Gene Therapy Studies of Adenoviral IL-10 Transduced Dendritic Cells in Allotransplantation

A Thesis submitted to the University of Adelaide

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Doctor of Philosophy

by

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Thesis Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been given in the text. I give my consent for this copy of my thesis, when deposited in the University library being available for loan or photocopy.

Toby Coates

Pittsburgh,

Pennsylvania,

November 2001.

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My father Dr John Coates and my "immunology" brother Dr Nick Coates have both been scientific role models for me throughout the course of my PhD studies. Finally it goes without saying that this work could not have been undertaken without the love and support of my wife Penny and my children Eleanor and Hugh. To my patient and long-suffering family as tiny recompense for many Sunday mornings I dedicate this thesis.

"In the midway of this our mortal life,

I found me in a gloomy wood, astray

Gone from the path direct: and e'en to tell,

It were no easy task, how savage wild

That forest, how robust and rough its growth,

Which to remember only, my dismay

Renews, in bitterness not far from death.

Yet, to discourse of what there good befel"

Inferno, Canto I , 1-8

The Divine Comedy

Dante Alighieri (1265-1321)

The Harvard Classics. 1909-1914.

Thesis Abstract

The search for novel means of inducing permanent allograft acceptance without recourse to ongoing immunosuppressive therapy is a major goal for Transplantation Immunologists. Recent advances in immunology have identified Dendritic Cells (DC) as initiators and modulators of the alloimmune response to transplanted organs. As such they are potentially novel targets for therapeutic intervention to promote allograft acceptance. Under the influence of regulatory cytokines DC can behave in either a tolerogenic or immunogenic manner. Using a gene therapy strategy to target donor DC with immunosuppressive cytokines is a novel means of inhibiting the alloimmune response. The principal aim of this thesis was to study the capacity of DC transduced with the immunosuppressive gene construct adenoviral interleukin-10 (AdV IL-10) to inhibit alloimmune responses in both small and large animal transplantation models.

The first results chapter of this thesis describes the generation of human DC from monocyte precursors using the recombinant human cytokines IL-4 and GM-CSF. These cells were then transduced with AdV IL-10 and their *in vitro* allostimulatory properties studied. AdV IL-10 transduced DC showed down regulation of the co-stimulatory molecules CD80 / CD86 and impaired secretion of the pro-inflammatory cytokine IL-12. AdV IL-10 transduced DC were potent inhibitors of the alloimmune response in the MLR.

In chapter 4 a chimeric human-immunodeficent mouse skin transplantation model was used to test the capacity of AdV IL-10 transduced DC to modify human skin

graft rejection. DC transduced with AdV IL-10 inhibited skin graft rejection in comparison to DC transduced with the control gene construct adenoviral MX-17 (AdV MX-17) or fibroblasts transduced with AdV IL-10 indicating specificity of the AdV IL-10 DC effect.

Chapter 5 describes the characterization and transduction of pseudoafferent ovine DC with adenoviral gene constructs. Ovine DC were collected via cannulation of a pseudoafferent lymphatic vessel. Using the conditions derived from human DC experiments, ovine DC were transduced with AdV IL-10 and showed similar *in vitro* allo-inhibitory properties to human DC.

Chapter 6 describes migration studies of allogeneic ovine DC within an ovine system. For local migration to draining lymph nodes, AdV IL-10 DC were labeled with the fluorochrome PKH-26 and injected into allogeneic recipients. Dendritic cells migrated to draining lymph nodes and co-localized with endogenous CD83 positive cells. The transcript for AdV IL-10 could be detected by polymerase chain reaction analysis from the draining lymph node. Untransduced ovine DC were also labeled with ^{111}Indium-oxine and the systemic distribution followed by gamma camera for up to 7 days post intravenous or intra-dermal injection.

Chapter 7 describes renal transplantation experiments in the ovine heterotopic renal allograft model. Kidney donor DC transduced with either AdV IL-10 or AdV MX-17 were administered to recipient sheep either pre-transplant (day-7 and day -1) or daily post transplant for 7 days as sole immunosuppressive therapy. Neither regimen was associated with prolonged allograft survival beyond 7 days.

These studies have shown promising *in vitro* evidence for gene therapy to modify DC function, which in small animal models can modify skin graft rejection. In large animals, despite promising *in vitro* and *in vivo* data genetically modified DC alone were not capable of prolongation of allograft survival, suggesting that these cells may require adjuvant immunosuppressive therapy to be used in future protocols.

Publications and Presentations

Publications arising from this thesis:

Coates T., Krishnan R., Russ GR, "Dendritic cells, Tolerance and Transplantation" Nephrology 2000 :5 :123-129

Coates T., Krishnan R., Chew G, Kireta S., Johnston J., Kanachanabat B, Russell CH, Siddins M, Russ GR "Dendritic cell TH2 cytokine gene therapy in sheep" Transplant Proc 2001:33;180-181

Coates T., Krishnan R., Kireta S., Johnston J. and Russ G.R. "Human Myeloid Dendritic Cells transduced with an Adenoviral Interleukin 10 Gene construct inhibit human skin graft rejection in chimeric mice" Gene Therapy 2001;8:1224-1233

Coates T., Thomson, AW "Dendritic Cells and Tolerance Induction" American Journal of Transplantation (submitted)

Coates T., Krishnan R., Chew G., Kireta S., Johnston J., Kanachanabat B, Russell C, and Russ GR " Dendritic cells transduced with adenoviral IL-10 inhibit alloimmune response in an ovine renal transplant model (manuscript in preparation)

Published Abstracts:

Coates T., Krishnan R., Russ GR. Characterisation and Transfection of human dendritic cells with an adenovirus B galactosidase gene construct and an adenovirus interleukin 10 viral construct Imm Cell Biol 1998, 76;2:A15 (abstract)

Coates T., Krishnan R., Russ GR., Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit the Mixed Lymphocyte Culture. J Leuk Biol (Suppl 2)1998 B59 (abstract)

Coates T., Krishnan R., Russ GR., Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Construct Strongly Inhibit the Mixed Lymphocyte Culture (MLC) J Amer SocNeph 1998;9: 648A (abstract)

Coates T., Krishnan R., Kireta, S., Johnston, J., Russ, G.R., Human myeloid dendritic cells transfected with an adenoviral interleukin 10 gene construct inhibit human skin graft rejection in a chimeric human-NOD/SCID mouse transplant model. Imm Cell Biol 1999,77; 3: A11 (abstract)

Presentations:

Oral presentation President's Prize Symposium "Characterisation and Transfection of Human Dendritic cells with an adenovirus B galactosidase construct and an adenovirus interleukin 10 construct" Transplantation Society of Australia and New Zealand 16th Annual Scientific Meeting, Canberra, Australia 1998.

Poster Presentation 5th International Symposium on Dendritic Cells in Fundamental and Clinical Immunology "Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit the Mixed Lymphocyte Culture" Pittsburgh, USA September 1998.

Oral Presentation "Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit the Mixed Lymphocyte Culture" American Society of Nephrology , Philadelphia, USA October 1998.

Oral Presentation "Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit Human Skin Graft Rejection in chimeric Human/Nod-scid mice" Australian and New Zealand Society of Nephrology, Brisbane, Australia 1999.

Oral Presentation President's Prize Symposium "Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit Human Skin

Graft Rejection in chimeric Human/Nod-scid mice" Transplantation Society of Australia and New Zealand Canberra, Australia 1999.

Oral Presentation President's Prize Symposium "Dendritic Cell Based TH2 Cytokine Gene Therapy in Sheep" Transplantation Society of Australia and New Zealand Canberra, Australia 2000

Poster Presentation "Dendritic Cell Based TH2 Cytokine Gene Therapy in Sheep" 6th International Symposium on Dendritic Cells, Port Douglas, Australia 2000

Combined Oral-Poster Presentation "Dendritic Cell TH2 Cytokine Gene Therapy in Sheep" XVIIIth International Meeting of the Transplantation Society, Rome, Italy 2000

Poster Presentation "Human Dendritic Cells Transfected with an Adenoviral Interleukin 10 Gene Construct Inhibit Human Skin Graft Rejection in chimeric Human/Nod-scid mice"XVIIIth International Meeting of the Transplantation Society, Rome, Italy 2000

Poster Presentation " Transduction of ovine DC with Adenoviral IL-10 inhibits the alloimmune response but does not prolong allograft survival in sheep" Transplant 2001, Chicago, USA 2001

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Chapter 1

Literature Review

1.1 Introduction:

Dendritic cells (DC) are a heterogeneous population of interstitial and blood borne leukocytes that are critically involved in the regulation of the immune system and initiation of the alloimmune response (Steinman 1991; Hart 1997; Banchereau et al. 1998). These leukocytes have a unique place in the immune system as they act as professional antigen presenting cells that are capable of directly activating T cells. Formal studies of DC have been difficult in the past because of their comparative rarity within the body and a lack of specific cell markers to aid their detection. Recently however, DC have been successfully propagated *in vitro* using recombinant cytokines (Caux et al. 1992; Romani et al. 1994; Strunk et al. 1996) and new DC specific monoclonal antibodies (Zhou et al. 1996; Fearnley et al. 1997; Hock et al. 1999; Dzionek et al. 2000) have been developed to facilitate their study. These new reagents have allowed easier identification and isolation of these DC *in vivo*, and the ability to study their properties *in vitro*. Many groups are looking at DC properties as nature's "adjuvant" and are developing strategies to use these cells for immunotherapy of cancers and autoimmune diseases (Mayordomo J.I. 1995; Lotze et al. 1996; Lee et al. 1997).

The particular focus of this literature review is DC biology and its relationship to organ transplantation.

1.2 Dendritic Cell Biology

In 1973, cells of dendritic morphology were identified within murine spleen by Steinman and Cohn (Steinman et al. 1973). The term 'Dendritic Cell' was derived from the Greek word '*dendros*' or tree and referred to the hallmark appearance of long cytoplasmic projections that resembled the branches of a tree (see figure 1.1). Early experiments identified the ability of these cells to stimulate the primary immune response (Steinman et al. 1978). Although initially identified within the mouse, these cells were subsequently identified within humans and other animals (Hart et al. 1983; Bujdoso 1989). It appears now that DC are of at least 2 lineages that arise from separate precursor cell types. These lineages, termed myeloid and lymphoid, demonstrate different properties. The myeloid DC (or DC1) lineage arises from a bone marrow precursor (CD34+). These cells make up the majority of the interstitial DC that reside within organs, and can be grown *in vitro* by the use of the recombinant cytokines interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF). The predominant function of these tissue derived DC is immune surveillance. In contrast, lymphoid DC (DC2)(Risson et al. 1999; Siegal et al. 1999) are typically resident within the thymus and other lymphoid organs, where they appear to have immuno-regulatory functions (Brocker et al. 1997). These cells arise from a lymphoid stem cell and can be grown *in vitro* without the use of GM-CSF.

1.3 Myeloid Dendritic Cells (DC1)

Myeloid DC are the most frequent type of DC within body tissues and function principally as immunosurveillant cells. Myeloid DC can be grown in vitro from $CD34^+$ stem cells (Caux et al. 1992; Strunk et al. 1996) or monocytes by culture

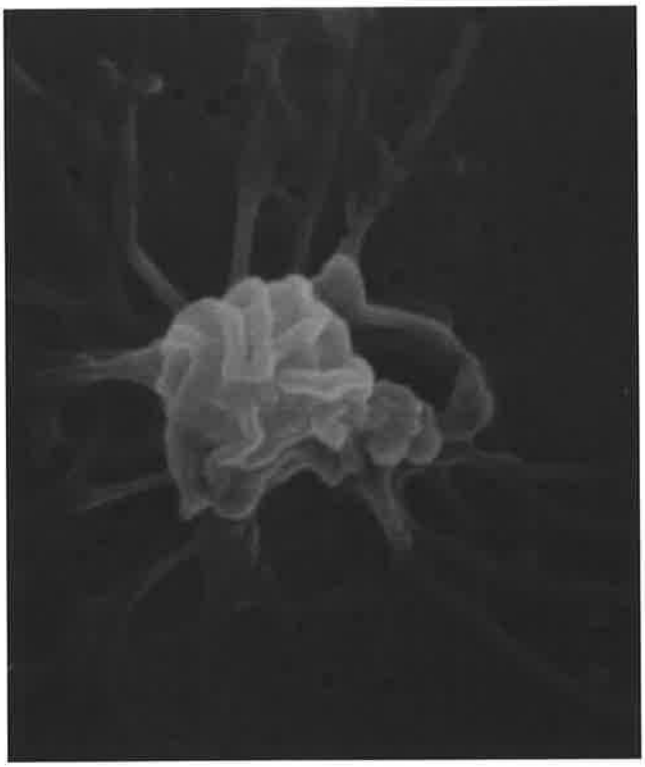


Figure 1.1

Scanning Electron Micrograph of a mature freshly isolated mammalian dendritic cell, showing typical extensive cytoplasmic processes (15,000 x)

using different combinations of cytokines (Romani et al. 1994). In the resting state these cells avidly take up and process antigens within the body tissues via pinocytosis and phagocytosis (Sallusto et al. 1995). Processed antigen is expressed on the DC cell surface as peptide in association with MHC class II molecules, the production of which can be rapidly up-regulated for the purpose of display of antigenic material on the cell surface. The peptide-MHC class II complex is then presented to $CD4^+$ T cells. Depending upon presence of other stimuli (e.g. interleukin 1,TNF-α, bacterial lipopolysaccharides, GM-CSF), DC maturation may then occur (Buelens et al. 1997a). Upon maturation, DC lose the ability to take up fresh antigens and up-regulate their cell surface expression of co-stimulatory molecules CD80 (B7.1), CD86 (B7.2) and CD40. Dendritic cells can also promote activation of cytotoxic T cells via class I-restricted antigenic presentation. This is dependent upon T helper cell-induced activation of the CD40 receptor on the DC (Bennett et al. 1998; Lanzavecchia A 1998).

The trafficking of the immature DC to sites of inflammation and subsequent migration of the mature DC to lymphoid organs is under the control of chemokines. Immature DC express receptors for the inflammation-associated chemokines (CCR1, CCR2, CCR5 and CCR6), which allows recruitment of DC into sites of inflammation. Upon exposure to a maturational stimulus, DC down-regulate their expression of receptors for inflammatory chemokines and express a different repertoire of chemokine receptors (CXCR4 and CCR7) which promote the efflux of these cells into lymph channels and subsequently to lymphoid organs (Lane et al. 1999; Sallusto et al. 1999b). In addition to the role of chemokines in determining DC trafficking patterns, the expression of variant CD44 isoforms

(particularly CD44 variant 6) has also been shown to influence DC migration (Weiss et al. 1997).

Once the DC arrives in the lymphoid organ it is capable of eliciting a T cell response. DC activate T cells by the delivery of 3 specific signals (see figure 1.2). The first signal is an interaction between an MHC class II-peptide complex on the DC and the T cell receptor on the T cell (signal 1). Following this interaction, CD40 ligand expression is up-regulated on the surface of the T cell, which binds to CD40 on the DC. This in turn results in further up-regulation of co-stimulatory molecules (especially CD80 and CD86) on the surface on the DC (signal 2). These co-stimulatory molecules then engage with CD28 on the T cell and thereby augment the transcription of interleukin-2 within the T cell. Interleukin-2 driven T cell proliferation then ensues. The third signal for T cell activation is that of secretion of the proinflammatory cytokine interleukin-12 by the DC, which has autocrine effects on the DC and paracrine effects on T cell proliferation.

Table 1.4.1 Comparison of putative murine and human myeloid Dendritic Cells

Property	murine	human
Cell Phenotype	$CD11c^+$	$CD83^+CD11c^+$
Precursor	?myeloid precursor	$CD34^+$
Phagocytosis	yes	yes
Interferon -γ secretion	negative	negative
Migration	yes	yes
TH1/TH2	TH1/TH2	TH1
Interleukin-12 secretion	+/-	++++
Cytokine Requirement	GM-CSF	GM-CSF

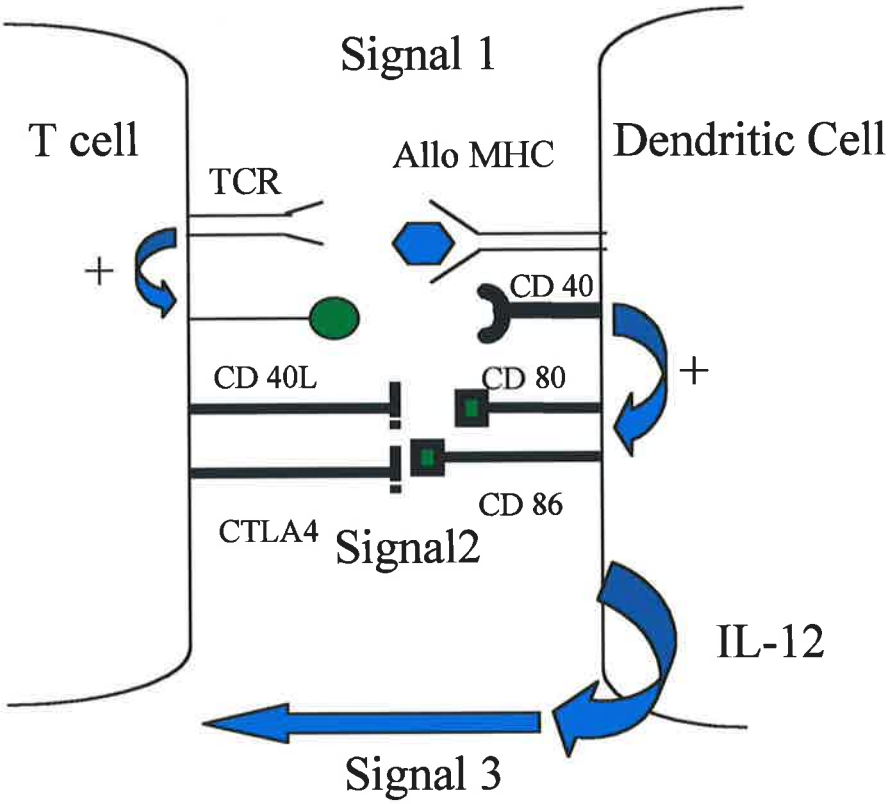


Figure 1.2

DC deliver 3 signals for T cell activation. Signal 1 is the interaction between antigen presented via DC allo MHC Class II to the T cell via the T cell Receptor (TCR). This in turn upregulates the expression of CD40 ligand on the T cell surface which binds to CD40 on the DC. Binding of CD40-CD40L results in DC production of the costimulatory molecules CD80 and CD86 (signal 2). These molecules in turn bind to CD28/CTLA4 on the T cell in turn inducing interleukin-2 (IL 2) production and T cell proliferation. Secretion of the proinflammatory cytokine interleukin 12 (IL-12) by DC amplifies the immune response (signal 3).

(modified from (Faratian et al. 2000) (Pulendran et al. 2001))

1.4 Lymphoid Dendritic Cells (DC2)

DC2 cells are cells that play a predominantly regulatory role within the immune system. During development of the T cell repetoire within the thymus, these DC2 cells bind to self-reactive T cells and delete these cells from the individual's repertoire (Ardavin et al. 1993; Winkel et al. 1994). Within the murine system DC2 are characterized by the expression of CD8α. Within the murine system, 3 subsets of dendritic cells have been described. These are $CD8\alpha^+$ DC, $CD8\alpha^-$ "myeloid" DC and Langerhans cell derived DC. All 3 subsets express the cell surface markers CD11c, MHC Class II and CD40 but have important differences in several other parameters. The $CD8\alpha^+$ DC is localized to the T-cell zones of the lymphoid organs, whereas the $CD8\alpha^-$ DC are found in the marginal zone of the spleen and in the sub-capsular sinus of lymph nodes. The conventional belief has been that murine $CD8\alpha^+$ DC are of lymphoid origin, although this view has been recently challenged by the finding that the $CD8\alpha^+$ DC may be generated from myeloid precursors (Traver et al. 2000). A population of murine DC has been identified within mouse spleen that expresses $CD8\alpha^+$ and is capable of deletion of $CD4^+$T cells via Fas Ligand in the periphery (Suss et al. 1996; Shortman et al. 1997). It appears that within the murine system, $CD8\alpha^+$ and CD $8\alpha^-$ may direct the development of $CD4^+$T cells with different cytokine profiles (Maldonado-Lopez et al. 1999). Furthermore, $CD8^+$ DC produce more interleukin-12 than do $CD8^-$ DC (Pulendran et al. 1999). Thus within murine systems, $CD8^+$ DC promote the generation of a TH1 response (Maldonado-Lopez et al. 1999). These same cells are

capable of regulating $CD8^+$ T cells by restricting their IL-2 production (Kronin et al. 1996).

Within the human system, further complexity arises with the latest phenotypic and functional analysis showing considerable differences between human and murine DC. At least 4 types of human DC have been described: (a) "plasmacytoid" DC (DC2) which express $CD11c^-$,$CD1a^-$ and $CD123^+$ (IL-3 receptor-α), (b) myeloid DC (DC1), (c) Langerhans cell derived DC and (d) follicular DC (believed to play an important role in immune memory). In contrast to the murine DC these 3 DC subsets do not all express CD11c, but they do all share cell surface expression of MHC Class II, CD4, CD40 and DC-LAMP. A cell of "plasmacytoid" morphology can be isolated from human tonsil and blood and be differentiated into a DC2 when cultured with interleukin-3 and CD 40 ligand. The evidence that these cells are of true lymphoid origin rests upon the expression of the IL-3Rα (CD 123)(Grouard et al. 1997). Such DC2 cells induce T helper 2 differentiation in T cells and are capable of producing 200-1000 times more interferon-α than other cell types in the blood and probably play a critical antiviral and anti-tumor role (Siegal et al. 1999). Plasmacytoid DC (or DC2) induce differentiation of TH2 cells, and MDC (DC1) induce a TH1 response. Functional differences between the plasmacytoid DC and the myeloid DC have also been described with plasmacytoid DC but not myeloid DC being capable of production of interferon-α. The precise role that these putative LDC might play in the maintenance of tolerance and in allotransplantation in humans has yet to be fully defined. The functional relationship between human DC2 and murine $CD8\alpha^+$ DC remains controversial.

Table 1.4.2 Comparison of putative murine and human Lymphoid DC

Property	Murine	Human
Cell Phenotype	$CD11c^{+}$ /$CD8\alpha^{+}$	$CD\ 123^{+}$, $CD11c^{low}$
Cell Precursor	?lymphoid	$CD34^{+}$
Phagocytosis	negative	negative
Interferon production	IFN-γ	IFN-α
Migration	yes	yes
Interleukin-12 secretion	++++	+/-
T cell polarization	TH1	TH2
Cytokine Requirement	IL-3	IL-3

(modified from (Faratian et al. 2000; Pulendran et al. 2001))

1.5 Dendritic Cells and Self- Tolerance

Dendritic cells are capable of induction of the primary immune response to foreign antigens, and are thus important in the immune response to infection and transplantation. In addition to this well recognized role in induction of immunity, these cells may also induce a tolerogenic response to antigens (Finkelman et al. 1996). As indicated above, certain specific DC play an important role in selection of the immune system T cell repertoire within the thymic environment (Matzinger et al. 1989). It has been proposed that DC may also play an important role in peripheral tolerance to antigens (Fazekas de St Groth B 1998). Albert *et al* have shown that immature DC1 have high capacity for phagocytosis of apoptotic bodies that is approximately 100 fold greater uptake of other exogenous antigens. In the normal resting non-inflamed situation, the scavenging of apoptotic bodies by

immature DC1 and presentation of self antigens to lymphoid areas provides a pathway by which the immune system could be constantly re-educating itself about the nature of self antigens. These DC1 could then traffic to the lymphoid organs where self-antigens could be presented (possibly by transfer of antigens from DC1 to resident DC2) to the host immune system in order to maintain tolerance to self antigens (Inaba 1997; Albert et al. 1998; Inaba et al. 1998; Sallusto et al. 1999a). This hypothesis is attractive as it provides a way by which tolerance to "mature" antigens can be maintained in the adult without these antigens necessarily having been presented 'thymically' during the neonatal period. As antigen processing *per se* is incapable of distinguishing self from foreign peptides, an immunoregulatory cell, such as the DC2, within the lymph node could play an important regulatory role in prevention of inappropriate T cell activation to self antigens (Steinman et al. 1999).

Dendritic cells have also been implicated in the maintenance of peripheral tolerance via 'cross presentation' of exogenous antigens. Cross presentation is a process by which exogenous antigen may be presented via a professional antigen presenting cell in association with MHC class I molecules to $CD8^+$ T cells, in order to provoke a cytotoxic T cell response to that antigen. In a similar manner it has been suggested that tolerance to peripheral antigens may be induced by a 'cross tolerant' antigen presenting cell. It has been speculated that DC may fulfill the critical role as the antigen-presenting cell capable of induction of 'cross-tolerance' to self-antigens (Heath et al. 1998). Evidence for DC playing this role comes from the studies in the rat by Huang *et al.* These authors found a migratory population of DC within mesenteric lymph nodes that had ingested apoptotic fragments of

intestinal epithelial cells (Huang et al. 2000). Furthermore, Sauter *et al* have demonstrated that DC respond differently to apoptotic bodies and necrotic cells. Uptake of necrotic tumour cells by DC initiated DC maturation whereas uptake of apoptotic bodies did not (Sauter et al. 2000) (see figure 1.3). In mechanistic terms activation induced cell death (AICD) may well play a role in the maintenance of peripheral tolerance (Li et al. 1999b; Wells et al. 1999). It has been suggested that DC may play a role in this process through their capacity to express FasL and thereby trigger AICD.

1.6 Dendritic Cells and Transplantation

Overcoming the immunogenicity of transplanted organs is one of the main aims for clinical transplantation immunologists. Central to achieving this was the identification of the immunogenic components of transplanted organs. In the 1970s, Lafferty *et al* proposed that populations of cells within the transplanted organ, known as 'passenger leucocytes', were the principle immunogenic cells within the transplanted organ (Talmage et al. 1976; Lafferty et al. 1983). Migration of these cells from the grafted organ into the recipient lymphoid tissue activates the recipient's immune system via the 'direct pathway' of allorecognition (see figure 1.4). Direct allorecognition refers to activation of the recipient T cells by peptide-foreign MHC complexes presented by donor antigen presenting cells in the passenger leukocyte population. It has been shown that DC are a significant percentage of this population within solid organs (Hart et al. 1982; Hart et al. 1983; McKenzie et al. 1984). In 1983 Lechler and Batchelor demonstrated that depletion of rat renal allografts of their passenger leukocyte DC populations was an effective strategy for promotion of tolerance to rat renal transplantation. This tolerance

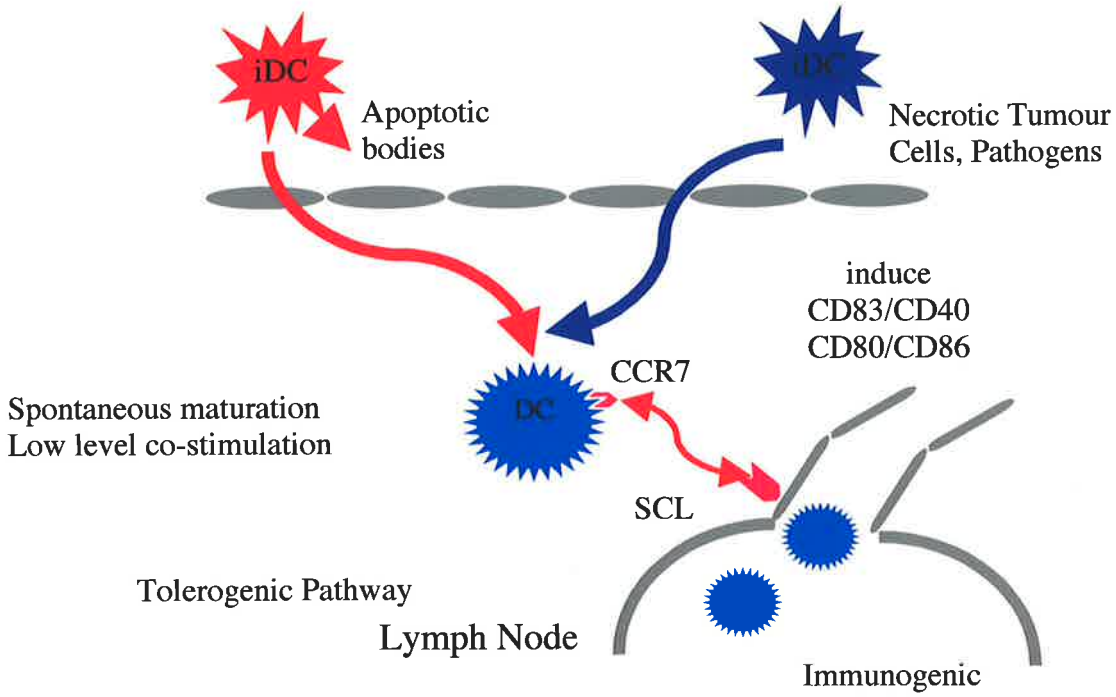


Figure 1.3

Two pathways of DC maturation to induce alternative immunological responses. Immature DC (iDC) may take up apoptotic bodies, produced as a consequence of cell turnover. These DC migrate towards the draining lymph node (DLN) without upregulation of cell surface co-stimulatory molecule expression. By way of contrast, iDC encountering either necrotic cell death or pathogens rapidly up regulate cell surface co-stimulatory molecules necessary for full induction of a T cell response. Migration to the DLN is mediated by DC expression of the chemokine receptor CCR7 in response to its ligand SCL. (figure modified from Sallusto et al 1999)

could be broken by infusion of DC from the original rat into the recipient (Batchelor et al. 1979) (Lechler et al. 1982). Subsequent observations have demonstrated that transplantation of solid organs (kidney, heart, liver) is also associated with a rapid flux of donor derived DC from the transplanted organ into the recipient lymphoid tissue (Austyn et al. 1990; Larsen et al. 1990b). Attempts have been made to target DC populations within the organ donor in order to modify the fate of the transplanted organ, principally using monoclonal antibodies directed against the resident DC populations, largely without success (Brewer et al. 1989; Iwai et al. 1989).

Whilst kidneys may undergo prompt 'direct pathway' mediated rejection in the manner indicated above, the same is not necessarily so for other commonly transplanted organs. Liver transplants are less likely to be associated with such rejection. Indeed, combined liver-kidney transplantation has been associated with a reduced risk of rejection of either transplanted organ (Rasmussen et al 1995). This has lead to the suggestion that the liver in some way may be tolerogenic (Garnier et 1965, Calne et al 1969 ,Morelli et al. 1999). It has been suggested that the tolerogenicity of liver transplants may in part be related to the high percentage of passenger leukocytes, in the form of immature dendritic cells, resident within the liver (Thomson et al. 1995b; Thomson et al. 1999). Alteration of the balance in favor of maturity between immature and mature liver DC by the administration of fms-like tyrosine kinase 3 ligand (Flt-3L) can produce acute liver allograft rejection in otherwise spontaneously tolerant murine models (Steptoe et al. 1997). Apoptosis of graft infiltrating T cells in "spontaneously" tolerant murine liver transplant models has been described and a role for donor DC in this process suggested (Qian et al.

1997). In a study of spontaneously tolerant rat liver transplants by Sharland *et al* the rapid migration of donor derived DC to recipient lymphoid tissue was noted to be accompanied by the upregulation of mRNA for IL-2 and IFN-γ. Markers for apoptosis indicated extensive apoptosis occuring within recipient lymphoid tissue in spontaneously liver grafts that did not occur in renal allografts (which are promptly rejected) within the same system. This suggested that an active process akin to "activation induced cell death" was occuring to facilitate successful liver transplantation (Sharland et al. 1999).

Another mechanism by which dendritic cells might modulate response to allo-antigens within recipient lymphoid tissue may be via the induction of T regulatory cells. A subset of T regulatory cells called Tr1 cells was found after cloning of allo-antigen activated human T cells exposed to the presence of IL-10 (Groux et al. 1997). These cells secrete IL-10, TGF- β but low levels of IL-2 and no IL-4 (Roncarlo et al. 2001). Under non-inflamed conditions, antigen transported to the draining lymph node via DC may be presented to T cells in the absence of DC co-stimulatory molecule expression and Tr1 like cells be produced. Evidence for this was recently shown in humans where a single injection of immature monocyte-derived DC pulsed with influenza matrix protein resulted in the generation of "regulatory" influenza matrix-protein specific IL-10 producing cells (Dhodapkar et al. 2001). The important implication of this study for allo-transplantation is that immature DC are capable of induction of antigen-specific hyporesponsiveness via an active regulatory mechanism. Conversely it also carries an important caveat for DC cancer therapy in that presentation of tumour antigen by immature DC may unintentionally inhibit an anti-tumour response (Roncarlo et al. 2001).

Dendritic cells of recipient origin also play an important role in rejection, mediating the 'indirect presentation' of allorecognition (see figure 1.4). Specifically, DC of recipient origin migrate into the transplanted organ where they are capable of taking up donor-derived MHC peptide for presentation to the recipient immune system. Recipient-derived DC can also generate cytotoxic T lymphocytes as mentioned above in response to donor derived minor histocompatiblity antigens via cross priming (Bennett et al. 1997; Heath et al. 1999). The 'indirect pathway' has been implicated in the aetiology of chronic allograft rejection (Gudmundsdottir et al. 1999; Murphy et al. 1999).

1.7 Chimerism and Transplantation

In 1992 Starzl *et al* detected persistent donor origin cells, of dendritic morphology in the non-graft tissues of long standing transplant recipients. He introduced the concept of 'microchimerism' as a possible mediator of long-term allograft acceptance (Starzl et al. 1992a; Starzl et al. 1992b; Thomson et al. 1995a; Starzl et al. 1997). The persistence of donor-derived cells has also recently been shown to correlate with stable long-term lung allograft function (O'Connell et al. 1998). In a study of rat cardiac allografts, both donor-specific transfusion and the presence of donor DC were required to develop organ transplant tolerance (Josien et al. 1998). The persistence of donor-derived DC within long term tolerant primate renal allografts was recently demonstrated using deoxyspergualin (an inhibitor of NF–κβ). In these studies, deoxyspergualin pretreatment was given to kidney donors immediately preceding transplantation (Thomas 1999a). Although several studies now exist correlating the presence of donor-derived cells with successful

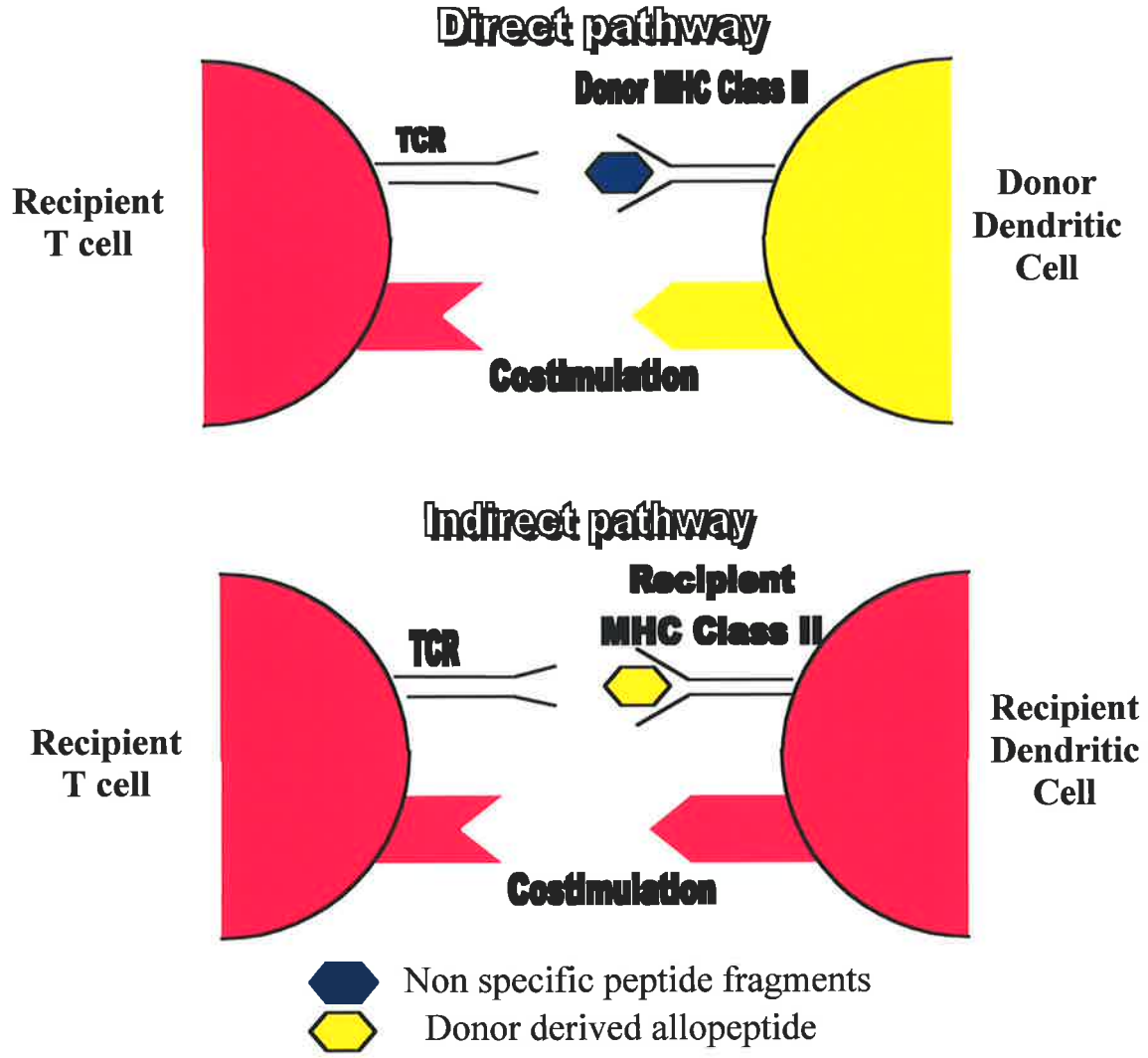


Figure 1.4

Donor-derived DC migrate from transplanted organs into the T cell rich lymphoid areas of the transplant recipient where they are capable of directly activating recipient T cells via the direct pathway of allorecognition. Non specific peptide fragments are presented to recipient T cells in association with donor MHC class II molecules. Indirect allorecognition involves recipient DC moving into the allograft and processing donor-derived alloantigens for presentation to recipient T cells in association with recipient MHC class II molecules

long-term transplant outcome (Starzl et al. 1993; Burlingham 1996; Bishop et al. 1997; Elwood et al. 1997; O'Connell et al. 1998), a body of literature also exists showing the converse (Suberbielle et al. 1994; Ishida et al. 1996; Sivasai et al. 1997). Thus, whether or not microchimerism is a cause, a consequence or a prerequisite for long-term allograft acceptance remains controversial.

