there has been an increase in total number of such organs being utilized, one can extrapolate that current belief may be more favourable.

3.7. Role of Immunosuppression. One of the worries about transplantation of tumour affected kidneys is the potential of tumour recurrence and growth in state of potential immune inattention due the immunosuppressive therapy. Renal cell carcinoma is known to be an immunogenic tumour [21] but in the presence of immunosuppression, if there was any transplantation of tumour cells in the host, then the potential of continued growth will be higher in a host with a compromised immune system. Furthermore, immunosuppression in itself has been known to increase the incidence of de novo malignancy [41, 42]. Because of these concerns, an immunosuppressive agent with no potential to increase de novo malignancy and better still to have antitumour activities would probably be ideal. Rapamycin has shown some promise as being a protective agent against RCC progression [21, 43, 44].

4. Discussion

Incidence of RCC has increased in Western countries in the last few years owing to the widespread use of US and CT scanning [45, 46]. Most RCC are now picked up at an early stage on investigations done for other reasons [47]. Furthermore the incidence of RCC in allografts will continue to increase as older people donate organs and graft survival is improved by better immunosuppression. Longitudinal studies have shown that many small tumours have a slow growth pattern with low metastatic spread in tumours of <3 cm [48, 49]. Autopsy studies have shown that RCC are present in 1%–20% of patients dying from unrelated cases, meaning that many of the tumours will not prove to be clinically significant in the course of patient's life [50, 51].

The gold standard treatment of resectable renal cell carcinoma has been radical nephrectomy. Recent evidence has changed this practice dramatically as survival after radical nephrectomy (RN) and partial nephrectomy (PN) has shown to be comparable [52]. Favourable outcomes

have been observed after NSS for <4 cm RCCs and RN has been described as “surgical overkill” [53] for these tumours. Furthermore, local recurrence after NSS has been reported to be <5% with recurrences mostly associated with large and multifocal tumours.

A significant risk of dying in patients on dialysis particularly in older patient has been one of the driving forces to increase the number of kidney donors. Renal transplantation seems to confer a substantial survival advantage over dialysis in patients with end-stage renal failure [2]. A significant number of patient accepted for dialysis are older patients, who have a mortality risk of 25%. With longer waiting times for a transplant, it is inevitable that many of the patients will die before they can receive a transplant which would have improved their quality of life and longevity [2]. Furthermore 16% to 23% of suspicious lesion resected from kidneys are either benign or of low malignant potential [53–55] and not using these kidneys with small tumours after partial nephrectomy for transplantation seems wastage of precious organs when one considers the benefits of transplantation over dialysis.

A suspicious lesion found at multiorgan retrieval should have an excision biopsy and histological confirmation of clear margins before any of the organs can be transplanted. A malignant lesion in the kidney when unrecognized and transplanted continues to grow under the immunosuppression carries high risk of metastasis and can result in fatal outcome. If the biopsy confirms clear margins with favourable histology then these organs could be used for transplantation as risk of recurrence is very low. Situation is more complex when it comes to using restored organs from live (related/unrelated) renal cell carcinoma patients. Major difference being that these are live cancer patients first and therefore must never be treated primarily as potential organ donors to prevent any bias in treating their primary problem which may lead to provision of less than optimal treatment and ultimately harm to these patients [8, ed]. This is shown by Takahara et al. [56] in their review of Mannami et al. series concerning ureteric carcinoma patient, where adherence to standard practice for treating these tumours was not practiced with disastrous consequences. With changing trends, radical nephrectomy is now regarded as an alternate standard of care to partial nephrectomy for T1a tumours when partial nephrectomy is not technically feasible. This is due to the comparable oncological outcomes after partial nephrectomy and evidence that radical nephrectomy is an independent predictor of low GFR. A positive outcome for a recipient can never justify harm to a live donor; on the contrary, for a transplant with a live donor to be regarded as a success means that both the recipient and the donor have done well [57]. Live related donors in Nicol et al. series were given the options of observation, radical or partial nephrectomy without any mention of the possibility of use of organs for transplantation. Only after the patients had decided to opt for radical nephrectomy possibility of domino donation was discussed. This approach has the benefit of making sure that patients make their own decisions without any pressure from clinicians. Other important factor is to make sure that beliefs of the clinician do not affect patient’s treatment choices.

Importance of detailed informed consenting cannot be over emphasised for the recipients of such restored organs. All the relevant information especially of the origin of the organ and potential of recurrence and associated risk must be discussed fully and patients understanding checked.

Routine followup of the patients with annual US have been suggested to make sure any recurrence is diagnosed as early as possible. Tumours have been detected at early stage with better outcomes because of regular followups. If one kidney is found to have a tumour it is important that the other kidney is closely followed up. It is easier in the live donor setting when the donor can be carefully followed up but in cadaveric donation there has to be a central database for tracking the contralateral kidney [22] which might be transplanted into a recipient in a different unit.

Immunosuppression is essential after transplant and unfortunately this has been associated with the higher incidence of cancers in recipients as opposed to the general population [42]. Certain newer immunosuppressive agents have anti tumour [58] activity and their use can, in theory not only reduce the chances of recurrence but they can also be used to treat patient should a recurrence occur.

Furthermore Human Tissue Act 2004 [59] that covers the use of organs for transplant in the UK allows anyone to be a donor including live related and unrelated (altruistic donor) provided there is adequate consenting. This means that donation can also occur from patients suffering from small renal cell carcinoma who have radical nephrectomy as primary treatment provided measures are taken to ensure that these patients are treated appropriately in the first place and both donor and recipients had given informed consent.

5. Conclusion

To increase the donor pool new sources have to be exploited. Use of kidneys after tumour resection seems a feasible source. There are several important issues in using such marginal and potentially dangerous organs; patients should have complete understanding of the implications of the type of organ they are donating and receiving, good surgical technique and rigorous pathological testing of the resected tissue to make sure there is no tumour left behind, regular followup with adequate investigations, and a reliable organ tracking system to investigate the recipient of contralateral organ should one organ develop a recurrence. On top of this, transplant surgeons and nephrologists should be comfortable in using such organs. Usage of such organs is still in its infancy, and for a much wider acceptance of this source to occur, there is need for more research. One interesting area will be to explore the new immunosuppressive agents with antiproliferative properties on such recipients with the potential to reduce recurrence rate or better still to prevent it altogether while either replacing standard immunosuppressive agents or reducing their required dose thereby reducing side effects.

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The developing concept of using elective benign and malignant kidneys for renal transplantation

COMMENTS

THE DEVELOPING CONCEPT OF USING ELECTIVE BENIGN AND MALIGNANT KIDNEYS FOR RENAL TRANSPLANTATION

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Renal transplantation confers improvement in quality of life and survival when compared with patients on dialysis. There is a universal shortage of organs, and efforts have been made to overcome this shortage by exploring new sources. One such area is the use of kidneys with benign pathologies or containing small tumours after resection of the neoplasm. Our comment investigates the possibility of using either of these two potential valuable sources.

There is still a large gap between the number of renal grafts available for transplantation and the number of patients on waiting lists. A significant number of patients awaiting renal transplantation either die or become too unwell from complications of chronic renal insufficiency on long-term dialysis before they can get a transplant [1]. Over the years many different sources of renal allografts have been exploited with variable success but the fundamental problem of low donor numbers persists. One potential source of renal allografts could be transplantation with kidneys by urologist for benign and malignant pathologies at the time of nephrectomy.

