Overcoming Skin Rejection in Composite Tissue Allotransplantation

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DECLARATION CONCERNING THESIS PRESENTED FOR DEGREE OF DOCTOR OF PHILOSOPHY

I, Benjamin Michael Horner, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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DEDICATION

This thesis is dedicated to the patients that I have been involved in treating, who could not be adequately reconstructed using standard techniques. The adversity these people faced, and continue to face, inspired me to undertake research in this area. I hope that the insights contained in this thesis will make some contribution in developing better reconstructive solutions.

ABSTRACT

The application of composite tissue techniques is constrained by the susceptibility of skin to rejection. The aim of this thesis is to improve our understanding of skin rejection and find ways to avoid it, in order to enable expansion of the application of composite tissue transplantation techniques.

The first part of the thesis explores the consequences and mechanism of skin rejection in rat models. These studies indicate that in the event of allograft failure, there is minimal damage to the vascular pedicle of a composite tissue allotransplant, even after full rejection, making retransplantation possible. Furthermore, there is only mild damage to the recipient tissues, indicating that the second transplant would not be limited in form or function by recipient tissue bed damage. Finally, the studies indicate that there are significant differences between the mechanism of rejection of skin in composite tissue transplants and conventional skin grafts. This means that much of the historical data relating to skin graft rejection is not necessarily relevant to composite tissue allotransplantation.

The second part of the thesis uses swine models to explore ways to overcome skin rejection while avoiding the toxicity of chronic systemic immunosuppression, through tolerance induction, and site specific therapy. Previous experience in organ and composite tissue allotransplantation models are analysed to develop the hypothesis that high-level chimeras are tolerant to vascularised skin allotransplants. *In utero* and adult chimerism induction models are then used in an attempt to attain moderate-level chimeras. A vascularised skin allotransplant model is developed. Finally, the hypothesis is confirmed with the transplantation of a vascularised skin allotransplant on to moderate-level chimeras with the achievement of tolerance. In addition, site-specific therapy is used in an attempt to avoid the side-effects of chronic high-dose systemic immunosuppression. This led to prolongation of skin survival, but eventual skin rejection.

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TABLES AND FIGURES

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ABBREVIATIONS

@	Anti
Ab	Antibody
Allo	Allogeneic
ATG	Anti-thymocyte globulin
BFU-E	burst-forming unit-erythroid
BMC	Bone marrow cell
BM-CFUs	Bone marrow colony forming units
bp	Base pair
CAFC	Cobblestone area-forming cell
CFU	Colony forming unit
CFU-GEMM	Colony forming unit granulocyte-erythroid-monocyte-megakaryocyte
CFU-GM	Colony forming unit granulocyte-monocyte
cGy	Centi-Gray
Chim	Chimerism
CM	Cytokine mobilised
CML	Cell mediated lymphocytotoxicity
CO_2	Carbon dioxide
Cpm	Counts per minute
C-section	Caesarian section
CSG	Conventional skin graft
CTA	Composite tissue allotransplantation
CyA	Cyclosporine-A
D	Day
DC	Dendritic cell
dDC	Donor dendritic cell
DLI	Donor leukocyte infusion
DNA	Deoxyribonnucleic acid
E:T	Effector:target
FACS	Fluorescence activated cell sorting
Fig	Figure
FN	False negative
FP	False positive
FT	Full thickness
G	Grade

g	Gram
GA	Gestational age
GFP	Green fluorescent protein
GvHD	Graft-versus-host disease
H&E	Heaematoxylin and eosin
HBSS	Hanks balanced salt solution
HCT	Haematopoietic stem cell transfer
HSCs	hematopoietic stem cells
I.P.	Intra-peritoneal
Ig	Immunoglobin
IL	Interleukin
Iso	Isogeneic
kg	Kilogram
Leuko	Leukapheresis
Lew	Lewis
Lew-GFP	Lewis GFP transgenic
M	Molar
MACS	Magnetic activated cell sorting
mg	Milligram
MGH	Massachusetts general hospital
MHC	Major histocompatibility complex
Min	minute
MLR	Mixed lymphocyte response
ng	Nanogram
NIH	National institute for health
NK	Natural killer
NPV	Negative predictive value
PAA	Pig allelic antigen
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PPV	Positive predictive value
PSL	Percent specific lysis
PT	Partial thickness

qPCR	Quantitative PCR
rDC	Recipient dendritic cell
RPMI	Roswell park memorial institute media
RRRC	Rat resource and research center
RT-1	RT-1 complex (rat MHC)
rt-PCR	Reverse transcriptase PCR
SB	Southern blot
SCF	Stem cell factor
SCTT	Skin within a composite tissue allotransplant
SI	Stimulation index
SLA	Swine leukacyte antigen (swine MHC)
TBRC	Transplantation biology research center
TN	True negative
TP	True positive
Treg	Regulatory (CD4+CD25+FoxP3+) T cell
Tx	Transplant
U	Unit
US	Ultrasound
USS	Ultrasound scan
WBC	White blood cell
WF	Wistar Furth
w/v	Weight/volume
YUC	Yucatan

CHAPTER 1: INTRODUCTION

1.1 THE FIRST CLINICAL APPLICATIONS OF COMPOSITE TISSUE ALLOTRANSPLANTATION

The primary aim of reconstructive surgery is to "replace like with like"[1]. However, many people have physical defects (e.g. severe facial burns or limb amputations) for which there are limited reconstructive surgery options using tissue from parts of their own bodies. In these cases, transplanting tissue (e.g. hand and face) from other people offers an effective way to reconstruct the defect.

The development of microsurgical techniques made possible the first composite tissue allotransplants in animal models more than a century ago (Carrel, A. et al. 1906). However, it was not until the advent of modern transplant immunology after World War II that clinicians seriously started to consider clinical composite tissue allotransplantation (CTA). The first recorded attempt was a hand allotransplant performed in Ecuador in 1964. However, this was lost early to acute rejection. The first successful reconstruction using a composite tissue allotransplant was a hand transplant performed in France in 1998. The initial success of this transplant paved the way for more procedures with 38 reported hand transplants (Lanzetta, M. et al. 2007), 8 abdominal wall transplants (Levi, D. M. et al. 2003), scalp (Jiang, H. Q. et al. 2005), 5 knee transplants and 3 face transplants (Kanitakis, J. et al. 2006) reported to date.

This introduction will examine the problem of risk associated with the clinical use of composite tissue allotransplantation techniques, and then outline how this study aims to address a key element of this risk.

1.2 THE PROBLEM

Skin makes up a central element of many composite tissue allotransplants and is considered to be the most antigenic of all the tissues (Lee, W. P. et al. 1991). The success of the reconstructive allotransplantation programme has been possible because of the efficacy of modern chronic immunosuppression regimens preventing immunological rejection of skin and other elements within the allograft. However, these medications have significant side-effects including predisposition to skin cancers, kidney damage and metabolic disorders. Furthermore, there still is the risk of chronic

[1] Attributed to Harold Gilles

rejection several years after transplantation. These risks are also relevant for recipients of other types of allograft such as bone marrow, organs and islets. There are three issues that specifically affect the assessment of potential risks of reconstructive transplantation: these procedures improve quality of life but do not directly extend life, there is a risk of chronic rejection, and there are significant psychological risks as well as benefits:

1.2.1 Quality not quantity of life

For a patient to consider it worthwhile to receive a transplant, the potential benefits following a procedure have to at least balance the risks associated with it. For life-saving transplants (e.g. heart), even if there are significant risks associated with the procedure, it may be still be considered more beneficial to undergo the procedure than to face the consequences of not receiving the transplant. Other organ allografts (e.g. kidney) are not immediately-life saving but are usually life prolonging. Therefore, the benefits will outweigh the risk of serious consequences. Composite tissue allotransplantation (CTA) can profoundly enhance quality of life but not directly prolong life. In such cases, the possibility of serious consequences, such as skin cancers and diabetes, may outweigh the possible benefits of receiving the transplant for some people. However, there have already been cases where the risk-benefit analysis has been considered supportive of performing CTA. It can be even beneficial to perform transplantation when the risk-benefit ratio is even smaller than in CTA: islet cell transplantation is similar to composite tissue allotransplantation in that it may not prolong life, but has the potential to significantly improve the quality of the recipient's life by curing them of diabetes. However, modern insulin regimens offer a good alternative to islet cell transplantation. In contrast there is no comparably good alternative to many composite tissue allotransplants.

1.2.2 Chronic Rejection

The risk of chronic rejection is unknown in composite tissue allotransplantation because it is still in its infancy. However, it is possible that chronic rejection may be more frequent and have more serious consequences for CTA than other forms of allotransplantation. Many recipients of organ allografts may have significant co-morbidity and so die before their allograft develops chronic rejection, or before they can develop significant consequences from it. However, recipients of composite tissue allotransplants will often have a life-expectancy of several decades allowing more time

for chronic rejection to develop. Furthermore, the effects of chronic rejection may have more severe consequences for composite tissue allotransplants than many organ allografts: a kidney allograft can function adequately even if half of it has become scarred by the chronic rejection process, whereas a hand allotransplant would only retain very limited function if half of its tissues were non-functional.

1.2.3 Psychology

Some of the major potential benefits from CTA may be psychological; for example, from reducing physical impairment and social stigma. However, the psychological risks associated with CTA may be higher than with many other forms of transplantation. The hand, and particularly the face, are important elements in our definition of personhood. Transplantation of tissues that significantly change one's appearance could have a major effect on sense of self and self-worth. There can also be secondary effects on the recipient due to the response of friends and family: two of the three US hand transplant recipients have divorced within 3 years of receiving a hand transplant, and one of these has also developed alcohol dependency. Furthermore, in the possible event of a composite tissue allotransplant failure, there may profound psychological effects. Most hand transplant recipients have incorporated their transplant into their image of personhood within 3-6 months, referring to the transplant as "my hand" instead of "the hand". In the event that a composite tissue allotransplant recipient lost the same part of their body for a second time, it is possible that this would be more difficult to deal with psychologically than the loss of a non-life sustaining organ transplant. The recipient would actually see the effects of their own body attacking the allograft, which may negatively affect their sense of wholeness and identity.

1.3 STUDY AIMS

The aims of this study are: (1) to examine the rejection process of skin and composite tissues and its effects, and (2) to explore ways to overcome skin rejection while avoiding the toxicity of chronic systemic immunosuppression. These questions were examined separately in parts A and B of the thesis.

1.3.1 Part A. The mechanism and consequences of rejection of skin and other composite tissues

In the first part of the thesis, questions relating to the consequences of rejection of skin and other composite tissues and the mechanism of skin rejection are explored.

The consequences of rejection of many composite tissue allotransplants are not known. For some composite tissue allotransplants (e.g. hand) the failed transplant can simply be amputated. This is not possible for other composite tissue allotransplants (e.g. face and abdominal wall) because the resulting defect cannot be left unreconstructed. The plan in many cases is to replace the failed composite tissue allotransplant with another one. However, it is possible that the underlying tissues will be so damaged by the rejection process that it would not be possible to perform another transplant. In Chapter 4 the consequences of composite tissue allograft rejection on the recipient tissues are examined in a rat model.

Much of the previous data relating to the mechanism of skin rejection was obtained from observations of conventional skin graft rejection. Conventional skin grafts only pick up a blood supply over the first few days after transplantation. In contrast, the skin within composite tissue allografts has a blood supply immediately following transplantation. The effects of vascularisation on the mechanism of rejection have not previously been examined. In Chapter 5 the mechanisms involved in the rejection of vascularised skin with a composite tissue transplant and a conventional skin graft are examined in a rat model using *in vivo* confocal microscopy to follow cell trafficking.

1.3.2 Part B Prevention of skin rejection while avoiding the risks of chronic high-dose systemic immunosuppression

The ultimate aim of transplant immunology research is to induce a state of selective immunological acceptance of the allograft without the need for chronic immunosuppression (i.e. tolerance). There has been some success clinically with renal allografts. However, skin tolerance across major histocompatability barriers has so far only been achieved in small animals. Previous work in a swine chimerism induction model achieved only prolonged survival of the skin element of a composite tissue allograft, despite achieving tolerance to the musculoskeletal element (Hettiaratchy, S. et al. 2004). The first part of this section (Chapter 6a) reviews the more extensive experience in organ allotransplantation in the swine chimerism induction model to identify predictors for achievement of tolerance. These predictors are then applied to the previous swine composite tissue allotransplant data to understand further why skin tolerance was not achieved and to develop a hypothesis on how to achieve skin tolerance (Chapter 6b). To test the resulting hypothesis two elements were required: a

vascularised skin allotransplant model in swine, and high-level chimeras to receive the allotransplant. A reliable skin flap model is developed (Chapter 7), and methods to boost chimerism to achieve high level-chimeras using Donor Leukocyte Infusion is examined (Chapter 8). *In utero* (Chapter 9) and adult (Chapter 10) chimerism induction models are then used in an attempt to attain high-level chimeras on which to perform a vascularised skin allograft. Finally, the use of site-specific therapy to try and prevent skin rejection while avoiding the side-effects of chronic high-dose systemic immunosuppression is examined (Chapter 11).

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 INTRODUCTION

In 1943 Thomas Gibson and Peter Medawar opened the modern era of transplantation research with a paper on the problem of skin allograft rejection (Gibson, T. et al. 1943). Ten years later, taking into account the observations by Owen that naturally occurring chimeric twin calves accepted reciprocal skin grafts (Owen, R. D. 1945), Billingham, Brent and Medawar went on to demonstrate that it was possible to induce selective immune acceptance of skin grafts in mice: a state of tolerance (Billingham, R. E. et al. 1953). After over six decades, however, the precise mechanism of skin allograft rejection is still ill-defined. Furthermore, it has not been possible to reliably achieve clinical tolerance, which would allow the widespread application of skin allotransplantation techniques.

This chapter summarizes the alternatives to enlarge the scope of skin allotransplantation techniques, the current understanding of mechanisms of skin rejection, and the utility and limitations of animal models used to study skin rejection and tolerance induction. Finally, the manipulation strategies that have been explored to achieve skin tolerance are outlined.

2.2 ROUTES TO WIDESPREAD APPLICATION OF SKIN ALLOTRANSPLANTATION TECHNIQUES

There are three options to overcome the difficulties limiting the expansion of the use of skin allotransplantation: A) reduction of the toxicity of chronic immunosuppression, B) reduction of the dose of immunosuppression by induction of a less alloreactive state, and C) obviating the requirement for immunosuppression by tolerance induction.

2.2.1 Reduction of chronic immunosuppression regimen toxicity

The development of novel and less morbid immunosuppressants opened the way for the successes achieved so far in composite tissue allotransplantation. In the short term, reduction of toxicity of chronic immunosuppression regimens may be the most easily achievable with more specific systemic immunosuppressive therapies, or the use of site-specific therapies with reduction or removal of systemic immunosuppression.

Future immunosuppressants are likely to offer only modest toxicity reduction on current medications as it is difficult to selectively suppress the graft alloresponse without influencing immune response to other stimuli. Site-specific therapies have been used with some success to treat early rejection episodes in some of the hand transplant recipients (Lanzetta, M. et al. 2005). However, it is unclear whether this is an effective clinical strategy for reducing the maintenance dose of systemic immunosuppression: in small animal models, indefinite skin survival has not been achieved using site-specific therapies (Fujita, T. et al. 1997; Inceoglu, S. et al. 1994; Yuzawa, K. et al. 1996).

2.2.2 Induction of a less alloreactive state

Some have speculated that the initial hand transplants may have coincidentally induced a less alloreactive state due to the donor bone marrow in the graft: hand transplant recipients have required less immunosuppression than was initially expected, with stable graft function using dosage regimens comparable to renal allotransplants despite the presumed higher antigenic load due to the inclusion of skin in the transplant. In addition, cells with a regulatory phenotype (CD4+CD25+FoxP3+) were detected in the allograft dermis of one of the French hand transplant recipients (Eljaafari, A. et al. 2006), although the functional significance of this is unclear as the patient was still on immunosuppression.

Interventions to reduce alloreactivity have not been effective for skin allotransplantation. Anti-thymocyte globulin (ATG) and anti-CD25 monoclonal antibodies were administered in two of the hand transplants (Lanzetta, M. et al. 2005), anti-CD52 mAb in abdominal wall allograft transplants, and post-transplant bone marrow infusion in the first French face transplant (based on regimens used in organ transplants (Ricordi, C. et al. 1997), all with no measurable success.

2.2.3 Tolerance

The ultimate goal for skin transplantation is to achieve donor specific tolerance. This will avoid risks from chronic medication, and possibly the risk of chronic rejection. This goal has been shown to be clinically achievable in renal transplantation (Fehr, T. et al. 2004), with further work required to improve the reliability of the regimen. Skin holds the unenviable title of being the most difficult of all transplanted tissues to achieve a state of tolerance towards. However, there are anecdotal reports of

achievement of skin tolerance in patients (Achauer, B. M. et al. 1986; Woodruff, M. F. et al. 1959), indicating that clinical skin tolerance is achievable.

2.3 MECHANISMS OF SKIN REJECTION

It has long been thought that transplanted skin is more susceptible to rejection than other tissues (Lee, W. P. et al. 1991; Sheil, A. G. et al. 1964). Four factors that may contribute to skin's particular susceptibility to rejection are its usual mode of transplantation, skin specific alloantigens, its composition, and allograft size; each of these will now be examined in more detail:

2.3.1 Mode of transplantation

The method of skin allograft transfer may influence their immunogenicity: primarily vascularised skin allografts have a small survival advantage over secondarily vascularised skin allografts in some studies (Bushell, A. et al. 1995; Steinmuller, D. 1998). Possible mechanisms for the difference in immunogenicity between primarily and secondarily vascularised skin allografts are initial post-transplant ischemic damage and the route of interaction of the allograft with the recipient immune system.

2.3.1.1 Ischemic damage

In a primarily vascularised allograft, vessels supplying the skin are anastomosed to recipient vessels establishing an immediate blood supply to the skin and minimizing any ischemic damage. In contrast, in a secondarily vascularised graft there is a period of relative ischaemia for the first 48-72 hours until the microvasculature connects to vessels in the wound bed. This causes degeneration and even death of the epidermis (Medawar, P. B. 1944; Steinmuller, D. 1962) stimulating an inflammatory response within the graft which could be a trigger for rejection.

2.3.1.2 Route of immune interaction

It is likely that the trafficking of immune cells differs radically between primarily and secondarily vascularised skin allografts immediately following transplantation.

There is little data regarding primarily vascularised skin allografts. However, extrapolating from primarily vascularised heart transplant data in mice it is likely that initial influx and efflux is mainly via the bloodstream involving both recipient and donor dendritic cells (Saiki, T. et al. 2001). In contrast, initial cell trafficking in

secondarily vascularised skin allografts is via lymphatics, as demonstrated by the prolonged survival of secondarily vascularised alymphatic skin allografts (Barker, C. F. et al. 1968; Tilney, N. L. et al. 1971), with no evidence of recipient dendritic cell involvement (Larsen, C. P. et al. 1990a).

It is possible that it is not the route of sensitisation itself that is a cause for a difference in immunogenicity between primarily and secondarily vascularised skin allografts. Rather the route of sensitisation affects the maturity, function and final destination of the dendritic cells, which in-turn are the cause the immunological difference between primarily and secondarily vascularised skin allografts (Emmanouilidis, N. et al. 2006; Moser, M. 2003; Ochando, J. C. et al. 2006).

Both the route of immune interaction and ischaemic damage may contribute to skin's antigenicity. However, the mode of transplantation does not fully explain skin's immunogenicity as primarily vascularised skin is still more easily rejected than other tissues (Perloff, L. J. et al. 1979).

2.3.2 Skin specific antigens

The proposal that the susceptibility of skin to rejection is due to expression of tissue specific antigens (Boyse, E. A. et al. 1968; Silverman, M. S. et al. 1962) was based on the observation that in certain chimeric rodent models, allogeneic donor bone marrow was accepted while skin was rejected. Three skin specific antigens have been described in mice: Skn-1, Skn-2 and Epa-1.

2.3.2.1 Skn antigens

Skn antigens (Skn-1 and Skn-2) seem to be truly skin specific. However, some chimeras accept skin grafts despite making Skn antibodies (Scheid, M. et al. 1972). The reason for this disparity may be that Skn antigens are not transplantation antigens: acute rejection is T cell mediated, whereas Skn antigens are primarily serologically defined, with incomplete evidence that they can stimulate a T cell response.

2.3.2.2 Epa-1 antigen

Epa-1 antigen can stimulate T cell mediated skin rejection, and has a possible homologue in humans; however, it is not skin specific. Consequently, Epa-1 can trigger rejection of other tissues (e.g. heart) (Steinmuller, D. 1998). The other tissues on which

Epa-1 is expressed are less immunogenic than skin, suggesting that the cause of skin antigenicity is not just Epa-1.

2.3.3 Skin composition

Skin is a barrier to the outside world. It is conceptually coherent that for skin to act as an effective first line of defence to any pathogen it is biased towards a rejection response. The intestine and the lungs perform a similar barrier function and are also particularly susceptible to rejection (Goss, J. A. et al. 1993; Prop, J. et al. 1985; Zhang, Z. et al. 1996). The cells that make up the skin and dermal structure may both play a role in the particular susceptibility of skin to rejection.

2.3.3.1 Cells within the skin

The term "Skin Immune System" was coined by Bos (Bos, J. D. et al. 1997) to indicate that skin is an immunological organ, with approximately half of its cells having immunological function. Of the many specialized immune cells within the skin Langerhans cells are likely to be the most important: the immunogenicity of skin allografts correlates directly with the density of Langerhans cells they contain (Bergstresser, P. R. et al. 1980; Chen, H. D. et al. 1983; Mathieson, B. J. et al. 1975; Sena, J. et al. 1976). However, skin allografts from class II knockout mice are acutely rejected at the same rate as wild type skin grafts (Illigens, B. M. et al. 2002) demonstrating that direct stimulation by donor class II expressed on these cells is not the sole cause of skin's susceptibility to rejection.

2.3.3.2 Dermal structure

The dermis is composed predominantly of collagen and glycosoaminoglycan matrix, which are only weakly immunogenic (Hoffman, D. K. et al. 1994; Wu, J. et al. 1995). However, this highly structured environment contains a high concentration of lymphocyte adhesion molecules, thereby making an ideal platform from which effector cells can mount an immune response. Furthermore, the dermis is highly vascular which allows for rapid immune cell trafficking to the skin.

2.3.4 Graft size

The volume of tissue within the allograft may affect the immune response. Evidence for this comes from both murine models and clinically. In a minor mismatch mouse transplant model, smaller skin and cardiac grafts are rejected acutely whereas larger

grafts can avoid acute rejection and are rejected more slowly (He, C. et al. 2004). In the clinic it has been observed that there is a lower incidence of acute cellular rejection in recipients of larger volume kidney allografts (Poggio, E. D. et al. 2006; Sanchez-Fructuoso, A. I. et al. 2001). The correlation between allograft size and avoidance of acute rejection puts transplanted skin at a relative disadvantage as the average skin allograft contains a lot less tissue than the average organ allograft. Additionally, the volume of skin required to avoid acute rejection may be proportionately more than other tissues (Jones, N. D. et al. 2001).

The difference in the speed of rejection between large and small grafts appears to be at the effector stage rather than the priming stage (He, C. et al. 2004), and may be due to immunomodulation as well as the influence of graft volume:donor-reactive T cell ratio. A larger graft may stimulate a stronger regulatory T cell response than a small graft, these in turn may down-modulate the rejection response (Sho, M. et al. 2002). Graft size can also influence the speed of rejection by changing the ratio of graft volume to number of donor-reactive T cells. Immediately following transplantation, a threshold number of donor-reactive T cells has to be reached to acutely reject an allograft of a certain size (He, C. et al. 2004; Jones, N. D. et al. 2001). A graft recipient may have enough donor-reactive T cells to reach the threshold required to cause acute rejection of small graft, but this may only be sub-threshold for rejection of a larger graft.

Graft size may play a role in making skin more susceptible to acute rejection, particularly in MHC-matched minor-mismatch models. However, the influence of graft size is limited to acute rejection; there is no evidence that larger grafts have a lower incidence of chronic immune damage.

In summary, no single dominant mechanism for skin's antigenicity and susceptibility to rejection has been identified. The mode of transplantation, skin specific antigens, the composition of skin and the allograft volume may all contribute, but more research is required to further understand their specific roles.

2.4 UTILITY OF ANIMAL MODELS IN THE DEVELOPMENT OF A STRATEGY FOR CLINICAL SKIN TOLERANCE INDUCTION

Skin tolerance can be reliably induced across major histocompatibility complex barriers in several small animal models; this has not been possible in large animals or humans.

The difficulty in translating between small and large mammals is likely to be due to differences between the models including resilience to toxic induction regimens, endothelial MHC Class II expression, and bystander activation.

2.4.1 Resilience to toxic induction regimens

Rodents are resilient to treatments that cause significant morbidity and mortality in large animal models and the clinic (e.g. lethal irradiation) (van Bekkum, D. W. 1984). This may be partly due to extrinsic factors such as the highly controlled environment small animals are kept in, with minimal exposure to infections, as well as their much shorter lifespan, with death occurring before many complications can develop. However, they are also intrinsically less susceptible to certain complications, such as thrombo-embolism following co-stimulatory blockade (Kawai, T. et al. 2000).

2.4.2 Vascular endothelium immune function

Vascular endothelium is a likely principle target for the host-anti-graft response. There are significant differences in the expression of molecules involved in the immune response on rodent versus human and large animal endothelial cells (Pober, J. S. et al. 2003). For example, large animals constitutively express MHC Class II on their endothelium, where as in rodents it is only inducible (Houser, S. L. et al. 2004; Kreisel, D. et al. 2001). This difference in Class II expression may not actually lead to a difference in a transplant scenario, as MHC Class II expression may be induced on rodent endothelium by the act of transplantation. It is also possible that endothelial class II MHC has different functions in small animals compared to large animals. In mice, endothelial class II MHC does not activate alloreactive CD4+ cells (Grazia, T. J. et al. 2004), and may even induce the generation of CD4+25+FoxP3+ regulatory cells (Krupnick, A. S. et al. 2005); this has not been examined in large animals.

2.4.3 Bystander activation

Large animals and humans are exposed to a variety of antigenic stimuli to which they mount an immune response with the consequent formation of memory cells. One or more clones of these memory cells may also be activated by the allograft due to antigenic similarity between the original stimulus and the graft ("heterologous immunological memory") (Adams, A. B. et al. 2003; Koyama, I. et al. 2007). In contrast, small animals are often bred in controlled environments and therefore are less likely to have previously formed memory cells that can be activated by the allograft.

In summary, differences between large and small animals mean that it is possible to induce skin tolerance in many small animal models, but rarely in large animals or humans. So although small animals are useful for outlining new approaches and for mechanistic studies, large animals, with their greater physiological and immunological similarity to humans, possibly better simulate the human condition and the development of clinically translatable protocols.

2.5 STRATEGIES FOR SKIN TOLERANCE INDUCTION

A tolerance induction strategy involves two elements. Firstly, the stage of immune development to induce tolerance is selected. Secondly, the method of immune manipulation used to induce one or more tolerance mechanisms is chosen. Each of these elements will be considered in turn.

2.5.1 Stage of immune development

Tolerance can be induced *in utero*, or during neonatal or adult life. Less manipulation is required to induce donor-specific tolerance in the immature immune system of *in utero* models. Adult tolerance induction models often require more aggressive manipulation, however they have a much wider scope of application as they can be used to treat acquired disorders not present *in utero* and avoid risk of triggering abortion by *in utero* manipulation. Neonatal models theoretically combine advantages of both *in utero* and adult models, with minimal manipulation required of the still developing immune system to achieve tolerance without risk of abortion. Initial work in small animal neonatal models was successful at achieving donor tolerance across a major MHC barrier to a delayed musculoskeletal allograft with the infusion of bone marrow cells (Butler, P. E. et al. 2000). However, similar strategies to induce skin tolerance with neonatal injection of bone marrow into the thymus (Cober, S. R. et al. 1999) or the simple intra-peritoneal injection of bone marrow with or without epithelial cells (Petit, F. et al. 2004) only resulted in modest prolongation of skin graft survival. There has been no improvement in induction of skin tolerance neonatally since Boyse and Old's successful neonatal skin tolerance radiation mouse model (Boyse, E. A. et al. 1973) which was no less toxic than successful regimens used in adult models. The theoretical advantage of the neonatal model does not seem to be bourne out in practice for skin tolerance induction.

2.5.2 Method of immune manipulation

Manipulations that have been used for skin tolerance induction attempts can be divided into two groups: those that involve donor haematopoietic stem cell transfer (HCT) and those that do not (non-HCT) (see table 2.1).

Method	Additional Procedure
Non-HCT	Immunosuppression
	T-cell depletion
	Costimulatory blockade
HCT	HCT alone
	Immunosuppression
	T cell/lymphocyte depletion
	Costimulatory blockade
	Dendritic dells

Table 2.1: Methods of immune manipulation to achieve tolerance

The transfer of donor haematopoietic stem cells (HSCs) to the recipient as part of tolerance induction has fundamental effects on the mechanism of tolerance induction. HSCs have the ability to indefinitely replicate as well as differentiate into cells of all lympho-haematologic lineages. If donor HSCs stably engraft in the recipient they will provide donor antigen to the thymus allowing life-long negative selection of newly arising donor-reactive thymocytes ('Central Deletion') (Sykes, M. 2001) and creation of naturally occurring regulatory cells. It is likely that with near complete replacement of recipient by donor HSCs central deletional mechanisms are dominant. However, at lower levels of HSC chimerism the mechanism of tolerance induction may not be very different from non-HCT approaches with regulatory cells having a greater role (Bemelman, F. et al. 1998; Domenig, C. et al. 2005; Kurtz, J. et al. 2004). Regulatory cells can be 'naturally occurring' thymic derived or be 'inducible' in the periphery (Waldmann, H. et al. 2006). Inducible regulatory T cells can stimulate mature T cells to change to a regulatory phenotype ('Infectious Tolerance' (Qin, S. et al. 1993)).

To attain tolerance it has been reported that regulatory T cells may only be required to a small number of antigens in an allograft; cells within the allograft expressing other

antigens attain protection by 'linked' or 'bystander' suppression (Chen, Z. K. et al. 1996; Davies, J. D. et al. 1996). However, it is unlikely that this mechanism will be relevant to attaining skin tolerance within a composite tissue allograft because bystander suppression appears to require both regulatory cells and bystander cells to exist in the same tissue and not just adjacent to each other (Chen, Z. K. et al. 1996). This is supported by the observation of "split tolerance" in composite tissue allotransplantation models with tolerance to the musculoskeletal element but eventual rejection of the skin element of the allograft (Hettiaratchy, S. et al. 2004).

It is possible that small numbers of donor cells are transferred in some non-HCT approaches with the achievement of microchimerism (i.e. detectable only by polymerase chain reaction). Some have suggested that a microchimeric state can lead to tolerance (Starzl, T. E. et al. 1992), and there is evidence of central deletion with microchimerism (Bonilla, W. V. et al. 2006). However, microchimerism and tolerance do not always correlate (Elwood, E. T. et al. 1997; Fuchimoto, Y. et al. 1999b; Wood, K. et al. 1996). The apparent disparity may be because the term 'microchimerism' is often used without specifying the donor cell type or their location (e.g. peripheral blood, bone marrow, thymus), meaning there are differing immunological processes occurring in different models all demonstrating 'microchimerism'.

HCT approaches are of particular interest in CTA because many allografts contain vascularised bone marrow. Donor marrow cells within CTAs may contribute to tolerance induction (Ozmen, S. et al. 2006). However, it is unclear what role they have in the maintenance of tolerance: Siemionow found that recipient marrow cells are substituted by donor cells over time in a rat model (Klimczak, A. et al. 2006); however, Mathes found in a pig model that the presence of donor cells within the allograft diminished over time with no evidence for donor substitution in recipient marrow (Mathes, D. W. et al. 2002). In addition, vascularised bone marrow may have limited application clinically: the bones contained within a hand transplant have minimal haematopoietic activity in adult life, and face transplants will contain little, or no, bone marrow. To counteract the possible effect of lack of bone marrow within the transplant, donor bone marrow infusions were given to the first facial allotransplant recipient (Kanitakis, J. et al. 2006).

2.5.2.1 Non-HCT approaches

2.5.2.1.1 Short course of immunosuppression

There are clinical reports of skin tolerance following just a short course of immunosuppression (Achauer, B. M. et al. 1986; Frame, J. D. et al. 1989). However, these were not formally studied to confirm pre-transplant alloreactivity or their immune status post-transplant.

A short course of FK506 in the MGH miniature swine induced tolerance to kidney allografts across full double-haplotype MHC barriers (Utsugi, R. et al. 2001). However, subsequently applied donor skin grafts were rejected, without rejection of the organ allograft.

2.5.2.1.2 T-cell depletion

Depletion of alloreactive T cells reduces the initial alloreactive response allowing development of peripheral tolerance mechanisms. This is often combined with a short course of immunosuppression to give further bias towards a tolerogenic versus an alloreactive state. This has been successful in small animals. Siemionow demonstrated prolonged survival of vascularised skin allografts in rats treated with αβTCR Ab and a short course of cyclosporine or FK506 (Demir, Y. et al. 2005). Strom attained skin graft tolerance across MHC barriers using rapamycin with an IL2-IL15 fusion protein that depleted cytopathic T cells while sparing regulatory T cells (Zheng, X. X. et al. 2003). In murine models CD4 and CD8 antibody blockade without T-cell depletion can achieve tolerance to class 1 MHC mismatch as well as minor mismatched skin allografts (Qin, S. X. et al. 1989) indicating that T cell blockade rather than actual depletion is important in achieving skin tolerance via peripheral mechanisms in small animals.

T-cell depletion has been less successful in large animals with only prolonged skin allograft survival (from 9.25 to 22-26 days) achieved in non-human primates by the administration of ATG (Preville, X. et al. 2001).

2.5.2.1.3 Costimulatory blockade

Costimulatory blockade is usually considered to act by preventing activation of alloreactive T cells. However, there is evidence that anti-CD154 may heighten the suppressive activity of regulatory cells as well (Jarvinen, L. Z. et al. 2003). Tolerance to

skin allografts has been achieved using costimulatory blockade in mice (Larsen, C. P. et al. 1996).

Simple costimulatory blockade has not been as successful in achieving skin tolerance in large animal models. Repeated intravenous injection of anti-CD154 achieved only a modest increase in skin allograft survival (7.3 to 13.3 days) across MHC barriers in primates. Survival was significantly prolonged with the addition of both rapamycin and DST (mean: 142.7 days) (Xu, H. et al. 2003). Also, repeated anti-CD154 antibody treatment given both intravenously and into the graft bed achieved markedly prolonged skin allograft survival to greater than >202 days (Elster, E. A. et al. 2001a) with only a marginal increase in survival (>236 days) with the addition of DST (Elster, E. A. et al. 2001b).

Other co-stimulatory molecules, including CD28 (Larsen, C. P. et al. 1996), CD134 (Habicht, A. et al. 2007) and OX40 (Demirci, G. et al. 2004), have all been shown to prolong skin allograft survival in murine MHC mismatch models. The utility of these in large animal models has not yet been reported on.

2.5.2.2 HCT approaches

2.5.2.2.1 HCT alone

The permissive immunological environment of the foetus *in utero* allows for HCT and engraftment without additional therapy. Tolerance is attained by central deletion, with peripheral mechanisms to control alloreactive T cells that escape thymic processing (Hayashi, S. et al. 2002). *In utero* induction of skin tolerance in mice was first demonstrated by Medawar (Billingham, R. E. et al. 1953). However, this was in part due to a fortuitous strain combination with only a MHC class 1 mismatch (CBA→A). *In utero* induction of skin tolerance has subsequently been attained, in the small animal, across MHC class 1 and 2 barriers (Kim, H. B. et al. 1998).

Skin grafts showed only prolonged acceptance (27 days vs. 7-9 days for controls) in swine with stable low-level multilineage chimerism (Mathes, D. W. et al. 2005). Interestingly, these animals did not demonstrate a second set reaction, or develop antibodies upon regrafting from the same donor; it is possible that this may have been

due to regulatory tolerance mechanisms that limited accelerated rejection following regrafting, but were not strong enough to completely prevent rejection.

Marginally prolonged secondarily vascularised skin allograft survival was demonstrated in primate models following donor leukocyte (Jonker, M. et al. 2001) and antigen (van Vreeswijk, W. et al. 1980) infusions. The mechanism of prolongation may be similar to following donor specific transfusion with stimulation of a regulatory response (Bushell, A. et al. 2003).

2.5.2.2.2 HCT and a short course of immunosuppression

In the MGH miniature swine model, administration of a 12-day course of cyclosporine induced tolerance to MHC-matched, minor mismatched musculoskeletal allografts (Lee, W. P. et al. 2001). Biopsies demonstrated non-inflammatory graft infiltrating lymphocytes indicating a possible regulatory mechanism (Baron, C. et al. 2001a). However, subsequent skin grafts (non-vascularised) from the donors were rejected, without breaking of tolerance to the musculoskeletal graft (Lee, W. P. et al. 1998) (a state of "split tolerance") demonstrating the skin's susceptibility to rejection.

In further development of this approach, a vascularised hind limb allograft which included a skin paddle was transplanted across a MHC-matched minor-mismatched barrier in six animals (Mathes, D. W. et al. 2003). The musculoskeletal element was accepted in all animals. In addition, one animal accepted the skin element of its vascularised graft with the others demonstrating split tolerance. This acceptor animal received a cryopreserved donor skin graft 120 days later. The skin graft was rejected by 60 days with simultaneous rejection of the epidermal element of the hind limb graft.

This finding suggests three things. Firstly, skin tolerance can be achieved across a minor mismatch barrier using this approach. The variability in success may have been due to a more close matching of minor antigens (although the skin graft rejection demonstrated that they were not completely matched), or may have been due to the recipient having a tolerant phenotype (Roussey-Kesler, G. et al. 2006). Secondly, the mode of transplantation may affect the outcome of skin transplantation, with acceptance of immediately vascularised skin while rejecting the skin graft. Thirdly, tolerance to the epidermis in this model can be broken more easily than to the dermal and musculoskeletal elements.

2.5.2.2.3 HCT and T cell/lymphocyte depletion

Transplanted donor HSCs (i.e. not transferred in the bone marrow contained in the graft) will not engraft in an adult without manipulation of the immunological environment. Some regimens have used high-dose irradiation to deplete alloreactive T cells and create "immunological space" to allow the donor HSCs to engraft in the recipient bone marrow. This has led to skin graft tolerance across MHC barriers in rodent models (Main, J. M. et al. 1955), and across a minor histocompatibility barrier in dog models (Tillson, M. et al. 2006; Yunusov, M. Y. et al. 2006). Other regimens have achieved HSC engraftment with lower doses of irradiation by the addition of T cell depleting antibodies. This approach has achieved skin graft tolerance across MHC barriers in the mouse (Cobbold, S. P. et al. 1986). In the MGH miniature swine, tolerance to skin grafts between MHC-matched, minor-mismatched animals was achieved in two out of six cases (Fuchimoto, Y. et al. 2001). The others rejected their skin grafts despite showing prior tolerance to a cardiac allograft (a state of split tolerance). Tolerance to the cardiac graft was not broken by rejection of the skin.

Some regimens have used T cell depleting antibodies without irradiation. Siemionow demonstrated tolerance to a hind-limb allograft (containing both vascularised bone marrow and skin) across a MHC barrier in rats conditioned with either anti-lymphocyte serum (Ozer, K. et al. 2003) or αβTCR Ab (Siemionow, M. et al. 2002; Siemionow, M. et al. 2004; Siemionow, M. Z. et al. 2003) followed by a short course of cyclosporine. The mechanism of tolerance induction was thymus dependent (Siemionow, M. et al. 2006), indicating a role for either central deletion and/or naturally occurring regulatory cells. In contrast, Waldmann achieved skin tolerance in murine MHC class 1 mismatch models with bone marrow transplantation following CD4 and CD8 antibody blockade instead of T-cell depletion. A peripheral tolerance mechanism is likely in this model as the mature T cells are not removed (Qin, S. X. et al. 1990). .

In the MGH miniature swine, tolerance was achieved to the musculoskeletal elements of a limb transplanted immediately following T-cell depletion with a porcine CD3 immunotoxin, pCD3-CRM9 (Huang, C. A. et al. 1999b) under the cover of a short course of cyclosporine across a full MHC mismatch barrier. However, the skin only showed prolonged acceptance of between 42-70 days (immunosuppression was stopped on day 30) (Hettiaratchy, S. et al. 2004). In two of the five long-term survivors, just the epidermis was rejected, with full-thickness skin rejection in the other three cases.

Peripheral mechanisms are likely to be involved in tolerance induction in this model because the induction regimen does not completely T cell deplete the recipient. The involvement of the dermis in skin rejection may, in some cases, be due to selective epidermal alloresponse with the secondary destruction of dermal bystander cells in an antigen non-specific manner (Doody, D. P. et al. 1994).

2.5.2.2.4 HCT and costimulatory blockade

Costimulatory blocking agents to the CD40/CD154 or CD28/B7.1/B7.2 pathway, and an increased HCT dose can achieve skin graft tolerance across MHC barriers without the need for irradiation or T-cell depletion in mouse models (Durham, M. M. et al. 2000; Seung, E. et al. 2003; Wekerle, T. et al. 1999; Wekerle, T. et al. 2000). In these models anergy, suppression and peripheral deletion are important in the induction of tolerance with central deletion being the dominant mechanism in the long-term maintenance of tolerance (Kurtz, J. et al. 2004; Wekerle, T. et al. 2002).

2.5.2.2.5 HCT and dendritic cells

Both recipient and donor dendritic cell infusions have been used in protocols attempting to achieve skin tolerance. Unactivated recipient dendritic cells loaded with donor antigen and injected prior to transplantation of a hind limb allograft across a major MHC barrier in rats achieved only a small increase in survival (8 vs. 5 days) (Nguyen, V. T. et al. 2007). This may have been via a thymic dependent mechanism (Garrovillo, M. et al. 2001). Beriou achieved tolerance to skin transplanted across a major allogeneic barrier in mice that were already tolerant to a cardiac transplant following infusion of immature bone marrow-derived recipient dendritic cells with a short course of a deoxyspergualin analogue (LF 15-0195) (Beriou, G. et al. 2005).

The use of donor dendritic cells has only achieved prolonged skin graft survival. Markees showed rapid rejection of major mismatched allogeneic skin grafted on to mice treated with Flt3-ligand induced donor dendritic cells, and only prolonged survival with the addition of anti-CD154 (61 vs. 7 days) (Markees, T. G. et al. 1999).

2.6 CONCLUSIONS

The widespread use of skin allotransplantation techniques would transform the field of reconstructive surgery. The risk-benefit ratio of immunosuppression is still an issue. Part A of this study attempts to further understand the risks involved in CTA.

For many a tolerogenic process would rebalance the risk-benefit ratio in favour of CTA. The methods used so far to induce tolerance have not achieved clinical tolerance against skin. The mode of skin transplantation, skin specific antigens and skin's composition may all contribute to the susceptibility of skin to rejection. Although there has been success in small animal models in achieving indefinite skin survival across MHC barriers, tolerance in the large animal model has only been attained across minor antigen barriers with prolonged survival between MHC mismatched animals.

It may not be always necessary to reach the ultimate goal of true tolerance to achieve a favourable risk-benefit ratio required for a wider-spread application of CTA techniques. Adequate reduction of systemic immunosuppressive toxicity may be possible with novel immunosuppressive therapies, site-specific adjuvant treatments, or by the induction of a less alloreactive state.

However, induction of skin tolerance offers the potential for transplantation free from immunological risk. This would facilitate the widest possible application of reconstructive transplantation techniques. Fifty-four years after Medawar first demonstrated that it was possible to induce tolerance to skin in a murine model, the Holy Grail of clinical skin tolerance has yet to be unearthed. Furthermore the barrier of transferring techniques that are effective in the small animal to large animal models is largely unbreached. Part B of this study explores an approach to induce tolerance to allotransplanted skin in a large animal model.

CHAPTER 3: MATERIALS AND METHODS

3.1 ANIMALS

All studies were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (MGH) and followed the policies outlined in the National Institutes for Health (NIH) *Guide for the care and use of laboratory animals.*

3.1.1 Rats

All procedures were performed using 8-12 week-old Lewis (LEW; RT-1^l) and Wistar Furth (WF; RT-1^u) rats (180-220g) obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN) as donor animals in the study examining the mechanism of skin rejection (Chapter 5), and as both donor and recipient animals in the study assessing recipient tissue damage following allograft failure (Chapter 4). Recipient animals in the study examining the mechanism of skin rejection were 8 week to 6 month old Lewis GFP transgenic rats (Rat Resource and Research Center (RRRC), Columbia, MO); these rats were derived from Lewis rats obtained from Harlan. Syngenicity between the donor Lewis rats (from Harlan) and recipient Lewis GFP transgenic (Lew-GFP) rats (from RRRC) was confirmed by observing conventional skin graft survival between the two strains (Lew→ Lew-GFP) for >100 days.

3.1.2 Swine

Three breeds of swine were used in the experiments: Massachusetts General Hospital (MGH) MHC-inbred miniature swine, Hanford mini-swine, and Yorkshire outbred swine.

3.1.2.1 Massachusetts General Hospital (MGH) MHC partially-inbred miniature swine

Donor and recipient animals for the adult chimerism induction (Chapters 6, 7 and 10), skin flap model (Chapter 8) and site-specific therapy studies (Chapter 11), and donor animals for the *in utero* chimerism induction study (Chapter 9) were selected from our herd of MGH partially-inbred, MHC-defined miniature swine; more detailed immunogenetic characteristics of the herd have been previously reported (Sachs, D. H. et al. 1976; Sachs, D. H. 1992). This is a unique herd of partially inbred animals that have been developed over the last 30 years. The MHC loci of these animals have been fixed for both class I and II by inbreeding. Three main lines are maintained and are

referred to by their swine leukocyte antigen (SLA (the swine equivalent of human leukocyte antigen (HLA)) types A, C and D. By interbreeding, any combination of these three haplotypes can be created (fig 3.1). Only the MHC has been fixed in each line, with non-MHC minor antigen differences being maintained. This allows transplantation to be performed across a range of MHC and non-MHC minor disparities, simulating potential clinical immunological mismatch combinations.

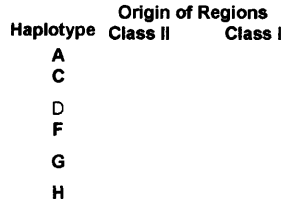


Figure 3.1 Massachusetts General Hospital (MGH) miniature swine. The immunogenetics of the MGH miniature swine (a) show the different major histocompatibility complex (MHC) types for both class I and II. A three month old animal is shown in (b).

One line of the SLA^{dd} haplotype pigs has been further inbred in an attempt to eliminate minor antigen differences. MGH MHC defined inbred miniature swine of SLA^{dd} (class I^d/II^d) between 2 and 6 months were used as bone marrow donors for the *in utero* study (Chapter 9). These animals were offspring of at least 12 generations of inbreeding with a coefficient of inbreeding of >94%. Tolerance to reciprocal skin grafts has been observed since reaching 7 generations of inbreeding (Mezrich, J. D. et al. 2003).

Donors ranged in age from 6 months to 2 years. Recipients for the adult chimerism induction studies were from 8 to 12 weeks at the time of HCT. Recipients for the skin flap model and site-specific therapy studies ranged in age from 3 months to 2 years.

In the adult chimerism induction studies, donors and recipients were chosen to differ by at least one haplotype at both class I and class II loci. All donor-recipient combinations were chosen so that the donors were either heterozygous or homozygous for the SLA^c haplotype, whereas the recipients contained only SLA^a or SLA^d haplotype combinations. To facilitate the detection of chimerism, all donors were chosen to be positive for Pig Allelic Antigen (PAA), a non-histocompatibility cell-surface antigen that is present on all differentiated haematopoietic cells in animals that express this allele (Fuchimoto, Y. et al. 1999a). All recipients were chosen to be PAA-negative.

3.1.2.2 Hanford mini-swine and Yorkshire swine

Unborn litters of pregnant sows selected from either Hanford mini-swine (Sinclair Research Center, Columbia, MO) or Yorkshire swine (Parsons Farm, Springfield, MA) were used as recipients for the *in utero* study (Chapter 9). They were selected because they are genetically and immunologically distinct from the MGH miniature swine. Consequently, both breeds could be used as recipients from MGH miniature swine donors to test tolerance induction across a full MHC barrier. Both Hanford and Yorkshire breeds are outbred and immunologically heterogeneous; to confirm a full MHC mismatch to MGH miniature swine, the animals selected for the *in utero* study had to be screened by mixed lymphocyte response (MLR).

3.2 HAEMATOPOIETIC STEM CELL TRANSFER (HCT) AND DONOR LEUKOCYTE INFUSION (DLI) PROTOCOLS

The HCT protocol to induce chimerism consisted of irradiation, T-cell depletion and haematopoietic cell infusion from a MHC mismatched donor, with cyclosporine cover for the peri/post infusion period; the amount and combination of these were varied during the time period analysed as the protocol was refined (see table 3.1) (Cina, R. A. et al. 2006; Fuchimoto, Y. et al. 2000; Horner, B. M. et al. 2006; Huang, C. A. et al. 2000).

PROTOCOL	Irradiation (Location:Dose)	T-cell depletion (Yes/ No)	Cyclosporine (Course Length)
A	Thymic: 700-1000 cGy	Yes	30 days
B			60 days
C	None	Yes	30 days
D			60 days
E	Whole Body: 100 cGy	No	45 days
F		Yes	45 days
G			30 days

Table 3.1 HCT protocols. Summary of the amount and combination of each of the elements within the different HCT protocols used in the animals analysed.

Irradiation and T-cell depletion were administered two days prior to HCT. Irradiation was delivered from a Cobalt source (Fuchimoto, Y. et al. 2000; Gleit, Z. L. et al. 2002b). T-cell depletion was achieved using a single intravenous dose of 0.05mg/kg pCD3-CRM9 immunotoxin two days prior to HCT. This conjugate toxin consists of an antibody subunit that selectively binds to porcine CD3 and a diphtheria toxin subunit which then kills the bound cells (Huang, C. A. et al. 1999b).

Donor animals haematopoietic cells were cytokine mobilised for 5 to 7 days with either recombinant porcine interleukin-3 (IL-3) and porcine stem-cell factor (SCF) (each at a dose of 0.1mg/kg for the first 30 kgs, and 0.05 mg/kg for each additional kg (Immerge Biotherapeutics, Cambridge, Massachusetts)), or by recombinant human granulocyte-colony simulating factor (10 μg/kg (Filgrastim, Amgen). Peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis (COBE BCT Inc., Lakewood, Colorado, USA) beginning on the fifth day of cytokine therapy and continuing until the target cell number was attained. Following the initial leukapheresis, $1x10^9$ to $15x10^9$ PBMCs per kg were infused intravenously daily until the target dose was achieved.

Enteral cyclosporine-A (CyA; Sandimmune) was administered via a gastrostomy tube, beginning one day prior to the mobilised PBMC infusion and continuing for 30 to 60 days. CyA whole blood levels were maintained between 300-800ng/mL for the first 30 days before being tapered in the animals receiving a longer course.

Some animals subsequently received a DLI. Non-mobilised leukocytes were collected by leukapheresis from either the original haematopoietic cell donor or a MHC-matched animal and then infused intravenously into the recipient at a dose adjusted to include $5x10^7$ donor T cells/kg of recipient body weight. One animal received a sensitised DLI: the DLI donor was pre-sensitised with a skin graft from a MHC recipient matched animal (i.e. MHC-mismatched to the donor) 10 weeks prior the DLI. DLI was defined as ineffective if there was no sustained increase in peripheral blood chimerism, thereby excluding the small, transient rise in lymphocyte chimerism seen immediately after DLI infusion due to the donor cells within the infusion.