1.8 Dendritic Cells and Transplantation Tolerance Induction

There are a number of potential ways in which DC may play a role in the induction of tolerance for allotransplantation. Firstly, allogeneic DC may induce T cell anergy within the recipient T cell compartment by presentation of donor MHC antigens with low levels of co-stimulatory molecules, thereby failing to produce full T cell activation.

Secondly, DC may produce immune deviation amongst the T cell population within the allograft recipient from TH1 towards TH2 T cell phenotype. Evidence that immune deviation from TH1 towards TH2 as a mechanism may be efficaceous in modifying disease processes itself comes from studies of murine experimental autoimmune encephalomyelitis (EAE) in which a switch in T cell phenotype from TH1 towards TH2 was successful in ameliorating established EAE (Kuchroo et al. 1995). Whether or not immune deviation, by itself, is capable of modifying allograft survival is as yet unclear (Piccotti et al. 1997). The capacity of human G-CSF mobilized DC (present within the bone marrow harvested from donors treated with G-CSF) to preferentially induce the DC2 subset and produce immune deviation towards TH2 has been recently described (Arpinati et al. 2000). It has

been suggested that the DC2 subset may be playing a role in reduction of graft-versus-host disease in these patients.

Thirdly, DC may induce clonal deletion of alloreactive T cell clones. As mentioned, central thymic deletion of auto-reactive T cells is the mechanism by which auto-reactive T cells are deleted from the circulating T cell population during T cell ontogeny. The thymic selection process depend upon the strength of T cell receptor (TCR) and major histocompatibility complex-peptide interactions. High affinity binding results in negative selection of a T cell clone, whereas low avidity binding results in positive selection. As DC are highly effective antigen presenting cells, the use of host DC pulsed with *allo* MHC peptides injected intra-thymically would expose developing T cells to allo-peptide and potentially results in negative selection of clones responsive to that peptide MHC complex. Utilizing surrogate thymic DC to regulate the T cell population for induction of antigen-specific hyporesponsiveness is another novel strategy. This strategy has been tested in a pancreatic islet transplantation model. Injection of bone marrow-derived DC pulsed with an MHC class I peptide into recipient thymus of streptozotocin-induced diabetic Wistar-Furth rats in combination with anti-lymphocyte serum, followed by islet transplantation resulted in permanent (>200 days) islet allograft survival in some animals (Ali et al. 2000). Furthermore, when peptide-pulsed DC were administered without anti-lymphocyte serum 2/5 animals developed >200 day graft survival. The underlying mechanism for this acquired thymic tolerance is as yet unknown, but may be linked epitope suppression. As the allopeptide used in this study is presented by syngeneic DC, this study also indicates the importance of the indirect pathway of allorecognition in acquired thymic tolerance.

As mentioned in 1.5, DC may also promote generation of regulatory cells or veto cells. The veto effect is a functional inactivation of an alloantigen responsive Cytotoxic T cell precursor (CTLp) and is mediated by an allo-antigen expressing cell. The alloantigen expressing cell is called a veto cell and in both rhesus and murine studies, this cell type expresses CD8α^+. It appears that the CD8 molecule itself may play an important role in the mediation of the veto effect (Zhang et al. 1995; Asiedu et al. 1999). Recently a murine cell bearing CD8 α^+ with morphological similarities to a DC has been reported to promote bone marrow engraftment (Gandy et al. 1999).

1.9 Dendritic Cell Subsets and Transplantation

The existence of DC subsets and the speculation that specific subsets may play a role in peripheral tolerance has encouraged studies of the purified subsets with allotransplantation. To date there have been no specific trials of DC based therapy in human organ transplantation. However studies of the effect of adjuvant infusion of donor-derived bone marrow upon organ transplantation have been reported (Salgar et al. 1999). Donor bone marrow contains a significant population DC and part of the postulated immunomodulatory effect of donor bone marrow infusion is the potential tolerogenic effect of the resident DC population. Preliminary studies in certain organ transplants has suggested a difference in some clinical outcomes favoring the bone marrow augmentation treatment arm in pancreatic transplantation (Corry et al. 1999), cardiac transplantation (Pham et al. 2000) and lung transplantation (Pham et al. 2000). It remains to be seen if long-term significant clinical differences will emerge between patients treated with adjuvant bone marrow and those treated with standard therapy. Clinical studies of the effect

of administration of cadaveric bone marrow post transplantation has suggested some tolerogenic effect, and this has been suggested to be as a consequence of "lymphoid" DC within bone marrow fractions.

Within human bone marrow transplantation, the administration of granulocyte-colony stimulating factor (G-CSF)to bone marrow donors promotes immune deviation towards TH2 via preferential induction of DC2 subsets within the donor marrow. Human subjects treated with G-CSF show a significant increase in DC2 in peripheral blood. When recipients of G-CSF mobilized unmodified blood stem cells were analyzed, it was apparent that these patients received a greater dose of DC2 cells than those patients receiving unmodified bone marrow without G-CSF (Arpinati et al. 2000). The authors speculate that the presence of DC2 within peripheral blood stem cells of G-CSF mobilized bone marrow donors plays a significant role in the prevention of graft-versus-host disease in bone marrow transplant recipients. Whether such an enhanced anti-rejection effect would be seen with infusions of purified DC subsets in other organ transplants is currently unknown.

The effect of $CD8\alpha^+$ DC upon murine cardiac allograft survival has recently been studied and reported. As DC subsets have differential effects of T cell activation the manipulation of dendritic cell subsets to prolong allograft survival is another potentially attractive means to modify organ transplant outcome. When $CD8\alpha^+$ DC were isolated from the spleens of C57B/10J ($H2^b$) mice treated with the potent DC mobilizing cytokine Flt-3L were administered intravenously to C3H mice 7 days prior to vascularized allogeneic cardiac allografting, mean allograft survival time was prolonged from 11 days (controls) to 23.5 days ($P<0.001$)(O'Connell et al. 2001)

1.10 Interleukin 10 and Transplantation

Interleukin-10 (IL-10) is a cytokine that antagonizes the effects of TH1 cytokines (IL-12 and IFN-γ). It was first described in 1989 as a factor that inhibited cytokine production in co-cultures of TH1 cells, antigen presenting cells and antigen by (Fiorentino et al 1989 , Mosmann 1993). It has subsequently been shown to have multiple immunological effects upon a wide variety of cell types. These effects include inhibition of cytokine production within macrophages in response to a variety of stimuli, inhibition of cytokine production by NK cells and importantly inhibition of cytokine secretion by TH1 and $CD8^+$ cells (Mosmann 1993). IL-10 also has a number of important effects upon DC function that favour its use to modify DC function. IL-10 can prevent differentiation of DC from monocytes (Buelens et al. 1997b). IL-10 prevents upregulation of DC co-stimulatory molecules (Enk et al. 1993). From the transplantation point of view, one of the most important of these effects is inhibition of the potent allo-stimulatory cytokine IL-12 (De Smedt et al. 1997). The effects of IL-10 upon the DC and T cell are summarized in figure 1.5 and 1.6.

In view of these effects it has been proposed that IL-10 would be a suitable agent to subvert the immune response away from TH1 and towards a TH2 response. *Ex vivo* gene transfer of this cytokine to transplanted tissues has been studied. Viral IL-10 DNA (see below) was introduced into murine cardiac allografts by DeBruyne et al using cationic liposomes and resulted in prolongation of Mean Survival Time from 8.28 days to 16 days) (DeBruyne et al. 1998). Similar modest prolongation of allograft survival was found when an adenoviral IL-10 gene

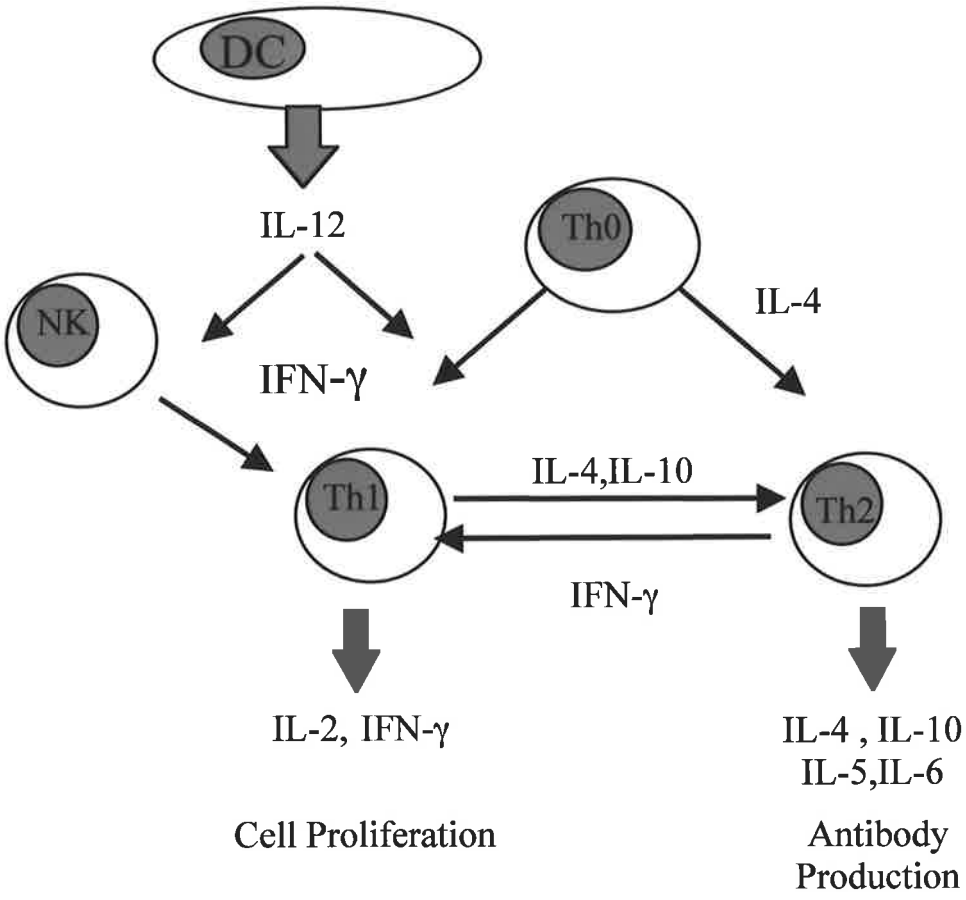


Figure 1.5

The TH1/TH2 paradigm as it pertains to allotransplantation indicating the predominant role that IL-12 secreted by dendritic cells (DC) has in polarization of the T cell response towards a TH1 response. The arrows adjacent to named cytokines indicate the effect those cytokines have upon the T cell populations. Thus IL-10 and IL-4 have diametrically opposed actions to interferon-gamma (IFN-γ).

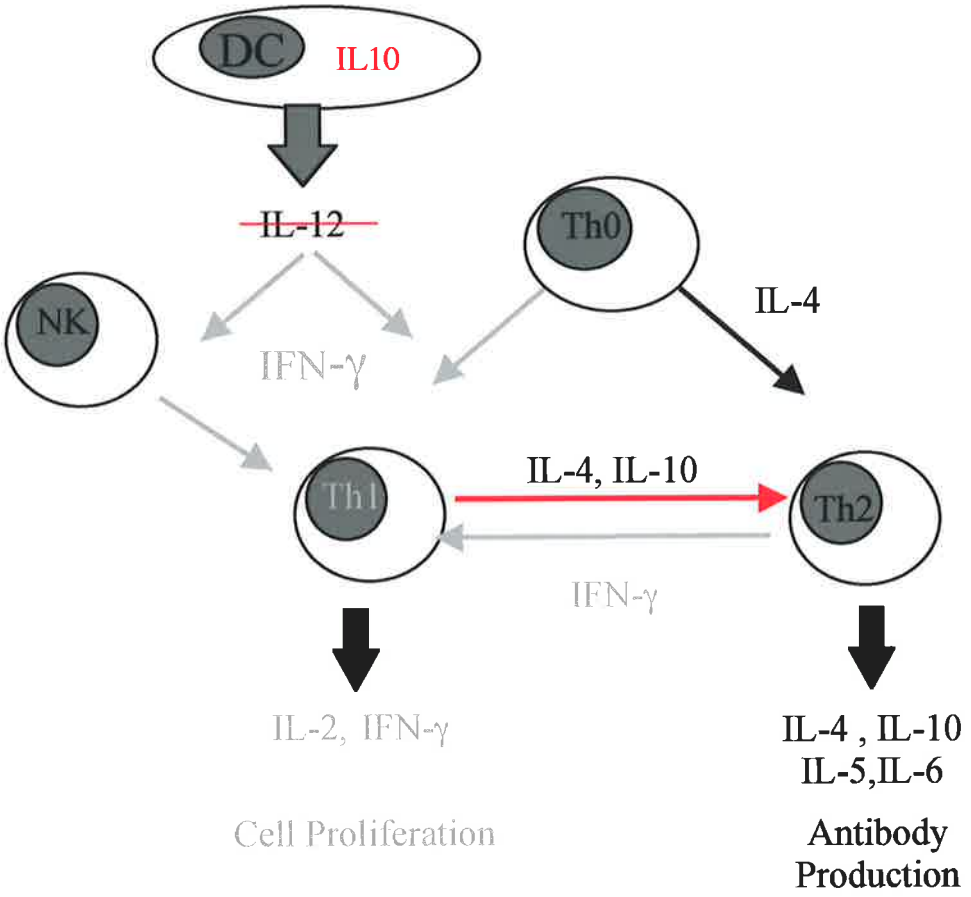


Figure 1.6

The predicted effects of AdV IL-10 transduction into DC upon the TH1/TH2 cell subsets. The inhibited pathways are shown in grey and the effects of IL-10 upon T cell polarization are shown in red. IL-10 antagonizes the effect of INF-γ and polarizes the TH response towards TH2. Note the inhibition of IL 2 induced TH1 clone proliferation by IL-10.

construct was used to deliver IL-10 directly into rat cardiac allografts (David et al. 2000). However it appears that the form of IL-10 is also critical in the outcome of the immune response. Two well characterized forms of IL-10 have been described. The first form is the mammalian or cellular IL-10 (Fiorentino et al. 1991). The second commonly studied form of IL-10 is viral IL-10. This protein was originally identified within the genomic sequence of Epstein-Barr virus (EBV) and had high sequence homology with murine and human IL-10 sequence. As IL-10 has inhibitory properties on T cells and macrophages it has been suggested that acquisition of an "IL-10 like gene" from mammalian cells would be advantageous to a virus (such as EBV) seeking to evade immune surveillance (Moore et al 1990,Mosmann 1993). The key difference between mammalian IL-10 and viral IL-10 are that under certain conditions, mammalian (cellular) IL-10 may be allostimulatory, whereas viral IL-10 may be more uniformly immunosuppressive.

1.11 Gene Therapy

Gene therapy is the process by which genetic material encoding useful function is introduced into biological systems for the purpose of induction of beneficial biological effect (Anderson 1998). Within the field of transplantation, gene therapy offers the potential to either modify the immunogenicity of the donor organ or promote donor specific hyporesponsiveness within the transplant recipient (including tolerance induction).

There are several choices of gene delivery vectors for gene therapy (Gojo et al. 2000). These include viral and non-viral gene delivery methods. The currently available viral vectors include retrovirus, adenovirus and adeno-associated virus. Most of the modulation of the allograft studies have involved testing of a variety of

genes delivered by adenoviral or retroviral gene therapy vectors. Genes targeted include viral IL-10 (Takayama et al. 1999), IL-12-p40 (Kato 1996), Transforming Growth Factor beta (TGF-β) , CTLA-4-Ig (Lu et al. 1999) and FasL (Zhang et al. 1998; Min et al. 2000).

1.12 Genetic Engineering of Dendritic Cells for Transplantation

The recognition that DC could present antigens in either a tolerogenic or immunogenic manner has lead to the concept that manipulation of DC function may promote allograft acceptance (Finkelman et al. 1996). As *in vitro* generated DC are capable of migration to sites of antigen presentation within the body (Barratt-Boyes et al. 1997), this suggests that genetically modified DC offer the potential to deliver alloantigens to the recipient immune system in the context of a tolerogenic stimulus. This has the clear advantage of avoiding or reducing systemic administration of immunosuppressants and potentially providing MHC-specific immunosuppression. Using DC as targets for transplantation gene therapy has a number of theoretical advantages. Firstly these cells could be manipulated *ex vivo* with the gene delivery vector thus limiting the amount of viral vector needed to target the desired cell type and avoiding the toxicity of exposure of the organism to the viral vector. Secondarily, *ex vivo* gene transduction might reduce the exposure of the immune system to adenoviral proteins against which an immune response might be mounted, that would occur upon systemic administration of a gene therapy product. At least theoretically this could enhance the usefulness of adenoviral vectors. Hence targeting the key antigen presenting cell using gene therapy in this manner has lead to the potential for DC to act as 'Trojan Horses' for the graft.

Experimental evidence for the potential of manipulating DC for transplantation comes from studies of the in vivo effects of the potent DC growth factor, fms-like tyrosine-kinase 3 ligand (Flt-3 ligand). The expansion of mature DC within mice treated with Flt-3 ligand is associated with enhanced immune responses against soluble antigens (Pulendran et al. 1998). When Flt-3 ligand is given to mice prior to organ transplantation, augmented allograft rejection is observed, and administration of Flt-3 ligand is capable of reversing the acceptance of spontaneously tolerant murine liver transplants, via induction of allostimulatory cells (Steptoe et al. 1997).

Evidence that DC may be manipulated for DC in experimental transplantation comes from cardiac allografts transplanted from donor mice rich in immature dendritic cells (with low co-stimulatory molecule expression) which showed prolonged allograft survival (Fu et al. 1996b). The fact that these allografts were still rejected was attributed in part to maturation of these cells within the recipient post transplantation. This lead to the speculation that genetic modification of donor DC to block maturation within the recipient might be a successful transplantation strategy. Similar findings have been reported by giving DC precursors in the Lewis rat cardiac transplant model (Hayamizu et al 1998).

Presentation of donor-derived antigens by DC with low expression of co-stimulatory molecules can induce hyporesponsiveness (anergy) to donor antigens (Lu et al. 1995). Using gene therapy to genetically engineer DC to prevent co-stimulatory molecule expression is thus an attractive means of modifying DC

function to favor tolerance induction (Giannoukakis et al. 1999). Both adenoviral and retroviral gene therapy vectors have been used to introduce a variety of genes into DC for modification of DC function (Aicher et al. 1997a; Dietz et al. 1998; Zhong et al. 1999). Genetic modification of DC to reduce expression or inhibit function of co-stimulatory molecules expression has been achieved using genes for IL-10, transforming growth factor beta (TGF-β) and CTLA4Ig (Zhong et al. 1998; Lu et al. 1999). Dendritic cells have also been transduced with other cell death promoting molecules including CD95L (FasL) cDNA in order to generate "killer" DC which have been capable of induction of antigen specific immunosuppression (Matsue et al. 1999). This study is particularly important as it shows for the first time that engineering of DC with immunosuppressive molecules enhances their survival in allogeneic hosts.

The critical role of donor DC maturational status in allograft outcome was shown by studies in the rhesus monkey renal transplantation model. A novel approach to preventing DC maturation using the NF-κB inhibitor deoxyspergualin (DSG) was undertaken at the University of Birmingham, Alabama. In these experiments, alloreactive rhesus macaques monkeys were treated with a combination of anti-CD3 immunotoxin and a 15 day course of DSG commencing 4 hours pre-transplant. This combination was associated with the development of long -term (>3 years) kidney allograft survival without evidence of chronic allograft nephropathy. Split skin grafts from 3rd party animals were promptly rejected indicating donor specificity of the DSG therapy. Treatment of recipient animals with DSG acts to inhibit nuclear translocation of NF-κB from the cytoplasm and into the DC nucleus and thus "stun" the donor-derived and recipient DC-

preventing their activation and keeping them in a immature state. Immunotoxin therapy administered at the time of transplantation depleted memory T cells and thus the recipient T cell population encountered only DC that were functionally immature. When peripheral lymph nodes from treated animals were sampled and resident DC examined by immunohistochemistry, a marked reduction in mature DC was observed in animals treated with DSG (Thomas 1999a).

Further weight has most recently been given to the concept that immature DC can promote prolonged allograft survival by the work of Lutz and Austyn. Bone marrow-derived dendritic cells (called GM^{low} DC), were generated from B10 mice with low dose GM-CSF (20 U/ml) for 8 days and were shown to retain immature DC phenotype and be resistant to the maturational stimuli such as LPS, TNF-α and CD40 ligation. Furthermore when $5x10^5$ GM^{low} DC administered 7 days pre-transplant to CBA recipient mice they showed impressive ability to prolong murine cardiac allograft survival (>100 days)(Lutz et al. 2000). These studies were also remarkable in that the effect on allograft survival was lost when the cells were administered at day -3, day-14 or day-28. The reason for this is currently unknown.

Another approach to modification of DC function to retain an immature state is targeting the NF-κ B cell activation pathway by anti-sense oligonucleotide therapy. Short oligodeoxynucleotides (ODN) with consensus binding sequences to NF-κB inhibit DC allostimulatory capacity by blocking nuclear translocation of NF-κ B which in turn inhibits cell surface co-stimulatory molecule expression. Bone marrow DC from B10 mice treated with NF-κ B ODN for up to 36 hours and administered as a single dose intravenously 7 days prior to transplantation into

C3H recipient mice significantly prolong murine B10 heart allografts survival (Giannoukakis et al. 2000).

The potential for genetic modification of DC to prolong allograft survival has been shown by a number of small animal transplant studies. O'Rouke *et al* engineered murine DC with CTLA4 and prolonged pancreatic islet survival (O'Rourke 2000). In these studies a DC cell line was modified with the immunoregulatory molecule CTLA4. Genetically modified DC were administered intravenously on the day of transplantation and again intravenously 6 days after transplantation with significant prolongation of islet allograft survival. Min *et al* engineered DC with FasL (Min et al. 2000) and Giannoukakis with the NFKB oligonucleotide decoy molecules (Giannoukakis et al. 2000). Lu *et al* have demonstrated that murine DC may be effectively transduced with adenoviral encoded CTLA4Ig. These cells transduced with this immunomodulatory molecule demonstrated profound alloinhibitory properties and showed enhanced survival within allogeneic recipients (Lu et al. 1999).

Although the modification of DC with immunosuppressive cytokines has shown great promise *in vitro* and *in vivo* in small animal models, *in vivo* large animal studies are needed to clarify the potential for these cells to prolong allograft survival. Whether genetic modification of DC alone will prove to be sufficient to modify the course of transplanted organs remains to be determined.

1.13 Summary

Basic immunology has given insights into the factors that control DC biological functions. It is clear that DC play a central role in tolerance and immunity to a wide variety of antigens and may well be involved in maintenance of peripheral tolerance at later time points. Manipulating DC function or DC subsets holds great promise as possible new biological therapy for transplantation that may allow a reduction in conventional immunosuppression and potentially long term donor specific tolerance. A number of genes can alter the allo-stimulatory properties of DC and have been successful at prolonging allograft survival. However many questions still remain to be answered. There is no profoundly convincing small or large animal data to suggest that one single gene introduced into DC may be capable of inducing *permanent* allograft acceptance either alone or in combination with other therapy. Nevertheless exploiting the capacity of donor DC to modify recipient T cell function offers a potentially fruitful avenue for transplantation research.

Thesis Hypothesis

The principal hypothesis to be tested in this thesis is that transduction of immature dendritic cells with an adenoviral gene construct encoding the cytokine gene interleukin-10 will inhibit the allostimulatory properties of the dendritic cell. As interleukin-10 is a cytokine that inhibits dendritic cell maturation, the underlying assumption is that dendritic cells transduced with adenoviral interleukin-10 will remain functionally immature. The phenotype of immature dendritic cells is characterized by low cell surface expression of costimulation molecules CD80 and CD86. It is known that T cells stimulated by dendritic cells in the absence of full expression of dendritic cell costimulation molecules become anergic. It is hypothesized that allogeneic T cells encountering interleukin-10 transduced dendritic cells (with low level costimulation molecule expression) will fail to become activated. In the context of allotransplantation, it is hypothesized that donor-derived dendritic cells genetically modified with an interleukin-10 gene contruct will inhibit activation of allogeneic T cells and that this will be a means to prolong survival of an allograft.

Chapter 2

Materials and Methods

2.1 Mononuclear Cell Protocols

2.1.1 Isolation Of Peripheral Blood Mononuclear Cells (PBMNC)

Whole blood was collected from donors in heparinized tubes, centrifuged at 250*g* for 10 min and the platelet-rich plasma removed and discarded. The remaining blood was diluted 1 in 4 in PBS, underlaid with 2 mls of Lymphoprep (Nycomed, Norway) and centrifuged at 920*g* for 20 min. The mononuclear cell layer at the interface was aspirated, pelleted at 1300*g*, washed twice in PBS and resuspended tissue culture medium (see section 2.9.1) supplemented with 10% fetal calf serum (CSL, Australia) at a density of 2×10^6 cells/ml.

Ovine mononuclear cells were isolated using the same protocol, except that the centrifuge was run at 1000*g* for 30 minutes.

When human PBMNC were isolated from buffy coat product, a modified procedure was followed in which 10ml of buffy coat was diluted with 30ml of PBS in 50ml sterile V bottom tubes. The cell suspension was then underlaid with 10ml of Lymphoprep and the identical centrifugation protocols followed as above.

2.1.2 Two way Mixed Leukocyte Culture

PBMNC from two donors were plated out in complete tissue culture medium (in a 1:1 ratio) into 96-well round bottomed tissue culture plates (Corning, USA) to a final cell number of 1 x 10^5 cells per well. Each well volume was increased to 200 µl with tissue culture medium or with an appropriate dilution of monoclonal antibody in tissue culture medium. The plates were incubated at 37^{0}C in a 5% $C0_2$ enriched and humidified atmosphere for 2 or 4 days. Individual wells were then pulsed with 1 µCi (37mBq) of tritiated thymidine and incubated for a further 20 hours under the same conditions. The cells were harvested using a Microtitre TomTek Cell Harvester (Torku, Finland) onto glass fibre filter mats and tritium incorporation was measured in the liquid phase using MicroBeta Liquid Scintillation Counter (Wallac, Finland). Results are expressed as the mean of triplicate wells +/- standard deviation.

2.2.2 One-way Mixed Leukocyte Culture

Stimulator cell populations (2 x 10^5 cells/ml) were treated with 0.1 mg/ml of Mitomycin C (Sigma, Australia) dissolved in tissue culture medium and incubated at 37^{0}C for 30 min. Cells were washed twice in PBS and once in tissue culture medium prior to being resuspended at a density of 2 x 10^6 cells/ml in tissue culture medium. All subsequent steps were as described for the two-way MLC. Stimulator:responder ratios were calculated starting at a ratio of 1:1 and ranging to 1:1000 cells.

2.2.3 Concanavalin A (Con A) Stimulation Of PBMNC to generate blast cells

Isolated ovine PBMNC were resuspended at a density of 1 x 10^6 cells/ml in tissue culture medium. Cells were aliquoted into tissue culture flasks (Dow Corning,

USA) and were stimulated with 5 μg/ml Con A for 48 hours. After resuspension cells were pelleted at 1200*g*. Every 48 hours the cell culture was supplemented with tissue culture supernatant derived from cultures of monkey MLA-144 cell line (a source of IL2).

2.3 RNA Extraction

Total RNA was prepared using a modification of the acid-guanidium thiocyanate-phenol-chloroform extraction method described by (Chomczynski et al. 1987). Tissue or cells were extracted into Solution D and vortexed thoroughly. To 0.5 ml of extract was added sequentially:

- 50 μl 2M sodium acetate (pH 4.0)
- 500 μl water-saturated phenol
- 100 μl chloroform: iso-amyl alcohol (49:1)

with the mixture vortexed between each addition. The solution was placed on ice for 15 min and centrifuged at 13,500*g* for 20 min at 4^{0}C. The RNA in the upper aqueous phase was removed, a volume of isopropanol equal to the aqueous volume added and the mixture vortexed prior to precipitation at -70^{0}C for 15 min. After centrifugation at 13,500*g* for 20 min at 4^{0}C the supernatant was discarded and the pellet was dissolved in 300 μl of Solution D. An equal volume of isopropanolol was added and the mixture vortexed and re-precipitated for 15 min at -70^{0}C. The sample was centrifuged again at 13,500*g* for 10 min at 4^{0}C, the supernatant discarded and the RNA pellet washed twice in 75% ethanol. The purified RNA pellet was air dried, resuspended in 10 μl of DEPC water and heated to 65^{0}C for 5 min to ensure complete dissolution of the pellet.

The concentration of RNA was measured by preparing a 1 in 50 dilution of the sample in DEPC water and measuring the spectrophotometric absorbance at 260 nm (Beckman, USA). An optical density unit of 1 corresponded to 40 μg/μl of total RNA. Stock RNA samples were then diluted with an appropriate volume of water to achieve a final RNA concentration of 1 μg/μl.

2.4 Reverse Transcription (RT)

First strand complementary DNA (cDNA) was synthesised by reverse transcription using a modification of the method of Montgomery and Dallman (1991). One microgram of total RNA was added to 4 μg of oligo dT (Pharmacia, Sweden) and incubated at 65^{0}C for 5 min. The mixture was snap cooled on ice prior to the addition of:

- 40 U of RNAsin (Promega, USA)
- 200 U of Moloney Monkey Leukaemia Virus (GibcoBRL, USA) reverse transcriptase in a final concentration of 10 mM dithiothreitol
- 1 x first strand reverse transcriptase buffer (GibcoBRL, USA)
- 4 mM total deoxynucleotide triphosphates (dNTPs, Promega, USA)

After 60 min incubation at 37^{0}C the reverse transcriptase was inactivated by heating to 70^{0}C for 10 min and snap chilling on ice. Finally the volume of cDNA was increased to 100 μl with sterile distilled water. Samples were stored at -20^{0}C.

2.5 Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a TC1 Perkin Elmer DNA Thermal Cycler (Perkin Elmer, USA). Oligonucleotide primer sequences used for PCR amplification in were either designed *de novo* from published sequence data and

verified using the GeneBank Database or taken from literature sources as indicated in table 2.5.1.

For each PCR reaction a volume of 2.5 µl of stock cDNA was added to:

- 1 x PCR buffer (see General Buffers)
- 1 µM forward primer
- 1 µM reverse primer
- 800 µM total dNTPs
- 0.5 units AmpliTaq DNA polymerase
- water to a final volume of 22.5 µl.

The mixture was sealed with a single drop of mineral oil to prevent evaporation losses. Relevant positive (cDNA from Con A stimulated PBMNC) and negative (reverse transcribed distilled water) controls were included in all experiments

Table 2.5.1 Primer Sequences for Polymerase Chain Reaction

gene	Sequence
B actin F	ATC ATG TTT GAG ACC TTC AA
B actin R	CAT CTC TTG CTC GAA GTC CA
ovIL-2 F	AAC TCT TGT CTT GCA TT
ovIL-2 R	GAT GCT TTG ACA AAA GGT
IL-4 F	TTA ATG GGT CTC ACC TCC C
IL-4 R	TTC CAA GAG GTC TCT CAG CG
ovIL-10 F	GCT GTT GAC CCA GTC TCT
ovIL-10 R	AGA AAA CGA TGA CAG CG
ovIL-12p35 F	GCA ACA CGA TTC AGA AGG CCA
ovIL-12p35 R	CCT CTT AGG ATC CAT CAG AAG C
ovIL-12p40 F	TTT GGA GAT GCT GGG CAG TAC A
ovIL-12p40 R	GAT GAT GTC CCT GAT GAA GAA GC

2.6 Gel Electrophoresis

PCR products were electrophoresed through 2% agarose gels in a Bio-Rad Minigel apparatus. One lane in each gel was loaded with 1 μg of PUC19 (Bresatec, Australia) pre-mixed with 2 μl of 6 x loading buffer (see section 2.9.4) as a molecular weight marker. Sample lanes were loaded with 10 μl of PCR product pre-mixed with 2 μl of the same loading buffer and electrophoresed for approximately 45-50 min at 90 volts. Each gel was then stained with ethidium bromide (1.25 μg/ml) for up to one hour and photographed under UV light using Kodak Instant Film (Kodak 667 instant film, Kodak, Australia).

2.7 Flow Cytometric Analysis (FACS)

All washes were performed by resuspending the cells in cold FACS wash buffer (see General Buffers) and pelleting by centrifugation at 200*g* for 5 min at 4^0C.

2.7.1 One colour analysis

After two washes the cells were resuspended at a density of 5×10^6 cells/ml in FACS wash buffer and 100 µl aliquots dispensed into FACS tubes. Heat inactivated rabbit serum was added to each tube to a final concentration of 10% (v/v) to block non-specific Fc receptor binding by antibody. Samples were incubated for 10 min before the addition of a single conjugated or unconjugated primary antibody was used. When the primary antibody was unconjugated, the cells were washed after the 30 minute incubation and an appropriate secondary antibody was added in a final dilution of 1 in 500 in FACS wash buffer. The samples were then incubated for 20 min at 4^0C and a further 5 min at room temperature prior to washing. The cells were then lysed by the addition of 1 ml of fresh 10% FACS lysing solution and incubation at room temperature for 20 min. Prior to analysis the cells were resuspended in 400µl of FACS wash buffer.

2.7.2 Two colour analysis

Cells were harvested using described techniques. An identical procedure was followed as for 1 color analysis. After the primary FITC steps were performed a PE-conjugated primary antibody was added and further incubation for 30 min performed. The cells were then lysed by the addition of 1 ml of fresh 10% FACS lysing solution and incubation at room temperature for 20 min. After two further

washes the cells were resuspended in 350 µl of filtered saline and stored at 4^0C in darkness until analysed using a Becton Dickinson FACScan flow cytometer (USA).

2.8 Electron Microscopy

Fresh cells were collected into sterile 15ml tubes containing the following fixative.

Fixative	4% formaldehyde 1.5% glutaraldehyde in 0.1M Sodium cacodylate

2.9 Buffer Solutions

2.9.1 General buffers

FACS washing buffer	1 x PBS, 0.1% sodium azide, 2% FCS
Phosphate buffered saline (PBS)	4 mM sodium dihydrogen orthophosphate, 120 mM sodium chloride, 16 mM sodium hydrogen orthophosphate
Tissue culture medium	RPMI 1640 supplemented with 50 units/ml of penicillin/streptomycin, 100 mM sodium pyruvate,0.2% sodium bicarbonate, 10 mM HEPES and 2 mM L-glutamine. pH adjusted to 7.3 by bubbling through CO_2

2.9.2 RNA buffers

Chloroform/iso-amyl alcohol	Solution of 49 parts chloroform to 1 part iso-amyl alcohol
DEPC water	redistilled water stirred with 0.1% v/v DEPC for 30 min at RT and autoclaved
Phenol (water equilibrated)	hydroxyquinolone was added to colour the phenol yellow, to prevent oxidation and to inhibit RNases
Stock denaturing solution	4 M guanidine thiocyanate salt, 25 mM sodium citrate pH 7.0, 0.5% N-lauroylsarcosine dissolved at 65^0C
Solution D	β–mercaptoethanol to a final concentration of 0.1 M in stock denaturing solution

2.9.3 PCR buffers

Standard 5 x PCR buffer	250 mM KCl, 50 mM Tris (pH 8.3), 12.5 mM $MgCl_2$, 0.5 mg/ml gelatin

2.9.4 Gel electrophoresis buffers

1 x TAE	32 mM Tris, 16 mM sodium acetate, 0.8 mM sodium EDTA pH 7.2
6 x loading buffer	1 x TAE, 50% glycerol, 24% saturated bromophenol blue

2.10 Reagents

ABC staining kit	Vector Laboratories, USA

Acetone	Ajax, Australia
Agarose	Progen, Australia
Ammonium sulphate	BDH, Australia
β-mercaptoethanol	Sigma, USA
Biotin	Calbiochem-Novabiochem,
Biotin blocking kit	Vector Laboratories, USA
Biotinylated horse anti-mouse IgG	Vector Laboratories, USA
Biotinylated rabbit anti-rat IgG	Vector Laboratories, USA
Calcium chloride	Ajax, Australia
Chloroform	Ajax, Australia
Concanavalin A type IV	Sigma, USA
Diaminobenzidine tetrahydrochloride	Sigma, USA
Diethylpyrocarbonate	Sigma, USA
Dimethyl sulphoxide	Ajax, Australia
Disodium hydrogen orthophosphate	Ajax, Australia
dNTP kit	Promega, USA
1,2 Epoxypropane	BDH, UK
EDTA (ethylenediamine tetraacetic acid)	Sigma, USA
Ethanol	BDH, Australia
Ethidium bromide	Sigma, USA
FACS lysing solution	Becton Dickinson, USA
Fetal Calf Serum	CSL, Australia
Fluothane	Zeneca, UK
Formalin (buffered 10%)	Orion, Australia
Frusemide	Alphapharm, Australia
Gelatin (porcine skin)	Sigma, USA

Glacial acetic acid	BDH, Australia
Glutaraldehyde	ProSciTech, Australia
Glycerol	Ajax, Australia
Guanidine thiocyanate salt	Sigma, USA
Harris' modified haematoxylin	Sigma, USA
Heparin (porcine mucus)	David Bull, NSW Australia
HEPES	Sigma, USA
8-Hydroxyquinolone	Sigma, USA
Indium oxine	Amersham, UK
Isoamyl alcohol	Sigma, USA
Isopropanol (anhydrous)	Sigma, USA
L-glutamine	Cytosystems, Australia
Lipopolysaccharide	Sigma, USA
Magnesium chloride	Sigma, USA
Mannitol	Baxter, Australia
Methanol	Ajax, Australia
Methyl ^{3}H thymidine	Amersham, UK
MMLV reverse transcriptase	GibcoBRL, USA
N-lauroylsarcosine	Sigma, USA
Nembutal	Boehringer Ingelheim, Australia
Normal rabbit serum	ICN Biomedicals, USA
Normal saline (0.9%)	Baxter, Australia
Osmium tetroxide	ProSciTech, Australia
Patent Blue V	Sigma, Australia
Penicillin/streptomycin	Cytosystems, Australia
Penstrep	Troy Labs, Australia

Phenol	Progen, Australia
Procure Resin	Electron Microscope Sciences
Potassium acetate	BDH, Australia
Potassium chloride	Ajax, Australia
Potassium dihydrogen orthophosphate	Ajax, Australia
Recombinant human interleukin-4	Peprotech, USA
Recombinant human GM-CSF	Schering Plough, USA
Ross kidney perfusate	Baxter, Australia
RPMI-1640	Sigma, USA
Sodium acetate	Ajax, Australia
Sodium bicarbonate	M&B, Australia
Sodium carbonate (anhydrous)	Ajax, Australia
Sodium chloride	M&B, Australia
Sodium citrate	BDH, Australia
Sodium dihydrogen orthophosphate	Ajax, Australia
Sodium hydrogen orthophosphate	Ajax, Australia
Sodium hydroxide	Ajax, Australia
Sodium pyruvate	ICN Biomedicals, USA
Taq polymerase	Perkin Elmer, USA
Water (pyrogen free)	Baxter, Australia

Chapter 3

Characterization and Transduction of Human Monocyte-Derived Dendritic Cells with Adenoviral Gene Constructs

3.1 Introduction

Advances in immunosuppressive therapy have made organ transplantation one of the outstanding achievements of modern medicine. Yet despite the improvement in organ and patient survival in the past 15 years, transplant rejection and infection remain as major causes of organ loss. The current arsenal of immunosuppressive medications available for the prevention of allograft rejection brings with it an extensive list of side effects and prescription of these medications is costly both to the patient and the community. Furthermore in contrast to the early years of transplantation when patients were usually maintained upon a combination of two agents each given once daily, modern immunosuppressive therapy usually involves a combination of up to four medications with different dosage regimens. The risk of immunosuppression related secondary malignancy rises with each year post transplant and the demand for organs for transplantation continues to outstrip supply. Clearly, therefore strategies that may allow enhanced allograft

acceptance and that may reduce allograft loss without excessive and costly drug therapy are attractive .