Recent evidence suggests that small renal cell tumours (T1a) could be safely treated by nephron-sparing surgery (NSS), such as partial nephrectomy (PN), with comparable outcomes to radical nephrectomy (RN) [2]. Current guidelines recommend a PN for small renal tumours, but the procedure itself has a steep learning curve and many centres continue to perform RN for small RCCs [3]. Despite the recommendation from European Association of Urology guidelines [4], many patients with localised RCC still undergo RN

[5]. In view of the above, there is a potential to use these organs as renal grafts for transplantation once the tumour has been resected *ex vivo* and confirmation with frozen section of clear margins followed by transplantation in selected groups of patients.

In 1995 Israel Penn [6] published the results from Cincinnati transplant tumour registry with 14 patients receiving restored organs after *ex vivo* resection of small tumours with no recurrence in a follow up period ranging up to 210 months. A further series by Buell *et al.* [7], again from Cincinnati in 2004 reported similar results. Nicol *et al.* [8], from Brisbane published their experience over an 11-year period from 1996–2007, where 31 patients received RCC affected kidneys after tumour resection with only one possible recurrence 9 years after transplantation (away from the location of primary tumour resection). There has been another case series from Japan [9] with eight such patients and at least seven published case reports [10]. The possibilities of using benign kidneys continues to be enormous. At our institution we have recently transplanted a kidney from a patient with a ureteric injury into a recipient successfully, with the donor being aware of the possibility of auto-transplantation.

Despite these published cases, before such a source could be widely popularised there are a few important practical issues that will need addressing. Patients with small RCCs must be informed of the option of NSS and RN clearly with associated risks involved with both techniques. Once the patients have made the decision to undergo RN, only then the potential of transplantation should

be discussed. This approach avoids the potential bias towards RN and use of kidney for transplantation from clinicians and this was the approach employed by Nicol *et al.* [8] in their series. The consenting of the potential recipients must be very thorough as well to ensure that they understand the origin of these kidneys and the potential risks involved. There has to be general acceptance of the idea especially among the potential recipients before the wide spread use of such organs could occur.

In our centre we have completed a regional questionnaire to potential recipients (patients on transplant waiting list) in North East of England, to see if they will accept such organs and 59% responded positively [10]. With current guidelines recommending NSS and new evidence suggesting better long-term GFR with NSS, the biggest criticism of this approach is subjecting patients to RN for small RCCs. But despite clear recommendations and current evidence favouring NSS, still a large proportion of patients undergo RN for various reasons. In an ideal world the number of RN should be very small meaning that exploring such a new source will not be feasible as the number of potential organs generated will be very small. But the reality is quite the opposite. Data from the USA and UK suggest that more patients undergo RN than PN for these small tumours. Whether it is due to patients' wishes, technically difficult cases, lack of facilities or expertise, is irrelevant as these precious organs are potentially wasted when they could have been transplanted. The other big question is who should receive these potentially 'dangerous' organs. Most of the modern practice of medicine is a balance between the risks and benefits to the patients and this area should not be any different. These organs should be transplanted to patients who are at the highest risk of dying on long-term dialysis without a renal transplant. After transplantation there should be strict follow-up of these patients to detect any new growth at a very early stage.

Under the Human Tissue Act 2004 anyone could be a donor (live related or unrelated), so these kidneys after resection of tumour can be used for transplantation under the umbrella of domino donation. With ≈7000 new cases of RCCs diagnosed each year in the UK, more than half of which are T1a and with still most of these undergoing RN,

COMMENTS

there is a huge potential to increase the organ-donor pool with these marginal organs, if the ethical issues involved could be addressed appropriately.

CONFLICT OF INTEREST

None declared.

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Abbreviations: NSS, nephron-sparing surgery; (P)(R)N, (partial) (radical) nephrectomy.

Therapeutic kidney donation: A potential source of precious organs

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Therapeutic kidney donation: A potential source of precious organs

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Abstract The number of patients awaiting a renal transplant considerably exceeds the number of organ grafts available. A successful kidney transplant is the most clinically and cost effective treatment for patients with end stage renal disease. A proportion of patients currently die awaiting a renal transplant as their continues to be a global deficiency of renal allografts. Efforts continue to be made in order to improve the current situation of waiting lists and there is now an urgent clinical need to explore potential new sources to increase the number of renal allografts for transplantation. We describe a successful case of a renal transplant with a kidney removed for benign aetiology and transplanted into a patient who was on the cadaveric renal transplant waiting list for 6 years. We predict that this of 'Altruistic domino donation' concept could potentially reduce the waiting list for cadaveric renal transplantation and more importantly become a valuable source for new renal allografts.

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Case report

A 59-year-old male underwent a resection of a large retroperitoneal 'tumour' which involved the left colon for a pre-operatively diagnosed sarcoma. Final histology confirmed "Benign Fibromatosis"

which is associated with an optimistic prognosis. The patients subsequently underwent a reversal of colostomy and intraoperatively sustained an iatrogenic injury to the left mid ureteric injury which presented on the 10th post-operative day as loin pain and sepsis. An initial nephrostomy and drainage of urinoma was performed followed by an unsuccessful attempt at antergrade and retero-grade stent insertion. A defect measuring >4 cm was identified in the mid-ureter. A Renogram confirmed a differential function of 41% on left side and the

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patients glomerular filtration rate (GFR) was calculated at 68 ml/min/1.73 m².

The patient was counselled with the options of either a ureteric repair (Uretero-ureterostomy, Transuretero-ureterostomy plus Boari-flap and reimplantation) or auto-transplantation (AT). The risks of all options were discussed with patient including the potential of an on table nephrectomy and unsuccessful repair. The patient was not keen to pursue reconstructive surgery and opted for a nephrectomy. We then discussed the possibility of 'Altruistic domino donation' with the patient. Permission for using the left kidney for potential transplantation was approved by UK Transplant (UKT) under the category of "domino donation" according to the Human Tissue Act [1].

A 70-year-old recipient on thrice weekly haemodialysis for 6-years was identified. The patient had ESRF secondary to hypertension and had concomitant ischemic-heart-disease. His mismatch was 1:1:1. This recipient had waited for 6 years for a cadaveric renal transplant and had no prospect of a live donor transplant. It would have been unlikely for him to receive a cadaveric transplant during his lifetime based on his performance status and co-morbidities making him completely dialysis dependent for life.

Both donor and recipient were scheduled for surgery on the same day and did not meet each other. An open nephrectomy was performed in view of the patient's previous extensive open surgery and adhesions via a loin incision and the kidney flushed with cold preservation solution. The kidney was prepared for transplantation after a thorough examination by the transplant team (Figs. 1 and 2).

The donor organ was implanted into the right iliac fossa and the ureter was successfully anastomosed into the bladder over a stent. Post-operative recovery was unremarkable for both the donor and the recipient. Donor was discharged on 2nd post-operative day with creatinine of 1.25 mg/dl. Recipient had immediate graft function and creatinine on discharge was 1.53 mg/dl. Donor and recipient have subsequently communicated anonymously with each other by letter and are doing well at 8-month follow-up.

Discussion

Kidneys removed for small renal tumours have been successfully transplanted after ex vivo tumour resection with extremely low recurrence rates



Figure 1 Left kidney after back benching. Artery, vein and ureter demonstrated.