In Chapter 10, animals underwent leukapheresis prior to DLI. This was performed in the same way as leukapheresis to collect the cytokine mobilised PBMCs.

3.3 *IN UTERO* BONE MARROW TRANSPLANTATION PROTOCOL

Bone marrow was harvested from the inbred SLA^{dd} donor pig. The inoculum was prepared by T-cell depletion of a portion of the bone marrow and then addition of unmanipulated bone marrow to attain a T cell level of 1.5%. The inoculum was then injected into the foetuses.

3.3.1 Bone Marrow Harvest and Processing

The donor animal was exsanguinated and long bones and vertebrae were harvested sterilely. Bone marrow fragments were removed and processed into a single cell suspension. The cells were washed with Roswell Park Memorial Institute media (RPMI) supplemented with 5% DNAase, lysed with ammonium chloride potassium lysing solution and stored in media consisting of RPMI 1640 supplemented with 5% donor animal serum, 1mM glutamine, 50 U/ml penicillin, 50μg/mL streptomycin, 50μg/mL gentamicin and 5% DNAase.

3.3.2 T-cell depletion

Bone marrow cells were coated with murine antibody 898H2-6-15 (IgG2a anti-swine CD3) for 30 minutes. Cells were then washed in Hanks balanced salt solution (HBSS) three times and incubated with magnetic beads coated with rat, anti-mouse IgG2a/b antibodies for 15 minutes. Cell were then washed three times and passed through a magnetic separation column to remove CD3+ cells.

3.3.3 *In vitro* confirmation of T cell composition of inoculum

Harvested bone marrow was assessed for its T cell content using flow cytometry prior to manipulation, after T-cell depletion, and following reconstitution to make up the inoculum. The T cell depleted bone marrow was mixed with unmanipulated bone marrow with the aim of attaining an inoculum containing 1.5% T cells (from now marrow that has undergone this manipulation is described as 'Add Back' bone marrow).

3.3.4 *In utero* bone marrow transplantation

Bone marrow cells were harvested about 24 hours prior to injection. Bone marrow processing commenced immediately following harvest, with injection into foetuses within hours after completion of processing. Transplantations were performed during mid-gestation (day 55-56 of a full gestation of approximately 113 days) in each pregnancy. The pregnant sow was brought to the operating room and underwent a laparotomy to expose the uterus. Under ultrasound guidance, the bone marrow inoculum was delivered into the hepatic vein of each foetal pig via transuterine injection. Each bone marrow inoculum consisted of $1.3\text{-}5\text{x}10^8$ cells suspended in 1 ml normal saline aiming for a total dose of $2\text{x}10^9$ cells per kilogram. Injections were performed using a 3-cc syringe with a 25-gauge spinal needle using live stream ultrasonography. At the end of the procedure the sow's abdomen was closed and the animal returned to her cage for recovery. All sows received 22mg oral progestin (Regumate, Hoechst Roused Vet, Warren, NJ, USA) per day beginning 3 days prior to injection, continuing until at least gestation day 100.

3.3.5 Freezing and thawing of bone marrow

Bone marrow remaining after each round of *in utero* injections was frozen. The bone marrow cells were mixed sterilely at a concentration of $5\text{x}10^7$/ml in media consisting of IMDM, 20% foetal porcine serum, 10% DMSO, 40ug/ml α-tocopherol acetate,

100ug/ml catalase, 80ug/ml ascorbic acid. Two 1ml aliquots were also frozen as test vials.

Frozen bone marrow was thawed in a 37°C water-bath and then immediately diluted with HBSS supplemented with DNAse 1mg/ml at a ratio of 1:12 cells:solution. The cells were then washed 3 times in 0.9% saline.

3.3.6 *In vitro* comparison of haematopoietic growth potential of bone marrow

Different preparations of bone marrow were analysed using CFU and CAFC assays as described below. The preparations of bone marrow included 1) unmanipulated bone marrow 2) T cell depleted bone marrow 3) T cell depleted bone marrow with unmanipulated bone marrow added back to bring the T cells to 1.5% (as in the inoculum for *in utero* injection).

3.3.6.1 Cobblestone area-forming cell (CAFC) assay to assess in vitro growth potential of the bone marrow inoculum

This assay has been described in detail (Ploemacher, R. E. et al. 1989). Briefly, bone marrow cells from different preparations were plated over a series of dilutions in 96-well plates on pre-established confluent murine stromal cell lines and cultured at 37°C and 5% $C0_2$ for 5 weeks in medium consisting of Myelocult (H5100; Stem Cell Technologies) supplemented with 25 ng/ml porcine stem cell factor, 2ng/ml porcine interleukin-3, and 10^{-6}M hydrocortisone (Sigma, St Louis, MO, USA). Percentages of wells with at least one phase-dark haematopoietic colony of at least five cells (i.e. cobblestone area) beneath the stromal layer were determined bi-weekly. Frequencies were calculated using 'L-Calc' (Stem Cell Technologies), and were compared among the different bone marrow preparations.

3.3.6.2 Colony forming unit (CFU) assay to assess in vitro growth potential of the bone marrow inoculum

Mononuclear cells from the initial bone marrow were plated at concentration of 2.5 x 10^4 cells in 35-mm Petri dishes in a total volume of 1.5mL methylcellulose-based medium (Methocult H4230; Stem Cell Technologies, Vancouver BC, Canada) supplemented with 11 ng/ml recombinant porcine stem cell factor, 0.85 ng/mL recombinant porcine interleukin-3, 1ng/mL recombinant porcine granulocyte-colony stimulating factor, and 0.85 U/mL recombinant human erythropoietin (Amgen Inc,

Thousand Oaks, CA, USA). Following 10 to 14 days incubation in 5% CO_2 at 37°C, each culture dish was visually scored through an inverted microscope and evaluated for presence of burst-forming unit-erythroid (BFU-E), colony-forming unit granulocyte-monocyte (CFU-GM), and colony-forming unit granulocyte-erythroid-monocyte-megakaryocyte (CFU-GEMM). Only colonies containing >50 cells were counted and classified based on morphology

3.4 RAT SURGICAL PROCEDURES

Both donor and recipient animals were deeply anesthetized with an intra-peritoneal injection of sodium pentobarbital (50mg/kg). All animals were monitored continuously for heart rate, respiration, body temperature and adequate analgesia while under anaesthesia. The abdomen and the lower limb of the donor and recipient were shaved, and the donor was also depilated with a commercially available depilatory ointment, Nair® (this facilitated easy differentiation of donor and recipient skin during confocal imaging). The surgical area was prepped with povidone iodine solution. All procedures were performed using sterile technique.

3.4.1 Musculocutaneous flap transplant

In donor animals, the composite musculocutaneous flap, containing both the epigastric skin flap and gastrocnemius muscle, was raised based on the femoral vessels. An oblique quadrangular skin flap was marked out (as described by Nishikawa (Nishikawa, H. et al. 1991)), raised with the underlying subcutaneous tissues, and isolated on the epigastric vessels. The gastrocnemius muscle was isolated on the sural vessels, which in turn were isolated on the femoral vessels. The composite flap was removed from the donor by dividing the femoral vessels at the inguinal ligament. The flap was flushed with 1ml of heparinised saline solution. The donor animal was then euthanized while still anesthetized with an overdose of pentobarbital (200mg/kg i.p.).

In recipient animals an incision was made in the groin crease on the contra-lateral side to which the musculocutaneous flap had been harvested from the donor. The femoral vessels were exposed distal to the inguinal ligament, isolated, clamped with haemoclips, and divided proximal to the origin of the epigastric vascular pedicle. The vessels were flushed with heparinised saline solution. Using microsurgical technique the donor and recipient femoral vessels were anastomosed end-to-end using 10-0 nylon suture under magnification of 6-25x as required. An average of eight sutures was used for both the

artery and the vein. After completing the anastomosis the venous clamp and then the arterial clamp were released.

In the experiment examining the mechanism of skin rejection (Chapter 5) the transplanted flap was simply secured into place. In the experiment assessing damage to recipient tissues (Chapter 4) the recipient tissue bed for the transplanted flap was prepared so that the composite tissue allograft would overlie recipient tissues commonly found in the bed of clinical composite tissue allografts (i.e. muscle, fascia and cartilage; see fig 3.2a).

a. b.

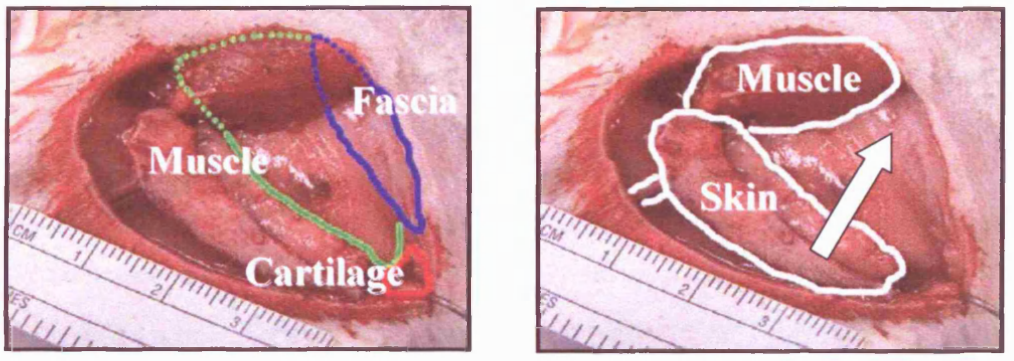


Figure 3.2 Positioning of the rodent musculocutaneous flap. (a) Recipient tissues are prepared exposing rectus fascia (blue outline), abdominal muscles (red outline) and cartilage (green outline) of the inferior pubic ramus. (b) Muscle and skin elements (white outlines) of the allograft are carefully positioned and then secured so that they overlay the different recipient tissues commonly found in contact with each of those elements following clinical composite tissue transplantation (arrow indicates final position of medial end of skin flap element).

An ellipse of skin was excised from the superio-medial aspect of the recipient's groin wound exposing the rectus sheath. The superior edge of the wound was dissected off the underlying abdominal muscles and rectus fascia creating a subcutaneous pocket. Muscles inserting into the anterior pubic ramus were dissected back to expose the pubic bone, which is still cartilaginous in the young rats used in these studies. The composite musculocutaneous allograft was carefully positioned and secured with 6.0 prolene sutures (see fig 3.2b). The gastrocnemius was placed to lie in the subcutaneous pocket overlying recipient abdominal muscles and rectus fascia, and underlying recipient skin. The skin flap was placed so that it overlay abdominal muscles, rectus fascia and

cartilaginous pelvis to which it was secured with a single 5.0 prolene stitch looping around the inferior pubic ramus passing through the pubic ring. All animals received buprenorphine 0.03mg/kg subcutaneously 12 hourly for 3 days post-operatively.

3.4.2 Conventional skin graft transplant

On both donor and recipient animals a 2x2cm rectangular piece of skin was raised over the posterior thorax, taking care to remove the panniculus carnosis. The skin raised from the donor was then secured to the margins of the wound bed of the recipient using 5-0 prolene interrupted sutures with the ends left long; these stitches were then used to secure a gauze tie-over dressing in place.

3.4.3 Injection of Evans blue dye

To visualize blood vessels within the transplanted skin, Evans blue dye was injected. The Evans blue dye within the vessel is distinguished from GFP positive recipient cells because it is detected at a different wavelength on confocal microscopy, this facilitates the assessment of recipient cell clustering around the vasculature. A groin incision on the contralateral side to the flap was made to expose the femoral vessels. A 1% (w/v) solution of Evans blue dye in phosphate buffered saline was mixed in equal parts with a 5% (w/v) solution of bovine albumin. A 2.5ml aliquot of the solution was injected into the right femoral vein with a 30G needle under magnification. The groin wound was closed directly with 4.0 Vicryl.

3.5 SWINE SURGICAL PROCEDURES

The swine composite tissue allotransplantation procedures used in these studies were developed as part of this study and are included in Chapter 7 as part of the results.

3.6 SITE SPECIFIC THERAPY: TOPICAL FK506

Some skin allograft recipients received topical 0.1% FK506 ointment (Astellas, Deerfield, IL) applied daily. No dressing was placed over the treated area. Systemic FK506 levels were monitored weekly.

3.7 ASSESSMENT OF REJECTION AND TOLERANCE

In rats, rejection and its consequences were followed *in vivo* with confocal microscopy and subsequent histological analysis. Recipient-anti-donor rejection response was assessed in rats using MLR and antibody assays.

In swine, tolerance was tested both *in vivo* with placement of an allograft, and *in vitro* by combination of CML, MLR and antibody cytotoxicity assays.

3.7.1 *In vivo* video rate confocal microscopy

Recipient cell trafficking in allotransplanted skin flaps and grafts were analysed using fluorescence confocal microscopy. Animals were anaesthetized and placed on the stage of a video-rate scanning laser confocal microscope platform (Sipkins, D. A. et al. 2005). High-resolution images were obtained at 30 frames-per-second, with 30 frame averaging, through intact rat skin at depths of up to 275μm from the surface using a 30x 0.90NA (Lomo, St. Petersburg, Russia) and a 60x 1.2NA water-immersion objective lens (Olympus, Melville, NY) providing a field width of either 330μm or 660μm. At each location a stack of images at depth increments of 25μm from the skin surface were obtained. In some animals Evans blue dye was injected (as described above) to visualize the vessels. GFP positive recipient cells were excited with a helium neon laser at 491nm (Dual Calypso, Cobolt AB, Stockholm, Sweden) and Evans blue dye at 638nm (Radius, Coherent Inc., Santa Clara, CA); these were then detected with a photomultiplier tube at 507nm and 610nm respectively through a +/- 27.5nm bandpass filters transmitting 500-550nm (Chroma, Rockingham, filter (Omega Optical, VT) and 667.5-722.5nm (Omega Optical, Brattleboro, VT), respectively.

Images were attained within 4 hours of transplantation, on days 1, 2, 3, 4 and then 3 times over the following week. Isogeneic transplants were imaged up to at least 10 days after transplantation. It was not possible to attain images from allogeneic transplants at timepoints later than 4 days because of autofluorescence on the skin surface due to cell death caused by the rejection process.

Ischaemia-reperfusion studies have demonstrated that initially the distal edges of a primarily vascularised skin flap have the poorest blood supply (Carroll, W. R. et al. 2000; Kuntscher, M. V. et al. 2002). This property was utilised to assess the importance of the vasculature as a route for cellular influx. Image stacks were obtained from areas at the centre of the transplanted skin as well as areas along the edges furthest from the pedicle at each timepoint. Comparison was then made between the centre and edge of the transplanted skin for variations in cellular influx secondary to differences in blood flow.

Evaluation of infiltrating cell numbers was made by identifying the depth of greatest cell density and then counting the number of cells per field at that depth. Evaluation of clustering of infiltrating cells around vasculature and hair follicles was performed by first merging the location of all vessels and hair follicles in the stack on to a single image. Vessels and the number of cells within 20μm of hair follicles within 40μm of each other were eliminated to avoid double-counting. The number of cells within 20μm of a hair follicle or part of a vessel were counted at the depth best visualization of the structure and compared to the number of cells in an immediately adjacent area of exactly the same size that was not within 20μm of another blood vessel or hair follicle (again, to avoid double-counting).

3.7.2 Histological assessment of rat biopsies

In the recipient tissue damage study (Chapter 4) biopsies were taken of the donor and recipient tissues including each interface at which they came into contact with each other; in the confocal study (Chapter 5) centre and edge biopsies were taken from donor tissues of each experimental animal at sacrifice. These were placed immediately into either cryomedia (Tissue-Tek®. Sakura Finetek U.S.A, Inc. Torrence, CA), and then kept at -80°C, or 4% paraformaldehyde solution for 24 hours and then transferred to phosphate buffered saline before processing. Haematoxylin and eosin stains were obtained for all specimens. Donor skin and muscle rejection was confirmed histologically. An objective histological grading scale was constructed to score recipient tissue damage for the experiment described in Chapter 4 (outlined in table 3.2, with examples of different grades of recipient muscle damage in figure 3.3). The recipient tissue at the interface between the donor and recipient was then examined by a qualified pathologist in a blinded fashion and scored for damage on the histological grading scale. Haematoxylin and eosin stains were obtained for all specimens. Some specimens received immunostaining with mouse monoclonal antibodies [3D6] to MHC Class II and [15-11C5] to CD8 (Abcam, Cambridge, MA). The slides were examined by a qualified pathologist in a blinded fashion.

	GRADE OF RECIPIENT TISSUE DAMAGE			
	G0 (No Damage)	**G1 (Mild)**	**G2 (Moderate)**	**G3 (Severe)**
Recipient Muscle	Undamaged	Oedema Wavy fibres, Decreased striations	Myocyte dropout, Focal mononuclear infiltrate	Multifocal Necrosis
Recipient Cartilage	Undamaged	N/A[a]	Focal Chondromalacia	Multifocal Chondramalacia
Recipient Fascia	Undamaged	N/A[a]	Focal Mononuclear infiltrate	Multifocal Destruction
Recipient Skin	Undamaged	Oedema	Mononuclear infiltrate Focal necrosis	Multifocal Necrosis

Key

a Mild (G1) damage to recipient cartilage and fascia was not identifiable histologically

Table 3.2 Histological grading scale for recipient tissue damage in rats

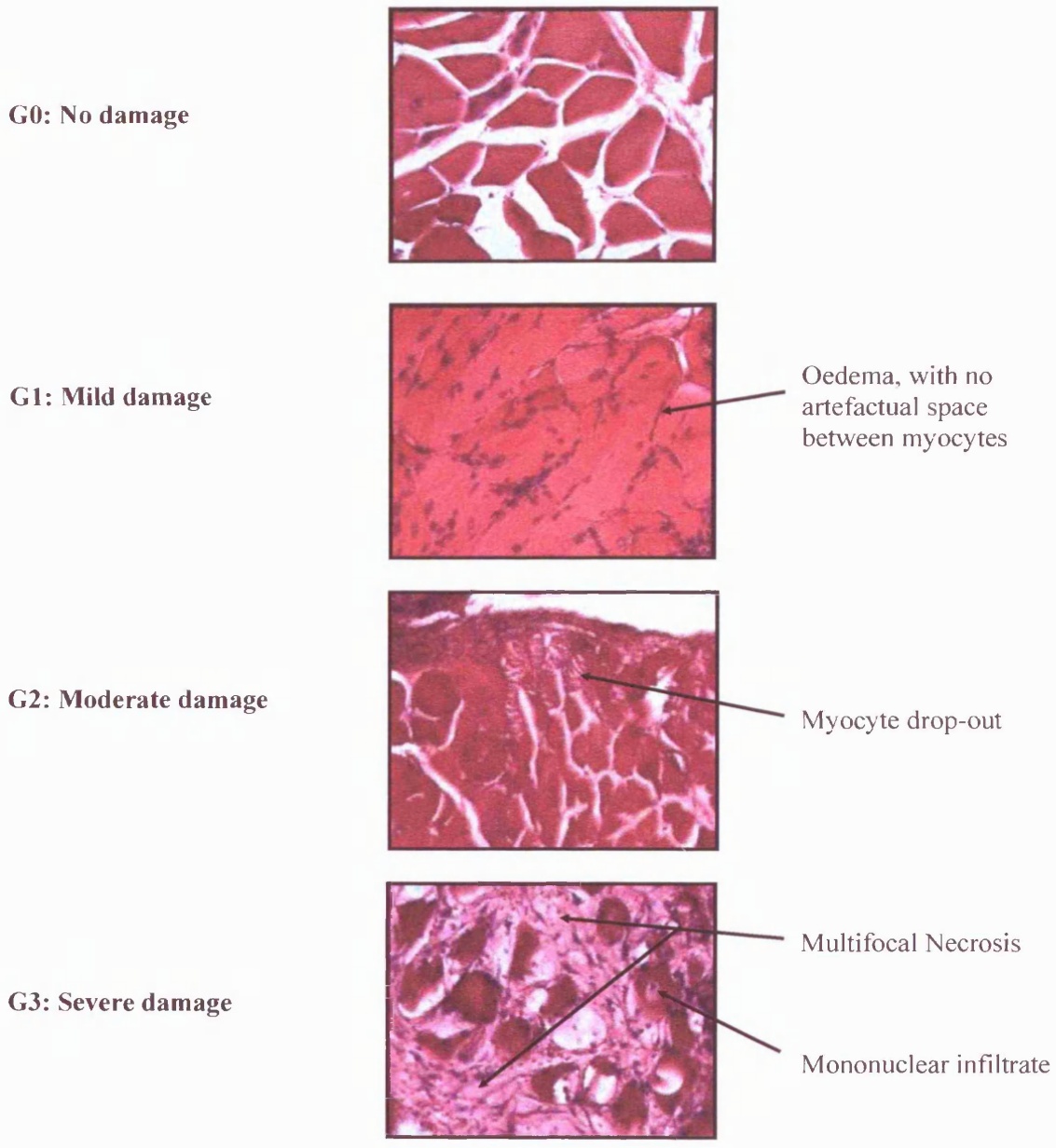


Figure 3.3 Examples of each grading of recipient muscle damage using the histological grading scale (x25 magnification)

3.7.3 Assessment of donor-anti-recipient alloreactivity in rats

3.7.3.1 Antibody detection by flow cytometry analysis

Antibody response to the composite tissue transplant was assayed by flow cytometry using sera collected at full rejection (or the time of biopsies in the isografted animals) to stain peripheral blood mononuclear cells (PBMC) MHC matched to the donor. Briefly, 10 µl of serum from each recipient was added to $1x10^6$ cells of WF ($RT1\text{-}1^u$) and/or Lew ($RT1\text{-}1^l$) PBMC. Following 30 minutes incubation with serum, cells were washed twice and incubated with a fluorescein conjugated secondary antibody (FITC goat anti-rat IgM and IgG, 3010-02, Southern Biotech, Birmingham, AL). Sera from a previously skin grafted animal was used as a positive control. Detection of antibody was reported as a difference in mean fluorescence intensity when compared to the reaction against recipient-matched PBMCs. The level of detectable antibody was also titered by serial dilutions of the sera samples.

3.7.3.2 Mixed Lymphocyte Response (MLR) assay: responder and stimulator cells were harvested from spleens taken from naïve rats and recipient rats at the time of allograft rejection (or at a matched time point in the isografts). Responders ($4x10^5$) and stimulators ($4x10^5$) irradiated with 25 Gy were cultured together in 200 µl of MLR media (RPMI 1640, hepes, gentamicin, NEAA, L glutamine, 2ME and foetal calf serum) in triplicate, and incubated for 3 days at 37°C in 5% CO_2 and 100% humidity. On the third day 1 µCi of 3[H] thymidine was added to each well and the cultures were incubated for 18 hours. Proliferation of responder cells was assessed by the uptake of ^3H thymidine and measured on a beta counter as counts per minute (cpm). Proliferation of recipient responder cells to donor stimulator cells was quantified with a stimulation index (calculated as the ratio of the proliferation of recipient cells to donor (cpm): recipient cells to self (cpm)).

3.7.4 *In vivo* assessment of tolerance in Swine

Tolerance in chimeras in Chapter 6a was tested *in vivo* by the acceptance or rejection of an organ or tissue allograft. Kidney and heart allografts were used in the chimeras assessed for the development of the hypothesis to induce skin tolerance (Chapter 6). A vascularised skin flap allotransplant was used to test the hypothesis (Chapter 10). Transplantation of organ allograft was performed using previously described techniques (Kirkman, R. L. et al. 1979; Madsen, J. C. et al. 1996). The technique of skin flap

transplantation is described in more detail in Chapter 7. Allografts were regularly biopsied for histological assessment. Samples were prepared for light microscopy using standard techniques. A board-certified pathologist assessed the histological slides in a blinded fashion. In addition, organ transplant function was assessed as an indicator of rejection: serum creatinine was measured initially daily and then when clinically indicated in renal allografts, and electrocardiographic analysis was performed weekly on heart allografts (Madsen, J. C. et al. 1996). Tolerance was defined as acceptance of the allograft, with no histological findings of rejection, and, in the case of the organ transplants, stable function (Kidney: Cr<2; heart: normal ECG), for greater than 3 months.

3.7.5 *In vitro* assessment of tolerance in swine

CML and MLR assays were used to assess T lymphocyte tolerance (i.e. unresponsiveness) to donor in vitro. Yucatan peripheral blood mononuclear cells were used to assess 3rd party response. These assays were performed as previously described (Fuchimoto, Y. et al. 2000; Gleit, Z. L. et al. 2002a; Thistlethwaite JR, J. r. et al. 1984). Antibody cytotoxicity assays were used to assess B-cell tolerance.

3.7.5.1 Mixed Lymphocyte Response (MLR) assay

Primary MLR and primary coculture MLRs were performed. In primary MLR, responders ($4x10^5$ cells) and stimulators ($4x10^5$; irradiated with 25 Gy) were cultured together. In primary coculture MLR, $2x10^5$ responders were incubated with $4x10^5$ stimulators; suppression was tested by adding $2x10^5$ PBMC from a mixed chimera to the baseline cultures thereby increasing the total number of responders to $4x10^5$. The responders and stimulators were plated at 200µl/well in triplicate, and incubated for 5 days at 37°C in 5% CO_2 and 100% humidity. Proliferation of responder cells was assessed by measuring the uptake of H^3 thymidine after 5 hours of incubation.

3.7.5.2 Cell-Mediated Lymphocytotoxicity (CML) assay

Responders and stimulators ($4x10^6$, irradiated with 2,500 cGy/mL) were cultured together and incubated in 2 ml of medium for 6 days at 37°C in 5% CO_2 and 100% humidity. Targets were labelled with ^{51}Chromium (^{51}Cr) (Amersham Pharmacia, Arlington Heights, IL) and plated in 96-well round-bottomed plates with effector cells at four effector:target (E:T) ratios (100:1, 50:1, 25:1, 12.5:1). ^{51}Cr release in the supernatant was determined using a gamma counter and compared to background and

maximum release (expressed as percent specific lysis (PSL)). An animal was considered responsive if PSL was >15% over background at 100:1 E:T ratio with reduction on two successive E:T titrations; hyporesponsive if PSL was 5-15% over background at 100:1 E:T ratio with reduction over two successive titrations; and unresponsive if PSL was <5% over background with no significant change over successive titrations.

3.7.5.3 Antibody cytotoxicity assay

This assay was used to assess a group of SLA^{ad} MGH miniature swine chimeras that had undergone chimerism induction and organ transplantation from SLA^{ac} donors. Target cell suspensions (SLA^{ac} PBMC) in medium 199 (Cellgro, Herdon, VA), supplemented with 2% foetal calf serum (culture medium), were incubated with serially-diluted heat-inactivated experimental animal serum samples, or foetal porcine serum (negative control), for 15 min at 37°C, and then with diluted rabbit complement (1:8 in medium 199). Dead cells were counted by 7-Aminoactinomycin (Sigma-Aldrich, MO). Serum cytotoxicity was considered positive if the percentage of cells lysed was >20% more than negative control in 1:8 serum dilution with reducing toxicity over at least three successive dilutions of sera.

3.8 ASSESSMENT OF CHIMERISM

Animals were assessed for peripheral blood donor chimerism by fluorescence activated cell sorting (FACS), thymic donor macrochimerism (defined as detectable by FACS) and/or donor microchimerism (defined as only detectable by polymerase chain reaction (PCR) and Southern Blot analysis). Mouse data indicates that detection of bone marrow colony forming units (BM-CFUs) longer than 12 weeks after HCT correlates with engraftment of HSCs (Christensen, J. L. et al. 2001). Consequently, presence of donor-derived BM-CFUs (detected by PCR analysis) greater than 12 weeks after PBMC transplantation, was used to indicate the presence of haematopoietic stem cells. As part of this study (Chapter 6a) it was found that thymic chimerism and multilineage peripheral blood chimerism present at 14 weeks always correlates with presence of BM-CFUs (Horner, B. M. et al. 2006). Consequently, from Chapter 7 onwards engraftment was defined as the presence of any of these three markers (donor-derived BM-CFUs, thymic chimerism or multilineage peripheral blood chimerism) at 14 weeks post HCT.

3.8.1 Flow cytometry

Flow cytometry was performed on a Becton-Dickinson FACS scanner (San Jose, Ca), as previously described (Fuchimoto, Y. et al. 1999a). A full range of SLA class specific mouse anti-swine monoclonal antibodies have been developed, allowing differentiation to be made between donor and recipient tissues and cells in the MGH miniature swine. The following swine specific antibodies were used: CD3ε (898H2-6-15, mouse IgGaK)(Huang, C. A. et al. 1999a), CD4 (74-12-4, Mouse IgG2bK), CD8α (76-2-11, mouse IgG2aK), CD172 (744-22-15, mouse IgG1K) (Pescovitz, M. D. et al. 1984; Pescovitz, M. D. et al. 1985; Saalmuller, A. et al. 1994; Smith, R. E. et al. 2003), CD5(Pescovitz, M. D. et al. 1998), CD1 (76-7-4, mouse IgG2aK), CD16 (G7, mouse Ig), CD25 (231-3B2, mouse IgG1) , SLA^{d} Class 1 (2.12.3A, mouse IgM), PAA (1038H-10-9, IgMK)(Fuchimoto, Y. et al. 1999a) and FoxP3 (FJK-165, eBioscience), with human CD16 (3G8, BD Pharmingen) used as a negative control. For assessment of chimerism, PAA staining was used to distinguish donor- and recipient-origin cells (Sachs. D. H. 1992). Monocyte and granulocyte chimerism was determined by gating on CD172-positive mononuclear cells and granulocytes respectively. Three colour staining was used to determine the proportion of CD4+CD25+ FoxP3+ cells. The absolute number of CD4+CD25+FoxP3+ cells in the animal was then calculated from the absolute white cell count of the animal at that time point. Peripheral blood chimerism was considered detectable if the percent of PAA staining cells was at least 0.5% above the background staining seen with the isotype matched control.

3.8.2 PCR detection of donor-derived DNA

Assessment of donor chimerism was performed in rats and swine. In rats PCR analysis was used in the mechanism of skin rejection study (donor Wistar Furth→recipient Lewis rats). In swine PCR analysis was used in the in utero study (donor SLA^{dd}→recipient outbred swine), and the adult chimerism induction study (donor SLA^{ac} →recipient SLA^{ad}). For all PCR reactions genomic DNA was isolated with the DNeasy Kit (Qiagen, Valencia, CA) according to manufacturer's directions.

3.8.2.1 Wistar Furth and Lewis DNA microsatellite repeat PCR Analysis

Biopsies of recipient tissues (allograft, bone marrow, thymus, lymph node, spleen, blood) were taken at sacrifice. Donor origin (Wistar Furth; Harlan) and recipient origin (Lew-GFP; RRRC) cells within recipient tissues were differentiated by PCR analysis of

two microsatellite repeat regions which differ in length between each strain. The microsatellite repeat D1Mgh14 is 128 base-pairs (bp) long in Wistar Furth and 156bp long in Lewis; it was amplified with the primer pair Fam-CCGCACTGAGCTCTCAGAG (F) and CCCAACCATTGAGCTAGTAAGG (R). The microsatellite repeat D11Mgh3 is 180 bp long in Wistar Furth and 140bp long in Lewis; it was amplified with primer pair Fam-GGAGCTGAAATACGAGAGAAATAA (F) and GTCCTGCTGGCTGTGCAT (R).

PCR amplification was performed using a PTC-100 programmable Thermal Cycler (MJ Research Inc., Watertown, MA) with template denaturation at 94°C for 15 minutes, and 44 cycles of melting at 94°C for 15 seconds, annealing at 54°C for 15 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for ten minutes. A mixing assay indicated that the strains could be distinguished down to a relative concentration of 1% WF:99% Lew-GFP.

3.8.2.2 MHC class I^d and Class II^d DNA PCR analyses

For Class I^d PCR primers GAGGCCCTGGAGCAGAAG (sense 5') and GCCTTCCTCTATCTGGTAGTTGTG (antisense 3') were used. For Class IId PCR primers 569 (CGAGTGCTACTTCTACAACGGA exon 2) and 571 (GTCGTGCCTTCCTCTATCTGGTAG exon 2 (reverse)) were used. Genomic DNA (50ng) was amplified in a reaction mix consisting of 1x HotStart Taq buffer (Qiagen), 1μM of each primer, 80μM each dNTP and 2.5U HotStart Taq polymerase (Qiagen) in a final volume of 50μl. PCR amplification was performed using a PTC-100 programmable thermal cycler (MJ Research Inc., Watertown, MA) with template denaturation at 94°C for 15 minutes, and 44 cycles of melting at 94°C for 15 seconds, annealing at 54°C for 15 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for ten minutes. The DNA-PCR product was then digested with Alu I restriction enzyme producing 184bp and 47bp products which distinguishes SLA^d from other haplotypes.

3.8.2.3 MHC class I^d quantitative PCR (QPCR) analysis

MHC Class I^d DNA was quantitatively assessed by real-time PCR analysis. The reaction mix included DNA template, 800nM of forward primer GAGGCCCTGGAGCAGAAG, 800nM reverse primer GCCTTCCTCTATCTGGTAGTTGTG, with 200 nM of labelled probe

TTTBCACACAGTTGTCCA and 'Absolute QPCR Rox Mix' (Abgene, Rochester, NY). Amplification was performed with a Stratagene Mx 3005 system (La Jolla, CA) with cycling conditions of 95°C for 10min, and 50 cycles of 95°C for 30s, 55°C for 1 min, and 72°C for 30s. Class I quantification was based on amplification of a plasmid reference standard, from 10^8-10^1 copies.

3.8.2.4 MHC class I^c DNA PCR analysis

PCR analysis was performed according to previously established methods (Lima, B. et al. 2003). Negative controls were extracted from whole blood of SLA^{dd} and SLA^{aa} animals, and positive controls from SLA^{cc} animals. The primers used in the PCR amplification were: no. 136 (CACTCCCTGAGCTATTTC), no.138 (GCTCTGGTTGTAGTAGCC), and no.146 (GTGTCCCTTTGTATCTGTGTC). The primer combination 136/138 amplified a 254-base pair (bp) segment of the SLA class I gene common to the A, C and D SLA haplotypes; this served as a positive control. The primer pair 136/146 amplified a 199-bp segment of the SLA class I gene unique to the SLA^c haplotype (SLA Class I ^{c}) which was only present on donor cells.

PCR amplification was performed using a PTC-100 programmable Thermal Cycler (MJ Research Inc., Watertown, MA) with template denaturation at 94°C for 15 minutes, and 45 cycles of melting at 94°C for 15 seconds, annealing at 53°C for 15 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for ten minutes. Southern blot analysis was performed on 136/146 amplification products using an internal oligonucleotide probe, no.162 (TACGTCGACGACACGCAGTTCG), specific for Class I of SLA^c.

3.9 ASSESSMENT OF ADVERSE EFFECTS

3.9.1 GvHD

Animals were monitored for development of GvHD by daily clinical examination, blood counts and liver function tests. Baseline skin and large bowel biopsies were obtained prior to conditioning and biopsies were repeated at monthly intervals. Biopsies were also taken at the first sign of possible GvHD. Biopsy samples were evaluated by a board-certified pathologist in blinded fashion. Severity of GvHD was scored according to Glucksberg-Seattle criteria (Glucksberg, H. et al. 1974).

3.9.2 Vascularised skin flap viability

Skin flaps were monitored daily for viability by direct observation of colour, temperature and capillary refill.

3.10 MAGNETIC BEAD DEPLETION

Magnetic bead depletion was used for two purposes: depletion of CD3+ cells from bone marrow for preparation of the inoculum for *in utero* induction of chimerism (Chapter 9), and depletion of CD25+ cells from PBMCs for use in *in vitro* assays.

3.10.1 Depletion of CD3+ cells

CD3+ cell depletion is described in the bone marrow transplant protocol (Section 3.3.2)

3.10.2 Depletion of CD25+ cells

PBMCs were coated with the anti-porcine CD25 antibody 231.3B2 (murine IgG1) (Denham, S. et al. 1994) for 30 minutes in magnetic activated cell sorting (MACS) buffer. Cells were washed in HBSS three times and incubated with magnetic beads coated with rat-anti-mouse IgG1 antibodies (Miltenyi Biotech) for 15 minutes. Cells were washed three times and passed through a magnetic separation column to remove CD25+ cells (according to the manufacturer's directions).

3.11 STATISTICAL METHODS

For parametric distributions student t-test (paired or unpaired as appropriate) was used to calculate statistical significance. For non-parametric distributions Fisher's exact test was used to calculate statistical significance for categorical data and Mann-Whitney U for ordinal and continuous data. R2 statistic was used to assess correlation in bivariate analyses.

Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were used to compare the predictive accuracy of assays in Chapter 6a:

PPV = True Positive (TP)/ (TP + False Positive (FP))

NPV = True Negative (TN)/ (False Negative (FN) + TN)

PART A:

IMMUNOLOGICAL

REJECTION

CHAPTER 4: CONSEQUENCES OF REJECTION

Recipient tissue damage following musculocutaneous transplant rejection

4.1 INTRODUCTION

The aim of this study was to assess if recipient tissue damage following composite tissue allotransplant failure would limit the function of a second transplant, or even make it impossible to perform a retransplant.

In the event of allograft failure the current plan, in many cases, is to replace the allograft with another allograft. This is particularly relevant for allografts such as a face transplant, because it is not possible to simply amputate the failed allograft without reconstructing the defect. However, it is not known whether retransplantation would even be possible: allograft failure may damage the underlying vessels and tissues to such an extent that it may be impossible to repeat the procedure. Even if it is possible to retransplant, there may be underlying tissue changes that would restrict the function of a retransplant and make any further procedures more complex to perform.

The risk of composite tissue allograft failure is unknown as the clinical composite tissue program is still in its infancy. However, experience from CTA and from organ transplantation indicates that both acute and chronic rejection could be important causes of graft failure. There have been acute rejection episodes in the majority of the hand and face transplants, with graft failure in some recipients (Kanitakis, J. et al. 2003; Lanzetta, M. et al. 2007). In renal transplantation chronic rejection is the cause for loss of the majority of grafts after the first year (Magee, J. C. et al. 2007); it is possible that this will be the case for composite tissue allotransplantation also.

Previous studies have focused on the consequences of rejection on the donor tissue (Buttemeyer, R. et al. 1996; Lee, W. P. et al. 1991). Damage to the recipient tissues has not been formally examined, even though it is of central importance for further reconstruction in the event of allograft failure. This study assesses the damage following composite allograft failure by: (1) examining the damage to the recipient blood vessels supplying the allograft to assess whether retransplantation is possible, and (2) quantifying the damage to the recipient tissue bed and identifying the factors that contribute to that damage.

4.2 OVERVIEW OF EXPERIMENT

This study used 24 donor and 24 recipient animals according to the experimental outline (table 4.1). A further 3 donor and 3 recipient animals were used during the development of the immunosuppression regimen in this model and other animals were used for the set-up of the immunological assays.

Group	n	Immune Barrier	Tapered FK506
I	6	Allograft (WF→Lew)	Yes
II	7	Allograft (WF→Lew)	No
III	5	Isograft (Lew→Lew)	No
IV	6	Isograft (Lew→Lew)	Yes

Table 4.1 Outline of recipient tissue damage experimental design

Animals were divided into four experimental groups (table 4.1). All recipient animals received a musculocutaneous flap allotransplant which was carefully positioned so that the transplanted tissue overlaid recipient tissues commonly found adjacent to composite tissue allotransplants clinically (i.e. muscle, fascia and cartilage; see section 3.4.1 for more details).

Group I was designed to model the clinical situation, in which a CTA is rejected following healing in while the recipient is still taking immunosuppression. This group received a composite musculocutaneous allotransplant (WF→Lew) with FK506 immunosuppressive cover to permit incorporation of the flap before being tapered to a subtherapeutic level to allow rejection. Initially a single 5mg/kg intramuscular dose of FK506 was used on day 1 post operatively; this was based on a previous paper by Godha et al (Gohda, T. et al. 2003) in a rat limb allotransplantation model, which achieved a median of 49 days survival. However, this regimen did not significantly prolong survival in our model (n=3; full skin rejection: day 7, 8, 9). Consequently, a tapering immunosuppression regimen was developed consisting of intramuscular administration of FK506 at a dose of 2mg/kg daily

from Day 0-7, 2mg/kg on alternate days from days 8-14, 1mg/kg 2x/week from days 15-21, and then 1mg/kg weekly from day 21 onwards. With this regimen we achieved a median of 41 days survival (range 37-43 days), compared to a median of 8 days survival (range 7-9 days) without immunosuppression. The recipient tissues were biopsied at the time of full rejection of the allograft. The definition of full rejection was when the entire surface of the skin flap was escharified (see fig 4.1)

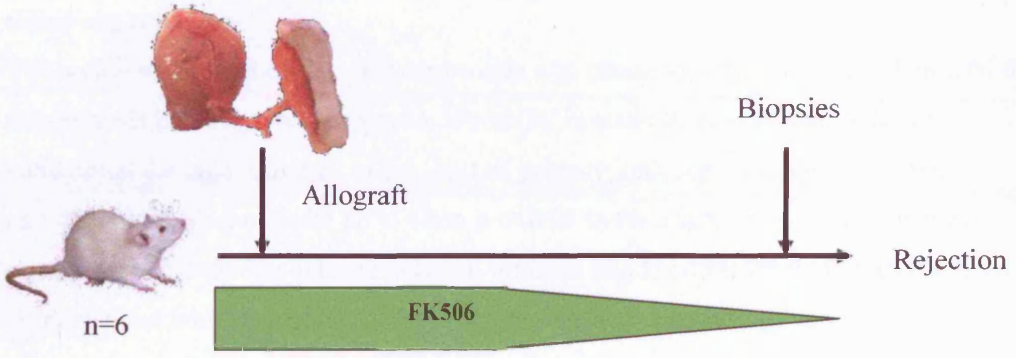


Figure 4.1 Group 1 experimental summary

The three other groups were designed to delineate the contributions of different elements to recipient tissue damage.

Group II received a composite musculocutaneous allotransplant (WF→Lew) without immunosuppression to assess the effect of the rejection process on recipient tissues. The recipient tissues were biopsied at the time of full rejection of the allograft. One animal in group II received a retransplant. Instead of biopsying the recipient tissues the first transplant was carefully removed from the recipient at full rejection, an isogeneic musculocutaneous graft was then transplanted.

Group III received a composite musculocutaneous isotransplant (Lew→ Lew) without immunosuppression to assess the effect of the healing process on recipient tissues. The recipient tissues were biopsied at time points matched to group II.

Group IV received a composite musculocutaneous isotransplant (Lew→Lew) with the same tapered FK506 immunosuppressive regimen as group I to assess the influence of

immunosuppression on the recipient tissues. Biopsies were performed at time points matched to group I. Recipient tissue biopsies were graded for tissue damage based on an objective histological grading scale ranging from '0' for undamaged tissue to '3' for multifocal necrosis (see table 3.2).

4.3 RESULTS

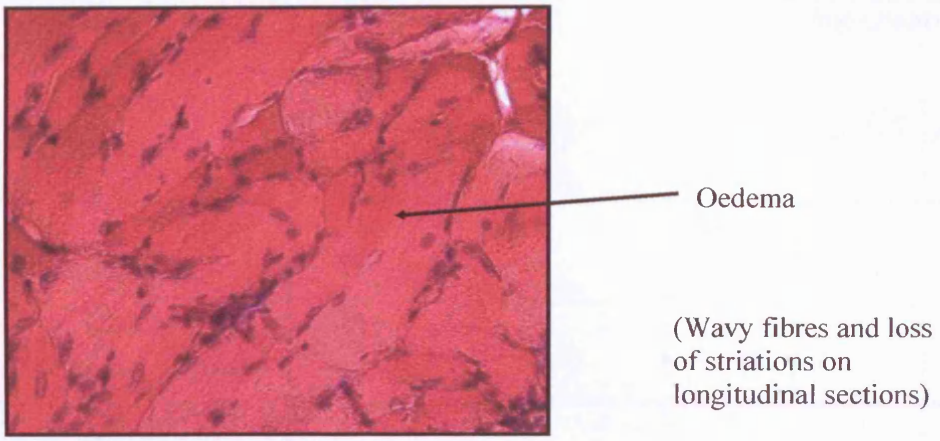
4.3.1 Recipient vascular pedicle is viable making retransplantation possible following rejection

The recipient element of the vascular pedicle was patent to within 1mm (+/-1mm) of the anastomosis in all rejected allografts. However, in some there was macroscopic endothelial damage proximal to the level of patency with separation of the vessel endothelium from the media up to 4mm proximal to the anastomosis. There was no significant difference in damage between groups I and II (allotransplant with FK506 cf. allotransplant without FK506; $p>0.5$). In two rejected allografts the donor femoral vessels were still patent to the origin of the epigastric vessels. To confirm that the recipient vascular pedicle findings are a valid indication that retransplantation is possible, a retransplant was performed in one animal from the group II. The second graft healed in promptly, being indistinguishable from the primary allografts at equivalent timepoints, and went on to survive long-term (>45 days).

4.3.2 Minimal recipient tissue bed damage following rejection under subtherapeutic FK506 cover

There was mild damage (grade 1; fig 4.2a) to recipient animals receiving an allotransplant with FK506 taper (group I) following rejection (see table 4.3). This damage occurred to the bed underlying the donor muscle, which was at an advanced stage of rejection. There was no damage to any other recipient tissues adjacent to donor muscle or any recipient tissues underlying donor skin.

a.



b.

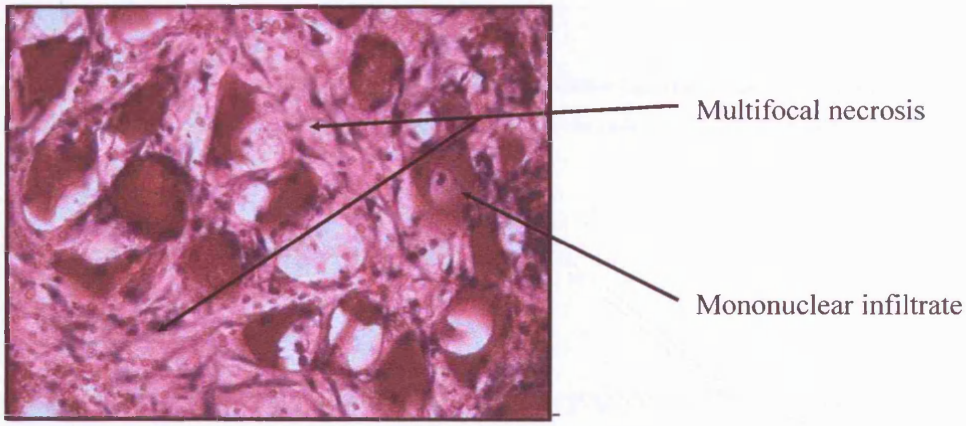


Figure 4.2 Histology of damage to recipient muscle following rejection. Biopsies taken from the recipient tissue bed following full rejection of the allograft. There is only mild damage to the recipient muscle (a) in animals receiving an allograft with FK506 that was subsequently tapered to a subtherapeutic level allowing rejection (group I). In contrast, there was severe (grade 3) damage to the recipient muscle (b) in animals following rejection of an allograft without FK506 (group II) (x25 magnification).

Donor Tissue	Donor Muscle			Donor Skin		
Recipient Tissue	**Skin**	**Muscle**	**Fascia**	**Muscle**	**Fascia**	**Carti-lage**
Group I (Allo; FK506 Taper)	0	1	0	0	0	0
Group II (Allo; No FK506)	**2***	**3***	0	**3***	0	0
Group III (Iso; No FK506)	0	0	0	1	0	0.5
Group IV (Iso, FK506 Taper)	0	0	0	0	0	0

Key

* Significantly more severe damage ($p<0.05$) at that tissue interface than in any other group

NOTE: Scores represent median grade of recipient tissue damage for that group (see table 3.2 for details on grading).

Table 4.2 Scoring of recipient tissue damage

4.3.3 Rejection process is the major cause of recipient tissue damage

Three groups were used to delineate the contribution of different elements to the damage to the recipient tissues (see table 3.2 for scoring system and table 4.2 for results). There was severe damage (grade 3) to the recipient muscle (fig 4.2b), and moderate damage (grade 2) to the recipient skin in animals receiving an allotransplant without immunosuppression (group II). In contrast, there was only mild damage to the recipient muscle and mild-to-no damage to the recipient cartilage in animals receiving an isotransplant without immunosuppression (group III). Similarly, there was no damage to any recipient tissues in animals receiving an isotransplant with immunosuppression (group IV).

The animal that received a second allograft did not undergo repeat biopsies because of the potential of inadvertent inclusion of an area damaged from the first biopsies.

4.3.4 Subtherapeutic immunosuppression prevents recipient tissue damage

There was significantly more damage to the underlying recipient muscle and overlying recipient skin in animals in group II than group I ($p<0.05$). Both groups I and II received a musculocutaneous allotransplant across the same major immunological barrier. The only difference between groups I and II was that group I received immunosuppression which was then tapered down to subtherapeutic levels whereas the group II received no immunosuppression.

In vitro assays were performed to investigate why immunosuppression was associated with less damage to the recipient tissues. Mixed lymphocyte response in group II at the time of rejection revealed normal alloresponsiveness with a stimulation index of 7 (fig 4.3a). However, there was no response in group I (fig 4.3b). Furthermore, antibody FACS at the time of rejection revealed over one hundred-fold stronger response in group II (fig 4.3c) than in group I (fig 4.3d).

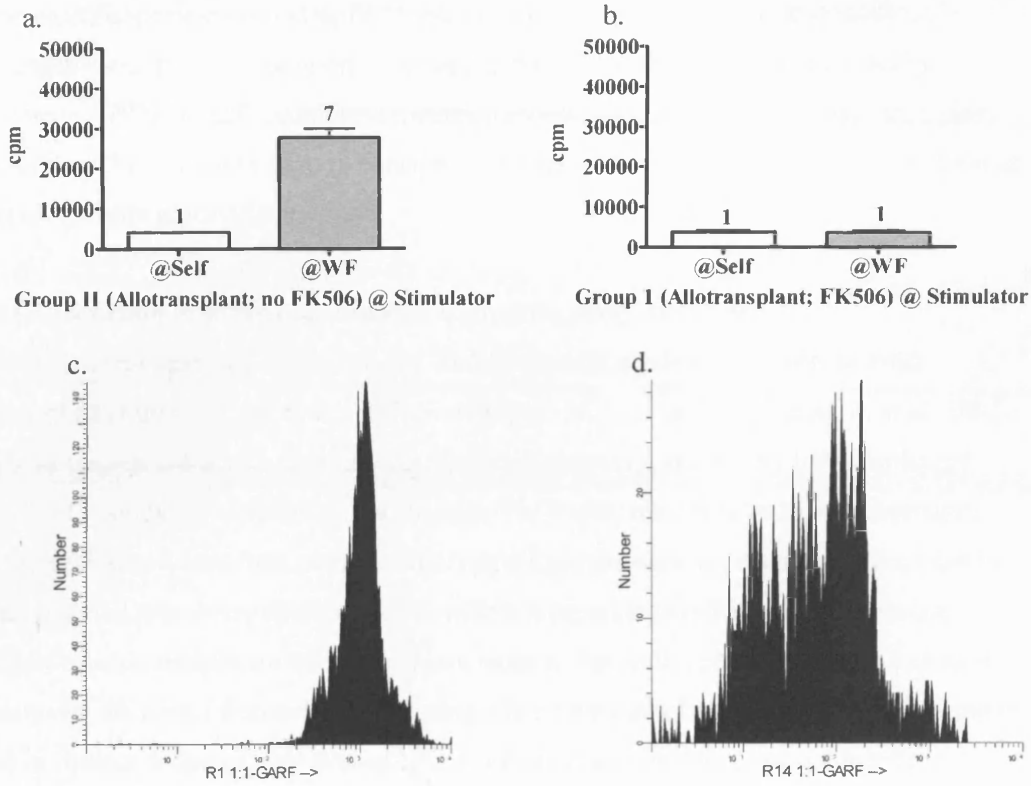


Figure 4.3 *In vitro* assessment of immune response to donor. There was strong alloreactivity to donor at the time of full allograft rejection (a: shaded bar; "@WF"; stimulation Index=7) in a mixed lymphocyte response in animals (Lew) receiving an allograft without immunosuppression (group II). In comparison, there was no alloreactivity to donor (b: black bar; "@WF"; stimulation index=1) in animals (Lew) receiving an allograft with subtherapeutic FK506 (group I) despite full allograft rejection. Flow cytometry analysis of antibody in both groups at the time of allograft rejection demonstrated of anti-donor antibody production in both group I (c) and group II (d). However, titration of the antibody level indicated that antibody production was >x100 less in group I compared to group II.