There have also been considerable advances in the field of immunology and in particular the understanding of the factors that are important in rejection of transplanted organs. One area that has been expanding area of knowledge regarding the biology of dendritic cells (DC) and the pivotal role that these cells play in terms of rejection or acceptance of transplanted organs. Dendritic cells are sentinel cells of the immune system that have the unique capacity to initiate T cell activation without so called "T cell help"(Banchereau et al. 2000). They reside within tissues as immature cells capable of uptake of antigenic material, which induces DC activation. Once activation has occurred they migrate towards draining lymphoid tissue where they can prime T cells and amplify the immune response via expression of co-stimulation molecules. They have been identified as one of the main components of the passenger leukocyte population within allografts. Migration of donor-derived passenger leukocytes from the transplanted organ into the lymphoid system of the recipient initiates the direct pathway of allo-recognition, and primes the recipient's immune system to generate allo-specific CTL's. The factors that control DC function and the ability to activate T cells have been the subject of much recent work (Banchereau et al. 2000). Whilst the role of DC in initiation of the immune response is well recognized, there is also an emerging body of evidence that DC might also be important in the maintenance of tolerance to self antigens (Fazekas de St Groth B 1998).

Using the recent advances in knowledge concerning the factors that promote the tolerogenicity of DC and gene transfer technology, it should be possible to genetically engineer DC to enhance a tolerogenic outcome of the DC-T cell

interaction. By generating the target cell population (in this case the DC) *ex vivo* and performing gene transfer *in vitro*, delivery of the target gene to the cell of interest can readily be achieved, without the potential for unwanted exposure of the gene construct to other cell types.

A critical cytokine that modifies DC function is IL-10. IL-10 is a TH2 cytokine, originally described in 1989 as a factor capable of switching off cytokine production by T cells (Mosmann 1993). It is expressed by monocytes, mast cells, T regulatory cells and B cells after activation. Its principal effects upon T cells include inhibition of cytokine production and T cell proliferation. The activation of T cells in the presence of IL-10 results in T cell anergy which cannot be reversed by IL-2 or by anti-CD3 monoclonal antibodies (de Waal Malefyt et al. 1999). IL-10 is an immunosuppressive cytokine with multiple immunomodulatory effects upon DC function (Fiorentino et al. 1991). IL-10 down-regulates cell surface expression of the DC co-stimulatory molecules CD80 and CD86 and reduces the ability of DC to capture and process antigens (Morel et al. 1997). IL-10 also inhibits DC production of the potent pro-inflammatory cytokine IL-12 (De Smedt et al. 1997). When added to culture medium, IL-10 prevents the final maturational phase of DC growth (Buelens et al. 1997b; Steinbrink et al. 1997). Recently it has been shown that autocrine IL-10 plays an important role in limiting maturation of monocyte-derived DC and their capacity to produce TH1 responses (Corinti et al. 2001). Therefore, IL-10 is an attractive cytokine to modify DC function to promote a TH2 response within T cells. By using IL-10 to inhibit DC maturation the generation of T regulatory cells could theoretically be enhanced (Roncarlo et al. 2001).

To deliver the IL-10 gene into DC there are several possible experimental approaches. Retroviral mediated gene transfer and adenoviral gene transfer are two of the most studied forms of gene delivery. One of the most effective delivery systems currently available are the adenoviral vector systems (Gojo et al. 2000). These vectors are capable of infection of a wide variety of cell types, and have the specific ability to infect non-dividing cells with close to 100% gene transfer (Anderson 1998). Retroviral vectors, on the other hand, can at best offer only 40% transduction into the target cell line and require the target cell line to be dividing. Once gene transduction has occurred, then there is permanent incorporation into the daughter cells. Dendritic cells are terminally differentiated and thus retroviral gene constructs would not be efficaceous. Furthermore, as DC are such potent stimulators of allo-immunity, maximal therapeutic gene transduction is required to ensure as many DC as possible become transduced with the gene of interest. For these reasons, adenoviral gene constructs were used to deliver IL-10 into DC.

The specific aims of this chapter were to a) generate functional human DC from monocyte precursors using recombinant human interleukin-4 (rh IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) b) transduce human monocyte-derived DC with adenoviral interleukin-10 (AdV IL-10) and in order to study their allostimulatory behaviour *in vitro*.

3.2 Materials and Methods

3.2.1 Growth of Monocyte-Derived Dendritic Cells

Immature DC were grown using the recombinant human cytokines IL-4 and GM-CSF, based upon the protocols of Caux and Romani (Caux et al. 1992; Romani et

al. 1994). Human PBMNC were isolated from buffy coats of healthy blood donors (Australian Red Cross Blood Transfusion Service, Adelaide) by Lymphoprep® (Nycomed, Oslo, Norway) gradient density separation. In a 50ml sterile container, 10ml of fresh buffy coat was diluted with 30ml of phosphate buffered saline (PBS) and underlayered with 10 ml of Lymphoprep®. The cell suspension was then centrifuged for 20 minutes at 800*g* without application of a braking force. The resultant mononuclear cell layer was resuspended in 10 ml of fresh phosphate buffered saline (PBS) and subsequently centrifuged at 300*g* for 10 minutes. This step was repeated twice. In order for monocyte adherence to then occur the cell pellet was resuspended in 3ml of complete medium and then plated onto 3 cm well plates (Falcon) at a concentration of 5 x10^6 PBMNCs per ml for 2 hours at 37°C. After incubation the non-adherent cells were washed off and the adherent monocyte fraction was subsequently cultured in 3 ml fresh complete medium, supplemented with the rhIL-4 (400 IU/ml) and rhGMCSF (800 IU/ml). Cells were grown for 5 days after which they were used for *in vitro* gene transfer studies. By day 5 the cells were no longer adherent to plastic and were floating freely in medium, from which they could be easily collected by gentle aspiration. For maturation the cells were exposed to rhTNF–α at 10ng/ ml of complete medium for a further 48 hours.

3.2.2 Flow Cytometric Analysis

For immunocytochemical staining of DC, cells were first incubated with 10% normal rabbit serum and 10% EMA (Molecular Probes, Eugene OR) for 20 minutes at 4°C. For the analysis of cell surface antigens a panel of mouse anti-human monoclonal antibodies was used which included antibodies W6/32 MHC Class I (American Tissue Culture Collection, Rockville, MD), MY-4 (FITC anti CD-14)

Coulter Corporation (Hialeah, FL), CD 80, CD 83, CD 86 (Serotec, Oxford). Isotype matched negative controls included X63 (IgG1 gift of Dr Heddy Zola, Flinders Medical Centre, Adelaide), 1D4.5 (IgG2a gift of Dr Leonie Ashman, Hanson Cancer Centre, Adelaide). The antibody RM3.54 (anti human MHC Class II) was generated within the host laboratory. The secondary antibody used to stain unconjugated primary antibodies was FITC conjugated anti-mouse IgG (Silenus, Hawthorn,Victoria). After staining the cells were fixed in 2% paraformaldehyde (BDH, Australia) and analysed using a Becton Dickinson FACScan® (San Jose, CA).

3.2.3 Transduction of Human Dendritic Cells with Adenoviral Gene Constructs

Dendritic cells were transduced using a Lipofectamine based strategy derived from published protocols (Dietz and Vuk-Pavlovic 1998). Monocyte-derived human DC were collected at day 5, centrifuged at 300*g* for 10 minutes, counted and then resuspended at a concentration of $1x10^5$ cells/100µl serum free medium. Subsequently $5x10^8$ adenoviral particles (5,000 particles per cell) were resuspended in 100µl of serum free medium and 1.25µg of the cationic lipid Lipofectamine® (Gibco BRL) and gently agitated for 15 minutes prior to transduction of the DC for 2 hours at 37°C. Serum-free conditions were used to minimize non-specific entry of other serum-derived molecules into the cells in competition with the adenovirus. During the transduction period the cells and the adenovirus were gently mixed by aspiration every 15 minutes in order to ensure maximal cell exposure to the adenoviral particles. After transduction the DC were resuspended in 3ml of fresh serum-replete medium supplemented with rhGM-CSF and rhTNF-α for a further 48 hours prior to their use in further studies.

3.2.4 Cytokine Analysis of IL-10 Transduced DC in MLC

Human DC were grown using standard cytokine conditions and transduced with adenoviral vectors as described. After transduction the cells were collected and were washed three times with PBS. The transduced DC were then incubated for a further 48 hours in fresh medium supplemented with rhGM-CSF (800 IU/ml) and rhTNF -α(10ng/ml). These DC were then incubated with allogeneic PBMNC collected from buffy coats of random blood donors (Red Cross Adelaide) at a stimulator: responder ratio of 1 to 100. After 6 hours the cells were harvested and 1μg of messenger RNA was analysed by reverse transcription-polymerase chain reaction (RT-PCR) for cytokine species. All experiments were performed in triplicate.

3.2.5 Metabolic Labeling and Immunoprecipitation of IL-10 by ^{35}S Methionine

In order to demonstrate secretion of IL-10 from AdV IL-10 transduced DC, metabolic labeling and immunoprecipitation of IL-10 from transduced DC was performed. Immature DC were differentiated from monocytes and transduced with adenoviral vectors (as described in 3.2.3). AdV IL-10 transduced DC were then centrifuged for 10 minutes at 800*g* to remove any medium and resuspended in 1ml of methionine/cysteine free RPMI. Equal numbers of cells were then recentrifuged and 100μCi/ml of ^{35}S was then added and the cells incubated for a further 4 hours prior to immunoprecipation using a anti human IL-10 polyclonal antibody and *Staph. aureus*. The immunoprecipitated product was then run on a 15% SDS-PAGE gel.

3.3 Results

3.3.1 Growth of human monocyte-derived dendritic cells

Human monocyte-derived DC (MoDC) were readily grown using the recombinant human cytokines IL-4 and GM-CSF. By day 2 post the addition of the cytokines, clumps of cells with irregular cytoplasm were visible in the culture plates. When daily cytospin examinations were performed a characteristic evolution from monocytoid appearance into typical immature DC appearance was observed (figure 3.3.1).

3.3.2 IL-4/GM-CSF treated monocytes develop into functional immature dendritic cells

The capacity of IL-4/GM-CSF (day 5) generated DC to induce proliferation in allogeneic mononuclear cells was assessed by 5 day MLR. Typical proliferative counts of greater than 4 fold increase upon single cell counts were observed. A representative experiment (showing proliferation at a stimulator:responder ratio of 1:100) is shown in figure 3.3.2.

3.3.3 Addition of TNF-α enhances maturation of immature dendritic cells to mature dendritic cells

In order to enhance maturation from immature DC into mature MC the maturational agent tumour necrosis factor alpha (rhTNF-α) was added at 10 mcg/ml for 48 hours. Under these conditions human DC underwent changes in their cell surface markers, consistent with "maturation". Specifically, they acquired the DC marker CD83 (data not shown). In addition, the exposure of human immature human DC to rhTNF-α was also associated with increased proliferation

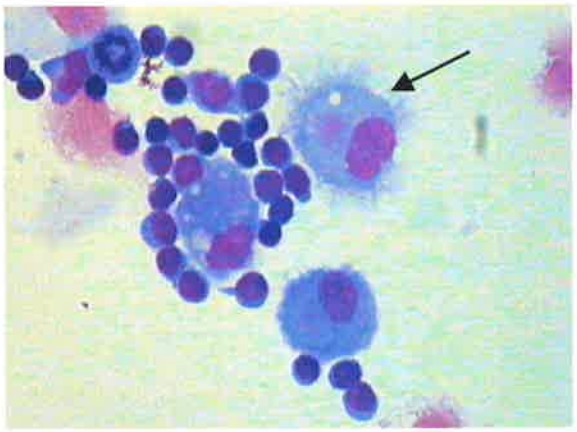


Figure 3.3.1

Monocyte-derived DC after five days culture with GM-CSF / IL4 and matured with TNF-α for 2 days prior to cytospin slide preparation. Typical DC morphology of enlarged cell nuclei and fine cytoplasmic processes is indicated(arrow). *Giemsa* stain x 100.

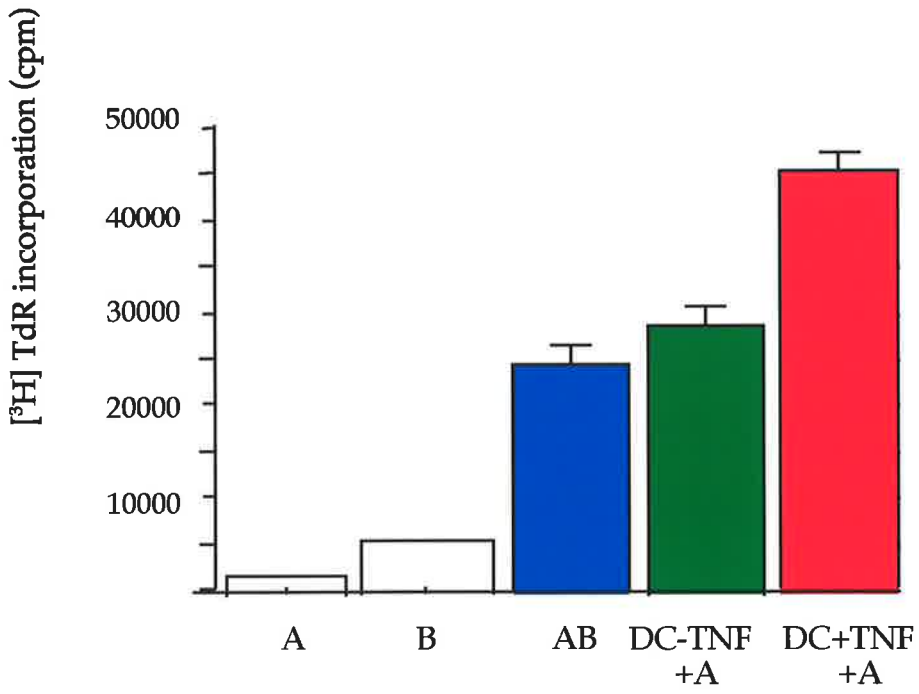


Figure 3.3.2

Mononuclear cells from individual A and B were cultured together (AB) in the standard 2 way MLR. DC generated from donor B with (red bar) and without (green bar) the use of rhTNF-α and were cultured with cells from donor A at a stimulator responder ratio of 1DC to 100 MNC . Mature DC (red) induced significantly greater proliferation compared to immature DC (green). Proliferation was assessed after 5 days using tritiated thymidine incorporation. Figure 3.3.2 shows one experiment representative of 3.

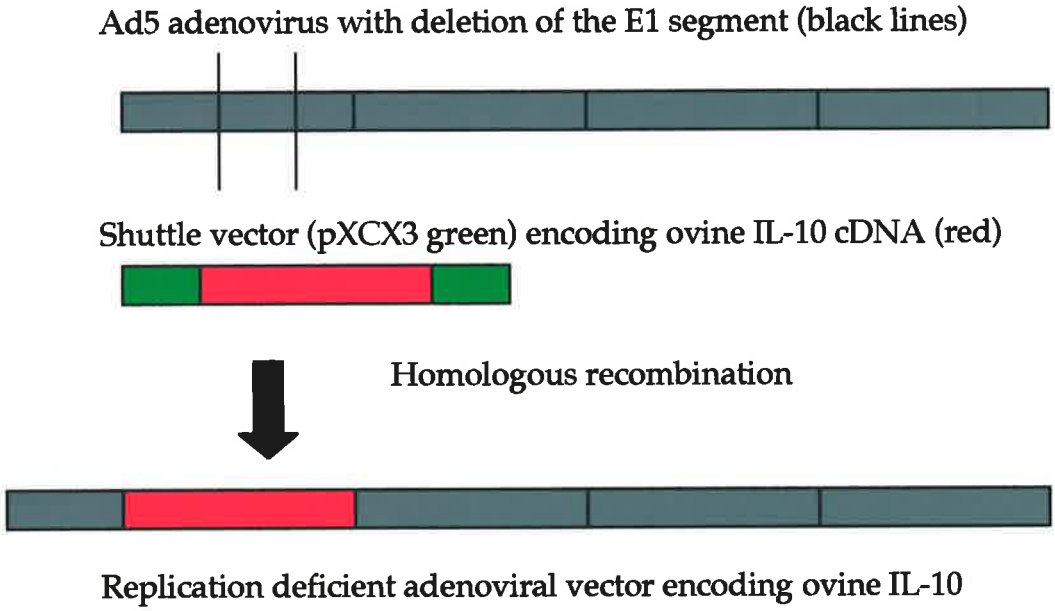


Figure 3.3.2b

A schematic map of the AdV IL-10 gene construct used in these studies. The AdV IL-10 gene construct (in red) was prepared by the homologous recombination of ovine IL-10 cDNA (Martin et al 1995) into an E1-deleted (indicated by double black lines) replication-deficient Ad5 adenovirus using the shuttle vector (pXCX3 shown in green). The ovine IL-10 gene was under the control of a CMV promoter.

in the mixed leukocyte reaction (figure 3.3.2). This proliferative response was significantly increased compared to immature DC. Figure 3.3.2 shows 1 representative experiment of 4. (NB there is no figure 3.3.3 - it is combined with 3.3.2)

3.3.4 Transduction of human monocyte-derived DC with adenoviral gene constructs

Human DC were transduced with adenoviral gene therapy vectors using the Adenoviral β galactosidase (AdV β–gal) gene construct. Initial multiplicity of infection was 100 viral particles per cell. Using this MOI there was only less than 5% of the DC transduced (data not shown). Increasing multiplicity of infection was associated with increasing percentage of gene transfer (data not shown). However, maximal gene transfer (>90%) was only achieved by the addition of the cationic lipid Lipofectamine® using the protocols derived from Dietz *et al* (Dietz and Vuk-Pavlovic 1998). Subsequently human DC were transduced with AdV GFP at MOI 5000 (figure 3.3.4a).

3.3.5 Transduction of human DC with AdV IL-10 inhibits the alloimmune response

Using the conditions determined by the AdV β–gal gene and confirmed by AdV GFP vector human DC were transduced with AdV IL-10. AdV IL-10 transduced DC were maintained in complete medium for up to 48 hours prior to their use in MLR. Transduced DC showed only minimal cellular toxicity (as assessed by Toluene blue staining and on flow cytometric analysis - data not shown). Transduction of human DC was confirmed by polymerase chain reaction using AdV-IL10 specific primers (figure 3.3.4b). Transduced human DC were then

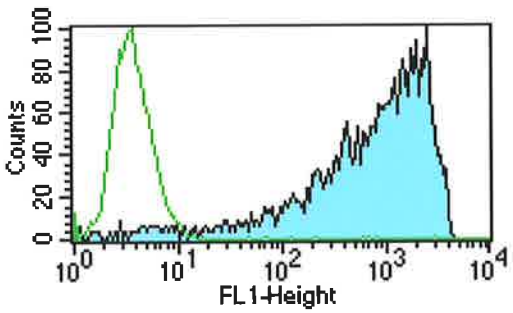


Figure 3.3.4 a

Human monocyte-derived DC transduced with AdV GFP (MOI 5000). The blue histogram shows >90% gene transduction, green histogram represents control cells. Experiment representative of 3 separate experiments.

Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6 Lane 7

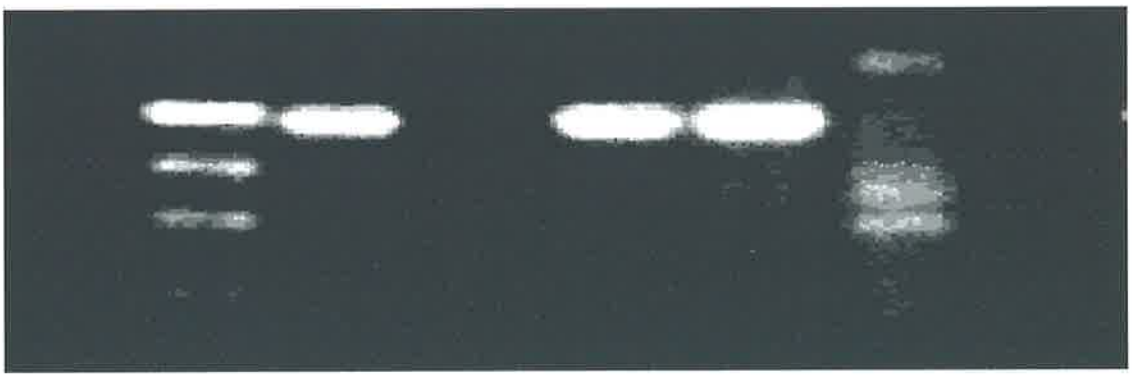


Figure 3.3.4 b

Adenoviral IL-10 specific PCR demonstrates effective transduction into human DC. Lane 1 molecular weight marker, lane 2 PCR positive control, lane 3 PCR negative, lane 4 and 5 AdV IL-10 transduced DC at MOI 5000, lane 6 AdV MX-17 transduced DC at MOI 5000 and lane 7 untransduced DC.

cultured with allogeneic human mononuclear cells in standard human 2 way MLR at a variety of stimulator: responder ratios. Strong inhibition of proliferation was observed across a range of stimulator: responder ratios (from 1:1 to 1:1000) (figure 3.3.5 shows 1 representative experiment of 5).

3.3.6 Human monocyte-derived DC Transduced with AdV IL-10 secrete IL-10

In order to demonstrate that monocyte-derived DC transduced with AdV IL-10 secrete IL-10 metabolic labeling and immunoprecipitation were used. The secretion of IL-10 was studied in IL-4/GMCSF generated DC and the cells transduced with adenoviral gene constructs by immunoprecipitation of ^{35}S metabolic labeled culture supernatant. Immature myeloid DC were shown to secrete IL-10 into tissue culture supernatant by immunopreciptation whereas DC matured with rhTNF-α showed no detectable IL-10 (figure 3.3.6). Human DC transduced with AdV MX-17 showed low levels of IL-10 secretion but AdV IL-10 transduction was associated with significantly greater secretion of IL-10 into tissue culture supernatant. In order to test the biological activity of the secreted AdV IL-10, the supernatant was then added to a standard 2 way MLC. Tissue culture supernatant derived from AdV IL-10 transduced DC was added to standard 2 way MLR performed between 2 unrelated individuals. At either 10% or 50%, conditioned medium was capable of inhibition of the day 5 proliferative response. The specificity of this response was demonstrated using a polyclonal anti- IL-10 antibody, which substantially reversed the inhibitory effect (see table 3.3.6)

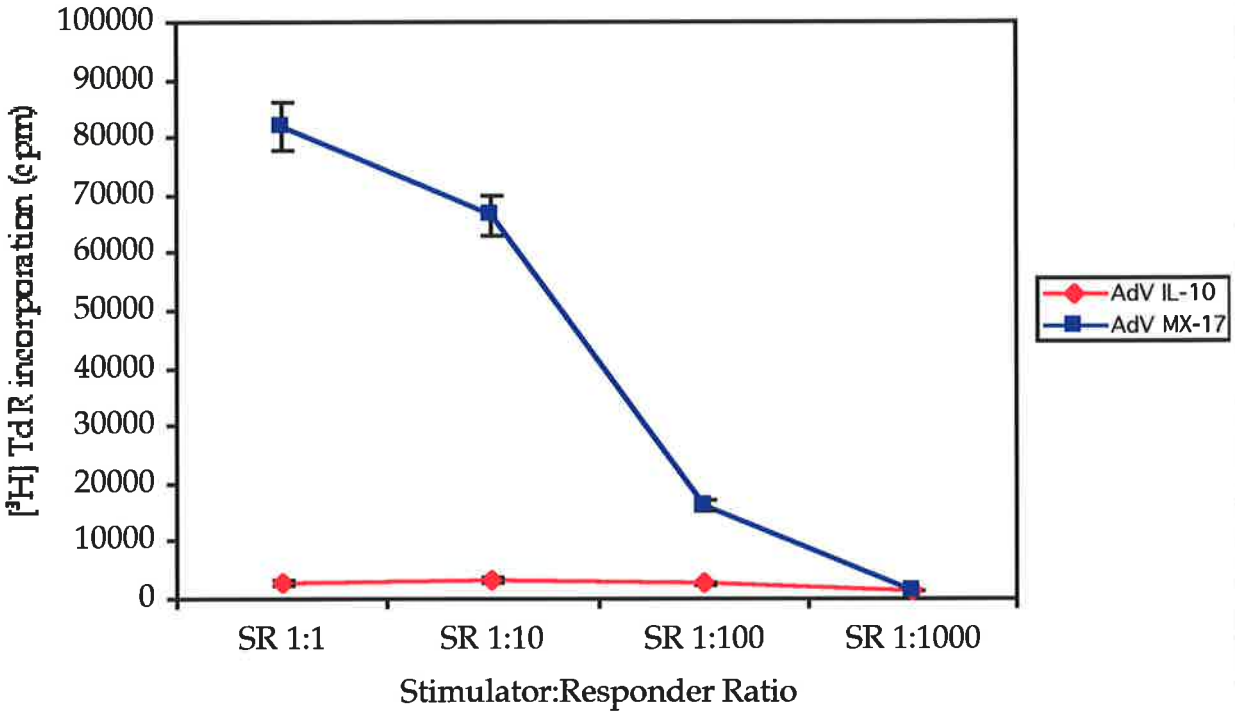


Figure 3.3.5

Mean proliferative response (counts per minute) of mononuclear cells incubated with DC transduced with either AdV IL-10 (red) compared to DC transduced with AdV MX-17 (blue) in 2 way MLR at 5 days. Error bars show 1 standard deviation. At S:R ratios greater than 1:100 AdV MX-17 DC show significantly greater proliferation than AdV IL-10 DC ($p<0.005$ Mann Whitney -U).

Lane 1 Lane 2 Lane 3 Lane 4

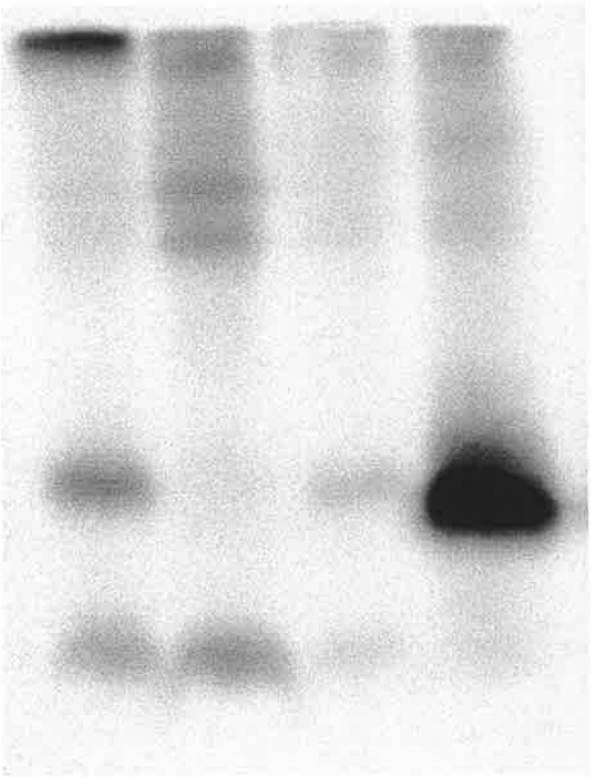


Figure 3.3.6

Immunoprecipitation of IL-10 species from tissue culture supernatant with ^{35}S from monocyte-derived DC under a variety of conditions. Under baseline conditions, immature untransduced day5 monocyte-derived DC secrete low levels of IL-10 (lane 1). Upon maturation with TNF-α, the secretion of IL-10 into the tissue culture supernatant is lost (lane 2). Transduction with AdV MX-17 results in minimal secretion of IL-10 (lane 3) whereas transduction with AdV IL-10 produces a profound secretion of IL-10 (lane 4).

AdV IL-10 Supernatant	AdV IL-10 Supernatant + anti IL-10	AdV MX-17 Supernatant
2,427+/-280	65,903+/-1,739	76,317 +/-6,155

Table 3.3.6

Conditioned medium from AdV IL-10 transduced DC inhibits the 2 way MLC and may be reversed by the addition of a polyclonal anti IL-10 antibody (mean counts per minute). Supernatant from DC transduced with AdV IL-10 was added to standard 2 way MLC at the initiation of the reaction. A polyclonal rabbit anti human IL-10 antibody was added at the same time as the AdV IL-10 conditioned medium. Results expressed as mean counts per minute of triplicate experiments +/- SD.

3.3.7 Transduction of monocyte-derived DC with AdV IL-10 downregulates DC cell surface expression of co-stimulatory molecules CD80-CD86

Human monocyte-derived DC were transduced with AdV IL-10 and transduced cells were studied using flow cytometric analysis. After 5 days of culture immature DC showed a typical pattern (Class I^{high}, class II^{high}, $CD14^{low}$, $CD33^{high}$, $CD1a^{high}$, $CD83^{low}$) (figure 3.3.7a). Dendritic cells transduced with either AdV IL-10 or AdV MX-17 and untransduced DC were then exposed to TNF-α (10ng/ml) for 48 hours prior to flow cytometric analysis. In figure 3.3.7a, the flow cytometric profile of untransduced "immature" human monocyte-derived DC *pre* treatment with TNF-α is shown. Dendritic cells transduced with the control virus (AdV MX-17) cells showed upregulation of cell surface expression of MHC Class I and II and the co-stimulatory molecules CD80 and CD86 and acquisition of the DC maturation marker CD83 (figure 3.3.7b). When DC were transduced with AdV IL-10 a down-

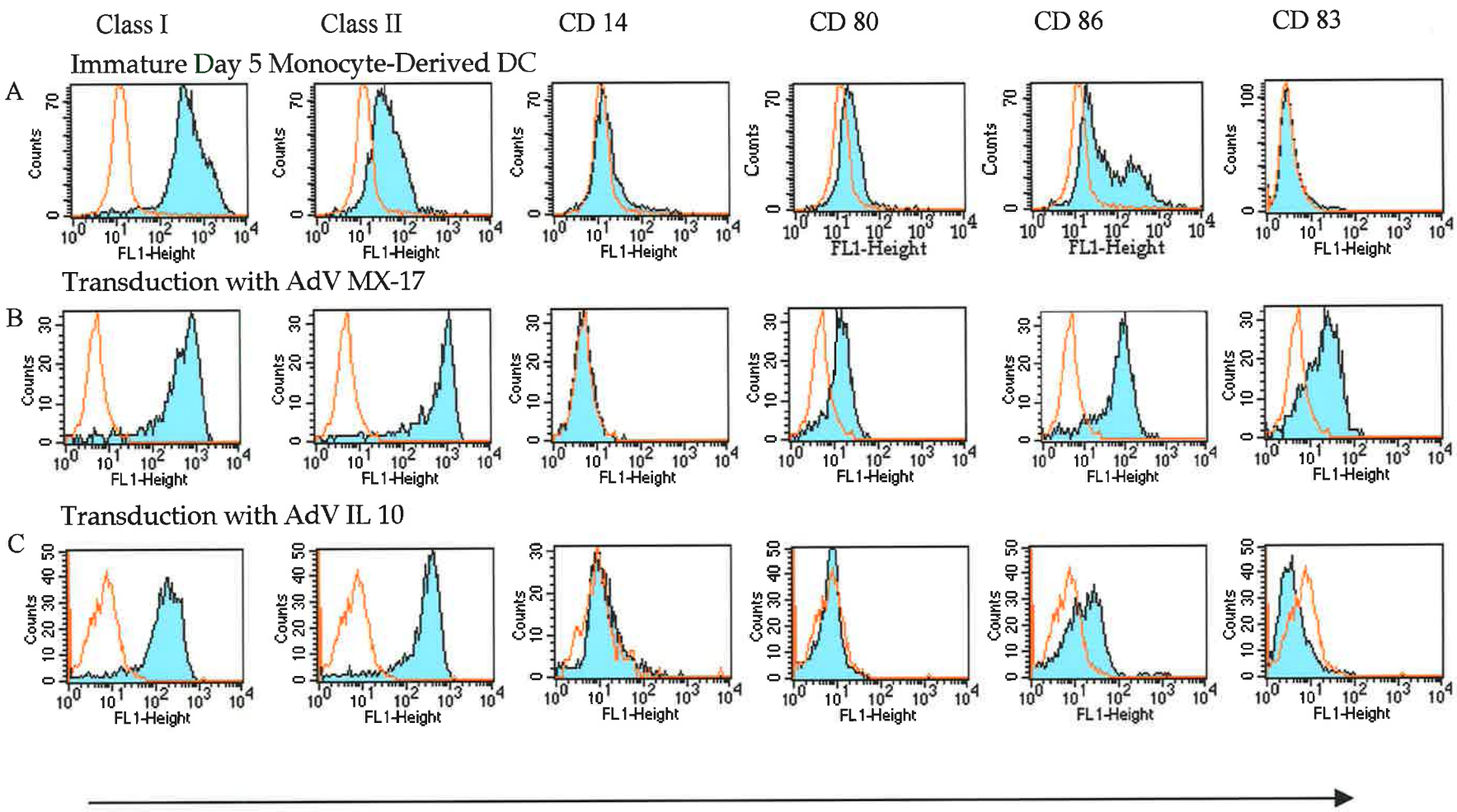

Class I
Class II
CD 14
CD 80
CD 86
CD 83
Immature Day 5 Monocyte-Derived DC
A
Transduction with AdV MX-17
B
Transduction with AdV IL 10
C
Counts
FL1-Height
Fluorescence Intensity

Figure 3.3.7

Figure 3.3.7

Flow cytometric analysis of cell surface markers (Class I, Class II, CD14, CD80, CD86 and CD83) on human day 5 myeloid DC. Shaded histogram represents fluorescence intensity of fluorescein stained cells, unshaded histogram shows the fluorescence intensity of an isotype matched negative control antibody.

A] Day 5 human myeloid IL-4/GM-CSF generated DC. These cells showed cell surface markers consistent with immature myeloid DC, without the expression of the DC maturational marker CD83.

B] DC transduced with the AdV MX-17 show upregulation of Class I, Class II, CD 80, CD86 and acquisition of the DC specific marker CD83.

C] DC transduced with AdV IL- 10 show lower expression of class II molecules and down regulation of CD80 / CD86 with failure to acquire the DC maturation associated cell surface marker CD83.

regulation of CD80 and CD86 and a failure to acquire the DC associated maturation marker CD83 was observed (see figure 3.3.7c)

3.3.8 Transduction of monocyte-derived DC with AdV IL-10 inhibits interleukin 12 production in the MLR

Dendritic cells transduced with AdV IL-10 were cultured with allogeneic mononuclear cells at a stimulator:responder ratio of 1 to 100 (DC to allogeneic cells). These cells were then harvested at 4 hours and 24 hours post the initiation of mixed leukocyte culture. The cytokine species were studied using reverse transcription polymerase chain reaction. At non-saturating conditions, the two biologically active species IL-12p35 and IL-12p40 were studied. At non-saturating PCR cycling conditions DC transduced with AdV IL-10 showed absent expression of IL-12p40 at the messenger RNA level (fig 3.3.8). In those cells transduced with the control virus AdV MX-17, strong mRNA expression of IL-12p40 was demonstrated, indicating the ability of DC to produce biologically active IL-12 had been interrupted by the transduction of the AdV IL-10 gene.

Lane 1 2 3 4 5 6 7

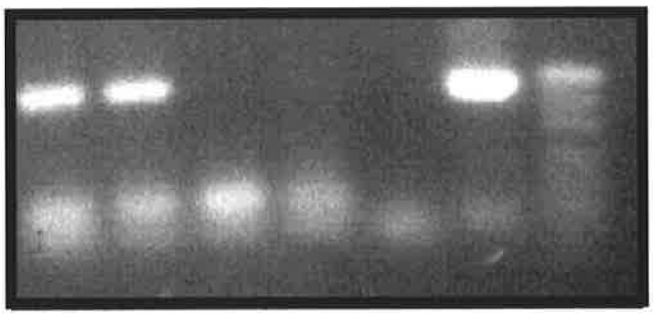


Figure 3.3.8

Human myeloid DC transduced with AdV IL-10 or MX-17 ($5x10^3$) were cultured with allogeneic MNC ($5x10^5$ cells/well) at a stimulator:responder ratio of 1:100 for 6 hours in a 2 way MLC. The transduced cells/allogeneic cells were then harvested and the mRNA species were analysed by reverse transcription polymerase chain reaction for the cytokine subunit IL-12p40 (35 cycles). In the cells transduced with AdV MX-17, mRNA for IL-12p40 could be detected (Lane 1 and 2) however in cells transduced with AdV IL-10 m RNA for IL-12p40 could not be detected under non saturating conditions (lanes 3 and 4). Lane 6 shows positive pcr control and lane 7 shows the marker puc-19. Figure 3.3.8 shows equivalent results for 2 separate experiments run in parallel.

3.4 Discussion

In this chapter the transduction of human monocyte-derived DC with adenoviral gene constructs encoding a variety of genes was studied. As a prelude to the gene transfer studies, DC were differentiated from monocytes using recombinant human cytokines. The main factor that has allowed easy study of DC function is the ability to generate large numbers of monocyte-derived so called "immature" DC using recombinant cytokines IL-4 and GM-CSF. Prior to this development, studies were limited to collection of freshly isolated DC from organs. Monocyte-derived DC clearly offer the advantage of ready access to an immature source of DC for use in *in vitro* and *in vivo* studies. This ability to produce large numbers of DC *in vitro* has important implications as it would allow generation of sufficient DC for human therapeutic application. One of the critical issues regarding these *in vitro* generated cells is their relationship to the 'gold standard' tissue-derived DC. As these cells require the presence of the purified recombinant cytokines IL-4 and GM-CSF, questions arise regarding the physiological relevance of the cells produced by application of these cytokines and indeed whether these cells are likely to be produced *in vivo* by these pathways (Hart 1997). From the therapeutic point of view, whether or not such IL-4/GM-CSF pathways exist *in vivo* is a moot point. The critical issue is the ability to generate enough DC or DC like cells for clinical use.

