STUDIES OF CLINICALLY APPLICABLE HUMAN TOLEROGENIC DENDRITIC CELLS AND PD-L2
GENETIC MODIFICATION OF HUMAN ISLET ALLOGRAFT TO PROMOTE GRAFT TOLERANCE

A thesis submitted in fulfilment of the PhD degree

In

The Department of Medicine
Faculty of Health Sciences
The University of Adelaide

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Contents

Thesis Summary

Islet transplantation is a developing therapy for type 1 diabetic patients (T1D), which has been limited by problems associated with hypoxia, poor revascularisation and allograft rejection. Immunosuppressive agents used to prevent rejection are associated with severe side effects including islet toxicity, increased susceptibility to the development of cancer, infections and cardio-vascular problems. In order for islet transplantation to be used widely as a potentially curative treatment for T1D there is a need to develop novel therapies to treat allograft rejection without the use of immunosuppressive agents.

In chapter 3, the immunomodulatory effects of IFN-γ on human monocyte-derived DC were investigated, using a standard 7-day *in vitro* DC propagation protocol. IFN-γ was shown to exert its immunomodulatory function on monocytes early during DC differentiation (IFNγ-DC_{D0}), resulting in an immature DC (iDC) phenotype with reduced expression of maturation markers CD83 and RelB. IFNγ-DC_{D0} induced a state of T-cell hyporesponsiveness in a MLR, whilst IFN-γ treatment at day 5 (IFNγ-DC_{D5}) did not modulate DC function. The ability of IFN-γ to promote the generation of maturation arrested DC, could potentially serve as a cellular therapy for transplant rejection. However DC propagation using the standard 7-10 day protocol is not clinically applicable in the islet transplant setting.

In chapter 4, a 'FAST-DC' protocol to promote the rapid generation of tolerogenic DC was investigated and used to generate IFNγ modulated DC in 48h. These IFNγ-DC featured an iDC phenotype similar to that seen in chapter 3. Maturation arrested IFNγ-DC caused significant T-cell hyporesponsiveness and promoted a higher frequency of $CD4^{+}CD25^{+}$ $Foxp3^{HI}$ T-regulatory cells. IFNγ-DC primed T-cells were shown to be functionally suppressive in an antigen specific manner. It was also confirmed that IFN-γ reduced the phosphorylation of IL-4 activated STAT-

6, which in turn affected the downstream gene expression of Interferon regulatory factor 4 (IRF4). IFNγ-DC were also investigated *in vivo*, where a humanised model of islet allo-transplantation model was developed. Diabetic NOD-SCID mice were transplanted with human islets and challenged with donor-derived DC and allogeneic PBMNC. After 21 days post transplantation, there was no significant change to euglycaemic state, between the tested groups.

Genetic modification of the allograft is an alternative therapy to protecting the graft from the recipient's immune system. In chapter 5, human islets were genetically modified with programmed cell death ligand 2 (PD-L2), an inhibitory molecule known inhibit T-cell immune responses. Two recombinant adenovirus constructs carrying the PD-L2 gene were generated. One construct encoded a soluble isoform, while the other expressed a full transmembrane PD-L2 molecule. Adenoviral transduction did not affect the viability or insulin producing capacity of islets. Interestingly, soluble PD-L2 was more efficient at inducing signalling by 1000 fold, compared to the transmembrane isoform.

In summary, this thesis demonstrated the timing of IFN-γ exposure is crucial in determining the function of DC and their maturational state, where IFN-γ exposure only during DC differentiation resulted in the inhibition of DC maturation. Secondly, the combination of IFN-γ and a FAST-DC protocol, enabled the generation of tolerogenic DC in 48h, making DC therapy more clinically applicable. Finally, the induced expression of soluble PD-L2 by human islets potently signals through human PD-1, which may provide the basis for the protection of islets from allo- and auto T-cell responses.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Darling Rojas and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (as listed below*) resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

* **Rojas D**, Krishnan R. IFN-gamma generates maturation-arrested dendritic cells that induce T cell hyporesponsiveness independent of Foxp3(+) T-regulatory cell generation. Immunology letters; **132**:31-7. (See Appendix F)

Signed Darling M Rojas-Canales

Publications

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Presentations

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Abbreviations

AdV – adenovirus

AICD- activation induced cell death

APC – antigen presenting cells

BGL- blood glucose levels

CPM- counts per minute

DC- dendritic cell

DMEM- dulbecco's modified eagle medium

FACS – fluorescence activated cell sorting

FCS- foetal calf serum

g – gravity

GM-CSF- granulocyte macrophage colony stimulating factor

Gy – greys

H&E hematoxylin and eosin

HEK – human embryonic kidney

HLA – human leukocyte antigen

iDC – immature dendritic cell

IDO – inodoleamine 2,3-dioxygenase

IEQ – islet equivalents

IFN- interferon

IL- interleukin

ILT- immunoglobulin-like transcript

mAb – monoclonal antibody

MFI – mean fluorescence

MHC- major histocompatability complex

MLR – mixed lymphocyte reaction

NOD- non obese diabetic

PBMNC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCR – polymerase chain reaction

RPM – revolutions per minute

RPMI – roswell park memorial institute

SCID – severe combined immunodeficiency

STZ- streptozotocin

TH – T helper

TOL-tolerogenic

Treg- T regulatory

T1D – Type 1 diabetes

CHAPTER 1 - Background

1.1 Introduction

Type 1 diabetes (T1D) is a debilitating autoimmune disease, which is currently treated with exogenous insulin administration. This approach however is limited by its inability to achieve physiological blood glucose levels. Islet allograft transplantation is an emerging potentially curative therapy, which is limited by many factors including alloimmunity and reoccurrence of autoimmunity. Islet allograft recipients are required to use toxic immunosuppressive agents to prevent the destruction of the islet allograft. Unfortunately, immunosuppressant therapy is non-specific and associated with severe side-effects, which in most cases outweighs the benefits of islet transplantation. Consequently, only patients with severe episodes of hypo-unawareness have access to allogeneic islet transplantation. In order for islet transplantation to become a standard therapy for T1D, there is a need to develop novel therapies that target auto- and allo-immune responses concurrently.

This chapter aims to review the current literature and the background for the basis of this thesis. In particular it focuses on the function of dendritic cells to act either as an 'immunogenic' or 'tolerogenic' antigen presenting cell in allograft responses. The review then discusses the application of gene therapy of the graft as an approach to achieve transplant tolerance. Finally the negative regulatory pathway of Programmed death 1 and its ligands is extensively reviewed for its involvement in peripheral tolerance and its potential to target both allo- and auto immune responses.

1.2 Type 1 Diabetes

Type 1 diabetes mellitus (T1D) is an autoimmune disease, where self reactive T-cells mediate the destruction of the insulin producing beta-cells of the pancreas [1-3]. The destruction of beta-cells affects the normal control of blood glucose levels, resulting in hyperglycaemia. This condition is commonly diagnosed in childhood, but can develop at any age including later in life. T1D affects over 120,000 people in Australia, with an expected 3% increase globally each year. (International diabetes federation, Diabetes Atlas 3rd edition IDF 2007). Figure 1.2.1 depicts the progression and development of the disease in association with beta-cell mass. Usually at the time of diagnosis more than 80% of islets have been destroyed [4], with minimal to no secretion of insulin. The current gold standard treatment for T1D is life-long use of exogenous insulin replacement therapy, which is limited by its inability to promote physiological glucose levels. Poor glucose control can result in ketoacidosis and hypoglycaemia, both of which are life threatening conditions. The Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications studies have demonstrated that poor glucose control in T1D patients is associated with the onset and progression of micro-vascular (retinopathy, nephropathy and neuropathy) and macrovascular (cardiovascular, cerebro-vascular and peripheral vascular disease) complications [5-10]. The development of T1D has been associated with genetic and environmental factors [2].

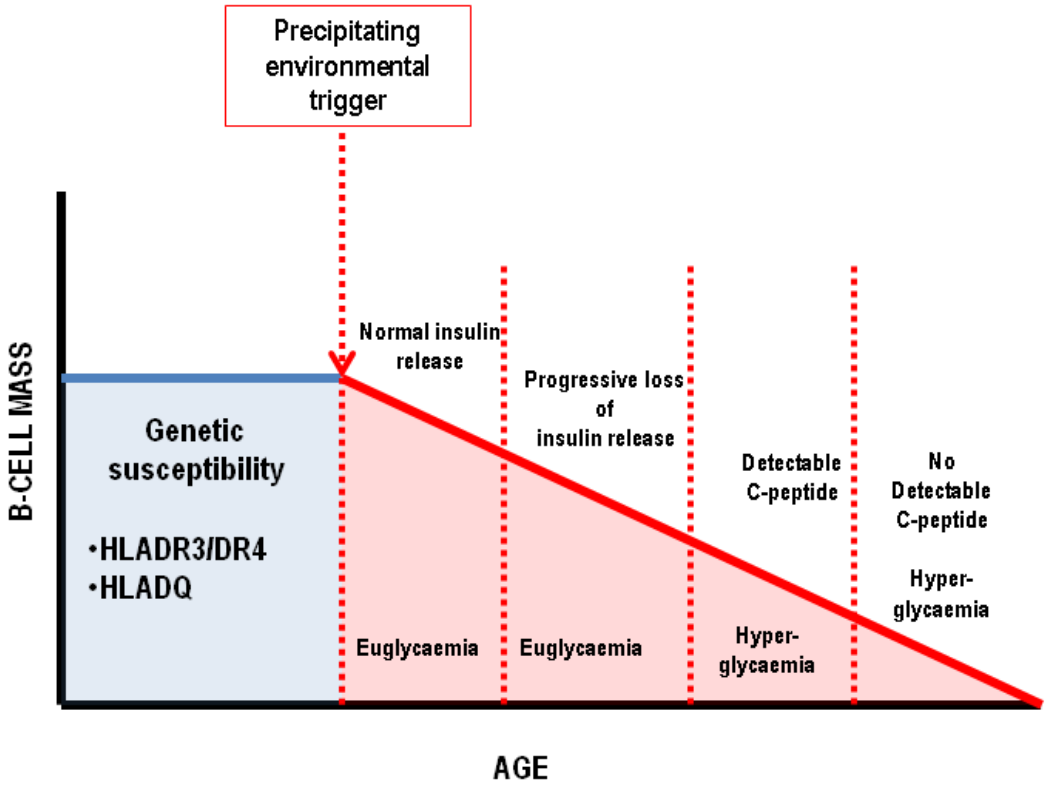

Figure 1.2.1 Developmental stages of Type 1 Diabetes. Most patients that develop Type 1 diabetes have a genetic disposition which is primarily associated with HLADR3, HLADR4 and HLADQ. An environmental trigger is believed to precipitate the initiation of autoimmune responses that results in beta mass loss. Initially the disease is asymptomatic with normal insulin release and glucose control. Over time with progressive loss of insulin, the disease manifests itself promoting hyperglycaemia with over 80% of the beta cell mass being destroyed. A modified adaptation from Eisenbarth GS, Jeffrey J. The natural history of type 1A diabetes. Arquivos Brasilerios de Endocrinologia Metabologia 2008; volume **52**: page 146-155.

The major genetic determinants of T1D are the genes within the major histocompatibility complex (otherwise known as human leukocyte antigen in humans) class II. Most T1D patients express HLA-DR3 or HLA-DR4 class II alleles, while 30-40% are heterozygous and carry the high risk genotype of HLA-DR3/DR4 [11]. However the strongest association with disease susceptibility is HLA-DQ [12]. HLA-DQ a heterodimer (α and β chains), which controls immune recognition and antigen presentation to CD4+ T cells. The HLA-DQ may therefore influence the development T1D by determining immune responses that lead to beta-cell destruction.

1.2.1 Islets of langerhans

The pancreas consists of exocrine and endocrine tissue that play a crucial role in aiding digestion and regulating blood glucose levels. The exocrine tissue is involved in the secretion of digestive enzymes, whilst the endocrine tissue produces hormones like insulin to control blood glucose levels. The endocrine cells of the pancreas are structured into cell clusters known as islets of langerhans that are scattered throughout the exocrine tissue. Islets take up only 1-2% of the total pancreas mass [13]. The size of islets can vary in size from 20 to 250 μm in diameter. Islets of langerhans cell clusters consist of insulin producing β-cells and α-cells that produce glucagon that stimulates the release of glucose from the liver. δ-cells are also present and are involved in secreting somatostatin and pancreatic polypeptide [14]. To a lesser extent other cell types are also found in islet clusters, such as stromal cells, blood vessels, neurons and immune cells such as dendritic cells [15]. In order to produce insulin, β-cells consume large amounts oxygen to produce ATP, accordingly to maintain an adequate supply of oxygen islets are inherently highly vascularised, making them highly sensitive to hypoxic conditions [16]. Proinsulin is an insulin precursor that is made up by an A and B chain

connected by a connecting peptide (c-peptide). Subsequently, c-peptide is a diagnostic marker for insulin secretion [17].

1.2.2 Islet transplantation – a potentially curative therapy for type 1 diabetes

Whole pancreas transplantation is an attractive therapy for T1D patients, which re-establishes normal glucose metabolism and is associated with complete and sustained normalization of glycosylated HbA_{1c} [18]. However pancreatic transplantation involves a large surgical procedure which is associated with a high degree of morbidity. Islet transplantation provides a less invasive alternative surgical procedure, which involves the infusion of isolated islets into the portal vein of the liver as demonstrated by figure 1.2.2. Islet transplantation is considered to be a relatively safe procedure in comparison to whole pancreas transplantation [19].

The isolation of islets was developed over 30 years ago and since has provided the basis for developing islet transplantation as a potentially curative therapy for T1D patients [13]. In 1990 the first islet transplanted patient reached insulin independence for 22 days prior to graft failure [20]. Despite improvements in protocols, less than 12% of patients were insulin independent one year post transplant, between the year 1990-2000 [21]. In 2000 Shapiro and colleagues reported the successful islet transplantation of 7 patients whom became insulin independent [22]. Their success was primarily due to the development of the Edmonton protocol, which now has been standardised worldwide [23]. The success of the Edmonton protocol was associated with an optimised immune therapy regime that consists of a combination of anti-Il2 antibody, Sirolimus and low dose tacrolimus. Despite these promising advances, only 10% of islet transplant recipients are insulin independent 5 years post-transplantation. However 80% of recipients remain C-peptide positive [22, 24]. Partial graft function has been shown to

remarkably improve the quality of life of T1D, by reducing the episodes and severity of hyperawareness[25].

1.2.3 Immunosuppression – a barrier to the success of islet transplantation

Cell and organ transplant recipients in a majority of cases require life-long use of immunosuppressive regimes in order to prevent acute allograft rejection. Immunosuppressive reagents are highly non-specific and are associated with a variety of side effects. In particular agents that target T-cell proliferation and cytokine production like, cyclosporine, azathioprine, mycophenolate mofetil and rapamycin, anti-CD3 and anti-IL-2 antibodies [26] increase the susceptibility of recipients to develop infections and malignancies [27]. The organo-toxicity of these drugs have also been associated with the development of cardiovascular complications giving rise to a high prevalence of hypertension, hyperlipidemia and to a lesser extent the onset of diabetes with long-term use [28]. Some immunosuppressive agents are significantly toxic and contribute to loss of islet cell mass post-transplantation [29-31]. The development of the Edmonton protocol, consisting of a corticosteroid free regime, with the use of sirolimus, low dose tacrolimus and induction therapy with anti-Interleukin 2 (IL-2) receptor antibody, has greatly improved the outcomes of islet transplantation. However, recipients are still susceptible to the non-specific side effects of long term immunosuppression. Subsequently islet transplantation is currently only available to adults that suffer from severe episodes of hypo-unawareness. In order for islet transplantation to be widely used including in children, novel therapies that target both the allo and autoimmune reactions need to be developed, which promote tolerance without the use of immunosuppression.

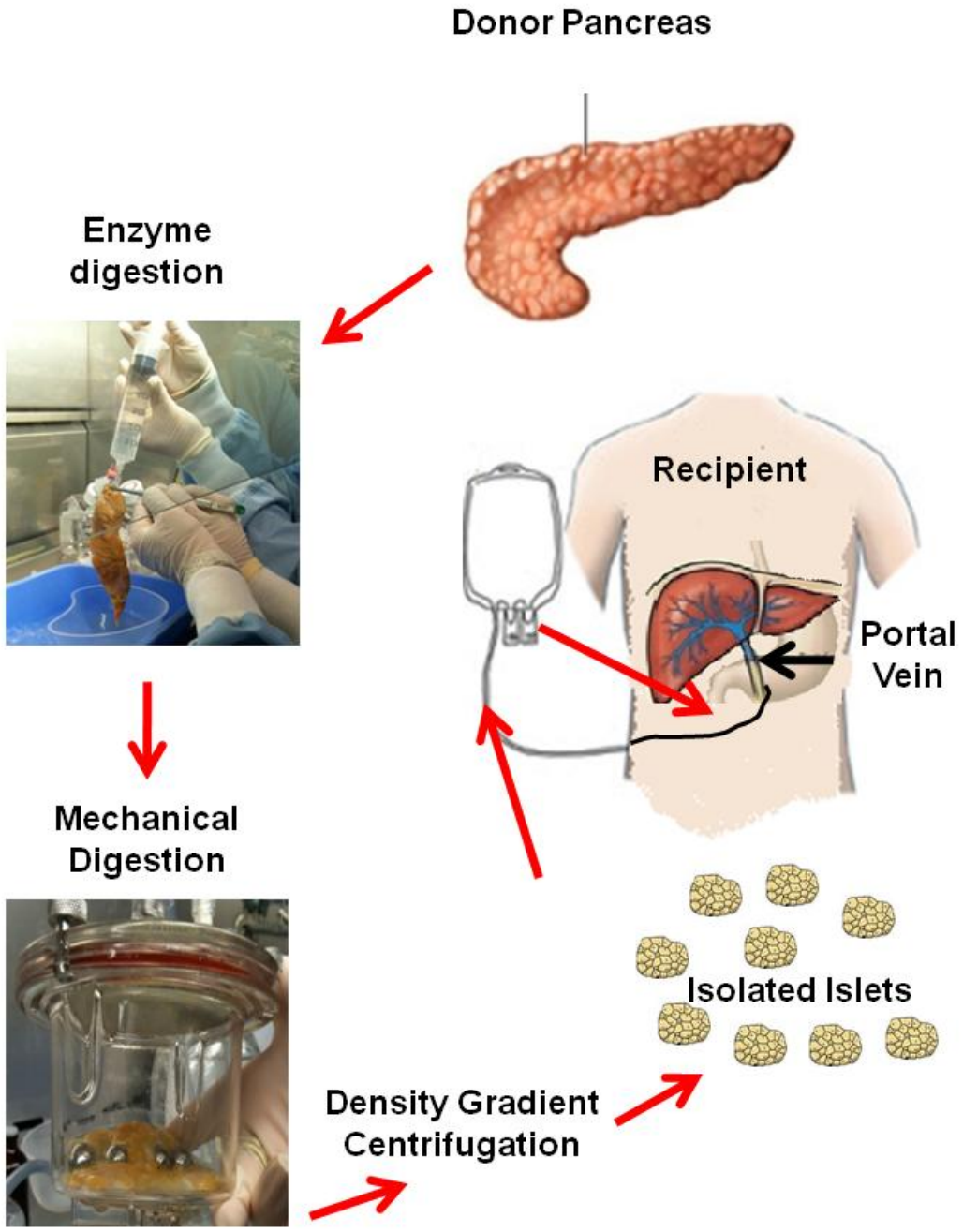

Figure 1.2.2: Islet Transplantation. The donor pancreas is inflated with digestive enzymes prior to the transfer into a Ricordi chamber which mechanically breaks down the tissue with metal marble bearings. Islets are then purified by density gradient centrifugation. Purified isolated islets of high quality that are then infused into the portal vein of the recipient's liver.

1.3 Transplantation immunology

1.3.1 Allogeneic T-cell Activation and priming

Allograft rejection involves co-ordinated immune responses, which recognise the allograft as foreign and subsequently cause allograft failure. Antigen presenting cells (APC) and in particular dendritic cells (DC) play a key role in presenting donor antigens to T-cells, which initiates the allo-immune response. At the molecular level, T-cells require multiple signals of activation to initiate cellular-mediated allograft rejection, however there are three major signals that are essential for T-cell activation. As depicted in figure 1.3.1A signal one of activation is delivered by the T-cell Receptor (TCR) that binds to the DC Major Histocompatibility (MHC) or otherwise known as the Human Leukocyte Antigen (HLA) in human, peptide complex. This mediates the recognition of specific epitopes of the antigen presented in combination with the presented MHC molecule [32]. However, this signal alone is unable to initiate sustained T-cell proliferation, resulting in T-cell apoptosis, or T-cell anergy [33] (an unresponsive T-cell state - to be later discussed in section 1.2.3). Full T-cell activation requires signalling from DC co-stimulatory molecules that provide signal 2 of activation [34]. There are a growing number of characterised positive and negative co-stimulatory molecules [35], some of which have been summarised in figure 1.3.1B. In addition to co-stimulation, T-cells require cytokine production (signal 3) to drive their clonal expansion, DC for example produce IL-12 and T-cells secrete IL-2 to drive a Th1 immune response [36, 37].

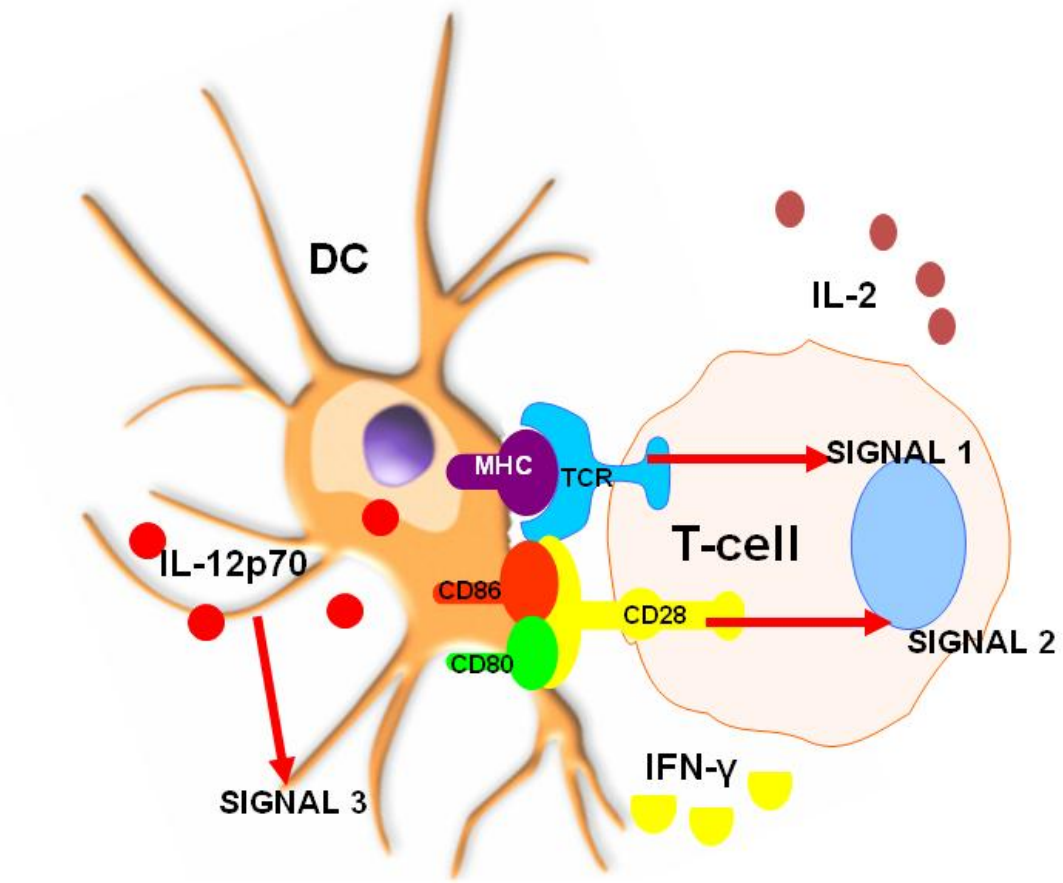

Figure 1.3.1: T-cell Activation. The activation of T-cells requires multiple signals of activation. The binding of MHC-peptide complex presented by DC to the TCR, this interaction initiates signal 1. Signal 2 is produced by the association of co-stimulatory signals CD80/CD86 with CD28 expressed on T-cells. Signal 2 amplifies MHC and TCR interaction to produce, IL-12 and IL-2 (signal 3).

Signal	Co-stimulatory molecule	Ligand
Positive	CD28	CD80/86 (B7-1/2)
Positive	CD27	CD70
Positive	HVEM	LIGHT, BTLA
Positive	ICOS	ICOS-L
Positive	OX40 (CD134)	OX40L
Positive	4-1BB (CD137)	4-1BBL
Positive	CD30	CD30L (CD153)
Positive	SLAM (CD150)	SLAM
Negative	BTLA	HVEM
Negative	CTLA-4	CD80/86 (B7-1/2)
Negative	PD-1	PD-L1 / PD-L2
Negative	Unknown	B7-H4

Figure 1.3.1B: This table illustrates the many positive and negative co-stimulatory pathways involved in T-cell activation. Those interactions highlighted in green promote positive signalling and T-cell proliferation. Those in interactions in red promote negative regulation of T-cell activation.

1.3.2 Allorecognition

The allorecognition of allograft MHC complex antigens by recipient T-cells is the primary event which promotes allograft rejection. The term allorecognition refers to the T-cell recognition of MHC glycoprotein genetic polymorphisms, which are highly variable between individuals [38]. There are two classes of MHC molecules, class I and II. MHC class I molecules otherwise known as human leukocyte antigens (HLA) A, B and C is constitutively expressed on a variety of nucleated cells. MHC class II (HLA-DR, DP and DQ) on the other hand is restricted to bone marrow derived APC, such as DC, macrophage, B lymphocytes and thymic epithelial cells [39]. Crystal structures have demonstrated that MHC molecules carry peptides on a 'Groove' which allows the peptides to bind and thus be presented to the TCR [40]. CD8+ T-cells are known to recognize peptides presented by MHC class I, which carry peptides that originate from intracellular proteins. CD4+ T-cells on the other hand recognise MHC class II peptide complexes, that present extracellular protein derived peptides [39]. Recipient DC capture and process donor antigens to elicit an immune response, known as the indirect pathway of allorecognition. Passenger leukocytes also promote an immune response by activating T-cells with their polymorphic MHC molecules named the direct pathway of allorecognition. Recipient DC can also take-up donor antigen and MHC, via a semi-direct pathway of allorecognition, through membrane lipid raft transfers. Each pathway is described below.

Direct allorecognition

The direct pathway of allorecognition as shown in figure 1.3.2 involves donor derived passenger leukocytes resident in the transplanted tissue. These tissue resident immature DC subsequently mature and migrate in response to proinflammatory signals produced by tissue injury during surgery [41-43]. Maturing DC migrate to secondary lymphoid tissue to encounter and present foreign MHC to recipient T-cells [44, 45]. DC are predominantly responsible for the activation of recipient T-cells [46], where 1 - 7% of the recipient T-cell repertoire respond to foreign MHC via the direct pathway of allorecognition [47]. Donor-derived DC die within a few weeks after transplantation, resulting in the decrease of direct allorecognition and thus a decline of donor-antigen specific T-cells over time [48-50].

Indirect allorecognition

The indirect pathway of allorecognition involves recipient DC that migrate to the site of transplantation. Recipient iDC capture and process donor antigens after shedding from the allograft. They mature and migrate to secondary lymphoid tissue, causing T-cell activation with allo-peptides derived from polymorphic sequences of allogeneic MHC molecules bound to self-MHC molecules[46, 51, 52] [53].The indirect pathway plays an important role in the development of chronic allograft rejection. This is evidenced by the elevated frequencies of T-cells with indirect anti-donor specificity seen in patients with chronic heart, kidney and lung transplant rejection [54-61]. Thus the indirect pathway is believed to take precedence, when the direct pathway becomes exhausted. However animal studies have demonstrated that the indirect pathway is able to promote acute rejection in the absence of the direct pathway of allorecognition [62].

Semi-direct allorecognition

The semi-direct pathway, involves the transfer of lipid rafts from the cell membrane of donor DC to recipient DC. Accordingly, intact surface molecules including allogeneic MHC molecules can be transferred from donor to recipient DC and vica-verse, by direct cell to cell contact [63, 64]. The release and uptake of exosomes have also been proposed as a mechanism for DC in acquiring and sharing foreign cell molecules [65]. As a result recipient DC can acquire donor MHC molecules promoting T-cell activation via the direct pathway of allorecognition. Recipient DC can also take up other donor DC cell surface molecules in this manner, which are processed and then loaded onto self-MHC molecules, resulting in the presentation of allo-antigens via the indirect pathway.

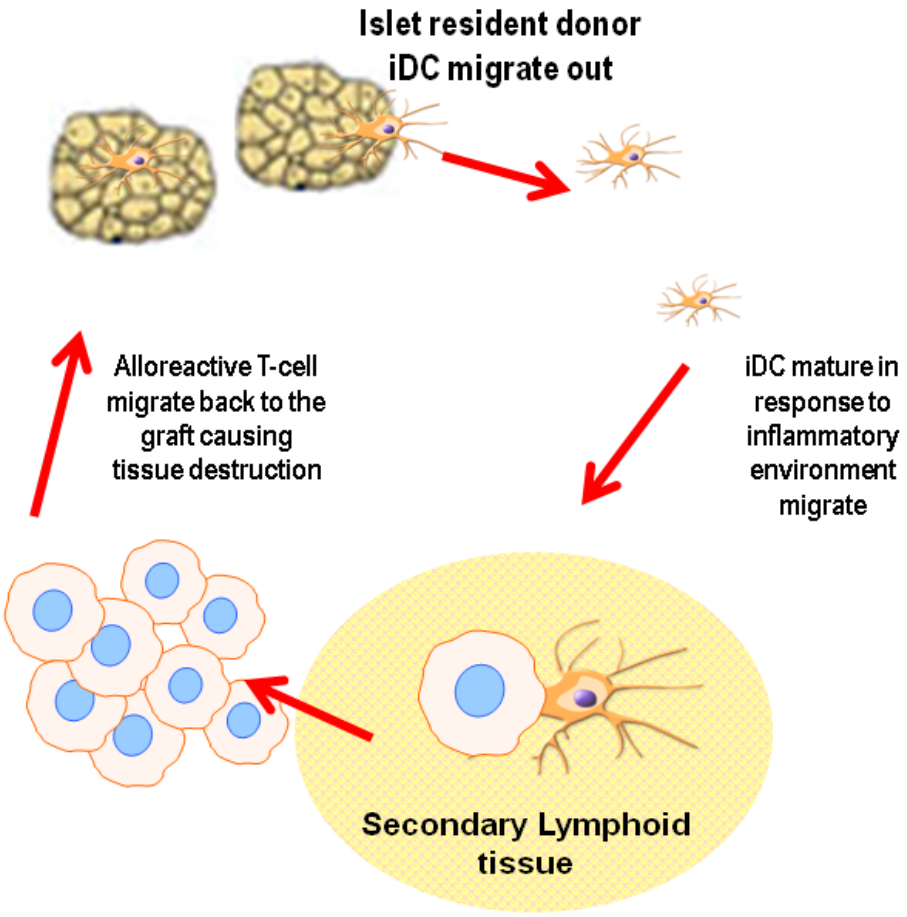

Figure 1.3.2: The direct pathway of allorecognition. Immature 'passenger' DC migrate out of the islet graft in response to the proinflammatory environment. DC subsequently mature and migrated to secondary lymphoid tissue, where they activate recipient T-cells causing the clonal expansion of alloreactive T-cells, which leads to allograft tissue destruction.

1.3.3 T-cell subtypes

T-cell subtype differentiation is defined by their expression of cytokines and signalling pathways from which their differentiation was mediated. Currently there are three main sub-types that have been commonly described and characterised. These subsets include T helper type 1 (Th1), Th2 and Th17, which are described below and summarised in figure 1.3.3.

Th1

Th1 subtype of effecter cells are characterised by their production of pro-inflammatory cytokines IL-2, IFN-γ and TNF-α. This cell type is known by its unique ability to express the transcription factor T-bet, which signals through STAT1 and STAT4 [66]. The binding of IFN-γ promotes the expression of T-bet, which upregulates the beta-2 chain of the IL-12R which in turn potentiates the expression of IFN-γ [67] [68] [69]. This forms a positive feed-back loop, where increased IFN-γ production reinforces Th1 differentiation, as DC secreted IL-12 binds to the IL12R and in combination with TCR signalling increases the transcription of IFN-γ and T-bet [68, 70].The development of this T-cell sub-type is associated with cellular immunity and allograft rejection.