4.4 DISCUSSION

Composite tissue allotransplantation techniques offer the possibility of reconstructing tissue defects for which there are no other good reconstructive solutions. However, it is possible that the composite tissue allograft may fail necessitating its removal. Extrapolating from other organ transplant programs, it has been estimated that the risk of chronic rejection may be as high as 30-50% at 5 years in composite tissue allotransplants (Morris, P. et al. 2007). In addition, composite tissue allotransplants may be at an increased lifetime risk of failure compared to organ transplants because they are often performed on young recipients who have an otherwise normal life expectancy and no co-pathology. Although only in a small animal model, this study

suggests that in the event of clinical allograft failure, there would be minimal damage to the vascular pedicle even after full rejection, making retransplantation possible. Furthermore, there was only mild damage to the recipient tissues in the clinically relevant subtherapeutic immunosuppression group, suggesting that a second transplant would not be limited in form or function by recipient tissue bed damage. These findings have not been reported previously.

4.4.1 Selection of musculocutaneous composite allograft model

Several orthotopic face transplant and limb transplant models have already been described (Lipson, R. A. et al. 1981; Siemionow, M. Z. et al. 2005; Unal, S. et al. 2005). These models are useful for studying functional recovery, but do not have particular utility for studying recipient tissue damage. The model used here has two advantages over previously described models. Firstly, it allows for semi-independent placement of the skin and muscle components. This makes it possible to individually assess the effect of each component on the recipient tissues. Secondly, both the skin and muscle elements are a significant size, facilitating a large amount of each type of donor tissue to be in contact with recipient tissues. A limitation of this model is that the interfaces between donor and recipient bone, and donor and recipient nerve, were not included. However, it is likely that damage to recipient bone would have been minimal as in fascia and cartilage, which are similarly robust and quiescent tissues. Furthermore, in most cases, any damage to the recipient nerve adjacent to the first anastomosis could be excised, and a fresh undamaged stump exposed for anastomosis to the second transplant.

4.4.2 Retransplantation is possible with no likely functional limitation due to recipient damage

In this model there was little damage to the recipient vascular pedicle supplying the allograft, with retransplantation possible even after full allograft rejection. Furthermore, there was minimal damage to recipient tissues in animals receiving an allotransplant with immunosuppression that is subsequently tapered allowing rejection (group I), indicating that the second transplant would not be limited in function by damage to the adjacent recipient tissues.

This is a particularly stringent model, with full rejection occurring before the tissues are examined and retransplantation is attempted. It is likely that in a clinical scenario

retransplantation may occur well before the level of rejection seen in this model is reached, because chronic rejection would cause functional allograft failure long before full rejection occurred. Consequently, at the time of retransplantation in patients there may be less damage to the recipient vascular pedicle and tissues than observed in this model.

A limitation of this model is that there may be different mechanisms involved in the rejection process of clinical composite tissue allotransplant failure. Graft loss in clinical composite tissue allotransplantation is likely to be largely due to chronic rejection, whereas in this model, rejection occurs due to tapering of immunosuppression a subtherapeutic level. These different rejection processes may in turn influence the level of recipient tissue damage. However, similar findings to this model in the organ transplantation program regarding vascular pedicle damage, support the relevance of this model. In organ transplants where repeat transplantation has to be performed on to the same vascular pedicle (e.g. heart and lung), damage to the pedicle has not been a significant limitation on repeat transplantation (Magee, J. C. et al. 2007). Recipient tissue damage is less important in organ transplants as this has little impact on their function, and consequently, to our knowledge, has not been reported on.

Even in the event of successful CTA retransplantation there may be an increased likelihood of rejection of the second transplant. This has been the case in repeat kidney, liver, heart and lung transplants (Magee, J. C. et al. 2007). The cause of this is thought to be heterologous immunological memory (Adams, A. B. et al. 2003; Koyama, I. et al. 2007): clones of memory cells to the first allograft are activated by the replacement allograft due to antigenic similarity between the two allografts. In kidney transplantation, some have investigated delaying transplantation of the replacement allograft following removal of the failed allograft in the hope that this would allow time for desensitisation of the recipient. This actually results in a spike in panel reactive antibodies (Smak Gregoor, P. J. et al. 2001) following removal of a failed renal transplant, indicating that the renal allograft may be performing the useful function of acting like a sponge for circulating antibody due to sensitisation. However, the significance of this is not clear: some of the worst outcomes from renal retransplantation have been in patients that had their first failed renal allograft removed before retransplantation (Abouljoud, M. S. et al. 1995), while others have reported no

difference in outcomes with raised circulating antibodies (Douzdjian, V. et al. 1996; Sumrani, N. et al. 1992).

Rejection of a second CTA transplant may also cause more damage than rejection of the first transplant due to a more intense rejection response caused by the heterologous immunological memory. The increased damage could have a significant impact on any further transplants. This was not examined in this study and is an avenue for future research.

4.4.3 Subtherapeutic immunosuppression protects against recipient tissue damage despite not preventing donor tissue rejection

In this model, the major cause of damage to the recipient tissues appeared to be the rejection process. The healing process caused minor damage, with immunosuppression having a protective effect. Both groups I and II received allografts across a MHC barrier; the only difference between the groups was that the group I received FK506, which was tapered down to a subtherapeutic level allowing rejection after the allograft had healed in, whereas group II did not receive immunosuppression. These groups differed in the severity of recipient muscle and skin damage due to the rejection process: there was severe damage to recipient muscle and skin in group II with only mild damage to the muscle and no damage to the skin in group I. This difference between the two groups was due to the subtherapeutic immunosuppression, which had a protective effect on group I.

The *in vitro* data suggests a reason for this protective effect. The MLR and flow cytometry analysis of antibody production indicated that the strength of the immune response in animals on subtherapeutic immunosuppression (group I), although present, was greatly diminished in comparison to animals not receiving immunosuppression (group II). It is unclear whether this protective effect is specific to the type of immunosuppression used or would be present whichever immunosuppressant is used. This finding may be important for clinical CTA as all patients are on some form of immunosuppression.

4.5 CONCLUSIONS

In conclusion, in the event of an allograft failure, these results suggest that the recipient vasculature would be intact making retransplantation possible. Furthermore, the

recipient tissue bed would have minimal damage meaning that the retransplant would not be limited in form or function. These findings indicate it is realistic to plan to perform a retransplant in the event of allograft failure. However, it is still possible that sensitisation to the first transplant may increase the risk of rejection of the second transplant. This could pose a significant problem, as restricting the donor pool to an immunological subtype would make it even more difficult to find donors.

The finding that retransplantation may be a realistic possibility, in the event of allograft failure, is encouraging. However, it would be better if the allograft did not fail in the first instance. Skin has been the tissue most susceptible to rejection in composite tissue allotransplants so far. A better understanding of the mechanism of skin rejection may help to guide future research to avoid rejection episodes. This will be the focus of the next chapter.

CHAPTER 5: MECHANISM OF SKIN REJECTION

In vivo observations of cell trafficking in allotransplanted vascularised skin flaps and conventional skin grafts

5.1 INTRODUCTION

The aim of this study is to objectively assess *in vivo* if there is a difference in the immune response to conventional skin grafts and skin within composite tissue transplants. In addition, this study aims to identify unique characteristics of the immune response to skin within composite tissue transplants that may be useful for directing the development of approaches to overcome composite tissue allotransplant rejection. The role of the vasculature, cell types involved, and the target of the immune response are examined.

Much of the understanding of skin rejection has been derived from histological studies examining the mechanism of rejection of conventional skin grafts (CSG). However, both the method of observation and the type of transplant used, limit the application of these findings to skin within composite tissue transplants (SCTT). The use of histological specimens to examine the rejection mechanism is restricted by artefact from fixation techniques, and the frequency that biopsies can be taken. Consequently, only subjective comparisons can be made as there are not enough observations to reach statistical significance. In addition, it is possible that there are differences between CSG and SCTT in their interaction with the immune system, making observations of CSG not directly applicable to SCTT. For example SCTT differ from CSG in the timing of vascularisation. The blood vessels in SCTT are anastomosed directly to recipient vessels resulting in immediate restoration of a blood supply to the skin. In contrast, CSG are not vascularised primarily, and survive initially by absorbing oxygen and nutrients from plasma in the graft bed. This is likely to have effects on the speed and route of trafficking of immune cells involved in the rejection response to and from the skin.

This study assessed *in vivo* the immune response to allotransplanted CSG and SCTT in a rat model using confocal microscopy.

5.2 OVERVIEW OF EXPERIMENT

The experiment is summarized in figure 5.1. *In vivo* confocal microscopy was used to observe cell trafficking into and targeting within the skin. This non-invasive *in vivo* technique avoids artefacts associated with histological fixation of biopsies, and

facilitates attainment of sufficient data at multiple timepoints and locations to make statistically significant quantitative observations. PCR was used to track cellular efflux from allografts.

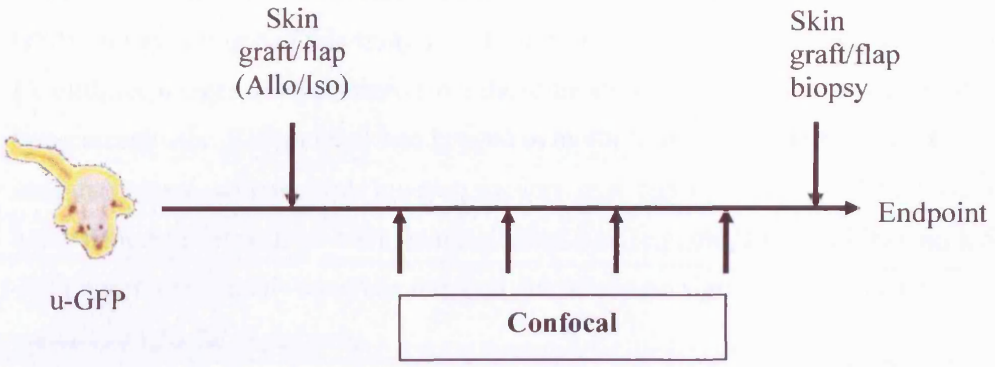


Figure 5.1 Experimental overview

Skin was transplanted from non-fluorescent donor rats to recipient rats that were transgenic for GFP so that all cells within the animal fluoresced. This allowed for selective imaging of fluorescent recipient cells infiltrating the non-fluorescent transplanted skin using confocal microscopy. A total of 18 rats were used in this study. Donor animals (n=9) were Lewis (LEW; RT-1^l) and Wistar Furth (WF; RT-1^u) rats. Recipient animals (n=9) were Lewis GFP transgenic rats.

To compare SCTT and CSG transplanted across isogeneic and allogeneic barriers, animals were divided into four experimental groups (see table 5.1).

GROUP	No. of rats	No. of images acquired	SCTT/CSG	Transplant Barrier
I	3	1012	SCTT	Lew→ Lew-GFP
II	2	962	SCTT	WF→ Lew-GFP
III	2	717	CSG	Lew→ Lew-GFP
IV	2	732	CSG	WF→ Lew-GFP

Table 5.1 Experimental groups

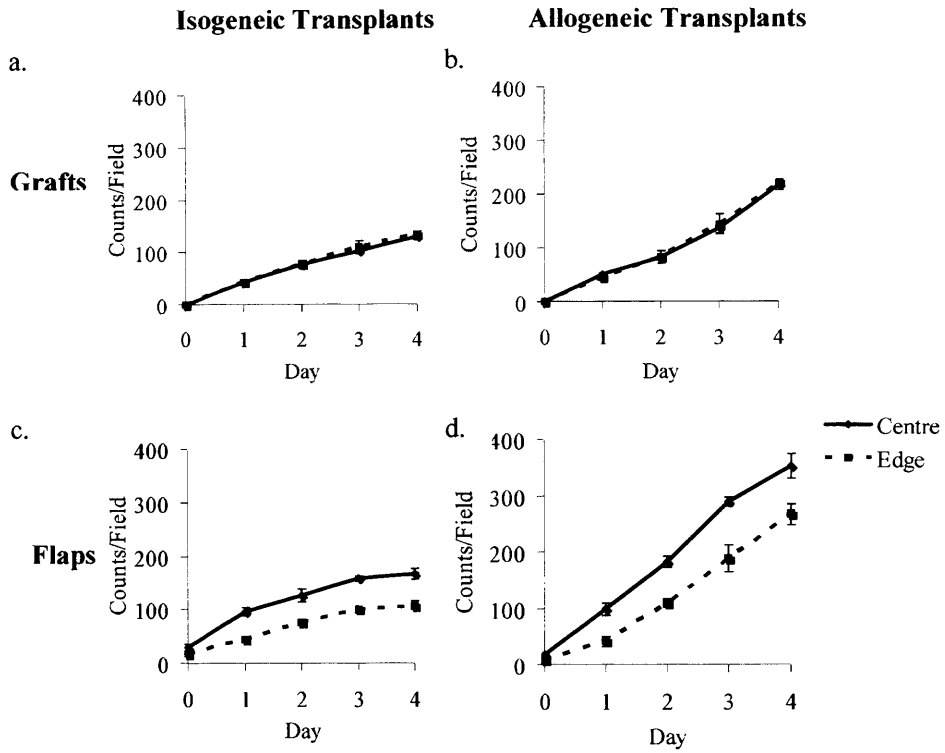
Group 1 (n=3) received a SCTT across an isogeneic barrier (Lew→Lew-GFP). Group 2 (n=2) received a SCTT across a full MHC mismatched allogeneic barrier (WF→Lew-GFP). Group 3 (n=2) received a CSG across an isogeneic barrier (Lew→Lew-GFP). Group 4 (n=2) received a CSG across a full MHC mismatched barrier (WF→Lew-GFP). A key strength of this study was the use of non-invasive imaging. This allowed for multiple images to be obtained from the same animal, controlling for variability between animals. Each animal was imaged in multiple areas and depths in each imaging session, with multiple imaging sessions over consecutive days. To obtain an equivalent data-set with invasive imaging techniques (e.g. biopsies and histology), 50-100 times more animals would be required, introducing a significant source of variability into the experiment.

Images were obtained at multiple timepoints after allotransplantation. Evans Blue Dye (detectable on confocal microscopy at a different wavelength to GFP) was injected intravascularly into some animals to visualize the blood vessels within the flap.

5.3 RESULTS

The influx of cells into transplanted skin, the target of the infiltrating cells and the efflux of donor cells was examined.

5.3.1 Influx of infiltrating cells



Key

Error bars indicate standard error of the mean

Figure 5.2 Representative examples of recipient cell influx into skin grafts and skin flaps transplanted across isogeneic and allogeneic barriers

5.3.1.1 More recipient cells infiltrate SCTT than CSG due to immediate vascularisation

There were up to twice as many cells infiltrating the centre of each isogeneic and allogeneic SCTT than the respective isogeneic (figs 5.2a cf.5.2c; $p<0.03$) and allogeneic (5.2b cf.5.2d; $p<0.01$) CSG at each timepoint.

To investigate why there was more cellular influx in SCTT compared to CSG, the influx at the centre and edge of each CSG and SCTT were compared. CSG, in which there is no blood supply initially, showed no significant difference in recipient cell numbers at the centre compared to the edge at all timepoints (figs. 5.2a & 5.2b; $p<0.1$). In contrast, all SCTT had significantly more infiltrating recipient cells at the centre (solid line) than

the edge (dotted line) from day 1 onwards (figs 5.2c & 5.2d; $p<0.05$) indicating that the vasculature (which supplies the centre more richly than the edge) was a major route for recipient cellular influx.

5.3.1.2 There is greater cellular trafficking into allografts than isografts

Greater numbers of recipient cells infiltrated allografts compared to isografts. In SCTT there were greater number of recipient cells in all allotransplants compared to isotransplants from two days after transplantation ($p<0.01$). In contrast, in CSG there were only greater numbers of recipient cells in allotransplants compared to isotransplants by three days after transplantation ($p<0.02$; fig 5.2).

a. b.

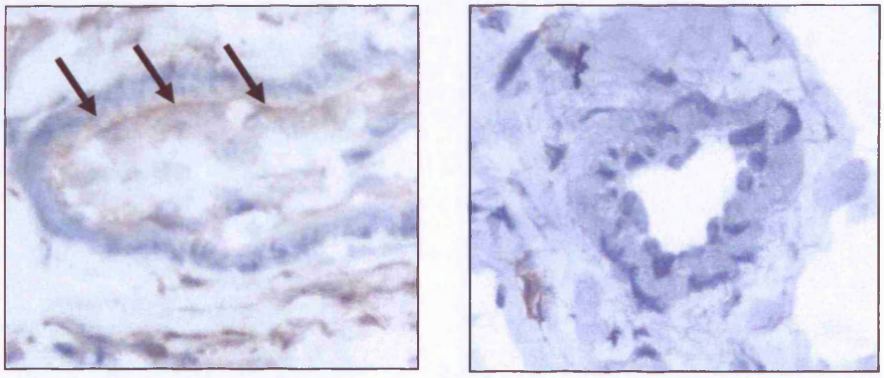


Figure 5.3 Endothelial class II expression on vasculature of rejecting skin. MHC Class II expression was observed on immunohistochemical staining of biopsies taken from allogeneic SCTT taken 4 days after transplantation (a), but not on isogeneic SCTT (b) (x40 magnification).

5.3.1.3 MHC II was only expressed on rejecting SCTT dermal vascular endothelium

Endothelial MHC class II expression was examined to investigate reasons for greater infiltrate in allogeneic SCTT compared to isogeneic SCTT. Staining revealed Class II MHC was expressed on the endothelium of all allogeneic SCTT but not on isogeneic SCTT or CSG four days after transplantation (fig 5.3).

The immune cell types infiltrating the graft were characterised by immunohistochemical staining of biopsy samples.The cells infiltrating isogeneic and allogeneic CSG and SCTT included MHC class II positive and CD8 positive lymphocytes (fig 5.4 a & b).

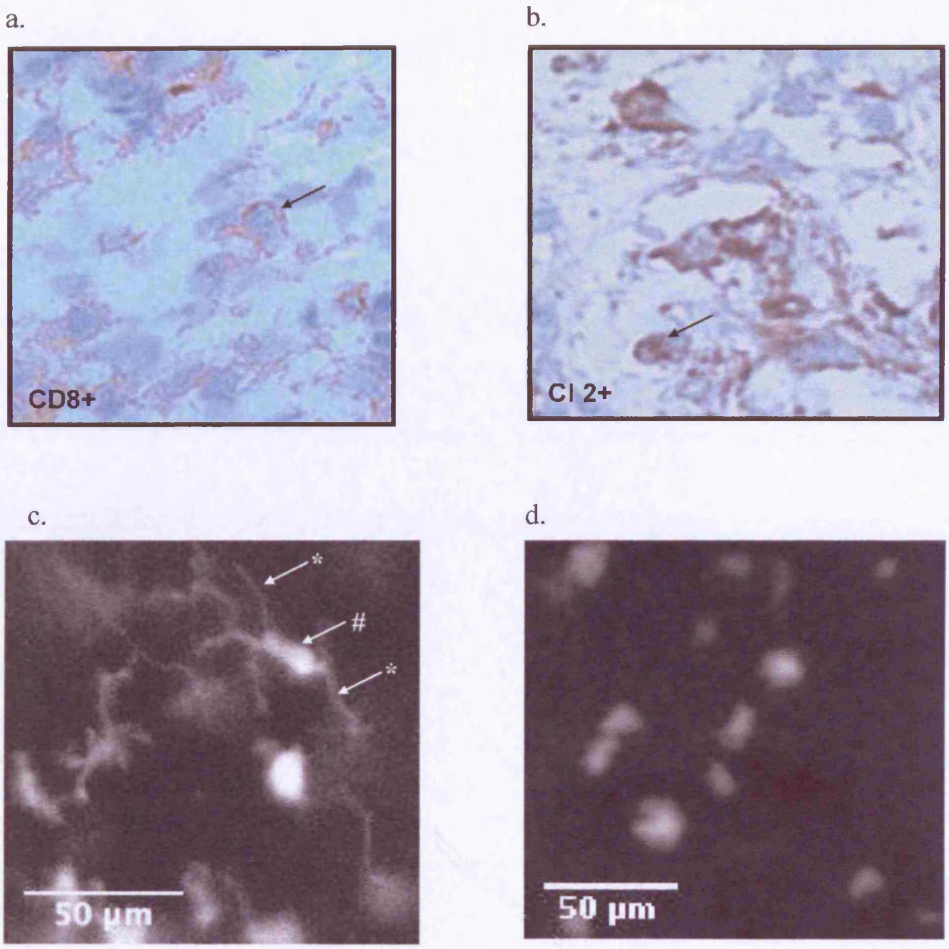


Figure 5.4 Recipient cell types within transplanted skin. Infiltrating cells included both CD8+ (a) and Class II positive (b) lymphocytes, which were identified in allogeneic and isogeneic CSG and SCTT on immunohistochemical staining (x40 magnification). Infiltrating recipient cells included dendritic cells (# - cell body; * - dendrite), which were observable on confocal microscopy in allogeneic flaps from two days after transplantation (c), but not observable at any timepoint in CSG (d).

5.3.1.4 Recipient dendritic cells are present in allogeneic SCTT, but not allogeneic CSG

Recipient cells infiltrating the transplanted skin could also be identified and characterised *in vivo* with confocal microscopy by virtue of their fluorescence. Recipient dendritic cells (rDCs) were observed in the dermis in allogeneic SCTT two days after transplantation (fig 5.4a), but not in allogeneic CSG at any timepoint (fig

5.4b). In contrast, rDCs were observable in both isogeneic SCTT from seven days after transplantation, and isogeneic CSG from eight days after transplantation[2].

5.3.2 Target of infiltrating cells

5.3.2.1 Infiltrating cells cluster around vessels and hair follicles in the superficial dermis of allotransplants

Rat epidermal thickness was determined to be ~40μm from measurements of biopsy samples. *In vivo* imaging revealed minimal cellular infiltrate within 50μm of the surface of the skin in all animals at all timepoints compared to the dermis ($p<0.01$).

Hair follicles and blood vessels were identified *in vivo* within the skin using confocal microscopy. Hair follicles could be localized by auto-fluorescence of the hair within the follicle, and blood vessels could be identified by injection of Evans blue dye.

SCTT were examined four days after transplantation for evidence of clustering of infiltrating cells. There was clustering of infiltrating cells around both hair follicles (fig 5a; $p<0.01$) and blood vessels (fig 5b; $p<0.01$) in allogeneic SCTT, but no significant clustering around either structure in isogeneic SCTT ($p>0.1$).

There was clustering of infiltrating cells around hair follicles in allogeneic CSG ($p<0.01$; fig 5c), but no significant clustering in isogeneic CSG ($p>0.1$). Only scattered blood vessels were visible in skin grafts at four days after transplantation following injection of Evans blue dye. Confirmation that sufficient dye had been injected was provided by imaging blood vessels in the ear (fig 5d). Due to the limited number of blood vessels visible in CSG, clustering of infiltrating cells was only examined around hair follicles.

[2] There is no immunohistochemical stain that is specific for dendritic cells. A standard method to distinguish DCs by their unique morphology (from which they derive their name) was used to identify DC.

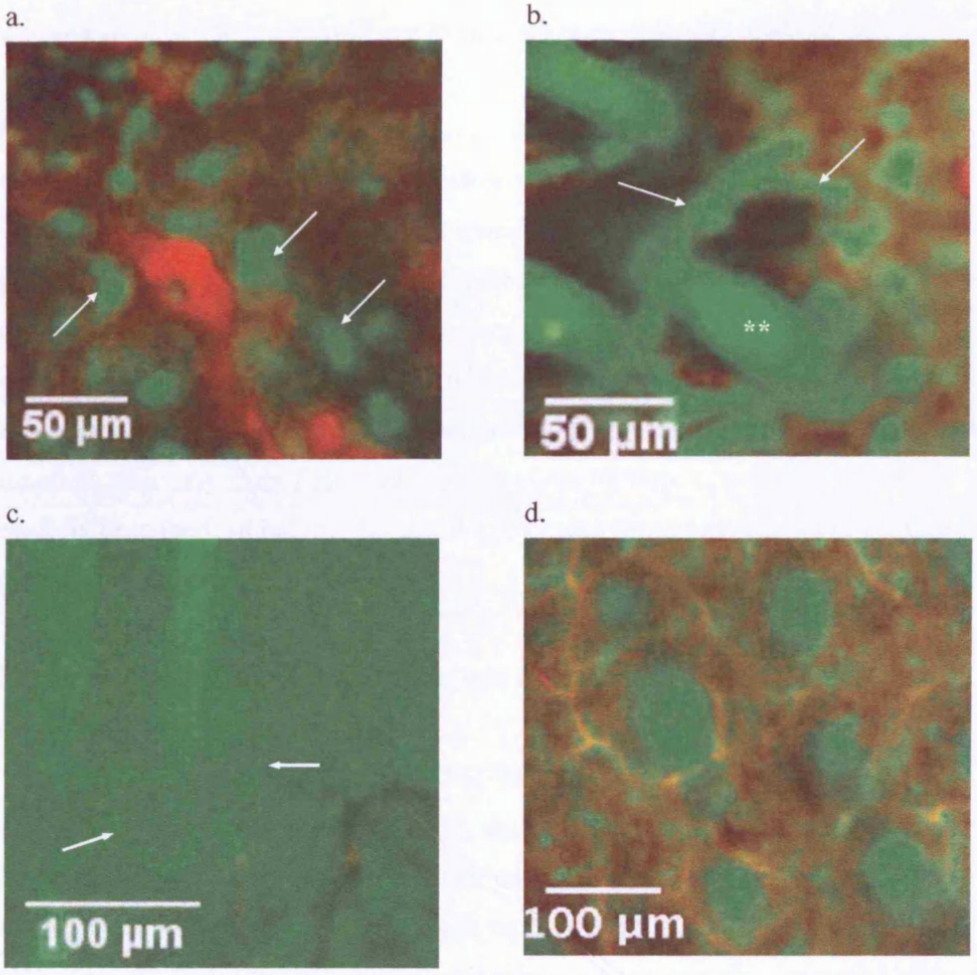


Figure 5.5 Clustering of infiltrating cells observed around blood vessels and hair follicles in allogeneic skin transplants. Clustering of infiltrating cells was observed around blood vessels (a; vessels coloured red, arrows indicating perivascular cell clustering; $p<0.01$) and hair follicles (b; arrows indicating cells pallisading around hair base; * indicating a hair ; $p<0.01$) in allogeneic SCTT, and around hair follicles in allogeneic CSG (c; arrows indicating cells pallisading around hair base; $p<0.01$), but not in isogeneic SCTT ($p>0.1$) and CSG ($p>0.1$), 4 days after transplantation. Adequate Evans blue dye injection to image CSG vessels was confirmed by good vessel detection in the ear (d; vessels coloured red). However, few vessels were visible in CSG 4 days after transplantation, preventing assessment of cell clustering around CSG vessels.

5.3.3 Efflux of donor cells

The efflux of donor cells from the skin to recipient tissues was assessed by PCR analysis of characteristic donor microsatellite repeats at day 4 following transplantation.

There was no evidence of donor cells in the bone marrow, thymus, draining lymph nodes, spleen or peripheral blood in recipients of either allogeneic CSG or SCTT.

5.4 DISCUSSION

Much of our knowledge about the mechanism of skin rejection has been gathered from *in vitro* observations of fixed histological specimens taken from rejecting CSG. However, this may not accurately represent the *in vivo* clinical situation for SCTT. This study indicates there are significant differences between SCTT rejection and CSG rejection, limiting the relevance of much of the historical data on skin graft rejection when applied to composite tissue allotransplantation. Furthermore, using novel *in vivo* techniques, this study identifies characteristics of the immune response to skin not previously described, which may be useful in directing future approaches to overcoming skin rejection.

5.4.1 Influx includes the vascular route, and is earlier and more intense in skin flaps compared to skin grafts

This study indicates that, in contrast to CSG, the vascular route of cellular influx is important for primarily vascularised SCTT, accounting for up to half of the recipient cells found within the skin over the first four days after transplantation. Consequently there are significantly more infiltrating cells within allogeneic SCTT than CSG. Furthermore, there was an earlier observable rejection response with increased cellular infiltration of allogeneic SCTT from day two post-transplantation compared to day three for CSG.

The earlier, more intense rejection response seen in SCTT compared to CSG at first seems to contrast with previous reports that allogeneic primarily vascularised skin transplants may have a slight survival advantage over CSG (Bushell, A. et al. 1995; Steinmuller, D. 1998). One explanation may be that many of the infiltrating cells in SCTT are not involved in the rejection response. This is supported by the observation of large numbers of cells infiltrating isogeneic SCTT, which are not involved in rejection. Additionally, SCTT may be more resistant to the effects of rejection than CSG due to SCTT having more extensive vasculature initially. This possibility is supported by the observations that the rejection response in CSG is primarily due to infarction of the microvasculature (Dvorak, H. F. et al. 1979), whereas vessel infarction

has only been seen in cases of severe rejection in human composite tissue allotransplants (Cendales, L. C. et al. 2006).

5.4.2 MHC class II was expressed only on SCTT, not CSG dermal vascular endothelium

MHC class II was expressed only on the dermal vascular endothelium of SCTT, not CSG. This difference may be a function of the later vascularisation of CSG compared to SCTT.

This is the first time that MHC class II has been observed on endothelium in rat dermis. However, it is consistent with previous studies indicating that rat endothelium does not express MHC class II constitutively (Choo, J. K. et al. 1997), but expression can be induced in retina, brain, liver, kidney (Ustinov, J. et al. 1994; Wang, Y. et al. 1995) and on heart allotransplants (Forbes, R. D. et al. 1991).

It is possible that the induced expression of MHC class II observed on the endothelium during rejection of allogeneic SCTT is a reason for more cellular infiltrate in allogeneic SCTT compared to isogeneic SCTT. This is supported by the observation that human dermal endothelial cells can present antigen for effector T cells homing to skin (Pober, J. S. et al. 2001).

5.4.3 Presence of rDCs in SCTT, but not CSG

A major difference between allogeneic SCTT and CSG was that rDCs were observed by day 2 in SCTT but not at any timepoint in allogeneic CSG. This has not been previously reported. It is possible that the early presence of rDC in rejecting SCTT may be able to be exploited to achieve skin tolerance in SCTT. In murine bone marrow transplant tolerance induction models recipient dendritic cells have been used to achieve skin tolerance (Beriou, G. et al. 2005), whereas only prolonged skin survival has been achieved with donor dendritic cells (Markees, T. G. et al. 1999), indicating that recipient dendritic cells can also have a crucial role in preventing the rejection process.

5.4.4 The target of the rejection response is the vasculature and adnexae

There was clustering of infiltrating cells in allogeneic transplants around vasculature and hair follicles, but not the epidermis. Previous explanations for the susceptibility of skin to reject have often focused on skin specific antigens. Skin specific antigens have

been described in mouse allotransplantation (Boyse, E. A. et al. 1968) and rat to mouse xenotransplantation (Silverman, M. S. et al. 1962) models. However, skin specific antigens have only been identified on epidermal cells in the skin, not around adnexal structures or blood vessels in the dermis. The observation of no epidermal clustering suggests that skin specific antigens may not be a complete explanation for skin's susceptibility to rejection. The observation of statistically significant clustering around hair follicles and blood vessels also objectively confirms previous subjective observations that skin rejection may be focused in the superficial dermis, around adnexal glands and the vasculature (Cendales, L. C. et al. 2006; Bhan, A. K. et al. 1982; Dvorak, H. F. et al. 1980).

5.4.5 Donor immune cell efflux is below the limits of detection using microsatellite repeat PCR

No efflux of donor cells was detected in the lymph node, spleen, bone marrow, thymus or peripheral blood in this study with an assay sensitivity of 1% donor chimerism. The inability to detect donor chimerism is in line with previous studies. Ozmen could not detect donor chimerism in the peripheral blood (sensitivity 0.5%) in rats receiving an SCTT across a MHC barrier without treatment (Ozmen, S. et al. 2006). Therefore, it is very likely that there was low-level efflux of donor cells into the recipient, but the resultant level of donor chimerism was below the level of detection.

Donor chimerism has been at detectable levels in composite tissue allotransplantation models incorporating immunosuppression. In a hemifacial allotransplant chronic immunosuppression model Siemionow demonstrated chimerism with dendritic cell morphology donor-derived cells in the lymph nodes and spleen of the recipient (Siemionow, M. et al. 2005).

It is likely that recipient cells as well as donor cells will efflux from the composite tissue allotransplant. Effluxing donor dendritic cells (dDCs) and rDCs are likely to have different functions, possibly due to their activation status (Fiorina, P. et al. 2007). Previous work indicates that the initial efflux of dDC from CSG probably occurs via the lymphatics (Larsen, C. P. et al. 1990b). Once in the lymphatics dDCs have been shown to migrate to the spleen (Fossum, S. 1988) and lymph nodes (Austyn, J. M. et al. 1988) leading to sensitisation and rejection. Intravascular efflux from CSG occurs only later, as demonstrated by the prolonged survival of secondarily vascularised alymphatic CSG

(Barker, C. F. et al. 1968; Tilney, N. L. et al. 1971). There is little direct data pertaining to immune cell efflux from primarily vascularised SCTT. In murine primarily vascularised heart allografts, both dDCs and rDCs preferentially migrate via the bloodstream to the spleen and regional lymph nodes stimulating sensitisation (Saiki, T. et al. 2001).

5.5 CONCLUSIONS

In conclusion, this study identifies differences in cell trafficking into and within CSG in comparison to SCTT. In contrast to CSG, the vascular route is important for initial cellular influx, there is trafficking in of rDCs, and there is an earlier and more intense cellular infiltrate seen in SCTT. In addition, there is an early presence of rDC in rejecting SCTT but not CSG. Finally, the rejection response has dermal targets rather than the epidermis as has been often assumed previously. These observations challenge previous dictums that have directed skin transplant rejection research, and also give direction to future research into ways to avoid skin rejection in CTA.

This study, and the previous study (Chapter 4), analysed the mechanism and consequences of skin rejection using rat models. Small animal models are very useful in examining issues related to rejection. However, as outlined in Chapter 2, there is difficultly in translating success in achieving skin allotransplant survival in small animal studies to the large animal. Consequently, in Part B, attempts to achieve skin survival across a MHC barrier will be examined in a large animal model.

PART B:

PREVENTION OF SKIN

REJECTION

CHAPTER 6

The aim of this study is develop a hypothesis of how to achieve skin tolerance in a large animal model. In Chapter 6a the more extensive experience with organ allotransplantation is examined to identify variables that predict subsequent organ allograft tolerance; then in Chapter 6b these predictors of tolerance induction are used to interpret the results from a smaller series of animals that previously underwent chimerism induction with CTA with identification of reasons why tolerance to skin was not achieved. On the basis of these findings a hypothesis of how to achieve skin tolerance is formed.

CHAPTER 6A: PREDICTORS OF TOLERANCE IN ORGAN TRANSPLANTATION

6.1 INTRODUCTION

Based on Ray Owen's initial observations that naturally occurring chimeric twin calves were tolerant to reciprocal skin grafts (Owen, R. D. 1945) and the early work of other researchers (Billingham, R. E. et al. 1953), it was thought that 'chimerism leads to transplant tolerance'. More recently this simple paradigm has been challenged. Although full haematocytic chimerism always leads to tolerance (Sayegh, M. H. et al. 1991), the relationship between chimerism and tolerance in mixed chimeras has been variable, with reported cases of long-lasting T cell chimerism being possible without tolerance (Umemura, A. et al. 2001), and stable tolerance achievable after only transient chimerism (Buhler, L. H. et al. 2002).

Some, in the most extreme hypothesis relating to this phenomena, have proposed low-level chimerism ('microchimerism') as the basis of all cases of long-term organ allograft survival, including those induced by chronic immunosuppression (Bonilla, W. V. et al. 2006; Starzl, T. E. et al. 1992; Starzl, T. E. 2004). However, this relationship has been disputed (Elwood, E. T. et al. 1997; Fuchimoto, Y. et al. 1999b; Wood, K. et al. 1996).

Previously Gleit et al. observed variability in the association of peripheral blood chimerism and tolerance following non-myeloablative chimerism induction protocols in

the MGH miniature swine pre-clinical model of haematopoietic cell transplantation (Gleit, Z. L. et al. 2002b).

This study aims to determine whether this variability of tolerance induction in the presence of peripheral blood chimerism might be the result of differences between engraftment status of haematopoietic cells in tolerant versus non-tolerant animals. For this purpose, the possible relationship between donor organ acceptance and the presence of donor-derived cells in the bone marrow, thymus and peripheral blood at the time of solid organ transplantation is examined in a series of haematopoietic cell transplant (HCT) recipients.

6.2 OVERVIEW OF EXPERIMENT

This is a combined retrospective and prospective analysis of animals from different protocols all directed toward induction of tolerance through mixed chimerism. All miniature swine from the Transplant Biology Research Center (TBRC) that had received HCT between 1998 and 2004 and then a delayed organ transplant were analysed. Animals were excluded if it was not possible to assess organ transplant tolerance due to technical problems, early animal death, or copathology.

Peripheral blood and bone marrow chimerism, as well as *in vivo* and *in vitro* (by CML and MLR) assessments of tolerance, were all assessed prospectively by the particular researcher using that animal. Thymic microchimerism, antibody cytotoxicity, collation and analysis of all data were performed retrospectively as part of this study.

Engraftment can be strictly defined as the long-term presence of donor haematopoietic stem cells in the bone marrow. However, there are not any specific markers for stem cells in pigs yet. Mouse data indicates that detection of donor bone marrow colony forming units (BM-CFUs) longer than 12 weeks after haematopoietic cell transplantation (HCT) correlates with engraftment of HSCs (Christensen, J. L. et al. 2001). Consequently, presence of donor-derived bone marrow colony forming units (BM-CFUs; detected by PCR analysis) greater than 12 weeks after PBMC transplantation, was used to indicate the presence of haematopoietic stem cells.

Chimerism was assessed in the peripheral blood, and in the bone marrow progenitors and thymus. Peripheral blood chimerism was assessed in the lymphocyte, monocyte

and granulocyte lineages individually by FACS analysis. Bone marrow progenitor chimerism was assessed by detection of donor-derived BM-CFUs, as described above. Thymic macrochimerism was assessed by FACS, and microchimerism by PCR with Southern Blot confirmation.

Tolerance was assessed *in vivo* by acceptance or rejection of an organ allograft, and *in vitro* by CML, MLR and antibody cytotoxicity assays. Organ transplants were performed from the PBMC donor (n=15) or donor-matched animal (n=7). All animals received a kidney transplant, except for animal 14980, which received a heterotopic heart transplant. The surgical procedures were performed, without immunosuppression, at least one month after cessation of cyclosporine.

6.3 RESULTS

Twenty-two animals that had undergone the mixed chimerism protocol and then received a MHC-mismatched organ allograft were analysed (10 retrospectively and 12 prospectively) (table 1). Four animals were excluded because allograft tolerance was indeterminate due to early death (animals 13810, 14224), technical failure (14529), and an unrecognized lymphocoele obstructing the ureter (animal 13635[3]).

6.3.1 Presence of donor-derived bone marrow CFUs at the time of organ transplantation correlate with tolerance

Presence of donor-derived progenitor cells in the bone marrow at the time of organ transplant was determined by measuring CFUs in 14 animals. This finding correlated precisely with organ tolerance ($p<0.001$) as all animals with detectable donor-derived BM-CFUs at 90 days or more after HCT accepted delayed donor matched organ allografts (n= 9; PPV = 100%). In one animal (15401) CFUs became undetectable by day 512 despite being detectable at day 90 after HCT. All animals with undetectable donor-derived BM-CFUs at 90 days after HCT (or the nearest timepoint after this) rejected their organ (n= 8; NPV = 100%) (table 6.1).

[3] This animal was previously reported as rejecting its organ (Gleit, Z. L. et al. 2002a; Gleit, Z. L. et al. 2002b)

6.3.2 Presence of thymic chimerism at the time of organ transplantation correlates with tolerance

Thymic microchimerism (detectable by PCR/Southern Blot; n=15) was assessed as well as macrochimerism (detectable by FACS; n=19) so that any cases with low-level thymic chimerism would still be identified. Thymic chimerism (micro- or macro-) at the time of organ transplantation correlated precisely with allograft acceptance ($p<0.001$; n=9;PPV=100%). Only one animal (14040) had detectable thymic microchimerism without macrochimerism[4]. All animals with undetectable thymic microchimerism rejected their allograft (n=13; NPV = 100%) (table 6.1).

[4] Animal 14040 had thymic microchimerism without macrochimerism 49 days before kidney transplantation (61 days after PBMC transplant); it was not possible to ascertain the status of thymic microchimerism at the time of transplantation from the samples obtained.

Animal Number	SLA Barrier (Donor-Host)	Days between PBMCs and Organ	Protocol	Chimerism				In Vitro Response[c]			
				Periph Blood[b] (Day[a])	Thymus		BM-CFU (Day[a])	MLR (Day[a])	CML (Day[a])	Ab Cytotoxicity	
					FACS (Day[a])	PCR/SB[d] (Day[a])				Post PBMC	Post Organ
Accepted Organ											
[A]13101[Ø]	AC-AD	190	A	M *(0)*	Y *(0)*	Y *(0)*	Y *(519)*	N *(-110)*	N *(-50)*	N	N
[B]13272	AC-AD	156	B	M *(6)*	Y *(57)*	Y *(7)*	Y *(875)*	N *(53)*	N *(53)*	N	N
[A]13476[Ø]	CD-AD	98	A	L *(7)*	Y *(0)*	Y *(0)*	Y *(482)*	N *(-28)*	N *(-28)*	N	N
[C]14225[Ø]	AC-AD	85	C	L *(180)*	Y *(21)*	€	Y *(180)*	N *(-23)*	N *(-23)*	N	N
[F]**14980***	AC-AD	693	F	M *(87)*	Y *(104)*	€	Y *(608)*	N *(3)*	N *(3)*	N	N
[E]**15401**	AC-AD	212	E	M *(110)*	Y *(301)*	Y *(184)*	Y *(90)*	N *(0)*	N *(0)*	N	N
[F]**15403**	AC-AD	212	F	M *(110)*	Y *(186)*	Y *(186)*	Y *(90)*	N *(0)*	N *(0)*	N	N
[F]**15641**	AC-AD	203	F	L *(86)*	Y *(-107)*	Y *(44)*	Y *(0)*	N *(155)*	N *(144)*	N	N
[B]14040	AC-AD	115	B	N *(-80)*	N *(-49)*	*(Y)* *(-49)*	Y *(0)*	Y *(0)*	Y→H *(0) (32)*	Y	€
Rejected Organ											
[B]13583[Ø]	CC-AD	119	B	N *(-109)*	N *(0)*	N *(0)*	€	Y *(Pre Tx)*	Y *(Pre Tx)*	N	€
[B]13584[Ø]	CC-AD	119	B	N (-109)	N (0)	N (0)	€	Y *(Pre Tx)*	Y *(Pre Tx)*	Y	€
[D]14041*[Ø]	AC-AD	110	D	L *(2)*	N *(0)*	€	€	€	€	Y	Y
[C]14143[Ø]	CC-DD	131	D	L *(0)*	N *(0)*	N *(0)*	N *(0)*	€	H *(0)*	Y	Y
[A]14145[Ø]	CC-DD	131	B	N *(-118)*	N *(-98)*	N *(0)*	€	€	H *(-54)*	Y	€
[C]**14682**[Ø]	CC-DD	203	A	N *(-141)*	N *(-169)*	N *(-132)*	N *(-169)*	Y *(-132)*	Y *(-132)*	Y	€
[C]**14683**	CC-DD	204	C	N *(-143)*	N *(-69)*	N (-33)	N (0)	Y (-33)	Y *(-33)*	N	€
[C]**14805**	AC-AD	104	C	N *(-1)*	N *(-34)*	€	N *(-34)*	Y (-49)	Y (-49)	€	€
[C]**14833**	AC-AA	204	C	L *(39)*	€	€	N *(-141)*	€	Y (-99)	€	€
[C]**14917**[Ø]	AC-AA	55	C	L *(1)*	€	€	€	€	N *(0)*	N	€
[E]**15638**	AC-AD	203	E	L *(15)*	€	€	N *(-31)*	€	N *(-24)*	N	Y
[E]**15704**	AC-AD	125	E	N *(0)*	N *(-36)*	N *(-30)*	N *(0)*	Y *(0)*	H *(0)*	N	Y
[F]**15770**	AC-AD	125	F	N *(-46)*	N *(-66)*	N *(-54)*	N *(-54)*	Y *(0)*	Y *(0)*	N	€

Key:

Bold animal no. Prospectively analysed

a Day of assay in relation to organ transplantation (transplantation day= 0; pre-transplantation= Pre Tx)

b Peripheral blood chimerism: recorded as Multilineage ('M'), Lymphoid ('L') or Not detectable ('N')

c In vitro responses: recorded as responsive ('Y'), hyporesponsive ('H') or unresponsive ('N')

d 'SB'=Southern Blot Animal number prefix = Induction Protocol (see table 3.1)

Ø Thymic biopsy at kidney transplant

* Pretransplant DLI

€ Value not ascertained at the relevant time point

Table 6.1 Summary of chimerism induction and delayed organ transplant animals

6.3.3 Multilineage peripheral blood chimerism correlates with organ tolerance

Previously we have noted that there was a variable relationship between peripheral blood lymphoid chimerism and tolerance (Gleit, Z. L. et al. 2002b). In this study, we separately analysed animals with donor-derived peripheral blood lymphoid and myeloid cells versus animals with donor-derived lymphoid cells only.

Multilineage chimerism, when present at the time of organ transplantation (n=5), always correlated with tolerance ($p<0.005$; PPV= 100%). As in previous studies, isolated lymphoid chimerism, at the time of organ transplantation (n=8) correlated poorly with tolerance ($p<0.3$; PPV=38%). One animal had no detectable peripheral blood chimerism at the time of organ transplantation but was tolerant (animal 14040). Analysis of the donor-derived cell populations contributing to chimerism revealed that isolated lymphoid chimerism consisted of only T lymphocytes (Gleit, Z. L. et al. 2002b) whereas multilineage chimerism included B and T lymphocytes, granulocytes, monocytes and natural killer (NK) cells (chimerism levels in representative animals are outlined in table 6.2).

	Peripheral Blood Chimerism on Day of Organ Allograft Transplantation				
	T cell (%)	B Cell (%)	Granulocyte (%)	Monocyte (%)	NK Cell (%)
High Multilineage Chimera (15403)	41.6	49.5	84.6	97.1	95.7
Low Multilineage Chimera (15401)	3.1	0.8	0.6	1.1	1.9
Isolated Lymphoid Chimera: Acceptor (14225)	10.0	ND	ND	ND	ND
Isolated Lymphoid Chimera: Rejector (14833)	5.5	ND	ND	ND	ND

Key:

NK Natural Killer

ND 'Not detectable'; taken as <0.5% above background staining on FACS assessment.

Table 6.2 Chimerism in each peripheral blood lineage at organ transplantation

6.3.4 Organ tolerance

6.3.4.1 In vivo assessment: organ T cell infiltrate in mixed chimeras but no T cell infiltrate in full chimeras

Nine of 22 animals (41%) were tolerant to their organ as assessed by biopsy histology and functional assessments (creatinine level for kidney allografts and electrocardiography for heart allografts). Histological analysis of transplant biopsies taken from acceptor animals revealed normal structure of the transplanted organ. However, there was significant cellular infiltrate (fig 6.1a) in all but two animals (14980 and 15403, the only near full chimeras at organ transplantation): these two had histological appearances identical to that of a naïve organ following transplantation (fig 6.1b).

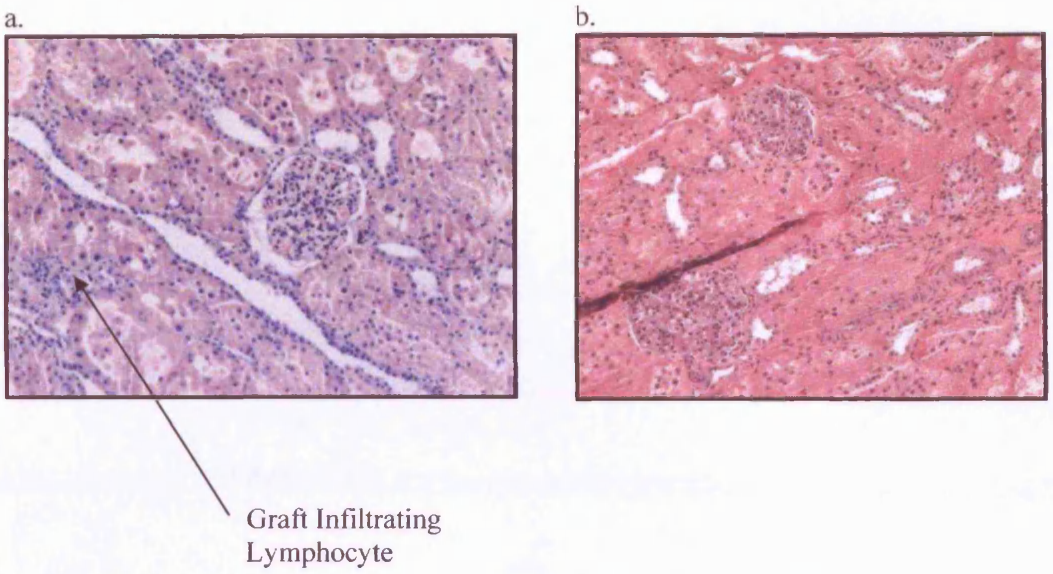


Figure 6.1 Histology of accepted organs. Representative histology of accepted organs: animals with mixed chimerism have graft infiltrating lymphocytes without evidence of inflammation or rejection (a; animal 13272). These infiltrating cells may have a regulatory function. In contrast, animals with full chimerism have no infiltrate in their organ graft (b; animal 15403).

6.3.4.2 In vitro assessment: assays do not always correlate with in vivo organ tolerance

CML, MLR and antibody cytotoxicity assay results were close, but not exact, correlates of *in vivo* allograft tolerance ($p<0.002$ for each). CML and MLR both showed donor unresponsiveness whilst maintaining robust 3rd party responses, and no antibody cytotoxicity was detected in any tolerant animal except animal 14040. Prior to organ allografting animal 14040 had detectable cytotoxic antibody to donor (fig 6.2a), and at the time of organ placement had a normal alloresponse to donor on CML (fig 6.2b) and MLR. However, on day 42 after organ transplantation the CML assay showed donor specific unresponsiveness at high ratios of effectors to target cells, tending towards hyporesponsiveness at increased effector to target cell dilution (fig 6.2c).

CML was unresponsive in two animals which rejected their kidney transplants: 14917 and 15638. MLR was not assessed in these animals, but was found to be responsive in all non-tolerant animals where it was assessed at an appropriate time point (n=9).