The protocols used to generate immature DC from monocytes are now well accepted and form the basis of many research publications (Romani et al. 1994). Typically, at the onset of culture, cells had an irregular appearance when assessed by Giemsa staining. The monocyte marker CD14 was usually present at the start of culture, but was rapidly lost as culture proceeded. Over a five day culture period

the percentage of CD14 positive cells fell such that by the end of 5 days very few CD-14 positive cells remained. The evidence that treatment of human monocytes with IL-4 and GM-CSF can generate immature DC can be summarized as follows. Firstly the cells have dendritic morphology, both at the light microscopic and ultrastructural levels (figure 3.3.1). Secondarily, the monocyte-derived DC show characteristic cell surface phenotype, including the acquisition of DC specific cell surface markers upon maturation with TNF-α (CD83) but also CMRF 44 and CMRF 56 (data not shown). Finally, functionally the immature monocyte-derived DC may induce significant proliferation within the 5 day allo-MLR (figure 3.3.2).

Having generated monocyte-derived DC, the next step was to engineer the cells to promote T cell responses favorable to allo-transplantation. One of the critical factors that modifies DC behavior is the environment in which DC maturation has occurred. A variety of stimuli may induce the activation and maturation of DC as well as encounter with antigenic material. Such non-immunological environmental factors may be of importance in the transplant setting (for example in the context of prolonged cold ischaemia time in renal transplantation where the presence of significant tissue injury, hypoxaemia and free radical formation are all factors that could induce DC maturation and up-regulation of DC co-stimulatory molecules). One of the factors that modifies T cell response to DC within the lymphoid system is the cytokine milieu to which DC are exposed during their maturation. For example, when myeloid DC are matured in the presence of prostaglandin E2 at concentrations between 10^{-9} to 10^{-6} M, the DC loses the capacity to produce IL-12 and the T cell population produced by these PGE-2 treated DC produce high levels of IL-4 and IL-5 (Kalinski et al. 1997). There is also *in vivo* evidence to support the concept that regional environments might have an effect on DC function. Dendritic

cells isolated from bronchial secretions have an impaired ability to produce IL-12 and show reduced stimulatory capacity (Stumbles et al. 1998). Peyers' patch DC produce higher levels of IL-10 than splenic DC and promote a TH2 response within T cells (Iwasaki et al. 1999). These studies have lead Kalinski *et al* to propose that there may be 3 types of DC based upon the final maturational exposure of DC to cytokine environments. The type 1 DC exposed to IFN-γ during final stages of maturation has high co-stimulatory molecule expression, high capacity to produce IL-12 and promotes a TH1 response within naïve T cells. The type 2 DC (exposed to factors such as PGE-2 during final maturation) shows high co-stimulatory molecule expression but low IL-12 and produces TH2 biased T cells. The type 3 DC is one which has been exposed to IL-10 during the final maturation and shows low levels of co-stimulatory molecule expression, low levels of IL-12 production and fails to induce significant proliferation within naïve T cells (Kalinski et al. 1999). The generation of these 'type 3 'DC by transduction of DC with AdV IL-10 has the potential advantage that the secreted product may have paracrine effects upon untransduced DC. Furthermore, such an approach is attractive as it allows a gene therapy strategy that is clinically feasible, by exposing the target cell population to the gene construct in an *ex vivo* situation, avoiding exposure of other cells to the gene construct.

In order to introduce the gene for IL-10 a variety of gene delivery strategies are available. Strategies for the introduction of genes into DC include adenoviral delivery (Zhong et al. 1999), retroviral delivery (Gabrilovich et al. 1994; Aicher et al. 1997b), electroporation and chemical means. Of the current options for transduction, replication deficient adenoviral delivery offers a means for achieving

high efficiency gene transfer into DC with the advantage of multiple copies of the gene of interest being inserted as compared to a single copy using the retroviral system. As a consequence it is possible to achieve close to 100% gene transduction using adenoviral gene delivery as compared to 30-40% using the retroviral techniques (Gojo et al. 2000). In these studies a replication deficient adenoviral vector was used to introduce the marker gene β–galactosidase into monocyte-derived dendritic cells. Dendritic cells could be efficiently transduced with adenoviral gene constructs, but this was achieved by the use of the cationic lipid Lipofectamine.

Using the reporter gene green fluorescence protein (AdV GFP) a high level of gene transduction at a multiplicity of infection of 5000:1 was demonstrated (figure 3.3.4). An adenoviral gene transfer strategy was then used to introduce the gene for IL-10 into human monocyte-derived DC. The results of other groups were confirmed using adenoviral vectors to get high efficiency gene transfer, without significant cell toxicity. Essential to this effective transduction was the addition of the cationic lipid Lipofectamine®, which considerably enhanced the adenoviral gene transduction into human DC and is consistent with the observations of others (Dietz and Vuk-Pavlovic 1998). The reason that Lipofectamine® enhances the transduction efficiency of adenoviral particles relates to the molecular mechanism of viral attachment to target cells. Adenovirus gains entry to the cell by binding to the αVβ3 integrin. Human monocyte-derived DC express this integrin only at low levels on the cell surface (data not shown). By coating the adenoviral particles with the cationic lipid, this allows the lipid coating to fuse with the DC cell surface membrane and thus partially bypass the integrin pathway.

The adenoviral gene construct delivery system was then used to introduce a cytokine gene into DC. The gene encoding IL-10 was chosen as the target gene. The rationale for this approach was that IL-10 had shown multiple immunomodulatory effects upon DC function, including inhibition of DC maturation. Previous studies with immature DC had shown allograft survival could be prolonged using co-stimulatory molecule deficient DC, but that rejection was still a problem as immature DC matured within the allograft recipient (Fu et al. 1996b). As IL-10 inhibits DC maturation the transduction of donor DC with AdV IL-10 in these studies was designed to prevent the *in vivo* maturation of donor DC.

Transduction of human monocyte-derived DC with AdV IL-10 was performed using the conditions derived from the reporter gene construct experiments. The AdV IL-10 transduced DC showed minimal cellular toxicity and retained culture appearances similar to non-transduced day 5 monocyte-derived DC. AdV IL-10 transduced DC were then studied using polymerase chain reaction to confirm transduction of the specific gene within the DC (figure 3.3.4). As the primers used for the PCR recognised only regions within the sequence of the AdV IL-10 specific gene transduction for the construct and not endogenous IL-10 was indicated. This and the reporter gene construct studies confirmed the conditions for adenoviral gene transduction that have been described by others (Dietz and Vuk-Pavlovic 1998).

The cell surface molecule profile of the AdV IL-10 transduced DC is shown in figure 3.3.7c. Transduction of human monocyte-derived DC with AdV IL-10 resulted in down-regulation of the co-stimulation molecules CD80 and CD86 when

compared to DC transduced with AdV MX-17 (figure 3.3.7b). Importantly transduction of human monocyte-derived DC with AdV IL-10 inhibited the acquisition of the DC specific marker CD83. This together with the failure of the DC to up-regulate co-stimulatory molecules CD80 and CD86 despite exposure of the transduced DC to the potent maturational agent rhTNF-α for 48 hours indicated that the "immature" phenotype had been maintained.

To study the effect of AdV IL-10 transduction upon DC function, the AdV IL-10 transduced DC were then stimulated in the 5 day mixed leukocyte reaction with allogeneic mononuclear cells. These AdV IL-10 DC showed a lack of stimulation in the MLR (see 3.3.5) when compared to DC transduced with the control virus AdV MX-17. Despite transduction with AdV IL-10, DC remained viable, with stable DC phenotype. The transduced DC showed a high level of expression of class I and class II molecules (the molecules that deliver signal 1) but down-regulation of the co-stimulatory molecules CD80 and CD 86 (signal 2) as shown in figure 3.3.7. The AdV IL-10 transduced DC were capable of secretion of IL-10 as evidenced by metabolic labeling with ^{35}S –methionine and immunoprecipitation studies (shown in 3.3.6). Furthermore, studies using the tissue culture supernatant demonstrated that the secreted product was biologically active, with inhibition of an allogeneic MLR being reversible by addition of a polyclonal anti-IL-10 neutralizing antibody (table 3.3.6). The AdV IL-10 DC cell surface molecule phenotype was consistent with published data regarding the effect of exogenous IL-10 upon DC maturation and function.

To further analyze the effect of AdV IL-10 transduction upon the capacity of DC to deliver signal 3 (IL-12 secretion), RT PCR was performed upon cells extracted from

the DC MLR. These PCR studies showed disruption of production of IL-12p70 by transduction with AdV IL-10. The cytokine IL-12 is a heterodimer composed of 2 subunits, IL-12 p40 and IL-12 p35. Inhibition of either chain prevents the formation of the biologically active heterodimer. At both time points studied (6 and 20 hours), there was inhibition of IL-12 p40 demonstrated at the mRNA level. Quantification of this inhibition could have been performed using commercially available human ELISA kits for IL-12. At the time of undertaking these studies these reagents were not available and therefore this quantification was not performed. It seems very likely that in the absence of the IL-12 p40 subunit chain as demonstrated at the mRNA level that biologically active IL-12 p70 would be inhibited.

In summary, human monocyte-derived DC could be easily and reproducibly generated using a combination of recombinant human IL-4 and GM-CSF. These DC showed cell surface phenotype consistent with immature DC and could be matured using rhTNF-α to produce potent DC that had acquired the DC specific cell surface marker CD83. Immature DC could be transduced with adenoviral gene constructs encoding reporter genes and therapeutic genes. When monocyte-derived DC, were transduced with AdV IL-10 the cells remained in an immature stage and were resistant to maturation with rhTNF-α, and acquired alloinhibitory properties in the MLR. AdV IL-10 transduced DC showed downregulation of CD80 and CD86 (signal 2) and impaired secretion of IL-12 (signal 3).

Chapter 4

An Experimental *In Vivo* Model System to Study the Capacity of Genetically Modified DC to inhibit Human Skin Graft Rejection in chimeric Humanized NOD-scid Mice

4.1 Introduction

Research to develop new reagents for the treatment of human diseases is hampered by the fact that very little research may be carried out using human material. For obvious practical and ethical reasons, it is not possible to use human subjects to test the early development of novel immunosuppressive agents. As a prelude to studies in larger animals many groups have turned to inbred strains of severe combined immunodeficient mice in order to develop smaller and less costly models in which to test treatment strategies. One such immunodeficient mouse is the severe combined immunodeficient (*scid)* mouse. These mice have a spontaneously occurring genetic mutation that prevents the successful rearrangement of immunoglobulins and T cell receptors, leading to the development of severe immunodeficiency involving both humoral and cell mediated immunity. In the absence of their own T and B cells, these mice may be adoptively transferred with human leukocytes to produce a humanized mouse, with an intact immune system (Mosier et al. 1988).

As a result, chimeric human-*scid* mice have been used to investigate the human alloimmune responses *in vivo* (Alegre et al. 1994; Murray et al. 1994b); (Shiroki et al. 1994; Sultan 1997). However, the *scid* mouse host does not provide a uniformly passive immunological microenvironment. Functionally active endogenous NK-cell and myeloid populations reduce the success of adoptive transfer and murine leukocytes can be induced to migrate into human skin grafts injected with rh TNF-α (Czitrom et al. 1985) (Dorshkind et al. 1984; Yan et al. 1993). In contrast, the NOD-*scid* mouse is more immunologically inert. The NOD-*scid* mouse is a severely immunodeficient mouse bred by cross mating of *scid* and NOD mice for 10 generations. This strain of mice, exhibits defective T- and B-cell function, defective NK-cell and macrophage populations and is deficient in circulating complement (Kataoka et al. 1983; Jacob et al. 1990; Serreze et al. 1993). This combination of immunological features makes these mice ideal recipients of allogeneic material in transplantation studies. In essence, these mice accept xenogeneic material but act as an inert *in vivo* background for allogeneic reactions.

In order to test the capacity of genetically modified human DC to modify allograft rejection, a chimeric human skin NOD-*scid* mouse transplant model was developed. This model is similar to humanized *scid* mouse transplant models (Murray et al. 1994a; Briscoe et al. 1999). In these models split human skin is engrafted onto an immunodeficient mouse. After acceptance of the graft, the mouse is reconstituted with human peripheral blood mononuclear cells from a blood donor allogeneic to the skin donor to induce skin allograft rejection (details in figure 4.3.1).

The specific aims of this chapter were to a) generate an immunodeficient human-murine chimeric skin transplant model b) to test the biological effect of human DC transduced with adenoviral gene constructs to modify skin allograft survival.

4.2 Materials and Methods

4.2.1 NOD-*scid* Mice

NOD-scid mice were purchased from the University of Adelaide Central Animal Storage Facility, and housed in a pathogen-free environment. All animals were allowed free access to food and water during the duration of the experiments.

4.2.2 Skin Grafting

Human split skin was obtained from health skin donors undergoing plastic surgical procedures within the Department of Surgery, The Queen Elizabeth Hospital. Human skin was stored on mesh at 4°C prior to engraftment.
Mice were then anaesthetized using a mixture of oxygen and halothane (Astra-Zeneca, UK). Initially the murine skin was shaved and two defects (approximately 10mm x 10mm) were created on the dorsal aspect of the mouse thorax. Fresh human split skin grafts were then grafted onto the subcutaneous tissue. The wounds were then covered with Sorbsan Calcium Alginate dressings (Steriseal, England) soaked with complete medium and then dressed with Tegaderm (3M Australia) bandages. After 7 days the dressings were removed and the wounds allowed to heal. Subsequently after a total of 14 days post skin engraftment the mice were challenged by intra-peritoneal injection of allogeneic mononuclear cells (see figure 4.3.1).

4.2.3 Human Leukocyte Isolation and Reconstitution of the NOD-*scid* mouse and processing of skin grafts

Human mononuclear cells were obtained from healthy blood donors buffy coat products (Australian Red Cross Blood Transfusion Service, Adelaide). Mononuclear cells were separated by centrifugation over a Lymphoprep® gradient (see Methods 3.2.1 for details)

Based upon previous studies each animal received between 2.5 and 3.0 $x10^8$ allogeneic mononuclear cells resuspended in 0.5ml complete medium which was given via intra-peritoneal injection (Patrick 1998; Briscoe et al. 1999). Animals were observed daily after surgery. At 7 days post humanization, the first skin graft was removed under anaesthesia and the skin deficit closed using 6/0 proline sutures. The second skin graft was removed at day 14 post humanization and the animals were euthanased.

After skin biopsy harvesting, samples were divided into two equal portions for immunohistology and conventional histology. Samples for immunohistology were embedded in OCT compound (Tissue-Tek®, Sakura-Finetek, Torrance, USA) and snap frozen in liquid nitrogen at -70°C prior to 4µm sections being cut. For cytokine analysis, 10µm sections were cut and dissolved in Solution D prior to RNA extraction and RT-PCR. Cut sections were dried overnight and fixed in acetone at 4°C. These sections were then rehydrated with 3% serum appropriate to the origin of the secondary antibody and then labeled with individual antibodies. Subsequently the samples were incubated with a biotinylated secondary antibody (horse anti-mouse IgG), the reaction was amplified by the use of Vectastain ABC-HRP (Vector laboratories, USA) and developed with diaminobenzidine substrate. Samples for haematoxylin and eosin staining were embedded in parafin and

processed according to standard protocols (Department of Histopathology, The Queen Elizabeth Hospital).

4.2.4 Rejection scoring

The diagnosis of skin graft rejection was based upon the scoring system published by Murray (Murray et al 1998). Skin sections were graded by 2 observers blinded to the experimental protocols according to the following skin graft grading system (grade 0- rare leucocytes comparable to normal skin, grade 1-sparse perivascular leucocytes, grade 2-dense perivascular leucocytes, grade 3-dense perivascular leucocytes with modest infiltration of dermis grade 4-dense perivascular leucocytes with dense infiltration of dermis.

4.2.5 Statistical Analysis

The difference between skin graft scores (as derived in 4.2.4) was assessed using a non parametric statistic, the Wilcoxon Rank statistic. Analysis was performed on an Apple Macintosh® G3 powerbook using the statistics program Statview 4.5 (Abacus Concepts®) .

4.3 Results

4.3.1 NOD-*scid* mice engrafted with human skin will accept skin grafts indefinitely in the absence of allogeneic cells (>100 day survival)

Figure 4.3.1 shows the grafting scheme for humanized NOD-*scid* mice. In order to assess the long-term survival of the skin grafts, a group of animals (n=4) was transplanted with human skin and allowed to heal. In the absence of allogeneic cells all human skin grafts remained pink and viable without evidence of rejection for greater than 100 days. Grafted human skin showed that the dermo-epidermal junction remains intact, epidermal blood vessels are patent and there is no dermal infiltrate (figure 4.3.1a).

4.3.2 Injection of allogeneic mononuclear cells produces rejection of human skin grafts

In order to induce rejection of human skin grafts, the mice were reconstituted with allogeneic mononuclear cells via intra-peritoneal injection. Between 2.5 – 3.0 x 10^8 allogeneic mononuclear cells were injected into each mouse via the intra-peritoneal route. Evidence of skin graft rejection was detectable at 7 days, with dermal infiltration and dermato-epidermal junction destruction at an early stage (4.3.2a). Histological evidence of skin graft rejection, in the absence of DC was maximal between 14-21 days post humanization, with dermal infiltration, vessel thrombosis and dermato-epidermal junction destruction (see figure 4.3.2b). Figure 4.3.2 a and b show representative *H&E* sections from 3 separate experiments mice (n=6).

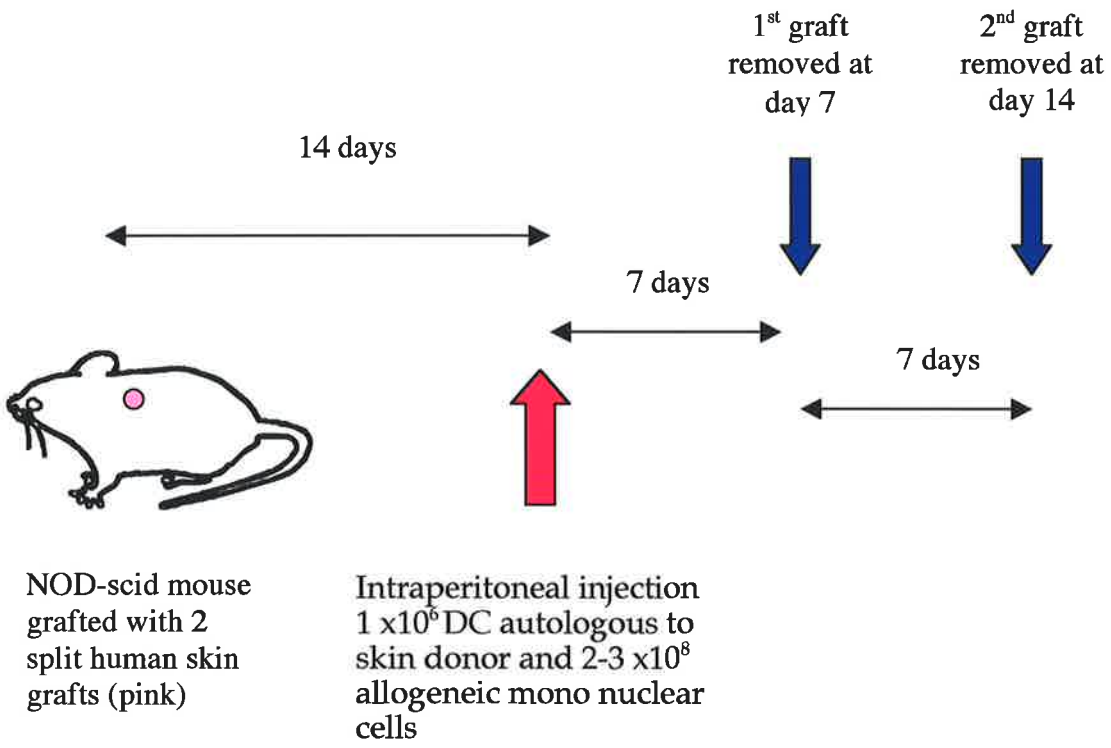


Figure 4.3.1

Human skin grafts were engrafted onto NOD-*scid* mice. Subsequently the mice were reconstituted with 2-3 x 10^8 allogeneic mononuclear cells and 1 x10^6 DC autologous to the skin graft 14 days after engraftment (red arrow). At 7 and 14 days post reconstitution, the skin grafts were removed and processed for histological analysis and cytokine analysis as required.

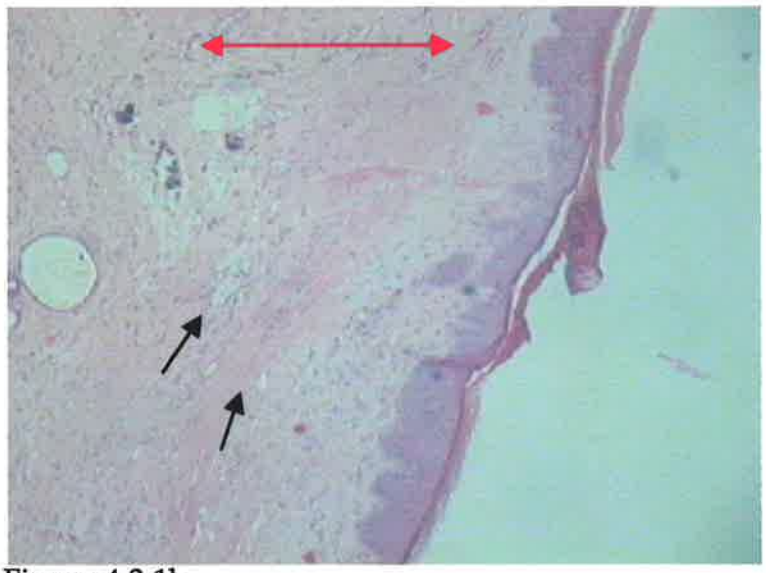


Figure 4.3.1b

Human skin grafted onto NOD-*scid* mouse (>100 days). The dermis remains free of infiltrate (red arrow), and the dermal vessels are patent (black arrows).

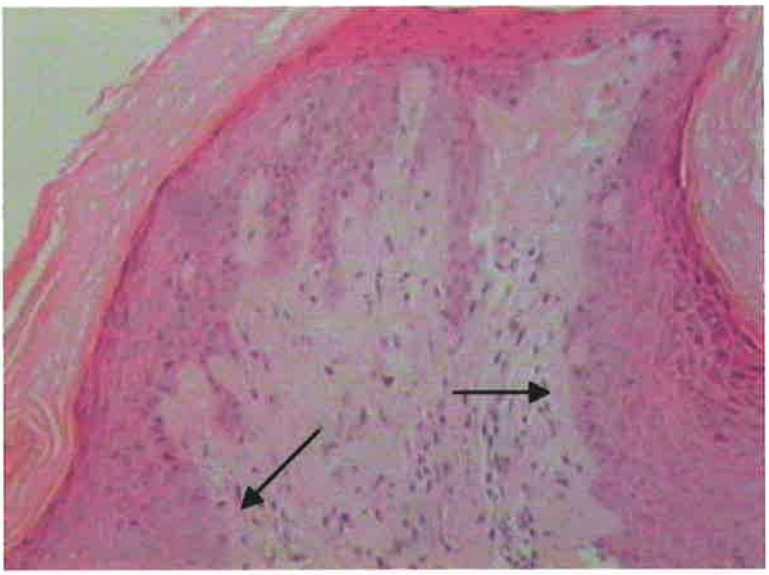


Figure 4.3.2 a

A typical appearance of a human skin graft 7 days after reconstitution with allogeneic mononuclear cells *without* autologous DC. Note the minor degree of cellular infiltration and destruction of the dermato-epidermal junction (arrows). (Murray grade 1)*H&E x40*

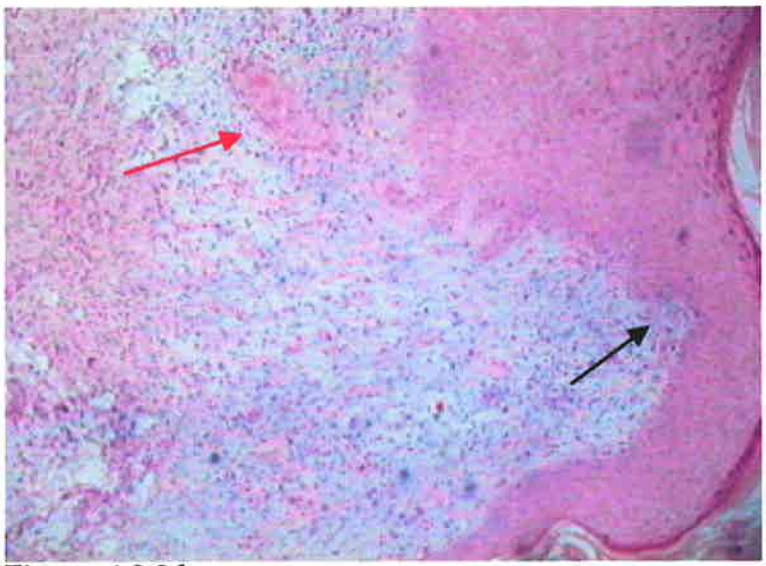


Figure 4.3.2 b

By day 21 post reconstitution with allogeneic mononuclear cells *without* autologous DC, a marked dermal infiltrate is evident, with small vessel thrombosis(red arrow). Infiltration of the dermato-epidermal junction is established (black arrow). (Murray grade 3) *H&E x40*

4.3.3 Injection of autologous mononuclear cells does not produce rejection of human skin grafts

In order to clarify that allogeneic cells were inducing rejection changes we studied the effect of autologous cells upon human skin engraftment. Mice (n=4) were engrafted with human skin and reconstituted with human mononuclear cells from the same skin donor (collected from peripheral blood). Skin grafts showed infiltration by mononuclear cells, but no evidence of dermo-epidermal destruction or up-regulation of MHC class II expression that were a feature of allogeneic treatment. Figure 4.3.3 shows a representative *H&E* section.

4.3.4 Co-Injection of Dendritic Cells autologous to the skin graft produces accelerated human skin graft rejection

In order to investigate the effect of the addition of autologous exogenous antigen presenting cells upon allograft survival, skin transplant experiments were performed with either a) immature DC (n=4) or b) mature DC (n=10). Seven days prior to humanization, 30-40ml of human peripheral blood was drawn from the skin donors, and human DC were grown *in vitro* using recombinant cytokines rhIL4/rhGM-CSF for 5 days. The animals receiving immature DC that were autologous to the skin graft were injected with day 5 DC that had been generated with rhIL-4/rhGM-CSF without further maturation. For the animals treated with mature DC, day 5 immature DC were exposed to rhTNF-α (10 ng/ml) for a further 2 days. The phenotype of injected cells was confirmed by flow-cytometric analysis. Flow cytometric analysis of injected human DC showed a "mature" phenotype with high expression of MHC class I, class II, CD 80, CD86 and the DC marker CD83. Autologous human DC ($1x10^6$ cells in 100 µl complete medium) were then given to the mouse via intra-peritoneal injection at the time of reconstitution with

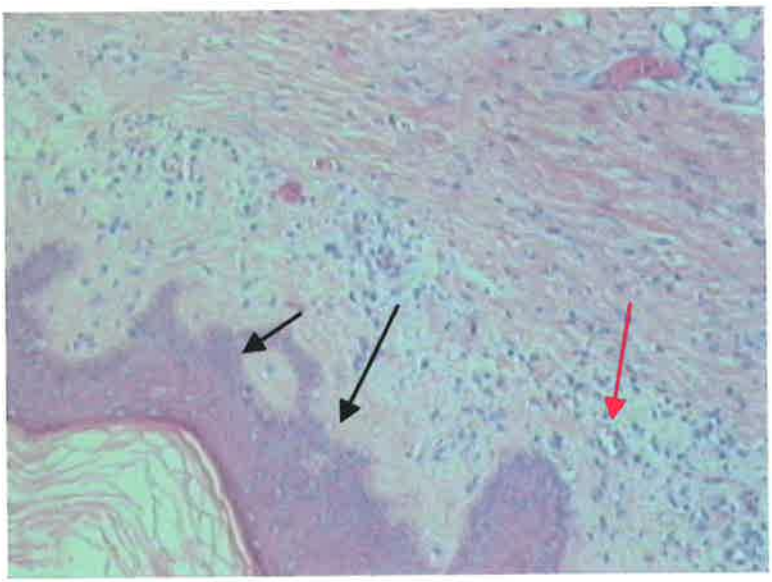


Figure 4.3.3
Day 7Mice reconstituted with autologous mononuclear cells without DC show skin graft infiltration but dermato-epidermal junction is intact (black arrow) and dermal blood vessels remain patent (red arrow). (Murray grade 1) *H&E x40*

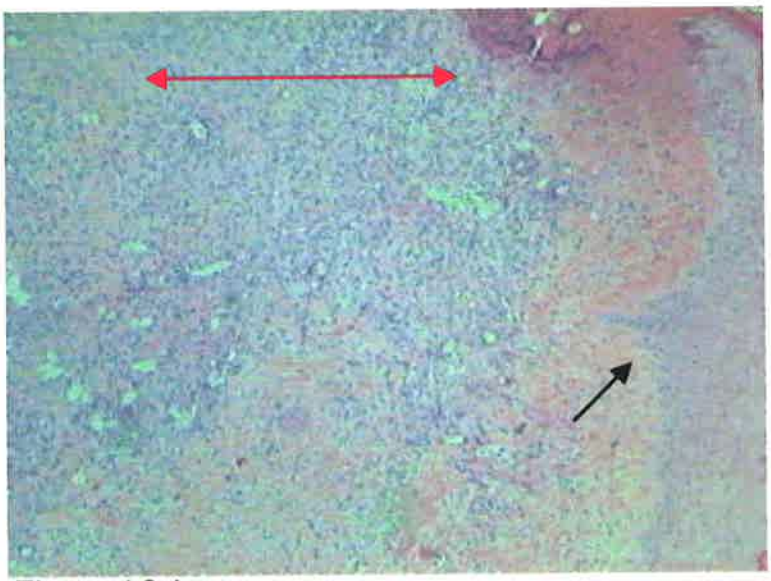


Figure 4.3.4
Day 7 Mice reconstituted with DC autologous to the skin graft and allogeneic mononuclear cells. Note the marked dermal infiltrate (red arrow) and destruction of the dermato-epidermal junction (black arrow). (Murray grade 4) *H&E x40.*

allogeneic mononuclear cells. In contrast to the animals given allogeneic mononuclear cells alone, enhanced skin graft rejection was evident by 7 days (figure 4.3.4), with discoloration of the human skin graft evident.

In order to compare the capacity of freshly isolated DC upon skin graft survival, 2 animals received freshly isolated human myeloid DC. (isolated by flow-cytometric sorting). The rejection changes seen in these 2 animals were identical to those seen with the monocyte-derived DC (data not shown).

4.3.5 Injection of Dendritic Cells transduced with AdV IL-10 protects human skin grafts from rejection

In order to assess the *in vivo* effect of transduction of human DC with adenoviral gene constructs, human DC that had been modified by transduction with AdV IL-10 (n=6 mice) or the control virus AdV MX-17 (n=6 mice) were injected via the intra-peritoneal route. In animals that received DC transduced with AdV MX-17 vigorous skin rejection was detected by day 7 which was associated with destruction of the dermo-epidermal junction and infiltration of the subepidermal region (figure 4.3.5 a). In contrast, animals that received DC transduced with AdV IL-10 showed preservation of the skin architecture, with only minimal skin graft infiltration (figure 4.3.5 b). The skin grafts were also examined using immuno-histology for the presence of MHC class II molecules. In the animals treated with AdV IL-10 there was a reduction in skin graft expression of MHC class II molecules compared to animals treated with AdV MX-17 transduced DC (figure 4.3.5 c and d).

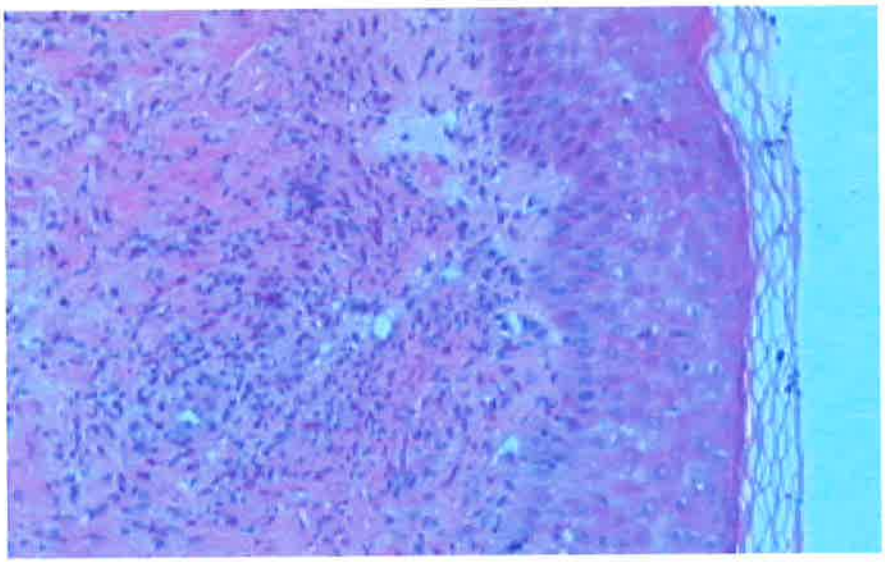


Figure 4.3.5 a) AdV MX-17 day 7 *H&E x 100*

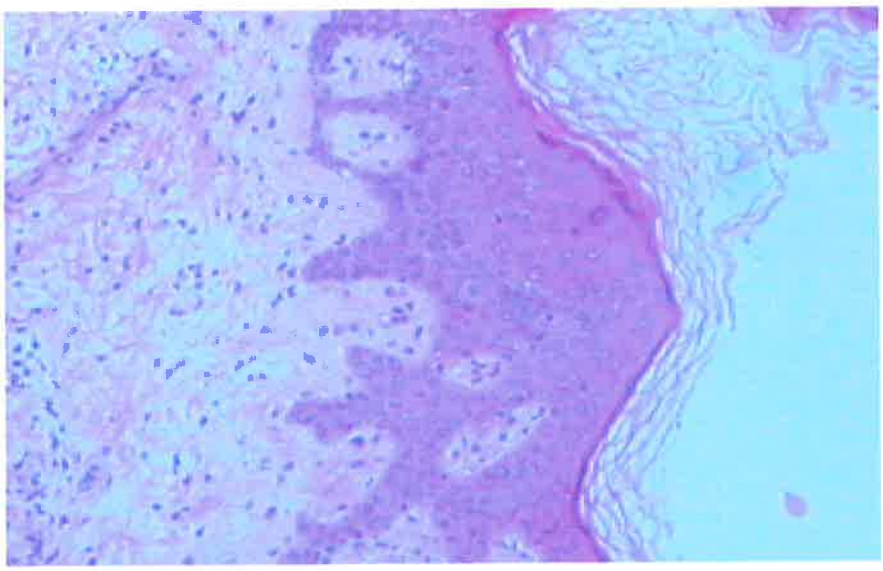


Figure 4.3.5 b) AdV IL-10 day 7 *H&E x100*

Day 7 post reconstitution human skin biopsies. NOD-scid mice engrafted with human skin and administered either AdV MX-17 DC (figure 4.3.5 a) or AdV IL-10 (figure 4.3.5 b) and allogeneic mononuclear cells. Note marked infiltration seen in animals treated with AdV MX-17 DC compared to near normal architecture seen in those treated with AdV IL-10 DC.

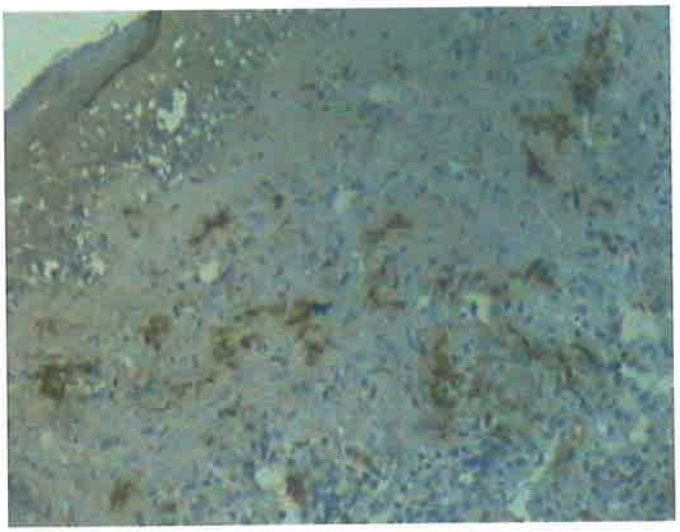


Figure 4.3.5 c) AdV MX-17 Day 7 *Immunoperoxidase x100*

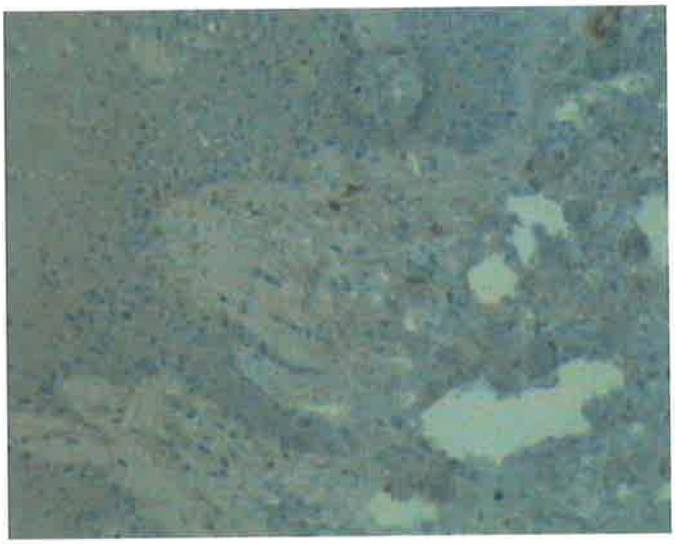


Figure 4.3.5 d) AdV IL-10 day 7 *Immunoperoxidase x100*

Immunoperoxidase study of human skin grafts treated with AdV MX-17 (4.3.5 c) DC or AdV IL-10 DC (4.3.5 d). Animals treated with AdV MX-17 DC showed upregulation of MHC class II molecules within skin grafts compared to animals treated with AdV IL-10 DC.