[2,3,7]. Kidneys removed for benign aetiologies are however not associated with any risk of tumour transmission. Transplantation with renal grafts from benign aetiologies can be potentially linked to the established concept of altruistic donation. With the "donor" being treated for their primary pathology with nephrectomy, such donations are regarded as Altruistic domino donation in the United Kingdom (UK) under Human Tissue Act (HTA) [4].

In current clinical practice the commonest indications for simple nephrectomy (SN) include intractable loin pain, renal artery aneurysm [5], ureteric injury [6] and refractory nephrotic



Figure 2 Implantation in right iliac fossa. The ureter being anastomosed to bladder over a stent.

syndrome [5]. Auto transplantation (AT) can be an option for these patients (excluding nephrotic syndrome) but on occasions this may not be the appropriate treatment in clinical situations such as extensive previous surgery, increasing risk of unsuccessful ureteric repair leading to post-operative complications, medical co-morbidities or patient choice.

Counselling of patients undergoing SN and recipients of these kidneys is extremely important. The recipient should be fully aware of the origin and quality of the organ. This technique has the advantage of favourable ischaemic times. Using this approach we successfully used the renal graft from our donor for transplantation. In normal clinical circumstances the graft would have ended up as a nephrectomy specimen and the donor would have been continued to be on thrice weekly haemodialysis. Our approach was psychologically very rewarding for the donor who viewed this as a positive outcome from a complex surgical scenario and the recipient who is now dialysis free.

Furthermore these organs should be transplanted locally as they may pose specific technical problems (short vessels, ureter, etc.) and transplanting them into local recipients may minimise the discard rates and potential risk of complications, a concept endorsed by NHS Blood and Transplant (NHSBT). We aim to highlight the importance of this new concept to urologists in the UK and worldwide as this could be a

very important source of precious renal allografts worldwide.

Conflicts of interest

None declared.

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Behaviour of transplanted tumours and role of matching in rejection

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Behaviour of transplanted tumours and role of matching in rejection

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ABSTRACT

Background: Tumour transfer/development is one of the more serious risks associated with transplant behaviour of a tumour can be unpredictable in immunosuppressed recipients. We report a high method to monitor tumour behaviour in real time in a rodent tumour transplant model. This paper al the effect of MHC matching on tumour growth among control and immunosuppressed hosts.

Methods: Luciferase expressing Wistar rat kidney tumour cells were transplanted into either Wist recipients which mimic a well and poorly matched combination to assess the effects of MHC m transplanted tumour cells. Experimental groups included controls with no immunosuppression a immunosuppressed with cyclosporine. The latter group was further divided into a continuous treat which received four weeks of immunosuppression and a treatment withdrawal group where immu sion was stopped after two weeks to assess the effects of rejection on tumour growth.

Results: All the tumour cells were rejected in the control animals that received no immunosuppress 2 weeks among well-matched combination and within one week in the poorly matched co (p 0.001). The transplanted tumour cells continued to grow in both well-matched and poorly matc who were treated with cyclosporine, but growth was significantly faster in the well-matched co (p 0.033). After treatment withdrawal the tumour cells were rejected in all the animals of the poor group compared to 50% in well matched animals within the four-week study period (p 0.039).

Conclusion: In the absence of immunosuppression the hosts reject the transplanted tumour cells, an tumour response is stronger when there is a greater mismatch in MHC with the recipient. In the f cyclosporine immunosuppression the tumour continues to grow, however, after withdrawal of the in pression, tumour clearance is quicker in the poorly matched background. This data supports t expansion of the donor pool by using kidneys after ex vivo resection of small renal tumours and that t should be transplanted into a less well-matched HLA recipient. We hypothesise that should a tumour occur a poorly matched recipient could clear the tumour through withdrawal of immunosuppressio

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1. Background

Transplantation has revolutionised the treatment of patients with renal failure. It not only improves quality of life but also has a significant survival advantage compared with dialysis [1]. Although graft survival and the absolute number of allografts have increased over the past couple of decades, there remains a large gap between the number of organs available and potential recipients [2]. Over the years new sources of organs have been explored but the problem persists and there is still a need to increase donor numbers.

There is a large body of evidence that patients with small carcinomas (RCC) can be treated with nephron sparing surg with comparable outcomes to the previous gold standard of r phrectomy [3,4]. Consequently for a patient electing to have th kidney removed for a small RCC there is a potential for the r the tumour and then allotransplantation of the remaining ki approach has been utilised by a few groups with good resu One of the most important and perhaps potentially dangerc ences between a urology patient that has undergone NSS fi RCC and a potential allograft recipient of an NSS kidney is t plant recipients are on lifelong immunosuppression. Immun sive agents inhibit the natural checks on cancer cells by the system. It is not known how tumour cells will behave in a HI patible immunosuppressed host, if there is any inadvertent tra tion along with such restored kidneys.

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In the absence of any immunosuppression the allograft is rejected. Theoretically any tumour cells transplanted along with the allograft should be rejected as they both originate from the same donor. However, cancerous cells have the ability to make themselves less immunogenic thereby evading the donor immune system in the first instance and it is not clear how they will behave in a new host [10].

2. Objectives

The aim of this study was to establish a rodent tumour transplant model and study the effects of immunosuppression on tumour growth. The other main aim was to study the effects of acute rejection on tumour cells in a transplantation setting.

3. Study design and methods

3.1. Cell culture

The tumour cell line, BP36b was acquired from Riken Bio Resource Centre (BRC) Cell Bank© Japan. This is a rat kidney tumour cell line derived from male Wistar rats that received N-ethyl-N-hydroxyethylnitrosamine (NHEN) in drinking water to induce tumour growth. The cell line is stable and maintained its characteristics after 100 passages over a 3 year period [11]. Cells were grown in RPMI 1640 supplemented with glutamine and antibiotics (penicillin 10,000 units ml⁻¹, streptomycin 10 mg ml⁻¹, gentamicin 50 µg ml⁻¹ and amphotericin B 25 µg ml⁻¹). The doubling time of the cell line was consistent with the reported time in the literature (17 h) [11].

3.2. Transfection

For real time in vivo imaging of the tumour cells, the cell line was transfected with a commercial lentiviral construct that is stably integrated and constitutively expresses the enzyme luciferase for bioluminescence and green fluorescent protein (GFP) for fluorescence [12,13]. Puromycin (10 µg ml⁻¹) was used for selection of stable transfectants.

Puromycin supplemented media was replaced every 48–72 h to select for single colonies of stable transfectants. Transfectants were initially assessed by the expression of GFP by fluorescence microscopy. Bioluminescence was determined initially by a luminometer and then by direct visualisation using the IVIS® spectrum imaging system (Caliper Inc.) (Fig. 1).

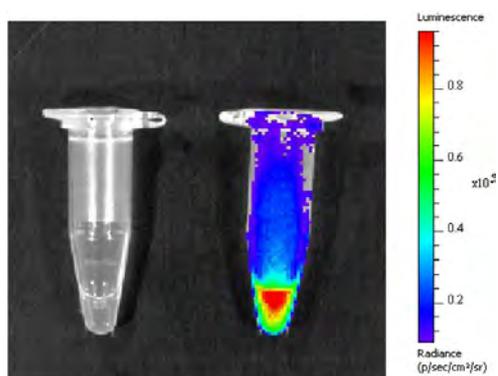


Fig. 1. IVIS spectrum image of non-transfected cells (left) and transfected cells (right). The system produces a heat map image that can be compared to the scale seen to the right of the image and the intensity of the luminescence calculated (P/s/cm²/sr).