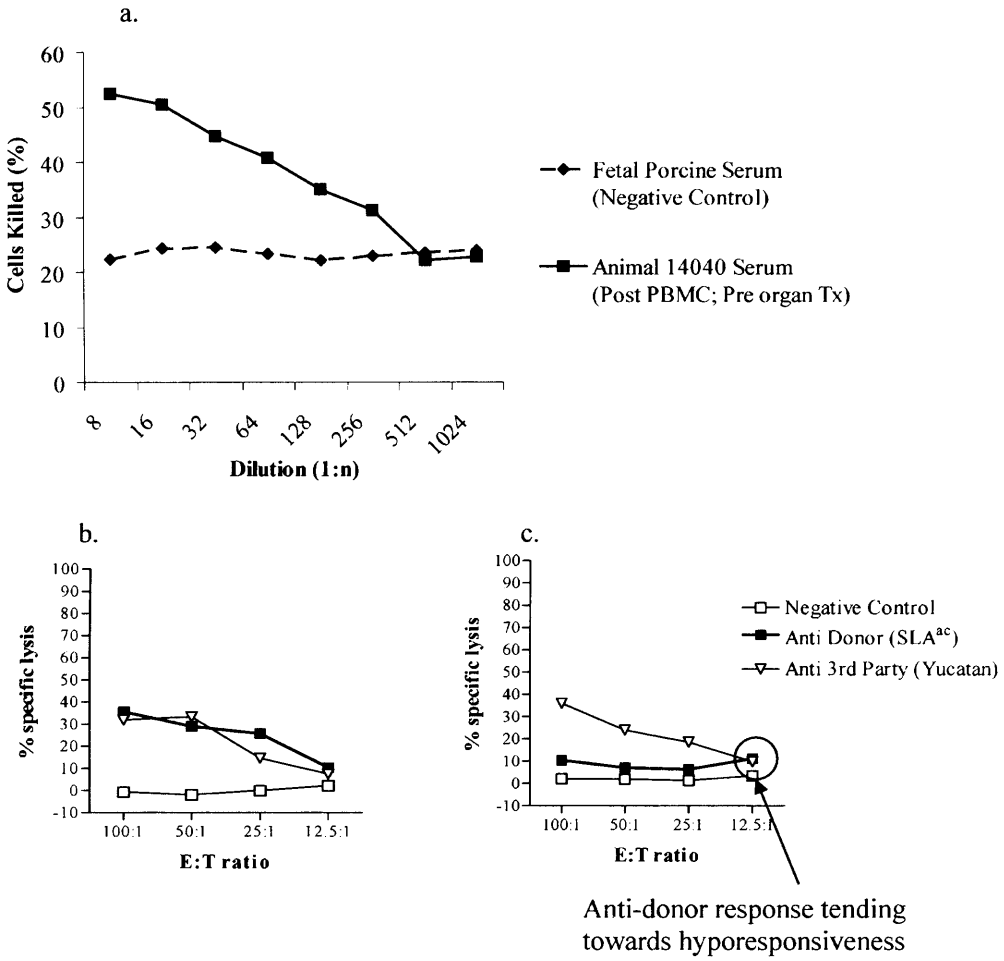


Figure 6.2 Animal 14040's immune alloresponse. Prior to transplantation this animal had detectable cytotoxic antibody to donor (a) and was responsive on CML ((b) day 112: day of organ transplantation). Following organ transplantation the CML was unresponsive at high effector to target (E:T) ratios however this tended towards hyporesponsiveness with increasing E:T dilutions ((c) day 154: 42 days after organ transplantation).

6.3.5 Minimal adverse effects

No animal in this study had significant complications related to treatment except animal 13101 which developed GvHD following HCT. This was mild and resolved with steroid treatment.

6.4 DISCUSSION

Presence of detectable levels of donor BM-CFUs (by PCR), thymic chimerism (by PCR/Southern Blot) and multilineage peripheral blood chimerism at time of delayed organ allograft placement are consistent markers of stable organ allograft tolerance in this chimerism induction protocol. The strong relationship of each of these three markers to tolerance in a clinically relevant large animal tolerance model has not been described previously. This relationship is important, both for directing tolerance induction research using HCT, and as a marker of the achievement of tolerance prior to organ allografting in future clinical HCT tolerance induction protocols.

6.4.1 Donor-derived bone marrow colony forming units are a reliable predictor of tolerance

Presence of donor-derived BM-CFUs at 90 days were used as a marker for engrafted stem cells. However, the detected BM-CFUs may also have originated from donor progenitor cells. One of the animals (15401) in this study lost evidence of BM-CFUs greater than 90 days following HCT. Other animals receiving the same HCT protocols without an organ allograft lost evidence of BM-CFUs up to 22 weeks after PBMC infusion in swine (data not shown). This is much longer than the 10-12 weeks demonstrated by Weissman in mice (Christensen, J. L. et al. 2001).

Rejector animals lost evidence of donor-derived BM-CFUs before 12 weeks. Antibody cytotoxicity data (table 6.1) indicates that this was due to graft rejection in five cases. However, in the majority (six cases) there was no evidence of antibody cytotoxicity to donor implying that the graft was not lost due to rejection in these animals.

Whatever the origin of the BM-CFUs, it is likely that they need to be present at least until the time of organ allografting. In tolerant animals assayed at relevant timepoints (at the time, or after organ transplantation) BM-CFUs were detected; the exception to this was 15401, however it did not have a relevant CFU assessment until 12 months after organ transplantation.

6.4.2 Donor thymic chimerism is an accurate predictor of tolerance

Presence of thymic chimerism is as closely associated with organ tolerance as the presence of BM-CFUs. This may be because both donor-derived BM-CFUs and cells in the thymus originated from haematopoietic stem and progenitor cells from the HCT.

The donor cell type in the thymus was not examined in this study. However, previously the presence of donor cells expressing MHC class II, without epithelial surface markers, and showing the morphology of dendritic cells in the thymus have been identified in a tolerant animal that underwent this protocol (Fuchimoto, Y. et al. 2000). The other possible source of these donor cells in the thymus is from the initial PBMC infusion. Tian found, in mice, that infusion of mature T cells into a conditioned host led to donor specific tolerance due to the T cells migrating to the thymus and mediating central deletion of alloreactive thymocytes (Tian, C. et al. 2004). Peripheral blood contamination was not the likely source of the donor cells detected in the thymus as thymic chimerism was absent in the presence of 7% peripheral blood chimerism in animal 14143.

The relationship between thymic chimerism and organ tolerance was unaffected by thymic biopsy on the day of transplantation: three animals had thymic biopsies on the day of organ transplantation with no adverse effects on tolerance (see table 2). The neutral effect of thymic biopsy is in contrast to the effect of thymic biopsy during establishment of renal allograft tolerance by peripheral mechanisms (using a short course of FK506) in which renal tolerance was not achieved if thymic biopsy is taken 21 days prior, or on the day of organ transplantation (Vagefi, P. A. et al. 2004). A likely reason for this is that tolerance is already established by the time of organ transplantation in this model whereas it is being induced following organ transplantation in the peripheral tolerance model.

The association of thymic chimerism and organ tolerance has limited utility as a predictor of subsequent allograft acceptance in tolerance induction regimens because it is not practicable to perform a thymic biopsy in a clinical context. However, a bone marrow aspirate for BM-CFUs would be relatively easy to obtain and both have equal positive predictive value.

6.4.3 Multilineage peripheral blood chimerism is a reliable predictor of tolerance

This study demonstrates that multilineage peripheral blood chimerism at the time of organ transplantation was predictive of organ tolerance. Previously, a variable relationship between peripheral blood chimerism and tolerance was described (Gleit, Z. L. et al. 2002b). However, this was in relation to simple peripheral blood chimerism (i.e. either lymphoid or multilineage). Establishing a clear relationship between

chimerism and tolerance is helpful in understanding the mechanism of tolerance induction with HCT. Whether the relationship of BM-CFU, thymic and multilineage peripheral blood chimerism with each other and with organ tolerance is causative or an epiphenomenon is the focus of ongoing research. It is interesting to note that multilineage peripheral blood chimerism is not necessary for tolerance.

The *in vitro* tolerance data did not correlate with organ tolerance in all animals. BM-CFU, thymic and multilineage peripheral blood chimerism are more reliable and consequently should be used in preference as predictors of tolerance. One possible reason for the predictive limitations of *in vitro* assays is that they do not mirror the conditions *in vivo*, with limited cell populations in non-physiologic relative concentrations. If a regulatory mechanism is involved in tolerance induction, then any regulatory cells may not be at the same relative concentration to effector cells as *in vivo* where they exert their physiologic effects.

6.4.4 Regulatory and deletional tolerance mechanisms

There was indirect evidence for the involvement of regulatory mechanism as well as a central deletional mechanism in organ allograft tolerance for some animals. For example, allograft histology in the near full chimera acceptor animals (e.g. animals 14980 and 15403) demonstrated a naïve looking organ, with no evidence of rejection or T cell infiltrate, as would be expected in a central deletional picture where there are no alloreactive T cells. However, in animals with lower levels of chimerism there was a T cell infiltrate without evidence of rejection. Previous analysis of these cells has indicated a likely regulatory function (Baron, C. et al. 2001a; Torrealba, J. R. et al. 2004).

Furthermore, the *in vitro* data from animal 14040 is consistent with a predominantly regulatory mechanism: following chimerism induction it became responsive to donor by *in vitro* assays. It was not initially tolerant to its transplanted organ but recovered after an initial rejection crisis. This pattern suggests that a deletional mechanism was not dominant. The animal then went on to become unresponsive to donor type cells on CML, becoming hyporesponsive on reducing concentrations of effectors suggestive of dilution of regulatory cells (figs. 6.1 c & 6.1 d).

Different tolerance mechanisms may dominate, depending on chimerism levels. In high-level chimeras, central deletional mechanisms are dominant, but with decreasing levels of chimerism there is decreased thymic deletion of the repertoire of developing T cells by donor-derived cells. This results in the emergence of more alloreactive T cells, and so increasing importance of regulatory mechanisms to achieve tolerance (fig 6.3). This has been demonstrated in mice (Domenig, C. et al. 2005; Kurtz, J. et al. 2004), but has not been suggested in a large animal model before.

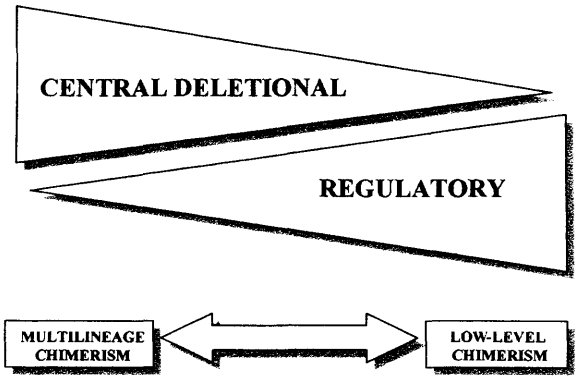


Figure 6.3 Both central deletional and regulatory mechanisms involved in tolerance induction in chimeras

6.5 CONCLUSIONS

The aim of this study was to identify reliable predictors of tolerance. Simple presence of peripheral blood chimerism and *in vitro* assays of responsiveness do not appear to predict tolerance with complete accuracy. This study indicates that the presence of donor-derived BM-CFUs, thymic microchimerism and multilineage peripheral blood chimerism correlate with subsequent tolerance to an organ transplant. Furthermore, this study suggests that high-level chimeras appear to have a predominantly central deletional mechanism of tolerance, with regulatory mechanisms becoming more important in lower level chimeras. These findings are applied retrospectively to a previous CTA allotransplant study in Chapter 6b to further understand why skin tolerance was not achieved and to formulate a hypothesis to induce skin tolerance.

CHAPTER 6B: APPLICATION OF ORGAN TOLERANCE FINDINGS TO CTA

6.6 INTRODUCTION

The aim of this part of the study was to develop a hypothesis to achieve skin tolerance in a preclinical swine model. Previously, a swine chimerism induction with CTA transplant model achieved tolerance to the musculoskeletal element of a composite tissue allograft but only prolonged survival of the skin element. This work is reviewed in the light of the findings gained from the more extensive experience of swine chimerism induction with an organ transplant model described in Chapter 6.

6.7 SUMMARY OF PREVIOUS CHIMERISM INDUCTION AND LIMB ALLOTRANSPLANT STUDY

6.7.1 Chimerism induction and limb allotransplant study outline

A previous study attempted to induce tolerance to a limb transplant using chimerism induction in swine. Tolerance was achieved to the musculoskeletal element but not to the skin (a state of split tolerance). Seven animals (previously reported on by Hettiaratchy (Hettiaratchy, S. et al. 2004)) underwent chimerism induction with T-cell depletion with immunotoxin, and infusion of either bone marrow cells (BMC) or cytokine-mobilised PBMCs (CM PBMCs) from the donor, followed by a course of cyclosporine until day 30. A limb transplant from either the donor or a donor matched animal was placed heterotopically in a subcutaneous abdominal pocket with a window to expose the donor skin (see fig 6.4) either on day 0 (n=6) or at day 52 (n=1). A control animal received immunotoxin and cyclosporine with a limb transplant, but did not receive haematopoietic cells.

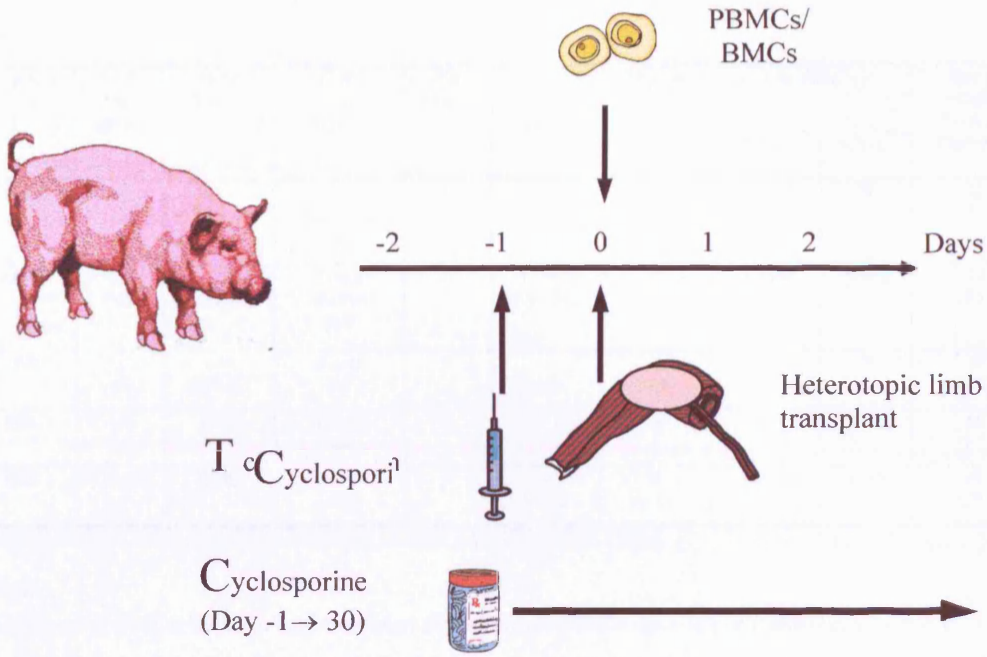


Figure 6.4 Chimerism induction and limb transplant study outline

6.7.2 Results from chimerism induction and limb allotransplant study

Two of the experimental animals died without rejecting their limb transplant but before tolerance could be formally assessed (i.e. before 90 days post transplant). All of the remaining five animals were analysed for tolerance *in vitro* by CML and MLR. They were also assessed for thymic and peripheral blood chimerism by FACS. Some animals (n=3) were analysed for presence of engraftment as indicated by donor bone marrow CFUs; these results have not been reported previously (see table 6.3). All animals rejected the epidermal portion of the skin on their limb allograft, with variable dermal involvement, but accepted the musculoskeletal elements (a state of split tolerance). The control animal died on post operative day 17 with evidence of skin rejection but no rejection of the musculoskeletal elements.

Animal No.	SLA Dis-parity	HCT Source	Limb Source (Day[a])	PB Chim[b]	BM-CFUs (Day[a])	Thymic Chim[c]	In Vitro[d]		Day of Skin Rejectn[a] (FT/PT)
							MLR	CML	
14831	AC-AA	CM PBMC	Donor Matched *(0)*	L	Y *(30)*	Y *(78)*	N	N	42 (PT)
14918	AC-AA	CM PBMC	Donor Matched *(52)*	L	Y → N *(30) (60)*	Y *(150)*	N →Y	N→Y	70 (FT)
15024	AC-AD	CM PBMC	Donor *(0)*	L	-	Y (148)	N	N	60 (PT)
15067	AC-AA	BMC	Donor *(0)*	N	-	N	Y	Y	60 (FT)
15022	CC-AD	BMC	Donor *(0)*	N	N *(180)*	N	N →Y	N→Y	60 (PT)

Key:

a Day of assay in relation to limb transplantation (transplantation day= 0; pre-transplantation= Pre Tx)

b Peripheral blood chimerism: recorded as Multilineage ('M'), Lymphoid ('L') or Not detectable ('N')

c Presence ('Y') or absence ('N') of thymic chimerism on FACS assessment (chimerism defined as >0.5% above background)

d *In vitro* responses: recorded as responsive ('Y'), hyporesponsive ('H') or unresponsive ('N'). In some the limb was removed and in vitro responses were reassessed (indicated by: result before limb removal '→' result following limb removal).

Table 6.3 Summary of the outcomes and assay results from chimerism induction and limb transplant study

6.8 REVIEW OF THE RESULTS OF THE LIMB ALLOTRANSPLANT STUDY IN LIGHT OF THE ORGAN TRANSPLANT FINDINGS

At the time of the limb allotransplantation study it was unclear precisely why tolerance to skin was not achieved in the limb allotransplants. In Chapter 6a predictive markers for delayed organ allograft tolerance following chimerism induction were identified. These are:

(1) Evidence of donor-derived bone marrow stem cell engraftment as measured by bone marrow CFUs at 14 weeks

(2) Evidence of thymic chimerism at transplantation

(3) Multilineage peripheral blood chimerism at transplantation

Furthermore there was indirect evidence that with decreasing levels of chimerism, regulatory mechanisms are more dominant and central deletional mechanisms less dominant in maintaining tolerance.

6.8.1 Predictors of tolerance

The following observations were made when the predictors of tolerance from the organ allotransplant study were applied to the limb allotransplant study:

(1) There is no evidence of engraftment of donor-derived bone marrow stem cells in either bone marrow cell or CM-PBMC recipients

(2) There is no thymic chimerism in the bone marrow recipients, and only low level (<2%) thymic chimerism in the CM-PBMC recipients beyond 14 weeks

(3) There is no peripheral blood chimerism in the bone marrow recipients, and falling isolated lymphoid chimerism in the CM-PBMC recipients

These results would predict that the limb transplant would be rejected. In the bone marrow recipients there were no predictors of tolerance, and even in the CM-PBMC recipients not all the essential predictors (presence of donor-derived BM-CFUs and thymic chimerism) were fulfilled. Interestingly, although the skin was eventually rejected, the musculoskeletal element was accepted in all animals, which would not be predicted with these criteria; there was evidence this may have been due to regulatory mechanisms.

6.8.2 Evidence of regulatory mechanisms maintaining musculoskeletal tolerance and prolonging skin survival

There is indirect evidence both from the histological findings and *in vitro* data indicating active regulatory mechanisms in the limb allotransplants. Histology revealed a non-inflammatory perivascular lymphocytic infiltrate within the musculoskeletal element of the limb transplants. This type of infiltrate has previously been demonstrated to have a regulatory phenotype in organ transplants (Baron, C. et al. 2001a; Torrealba, J. R. et al. 2004).

The MLR results also indicated the presence of a regulatory mechanism that was maintained within the limb allograft itself. Following allografting, animals showed donor specific unresponsiveness to the limb allograft [5]. Two animals subsequently had the limb removed with return of responsiveness to donor-type cells.

6.8.3. Reasons for failure to achieve engraftment but still attainment of musculoskeletal tolerance

This study used the same general approach to induce chimerism as in the animals receiving organ transplants outlined in Chapter 6a. However, there were several elements that were specific to this protocol which may have contributed to the outcome in this study:

(1) No irradiation was given to the recipients. The rationale for this was to attempt to reduce the regimen's toxicity and so make it more widely applicable. Irradiation is thought to create an "immunological space" to allow engraftment of the donor haematopoietic stem cells. It has been possible to omit this element from chimerism induction protocols in small animals (see Chapter 2). However, this has required profound T-cell depletion or disablement, which is not currently possible in large animal models. This may have been a key factor in the failure to engraft and the low to non-existent peripheral blood and tissue chimerism levels.

(2) Bone marrow cells were given to some of the recipients (n=2). It is not possible to attain as high a number of cells for transplantation with bone marrow so only $7.5x10^6$ cells/kg were administered compared to $1.5x10^9$ CM-PBMC. A lower number of donor cells could decrease the likelihood of achieving engraftment, and this may have been the reason that no chimerism was observed in the bone marrow recipients.

(3) The animals received a limb instead of an organ transplant. This may have had several consequences. Firstly, the predictors of organ tolerance may not apply to the tissue in a limb: skin is generally considered more difficult to induce tolerance to than other tissues, and the musculoskeletal tissue in a limb may actually be more tolerogenic than many organs. Secondly, the donor marrow in the transplanted limb could be an ongoing source of donor cell chimerism. However, there was no chimerism in the bone

[5] One animal was responsive on MLR despite being tolerant to the musculoskeletal element of the limb. There was evidence that this was due to sensitisation to non-MHC minor antigens.

marrow recipients indicating that any donor bone marrow in the transplanted limb was not particularly active. Finally, the bone marrow in the transplanted limb could be a target for engraftment of the infused HSCs at chimerism induction. This may facilitate engraftment of donor HSCs even with less stringent conditioning of the recipient, and is a possible location of engraftment in the animals receiving CM-PBMC.

These factors may explain why there was no clear evidence of HSC engraftment with rejection of the donor skin, but acceptance of the musculoskeletal element of the allograft. There is no evidence that the timing of progenitor cell infusion had any effect on tolerance. In four cases the limb was transplanted on the day of bone marrow cell/ PBMC infusion, and in one case the limb transplant was delayed by 52 days. Despite this difference, all animals had the same outcome of split tolerance.

6.9 HYPOTHESIS FOR ACHIEVEMENT OF SKIN TOLERANCE

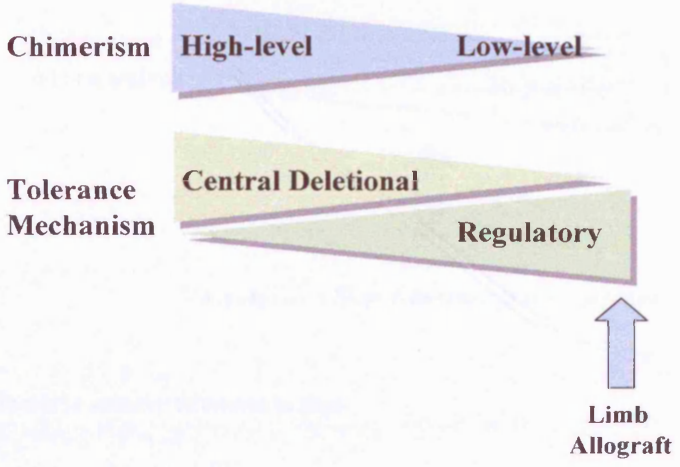


Figure 6.5 Likely mechanism of tolerance induction for limb allografts

The findings in the chimerism induction and limb allograft model are consistent with a predominantly regulatory mechanism of tolerance, which was strong enough to achieve tolerance to the musculoskeletal element of the limb allograft but did not induce tolerance to skin (see fig 6.5).

Central deletional tolerance is considered more robust than regulatory tolerance, which may explain why skin was rejected in this model. However, a purely central deletional

mechanism may not achieve skin tolerance because it may not be able to prevent skin rejection via skin specific antigens. There is evidence that skin specific antigens can cause skin rejection. These skin specific antigens may not be represented in the repertoire of the dendritic cells originating from the donor bone marrow that migrate to the thymus to take part in negative selection of thymocytes. In a purely central deletional model it is possible that lymphocytes specific for those skin specific antigens could be produced despite full chimerism. A moderate-level engrafted chimera may combine the advantages of both a robust central deletional mechanism with significant skewing of the T cell repertoire away from a rejection, and a strong regulatory mechanism that may prevent rejection due to skin specific antigens.

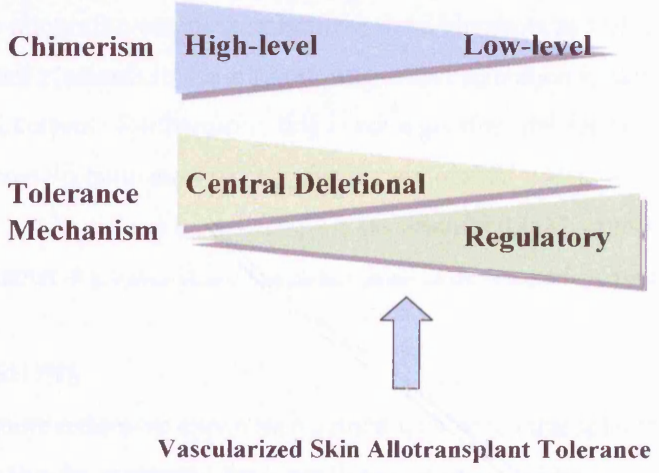


Figure 6.6 Hypothesis to achieve tolerance to skin

Based on these findings, the following hypothesis can be formulated: tolerance can be achieved to primarily vascularised skin transplanted across a single haplotype MHC barrier in a preclinical swine model, by induction of haematopoietic stem cell engraftment with moderate levels of mixed chimerism as measured in the thymus and peripheral blood. This will achieve a mixture of both central deletional and regulatory tolerance (see fig 6.6).

6.9.1 Conditions to be fulfilled to test hypothesis

To be able to test this hypothesis in an ideal model two conditions need to be fulfilled:

(1) Engraftment with a moderate level of chimerism needs to be achieved. Two methods of achieving a moderate level engrafted chimera are explored: adult and *in utero* chimerism induction. The reliable achievement of moderate chimerism is difficult with adult chimerism regimens, since most animals in previous chimerism induction experiments only achieved low levels of chimerism (thymic chimerism<10%). Consequently, a method to boost chimerism using donor leukocyte infusions is examined in Chapter 7. An alternative to adult chimerism induction that has been successful in achieving moderate-to-high levels of chimerism is *in utero* chimerism induction. Therefore, in Chapter 9 *in utero* chimerism induction protocols are used to attempt to induce moderate level chimeras.

(2) A vascularised skin allograft model is required. The previous study used a composite tissue allograft containing musculoskeletal elements as well as skin. However, the other elements in the allograft may affect tolerance to skin making the data difficult to interpret. Furthermore, this is not a good model for many CTAs that do not necessarily contain bone marrow (e.g. face or abdominal wall). A non-vascularised skin graft would not be a good model for CTA (as described in Chapters 2 and 5). Therefore, in Chapter 8 a vascularised skin allograft is developed in swine.

6.10 CONCLUSIONS

Analysis of the more extensive experience gained with organ transplantation in chimerism induction demonstrated three predictors of organ tolerance, as well as indicating that both central deletional and regulatory mechanisms can be involved in the achievement of tolerance. The application of these findings to the chimerism induction with limb allotransplant data indicates that not all the predictors were met. Furthermore, the data suggests that active regulatory mechanisms enabled acceptance of the musculoskeletal element. The hypothesis developed for the achievement of skin tolerance requires the induction of moderate levels of mixed chimerism (i.e. thymic chimerism of 10-80%). However, this level of chimerism is not reliably achieved in the adult chimerism induction model. Furthermore, a vascularised skin allograft transplant is required to test this hypothesis in an easily interpretable model. Chapter 7 will examine the use of donor leukocyte infusion to boost chimerism following adult chimerism induction, and Chapter 8 will describe the development of a vascularised skin allotransplant model in swine.

CHAPTER 7: BOOSTING CHIMERISM WITH DONOR LEUKOCYTE INFUSION

7.1 INTRODUCTION

The hypothesis laid out in Chapter 6 is that engraftment with at least a moderate level of thymic and peripheral blood chimerism is required to achieve tolerance to skin allotransplanted across a full MHC barrier in a swine model. The most reliable chimerism induction regimen outlined in Chapter 6a achieved engraftment in all long-term surviving animals, however only low-level chimerism was achieved in half of the animals (Cina, R. A. et al. 2006). One method used to increase donor cell chimerism in low-level chimeras is to perform a donor leukocyte infusion (DLI) following HCT, an approach that has been promising both in animal models (Baron, F. et al. 2006b; Billiau, A. D. et al. 2002; Georges, G. E. et al. 2000) and in clinical settings (Spitzer, T. R. et al. 2000). Unfortunately, overall results from DLI studies remain variable and GvHD often develops in patients receiving DLI.

This study reviews the previous experience with DLI in this model. Mechanisms controlling the effectiveness of DLI at boosting chimerism are investigated. Finally a strategy for improving the effectiveness of DLI is developed. This strategy is then tested in Chapter 11.

7.2 OVERVIEW OF EXPERIMENT

All miniature swine from the Transplant Biology Research Center (TBRC) that had received a non-myeloablative HCT across a single haplotype major MHC barrier between 1998 and 2006 were analysed. Animals were excluded if the HCT protocol was not completed. Forty-seven animals that had undergone HCT, including 15 chimeric animals that received DLI, were analysed. All animals had detectable levels of peripheral blood chimerism by flow cytometry at the time of DLI.

DLI was defined as ineffective if there was no sustained increase in peripheral blood chimerism so as to exclude the small rise in lymphocyte chimerism seen immediately after DLI infusion due to the donor cells within the infusion.

The engraftment status (presence of donor haematopoietic stem cells) was assessed in the animals included in the study. Engraftment was indirectly assessed by the presence

of donor-derived bone marrow colony forming units (BM-CFUs; detected by PCR analysis), thymic chimerism or multilineage peripheral blood chimerism beyond 12 weeks after PBMC transplantation, as previously described in Chapter 6a.

Animals were monitored for symptoms of GvHD indicated by the presence of a skin rash, abnormal liver function tests and/or gastrointestinal disturbances as well as the clonal expansion of alloreactive donor T cells.

7.3 RESULTS

7.3.1 Donor leukocyte infusion failed to increase chimerism in the majority of chimeric recipients

Fifteen chimeric animals received one or more DLIs, either from the original donor or from an animal MHC-matched to the original donor (summarized in table 7.1). Twelve of these recipients (80%) showed no response to DLI as measured by increase in peripheral blood chimerism.

Only three animals showed a sustained increase in peripheral blood chimerism following DLI. Animal 14980 converted to full chimerism (i.e. 100% donor cells) without GvHD. This animal had high-level multilineage chimerism (Lymphocyte (L): 50%; Monocyte(M): 60%; Granulocyte (G): 74%) prior to receiving DLI. The two other animals, 13101 and 15204, had low-level chimerism[6] (13101 L:49; M:1; G:2, 15204 L:25; M:0; G:3) prior to DLI and developed GvHD following DLI leading to their subsequent sacrifice. All three animals had evidence of haematopoietic stem cell engraftment in the bone marrow and multilineage peripheral blood chimerism at the time of DLI. Donor stem cell engraftment in the bone marrow was detected in 6 of the 12 animals that did not respond to DLI, with engraftment not present (n=2) or not assessed (n=4) in the others.

[6] Lymphoid chimerism is not a time-sensitive measure of haaematopoietic chimerism due to the long life-span of lymphoid cells. Chimerism in the shorter surviving myeloid lineages (monocyte and granulocyte) provides a more accurate measure.

ANIMAL	HAPLOTYPE MISMATCH	DLI Day[a]	Donor Stem Cell BM Engraft-ment[d]	PB CHIMERISM AT DLI % [e] (Day[a])	GvHD[f]	PB CHIMERISM ~4WK POST –DLI (Day) %[f]
Chimerism Boost						
13101	AC-AD	709	Y	*L:49 M:1 G:2* (709)	Y	*L:95 M:79 G:99* (742)
14980	AC-AD	219	Y	*L:50 M:62 G:74* (217)	N	*L:57 M:85 G:79* (246)
15204	AC-AD	111	Y	*L:25 M:0 G:3* (104)	Y	*L:9 M:41 G:1* (134)
No Chimerism Boost						
13272	AC-AD	745[b]	Y	*L:13* (745)	N	
		984[b]	Y	*L:7* (984)	N	
13476	CD-AD	482[b]	Y	*10* (469)	N	
13810	AC-AD	82	U	*L:14* (82)	N	
14225	AC-AD	110	Y	*L:10 M:0 G:0.5* (110)	N	
14375	AC-AD	35	U	*L:59 M:25 G:40* (34)	N	
14376	AC-AD	35	N	*L:33 M:18 G:25* (35)	N	
		252[c]	N	*L:10* (252)	N	
14529	AC-AA	35	N	*L:35 M:24 G:31* (34)	N	
14547	AC-AD	35	U	*L:30 M:11 G:20* (34)	N	
14548	AC-AD	35	U	*L:34 M:10 G:18* (34)	N	
16558	AC-AD	152	Y	*L:10 M:0 G:1* (151)	N	
16626	AC-AD	153	Y	*L:6 M:3 G:4* (153)	N	
		566[b]	Y	*L:3 M:3 G:2* (566)	N	
17017	AC-AD	150	Y	*L:17 M:8 G:13* (153)	N	

Key

a Day in relation to original PBMC infusion to induce chimerism

b Donor Matched Leukocyte infusion

c Sensitised DLI

d 'Y'=Engrafted, 'N'=Not engrafted, 'U'=Engraftment undetermined at time of DLI

e Percentage of peripheral blood donor chimerism in each lineage (L-lymphocyte; M-monocyte; G-granulocyte). Monocyte and granulocyte levels not given if only lymphoid chimerism present

f 'Y'=presence, 'N'=absence of GvHD

Table 7.1 Donor leukocyte infusion outcomes

7.3.2 Evidence of suppression of the DLI donor-vs.-chimera effect by CD25+ cells

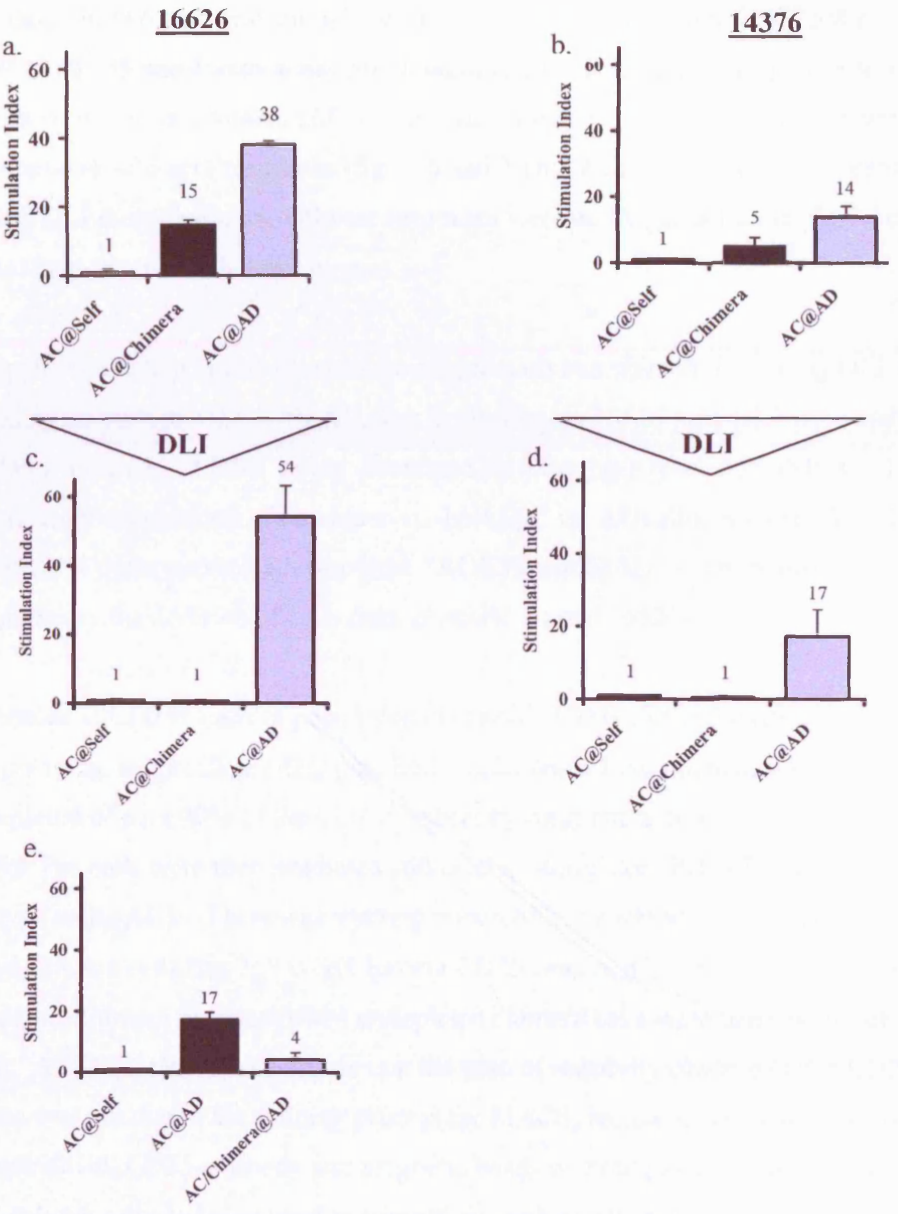


Figure 7.1 Suppression of graft versus host alloresponses following DLI in chimeric recipients[7]. Suppression of donor T cell alloresponses by two chimeric recipients (16626 and 14376) were assessed by MLR before (a & b) and after DLI (c, d & e).

[7] Mean background count for 16626 pre-DLI MLR= 305 counts per minute (cpm); 2 weeks post-DLI MLR =136 cpm, 2 weeks post-DLI coculture MLR=335. Mean background count for 14376 pre-DLI MLR=149 and 2 weeks post DLI MLR=136.

Suppression of the donor-vs.-chimera effect of DLI is one possible reason that DLI failed to increase chimerism in the majority of animals. Donor-vs.-chimera suppression was assessed in two chimeric animals before and after DLI treatment (14376 was assessed at day 35 and 16626 at day 566 following chimerism induction). Prior to DLI there was no donor-vs.-chimera (AC vs. AC/AD)[8] suppression in either animal, with strong donor-vs.-chimera responses (fig 7.1a and 7.1b: "AC@Chimera"). In contrast, following DLI these donor-vs.-chimera responses were no longer detected (fig 7.1c and d), indicating donor-vs.-chimera suppression.

Confirmation of active donor-vs.-chimera suppression in a chimera following DLI was obtained by co-culture MLR. PBMC taken from chimera 16626 post-DLI were added to naïve donor-type (AC) PBMC being stimulated by naïve host-type (AD) PBMC. There was significant suppression of the donor-vs.-host (AC vs. AD) alloresponse (fig 7.1e: "AC/-@AD" - unsuppressed alloresponse; "AC/Chimera@AD" - suppression of alloresponse by the addition of cells from chimeric animal 16626).

To determine if CD25+ cells (a population containing CD4+CD25+FoxP3+ Treg cells), contribute to the suppressive effect post DLI. Cells taken from chimera 16626 post-DLI were depleted of over 90% of the CD25+ subset by magnetic activated cell sorting (MACS). The cells were then irradiated and used as stimulators in MLR for naïve donor-type cells (AC). There was a strong donor(AC) -vs.-chimera response to CD25 depleted chimera cells (fig 2: "AC@Chimera CD25 Depleted"). In contrast, there was no donor-vs.-chimera response when undepleted chimera cells were used as stimulators (fig 7.2: "AC@Chimera"). To ensure that the gain of reactivity observed after CD25 depletion was not due to the staining process for MACS, recipient cells from 16626 were coated with CD25 antibody and magnetic beads without passage through the MACS column prior to being used as stimulators with no alloresponse detected (fig 7.2: "AC@Chimera Stained").

[8] the majority of their leukocytes in these mixed haaematopoietic chimeras were AC (i.e. host) phenotype (>66% in 14376 and >94% in 16626), with the remainder AD (i.e. donor) phenotype.

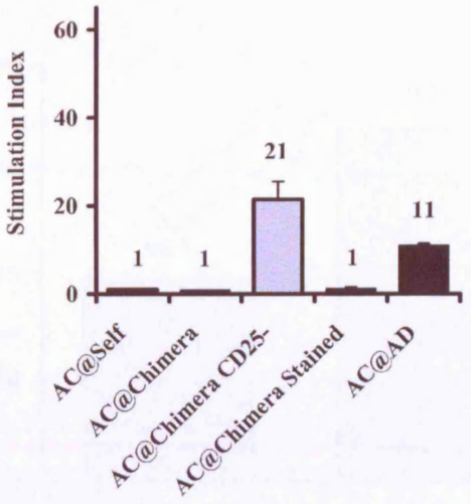


Figure 7.2 Suppression of donor-vs.-chimera response may be mediated by CD25+ cells in chimeric recipients[9]

A further indication that CD4+CD25+FoxP3+ Treg have a role in donor-vs.-chimera suppression post DLI was provided by observation of the change in the absolute number of Treg before and after DLI in animal 16626. There was a 74% increase in the number of Treg 2 weeks after DLI compared to the day of DLI (96.5 → 168 Treg/μm^3), this increase was sustained at 4 weeks (172 Treg/μm^3) (fig 7.3).

[9] Mean background count = 84.

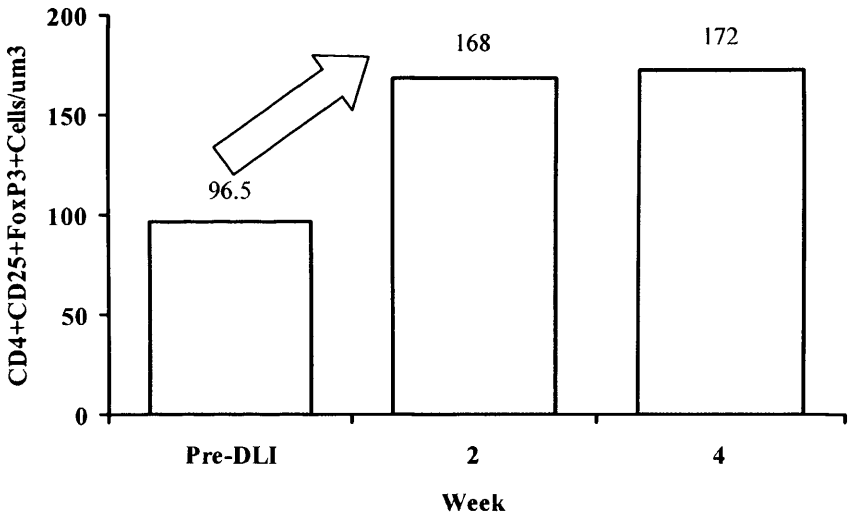


Figure 7.3 Absolute levels of CD4+CD25+FoxP3+ cells increase post DLI

Prior to DLI the level of CD4+CD25+FoxP3+ T cells in the animal 16626's blood was 96.5/um^3 ('Pre-DLI' left-hand column). Following DLI the level of CD4+CD25+FoxP3+ T cells increased to 168/um^3 (middle column), an increase of 74%. The level remained raised at 4 weeks following DLI at 172/um^3 (right-hand column). NOTE: It is possible that up to 30% of this increase was due to donor CD4+CD25+FoxP3+ T cells within the DLI.

7.4 DISCUSSION

The non-myeloablative chimerism induction regimen used in this model results in a low incidence of GvHD, but does not reliably achieve high enough levels of chimerism to test the hypothesis that a moderate level engrafted chimera will be tolerant to a vascularised skin allotransplant. DLI is able to boost chimerism in chimeras, but is largely ineffective in this model. The low incidence of GvHD in this model may be due to donor-vs.-chimera suppression. However, donor-vs.-chimera suppression may also make DLI ineffective at increasing chimerism.

7.4.1 DLI is rarely effective due to donor-vs.-chimera suppression

DLI was not effective at increasing donor chimerism in the peripheral blood of most chimeric animals studied. These findings are in agreement with those of the Storb group who showed that repeated DLI neither facilitated conversion to full donor chimerism after HCT nor prevented rejection in dogs receiving a non-myeloablative single-haplotype mismatch HCT (Fukuda, T. et al. 2006). They had previously

demonstrated success with DLI in MHC-matched minor antigen-mismatched transplants following sensitisation of the DLI donor (Georges, G. E. et al. 2000) and even some success without prior donor sensitisation (Baron, F. et al. 2006b). It is possible that the difference in the DLI effects between donor/host combinations that differed by minor antigen versus single MHC haplotypes may be because there is greater immunological similarity in minor mismatched animals and so not as much donor-vs.-chimera down-modulation is stimulated.

One possible weakness in this study is that a donor-matched DLI was used in two of the animals that did not respond to DLI opening up the possibility that minor antigen differences could stimulate anti-donor responses from the original donor cells in the chimera.

In this study *in vitro* evidence of donor-vs.-chimera suppression was only obvious within 4 weeks following DLI. The origin of the regulatory cells mediating donor-vs.-chimera regulation is unclear. These regulatory cells were either present in the recipient prior to DLI and their numbers and/or activity were boosted by the DLI, or were donor-derived and were infused with the DLI. It has been previously demonstrated that regulation is present in animals prior to DLI (Kunisaki, S. M. et al. 2001), indicating that these regulatory cells were not infused with the DLI, rather the DLI actually boosts the activity of the regulatory cells. Whether these donor-vs.-chimera regulatory cells are of recipient or donor origin (i.e. from donor PBMCs given for initial chimerism induction) has not been determined. Johnson et al. attributed the donor-vs.-chimera suppressive effect to donor cells (Johnson, B. D. et al. 1999); however, Blazar et al demonstrated that recipient cells can also mediate the donor-vs.-chimera suppressive effect (Blazar, B. R. et al. 2000).

In this non-myelablative model, recipient cells are exposed to donor T cells following HCT induction. It is therefore possible that recipient T cells are then stimulated to contribute, at least in part, to the suppressive effect. This is supported by the fact that one of the animals (14376) analysed for evidence of donor-vs.-chimera regulation of DLI was not engrafted, and so would have had limited capacity to produce any donor origin regulatory cells following chimerism induction. However, this animal still had a donor-vs.-chimera regulatory mechanism that was boosted by DLI.

In this model the cell type mediating the suppression appeared to be CD25+ cells. This finding correlates with rodent data demonstrating that regulatory T cells can suppress GvHD (Cohen, J. L. et al. 2002; Hoffmann, P. et al. 2002; Taylor, P. A. et al. 2002; Trenado, A. et al. 2006; Zeng, D. et al. 2004). It is possible that there are other cells which contribute to suppression, and that these were removed by non-specific binding to the column during magnetic bead depletion. CD4+ T cells (Johnson, B. D. et al. 1999), veto cells (Weiss, L. et al. 1999) and NK cells (Asai, O. et al. 1998; Sykes, M. et al. 1990a) have all been shown to suppress donor-vs.-chimera responses in mouse models. However, the coincident increase in T cells with a regulatory phenotype in the peripheral blood of recipients following DLI provides *in vivo* evidence supporting the hypothesis that CD25+ cells contribute to the regulatory mechanism.

7.4.2 Possible approaches to make DLI effective

DLI appears not to be effective at increasing chimerism in this model due to donor-vs.-chimera suppression. An approach to achieving increased chimerism following DLI would be to prevent donor-vs.-chimera suppression. This could be achieved by either preventing the development of donor-vs.-chimera suppression or removing donor-vs.-chimera suppression.

Prevention of the development of donor-vs.-chimera suppression has been achieved in murine models by complete long-lasting T-cell depletion that continues until after the HCT thereby achieving a donor-vs.-chimera and chimera-vs.-donor free platform, has been use to prevent donor-vs.-chimera suppression and make DLI more effective. This allows engraftment without inflammation, which can be subsequently boosted by DLI (Pelot M.R. et al. 1999). However, the success in rodents has not been replicated in large animals or the clinic. Reasons for this may be that, in contrast to rodent models, it is not possible to achieve complete T-cell depletion without significant toxicity, nor can such high T cell doses be administered in the DLI in large animals and the clinic.

A second approach to prevent donor-vs.-chimera suppression and make DLI more effective is to remove the suppression. This study demonstrates that this is possible *in vitro*. It may also be possible to achieve this *in vivo* by either global T-cell depletion or by selective depletion of CD25+ cells with specific reagents.

A possible limitation of both approaches for removal of donor-vs.-chimera suppression (prevention or depletion) is that in addition to the increased chimerism (due to donor-vs.-chimera response), GvHD may develop. Separation of donor-vs.-chimera response from GvHD is central to the achievement of high-level chimerism without significant morbidity and death. Donor-vs.-chimera response without GvHD was achieved in only one of the three animals that responded to DLI in this study. In mouse models it has been possible to consistently separate lympho-haematopoietic donor-vs.-chimera response and GvHD effects for the treatment of leukaemia (Bortin, M. M. et al. 1979; Sykes, M. et al. 1989; Sykes, M. et al. 1990b). However, it has not been possible to reliably replicate this in the clinic in either HLA mismatched (Sykes, M. et al. 1999) or HLA matched transplants (Dey, B. R. et al. 2003; Spitzer, T. R. et al. 2000). This may indicate that this large animal model provides a closer clinical representation than rodent models for studies separating donor-vs.-chimera response and GvHD.

The mechanism for separation of donor-vs.-chimera response and GvHD has not been fully elucidated. However, it is likely that the T cells mediating donor-vs.-chimera response also mediate GvHD (Horowitz, M. M. et al. 1990). Donor-vs.-chimera regulation may provide a mechanism by which it is possible to isolate donor-vs.-chimera response from GvHD. Edinger et al. demonstrated that CD4+CD25+ regulatory T cells can suppress the expression of IL-2 receptor alpha chain by donor-vs.-chimera alloreactive T cells as well as decrease their ability to mediate GvHD, without affecting donor-vs.-chimera response. This occurs through the perforin lysis pathway (Edinger, M. et al. 2003).

In addition to removing donor-vs.-chimera suppression, HSC engraftment may be necessary for DLI to be effective at increasing chimerism. In our model all three animals that responded to DLI with an increase in donor chimerism had evidence of HSC engraftment. However, several animals that did not respond to DLI also had evidence of engraftment, so engraftment alone is not sufficient for DLI to be effective. The level of engraftment may also be of consequence: it is possible that with greater levels of engraftment, regulatory mechanisms play less of a role and deletional mechanisms are more important (Domenig, C. et al. 2005; Kurtz, J. et al. 2004). It is possible that DLI could facilitate engraftment; Baron found that an early DLI following non-myeloablative chimerism induction in a dog model correlated with increased incidence of long-term mixed peripheral blood chimerism (Baron, F. et al. 2006b).

7.5 STRATEGY TO INCREASE THE EFFECTIVENESS OF DLI IN THIS MODEL

The model described is minimally toxic with a low incidence of GvHD but often achieves a level of chimerism too low to be useful for testing the hypothesis that a moderate level engrafted chimera will be tolerant to a vascularised skin allotransplant. DLI is one possible method to increase chimerism. However, it only achieves boosting of chimerism in this model in a minority of cases. This may be due to donor-vs.-chimera suppression.

Possible ways to achieve boosting of chimerism are either to prevent development of donor-vs.-chimera suppression, or to remove/reduce suppression once it has developed. Even if it is possible, it may not be desirable to prevent the development of donor-vs.-chimera suppression in a large animal as this may result in a high incidence of GvHD. Another option is to remove or reduce donor-vs.-chimera suppression; there are several ways that donor-vs.-chimera suppression could be reduced:

(1) Depletion of all white blood cells (WBCs), including the suppressor T cells. WBC depletion could be achieved by leukapheresing the animal immediately before administration of the DLI. This approach has the added advantage that the leukoproduct can be frozen and saved. If the animal subsequently develops GvHD following DLI the leukoproduct (containing donor-vs.-chimera suppressor cells) could be given back to treat the GvHD.