Th2

The differentiation of T helper 2 subtype (Th2) T-cell sub-set is characterised by the production of IL-4, IL-15 and IL-13 and the expression of GATA-3 and STAT-6 [71], which is primarily involved in humoral immunity, by providing B-cell help. Th2 T-cell responses are antagonistic to the development of the Th1 population [72] [73], thus historically Th2 responses were thought to protect foreign tissue. However this is no longer strictly considered to be the case, as Th2 T-

cells have been shown to mediate the rejection of MHC-mismatched allografts [74] and is believed a primary role in chronic allograft rejection.

Th17

The Th17 subtype is defined by its ability to produce proinflammatory cytokine IL-17A [75, 76], which forms part of a IL-17 family of cytokines (A-F)[77-81]. IL17A predominately is secreted by activated CD4+ T-cells, however other studies have also demonstrated that IL17A is expressed by γδ T-cells, CD8+ memory T-cells, eosinophils, neutrophils and monocytes [82-88]. This cytokine plays an important role in the activation and recruitment of neutrophils and monocytes to sites of inflammation [89-92], whilst also enhancing granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) promoting the growth and maturation of myeloid cells. Th17 cells have been implicated in pathogenesis of autoimmune diseases and allograft rejection. Elevated expression of IL17 mRNA and protein have been found in graft infiltrating mononuclear cells in acute renal graft rejection both in rat models and in humans[93]. Other studies in lung and cardiac rejection have also supported the role of IL17 in acute rather than chronic allograft rejection [94-96]. Th17 differentiation is characterized by its unique signalling pathways involving receptor-related orphan receptor (ROR C2 or RAR-alpha) and STAT3 (as summarised in figure 1.5) [97, 98]. In humans IL1-β is important in promoting Th17 differentiation alone and in combination with IL-23, which enhances the conversion of human Tregs to Th17 T cells. PGE_2, a key mediator of tissue inflammation, up regulates IL-23R and IL-1R, which also promotes Th17 differentiation and expansion in humans. Th17 are unique in their ability to block the generation of Tregs, through the production of IL-6, IL-1 and TGF-β, however high concentrations of IL-6 can also inhibit Th17 differentiation. In vitro studies have also shown that IFN-y can negatively regulate Th17 differentiation [99].

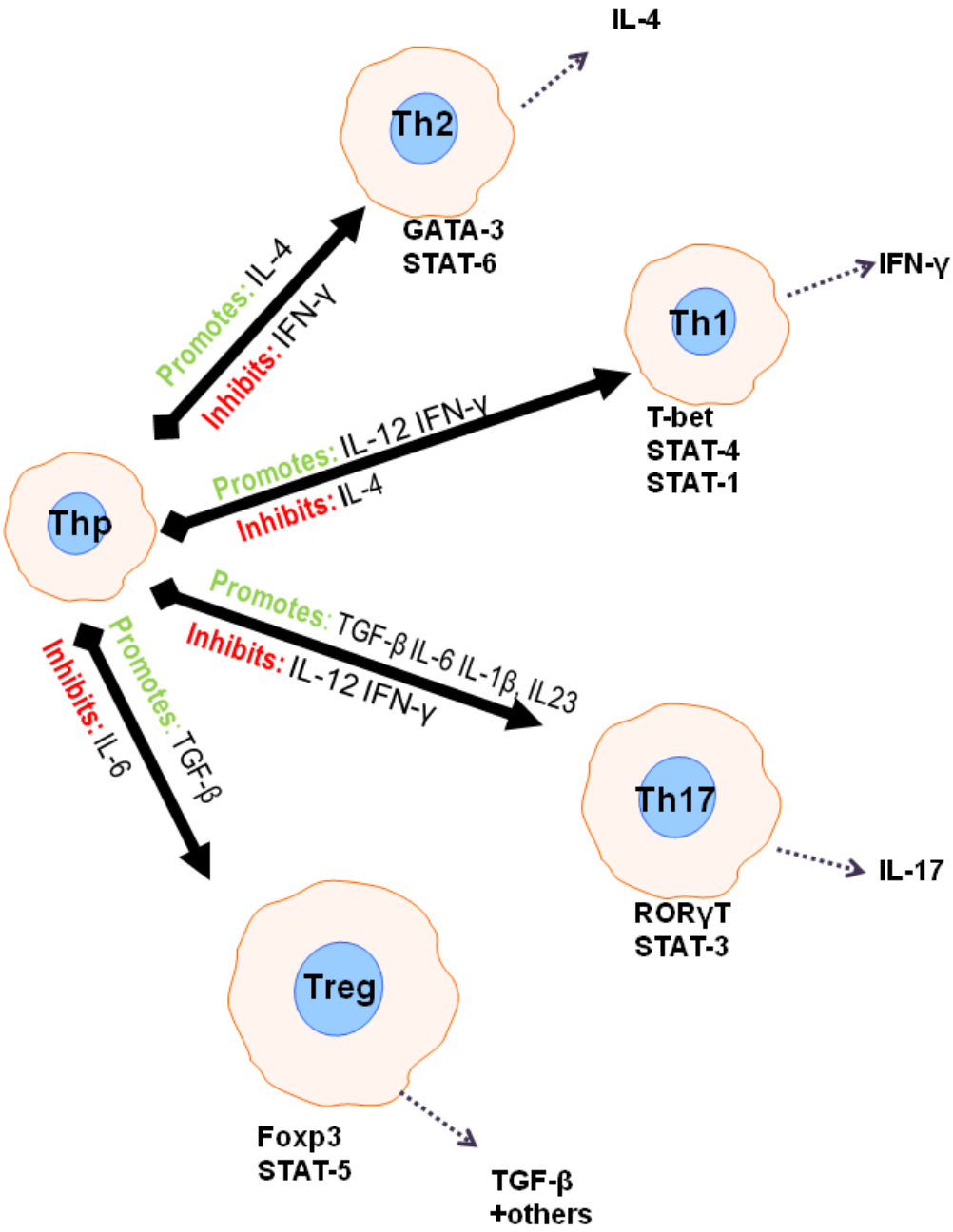

Figure 1.3.3: Differentiation of naive T-cells. The differentiation of naive CD4+ T-cells is dependent on proinflammatory milieu. The early secretion of Il-4 promotes the differentiation of Th2 T-cell subset. However the production of IL-12 inhibits Th2 and promotes the differentiation of Th1 cells, whilst the production of TGF-β promotes the generation of Tregs. Reciprocally IL-6 inhibits Treg generation, while helping promote Th17 differentiation with the help of IL-1β and IL-23.

1.3.4 Tolerance mechanisms

Central and peripheral tolerance mechanisms exist as homeostatic measures to ensure that the immune system can adequately recognise self and prevent autoimmune responses. Central tolerance or otherwise known as recessive tolerance involves the negative selection of thymocytes with a high affinity for self-peptide/MHC complexes, which occurs in the thymus [100]. Negative selection can arise from various mechanisms including receptor editing, clonal diversion and clonal deletion, the later being the predominant form of negative selection [101]. Peripheral tolerance on the other hand controls immune responses in the periphery outside the thymus once T-cells have matured, which can involve different mechanisms as described below.

T cell anergy

T cell anergy is a tolerance mechanism defined by an unresponsive T-cell state as shown in figure 1.3.4A. Presentation of MHC to the TCR in the absence of co-stimulation, such as CD80/CD86, renders T-cells anergic. Subsequently, these T-cells become unresponsive to the presented allo-peptide, even upon re-counter with the same peptide presented by an equipped APC expressing co-stimulation [102]. Cytotoxic T lymphocyte associated antigen (CTLA-4) is a example of a inhibitory molecule, which blocks co-stimulation signalling between CD80/CD86 expressed by APC and CD28 expressed by T-cells to induce T-cell anergy [103].

Activation induced cell death

Immune responses are also regulated by a mechanism known as activation-induced cell death (AICD) (Figure 1.3.4B), after repeated encounters with a specific antigen at a high

concentrations [104]. AICD is mediated by the surface expression of FAS (CD95) and its ligand (FASL or CD95L), The interaction of FAS/FASL between proliferating cells activates a cascade of caspase enzymes that induce apoptosis induced cell death [105]. Gene disruptions to FAS and FASL result in lymphoproliferative disease in mice and autoimmune lymphoproliferative syndrome in humans [106].

T regulatory cells

Immune regulation via suppression by T regulatory cells (Tregs), is a concept that was initially proposed in the late 1960s, but failed to be accurately defined. However over recent years their existence has been proven by numerous studies. In particular, the description of CD4+ CD25+ Foxp3+ Tregs have been of the greatest interest. Foxp3 or otherwise known as forkhead box P3 from the forkhead family of DNA binding factors, is the master gene associated with the differentiation of these Tregs [107]. The function of Foxp3 as a primary regulator of Treg function was displayed in the scurfy mouse, where mutations to Foxp3 resulted in a fatal autoimmune and inflammatory disorder [108]. A similar disorder is also known in humans, called IPEX (immune dysregulation, polyendocrinopathy, enteropathy and X-linked) syndrome, which is associated with poor T-cell activation and impaired suppressive function of CD4+CD25+ T-cells [109]. The Treg pathway reciprocally develops to the Th17 T-cell sub-set as explained in section 2.3. Foxp3 Tregs also require TGF-β to differentiate, while IFN-γ has been shown to be crucial in the generation of functional alloantigen reactive Tregs [110].

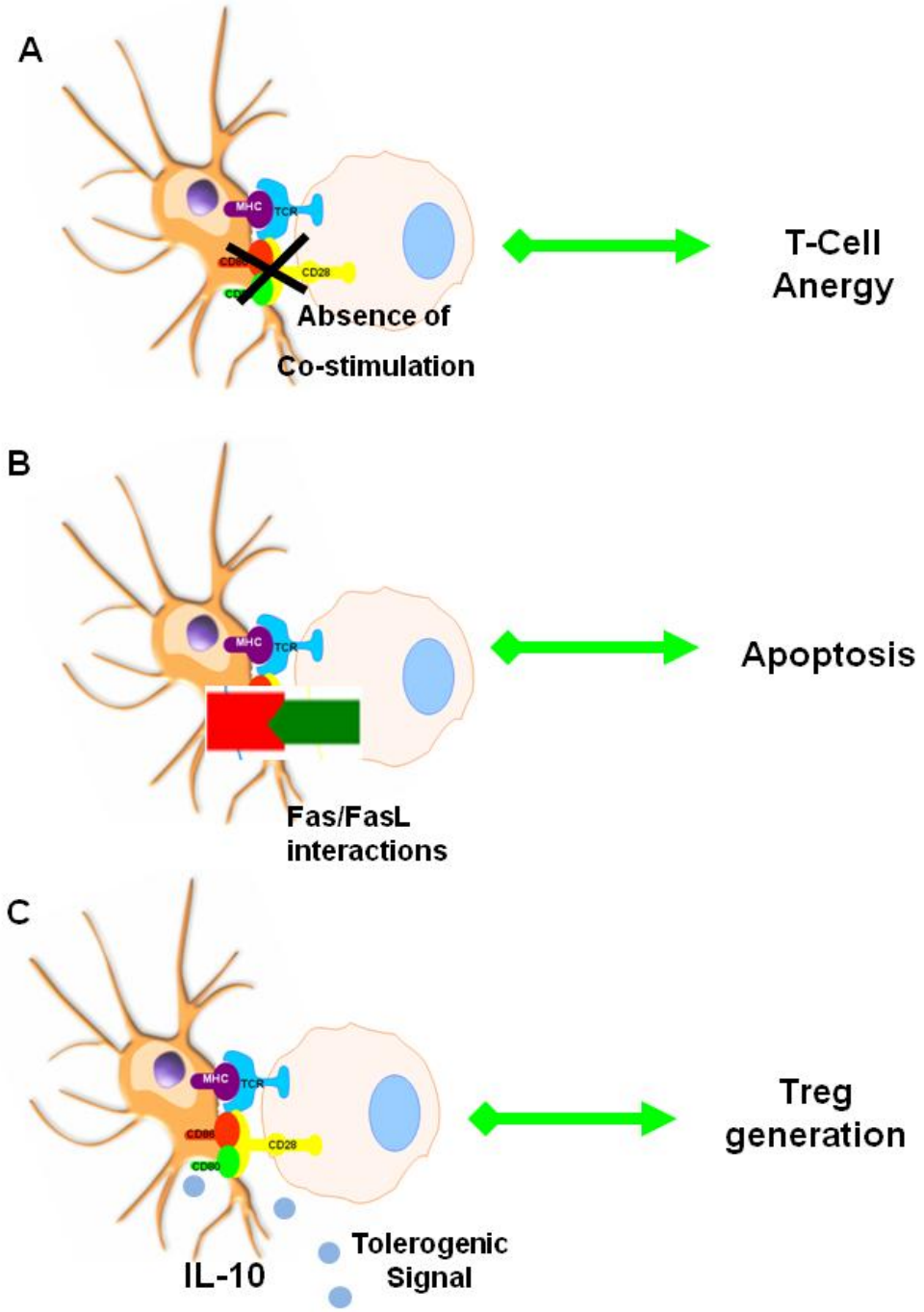

Figure 1.3.4: Tolerance Mechanisms. Tolerance induction can be mediated through three major cellular mechanisms. A) Priming of T-cell in the absence of co-stimulatory signals generates anergic T-cells, rendering reactive T-cells no longer responsive. B) Apoptosis of antigen primed T-cells can be mediated through the interaction of Fas and Fas L, thus causing its deletion. C) Tolerogenic signals can convert reactive T-cells to generate CD4+ CD25+ FOXP3+ T-regs, which have suppressive properties that can inhibit other effector T-cells.

1.3.5 Immune reactions to islet allograft

Loss of islet cell mass post transplantation is the leading cause of islet graft failure in recipients. Only 10-20% of the original mass remains post transplantation, therefore recipients require multiple islet infusions before they become insulin independent. Graft survival is dependent on the number of transplanted islets, quality of islets post mechanical and enzymic digestion, re-vascularisation post transplant and the toxicity of immunosuppressive drugs. Moreover, allogeneic islets are susceptible to the innate, adaptive and recurrent autoimmune response, which affects graft function and survival.

Innate

The innate immunity is responsible to the loss of islet cell mass immediately post transplantation. The infusion of islets into the portal vein of the liver, places islets into direct contact with blood. This initiates the 'instant-blood mediated immune response' (IBMIR), and within minutes of transplantation there is a rapid activation and binding of platelets trapping islets in clots, activation of the coagulation and complement systems, causing the rapid infiltration of leukocytes, [111, 112]. This reaction is primarily driven by the inflammatory factors that are endogenously produced by human islets such as tissue factor, Interleukin 1-β, Interleukin-8, MIP-2, MIF and Monocyte Chemotactic protein-1[113, 114]. Accordingly the presence of cytokines in the local environment of islets are known to cause islet cell death [115, 116].

Adaptive

Islet transplantation like any other allogeneic transplantation is susceptible to allorecognition as described in section 1.3.2. However the non-specific inflammation driven by the innate immune response and in particular the 'cytokine storm' potently activates the adaptive immune response. Subsequently the release of cytokines such as Tumour Necrosis-alpha, Interleukin-1 and Interferon gamma activates donor (resident in islets) and recipient APC promoting T cell activation as described in section 1.3.1. The adaptive immune response plays a crucial role in promoting the destruction of the islet cells as evidenced in many mouse models of islet allograft transplantation, which have shown that the blocking of co-stimulation prolongs allograft survival [117-121]. Moreover, in human islet transplant recipients that reach insulin independence is associated with a T-regulatory cytokine profile [122], thus demonstrating the key role of the adaptive immunity in regulating the success and survival of islet allografts.

Recurrent autoimmunity

The autoimmunity that initially destroys the islets of T1D patients creates the problem of recurrent autoimmunity to the newly transplanted islets. Studies have shown that auto-reactive T-cells and auto-antibodies play an important role in the dysfunction and loss of islets, which prevents receipts reaching insulin independence [123]. The use of immunosuppressive drugs, therefore appears to be more effective in controlling allo-immune responses. Accordingly there is need to develop therapies that target both the allo-immune and auto-immune responses, to promote allograft tolerance.

1.4 Immune modulation towards tolerance induction

The severe side-effects associated with the use of immunosuppressive drugs have prompted the explosion of research into the development of novel therapies to target allograft rejection. In particular, research has aimed to achieve the holy grail of 'transplant tolerance', by promoting allograft acceptance without the use of systemic immunosuppression. Islet transplantation has the added task of developing therapies that target both the allo- and auto- immune responses concurrently. The immunomodulation of cellular-mediated rejection with the use of 'tolerogenic DC' (Tol-DC) and the genetic modification of allografts, are novel strategies that have great potential to promote tolerance induction as discussed below.

1.4.1 DC cellular immunotherapy – a key to tolerance induction

In 1973, Steinman and Cohn described a novel cell type with distinct morphological features which they termed dendritic cells (DC) [124]. Since their identification, DC have been shown to play a crucial role as sentinels of our immune system [125-128]. Despite taking up less than <0.1% of the total cell population, they play an important role as potent antigen presenting cells which can promote either 'immunogenic' or 'tolerogenic immune responses. It is this plasticity, which make them great candidates for tolerance induction.

Dendritic cell origin and function - mature vs immature

DC differentiate from precursor cells derived from haematopoietic CD34+ bone marrow progenitor cells. DC precursors migrate from the bone marrow to nonlymphoid / peripheral tissues, including commonly transplanted organs where they reside as immature DC (iDC) [126, 129]. These tissue resident iDC are specialised in antigen uptake and processing, where they use receptor dependent and independent endocytic mechanisms to do so. Immature DC have low surface expression of co-stimulatory and MHC molecules making them poor

stimulators of T-cell activation [130, 131]. Microbial products like bacterial lipopolysaccharide (LPS), unmethylated cytosine poly-guanine (CpG) motifs and double stranded RNA can stimulate the maturation of iDC. Other danger signals such as pro-inflammatory cytokines GM-CSF, IL-1β, TNF-α and IFN-α can also initiate the maturation of resident iDC [132-134], Necrotic death and ischemia/reperfusion injury during transplantation induce the release of a variety of factors including pro-inflammatory cytokines that promote DC maturation of passenger iDC transferred with the allograft[135, 136]. This cytokine and chemokine insult also recruits recipient DC-precursors and iDC to the site of injury. Donor and recipient iDC subsequently take up allo-antigens and mature to potent antigen presenting cells.

Maturation of DC is defined by a variety of changes both phenotypic and functional. Immature DC switch from an antigen capturing cell to antigen presenting cells by losing their endocytic activity [137]. Immature DC function by loading antigen-peptides to their MHC molecules forming MHC-peptide complexes, which translocate to the cell surface. As iDC mature they rapidly increase their expression of co-stimulatory molecules and migrate to draining lymphoid tissues, where they activate and prime T-cells causing clonal T-cell expansion [138, 139]. However resting iDC can prime T-cells in the absence of co-simulation to promoting antigen-specific tolerogenic immune responses [140]. The terminal arrest of DC maturation may therefore provide a platform from which allograft tolerance can be induced.

DC maturational arrest

A large amount of research has gone into the development of novel strategies to arrest DC maturation, as a potential way of inducing tolerance. In particular, pharmacological agents have been used to generate maturation arrested DC for the treatment of autoimmunity and allograft rejection. Glucocorticoids (GC) are potent immunosuppressive agents, initially described for their ability to inhibit T-cell responses. However GC also has a profound effect on the production of proinflammatory cytokines by monocytes and macrophages. Moreover GC have the distinct ability to modify DC function [141]. Dexamethasone (Dex) is the most widely investigated GC, which down regulates components of NF-κB signalling molecules involved in DC maturation including RelB [142]. Vitamin D_3 [1,25(OH)2D_3] has shown similar effects to induce the differentiation of phenotypically immature DC, which do not mature in response to maturation stimuli. These phenotypically tolerogenic DC also have a reduced capacity to stimulate T-cells [143, 144]. Rapamycin also has been extensively investigated in its ability to promote the generation of iDC. Unlike Dex and Vitamin D_3, rapamycin inhibits the mammalian target of rapamycin (mTor), which acts as a serine/threonine protein kinase. It has a diverse role as a regulator of cell growth and proliferation, transcription, ribosomal biogenesis, vesicular trafficking, autophagy, cytoskeletal organization and cell size [145]. Studies using bone marrow derived DC have shown that rapamycin down regulates the IL-4 receptor complex, suppressing IL-4 dependent DC maturation. This in turn results in the inhibition of co-stimulatory molecule expression and the production of IL-4 induced IL-12 and TNF-α [146]. The effects of rapamycin on DC maturation have also been demonstrated in human studies, where monocyte-derived and CD34+progenitor-derived DC fail to mature [147, 148]. Cytokines also have a role in producing maturation arrested iDC, for example IL-10 treatment of DC-precursors during differentiation results in phenotypically immature DC. Similarly, these DC have an inhibited capacity to up regulate the expression of CD86 and CD83, which reduces their capacity to

stimulate CD4+ T-cells, resulting in the induction of antigen-specific T cell anergy [149]. Many other reagents and strategies have been used to generate tolerogenic iDC including siRNA KO of RelB, the use aspirin and curcumin to name a few, which have been proven to be tolerogenic *in vitro* and *in vivo*[150-152]. Moreover, in the NOD mice the use of maturation arrested DC generated by targeting CD40, CD80 and CD86 with anti-sense oligonucleotides, has been proven successful in delaying the onset of diabetes [153]. So successful in fact the University of Pittsburgh is running a phase I clinical safety study, the first of its kind to use maturation arrested autologous DC. The randomized trial proposes to evaluate the safety of using autologous tolerogenic monocyte-derived DC in type 1 diabetic patients (www.clinicaltrials.com – Identifier NTC00445913). The tolerogenic DC accordingly are generated from the recipient's monocytes and treated ex vivo with antisense phosphorothioate-modified oligonucleotide targeting CD40, CD80 and CD86 costimulatory molecules. Similarly the University of Queensland plans to run a Phase I trial using monocyte-derived DC for the treatment of rheumatoid arthritis (RA). Whist in England the University of Newcastle plan to undergo second phase I trial using Vitamin D_3 maturation arrested DC for also the treatment of RA [154]. These translational studies will further define the applicability of using tolerogenic vaccines in humans as a therapy for autoimmune diseases and allograft rejection.

Donor vs Recipient Dendritic cells

Tolerogenic DC research may ultimately be used in 'negative vaccination' protocols. This concept is still in early development, however it may hold great promise in the field of transplantation as a way to promoting allograft tolerance [155]. The use of either donor-derived or recipient-derived DC and timing of DC treatment still remains debatable. Using donor-derived DC is advantageous as it targets the direct pathway of allorecognition as described in section 1.3.2, thus playing a critical in preventing acute allograft rejection and promoting donor-specific tolerance. However, many models describing the use of donor-derived Tol-DC, administer DC therapy 7 days prior to transplantation and current protocols for generating human monocyte-derived DC takes 7-10 days [155, 156]. This type of cellular therapy would only be available in cases of living donor transplantation. On the other hand modulated recipient-DC have been shown to be affective in promoting tolerance, even if administered at the day of transplantation. There is also evidence emerging that donor-derived DC therapy may provide a source of alloantigen in the draining lymph nodes promoting indirect allorecognition and the risk of sensitization [157, 158], this however still remains to be fully characterised especially in larger non-human primate models. Moreover there is a great body of evidence demonstrating that donor-derived Tol-DC can promote allograft tolerance in *vivo* rather than immunity.

1.4.2 Immunomodulation of the graft – a gene therapy approach

The introduction of genes into the allograft itself has been among the popular approaches to protect the allograft from rejection. Several inhibitory molecules have been investigated using this gene therapy approach. Cytotoxic T lymphocyte associated antigen-4 (CTLA4) for example is an immunomodulatory molecule involved in blocking the interaction of CD86/CD80 with CD28. Its induced expression as CTLA-4Ig (fused to Fc portion of human IgG1) in islets

improves allograft survival as shown by numerous *in vivo* models [159]. The successes of CTLA4Ig to prevent T-cell activation has permitted its use in the treatment of rheumatoid arthritis, while a second generation drug known as Belatacept is currently in phase III clinical trials for renal transplantation [160]. Th2 cytokine IL-10 viral vector mediated systemic production in an islet transplant model, is also successful in prolonging allograft survival by suppressing autoimmune responses [161]. In NOD mice, adeno-associated virus (AdV) mediated gene expression of IL-4 by islets, prevents the development of hyperglycaemia and reduces the severity of insulitis. Thus preventing the destruction of islets and inhibiting autoimmune processes, by regulating T-cell function [162].

Programmed cell death 1 (PD-1) is also an inhibitory molecule known to regulate the onset of diabetes in the NOD mouse [163] and thus important in maintaining peripheral tolerance [164]. Stimulation of the PD-1 pathway in an islet allograft transplantation model, promoted allograft survival in presence of co-stimulation blockade [165]. So far there have been lack of human studies which have examined the effects of the over expression of PD-1 ligands in the survival of human islet allograft, which warrants investigation given the important role of PD-1 pathway in regulating auto-immune and allo-immune responses.

1.5 The Programmed Cell Death 1 (PD-1) Pathway

1.5.1 Programmed Cell Death-1: a negative regulator of T-cell activation

Programmed cell death 1 (PD-1) is a T-cell receptor known to negatively regulate T-cell proliferation. PD-1 binds to PD-1 ligands 1 and 2 (also commonly known as B7-H1 and B7-DC respectively). This emerging negative co-stimulatory pathway is of great interest and is being heavily investigated as a novel avenue for the development of therapies to target autoimmunity, cancer, opportunistic infection, and of interest to this study, alloimmunity.

1.5.2 Programmed Cell Death-1 receptor

PD-1 was initially described by Ishida and colleagues in 1992 when isolating the murine PD-1 gene. Its expression was upregulated in a T-cell hybridoma that was undergoing cellular death, thus named programmed cell death 1. PD-1 shares amino acid homology with other members of the CD28/CTLA4 immunoglobulin super family. Despite its given name, PD-1 it is not involved in classical programmed cell death [166]. Rather PD-1 was found to be responsible for arresting the cell-cycle of proliferating T-cells at the Go/ G1 phase [167].

1.5.3 PD-1 protein and gene structure

The murine PD-1 receptor is a 50 to 55 kDa type 1 monomeric transmembrane glycoprotein. Within its immunoglobulin (Ig) super family member it shares approximately 20% sequence homology with other members such as CD28, CTLA-4 and ICOS (inducible costimulatory), however unlike CTLA-4 and ICOS, PD-1 lacks the extra cellular cysteine which allows homodimerization [168]. Human PD-1 shares 70% nucleotide homology and 60% amino acid sequence homology with murine PD-1. The human 288 amino acid PD-1 protein consists of an

Ig variable type extracellular domain, a transmembrane domain and a cytoplasmic domain [169]. Stimulation through the TCR or BCR (B-cell receptor) induces the expression of PD-1 and the translocation of compartmentalized PD-1 to the cell surface of T-cells and B-cells respectively [169, 170].

1.5.4 Signalling through PD-1 mediates the inhibition of TCR activation

Functional studies of PD-1 show that the cytoplasmic tail of this receptor has two signalling tyrosine residues. The tyrosine residue proximal to the membrane is located in an immunoreceptor tyrosine-based inhibitory motif (ITIM), while the distal tyrosine is found in an immunoreceptor tyrosine-based switch motif (ITSM). Mutation studies have demonstrated that the inhibitory action of PD-1 is mediated by the ITSM rather than the ITIM [171]. Engagement with PD-L1 or PD-L2, induces the ITSM to recruit Src homology region 2 domain-containing phosphotase -1 (SHP-1) and SHP-2 to the PD-1 cytoplasmic tail [167]. Subsequently T-cell effector molecules are phosphorylated by SHP-1 and SHP-2 to inhibit T-cell activation. Similarly in B-cells SHP-2 is recruited upon PD-1 engagement to inhibit B-cell receptor mediated activation [172]. Signalling through PD-1 receptor also inhibits Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase (PI3K), which is dependent on the PD-1 ITSM [173]. Subsequently, causing a decrease T-cell proliferation, cell survival and cytokine and protein production, as demonstrated by figure 1.5.1.

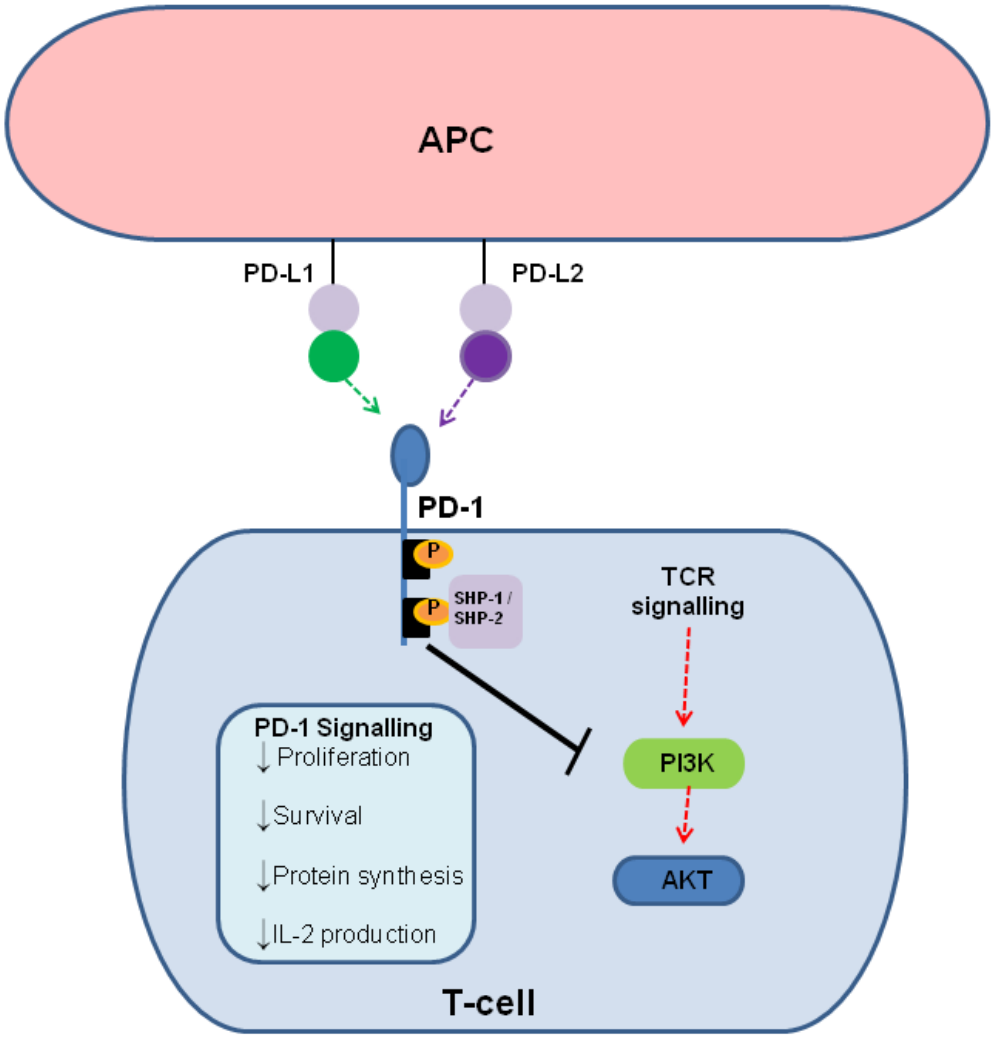

Figure 1.5.1: Ligation of PD-1 Ligands expressed by APC promotes PD-1 signalling. Binding of the ITSM of the PD-1 cytoplasmic tail by SHP-1 /SHP-2 causes the dephosphorylation of proximal signalling molecules. This results in the reduction of PI3K activation subsequently affecting the AKT pathway. PD-1 signalling can therefore decrease T-cell proliferation, cell survival and cytokine and protein production.

1.5.5 PD-1 deficient mice develop auto-immune diseases

Animal functional studies have demonstrated that PD-1 plays a vital in maintaining peripheral self tolerance. PD-1 deficient C57BL/6 (B6) mice develop autoimmune diseases, such as lupus-like proliferative arthritis and glomerulonephritis [174]. The disruption of PD-1 gene in BALB/c mice, similarly mediates the development of autoimmune dilated cardiomyopathy [175]. In the NOD mouse PD-1 pathway blocking studies have also demonstrated the inhibitory role of PD-1 in regulating the onset of diabetes. It was demonstrated that blocking of the PD-1 pathway resulted in the rapid development of diabetes regardless of the mouse age. Moreover, blocking significantly increased the frequency of Interferon gamma producing GAD-reactive splenocytes [163]. Likewise in PD-1 ligand KO models, have demonstrated that the PD-1 pathway specifically regulates CD4+ auto-reactive T-cell mediated tissue destruction and cytokine production in the development of diabetes [164]. These *in vivo* functional studies demonstrate that PD-1 is important in the establishment of self peripheral tolerance.