3.3. Tumour transplantation

Animals were injected with a fixed number of cells (1.8×10^7) into the right flank under Isoflurane anaesthesia after shaving the fur. The animals were anaesthetised in the induction chamber of the IVIS spectrum imaging system and then transferred into the dark chamber where they were scanned for varying lengths of time (60–300 s). Animals were kept anaesthetised in the imaging chamber to enable long exposure times required to detect even very faint bioluminescent signals. Luciferin was injected intraperitoneally at the dose of 150 mg/kg 10–15 min before scanning to allow circulatory distribution throughout the animal before detection. Timing of luciferin injection was calculated by plotting the kinetic curve prior to the experiments. To compensate for variations in luciferin distribution, 2–3 images were taken of each animal at different time points and the only image with the strongest signal used for further analysis. Regions of interest (ROIs) were the areas of cell injection and any other areas with positive signals. The background luminescence was calculated for each animal and signal intensity was calculated by subtracting this from the ROI value to get the accurate value of signals from the transplanted tumour cells (Fig. 2).

3.4. Experimental groups

To study the effects of matching on transplanted tumour growth two different strains of rats, Wistar and Lewis were used. Since tumour cells were of Wistar origin, when injected into Wistar rats (outbred) [14] this combination served as a well-matched group as both the animals were of the same strain. Despite the similarities between the tumour cell line and the recipients, these animals were not true syngeneic to the tumour cells due to being outbred [15]. The other group was of inbred Lewis animals that served as a poorly matched group due to transplantation across the strain, leading to more marked immunological differences.

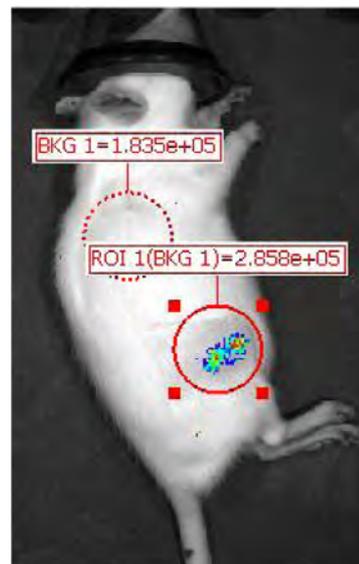


Fig. 2. Day 0 IVIS spectrum image of Wistar rat after injection of transfected tumour cells into the right flank. Imaging was performed 15 min after intra-peritoneal injection of luciferin for maximum signal intensity. Region of interest (ROI, solid red circle) is the area of positive signals from the injection site while the background bioluminescence (dotted red circle) is calculated for each image to calculate bioluminescence.

To keep the variables to a minimum, only male Wistar or Lewis rats were used for experiments as follows:

Controls; not receiving any immunosuppression and cyclosporine (Cyc) group; receiving 25 mg/kg of Cyc daily via oral gavage. The cyclosporine group was further divided into *treatment continue group* receiving four weeks of continuous immunosuppression and the *treatment withdrawal group* where immunosuppression was stopped after 2 weeks to study the effects of rejection on the transplanted tumour cells. All the animals were kept in a clean air conditioned rodent area with 12 hour dark/light cycle and were fed standard rodent blocks and with free access to tap water. Animals were weighed weekly to adjust the doses of cyclosporine and luciferin.

3.5. Statistical analysis

To detect a five-fold difference in tumour size with a standard deviation of 0.2 with a 90% certainty and alpha of 0.05 we calculated a sample size of 6 rats per group. Statistical analysis was performed with the PASW 18.0.0 (IBM Inc. 2009) and GraphPad Prism (Version 5.04 GraphPad Inc.) softwares. The normality of the data was tested prior to performing either ANOVA or the non-parametric Mann-Whitney tests accordingly.

4. Results

4.1. Controls

The kinetics of tumour rejection was first studied in the absence of immunosuppression. With *well-matched animals* there were still good signals at week one, but all the animals subsequently rejected the tumour cells and lost signal, even after long exposure, at week two. All *poorly matched animals* rejected the tumour cells and lost signal within the first week ($p < 0.001$ at week 1) (Fig. 3).

4.2. Cyclosporine treatment

The effect of Cyclosporine on the rate of rejection was then studied in well-matched and poorly matched groups. These groups were further sub-divided into the animals receiving the immunosuppression for a full four weeks and the animals receiving the treatment for 2 weeks followed by treatment withdrawal. The rats in the treatment

withdrawal group were scanned as normal for the study period of four weeks before euthanasia.

The tumour continued to grow in both the well and poorly matched animals when immunosuppressive treatment was continuous. There was no significant difference in the growth of the transplanted tumour cells in the initial three weeks of the study, however growth was significantly faster in the well matched Wistar animals compared to the poorly matched Lewis rats ($p 0.033$) by week 4 (Fig. 4).

In 4 of 8 animals of the well-matched Wistar rats after treatment withdrawal tumour signal could still be detected at 2 weeks post-treatment withdrawal. However, in the poorly matched Lewis animals the whole group had rejected the tumour by the end of the study period (two weeks post-treatment withdrawal) ($p 0.039$) (Figs. 5 & 6).

5. Discussion

Better immunosuppressive therapies have resulted in long allograft survival with reduced side effects. The risk of cancer development, however, even from standard allografts without any obvious donor malignancy still persists. The initial results of function and recurrence rates from transplanting restored organs after ex vivo resection of tumour remain favourable [16] from the limited data available so far. However, there remain some serious questions regarding the safety of such an approach in immunocompromised hosts. The behaviour of a tumour in a transplant setting can be unpredictable since all patients will be immunocompromised to some degree in order to prevent graft rejection. Consequently any study, which investigates the effect of tumour cell growth in a transplant model to investigate whether the immunosuppressive treatment, or MHC mismatch has any bearing on tumour growth is worthwhile.

The stability of the tumour cells (BP36B) used for our study has been demonstrated by the observation that the cells retained their properties after multiple passages [11]. The cells being of Wistar origin made it possible for us to study the effects of tissue matching on the tumour behaviour by using outbred Wistar and inbred Lewis strains for implantation. When these cells were injected in the Lewis animals, they behaved as a poorly matched group as the transplantation was between two different strains with marked immunological differences. When these cells were injected into the Wistar rats, they behaved as relatively well-matched combination when compared to the Lewis animals but strictly speaking they could not be classified as syngeneic transplantation. This is because of inter-individual variations in RT1 (rat major histocompatibility complex) among any outbred strain of the rats [15,

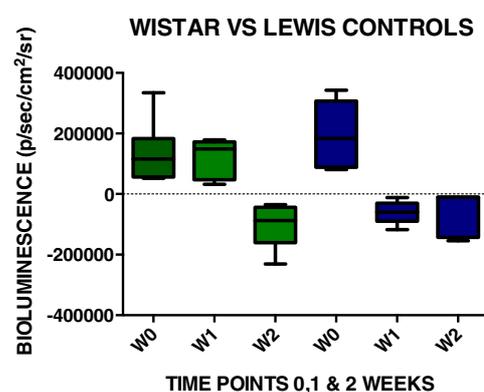


Fig. 3. Graph showing the time taken for rejection of tumour cells among control animals without immunosuppression. Well-matched Wistar animals (green) took two weeks to reject the tumour load while poorly matched Lewis (blue) animals rejected the tumour load within one week ($P < 0.001$) (the error bars representing standard error of mean).