(2) Depletion of all T cells including the suppressor T cells. The only option for achieving this in swine would be with the anti-CD3 immunotoxin used in the chimerism induction protocol. However, at the time of these experiments there is only enough immunotoxin available for chimerism induction when the animals are smaller, but not enough for the subsequent depletion T cells at the time of DLI when the animals have grown larger.

(3) Selective depletion of the suppressor cells. Removal of just one cell type is an attractive experimental approach because it allows the study of the effect of that cell type in isolation. Options for achieving this are use of anti-CD25 or anti-CD8 antibodies (swine Tregs are CD4/CD8 double positive). However, these were not available in large enough quantities to deplete *in vivo* at the time of this study. Another

option was to use Ontak®, a human IL2R-Diptheria toxin fusion protein. Ontak® binds to the IL2R (which is CD25) on T cells and is internalized. The dipheria toxin then blocks elongation factor 2 leading to cell death. The IL2R is conserved between pigs and humans making it highly likely Ontak® would work in pigs. Several routes were explored to acquire Ontak at an affordable price but all were unsuccessful.

On the basis of these constraints, in Chapter 10 animals underwent a pre-DLI leukapheresis in an attempt to make DLI effective at boosting chimerism.

CHAPTER 8: PIG COMPOSITE TISSUE ALLOTRANSPLANTATION MODELS

8.1 INTRODUCTION

The aim of this study is to develop a swine vascularised skin transplant model to test the hypothesis that an engrafted moderate-level chimera will be tolerant to a vascularised skin allotransplant. A secondary aim that would be useful for future research is to incorporate the possibility of testing allograft functional recovery following allotransplantation.

The two elements required for a useful vascularised skin flap model to test the skin tolerance hypothesis is that it should contain no other tissues except skin, and that it is primarily vascularised. There are several composite tissue allotransplant models already described. Many of these CTA models contain vascularised bone and/or muscle (Eduardo Bermu, Dez L. et al. 2002; Lee, W. P. et al. 1991; Mathes, D. W. et al. 2003; Xudong, Z. et al. 2006; Yazici, I. et al. 2006). The inclusion of other tissues in the allograft may affect tolerance to skin making the data difficult to interpret. For example in a rodent model, it has been observed that the presence of vascularised bone marrow extends allograft survival (Ozmen, S. et al. 2006). An alternative to these CTA models are conventional skin grafts which do not contain other tissues. However, conventional skin grafts would not be a good model for composite tissue allotransplantation due to the differences in the interaction with the immune system compared to primarily vascularised skin found in CTA (as described in Chapters 2 and 5).

Functional recovery following allotransplantation can include both motor and sensory elements. The ideal CTA model for testing the skin tolerance hypothesis would include only skin and so motor recovery could not be assessed. However, identification of the sensory innervation of the skin and anastomosis to the donor in the transplant would allow assessment of sensory recovery. A musculocutaneous allotransplant model would be required to assess both motor and sensory recovery. There have been no previous models described in swine that allow for assessment of motor and/or sensory recovery.

It was not possible to develop a single model to address all the limitations of previous models. Consequently, both cutaneous and musculocutaneous vascularised CTA swine models that allow for assessment of functional recovery are developed in this study.

8.2 OVERVIEW OF EXPERIMENT

Possible swine CTA models for orthotopic or heterotopic placement were explored on cadaveric swine (two animals per model). These models were then developed in live animals, by either simple dissection to confirm cadaveric findings, or by isolating the CTA on its vascular pedicle to confirm reliability of the blood supply. Finally, the most useful CTA models were transplanted, either orthotopically or heterotopically, to ascertain the best strategy technically for transplantation.

8.3 RESULTS

Five different CTA models were explored (as outlined in table 8.1):

Flap	Flap Type (Recipient site)	Number performed	Technical Success n	Technical failure n (Day; Reason)
Facial	Pedicled	1	NA	NA
Radial Artery	Pedicled	2	0	2 (D0; not perfused)
Groin	Pedicled	2	2	0
Saphenous	Pedicled	3	2	1 (D3; Infection)
	Free (orthotopic)	1	1	1 (D4; Infection)
	Free (heterotopic)	4	3	1 (D1; thrombosis)
Gastrocnemius	Free (heterotopic)	1	1	0

Table 8.1 Swine CTA models and outcomes

8.3.1 Facial flap

On cadaveric dissection the facial vein was easily identified lying in a similar location as in humans, originating at the medial canthus before descending obliquely across the face, crossing the inferior border of the mandible and terminating in the internal jugular vein. However, it was not possible to identify a facial artery. The motor and sensory innervation of the facial region was also examined. The facial nerve was identified just postero-superior to the angle of the jaw, and the dorsal buccal branch was easily followed to the snout. In addition, the auriculotemporal nerve was also identified running superiorly behind the posterior ramus of the mandible.

To confirm the findings on cadaveric dissection, a dissection of the facial vasculature was performed on a live animal. The facial veins were again easily identified bilaterally. However, no facial artery was identified. Consequently, this model was not developed further.

8.3.2 Radial artery flap

The radial vessels were easily identified on cadaveric dissection running along the interosseus membrane. The overlying skin flap was isolated on distal branches from these vessels on live dissection (fig 8.1a). However, only a small area of skin was viable by 48 hours following operation (fig 8.1b). In addition, no single sensory nerve supplying the skin within the flap could be easily identified. Consequently, this model was not developed further.

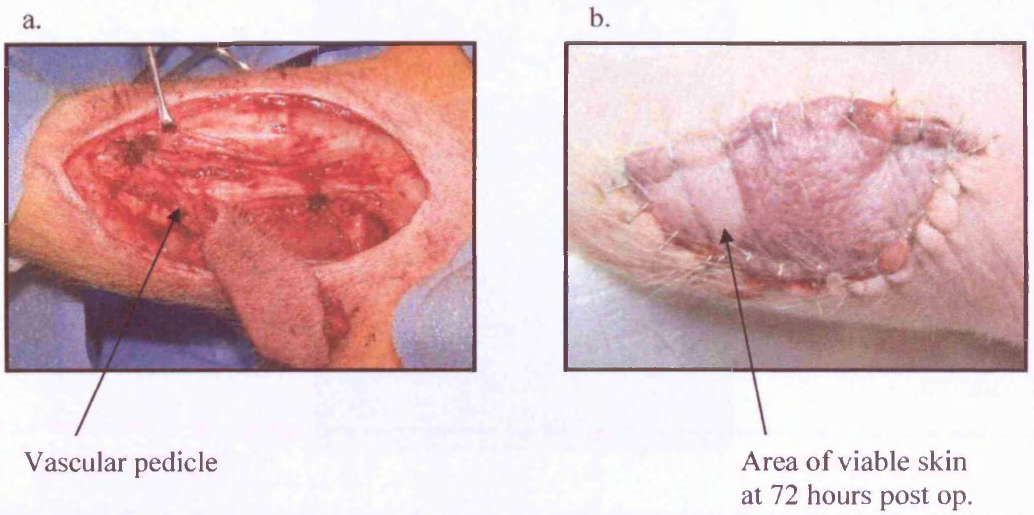


Figure 8.1 Radial artery flap. A radial artery flap was raised on a pedicle in two live dissections (a). However, by 48 hours it was apparent that only a small area of the flap was actually supplied by the pedicle (b).

8.3.3 Groin flap

A groin flap based on the superficial epigastic artery was developed. An elliptical skin incision, based over the lateral half of the groin crease, was made (fig 8.2). The abdominal cavity was not opened. The superior edge of the skin flap was raised deep to Scarpa's fascia. The superficial epigastric vessels were identified and isolated retrograde to the femoral vessels. Two pedicled groin flaps were raised. Although both flaps survived, the dissection of the inferior epigastric vessels was difficult: the vessels in the pedicle were small (1-2mm diameter) with a pedicle length of over 12 cm. Consequently, the flap was not developed further.

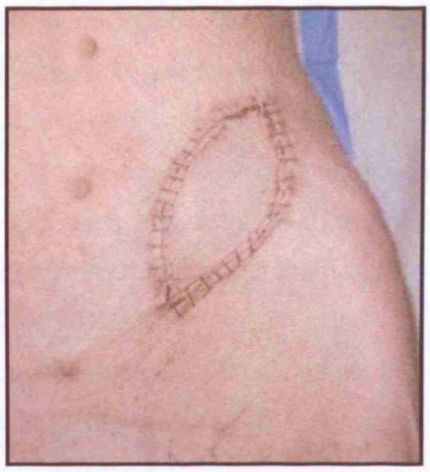


Figure 8.2 Groin flap: technically possible, but long pedicle with narrow calibre vessels. A groin flap 1 week post operatively indicating the position of the skin paddle centred over a line running from the anterior-superior iliac spine to the pubic tubercle.

8.3.4 Saphenous fasciocutaneous flap

A flap based on the saphenous vessels was developed. The medial saphenous artery was palpated and marked. An elliptical skin incision, based around the medial saphenous artery, was made on the medial aspect of the knee and extending inferiorly (fig 8.3a). The flap was raised deep to fascia lata with identification of the neurovascular pedicle (fig 8.3b). Isolation was straightforward, requiring minimal dissection of the recipient tissues. The saphenous vessels were divided inferiorly and then the neurovascular pedicle was dissected superiorly. The neurovascular pedicle was larger in diameter than the groin flap (arterial diameter 3mm) with a vascular pedicle length of 8-10 cm. The saphenous vessels (usually one artery and two veins) were isolated along the inferior edge of sartorius back to the femoral vessels.

The saphenous fasciocutaneous flap receives cutaneous innervation from the saphenous nerve. This was easily identified and preserved as it separated from the vessels approximately 6-8cm proximal to the skin flap. Identification of this nerve indicates that functional assessment of return of cutaneous sensation is possible in this model if the nerve is anastomosed to a cutaneous nerve on the recipient (e.g. the auriculotemporal nerve).

The initial live dissections of the saphenous flap indicated that this model fulfilled the criteria of being composed of just skin (and associated connective tissue), and having an identifiable sensory innervation. To develop this model further, the flap was transplanted orthotopically. In these free flaps the vessels were divided including a 2cm cuff of the femoral vessels superiorly. Following removal of the flap it was possible to close the donor site directly without a drain (fig 8.3c) and recover the animal. The recipient femoral vessels were isolated and divided just proximal to the saphenous vessels. The femoral vessels in the cuff on the flap were anastomosed end-to-end to the recipient femoral vessels. A suction drain was placed.

Two orthotopic flaps (one isolated on the neurovascular pedicle and one orthotopic transplant) underwent thrombosis. In both cases, at necropsy, the vein was found to be thrombosed, with significant of inflammation in the flap and surrounding tissues, and an infected seroma underlying the flap.

Due to the complications with the orthotopically transplanted flaps, further flaps were transplanted heterotopically in a cervicofacial position. The vessels of the flap femoral vessel cuff were anastomosed, end-to-end, to the recipient common carotid artery and internal jugular vein. An ellipse of skin was excised from the angle of the jaw and superior cervical area and the flap secured in place (fig 8.3d). These heterotopically placed flaps had a lower complication rate compared to the orthotopic flaps.

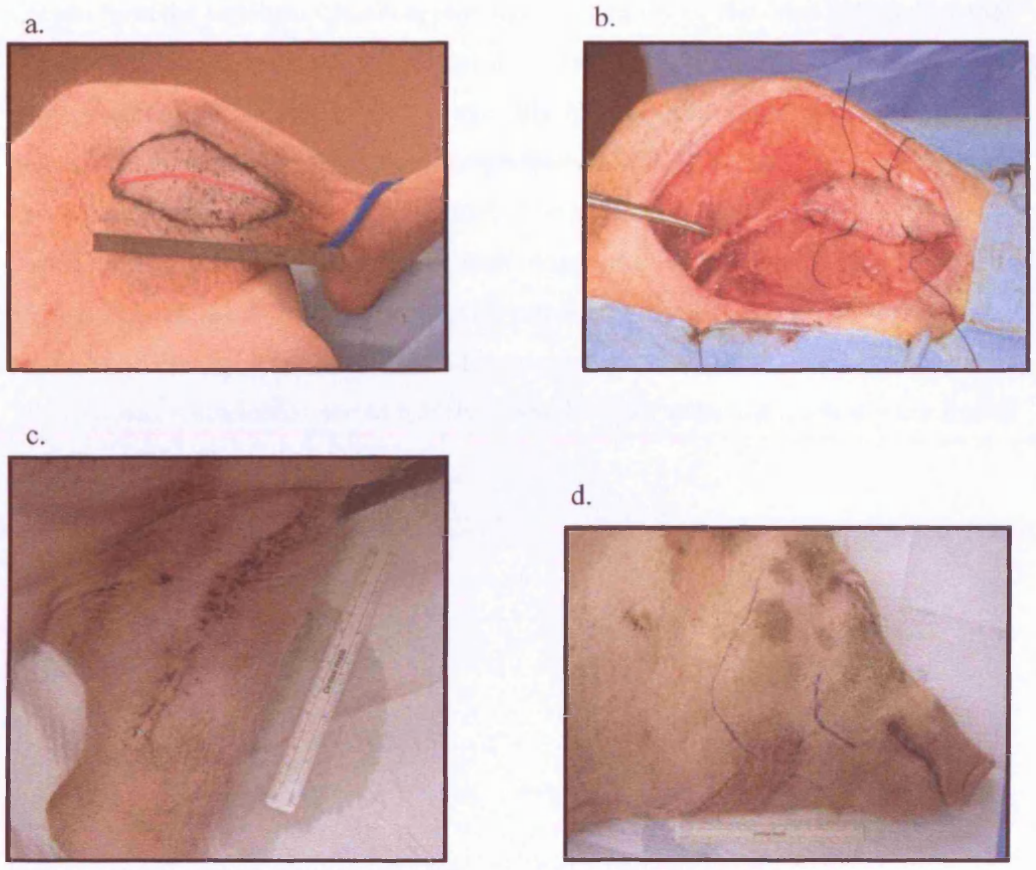


Figure 8.3 Saphenous flap: identifiable sensory nerve, with minimal dissection allowing recovery of the donor. a: The flap was marked out (marked in black) overlying the course of saphenous artery (marked in red). b: The flap was then isolated on its neurovascular pedicle with minimal dissection of the surrounding tissues. c: In flaps that were transplanted the donor site was closed directly with good healing (picture shows donor site at one week post op) allowing for post operative recovery of the donor and use in follow-up immunological assays. d: Due to complications on orthotopic placement of the flap, later flaps were transplanted to the cervicofacial region.

8.3.5 Gastrocnemius musculocutaneous flap

The saphenous flap contained no muscle for assessment of return of motor innervation and function. Therefore, a myocutaneous flap based on the gastrocnemius muscle was developed. This included muscle, with its identified motor nerve (tibial nerve), as well as skin with the identified sensory nerve (saphenous nerve) to allow for assessment of functional recovery on transplantation. An elliptical skin incision was made centred over the gastrocnemius muscle, extending from the popliteal fossa to the tendo-achilles (fig 8.4a). The medial sural vessels were dominant, branching with the lateral sural

vessels from the popliteal vessels approximately 2cm below the knee joint before they both almost immediately entered the muscle. The popliteal vessels were both 4-5mm in diameter and were isolated for 5cm proximally before passing through the adductor hiatus. The branch of the tibial nerve to gastrocnemius (fig 8.4b) and the saphenous nerve was identified, isolated, and divided. The tendo-achilles was divided and the gastrocnemius was separated from the underlying muscles. The medial and lateral sural vessels were isolated, followed back to the popliteal vessels overlying the knee joint capsule and divided just distal to the adductor hiatus.

The flap was transplanted across a MHC Class 1 barrier with histologically confirmed rejection of the flap at 8 days.

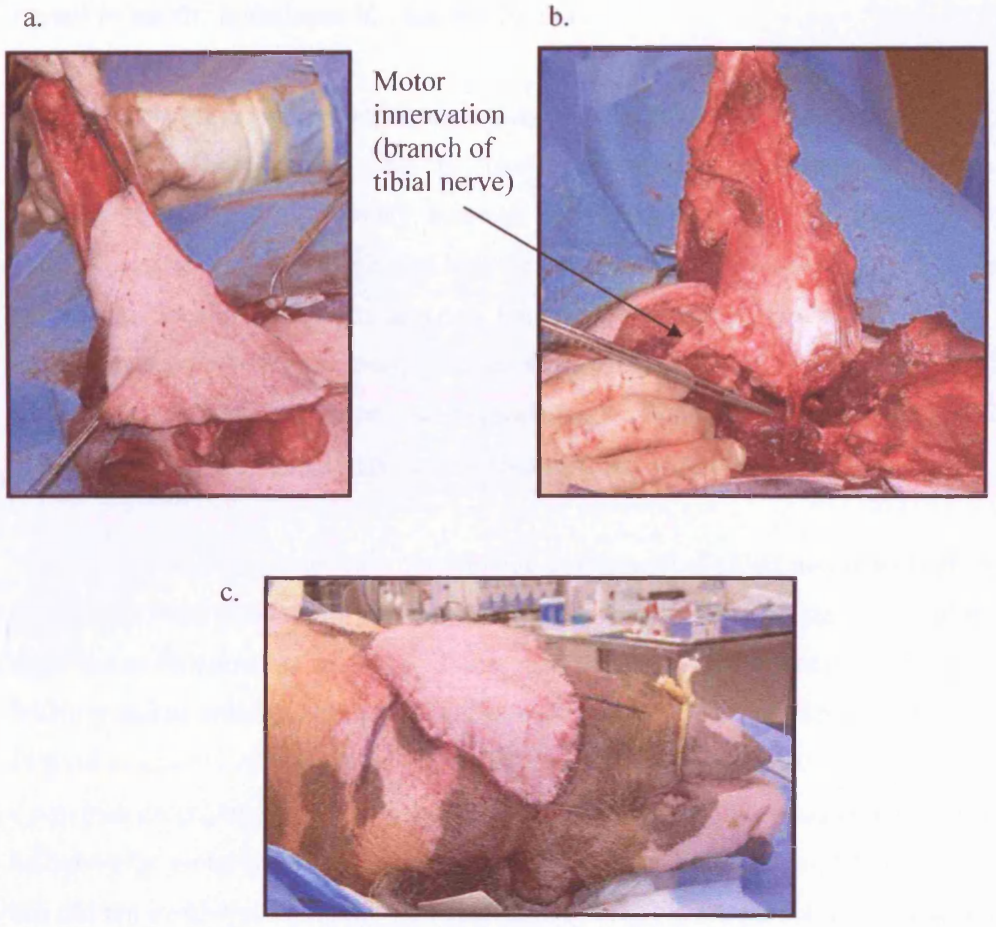


Figure 8.4 Gastrocnemius myocutaneous flap: identifiable motor and sensory nerves, with utility for functional recovery assessment. a: The flap was raised centred over the gastrocnemius muscle. b: The flap was isolated on the popliteal vessels with identification of the tibial and sural nerves. c: The flap was then transplanted to a heterotopic cervicofacial position overlying the jaw (outline marked in blue) and cheek.

8.4 DISCUSSION

This study has developed a skin allotransplantation model in swine based on the saphenous vessels which can be used to assess the hypothesis developed in Chapter 6 for the attainment of skin tolerance by creating an engrafted moderate level chimera. This saphenous skin flap model is particularly useful because skin is the only major tissue type, thereby avoiding effects from other tissues. In addition, the saphenous flap is harvested with minimal morbidity allowing for recovery of the donor animal, which

can then be used in follow-up immunological assays. Consequently the saphenous flap is used to test the hypotheses in Chapters 10 and 11.

As well as preventing rejection, functional recovery is of central importance for a good outcome in CTA. Functional recovery in most composite tissue allotransplants require at least moderate sensory recovery, however not all require good motor recovery. In hand allotransplants the transplanted long flexor and extensor tendons attach proximally to innervated recipient muscles, and only intrinsic muscle function is dependent on motor nerve growth into the transplant. In contrast, motor function may be essential in some face allotransplants for perioral musculature to maintain oral continence and for communication, and for periorbital musculature to achieve eyelid closure.

The models described here allow for function assessment of either just sensory recovery (saphenous flap) with recovery of the donor for further immunological assays, or motor and sensory functional recovery (gastrocnemius flap). It is important to be able to study both motor and sensory recovery as unfortunately, motor nerve recovery is not always as good as sensory recovery in allotransplants (Lanzetta, M. et al. 2005). There has been a previous description of bilateral orthotopic replantation of the rectus femoris muscles including the motor nerve and vascular pedicles in dogs (Hua, J. et al. 1996). However, this did not involve skin, limiting the relevance of it as a CTA model due to reduced immunogenicity.

The facial allograft models described here are heterotopic, which is a possible limitation as they could not be used to study the technical aspects of a face transplant. Our findings indicate that it would be difficult to develop an orthotopic model in swine based on the vascular pedicle that would be commonly used clinically. Previously, orthotopic facial transplant animal models have been described (Ulusal, B. G. et al. 2003; Zhang, X. D. et al. 2006); however, these have differing anatomy from humans and so will have limited utility in further improving clinical operative technique. Furthermore, the initial successes of the facial transplants, and previous facial replants, have demonstrated that technical difficulty is not a limitation.

8.5 CONCLUSIONS

The saphenous flap achieves the primary aim of this study: defining a vascularised skin flap, not containing other tissues, to be used to test the skin tolerance hypothesis

developed in Chapter 6. Consequently, the saphenous flap is used to test the hypothesis that skin tolerance can be achieved in an engrafted moderate level chimera in Chapter 10 and to examine site-specific therapy in Chapter 11.

CHAPTER 9: *IN UTERO* INDUCTION OF CHIMERISM

9.1 INTRODUCTION

The primary aim of this study was to obtain an engrafted moderate-level chimera in which to test tolerance of vascularised skin allografts transplanted across a full mismatch barrier. The *in utero* chimerism induction model has previously proven to be a robust model for achieving moderate-level chimerism in swine (Lee, P. W. et al. 2005b; Mathes, D. W. et al. 2001; Rubin, J. P. et al. 2001) and was therefore selected for this study.

In previous studies SLA^{cc} MGH miniature swine were used as bone marrow donors and Yorkshire outbred swine as recipients. SLA^{cc} MGH miniature swine were selected because, at the time, more reagents were available to phenotypically type immune cells in SLA^{cc} cells than other SLA subtypes. Yorkshire swine were chosen as recipients because they are large, which is helpful in correctly locating the *in utero* inoculation, and also they are robust with low abortion rates.

In this study two modifications were made from previous experiments. Firstly, the MGH miniature swine inbred line of SLA^{dd} animals are used as bone marrow donors, instead of partially inbred SLA^{cc} miniature swine. This eliminates minor antigen differences between the bone marrow donor and the skin donor. The bone marrow donor is sacrificed to harvest bone marrow for *in utero* injection, then an animal from the same line is used for donation of the skin flap allotransplant. One of the mechanisms of skin rejection may be via skin specific minor antigens. Partially inbred SLA^{cc} miniature swine are identical at the MHC but may have non-MHC minor antigen mismatches making these animals unsuitable donors. The "inbred" SLA^{dd} line is not fully inbred and so still may have some minor antigen differences. However, previous studies have demonstrated that they accept reciprocal skin grafts from animals in the same line (Mezrich, J. D. et al. 2003) making these animals suitable donors.

The second modification from previous studies was the use of Hanford mini-swine instead of Yorkshire swine as recipients. The rationale for this was that some of the resultant chimeras would later have received renal allotransplants as well to assess tolerance to organ allografts in the event of vascularised skin allograft rejection. Previous studies have demonstrated initial tolerance to renal transplants (Lee, P. W. et al. 2005a; Mathes, D. W. et al. 2005); however, the kidneys eventually failed. The

reason for the kidney allograft failure is unclear but one possibility is that the size mismatch between the donor kidney (from a miniature swine) and the recipient (a full-sized swine) eventually became too great for the kidney to be life-supporting. This size mismatch issue would not be a problem with mini-swine and so Hanford mini-swine were selected because of their large litter sizes and robustness in pregnancy in comparison to the MGH mini-swine.

It was noted in the previous studies that better chimerism was achieved when unmanipulated bone marrow was added back to the T cell depleted fraction to achieve the desired percentage of T cells (Rubin, J. P. et al. 2001). The secondary aim of the current study was to further understand the effect of bone marrow manipulation and T-cell depletion on progenitor cell function using cobblestone area forming cell (CAFC) assay and bone marrow colony forming unit (CFU) assays.

9.2 OVERVIEW OF EXPERIMENT

The experiment is summarized in figure 9.1. Potential recipient animals were screened by MLR to confirm alloreactivity to donor MHC, and by PCR and FACS to confirm that donor and recipient could be phenotypically distinguished. An inbred SLA^{dd} donor animal was sacrificed and bone marrow was harvested. Some of the bone marrow was T cell depleted by magnetic bead cell sorting (MACS). Unmanipulated bone marrow was then added back to the depleted marrow to bring the T cell count up to 1.5 -1.9%. The bone marrow harvest was timed to coincide with day 55 or 56 of gestation for the foetuses within the pregnant recipient. The bone marrow was then injected into the foetuses at a dose of $2x10^9$ cells/kg. The recipient sow received daily progestin (Regumate®) until day 100 of pregnancy in an attempt to prevent spontaneous abortion. The chimeric animals were then to receive a donor-type skin allotransplant after birth.

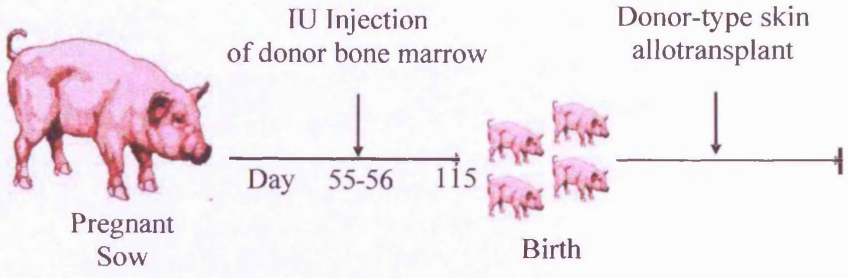


Figure 9.1 Summary of *in utero* experimental outline

9.3 RESULTS

Four rounds of *in utero* injections were performed

9.3.1 Near full viability of bone marrow cells at injection following manipulation

To assess the viability of the bone marrow injected *in utero* an extra syringe was prepared in rounds 2, 3 and 4 at the time of *in utero* injection with the 'Add back' bone marrow in exactly the same way as for the actual injections. The bone marrow from this syringe was then analysed for cell viability using trypan blue exclusion method. There was >99% cell viability for each round.

9.3.2 Bone marrow progenitor cell growth decreased following T-cell depletion by MACS

To assess the effect of T-cell depletion and subsequent adding back of T cell replete unmanipulated bone marrow on the growth potential of the bone marrow progenitor cells *in utero* samples were saved during bone marrow processing and then tested *in vitro*.

The growth potential of bone marrow progenitors used in the first round of *in utero* injections was assessed using cobblestone area forming cell assay. 'Add back' bone marrow had significantly better CAFC growth at 10 weeks (fig 9.2 'Add back'; cream column) than either the unmanipulated bone marrow or the T cell depleted bone marrow; there was no significant difference between the CAFC growth in unmanipulated and T cell depleted bone marrow.

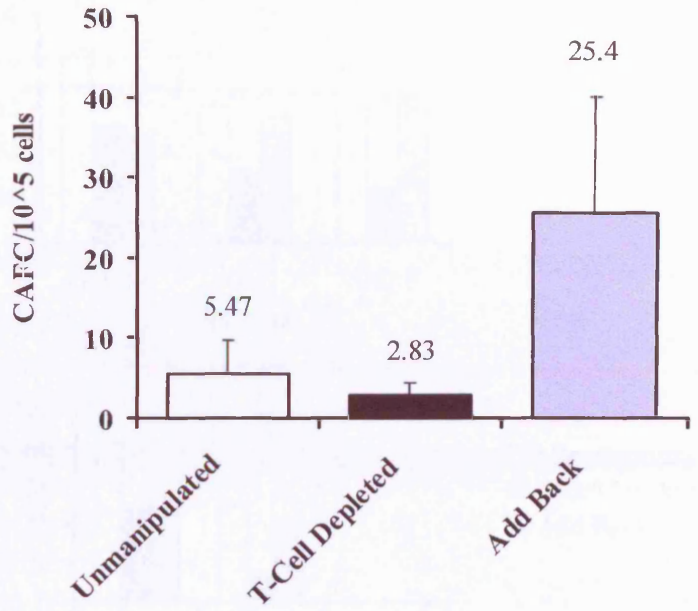


Figure 9.2 CAFC growth after 10 weeks in unmanipulated, T-cell Depleted and T-cell 'Add back' bone marrow from round 1

The growth potential of bone marrow progenitors used in rounds 2, 3 and 4 of *in utero* injections were assessed by the growth of bone marrow colony forming units (BM-CFU) in the granulocyte erythrocyte monocyte megakaryocyte (GEMM), granulocyte monocyte (GM) and blast forming unit erythrocyte (BFU-E) lines (fig 9.3). Following removal of T cells there was significantly worse growth in T cell depleted bone marrow ($p<0.01$; paired t-test) with restoration of growth on adding back of unmanipulated bone marrow to a reach a T cell level of 1.5% ($p<0.02$). There was no significant difference in growth between unmanipulated BM-CFUs and add back BM-CFUs ($p<0.08$, paired t-test).

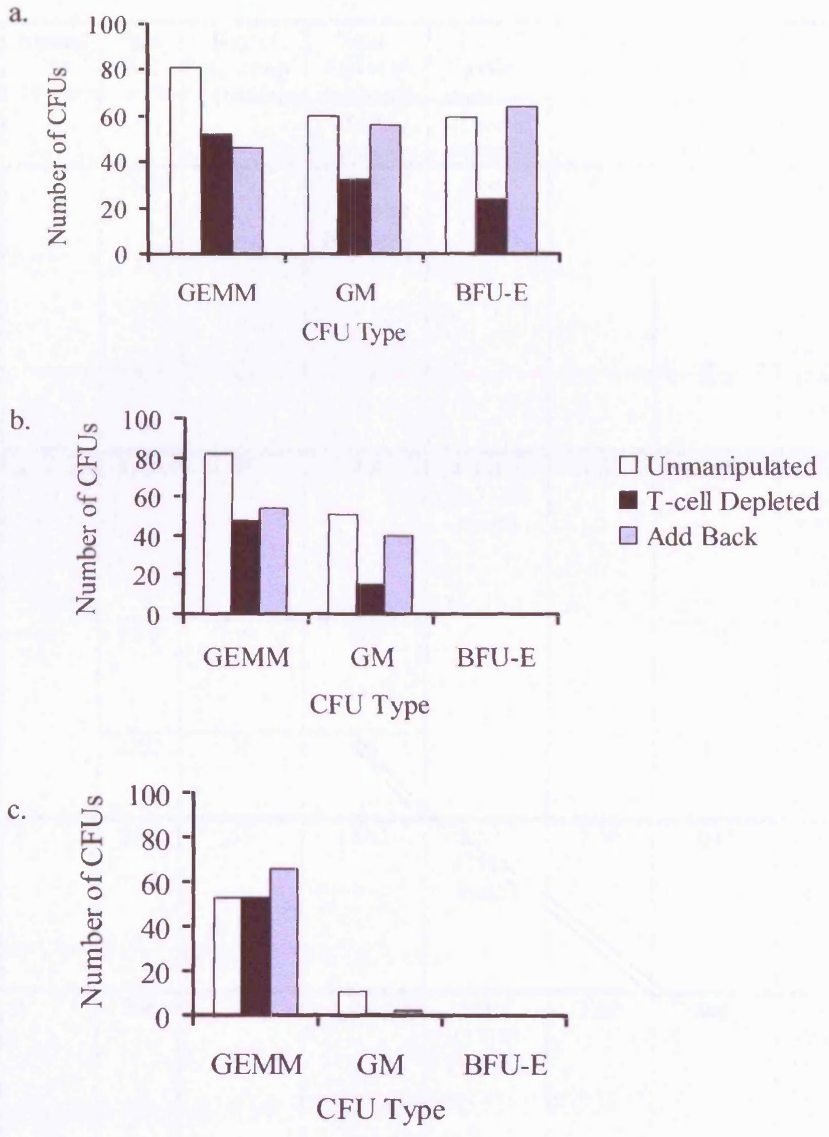


Figure 9.3 CFU growth in unmanipulated, T-cell depleted and T-cell 'Add back' bone marrow.
There was reduced growth of CFUs in T-cell depleted bone marrow in round 2 (a), round 3 (b) and round 4 (c).

Round	Sow No.	Day of injection (GA[a])	No. of foetuses injected/ Total foetuses	No. of cells injected[b] (Donor animal[c])	T cell content (%)	Outcome (Live Births/ Foetuses injected)	Comments
1	1688	55	10/11 (11th had hydrops)	$5x10^8$ (17042 Fresh)	4.5	0/10	**GA 58** Spontaneous abortion: 4 decaying foetuses *1688-1, 1688-3*[d], *1688-4, 1688-5* 1 fresh foetus *1688-2* (82g)
	14-1	55	6/6			0/6	**GA 120** C-section: 6 mummified foetuses[d]
2	2406	55	8/8	$1.3x10^8$ (17139 Fresh)	1.50	1/8	**GA 93**: Transabdominal doppler US probe: 2 foetal heart-beats detected **GA 117**: 1 live birth *2406-1*; 6 mummified foetuses[d,e]
	22-2	56	5/5			0/5	**GA 96** Spontaneous abortion: 1 still-born *22-2-1*, 4 mummified foetuses[d]
	2252	56	5/5			1/5	**GA 108**: 1 live birth *2252-1*, 3 mummified foetuses[d,e]
3	263	55	11/11	$5x10^8$ (17141 Fresh)	1.50	1/11	**GA 90**: Transabdominal USS: 7 foetuses, all with heart beats **GA 103** Farrowed: one live foetus *263-1*, 9 mummified foetuses[d,e]
4	368	56	4/4	$5x10^8$ (17141 Frozen)	1.60	0/4	**GA 62** Transuterine USS: Heart beats in all 4 foetuses **GA 68** Transabdominal USS: Heart beats in all 4 foetuses **GA 99** Vaginal Discharge **GA103** C-section: 4 mummified foetuses[d]

Key:

a 'GA' Gestational Age

b Number of bone marrow cells injected per foetus.

c Bone marrow was either freshly prepared for injection ('Fresh') or was frozen, stored and then thawed before injection

d Foetus too decayed to obtain tissue for analysis

e Swine can resorb foetuses during gestation so all foetuses may not be accounted for at birth

Table 9.1 Outcomes from *in utero* injections

9.3.3 The majority of foetuses died *in utero*

Forty-nine foetuses were injected with bone marrow in utero (reviewed in table 9.1). Only three piglets (6%) were born alive at full-term. Modifications were made in each round with the aim of improving the survival rate. In the first round, due to a technical error, the T cell percentage in the add-back bone marrow was 4.5% instead of the planned 1.5-1.9%; there were no survivors. Furthermore, the abortion of sow 1688 three days after injection revealed that the foetuses were only a third of the estimated size pre-injection (82g vs. estimated 250g).

The T cell percentage in the bone marrow injection and the smaller than expected size of Hanford foetuses were taken into account in the second round of injections: the T cell percentage was kept to 1.50% and the total number of cells injected per foetus was only 1.3 $x10^8$ cells (i.e. an estimated $2x10^9$ cells/kg). Additionally, in an attempt to monitor the foetuses *in utero* one of the sows underwent trans-abdominal Doppler ultrasound assessment at gestational age day (GA) 93, which was 38 days after *in utero* injection. Two foetal heart-beats were detected on Doppler ultrasound, but this was at the limit of detection for the probe and so no conclusions could be made about the status of the other foetuses. Even with the modifications made in round 2 only two piglets from the 18 injected foetuses were born alive (survival rate: 11%).

The two live piglets from round 2 (2252-1 and 2406-1) were born prematurely and despite maximal supportive therapy both animals died at five days old. Piglet 2252-1 succumbed to sepsis secondary to Klebsiella and E.Coli gastrointestinal infection. Piglet 2406-1 died from sepsis due to segmental necrosis of the distal small bowel, presumably secondary to a volvulus that resolved before death.

It was hypothesized that the low live birth rate in round 2 may have been because the Hanford breed is less robust than the larger Yorkshire pigs that had been used by others in previous experiments. So, in the third round, the breed of pig was changed from Hanford to Yorkshire. The foetuses were monitored at GA 90 using trans-abdominal Doppler ultrasound scanning; 7 foetal heart-beats were detected before the mother became non-compliant preventing further examination. However, at term there was only one live birth from the 11 foetuses injected in round 3 (survival rate: 9%). This piglet (263-1) was assessed not to be chimeric and so was sacrificed at 7 days old.

As there were no significant differences between the methods used in round 3 and previous experiments it was planned to use the same conditions in round 4 that had been used in round 3. The bone marrow for the *in utero* injection was harvested from the donor (animal 17302). Unfortunately, the bone marrow was then irretrievably contaminated. As an alternative, bone marrow from round 3 that had been frozen was defrosted and used. The viable cell yield after defrosting was 31.4%. The foetuses were monitored with transuterine ultrasound one week after injection, and trans-abdominal ultrasound two weeks after injection with heart-beats detected in all foetuses. However, at term there were no live births.

9.3.4 Chimerism was achieved both *in utero* and perinatally

Microchimerism (via PCR) was assessed in foetuses, and both macrochimerism (via FACS) and microchimerism was assessed in piglets within one week of birth.

In utero assessment of microchimerism was facilitated by the spontaneous abortion of sow 1688 3 days after injection. Microchimerism was detected in the livers of four foetuses assessed using a PCR specific for the Class 1 MHC of SLA-D (fig 9.4).

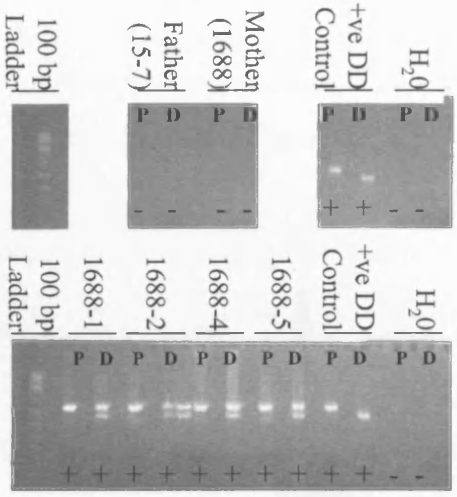
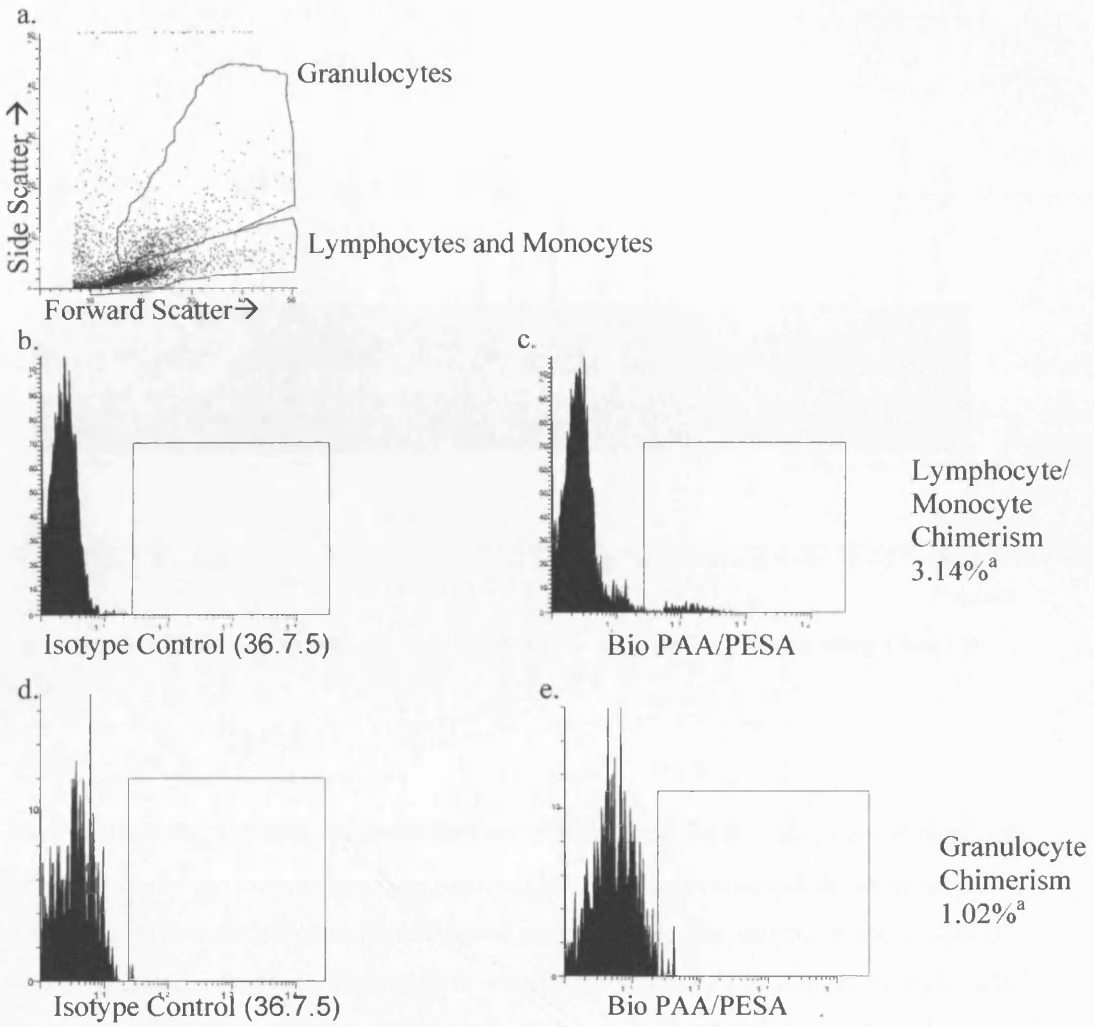


Figure 9.4 Microchimerism in the liver of foetuses three days after *in utero* injection. To achieve specificity of the PCR for MHC Class II of D, an aliquot of each PCR product (P) was digested (D). Each digest was run on the gel adjacent to its originator product. There was evidence of SLA-D DNA in all four foetuses examined (1688-1, 1688-2, 1688-4 and 1688-5) indicating presence of donor DNA originating from the bone marrow injection. No SLA-D DNA was detected in either parent (1688 and 15-7) indicating that the foetal findings were not due to cross-reactivity.

Peripheral blood macrochimerism was assessed in the three live-born piglets. Only piglet 2252-1 had detectable macrochimerism with lymphocyte/monocyte chimerism of 3.14% and granulocyte chimerism of 1.02% (fig 9.5). Blood from piglet 2406-1 did not stain with any antibody including the positive control (antibody 74-22-15 against myeloid cells), so it was not possible to determine if there was macrochimerism. Piglet 263-1 had no evidence of peripheral blood macrochimerism on FACS.



Key:

[a] Percentage chimerism above isotype control

Figure 9.5 Peripheral blood chimerism detectable by FACS perinatally in piglet 2252-1

Microchimerism was assessed in all four piglets available for perinatal assessment (the three live-born piglets and one still-born piglet) using a PCR specific for class II MHC of SLA-D (fig 9.6).

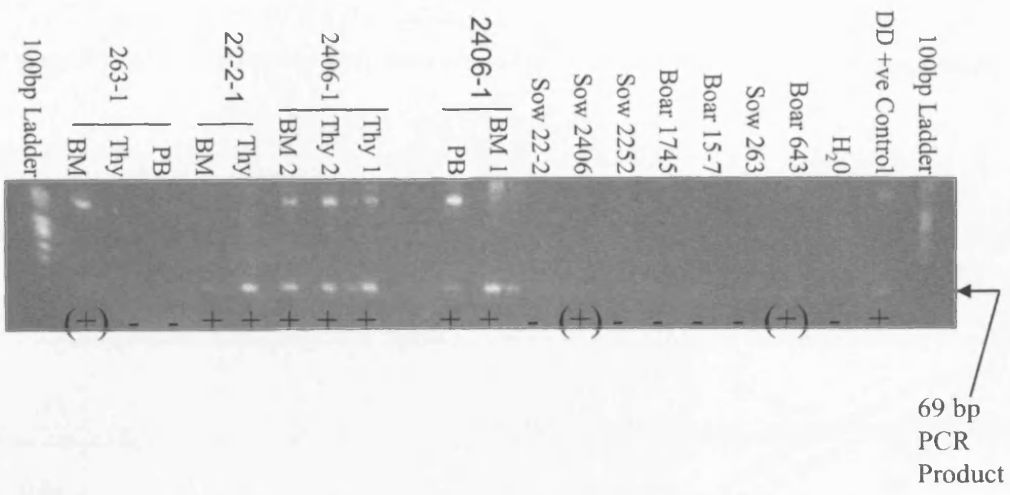


Figure 9.6 rt-PCR assessment of microchimerism in live- and still-born piglets using Class I D primers

Microchimerism was detected in the tissues (bone marrow and/or thymus) of all piglets. To confirm that the microchimerism detected was due to presence of donor DNA and not due to cross-reactivity, peripheral blood samples from the parents of these piglets were also tested. Parents of two piglets were cross-reactive. The mother of piglet 2406-1 was weakly positive (fig 9.6: Sow 2406). However piglet 2406-1 was strongly positive in bone marrow, thymus and peripheral blood making it likely that the chimerism detected was real. The father of piglet 263-1 was weakly positive (fig 9.6; Boar 643) with only piglet 263-1 also being only weakly positive in the bone marrow (fig 9.6: 263-1 BM) indicating possible cross-reactivity of host DNA to MHC class II SLA-D PCR. To investigate this further quantitative PCR (qPCR) was performed (fig 9.7). The level of amplification of DNA extracted from the peripheral blood of 263-1 and her parents was compared. DNA from the peripheral blood of an SLA^{dd} animal was used to define 100% amplification. There was 0% amplification of DNA from the mother sow and 3.7% non-specific amplification of DNA from the father boar. The amplification in the father was greater than the 0.82% amplification seen in piglet 263-1 indicating that the microchimerism detected was likely to be due to cross-reactivity inherited from the father.

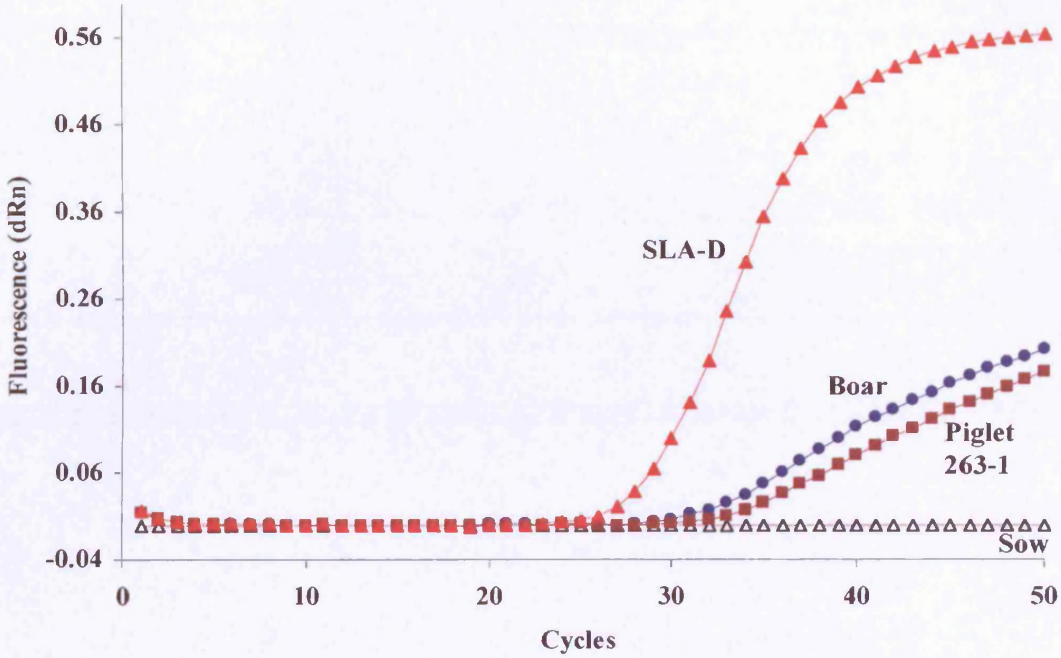


Figure 9.7 q-PCR assessment of chimerism in piglet 263-1 using Class II D primers/probe

9.4 DISCUSSION

The primary aim of this study was to achieve robust chimerism with engraftment in a swine model for the purpose of testing tolerance to a vascularised skin allotransplant: this was not achieved. Chimerism was achieved both *in utero* and in the perinatal period. However, no animal survived long enough to receive a vascularised skin allotransplant. Despite lack of success to achieve the primary aim, useful data was acquired regarding the influence of MACS depletion of T cells on progenitor cell growth.

9.4.1 Decreased *in vitro* growth of bone marrow progenitors following MACS depletion of T cells

This study identified reduced growth in both CAFC and CFU assays of bone marrow progenitor cells following bone marrow T-cell depletion by MACS. This is in contrast to a previous report on *in vitro* growth potential of swine bone marrow progenitor cells, which found no difference in growth potential in either CAFC or CFU assays (Lee, P. W. et al. 2005b). A reason for this difference is was that growth was assessed until 10

weeks in this study compared to only 6 weeks in the previous study (in this experiment, the growth difference had not yet reached statistical significance at 6 weeks).

The CAFC assay identified the best progenitor cell growth potential in the 'Add back' bone marrow, whereas in the CFU assay both unmanipulated bone marrow and 'Add Back' bone marrow had similarly improved growth potential compared to 'T Cell Depleted' bone marrow. This difference between the CAFC and CFU assays may be because of a differential growth rate of the more primitive progentiors in the CAFC assay compared to later progenitors in the CFU assay. An alternative possibility for the difference in growth potential seen between the CAFC assay and the CFU assays is the much higher T cell percentage in the 'Add Back' bone marrow in the CAFC assay (4.5%), which used marrow from round 1 of the injections, compared to the CFU assays (1.5%), that used marrow from rounds 2, 3 and 4. It would have been useful to compare CAFC and CFU growth from the same inoculum. Unfortunately, the CAFC assay set up for round 2 became contaminated, and there was not enough time to set up the assay for rounds 3 and 4 (up to 4 weeks are required).

These results indicate that there is a decrease in growth *in vitro* following T-cell depletion by MACS. The relatively poor growth of bone marrow progenitor cells following MACS T-cell depletion correlates with previous *in vivo* findings indicating that *in utero* injection of T cell depleted bone marrow does not achieve chimerism (Crombleholme, T. M. et al. 1990; Rubin, J. P. et al. 2001). This decreased growth potential following T-cell depletion by MACS may be due to depletion of the T cells. Support for this possibility comes from previous murine studies, which indicate that the resistance to engraftment induced by T-cell depletion may be due to a direct role T cells have in haematopoiesis (Sharkis, S. J. et al. 1978; Wiktor-Jedrzejczak, W. et al. 1977). However, it is also possible that the effect of MACS on progenitor cell growth may not just be due to T-cell depletion; other cell populations could be removed, activated or down-regulated during the staining or on passaging through the magnetic column.

9.4.2 Chimerism achieved

Chimerism was achieved in this model. Macrochimerism (detectable by FACS) was only able to be assessed in one animal (2252-1). However, donor derived DNA was detected by PCR in one other piglet (2406-1) and in four foetuses three days after injection. Detection of chimerism by PCR in isolation is referred to as microchimerism.