1.6 PD-1 Ligands

1.6.1 PD-L1

Discovery

In 2000 the first ligand for PD-1 was described, which was found to induce the inhibition of T-cell receptor mediated T-cell proliferation [176]. The human and murine Programmed death ligand 1 (PD-L1) was identified through B7 homology-based searches of the NCBI database. The identified PD-L1 of 290 residues however, was also described as B7-H1 by Dong and colleagues the previous year in 1999, where they conversely showed this molecule to have a positive stimulatory capacity [177]. However subsequent data indicate that PD-L1 has a dominant role as a negative regulator of T-cell activation. More recently it was discovered that PD-L1 also binds to positive co-stimulatory molecule CD80, otherwise known as B7-1, a phenomenon present in murine models and humans [178]. The interaction of CD80 and PD-L1 appears to be at intermediate affinities compared to that of CD80 to CD28 and to CTLA-4. The area which PD-L1 binds with CD80 overlaps with the area used to bind PD-1. Vice-versa the area of CD80 binding to PD-L1 overlaps with the area used to bind CD28. Furthermore the interaction between PD-L1 and CD80 leads to the specific inhibition of T-cell activation [179], demonstrating the ability of PD-1 ligands to work independently to PD-1 to also inhibit T-cell proliferation.

Gene and protein structure

Analysis of PD-L1 sequences has shown that this B7-family member has a IgV and IgC domain in its extra cellular region with a hydrophobic transmembrane domain and a short charged intracellular region [176, 177]. Although PD-1 is structurally similar to CTLA-4, PD-L1 specifically binds to PD-1 and not CTLA-4. Furthermore the human PD-L1 is also able to bind to the murine PD-1 orthologue [176], demonstrating high evolutionary conservation of PD-1

between species. Overall many studies have demonstrated that PD-L1 binding with PD-1 mediates the inhibition of T-cell proliferation [176, 180].

1.6.2 Discovery of PD-L2 and protein structure

Latchman and colleagues in 2001 were the first to identify the second ligand for PD-1 Programmed Death Ligand 2 (PD-L2), which also inhibits T cell activation [167]. Similar to the discovery of PD-L1, PD-L2 was found though sequence homology searches on GenBank, which identified a mouse PD-L1 homologue. Likewise human cDNA was also identified, which shares a 70% amino acid identity with murine PD-L2. The B7 family members therefore share a 21 to 27% amino acid homology and are structurally similar in their organization. Unlike PD-L1 there is poor conservation of the PD-L2 cytoplasmic tail between the human and murine orthologue. Accordingly the human PD-L2 has a longer cytoplasmic tail, which may be responsible for some functional differences between the human and murine PD-L2. PD-L1 and PD-L2 are closely located to each other on the human chromosome 9p24.2 separated by 42kb.

1.6.3 Tissue distribution of PD-1 Ligands

Messenger RNA

PD-1 Ligand mRNA in human and murine tissues have similar mRNA expression patterns. PD-L1 appears to have high expression levels in placenta, heart, skeletal muscle and lung, however lower expression is also found in the thymus, spleen, kidney and liver [167, 177]. PD-L1 mRNA however is not expressed in human monocytes unless stimulated with pro-inflammatory cytokine IFN-γ, where it is quickly up-regulated [167]. PD-L1 mRNA is constitutively expressed on DC and macrophage [181]. The mRNA expression of PD-L2 is similar to that of PD-L1; however mRNA transcripts do not correlate with the protein expression of these ligands, as the protein expression of PD-L1 is broadly expressed compared to PD-L2 as described below.

Protein expression

PD-L1 protein is broadly expressed compared to PD-L2. PD-L1 is constitutively expressed on a wide variety of hematopoietic and non-hematopoietic cells including mononuclear small intestine cells, placental syncytiotrophoblasts, vascular endothelium, epithelia, pancreatic islet cells, astrocytes, neurons and lung macrophage [182, 183]. Dendritic cells also have high protein expression of PD-L1. PD-L2 protein expression however is restricted to macrophage and DC [181, 182, 184], and more recently B1 cells, B memory cells, bone marrow-derived mast cells were also found to express PD-L2 [183, 185]. PD-L2 is also strongly expressed on placental endothelial cells rather then syncytiotrophoblasts [184]. Thus the expression of PD-L1 and PD-L2 in the placenta suggests that these ligands may play an active role in the establishment of fetal-maternal tolerance [186].

1.6.4 Regulation of PD-1 Ligand expression

The expression of PD-L are largely dependent on the inflammatory environment, subsequently cytokines play an important role in the stimulation of their expression. Both type 1 and type 2 interferons (IFN) and TNF-α induce the expression of PD-L1 on T-cells, B-cells, endothelial cells and epithelial cells [187]. Common γ chain cytokines such as Il-2, Il-7 and Il-15 have all been shown to upregulate PD-L1 expression on human T-cells, whereas Il-21 can induce its expression on PBMNC CD19+ B cells but not on T-cells[188]. IFN-γ also upregulates the expression of PD-L1 and PD-L2 in human monocytes, however it appears to have more profound effect on PD-L1 rather than PD-L2. [181, 184]. The up-regulation of PD-L2 protein expression in macrophage is highly dependent on IL-4, which suggests that PD-L1 and PD-L2

are differentially regulated by Th1 and Th2 immune responses respectively [181]. Thus PD-L1 and PD-L2 may function differently in the setting of transplantation. The transcription of the PD-L1 is thought to be regulated by IRF-1, which is involved in its constitutive and inducible expression [189]. Lack of STAT3 activity by siRNA has demonstrated that STAT3 is required for the expression of PD-L1 [190]. Little is known about the transcriptional regulation of PD-L2, however there is evidence that shows IFN-y induced expression of PD-L2 is partially dependent on the NF-κB-binding sites, situated upstream of the transcriptional start site[182].

1.6.5 Evidence of second receptor for PD-1 Ligand

In 1999 PD-L1 was identified by Dong *et al* and described as a third B7 family member that stimulates T-cell proliferation and IL-10, however they still showed the inhibition of IL-2 production, thus concluding that PD-L1 molecule may also have negative regulatory properties. Also in 2001 Tseng and colleagues, claimed the discovery of B7-DC, which is identical to PD-L2. In a murine model, PD-L2 was shown to have potent co-stimulatory properties causing the activation of T-cells [191]. In a separate study, PD-L2 had positive co-stimulatory properties, however they were independent to the engagement of PD-1, as PD-L2 was able to co-stimulate PD-1 deficient CD4+ T-cells, providing evidence of a possible second ligand for PD-L2 which mediates the stimulatory response rather than negative one [192]. However in other mouse models both PD-L1 and PD-L2 appear to mediate inhibitory signals. A study using C57BL/6 mice showed that blocking of PD-L1 and PD-L2 expressed by iDC, significantly increased the stimulatory capacity of iDC [193]. A human study demonstrated similar results backing the concept that PD-L are vital inhibitory molecules. More importantly this study described PD-L2 as the more potent PD-1 ligand compared to PD-L1 [184]. The different reported functions of PD-L to either act as a stimulatory or inhibitory molecule are still currently under debate and may be a reflection of the background of the mice used in those studies. Never the less some

studies have demonstrated that PD-L1 can increase T-cell activation by inhibiting IFN-γ induced nitric oxide [194]. Further to that there is evidence which suggests that PD-L1 may be bi-directional in its signalling capacity [178]. However there is a general consensus in the literature that PD-L1 and PD-L2 play an important inhibitory role in immune regulation [183].

1.6.6 Functional differences between PD-L1 and PD-L2

Although many studies suggest that PD-L1 and PD-L2 have overlapping functions in mediating negative regulation of T-cells, other studies have also revealed distinct functional differences between the two ligands. Comparative molecular modelling and site directed mutagenesis was used to demonstrated that the A'GFCC'C" protein face, which interacts with PD-1 has poor residue conservation between the two ligands. Furthermore these ligands differentially bind to different locations on the PD-1 receptor. Accordingly through the use of flow cytometry and surface plasmon resonance it was established that PD-L1 and PD-L2 both have different affinities for the PD-1 receptor. PD-L2 has a 2-6 times higher affinity for the PD-1 receptor compared to PD-L1, indicative that these two ligands make a differential contribution to immune responses [195] PD-L2 in humans appears to be a far more potent negative regulator of T-cell activation, as blocking of PD-L2 in a mixed lymphocyte reaction was able to significantly increase the proliferation of CD4+ T-cells, whilst the blocking of PD-L1 only had a modest effect almost comparable to its controls. Furthermore PD-L2 blockade was able to reverse the tolerogenic effects of IL-10 treated DC, even though its expression levels found in these cells are considerably low, thus clearly demonstrating its potent negative regulatory properties in humans [184].

1.7 The PD-1 pathway and immunological tolerance

As highlighted previously, PD-L interaction with PD-1 mediates inhibitory signalling that results in T-cell hyporesponsiveness, which is vital in the induction of self tolerance. PD-L signalling through PD-1 results in the inhibition of the production of cytokines such as IFN-γ and IL-10, IL-4 and also IL-2, which are required for the proliferation of T-cells [167, 176, 180].

1.7.1 PD-1 pathway and peripheral tolerance

In vivo animal studies have further substantiated the role of the PD-1 and PD-L pathway as a negative regulator of T-cell mediated immune responses and its crucial role in immunological tolerance. Both PD-L1 and PD-L2 deficient mice have shown dramatic increases in the proliferation of CD4+ and CD8+ T cells both *in vitro* and *in vivo* [196, 197] via the increased ability of these cells to produce IL-2 and IFN-γ [164]. Furthermore in an experimental autoimmune encephalomyelitis resistant strain, PD-L1 deficiency causes this strain to become susceptible to autoimmune disease [196]. However the blocking of the PD-1 pathway does not appear to be as severe as the KO of CTLA-4. PD-L1 also appears to be important in the maintenance of semi-allogeneic pregnancy, as PD-L1 deficient mice have poor fetal survival due to the increased ability of these mice to reject allogeneic foetus [186]. PD-L2 is also vital in tolerance induction, as CD4+ T-cells of PD-L2 deficient mice show enhanced T-cell activation when co-cultured with antigen presenting cells (APC) compared to WT mice, which also feature increased production of IFN-γ and IL-4. Furthermore, the immunization of PD-L2 deficient mice with chicken ovalbumin, increases the activation of CD4+ and CD8+ T cells *in vivo* compared to their WT controls. Subsequently the lack of PD-L2 in these mice abrogated tolerance to oral antigens, thus further supporting the role of PD-L2 as a critical ligand in the regulation of T-cell

tolerance [197]. Although there is significant evidence to suggest PD-Ls are important in the maintenance of tolerance, there is a clear lack of studies using models that are more clinically relevant to the human setting.

1.7.2 PD-1 pathway and allograft transplantation

The function of PD-1 to negatively regulate T-cell clonal expansion and tolerance has encouraged investigations in the field of transplantation. The modulation of alloimmune responses via the PD-1 and PD-1 ligand pathway may therefore serve as a therapeutic tool in the prevention of allograft rejection.

Kinetics of PD-1 and its ligands in allograft transplantation

PD-1 and its ligands are known to be upregulated during allograft rejection of fully MHC mismatched cardiac transplantation [198]. Interestingly PD-1 mRNA expression is observed late in the rejection response. PD-L1 on the other hand increases significantly the day after transplantation, whilst PD-L2 had a similar expression pattern to PD-1. The expression of these molecules continued to be expressed despite the treatment with immunosuppressive agents [198].

PD-1 Ligand 1 fusion proteins prolong allograft survival

Systemic administration of PD-L1Ig fusion protein is able to prolong allograft survival in the absence of CD28 co-stimulation and in conjunction with the administration of immunosuppressive agents. PD-L1Ig therefore acts synergistically with co-stimulatory blockade to induce long-term islet allograft survival [165]. Furthermore, PD-L1 expressed in corneal endothelial cells induces the apoptosis of effector cells within the cornea to maintain long-term acceptance of corneal allografts [199]. PD-L1Ig transduced rat cardiac allografts also prolong

allograft survival compared to controls. The synergistic effects of a sub-therapeutic immunosuppressant regime were also observed in this study, as the combination of treatments was better than immunosuppressive treatment and gene transfer alone. PD-L1Ig genetic modification of the graft reduced CD4+ T-cells, monocyte and macrophage infiltration [200].

PD-1 and ligands required for allograft acceptance

MHC class II mismatched skin grafts *in vivo*, result in accelerated rejection with the blockade of PD-L1, which is similar to the rejection seen in CTLA-4 blocking studies [201]. In a separate study the targeting of PD-1 in fully MHC mismatched transplantation models also accelerates the rejection of allografts [202]. Furthermore interruption of the PD-1 and PD-L1 interaction is associated with the regulation of Graft Arterial disease in cardiac allografts [203]. These studies demonstrate that PD-1 and PD-L1 is associated with induction of allograft acceptance. However the literature in this area lacks the description of PD-L2 and its association with allograft tolerance. A recent study using human PD-L transduced pig B-cell line, demonstrated that both PD-L1 and PD-L2 over expression in pig antigen presenting cells was able to significantly inhibit the proliferation of human CD4+ T-cells, which was accompanied with the reduced production of IL-2, IFN-γ, TNF-α, Il-4 and IL-5. Moreover, the induced expression of PD-L promoted the generation of $CD4+CD25^{HI}Foxp3+$ T-cells [204]. Thus, suggesting that human PD-1 ligands could also be potentially used in the prevention of xenogeneic immune responses.

8.1 Thesis study rationale

The success of islet transplantation as a curative therapy for T1D relies on the development of novel strategies to preserve islet cell mass post transplantation. Targeting both the auto- and allo- immune responses concurrently may prolong the survival and function of allogeneic islets long-term. However the currently available immunosuppressive regimes are associated with severe side-effects. Moreover they cannot inhibit the reoccurrence of autoimmunity or chronic rejection. As reviewed in this chapter, maturation arrested DC have potential to promote tolerogenic responses. The use of 'tolerogenic vaccines' in human clinical trials enforces the translational potential of developing DC based therapies for islet transplantation. Currently these clinical trials are focused on using autologous monocyte-derived DC. In order for 'tolerogenic vaccines' to be used in allotransplantation, to target the direct pathway of allorecognition, it requires the use of allogeneic tolerogenic DC derived from the donor, which adds to the complexity of DC based therapies in transplantation. Nevertheless, they hold great potential in promoting allograft acceptance. To overcome this problem and in particular in the setting of deceased organ donor, the development of a novel propagation strategy is also required. Like many therapeutic regimes, a combination of therapeutic strategies may be required to promote long-term graft acceptance. The genetic modification of the graft with inhibitory molecules is novel approach to confer direct protection to the graft from allo- and auto- reactive T-cells.

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9 Thesis aims and hypothesis

The work presented in this thesis aimed to investigate novel therapies that may promote allograft tolerance without the use of immunosuppressive agents in the context of islet transplantation. In order to do this the following aims and hypothesis were investigated:

Aim 1 - To investigate the immunomodulatory effects of IFN-γ on the development and function of human monocyte-derived DC using *'standard'* propagation techniques (Chapter 3)

Hypothesis:

IFN-γ mediates its regulatory effect by inhibiting allogeneic T-cell activation through the modification of dendritic cell function

Aim 2 - To examine the propagation of tolerogenic DC in a short-time frame using a *'Fast-DC'* approach to make tolerogenic DC therapy more clinically applicable to islet transplantation. (Chaper4)

Hypothesis:

The combination of IFN-γ and a FAST-DC protocol would generate DC with tolerogenic function in 48h

Aim 3- To induce the transgenic expression of inhibitory molecule PD-L2 by human islets and examine the potential benefits of signalling through the PD-1 receptor (Chapter 5).

Hypothesis:

The induced expression of human PD-L2 by human islets may promote signalling through inhibitory PD-1 pathway causing the inhibition of T-cell activation

CHAPTER 2 - Methods and Materials

2.1 CELL ISOLATION PROTCOLS

2.1.1 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMNC) were isolated from healthy human blood donors (Australian Red Cross Blood Service, Adelaide, South Australia). Ten millilitres (mL) of buffy coat was diluted with 25mL of PBS. Diluted blood was under laid with 12mL of Ficoll Paque. Samples were spun at 600g (No brake) for 20min at RT. Monolayer was harvested and washed with PBS at 300g for 10min at 4°C, this was repeated 3 times.

2.1.2 Nylon Wool T cells

Approximately 10g of teased nylon wool was packed into sterile 10ml syringes after removing the plunger. All nylon wool columns (NWC) were autoclaved for sterility and allowed to dry. Prior to purification NWC was equilibrated with 7ml of RPMI at 37°C for 20min. PBMNC were panned as described in 2.2.1, however the non-adherent fraction was collected. Approximately 10^8 non-adherent cells in s10g (10% FCS in RPMI containing 1% glutamine – see section 15) placed into RPMI equilibrated NWC. Columns were incubated in an up-right position at 37°C / 5% CO_2 for 30min. Nylon wool T-cells were then eluded with 12.5ml s10g.

2.1.3 $CD4^+$ T-cells

CD4+ T-cells were purified using a magnetic bead depletion kit by stem cell technologies (catalogue 19052) according to the manufacturer's instructions. In brief PBMNC were isolated as described as in 2.1.1. Cells were resuspended in recommended suspension media (PBS with 2% FCS) at a concentration of $5x10^7$/ml and max of 2ml was transferred to sterile polystyrene FACS tube. Recommended volume (50ul/ml) of human CD4+ T-cell enrichment

cocktail was added to tube and incubated at RT for 10min. Then 100ul/ml of provided magnetic beads (EasySep magnetic particle D) was mixed into cells and allowed to incubate for 5min at RT. Mixture of cells and magnetic beads were resuspended and transferred to the EasySep magnet (catalogue# 18000) and incubated for 5min. Enriched CD4+ T cells were decanted into clean tube and counted.

2.1.4 $CD3^+$ T cells

Post primary MLR T-cells were separated from DC using the magnetic bead depletion kit by stem cell technologies (catalogue# 18051) according to the manufacturer's instructions. In brief cells were harvested from 96well plates and pooled into a 25ml tube. Cells were centrifuged for 5min at 600g and resuspended in recommended media (PBS with 2% FCS) at 10^8/ml. A maximum of 2.5ml was transferred into sterile polystyrene FACS tubes and incubated with 100ul/ml of EasySep positive selection cocktail for 15min at RT. Then 100ul/ml of magnetic nanoparticles was added thoroughly mixed and incubated for 10min at RT. Suspension was topped-up to 2.5ml and transferred to the EasySep magnet for 5min. Non CD3+ cells were discarded in one swift motion with tube being inverted for 2-3 seconds. Tube was removed from magnet and CD3+ cells were resuspended with 2.5ml of recommended media. To increase purity cell suspension was returned to the magnet for a further 5 minutes. Once again non CD3+ cells were discarded. CD3+ cells were resuspended in s10g media and counted.

2.1.5 Positive CD25 selection

CD25+ T-cells were isolated using Easysep positive selection kit according to manufacturer's instructions. In brief, cells were resuspended in EasySep buffer (PBS with 2% FCS) and

incubated with CD25+ cocktail for 15min at RT (5ul/100ul). Then magnetic beads were added and incubated at RT for 10min (5ul/100ul of cell suspension). Suspension was then topped up to 2.5ml and tube was then placed into magnet for 5 min at RT. Non CD25+ were decanted and fresh 2.5ml of Easysep buffer was added to the tube. This was repeated 2 times to enrich CD25+ cells or 1 time if depleting CD25+ T cells.

2.2 IN VITRO DC GENERATION

2.2.1 Standard- 7 day generation protocol of human monocyte-derived dendritic cells

PBMNC were prepared as per 2.1.1. and resuspended in RPMI supplemented with 1% FCS at a concentration of $5x10^6$ per ml. Approximately $5x10^7$ PBMNC were transferred into 75-cm^2 and incubated for 1h at 37°C under 5% CO_2. Non adherent cells were thoroughly washed away with PBS. Adherent monocytes were cultured in RPMI 1640 containing 10% FCS, 800U/ml ($1.2x10^7$ U/mg) of granulocyte colony stimulating factor (GMCSF)-Leucomax™ and 400U/ml ($1x10^7$ U/mg) of IL-4, monocytes were allowed to differentiate into immature DC for 5 days at 37°C under 5% CO_2. Immature DC were matured with 10ng/ml of TNF-α for 2 days

2.2.2 Fast – 2 day generation protocol of human monocyte-derived dendritic cells

PBMNC were prepared as per 2.1.1. and resuspended in RPMI supplemented with 1% FCS at a concentration of $5x10^6$ per ml. Approximately $5x10^7$ PBMNC were transferred into 75-cm^2 and incubated for 1h at 37°C under 5% CO_2. Non adherent cells were thoroughly washed away with PBS. Adherent monocytes were cultured in RPMI 1640 containing 10% FCS, and a higher concentration of GMCSF-Leucomax™ (1000U/ml) and IL-4 (500U/ml). Monocytes were

allowed to differentiate into immature DC for 24h at 37°C under 5% CO_2. Immature DC were matured with 10ng/ml of TNF-α and 1μM PGE_2 for 24h.

2.3 Fluorescence Activated Cell Sorting (FACS)

2.3.1 Surface staining Conjugated mAb

Cells were thoroughly washed with PBS. FACS wash was used to resuspend cells at approximately $2x10^6$/ml. Rabbit serum (10%) was used as a blocking agent for 20min at 4°C. Then 100μl of cell suspension and recommended amount of mAb was aliquoted into FACS tubes and incubated at 4°C for 30min. Samples were equilibrated to room temperature for 5 min. One ml of FACS lysing solution was then added to fix cells. Samples are then washed with FACS wash and spun at 300g for 5 min. Cells were resuspended with 200μl of FACS wash.

2.3.2 Primary mAb staining with secondary antibody detection

Cells were thoroughly washed with PBS. FACS wash was used to resuspend cells at approximately $2x10^6$/ml. Rabbit serum (10%) was used as a blocking agent for 20min at 4°C. Then 100μl of cell suspension, mAb was added into FACS tubes and incubated at 4°C for 30min. Cells were then washed with FACS wash and spun at 300g for 5min at 4°C, supernatant was decanted and cells resuspended. Appropriate amount of conjugated secondary antibody (50ul of 1:50) was added and incubated for 25min at 4°C. Samples were equilibrated to room temperature for 5 min. One ml of FACS lysing solution was then added to fix cells. Samples are then washed with FACS wash and spun at 300g for 5 min. Cells were resuspended with 200μl of FACS wash.

2.3.3 Intracellular staining of *Foxp3*

T cells were stained for intracellular expression of Foxp3, using Foxp3 staining kit as per manufacturer's instruction. Briefly, cells were permeabilised and fixed before blocking with normal rat serum. Cells were then incubated with anti-human Foxp3 PE-conjugated mAb (PCH101) for 30min at 4°C. Expression of Foxp3 was assessed by flow cytometry (FACS CANTO).

2.3.4 Intracellular staining of phosphorylated STAT-6

Approximately $5x10^5$ cells were incubated with IL-4 (500U/ml) and GMCSF (1000U/ml) plus or minus IFN-γ (500U/ml) in 0.5ml RPMI for 10 minutes in FACS tubes at 37°C. BD cytofix was pre-warmed at 37°C and then 0.5ml was added to treated tubes. Tubes were then incubated for a further 10min in 37°C water-bath. Cells were surfaced stained with CD14-PE for 25min at 4°C. Then 1ml of BD permeabilisation buffer was added and tubes were incubated for 30min on ice. Cells were washed with FACS wash and spun for 5min at 300g 4°C. Cells were stained with 5ul of anti-STAT-6 antibody for 30min at RT in the dark. Cells were finally washed with FACS wash and spun for 5min at 300g 4°C and resuspended in 200ul of saline.

2.3.5 PI Staining

Islet cells were stained with propidium iodide (20µg/ml) in 100µl for 15min on ice and then immediately analysed on FACS CANTO II (BD Bioscience, USA).

2.3.6 Cytometric Bead Array (CBA)

A human TH1/TH2 BD bioscience CBA cytokine kit was used to determine the concentration of IFN-γ, IL-2, IL-10 and IL-4 in supernatants from DC-T cell co-cultures. DC were co-cultured with T-cells and 50ul of supernatant from each sample was assayed according to manufacture's instructions

2.4 MIXED LYMPHOCYTE REACTION (MLR)

2.4.1. Primary MLR

DC were washed with PBS three times, irradiated (30Gy) and used as stimulators in the MLR. DC were co-cultured with T-cell responders in 96-well round bottom plates, at stimulator to responder ratios of 1:10, 1:100 and 1:1000. After 4 days cells were pulsed with 1μCi [^{3}H]-Thymidine for 18 h and then harvested onto glass-fibre filters and counted in β-scintillation fluid using a Wallac Microbeta Counter. Proliferation was expressed as counts per minute (mean of 5 replicates, +/- SD).

2.4.2 Suppression assay by [^{3}H]-Thymidine

CD4$^+$ Naïve T-cells and DC were co-cultured at a ratio of 10:1 for 5 days. Then CD3$^+$ primed T-cells were isolated (Stem Cell Technologies, Vancouver, Canada) and co-cultured with autologous un-primed CD4$^+$ T-cells at varying ratios and UT-DC were used as stimulators. After 4 days cells were pulsed with 1μCi [^{3}H]-Thymidine (Amersham, Biosciences LTD, Bucks, UK) for 18 h and harvested onto glass-fibre filters and counted on a Wallac Microbeta Counter (Turku, Finland). Proliferation was expressed as counts per minute (mean of 5 replicates, +/- SD).

2.5 Immunohistology

Slides were fixed with cold acetone for 5 min and air dried. Slides were washed with PBS for 5min at RT. Primary antibody, was applied after blocking with 3% goat serum (30min at RT) for 2h at RT or O/N at 4°C. Slides were washed with PBS for 5min at RT. Optimal concentration of conjugated secondary antibody was applied and incubated at RT for 1h. Blocking serum used was adjusted according to the animal for which the secondary antibody was raised in. All antibody dilutions were prepared in blocking solution.

2.6 ELISA

2.6.1 IL-12 ELISA

DC were in 24 well plates ($1x10^6$ cells/ml) were stimulated with CD40L (500ng/ml) and IFN-γ (1000U/ml) for 48h as previously published methods [205]. Supernatants were harvested and assayed for the biologically active human IL12p70, using Ready-SET-go® ELISA Kit according to manufacturer's instructions, sensitivity 4pg/ml-500pg/ml (eBiosciences, San Diego, CA, USA).

2.6.2 IL-2 ELISA

Transduced human islets (10 IEQ) were co-cultured with hPD-1/mCD28 chimera T-cell murine hybridoma ($2x10^4$) per well in a 96 well plate at an approximate 1:1 islet cell to T-cell ratio. Supernatants were harvested after 3 days of culture in 37˚C / CO_2. Samples were stored at -80°C prior to analysis. Mouse IL-2 ELISA Ready set go kit was used according to manufacturer's instructions. The ability of transduced islets to induce signalling though PD-1 was measured as depicted in Appendix D.

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2.7 CELL LINES

2.7.1 HEK 293

Human embryonic kidney 293 cells were obtained from Cell Biolabs (Catalogue# AD-100). Thawed and expanded in DMEM complete media. Cells were allowed to a maximum of 70-80% confluence. To split cells, adherent cells were washed with PBS and 1ml/75cm^2 of pre-warmed Accutase (Sigma, St Louis, USA) was applied. Cells were incubated at 37°C for 2-3min. Light tapping of the sides of the flask results in HEK-293 detachment. Cells were centrifuged for 10 min at 400g. Cell aggregates were dispersed using a 18G blunt needle and a 30ml syringe. Cells were resuspended and seeded into 75cm^2 flasks at concentration of 2x10^5/ml.

2.7.2 PD-1/CD28 murine T-cell hybridoma

Murine T-cell hybridoma cell line expressing human PD-1 extracellular portion fused to murine origin intracellular portion of CD28 (DO.11.10) was obtained from Dr. Simon Davis from the Weatherall Institute of Molecular Medicine part of The University of Oxford. Cells were thawed and expanded in JMEM media. In general DO11.00 cells were cultured at concentration of 10^4-10^5/ml. These cells grow in suspension, when of 60 - 80% confluent cells were split 1 into 3 flasks, i.e. 3ml of cell suspension into 6ml of fresh media per 75cm^2 flask.

2.8 MOLECULAR BIOLOGY METHODS

2.8.1 RNA extraction

Total RNA was extracted using RNAspin mini kit (GE Healthcare, UK) according to manufacturer's instructions. In brief cells were washed with PBS to remove traces of FCS. A maximum of 5x10^6 cells were transferred to a 1.5ml eppendorf and micro-centrifuged at 13,000rpm for 5min. Cells were resuspended in 350ul of lysis buffer and 3.5ul of β-

mercaptoenthanol and vortexed until suspension was homogenous. Samples were stored at -80°C until multiple samples were accrued. When sufficient samples were collected, samples were allowed to equilibrate to RT, as the rest of the protocol is performed at RT. Samples were then placed through a RNAspin mini filter and micro-centrifuged at 11,000g for 1min. The filtrate was transferred to a 1.5ml eppendorf and mixed with 350ul of 70% ethanol after careful vortexing (2x 5sec), sample was placed into a RNAspin mini column and spun at 8000g for 30sec. Filter with bound RNA was transferred to a new collection tube and 350ul of desalting buffer was added and centrifuged at 11000g for 1 min. To digest any present DNA, 100ul of DNase1 was added directly to the filter membrane and allowed to digest for 15min. Filter was then washed with 200ul of wash buffer 1 and spun at 11000g for 1min. Then filter was washed with 600ul of wash buffer II and centrifuged. Finally filter is washed with 250ul of wash buffer II and spun at 11000g for 2min. RNA was eluded with 25-100ul of RNase-free water after spinning at 11000g for 1min. This step was repeated to improve yield. RNA quantity and quality was determined by NanoDrop 1000 absorbance at 260nm.

2.8.2 Reverse Transcription using Oligo dT

RNA samples of good quality were chosen for cDNA synthesis. In order to do this 1ug of total RNA was mixed with 4ul of Oligo dT and headed for 5 minutes at 60°C. Samples were transferred to ice and cooled. Then 14ul of master mix (as per table 2.1) was added. Finally nuclease free H_20 was added giving a final volume of 40ul per tube.

Table 2.1: Reverse Transcription Master mix

Reagent	Volume per reaction (µl)
5x First Strand Buffer	8
40nM dNTP	4
RNAsin	1
MMLV reverse transcriptase	1
TOTAL	**14**

Samples were mixed well by vortex. Pulsed samples were incubated for 60min at 37°C. Sample inactivation was performed at 70°C for 10min and then transferred directly to ice. Samples were pulsed to collect precipitate, then 60ul of nuclease free water was mixed into each samples. All samples were stored at -80°C.

2.8.3 Reverse Transcription using Random Hexamers

Reverse transcription using random hexamers was used to reverse transcribe, RNA extracted from mouse transplant tissue, according to the following table:

Table 2.2: Reverse Transcription Master mix using random hexamers

Reagent	Volume per reaction (µl)
10x Buffer	2
dNTP (5mM each)	2
Random hexamers (10µM)	0.9
RNase inhibitor	1
Omniscript Reverse Transcriptase	1
RNA 1µg	2*
Nuclease free water	11.1**
TOTAL	**20**

* varies according to RNA concentration

** Varies according to volume of RNA added.

Samples were prepared in 0.2ml PCR tubes and incubated at 37°C for 1h. Samples were then transferred to ice and pulsed, prior to adding 80µl of nuclease water. Throughly mix samples were then stored at -20°C.

2.8.4 Western Blot

Samples were harvested and washed well with PBS to remove FCS residue. Samples were then lysed with western blot lysis buffer (refer to section 2.15). Vortex was used to homogenize sample. Samples were stored at -80°C prior to analysis. Samples were thawed at RT and then mixed with 5µl of loading buffer and 2µl of reducing agent and run through invitrogen pre-cast gels as per manufacturer's instructions at 200mV for 45min. Chemiluminescence kit was used to visualise blot, in brief membrane was incubated with 1:1000 anti-mouse HRP for 1h at RT and then visualised with kit as per manufacturer's instructions.

2.9 POLYMERASE CHAIN REACTION

2.9.1 Standard PCR

PCR master mixes were prepared as per table 2.2 in a strictly DNA free area of laboratory. Master mix (22.5µl) was aliquoted per reaction into DNA free 0.5ml PCR tubes. In order to prevent liquid evaporation 1 drop of sterile mineral oil was carefully placed on top of master mix. 2.5µl of cDNA as per prepared in section 2.8.2 was mixed into master mix. Tubes were vortex mixed and then pulsed. Standard PCR were run in Perkin Elmer DNA Thermal Cycler.