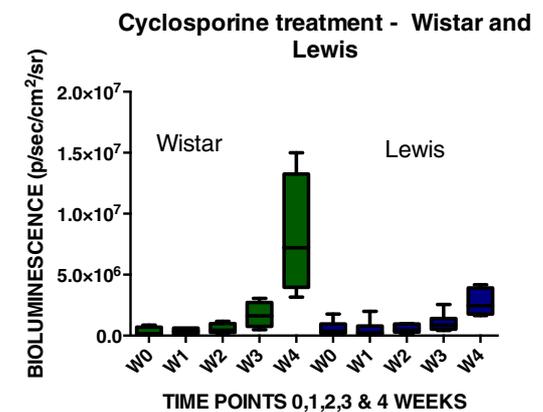


Fig. 4. Cyclosporine treatment continue group: Comparison between well matched Wistar (green) and poorly matched Lewis (blue) groups. Transplanted tumour cells continued to grow between both the groups but again the growth was stronger in well-matched animals compared to the poorly matched combination.

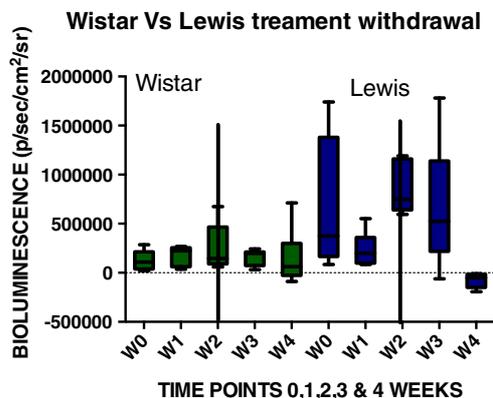


Fig. 5. Effect of treatment withdrawal in well matched and poorly matched combination groups. All the poorly matched animals (blue) rejected the transplanted tumour cells within four weeks of study period as opposed to only 50% in well-matched Wistar (green) during the same study period.

17,18]. This slight variation made our tumour model closely reflective of scenarios in human transplantation; as even the very well matched individuals (excluding identical twins-syngeneic transplantation) would have subtle differences in histocompatibility loci due to the very wide variations in the HLA haplotype [19].

Tumour cells injected into hosts normally take a long time to become palpable. Even cells with short doubling times often take a long time to become clinically significant and enable accurate measurements. The BP36B cells, used in our study took two months to establish when transplanted in immunocompromised nude mice [11]. Consequently we decided to transfect the tumour cells with luciferase in order to detect and monitor tumour growth by sensitive bioluminescent imaging techniques. Furthermore, the quantitative measurements made by this method were objective and less susceptible to human error and bias since tumour load was calculated computationally by signal intensity from the injected tumour cells rather than the more subjective method of visually grading the tumour size.

The behaviour of well and poorly matched transplanted tumour cells under conditions of immunosuppression and rejection (treatment withdrawal) has potential important clinical implications. The tumour cells were, as expected, rejected in the absence of any immunosuppression in both groups of animals since there are likely to be some differences between the donor and recipient even in the well-matched combination. However, the time taken for the poorly matched animals

to reject the tumour was significantly shorter ($p < 0.001$), and it is likely that this was due in part to the stronger allogeneic response having an anti-tumour effect. Similar results were noted when the immunosuppression was withdrawn midway in the study period to monitor the effects of rejection ($p < 0.039$). All the Lewis animals rejected the tumour two weeks after withdrawal while only half in the well-matched group did so. The clinical significance of this finding is that if we were to transplant kidneys after ex vivo resection of T1a tumours, then perhaps choosing a less well-matched donor recipient combination would be preferable. This would mean, should a recurrence occur in the recipient, simply withdrawing the immunosuppression (with transplant nephrectomy) may aid “rejection” of extra renal tumour cells [20]. This was the approach utilised by Nicol et al. in their series, although they were not able to test this hypothesis as the only patient developing recurrence in their series declined any further treatment [7].

The other clinically significant implication is the fact that under standard immunosuppression the tumour continued to grow. There were subtle but statistically significant ($p < 0.033$) differences in the rate of growth, with higher rate of tumour growth in well-matched animals. However, in both strains by the end of study period the signal intensity was high and in the majority of immunosuppressed rats the tumours were palpable. Therefore, the risk of unchecked tumour growth and perhaps metastasis would be a real concern should a tumour be transplanted inadvertently with a restored organ. The behaviour of tumours with immunosuppression using more contemporary immunosuppressants that have reported anti-neoplastic activity, such as rapamycin and leflunomide, needs to be investigated. Such immunosuppressive agents may prove to be effective in preventing recurrence or eliminate the cancer cells should they be transplanted inadvertently. A strategy to transplant these kidneys into less well-matched recipients and to use non-calcineurin inhibitor immunosuppression may provide the best outcomes.

6. Conclusions

Subtle variations in the growth of the tumour cells based on MHC-dependent differences in various experimental conditions were detected with great accuracy using the IVIS spectrum imaging system. There are two clinically relevant deductions of our experiments. Firstly, transplanted tumour cells continue to grow unchecked in immunosuppressed hosts. This finding makes it of paramount importance that any kidney transplanted after ex vivo resection must be devoid of any tumour load. Secondly, poorly matched combination of donor and hosts were significantly better in rejecting any donor-derived tumour if immunosuppression was withdrawn in this animal model. *Should a recurrence occur in a clinical situation after such transplants, it might be better to have less well matched donor recipient combination so that host's own immune system can be used at least in part to reject the transplanted tumour by withdrawal of immunosuppression.*

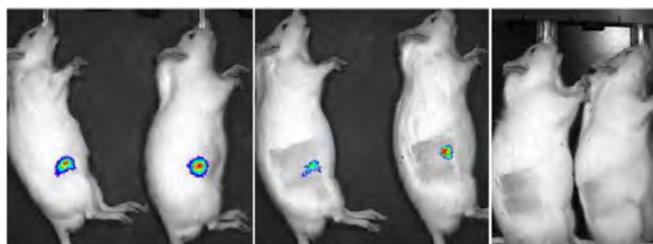


Fig. 6. Serial IVIS scans of Lewis rats: At the end of 2 weeks of immunosuppression (left), 1 and 2 week post-treatment withdrawal (middle and right). Tumour continued to grow when animals were kept on cyclosporine immunosuppression. With treatment withdrawal there has been steady rejection of tumour cells till all the injected cells were destroyed (significantly stronger rejection than well matched combination of Wistar animals).

List of abbreviations

RCC	renal cell carcinomas
NSS	nephron sparing surgery
NHEN	N-ethyl-N-hydroxyethylnitrosamine
HLA	human leukocyte antigen
GFP	green fluorescent protein
ROIs	regions of interest
Cyc	cyclosporine
MHC	major histocompatibility complex

Competing interests

No author has any competing interest to declare.

Author contributions

Khurram (MAK): manuscript, experiments and concept.
 Stamp (SS): experiments and manuscript review.
 Sheerin (NS): concept development and manuscript review.
 Rix (DR): concept development and manuscript review.
 Cunningham (AC): concept development, cell culture and manuscript review.
 Carter (NC): concept development, cell culture, transfection and manuscript review.
 Talbot (DT): concept and experiment development and manuscript review.