The literature is unclear whether microchimerism has significance for tolerance in several models (as discussed in chapter 2); this is also true for *in utero* induction of chimerism (Donahue, J. et al. 2001; Hedrick, M. H. et al. 1994). It is possible that there was also macrochimerism present in these animals but this could not be analysed, either because it was not possible to obtain live cells (from the foetuses), or because the animal's cells did not stain with the control antibodies (piglet 2406-1).

The foetuses in which microchimerism was detected were obtained only 3 days after *in utero* injection. The detection of donor DNA in these animals may not necessarily indicate that these animals were chimeric. The donor bone marrow cells may have died leaving behind donor DNA that had not yet been degraded, and this was detected on PCR. Analysis of RNA would have circumvented this problem as it degrades much faster. The detection of donor DNA by PCR in piglet 2406-1 is likely to represent true chimerism: any residual DNA from dead cells would have been degraded by this timepoint (67 days after injection).

9.4.3 Reason for low survival:

A 15-40% embryonic mortality is to be expected even in a normal swine pregnancy (Legault, C. 1985). However, there was a 94% mortality rate in the injected foetuses. The reasons for the low survival seen in this model are unclear; possibilities include (1) trauma at the time of injection, (2) contamination of the bone marrow, and (3) Graft-versus-Host Disease.

9.4.3.1 Trauma at the time of injection

It is possible that the high mortality rate was due to the injection procedure itself. All injections in this experiment were performed by the same person (Injector C); this was a different person to previous experiments in this model. The method used to inject cells in this model is technically more difficult than the intra-peritoneal route used in most other models (Carrier, E. et al. 1997; Crombleholme, T. M. et al. 1990; Hedrick, M. H. et al. 1994; Kim, H. B. et al. 1998; Mychaliska, G. B. et al. 1997; Pallavicini, M. G. et al. 1992; Rice, H. E. et al. 1994; Shields, L. E. et al. 2003). The intravenous injection involves insertion of a needle trans-abdominally through the liver parenchyma to the hepatic vein. Additionally, the needle entry point depends on the orientation of the foetus in the uterus, so adjacent organs (e.g. spleen, kidney, bowel, etc.) can also be punctured. It is possible that the process of injecting the foetuses *in utero* caused fatal

trauma to the foetus. Depending on the nature of the damage, this could have resulted in immediate death or caused damage to a vital organ with death occurring at a later timepoint.

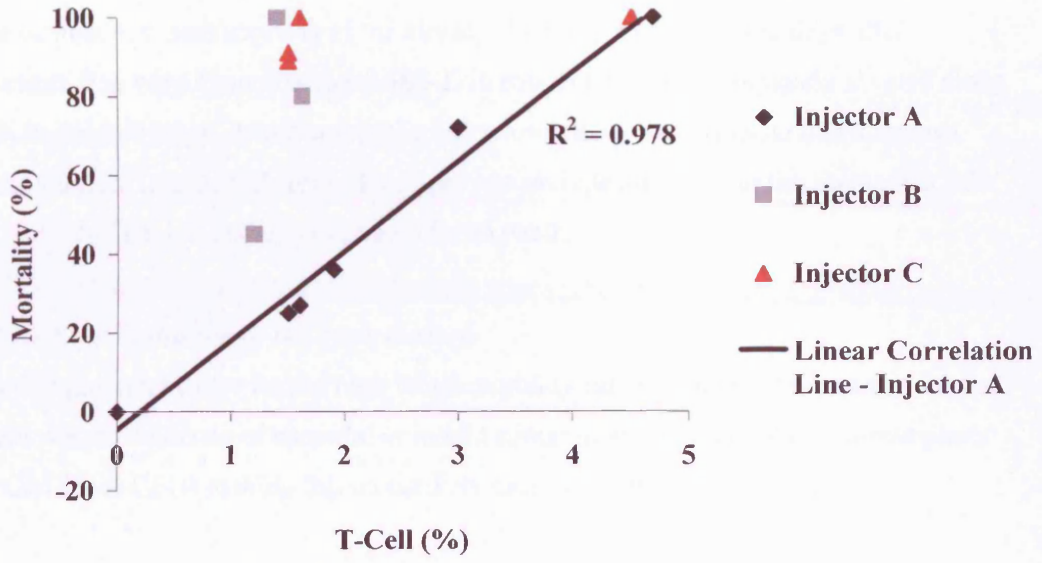


Figure 9.8 Relationship between mortality and T cell percentage in the inoculum for injections performed by different operators in previous experiments

The best way to assess whether the injection procedure itself was a factor in the high mortality in this experiment is to perform saline injections into the foetuses of a pregnant sow as has been done previously (Rubin, J. P. et al. 2001). However, a saline injection was not performed in this case. An indirect method to assess the effect of the injection procedure is to compare the outcomes of the person performing the injection (Injector C) to previous injectors in the same model. There have been two previous operators (Injector A and Injector B). Data from previous published (Lee, P. W. et al. 2005b; Mathes, D. W. et al. 2001; Rubin, J. P. et al. 2001) and unpublished (Mathes et al.) swine *in utero* experiments in this laboratory were reviewed. The T cell percentage at the time of injection was also included in the analysis as previous studies have indicated that this can also have a significant impact on mortality due to GvHD (Crombleholme, T. M. et al. 1990; Shields, L. E. et al. 2003). There was a very close correlation between mortality rate and T cell percentage for Injector A (r^2=0.978; fig 9.8, blue dots). There was a significantly lower mortality in the pigs injected by Injector A than either Injector B ($p<0.04$; paired t-test) or Injector C ($p<0.04$; paired t-test).

There was an increased mortality in pigs injected by Injector C compared to Injector B, but this did not reach statistical significance ($p>0.1$; paired t-test).

This data suggests that the injection procedure may have been one factor in the high mortality rate. It is likely that four of the foetuses of sow 1688 were fatally injured at the time of injection with abortion of the already decaying foetuses three days after injection. The only intact foetus (#1688-2) in sow 1688 was consequently aborted along with its dead siblings. In several of the other sows there was ultrasound evidence of foetal survival to near full-term. This does not exclude an injury to the foetus that was not initially fatal but eventually caused foetal death.

9.4.3.2 Contamination of the bone marrow

Another potential cause for the high foetal mortality rate is bone marrow contamination. There was no evidence of bacterial or mould contamination on the bone marrow plated for CAFCs or CFUs making this an unlikely cause of foetal death.

9.4.3.3 Graft versus Host Disease (GvHD)

A third possibility is that the foetuses developed GvHD *in utero* causing foetal death. It was impossible to analyse the mummified foetal tissue for GvHD so this cannot be excluded. GvHD was cited as the likely cause for foetal loss in a previous *in utero* experiment (Rubin, J. P. et al. 2001), but no evidence was provided. In previously described models GvHD correlates to T cell dose. In round 1 the T cell percentage was 4.5% and the bone marrow cell dose was three times as high as planned (due to over-estimation of foetal weight) with a resulting absolute T cell dose 6 times higher than considered optimal. Therefore it is possible that the foetuses of sow 14-1 in round 1 succumbed to GvHD. However, it is less likely that GvHD was the cause of the high mortality in rounds 2, 3 and 4 because the T cell dose was comparable to previous studies that achieved much lower mortality (Mathes, D. W. et al. 2005).

9.5 CONCLUSIONS

This study did not achieve its primary objective of providing a robust large animal model for achieving high-level chimerism with engraftment in which to test skin tolerance. However, useful data was obtained indicating that the process of MACS depletion of T cells results in poorer growth of bone marrow progenitors. Furthermore, a direction for future studies has been outlined: (1) Sham injection of saline instead of

bone marrow to assess the injection procedure; (2) plan foetal sacrifice at various times throughout pregnancy to obtain live tissue samples for assessment to exclude GvHD; and (3) plating of both CAFCs and CFUs from the same inoculum to further define the role of T cells in progenitor cell growth, as well as experimenting with different levels of T cells.

In Chapter 10 adult induction of chimerism is explored as an alternative model to achieve a moderate level engraftment chimera to test the skin tolerance hypothesis.

CHAPTER 10: INDUCTION OF SKIN TOLERANCE

10.1 INTRODUCTION

The primary aim of this study is to test the hypothesis developed in Chapter 6. The hypothesis states that an engrafted moderate-level chimera will be tolerant to vascularised skin transplanted from the HCT donor across a single haplotype MHC barrier. The secondary aim of this study is to assess the effectiveness of DLI with prior leukapheresis to boost chimerism.

In Chapter 9 an *in utero* model was used in an attempt to obtain an engrafted moderate-level chimera to test the skin tolerance hypothesis. However, this experiment was not successful in attaining long-living chimeras. Consequently, the approach of adult induction of chimerism was used in this experiment. The experiment required two separate phases: induction of an engrafted moderate level chimera, and then performance of vascularised skin flap allotransplantation.

10.1.1 Induction of an engrafted moderate level chimera

The method used successfully by Cina (Cina, R. A. et al. 2006) to non-myeloblatively induce chimerism with engraftment across a single haplotype MHC barrier in pigs is used in this study. This involves low-dose whole body irradiation, T-cell depletion with immunotoxin, haematopoietic cell transplantation (HCT) of cells from a single-haplotype MHC mismatched donor mobilised with SCF and IL3, and cyclosporine cover until day 45.

Previously, this protocol achieved engraftment in all surviving animals, but only fifty percent had high enough levels of chimerism to test the skin tolerance hypothesis. Consequently, in this study animals that do not achieve high enough levels of chimerism with the protocol will receive a DLI with a prior leukapheresis. The use of leukapheresis prior to DLI is based on the hypothesis developed in Chapter 7: leukapheresis will deplete suppressor cells (along with all other white cells) in the chimera which normally prevent DLI from being effective.

10.1.2 Vascularised skin flap allotransplantation

Once a moderate level of chimerism is achieved in an engrafted chimera, the animals receive a vascularised skin flap from the HCT donor. Engraftment has been previously

defined as presence of donor bone marrow colony forming units (BM-CFUs) beyond week 14 after HCT. However, it has previously been observed with this chimerism induction protocol that moderate and high-level multilineage peripheral blood chimerism (i.e. myeloid lineages >10% chimerism) at day 60 correlates with presence of donor-derived BM-CFUs beyond week 12, so this was used as a marker of engraftment as well. The use of the actual HCT donor aims to avoid a non-MHC minor antigen mismatch between the HCT donor and a MHC matched skin donor. This non-MHC minor antigen mismatch has not been a problem in previous organ allotransplants but may be significant in skin allotransplantation due to skin's susceptibility to rejection.

10.2 OVERVIEW OF EXPERIMENT

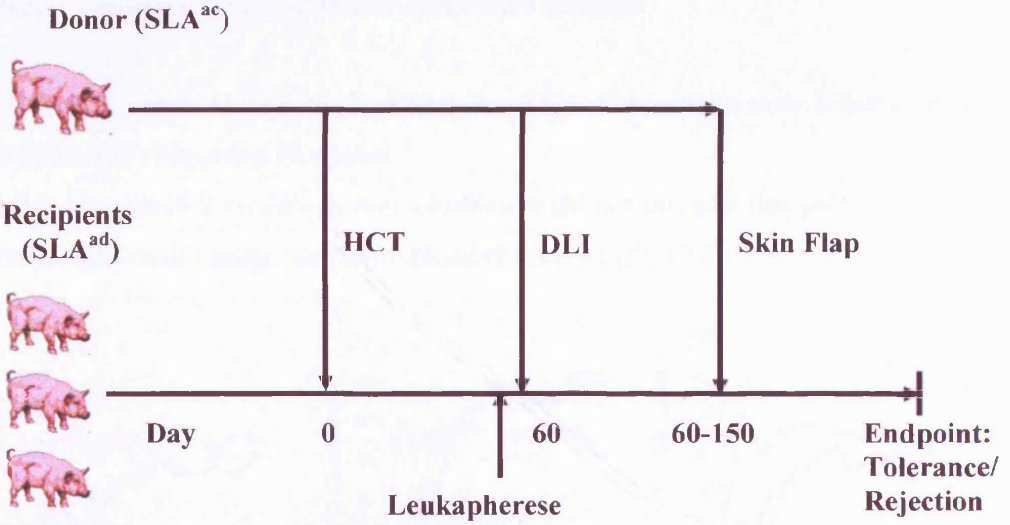


Figure 10.1 Summary of experimental plan

The experimental is summarized in figure 10.1. Three sibling SLA^{ad} animals (17467, 17468 and 17469) underwent HCT with non-myeloablative chimerism induction (summarized in table 3.1 protocol F).

Sixty days after HCT (which is two weeks after completion of the 45 day initial induction period) animals were allocated to receive a leukapheresis and DLI, if required, to achieve moderate level of chimerism needed to test the skin tolerance hypothesis. To allow for possible later downward drift of chimerism, even moderate level chimeras that were not approaching near full chimerism were allocated for a leukapheresis and DLI. The application of leukapheresis and DLI was staggered between animals so that an

animal receiving a delayed leukapheresis and DLI could act as a control for an animal receiving immediate leukapheresis and DLI. Following achievement of engraftment and moderate level chimerism a vascularised saphenous skin flap was allotransplanted from the HCT donor. A control animal was included that did not undergo chimerism induction but received a vascularised saphenous skin flap across a single-haplotype barrier.

10.3 RESULTS

The results from the study are in two parts. Firstly, the findings following leukapheresis and DLI; and secondly, the outcome following vascularised skin allotransplantation.

10.3.1: Leukapheresis and Donor Leukocyte Infusion

10.3.1.1 Moderate to high levels of peripheral blood chimerism were achieved at day 60 following chimerism induction

Following completion of the non-myeloablative chimerism induction protocol all three animals had multilineage peripheral blood chimerism (fig 10.2).

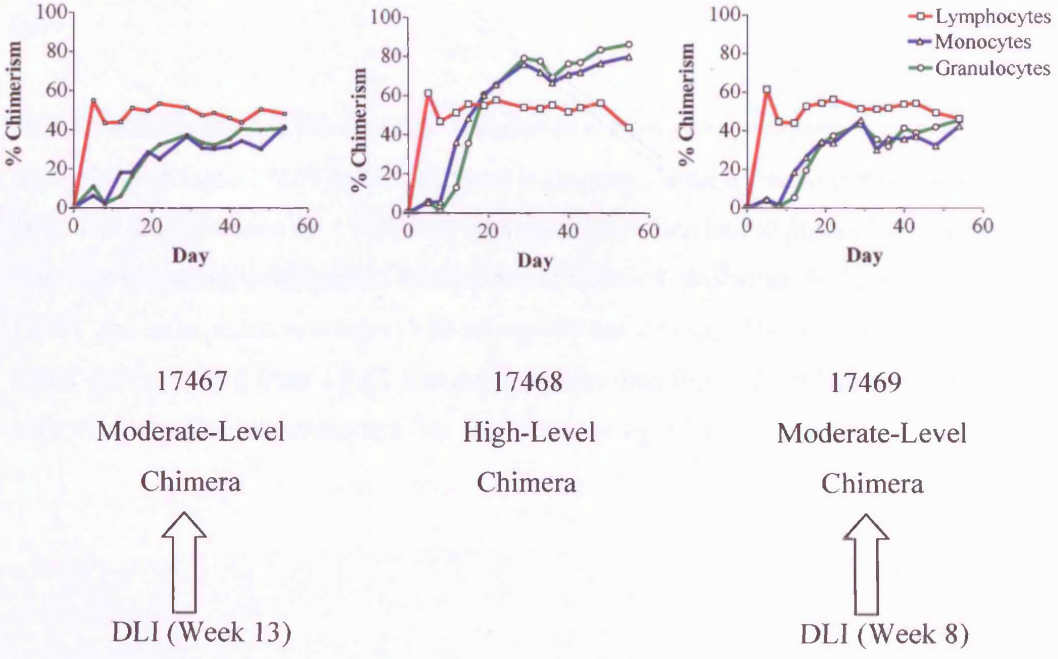


Figure 10.2 Peripheral blood chimerism following chimerism induction protocol

The presence of multilineage peripheral blood chimerism indicated haematopoietic stem cell engraftment in all three animals. This was confirmed at week 14 with evidence of donor-derived BM-CFUs and thymic chimerism.

The level of multilineage chimerism was then assessed for allocation of animals to receive a leukapheresis and DLI to achieve moderate level chimerism if required. Animal 17468 had 70-80% chimerism in the myeloid (monocyte and granulocyte) lineages, with chimerism still rising. The lymphocyte lineage was lower at 40%. However, lymphocyte chimerism is not as sensitive a marker of production of donor cells in the recipient due to the long-life of lymphocytes. Due to the high and rising myeloid levels of peripheral blood chimerism in 17468 it was decided not to perform a leukapheresis and DLI on this animal.

Animals 17467 and 17469 had similarly moderate levels of peripheral blood multilineage chimerism. To protect against possible later downward drift to low-level chimerism both animals were allocated to undergo leukapheresis and DLI. Animal 17469 underwent leukapheresis and DLI immediately with 17467 initially used as a control animal for comparison. Animal 17467 subsequently underwent a DLI 5 weeks later.

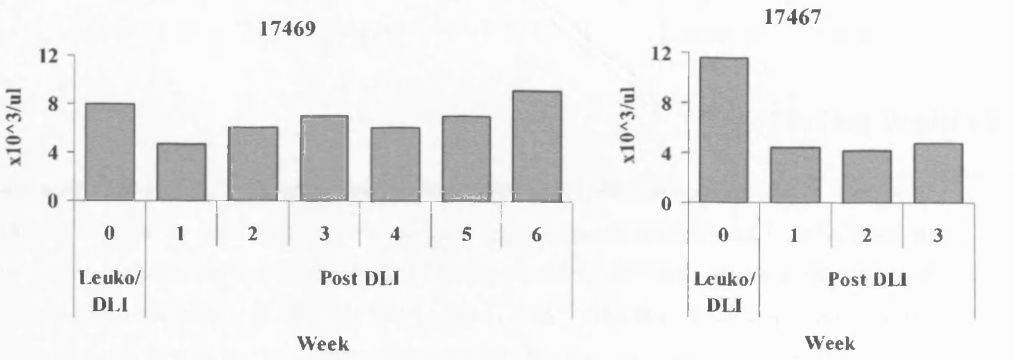
10.3.1.2 Leukapheresis prior to DLI resulted in the temporary depletion of Tregs

Animals 17467 and 17469 both underwent leukapheresis for a 7-hour period prior to DLI. The leukapheresis on 17469 was interrupted and then had to proceed at a slower flow rate (20ml/min) for the last 4 hours due to technical problems; leukapheresis on 17467 was unimpeded running at >30 ml/min for the 7 hours. The number of white blood cells removed from 17467 was much greater than from 17469 (see table 10.1), with a more profound subsequent lymphopenia (see fig 10.4)

	Total WBCs ($x10^{10}$)	
	17469 (22kg)	17467 (16kg)
WBC in peripheral blood pre-leukapheresis	0.98	1.19
WBCs in leukoproduct	1.11	4.57
WBC in peripheral blood post-leukapheresis	Not measured	1.74

Table 10.1 White blood cells removed by DLI, and in the peripheral blood before and after DLI

The number of circulating peripheral white blood cells prior to leukapheresis was similar in 17469 and 17467 despite the difference in animal weights (22kg vs.16kg). Leukapheresis was technically better in 17467 with removal of approximately four times as many white cells as 17469. Despite the large numbers of white cells removed, the peripheral blood white cell count actually rose in 17467 by the end of leukapheresis.



42% decrease in lymphocytes

62% decrease in lymphocytes

Key

'Leuko/DLI' timepoint is immediately before leukapheresis

Figure 10.3 Lymphocytes in the peripheral blood before and after DLI. Measurement of the absolute concentration of lymphocytes before and after leukapheresis and DLI revealed that there was a significant decrease in lymphocyte count in both 17469 and 17467. The decrease was larger in 17467 than 17469 (62% vs. 42%). There was only a minimal resolution of post leukapheresis relative lymphopenia by 3 weeks in 17467, and it took 6 weeks for the lymphocyte count to rise to pre-DLI levels in 17469.

Despite the larger number of cells removed from 17467 compared to 17469, there was a similar decrease in CD4+CD25+FoxP3+ T cells (Tregs) from pre leukapheresis/DLI to afterwards. This depletion of Tregs was only temporary, with return to pre-leukapheresis levels in 17469 within 4 weeks (fig 10.3).

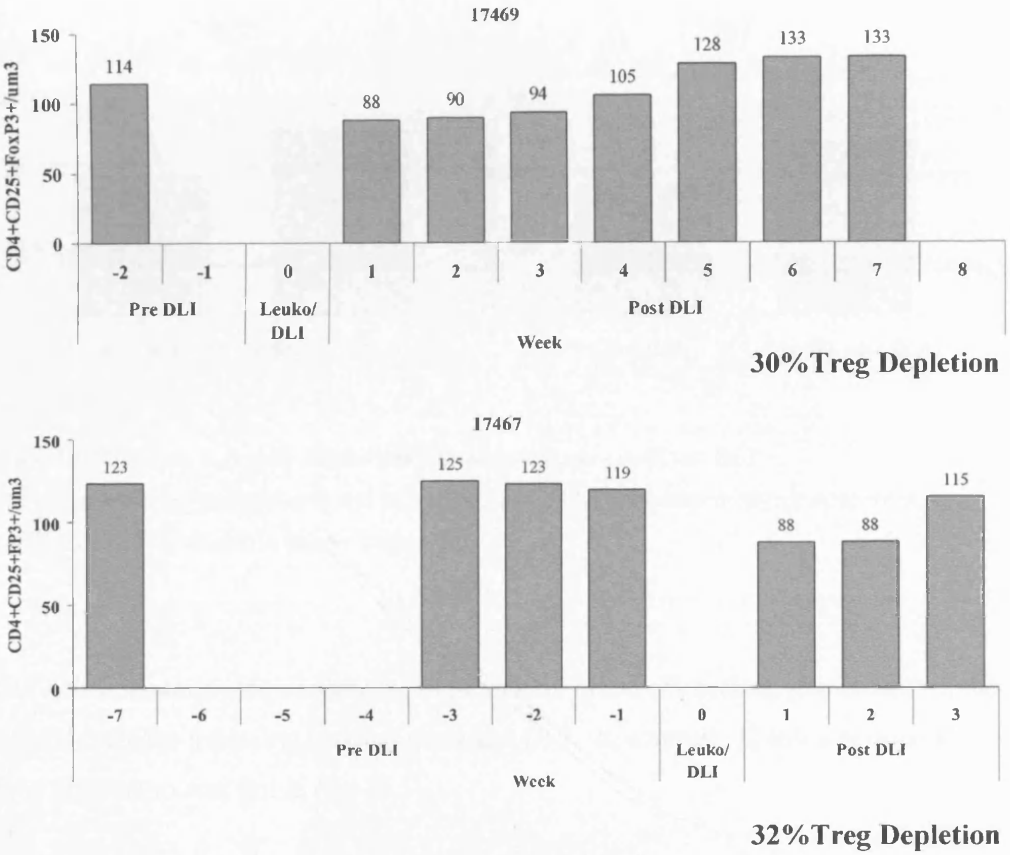


Figure 10.3 CD4+ CD25+ FoxP3+ cell levels pre and post leukapheresis and DLI. Graphs of CD4+CD25+FoxP3+ cell (Treg) levels over the same time period in 17469 and 17467 aligned for comparison of contemporaneous timepoints. Treg levels fell by 30% following leukapheresis and DLI in 17469, with no decrease over the same time period in 17467. Similarly, Treg levels fell by 32% in 17467 following leukapheresis and DLI with no decrease over the same time period in 17469.

10.3.1.3 Increased chimerism at 4 weeks following DLI

Chimerism levels following leukapheresis and DLI were monitored in the thymus at 4 weeks after DLI and in the peripheral blood several times a week. Both 17467 and 17469 demonstrated increased thymic chimerism at 4 weeks after DLI compared to the immediate pre-DLI level (fig 10.4). 17467 received its leukapheresis and DLI at week 14, later than 17469. 17467 demonstrated no increase in thymic chimerism over the

time-period (weeks 8-12 post HCT) that 17469's thymic chimerism increased. Similarly, no historical control animals (n=5) that had undergone the same chimerism induction protocol but no DLI at this time point, showed an increase in thymic chimerism over the same period.

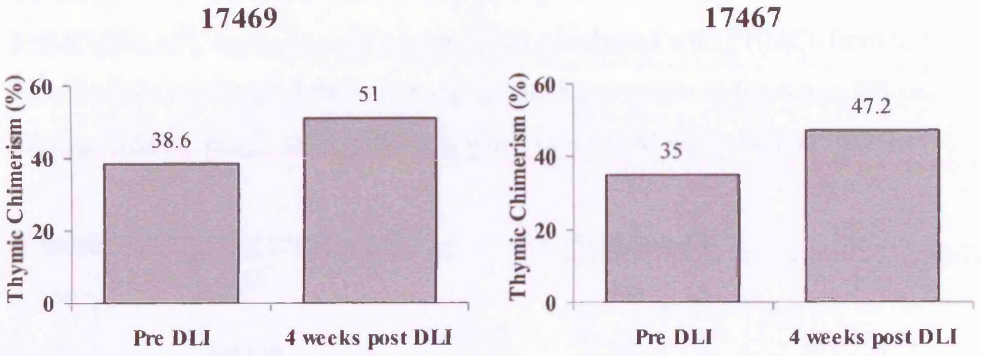


Figure 10.4 Increase in thymic chimerism following leukapheresis and DLI
Four weeks following leukapheresis and DLI, 17469 had a 12.4% increase in thymic chimerism, and 17467 had a 12.2% increase in thymic chimerism.

17467 also demonstrated an increase in peripheral blood chimerism, particularly in the myeloid lineages following leukapheresis and DLI. In contrast, 17469's peripheral blood chimerism was stable (fig 10.5).

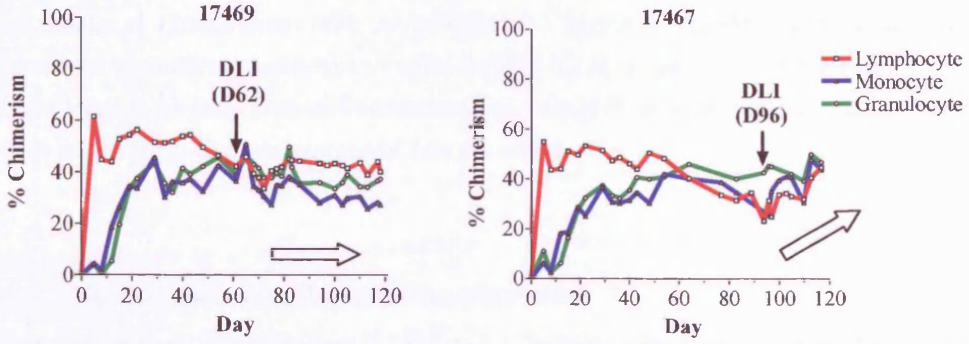


Figure 10.5 Peripheral blood chimerism following leukapheresis and DLI. Peripheral blood chimerism was unchanged in 17469 following leukapheresis and DLI. In contrast peripheral blood chimerism increased in 17467 over the 4 weeks following leukapheresis and DLI (lymphocytes ↑14%, monocytes ↑19% and granulocytes ↑2%).

10.3.1.4 Evidence for increased donor-vs.-chimera suppression by 4 weeks post DLI

Animals 17467 and 17469 underwent *in vitro* assessment of donor-vs.-chimera suppression prior to leukapheresis and DLI. 17469 also underwent *in vitro* assessment 4 weeks after DLI (see fig 10.6) (17467 unfortunately died before post DLI *in vitro* assessment could be performed). Prior to DLI, in both 17467 and 17469, PBMCs from the donor (SLA^{ac}; marked 'nAC') showed a normal alloresponse to recipient type PBMCs (SLA^{ad}; marked 'nAD'), even when cocultured with PBMCs from the chimera. However, following DLI there was significant suppression of the donor PBMC alloresponse to recipient-type PBMCs when cocultured with chimera PBMCs.

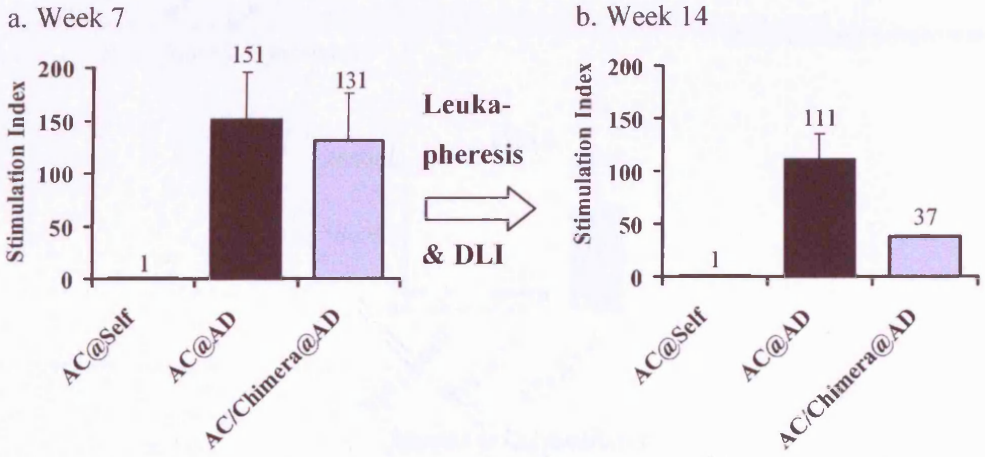


Figure 10.6 *In vitro* assessments of donor-vs.-chimera suppression pre and post leukapheresis and DLI. Animal 17469 was assessed both just prior (a), and 4 weeks following DLI (b), for evidence of active donor-vs.-chimera suppression by primary coculture MLR. Prior to leukapheresis and DLI, addition of non-irradiated peripheral blood mononuclear cells (PBMCs) from 17469 to the donor ('nAC') did not inhibit the donor's normal alloresponse (a; normal alloresponse: 'nAC@nAD' cf. alloresponse with addition of PBMCs from 17469: 'nAC/469@nAD'). However, following DLI there was significant suppression of the donor alloresponse on addition of PBMCs from 17469 (b). Animal 17467 demonstrated no evidence of donor-vs.-chimera suppression at the same timepoints (data not shown), which were both pre-leukapheresis and DLI for this animal.

10.3.2 Vascularised skin flap allotransplantation

Only two of the three chimeras (17468 and 17469) survived long enough to receive a vascularised skin flap allotransplant from the HCT donor. One control animal (17519) was included, which underwent skin flap allotransplantation across a single haplotype MHC barrier without prior chimerism induction. The chimeras were tolerant to the

MHC on *in vitro* assessment by MLR prior to skin flap allotransplantation; in contrast the control animal was responsive. (fig 10.7).

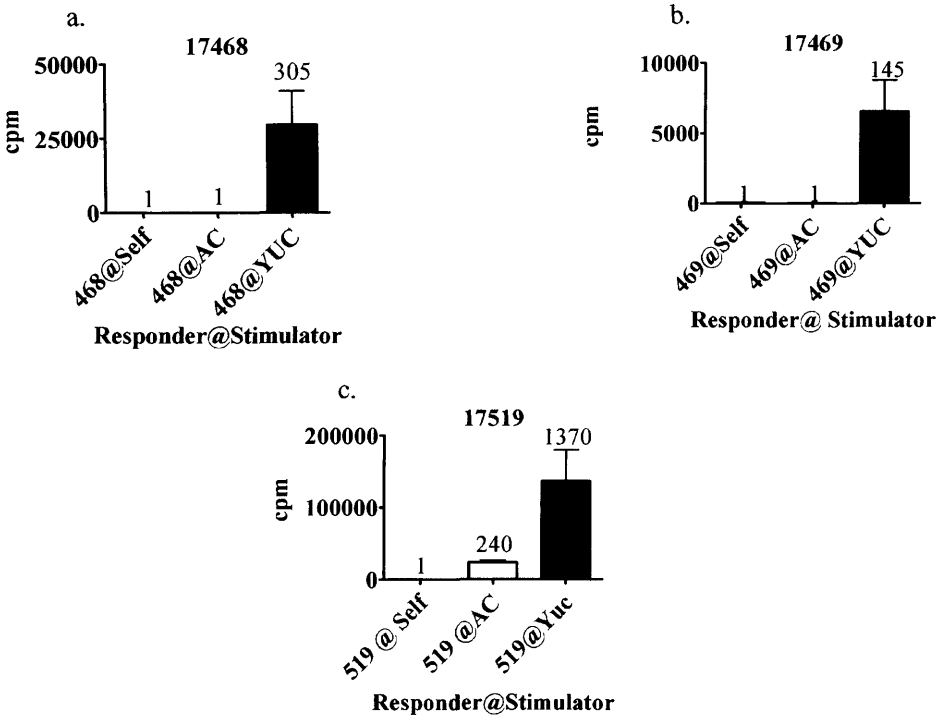


Figure 10.7 Assessment of *in vitro* responsiveness to the donor prior to skin flap allotransplantation. MLRs performed prior to skin flap transplant demonstrating tolerance to the MHC in both chimeric animals (a, b), but alloresponsiveness in the control animal (c).

Both chimeras and the control animal received a vascularised saphenous skin flap allotransplant across a single haplotype barrier without immunosuppression. The results of skin flap allotransplantation are summarized in Table 10.2.

Animal	Immune Barrier	Engrafted Chimera	Skin Survival
17519 (Control)	AC→AD	No	6 Days
17468	AC→AD	Yes	>46 Days
17469	AC→AD	Yes	>130 Days

Table 10.2 Summary of results of vascularised skin flap allotransplantation

10.3.2.1 Tolerance is achieved to vascularised skin in a moderate level chimera

The control animal rejected the allotransplanted skin flap by day 6 (fig 10.8 a & b). In contrast, both animals 17468 and 17469 showed no signs of rejection of their allotransplanted flaps at any timepoint. Animal 17468 died 46 days after skin transplantation from an unrelated cause[10] with no evidence of skin flap rejection (fig 10.8 c & d). Animal 17469 survived long-term with no evidence of skin flap rejection (fig 10.8 e & f). Tolerance (i.e. donor-specific unresponsiveness) was confirmed in animal 17469 with *in vitro* assays (fig 10.9).

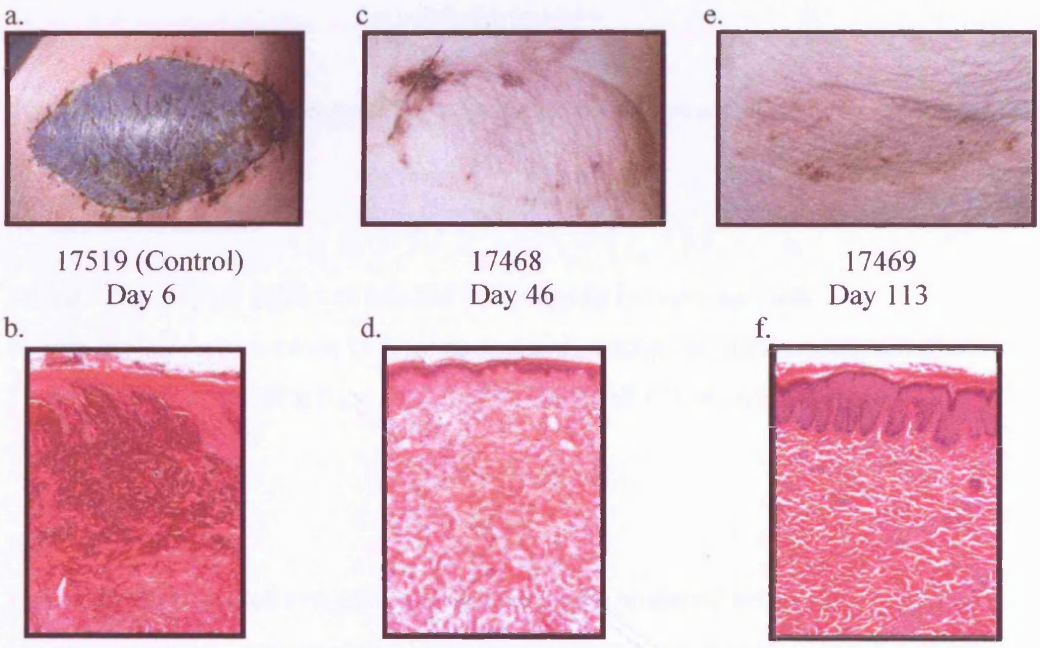


Figure 10.8 Skin survival outcomes: photographs and histology. Photographs and incisional biopsies from the allograft skin flap allografts were taken. Histological examination of the biopsies was performed (haematoxylin and eosin stain, x80 magnification shown). Animal 17519 (control animal) had visible skin necrosis of its flap at 6 days after transplantation with histological evidence of epidermal necrosis and dermal mononuclear cell infiltrate. Animals 17468 and 17469 had viable skin flaps with no visual or histological evidence of skin rejection at any timepoint (17468 died at day 46, 17469 survived to >130 days).

[10] Animal 17468 caught a multi-drug resistant bowel infection from an animal in an adjacent cage. This infection became systemic and eventually caused formation of an infected thrombus around a central line, from which the animal subsequently died.

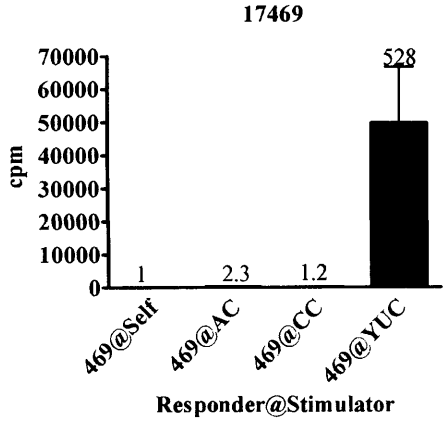


Figure 10.9 Animal 17469 had donor (AC) specific unresponsiveness 91 days after skin flap allotransplantation

10.3.2.2 No cellular infiltrate into the skin flaps in tolerant animals

In contrast to previous swine composite tissue allotransplantation models, there was no T cell infiltrate of the skin flaps of animals 17468 and 17469 on histological examination.

10.4 DISCUSSION

This study consisted of two parts: (1) induction of a moderate level chimera by HCT and subsequent leukapheresis and DLI, in which to test the skin tolerance hypothesis; and (2) skin allotransplantation across a single haplotype MHC barrier from the HCT donor with the aim of achieving tolerance.

Both elements of the study were successful. Chimerism was achieved following HCT with subsequent boosting of chimerism to moderate levels by leukapheresis and DLI. In addition, tolerance to skin transplanted across a single haplotype MHC barrier was achieved.

10.4.1 Leukapheresis and DLI is effective at increasing chimerism

In this study, the strategy developed in Chapter 7 was tested: DLI with prior leukapheresis will be effective at boosting chimerism. Both animals that underwent leukapheresis and DLI showed increased thymic chimerism by 4 weeks after the procedure, and one had increasing levels of peripheral blood chimerism. The aim of

leukapheresis was to reduce all white blood cell populations, including any causing donor-vs.-chimera suppression thereby preventing the DLI from being effective. Simple measurement of the total number of white blood cells in the peripheral blood did not give a good estimate of the proportion of total white blood cells removed by leukapheresis, with the actual number increasing after removal of four times the number of white blood cells initially present in the peripheral blood in animal 17467. The rapid replenishment of peripheral blood white blood cells by the completion of leukapheresis is likely to be largely due to release of large numbers of leukocytes from lymphoid tissues, with expansion of residual leukocytes as well as production of new white blood cells in the bone marrow.

Measurement of the peripheral blood lymphocyte count over time indicated that the leukapheresis procedure was effective at temporarily depleting up to almost two-thirds of the lymphocyte population. This is consistent with clinical studies that have found that leukapheresis can result in a reduction of peripheral blood lymphocytes (Nicolini, F. E. et al. 2004; Prior, C. R. et al. 1991; Strauss, R. G. et al. 1980).

Recovery of both the lymphocyte and the Treg population to pre-leukapheresis and DLI levels took six weeks in 17469. The time required for replenishment of the lymphocyte blood count has not been directly examined in the pig before. Huang followed T cell recovery following profound depletion with anti-CD3 immunotoxin (Huang, C. A. et al. 1999b), noting return of peripheral T cell levels by 6-11 days. However, this was only partial recovery and the levels appeared to be already plateauing in the two animals followed to 11 days indicating that return to pre-depletion levels may have taken a lot longer. In contrast, Suzuki found that depletion using anti-CD8 antibody resulted in the return of CD8+ cells to pre-depletion levels in the peripheral blood within 8 to 12 days (Suzuki, T. et al. 1990). However, it is not clear that this was due to replenishment with new CD8+ cells. This study did not exclude the possibility that CD8+ cells in lymphoid tissues were not fully depleted. Furthermore, it is possible that many of the CD8+ cells were not actually depleted but just temporarily down-regulated CD8 from their cell surface.

There have been few relevant clinical studies analysing replenishment of the lymphocyte pool after depletion. In patients donating at least one DLI following a previous bone marrow or cytokine mobilised progenitor cell donation Nicolini found

that 22% of donors became leukopenic following their first DLI. Furthermore, donors stayed leukapenic for a median of 3.7 months (range 1.6 to 43 months) following DLI (Nicolini, F. E. et al. 2004). However, these patients already had depleted levels of lymphocytes prior to DLI due to the previous bone marrow or progenitor cell donation.

Following leukapheresis and DLI there was an initial reduction of 30-32% in the number of CD4+CD25+FoxP3+ cells in the peripheral blood. This was associated with an increase in thymic chimerism (and peripheral blood chimerism in 17467) by 4 weeks after DLI. It was hypothesized that leukapheresis would deplete cells mediating Donor-vs.-chimera suppression thereby making DLI effective. It is possible that CD4+CD25+FoxP3+ cells are mediating the suppression and their temporary depletion provides a window of opportunity for the DLI to be effective. However, depletion of CD4+CD25+FoxP3+ cells may not be relevant for the effectiveness of DLI following leukapheresis because there may be other cell types mediating suppression. Additionally, it is possible that this approach is effective because global depletion of the white blood cell population by leukapheresis renders the recipient partly immuno-incompetent thereby creating "immunological space" in which donor bone marrow progenitor cells can expand.

The absolute number of CD4+CD25+FoxP3+ cells increased to greater than pre-leukapheresis/DLI levels in 17469 by five weeks after the procedure. This was associated with donor-vs.-chimera suppression in vitro, which was not evident prior to leukapheresis and DLI. This correlation suggests that the increase in CD4+CD25+FoxP3+ cells may have this boosted donor-vs.-chimera suppression. However, this was not confirmed. Furthermore, previous work indicates that increase suppression is not just due to CD4+CD25+FoxP3+ cell expansion (Koenen, H. J. et al. 2005), but also to changes in their activation status (Yates, J. et al. 2007).

10.4.2 Skin tolerance is achieved in a moderate-level engrafted chimera

Composite tissue allotransplantation techniques are the best reconstructive option for many severe defects. Induction of tolerance to skin and other tissues in composite tissue allotransplants would remove many of risks associated with composite tissue allotransplantation techniques, further widening their applicability. Several methods have been successful in achieving tolerance to skin in rodents, as outlined in Chapter 2. However, this success has not been translated to the large animal or the clinic.

Tolerance to musculoskeletal elements of a CTA has already been achieved across a MHC mismatch in large animal models (Hettiaratchy, S. et al. 2004); however, tolerance to skin has only previously been achieved across non-MHC minor mismatch barriers (Mathes, D. W. et al. 2003; Tillson, M. et al. 2006; Yunusov, M. Y. et al. 2006). This study confirms the hypothesis that a moderate-level engrafted chimera will be tolerant to skin allotransplanted from the HCT donor across a single-haplotype MHC barrier. This is the first time that tolerance to skin transplanted across a MHC barrier has been achieved in a large animal model.

The achievement of tolerance to allotransplanted skin in a large animal model indicates that it may be also possible to achieve skin tolerance clinically. However, there are limitations to this experimental protocol, which may affect the application of this approach to clinical composite transplantation.

A limitation in the design of this experiment is that transplantation was performed across just a single haplotype MHC barrier; this is equivalent to transplantation between siblings or between parent and child. Until now, due to a lack of donors, there has been no deliberate matching between donors and recipients, limiting the use of this technique to fortuitous HLA matching. However, as composite tissue allotransplantation becomes more common-place, people may be more willing to be donors. So, it may be possible to get at least single haplotype HLA matching.

There are also several other elements of the induction protocol which would not be directly transferable to the clinical situation. For example, the delay following chimerism induction before skin allotransplantation would not be possible clinically because the donor would be brain dead and so could not be kept alive for an extended period of time. The barriers to the clinical application of each element of the protocol, and approaches to overcome these barriers are discussed further in Chapter 12.

Another limitation in this experiment is that only two animals (17468 and 17469) underwent chimerism induction and vascularised skin allotransplantation. Although both animals accepted their skin flap transplant, this is still a small number. Furthermore, animal 17468 died 46 days after skin allotransplantation. So, it is possible that this animal would have gone on to reject its transplant. However, even survival to day 46 without immunosuppression is longer than any previous vascularised skin

allotransplant. Hettiaratchy (Hettiaratchy S. et al 2004) achieved limb allotransplant (which included vascularised skin) survival of 46-70 days. However, these animals were given high-level systemic cyclosporine for the first 30 days following transplantation. Consequently, survival without immunosuppression only ranged between 16-40 days. Despite the long survival of 17468's flap, it is still possible that it could have been rejected.

Animal 17469 demonstrated long-term acceptance of the allotransplanted skin flap to greater than 100 days. This is significant because it is far beyond the time period when rejection due to non-MHC minor antigens usually occurs (10-50 days (Huang C. A et al 2000; Fuchimoto Y et al. 2001)), indicating tolerance. Skin has been considered one of the most difficult tissues to achieve tolerance to, and it was not known whether it would be even possible to achieve tolerance in a large animal model across a MHC mismatch. This experiment demonstrates that it is possible.

10.4.3 Mechanism of skin tolerance

This study confirmed the hypothesis that an engrafted moderate level chimera would be tolerant to a vascularised skin allograft. The assumption underlying this hypothesis was that a moderate level chimera would achieve tolerance by combined central deletional and regulatory mechanisms. The central deletional mechanism provides robust tolerance to the MHC. The central deletional mechanism is supplemented by regulatory mechanisms which provide tolerance to any skin specific antigens which are are not covered by the central deletional mechanism because they are not represented on bone marrow derived cells.

The role of central deletional mechanisms was not directly examined in this model. However, the animals had evidence of haematopoietic stem cell engraftment. Engraftment has previously been associated with evidence of the presence of central deletional mechanisms achieving donor specific transplant tolerance (Fuchimoto Y. et al. 2000), and so it is likely that deletional mechanisms contributed to the achievement of skin tolerance in this study.

The presence of regulatory mechanisms involved in the achievement of skin tolerance was only indirectly examined. There was no regulatory T cell infiltrate into the skin flap. This finding contrasts with the previous split tolerance composite tissue

allotransplant models, as well as the kidney transplant model described in Chapter 6. In these models there was a perivascular non-inflammatory T cell infiltrate which was likely to have a regulatory function. One reason for this difference is that the previous models achieved tolerance by almost entirely peripheral regulatory mechanisms (see Chapters 6a and 6b), whereas in this model a central deletional mechanism is likely to have made a significant contribution to the achievement of tolerance.

If regulation is important in the achievement of skin tolerance, the mechanisms involved may only function across a single haplotype MHC mismatch between donor and recipient. There is evidence that the induction of Treg cells involves the presentation of antigenic peptide on a self MHC class II molecule (LeGuern, C. 2003). This would occur in transplantation across a single haplotype MHC barrier because of the shared MHC on the second haplotype. In contrast, a full double-haplotype MHC mismatch would not have a shared MHC and so tolerance may not be able to be achievable. However, there are peripheral tolerance models that achieve organ tolerance across a full double-haplotype mismatch barrier (Utsugi 2001), indicating there are mechanisms not limited to the sharing of MHC class II.

10.5 CONCLUSIONS

Billingham, Brent and Medwar were the first to describe a method of skin tolerance induction in a small animal model (Billingham, R. E. et al. 1953). Fifty-five years later this experiment finally takes the next step towards clinical skin tolerance induction, achieving tolerance to a vascularised skin allotransplant in a preclinical large animal model. The achievement of clinical skin tolerance could significantly lower the risks of composite tissue allotransplantation techniques, facilitating their widespread use, and also allow expansion into new applications; for example, CTA techniques could replace many current reconstructive techniques, thereby removing the problem of donor site morbidity.

CHAPTER 11: THE USE OF SITE-SPECIFIC THERAPY TO PREVENT SKIN REJECTION

11.1 INTRODUCTION

The aim of this study is to examine the ability of topical FK506 to prevent skin rejection in skin transplant recipients both with normal and reduced alloreactivity.

The main goal of CTA transplantation immunology research is to achieve widespread application of CTA techniques. To achieve this goal there needs to be long-term survival of skin and other tissues transplanted across MHC barriers while avoiding the toxicity of systemic immunosuppression. As discussed in Chapter 2, there are several strategies that can be used to achieve this:

(A) Tolerance induction: this would obviate the requirement of chronic immunosuppression

(B) Reduction of the toxicity of chronic immunosuppression

(C) Induction of a less alloreactive state with consequent reduction in immunosuppression

The induction of skin tolerance (option A) has been investigated in previous chapters. However, skin tolerance is not necessarily required. A reduction of immunosuppression toxicity (option B) or induction of a less alloreactive state (option C) could also achieve the goal of long-term survival of skin without immunosuppression toxicity. Site-specific therapy is one modality that could be used in the accomplishment of options B and C. The use of site-specific treatment could facilitate a decrease in the levels of systemic immunosuppression required in a recipient with normal alloreactivity, with consequent reduction in medication toxicity. Alternatively, site-specific monotherapy may prevent skin allotransplant rejection following induction of a less alloreactive state.

The use of topical FK506 has been demonstrated to double the length of skin survival in a rat model (Fujita, T. et al. 1997; Yuzawa, K. et al. 1996). Furthermore, topical steroids have been used to achieve prolonged limb allotransplant survival in a rat model (Inceoglu et al. 1994). There has also been some success using site-specific treatments

to treat acute rejection episodes clinically. The Louisville group achieved clinical resolution of grade 3 histological skin rejection in a hand transplant by alternating topical tacrolimus and topical steroid treatment with change to the systemic immunosuppressive regimen. However, topical tacrolimus was not sufficient to reverse rejection responses in either an Austrian hand transplant (Lanzetta, M. et al. 2007) or the first French face transplant (Dubernard et al. 2007).

The clinical use of site specific therapy has not been studied in a systematic way so it is not possible to draw firm conclusions about its effect. This study aims to examine the effect of site-specific therapy in a clinically relevant large animal model. FK506 was selected as the topical therapy because it is very effective in treating several immune mediated dermatological complaints, has minimal systemic toxicity (Munzenberger et al. 2007), and avoids the local side effects of topical steroids (e.g. skin atrophy, striae, telangectasia) that limit its prolonged use.

11.2 OVERVIEW OF EXPERIMENT

This is a combined prospective and retrospective study examining the effect of topical FK506 on allogeneic skin transplant survival. The effect of topical FK506 in animals both with normal alloreactivity and reduced alloreactivity is examined.

11.2.1 The effect of FK506 in animals with normal alloreactivity

To ascertain if topical FK506 could be used as a substitute for systemic immunosuppression, the effect of FK506 on survival of both conventional skin grafts and vascularised saphenous skin flaps transplanted across a MHC barrier in naïve animals was examined (table 11.1; Groups I and II). Experimental animals received FK506 daily starting on the day of skin allotransplant. Controls received either a conventional skin allograft or a vascularised saphenous skin flap transplanted across a MHC barrier without immunosuppressive treatment.