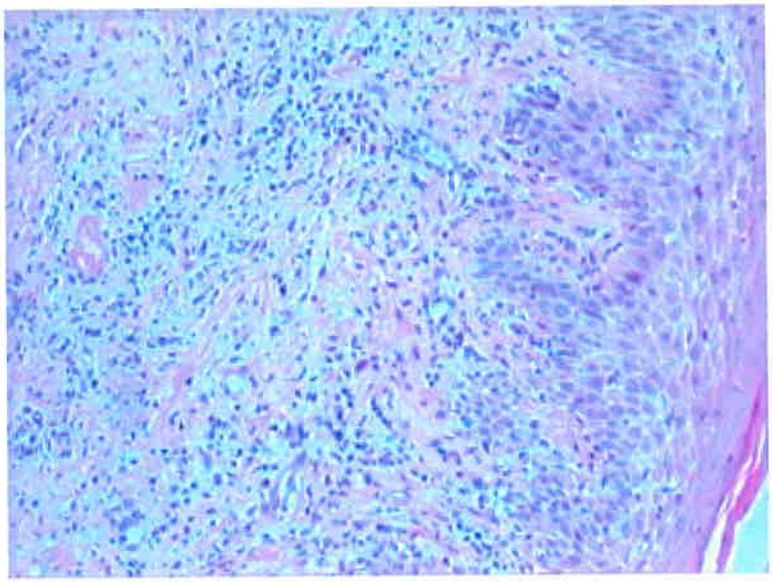


Figure 4.3.5 e) AdV MX-17 Day 14 *H&E x100*

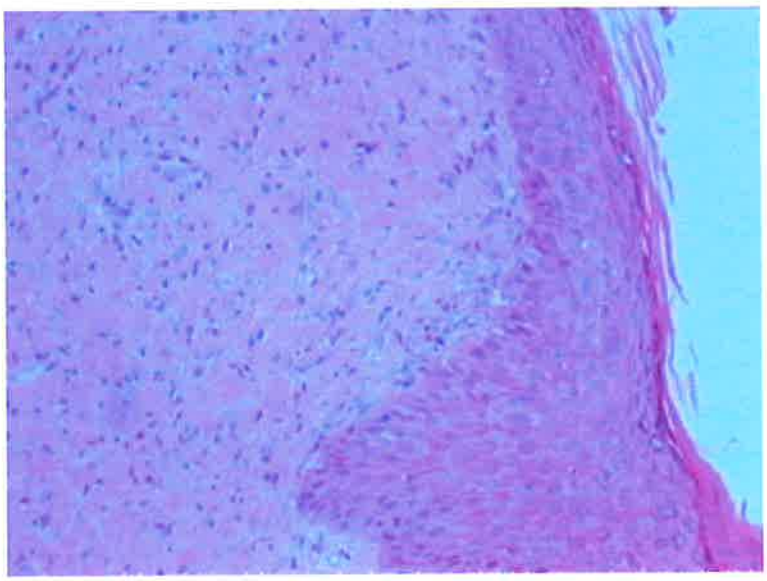


Figure 4.3.5 f) AdV IL-10 Day 14 *H&E x100*

Effect of intra-peritoneal DC transduced with AdV IL-10 or AdV MX-17 at day 14. Animals treated with AdV IL-10 showed reduced skin graft infiltration and DEJ destruction compared to animals treated with AdV MX-17.

The anti-rejection effect was enhanced by day 14 (figure 4.3.5 e and 4.3.5 f) with animals treated with AdV IL-10 DC showing near normal skin architecture compared to those receiving DC transduced with the control virus AdV MX-17. As the effective lifespan of reconstituted mice is between 14-21 days, time points beyond 14 days were not examined (Patrick 1998). To quantify the rejection process, the biopsies were graded by 2 observers blinded to the status of the animals and a rejection score calculated. The rejection scores for AdV IL-10 transduced DC and AdV MX-17 transduced DC are shown in figure 4.3.8. The mean rejection score for AdV IL-10 transduced DC treated animals was 1.2 +/- 0.97. The mean rejection score for AdV MX-17 transduced DC treated animals was 3.75 +/- 0.45 (p=0.0077 Wilcoxon Rank statistic).

4.3.6 Dendritic cells tranduced with Adenoviral β Galactosidase given via intraperitoneal injection migrate to the skin graft

In order to determine migration of human DC within the NOD-scid model, human DC were transduced with Adenoviral β galactosidase (AdV β–gal), prior to being infused into the peritoneum of NOD-*scid* mice. Subsequently the skin grafts were removed and processed for immunohistochemistry and routine light microscopy. In 2 separate experiments labeled DC were only identified in skin grafts. Liver and spleen were also processed but no labeled cells were isolated within these organs (figure 4.3.5).

4.3.7 Skin Donor Derived Fibroblasts Transduced with AdV IL-10 do not inhibit skin graft rejection in NOD-*scid* mice

To clarify the mechanism of AdV IL-10 DC protection of human skin grafts we transduced donor-derived fibroblasts with AdV IL-10 at identical MOI used for

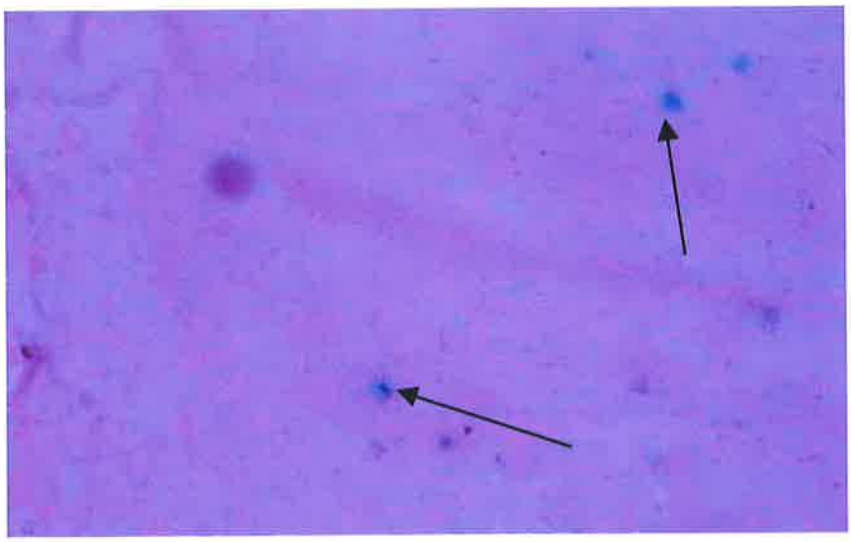


Figure 4.3.6

1x 10^6 DC autologous to the skin graft were transduced with AdV β-gal (blue) and were injected into the peritoneal cavity. Blue labeled cells were detected within human skin grafts within 48 hours of injection indicating migration from the peritoneal cavity (*x40 Fuscin counter stain*)

transduction of DC. Human skin grafts were removed at 7 and 14 days post engraftment and studied histologically for rejection. Mice (n=4) were injected with 1 x10 ^{6} donor-derived fibroblasts transduced with AdV IL-10, reconstituted with allogeneic mononuclear cells and were unable to protect skin grafts from rejection (figure 4.3.8). The mean rejection score for animals treated with fibroblasts transduced with AdV IL-10 was 4 and was not statistically different to animals treated with AdV MX-17 or immature DC (Wilcoxon rank).

4.3.8 Rejection Associated Cytokine Analysis of Human Skin Grafts

To further assess the immunological evidence of skin graft rejection, skin grafts were analyzed for the presence of rejection associated cytokines by reverse transcription polymerase chain reaction (RT-PCR). At non-saturating cycling conditions skin biopsies from animals treated with DC transduced with AdV MX-17 showed expression of FasL , whereas animals treated with DC transduced with AdV IL-10 showed absent messenger RNA for this cytokine (figure 4.3.7). Figure 4.3.7 shows one representative experiment of three.

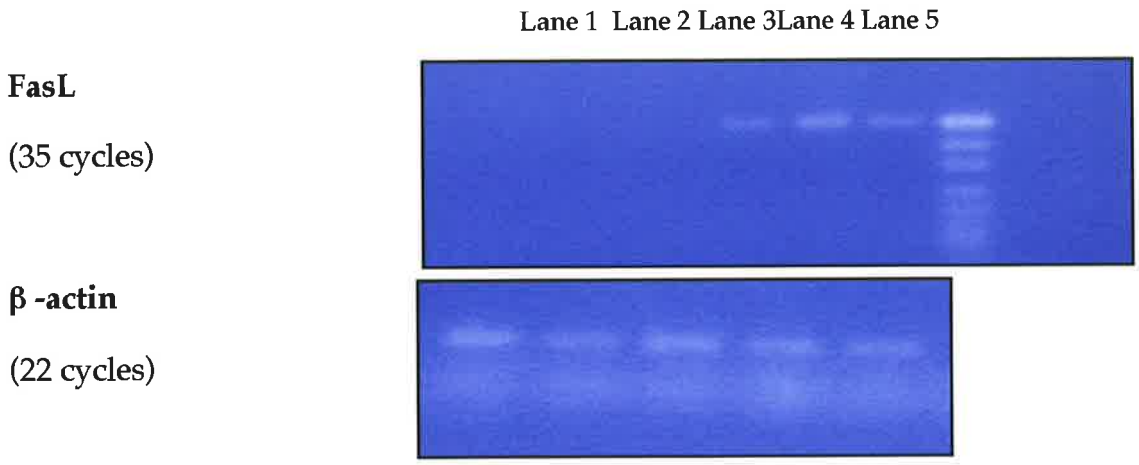


Figure 4.3.7 Analysis by PCR of human skin grafts for FasL. Top panel shows 500 base pair product. Lane 1 and 2 contain product from skin grafts treated with AdV IL-10 transduced DC. Lane 3,4 and 5 contain product from skin grafts treated with AdV MX-17 transduced DC. The gene β-actin was detected at 22 cycles with product of 317 base pairs.

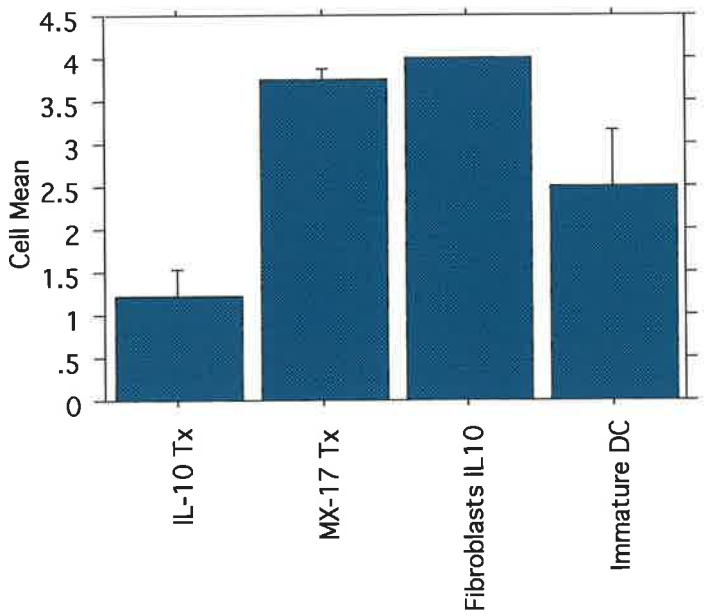


Figure 4.3.8

Pooled results scores shown for AdV IL-10, AdV MX-17, fibroblasts transduced with AdV IL-10 and immature myeloid DC treated animals at 2 time points day 7 and day 14 post reconstitution were expressed as mean score +/- one standard deviation. A rejection score for each biopsy was calculated using the following score: grade 0 rare leucocytes comparable to normal skin, grade 1 sparse perivascular leukocytes, grade 2 dense perivascular leukocytes, grade 3 dense perivascular leukocytes with modest infiltration of dermis grade 4 dense perivascular leukocytes with dense infiltration of dermis. The rejection scores for AdV IL-10 transduced DC treatment was significantly lower than AdV MX-17 or AdV IL-10 fibroblasts ($p=0.0077$ Wilcoxon rank statistic).

4.4 Discussion

In these experiments we studied the ability of human DC transduced with AdV IL-10 to modify human skin graft rejection in a small animal transplantation model. The model we used was the profoundly immunodeficient NOD-*scid* mouse model. When human skin is engrafted onto these mice indefinite skin graft survival is achieved (figure 4.3.1a). Engrafted skin remains pink and healthy for the lifespan of the mouse with grafts removed up to 12 months post engraftment remaining rejection free. Specifically the blood vessels remained patent, and the dermo-epidermal junction was free of infiltrating cells (figure 4.3.1b). This model differs only slightly from the humanized scid mouse transplantation model. The main advantage of the NOD-*scid* model is the lack of endogenous circulating NK cells, thus averting the need for administration of a NK cell depleting antibody such as anti-aislo-1 antibodies.

Engraftment of human split skin grafts was readily achieved and paralleled the experience of engrafting this strain of mice with ovine split skin (Patrick 1998). Studies in the sheep indicated that these skin grafts have a dual blood supply with ovine vessels growing out into the murine subcutaneous tissues and murine vessels that grow into the human skin graft. Thus allograft rejection when it occurs does not result in skin graft necrosis as murine vessels continue to supply the skin graft. Rejection of the grafts is however associated with graft discolouration, which was also observed in these studies. This is not surprising when the histology of rejected skin is reviewed showing small vessel thrombosis. Reconstitution of the murine immune system with allogeneic mononuclear cells given via intra-peritoneal injection causes rejection of engrafted human skin to occur. Based upon

previous humanized *scid* models we chose to reconstitute mice with between 2 and 3×10^8 allogeneic mononuclear cells derived from buff coats obtained from the Australian Red Cross.

In this transplantation model the rejection changes are histological, predominantly associated with infiltration of the dermato-epidermal junction by allogeneic cells. Small blood vessels that remain patent in the absence of allogeneic mononuclear cells develop thrombosis and therefore contribute to skin graft discolouration. In comparison to reconstitution of mice with allogeneic mononuclear cell cells, the administration of autologous cells does not result in skin graft rejection.

Previous studies in the *scid* mouse human skin graft model have shown that maximum rejection occurs within 16-21 days post reconstitution (Murray et al. 1994b; Murray et al. 1995; Murray 1998). Similar results were obtained in these studies with rejection being well established by day 21 post reconstitution. The addition of skin donor-derived professional antigen presenting cells (DC) via intra-peritoneal injection was associated with an enhanced rejection response with changes seen at 7 days post reconstitution that were typical of 21 days in the absence of extra DC. Antigen presentation occurs within the peritoneum where skin-donor derived DC may encounter allogeneic mononuclear cells. Addition of exogenous professional antigen presenting cells produced enhanced skin graft rejection by "direct pathway" presentation of donor derived antigens to allogeneic mononuclear cells. Skin graft rejection then depends upon migration of MNC to the graft. The maturational state of the DC population was found to be critical in determining the response. Previous studies have indicated the importance of DC maturation. When cardiac allografting is performed using donor animal strains

enriched for immature DC - prolonged, but not permanent allograft survival is observed (Fu et al. 1996; Hayamizu et al. 1998). This may be attributable to maturation of immature DC precursors within the allograft recipient.

In order to assess the effect of DC maturation upon skin graft survival the effect of IL-4/GM-CSF generated DC without use of the maturational stimulus rhTNF-α ("immature DC") with a similar population treated with rhTNF -α("mature DC"). The rejection score was lower in the immature DC-treated animals compared to the mature DC treated animals (see figure 4.3.8). This effect upon rejection paralleled the effect of proliferation observed in the mixed leukocyte reaction. As indicated in Chapter 3 this effect is likely to be a result of enhanced co-stimulation molecule expression upon the dendritic cell surface thus readily providing the necessary signal2 for optimal cell proliferation.

The *in vitro* experiments outlined in chapter 3 suggested that transduction of human IL-4/GM-CSF generated DC with AdV IL-10 was a promising strategy to induce hyporesponsiveness in allogeneic mononuclear cells. In order to test these cells in an *in vivo* model we injected $1x10^6$ DC into NOD-*scid* mice engrafted with human skin. Mice that received AdV IL-10 transduced DC showed significantly less rejection than those mice receiving DC transduced with the control adenovirus AdV MX-17 (figure 4.3.5 a-f and figure 4.3.8). The *in vitro* studies suggested that the mechanism of inhibition of skin allograft rejection was via effects on signal 2 (co-stimulation molecule expression) and signal 3 (production of interleukin-12). Another possible mechanism for this effect is the effect of transduction of AdV IL-10 upon the capacity of DC to induce cytotoxic T cells (Steinbrink et al. 1999). Steinbrink et al have studied the effect of exogenous IL-10 upon DC function in the

context of melanoma and found limitation of the capacity to produce melanoma-specific CTL. Whilst this was not formally studied in these experiments, it may be contributing to the protective effect.

As DC transduced with AdV IL-10 are capable of secretion of biologically active IL-10 (figure 3.3.6) it is possible that the mechanism of the protective effect observed in these studies may also be due to a local effect of IL-10. To test this hypothesis an equivalent number of human fibroblasts were transduced with AdV IL-10 and mice were reconstituted via intraperitoneal injection using the same protocol. Skin graft rejection at day 7 and day 14 was not modified by injection of AdV IL-10 transduced human fibroblasts (see figure 4.3.8). This indicates the necessity of antigen presentation via DC as well as the presence of biologically active IL-10.

In order to ascertain the site of action of the transduced DC, DC transduced with AdV β–gal were injected under the same conditions into the peritoneum. Blue labeled cells were subsequently detected within the skin graft within 24 hours of injection but not within the murine liver or spleen (figure 4.3.6). Although sections of the peritoneum were not formally studied it seems most likely that the site of action of the adenoviral transduced DC is within the peritoneum as well as the skin graft.

In summary, NOD-*scid* mice transplanted with human split skin grafts will accept grafts without difficulty in the absence of allogeneic mononuclear cells. When allogeneic mononuclear cells were injected into the peritoneal cavity, rejection was evident by day 21 and is associated with destruction of the dermato-epidermal

junction and upregulation of MHC class II molecules within the skin graft. This rejection process was accelerated by the addition of DC autologous to the graft to the mice. Administration of AdV IL-10 DC was associated with inhibition of the rejection response for up to 14 days compared to AdV MX-17 transduced DC.

Chapter 5

Characterization and Transduction of Ovine Pseudoafferent Dendritic Cells with Adenoviral Gene Constructs

6.0 Introduction

The recognition of the pivotal role of DC in the maintenance of central tolerance and possibly peripheral tolerance has focused attention on manipulation of DC function as a potential means of tolerance induction. Small animal transplantation models have suggested that DC based immunosuppressive strategies are a promising new technology for allotransplantation (Fu et al. 1996; Lutz et al. 2000; Min et al. 2000). However, prior to adoption of such technology for human transplantation it is necessary to test the therapeutic potential of DC in large animal pre-clinical transplant models.

Large animal models for transplantation have generally focused around dogs and pigs. The canine model was the mainstay of early transplantation research, with the notable development of the therapeutic agents Cyclosporin and Azathioprine largely based around this pre-clinical model. However the general availability of mongrel dogs for medical research is reducing and the genuine attachment felt by humans for dogs has made canine research much less acceptable now than in previous times. As an alternative large animal transplantation model the sheep has

many advantages. Sheep are less costly than other large animals (such as non human primates and pigs) and in general the animals are docile, tolerating experimental procedures well (Hecker 1974). The immunology of the sheep is well studied and a considerable array of sheep specific immunological reagents have been developed by the University of Melbourne, the host laboratory and other groups (Mackay et al. 1985; Maddox et al. 1985; Puri et al. 1987b; Puri et al. 1987a; Grooby et al. 1997). The sheep has also long been favoured as an experimental model to study lymphocyte migration (Young 1999). The particular advantage of the ovine model is that lymphatic vessels draining individual lymph nodes can be readily accessed and cannulated to collect lymph in a variety of pathological and non pathological situations. Lymph nodes within sheep do not occur in chains (as they do in humans) and the efferent lymphatic channel drains directly to the thoracic duct, making lymphocyte migration studies much easier to perform (Issekutz 1984; Issektuz 1985). Furthermore DC may also be readily extracted from ovine lymph using gradient density separation (Bujdoso 1989). The ovine heterotopic renal transplant model is also well established allowing the ability to test DC therapy in a relevant pre-clinical kidney transplant model (described in detail in chapter 7) (Pedersen et al. 1970; Pedersen et al. 1974) (Grooby et al. 1998).

As described in chapter 1, DC migrate from the peripheral tissues to draining lymph nodes and they make up approximately 10% of the cellular content of lymph (Young 1999). Excision of the pre-femoral lymph node of sheep results in the formation of a new lymphatic channel, the "pseudoafferent" lymphatic vessel. The cannulation of these pseudoafferent lymphatic vessels with heparin impregnated cannulas is a means by which unfractionated lymph may be collected from sheep. Such lymphatic channels may be easily cannulated and the lymphatic

flow maintained for prolonged periods. Dendritic cells may be enriched from the collected lymph using a 14% (w/v) Metrizamide® gradient. As sheep tolerate pseudoafferent cannulation well this model has been used to study long term DC biology with specific reference to topical application of carcinogens (Dandie et al. 1992; Ragg et al. 1995; Ragg et al. 1997). However, the generation of fresh monocyte-derived DC using recombinant cytokines akin to the manner described in chapter 3 has not been described. The recombinant ovine cytokine GM-CSF has been synthesized and enhances the survival of ovine afferent lymphatic DC in culture (Haig et al. 1995). However an ovine specific source of IL-4 is not commercially available. Prolonged efforts within the host laboratory to use a combination of recombinant human GM-CSF and recombinant human IL-4 to generate ovine DC from ovine monocytes were unsuccessful and therefore pseudoafferent lymphatic cannulation was used as the source of DC for these studies.

The specific aims of this chapter were to: a) establish stable collection of ovine dendritic cells b) transduce ovine dendritic cells with adenoviral IL-10 gene construct and study their *in vitro* properties to modify the alloimmune response prior to their use in a large animal renal transplantation model.

6.0 Materials and Methods

6.0.0 Cannulation of Pseudoafferent Ovine Lymphatic Vessels.

Two year old outbred Merino sheep were housed in the animal storage facility at The Queen Elizabeth Hospital and allowed free access to food and water. The pre-femoral lymph node was identified and excised under general anaesthesia. Subsequently the animals were returned to normal husbandry practice for 6-8

weeks whilst the pseudoafferent vessels were formed. The animals were then returned to the animal storage facility at The Queen Elizabeth Hospital. Under general anaesthesia the pre-femoral region was re-opened. Into the skin draining the pre-femoral region 1ml of Patent Blue V dye (Sigma, Australia) was injected intra-dermally. The enlarged pseudoafferent lymphatic was then identified as an enlarged blue channel and cannulated using 3Fr heparin impregnated cannulas (Carmeda, San Antonio, USA). The cannula was then externalised and sutured under the skin.

The lymphatic drainage was collected into sterile 100ml bags supplemented with heparin 5000 IU, penicillin 1.2 $x10^6$ units and gentamicin sulphate 40mg. On average 24 hour lymphatic volume was between 100ml and 150ml. The typical appearance of freshly isolated ovine DC is shown in figure 5.3.1.

5.2.2 Separation of Ovine DC from Ovine Lymph using Metrizamide®

Gradient Separation

Ovine lymph was centrifuged at 400*g* for 10 minutes. The resultant cellular pellet was resuspended in 7.5ml of complete medium and underlayered with 2.5ml Metrizamide® 14% w/v (in complete medium) in a sterile 10ml V bottom tube. The cell suspension was then centrifuged at 800*g* for 15minutes without the application of a braking force. The DC were collected at the interface between Metrizamide® and the cell suspension and washed twice in complete medium prior to use in further studies. Typical flow cytometric analysis of collected DC showed approximately 80-90% CD1a expressing cells, with the remaining cells being CD4 and CD8 cells (data not shown). These cells were used as the source of DC in further studies.

5.2.3 Electron Microscopy of Ovine Dendritic Cells

Freshly isolated Metrizamide® gradient separated ovine DC were collected into 1% glutaraldehye based fixative prior to transmission and scanning electron microscopy.

Scanning Electron Microscopy

Fixed DC were filtered onto a 200nm millipore filter and further fixed with 2% osmium tetroxide, dehydrated with graded ethanol and critical point dried. The filtered and dried cells were adhered to an SEM stub and coated with gold in a sputter coater. Cells were then examined in a Hitachi S-520 (Hitachi , Japan) scanning electron microscope.

Transmission Electron Microscopy

Fixed DC were post fixed in 2% osmium tetroxide, stained *en bloc* with osmium tetroxide and dehydrated through a series of ethanol washes. Cells were then placed in 1,2-epoxypropane. Subsequently the cells were further processed in a 50/50 mixture of 1,2-epoxypropane and procure 812 resin, and then, two changes of 100% resin. Cells and resin were transferred to TAAB embedding capsules and placed in an oven for 12 hours at 90°C. Between each step cells were centrifuged at 2000 rpm for 10 minutes (800*g*). The embedded cells were then thin-sectioned at approximately 90nm thickness on a Porter-Blum (Sorvall) ultramicrotome using a diamond knife. Sections were stained with Reynolds' lead citrate and examined in a Hitachi H-6000 transmission electron microscope (Hitachi, Japan).

5.2.4 Transduction of Ovine Dendritic Cells with Adenoviral Gene Construct: AdV GFP

Ovine DC were transduced with adenoviral gene therapy constructs using protocols derived from human monocyte-derived DC (see chapter 3). Ovine DC were washed in MEM and resuspended at 1 x 10^6 cells per 100µl. Adenoviral GFP (MOI 5000:1) was initially mixed with the cationic lipid Lipofectamine® 1.25µg for fifteen minutes prior to contact with ovine DC. DC were then incubated with the adenoviral-Lipofectamine® mixture for 90 minutes in humidified oxygen supplemented with 5% CO_2. After gene transduction the cells were then washed 3 times with complete medium, prior to their use in further studies.

5.2.5 Isolation of $CD8^+$ Dendritic cells by Positive Selection using Labeled Immuno-magnetic Beads

$CD8^+$ Dendritic cells were isolated from ovine pseudoafferent lymph using immunomagnetic bead separation. Fresh unconjugated 4µm beads (Detachabead®, Dynal, Oslo, Norway) were washed using PBS supplemented with 1% Bovine Serum Albumen. Ten million beads (25µl of stock concentration) was resuspended in 100µl of PBS and then incubated with fresh anti ovine CD8 ascites (SBU T8) for 30 minutes at room temperature. The beads were then washed twice using 1 ml of fresh PBS prior to being incubated for 15 minutes with freshly isolated Metrizamide® gradient purified ovine DC at a bead to target cell ratio of 5 beads to 1 cell at room temperature. The bead-cell complex was separated from the CD8 negative dendritic cells by use of a magnetic cell particle separator (MCP-1, Dynal, Oslo). The $CD8^-$ fraction was then washed twice using 1 ml PBS and used for other studies. The remaining $CD8^+$ fraction was separated prior to their use in further

studies from the Dynabeads® by incubation with a DNAase supplied by the manufacturer.

5.3 Results

5.3.1 Flow Cytometric Characterization of Ovine DC

Freshly isolated ovine DC were isolated from pseudoafferent lymph as indicated, and the cell surface marker profile of these cells was studied using a panel of ovine and human monoclonal antibodies (figure 5.3.1). Ovine DC stained strongly with SBU-I (class I), SBU-II (class II). These cells displayed low cell surface expression of ovine CD-14. The level of co-stimulatory molecule expression was assessed using the chimeric-human murine immunoglobulin-fusion protein CTLA4 with its control fusion protein MCD 40 (shown in green overlay on figure 5.3.1) Ovine DC showed low level CTLA4 staining consistent with an immature DC phenotype. As there are currently no commercially available ovine DC specific markers ovine DC were also stained with anti human CD83 (a specific marker for human DC). In humans immature monocyte-derived DC do not express CD83 unless they have been exposed to a maturational stimulus such as TNF-α (see 3.3.3). In contrast the resting ovine pseudoafferent DC also cross react with anti- human CD83.

5.3.2 Electron Microscopic Characterization of Ovine DC

Freshly isolated ovine DC were collected and resuspended in either glutaraldehyde based fixative or into the electron microscopic embedding medium LR white. Scanning electron microscopy of cells showed a typical veiled morphology (figure 5.3.2a).

The typical transmission electron microscopic appearance of ovine DC showed short cytoplasmic processes with large multilobed cell nuclei (see figure 5.3.2b). In

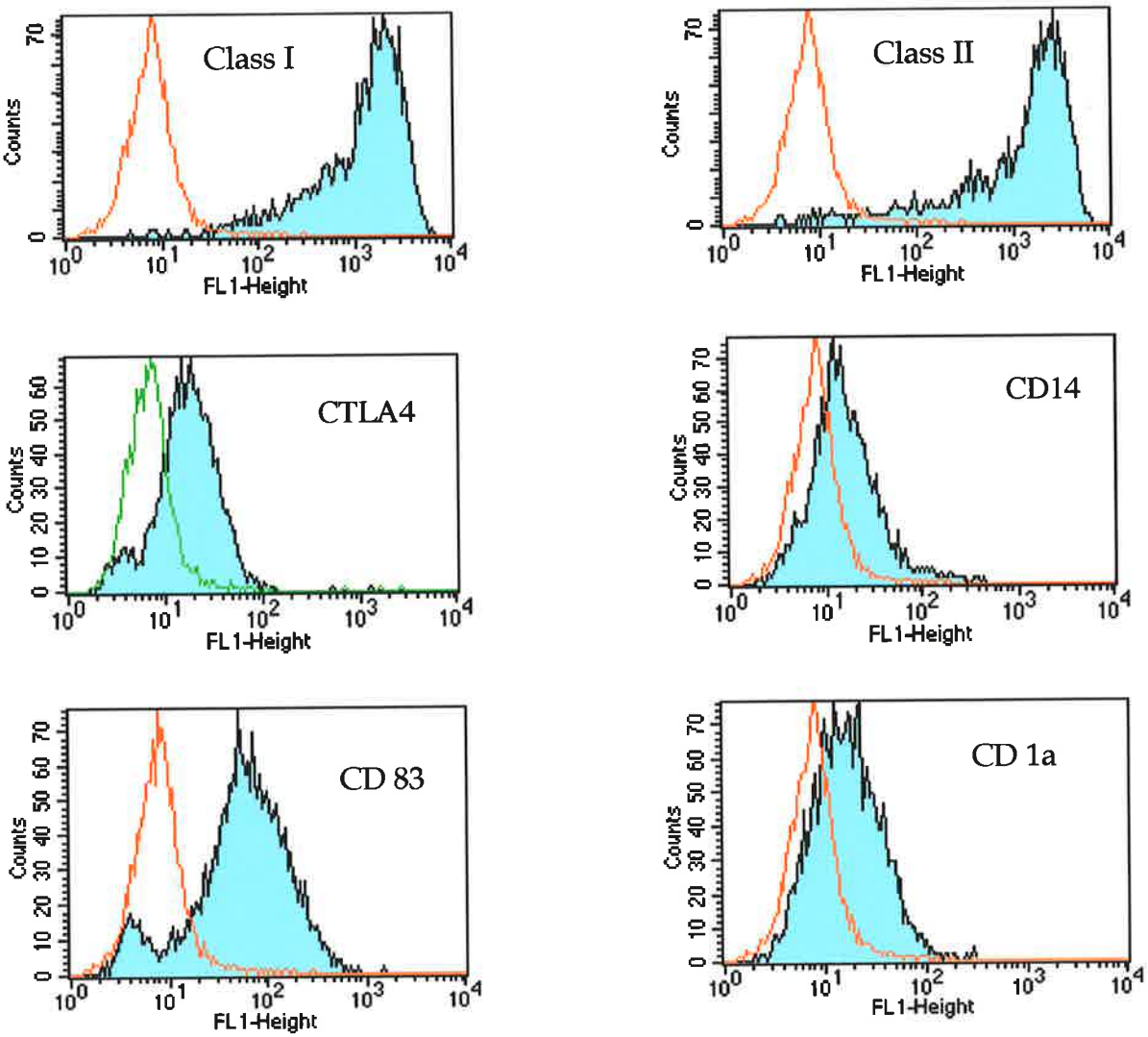


Figure 5.3.1
Flow cytometric analysis of ovine dendritic cells. The blue histogram represents the dendritic cell population for each antibody indicated. The brown or green histogram represents isotype matched irrelevant antibody. Results representative of at least 10 experiments.

order to further characterize ovine pseudoafferent lymphatic DC, the localization of class II molecules (SBU Class II) was studied using immunoelectron microscopy of labeled gold particles. Class II molecules were identified within cytoplasmic vesicles (see figure 5.3.2c)

5.3.3 Pseudoafferent Ovine DC are Capable of Uptaking FITC-dextran

In order to study the ability for ovine DC to take up antigens, freshly isolated ovine DC were incubated with fluorescein conjugated dextran (FITC- dextran) of molecular weight 40,000 and then studied using flow cytometric analysis. Ovine DC showed rapid uptake of FITC-dextran within 5 minutes of incubation in the presence of 1mg/ml FITC-dextran (figure 5.3.3). Samples were analyzed at room temperature (23°C) and control samples were maintained at 4°C. Figure 5.3.3 shows one representative experiment of three separate experiments.

5.3.4 Pseudoafferent DC are Potent Stimulators of the Mixed Leukocyte Reaction

The ability of freshly isolated ovine DC to stimulate a proliferative response in mixed leukocyte reaction was studied using various ratios of stimulator:responder cells. Figure 5.3.4 a) shows a representative experiment of 8 separate experiments. Maximal 5 day proliferative response as assessed by incorporation of tritiated thymidine of greater than 40,000 counts per minute were typical.

In order to assess the capacity of ovine DC to induce proliferation in autologous MNC in response to exogenous antigen, proliferation assays were performed. Dendritic cells were pulsed with the antigen ovalbumen (1mg/ml) for 30 minutes prior to culture with tritiated thymidine incorporation measured after 5 days

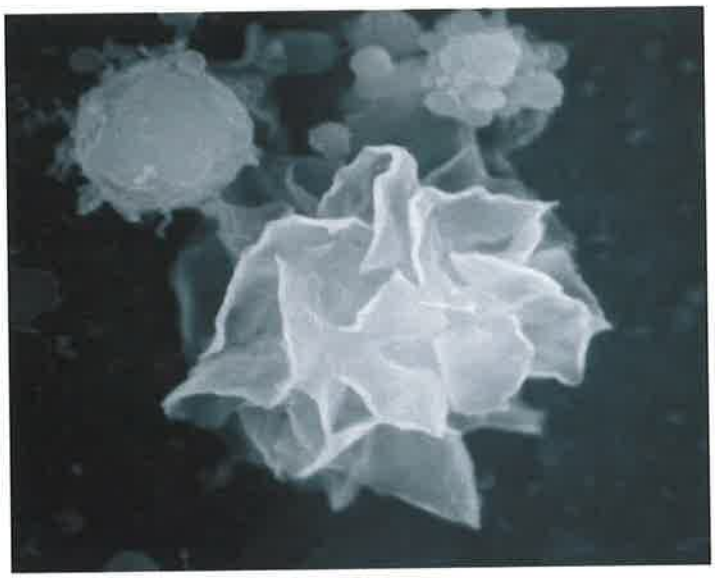


Figure 5.3.2a

Scanning electron micrograph of ovine DC showing characteristic "veils" that are shown in cross section in figure 5.3.2b.

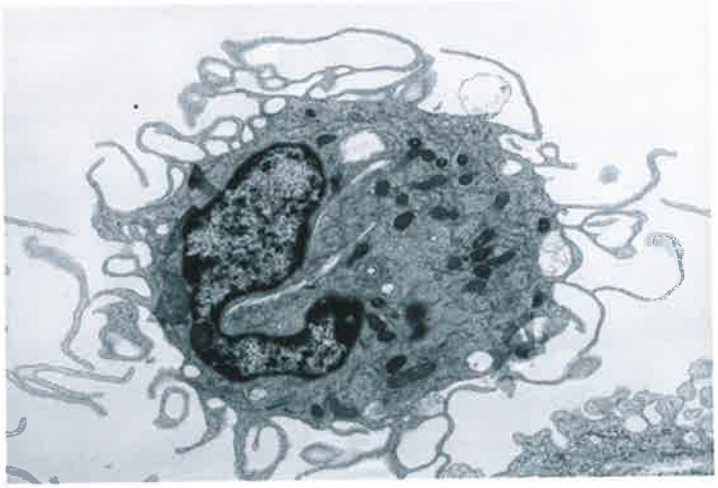


Figure 5.3.2b

The typical transmission electron micrograph showing an ovine DC with extensive cytoplasmic processes that form "veils" see figure 5.3.2a

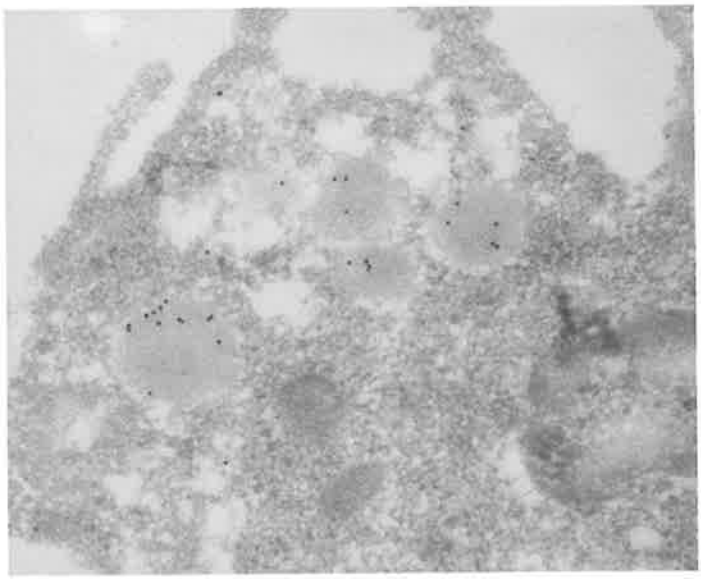


Figure 5.3.2c

Immunogold SBU II labelled beads (black dots) showing localisation of class II molecules within intra-cytoplasmic vesicles of ovine DC. Original magnification x 17,000.

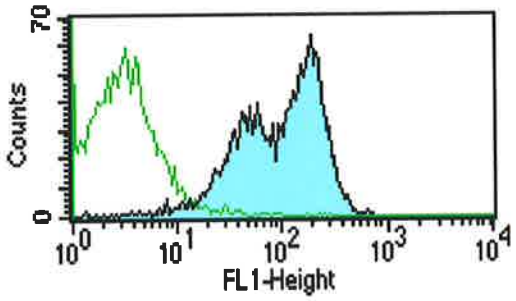


Figure 5.3.3

Flow cytometric analysis showing freshly isolated ovine DC and rapid uptake of FITC dextran (blue histogram) indicating the ability to take up exogenous antigenic material. X axis represents fluorescence intensity, Y axis represents cell numbers. Isotype matched irrelevant antibody shown in green.