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Chapter 9 Box charts – Comparative analysis

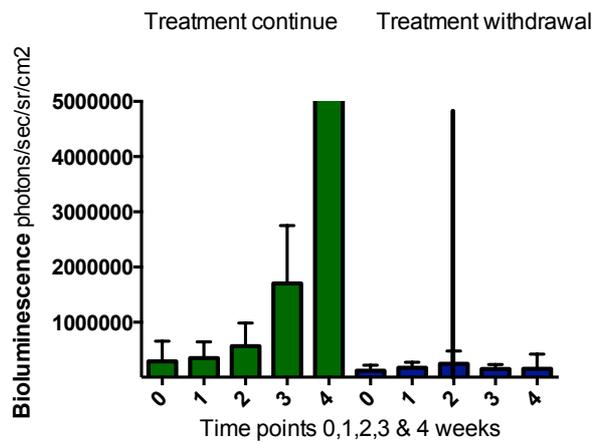


Figure 112 Cyclosporine immunosuppression: Direct comparison between treatment continue/withdrawal well matched animals

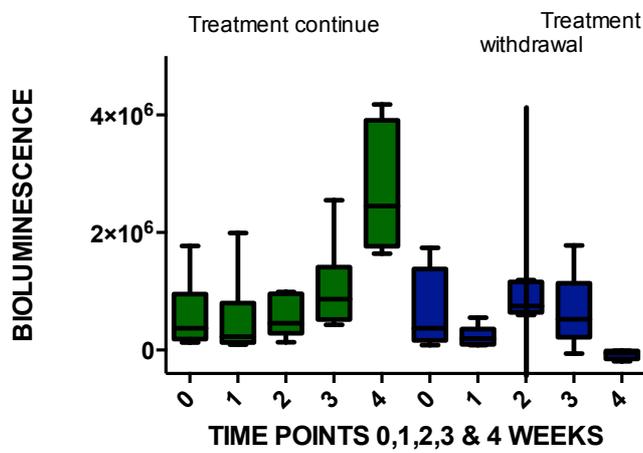


Figure 113 Comparison between treatment continue and withdrawal groups in CsA treated-Lewis animals.

As opposed to continued treatment, there was complete elimination of tumour load after treatment withdrawal.

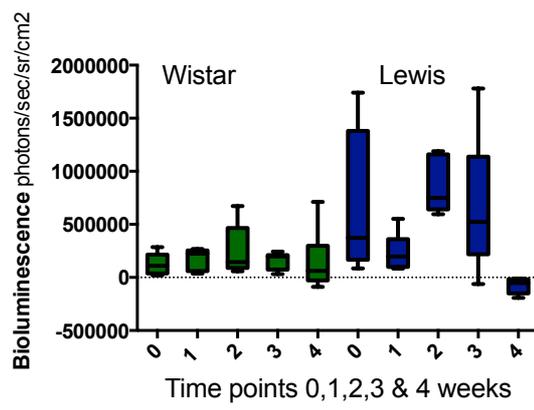
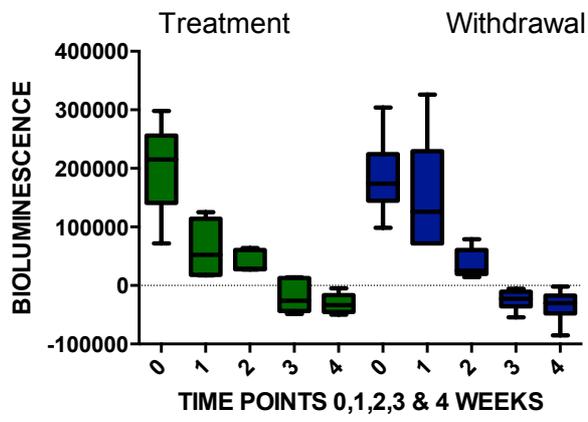


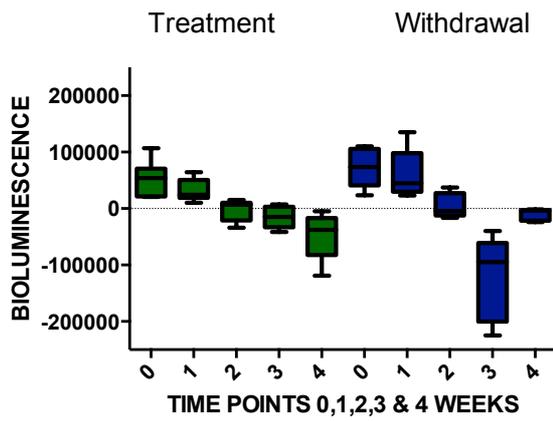
Figure 114

CsA treatment withdrawal: Wistar and Lewis animals

Sirolimus low dose Wistar treatment continue and withdrawal group



Sirolimus low dose Lewis treatment continue and withdrawal groups



Sirolimus low dose Wistar Vs Lewis
treatment withdrawal

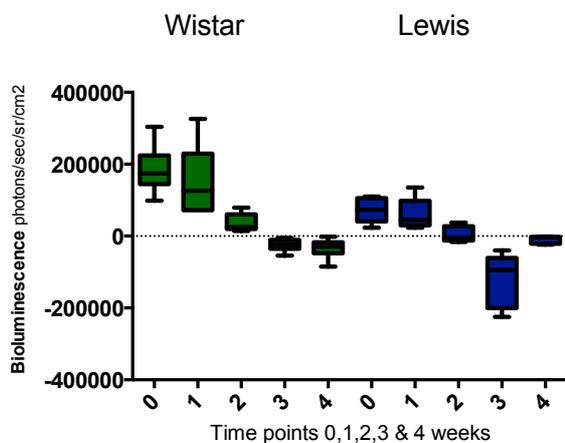


Figure 115 Sirolimus low dose. Comparison between treatment withdrawal and treatment continue groups in both strains and also direct comparison between Wistar and Lewis treatment withdrawal arm.

Wistar: Cyclosporine Vs Sirolimus high dose

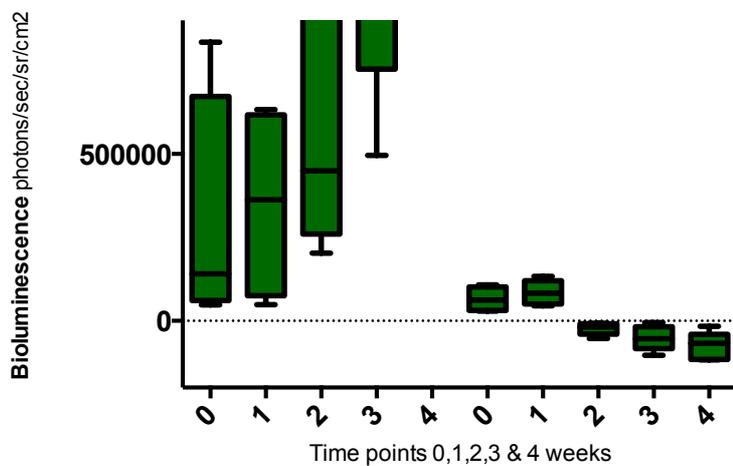


Figure 116 CsA vs. Sirolimus high dose Wistar animals. Direct comparison. Complete removal of all the tumour load in Sirolimus arm.