Group	Alloreactivity	Conventional Skin Graft/ Vascularised Skin Flap	Topical FK506	Animal	Transplant Barrier
I	Normal Alloreactivity	Graft	No	**17476**	DD→AC
				17506	DD→CC
			Yes	15129	AA→DK
				15132	AA→DK
II		Flap	No	**17519**[a]	AC→AD
			Yes	**17520**	AC→AD
III (Chimera)	Reduced Alloreactivity	Graft	No	13476[b]	CD→AD
			Yes	**16626**[b]	AC→AD

Key

Prospectively studied animals are **highlighted**

a. Animal 17519 was also used as a control animal in Chapter 10

b. Animal received an organ transplant (to which it was tolerant prior to skin allograft)

Table 11.1 Summary of experimental groups

11.2.2 Effect of topical FK506 in animals with reduced alloreactivity

It has been previously observed that induction of engrafted chimerism across MHC-matched minor mismatched barriers achieves a state of reduced alloreactivity with prolonged survival of conventional donor type skin grafts (Huang, C. A. et al. 2000). This model of a less alloreactive state achieved through chimerism induction was used to assess the effectiveness of FK506 to attain long-term skin allograft survival across a MHC barrier (table 11.1; Group III). Following non-myeloablative chimerism induction with engraftment across a single haplotype MHC barrier, experimental animal 16626 received the following conventional skin grafts: cryopreserved donor skin graft, fresh and cryopreserved donor-matched skin graft (from the same donor animal), and an autograft[11]. These skin grafts were then treated with daily topical FK506. The control animal underwent chimerism induction with engraftment, and subsequent application of

[11] It was not possible to use fresh donor skin as the PBMC donor had been sacrificed previously. Therefore, in addition to a cryopreserved donor skin graft, both fresh and cryopreserved donor-matched skin grafts were applied so that any possible effect of cyropreservation could be ascertained.

a fresh donor-matched skin allograft without application of topical FK506. The effect of topical FK506 on vascularised skin flaps in chimeras was not examined as tolerance can be achieved without topical FK506 in this model (see Chapter 10).

Animals were followed daily for evidence of skin rejection. Skin biopsies for histological assessment were taken at regular intervals and at the first signs of erythema possibly indicating skin rejection.

11.3 RESULTS

11.3.1 Conventional skin grafts, but not vascularised skin flaps, show prolonged survival with the application of topical FK506 in animals with normal alloreactivity

Alloreactivity to the MHC of the donor was confirmed by MLR prior to skin allotransplantation (not shown). The effect of FK506 on skin allotransplant survival is summarized in table 11.2

Group	Conventional Skin Graft vs. Vascularised Skin Flap	Topical FK506	Animal	Skin Survival (Days)
I	Graft	No	17476	8
			17506	7
		Yes	15129	18
			15132	17
II	Flap	No	17519	6
		Yes	17520	6

Table 11.2 Summary of skin allotransplant survival in animals with normal alloreactivity

Daily topical FK506 ointment prolonged skin graft survival by 10 days in animals receiving conventional skin allografts (17.5 vs. 7.5 days). In contrast, there was no prolongation of vascularised skin flap survival by the application of topical FK506 (6 days vs. 7.5 days).

11.3.2 Topical FK506 prolongs survival of conventional skin allografts in animals with a reduced alloreactive state

Reduced activity to the MHC of the donor was confirmed by MLR prior to skin allotransplantation (see fig 11.1). The outcomes following skin allografting are summarized in table 11.3.

In animals in a reduced alloreactive state, the daily application of topical FK506 ointment prolonged the survival of skin by 9 days of both donor and donor-matched skin, with rejection occurring 64 vs. 55 days after transplantation (see fig 11.2). There was no difference in the rejection times between fresh and frozen donor-matched skin.

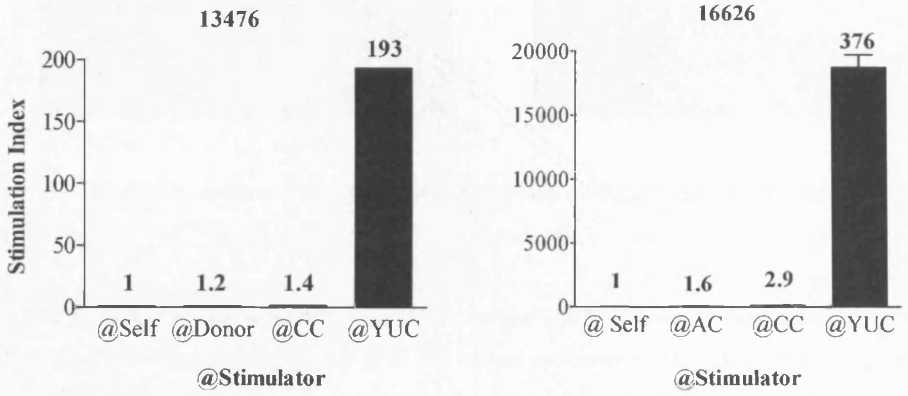
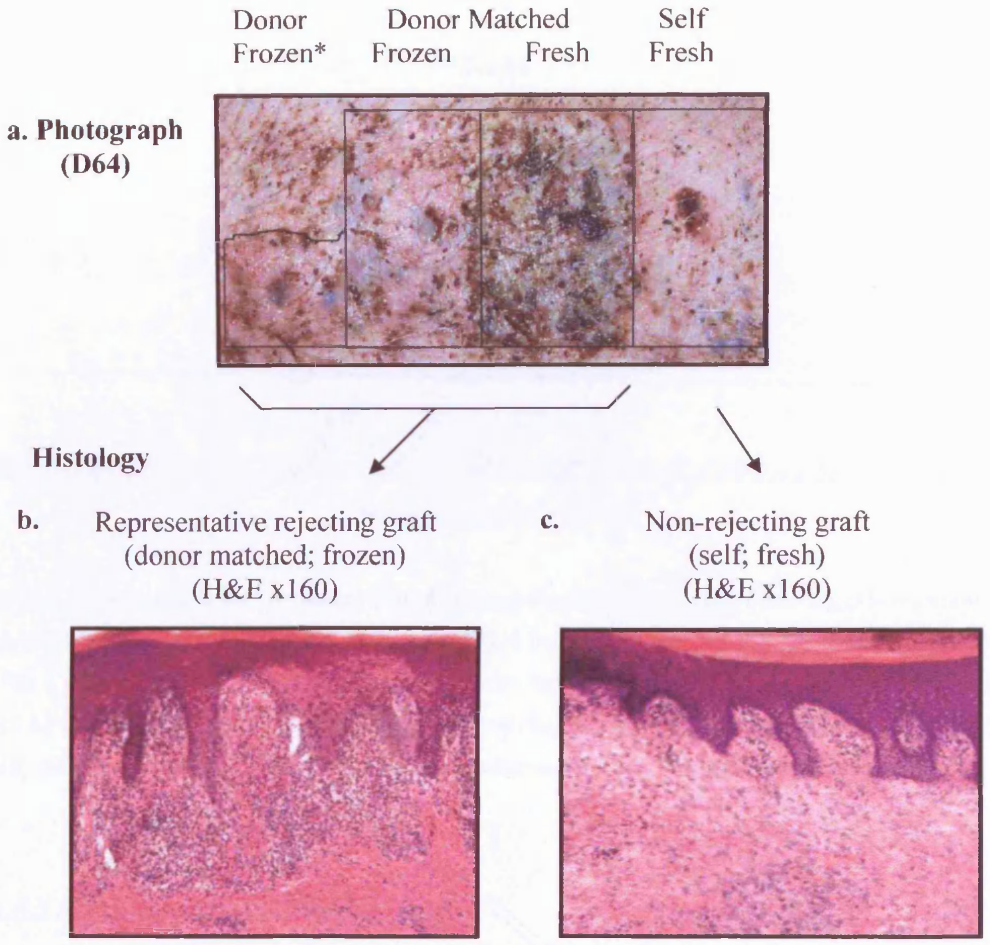


Figure 11.1 Unresponsiveness to donor MHC demonstrated in animals prior to skin grafting

Group	Topical FK506	Animal	Skin Survival (Days)
III	No	13476	55
	Yes	16626	64

Table 11.3 Summary of skin allograft survival in animals with reduced alloreactivity



Key

* Only part of the frozen donor graft became vascularised following grafting and survived to rejection (outlined).

Figure 11.2 Evidence of skin allograft rejection in animal 16626: photograph and histology. (a) Sixty-four days following grafting the self graft had normal appearance. However, there was generalized erythema of the donor (frozen) and donor matched (frozen and fresh) skin allografts, indicating rejection. Visual findings were confirmed on histological examination of punch biopsies: (b) Rejecting grafts demonstrated generalized inflammation with degeneration of rete pegs and epidermal necrosis. (c) In contrast, the self graft was viable with no inflammation.

In vitro assessment of animal 16626 was performed following skin graft rejection (fig 11.3). Animal 16626 was alloreactive against donor-type (AC) cells despite still receiving site-specific therapy. However, this may have been responsiveness to minor antigens as animal 16626 had a greater response against the single donor haplotype (AC) than the full donor haplotype (CC).

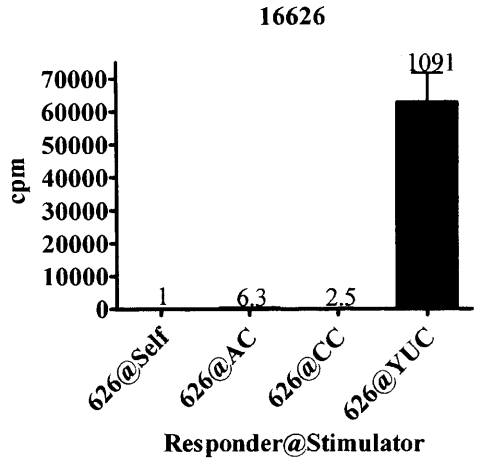


Figure 11.3 Responsiveness to donor MHC following skin rejection. MLR following skin rejection demonstrated that peripheral blood lymphocytes (PBLs) from 16626 had a stronger alloreactive response to PBLs with a single donor haplotype (AC; Stimulation Index (SI)=6.3)) than a double donor haplotype (CC; SI=2.5). This suggests that 16626 PBLs were reacting to non-MHC minor antigens rather than MHC antigens. There was a strong alloreactive response against the 3rd party YUC control.

11.3.3 Systemic effects of topical FK506

Systemic absorption of FK506 was measured weekly. The systemic levels of FK506 in skin grafts was greatest in the first two weeks following allotransplantation (maximum 7ng/ml), subsequently declining to a lower levels (consistently <2ng/ml from one month following grafting). Skin flaps did not survive to later timepoints; the early levels of FK506 in skin flaps did not reach as high a level as skin grafts (maximum 3ng/ml). No adverse local or systemic effects of FK506 were observed.

11.4 DISCUSSION

The use of site-specific therapies offers the hope of an effective way of reducing the systemic toxicity of current immunosuppressive regimens in a way that may be more immediately achievable than tolerance induction. Topical FK506 has been considered a particularly attractive site-specific therapy both because of its effectiveness in immune-mediated skin disorders and because it has minimal side-effects. This study indicates that topical FK506 did not prolong the survival of a vascularised skin flap transplanted across a MHC barrier. However, topical FK506 has some efficacy in prolonging conventional skin allograft survival transplanted across a MHC barrier both in animals with normal and reduced alloreactivity.

11.4.1 Topical FK506 has previously been demonstrated to be an effective inhibitor of the immune response to antigens in the skin

Topical FK506 is clinically proven in non-transplant scenarios to be an effective inhibitor of immune mediated inflammatory skin disorders without causing significant side-effects. Topical FK506 exerts its effects by acting on both the priming and the effector phases of the immune response.

The priming phase of the immune response to antigens in the skin is mediated by antigen presenting cells. These antigen presenting cells include dermal dendritic cells and epidermal Langerhans cells which migrate from the skin to activate naïve T cells in draining lymph nodes. Studies have indicated that topical FK506 affects several stages of the priming phase. *In vitro* studies demonstrated that FK506 significantly decreased the expression of MHC class I and II, CD25 and costimulatory molecules on human cultured Langerhans cells (Panhans-Gross, A. et al. 2001). *Ex vivo* studies have demonstrated that FK506 inhibits DC migration from murine skin (Bäumer, W. et al. 2005). Topical FK506 has also been shown to inhibit dendritic cell-lymphocyte synapse formation (Mijal, J. et al. 2002). This may explain why dendritic cells isolated from patients treated with topical FK506 for atopic dermatitis have reduced stimulatory capacity by skin MLR (Wollenberg, A. et al. 2001).

The effector phase of the immune response to antigens in the skin is mediated by T-lymphocytes that have been activated in the draining lymph nodes. These T cells migrate to the area of the antigenic stimulus due to a combination of expression of tissue-specific homing receptors, cytokines and adhesion molecules. Once they have infiltrated the area of the antigenic stimulus they secrete cytokines that potentiate the

immune response. Topical FK506 has been demonstrated to inhibit adhesion molecules involved in cutaneous lymphocyte migration (Caproni, M. et al. 2006) and reduce inflammatory cytokine production of infiltrating T cells in patients with atopic dermatitis (Simon, D et al. 2004).

11.4.2 Utility of topical FK506 for CTA recipients with normal alloreactivity

Despite achieving prolonged conventional skin allograft survival in naïve recipients in this study, daily topical FK506 application did not prolong vascularised skin allograft survival. This indicates that topical FK506 would be an ineffective monotherapy to prevent rejection of composite tissue allotransplants which include just skin.

Topical FK506 is more likely to be used in conjunction with systemic immunosuppression rather than as a monotherapy. The application of topical FK506 might allow reduction of the systemic medication to a less toxic level, while still preventing skin rejection. This study indicates that topical FK506 can delay skin rejection, so it is possible that FK506 ointment would achieve the goal of reduction in the amount of immunosuppression taken systemically in CTA. However, it is unclear whether the effect of FK506 is due to its systemic absorption or a loco-regional effect.

Topical FK506 may actually exert its main effects systemically. There were detectable levels of FK506 in all animals, indicating that FK506 is being absorbed systemically. These levels were in the clinical therapeutic range initially (i.e.>5ng/ml) in some skin allograft recipients. If prolonged skin survival is due to systemic absorption then the overall systemic levels of immunosuppression may not actually be reduced even though the oral dose will be less. However, even in this scenario, topical FK506 may still reduce systemic toxicity: the topically administered FK506 may be absorbed at a constant rate over a period of time thereby avoiding the more toxic peak levels associated with bolus dosaging.

Alternatively, topical FK506 may exert its primary effects at a loco-regional level. If much of the topically applied FK506 enters the lymphatics, it will lead to a higher concentration in the regional lymph node basin for a given systemic level than FK506 taken orally. This relatively higher level of FK506 in the draining lymph node system may lead to more effective prevention of the initiation of the rejection response.

The systemic absorption of topically applied FK506 was highest immediately after skin transplantation, and then decreased. Systemic FK506 levels were also higher when applied to conventional skin grafts rather than vascularised skin flaps. The difference in systemic absorption of FK506 seen in this model early after skin transplantation may be a reason for the differential effect of FK506 on conventional skin grafts compared to vascularised skin flaps. The inflammation in the period immediately after skin grafting may be the reason for the higher systemic levels of FK506 seen early after skin grafting, or in skin flaps. It has been observed clinically that there is greater absorption of topical FK506 through inflamed skin compared to normal skin (Reitamo, S. et al. 2002). Following transplantation, a skin graft goes through a period of relative ischemia which may stimulate an inflammatory reaction. This inflammatory reaction will settle down once revascularisation has occurred and will not be present in older skin grafts, possibly reducing FK506 absorption. Likewise, skin flaps are primarily vascularised and so do not pass through a pro-inflammatory period of relative ischaemia, which may reduce FK506 absorption.

11.4.3 Utility of topical FK506 for CTA recipients with reduced alloreactivity

In this study, topical FK506 prolonged skin allotransplant survival in a state of reduced alloreactivity. This has the potential for clinical relevance if the state of reduced alloreactivity that can already be achieved in organ transplants can be extended to CTA.

Calne was the first to report on a state of "Prope (almost) tolerance", in which low rejection rates of renal allografts were achieved with a low dose of cyclosporine after peri-transplant T-cell depletion with alemtuzumab (Calne, R. et al. 1998). Furthermore, groups in both Pittsburgh and Miami have used donor bone marrow infusions following organ allografting with conventional immunosuppression achieving survival with a lower incidence of acute and chronic rejection (Shapiro, R. et al. 2001; Tryphonopoulos, P. et al. 2005). T-cell depletion and bone marrow infusions have been used in some composite tissue allograft patients without measurable effect; however, this has been performed on an *ad hoc* basis and so it is difficult to draw any firm conclusions about the effectiveness of this approach.

There are limitations to this model which may restrict the clinical significance of prolonged skin allgraft survival with topical FK506. The conditioning regimen in this model does not correlate precisely with current clinical approaches to induce a state of

reduced alloreactivity, and so may involve different immunological mechanisms. Furthermore, conventional skin allografts were used in this model, not primarily vascularised skin, as used in clinical composite tissue allotransplantation.

It is possible that the use of conventional skin allografts may not actually have been a limitation of this model, but rather, they may have made this model even more stringent than the clinical situation using primarily vascularised skin. Skin rejection appeared to be mediated by non-MHC minor antigens in this model. It is likely that these minor antigens were tissue specific skin antigens. Skin specific antigens have been thought to be the cause of rejection in previous chimeras receiving a non-vascularised skin allograft (Fuchimoto, Y., et al. 2001). However, skin specific antigens do not cause vascularised skin allograft rejection, as described in Chapter 10. Consequently, site-specific therapies may be effective in indefinitely prolonging primarily vascularised skin survival under conditions of reduced alloreactivity, even if they only have a limited effect in prolonging secondarily-vascularised skin allograft survival.

11.5 CONCLUSIONS

Site-specific therapies have been used in several of the clinical composite tissue allotransplants because they offer the possibility of counter-acting rejection in composite tissue allotransplantation whilst avoiding significant systemic side-effects. However, the clinical benefits of using site-specific therapy have not yet been clearly demonstrated. This study indicates that there are minimal systemic side-effects from the topical FK506 in a large animal pre-clinical model. However, there is no measurable benefit in using topical FK506 on a vascularised skin allotransplant in a state of normal alloreactivity, and only marginal benefit in a state of reduced alloreactivity. These findings indicate that, under the conditions investigated here, topical FK506 is unlikely to have a central role in preventing clinical composite tissue allotransplant rejection.

CHAPTER 12: DISCUSSION

12.1 INTRODUCTION

The outcomes from clinical composite tissue allotransplantations have been much better than many predicted. It has been 10 years since the first successful clinical hand transplant was performed. Since then, allotransplantation techniques have been successfully used to reconstruct defects on many areas of the body. The greatest experience has been gained with hand allotransplantation. In the Western world, there have been 24 reported hand allotransplants, with loss of only one due to patient non-compliance with medication. The knee transplantation programme is the only application to date in which CTA outcomes have been suboptimal. It has been suggested that the high rates of rejection of this essentially musculoskeletal transplant may have been triggered by the sentinel skin flap included in the transplant for monitoring purposes. This may be another indication of the crucial role skin plays in the achievement of long-term survival of composite tissue allotransplants.

Skin forms a key element of many composite tissue allotransplants. Skin is more susceptible to immune rejection than other tissue within composite allografts, with more than two-thirds of recipients having at least one acute rejection episode within the first year. These skin rejection episodes have so far been overcome with increased levels of immunosuppression, but this has prevented tapering of the immunosuppressant dose to lower, less toxic levels. Consequently, significant side-effects are being observed: two hand recipients have already required hip replacements for avascular necrosis due to steroid medications, and the first face transplant recipient has suffered from renal toxicity.

The experiments described in this thesis aim to address many of the questions surrounding the process of skin rejection and ways to overcome it. This discussion is in two parts. Firstly, the implications of these experimental findings for clinical CTA are examined. Secondly, the possible future avenues of research will be discussed. The order followed in each section is as follows:

PART A: Skin rejection

(1) The consequences of skin rejection

(2) Cell trafficking in skin rejection

PART B: Prevention of skin rejection

(3) Tolerance induction to skin

(4) Reducing immunosuppressant toxicity

12.2 PART A: SKIN REJECTION

This thesis examined both the consequences of skin rejection in CTA, and cell trafficking in skin transplant rejection.

12.2.1 The consequences of skin rejection

There will be both social and biological consequences to skin rejection and composite tissue allograft failure. The support of the wider scientific community and the public is crucial for the expansion of CTA.

12.2.1.1 Social consequences of CTA allograft failures

Initial difficulties with the first heart allotransplants led to the whole programme being suspended. In the current hand allograft programme, there has been only one failure in the Western world, and much of the criticism of this case focused on inappropriate initial patient selection. In contrast, 15 of the 16 hands that have been transplanted so far in China have now failed. The Chinese experience has been largely ignored in the Western media because this was due to lack of funding leading to cessation of patient medications and so has limited relevance to the experience in the rest of the world. However, it is likely that more composite tissue allografts will fail in the West, and an open, self-critical approach will be important for a fair assessment of the benefits from CTA by society.

12.2.1.2 Consequences for the patient of CTA allograft failure

In the event of a composite tissue allograft failure, the best reconstructive option is likely to be replacement of the failed allograft. The findings in Chapter 4 suggest that in the event of a composite tissue allograft failure the recipient vessels would be intact making a retransplantation technically possible. Obviously, graft survival is the most important factor for good outcome following retransplantation. The experience from organ retransplantation is that graft survival rates are poorer for retransplants than for first transplants. It is likely that the experience in composite tissue retransplantation will mirror that of organ transplantation. Factors that may contribute to poorer

outcomes following retransplantation include prior allosensitisation to the donor making it harder to find a suitable second transplant.

12.2.1.3 Functional recovery of a retransplant is unknown

In the event of graft survival the findings in Chapter 4 indicate that there will not be significant damage to recipient tissues underlying the allograft, which could otherwise limit the allograft function. However, re-innervation is also essential for a good functional outcome. Motor and sensory functional recovery in hand allografts has been comparable to that of hand replantation, and some have suggested that systemic FK506 therapy may even promote nerve regrowth (Yang, R.K. et al. 2003). However, there could be less functional recovery in a second transplant due to damage to the nerve from the rejection of the first transplant.

12.2.2 Cell trafficking in skin rejection

Chapter 5 raises several issues regarding cell trafficking in skin rejection including the susceptibility of skin to reject, the focus of the rejection response and the use of *in vivo* imaging in other models.

12.2.2.1 Susceptibility of skin to reject

Transplanted skin has a particular susceptibility to be rejected, compared to other tissues. This has been a focus of research for many years. Several factors have previously been identified that may contribute to skin's tendency to reject, including the mode of skin transplantation. The findings in Chapter 5 indicate that recipient cell trafficking as part of the rejection response in secondarily vascularised conventional skin grafts is very different to primarily vascularised skin flaps. These findings indicate that conventional skin allograft data has to be interpreted with caution when developing new strategies to prolong skin survival in composite tissue allotransplantation.

12.2.2.2 Focus of the rejection response

In Chapter 5 it is observed that the rejection response appeared to be focused in the superficial dermis with limited infiltration of the epidermis. This has implications both for the interpretation of some previous studies, and for the role of skin specific antigens. The focus of rejection within the skin in some previous studies appeared to be the epidermis: in the previously described split tolerance swine model there was acceptance of the dermal element of the allograft but rejection of the epidermis. It is possible that

the findings in the rat rejection and the swine split tolerance models are not contradictory, and that it is the intense superficial dermal rejection response that leads to epidermal loss. In addition, the finding of rejection focused on the dermis indicate that skin specific antigens may not be as important as sometimes thought in the rejection response to skin.

12.2.2.3 The use of in vivo confocal imaging in other models

Current clinical methods to prevent skin rejection include various chronic immunosuppression regimens, with attempts to reduce the alloreactivity of the recipient in some cases by T-cell depletion or bone marrow infusion. The effects of these methods on immune cell entry, targeting and exit have not been fully examined. However, modifications to cell trafficking will be pertinent for the development of successful ways to improve the effectiveness of regimens to avoid skin rejection without toxicity. Chapter 5 demonstrates the usefulness of *in vivo* imaging for studying cell trafficking.

12.3 PART B: PREVENTION OF SKIN REJECTION

Part B of the thesis explored two approaches to prevent skin rejection in a swine model: tolerance induction, and reduction of immunosuppressant toxicity by the induction of a less alloreactive state and/or site-specific therapy. The swine model used, and both approaches to prevent skin rejection will be examined in turn:

12.3.1 Limitations of the swine model

This thesis uses a swine model to investigate methods to prevent skin rejection. This is a useful model because of its greater clinical relavence compared to small animals, the ability to repeat transplants across the same immune barrier because the herd is immunologically defined, and the availability of immunological reagents for investigation of the immune mechanisms involved. However, there are certain limitations to the swine model due to the use of the MGH miniature swine herd, and the use of young adult swine recipients.

The MGH partially-inbred miniature swine herd is one of the only immunologically defined large animal models worldwide, and so it is not easy to corroborate findings in another large animal model. It is therefore difficult to know if any observations in this model have global relevance or are specific to this herd due to inbreeding. Secondly, in

this thesis, skin tolerance was achieved across only a single haplotype MHC barrier. This is the equivalent of receiving a transplant from a parent or sibling. In composite tissue allotransplantation it is likely that suitable donors will be in short supply, and so HLA matching to this extent may not be possible. This model needs to be effective across a double-haplotype MHC barrier to maximize its clinical utility. However, a double-haplotype mismatch in this swine model, equivalent to the clinical situation is not even possible, since there are only three lines of swine (SLA^{aa}, SLA^{cc}, and SLA^{dd}). Furthermore, there is some sharing of MHC Class II DR between SLA^{aa} and SLA^{dd}. The closest equivalent to a clinical double-haplotype mismatch transplant is to do a transplant from SLA^{aa} to SLA^{cc}; this is actually a double single haplotype mismatch. To achieve a mismatch equivalent to a clinical double-haplotype mismatch would require another subline; for example if the SLA^{bb} subline still existed it would be possible to perform a double-haplotype mismatch transplant from SLA^{ab} to SLA^{cd}.

Young adult recipients were used in this thesis as recipients for haematopoietic cell transplantation. It is likely that the mechanism of tolerance induction in this model is thymic dependent. However, it has been observed in other thymic dependent tolerance induction protocols in this swine model, that tolerance can only be achieved in younger pigs. Once the thymus has started to atrophy in adult pigs, it is no longer possible to achieve tolerance. If this was true clinically, it would significantly limit the application of these techniques. Consequently, methods to rejuvenate the thymus are currently being examined by others to extend the application of these techniques to older recipients.

12.3.2 Tolerance induction

The hypothesis developed in Chapter 6, and subsequently tested in Chapter 10, is that a moderate level engrafted chimera would be tolerant to a vascularised skin allograft transplanted across a single haplotype MHC barrier. This hypothesis is correct, with the achievement of tolerance to MHC mismatched skin transplant for the first time in a large animal model. The achievement of this in a large animal model represents an important step towards clinical CTA tolerance induction.

12.3.3 Reducing immunosuppressant toxicity

For many, clinical tolerance induction is too distant a goal for composite tissue allotransplantation. Even if it were possible, the induction regimen might have to be

more aggressive than simple immunosuppression, increasing the risks in the initial perioperative period. However, induction of a less alloreactive state may provide many of the benefits of tolerance induction, such as reduced treatment-related toxicity and decreased risk of chronic rejection, without the initial risks from the induction regimen. Consequently several composite allotransplant recipients have received T-cell depletion or post-transplant bone marrow infusion in an attempt to induce a state of reduced alloreactivity. However, there is no evidence these approaches have worked.

A less alloreactive state has been achieved clinically in organ transplant recipients. However, it may require stronger, more toxic treatment to induce a state of reduced alloreactivity to a composite tissue transplant due to the tendency of skin to reject. It is possible that although this middle ground between high-dose chronic immunosuppression and true tolerance may exist, it may have little benefit, with only slightly less aggressive induction therapy required, but without the advantages of full tolerance.

Even with the achievement of a less alloreactive state in composite tissue allotransplantation, the use of site specific therapy may not be worthwhile. Topical FK506 only achieved prolongation of skin survival by a few days in a swine model; this would have little clinical use.

12.4 FUTURE DIRECTIONS OF RESEARCH

The findings of this thesis give the following direction to further possible research:

12.4.1 Consequences of skin rejection

In this study, the consequences of vascularised skin allograft failure while on immunosuppression were examined in a small animal model. The findings indicate that there is minimal damage to the vasculature and recipient tissues with sub-therapeutic immunosuppression still having a protective effect on recipient tissues.

To gain more clinically relevant information, there are two elements that could be added to the model. Firstly, a large animal model may give a closer approximation of damage to recipient tissues caused by the rejection process. Secondly, to assess fully the effect of allograft rejection on the function following retransplantation, a model is required that allows assessment of motor and sensory recovery. A large animal model would be

most useful as it is not possible to apply all clinical sensori-motor assessment techniques to a small animal. The gastrocnemius musculocutaneous allograft developed in Chapter 8 could be utilised to assess the effect of rejection on the functional outcome following retransplantation.

One other avenue for future research is that rejection of a second CTA transplant may also cause more damage than rejection of the first transplant due to a more intense rejection response caused by the heterologous immunological memory. The increased damage could have a significant impact on the outcome of any further transplants.

12.4.2 Mechanism of skin rejection

In this study, using *in vivo* confocal imaging, direct observations of recipient cell influx and targeting were performed; constituting the first steps in initiation of the immune response.

12.4.2.1 Immune cell targeting

In vivo imaging of antigen presentation, as well as cellular interactions within the allograft, has the potential to further characterise the elusive skin specific antigen(s). However, this would require a more complex model incorporating labelling of specific cell types. At present, there are no transgenic rat models available that would facilitate this.

Alternatives include *in vivo* use of fluorescent labelled antibodies in a rodent model, or transferring to a transgenic mouse model, but both these approaches have limitations. Relevant fluorescent antibodies are available that could be used *in vivo*. However, it would be difficult to obtain global staining of all cells of a particular subtype, particularly over a sustained period. Transgenic mouse models are another option with several available that have differential fluorescent labelling several relevant cell types. These models would make it possible to define the cellular interactions both within the allograft and lymphoid tissues *in vivo*. However, reliable vascularised skin flap transplantation in mice is technically challenging; this has limited the use of mouse models to study vascularised skin allotransplantation.

12.4.2.2 Characterisation of immune cell efflux from allotransplanted skin

The final element in the initiation of the immune response is cellular efflux and antigen presentation in lymphoid tissues. Donor cell efflux was examined via the more indirect method of PCR microchimerism analysis. As in previous studies, the effluxing donor cells were below the level of detection. To obtain a more complete picture of the mechanism of rejection, the efflux of donor and recipient cells from the allograft, precise trafficking patterns to lymphoid tissues, and antigen presentation to T cells all need to be examined.

To image trafficking patterns of donor and recipient cells, simple modifications to the model used in this thesis could be introduced. Efflux of donor cells could be imaged by performance of a vascularised skin allotransplant from a GFP positive rat to a GFP negative rat with *in vivo* imaging of target lymphoid tissues. Likewise, efflux of recipient cells targeting could be followed by injection of recipient type immature GFP labelled dendritic cells at the time of transplantation with *in vivo* imaging of the targeted lymphoid tissues.

12.4.2.3 Design and direction of future experiments examining skin rejection and ways to overcome it

Observations of significant differences in the rejection response to conventional skin allografts and vascularised skin allografts indicate that future CTA research should use vascularised skin allografts.

In addition, the importance of recipient dendritic cells in the immune response to a vascularised skin allograft is supportive of research manipulating recipient dendritic cells to overcome skin rejection. However, as the literature review in Chapter 2 indicates, simple manipulation of dendritic cells has not been successful at achieving skin tolerance in a large animal model. Consequently, it is possible recipient dendritic cell manipulation will only be successful at achieving large animal (and clinical) tolerance as part of a combination therapy regimen.

12.4.3 Possible approaches to induce skin tolerance

Chapter 10 contains the first description of the induction of tolerance to skin transplanted across a MHC barrier in a large animal model. Several steps are required to further develop this finding for clinical application:

12.4.3.1 Confirmation of current findings

In this thesis skin tolerance induction was performed in a small number of animals, with only one animal surviving long-term. The first step would be to repeat the experiment to confirm the findings.

12.4.3.2 Protocol schedule and immune barrier

As previously described, the protocol is not directly clinically applicable because of the time taken to mobilise the donor haematopoietic progenitor cells and the delay between chimerism induction and transplantation. Furthermore, transplantation was only performed across a single haplotype MHC barrier. The modified protocol would need to address these constraints.

12.4.3.3 Protocol medication

Certain medications used in the progenitor cell mobilisation and chimerism induction protocol are not available for clinical use (e.g. stem cell factor and the T-cell depleting agent). There are medications that have similar effects to those in clinical use. However, it is possible that these may significantly change the outcome necessitating the development of more closely correlating medications.

12.4.4 Reduction of immunosuppressant toxicity

Topical FK506 had minimal effect as a monotherapy to prolong skin flap survival in recipients with normal alloreactivity. However, it prolonged survival of skin allografted across a MHC barrier in both recipients with normal, and reduced alloreactivity. This has minimal direct clinical applicability because the effect was only seen in skin grafts, and also survival was only prolonged by a few days. However, it indicates that this general approach may warrant further investigation. There are several possible avenues for future research:

12.4.4.1 Induction of a less alloreactive state

Several methods have been used to induce reduced alloreactive states both in animal models and clinically (as described in Chapter 2). However, the efficacy of many of these techniques has not been tested on composite tissue allotransplants. It would be of particular interest to investigate some of these approaches in a clinically relevant model, particularly the ones that have had some efficacy in clinical organ transplant regimens.

12.4.4.2 Site-specific therapies

Topical FK506 has efficacy compared to other medications in treating immune-mediated skin disorders, with minimal side-effects. For these reasons FK506 was assessed as having the highest likelihood of preventing skin allograft rejection and so was used in this study. However, only marginal prolongation of skin allograft survival was achieved in this study. It is possible that topical FK506 is not as efficacious as other medications in composite tissue allotransplantation, despite its utility in non-transplant scenarios. Topical steroids, cyclosporine, pimecrolimus, and combination therapy are possible alternative site-specific regimens that could be used instead.

Topical steroids have been used for several decades and have a dominant role in immunosuppression for skin disorders. However, long-term use of topical steroids can cause a multitude of complications such as skin atrophy, telangectasia, osteoporosis and diabetes. Furthermore, topical FK506 appears to be more efficacious than hydrocortisone in treating atopic dermatitis (Reitamo, S. et al. 2002).

When introduced, systemic cyclosporine revolutionised immunosuppression in both transplantation, and treatment of inflammatory skin disorders. However, it has low topical activity (Lauerma, A.I. et al. 1994). Pimecrolimus is a macrolide with similar properties to FK506. However, in a head-to-head study topical FK506 was more efficacious at treating atopic dermatitis (Fleischer, A.B. Jr. et al. 2007).

Another alternative is to use several of these therapies in combination to maximize the immunomodulatory effect while avoiding side-effects. A rejection episode in one of the Louisville hand transplant recipients has already been successfully treating by combining topical tacrolimus and steroid treatments.

12.5 CONCLUSION

Many people still suffer defects that cannot be adequately reconstructed using standard techniques. The first successful clinical composite tissue allotransplants are already transforming the lives of their recipients and have demonstrated the potential of these techniques. However, the application of these techniques is likely to remain limited until further progress is made in reducing the risks associated with the technique.

It is possible that there will be other options for reconstruction in the future due to advances in robotics and tissue engineering. However, these are still a long way off. The growing interest generated by these first few cases will intensify research into composite tissue allotransplantation, making immanent achievement of the widespread application of these techniques a real hope. It is likely that the key breakthroughs will come from centres in which both clinical and research CTA activities are taking place.

REFERENCES

Abouljoud, M. S., Deierhoi, M. H., Hudson, S. L., and Diethelm, A. G. (1995). "Risk factors affecting second renal transplant outcome, with special reference to primary allograft nephrectomy" Transplantation 60(2): 138-144.

Achauer, B. M., Hewitt, C. W., Black, K. S., Martinez, S. E., Waxman, K. S., Ott, R. A., and Furnas, D. W. (1986). "Long-term skin allograft survival after short-term cyclosporin treatment in a patient with massive burns" Lancet 1(8471): 14-15.

Adams, A. B., Pearson, T. C., and Larsen, C. P. (2003). "Heterologous immunity: an overlooked barrier to tolerance" Immunol Rev 196147-160.

Asai, O., Longo, D. L., Tian, Z. G., Hornung, R. L., Taub, D. D., Ruscetti, F. W., and Murphy, W. J. (1998). "Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation" J Clin Invest 101(9): 1835-1842.

Austyn, J. M., Kupiec-Weglinski, J. W., Hankins, D. F., and Morris, P. J. (1988). "Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone" J Exp Med 167(2): 646-651.

Barker, C. F. and Billingham, R. E. (1968). "The role of afferent lymphatics in the rejection of skin homografts" J Exp Med 128(1): 197-221.

Baron, F. and Sandmaier, B. M. (2006a). "Chimerism and outcomes after allogeneic haematopoietic cell transplantation following nonmyeloablative conditioning" Leukemia 20(10): 1690-1700.

Baron, F., Sandmaier, B. M., Zellmer, E., Sorror, M., Storer, B., and Storb, R. (2006b). "Failure of donor lymphocyte infusion to prevent graft rejection in dogs given DLA-identical marrow after 1 Gy of total body irradiation" Biol Blood Marrow Transplant 12(8): 813-817.

Bäumer, W., Sülzle, B., Weigt, H., De Vries, V. C., Hecht, M., Tschernig, T., and Kietzmann, M. (2005). "Cilomilast, tacroliomus and rapamycin modulate dendritic cell function in the elicitation phase of allergic contact dermatitis" Br J Dermatol 153(1): 136-44.

Bemelman, F., Honey, K., Adams, E., Cobbold, S., and Waldmann, H. (1998). "Bone marrow transplantation induces either clonal deletion or infectious tolerance depending on the dose" J Immunol 160(6): 2645-2648.

Bergstresser, P. R., Fletcher, C. R., and Streilein, J. W. (1980). "Surface densities of Langerhans cells in relation to rodent epidermal sites with special immunologic properties" J Invest Dermatol 7477-80.

Beriou, G., Peche, H., Guillonneau, C., Merieau, E., and Cuturi, M. C. (2005). "Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression" Transplantation 79(8): 969-972.

Beschorner, W. E., Ren, H., Phillips, J., Pulido, H. B., Jr., Hruban, R. H., and Hess, A. D. (1991). "Prevention of syngeneic graft-versus-host disease by recovery of thymic microenvironment after cyclosporine" Transplantation 52(4): 668-674.

Bhan, A. K., Mihm, M. C., Jr., and Dvorak, H. F. (1982). "T cell subsets in allograft rejection. In situ characterisation of T cell subsets in human skin allografts by the use of monoclonal antibodies" J Immunol 129(4): 1578-1583.

Billiau, A. D., Fevery, S., Rutgeerts, O., Landuyt, W., and Waer, M. (2002). "Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-leukemia responses in mice receiving allogeneic bone marrow transplants" Blood 100(5): 1894-1902.

Billingham, R. E., Brent, L., and Medawar, P. B. (1953). "Actively acquired tolerance to foreign cells." Nature 172603-606.

Blazar, B. R., Lees, C. J., Martin, P. J., Noelle, R. J., Kwon, B., Murphy, W., and Taylor, P. A. (2000). "Host T cells resist graft-versus-host disease mediated by donor leukocyte infusions" J Immunol 165(9): 4901-4909.

Bonilla, W. V., Geuking, M. B., Aichele, P., Ludewig, B., Hengartner, H., and Zinkernagel, R. M. (2006). "Microchimerism maintains deletion of the donor cell-specific CD8 T cell repertoire" J Clin Invest 116(1): 156-162.

Bortin, M. M., Truitt, R. L., Rimm, A. A., and Bach, F. H. (1979). "Graft-versus-leukaemia reactivity induced by alloimmunisation without augmentation of graft-versus-host reactivity" Nature 281490-491.

Bos, J. D., Zonneveld, I., Das, P. K., Krieg, S. R., van der Loos, C. M., and Kapsenberg, M. L. (1987). "The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin" J Invest Dermatol 88(5):569-73.

Boyse, E. A., Carswell, E. A., Scheid, M. P., and Old, L. J. (1973). "Tolerance of Sk-incompatible skin grafts" Nature 244(5416): 441-442.

Boyse, E. A. and Old, L. J. (1968). "Loss of skin allograft tolerance by chimeras" Transplantation 6(4): 619-619.

Buhler, L. H., Spitzer, T. R., Sykes, M., Sachs, D. H., Delmonico, F. L., Tolkoff-Rubin, N., Saidman, S. L., Sackstein, R., McAfee, S., Dey, B., Colby, C., and Cosimi, A. B. (2002). "Induction of kidney allograft tolerance after transient lymphohaematopoietic chimerism in patients with multiple myeloma and end-stage renal disease" Transplantation 74(10): 1405-1409.

Bushell, A., Karim, M., Kingsley, C. I., and Wood, K. J. (2003). "Pretransplant blood transfusion without additional immunotherapy generates CD25+CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect" Transplantation 76(3): 449-455.

Bushell, A., Liddington, M. L., and Morris, P. J. (1995). "Directly vascularised skin grafts are more immunogenic than whole organ allografts: A novel study in a CD4 tolerance model" Abstracts of the 4th Basic Sciences Symposium of the Transplantation Society, Noordwijkerhout 400.

Butler, P. E., Lee, W. P., van de Water, A. P., and Randolph, M. A. (2000). "Neonatal induction of tolerance to skeletal tissue allografts without immunosuppression" Plast Reconstr Surg 105(7): 2424-2430.

Buttemeyer, R., Jones, N. F., Min, Z., and Rao, U. (1996). "Rejection of the component tissues of limb allografts in rats immunosuppressed with FK-506 and cyclosporine" Plast Reconstr Surg 97(1): 139-148.

Calne, R., Friend, P., Moffatt, S., Bradley, A., Hale, G., Firth, J., Bradley, J., Smith, K., and Waldmann, H. (1998). "Prope tolerance, perioperative campath 1H, and low-dose cyclosporin monotherapy in renal allograft recipients" Lancet 351(9117): 1701-1702.

Caproni, M., Torchia, D., Schincaglia, E., Volpi, W., Frezzolini, A., Schena, D., Marzano, A., Quaglino, P., De Simone, C., Parodi, A., Barletta, E., and Fabbri, P. (2006). "Expression of cytokines and chemokines receptors in the cutaneous lesions of erythema multiforme and Stevens-Johnson syndrome/toxic epidermal necrolysis" Br J Dermatol 55(4): 722-8.

Carrel, A. and Guthrie, C. C. (1906). "Complete amputation of the thigh with replantation" American Journal of Medical Sciences 297-301.

Carrier, E., Lee, T. H., Busch, M. P., and Cowan, M. J. (1997). "Recruitment of engrafted donor cells postnatally into the blood with cytokines after in utero transplantation in mice" Transplantation 64(4): 627-633.

Carroll, W. R. and Esclamado, R. M. (2000). "Ischemia/reperfusion injury in microvascular surgery" Head Neck 22(7): 700-713.

Cendales, L. C., Kirk, A. D., Moresi, J. M., Ruiz, P., and Kleiner, D. E. (2006). "Composite tissue allotransplantation: classification of clinical acute skin rejection" Transplantation 81(3): 418-422.

Chen, H. D. and Silvers, W. K. (1983). "Influence of Langerhans cells on the survival of H-Y incompatible skin grafts in rats" J Invest Dermatol 81(1): 20-23.

Chen, Z. K., Cobbold, S. P., Waldmann, H., and Metcalfe, S. (1996). "Amplification of natural regulatory immune mechanisms for transplantation tolerance" Transplantation 62(9): 1200-1206.

Choo, J. K., Seebach, J. D., Nickeleit, V., Shimizu, A., Lei, H., Sachs, D. H., and Madsen, J. C. (1997). "Species differences in the expression of major histocompatibility complex class II antigens on coronary artery endothelium - Implications for cell-mediated xenoreactivity" Transplantation 641315-1322.

Christensen, J. L. and Weissman, I. L. (2001). "Flk-2 is a marker in haematopoietic stem cell differentiation: a simple method to isolate long-term stem cells" Proc Natl Acad Sci U S A 98(25): 14541-14546.

Cina, R. A., Wikiel, K. J., Lee, P. W., Cameron, A. M., Hettiarachy, S., Rowland, H., Goodrich, J., Colby, C., Spitzer, T. R., Neville, D. M., Jr., and Huang, C. A. (2006). "Stable Multilineage Chimerism without Graft versus Host Disease Following Nonmyeloablative Haploidentical Haematopoietic Cell Transplantation" Transplantation 81(12): 1677-1685.

Cobbold, S. P., Martin, G., Qin, S., and Waldmann, H. (1986). "Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance" Nature 323(6084)164-166.

Cober, S. R., Randolph, M. A., and Lee, W. P. (1999). "Skin allograft survival following intrathymic injection of donor bone marrow" J Surg Res 85(2): 204-208.

Cohen, J. L., Trenado, A., Vasey, D., Klatzmann, D., and Salomon, B. L. (2002). "CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft- versus-host disease" J Exp Med 196(3): 401-406.

Crombleholme, T. M., Harrison, M. R., and Zanjani, E. D. (1990). "In utero transplantation of haematopoietic stem cells in sheep: the role of T cells in engraftment and graft-versus-host disease" J Pediatr Surg 25(8): 885-892.

Davies, J. D., Leong, L. Y., Mellor, A., Cobbold, S. P., and Waldmann, H. (1996). "T cell suppression in transplantation tolerance through linked recognition" J Immunol 156(10): 3602-3607.

Demir, Y., Ozmen, S., Klimczak, A., Mukherjee, A. L., and Siemionow, M. Z. (2005). "Strategies to develop chimerism in vascularised skin allografts across MHC barrier" Microsurgery 25(5): 415-422.

Demirci, G., Amanullah, F., Kewalaramani, R., Yagita, H., Strom, T. B., Sayegh, M. H., and Li, X. C. (2004). "Critical role of OX40 in CD28 and CD154-independent rejection" J Immunol 172(3): 1691-1698.

Denham, S., Shimizu, M., Bianchi, A. T., Zwart, R. J., Carr, M. M., and Parkhouse, R. M. (1994). "Monoclonal antibodies recognising differentiation antigens on porcine B cells" Vet Immunol Immunopathol 43(1-3): 259-267.

Dey, B. R., McAfee, S., Colby, C., Sackstein, R., Saidman, S., Tarbell, N., Sachs, D. H., Sykes, M., and Spitzer, T. R. (2003). "Impact of prophylactic donor leukocyte infusions on mixed chimerism, graft-versus-host disease, and antitumor response in patients with advanced haematologic malignancies treated with nonmyeloablative conditioning and allogeneic bone marrow transplantation" Biol Blood Marrow Transplant 9(5): 320-329.

Domenig, C., Sanchez-Fueyo, A., Kurtz, J., Alexopoulos, S. P., Mariat, C., Sykes, M., Strom, T. B., and Zheng, X. X. (2005). "Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol" J Immunol 175(1): 51-60.

Donahue, J., Gilpin, E., Lee, T. H., Busch, M. P., Croft, M., and Carrier, E. (2001). "Microchimerism does not induce tolerance and sustains immunity after in utero transplantation" Transplantation 71(3): 359-368.

Doody, D. P., Stenger, K. S., and Winn, H. J. (1994). "Immunologically nonspecific mechanisms of tissue destruction inX the rejection of skin grafts.X" J Exp Med 1791645-1652.

Douzdjian, V., Rice, J. C., Carson, R. W., Gugliuzza, K. K., and Fish, J. C. (1996). "Renal retransplants: effect of primary allograft nephrectomy on early function, acute rejection and outcome" Clin Transplant 10(2): 203-208.

Dubernard, J. M., Lengelé, B., Morelon, E., Testelin, S., Badet, L., Moure, C., Beziat, J. L., Dakpé, S., Kanitakis, J., D'Hauthuille, C., El Jaafari, A., Petruzzo, P., Lefrancois, N., Taha, F., Sirigu, A., Di Marco, G., Carmi, E,. Bachmann, D., Cremades, S., Giraux, P., Burloux, G., Hequet, O., Parquet, N., Francès, C., Michallet, M., Martin, X., and

Devauchelle, B. (2007). "Outcomes 18 months after the first human partial face transplantation" N Engl J Med 357(24):2451-60.

Durham, M. M., Bingaman, A. W., Adams, A. B., Ha, J., Waitze, S. Y., Pearson, T. C., and Larsen, C. P. (2000). "Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning" J Immunol 165(1): 1-4.

Dvorak, H. F., Mihm, M. C., Jr., Dvorak, A. M., Barnes, B. A., and Galli, S. J. (1980). "The microvasculature is the critical target of the immune response in vascularised skin allograft rejection" J Invest Dermatol 74(5): 280-284.

Dvorak, H. F., Mihm, M. C., Jr., Dvorak, A. M., Barnes, B. A., Manseau, E. J., and Galli, S. J. (1979). "Rejection of first-set skin allografts in man. the microvasculature is the critical target of the immune response" J Exp Med 150(2): 322-337.

Edinger, M., Hoffmann, P., Ermann, J., Drago, K., Fathman, C. G., Strober, S., and Negrin, R. S. (2003). "CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation" Nat Med 9(9): 1144-1150.

Eduardo Bermu, Dez L., Santamaria, A., Romero, T., and Caldero, D. F. (2002). "Experimental model of facial transplant" Plast Reconstr Surg 110(5): 1374-1375.

Eljaafari, A., Badet, L., Kanitakis, J., Ferrand, C., Farre, A., Petruzzo, P., Morelon, E., Dubosson, M., Tiberghien, P., Dubois, V., Martin, X., Miossec, P., and Dubernard, J. M. (2006). "Isolation of regulatory T cells in the skin of a human hand-allograft, up to six years posttransplantation" Transplantation 82(12): 1764-1768.

Elster, E. A., Xu, H., Tadaki, D. K., Burkly, L. C., Berning, J. D., Baumgartner, R. E., Cruzata, F., Patterson, N. B., Harlan, D. M., and Kirk, A. D. (2001a). "Primate skin allotransplantation with anti-CD154 monotherapy" Transplant Proc 33(1-2): 675-676.

Elster, E. A., Xu, H., Tadaki, D. K., Montgomery, S., Burkly, L. C., Berning, J. D., Baumgartner, R. E., Cruzata, F., Marx, R., Harlan, D. M., and Kirk, A. D. (2001b). "Treatment with the humanized CD154-specific monoclonal antibody, hu5C8, prevents acute rejection of primary skin allografts in nonhuman primates" Transplantation 72(9): 1473-1478.

Elwood, E. T., Larsen, C. P., Maurer, M. H., Routenberg, K. L., Neylan, N. F., Whelchel, J. D., O'Brien, D. P., and Pearson, T. C. (1997). "Microchimerism and rejection in clinical transplantation" Lancet 3491358-1360.

Emmanouilidis, N., Guo, Z., Dong, Y., Newton-West, M., Adams, A. B., Lee, E. D., Wang, J., Pearson, T. C., Larsen, C. P., and Newell, K. A. (2006). "Immunosuppressive and trafficking properties of donor splenic and bone marrow dendritic cells" Transplantation 81(3): 455-462.

Fehr, T. and Sykes, M. (2004). "Tolerance induction in clinical transplantation" Transpl Immunol 13(2): 117-130.

Ferrara, J. L., Levy, R., and Chao, N. J. (1999). "Pathophysiologic mechanisms of acute graft-vs.-host disease" Biol Blood Marrow Transplant 5(6): 347-356.