2.9.2 Quantitative Real-time PCR using standard primers

RNA was extracted as per section 2.8.1 and cDNA was produced by reverse transcription PCR (section 2.8.2). Quantitative Real-Time PCR based on a standard curve of copy numbers for each specific gene. PCR was conducted according to optimised conditions as shown in APPENDIX A. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene in order to verify RNA integrity and cDNA synthesis.

Table 2.3. Standard PCR Master mix

Reagent	Volume per Reaction (ul)
Sterile Water	15.9*
10x Tth Buffer	2.5
25mM $MgCl_2$	2.5*
dNTP (0.2 mM)	0.5
Forward Primer (50uM)	0.5*
Reverse Primer (50uM)	0.5*
Tth Polymerase	0.1
cDNA	2.5
TOTAL VOLUME	**25**

Table 2.4 Real-time PCR Master mix

Reagent	Volume per Reaction (ul)
Sterile Water	15.7*
10x Tth Buffer	2.5
25mM $MgCl_2$	2.5*
dNTP (0.2 mM)	0.5
Forward Primer (50uM)	0.5*
Reverse Primer (50uM)	0.5*
Tth Polymerase	0.1
cDNA	2.5
Syber green	0.8
TOTAL VOLUME	**25**

* Can very depending on gene being examined.

2.9.3 Quantitative Real-time PCR using TAQMAN primers

Taqman primers were used according to the manufacturer's instructions. Briefly, PCR reaction was prepared according to the following table:

Table:2.5 Taqman PCR reaction

Reagent	Volume per Reaction (µl)
Taqman master mix	5
Taqman primer	0.5
Water	3.7
cDNA	0.8
TOTAL	10

Samples were run on Corbbett Real-time machine according to manufacturer's recommended cycling conditions. Gene expression was determined according to a standard curve.

2.10 AGAROSE GEL ELECTROPHORESIS

2.10.1 PCR product Electrophoresis

2.5µl of PCR product was mixed with 6x Loading buffer to make a total of 15µl sample. Samples were loaded into a 2% agarose gel and run for approximately 1h at 80mV, using a Bio-Rad Mini-gel Apparatus.

2.10.2 Restriction digest product electrophoresis

Approximately 10µl of restriction digest products was mixed with 2ul of 6x loading buffer. Samples were run on a 1% w/v agarose gel.

2.10.3 Adenoviral digests product electrophoresis

All adenoviral digest products were run in a 0.8% w/v agarose gel.

2.10.4 DNA markers

SPP1/EcoRI and pUC19 were used as appropriate size markers according to product size that was expected in the gel. In general 2.5µl of markers were mixed with 10µl water (DNA free) and 2.5µl of 6x loading buffer.

2.11 GENERATION OF ADENOVIRAL CONSTRUCT

2.11.1 Bioinformatics and Primer Design

Primers were designed to incorporate an EcoR1 restriction site in the forward primer. Both full length and soluble isoforms of PD-L2 were amplified with the same forward primers but used different reverse primers. Both reverse primers had a BamHI restriction site incorporated into them. The soluble isoform was designed to amplify a truncated transcription product with the transmembrane region interrupted. Designing of primers were designed with the aid of bioinformatics software tools from 'The National Center for Biotechnology Information' website.

2.11.2 Endonuclease restriction enzyme digestion

Enzyme digestion reactions were conducted in 20µl volumes as demonstrated in the table below for 18 hours at 37°C.

Table 2.6: Single Digest Reaction

Reagent	Volume (µl)
10x Buffer	2
10xBSA	2
Enzyme	1 (10 Units)
H_20	5*
DNA	10 (1µg)*
Total	**20**

Table 2.7: Double Digest Reaction

Reagent	Volume (µl)
10x Buffer	2
10xBSA	2
Enzyme 1	1 (10 Units)
Enzyme 2	1 (10 Units
H_20	4*
DNA	10 (1µg)*
Total	**20***

*=Volume will vary according to DNA concentration

2.11.3 Agarose gel product restriction digest purification

Digested produced were run on 1% w/v Agarose gel. Digests were divided into equal amounts and run in separate well. The exterior wells flanking were removed and stained with gel red. These gel regions were visualised under UV light to identify band and marked by cutting gel piece out of where the band is situated. Unstained gel portion was covered with cling wrap and stained portions were re-aligned in the original position over the cling wrap. Using the mark from the stained gel and a new blade gel was cut to excise the ban from unstained gel. DNA was then purified according to section 2.11.4.

2.11.4 Purification of DNA from gels

DNA from gels was purified using the Ultraclean™ DNA purification Kit as per manufacturer's instructions.

2.11.5 DNA ligation

In order to conduct ligation reactions a mix as described in table below was prepared and incubated at RT for 2h or at 4°C for 18h.

Table 2.8: DNA Ligation Reaction

Reagent	**Volume (µl)**
10x Buffer	1
T4 Ligase Enzyme	1
H_20	3*
DNA insert	5*
Total	**10**

*=Volume will vary according to DNA concentration

2.11.6 Preparation of Competent E.*coli* TG1-alpha and DH10β cells

Competent E.*coli* were prepared as per standard laboratory protocol. In brief bacterial were taken from glycerol stocks and streaked on to LB agar plates. E.*coli* were grown for 18h at 37°C. Single colonies were selected and inoculated into LB media and were grown under shaking conditions at 37°C (200rpm) overnight. Overnight cultures were then transferred (1ml) into 25ml LB media. Then allowed to grow until they reached an OD_{600nm} of 0.42 (log phase of growth) under 37°C shaking at 200rpm. E.*coli* were transferred onto ice for 15min and then centrifuged for 280g for 10min at 4°C. Supernatants were discarded and cells were resuspended with 2ml of 0.1M $CaCl_2$ / 20mM $MgCl_2$ solution. E.*coli* were then incubated on ice for no less then 1h before use. All competent E.*coli* were used within 18h post competence was induced.

2.11.7 Transformation of competent E.*coli* cells

Competent E.*coli* (200ul) were transformed with 5ul of ligation reaction (as per prepared in section 2.11.5). Cells and ligation mix were incubated on ice for 30min and then were heat shocked for 90s at 42°C and snap cooled on ice for 10min. This mix was then transferred to V-bottom 20ml tubes, which contained 0.5ml LB media and incubated for 1h at 200rpm 37°C. Volume of cell suspension was reduced to 200-300ul and spread on LB agar supplemented with the appropriate antibiotics. Agar plates were incubated at 37°C for 18h.

2.11.8 Plasmid Mini-preparation

Positive single colonies were selected from plates generated in section 2.11.7. These colonies were placed into 2ml LB media supplemented with the appropriate antibiotics and incubated at 37°C under shaking conditions (200rpm) for 18h. Approximately 1.5ml of cell suspension was transferred into 2ml eppendorf and microcentrifuge at 11,600g for 1min. Pellet was

resuspended in 100ul of mini-prep solution-1 for 5min. Then 200ul of solution-2 was added and cells transferred for 5 min onto ice (causing cell lysis). Lysis was neutralized with 150ul of solution-3 and this was allowed to incubate for another 5 min on ice. Cells were centrifuged for 10min at 11,600g, supernatant was transferred into a new 2ml eppendorf tube. DNA phenol (225ul) and chloroform (225ul) were added to cell lysate and mixed thoroughly. Mixture was centrifuged for 10min (11,600g), then approximately 300-400ul of the upper phase layer was transferred into new tubes and mixed with 2 x volumes of 100% ethanol (600-800ul) to precipitate DNA plasmids. Precipitates were centrifuged for 10min (11,600g). DNA pellets were washed with 250ul of 70% ethanol and centrifuged for 10min (11,600g) an then air-dried. DNA was resuspended in 45ul of TE8 (TRIS-HCL EDTA Ph 8 refer to section 15) and 5ul of DNase inactivated RNase A (200ug/ml) and stored at -80°C.

2.11.9 Plasmid midi preparation

Single colonies were selected and transferred to 2mlof LB media containing corresponding antibiotics. Bacterial colony was grown at 37°C in a shaking incubator (200rpm) for 18h. This 2ml bacterial culture was transferred to 100ml of LB media (containing the same antibiotic) and incubated for a further 18h at 200rpm 37°C. Jetstar 2.0 plasmid midi kit was used to isolate plasmid according to the manufacturers' instructions. Approximately 1ml of the plasmid elution was transferred into eppendorf tubes. Then 700µl cold isopropanol was mixed in to precipitate plasmid. Precipitate was centrifuged at 4°C, 11600g for 30min. Plasmid pellet was washed with 1.5ml of 70% ethanol and centrifuged at 4°C, 11600g for 20min, this was repeated twice with drying of pellet between each wash. Pellet was resuspended in 30µl of sterile water. Plasmid concentration was determined by absorbance at 260nm on the Nanodrop 1000 (Thermo Scientific, USA).

2.11.10 DNA sequencing

Dideoxy based DNA sequencing with chain-terminating inhibitors was used for sequencing recombinant pShuttle vectors. Sequencing was performed by the Flinders Sequencing Facility, which is part of the Department of Haematology at the Flinders Medical Centre.

2.11.11 LipofectAMINE Transfection into HEK 293

HEK293 cells ($2x10^6$) were seeded into a $25cm^2$ flask 18-20h prior to transfection, allowing cells to get 50-70% confluent. Approximately 16-18h prior to transfection recombinant plasmid was digested with PacI (5U/1µg of Plasmid) in a 20µl reaction. Approximately 4-5µg of digested plasmid was used to transfect HEK-293. Digested plasmid was mixed with equal volume of LipofectAMINE 2000, this was then mixed with 500ul of DMEM media and incubated for 30min at RT. Cells were washed with serum free DMEM to remove residual FCS. Approximately 2.5ml of serum free DMEM was added to flask and then 0.5ml of DNA/LipofectAMINE was added drop wise to flask. Cells were transfected for 4h at 37°C at 5%CO_2. Transfection media was replaced with 7ml of complete DMEM media (see section 2.15). Transfected cells were incubated (at 37°C at 5%CO_2) for 10-12 days.

2.11.12 Adenoviral HEK-293 viral lysate production

Infected HEK-293 were used to produce viral lysates. All cells were scraped and spun at 600g for 10min. Pellet was then resuspended in 3ml of DMEM (serum free). Cell suspension was frozen using liquid nitrogen and then thawed in 37°C water bath until just thawed. Lysate was vigorously vortexed for 30-60sec. The lysate underwent freeze/thaw/vortex cycle for 3-4 times prior to spinning at 600g for 10min to remove cell debris. Viral lysates were stored at -80°C in 10% glycerol if not used immediately.

2.11.13 Viral particle up-scale

HEK-293 cells were used for virus production. Virus was amplified via multiple rounds of HEK-293 as described below:

Round 1:

HEK-293 cells at a confluence of 80-90% in a 25cm^2 was infected with 1/3 of viral lysate from cells prepared in section 2.11.12. In brief cells were washed to remove FCS residue. Lysate was carefully run down side of flask to avoid detaching cells. Then carefully the lysate was evenly spread to cover all cells and incubated for 4h at 37°C at 5%CO_2. Cells were then supplemented with 5ml of complete DMEM. When 50-70% of cells from round 1 have rounded and detached, cells were collected into 50ml tube. Viral lysate was then prepared as described in section 11.12. This round was repeated 3 times prior to moving on to round 2, with repeat the number of flasks were doubled.

Round 2:

HEK-293 of early passage (less then10) was used to prepare 6 x 75cm^2 of approximately 80-90% confluency. Viral lysate prepared from round 1 was used to infect these cells.
Approximately 3-4ml was used per flask. Cells were infected for 4h at 37°C at 5%CO_2 and then supplemented with 7-8ml of complete DMEM media. Cells were cultured until 50% of cells had rounded and detached. Cells were harvested and viral lysates were prepared as per section 11.12.

Round 3 (final):

HEK-293 cells of low passage were used to prepare 5x 175cm^2 of 80% confluency. Viral titres were determined by flow cytometry (see section 11.13) and an MOI of 10 was used to infect HEK-293. Cells were infected for 4h at 37°C at 5% CO_2. After this incubation cells were

supplemented with complete DMEM media. Cells were incubated until 50% of cells had rounded and detached. Lysate was prepared as per in section 11.12.

2.11.14 Viva pure virus purification and concentration

Lysate was collected from 5 T175 infected HEK-293 cells. Lysate was filtered through 5 µm, 1.2 µm and 0.8/0.2 µm filters prior to proceeding. Virus was purified with Cell biolabs Vivapure kit as per manufacturer's instructions. In brief, filtered lysate was treated with 12.5U/ml of benzonase nuclease and incubated for 30min at 37°C. Treated lysate was then loaded into the vivaclear maxi column and spun for 5min at 500g. Appropriately calculated volume of 10x loading buffer was added to lysate. The adenopack 20 maxi-spin column was equilibrated with 5ml of 1x wash buffer. Lysate was then transferred to equilibrated column and spun for 5min at 500g to allow virus binding to membrane. Column was then washed twice with 18ml of wash buffer (spun in between at 500g for 5min). Column was then transferred to a clean column holder provided by kit. Then 1ml of elution buffer was applied to the membrane of the column. Column was briefly centrifuged for 30sec at 500g, and then incubated for 10min, prior to complete elution with a 5min spin at 500g. Buffer exchange and concentration was performed using a concentrator column also provided in kit. Virus was loaded into buffer and concentration column chamber and topped up to 10ml with virus storage buffer (25mM Tris, 25mM NaCl, 2.5% glycerol, pH8). Centrifuged for 30min at 800g, concentrated virus was collected from the top chamber.

2.11.15 Adenoviral titre determination by flow cytometry

Serial dilutions of virus was prepared in 0.5ml and 80-90% confluent HEK-293 in 25cm^2 flask were infected for 4h at 37°C at 5%CO_2. Cells were then supplemented with complete DMEM and incubated for 24h. Cells were then harvested and approximately 10^6 cells were transferred into FACS tubes and fixed with 1ml FACS lysing solution. Samples were analysed on FACS CANTO. Viral titre was calculated according to previously published formula [206]:

$$\text{TITRE (GFU/ML)} = \frac{\%\text{INF} \times \text{TOTAL CELL} \times \text{DIL}}{100 \times \text{VOL}}$$

%INF = Percentage of positive cells

TOTAL CELL = The initial number of cells infected

DIL= dilution factor

VOL= Initial infection volume (during the 4h incubation)

2.11.16 Viral titre determination by ELISA

Once the virus had been purified and concentrated the viral titer was determined by ELISA using the QuickTiter™ Adenovirus Titer ELISA Kit. ELISA was conducted according to the manufacturer's instructions. In brief, HEK-293 cells at a concentration of 5x10^5/ml was seeded into 96 well plate provided (100µl/well). Cells were allowed to attach for 1h at 37°C. Serial dilution of positive control, which formed the standard curve and dilutions of purified virus were prepared as per instructions. Drop wise dilutions were placed into appropriate cells (50µl/well). Each dilution was performed in duplicate. Cells were incubated for 2 days at 37°C 5%CO_2. Then media was carefully removed without disturbing attached cells. The cells were fixed with 100µl of cold methanol and incubated at -20°C for 20min. Cells were washed with PBS 3 times

for 5min. BSA (1%) in PBS (200µl/well) was used to block for 1h at RT on a orbital shaker. Then 100µl of provided anti-hexon antibody was added and incubated at RT on orbital shaker for 1h. Plate was washed 3 times with PBS for 5min each time. Provided secondary antibody (HRP-conjugated) was used to detect the hexon antibody. One hundred micro litres was placed into each well and incubated for 1h at RT on orbital shaker. Plate was then washed 5 times with PBS for 5min each time. TMB solution was then added and incubated for 10min (100µl/well), the reaction was then stoped with the provided stop solution (100µl/well). Optical density was then measured at 450nm. Viral titre was calculated on the basis of the standard curve.

2.12 STATISTICS

*P*rism statistical software was used for statistical analysis, where *T*-test and ANOVA analysis were used. With statistical significance considered to $P < 0.05$.

2.13 ISLET ASSOCIATED CULTURES

2.13.1 Culture conditions

Human islets clusters were maintained in CMRL media supplemented with 10% human serum (Albumex 20) and 1% glutamine. Media exchange was performed daily, unless otherwise indicated. All use of human islet tissue has been approved by the Royal Adelaide Human Ethics Committee and was kindly given research consent by the donor families. All human islets were sourced from isolating centres that form the 'Australian Islet Consortium'. Prior to shipping, islets underwent clinical grade testing at the isolating centres with ethidium bromide / acridine orange staining and FACS analysis to determine islet and beta cell viability

respectively. Prior to experimental use islet quality and viability was determined by dithizone staining.

2.13.2 Islet dissociation

Islets were washed with PBS to remove serum and centrifuged at 300g for 2 min, supernatant was decanted. Accutase (0.5ml) was used to resuspend islets and incubated in water bath at 37°C in order to disaggregate islet cell clusters for 10min. Cells per agitated every 3-4min. Islets were further dissociated with gentle pipetting . Accutase was inactivated with the addition of 2ml of complete CMRL media. Cells were centrifuged to remove accutase (300g for 5min). Cells were passed through a 50uM mesh filter.

2.13.3 Viral transduction

Islets were transduced in a minimal volume to just resuspend islets in serum free CMRL. Islets were infected for 2-4h at 37°C at 5%CO_2. After incubation islets were supplemented with complete CMRL media.

2.14 NOD-SCID model of islet transplantation

2.14.1 Animal housing

NOD-SCID mice were housed in a pathogen free environment. Mice were purchased from the ARC, Perth. Mice were used in accordance and overseen by the animal ethics committee of the University of Adelaide and the IMVS approval number: M-2009-124 (The University of Adelaide), 91/09 IMVS.

2.14.2 STZ diabetes induction

Diabetes was induced in mice according to previously published data and according to the standard operating protocol of the Australian Islet Consortium. In brief, mice of a minimum of 20g were injected with 180mg/kg of STZ intra peritoneal. STZ was prepared immediately prior to reconstitution with sodium citrate buffer pH 7.8. All mice were injected within 10 minutes of reconstitution. Mice were monitored twice daily, with regular re-hydration with 0.5ml S.C or I.P saline, as required. Mice with 2 consecutive readings of high BGL were treated with insulin pellet or insulin injection of 1U.

2.14.3 Human islet transplantation

Mice were anaesthetised with ketamine (100mg/kg) and xylazine (6mg/kg) via IP, and kept on a heat mat for the entirety of the procedure. Eye ointment was applied to eyes to prevent drying out of the retina, while under anaesthesia. Under aseptic conditions and in a pathogen free environment, the left side at the cost vertebral angle was wiped and cleaned with sterile swabs. A 1cm incision on the left side was made to expose the kidney. With a sterile scalpel a small nick

was made to the kidney capsule and a glass rod was used to create a pocket for the islets. The kidney was covered with wet gauze during islet pellet preparation. Islets were prepared by gently spinning at 800 rpm for 1min. Islets were then transferred to a pre-loaded gel-foam tip and spun further at 1000rpm for 2min. Excess media was removed and tip was unplugged prior to insertion under the kidney capsule. Using an insulin syringe plunger, islets were gently and carefully pushed under the capsule. Islets were spread by gently rubbing with the glass rod. The kidney was returned to its natural position and the muscle and skin was sutured using 4.0 catgut suture. Plain relief (temegsic) was administered sub-cut for long lasting relief.

2.15 BUFFERS AND SOLUTIONS

DMEM complete media
DMEM (High Glucose)
10% FCS
1% Glutamine
1% Pen/step

CMRL complete
CMRL Media
10% Albumex 20
1% L-Glutamine
1% Pen/step

RPMI Complete media (s10g)
RPMI media
10% FCS
1% L-Glutamine
1%Pen/strep

GelRed ™ Nucleic Acid gel Stain (1x)
5ul of 10,000x stock GelRed
50ml of 0.1M Sodium Choride

Agarose gel per 100ml
0.8% = 0.8g

1% = 1g

2% = 2g

In a conical flask required amount was weight out and mixed with distilled water to measure 100ml. Agarose gel was heated in microwave until agarose gel had dissolved.

50x TAE per 100ml
Trizma base -193.8g [1.6M]
Sodium acetate – 65.6g [800mM]
EDTA – 14.9 [40.27mM]
pH to 7.2

6x loading Buffer per 10ml
200ul of 50x TAE
5ml glycerol (50%)
2.4ml Bromophenel Blue (24%)
2.4ml Water

TE8 per 100ml
0.5ml 2M TRIS-HCL pH 8
0.2ml 0.5M EDTA pH 8
Make up to 100ml with water

Luria Broth (LB) per 1L
5g Bacto-Yeast Extract
10g Bacto-tryptone
10g Sodium Chloride
Make up to 1L with water

LB Agar
15g Bacteriological Agar
LB to 1L

Autoclave mix prior to use. To melt agar heat in microwave. Then add 1ml of 30mg/ml kanamycin stock to bring final concentration of kanamycin to 30ug/ml. Make sure antibiotic is added when agar is 55°C or less.

1M $CaCl_2$ solution per 100ml
14.7g of $CaCl_2$
Mix with water and make up to 100ml autoclave the solution prior to use
1M $MgCl_2$ per 100ml
20.33g $MgCl_26H_20$
Mix with water and make up to 100ml autoclave the solution prior to use

0.1M $CaCl_2$/ 20mM $MgCl_2$ Transformation Solution per 10ml
1ml 1M $CaCl_2$
0.2ml 1M $MgCl_26H_20$
8.8ml water

Mini-preparation solution 1

50mM D-Glucose
25mM Tris-HCL
10mM EDTA
pH to 8

Mini-preparation solution 2
0.2M Sodium Hydroxide
1% SDS

Mini-preparation solution 3
3M Potassium acetate
11.5% Glacial acetic acid
Adjust pH to 4.8

Mini-preparation storage solution
200ng /5ml DNase inactivated RNaseA
5ml TE8

SOC Media per 1L
5g Bacto-Yeast Extract
20g Bacto-Tryptone
2ml 5M NaCl
2.5ml 1M KCl
Water make up to 1L
Adjust pH to 7

Western Blot lysis buffer
50mM HEPES (Ph 7.4)
150mM NaCl
1% Triton-X-100
1mM Na_3VO_4
30mM NaF
10mM $Na_4P_2O_7$
10mM EDTA

10x Tris buffered Saline (TBS)
24.2g Tris base
80g NaCl
pH to 7.6 with HCL

Western blot blocking buffer
1x TBS
0.1% Tween-20
5% w/v nonfat dairy milk

2.16 PRODUCT LISTS

2.16.1 Antibodies

Antibody Antigen	Conjugate	Clone	Source
CD83	n/a	HB15e	Serotec LTD, Oxford, UK
CD86	n/a	BU63	Serotec LTD, Oxford, UK
CD80	n/a	MAB104	Immunotech, Mareille, France
CD40	n/a	LOB7/6	Santa Cruz Biotechnology, Santa Cruz, CA, USA
ILT3	n/a	ZM3.8	Dr. Marco Colonna, Washington State University, St Louis, USA
mouse IgG	FITC	-	Southern Biotech, Birmingham, AL, USA
mouse IgG	PE	-	Southern Biotech, Birmingham, AL, USA
CD-14	FITC	MY-4	Beckman Coulter, Fullerton, CA, USA
ILT2	PE	HP-F1	Beckman Coulter, Fullerton, CA, USA
ILT4	n/a	42D1	gift of Dr. Marco Colonna, Washington State University, St Louis, USA
Foxp3	PE	PCH101	eBioscience, San Diego, USA
IL-17	FITC	Clone: eBio64DEC17	eBioscience, San Diego, USA
CD83	FITC	HB15e	eBioscience, San Diego, USA
CD86	FITC	FUN1	BD Bioscience,

			San Jose, California, USA
CD80	FITC	L307.4	BD Bioscience, San Jose, California, USA
DC-SIGN	FITC	DCN46	BD Bioscience, San Jose, California, USA
HLA-DR	PE-Cy5	G46-6	BD Bioscience, San Jose, California, USA
PD-L2	n/a	MIH18	eBiosciences, USA
PD-L1	n/a	MIH1	eBiosciences, USA
ILT4	n/a	42D1	Santa Cruz, USA
rat IgG	FITC	-	*Silenus*, Australia
pY641	Alexa488	clone: 18	BD Bioscience, California, USA
CD14	PE	M5E2	BD Bioscience, California, USA
CD25	PE-Cy7	M-A251	eBiosciences, San Diego, USA
CD4	Percp5.5	OKT4	eBiosciences, San Diego, USA
CD3	FITC	SK1	BD Bioscience, California, USA
RelB	n/a	polyclonal	Santa Cruz Biotechnology, USA
anti-rabbit IgG	FITC	-	Santa Cruz Biotechnology, USA
human F(ab')2	PE	-	Immunex France
CD124	PE	hIL4R-M57	BD Bioscience, California, USA
Asialo GM1	n/a	polyclonal	Wako, Japan
Insulin	n/a	polyclonal	Millipore, USA

2.16.2 Cytokines

IL-4 (eBiosciences, USA)

IFN-γ (eBiosciences)

Prostaglandin E_2 (*Sigma,* USA)

TNF-α (R&D Systems, USA)

CD40L (R&D Systems, Minneapolis, USA)

(GMCSF)-Leucomax™ (Sandoz Australia, Australia)

2.16.3 FACS Reagents

FACS lysing solution (BD Bioscience, USA)

Rabbit serum cytofix buffer (BD Bioscience, USA)

BD perm-buffer (BD Bioscience, California, USA)

2.16.4 Molecular Reagents

Agarose – DNA Grade (Progen, Australia)

Ampicillin (Roche, Germany)

Custom Oligo-nucleotides (Sigma-Genosys, USA)

BamH I restriction endonuclease (New England Biolabs, USA)

EcoR I restriction endonuclease (New England Biolabs, USA)

Kanamycin (Life Technologies, USA)

LipofectAMINE 2000™ (Invitrogen, USA)

$MgCl_2$ 25mM Solution (Fisher Biotech, USA)

Mineral Oil (Sigma-Aldrich, USA)

MMLV Reverse Transcriptase (Life Technologies, USA)

Not I Endonuclease (New England Biolabs, USA)

Oligo dT (Amersham, Australia)

Pac I restriction endonuclease (New England Biolabs, USA)

pUC19 (Bresatec, Australia)

RNase A (Sigma-Aldrich, USA)

RNAsin (Promega, USA)

Random Hexemers (Qiagen, USA)

Sal I restriction endonuclease (New England Biolabs, USA)

SPP1/*Eco*RI (Geneworks, Adelaide, Australia)

T4 Ligase (Promega, USA)

Tth Polymerase (Fisher Biotech, USA)

Tth PCR buffer (Fisher Biotech, USA)

GelRed™ Nucleic Acid Gel Stain (Biotium, USA)

2.16.5 Plasmid vectors

Pshuttle-CMV (Vogelstein, B, The John Hopkins University, Baltimore, USA)

pAdEasy-1 (Vogelstein, B, The John Hopkins University, Baltimore, USA)

pEGFP-N1 Vogelstein, B, The John Hopkins University, Baltimore, USA)

2.16.6 Tissue Culture Reagents

Accutase (Sigma-Aldrich, USA)

RPMI 1640 (Invitrogen, USA)

DMEM (Invitrogen, USA)

Albumex 20 (Australian Red Cross, Australia)

[^{3}H]-Thymidine (Amersham, Biosciences LTD, Bucks, UK)

CMRL (Invitrogen, USA)

2.16.7 Kits

CD4^{+} enrichment kit (Stem Cell Technologies, Canada)

CD3^{+} positive selection kit (Stem Cell Technologies, Canada)

RNAspin mini kit (GE Healthcare, USA)

IL12p70 ELISA (eBiosciences, San Diego, USA).

Human Insulin Elisa (Mercodia, Uppsala, Sweden)

Mouse IL-2 ELISA (eBiosciences, San Diego, USA).

Cytometric bead array kit, (BD Bioscience, USA)

Ultraclean™ DNA purification Kit (Mo Bio Laboratories, USA)

Jetstar 2.0 Plasmid midi kit (Genomed, Germany)

QuickTiter™ Adenovirus Titer Kit (Cell Biolabs, San Diego, CA, USA)

2.16.8 Other Reagents

Dimethyl sulphoxide (DMSO) (Ajax Chemicals, Australia)

Trypan Blue (BDH, Australia)

Tween-20 (Bio-Rad, USA)

Dithizone (Sigma-Aldrich, USA)

Streptozocin (Sigma-Aldrich, USA)

Propidium Iodine (Invitrogen, USA)

2.16.9 Equipment

Cytospin II Shandon (Thermo scientific, USA)

NanoDrop 1000 (Thermo Scientific, USA)

Perkin Elmer DNA Thermal Cycler (USA)

Bio-Rad Minigel Apparatus

Wallac Microbeta Counter (Turku, Finland)

FACS CANTO (BD Bioscience, San Jose, California, USA),

CHAPTER 3 - IFN-γ generates maturation-arrested dendritic cells that induce T-cell hyporesponsiveness

3.1 INTRODUCTION

Cytokines are small non-structural proteins that regulate the response to infection, inflammation, trauma and immune responses [207]. Classically cytokines that are known to promote disease severity are called 'proinflammatory' whereas those that promote healing are regarded as 'anti-inflammatory. Interferon-gamma (IFN-γ), a type II cytokine, classically considered as proinflammatory is the primary focus of this chapter [208]. IFN-γ (also known as macrophage –activating factor) plays an important role in macrophage stimulation, which induces anti-microbial and anti-tumour mechanisms. Its capacity to upregulate antigen processing and presentation pathways as well as supporting leukocytes attraction, growth, differentiation and maturation of many cell types, it is well known as a proinflammatory cytokine [209, 210]. Initially, IFN-γ was thought to be exclusively released by $CD4^+$ T-helper cell Type 1 (TH1) lymphocytes, CD8+ cytotoxic lymphocytes and natural killer cells [211, 212]. However B-cells and professional APC have also been found to produce IFN-γ. The secretion of IFN-γ by APC such as DC, may be important in self-activation and the activation of nearby cells in their local environment [213] [214-216]. IFN-γ release by NK cells is important in the early phase of infection defence mechanisms. T-cells on the other hand are the primary source of IFN-γ, in the adaptive immune response [213, 217].

IFN-γ is well known in the field of allograft transplantation for promoting antigen-specific Th1 differentiation involved in cell-mediated rejection. It does this by inhibiting the growth of Th2 T cell subsets and by promoting T-cell and APC interactions. Thus it upregulates the presentation and stimulatory capacity of APC, which increases CD4+ T-cell differentiation [218]. The presence of IFN-γ in the micro environment of naive T-cells during TCR engagement, is thought to heavily influence Th1 T-cell differentiation. Subsequently the production of IFN-γ and

IL12 further inhibits the secretion of Th2 cytokine IL-4, driving a Th1 response [219-221]. Thus the presence of IFN-γ at the graft site is associated with acute allograft rejection [222]. However there is a large body of work which suggests that IFN-γ has a paradoxical role as an anti-inflammatory cytokine.

Studies in the mid 1990s elucidated the unexpected protective role of IFN-γ in models of autoimmune disease. Initially the disruption of the IFN-γ gene in mouse models of experimental autoimmune encephalomyelitis and collagen induced arthritis, demonstrated that IFN-γ was required for protection against disease onset and severity rather than promoting the disease state [223-226]. In models of skin and cardiac allograft transplantation, it was observed that the supplementation of IFN-γ was essential for prolonging graft survival and inducing tolerance by co-stimulatory blockade [227-229]. More recently, in 2008 *in vitro* studies by Feng showed that the addition of IFN-γ into murine DC-allogeneic T-cell co-cultures promotes the enrichment of $CD4^+$ $CD25^+$ $Foxp3^+$ T-regulatory cells, by converting and eliminating T-effector cells [230], a phenomenon that contradicts the view that IFN-γ solely drives Th1 mediated immune responses. Moreover, IFN-γ KO models have demonstrated that, IFN-γ is required to negatively regulate the stimulatory capacity of DC, an occurrence that has only been vaguely described in humans.

In the past decade some human studies have also suggested that IFN-γ may negatively regulate monocyte differentiation into mature DC [231, 232]. These studies however failed to accurately phenotype the resultant APC and they did not investigate the effect of IFN-γ on inhibitory molecule or Lineage marker expression, nor did they assess the tolerogenic capacity of these APC. As discussed in section chapter 1.4.1, DC are crucial regulators of the immune responses and are capable of directly inhibiting allograft rejection by promoting tolerogenic

immune responses. Therefore the aim of this chapter was to investigate the tolerizing capability of IFN-γ to modulate DC function to promote tolerance.