Lewis: Cyclosporine Vs Sirolimus high dose

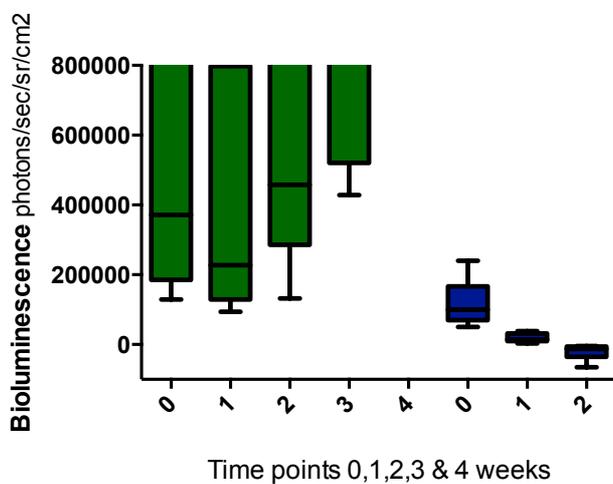


Figure 117 CsA vs. Sirolimus high dose Lewis animals. Direct comparison. Complete removal of all the tumour load in Sirolimus arm.

Cyclosporine Vs Sirolimus low dose
Well matched animals

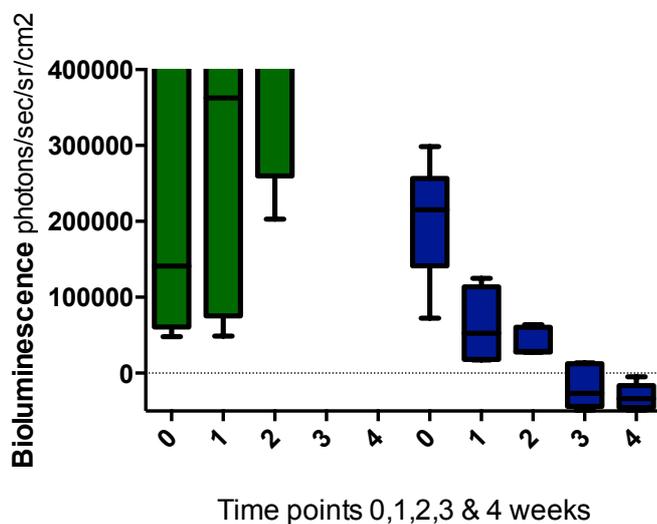


Figure 118 CsA vs. Sirolimus low dose Wistar animals. Direct comparison. Complete removal of all the tumour load in Sirolimus arm.

Cyclosporine Vs Sirolimus low dose
Poorly matched animals

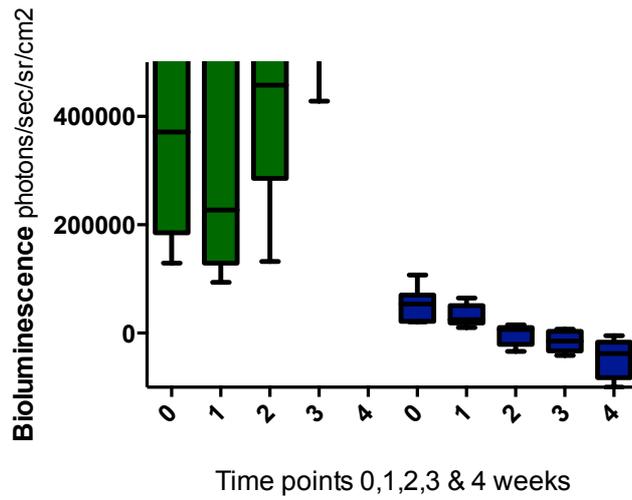


Figure 119 CsA vs. Sirolimus low dose Lewis animals. Direct comparison. Complete removal of all the tumour load in Sirolimus arm.

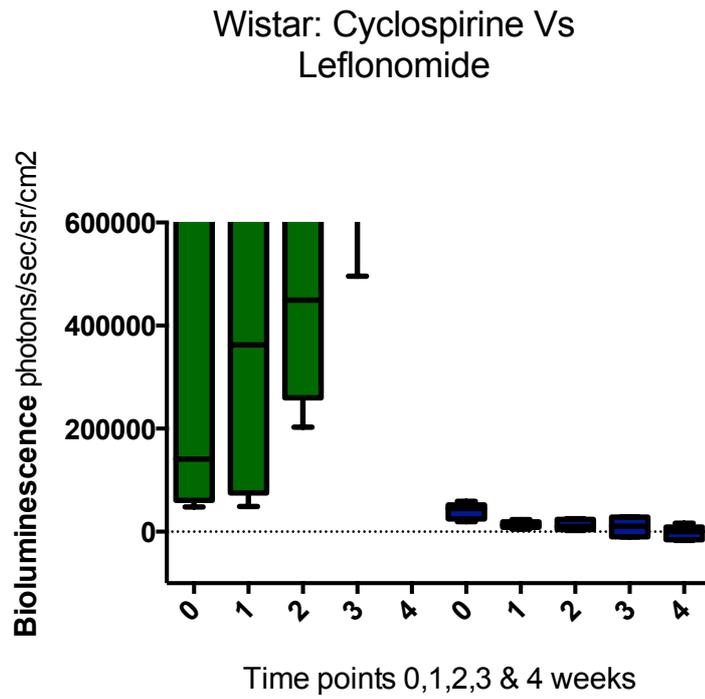


Figure 120 CsA vs. Leflunomide Wistar animals. Direct comparison. Continued growth with CsA immunosuppression while with leflunomide three out of four animals have complete elimination of the tumour load.

Chapter 10 FLOW CYTOMETRY DATA

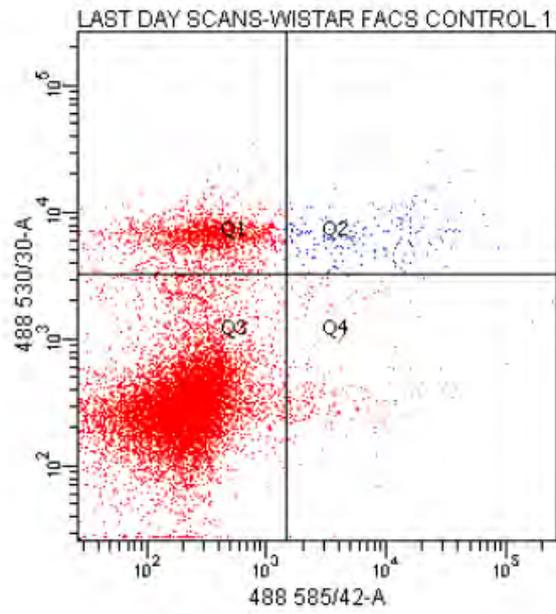


Figure 121
(PE) positive.

CD4+ cells. Q1 & Q2 are the total CD4 cells. The events in the Q2 are both CD4 (FITC) and CD25