Fiorina, P., Jurewicz, M., Tanaka, K., Behazin, N., Augello, A., Vergani, A., von Andrian, U. H., Smith, N. R., Sayegh, M. H., and Abdi, R. (2007). "Characterisation of donor dendritic cells and enhancement of dendritic cell efflux with CC-chemokine ligand 21: a novel strategy to prolong islet allograft survival" Diabetes 56(4): 912-920.

Fleischer, A.B. Jr., Abramovits, W., Breneman, D., and Jaracz, E. (2007). "US/Canada tacrolimus ointment study group.Tacrolimus ointment is more effective than pimecrolimus cream in adult patients with moderate to very severe atopic dermatitis" J Dermatolog Treat 18(3):151-7.

Forbes, R. D., Gomersall, M., Darden, A. G., and Guttmann, R. D. (1991). "Multiple patterns of MHC class II antigen expression on cellular constituents of rat heart grafts. Lack of correlation with graft survival, but strong correlation with vasculitis" Transplantation 51(5): 942-948.

Fossum, S. (1988). "Lymph-borne dendritic leucocytes do not recirculate, but enter the lymph node paracortex to become interdigitating cells" Scand J Immunol 27(1): 97-105.

Frame, J. D., Sanders, R., Goodacre, T. E., and Morgan, B. D. (1989). "The fate of meshed allograft skin in burned patients using cyclosporin immunosuppression" Br J Plast Surg 42(1): 27-34.

Fuchimoto, Y., Gleit, Z. L., Huang, C. A., Kitamura, H., Schwarze, M. L., Menard, M. T., Mawulawde, K., Madsen, J. C., and Sachs, D. H. (2001). "Skin-specific alloantigens in miniature swine" Transplantation 72(1): 122-126.

Fuchimoto, Y., Huang, C. A., Shimizu, A., Seebach, J., Arn, J. S., and Sachs, D. H. (1999a). "An allelic non-histocompatibility antigen with wide tissue distribution as a marker for chimerism in pigs" Tissue Antigens 54(1): 43-52.

Fuchimoto, Y., Huang, C. A., Yamada, K., Shimizu, A., Kitamura, H., Colvin, R. B., Ferrara, V, Murphy, M. C., Sykes, M., White-Scharf, M., Neville, D. M., Jr., and Sachs, D. H. (2000). "Mixed chimerism and tolerance without whole body irradiation in a large animal model" J Clin Invest 105(12): 1779-1789.

Fuchimoto, Y., Yamada, K., Shimizu, A., Yasumoto, A., Sawada, T., Huang, C. A., and Sachs, D. H. (1999b). "Relationship between chimerism and tolerance in a kidney transplantation model" J Immunol 1625704-5711.

Fujita, T., Takahashi, S., Yagihashi, A., Jimbow, K., and Sato, N. (1997). "Prolonged survival of rat skin allograft by treatment with FK506 ointment" Transplantation 64(6): 922-925.

Fukuda, T., Kerbauy, F. R., Gooley, T., Santos, E. B., Storb, R., and Sandmaier, B. M. (2006). "Dog leukocyte antigen-haploidentical stem cell allografts after anti-CD44 therapy and nonmyeloablative conditioning in a preclinical canine model" Transplantation 82(3): 332-339.

Garrovillo, M., Ali, A., DePaz, H. A., Gopinathan, R., Oluwole, O. O., Hardy, M. A., and Oluwole, S. F. (2001). "Induction of transplant tolerance with immunodominant allopeptide-pulsed host lymphoid and myeloid dendritic cells" Am J Transplant 1(2): 129-137.

Georges, G. E., Storb, R., Thompson, J. D., Yu, C., Gooley, T., Bruno, B., and Nash, R. A. (2000). "Adoptive immunotherapy in canine mixed chimeras after nonmyeloablative haematopoietic cell transplantation" Blood 95(10): 3262-3269.

Gibson, T. and Medawar, P. B. (1943). "The fate of skin homografts in man" J Anat 77(Pt 4): 299-310.

Gleit, Z. L., Cameron, A. M., Fuchimoto, Y., Melendy, E., Monajati, L., Coburn, R. C., Sachs, D. H., and Huang, C. A. (2002a). "Persistent Chimerism Despite Anti-Donor MHC *In Vitro* Responses In Miniature Swine Following Allogeneic Haematopoietic Cell Transplantation" Transplantation 74(9): 1260-1266.

Gleit, Z. L., Fuchimoto, Y., Yamada, K., Melendy, E., Scheier-Dolberg, R., Monajati, L., Coburn, R. C., Neville, D. M. Jr., Sachs, D. H., and Huang, C. A. (2002b). "Variable relationship between chimerism and tolerance following haematopoietic cell transplantation without myelosuppressive conditioning in miniature swine" Transplantation 74(11): 1535-1544.

Glucksberg, H., Storb, R., Fefer, A., Buckner, C. D., Neiman, P. E., Clift, R. A., Lerner, K. G., and Thomas, E. D. (1974). "Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors" Transplantation 18(4): 295-304.

Gohda, T., Iwasaki, N., Yoshioka, C., and Minami, A. (2003). "Effect of single-dose administration of FK-506 on allogeneic limb transplantation in rats: is there an optimal timing of its administration?" Plast Reconstr Surg 111(5): 1653-1658.

Goss, J. A., Nakafusa, Y., and Flye, M. W. (1993). "Prolongation of small bowel allografts after intrathymic injection of donor alloantigen and ALS" J Surg Res 54(5): 494-498.

Grazia, T. J., Pietra, B. A., Johnson, Z. A., Kelly, B. P., Plenter, R. J., and Gill, R. G. (2004). "A two-step model of acute CD4 T cell mediated cardiac allograft rejection" J Immunol 172(12): 7451-7458.

Habicht, A., Najafian, N., Yagita, H., Sayegh, M. H., and Clarkson, M. R. (2007). "New Insights in CD28-Independent Allograft Rejection" Am J Transplant 7(8): 1917-1926.

Hayashi, S., Peranteau, W. H., Shaaban, A. F., and Flake, A. W. (2002). "Complete allogeneic haematopoietic chimerism achieved by a combined strategy of in utero haematopoietic stem cell transplantation and postnatal donor lymphocyte infusion" Blood 100(3): 804-812.

He, C., Schenk, S., Zhang, Q., Valujskikh, A., Bayer, J., Fairchild, R. L., and Heeger, P. S. (2004). "Effects of T cell frequency and graft size on transplant outcome in mice" J Immunol 172(1): 240-247.

Hedrick, M. H., Rice, H. E., MacGillivray, T. E., Bealer, J. F., Zanjani, E. D., and Flake, A. W. (1994). "Haematopoietic chimerism achieved by in utero haematopoietic stem cell injection does not induce donor-specific tolerance for renal allografts in sheep" Transplantation 58(1): 110-111.

Hettiaratchy, S., Melendy, E., Randolph, M. A., Coburn, R. C., Neville, D. M., Jr., Sachs, D. H., Huang, C. A., and Lee, W. P. (2004). "Tolerance to composite tissue

allografts across a major histocompatibility barrier in miniature swine" Transplantation 77(4): 514-521.

Hoffman, D. K., Sibley, R. K., Korman, J. M., and Press, B. H. (1994). "Light microscopic and immunohistochemical features in serial biopsies of epidermal versus dermal allografts" Ann Plast Surg 33(3): 295-299.

Hoffmann, P., Ermann, J., Edinger, M., Fathman, C. G., and Strober, S. (2002). "Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft- versus-host disease after allogeneic bone marrow transplantation" J Exp Med 196(3): 389-399.

Horner, B. M., Cina, R. A., Wikiel, K. J., Lima, B., Ghazi, A., Lo, D. P., Yamada, K., Sachs, D. H., and Huang, C. A. (2006). "Predictors of organ allograft tolerance following haematopoietic cell transplantation" Am J Transplant 6(12): 2894-2902.

Horowitz, M. M., Gale, R. P., Sondel, P. M., Goldman, J. M., Kersey, J., Kolb, H.-J., Rimm, A. A., Ringdén, O., Rozman, C., Speck, B., Truitt, R. L., Zwaan, F. E., and Bortin, M. M. (1990). "Graft-versus-leukemia reactions after bone marrow transplantation" Blood 75555-562.

Houser, S. L., Benjamin, L. C., Wain, J. C., Madsen, J. C., and Allan, J. S. (2004). "Constitutive expression of major histocompatibility complex class II antigens in pulmonary epithelium and endothelium varies among different species" Transplantation 77(4): 605-607.

Hua, J. and En-tan, G. (1996). "Effect of postoperative treatment with a combination of chuangxiong and electret on functional recovery of muscle grafts: an experimental study in the dog" Plast Reconstr Surg 98(5): 851-855.

Huang, C. A., Fuchimoto, Y., Scheier-Dolberg, R., Murphy, M. C., Neville, D. M., Jr., and Sachs, D. H. (2000). "Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model" J Clin Invest 105(2): 173-181.

Huang, C. A., Lorf, T., Arn, J. S., Koo, G. C., Blake, T., and Sachs, D. H. (1999a). "Characterisation of a monoclonal anti-porcine CD3 antibody" Xenotransplant 5201-212.

Huang, C. A., Yamada, K., Murphy, M. C., Shimizu, A., Colvin, R. B., Neville, D. M., Jr., and Sachs, D. H. (1999b). "*In vivo* T-cell depletion in miniature swine using the swine CD3 immunotoxin, pCD3-CRM9" Transplantation 68(6): 855-860.

Illigens, B. M., Yamada, A., Fedoseyeva, E. V., Anosova, N., Boisgerault, F., Valujskikh, A., Heeger, P. S., Sayegh, M. H., Boehm, B., and Benichou, G. (2002). "The relative contribution of direct and indirect antigen recognition pathways to the alloresponse and graft rejection depends upon the nature of the transplant" Hum Immunol 63(10): 912-925.

Inceoglu, S., Siemionow, M., Chick, L., Craven, C. M., and Lister, G. D. (1994). "The effect of combined immunosuppression with systemic low-dose cyclosporin and topical fluocinolone acetonide on the survival of rat hind-limb allografts" Ann Plast Surg 33(1): 57-65.

Jarvinen, L. Z., Blazar, B. R., Adeyi, O. A., Strom, T. B., and Noelle, R. J. (2003). "CD154 on the surface of CD4+CD25+ regulatory T cells contributes to skin transplant tolerance" Transplantation 76(9): 1375-1379.

Jiang, H. Q., Wang, Y., Hu, X. B., Li, Y. S., and Li, J. S. (2005). "Composite tissue allograft transplantation of cephalocervical skin flap and two ears" Plast Reconstr Surg 115(3): 31e-35e.

Johnson, B. D., Becker, E. E., LaBelle, J. L., and Truitt, R. L. (1999). "Role of immunoregulatory donor T cells in suppression of graft-versus-host disease following donor leukocyte infusion therapy" J Immunol 163(12): 6479-6487.

Jones, N. D., Turvey, S. E., Van Maurik, A., Hara, M., Kingsley, C. I., Smith, C. H., Mellor, A. L., Morris, P. J., and Wood, K. J. (2001). "Differential susceptibility of heart, skin, and islet allografts to T cell-mediated rejection" J Immunol 166(4): 2824-2830.

Jonker, M., van den, Hout Y., Noort, R. C., Versteeg-van der Voort Maarschalk MF, Claas, F. H., Woude, F. J., Hollander, D., Perico, N., and Remuzzi, G. (2001). "Immunomodulation by intrathymic injection of donor leukocytes in rhesus monkeys" Transplantation 72(8): 1432-1436.

Jorgensen, J. L., Esser, U., Fazekas de St, Groth B., Reay, P. A., and Davis, M. M. (1992). "Mapping T cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics" Nature 355(6357): 224-230.

Kanitakis, J., Badet, L., Petruzzo, P., Beziat, J. L., Morelon, E., Lefrancois, N., Frances, C., Claudy, A., Martin, X., Lengele, B., Testelin, S., Devauchelle, B., and Dubernard, J. M. (2006). "Clinicopathologic monitoring of the skin and oral mucosa of the first human face allograft: Report on the first eight months" Transplantation 82(12): 1610-1615.

Kanitakis, J., Jullien, D., Petruzzo, P., Hakim, N., Claudy, A., Revillard, J. P., Owen, E., and Dubernard, J. M. (2003). "Clinicopathologic features of graft rejection of the first human hand allograft" Transplantation 76(4): 688-693.

Kawai, T., Andrews, D., Colvin, R. B., Sachs, D. H., and Cosimi, A. B. (2000). "Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand" Nat Med 6(2): 114.

Kim, H. B., Shaaban, A. F., Yang, E. Y., Liechty, K. W., and Flake, A. W. (1998). "Microchimerism and tolerance after in utero bone marrow transplantation in mice" J Surg Res 77(1): 1-5.

Kirkman, R. L., Colvin, M. W., Flye, G. S., Leight, S. A., Rosenberg, S. A., Williams, G. M., and Sachs, D. H. (1979). "Transplantation in miniature swine. VI. Factors influencing survival of renal allografts" Transplantation 2818-23.

Klimczak, A., Agaoglu, G., Carnevale, K. A., and Siemionow, M. (2006). "Applications of bilateral vascularised femoral bone marrow transplantation for chimerism induction across the major histocompatibility (MHC) barrier: part II" Ann Plast Surg 57(4): 422-430.

Koenen, H. J., Fasse, E., and Joosten, I. (2005). "CD27/CFSE-based ex vivo selection of highly suppressive alloantigen-specific human regulatory T cells" J Immunol 174(12): 7573-7583.

Koyama, I., Nadazdin, O., Boskovic, S., Ochiai, T., Smith, R. N., Sykes, M., Sogawa, H., Murakami, T., Strom, T. B., Colvin, R. B., Sachs, D. H., Benichou, G., Cosimi, A. B., and Kawai, T. (2007). "Depletion of CD8 memory T cells for induction of tolerance of a previously transplanted kidney allograft" Am J Transplant 7(5): 1055-1061.

Kreisel, D., Krupnick, A. S., Gelman, A. E., Engels, F. H., Popma, S. H., Krasinskas, A. M., Balsara, K. R., Szeto, W. Y., Turka, L. A., and Rosengard, B. R. (2002). "Non-haematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition" Nat Med 8(3): 233-239.

Kreisel, D., Krupnick, A. S., Szeto, W. Y., Popma, S. H., Sankaran, D., Krasinskas, A. M., Amin, K. M., and Rosengard, B. R. (2001). "A simple method for culturing mouse vascular endothelium" J Immunol Methods 254(1-2): 31-45.

Krupnick, A. S., Gelman, A. E., Barchet, W., Richardson, S., Kreisel, F. H., Turka, L. A., Colonna, M., Patterson, G. A., and Kreisel, D. (2005). "Murine vascular endothelium activates and induces the generation of allogeneic CD4+25+Foxp3+ regulatory T cells" J Immunol 175(10): 6265-6270.

Kunisaki, S. M., Haller, G. W., Fuchimoto, Y., Huang, C. A., and Sachs, D. H. (2001). "Peripheral regulation of graft-versus-host alloreactivity in mixed chimeric miniature swine" Transplantation 72(3): 523-526.

Kuntscher, M. V., Schirmbeck, E. U., Menke, H., Klar, E., Gebhard, M. M., and Germann, G. (2002). "Ischemic preconditioning by brief extremity ischemia before flap ischemia in a rat model" Plast Reconstr Surg 109(7): 2398-2404.

Kurtz, J., Wekerle, T., and Sykes, M. (2004). "Tolerance in mixed chimerism - a role for regulatory cells?" Trends Immunol 25(10): 518-523.

Lanzetta, M., Petruzzo, P., Dubernard, J. M., Margreiter, R., Schuind, F., Breidenbach, W., Nolli, R., Schneeberger, S., van Holder, C., Gorantla, V. S., Pei, G., Zhao, J., and Zhang, X. (2007). "Second report (1998-2006) of the international registry of hand and composite tissue transplantation" Transpl Immunol 18(1): 1-6.

Lanzetta, M., Petruzzo, P., Margreiter, R., Dubernard, J. M., Schuind, F., Breidenbach, W., Lucchina, S., Schneeberger, S., van Holder, C., Granger, D., Pei, G., Zhao, J., and Zhang, X. (2005). "The International Registry on Hand and Composite Tissue Transplantation" Transplantation 79(9): 1210-1214.

Larsen, C. P., Barker, H., Morris, P. J., and Austyn, J. M. (1990a). "Failure of mature dendritic cells of the host to migrate from the blood into cardiac or skin allografts" Transplantation 50294-301.

Larsen, C. P., Elwood, E. T., Alexander, D. Z., Ritchie, S. C., Hendrix, R., Tucker-Burden, C., Cho, H. R., Aruffo, A., Hollenbaugh, D., Linsley, P. S., Winn, K. J., and Pearson, T. C. (1996). "Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways" Nature 381(6581): 434-438.

Larsen, C. P., Steinman, R. M., Witmer-Pack, M., Hankins, D. F., Morris, P. J., and Austyn, J. M. (1990b). "Migration and maturation of Langerhans cells in skin transplants and explants" J Exp Med 1721483-1493.

Lauerma, A.I. and Maibach, H.I. (1994). "Topical FK506 clincial potential or laboratory curiosity?" Dermatol 188:173-6.

Lee, P. W., Cina, R. A., Randolph, M. A., Arellano, R., Goodrich, J., Rowland, H., Huang, C. A., Sachs, D. H., and Kim, H. B. (2005a). "In utero bone marrow transplantation induces kidney allograft tolerance across a full major histocompatibility complex barrier in Swine" Transplantation 79(9): 1084-1090.

Lee, P. W., Cina, R. A., Randolph, M. A., Goodrich, J., Rowland, H., Arellano, R., Kim, H. B., Sachs, D. H., and Huang, C. A. (2005b). "Stable multilineage chimerism across full MHC barriers without graft-versus-host disease following in utero bone marrow transplantation in pigs" Exp Haematol 33(3): 371-379.

Lee, W. P., Rubin, J. P., Bourget, J. L., Cober, S. R., Randolph, M. A., Nielsen, G. P., Ierino, F. L., and Sachs, D. H. (2001). "Tolerance to limb tissue allografts between swine matched for major histocompatibility complex antigens" Plast Reconstr Surg 107(6): 1482-1490.

Lee, W. P., Rubin, J. P., Cober, S., Ierino, F., Randolph, M. A., and Sachs, D. H. (1998). "Use of swine model in transplantation of vascularised skeletal tissue allografts" Transplant Proc 30(6): 2743-2745.

Lee, W. P., Yaremchuk, M. J., Pan, Y. C., Randolph, M. A., Tan, C. M., and Weiland, A. J. (1991). "Relative antigenicity of components of a vascularised limb allograft" Plast Reconstr Surg 87(3): 401-411.

Legault, C. (1985). "Selection of breeds, strains and individual pigs for prolificacy" J Reprod Fertil Suppl 33151-166.

LeGuern, C. (2003). "Regulation of T cell functions by MHC class II self-presentation" Trends Immunol. 24(12):633-8.

Levi, D. M., Tzakis, A. G., Kato, T., Madariaga, J., Mittal, N. K., Nery, J., Nishida, S., and Ruiz, P. (2003). "Transplantation of the abdominal wall" Lancet 361(9376): 2173-2176.

Lima, B., Gleit, Z. L., Cameron, A. M., Germana, S., Murphy, M. C., Consorti, R., Chang, Q., Down, J. D., LeGuern, C., Sachs, D. H., and Huang, C. A. (2003). "Engraftment of quiescent progenitors and conversion to full chimerism following nonmyelosuppressive conditioning and haematopoietic cell transplantation in miniature swine" Biol Blood Marrow Transplant 9(9): 571-582.

Lipson, R. A., Halloran, P. F., Kawano, H., and Langer, F. (1981). "A microsurgical model for vascularised bone and joint transplants in rats" Transplant Proc 13(1 Pt 2): 891-892.

Madsen, J. C., Sachs, D. H., Fallon, J. T., and Weissman, N. J. (1996). "Cardiac allograft vasculopathy in partially inbred miniature swine. I. Time course, pathology, and dependence on immune mechanisms" J Thorac Cardiovasc Surg 111(6): 1230-1239.

Magee, J. C., Barr, M. L., Basadonna, G. P., Johnson, M. R., Mahadevan, S., McBride, M. A., Schaubel, D. E., and Leichtman, A. B. (2007). "Repeat organ transplantation in the United States, 1996-2005" Am J Transplant 7(5 Pt 2): 1424-1433.

Main, J. M. and Prehn, R. T. (1955). "Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow" J Natl Cancer Inst 15(4): 1023-1029.

Markees, T. G., Phillips, N. E., Gordon, E. J., Noelle, R. J., Maliszewski, C., Mordes, J. P., Greiner, D. L., and Rossini, A. A. (1999). "Prolonged skin allograft survival in mice treated with Flt3-ligand-induced dendritic cells and anti-CD154 monoclonal antibody" Transplant Proc 31(1-2): 884-885.

Mathes, D. W., Randolph, M. A., Bourget, J. L., Nielsen, G. P., Ferrera, V. R., Arn, J. S., Sachs, D. H., and Lee, W. P. (2002). "Recipient bone marrow engraftment in donor tissue after long-term tolerance to a composite tissue allograft" Transplantation 73(12): 1880-1885.

Mathes, D. W., Randolph, M. A., Solari, M. G., Nazzal, J. A., Nielsen, G. P., Arn, J. S., Sachs, D. H., and Lee, W. P. (2003). "Split tolerance to a composite tissue allograft in a swine model" Transplantation 75(1): 25-31.

Mathes, D. W., Solari, M. G., Randolph, M. A., Gazelle, G. S., Yamada, K., Huang, C. A., Sachs, D. H., and Lee, W. P. (2005). "Long-term acceptance of renal allografts following prenatal inoculation with adult bone marrow" Transplantation 80(9): 1300-1308.

Mathes, D. W., Yamada, K., Randolph, M. A., Utsugi, R., Solari, M. G., Gazelle, G. S., Wu, A., Sachs, D. H., and Lee, W. P. (2001). "In utero induction of transplantation tolerance" Transplant Proc 33(1-2): 98-100.

Mathieson, B. J., Flaherty, L., Bennett, D., and Boyse, E. A. (1975). "Differences in the rejection of trunk skin and tail skin allografts involving weak histocompatibility loci" Transplantation 19(6): 525-527.

Medawar, P. B. (1944). "The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council" J Anat 78(Pt 5): 176-199.

Medawar, P. B. (1965). "Transplantation of Tissues & Organs: Introduction" Br Med J 2197.

Mezrich, J. D., Haller, G. W., Arn, J. S., Houser, S. L., Madsen, J. C., and Sachs, D. H. (2003). "Histocompatible miniature swine: an inbred large-animal model" Transplantation 75(6): 904-907.

Mijal, J., Galkowska, H., and Olszewski, W. L. (2002). "Formation of synapses between dendritic cells and lymphocytes in skin lymph in an allogeneic reaction" Annals of Transplantation 7(4): 36-41.

Morris, P., Bradley, A., Doyal, L., Earley, M., Hagen, P., Milling, M., and Rumsey, N. (2007). "Face transplantation: a review of the technical, immunological, psychological and clinical issues with recommendations for good practice" Transplantation 83(2): 109-128.

Moser, M. (2003). "Dendritic cells in immunity and tolerance-do they display opposite functions?" Immunity 19(1): 5-8.

Munzenberger, P. J., and Montejo, J. M. (2007). "Safety of topical calcineurin inhi bitors for the treatment of atopic dermatitis" Pharmacotherapy. 27(7): 1020-8.

Mychaliska, G. B., Rice, H. E., Tarantal, A. F., Stock, P. G., Capper, J., Garovoy, M. R., Olson, J. L., Cowan, M. J., and Harrison, M. R. (1997). "In utero haematopoietic stem cell transplants prolong survival of postnatal kidney transplantation in monkeys" J Pediatr Surg 32(7): 976-981.

Nguyen, V. T., Taieb, A., Sacks, J. M., Unadkat, J. V., Clavijo, J. A., Kim, H., Feili-Hariri, M., and Lee, W. P. (2007). "Allopeptide-pulsed dendritic cells and composite tissue allograft survival" Microsurgery 27(2): 105-111.

Nicolini, F. E., Wattel, E., Michallet, A. S., Bourgeot, J. P., Tremisi, J. P., Hequet, O., and Michallet, M. (2004). "Long-term persistent lymphopenia in haematopoietic stem cell donors after donation for donor lymphocyte infusion" Exp Haematol 32(11): 1033-1039.

Nishikawa, H., Manek, S., and Green, C. J. (1991). "The oblique rat groin flap" Br J Plast Surg 44(4): 295-298.

Ochando, J. C., Krieger, N. R., and Bromberg, J. S. (2006). "Direct versus indirect allorecognition: Visualization of dendritic cell distribution and interactions during rejection and tolerization" Am J Transplant 6(10): 2488-2496.

Owen, R. D. (1945). "Immunogenetic consequences of vascular anastomoses between bovine twins" Science 102400-401.

Ozer, K., Oke, R., Gurunluoglu, R., Zielinski, M., Izycki, D., Prajapati, R., and Siemionow, M. (2003). "Induction of tolerance to hind limb allografts in rats receiving cyclosporine A and antilymphocyte serum: effect of duration of the treatment" Transplantation 75(1): 31-36.

Ozmen, S., Ulusal, B. G., Ulusal, A. E., Izycki, D., Yoder, B., and Siemionow, M. (2006). "Trafficking of donor-derived bone marrow correlates with chimerism and extension of composite allograft survival across MHC barrier" Transplant Proc 38(5): 1625-1633.

Pallavicini, M. G., Flake, A. W., Madden, D., Bethel, C., Duncan, B., Gonzalgo, M. L., Haendel, S., Montoya, T., and Roberts, L. (1992). "Hemopoietic chimerism in rodents transplanted in utero with fetal human hemopoietic cells" Transplant Proc 24(2): 542-543.

Panhans-Gross A, Novak N, Kraft S, and Bieber T. (2001) "Human epidermal Langerhans' cells are targets for the immunosuppressive macrolide tacrolimus (FK506)" J Allergy Clin Immunol.107(2):345-52.

Pelot, M.R., Pearson, D.A., Swenson, K., Zhao, G., Sachs, J., Yang, Y.G., and Sykes, M. (1999) "Lymphohaematopoietic graft-vs.-host reactions can be induced without graft-vs.-host disease in murine mixed chimeras established with a cyclophosphamide-based nonmyeloablative conditioning regimen" Biol Blood Marrow Transplant. 5(3):133-43.

Perloff, L. J. and Barker, C. F. (1979). "The immediately vascularised skin allograft" Transplantation 28(2): 125-130.

Pescovitz, M. D., Book, B. K., Aasted, B., Dominguez, J., Bullido, R., Trebichavsky, I., Novikov, B., Valpotic, I., Tomaskovic, M., Nielsen, J., Arn, S., Sachs, D. H., Lunney, J. K., Boyd, P. C., Walker, J., Lee, R., and Saalmuller, A. (1998). "Analyses of monoclonal antibodies reacting with porcine CD5: results from the Second International Swine CD Workshop" Vet Immunol Immunopathol 60(3-4): 269-273.

Pescovitz, M. D., Lunney, J. K., and Sachs, D. H. (1984). "Preparation and characterisation of monoclonal antibodies reactive with porcine PBL" J Immunol 133368-375.

Pescovitz, M. D., Lunney, J. K., and Sachs, D. H. (1985). "Murine anti-swine T4 and T8 monoclonal antibodies: distribution and effects on proliferative and cytotoxic T cells" J Immunol 13437-44.

Petit, F., Minns, A. B., Nazzal, J. A., Hettiaratchy, S. P., Lantieri, L. A., Randolph, M. A., and Lee, W. P. (2004). "Prolongation of skin allograft survival after neonatal injection of donor bone marrow and epidermal cells" Plast Reconstr Surg 113(1): 270-276.

Ploemacher, R. E., van der Sluijs, J. P., Voerman, J. S., and Brons, N. H. (1989). "An in vitro limiting-dilution assay of long-term repopulating haematopoietic stem cells in the mouse" Blood 74(8): 2755-2763.

Pober, J. S., Bothwell, A. L., Lorber, M. I., McNiff, J. M., Schechner, J. S., and Tellides, G. (2003). "Immunopathology of human T cell responses to skin, artery and endothelial cell grafts in the human peripheral blood lymphocyte/severe combined immunodeficient mouse" Springer Semin Immunopathol 25(2): 167-180.

Pober, J. S., Kluger, M. S., and Schechner, J. S. (2001). "Human endothelial cell presentation of antigen and the homing of memory/effector T cells to skin" Ann N Y Acad Sci 94112-25.

Poggio, E. D., Hila, S., Stephany, B., Fatica, R., Krishnamurthi, V., del Bosque, C., Goldfarb, D., Herts, B., Dennis, V. W., Heeger, P. S., and Braun, W. (2006). "Donor kidney volume and outcomes following live donor kidney transplantation" Am J Transplant 6(3): 616-624.

Preville, X., Flacher, M., LeMauff, B., Beauchard, S., Davelu, P., Tiollier, J., and Revillard, J. P. (2001). "Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model" Transplantation 71(3): 460-468.

Prior, C. R., Coghlan, P. J., Hall, J. M., and Jacobs, P. (1991). "In vitro study of immunologic changes in long-term cytapheresis donors" J Clin Apher 6(2): 69-76.

Prop, J., Nieuwenhuis, P., and Wildevuur, C. R. (1985). "Lung allograft rejection in the rat. I. Accelerated rejection caused by graft lymphocytes" Transplantation 40(1): 25-30.

Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J., and Waldmann, H. (1993). ""Infectious" transplantation tolerance" Science 259974-977.

Qin, S. X., Cobbold, S., Benjamin, R., and Waldmann, H. (1989). "Induction of classical transplantation tolerance in the adult" J Exp Med 169779-794.

Qin, S. X., Wise, M., Cobbold, S. P., Leong, L., Kong, Y. C., Parnes, J. R., and Waldmann, H. (1990). "Induction of tolerance in peripheral T cells with monoclonal antibodies" Eur J Immunol 20(12): 2737-2745.

Reitamo, S., Van Leent, E.J., Ho, V., Harper, J., Ruzicka, T., Kalimo, K., Cambazard, F., Rustin, M., Taïeb, A., Gratton, D., Sauder, D., Sharpe, G., Smith, C., Jünger, M., de Prost, Y., and European /Canadian Tacrolimus Ointment Study Group (2002). "Efficacy and safety of tacrolimus oitment compared with that of hydrocortisone acetate ointment oin children with atopic dermatitis" J Allergy Clin Immunol 109(3): 539-46.

Rice, H. E., Hedrick, M. H., and Flake, A. W. (1994). "In utero transplantation of rat haematopoietic stem cells induces xenogeneic chimerism in mice" Transplant Proc 26(1): 126-128.

Ricordi, C., Karatzas, T., Nery, J., Webb, M., Selvaggi, G., Fernandez, L., Khan, F. A., Ruiz, P., Schiff, E., Olson, L., Fernandez, H., Bean, J., Esquenazi, V., Miller, J., and Tzakis, A. G. (1997). "High-dose donor bone marrow infusions to enhance allograft survival - The effect of timing" Transplantation 637-11.

Roussey-Kesler, G., Giral, M., Moreau, A., Subra, J. F., Legendre, C., Noel, C., Pillebout, E., Brouard, S., and Soulillou, J. P. (2006). "Clinical operational tolerance after kidney transplantation" Am J Transplant 6(4): 736-746.

Rubin, J. P., Cober, S. R., Butler, P. E., Randolph, M. A., Gazelle, G. S., Ierino, F. L., Sachs, D. H., and Lee, W. P. (2001). "Injection of allogeneic bone marrow cells into the portal vein of swine in utero" J Surg Res 95(2): 188-194.

Saalmuller, A., Aasted, B., Canals, A., Dominguez, J., Goldman, T., Lunney, J. K., Maurer, S., Pescovitz, M. D., Pospisil, R., and Salmon, H. (1994). "Analyses of mAb reactive with porcine CD8" Vet Immunol Immunopathol 43249-254.

Sachs, D. H. (1992). "MHC Homozygous Miniature Swine". In: Swindle, M. M., Moody, D. C., and Phillips, L. D., eds. Swine as Models in Biomedical Research. 1 ed. Ames, Iowa: Iowa State University Press. 3-15.

Sachs, D. H., Leight, G., Cone, J., Schwartz, S., Stuart, L., and Rosenberg, S. (1976). "Transplantation in miniature swine. I. Fixation of the major histocompatibility complex" Transplantation 22559-567.

Saiki, T., Ezaki, T., Ogawa, M., and Matsuno, K. (2001). "Trafficking of host- and donor-derived dendritic cells in rat cardiac transplantation: allosensitisation in the spleen and hepatic nodes" Transplantation 71(12): 1806-1815.

Sanchez-Fructuoso, A. I., Prats, D., Marques, M., Perez-Contin, M. J., Fernandez-Perez, C., Contreras, E., Blanco, J., and Barrientos, A. (2001). "Does renal mass exert an independent effect on the determinants of antigen-dependent injury?" Transplantation 71(3): 381-386.

Sayegh, M. H., Fine, N. A., Smith, J. L., Rennke, H. G., Milford, E. L., and Tilney, N. L. (1991). "Immunologic tolerance to renal allografts after bone marrow transplants from the same donors" Ann Intern Med 114954-955.

Scheid, M, Boyse, E. A., Carswell, E. A., and Old, L. J. (1972). "Serologically demonstrable alloantigens of mouse epidermal cells." J Exp Med 135(4): 938-955.

Sena, J., Wachtel, S. S., and Murphy, G. (1976). "A comparison of the survival of H-Y incompatible ear, tail, and body skin grafts" Transplantation 21(5): 412-416.

Seung, E., Mordes, J. P., Rossini, A. A., and Greiner, D. L. (2003). "Haematopoietic chimerism and central tolerance created by peripheral-tolerance induction without myeloablative conditioning" J Clin Invest 112(5): 795-808.

Shapiro, R., Rao, A. S., Corry, R. J., Valenti, M., Zeevi, A., Jordan, M. L., Scantlebury, V. P., Vivas, C. A., Jain, A., McCauley, J., Randhawa, P., Gray, E. A., Dvorchik, I., McMichael, J., Fung, J. J., and Starzl, T. E. (2001). "Kidney transplantation with bone marrow augmentation: five-year outcomes" Transplant Proc 33(1-2): 1134-1135.

Sharkis, S. J., Wiktor-Jedrzejczak, W., Ahmed, A., Santos, G. W., McKee, A., and Sell, K. W. (1978). "Antitheta-sensitive regulatory cell (TSRC) and haematopoiesis: regulation of differentiation of transplanted stem cells in W/Wv anemic and normal mice" Blood 52(4): 802-817.

Sheil, A. G., Moseley, R. V., and Murray, J. E. (1964). "Differential skin and renal homograft survival in dogs on immunosuppressive therapy" Surg Forum 15166-168.

Shields, L. E., Gaur, L. K., Gough, M., Potter, J., Sieverkropp, A., and Andrews, R. G. (2003). "In utero haematopoietic stem cell transplantation in nonhuman primates: the role of T cells" Stem Cells 21(3): 304-314.

Sho, M., Yamada, A., Najafian, N., Salama, A. D., Harada, H., Sandner, S. E., Sanchez-Fueyo, A., Zheng, X. X., Strom, T. B., and Sayegh, M. H. (2002). "Physiological mechanisms of regulating alloimmunity: cytokines, CTLA-4, CD25+ cells, and the alloreactive T cell clone size" J Immunol 169(7): 3744-3751.

Siemionow, M., Demir, Y., Mukherjee, A., and Klimczak, A. (2005). "Development and maintenance of donor-specific chimerism in semi-allogenic and fully major histocompatibility complex mismatched facial allograft transplants" Transplantation 79(5): 558-567.

Siemionow, M., Izycki, D., Ozer, K., Ozmen, S., and Klimczak, A. (2006). "Role of thymus in operational tolerance induction in limb allograft transplant model" Transplantation 81(11): 1568-1576.

Siemionow, M., Ortak, T., Izycki, D., Oke, R., Cunningham, B., Prajapati, R., and Zins, J. E. (2002). "Induction of tolerance in composite-tissue allografts" Transplantation 74(9): 1211-1217.

Siemionow, M., Ulusal, B. G., Ozmen, S., Ulusal, A. E., and Ozer, K. (2004). "Composite vascularised skin/bone graft model: a viable source for vascularised bone marrow transplantation" Microsurgery 24(3): 200-206.

Siemionow, M. Z., Demir, Y., Sari, A., and Klimczak, A. (2005). "Facial tissue allograft transplantation" Transplant Proc 37(1): 201-204.

Siemionow, M. Z., Izycki, D. M., and Zielinski, M. (2003). "Donor-specific tolerance in fully major histocompatibility major histocompatibility complex-mismatched limb

allograft transplants under an anti-alphabeta T cell receptor monoclonal antibody and cyclosporine A protocol" Transplantation 76(12): 1662-1668.

Silverman, M. S. and Chin, P. H. (1962). "Differences in the rejection of skin transplants and blood cells of donor marrow origin by radiation-induced chimeras" Ann N Y Acad Sci 99542-549.

Simon, D., Vassina, E., Yousefi, S., Kozlowski, E., Braathen, L.R., and Simon, H.U. (2004). "Reduced dermal infiltration of cytokine-expressing inflammatory cells in atopic dermatitis after short-term topical tacrolimus treatment" J Allergy Clin Immunol 114(4):887-95.

Sipkins, D. A., Wei, X., Wu, J. W., Runnels, J. M., Cote, D., Means, T. K., Luster, A. D., Scadden, D. T., and Lin, C. P. (2005). "In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment" Nature 435(7044): 969-973.

Smak Gregoor, P. J., Zietse, R., van Saase, J. L., op de Hoek, C. T., Ijzermans, J. N., Lavrijssen, A. T., de Jong, G. M., Kramer, P., and Weimar, W. (2001). "Immunosuppression should be stopped in patients with renal allograft failure" Clin Transplant 15(6): 397-401.

Smith, R. E., Patel, V., Seatter, S. D., Deehan, M. R., Brown, M. H., Brooke, G. P., Goodridge, H. S., Howard, C. J., Rigley, K. P., Harnett, W., and Harnett, M. M. (2003). "A novel MyD-1 (SIRP-1alpha) signaling pathway that inhibits LPS-induced TNFalpha production by monocytes" Blood 102(7): 2532-2540.

Spitzer, T. R., McAfee, S., Sackstein, R., Colby, C., Toh, H. C., Multani, P., Saidman, S., Weyouth, D. W., Preffer, F., Poliquin, C., Foley, A., Cox, B., Andrews, D., Sachs, D. H., and Sykes, M. (2000). "The intentional induction of mixed chimerism and achievement of antitumor responses after nonmyeloablative conditioning therapy and HLA-matched donor bone marrow transplantation for refractory haematologic malignancies" Biol Blood Marrow Transplant 6(3A): 309-320.

Starzl, T. E. (2004). "Chimerism and tolerance in transplantation" Proc Natl Acad Sci U S A 101 Suppl 214607-14614.

Starzl, T. E., Demetris, A. J., Murase, N., Ildstad, S., Ricordi, C., and Trucco, M. (1992). "Cell migration, chimerism, and graft acceptance" Lancet 339(8809): 1579-1582.

Steinmuller, D. (1962). "Passive transfer of immunity to skin homografts in rats" Ann N Y Acad Sci 99629-644.

Steinmuller, D. (1998). "The enigma of skin allograft rejection" Transplantation Reviews 12(1): 42-57.

Strauss, R. G., Maguire, L. C., Koepke, J. A., and Thompson, J. S. (1980). "Effect of intermittent-flow centrifugation leukapheresis on donor leukocyte counts" Acta Haaematol 63(3): 128-131.

Sumrani, N., Delaney, V., Hong, J. H., Daskalakis, P., and Sommer, B. G. (1992). "The influence of nephrectomy of the primary allograft on retransplant graft outcome in the cyclosporine era" Transplantation 5352-55.

Suzuki, T., Sundt, T. M., Mixon, A., and Sachs, D. H. (1990). "In vivo treatment with anti-porcine T cell antibodies" Transplantation 5076-81.

Sykes, M. (2001). "Mixed chimerism and transplant tolerance" Immunity 14(4): 417-424.

Sykes, M., Bukhari, Z., and Sachs, D. H. (1989). "Graft-versus-leukemia effect using mixed allogeneic bone marrow transplantation" Bone Marrow Transplant 4465-474.

Sykes, M., Hoyles, K. A., Romick, M. L., and Sachs, D. H. (1990a). "*In vitro* and *in vivo* analysis of bone marrow-derived $CD3^+$, $CD4^-$, $CD8^-$, $NK1.1^+$ cell lines" Cell Immunol 129478-493.

Sykes, M., Preffer, F. I., McAfee, S. L., Saidman, S. L., Weymouth, D., Andrews, D., Colby, C., Sackstein, R., Sachs, D. H., and Spitzer, T. R. (1999). "Mixed lymphohaematopoietic chimerism and graft-vs-lymphoma effects are achievable in adult recipients following non-myeloablative therapy and HLA-mismatched donor bone marrow transplantation." Lancet 3531755-1759.

Sykes, M., Romick, M. L., and Sachs, D. H. (1990b). "Interleukin 2 prevents graft-vs-host disease without diminishing the graft-vs-leukemia effect of allogeneic lymphocytes" Proc Natl Acad Sci U S A 875633-5637.

Taylor, P. A., Lees, C. J., and Blazar, B. R. (2002). "The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality" Blood 99(10): 3493-3499.

Teuffel, O., Schrauder, A., Sykora, K. W., Zimmermann, M., Reiter, A., Welte, K., and Schrappe, M. (2005). "The impact of cyclosporin A on acute graft-versus-host disease after allogeneic bone marrow transplantation in children and adolescents with acute lymphoblastic leukemia" Bone Marrow Transplant 36(2): 145-150.

Thistlethwaite JR, J. r., Auchincloss, H., Jr., Pescovitz, M. D., and Sachs, D. H. (1984). "Immunologic characterisation of MHC recombinant swine: role of class I and II antigens in in vitro immune responses" J Immunogenet 119-19.

Tian, C., Bagley, J., Forman, D., and Iacomini, J. (2004). "Induction of central tolerance by mature T cells" J Immunol 173(12): 7217-7222.

Tillson, M., Niemeyer, G. P., Welch, J. A., Brawner, W., Swaim, S. F., Rynders, P., Lenz, S. D., Dean, B., and Lothrop, C. D., Jr. (2006). "Haematopoietic chimerism induces renal and skin allograft tolerance in DLA-identical dogs" Exp Haematol 34(12): 1759-1770.

Tilney, N. L. and Gowans, J. L. (1971). "The sensitisation of rats by allografts transplanted to alymphatic pedicles of skin" J Exp Med 133(5): 951-962.

Torrealba, J. R., Katayama, M., Fechner, J. H., Jr., Jankowska-Gan, E., Kusaka, S., Xu, Q., Schultz, J. M., Oberley, T. D., Hu, H., Hamawy, M. M., Jonker, M., Wubben, J., Doxiadis, G., Bontrop, R., Burlingham, W. J., and Knechtle, S. J. (2004). "Metastable tolerance to rhesus monkey renal transplants is correlated with allograft TGF-beta 1+CD4+ T regulatory cell infiltrates" J Immunol 172(9): 5753-5764.

Trenado, A., Sudres, M., Tang, Q., Maury, S., Charlotte, F., Gregoire, S., Bonyhadi, M., Klatzmann, D., Salomon, B. L., and Cohen, J. L. (2006). "Ex vivo-expanded CD4+CD25+ immunoregulatory T cells prevent graft-versus-host-disease by inhibiting activation/differentiation of pathogenic T cells" J Immunol 176(2): 1266-1273.

Tryphonopoulos, P., Tzakis, A. G., Weppler, D., Garcia-Morales, R., Kato, T., Madariaga, J. R., Levi, D. M., Nishida, S., Moon, J., Selvaggi, G., Regev, A., Nery, C., Bejarano, P., Khaled, A., Kleiner, G., Esquenazi, V., Miller, J., Ruiz, P., and Ricordi, C. (2005). "The role of donor bone marrow infusions in withdrawal of immunosuppression in adult liver allotransplantation" Am J Transplant 5(3): 608-613.

Ulusal, B. G., Ulusal, A. E., Ozmen, S., Zins, J. E., and Siemionow, M. Z. (2003) "A new composite facial and scalp transplantation model in rats" Plast Reconstr Surg 112(5):1302-11.

Umemura, A., Morita, H., Chang, Li, X, Tahan, S., Monaco, A. P., and Maki, T. (2001). "Dissociation of hemopoietic chimerism and allograft tolerance after allogeneic bone marrow transplantation" J Immunol 167(6): 3043-3048.

Unal, S., Agaoglu, G., Zins, J., and Siemionow, M. (2005). "New surgical approach in facial transplantation extends survival of allograft recipients" Ann Plast Surg 55(3): 297-303.

Ustinov, J., Loginov, R., Bruggeman, C., Suni, J., Hayry, P., and Lautenschlager, I. (1994). "CMV-induced class II antigen expression in various rat organs" Transpl Int 7(4): 302-308.

Utsugi, R., Barth, R. N., Lee, R. S., Kitamura, H., LaMattina, J. C., Ambroz, J., Sachs, H., and Yamada, K. (2001). "Induction of transplantation tolerance with a short course of tacrolimus (FK506): I. Rapid and stable tolerance to two-haplotype fully mhc-mismatched kidney allografts in miniature swine" Transplantation 71(10): 1368-1379.

Vagefi, P. A., Ierino, F. L., Gianello, P. R., Shimizu, A., Kamano, C., Sachs, D. H., and Yamada, K. (2004). "Role of the thymus in transplantation tolerance in miniature Swine: IV. The thymus is required during the induction phase, but not the maintenance phase, of renal allograft tolerance" Transplantation 77(7): 979-985.

van Bekkum, D. W. (1984). "Conditioning regimens for marrow grafting" Semin Haematol 2181-90.

van Vreeswijk, W. and Balner, H. (1980). "Major histocompatibility complex matching and other factors influencing skin allograft survival in related and unrelated rhesus monkeys" Transplantation 30(3): 196-202.

Waldmann, H., Adams, E., Fairchild, P., and Cobbold, S. (2006). "Infectious tolerance and the long-term acceptance of transplanted tissue" Immunol Rev 212301-313.

Wang, Y., Calder, V. L., Lightman, S. L., and Greenwood, J. (1995). "Antigen presentation by rat brain and retinal endothelial cells" J Neuroimmunol 61(2): 231-239.

Weiss, L. and Slavin, S. (1999). "Prevention and treatment of graft-versus-host disease by down-regulation of anti-host reactivity with veto cells of host origin" Bone Marrow Transplant 23(11): 1139-1143.

Wekerle, T., Kurtz, J., Bigenzahn, S., Takeuchi, Y., and Sykes, M. (2002). "Mechanisms of transplant tolerance induction using costimulatory blockade" Curr Opin Immunol 14(5): 592-600.

Wekerle, T., Kurtz, J., Ito, H., Ronquillo, J. V., Dong, V., Zhao, G., Shaffer, J., Sayegh, M. H., and Sykes, M. (2000). "Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment" Nat Med 6(4): 464-469.

Wekerle, T., Sayegh, M. H., Ito, H., Hill, J., Chandraker, A., Pearson, D. A., Swenson, K. G., Zhao, G., and Sykes, M. (1999). "Anti-CD154 or CTLA4Ig obviates the need for thymic irradiation in a non-myeloablative conditioning regimen for the induction of mixed haematopoietic chimerism and tolerance" Transplantation 68(9): 1348-1355.

Wiktor-Jedrzejczak, W., Sharkie, S., Ahmed, A., Sell, K. W., and Santos, G. W. (1977). "Theta-sensitive cell and erythropoiesis: identification of a defect in W/Wv anemic mice" Science 196(4287): 313-315.

Wollenberg A., Sharma S., von Bubnoff D., Geiger E., Haberstok J., and Bieber T. (2001). "Topical tacrolimus (FK506) leads to profound phenotypic and functional alterations of epidermal antigen-presenting dendritic cells in atopic dermatitis" J Allergy Clin Immunol. 107(3):519-25.

Wood, K. and Sachs, D. H. (1996). "Chimerism and transplantation tolerance: Cause and effect" Immunol Today 17584-587.

Woodruff, M. F. and Lennow, B. (1959). "Reciprocal skin grafts in a pair of twins showing blood chimaerism" Lancet 2476-478.

Wu, J., Barisoni, D., and Armato, U. (1995). "An investigation into the mechanisms by which human dermis does not significantly contribute to the rejection of allo-skin grafts" Burns 21(1): 11-16.

Wustrack, K. O., Gruber, R. P., and Lucas, Z. J. (1975). "Immunological enhancement of skin allografts in the rat. Role of vascular and lymphatic reconstitution" Transplantation 19(2): 156-165.

Xu, H., Montgomery, S. P., Preston, E. H., Tadaki, D. K., Hale, D. A., Harlan, D. M., and Kirk, A. D. (2003). "Studies investigating pretransplant donor-specific blood transfusion, rapamycin, and the CD154-specific antibody IDEC-131 in a nonhuman primate model of skin allotransplantation" J Immunol 170(5): 2776-2782.

Xudong, Z., Shuzhong, G., Yan, H., Datai, W., Yunzhi, N., and Linxi, Z. (2006). "A hemifacial transplantation model in rabbits" Ann Plst Surg 56(6): 665-669.

Yang RK, Lowe JB 3rd, Sobol JB, Sen SK, Hunter DA, Mackinnon SE.(2003) "Dose-dependent effects of FK506 on neuroregeneration in a rat model" Plast Reconstr Surg 112(7):1832-40.

Yates, J., Rovis, F., Mitchell, P., Afzali, B., Tsang, J. Y., Garin, M., Lechler, R. I., Lombardi, G., and Garden, O. A. (2007). "The maintenance of human CD4+ CD25+ regulatory T cell function: IL-2, IL-4, IL-7 and IL-15 preserve optimal suppressive potency in vitro" Int Immunol 19(6): 785-799.

Yazici, I., Unal, S., and Siemionow, M. (2006). "Composite hemiface/calvaria transplantation model in rats" Plast Reconstr Surg 118(6): 1321-1327.

Yunusov, M. Y., Kuhr, C. S., Georges, G. E., Hogan, W. J., Taranova, A. G., Lesnikova, M., Kim, Y. S., Abrams, K., Hwang, B., Sale, G. E., Storer, B., Storb, R., and Nash, R. A. (2006). "Partial donor-specific tolerance to delayed skin grafts after rejection of haematopoietic cell graft" Transplantation 82(5): 629-637.

Yuzawa, K., Taniguchi, H., Seino, K., Otsuka, M., and Fukao, K. (1996). "Topical immunosuppression in skin grafting with FK 506 ointment" Transplant Proc 28(3): 1387-1389.

Zeng, D., Lan, F., Hoffmann, P., and Strober, S. (2004). "Suppression of graft-versus-host disease by naturally occurring regulatory T cells" Transplantation 77(1 Suppl): S9-S11.

Zhang, X. D., Guo, S. Z., Han, Y., Wang, D. T., Ni, Y. Z., and Zhang, L. X. (2006). "[A hemifacial transplantation model in hares]" [Article in Chinese] Zhonghua Zheng Xing Wai Ke Za Zhi 22(3):204-7.

Zheng, X. X., Sanchez-Fueyo, A., Sho, M., Domenig, C., Sayegh, M. H., and Strom, T. B. (2003). "Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance" Immunity 19(4): 503-514.

Zhang, Z., Zhu, L., Quan, D., Garcia, B., Ozcay, N., Duff, J., Stiller, C., Lazarovits, A., Grant, D., and Zhong, R. (1996). "Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations" Transplantation 62(9): 1267-1272.