It was hypothesised that:

- The capacity of IFN-γ to negatively regulate DC function lies with the timing of IFN-γ exposure during DC differentiation (day 0 treatment) and maturation (Day 5 treatment)

- The negative regulation of DC function is related to IFN-γ inducible negative co-stimulatory molecule expression

The capacity of IFN-γ to negatively regulate DC function was shown to be dependent on the timing of exposure during DC development. IFN-γ treatment only at day 0 of DC differentiation inhibited the capacity of DC to efficiency prime T-cells, thus causing T-cell hyporesponsiveness. Moreover, IFN-γ treatment at day 0 upregulated the expression of inhibitory Immunoglobulin-Like Transcript (ILT) family molecules and inhibited the expression of maturation marker CD83, which in part may have contributed to the poor capacity of DC to drive a Th1 response, whereas the latter exposure of IFN-γ had little effect on DC function.

3.2 MATERIALS AND METHODS

3.2.1. Generation of human monocyte-derived dendritic cells

Monocytes were isolated from healthy human blood donors as described in chapter 2 section 1.1 and 2.1. Monocytes were cultured in RPMI 1640 containing 10% FCS, 800U/ml ($1.2x10^7$ U/mg) of recombinant human granulocyte colony stimulating factor (GMCSF)-Leucomax™ (Sandoz Australia, Australia) and 400U/ml ($1x10^7$ U/mg) of recombinant human IL-4 (eBiosciences, USA) in the absence (UT-DC) or presence of 500U/ml of IFN-γ (eBiosciences, USA) (IFN-γ-DC_0) for 5 days to generate immature DC (iDC). At day 5 of culture, iDC were treated with 10ng/ml of TNF-α (R&D Systems, USA) for 2 days to generate mature DC (mDC). In some experiments iDC were matured with TNF-α (R&D Systems, USA)in the presence of IFN-γ (eBiosciences, USA) (500u/ml) (IFN-γ-DC_5). All cell cultures were incubated under 5% CO_2 at 37°C.

3.2.2 Phenotypic analysis of DC by flow cytometry

The phenotypic profile of DC was defined using the following primary mAb: anti-CD83 (HB15e) (Serotec Ltd, UK) and anti-CD86 (BU63) (Serotec Ltd, UK), anti-CD80 (MAB104) (Immunotech, France) anti-CD40 (LOB7/6) (Santa Cruz Biotechnology, USA), anti-MHC Class II (In house tissue supernatant), anti-ILT3 (ZM3.8) (Kind gift from Dr. Marco Colonna, Washington State University, St Louis, USA) anti-PD-L1(MIH1) and anti-PD-L2 (MIH18) (eBioscience, USA). Unconjugated mouse mAb were detected with FITC or PE conjugated anti-mouse IgG secondary antibody. Conjugated mAb were used to assess the expression of CD-14-FITC (MY4) (Beckman Coulter, USA), ILT2-PE (HP-F1) Beckman Coulter, USA), and DC-SIGN-FITC (DCN46) (BD Bioscience, USA). Rat anti-human ILT4 (42D1) (A gift of Dr. Marco Colonna, Washington State University, St Louis, USA) was also used as a primary mAb, whist FITC-

conjugated anti-rat IgG was used for detection. For antibody details please refer to chapter 2 section 16.1.

3.2.3 Mixed lymphocyte reaction

DC were washed with PBS three times, irradiated (30Gy) and used as stimulators in the MLR. Allogeneic T-cells were purified from PBMNC using nylon wool packed columns as described in chapter 2 section 1.2. DC were co-cultured with T-cell responders in 96-well round bottom plates, at stimulator to responder ratios of 1:10, 1:100 and 1:1000. After 4 days cells were pulsed with 1μCi [^{3}H]-Thymidine (Amersham, UK) for 18 h and then harvested onto glass-fibre filters and counted in β-scintillation fluid using a Wallac Microbeta Counter (Tuku, Finland). Proliferation was expressed as counts per minute (mean of 5 replicates, ± SD).

3.2.4 Messenger RNA expression analysis

RNA was extracted was as per described in the methods (Chapter 2 section 2.8.1) cDNA was synthesized using 1μg total RNA by reverse-transcription (Chapter 2, section 2.8.2). Quantitative Real-Time PCR, based on a standard curve of copy numbers for each specific gene was generated and used to analyse the expression of RelB, IL-12p40, IDO and HLA-G. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene in order to verify RNA integrity and cDNA synthesis. Please refer to appendix A for sequence details and cycling conditions.

3.2.5 T-cell cytokine and phenotype analysis

Purified T-cells were co-cultured with DC at a ratio of 100:1, for 6 days in RPMI 1640 supplemented with 10% FCS at 37ºC under 5% CO_2. T-cells ($5x10^5$) were stained for CD4 and intracellular expression of Foxp3 or IL-17A (eBioscience, USA). Briefly, cells were permeabilised and fixed (with buffer provided in kit) before blocking with normal rat serum. Cells were then incubated with anti-human Foxp3-FITC mAb or IL-17-FITC. $CD4^+$ T cells were gated and analysed on BD FACSDIVA Software, quadrant gates were set according to isotype control. In addition, supernatants from co-cultures were collected and assayed for the production of IL-2, IFN-γ, IL-4 and IL-10 by using a cytometric bead array kit (BD Bioscience, USA). Cells and supernatants were analysed by flow cytometry on a FACS CANTO (BD Bioscience, USA).

3.2.6 Statistical analysis

Student's *t*-test was conducted, with statistical significance at $P < 0.05$, using Microsoft Excel software.

3.3. RESULTS

3.3.1 IFN-γ modifies the expression of positive co-stimulatory molecules and negative regulatory molecules on DC during differentiation and maturation

In preliminary experiments a range of 50 to 1000U/ml of IFN-γ was used to determine the effects on DC differentiation and maturation from monocyte precursors. The concentration of 500U/ml was considered as optimal as no cytopathic effects were observed and 1000U/ml did not significantly improve the capacity of DC to further inhibit T cell proliferation (see appendix B), thus 500U/ml was used in subsequent experiments. IFN-γ treatment of monocytes at day 0 prior to DC differentiation or at day 5 during maturation caused phenotypic changes (Figure 3.3.1A). DC differentiation was observed by the loss of the monocyte marker and the high expression of the DC lineage marker DC-SIGN (>92%+ve cells for all groups). IFN-γ treatment at day 0 (IFN-γ-DC_0**)** however reduced the CD83 percentage positive population to 6%, which was considerably less compared to UT-DC, in which 74% of its population expressed CD83. IFN-γ-DC_0 also caused the down regulation of co-stimulatory molecules. CD86 saw a 9% reduction in the percentage of IFN-γ-DC_0 expressing this molecule. Moreover, only 33% of the IFN-γ-DC_0 population expressed CD80 compared to 93% of the UT-DC population (Figure 3.3.1A). Treatment with IFN-γ at day 5 (IFN-γ-DC_5) on the other hand did not modify the expression profile of co-stimulatory molecules compared to UT-DC.

Flow cytometric analysis of negative regulatory molecules showed that both IFN-γ-DC_0 and IFN-γ-DC_5 had induced expression of PD-L1 (Figure 3.3.1B). IFN-γ increased the MFI of PD-L1 to 325 in IFN-γ-DC_0 and 245 in IFN-γ-DC_5 from 136 in UT-DC. However, ILT4 expression was exclusively upregulated in IFN-γ-DC_0 from negative UT-DC to 22% of the IFN-γ-DC_0 population. The MFI of ILT2 and ILT3 were also upregulated in IFN-γ-DC_0 compared to UT-DC. IFN-γ-DC_5 and UT-DC had similar expression levels of inhibitory receptors ILT2, ILT3 and ILT4. Both IFN-

γ treated groups had reduced PD-L2 expression (68%+ve UT-DC vs 34% +ve IFN-γ-DC_5 vs 9%+ve IFN-γ-DC_0). The surface expression of HLA-G, an inhibitory ligand for ILT2 and ILT4 associated in regulating DC and T-cell responses, was not present in UT-DC and IFN-γ-DC_0, whilst IFN-γ-DC_5 showed a negligible upregulation to 3%. Overall IFN-γ-DC_0 appears to have an immature DC phenotype despite stimulation with maturing agent TNF-α. This coincided with reduced expression of positive co-simulation and the upregulation of inhibitory molecules a phenomenon not seen in UT-DC or IFN-γ-DC_5.

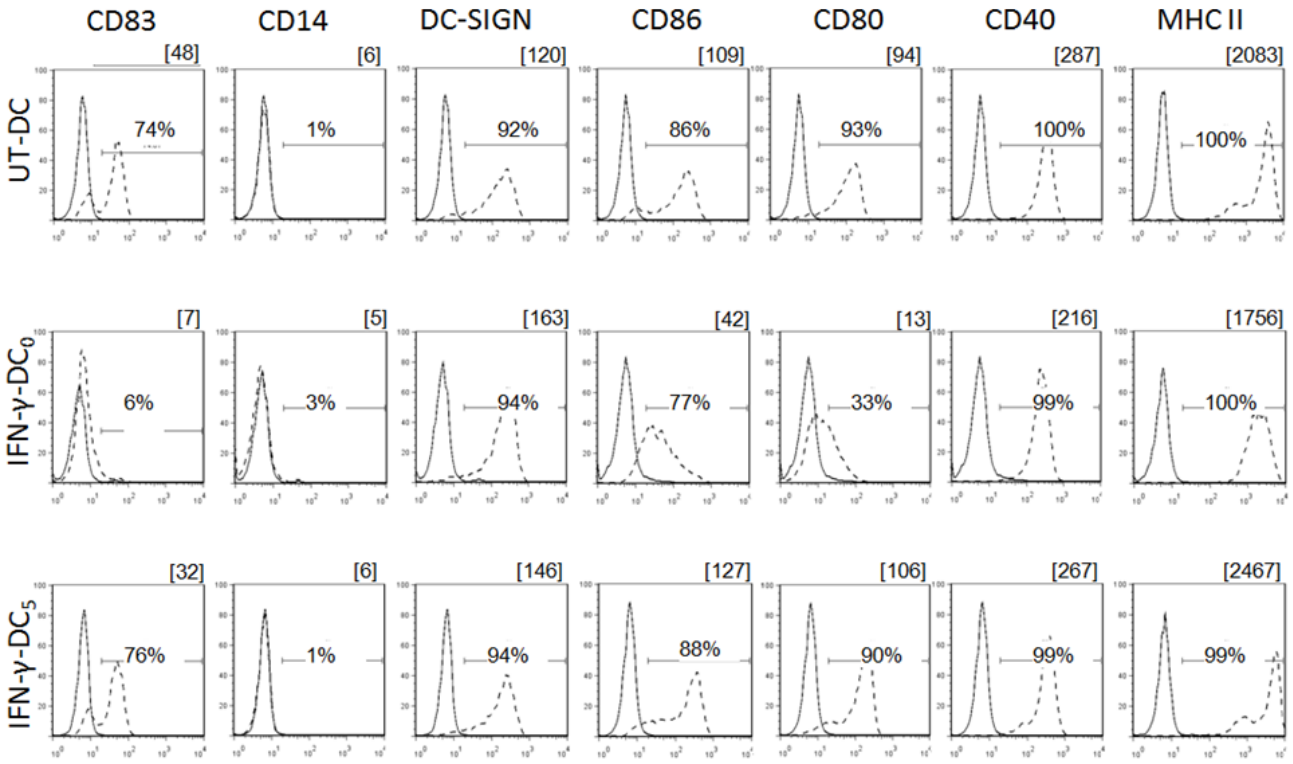

FIGURE 3.3.1A. ***IFN-γ modifies the expression of positive co-stimulatory molecules by DC during differentiation and maturation.*** DC were treated with IFN-γ at day 0 (IFN-γ-DC_0) and at day 5 (IFN-γ-DC_5) or cultured in the absence of IFN-γ (UT-DC) for 5 days, then matured with TNF-α for 2 days. Flow cytometry was used to analyse the phenotype of IFN-γ treated DC and compared to UT-DC. The following costimulatory and lineage markers were examined: CD83, CD86, CD40, MHC class II, DC-SIGN and CD14. The expression of all markers was analysed by flow cytometry. Solid line indicates the isotype control and dash line shows the tested mAb. Mean Fluorescence Intensity (MFI) is indicated in the top-right corner of each graph and percentage positive cells are shown below the MFI. Representative of 10 independent experiments.

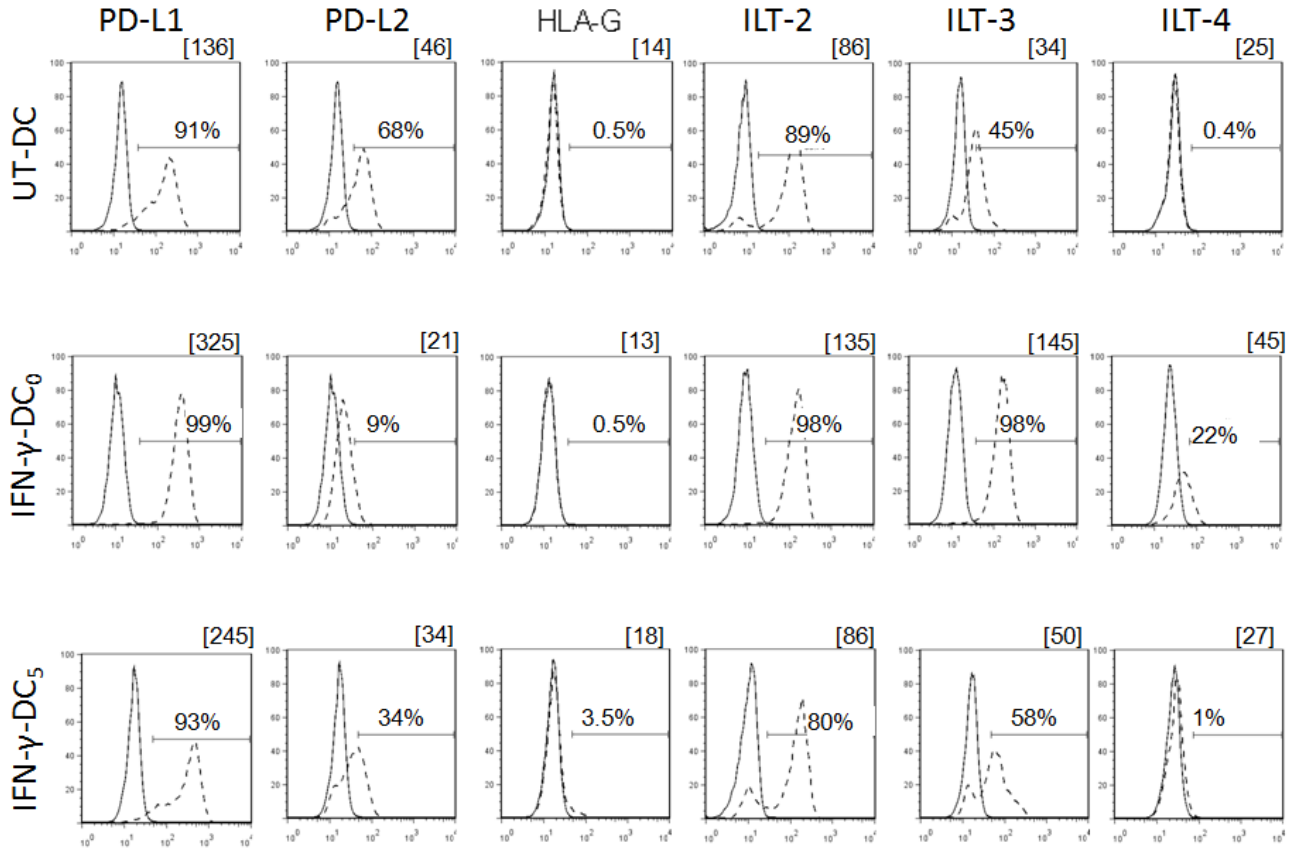

FIGURE 3.3.1B. ***IFN-γ upregulates the expression of inhibitory co-stimulatory molecules from the ILT family and PD-L1***. To further study the phenotype, the expression of negative co-stimulatory molecules was examined including: PD-L1, PD-L2, HLA-G, ILT-2, ILT-3 and ILT-4. The expression of all markers was analysed by flow cytometry. Solid line indicates the isotype control and dash line shows the tested mAb. Mean Fluorescence Intensity (MFI) is indicated in the top-right corner of each graph and percentage positive cells are shown below the MFI. Representative of 4 independent experiments.

3.3.2 Monocyte-derived DC differentiated in the presence of IFN-γ induce allogeneic T cell hyporesponsiveness

The effect of IFN-γ on the stimulatory function of DC, was examined in a DC mixed lymphocyte reaction (MLR). After DC maturation with TNF-α, cells were harvested and extensively washed to minimise the transfer of residual factors into the MLR. In comparison to UT-DC, IFN-γ-DC_0 inhibited the proliferation of T-cells by 33% (P=0.02) at a stimulator : responder ratio of 1:100 and 97% (P=0.007) at a 1:1000 ratio (Fig. 3.3.2A). The stimulatory capacity of IFN-γ-DC_5 remained unchanged compared to UT-DC. The addition of IL-2 (100U/ml) to T-cells co-cultured with IFN-γ-DC_0 (Figure.3.3.2B), did not revert T-cell proliferation, suggesting that T-cells co-cultured with IFN-γ-DC_0 are not anergic in nature [233].

3.3.3 IFN-γ treatment at day 0 prior to DC-differentiation produces maturation-arrested DC

In order to confirm the maturation status of phenotypically immature DC the gene expression of NF-κB transcription factor RelB and proinflammatory cytokine IL-12 were assessed by quantitative real-time PCR (Figure 3.3.3). Expression of RelB was significantly inhibited by 79% (P=0.01) in IFN-γ-DC_0 when compared to UT-DC. However exposure to IFN-γ at day 5 during DC maturation resulted in a significant increase of 48% in RelB expression, when compared to both other groups (UT-DC and IFN-γ-DC_0). IFN-γ-DC_0 failed to upregulate the gene expression of IL-12, instead a significant inhibition was observed by 97% (P=0.02) in comparison to UT-DC, whereas IFN-γ-DC_5 showed a significant increase of 50%.

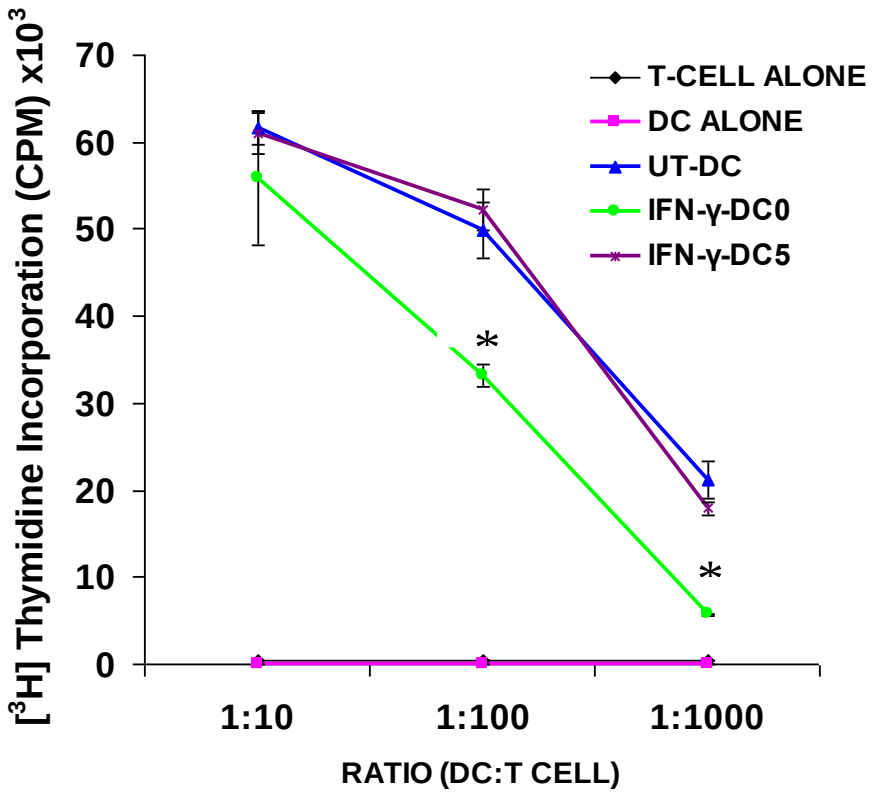

FIGURE 3.3.2A: ***Monocyte-derived DC differentiated in the presence of IFN-γ induce allogeneic T-cell hyporesponsiveness.*** DC were treated with IFN-γ at day 0 (IFN-γ-DC_0) and at day 5 (IFN-γ-DC_5) or cultured in the absence of IFN-γ (UT-DC) for 5 days, then matured with TNF-α for 2 days. T cells ($1x10^5$) were incubated with DC, at a DC to T-cell ratio of 1:10, 1:100 and 1:1000 for 5 days. Proliferation was determined by [^{3}H] thymidine incorporation (CPM). *=$p<0.05$. (Mean ± SD of 5 replicates within the experiment). Figure representative of 5 independent experiments.

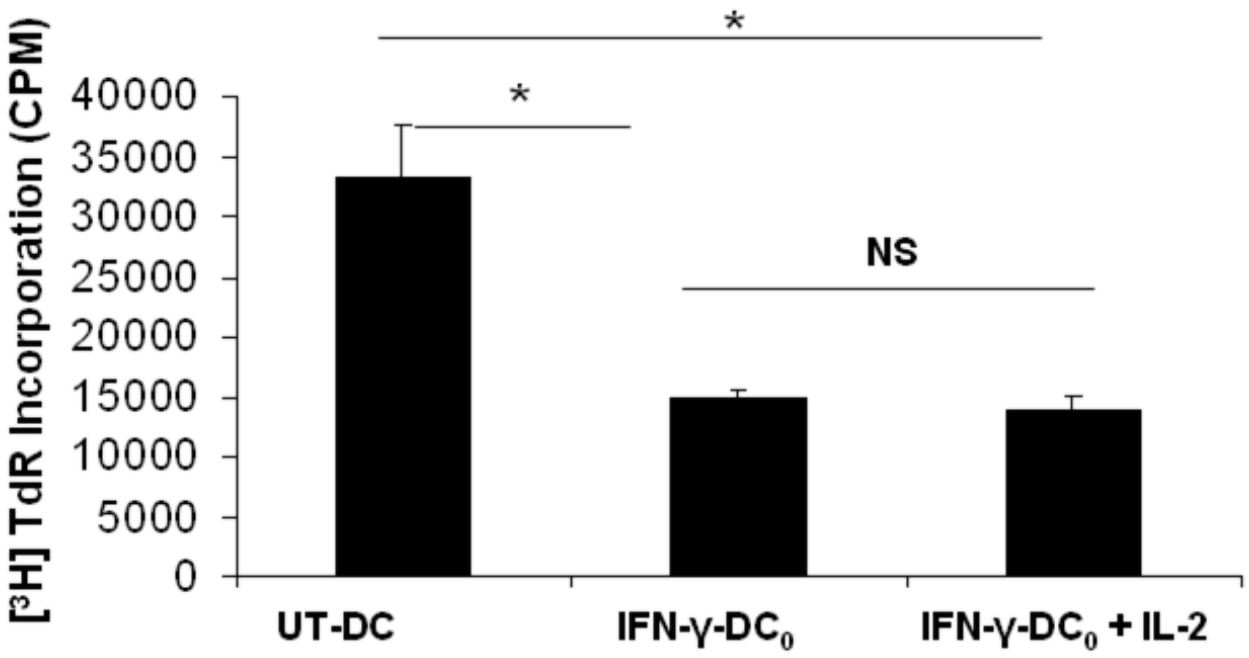

FIGURE 3.3.2B. ***IFN-γ-DC$_0$ cause T cell hyporesponsiveness independent of T cell anergy.*** To test the ability of IFN-γ-DC$_0$ to induce T cell anergy, 100U/ml of exogenous IL-2 was added to the co-culture of DC and T cell at a ratio 1:100 for 5 days. Proliferation was determined by [^{3}H] thymidine incorporation (CPM) (n=10) (B). *=$p<0.05$, NS= No Significance. ±SD of 5 replicates within the experiment. Figure representative of 5 independent experiments

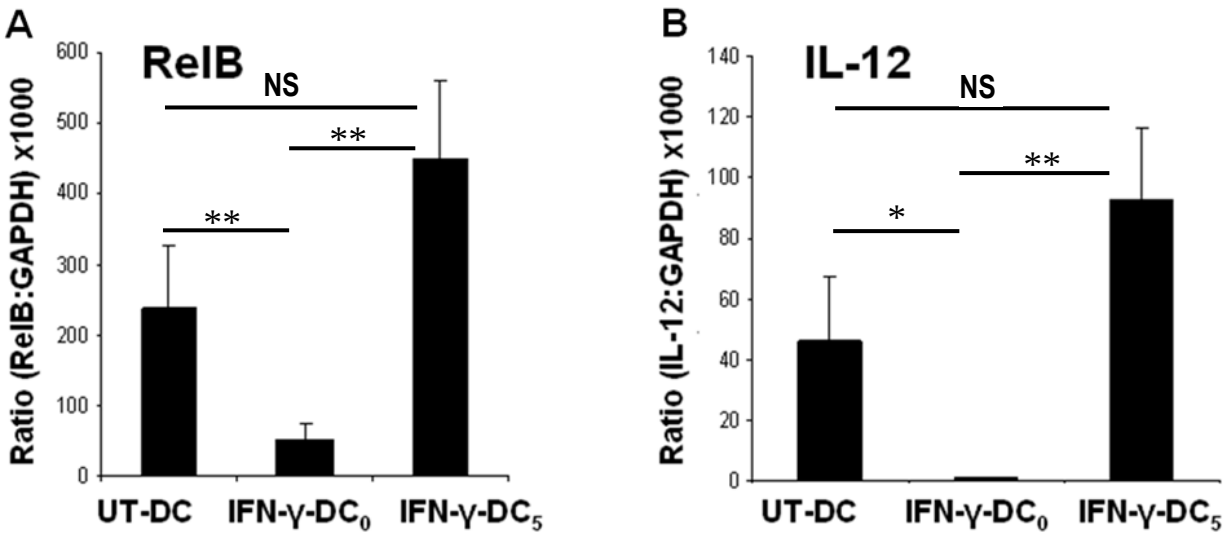

FIGURE 3.3.3: ***IFN-γ treatment at day 0 prior to DC-differentiation produces maturation-arrested DC***. DC were treated with IFN-γ (500U/ml) at day 0 (IFN-γ-DC_0) and at day 5 (IFN-γ-DC_5) or cultured in the absence of IFN-γ (UT-DC) for 5 days, then matured with TNF-α for 2 days. Quantitative real-time PCR was used to analyse the mRNA expression levels of NF-κB transcription factor RelB (A) and IL-12 (B) to confirm the maturation status of IFN-γ-DC_0 as immature. Mean ± SD of quadruplicates, *=$p<0.05$, **=$p<0.01$, NS= No Significance. Figure representative of 5 independent experiments

3.3.4 T-cell hyporesponsiveness induced by IFN-γ-DC_0 is independent of the generation of total $Foxp3^+$ T regulatory cells.

To investigate other possible mechanisms causing T-cell hyporesponsiveness by IFN-γ-DC_0, the ability of IFN-γ-DC_0 to generate $Foxp3^+$ T-regulatory cells and IL17-secreting TH17 cells was assessed. The proportion of $CD4^+Foxp3^+$ T-cells and $CD4^+IL17^+$ T cells (Figure 3.3.4A) after co-culturing DC and T-cell for 6 days was determined by FACS. UT-DC on average 64% of the CD4+ T-cell population were $Foxp3^+$ compared to 38% generated by co-cultures with IFN-γ-DC_0 ($p=0.017$ based on 4 independent experiments). The presence of IL17 producing $CD4^+$ T-cells in the resultant co-cultures was similar in both groups. IFN-γ-DC_0 promoted on average 4% of $CD4^+IL17^+$ T-cells which was not significantly different to UT-DC (mean = 4%) ($p=0.43$). Analysis of supernatants (Figure 3.3.4B) from IFN-γ-DC_0 and T-cell co-cultures had significantly reduced concentration of IL-2 ($p=0.0097$) and IFN-γ ($p=0.037$) production by 67% and 73% respectively, when compared to UT-DC. The production of IL-4 and IL-10 was not statistically different in IFN-γ-DC_0 co-cultures compared to UT-DC. IFN-γ responsive regulatory molecules indoleamine 2, 3-dioxygenase (IDO) and HLA-G (Figure 3.3.4C), were also examined as they are known to regulate T-cell responses. IDO and HLA-G mRNA expression in IFN-γ-DC_0 were not significantly upregulated compared to UT-DC. However IDO and HLA-G were upregulated in IFN-γ-DC_5 by 94% and 97%, respectively.

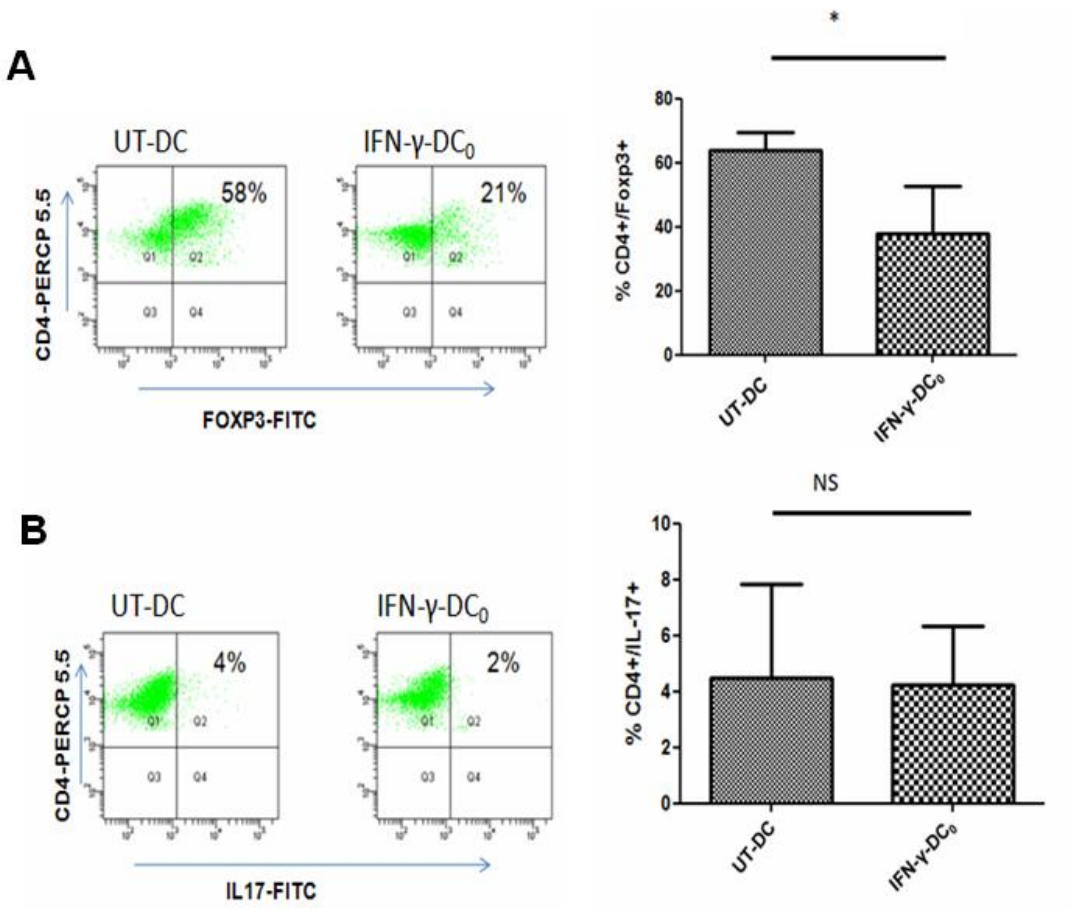

FIGURE 3.3.4A: ***T-cell hyporesponsiveness induced by IFN-γ-DC$_0$ is independent of the generation of Foxp3$^+$ T regulatory cells and the generation of IL-17 producing T-cells.*** DC were treated with IFN-γ at day 0 (IFN-γ-DC$_0$) and at day 5 (IFN-γ-DC$_5$) or cultured in the absence of IFN-γ (UT-DC) for 5 days, then matured with TNF-α for 2 days. IFN-γ-DC$_0$ were co-cultured for 6 days with 1x10^5 T-cells (1:100, DC to T-cell ratio), then stained for surface expression of CD4 and intracellular expression of Foxp3 (A) or IL17 (b) (n=4). *=p<0.05, NS= No significance (Mean ± SD of 4 independent experiments).