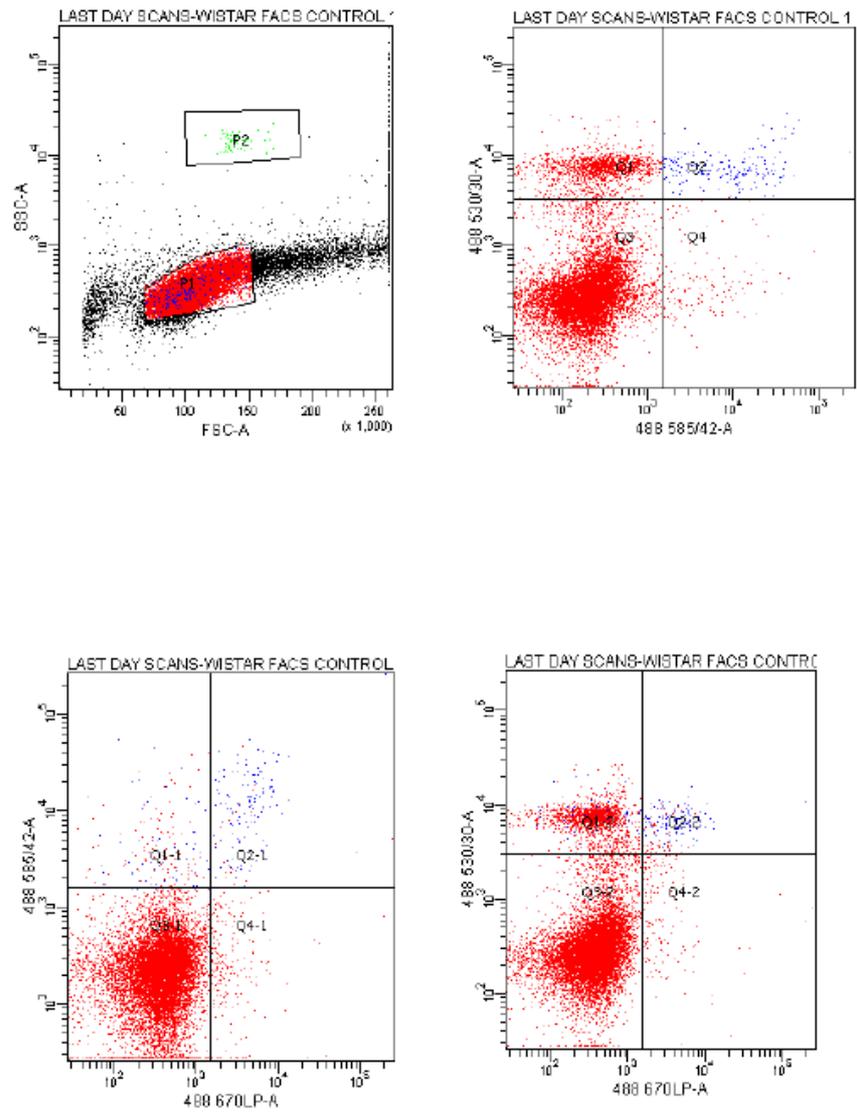


Figure 122 Estimation of regulatory T cells. The cells in the Q2 in the top right hand histogram are CD4 25 (PE fluochrome) positive while the cells in the Q2-2 (bottom right) are the CD4, FoxP3 (PE Cy 5 fluochrome) positive.

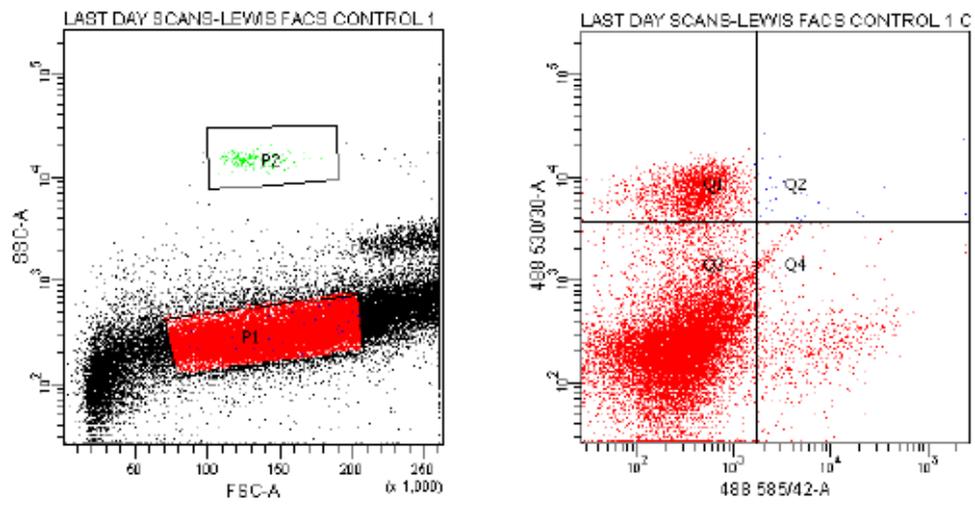


Figure 123

CD8+ T cells. FITC anti rat CD8 staining for estimation of CD8+ cells (Q1+Q2)

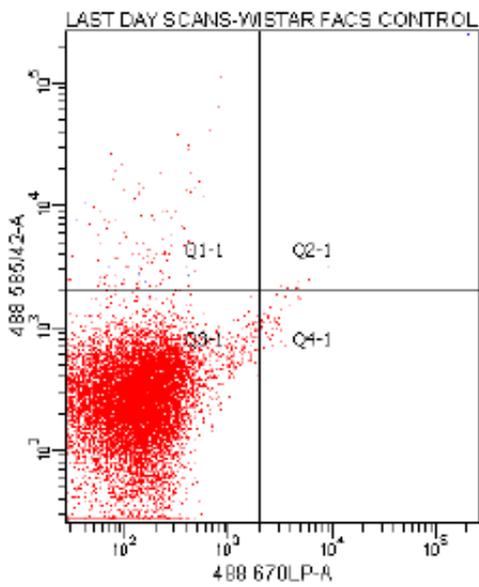
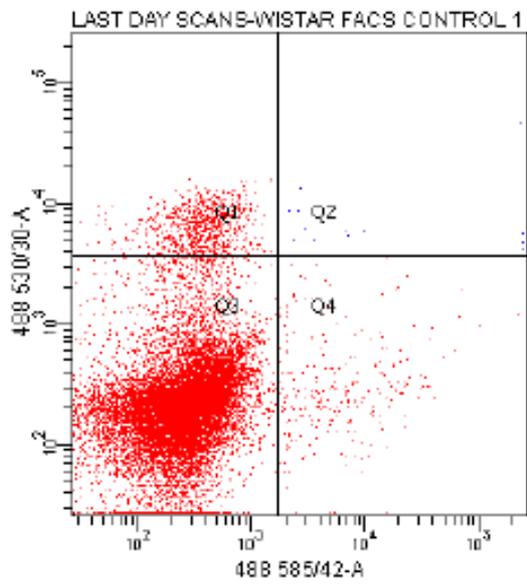


Figure 124 Activated CD8 T cells. Cells in Q2 are positive for both CD8 and CD25. The cells in Q1-1 are only positive for CD25.

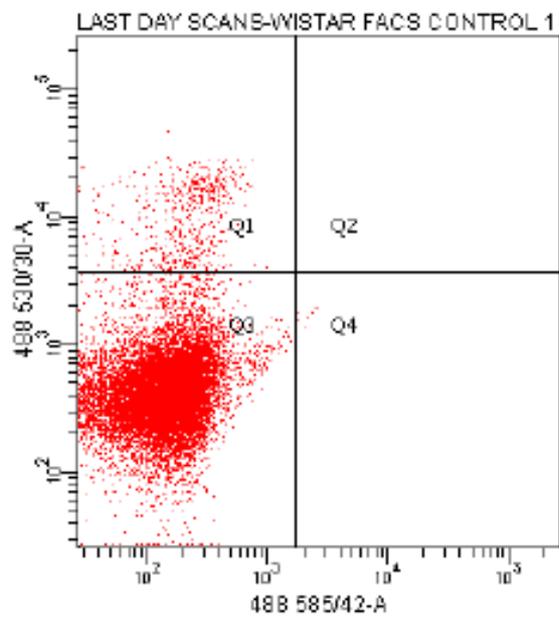


Figure 125 Natural killer cells (CD161+). FITC +ve in Q1.

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