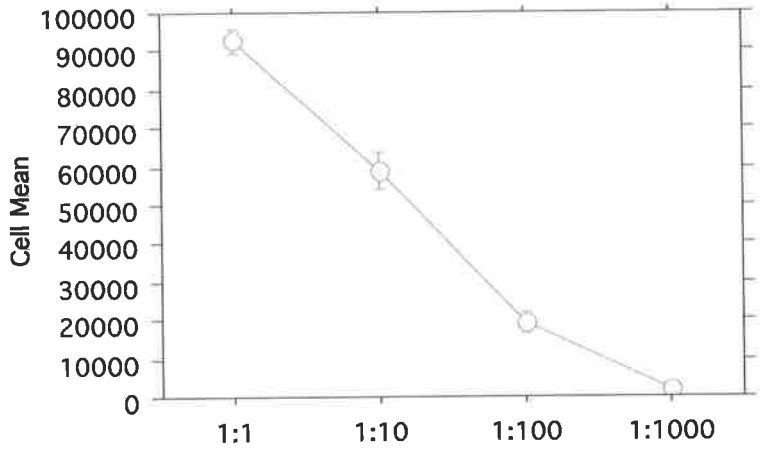


Figure 5.3.4a

Ovine DC show proliferation when incubated with allogeneic mononuclear cells in the 5 day MLR at variable stimulator responder ratios.

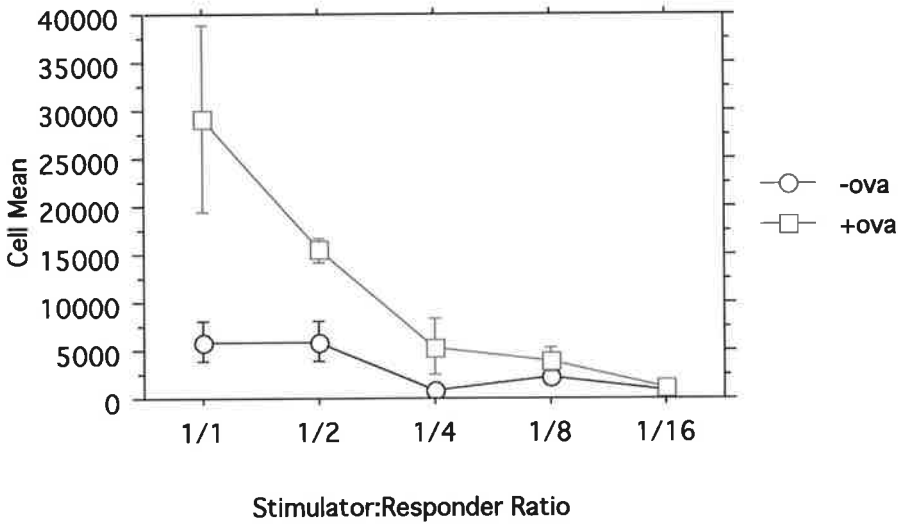


Figure 5.3.4 b)

Results of 5 day 1 way MLR of ovine DC isolated using Metrizamide® gradient separation and pulsed for 1 hour with (red line) or without (green line) ovalbumen prior to incubation with autologous mononuclear cells. Ovine DC were treated with mitomycin C to prevent proliferation in the MLR reaction. A marked increase in proliferation was seen in the cells exposed to exogenous antigen compared to unexposed cells indicating enhanced proliferation of autologous T cells in response to antigen-pulsed DC.

incubation. Figure 5.3.4b shows the capacity of ovine DC to induce proliferation in autologous mononuclear cells after culture with ovalbumen (experiment representative of 3 separate experiments).

5.3.5 Transduction of Ovine Pseudoafferent DC with Adenoviral Gene Constructs

Using the transduction protocols described in chapter 3 ovine pseudoafferent DC were transduced with the reporter gene AdV-GFP. The percentage of gene transduction was then assessed using flow cytometric analysis (figure 5.3.5). A multiplicity of infection of between 2500 and 5000:1 viral particles per cell gave consistently high gene transduction (>90%). As in human studies there was minimal cellular toxicity as assessed by EMA staining on flow cytometric analysis and cellular uptake of toluene B dye (data not shown).

5.3.6 Transduction of Ovine DC with AdV IL-10 inhibits the Mixed Leukocyte Reaction

Transduction of ovine DC with AdV IL-10 was performed under standard conditions (MOI 5000:1). Simultaneously ovine DC were transduced with the control gene construct AdV MX-17 under the same conditions and a third population of DC was kept untransduced. The cells were then placed in MLR at a variety of stimulator:responder ratios (figure 5.3.6). Figure 5.3.6 shows a representative experiment of 5 separate experiments. DC transduced with AdV IL-10 showed consistent strong inhibition of the MLR. Interestingly, in contrast to the human studies, the control virus MX-17 showed moderate inhibition of the MLR in comparison to untransduced cells upon proliferation within the 5 day MLR.

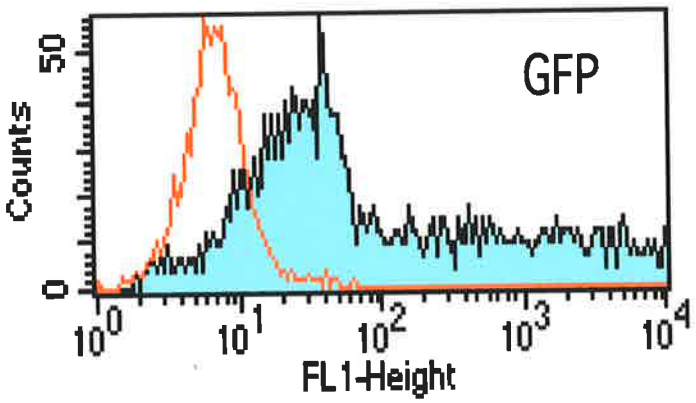


Figure 5.3.5

Flow cytometric analysis of ovine DC transduced with AdV GFP (blue histogram) at MOI 5000 show >90% gene transduction. X axis represents cell numbers, Y axis represents fluorescence intensity. Isotype matched negative control antibody shown in brown.

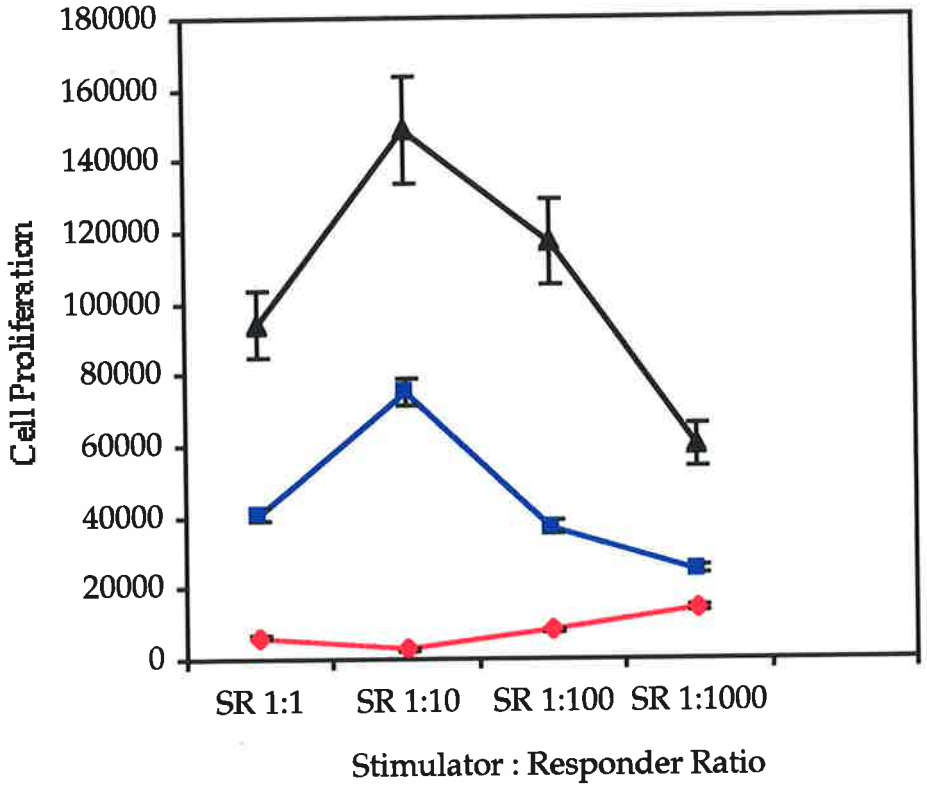


Figure 5.3.6

The mean stimulatory capacity of ovine DC in allogeneic MLR untransduced (black), transduced with AdV IL-10 (red) or AdV MX-17 (blue) at variable stimulator : responder ratios in the 5 day MLR. At all stimulator:responder ratios transduction of DC with AdV IL-10 was most effective at inhibition of proliferation.

5.3.7 Effect of Transduction of DC with AdV IL-10 upon cytokine production in the Mixed Leukocyte Reaction

In order to study the effect of AdV IL-10 transduction upon cytokines present within the MLR, reverse transcription polymerase chain reaction (RT-PCR) was performed. Ovine DC were collected in the manner described and transduced with AdV IL-10. Subsequently the transduced DC were cultured with allogeneic mononuclear cells at a stimulator:responder ratio of 1 DC to 100 MNC and incubated in a humidified atmosphere. After either 4 hours or 20 hours the cells were harvested, RNA extracted and the cytokine species studied by RT-PCR. The housekeeping gene β–actin was detectable at 23 cycles in all samples. When ovine IL-12 p40 and p35 were studied, mRNA for IL-12 p35 was suppressed at non-saturating conditions (figure 5.3.7). This indicated the capacity to produce biologically active hetero-dimer IL-12 (requiring the two components IL-12 p40 and IL-12 p35) had been down-regulated. Messenger RNA for ovine IL-2 was detectable in all samples.

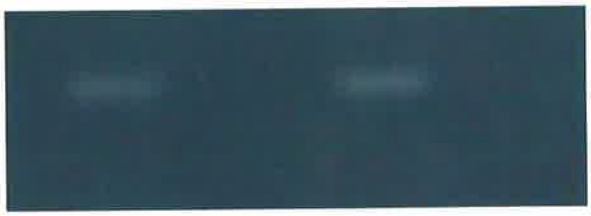
5.3.8 A population of ovine pseudoafferent dendritic cells express CD8

During the initial characterization of Metrizamide®-enriched ovine DC, a population of DC was noted within pseudoafferent lymph which expressed the DC marker CD83 and co-expressed CD8. These cells consistently and reproducibly represented between 1 and 5% of the total DC population at any given time point after pseudoafferent lymphatic cannulation. Figure 5.3.8 shows a representative dual labelling analysis of ovine DC. In light of recent research, publications concerning the potential tolerogenic nature of $CD8\alpha^{+}$

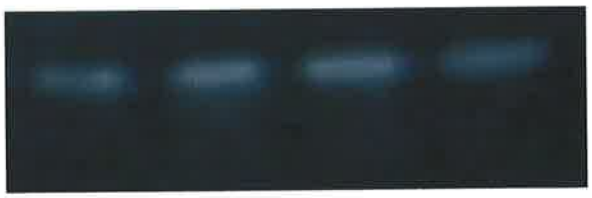
DC further enrichment and separation of these cells was undertaken using Dynabeads coated with SBU T8 supernatant (see 5.2.4).

Lane1 Lane 2 Lane 3 Lane 4

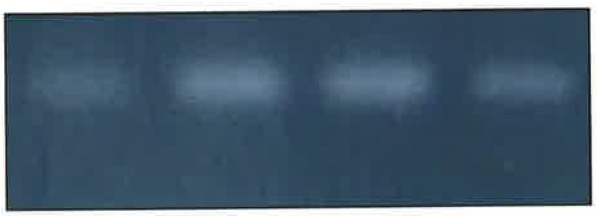
a ovine IL-12 p40 , 35 cycles (product 461 base pairs)



b ovine IL-12 p35 ,35 cycles (product 550 base pairs)



c ovine IL-2 ,35 cycles (product 409 base pairs)



d ovine β - Actin ,23 cycles (product 317 base pairs)

Figure 5.3.7

Analysis by PCR of mRNA from MLR. Lane 1 (L to R) :untransduced DC at 4 hours. Lane 2 :pcr products from AdV IL-10 transduced DC at 4 hours.Lane 3 shows untransduced DC at 20 hours. Lane 4 shows AdV IL-10 transduced DC at 20 hours. All samples showed detectable mRNA for ovine IL-12p40, but in samples from AdV IL-10 transduced DC the mRNA for ovine IL-12p35 was suppressed at non saturating conditions. IL-2 was detectable in all samples studied.

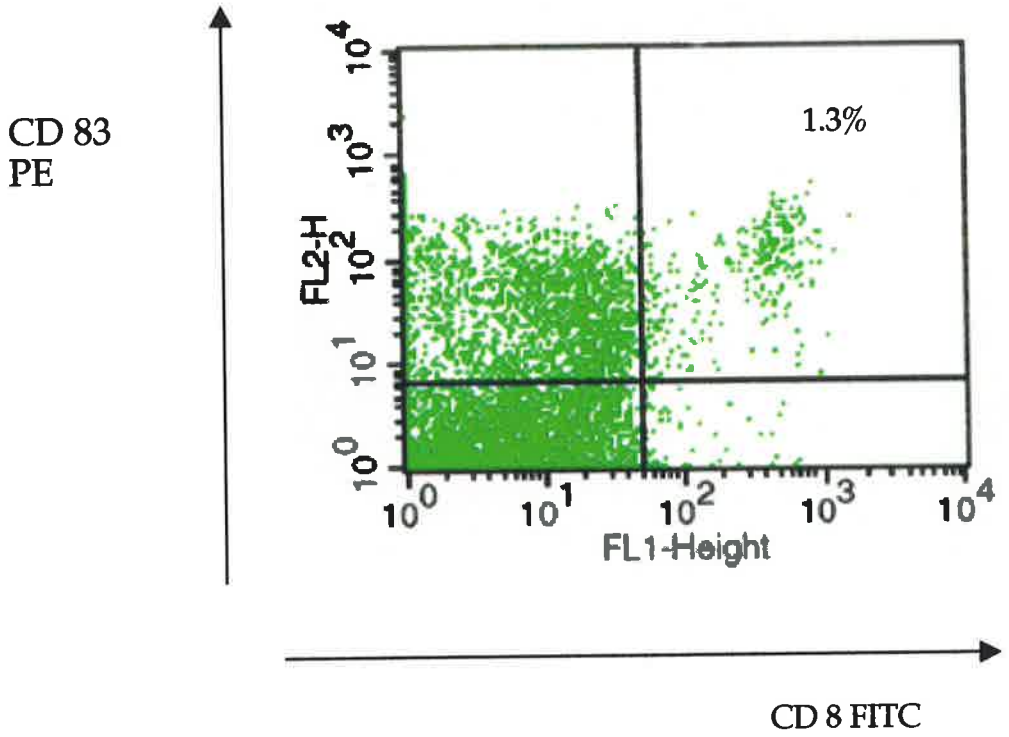


Figure 5.3.8

A population of ovine DC express CD8 and CD83 (1.3% right upper quadrant). X axis represents CD8 fluorescent channel, Y axis show CD83 phycoerythrin channel. Dual labeling shows a population of ovine DC expressing both cell surface markers.

Results representative of 3 separate experiments.

5.3.9 Electron Microscopic Analysis of Ovine $CD8^+$ Dendritic cells

Ultrastructural analysis of dendritic cells expressing CD8 was performed on freshly separated DC using scanning (SEM) and transmission electron microscopy (TEM). Dendritic cells expressing CD8 showed typical cell cytoplasmic projections. There was little difference in ultrastructural appearance between populations of sheep DC that were either $CD8^+$ or $CD8^-$. An electron micrograph demonstrating the binding of DC to a $CD8^+$ Dynabead® is shown in figure 5.3.9a. To confirm the cell surface expression of $CD8^+$ DC, a fresh population of pseudoafferent DC was studied using transmission electron microscopy and 10 μm immunogold particles labeled with SBU-T8. These studies confirmed that a population of sheep DC within pseudoafferent lymph express the ovine cell surface marker CD8 (figure 5.3.9b).

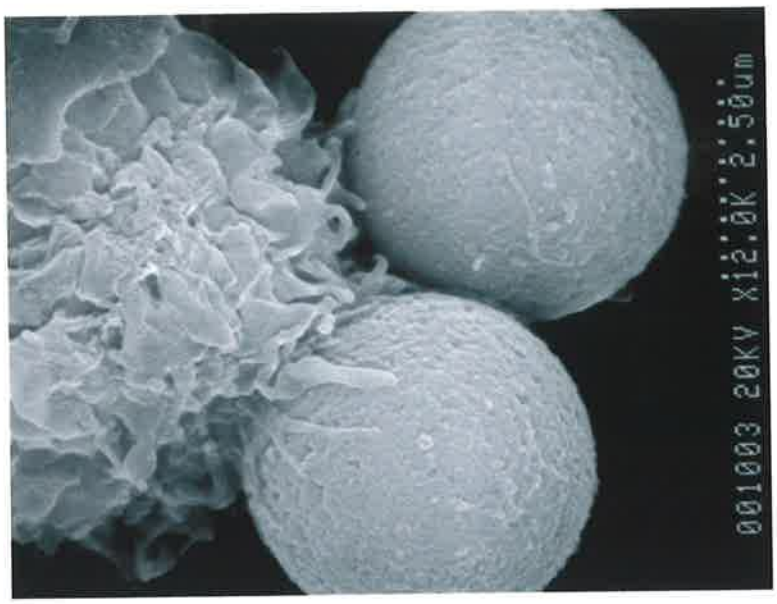


Figure 5.3.9a

Scanning electron micrograph showing Dynabeads® labelled with SBU T8 bind to ovine DC. SBU T8 labelled DC were then separated from the beads by use of a specific DNAase provided within the Dynabead kit.

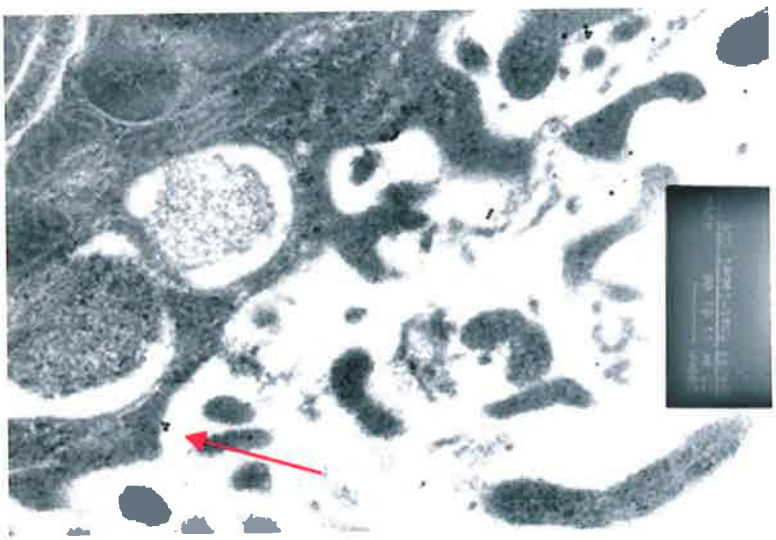


Figure 5.3.9b

Ovine DC labelled with SBU T8 immunogold particles (black dots red arrow), demonstrating a population of ovine DC expressing CD8 upon their cell surface (original magnification x15,000).

5.4 Discussion

This chapter describes 1) the enrichment of ovine DC from ovine lymph collected from sheep by pseudoafferent lymphatic cannulation 2) the identification of $CD8^+$ and $CD8^-$ subsets and the subsequent transduction of unfractionated ovine DC with the adenoviral gene constructs AdV IL-10 and AdV MX-17. Pseudoafferent lymphatic cannulation was chosen as the means of collecting ovine DC after several months of attempts to generate ovine DC from monocyte precursors had failed. Stable cannulation of the reformed pseudoafferent lymphatic vessel has meant that the composition of ovine lymph has been well studied (Haig et al. 1999; Young 1999). Separation of the DC fraction using Metrizamide® gradient density separation allows large numbers of fresh DC to be isolated on a daily basis. In common with other groups, we found the ability to maintain long-term lymphatic cannulation of up to 6 or more months relatively easily. There were several factors that allowed this to occur. These included the use of Patent Blue V® dye injected intra-dermally, which allowed a clear identification of the reformed lymphatic vessel and the practice of returning the animal to pasture during the recovery phase post surgery. In order to reduce the risk of catheter thrombosis, subcutaneous heparin was injected daily for at least the first two weeks post cannulation.

The morphology and phenotype of the cells isolated by Metrizamide® gradient separation was consistent with the descriptions of ovine DC within the literature (Ragg et al. 1995). In common with mouse and human DC, ovine DC express the cell surface marker CD1a. Morphologically they were large irregular cells with veiled cellular processes on light microscopy and electron microscopy (see figures 5.3.2 a and b). When immunoelectron microscopy was performed MHC class II

molecules were observed within DC vesicles - a feature typical of immature DC (figure 5.3.2c). These cells expressed the cell surface markers MHC class I, MHC class II, CD14, and low level co-stimulatory molecules. One unusual feature of these cells was cross reaction with the human monoclonal antibody CD83. CD 83 is a member of the immunoglobulin gene super family for which no specific function has yet been identified (Zhou and Tedder 1996). However CD83 is one of few specific human DC markers currently available that is usually expressed in mature DC (the others being CMRF-44 (Fearnley et al. 1997) and CMRF-56 (Hock et al. 1999)). Freshly isolated pseudoafferent ovine DC show consistent cross reaction with this human DC specific reagent.

The maturational status of these DC is important, as this influences their allostimulatory capacity. The evidence for an immature status is firstly with antigen uptake. Dendritic cells may take up antigenic material by phagocytosis and by a variety of membrane bound receptors (Hart 1997). Exogenous antigenic material once encountered undergoes degradation and proteolysis to produce peptides within the cytosol. During this phase, exogenous antigenic material is degraded within acidic lysosomes which fuse with vesicles containing freshly synthesized MHC Class II molecules (with binding sites protected by class II invariant protein or CLIP). Fusion of exogenous antigenic material with MHC class II enriched vesicles is accompanied by loss of the ability to process antigen (Stossel et al. 1989; Nijmann et al. 1995). Pseudoafferent DC are capable of taking up FITC dextran (figure 5.3.3) - an observation that has also been made by others (Sallusto et al. 1995). Secondarily, these MHC class II molecules can be demonstrated still within endosomes within the cytosolic compartment (figure 5.3.2 c). Finally,

exposure of ovine pseudoafferent DC to antigen (as evidenced by the effect of ovalbumen) results in increased capacity of DC to stimulate proliferation of autologous MNC (figure 5.3.4).

It is possible to reconcile the cell surface expression of the CD83 and the ovalbumen data if pseudoafferent ovine DC are considered to be "activated" DC (as evidenced by the surface expression of CD3) which become "super-activated" DC upon exposure within the MLR (Matzinger 1999).

Ovine DC were then transduced with adenoviral reporter gene constructs (AdV GFP and AdV β–Gal). The transduction conditions derived for human DC achieved similar levels of gene transduction in the ovine system with greater than 90% gene transduction consistently being achieved. Importantly there was minimal toxicity from the gene transduction as assessed by flow cytometric analysis or by Toluene Blue dye uptake. The introduction of the gene for IL-10 via the vector AdV IL-10 resulted in significant inhibition of the ability of DC to allostimulate when compared to either the control virus or un-transduced DC (figure 5.3.6). This inhibitory effect of transduction of ovine DC with AdV IL-10 was very similar to the effect seen when human monocyte-derived DC were transduced with the same construct.

The cytokine milieu of the MLR was then investigated using PCR. Of key interest was the effect of transduction of AdV IL-10 upon the potent immunostimulatory cytokine IL-12. Interleukin-12 is a heterodimeric cytokine produced by DC and macrophages but not by T lymphocytes (Macatonia et al. 1995). It is a key cytokine in induction of TH1 responses within T cell populations and it a critical cytokine

involved in proliferation within the MLR (Patrick 1998). At both time points studied (4 and 20 hours post initiation of the MLR), mRNA for IL-12 p40 was detectable in all samples studied, suggesting constitutive expression of the IL-12 p40 subunit. However, analysis for IL-12 p35 showed absent expression at both time points, suggesting that transduction of AdV IL-10 into DC had modified the ability of the DC to produce biologically active ovine IL-12. Biological activity of both human and ovine IL-12 is determined by the IL-12p35 subunit (Swinburne et al. 2000). The secretion of ovine IL-10 and other cytokines was not formally assessed as the appropriate ELISA assays were not available.

In order to compare the effect of AdV IL-10 transduction upon ovine DC cell surface molecules flow cytometric analysis was also performed. In human studies transduction of human monocyte-derived DC with AdV IL-10 resulted in significant down-regulation of CD 80 and CD 86 (see figure 3.3.7). Ovine DC transduced with AdV-IL10 showed low level co-stimulation molecule expression, consistent with their pre-transduction status. The antibody that was available to detect B7 molecules on sheep cells detected both B7.1 (CD80) and B7.2 (CD86) and therefore differences in the expression of these molecules could not be detected (data not shown). Therefore it cannot be definitively stated that the effect of transduction of ovine DC with AdV IL-10 results in down regulation of ovine CD80/CD86 (as it does in human monocyte-derived DC). However the interruption of the ability to produce IL-12 p35 subunit of ovine IL-12 at the mRNA level would skew the alloimmune response away from TH1 and towards TH2.

Within the other cytokine species present in the MLR, IL-2 could also be detected at the mRNA level. Previous studies tolerance induction have suggested that

transcription of IL-2 is a critical cytokine for tolerance, as activation induced cell death (AICD) probably plays a critical role in active immunoregulatory mechanisms such as deletion and therefore in the development of tolerance (Li et al. 1999b; Li et al. 2001).

The presence of a population of ovine DC within the pseudoafferent lymph expressing the ovine cell surface marker CD8 is of considerable interest. A population of $CD8^+$ DC was described by Suss and Shortman within the murine spleen that was capable of deletion of CD4 T cells via a Fas dependent mechanism (Suss and Shortman 1996). These DC had immunoregulatory properties and were capable of inducing cell death by virtue of high cell surface expression of Fas ligand (FasL). The characteristic of these cells was that they expressed the CD8α/α cell surface marker. Other authors have found the same population of cells and reported differential effects of $CD8^+$ /$CD8^-$ DC subsets upon T cell responses (Maldonado-Lopez et al. 1999; Morelli et al. 2000a; O'Connell et al. 2000b). The discovery of this cell population, with its capacity to induce apoptosis within murine spleen has lead to the suggestion that these cells might be implicated in the maintenance of peripheral tolerance. Others have speculated that this cell type (sometimes designated the LDC) that might also be involved in "cross tolerance" (Fazekas de St Groth B 1998; Sallusto and Lanzavecchia 1999a). Furthermore the recent demonstration that a population of $CD8^+TCR^+$ Bone Marrow cells (cells which have phenotypic similarity to CD8α/α lymphoid DC) can enhance haematopoietic stem cell engraftment across a fully allogeneic barrier provides further evidence that these cells may be important in the acceptance of allografts (Gandy et al. 1999). Preliminary evidence for the potential tolerogenic effect of

CD8 α^+ DC was reported at the 6th International Symposium on Dendritic Cells (Port Douglas, Australia). A CD8 α^+ DC population isolated from the spleen of Flt-3L treated B10 ($H2^b$) prolonged murine heterotopic cardiac allograft survival when administered intravenously 7 days pre-transplant compared to CD8 α^- DC, which induced acute rejection(O'Connell et al. 2000a). The potential tolerogenic nature of these lymphoid-related DC has recently been shown to be modified by ligation of CD40 in a P815AB tumour/self peptide model (Grohmann et al. 2001).

It is tempting to speculate that this population within ovine lymph is expressing a similar cell surface marker. However as the precise specificity of the ovine SBU T8 monoclonal antibody is unknown and therefore it is not valid to assume that these cells represent the ovine equivalent to those found in the mouse. Further detailed study of this population using a variety of monoclonal antibodies directed to other cell surface markers would be of interest. Nevertheless the function of $CD8^+$ DC within ovine lymph remains to be determined.

In summary, ovine DC may be readily obtained using pseudoafferent lymphatic cannulation. These cells show an immature phenotype with the ability to rapidly endocytose FITC-dextran and to take up exogenous antigens and present these to autologous T cells. These cells may be readily transduced with adenoviral gene constructs, and when transduced with AdV IL-10 these cells become allo-inhibitory in the MLR.

Chapter 6

Migration Studies of allogeneic ovine DC in the sheep model

6.1 Introduction

The ability of DC to migrate to sites of antigen presentation within allogeneic hosts is one of the key properties favouring their possible therapeutic use. Using this specific migration to sites of antigen presentation within the allograft recipient is a potential means of delivering allospecific therapy and limiting unwanted systemic distribution that may produce adverse effects. Migration of DC is controlled by chemokines and chemokine receptors (see chapter 1). A concern regarding the genetic modification of DC is that transduction with adenoviral gene constructs may alter the ability of DC to migrate to lymphoid sites. This is of particular concern with the gene for IL-10 as a critical role for IL-10 in the inhibition of migration of Langerhans cells via effects on IL-1 and TNF has been suggested (Wang 1999; Wang et al. 1999). Clearly, therefore, it is important to assess the effect of transduction of AdV IL-10 upon DC migration. In order to address this issue, genetically modified and unmodified DC were labeled with a fluorescent dye and migration to allogeneic draining lymph nodes of sheep was assessed.

In this chapter the *in vivo* properties of both adenoviral transduced and untransduced DC with specific reference to migration are investigated. In order to investigate microscopic localization of DC within allogeneic lymph nodes the

fluorescent dye PKH-26 (Zynaxis, USA) was used. The dye is a lipophilic membrane-inserting red fluorescent dye which is stable *in vivo* for up to 3 months. It has been used to track lymphocyte migration *in vivo* for periods of weeks to months (Parish 1999). It has also been successfully used to label sheep neutrophils for trafficking studies (Albertine et al. 1996). As the labeled cells stain red it is possible to simultaneously use a green fluorescent dye to detect other molecules of interest.

Whilst fluorescent dyes are useful for studying microscopic localization of cells within tissues at biopsy or autopsy, other means must be used to follow migration within living large animals. As the migration of allogeneic DC from the transplanted organ to lymphoid sites within the recipient is one of the events that initiates direct pathway of allorecognition, migration to the spleen following therapeutic DC administration is an important property of allogeneic DC (Larsen et al. 1990a). To investigate the migratory properties of untransduced allogeneic DC, the radio-pharmaceutical ^{111}Indium-oxine was utilized. Indium-oxine is a lipophillic compound that is readily taken up by phagocytic cells. Once engulfed the indium dissociates from the oxine component and the indium label remains trapped within the cell rendering the cell radioactive (Zakhireh et al. 1979). As ^{111}Indium-oxine has a long half life (2.81 days) its clinical use is to label white cells for scintigraphic studies of infection or inflammation. It is therefore an ideal choice to study migration of allogeneic DC for a period of several days after administration.

The specific aims of this chapter were therefore to: a) label AdV IL-10 transduced ovine DC with PKH-26 to study the microscopic localization of AdV IL-10 DC from intra-dermal injection to draining lymph node b) analyze the effect of administration of AdV IL-10 DC upon production of local effector cells within draining lymph nodes c) study migration profile of allogeneic untransduced DC labelled with ^{111}Indium-oxine administered either by intradermal injection or by intravenous injection.

6.2. Materials and Methods

6.2.1 Fluorochrome Labeling of Ovine DC and Microscopic Migration Studies

Ovine DC were labeled using the fluorescent dye PKH-26 (Zinexis, Pennsylvania). $5x10^6$ freshly isolated DC were suspended in 1000 µl of serum depleted medium and incubated at 25°C for 5 minutes with 2×10^{-6} M PKH-26 dye. To stop the dye reaction 1ml of complete medium supplemented with 1% bovine serum albumin was added. Cells were then washed three times with complete medium and centrifuged at 400*g* for 10 minutes between each wash. Finally the labeled cells were resuspended in 100µl of complete medium prior to their administration.

Migration of autologous and allogeneic dendritic cells labeled with PKH-26 was assessed by injection intra-dermally into the flank or neck of either autologous or allogeneic sheep. After 24 hours the draining lymph node was then removed and 5µm frozen sections were cut. The presence of PKH-26 stained DC was detected using fluorescence microscopy with a rhodamine filter. Serial cut sections were used in order to relate the location of PHK-26 labeled cells to other cell types within the draining lymph node. As PKH-26 labeled cells emit light in red under

fluorescence then FITC secondary antibodies (staining green) were used to study cell markers in close proximity to the red labeled DC.

6.2.2 Labeling of Ovine Dendritic Cells with ^{111}Indium oxine

Ten million ovine DC were incubated with 0.5 mCi ^{111}Indium Oxine for 1 hour at room temperature. The cells were then centrifuged at 450*g* for 10 minutes. The supernatant was then removed and saved, and its radioactivity counted. The cell pellet was then resuspended in 2 ml normal saline. The centrifugation and resuspension steps were repeated twice with the supernatant saved for counting on each occasion. The final cell pellet was resuspended in 2 mls of normal saline and the radioactivity counted. The labeling efficiency of DC with indium was determined by accounting for the activity in the supernatants in the 3 washes. Labeling efficiency was typically between 84-99%.

In each experiment 5 x 10^6 DC were administered either via either intravenous or intra-dermal route. Images were acquired within the Nuclear Medicine Department of the Queen Elizabeth Hospital.

6.2.3 Studies on the draining lymph node

In order to study the effect of AdV IL-10 transduced DC upon the T cells within draining lymph nodes, 5 x 10^6 allogeneic DC transduced with either AdV IL-10 or AdV MX-17 were administered intra-dermally to recipient sheep (n=3). The injection site was precisely marked. Forty-eight hours later the injection site was re-injected with intra-dermal Patent Blue V dye and the draining lymph node removed. The purpose of injection with patent Blue V dye was to clearly delineate the section of lymph node that drained the skin segment. The draining segment

was then processed either for enrichment of effector cells or for RNA extraction and PCR studies.

6.2.4 Enrichment of Effector Cells from Draining Lymph Nodes

Sections of draining lymph node were crushed using a fine mesh sieve and the resultant cell suspension washed and centrifuged at 900*g* three times with sterile PBS. In order to deplete the suspension of monocytes the cell suspension was incubated in plastic Petri dishes for 2 hours at 37°C. B lymphocytes were then removed by incubation at 37°C over a nylon wool column for 20 minutes. The cell suspension then underwent flow cytometric analysis to determine the enrichment of $CD8^+$ cells within the suspension. Effector to target cell ratios were then determined commencing at an effector:target ratio of 50:1.

6.2.5 Cytotoxic T Cell Assay

Cytotoxic T cells (CTL) activation was detected using Cr^{51}lysis. Effector cells were enriched from the lymph nodes of treated animals as per 6.2.4. $CD8^+$ cells were selected using Dynabeads® according to the manufacturer's instructions. Lymphoblast target cells were prepared from PBMNC of donor animals that were stimulated with Conconavalin A at 10µg/$1x10^6$ donor cells for 72 hours prior to use in the assay. At 48 hours post initial Con A treatment, the tissue culture was supplemented with supernatant from the monkey cell line MLA-144 (a source of IL-2). Target cells were labeled with 100µCi Cr^{51} for 90 minutes at 37°C. Effector cells were incubated with target cells at variable ratios beginning at a ratio of 100:1. The percentage specific lysis was calculated as a percentage of maximal lysis based upon forced maximal lysis (calculated by lysis with 5% Triton X-100 detergent)

6.2.6 Radioisotopic detection of ^{111}Indium-oxine labeled cells

Two methods of DC administration were studied. Firstly intra-dermal injection and migration to draining lymph node and secondarily intra-venous injection and systemic images were acquired with a large field of view gamma camera (using a medium energy parallel hole collimator; 128 X 128 matrix; two 20% windows were used centred at photopeaks of 173 and 247 KeV) in the following positions:

a. Supine position, anterior view of the lower chest / upper abdomen, acquire 1 frame per second for the first minute, then 1 minute acquisitions for 30 minutes.
b. Static anterior view of the chest : 10 minutes
c. Static anterior view of the abdomen : 10 minutes
d. Static left lateral view of the upper abdomen : 10 minutes

Steps b to d were repeated at 24 hours, 48 hours and 144 hours.

In order to obtain the images, animals were sedated via intravenous injection, underwent endotracheal intubation and were transported to the nuclear medicine department.

6.3 Results

6.3.1 Allogeneic Ovine DC migrate to T cell dependent areas within draining lymph nodes

Essential to the strategy of using DC to delivery immunosuppressive cytokines to large animals is the ability of DC to migrate to draining lymph nodes and interact with T cells. Initially we used autologous DC labeled with an immunofluorescent dye PHK-26 to study the localization of DC within draining lymph nodes. *In vitro* labeling of ovine DC showed strong labeling when viewed under rhodamine filter (figure 6.3.1a). PKH-26 labeled DC were then injected intra-dermally into the neck

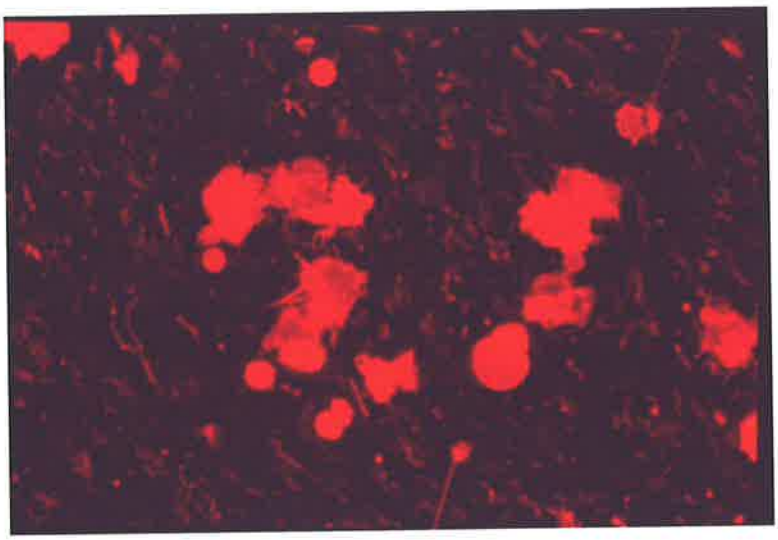


Figure 6.3.1 a

Freshly isolated ovine DC labeled with PKH-26 viewed under Rhodamine filter (x100)

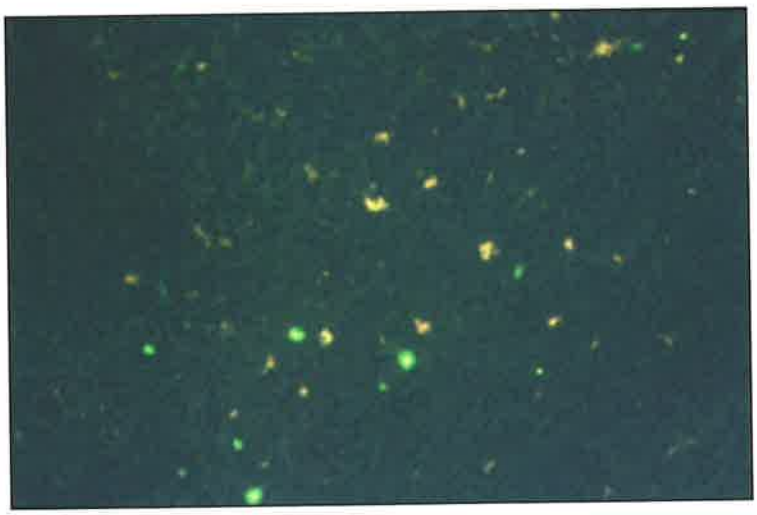


Figure 6.3.1b)

Allogeneic DC transduced with AdV IL-10 show homing to dendritic cell areas within draining lymph nodes. In orange are indicated allogeneic PKH-26 labelled DC. Endogenous CD83 labelled DC are stained green.