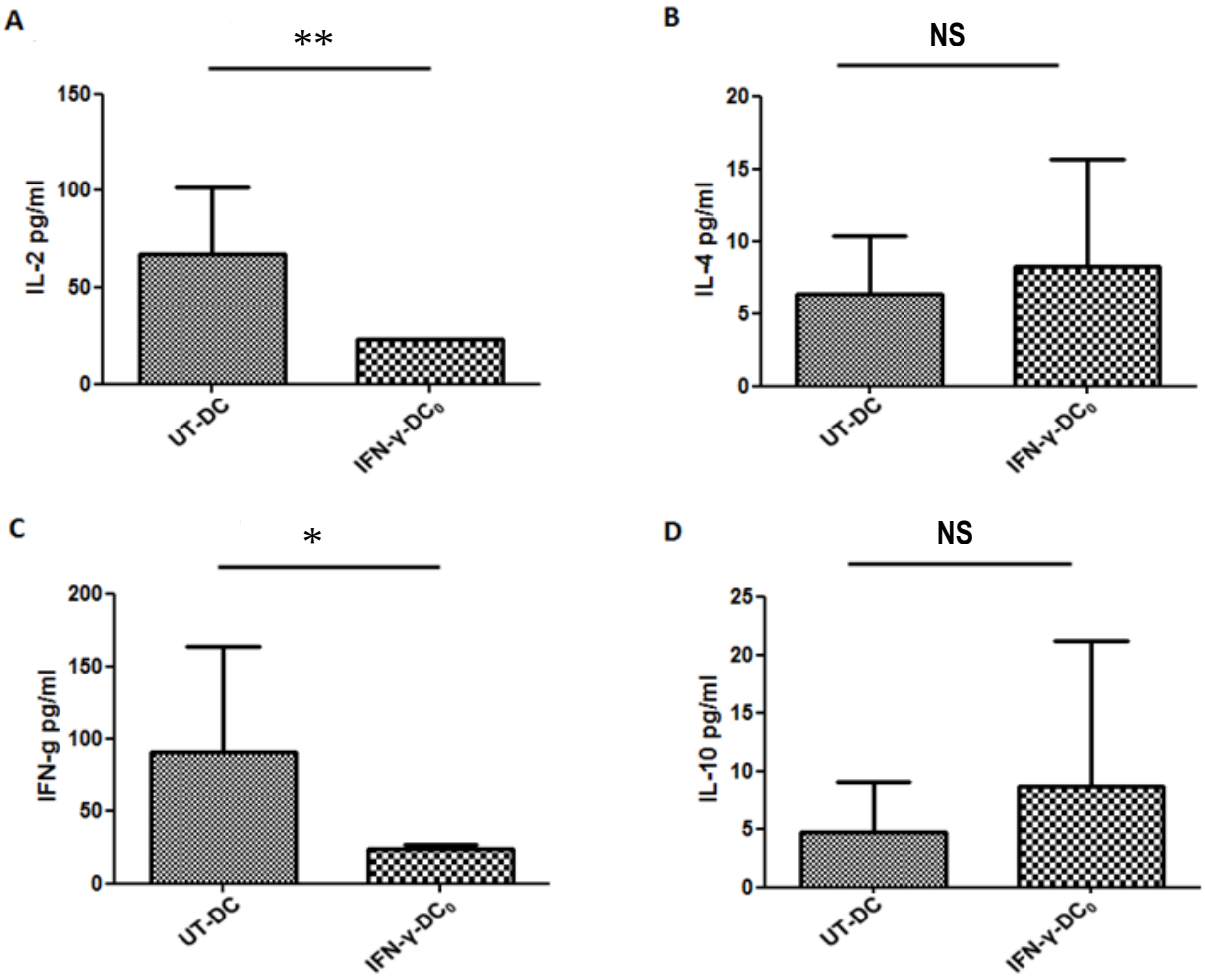

FIGURE 3.3.4B: ***IFN-γ-DC$_0$ co-cultured with T-cells inhibits the production of IL-2 and IFN-γ*****. DC were co-cultured for 6 days with 1x10^5 T-cells (1:100, DC to T-cell ratio).** Supernatants from co-cultures were harvested and analysed by cytometric bead array assay (as described in chapter 2.3.6) for the production of IL-2 (A), IFN-γ (B), IL-4 (C) and IL-10 (D) (Mean ± SD of 5 independent experiments). *=p<0.05, **=p<0.01, NS=No significance.

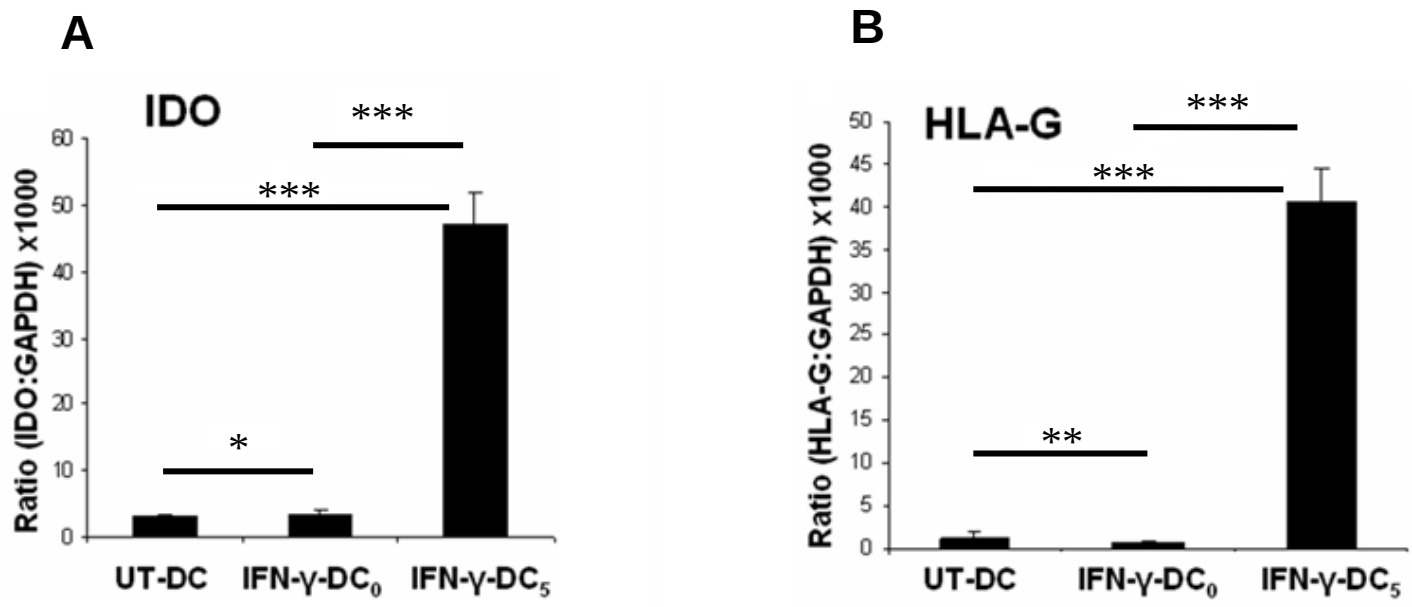

FIGURE 3.3.4C: ***IFN-γ-DC$_0$ do not have upregulated expression of soluble inhibitory molecules IDO and HLA-G.*** DC were treated with IFN-γ at day 0 (IFN-γ-DC$_0$) and at day 5 (IFN-γ-DC$_5$) or cultured in the absence of IFN-γ (UT-DC) for 5 days, then matured with TNF-α for 2 days. DC total RNA was extracted and reverse transcribed. The mRNA expression of immunomodulatory molecules IDO (A) and HLA-G (B) were determined by quantitative PCR (n=5). *=p<0.05, **=p<0.01, ***=p<0.001. Mean ± SD of quadruplicates. Figure representative of 5 independent experiments.

3.3.4. DISCUSSION

IFN-γ has classically been considered as a proinflammatory molecule known to primarily drive Th1 allo-responses [218]. In this chapter the timing of IFN-γ exposure during DC development was investigated, in order to examine its capacity to negatively regulate DC function. In this study an *in vitro* model of DC differentiation with GMCSF and IL-4 was used to assess the immunomodulatory effects of IFN-γ at day 0 and day 5 of DC propagation, to define its effects during DC differentiation and maturation. It was found that the anti inflammatory function of IFN-γ was time dependent. Treatment at day 0 (IFN-γ-DC_0), but not at day 5 down regulated positive costimulatory molecule expression and remarkably inhibited the expression of DC maturation marker CD83, which also known to play an important role in the activation of T-cells [234, 235]. This study also observed an up regulation of inhibitory molecules ILT2, ILT3 and ILT4, a finding which has not been previously described. It is speculated, that the known antagonistic effects of IFN-γ on IL-4 may play role in the mechanism, by which IFN-γ exerts its regulatory function.

IFN-γ is known to inhibit IL-4 activated STAT-6 phosphorylation, which reduces IL-4 receptor expression [236]. The promoter region of DC development factor IRF4 has a STAT-6 binding element, which helps to regulate its expression. Therefore it is speculated that the reduction of STAT-6 phosphorylation may inhibit the downstream expression of IRF4 [237, 238]. IRF4 also binds to PU.1 a negative regulator of ILT2/ILT4. Thus inhibited IRF4 expression may also reduce binding of PU.1 resulting in increased expression of ILT2 and ILT4. The expression of immunoglobulin like transcript (ILT) molecules and in particular ILT3 and ILT4 are important in regulating allo-immune responses. ILT4 and ILT2 are known to bind to HLA-G, which has

immunosuppressive functions relevant to transplantation [239], ILT3 on the other hand has no known ligand [240-242]. Tolerogenic dendritic cells have been shown to inhibit allogeneic T-cell proliferation in a ILT3 and ILT4 specific manner as shown by mAb mediated blocking. High expression of these molecules is also important in the induction of CD4+CD25+ Treg cells [243, 244]. Moreover, signalling through these receptors causes a calcium-dependent down regulation of NF-κB transcription factor [240, 242, 243], which may contribute to the reduced expression of RelB observed in IFN-γ-DC_0.

RelB is a NF-κB transcription factor, and its nuclear expression is related to DC maturation [245, 246]. RelB is known to target genes that control the expression of MHC class II, CD80, CD86, CD40 and CD83 [247-249]. The reduced expression of co-stimulatory molecules and CD83 in IFN-γ-DC_0, may therefore be a downstream effect of RelB inhibition. Inflammatory cytokine IL-12p40 mRNA expression was also significantly lower in IFN-γ-DC_0 compared to UT-DC, suggestive of decreased production of IL-12 protein. Previously published data have demonstrated that IL-12 protein production positively correlates with mRNA expression of IL-12 [250]. These factors combined render IFN-γ-DC_0 immature with a reduced capacity to activate T-cells causing T-cell hyporesponsiveness.

T-cell hyporesponsiveness induced by IFN-γ-DC_0 is supported by previously published studies, which reported similar effects of IFN-γ on monocyte-derived APC [231, 232]. In contrast to these studies, it was demonstrated that IFN-γ-DC_0 are of DC lineage, through the expression of DC specific marker DC-SIGN [251]. In this chapter we have extended this observation of induced T cell hyporesponsiveness to examine the polarization to either a Th2 or Th1 response. We did this by examining the secretion of Th1 and Th2 cytokines during a DC-MLR. We found that IFN-γ-DC_0 reduced the production of IL-2 and IFN-γ in comparison to UT-DC.

Suggesting a de-polarization from Th1 response, however there is no evidence to suggest that a Th2 response was present. The reduced production of IL-2 and IFN-γ may be a factor contributing to the inhibition of T-cell proliferation by IFN-γ-DC_0 affecting the polarization of Th1 responses [219, 252, 253]. Although this assay did not take into account the differences of final number of cells, the overall immune response was blunted by IFN-γ-DC_0 in comparison to UT-DC. However this also does not exclude the induction of T-cell anergy by IFN-γ-DC_0, as mechanism of inducing T-cell hyporesponsiveness.

T-cell anergy is characterized by the induction of an unresponsive state in T-cells, where T-cells become functionally inactivated following stimulation through the T-cell receptor, in the absence of co-stimulation [254]. Given that IFN-γ-DC_0 have reduced co-stimulation in comparison to UT-DC, it was hypothesised that IFN-γ-DC_0 may induce T-cell anergy. Surprisingly, the addition of exogenous IL-2 did not reverse the T-cell hyporesponsiveness, unlike other studies [233], suggesting that IFN-γ-DC_0 does not induce IL-2 reversible T-cell anergy. However, this does not exclude cell division-arrest, whereby the IL-2 receptor is not induced by TCR signals [255, 256]. In addition, there is no evidence to suggest that IFN-γ-DC_0 induced T-cell hyporesponsiveness, correlates with the total number of Foxp3+ T-cells or the deviation to a Th17 response.

T-cell mediated immunoregulation is the primary mechanism known to maintain antigen-specific tolerance *in vivo* [257]. In particular $CD4^+$ $Foxp3^+$ T-regulatory cells (Tregs), have been described as the most potent suppressive T-cell subtype. Initially described in murine models as $CD4^+CD25^+$ highly suppressive T-cells by Sakaguchi [258], this T-cell subtype was later also described in humans [259-264]. Foxp3 has been described as the master gene, involved in the development and function $CD4^+$ Tregs [107, 265] [266]. Recent studies however have shown

that, unlike in murine models Foxp3^{+} cells are not homogenous in their function to be suppressive. Various studies have now shown that TCR stimulation alone can induce Foxp3 expression in naive CD4^{+}Foxp3^{-} T-cells, without conferring any suppressive function [267-270]. More recently it was described that activated human CD4^{+} T-cells expressing low levels of Foxp3 fail to be suppressive and rather are cytokine producing effector T-cells, whereas activated CD4^{+} Foxp3^{HI} T-cells are highly suppressive [271]. Thus measuring the total CD4^{+}Foxp3^{+} population alone does not define the suppressive capacity of this T-cell population in humans. This suggests that the reduced capacity of IFN-γ-DC$_{0}$ to promote Foxp3^{+} cells is a reflection of the its reduced capacity to activate T-cells rather than their ability to promote the generation of suppressive Tregs. Therefore this study was limited in its capacity to determine the ability of IFN-γ-DC$_{0}$ to promote a suppressive Treg population. However the reciprocal relationship that exists between Foxp3 Tregs and Th17 cells, and the observation that IFN-γ-DC$_{0}$ depolarizes a Th1 response and do not induce a significant generation of IL-17, suggests that Foxp3 Tregs are more like to be promoted by IFN-γ-DC$_{0}$ rather than UT-DC. However this does not exclude the possibly of IFN-γ inducible inhibitory soluble molecules or enzymes playing a role in IFN-γ-DC$_{0}$ mediated T-cell hyporesponsiveness.

Immunomodulatory molecules Indoleamine 2,3-Dioxygenase (IDO) and HLA-G are upregulated by IFN-γ [272-274]. IDO is an enzyme that catabolises tryptophan to inhibit T-cell proliferation [274]. HLA-G is a ligand for ILT2 and ILT4, where binding to its receptors mediates its immunomodulatory effects [275, 276]. Surprisingly, mRNA of IDO and HLA-G was not induced in IFN-γ-DC$_{0}$, thus unlikely to play a role in IFN-γ-DC$_{0}$ induced T-cell hyporesponsiveness. Significant increases of HLA-G and IDO mRNA in IFN-γ-DC$_{5}$ did not affect their ability to stimulate T-cells when compared to UT-DC. A surprising result given the inhibitory function of these molecules, but not uncommon given that other studies have shown

the same phenomenon [277-279]. However, the regulatory effects of IDO and HLA-G may be overcome by the robust positive co-stimulation provided by IFN-γ-DC_5. Munn and colleagues have suggested that IDO can be also be present in enzymatically active and inactive states [278]. Accordingly, IDO may be present in IFN-γ-DC_5 in an inactive state, thus unable to contribute to the T-cell regulation.

In summary this chapter has demonstrated that the timing of IFN-γ exposure during DC development is important for its function as either a pro-inflammatory or anti-inflammatory cytokine. IFN-γ exposure only at day 0 during DC differentiation (IFN-γ-DC0) was able to down regulate the expression of positive co-stimulatory molecules and most importantly maturation marker CD83, a function not seen in IFN-γ-DC_5. It is speculated that early monocyte exposure to IFN-γ during differentiation, antagonistically inhibits IL-4 activated STAT-6 phosphorylation, and the downstream expression of IRF4, which precipitates in a cascade of events leading to maturation arrest. It is believed that IRF4 gene inhibition promotes the expression of ILT inhibitory molecules, which signal to inhibit RelB expression. Inhibited expression of RelB in turn arrests DC maturation resulting in CD80/CD86 down regulation and inhibition of CD83. Subsequently, the immature phenotypic features of these DC, in particular their poor stimulatory capacity contributes to the observed T-cell hyporesponsiveness. However this is speculative and warrants confirmation at the molecular level, in particular it is important to demonstrate that STAT-6 phosphorylation and IRF4 are inhibited in addition to RelB protein expression and translocation into the nucleus. The ability of IFN-γ-DC_0 to generate T-regs still remains elusive due to the limitations associated with the T-cell assay used in this chapter. Human Treg populations should therefore in future be defined according to recent publications, which have defined the $Foxp3^{HI}$ population as suppressive, in addition to *in vitro* suppression

assays to show functionality. Nevertheless IFN-γ-DC_0, may potentially serve as a tolerogenic DC for the cellular therapy of allograft rejection. The standard DC propagation protocol to generate IFN-γ-DC_0, which takes 7-10 days is suitable for living donor kidney transplantation, where there donor and recipient pairs are identified well in advance. However it is not applicable to deceased donor transplantation and in particular islet cell transplantation, where living donors are not suitable. Therefore, a novel propagation strategy to generate IFN-γ modulated DC in 48 hours was investigated in chapter 4.

CHAPTER 4 - IFN-γ inhibits STAT-6 signalling in human monocytes promoting the rapid generation of tolerogenic dendritic cells

4.1 INTRODUCTION

Dendritic cells (DC) play a key role as sentinels of the immune system for the detection of pathogens and disturbance of the immunological milieu [280]. All transplantable organs possess a cellular component of passenger leukocytes, which migrate from the allograft to initiate rejection via the direct pathway of allorecognition [126, 129, 281, 282]. DC are an important component of the passenger leukocyte population [135, 136, 283]. However, under certain conditions DC may be modulated to become tolerogenic and inhibit T-cell function. In particular, immature DC (iDC) have been shown to inhibit allogeneic T-cells proliferation [184, 284, 285], while the injection of antigen pulsed iDC block T-cell responses in humans [286]. However, iDC undergo maturation *in vivo* limiting their tolerogenic potential. The modification of DC to stably inhibit maturation has been extensively studied in recent years. A variety of pharmacological and immunological approaches including interleukin 10, vitamin D3, dexamethasone, aspirin and most recently the NF-κB inhibition by curcumin was shown to arrest DC in an immature state and promote tolerance *in vitro* and *in vivo*. [143, 144, 149, 150, 152, 287].

The use of tolerogenic DC to modify the recipient immune system to promote allograft acceptance is a potential alternative therapeutic approach, which has the advantage of not requiring conventional immunosuppressive therapy. However their use in the clinic has faced significant hurdles - current protocols to generate monocyte-derived DC take 7-10 days [288] while rejection begins within hours of transplantation and is well established within 7 days [289]. Clinically useful DC must be generated in a short period of time. In 2003, Dauer and colleagues published a 'FAST-DC' protocol to generate potent immunostimulatory mature

monocyte-derived DC for the purpose of cancer immunotherapy in 48hr [205]. In the present study a complementary approach to rapidly generate stable immature DC was developed.

IFN-γ is regarded as a potent proinflammatory cytokine and is secreted by $CD4^+$ T-helper type 1 (Th1) lymphocytes, $CD8^+$ cytotoxic lymphocytes and NK cells, which play key roles in allograft destruction [290, 291]. IFN-γ also plays an essential role in allograft acceptance. IFN-γ KO models of skin and cardiac allograft transplantation demonstrate that IFN-γ is essential for prolonging graft survival and inducing tolerance [227-229]. However, IFN-γ also has an immunomodulatory role as illustrated by knockout (KO) mouse models of experimental autoimmune encephalomyelitis and collagen induced arthritis where IFN-γ is required for protection against disease onset and severity [223-226]. Moreover, a recent study by Wu and colleagues showed IFN-γ was required for the negative regulation of DC migration and T-cell priming [292]. In chapter 3 it was demonstrated that the timing of IFN-γ exposure is critical in its function to either inhibit or promote the stimulatory potential of DC. It was shown that negative regulation of DC stimulatory capacity is only mediated by IFN-γ if exposure occurs early during DC differentiation at the monocyte level [156].

In this chapter the mechanism of action by IFN-γ, where by the maturation arrest of DC maturation was further examined, extending chapter 3 by developing a 'rapid' DC protocol to produce tolerogenic DC in the clinically applicable timeframe of 48 hours. Here it was demonstrated that the effect of IFN-γ is mediated by the inhibition of STAT-6 phosphorylation and NF-κB activation resulting in DC maturational arrest and the development of a tolerogenic DC phenotype. These rapidly generated IFN-γ modulated DC support the generation of antigen specific T-regulatory cells *in vitro*. These cells were then used in a surrogate NOD-

SCID chimeric model of islet transplantation. However poor human cell engraftment into the NOD-SCID mouse model, failed to demonstrate the tolerogenic potential of IFNγ-DC.

4.2 METHODS AND MATERIALS

4.2.1 Antibodies

The phenotypic profile of DC was defined using the following directly conjugated mAb: anti-CD83-FITC (HB15e) (eBioscience, USA), anti-CD86-FITC (FUN1) (BD Bioscience, USA), anti-CD80-FITC (L307.4) (BD Bioscience, USA), anti-DC-SIGN-FITC (DCN46) (BD Bioscience, USA), anti-HLADR-PE-Cy5 (G46-6) (BD Bioscience, USA), anti-PD-L2 (M1H18) (eBioscience, USA) and rat anti-human ILT4 (42D1) used as a primary mAb and FITC-conjugated anti-rat IgG was used for detection. STAT-6 phosphorylation was detected by intracellular staining, using anti-pY641-Alexa488 (Clone 18) (BD Bioscience, USA)and surface staining with CD14-PE (M5E2) (BD Bioscience, USA). T-cell phenotypes were determined using anti CD25-PE-Cy7 (M-A251) (eBioscience, USA), anti CD4-Percp5.5 (OKT4) (eBioscience, USA)and anti-human Foxp3 PE-conjugated mAb (PCH101) (eBioscience, USA). IL-4 receptor expression was detected using anti-human CD124 PE conjugated (hIL4R-M57) (BD Bioscience, USA). Anti-human RelB polyclonal antibody (Santa Cruz Biotechnology, USA) was used as the primary antibody to detect localisation of RelB by immunohistology. For further antibody details please refer to chapter 2 section 2.16.1.

4.2.2 Generation of 'FAST' human monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMNC) were isolated from buffy coat of healthy human blood donors (Australian Red Cross Blood Service, Adelaide, South Australia) by Ficoll Paque density gradient centrifugation as described in chapter 2 section 2.1.1. DC were rapidly generated as described in section 2.2.2, chapter 2. Monocytes were cultured in RPMI 1640 containing 10% FCS, 1000U/ml ($1.2x10^7$ U/mg) of granulocyte colony stimulating factor (GMCSF)-Leucomax™ (Sandoz Australia, Australia) and 500U/ml ($1x10^7$ U/mg) of IL-4 (eBioscience, USA) in the absence (UT-DC) or presence of 500U/ml of IFN-γ (eBioscience, USA) (IFNγ-DC) for 24h. Cells were then treated with 10ng/ml TNF-α (R&D Systems, USA) and 1 μM Prostaglandin E_2 (Sigma-Aldrich, USA) for a further 24h. All cell cultures were incubated under 5% CO_2 at 37°C.

4.2.3 FACS Analysis

*DC surface staining. (*Please refer to section 2.3.1 and 2.3.2 of chapter 2). To determine terminal differentiation of IFN-γ modulated DC, cells were harvested and extensively washed to remove all residual cytokines. Then DC were either analysed by FACS (Day 0 post wash-out) or were placed back into culture for 12 days in complete S10g media and then analysed (Day12 post wash-out).

Intracellular STAT-6 phosphorylation staining. Please refer to chapter 2 section 2.3.4. Monoclonal antibody targeting phosphorylated STAT-6 (pY641) (BD Bioscience, USA) was used to examine STAT-6 phosphorylation. Samples were analysed within 1hr of staining.

Treg phenotyping. For the enumeration of Foxp3+ cells, T-cells from the primary MLR were harvested and stained with directly conjugated mAb targeting CD25 (eBioscience, USA) and CD4 (eBioscience, USA) for 25 min at RT. Cells were stained for the intracellular expression of Foxp3 as per manufacturer's instructions. Briefly, cells were permeabilised and fixed (Fix/Perm buffer provided by kit) before blocking with normal rat serum to prevent non-specific binding. Cells were incubated with anti-human Foxp3. Numbers of $CD4^+CD25^+Foxp3^+$ T regulatory cells were assessed by flow cytometry. For detailed methods refer to section 3.1 and 3.3 of chapter 2.

Cytokine Cytometric Bead Array (CBA). A human TH1/TH2 CBA cytokine kit (BD Bioscience, USA) was used to determine the concentration of IFN-γ, IL-2, IL-10 and IL-4 in supernatants from DC-T cell co-cultures. DC were co-cultured with purified T-cells (1:10 stimulator to responder ratio) for 5 days in complete RPMI medium (10% FCS and 1% glutamine) in 96 round well bottom plates (under 5% CO_2 at 37°C). Supernatant was harvested and assayed, according to the manufacturer's instructions. The lower detection limits for the tested cytokines were: IL-2: 2.6 pg/ml, IFN-γ: 7.1 pg/ml, IL-4: 2.6 pg/ml, IL-10: 3.0 pg/ml.

4.2.4 Allogeneic mixed lymphocyte reaction assay

Primary MLR. DC were washed with PBS three times, irradiated (30Gy) and used as stimulators in the MLR. Allogeneic T-cells were purified from PBMNC using nylon wool packed columns (85% CD3+ T-cells) as described in section 2.1.2 chapter 2. DC were co-cultured with T-cell responders in 96-well round bottom, at stimulator to responder ratios of 1:10, 1:100 and 1:1000. After 4 days cells were pulsed with 1μCi [^{3}H]-Thymidine (Amersham, UK) for 18h and harvested onto glass-fibre filters and counted in β-scintillation fluid using a Wallac Microbeta Counter (Turku, Finland). Proliferation was expressed as counts per minute and expressed as the mean of 5 replicates, +/- SD.

Suppression Assay. Primary MLR were performed using naïve $CD4^+$ T-cells purified from PBMNC using a human $CD4^+$ T cell enrichment kit (Stem Cell Technologies, Canada) (95-98% CD4+ T-cells)(see methods chapter 2 sections 2.1.3). Naïve $CD4^+$ T-cells and IFNγ-DC were co-cultured at a ratio of 10:1. After 5 days, primed $CD3^+$ T-cells were harvested using a $CD3^+$ positive selection kit (Stem Cell Technologies, Canada) (see methods chapter 2 section 2.1.4). These IFNγ-DC primed T-cells were co-cultured with naïve $CD4^+$ T-cells (10^5 cells per well) at varying ratios of 1:1, 1:2, 1:4 or 1:8 from the same donor. In addition, at the 1:1 ratio an irrelevant non-related donor 3rd party DC was used to determine the antigen specificity of Treg cells. Cells were cultured for 5 days and thymidine incorporation was used to measure proliferation following an 18h pulse with 1μCi of [^{3}H] thymidine (Amersham, UK).

4.2.5 Gene expression analysis

RNA was extracted from DC using RNAspin mini kit (GE Healthcare, UK) (see methods 2.8.1 chapter 2) and cDNA was synthesized using 1μg total RNA by reverse-transcription using an oligo-dT primer (materials and methods 2.8.2 chapters 2). Quantitative Real-Time PCR based on a standard curve of copy numbers for each specific gene generated was used to analyse the expression of RelB, IL-12, IRF4 and GAPDH. PCR was conducted according to optimised conditions (Please refer to appendix A for PCR conditions and primer sequences. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene in order to verify RNA integrity and cDNA synthesis.

4.2.6 Immunohistology

DC ($2x10^5$) were spun onto slides at 20g (400 rpm) for 5 min using a Shandon Cytospin II (Thermo Scientific, USA). Staining conditions are described in section 5 chapter 2. Anti-RelB

polyclonal antibody was used as the primary antibody, and goat-anti-rabbit IgG-FITC (Santa Cruz Biotechnology, USA) was used to detect the protein localisation of NF-κB transcription factor RelB. Frozen spleen sections (5μm) from humanely killed mice islet transplanted mice, were stained with mouse anti-human CD3-FITC (20μg/ml) overnight in humid chamber at 4°C, after blocking (3% mouse serum in PBS) for 25min at RT.

4.2.7 IL-12 ELISA

DC were in 24 well plates ($1x10^6$ cells/ml) were stimulated with CD40L (500ng/ml) and IFN-γ (1000U/ml) for 48h. Supernatants were harvested and assayed for the biologically active human IL12p70 by ELISA (eBioscience, USA). For details please refer to section 2.6.1 in chapter 2.

4.2.8 Islet transplantation

NOD-SCID female mice of >20g of weight were I.P injected with 180mg/kg Streptozocin (STZ) (Sigma-Aldrich, USA) a dose previously published to be effective in inducing diabetes and optimised by our laboratory and shown to have an induction rate of 70% with low morbidity (see Appendix C). Diabetes was diagnosed by 3 consecutive readings of BGL of >16.6mmol/L. Diabetic mice were transplanted with approximately 3000IEQ of human islets 80% pure under the kidney capsule. All human islets used in this project had research consent given by donor families. This project was approved and over seen by the University of Adelaide (Approval number: M-2009-124) and IMVS (Approval number: 91_09) animal ethics committees.

4.2.9 Allogeneic challenge of islet transplant

DC were generated from monocytes obtained from the islet donor at the time of organ procurement. In order to so approximately 100ml of donor peripheral blood was collected into

9ml lithium heparin tubes (approximately 12 altogether). Blood was pooled and diluted 1 part blood 2 parts PBS, then 30ml of diluted blood was under laid with 15ml of ficoll (GE Healthcare, UK) and processed as described in section 2.2 (approximately 6-7 million DC were generated per donor). Prepared DC were mixed with allogeneic PBMNC (isolated as per chapter 2, section 2.1.1) at a 1:100 ratio (10^6 DC to 10^8 PBMNC) and resuspended with 300µl of sterile PBS, containing 50µl of anti-asialo GM1 (Wako BioProducts, Japan) as per manufactures' instructions. Using a 27 gauge insulin syringe mice were injected with DC: PBMNC via I.P 1 day post islet transplantation. Mice were sacrificed at 21 and 30days post allogeneic challenge. Spleen and transplanted kidney were harvested and placed into OCT. Approximately 200µl of blood serum was stored for human c-peptide analysis. Serum samples were analysed by the IMVS, Frome Road, Adelaide, which used IMMUNOLITE 2000 C-PEPTIDE, a solid phase two-site chemiluminescent immuno assay for the detection of human c-peptide (Siemens Healthcare, Llanberis, Gwyneed, UK).

4.2.10 mRNA analysis of transplanted islet tissue

Total RNA was extracted from frozen sections of tissue RNA spin column as per manufacturer's instructions (chapter 2, section 2.8.1). A total of 1µg of RNA was reverse transcribed using random hexamers (Qiagen, USA) (refer to chapter 2, section 2.8.3). Quantitative Real-Time PCR based on a standard curve using taqman primers according to the manufacturers' instructions (Applied Biosystems, USA) was used to analyse the mRNA expression of CD8A, CD4, IL-2 and IFN-γ (see chapter 2, section 2.9.3).

4.2.11 Statistical analysis

T-test and ANOVA statistical tests were conducted using Prism Statistical Software where appropriate. With statistical significance of $P < 0.05$.

4.3 RESULTS

4.3.1 IFN-γ in combination with the FAST-DC protocol generates phenotypically tolerogenic dendritic cells

The phenotype of monocyte-derived DC generated via the FAST-DC protocol (TNF-α and PGE_2 maturation) following pre-treatment with IFNγ (IFNγ-DC) was examined using flow cytometry (Figure 4.3.1A). Monocyte-derived DC that did not receive IFNγ pre-treatment (UT DC) were used as a control. Both groups showed marked upregulation of the DC-specific marker CD209 (DC-SIGN) and HLA-DR molecules. Pre-treatment with IFNγ, reduced the number of cells expressing the DC maturation marker CD83 to 22% (compared to 76% in UT-DC), without affecting CD209 expression. IFNγ-DC showed decreased expression of positive co-stimulatory molecules CD80 and CD86 by 17% and 10% respectively compared to UT-DC. The negative co-stimulatory molecule ILT4 was upregulated from 19% of UT-DC expressing ILT4 to 35% of IFNγ-DC, thus IFN-γ treatment increased ILT4 by 1.8 fold.

At the transcriptional level, quantitative real-time PCR demonstrated that IFNγ-DC produced 60% fewer transcripts of NF-κB transcription factor RelB, which was confirmed at the protein level (Figure 4.3.1B). Immunohistology also showed that UT-DC had distinct co-localisation of RelB in the nucleus; indicative of typical DC maturation in response to maturation stimuli. However, IFNγ-DC had reduced expression of RelB in the cytoplasm, with little to no translocation into the cell nucleus (Figure 4.3.1B). Similarly, pro-inflammatory molecule IL-

12p40 gene expression was markedly reduced in IFNγ-DC both at the level of messenger RNA (decreased by 86% compared to UT-DC) and protein (IFNγ-DC produced 62% less biologically active IL-12p70 compared to UT-DC) (Figure 4.3.1C).The ability of DC to terminally differentiate and stably maintain in their differentiated state is important for its applicability as a DC therapy. Thus the ability of IFNγ-DC to terminally differentiate was examined. IFNγ-DC were harvested after stimulation with maturation stimuli (after 24 hours) and washed extensively to remove all residue cytokines. IFNγ-DC were then returned to culture and maintained for 12 days in complete media without cytokines. After this wash-out period IFNγ-DC showed a marginal decrease in the expression of Lineage marker-DC sign by 2%, whilst CD83 expression changed by only 0.3% (Figure 4.3.1D). Thus, pre-treatment of human monocytes with IFN-γ prior to exposure to maturation stimuli, produces DC with a phenotype that has been associated with tolerogenic properties, shown by cell surface molecule expression, transcriptional profile and cytokine production.

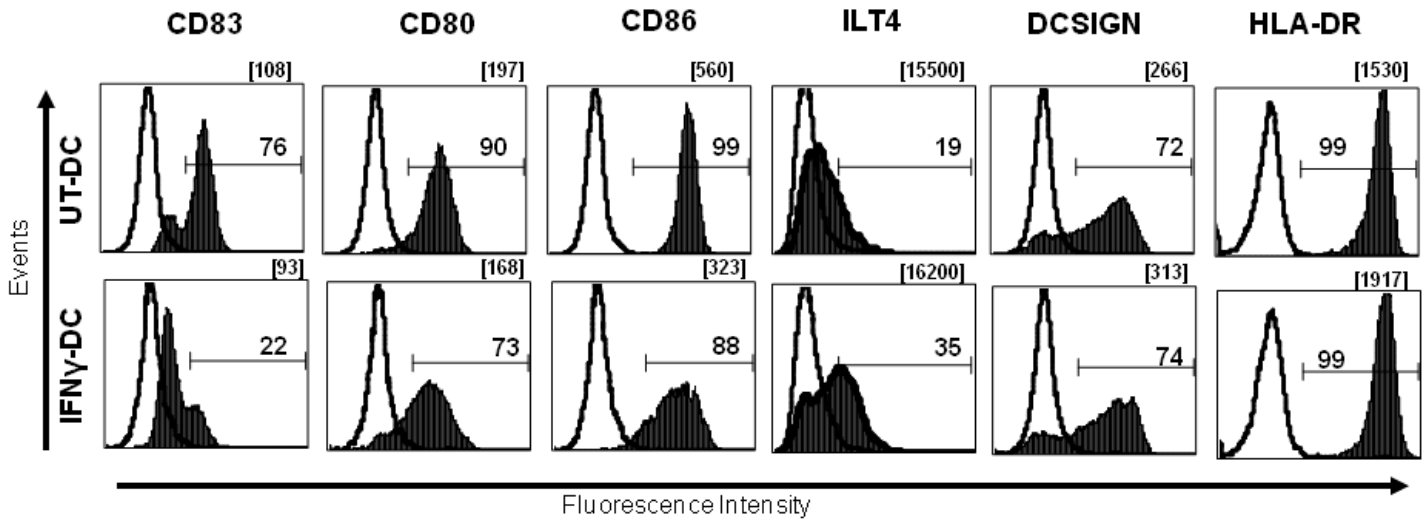

Figure 4.3.1A: IFN-γ in combination with the FAST-DC protocol generates phenotypically tolerogenic DC. Monocytes were isolated from peripheral blood and cultured in the without (UT-DC) or with IFN-γ (IFNγ-DC) in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml] for 24h. DC were subsequently treated with TNF-α and PGE_2 for another 24h period. Flow cytometry was used to analyse the phenotype of IFN-γ treated DC and compared to UT-DC. The following co-stimulator and Lineage markers were examined: CD83, CD80, CD86, HLA-DR and DC-SIGN. Solid line indicates the isotype control and solid histogram shows the tested mAb. This figure is representative of 6 independent experiments

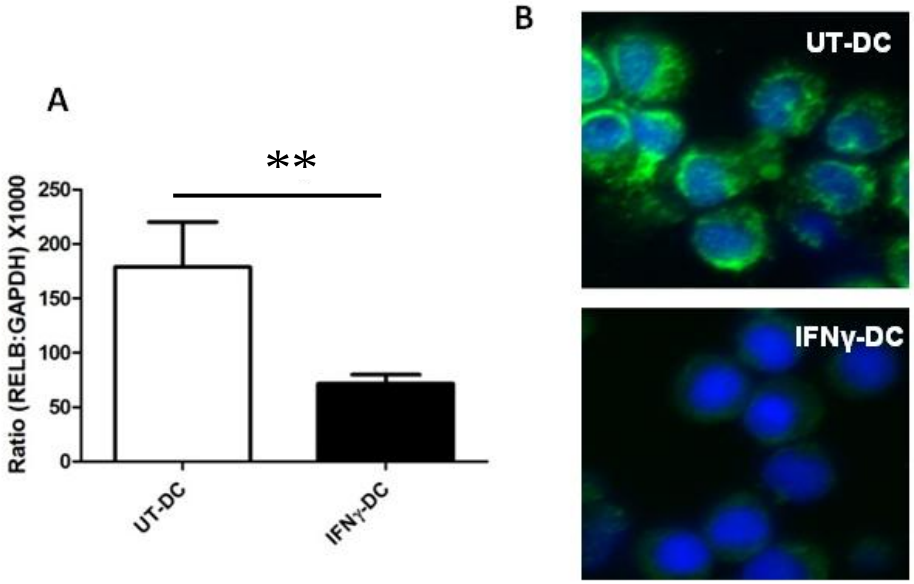

Figure 4.3.1B: IFN-γ treated DC have reduced expression and production of RelB. Isolated monocytes were treated with IFN-γ (IFNγ-DC) or without (UT-DC) in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml] for 24h and matured for another 24h period with TNF-α [10ng/ml] and PGE_2 [1μm]. (A) Quantitative real-time PCR was used to determine the gene expression of NF-κB transcription factor RelB. (B) Fluorescence microscopy was used to visualise the protein co-localisation of RelB, where blue depicts DAPI nucleus staining and green shows RelB expression. Mean ± SD **p<0.001. This figure is representative of 4 independent experiments

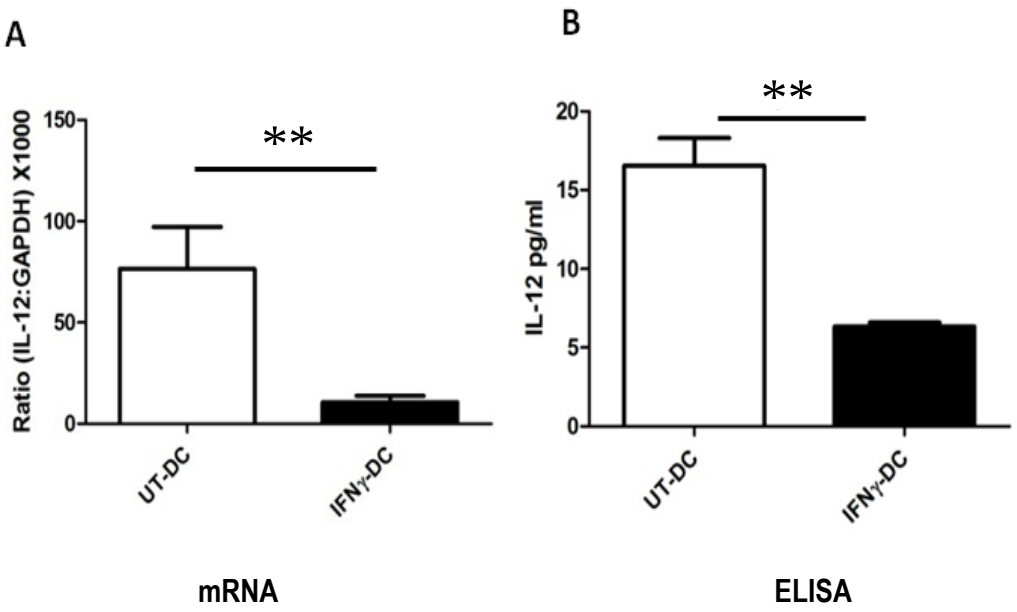

Figure 4.3.1C: IFN-γ treated DC have reduced expression and secretion of proinflammatory cytokine IL-12. Isolated monocytes were treated with IFN-γ (IFNγ-DC) or without (UT-DC) in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml] for 24h and matured for another 24h period with TNF-α [10ng/ml] and PGE_2 [1μm]. (A) Quantitative real-time PCR was used to determine the gene expression of IL-12. (B) ELISA was used for the detection of biologically active IL-12p70. Mean ± SD **=p<0.001. This figure is representative of 4 independent experiments

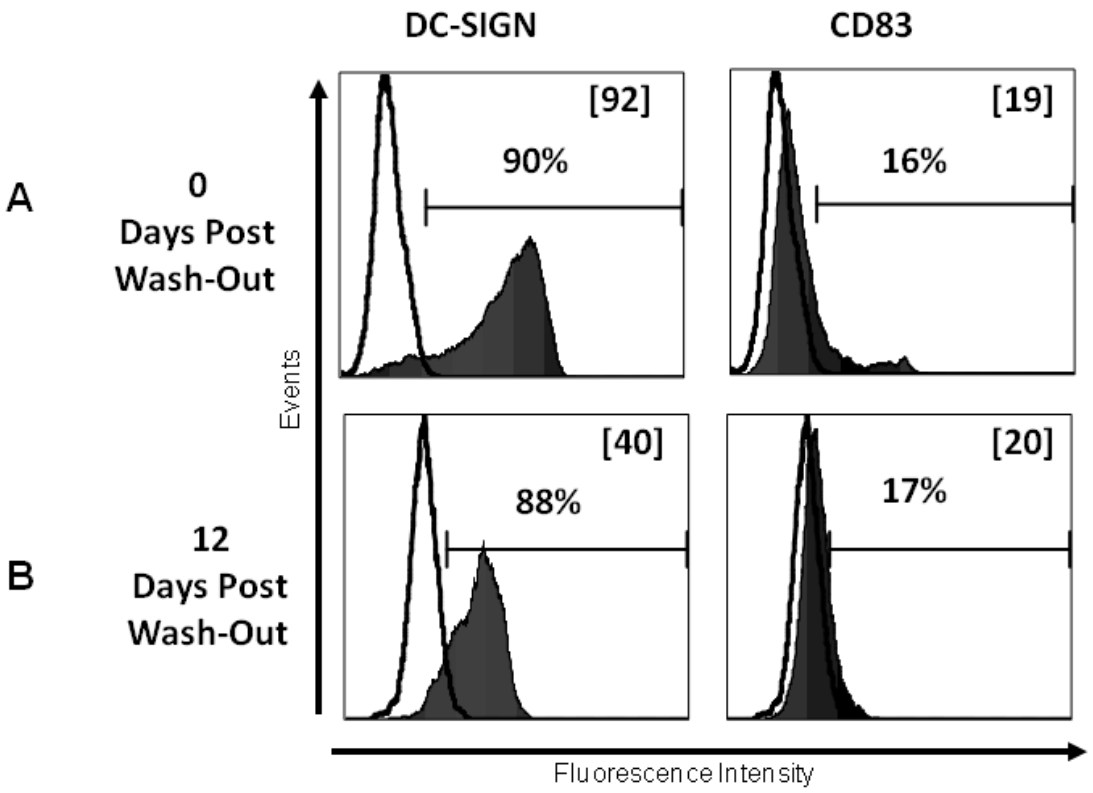

Figure 4.3.1D: IFN-γ treatment does not affect the ability of DC to terminally differentiate. Isolated monocytes were treated with IFN-γ [500U/ml] in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml] for 24h. DC were then stimulated with TNFα [10ng/ml] and PGE_2 [1μm]. After another 24h cells were harvested and washed to remove cytokines and returned to culture (wash-out period). Cells were surfaced stained for DC Lineage marker DC-SIGN and maturation marker CD83, at day 0 (A) and day 12 (B) post wash-out period. Black line represents isotype control and solid histogram depicts tested mAb. Representative of 5 independent experiments.

4.3.2 IFN-γ inhibits IL-4 driven STAT-6 in monocytes

To investigate the mechanism of IFNγ-mediated effects on monocyte-derived DC, human PBMNC were treated with or without IFN-γ in the presence of IL-4 and GMCSF to induce the activation mediated phosphorylation of STAT-6. Flow cytometric analysis showed that IFN-γ pre-treatment inhibited the phosphorylation of STAT-6 in monocytes by 65% compared to IL-4 and GMCSF alone (Figure 4.3.2A). STAT-6 is known to be involved in the transcription of IRF-4 molecule, which is upregulated during DC maturation [237]. Accordingly, the downstream effects of STAT-6 inhibition on the transcription of IRF-4 gene were investigated. PCR demonstrated that the inhibition of STAT-6 in IFNγ-DC reduced the expression of IRF4 by 78%-95% compared to UT-DC as determined by 6 independent experiments (Figure 4.3.2B). The IL-4α chain of the IL-4 receptor is essential in the recruitment and phosphorylation of STAT-6, which in turn regulates its own IL4α chain expression [293]. Therefore the cell surface expression of IL-4α receptor subunit was examined. IFN-γ down regulated the protein expression of IL-4α chain subunit in treated monocytes to 29% compared 42% in IL-4 and GMCSF alone. Monocytes alone had a baseline expression of 38% (Figure 4.3.2C).

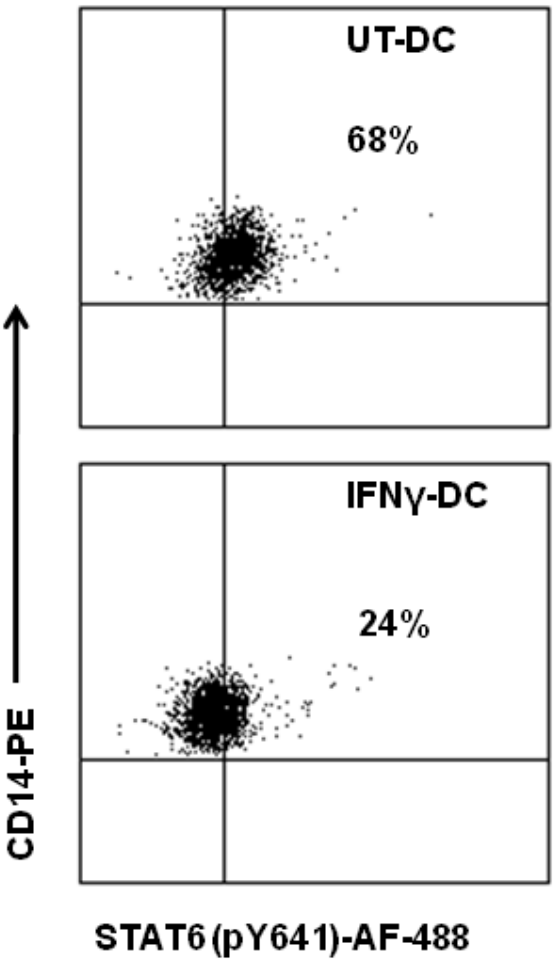

Figure 4.3.2A: IFN-γ treatment of monocytes inhibits the phosphorylation of STAT-6. Isolated PBMNC treated with or without IFN-γ [500U/ml] in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml] for 10 minutes. Cells were then immediately fixed and then permeabilised (using BD™ Cytofix (containing formaldehyde) and BD™ Phosflow perm buffer, see materials and methods section 2.3.4 - chapter 2). Cells were surface stained for CD14-PE and intra-cellularly stained for STAT-6(pY641)-AF-488, to determine STAT-6 phosphorylation. Representative of 6 independent experiments.

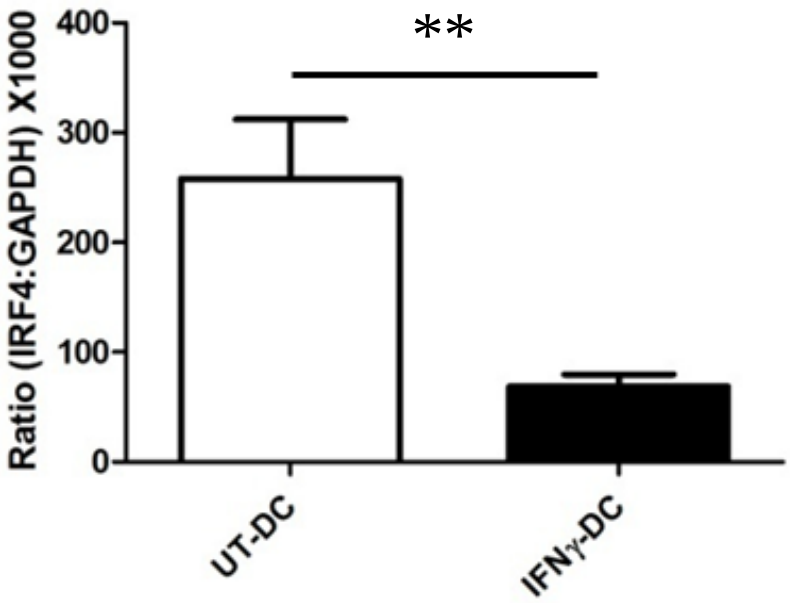

Figure 4.3.2B: IFN-γ treatment of monocytes inhibits the downstream gene expression of IRF4. Quantitative real-time PCR was used to analyse the mRNA expression of IRF4 in DC post maturation with [10ng/ml] and PGE_2 [1μm]. The mean ± SD of quadruplicate samples within experiment **=p<0.01. This figure is representative of 6 independent experiments.

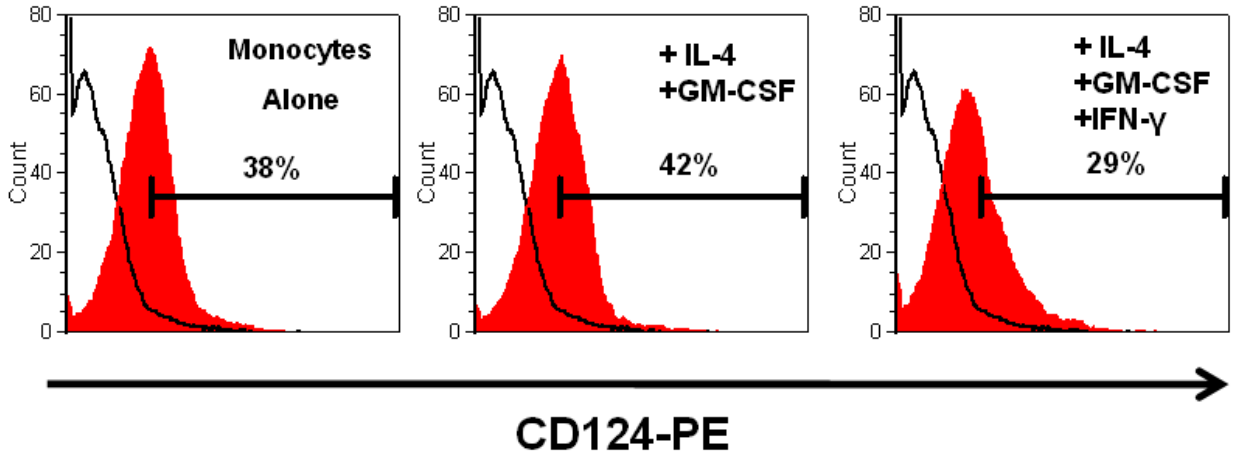

Figure 4.3.2C: ***IFN-γ regulates the expression of IL-4 receptor.*** IL-4 receptor expression was determined by FACS analysis using anti-CD124 24h post treatment with + or - IFNγ [500U/ml] in the presence of IL-4 [500U/ml] and GMCSF [1000U/ml]. Solid black line defined the isotype control, whilst the solid red histogram shows the test mAb. Representative of 3 independent experiments.

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4.3.3 IFNγ-DC *induce T-cell hyporesponsiveness that is associated with a reduction of IL-2 and IFN-γ*

The stimulatory capacity of rapidly generated DC to induce T-cell proliferation was measured in primary MLRs. IFNγ-DC significantly inhibited the proliferation of allogeneic T-cells by 71% at 1:10, 89% at 1:100 and 95% at 1:1000 DC to T-cell ratios (Figure 4.3.3A). Analysis of supernatants demonstrated that T-cell co-cultures with UT-DC contained significantly greater quantities of IFN-γ (UT-DC: 2300pg/ml vs IFNγ-DC: 240pg/ml, p=0.003) and IL-2 (UT-DC: 40pg/ml vs IFNγ-DC: 20pg/ml, p=0.02) compared to IFNγ-DC (Figure 4.3.3B).

4.3.4 IFNγ-DC promote the generation of antigen-specific T-regulatory cells

T-cells from a primary MLR were assayed for the enumeration of Tregs. IFNγ-DC increased the frequency of $Foxp3^{Hi}$ $CD4^{+}CD25^{+}$ T-cells from 7% by UT-DC to 17% by IFNγ-DC (Figure 4.3.4A). When IFNγ-DC primed T-cells were co-cultured with naïve T-cells at varying ratios, T-cell proliferation was suppressed at a 1:1 and 1:2 ratio, by 70% and 49% respectively (Figure 4.3.4B). Moreover at a 1:1 ratio, when stimulated instead with an irrelevant 3^{rd} party donor DC, there was a significant increase in proliferation compared to naïve T-cell alone by 30% (p=0.0004) indicating that IFNγ-DC generated T-regs were antigen specific in their suppressive function.

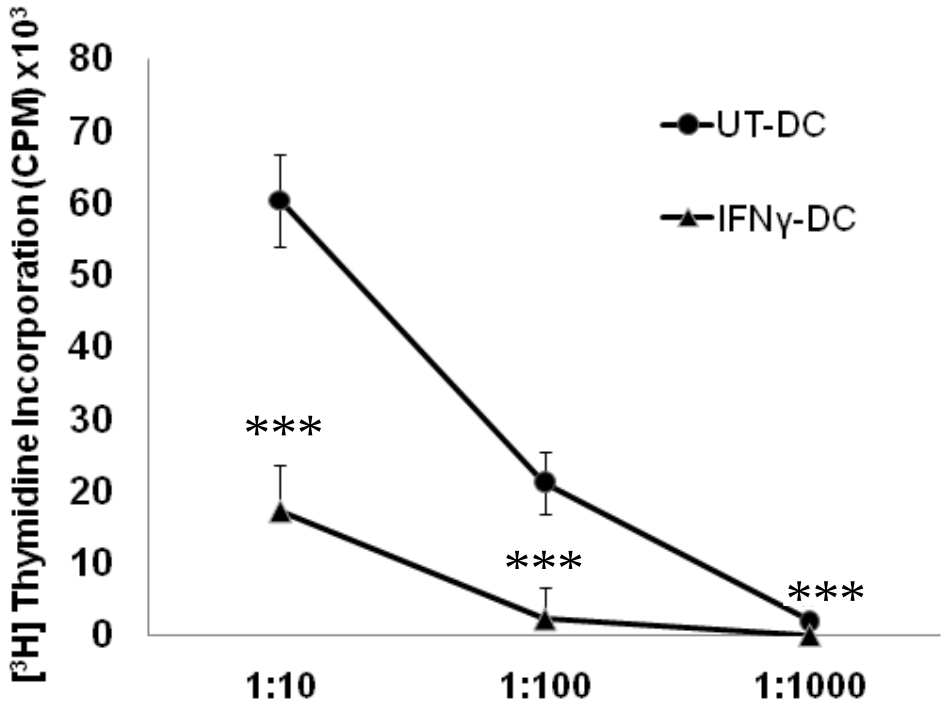


Figure 4.3.3A: IFNγ-DC induce T-cell hyporesponsiveness. Human monocytes were treated with IFN-γ (IFNγ-DC) or cultured in the absence of IFN-γ (UT-DC) for 24h in the presence of IL-4 and GMCSF, then matured with TNF-α [10ng/ml] and PGE_2 [1μm] for another 24h. Nylon wool T-cells ($1x10^5$) were incubated with DC, at a DC to T cell ratio of 1:10, 1:100 and 1:1000 for 5 days. Proliferation was determined by [^{3}H] thymidine incorporation (CPM). The mean ± SD ***=$p<0.001$ (This figure is representative of 10 independent experiments).

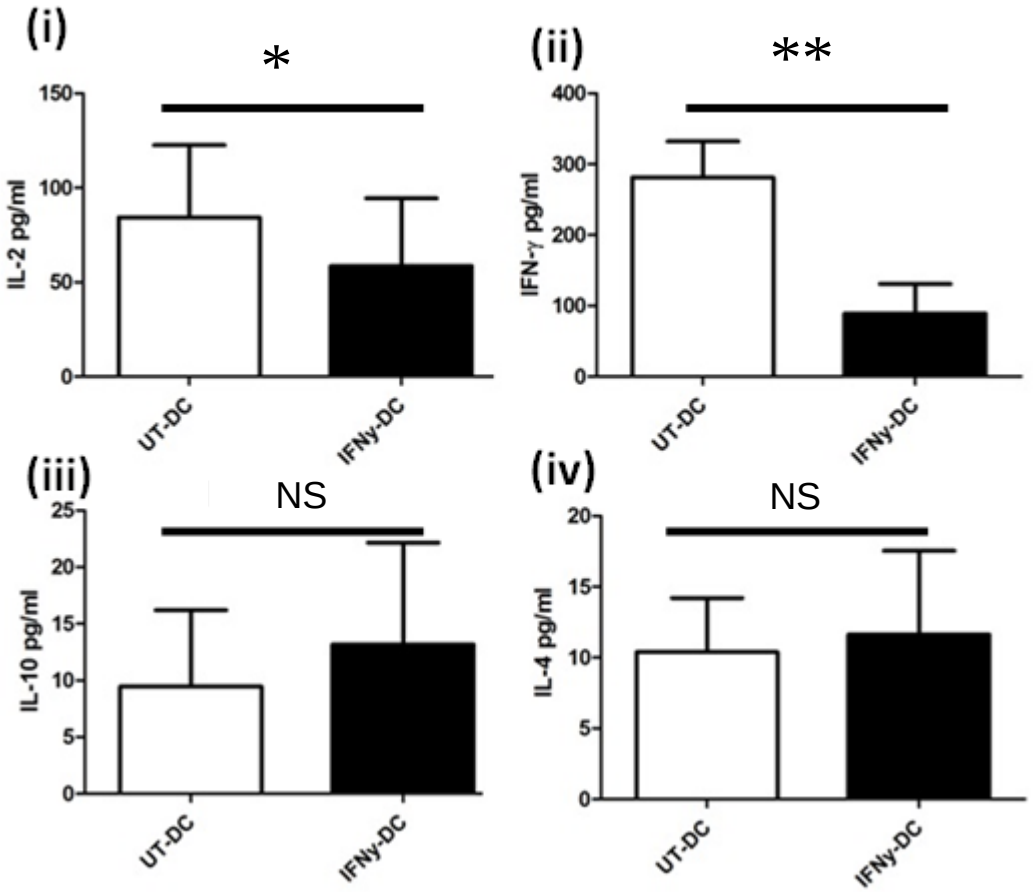


Figure 4.3.3B: ***IFN-γ modulated DC inhibit the production of IL-2 and IFN-γ.*** DC were co-cultured with purified T cells at 1:10 stimulator to responder ratios for 5 days. Supernatants from co-cultures were harvested and analysed by cytometric bead array for the production of IL-2 (i), IFN-γ (ii), IL-4 (iii) and IL-10 (iiii). The mean ± SD *p<0.02, NS= Not Significant. Representative of 4 independent experiments.