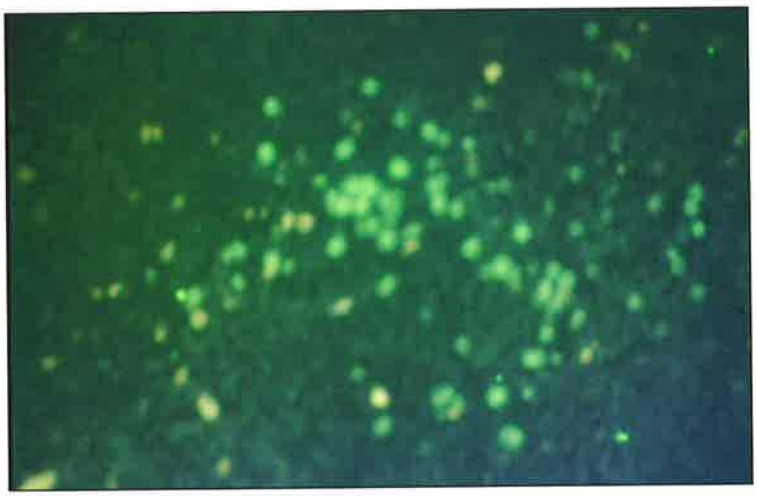


Figure 6.3.1c)

Allogeneic AdV IL-10 transduced DC (orange) migrate to $CD4^+$ rich T cell zone (green) within draining lymph nodes.

Figure 6.3.1 d)

A section of an ovine lymph node not exposed to AdV IL-10 transduced cells and stained with an irrelevant antibody - negative control (X63).

of an autologous recipient, and the draining lymph node removed 24 hours post injection (n=1). In subsequent experiments ovine DC transduced with AdV IL-10 and labeled with PKH-26 were used (n=3). Under plain rhodamine filtered light the labeled DC were clearly identified within the cortex of the draining lymph node of all 3 separate experiments. Using immuno-fluorescence for the cell surface marker CD83, PKH-26 labeled DC (stained orange) could be clearly seen in close proximity with the endogenous CD83 positive DC (stained green) within the lymph node (figure 6.3.1b). This indicated that DC injected intra-dermally were capable of migration to DC rich environments within the lymph node. Co-localization of ovine DC to $CD4^+$ cells was then demonstrated with the ovine monoclonal antibody SBU T4 (figure 6.3.1c).

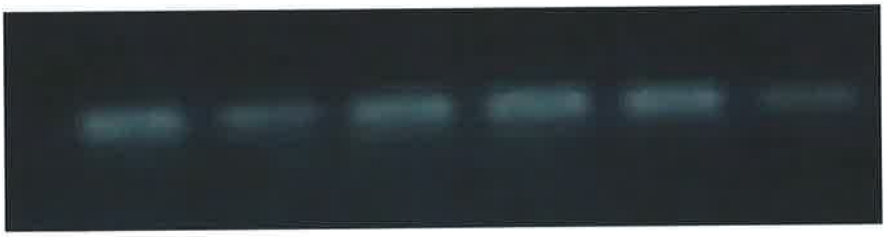
6.3.2 Cytokine Profiles within Draining Lymph Nodes

The cytokine environment of draining lymph nodes was investigated by reverse transcription polymerase chain reaction (RT-PCR). The draining lymph nodes from a number of sheep under a variety of conditions was studied. All lymph nodes were removed 48 hours after injection. Thin sections (10 μm) of draining lymph nodes that had received either allogeneic AdV IL-10 transduced DC (lanes 1 and 3), untransduced allogeneic DC (lanes 2, 5 and 6) were dissolved into RNA extraction solution (Solution D) and the cytokine species were studied by PCR. Within all lymph nodes studied mRNA for the cytokine species, ovine TNF-α (figure 6.3.2 a), ovine IL-2 (figure 6.3.2 b) and ovine IFN-γ (figure 6.3.2 c) were detectable. The gene β– actin was amplified as a control gene.

In lymph nodes receiving AdV IL-10 transduced DC, the gene could be readily detected using adenoviral IL-10 specific PCR primers (Figure 6.3.2 B e). When the

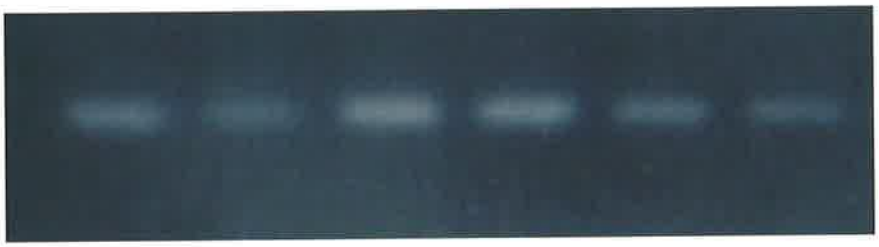
Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6

a) ovine TNF-α, 40 cycles (product size 338 base pairs)



b) ovine IL-2 , 40 cycles (product size 409 base pairs)

c) ovine IFN-γ, 30 cycles (product size 278 base pairs)



d) β - actin, 23 cycles (product size 317 base pairs)

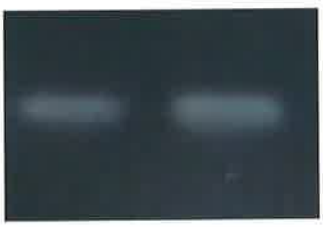
Figure 6.3.2A

A Ovine lymph node PCR for Cytokine Species (*Left - right)* Lane 1 AdV IL-10 DC, Lane 2 Untransduced DC, Lane 3 AdV IL-10 DC, Lane 4 No DC, Lane 5 and 6 Untransduced Allogeneic DC

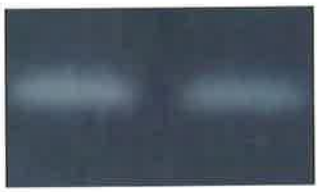
Lane 1 Lane 2

e) Adenoviral specific AdV IL-10 , 40 cycles (product 257 base pairs)

f) ovine IL-12p35 , 35 cycles (product 550 base pairs)



g) ovine IL-12p40 , 40 cycles (product 461 base pairs)



h) β- actin, 23 cycles (product size 317 base pairs).

Figure 6.3.2 B

Lymph Node PCR for AdV IL-10 and ovine IL-12 Cytokines (*Left to right*) Lane 1 Untransduced DC, Lane 2 AdV IL-10 transduced DC.

IL-12 species were studied within draining lymph nodes receiving AdV IL-10 transduced DC, mRNA for IL-12p40 (figure 6.3.2 B g) was readily detectable. However, mRNA for IL-12 p35 (figure 6.3.2 B f) was not detected at non-saturating conditions (35 cycles). As both subunits are required for the formation of IL-12p70 this indicated a down regulation of ovine IL-12 within the lymph nodes of animals treated with AdV IL-10 transduced DC.

6.3.3 AdV IL-10 Transduced DC inhibit the formation of donor specific CTL within draining lymph nodes

In order to study the effect of adenoviral transduced DC into draining lymph nodes, the draining lymph node from the neck of animals that received either 5 x 10^6 AdV IL-10, AdV MX-17 or untransduced cells were removed 48 hours after injection. Two animals that had received either AdV IL-10 or AdV MX-17 were studied (a total of 2 lymph nodes in each animal, in 2 separate experiments). The drainage of the skin segment to the draining lymph node was confirmed by intra-dermal injection of Patent Blue V dye. The segment of the lymph node to which the skin injected with DC (and Patent Blue V) was identified by virtue of blue staining and a cell suspension of this segment of the lymph node from within the draining segment. These cells were then enriched using a protocol detailed above in 6.2.4 to generate effector cells. Target blast cells were generated using Con A stimulation and were labeled with ^{51}Cr as described(figure 6.2.5). At variable effector:target ratios, cells enriched from draining lymph nodes induced significant target lysis, with the maximal lytic effect being seen within lymph nodes that received AdV MX-17 transduced cells. The percentage lysis of donor blasts is shown in figure 6.3.3.

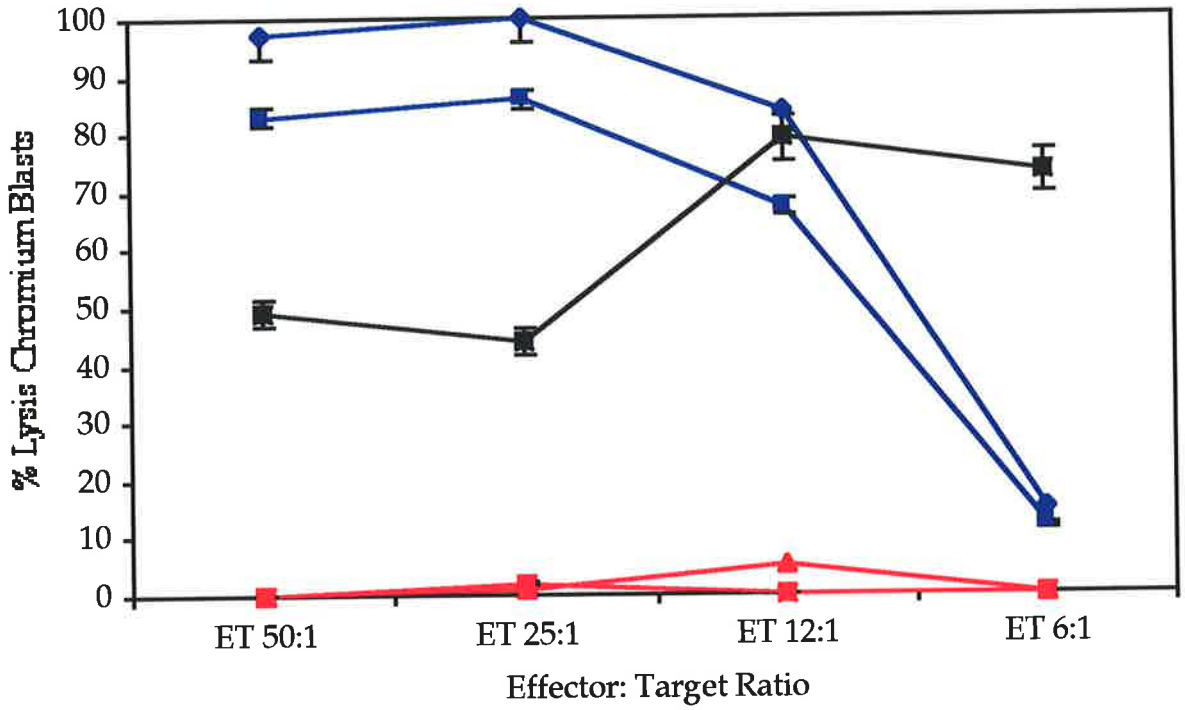


Figure 6.3.3

AdV IL-10 transduced DC inhibit donor specific CTL within draining lymph nodes. $CD8^+$ Enriched cells from ovine LN enriched from animals treated with AdV IL-10 DC (red) induce less lysis of chromium labeled donor blast cells compared to AdV MX-17 (blue) or untransduced DC (black). Each line represents the mean % (+/- 1 SD) of chromium lysis from a single lymph node from 2 separate experiments.

6.3.4 Migration pattern of allogeneic ovine Dendritic Cells injected via intra-dermal injection

^{111}Indium-oxine labeled allogeneic DC were administered via intra-dermal injection in the neck of a single sheep. By 35 minutes, labeled cells were noted in the sentinel node in the neck of the recipient sheep. The surface location of the node was marked in the lateral and anterior projections.

Subsequent scanning showed appearance of activity in the right upper quadrant of the abdomen. In contrast to intravenous injection there was absence of lung activity.

Day 1: Sentinel neck node showed increased activity. The right upper quadrant abdominal uptake also more prominent, and assumed the shape of liver. Marrow and kidneys are seen. Low grade activity was detectable in the lungs.

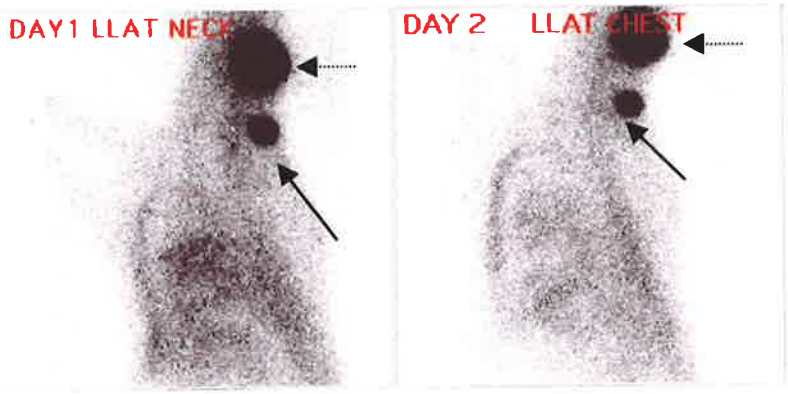
Day 2: Sentinel neck node remains hot. Hepatic activity increased in prominence and low grade spleen activity developed. Lungs, marrow and kidney were also clearly visible.

Day 6: The sentinel lymph node and liver remained detectable. There was no other visible activity in the other organs.

A representative nuclear medicine image is shown in figure 6.3.4

In order to quantitate the organ distribution of the labeled cells, the recipient animal was sacrificed at day 7 and the internal organs removed for quantification of radioactivity with a large volume counter (table 6.3.4). The radioactivity within a piece of recipient abdominal wall skin was measured for use as the background. The radioactivity of each organ per gram is expressed as multiples of the "background".

a) b)



c)

Figure 6.3.4 a)

Migration of untransduced DC labeled with ^{111}Indium-oxine following intradermal injection Left lateral view showing intradermal injection of untransduced allogeneic DC showing depot injection site (dotted arrow) and drainage to draining lymph node (solid arrow) within 35 minutes of injection. 6.3.4 b) Left lateral view 24 hours post injection the depot and draining lymph node remain clearly visible. 6.3.4 c) Anterior view showing day 7 radioactivity within the liver becomes detectable (red arrow)

Table 6.3.4 Radioactivity detected within ovine organs post intradermal injection

Organ	Radioactive Count relative to background
liver	10.4 X background
Spleen	2.9 X background
Lymph Node	331.4 X background
Lungs	2.21 X background
Marrow	3.6 X background
Injection Site	1420.6 X background
Kidneys	10.1 X background

These results indicated that maximal radioactivity remained within the intra-dermal injection site and within the draining lymph node. There was only minimal radioactivity detected within the remaining organs studied.

6.3.5 Migration pattern of allogeneic ovine Dendritic Cells injected via intravenous injection

^{111}Indium-oxine labeled DC were administered intravenously by injection into a central venous cannula placed into the right jugular vein into a single recipient sheep.

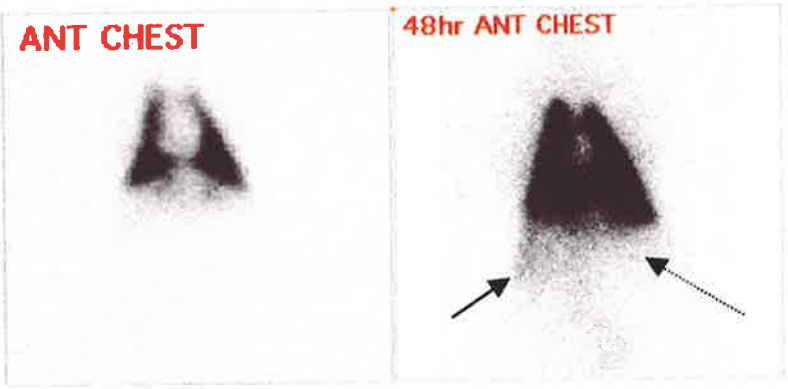
Day 1: ^{111}Indium-oxine labeled DC were rapidly detected within lungs.

Day 2: Labeled DC were found within the lungs with radiotracer activity detected within liver and spleen.

The recipient animal was sacrificed at day 3 and the internal organs processed for radioactivity as per 6.3.4.

A typical scan is shown in figure 6.3.5.

a) b)



c)

Figure 6.3.5 a)

Migration of allogeneic untransduced ovine DC following intravenous injection. Anterior view showing labeled allogeneic DC within the recipient sheep lungs within 35 minutes of intravenous injection. Figure 6.3.5 b) Anterior abdominal view showing uptake of radioisotope within liver (arrow) and spleen (dotted arrow). Figure 6.3.5 c) Magnified view of the anterior abdomen at 48 hours showing ongoing detectable activity within liver (arrow) and spleen (dotted arrow). Excretion of contrast is evident by the gall bladder (red arrow).

Table 6.3.5 Radioactivity detected in ovine organs after intravenous injection of ^{111}Indium-oxine labeled allogeneic DC

Organ	Radioactive count relative to background
Lungs	524 X Background
Heart	4.7 X Background
Liver	29 X Background
Spleen	10 X Background
Gall Bladder	3 X Background
Kidney	36 X Background

These results indicated that maximal radioactivity remained within the lungs (possibly sequestration of DCwithin lung capillaries). Radioactivity was detected in the spleen at higher level than with intra-dermal injection.

6.4 Discussion

In this chapter the migratory properties of both unmodified allogeneic ovine DC and those that had been transduced with AdV IL-10 were studied. The fluorescent dye PKH-26 was used to study microscopic cellular localization of AdV IL-10 transduced DC and the *in vivo* migratory characteristics of allogeneic DC were studied by labeling the cells with ^{111}Indium-oxine.

The migration of untransduced allogeneic DC as well as cells transduced with AdV IL-10 were firstly studied by intra-dermal injection of PHK-26 labeled dendritic cells. Autologous DC were studied initially and found to be capable of migration from the injection site into draining lymph nodes. Allogeneic AdV IL-10 DC were then also labeled with PHK-26 and injected according to the same protocol. At the microscopic level AdV IL-10 DC showed a similar pattern of migration compared to untransduced DC. They showed co-localization with CD83 positive endogenous cells - presumably endogenous DC. These results are similar to those obtained in autologous DC studied within murine cancer systems (Eggert et al. 1999). Others have suggested a critical role for IL-10 in the inhibition of migration (Wang et al. 1999). Wang et al studied the migration of Langerhans cells in IL-10 knock-out mice and concluded that IL-10 was a critical cytokine involved in Langerhans cell migration via effects on IL-1 and TNF (Wang 1999). Our observations show that IL-10 transduction does not impair the migratory capacity of DC as assessed by PKH-26 labeling. In our model these results may have an alternative explanation that is not in conflict with the studies of Wang - as the pseudoafferent DC are already migrating *towards* a draining lymph node they are

presumably already expressing the appropriate chemokine receptors to allow migration into draining lymph nodes. In which case, transduction of DC with AdV IL-10 may make little difference to the receptors already being expressed.

These migration studies are consistent with studies using *in vitro* generated monocyte-derived DC in a chimpanzee model. In these studies injected DC localized to lymph node paracortex within 24 hours of injection. The maturational status of the injected DC is an important determinant of cell migration. Barratt-Boyes et al found that immature DC showed 20 fold greater migration into draining lymph nodes compared to DC that had been matured *in vitro* (Barratt-Boyes et al. 2000). In contrast, mature DC remained within the dermis at the site of intra-dermal injection. This effect was likely to be due to the expression of immature DC chemokines. Analysis of recovered DC from draining lymph nodes in these studies was associated with an up-regulation of CD86 indicating maturation. In our studies, allogeneic DC transduced with AdV IL-10 migrated into the T cell dependent areas of draining lymph nodes and showed similar distribution compared to un-transduced cells injected simultaneously into contra-lateral lymph nodes (figure 6.3.1 b-c). Although there is a theoretical advantage of transduction of DC with AdV IL-10 in that preservation of an immature phenotype could enhance arrival at the draining lymph node, we were unable to detect any significant difference in cell numbers within nodes studied.

In all of the animals that received adenoviral transduced DC we were able to detect mRNA for the IL-10 gene using specific polymerase chain reaction (figure 6.3.2 B e). This shows the utility of DC to deliver candidate genes into sites of antigen presentation within allogeneic recipients. Previous studies in mice have shown that

the route of administered DC can alter the TH1/TH2 balance within lymphoid organs (Lappin et al. 1999). These studies showed that subcutaneous injection of KLH pulsed syngeneic DC polarized the T cell response within draining lymph nodes towards a TH1 response, whereas DC injected intra-venously were associated with a TH2 response within the spleen (Lappin et al. 1999). The resting cytokine mileau of the draining lymph node within healthy non immuno-compromised sheep showed a generalized cytokine upregulation of both TH1 and TH2 cytokine species in all nodes studied (figure 6.3.2 A). This pattern was observed in resting ovine lymph nodes, not challenged by DC as well as those receiving allogeneic dendritic cells (figure 6.3.2 A). When ovine IL-12 species were studied using PCR under *non saturating conditions* IL-12 p35 mRNA was not detected. As ovine IL-12 p35 is the critical determinant of ovine IL-12 p70's biological function this indicated an interruption of the normal IL-12 production (Swinburne et al. 2000). It is possible, however, that as the draining lymph nodes were removed at 48 hours post injection of allogeneic DC that transcription of IL-12 p35 had occurred at an earlier time point and was hence undetectable at this time. However, the fact that mRNA was detectable in the draining lymph node treated with untransduced DC argues against this (figure 6.3.2 B f). The expression of mRNA, however does not necessarily correspond to protein expression (not studied in these experiments) or biological action. In order to address biological function, $CD8^+$ T cells were enriched from draining lymph nodes and their capacity to lyse donor target blast cells assessed.

$CD8^+$ enriched T cells from draining lymph nodes that received AdV IL-10 transduced DC showed a significantly less cytolytic capacity than enriched cells from animals receiving either un-tranduced DC or adenoviral control transduced

DC. These results are consistent with biological activity of IL-10 from AdV IL-10 transduced donor DC. These findings are in accordance with the findings of Li *et al* who administered exogenous mammalian IL-10 to murine cardiac allograft recipients as *pre* transplant therapy and found a reduction in donor-specific CTL within the spleens of recipient animals. Animals pre-treated in this manner showed prolonged allograft survival (Li et al. 1999a). A key question raised by these experiments is the immunological outcome of the administration of genetically modified DC. It is possible that the DC transduced with AdV IL-10 simply failed to stimulate an immunological response relative to the effect of either un-transduced cells or cells transduced with the control virus. It is also possible that AdV IL-10 transduced DC have generated "suppressor cells" or regulatory T cells. The existence of such cells is notoriously difficult to prove *in vitro.* In order to investigate the possibility that this route of injection might enhance allograft survival, transplantation experiments to look at allograft survival were performed (see chapter 7).

In order to study the macroscopic distribution of DC in real-time, the radio-pharmaceutical ^{111}Indium-oxine was used to label allogeneic DC prior to administration.^{111}Indium-oxine has a number of advantages as a radiolabel. Initial DC labeling studies were attempted with Technetium (^{99m}Tc HMPAO), but this radio-pharmaceutical was associated with significant dissociation of tracer (data not shown). Therefore the images obtained could not be assumed to represent the true distribution of labeled DC. One of the main advantages of ^{111}In is long half life (2.81 days). The other significant difference was the stability of the tracer within the cells with low dissociation into the tissue culture supernatant over time. *In vivo* studies by Thakur *et al* indicate that at least 90% of circulating radioactivity

remains within human leukocytes labeled with ^{111}In (Thankur et al. 1977). In our own *in vitro* studies with ovine DC we found that at least 81% of radioactivity remained associated with DC at 72 hours post labeling (data not shown).

Two routes of administration were studied with indium labeled cells; intra dermal administration and intravenous administration. Radio-labeled allogeneic DC migrated from the depot site of injection into the draining lymph node (figure 6.3.4). In contrast radioactivity cleared from the draining lymph nodes receiving an equivalent dosage of free ^{111}Indium-oxine. These findings are similar to those found by Thomas *et al*, who studied migration of autologous monocyte-derived DC in melanoma patients. Using ^{99m} Tc-HMPAO labeled autologous DC injected into the skin of one thigh, they found migration from intra-dermal injection site to draining lymph node. When the contra-lateral thigh was injected with an identical dose of free ^{99m} Tc-HMPAO , the radiotracer failed to accumulate within the draining lymph node in contrast to the side receiving labeled DC (Thomas 1999b). Morse et al found similar findings using autologous *in vitro* generated DC (Morse et al. 1999). In these studies most of the radioactivity remained within the draining lymph node and within the injection site rather than within other organ systems (see table 6.3.4). This is not surprising, as these pseudoafferent DC are migratory towards draining lymph node. These cells would not be expected to form a part of efferent lymphatic drainage (Haig et al. 1999).

The intra-venous injection of allogeneic DC resulted in rapid uptake within ovine lungs. Over the subsequent 48 hours radio-tracer was detected within ovine liver and spleen indicating a gradual migration from lung to the lymphoid organs.

Similar findings in humans with autologous indium labeled DC injected intravenously of rapid lung uptake, followed by redistribution have been reported (Mackensen et al. 1999; Morse et al. 1999). Studies in small animals have also shown the phenomenon of splenic migration of intravenously injected DC (Oluwole et al. 1991). The importance of splenic migration of DC post transplantation has been clearly shown (Larsen et al. 1990a; Larsen et al. 1990b).

It is difficult to draw firm conclusions from the radioactivity data obtained at autopsy on day 7 post injection. In human clinical imaging with ^{111}Indium-oxine labeled white cells, scans are conventionally performed up to 48 hours post injection (although this may be extended to several more days in certain situations). ^{111}Indium-oxine labeled white cells have been used to obtain splenic images in sheep up to 48 hours post injection (Butcher et al. 1986). As the survival of allogeneic radio-labeled DC is not known *in vivo* the radioactive counts may be representing dead or non viable labeled cells or free^{111}Indium particles dissociated from DC rather than true viable DC. The splenic radioactive count at 48 hours post injection was greater in the intravenous injected animals than in the intra-dermal injected animals. By day 2 there was detectable splenic activity after intravenous injection which was not detected after intra-dermal injection. In order to determine the nature of the radioactivity within the spleen auto-radiography using ^{111}Indium-oxine labeled DC could be performed.

In summary, ovine pseudoafferent lymphatic DC transduced with AdV IL-10 were labeled with PKH-26 and were capable of migration from intra-dermal injection sites into draining lymph nodes. Furthermore, $CD8^+$ cells isolated from the

draining lymph node showed less capacity to lyse target donor chromium labeled blast cells than did cells derived from lymph nodes treated with AdV MX-17 transduced DC. Intra-dermal migration from local depot injection site into draining lymph node was also demonstrated by ^{111}Indium-oxine labeling. Allogeneic untransduced DC when administered intravenously showed migration into the recipient lung, liver and spleen.

Chapter 7

A Large Animal Renal Transplantation Model using Dendritic Cell Therapy

7.1 Introduction

The recognition of the pivotal role of DC in determining the balance between tolerance and immunity to antigens has lead to great interest in exploiting the tolerogenic potential of these cells as novel transplantation therapy (Thomson et al. 1998; Morelli and Thomson 1999; Morelli et al. 2000b). *In vitro* studies have shown that the capacity of DC to induce proliferation in allogeneic T cells is directly related to their maturity and in particular to expression of cell surface co-stimulatory molecules, therefore the maturational status of DC used as transplantation therapy is of critical importance. Specifically, "immature" DC show less capacity to induce T cell proliferation than "mature"DC. Enhanced allograft survival has been observed in a number of small animal transplantation models using DC based strategies. Three studies in particular have shown that prolonged allograft survival *in vivo* may be achieved by using DC strategies based predominantly on DC maturity. Fu et al demonstrated enhanced murine cardiac allograft survival using "immature" co-stimulatory molecule deficient dendritic cells administered as pre transplant conditioning (Fu et al. 1996). Tolerance to Lewis rat cardiac allografts may be enhanced by infusion of donor derived dendritic cell precursors after total lymphoid irradiation of recipients (Hayamizu et al. 1998). Most recently, Lutz *et al* have generated immature DC using low dose

GM-CSF that have the capacity to prolong allograft survival to greater than 100 days in fully mismatched vascularized cardiac allografts (Lutz et al. 2000).

However the potential maturation of donor-derived DC within the recipient is a factor that limits the use of these immature cells as potential tolerogenic agents. Prevention of maturation of donor-derived DC by chemical or other means is therefore a logical means to enhance the therapeutic usefulness of DC for transplantation. One such approach is to target the molecular signals that produce DC activation thus keeping DC in a functionally immature phenotype. An example of such a molecule the transcription factor NF-κB. The translocation of NF-κB from the cytoplasm into the DC nucleus is a critical step in DC activation and in the capacity of DC to induce T cell proliferation. Short oligodeoxynucleotides with consensus binding sequences to NF-κB inhibit DC allostimulatory capacity via effects on cell surface co-stimulatory molecule expression and significantly prolong cardiac allograft survival *in vivo* (Giannoukakis et al. 2000).

Another approach is the genetic modification of DC to prevent DC maturation or inhibit the DC signals required to promote a proliferative T cell response. A number of research groups have genetically modified DC to promote *in vitro* and *in vivo* T cell hypo-responsiveness using a variety of gene therapy vectors . These include viral IL-10(Takayama et al. 1998) CTLA4 Ig (Lu et al. 1999; Takayama et al. 2000) TGF-β (Lee et al. 1998), Fas ligand (Min et al. 2000). Recently O'Rourke *et al* generated a DC cell line that was modified to express CTLA4 immunoglobulin. These cells were capable of inducing allospecific immune hyporesponsiveness. When administered intravenously on the day of transplantation and again 6 days

after transplantation, these modified DC enhanced pancreatic islet cell survival in diabetic Balb/C mice (O'Rourke 2000).

Whilst genetically modified DC have been effective in modifying the *in vitro* alloimmune response and the *in vivo* alloimmune response in a variety of rodent transplantation models, there has been little work done in larger animals to investigate the effect that these cells might have upon allograft survival. The sheep renal transplant model offers the potential to study the effect of genetically modified DC in a pre clinical renal transplantation model. The biology of ovine DC is known (Bujdoso 1989; Dandie et al. 1992; Dandie et al. 1994) and ovine heterotopic renal transplantation may be readily performed (Pedersen and Morris 1974; Grooby et al. 1998). As experimental transplant animals sheep also offer a number of advantages. Firstly due to the size of individual recipient sheep and donor kidney, the operative transplant procedure is similar to operations performed in humans. Secondarily, placement of central venous cannulae allows easy access to the circulation for measurement of serum creatinine, as a measurement of allograft function. The positioning and size of the sheep kidney as well as the docile nature of the animals allows renal allograft biopsy to be performed as required.

The specific aim of this chapter was to investigate the effect of treatment of kidney allograft recipients with donor-derived DC transduced with the adenoviral gene constructs AdV IL-10 and the control construct AdV MX-17. Two protocols were tested. Protocol 1 was intra-dermal pre transplantation DC treatment with the aim of inducing donor specific hypo-responsiveness. Protocol 2 entailed post

transplantation intravenous DC administration as an immunosuppressive strategy where donor specific alloantigen was presented to recipient lymphoid tissue in the context of IL-10 at the time of transplantation. The end points of these studies were kidney allograft survival, serum creatinine measurements and renal allograft histology.

7.2 Methods

7.2.1 Heterotopic Renal Allografting

Two year old female Merino sheep were used as both kidney donors and recipients in a heterotopic renal transplant model. All ovine kidney donors had previously undergone surgery to cannulate pseudoafferent lymphatic vessels (chapter 5.2.1). Animals were fasted for 12 hours prior to surgery. Anaesthesia was induced using 0.4ml/kg Nembutal and maintained after endotracheal intubation by variable administration of inhalational Fluorothane and oxygen. In all recipient animals a central venous cannula (Arrow, USA) was placed into the left internal jugular vein.

Renal allografting was performed using a method as described by Pederson *et al* and modified in the host laboratory by Grooby *et al* (Pedersen and Morris 1970; Pedersen and Morris 1974; Grooby et al. 1998). Under general anaesthesia the recipient animal underwent bilateral nephrectomy through bilateral loin incisions. This resulted in the recipient animal being solely reliant upon the kidney allograft for renal function.

The donor animal underwent nephrectomy through a lumbar loin incision. The donor kidney was then immediately cooled to 4°C using a sterile ice slurry and perfused using a Hamilton- Bailey needle and cold Ross perfusion fluid (Baxter,

Australia) supplemented with 5000 IU heparin for up to 30 minutes prior to transplantation.

During cold perfusion of the transplanted kidney the recipient sheep neck vessels were explored. The donor renal artery was anastomosed to the right internal jugular artery by an end to end anastomosis. The allografted renal vein was anastomosed using an end to side anastomosis. Each animal was hydrated during the transplant operation with 1L saline (Baxter, Australia) and given 100 ml of 20% Mannitol (Baxter, Australia) to help prevent acute renal failure. The ureter was externalised through a cutaneous ureterostomy - patency of this stoma was maintained using a 20G indwelling cannula (InSite®). A tissue flap was raised to cover the transplanted kidney using standard surgical procedures. The appearance of a sheep post renal allografting is shown in figure 7.3.1a. At the end of each procedure the animals were treated with intra-muscular penicillin/streptomycin for infection prophylaxis and opiate for pain relief. Allograft function was monitored by daily measurement of serum creatinine by Jaffe method (Department of Clinical Chemistry, The Queen Elizabeth Hospital). Allograft biopsy was performed as required using an automated 18 Gauge Monopty™ biopsy needle (Bard, USA). Allograft biopsies were formalin-fixed, embedded in parafin, sectioned and stained with Haematoxyline and Eosin or alcian blue periodic acid-Schiff (ABPAS) by the Department of Histopathology, The Queen Elizabeth Hospital. Renal biopsies were scored blind by a renal histopathologist for rejection according to the 1997 Banff criteria (Solez et al. 1993). Renal allograft failure was deemed to have occurred when the serum creatinine was greater than 600μmol/litre.

Renal allografting procedures were approved by the Animals Ethics Committee of the Queen Elizabeth Hospital and of the University of Adelaide. Animals receiving genetically modified dendritic cells were housed under PC2 conditions within isolated metabolic crates. All experimental protocols involving genetically modified cells were performed with permission of the Genetic Manipulation Advisory Committee (GMAC) of Australia.

7.2.2 Pre Transplantation Measurements of Alloreactivity

In order to determine alloreactivity of kidney donor-recipients the mixed leukocyte culture was performed (see 2.2.1). All donor-recipient pairs were alloreactive with at least a 5 fold rise in proliferation above resting cell counts by day 5. HLA serotyping of sheep was not performed.

In order to exclude the presence of pre transplantation donor and recipient blood group incompatibility red blood cell cross matching by Immediate Spin Method was performed. In brief fresh blood from ovine kidney donor and recipient pairs was collected in 10ml EDTA anticoagulated tubes. Red cells and plasma were separated by centrifugation at 1200*g* for 5 minutes. Donor cells were diluted to a 3-5% concentration using phosphate buffered saline. One drop of diluted donor cells was aliquoted into a glass tube (12x75mm). Two drops of recipient plasma was added and haemolysis and agglutination detected using a light box.

7.2.3 DC Administration Protocols

In order to test the capacity of ovine DC to modify allograft survival, AdV IL-10 transduced ovine DC and control DC transduced with AdV MX-17 were administered to unrelated alloreactive sheep using two protocols. Ovine DC were

collected from the kidney donor sheep by pseudoafferent lymphatic cannulation as described in chapter 5 (5.2.1). Donor DC were then frozen and stored in liquid nitrogen before their use in DC protocols. On the day before administration, cells were thawed and then transduced with adenoviral gene constructs as described in chapter 5 (5.2.3). Transduced cells were cultured in complete medium for 24 hours prior to their *in vivo* use.

Protocol 1: The first protocol was a pre-transplantation conditioning regimen based upon intradermal administration of genetically modified DC. The dosage of DC transduced with either AdV IL-10 or AdV MX-17 and was $5x10^6$ cells given as two separate intradermal injections on day -7 and day -1 prior to transplantation. This dosage of DC was chosen because it approximates the number of DC in a unit of peripheral blood. DC within blood have been implicated as having major immunomodulatory effects (Josien et al. 1998).

Protocol 2: The second protocol was a post transplantation protocol in which genetically modified DC were given via daily intravenous injection. A dosage of $1x10^6$ DC was administered daily intravenously for the first 7 days post transplantation.

7.3 Results

7.3.1 Protocol 1: Pre-transplant administration of intra-dermal AdV IL-10 and AdV MX-17 transduced DC

Two allograft recipients were studied using donor DC and a kidney from the same sheep. One animal received donor DC transduced with AdV IL-10 and the other animal received DC transduced with the control virus AdV MX-17. In both procedures, renal allografting was uncomplicated with prompt allograft function post transplantation. The position of the allograft is shown in figure 7.3.1a. In both animals there was comparable warm ischaemia time (less than 3 minutes) and equivalent cold ischaemia time (less than 30 minutes). Results of daily serum creatinine measurement are shown in figure 7.3.1b. The baseline creatinine levels pre-transplant were within normal ranges expected, based upon previous studies within the host laboratory (Grooby 1996; Patrick 1998). On first post operative day there was a significant rise in serum creatinine in both animals. By day 4 serum creatinine values had exceeded 600 μmol/l, the value that has been previously used to define allograft failure in ovine renal transplantation (Grooby 1996). Renal allograft biopsies were performed on day 0,3,5 and at autopsy. The Banff criteria for allograft rejection were used to grade rejection and the results are shown in table 7.3.1. In both animals cellular and glomerular rejection were evident as early as day 3 with characteristic changes of severe vascular rejection seen by day 5 and at autopsy.

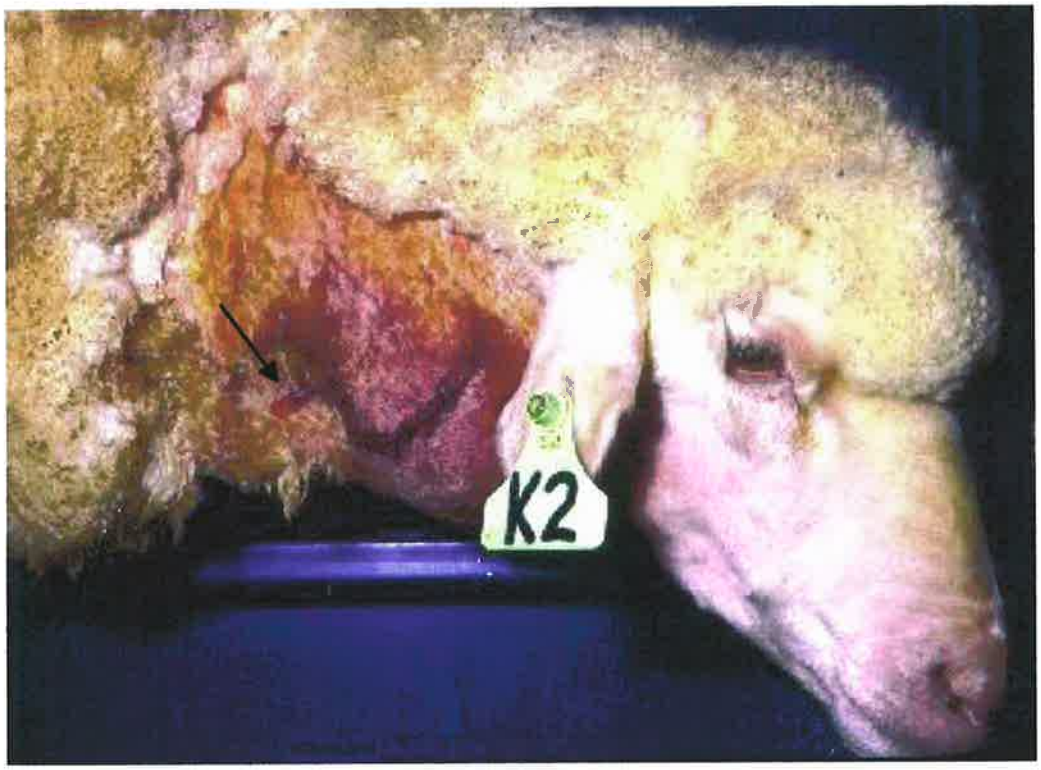


Figure 7.3.1a

Heterotopic renal allografting in sheep K2 (AdV IL-10 IV). The transplanted kidney is located under a skin flap on the right hand side of the sheep neck. The transplant ureter is exteriorized using a cutaneous ureterostomy with a 16g intravenous cannula (arrow).

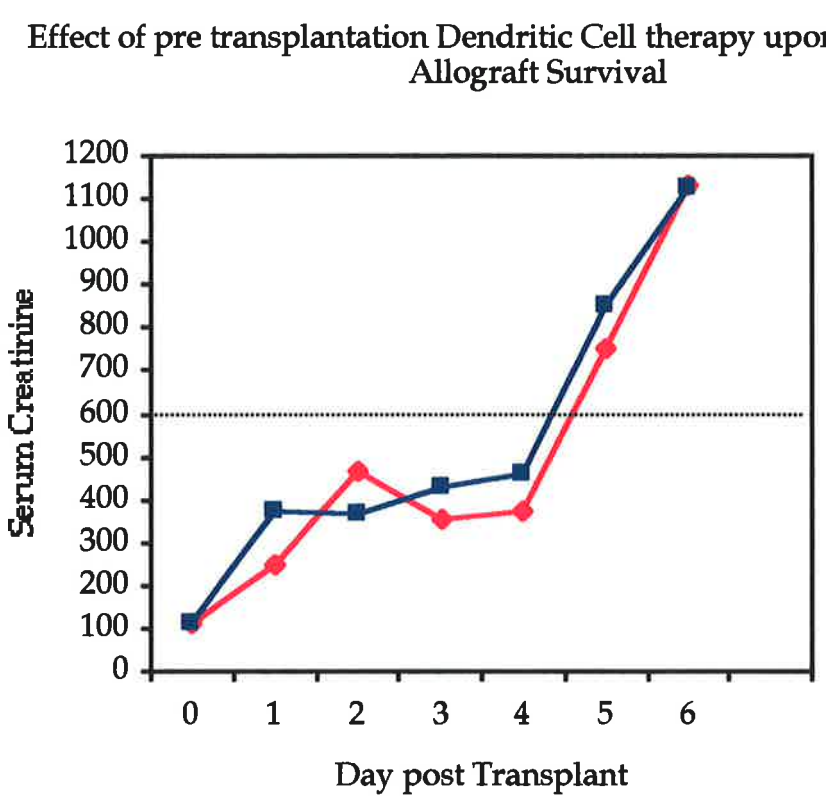


Figure 7.3.1b

Serum creatinine values in sheep D2 (AdV MX-17 in blue) and sheep E2 (AdV IL-10 in red) treated with pre-transplant DC protocol. Both allografts showed serum creatinine >200 on day 1 post transplant and failed (serum creatinine >600 as indicated by the dotted line) by day 5.

Table 7.3.1 Renal Biopsy Findings in Animals Receiving DC pre transplant (Banff scoring system)

Sheep	Treatment	Day 1	Day 3	Day 5	Autopsy
E2	AdV MX-17	$i^0, t^0 g^0$	i^1, t^0, g^0	$I^1, t^0 g^3, v^2$	i^3, t^3, g^3, v^3
D2	AdV IL-10	$i^1, t^0 g^0$	$i^3, t^0 g^3$	i^3, t^0, g^3, v^3	i^3, t^3, g^3, v^3

7.3.2 Protocol 2: Post-transplant administration of AdV IL-10 and AdV MX-17 transduced DC

A total of 6 animals were treated with protocol 2 (4 with AdV IL-10 and 2 with AdV MX-17). All donor-recipient pairs were alloreactive as assessed by the MLR and red blood cell cross match negative. Transplantation surgery was unremarkable for all animals with all kidney warm ischaemia times less than 3 minutes and cold ischaemia times of less than 30 minutes. The post transplant serum creatinine profile of sheep transplant recipients who were treated with intravenous genetically modified DC as therapy are shown in figure 7.3.2.

Clinical Course of Sheep Transplanted with Protocol 2

All animals showed an unremarkable immediate post-operative course, with animals eating within 3 hours of surgery. Specifically, the animals showed minimal signs of distress during the post-operative period. One animal (sheep Y) died on day 5 post surgery from wound related sepsis. Routine renal biopsy samples were taken at day 0, day 3 and at autopsy in all sheep. They were graded according to the Banff criteria and the results are recorded in table 7.3.2. Typical renal allograft biopsy appearances are also shown in figures 7.3.4 a-c and 7.3.5.

Effect of Daily Intravenous genetically modified DC upon Ovine Kidney Allograft Survival

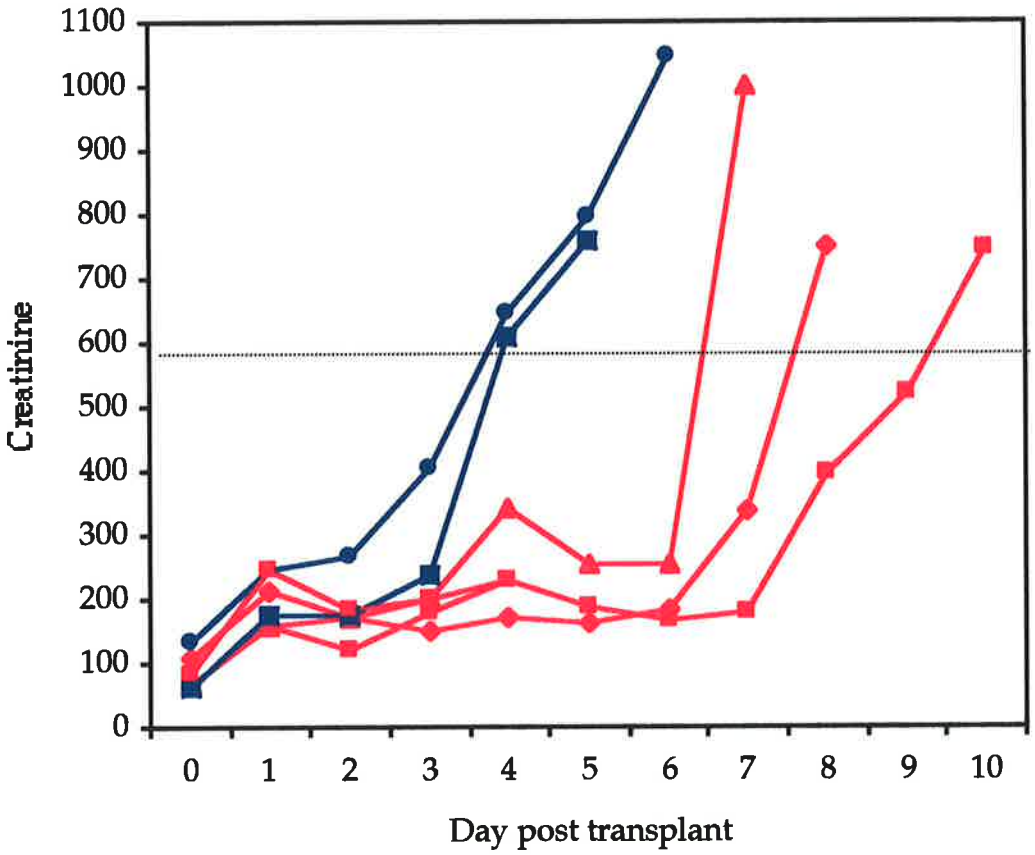


Figure 7.3.2

Sheep Serum creatinine values in animals treated with DC transduced with either AdV IL-10 (red) or AdV MX-17 (blue). Individual sheep K2 (AdV IL-10 in red diamond), sheep Q (AdV IL-10 red triangle), sheep N (AdV IL-10 red square) and sheep R(AdV MX-17 in blue square), sheep M (AdV MX-17 in blue circle) treated with an intravenous DC protocol. Sheep treated with intravenous AdV IL-10 DC showed longer allograft survival than sheep treated with AdV MX-17 DC.

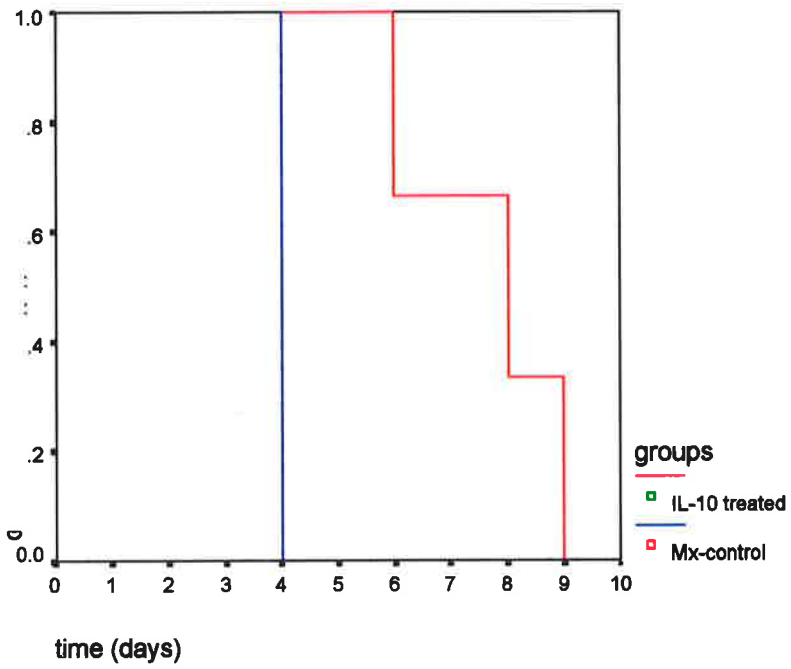


Figure 7.3.3

Kaplan-Meier curve for sheep kidney allograft survival stratified by treatment with daily intravenous DC therapy of either AdV MX-17 (blue) or AdV IL-10 (red). Log rank statistic p=0.045 for survival. Cumulative survival (%) is shown on the Y axis.

Table 7.3.2 Renal Allograft biopsy findings graded by Banff criteria.

Sheep	Treatment	Day 1	Day 3	Autopsy	
K2	AdV IL-10	i^0, t^0, g^0, v^0	i^1, t^0, g^0, v^0	i^3, t^0, g^0, v^0	Sacrificed Day 8
Y	AdV IL-10	i^0, t^0, g^0, v^0	i^3, t^0, g^0, v^0	$i^3 t^0 g^2 v^0$ (day 5)	Died sepsis Day 5
N	AdV IL-10	i^0, t^0, g^0, v^0	i^2, t^0, g^0, v^0	i^2, t^1, g^3, v^2	Sacrificed Day 10
Q	AdV IL-10	i^0, t^0, g^0, v^0	i^2, t^0, g^1, v^0	Corticol necrosis	Sacrificed Day 7
R	AdV MX-17	i^0, t^0, g^0, v^0	i^1, t^0, g^0, v^0	i^3, t^1, g^2, v^0	Sacrificed Day 6
M	AdV MX-17	i^0, t^0, g^0, v^0	i^2, t^0, g^3, v^1	i^3, t^0, g^3, v^0	Sacrificed Day 5

The sheep receiving AdV IL-10 transduced DC post transplantation (sheep Q, K2 and N) all showed allograft survival greater than 6 days (range 7-10). Sheep Y (AdV IL-10) died on day 5 of sepsis related to wound infection. In contrast both sheep R and sheep M received DC transduced with the control virus (AdV MX-17) and showed significant rejection by day 4 with creatinine greater than 600μmol/l. Figure 7.3.3 shows the Kaplan-Meier survival analysis for animals treated with either protocol 1 or protocol 2. There was a small survival advantage for animals treated with AdV IL-10 in comparison with animals treated with AdV MX-17 (log rank p=0.045).

Renal Biopsy Findings in AdV IL-10 Treated Sheep

All allograft biopsies showed evidence of transplant rejection by day 7, with typical changes being glomerulitis (figure 7.3.4 a-c) and mononuclear cell interstitial infiltration into the intersitium. In two allograft recipients (K2 and Q),

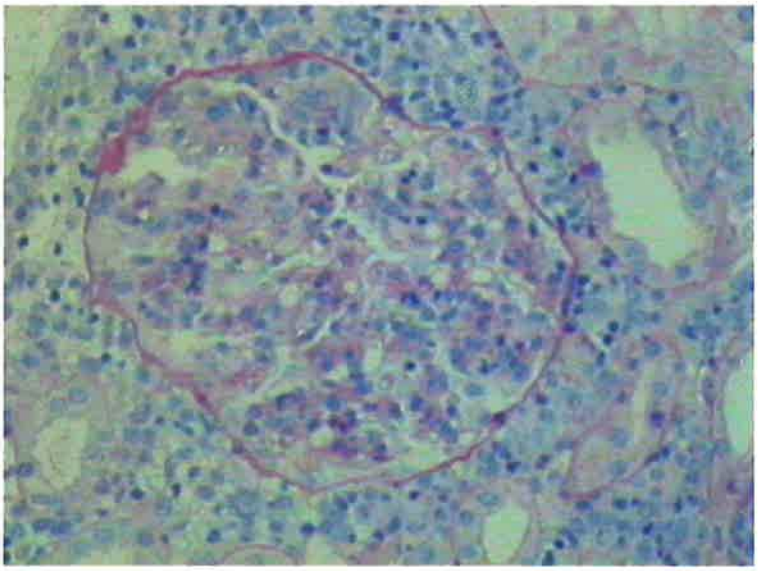


Fig 7.3.4a

Sheep D2 (AdV MX-17 IV) day 3 renal allograft biopsy showing a glomerular infiltrate of mononuclear cells and endothelial swelling - changes typical of glomerulitis. ABPAS stain x100.

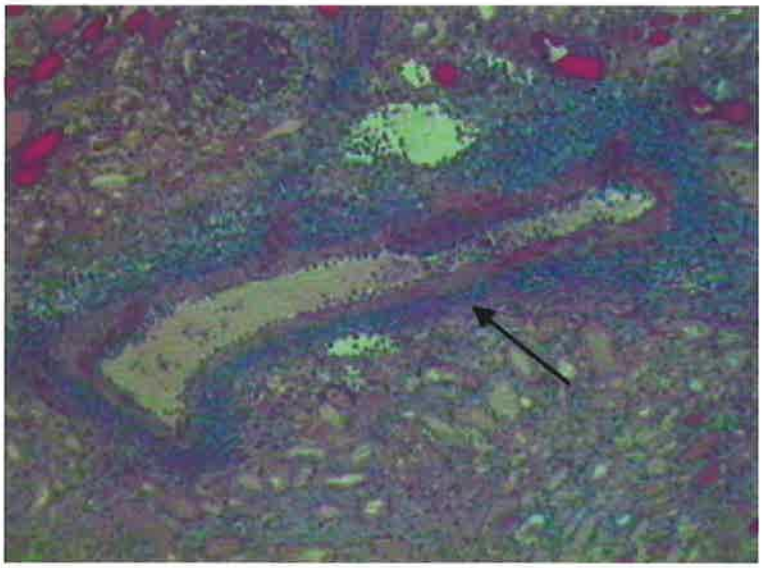


Figure 7.3.4b

Sheep E2 (AdV IL-10 IV)day 8 showing established vascular rejection in a small renal arteriole. The black arrow indicates intimal arteritis with 25% loss of luminal area (Banff grade v^2). ABPAS stain x 40.

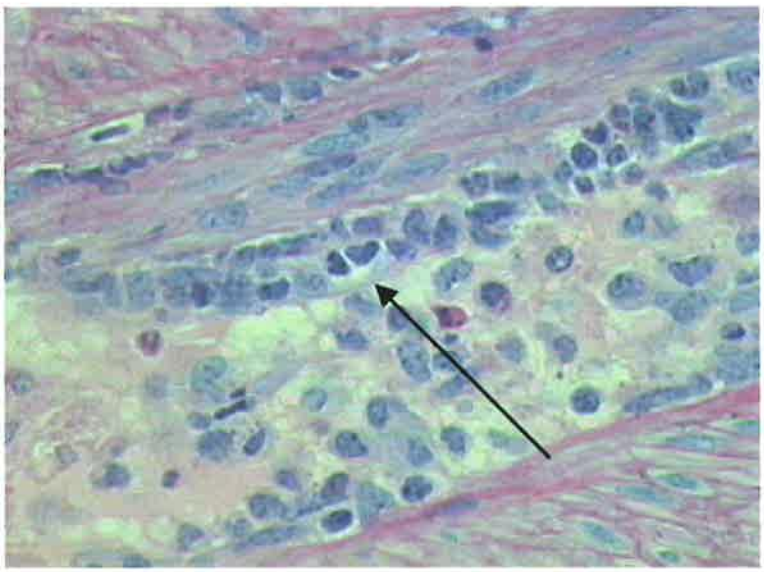


Figure 7.3.4c Sheep E2 (AdV IL-10 IV) day 8 showing a high power view of intimal arteritis seen in figure 7.3.4b. The black arrow indicates clearly visible mononuclear cells beneath the endothelial layer (Banff grade v^2 arteritis). ABPAS x 100.

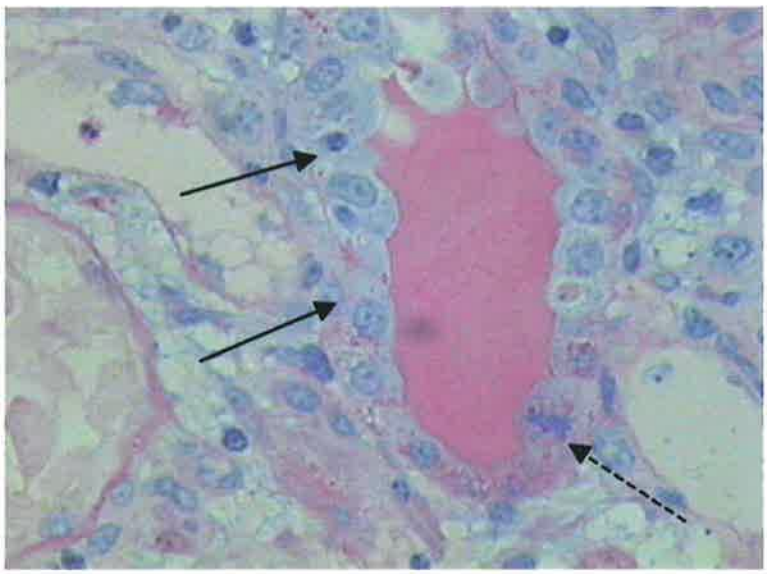


Figure 7.3.5

Sheep K2 (AdV IL-10 IV) day 3 showing changes of tubulitis. Solid arrows indicate mononuclear cells within the tubule. The dotted line indicates a tubular cell mitosis. Changes representative of t^1 (Banff criteria). ABPAS x100.

there were autopsy findings suggestive of humoral rejection that were not gradable by Banff criteria. These findings were interstitial haemorrhage and peritubular polymorphonucleocyte infiltration (sheep K2 day 5 and sheep Q at day 3). In sheep Q the autopsy specimen showed cortical necrosis. Renal biopsy findings in sheep Y at day 5 showed a significant infiltrate but no changes of glomerular or vascular rejection.

Renal Biopsy Findings in AdV MX-17 Treated Sheep

Both animals showed a significant early interstitial cellular infiltrate by day 3 post transplantation. At autopsy Sheep M (AdV MX-17) showed changes of glomerular thrombotic microangiopathy and glomerular mesangiolysis. Sheep R renal allograft biopsies showed marked interstitial infiltration with evidence of tubulitis (see figure 7.3.5) and a moderate glomerulitis at autopsy.

7.4 Discussion

In this chapter, the effect of two routes of administration and two different timings of administration of genetically modified DC upon allograft survival were studied using a renal transplant model. Protocol 1 involved the administration of genetically modified DC pre transplantation. The protocol entailed intra-dermal delivery of $5x10^6$ DC pre-transplantation by injection at day -7 and day -1. The rationale for this approach was based upon the data acquired in chapter 6. Firstly, this route was chosen based upon the Indium-oxine labeling studies that demonstrated migration of allogeneic DC from the site of injection into the draining lymph node, thus indicating that allogeneic genetically modified DC could migrate to appropriate lymph nodes within the recipient. Furthermore, PKH-26 labeled allogeneic DC could be detected within draining lymph nodes (see figure 6.3.1), and the delivered transgene identified using RT-PCR (figure 6.3. 2B). Finally and most importantly cells enriched from draining lymph nodes that received AdV IL-10 transduced DC showed reduced capacity to lyse chromium labeled donor blasts compared to nodes receiving AdV MX-17 transduced DC, suggesting that there had been an inhibitory immunological response within these lymph nodes (figure 6.3.3).

There are further rationale for the strategy of delivery of donor antigen to the recipient immune system in the presence of IL-10. One of the mechanisms of transplant tolerance induction has been shown to be the generation of active regulatory T cells (see chapter 1)(Waldmann et al. 2000). A well defined population of these cells designated Tr1 cells was first identified in the context of IL-10 expression and recently T regulatory cells have been generated by immature DC (Roncarlo et al. 2001). By administration of AdV IL-10 transduced DC pre-

transplantation, it was hoped that some form of regulatory cell might have been induced.

The effect of administration of exogenous IL-10 upon allograft survival has been investigated. Li *et al* found that *pre*-transplantation treatment of *recipient* C3H ($H2^k$) mice with mammalian IL-10 of over a range of doses inhibited rejection of B10 ($H-2^b$) murine cardiac allografts (mean survival time 17.6 days compared to control 10.7 days). Pre-transplant treatment was associated with a reduction in splenic anti-donor CTL response and a reduction in graft infiltrating $CD8^+$ cells (Li et al. 1999a). When exogenous mammalian IL-10 was administered *post* transplantation, accelerated cardiac allograft rejection was observed. The results seen in the sheep transplant experiments are not consistent with these observations.

In protocol 1 a dose of 5×10^6 DC transduced with either AdV IL-10 or AdV MX-17 was used. This dosage was chosen for 2 reasons. The first was on the basis of lymph node studies performed in chapter 6, where an inhibitory effect upon the lymph node environment was demonstrated by using this dosage. The second reason was that these cell numbers approximate the number of DC within a unit of human peripheral blood. The tolerogenic effect of blood transfusion on renal allograft survival is well documented and may be related to DC or DC subsets within blood (Blajchman 1998). The adenoviral transduced cells were administered via intra-dermal injection into the neck of the sheep in close proximity to the suprascapular lymph node (as identified on ^{111}Indium-oxine scanning see chapter 6). The cells were administered on 2 separate occasions pre transplantation (day -7 and day -1). Both transduction with AdV IL-10 and AdV MX-17 were associated

with allograft failure and prompt renal transplant rejection (creatinine >600μmol/l at day 5).

The rapid rejection seen in the animals treated with the pre-transplant DC therapy suggested that the treatment had induced sensitization to donor antigens. In previous studies of allograft survival in this model, rejection changes were visible on biopsy as early as day 3 but urine output and allograft failure (as measured by serum creatinine concentrations of greater than 600μmol/l) did not occur until day 6-9 (Grooby 1996). Both animals that received protocol 1 showed accelerated rejection with allograft failure evident by day 5 irrespective of treatment with either AdV IL-10 or AdV MX-17. As the direct spin cross match was negative, and the transplant insertion biopsy showed no significant abnormality it is unlikely that this represents hyperacute rejection. Renal biopsy changes showed mononuclear cell infiltration consistent with acute rejection changes.

It is paradoxical that treatment with AdV IL-10 DC produced accelerated rejection as *in vitro* studies of the draining lymph node had suggested that these DC were associated with an allo-inhibitory response (reduction in CTL activity) when administered to draining lymph node. It is possible that the IL-10 transduced DC were having an allo-stimulatory effect *in vivo*. Others have reported the allo-stimulatory properties of mammalian IL-10 (Qian et al. 1996). The allo-stimulatory properties of IL-10 depend upon a crucial amino-acid substitution (Ding et al. 2000). One of the critical changes at the amino acid level that determines biological activity of vIL-10 is the substitution of alanine for isoleucine at position 87. Ovine IL-10 is a mammalian form of IL-10, and the published sequence of ovine IL-10

contains an isoleucine at position 87 therefore allostimulatory behaviour would be predicted. The *in vitro* data presented in chapter 5 demonstrated that ovine IL-10 has immunosuppressive action on ovine DC in isolation. As human DC transduced with AdV IL-10 show significant secretion of IL-10 into tissue culture supernatant (3.3.6) *in vitro* it is probable that these cells are secreting significant IL-10 *in vivo*. The secreted IL-10 may be having an effect upon the recipient immune system. One possible explanation for the failure of AdV IL-10 transduced DC to inhibit kidney allograft rejection is that ovine IL-10 may have been having an allostimulatory effect upon other cell types such as monocytes and macrophages and that rejection may be mediated by these cells.

Another more mundane explanation for the occurrence of rejection is that AdV IL-10 transduction process may have been deficient and insufficient gene transduction occurred. This is unlikely based upon extensive experience with this regimen in terms of efficiency of gene transduction but this could potentially allow direct DC priming of the recipient T cell repertoire resulting in accelerated allograft rejection. The results obtained are consistent with this mechanism.

Protocol 2 involved the administration of genetically modified DC. Animals receiving genetically modified DC delivered intravenously post transplantation also showed allograft rejection. Neither AdV IL-10 or AdV MX-17 transduced DC administered intravenously in the dose of $1x10^6$ cells/day prolonged allograft survival beyond 10 days, although a small, non-sustained survival advantage was seen in the animals receiving AdV IL-10 (see figure 7.3.3). In the 2 animals treated with AdV MX-17 a rapid rejection response was observed compared to animals receiving AdV IL-10 DC. Nevertheless AdV IL-10 treatment was not effective in

prolonging kidney allograft survival past 10 days. Unmodified ovine renal allografts survive between 6-9 days (Grooby 1996).

There are a number of possible explanations for the failure of AdV IL-10 DC administered intravenously to prolong allograft survival. These relate to cellular dosage of cells and issues about the biological activity of AdV IL-10 transduced DC. Firstly the dose of cells (1×10^6 per dose) given may have been insufficient for a biological effect. The dosage was based upon the number of saved cells available for the study and the desire to treat for 7 days post transplantation. This explanation is unlikely as treatment with an identical number DC transduced with AdV MX-17 DC was associated with accelerated renal allograft rejection evident by day 4. It is possible that the AdV IL-10 DC were having an allo-inhibitory effect, but that the effect was only modest in size and insufficient to prolong survival beyond 10 days. As unmodified DC are potent T cell stimulators *in vitro* it would potentially only require a few cells to enhance the proliferative response against the allograft. The DC given in this study were also given once daily as a bolus injection. The migration of donor-derived DC from the allograft into the recipient lymphoid system begins immediately after transplantation and is probably a constant phenomenon. Therefore it is not inconceivable that a bolus dose of AdV IL-10 DC might be incapable of overcoming the effect of a constant migration of unmodified allostimulatory DC from the allograft. A more physiological means of delivery of genetically modified DC might be to give infusions of cells post transplantation.

Secondarily, the timing of DC administration may be inappropriate to see maximum benefit. In most other studies infusions of DC preparations are given pre-transplantation. Lutz *et al* found that GM-CSF generated DC could promote long term vascularized cardiac allograft survival only when administered 1 week prior to transplantation (Lutz et al. 2000). When the same cells were administered 3 days prior to transplantation the protective effect on allograft survival was lost. The mechanism of this protective effect in this small animal model is unknown. Theoretically administration of donor DC to allograft recipient to generate regulatory or veto cells could be undertaken *pre*-transplantation- if this is the case then a minimal time period, such as 7 days prior to the transplant may be critical. In our model we tested the capacity of these cells to modify allograft survival by post transplantation administration, akin to conventional immunosuppression. However, if AdV IL-10 DC are functioning as immunosuppressant cells then administration post transplantation would be appropriate. IL-10 polarizes the immune response towards the TH2 response which includes a B cell mediated allo-antibody response. The biopsy findings in the sheep kidney was typical of a humoral type reponse (predominantly glomerulitis). This pattern has been reported in human renal transplants in association with antibody against donor class I antigens (Trpkov et al. 1996). It is possible that the rejection changes seen in the glomeruli of sheep treated with AdV IL-10 represent a TH2 biased humoral mediated rejection response.

Finally, transduction of donor DC with AdV IL-10 is a strategy that targets the direct pathway of allo-recognition. The indirect pathway of allo-recognition is unaffected by such a strategy. Acute rejection may be mediated predominantly by indirect allo-recognition (Auchincloss et al. 1993). Recent studies have examined

the importance of indirect allorecognition in murine transplants and indicated a more important role for the indirect pathway of allorecognition than is usually assumed (Benchiou et al. 1999). It is possible that the prolongation of allograft function as evidenced by the difference in serum creatinine seen in the AdV IL-10 treated animals reflects partial inhibition of direct allo-recognition. The occurrence of rejection between day 6 and day 10 may be reflecting a greater indirect allo-recognition component than in sheep treated with AdV MX-17.

Although it is difficult to precisely define the reasons that administration of AdV IL-10 transduced DC did not impact upon allograft survival a number of useful points may be drawn from these studies. Firstly it seems likely that some other form of immunosuppressive therapy may need to be co-administered with genetically modified DC to enhance any survival benefit. An agent that could inhibit indirect allorecognition would be of theoretical interest. Combination therapy consisting of genetically engineered DC coupled with conventional immunosuppressive therapy (for example Rapamycin) could be considered. It would also be of interest to try dosage escalation of genetically modified DC and study the effect of pre-transplantation intravenous administration.

In summary, intra-dermal pre transplantation administration of genetically modified DC was incapable of prolonging ovine kidney allograft survival beyond 4 days. Intravenous post transplantation administration of AdV MX-17 DC was associated with prompt allograft rejection by day 4. In contrast animals receiving AdV IL-10 showed allograft survival up to 10 days. However historical control operations indicate that unmodified ovine renal allografts function for between 6-9 days. The DC numbers (5×10^6 pre transplantation intradermally and 1×10^6 post

transplantation intravenously) may be insufficient to show a definitive effect of transduction with adenoviral gene constructs.

Chapter 8

Concluding Remarks

In this thesis the effect of transduction of both human and ovine DC with adenoviral gene constructs encoding the cytokine IL-10 was examined. In the initial experiments human monocyte-derived DC were transduced with adenoviral IL-10 and strong allo-inhibitory properties were observed both *in vitro* and *in vivo* . Specifically, in the humanized NOD-*scid* mouse model, AdV IL-10 transduced DC inhibited allogeneic challenge. In the sheep, pseudoafferent DC were transduced by AdV IL-10 and showed alloinhibitory properties *in vitro* and *in vivo*. Despite promising preliminary data, AdV IL-10 transduced DC alone were incapable of prolongation of ovine heterotopic renal allograft survival. In terms of further studies of DC in transplantation there are several leads to follow from this work.

Gene Therapy

The gene therapy studies undertaken within chapter 7 failed to significantly prolong ovine kidney transplant survival. There are a number of potential explanations for this lack of efficacy. This failure underscores a major problem with current novel immunosuppressive strategies, namely the lack of a reliable *in vivo* test for tolerogenicity. There are several currently available tests that could be developed for use in the sheep model. One of the most promising tests is the Elispot® test. This assay is an assay for tolerance and allows for the detection of IFN-γ secreting T cells. It can be used to monitor the immunological response to novel therapies.

Another recently developed assay could also be readily applied to ovine studies. This assay is the trans-vivo DTH assay, in which an immunodeficient mouse is re-constituted with mononuclear cells from a "tolerant" animal. The mouse is then injected in the foot-pad with sonicated antigen of donor origin and the degree of swelling of the foot is then graded in reference to a recall antigenic stimulus (in human studies usually tetanus antigen)(VanBuskirk et al. 2000). The development of such assays will better allow selection of promising therapeutic strategies for pre-clinical evaluation in models such as the sheep. This would allow measurement of the immunological response to allogeneic DC before proceeding to transplantation.

With regard to AdV IL-10 transduced DC a number of further experiments should be carried out within the ovine model. The variable most likely to impact on allograft survival is the timing and route of administration. One strategy that could be followed would be administration of increased dosages of AdV IL-10 transduced DC as intravenous injection pre transplantation (at day -7). The *ex vivo* manipulation of DC remains an attractive means to minimize effects upon the recipient.

The sheep kidney transplant allograft model itself has much to offer. It is a large animal model for which many immunological reagents are available. Animal husbandry techniques and cost of animals are a major cost advantage for this model compared to the non-human-primate or even canine models. As such it provides a large animal transplantation model that may be used as an intermediary model for the testing of promising approaches.

The recent publications suggesting that immature DC may induce permanent allograft survival in murine models does suggest that DC based approaches for transplantation may be feasible. As it is unlikely that DC based therapy alone will ever be tried in the clinic, the investigation of combinations of DC with conventional immunosuppressive medication should be undertaken. The newer medications targeting the TOR pathway (eg Rapamycin) in particular would be theoretically very promising as they do not interfere with IL-2 transcription, that is thought to play an important part in activation induced cell death (AICD).

Dendritic Cell Subsets

One of the interesting findings was the presence of a population of ovine DC within lymph that expressed the cell surface marker CD8. As discussed in chapter 5 murine DC expressing CD8α/α have attracted considerable interest in the field of DC immunobiology. Although the exact molecular specificity of the ovine SBU T8 molecule is not known, the finding of an ovine DC population that expresses CD8 within afferent lymph raises a number of interesting questions. As human "plasmacytoid" DC do not express the cell surface marker CD8, the demonstration of a population of DC expressing this marker in a large animal has potential to shed light on the function of these cells. Clearly further characterization of the ovine CD8 expressing DC is warranted, with specific aim to determine its relationship to the murine $CD8^+$ DC and to its putative human counterpart - the "plasmacytoid" DC .

In order to further investigate the properties of these DC it must first be established what relationship these cells have to their murine counterparts. Isolation of these ovine CD8 DC was easily achieved by positive selection using

Dynabeads coated with SBU T8. An alternative selection technology would be the use of flow cytometric cell sorting analysis (eg to collect double positive CD8/CD83 expressing cells). As DC are actively phagocytic and as afferent lymph contains CD8 positive T cells non-specific isolation of cells other than those expressing CD8 as a cell surface marker is a potential problem with this magnetic bead technology. In general cell sorting analysis allows a greater degree of purity than is achieved by the use of magnetic bead technology. The purity of the DC collected is an important consideration in further characterization of these cells. Within the murine system, $CD8^+$ DC have been shown to express CD11c and CD40. The comparative characterization of ovine $CD8^+$ DC is limited by the lack of availability of monoclonal cell surface antibodies with these specificities. However, the murine $CD8\alpha^+$ DC can be localized to the T-cell zones of the lymphoid organs, therefore immunohistological studies by con-focal laser microscopy of ovine lymph nodes could be undertaken to determine co-localization within lymph nodes. Recent studies have shown that murine $CD8\alpha^+$ DC are capable of secretion of TH1 cytokine IL-12 and may induce protective immunity against viruses by production of cytotoxic T lymphocytes (CTL) via secretion of interferon-γ. A number of experiments to study interleukin-12 production could be undertaken. Firstly, analysis of messenger RNA species present within ovine $CD8^+$ DC could be performed under a variety of conditions, including treatment with lipopolysaccharidase (LPS) and an immunological stimulus (eg CD40L). Another approach to further characterize these cells would include intracellular cytokine staining for interferon gamma via flow cytometric analysis.

As well as providing further information regarding the immunobiology of DC, the studies of $CD8^+$ DC are of particular relevance for transplantation. The murine $CD8^+$ DC subset has recently been shown to be capable of cross priming (den Haan et al. 1999). These is considerable speculation that the same cell may be capable of presenting donor antigen in a tolerogenic context - so called "cross tolerance" (Fazekas de St Groth B 1998). There is also data now concerning the effect of administration of purified $CD8^+$ DC upon allograft survival within the murine system. When $CD8\alpha^+$ DC were isolated from the spleens of C57B/10J ($H2^b$) mice treated with the potent DC mobilizing cytokine Flt-3L were administered intravenously to C3H mice 7 days prior to vascularized allogeneic cardiac allografting in the absence of adjuvant immunosuppression, the mean allograft survival time was prolonged from 11 days (controls) to 23.5 days ($P<0.001$)(O'Connell et al. 2001). These studies, within a fully MHC mismatched transplant model strongly suggest that manipulation of DC subsets may be an efficacious means of prolonging allograft survival. If further work on the $CD8^+$ ovine DC suggests that this DC bears some of the characteristics of the murine $CD8^+$ DC then protocols with this subset could be developed.
Further studies investigating DC subsets and their capacity to modify allogeneic T cell function within large animals including human and non-human primates would allow the potential novel immunological studies to be performed.

Finally, in conclusion, therapeutic organ transplantation occurs under a unique immunological circumstances. Unlike auto-immunity the timing of initiation of the alloimmune response is precisely known, and in the case of live-related organ transplantation, can be manipulated to suit therapeutic purposes. This presents a

unique opportunity to use the enlarging understanding of the immune system, and the role that DC play within the immune system to develop immunologically based strategies for donor-specific hyporesponsiveness or tolerance induction.

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