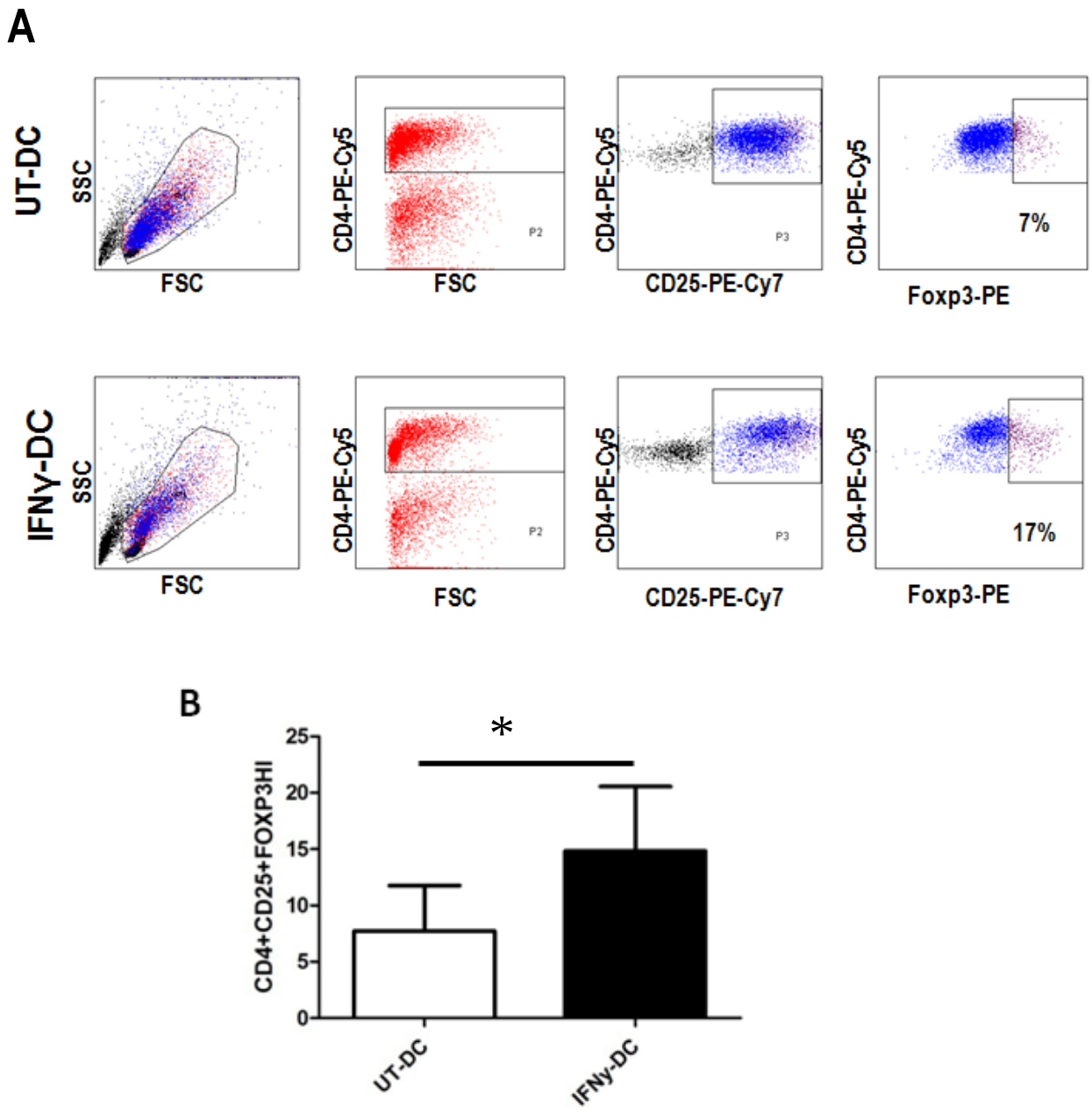


Figure 4.3.4A: ***IFNγ-DC promote the generation of antigen-specific Tregs.*** A, DC were co-cultured with CD3 T-cells for 5 days at a 1:10 DC to T-cell ratio and then stained for surface expression of CD4, CD25 and intracellular expression of Foxp3. Cells were gated for CD4+CD25+Foxp3^{HI} population. Samples were analysed by flow cytometry. B shows the percentage of positive cells for CD4+CD25+Foxp3^{HI} population (Mean ± SD of 4 independent experiments).*=$p<0.05$

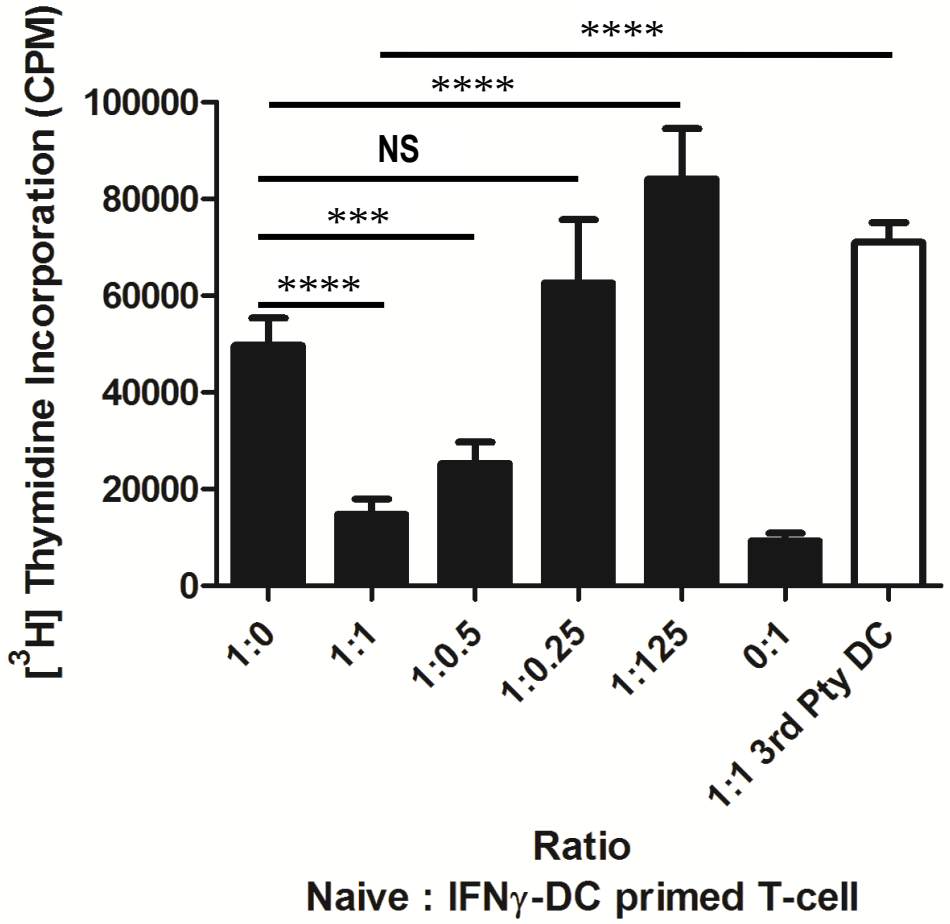


Figure 4.3.4B: ***IFNγ-DC primed T-cells are suppressive and antigen-specific Tregs.*** IFNγ-DC primed T-cells were isolated from primary MLR and co-cultured with unprimed $CD4^+$ T-cells at varying ratios of 1 naïve (remained constant at 10^3 per well) to 1 primed (1:1), 1:0.5, 1:0.25 or 1:0.125 and stimulated with UT-DC. In addition, at the 1:1 naive to primed T-cell co-cultures an irrelevant 3rd party DC was also used to determine the antigen specificity of Tregs. Cells were cultured for 5 days and proliferation was determined by [^{3}H] thymidine incorporation (CPM). Mean ± SD ***=p<0.001, ****=p<0.0001, NS=p>0.05. This figure is representative of 4 independent experiments

4.3.5 Humanised NOD-SCID mouse model of islet transplantation failed to show significant differences between UT-DC and IFNγ-DC *in vivo*

STZ dosages were batch tested, using a single dose range from 170-200mg/kg to determine the optimal dose (see appendix C). The STZ dose of 180mg/kg was chosen as it stably induced diabetes in 70% of animals tested, with the least amount of morbidity and a dose which has been successfully used by other groups [294]. Diabetes was established by hyperglycaemia (3 consecutive readings of >16.6 mmol/L), polyuria and weight loss. Isolated human islets of more then 80% purity and high viability as determined by dithizone staining (note all preparations used were of clinical grade, which were not used for human transplant due to insufficient numbers) were transplanted under the left kidney capsule of NOD-SCID mice (3000IEQ), which resulted in the reversal of the hyperglycaemic state to normal BGL. DC were propagated from donor-derived monocytes, on the day of pancreas procurement, approximately 100ml of donor blood resulted in the production of 6-7million donor monocyte derived-DC. The following day post transplantation, PBMNC from allogeneic donor were isolated and mixed at 1:100 DC to PBMNC ratio and administered via IP to transplanted mice. BGL were recorded on daily basis post transplantation. As demonstrated in figure 4.3.5A, from the 3 treatment groups there was no significant difference in BGL readings. Suggesting that graft function was similar between the groups, even 21 days post challenge. Plasma serum was taken from humanly killed mice and samples were analysed for c-peptide levels, a by-product of insulin production (Figure 4.3.5B). Although the mean value for the IFNγ-DC treated group was 10 and 20% higher than the mean value of UT-DC and PBMNC alone controls respectively, it failed to show significance ($p=0.988$ as determined by Kruskal-Wallis non-parametric test).

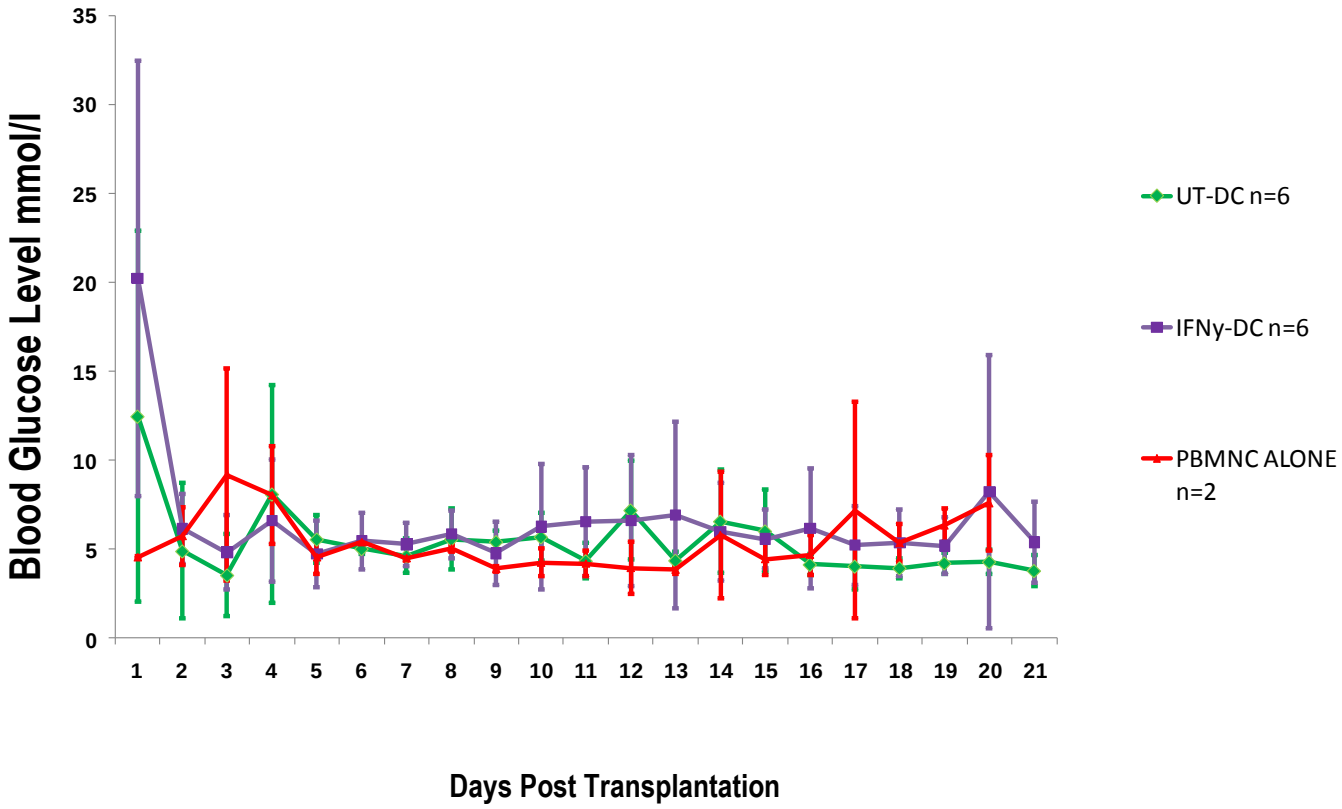


Figure 4.3.5A: ***Allogeneic challenge did not reverse euglycaemia 21 days post transplant.*** NOD-SCID mice were rendered diabetic by 180mg/kg of STZ via IP. Diabetic mice were transplanted with 3000IEQ of human islets under the kidney capsule. One day post transplant mice were challenged with allogeneic PBMNC and reconstituted with donor DC at a DC to PBMNC ratio of 1 to 100 via IP. Blood glucose levels were taken daily for 21 days post transplant. (±SD of daily BGL of mice within treatment group). Where n= number of mice per group.

A

ID	TREATMENT	C-peptide pmol/L	Mean	SD
166	UT-DC + allo-PBMNC	606		
185	UT-DC + allo-PBMNC	662		
173	UT-DC+ allo-PBMNC	307		
186	UT-DC + allo-PBMNC	616		
117	UT-DC + allo-PBMNC	165		
116	UT-DC + allo-PBMNC*	100	409	249
163	IFNy-DC- +allo-PBMNC	180		
182	IFNy-DC- +allo-PBMNC	1026		
187	IFNy-DC- +allo-PBMNC	847		
114	IFNy-DC- +allo-PBMNC*	100		
115	IFNy-DC- +allo-PBMNC*	100	450	449
122	PBMNC ALONE	516		
106	PBMNC ALONE	199	357	224.

B

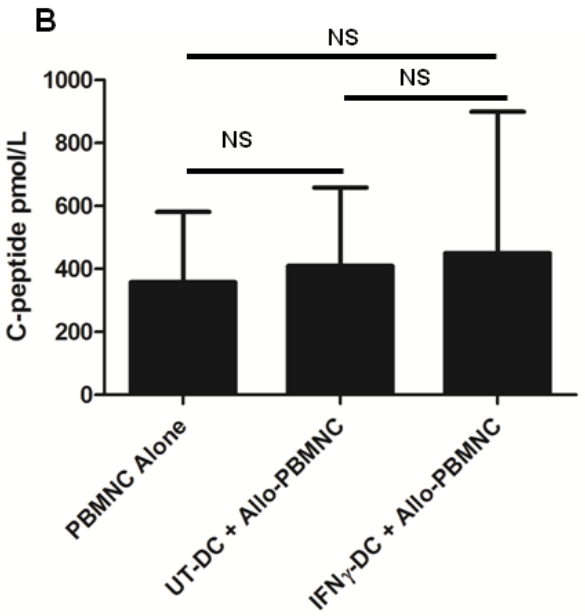


Figure 4.3.5B: On average IFNγ-DC treated mice had improved C-peptide serum levels but it failed to show significance. NOD-SCID mice were rendered diabetic by 180mg/kg of STZ via IP. Diabetic mice were transplanted with 3000IEQ of human islets under the kidney capsule. One day post transplant mice were challenged with allogeneic PBMNC. Plasma samples were isolated from blood samples and were analysed for C-peptide concentration, all results were tabulated in A. B, column graph of C-peptide serum levels mean ± SD of c-peptide level within treatment group. PBMNC (n=2), UT-DC (n=6) and IFNγ-DC (n=5). NS = no significance, 0.989 ($p>0.05$) as determined by Kruskal-Wallis non-parameteric test.

4.3.6 Human PBMNC engraftment evident in spleen and at graft site

Spleen from transplanted mice was stained with anti-human CD3-FITC antibody to detect PBMNC engraftment. Spleen sections from all treatment groups demonstrated the presence of human $CD3^+$ cells in the spleen (Figure 4.3.6A), of allo-challenged mice. Messenger RNA analysis of islet allograft by real-time PCR showed the presence of human CD8 and CD4 gene expression, indicative of T-cell infiltration of the graft (Figure 4.3.6B). Expression of these genes, varied considerably between animals. At day 21 post transplantation, mouse 166 (+UT-DC) showed significantly higher CD4 expression compared to 163 (+IFNγ-DC), whilst animal 165 (+UT-DC) did not. However 166 (+UT-DC) and 165 (+UT-DC) both had significantly higher CD8 expression compared to IFNγ-DC treated mouse 163 (Figure 4.3.6B). Day 30 post transplantation, there was no significant differences in the gene expression of CD4 and CD8 between mouse 116 (+UT-DC) and 115 (+IFNγ-DC) or 117 (+UT-DC) and 114 (+IFNγ-DC). The gene expression of Th1 cytokine IFN-γ and IL-2 were also examined (Figure 4.3.6 C). Both genes were present at day 21, where IFN-γ was significantly higher in mouse 166 and 165 (+UT-DC) compared to 163 (+IFNγ-DC). However at day 30 no IL-2 gene expression was observed, whilst contradictory to day 21 results mouse 115 (+IFNγ-DC) had significantly higher IFN-γ expression compared to 114 (+IFNγ-DC) and 117 (+UT-DC).

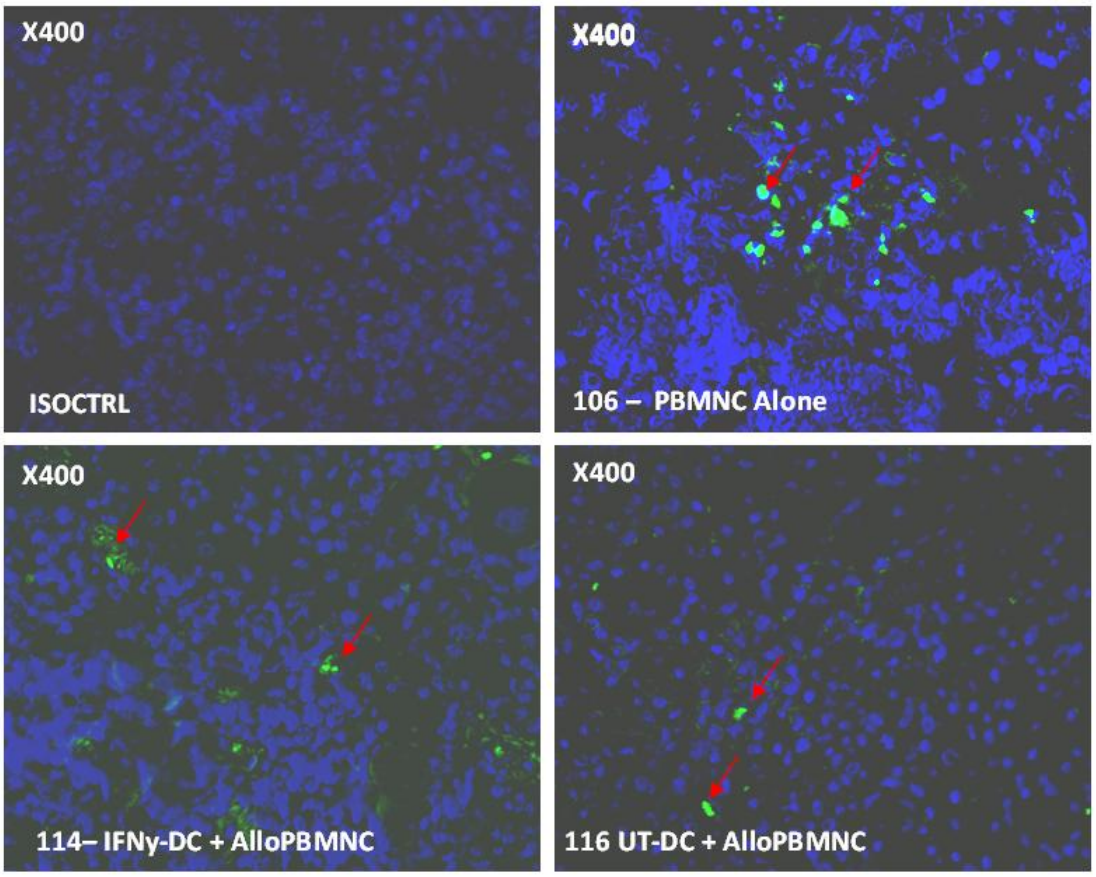


Figure 4.3.6A: Human CD3 positive cells found in the spleen of PBMNC reconstituted NOD-SCID mice. NOD-SCID mice were rendered diabetic by 180mg/kg of STZ via IP. Diabetic mice were transplanted with 3000IEQ of human islets under the kidney capsule. One day post transplant mice were challenged with allogeneic PBMNC and reconstituted with donor DC at a DC to PBMNC ratio of 1 to 100 via IP. Mice were sacrificed 30 days post transplantation and the transplanted kidney was snap frozen in OCT. Frozen sections of spleen transplanted mice were collected and stained with anti-human CD3-FITC conjugated mAb and DAPI for nucleus staining. Isotype control used as a negative control.

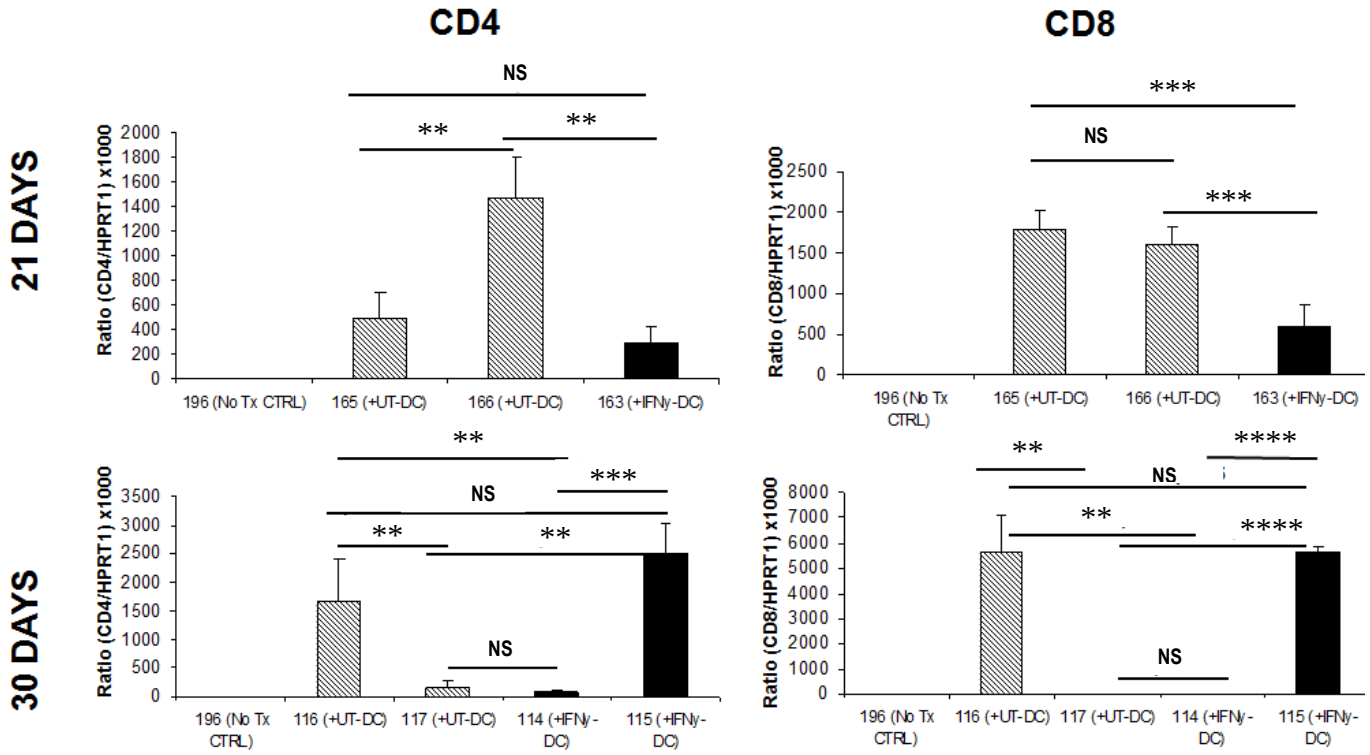


Figure 4.3.6B: Human CD4 and CD8 gene expression found at islet allograft site. NOD-SCID mice were rendered diabetic by 180mg/kg of STZ via IP. Diabetic mice were transplanted with 3000IEQ of human islets under the kidney capsule. One day post transplant mice were challenged with allogeneic PBMNC and reconstituted with donor DC at a DC to PBMNC ratio of 1 to 100 via IP. Mice were sacrificed either 21 days post transplantation or 30 days post transplantation and the transplanted kidney was snap frozen in OCT. Sections of islet allograft were collected and RNA was extracted and reverse transcribed. Taqman primers were used to detect the expression of human CD4 and CD8 by quantitative real-time PCR. Untreated /Un-transplanted control was used as a comparative control. HPRT1 was used as a house keeping gene. Mean ± SD of quadruplicate of sample **=p<0.01, ***=p<0.001, ****=p<0.0001, NS=p>0.05.

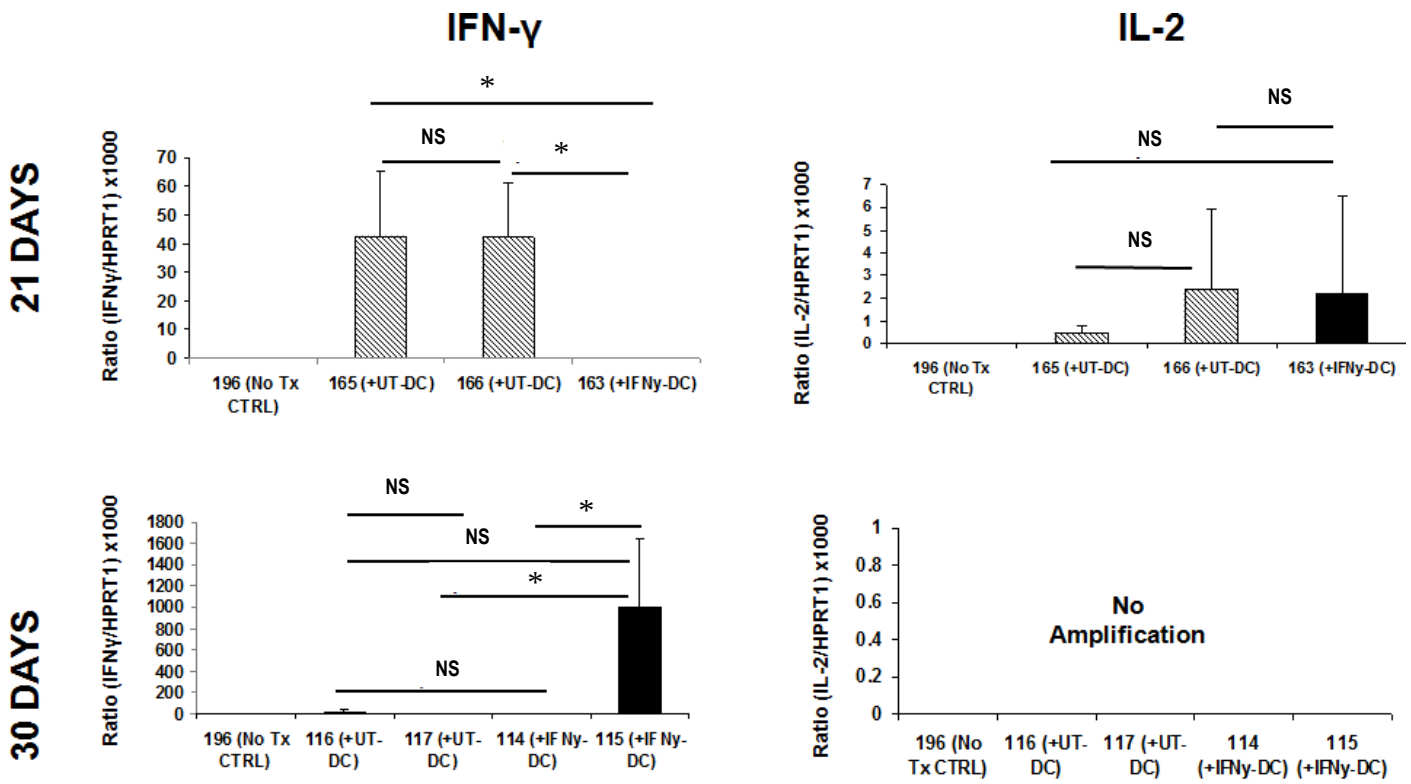


Figure 4.3.6C: Human proinflammatory cytokine gene expression observed at islet allograft site. NOD-SCID mice were rendered diabetic by 180mg/kg of STZ via IP. Diabetic mice were transplanted with 3000IEQ of human islets under the kidney capsule. One day post transplant mice were challenged with allogeneic PBMNC and reconstituted with donor DC at a DC to PBMNC ratio of 1 to 100 via IP. Mice were sacrificed either 21 days post transplantation or 30 days post transplantation and the transplanted kidney was snap frozen in OCT. Sections of islet allograft were collected and RNA was extracted and reverse transcribed. Taqman primers were used to detect the expression of human IFN-γ and IL-2 by quantitative real-time PCR. Untreated /Un-transplanted control was used as a comparative control. HPRT1 was used as a house keeping gene (Mean ± SD of quadruplicate of sample). *=$p<0.05$, NS= No Significance.

4.4 DISCUSSION

The toxicities inherent in modern immunosuppressive therapy has prompted efforts to develop novel drug free ways to manipulate or condition the immune system to promote allograft acceptance or tolerance. DC are a prime cell for manipulation through their unique ability to dampen immune responses and their known role in maintenance of self tolerance. For living donor transplantation, conventional 7 day protocols to generate DC may be used; however, for deceased organ donor transplants this is clearly not practical, in particular in the setting of islet transplantation.

In chapter 3 the immunomodulatory role of IFN-γ using a standard 7-day protocol was established. It was demonstrated that IFN-γ modulated monocyte-derived DC have a reduced capacity to induce T-cell proliferation. These DC featured a maturation arrested phenotype with increased expression of inhibitory molecules [156]. IFN-γ released in an allogeneic MLR, has also been previously shown to play a key role in promoting the generation of tolerogenic dendritic cells (Tol-DC) [295].

In this chapter the mechanism of IFN-γ modulated DC to suppress T cell responses was investigated in addition to the development a short-term DC protocol to promote the generation of maturation arrested Tol-DC in 48 hours. Rapidly generated IFNγ-DC express DC specific marker c-type lectin, CD209 (DC-SIGN) and HLA-DR, but have reduced CD83 and B7 molecule expression (Figure 4.3.1A). In 1995 CD83 was characterised as a maturation marker for DC [296]. Early studies demonstrated that herpes simplex virus type 1 infected iDC failed to induce the expression of CD83 during maturation without affecting the expression of CD80 and CD86, resulting in a poor capacity to stimulate T-cells. This was the first evidence to suggest that CD83 is essential in enhancing T-cell activation [234]. More recently the function of CD83

was further investigated, with the aid of siRNA knock down of CD83. Confirming further that CD83 functions as an enhancer of T-cell activation [235]. The inhibition of CD83 in IFNγ-DC therefore is an important factor contributing to their poor stimulatory capacity.

Mechanistically, IFN-γ reduced the expression NF-κB transcription factor RelB, which failed to co-localise into the nucleus of monocytes, this correlated with reduced RelB gene expression (Figure 4.3.1B). The nuclear translocation of RelB is a known hallmark of DC maturation [246]. The importance of the NF-κB pathway in DC development was also shown in knockout mice lacking components of NF-κB, where these mice failed to generate mature DC [297]. NF-κB transcription factors are known to interact with κB sites in the regulatory region of target genes that control the expression of MHC class II, CD80, CD86 and CD40. [247, 248]. In addition, NF-κB is known to regulate the expression of CD83 [249]. Thus the down regulation of positive costimulatory molecules including CD83 seen in IFNγ-DC may be a downstream effect of the inhibition of RelB expression and nuclear translocation. Moreover, studies using maturation resistant donor-derived RelB silenced DC, functionally induce antigen specific tolerance *in vivo*, thus prolonging murine heart allotransplantation [151]. Others have also reported the blocking of NF-κB using pharmacological agents, such as proteasome inhibitor PSI, LF 15-0195, aspirin, N-acetyl-cystine, cyclosporine and tacrolimus, which subsequently inhibit DC maturation [150, 298-301]. DC maturation arrest evident in IFNγ-DC was accompanied with low production of biologically active IL-12p70 secretion and IL12p40 gene expression (Figure 4.3.1C). Early administration of IFN-γ did not interfere with the ability of IFNγ-DC to terminally differentiate, subsequently remained maturation arrested and for positive for DC-SIGN 12 days post wash-out period of cytokines (Figure 4.3.1D).

The previously reported antagonist effects of IFN-γ on IL-4 were also observed by this study. IFNγ-DC had significantly reduced STAT-6 phosphorylation compared to UT-DC (Figure

4.3.2A). This is consistent with previous observations [236], showing that IFN-γ treatment of monocytes also reduced IL-4 activation of STAT-6, by suppressing the expression of the IL-4 receptor [236]. In this chapter it was also found that rapidly generated IFNγ-DC in the presence of IL-4 and GM-CSF also resulted in the downstream inhibition of the IL-4α receptor (Figure 4.3.2C). Transcription factor Interferon Regulatory Factor 4 (IRF4) a molecule also implicated in DC development and maturation was also down regulated by the inhibition of STAT-6 phosphorylation (Figure 4.3.2B). The IRF4 promoter region has a STAT-6 responsive element that specifically up-regulates IRF4 in response to IL-4. [237]. The poor expression of IRF4 may contribute to the lack of responsiveness of IFNγ-DC to mature. IRF4 also binds to transcription factor PU.1 to negatively regulate the expression of ILT4. Reduced expression of IRF4 may therefore contribute to sustained expression of ILT4 (Figure 1) [238]. As discussed in chapter 3, the signalling through these molecules is also known to inhibit NF-κB transcription, thus may also contribute to the reduced expression of RelB in these rapidly generated IFNγ-DC. Studies have directly demonstrated that the up-regulation of ILT3 and ILT4 is associated with the inhibition of NF-κB that render human monocytes and DC, tolerogenic [243].

The T-cell hyporesponsiveness induced by IFNγ-DC (Figure 4.3.3A) is due to reduced expression of positive co-stimulatory molecules and proinflammatory cytokines, that render IFNγ-DC poor T-cell stimulators, a characteristic that is associated with promoting a tolerogenic response [302]. Reduced concentration of IL-2 and IFN-γ in IFNγ-DC co-cultures (Figure 4.3.3B) is believed to contribute to the T-cell hyporesponsiveness. The ability of the IFNγ-DC generated by the fast-DC protocol however appeared to have a reduced capacity to stimulate T-cells compared to their equivalent generated by the standard DC protocol (figure 3.32A). This variability may be a reflection of differences of ILT4 expression, for instance 22% of standard IFNγ-DC expressed ILT4 verses 35% of fast-DC generated IFNγ-DC. T-cell phenotypes from

primary MLR were also examined by FACS (Figure 4.3.4A). IFNγ-DC promoted a higher frequency of $CD4^{+}CD25^{+}FOXP3^{HI}$ T-cells compared to UT-DC. Miyara *et al.* demonstrated that in humans the $Foxp3^{HI}$ subpopulation of $CD4^{+}CD25^{+}$ T-cells are highly suppressive, compared to the $Foxp3^{lo}$ subpopulation, which were characterised as cytokine producing effector T-cells. Moreover, IFNγ-DC primed T-cells were shown to be antigen specific in their ability to functionally suppress the proliferation of naive T-cells (Figure 4.3.4B). However it was observed in this assay that at 1:0.125 (Naïve: IFNγ-DC primed) T-cell ratio there was a significant increase in proliferation compared to the 1:0 ratio. This suggests that possibly the IFNγ-DC primed T-cells could be proliferating or may be acting as T-APC, which arises from a phenomenon known as 'trogocitosis' when DC share molecules such as HLA-DR to T-cells via lipid rafts allowing them to act as APC [63]. It may therefore be necessary to further characterise these IFNγ-DC primed T-cells by firstly placing them into a suppression assay where they are labelled with PKH and co-cultured with CFSE-labelled naïve T-cells, to discriminate between the proliferation of primed T-cells and naïve T-cells. Secondly, IFNγ-DC primed T-cells should be analysed for DC surface molecules such as HLA-DR, to determine their ability to act as T-APC. Never the less this non-specific proliferation was not seen at high ratios (1:1), where there was 8 times more primed T-cells per well compared to the 1:0.125. Suggesting that the suppression of the naïve T-cells was specific, and that the proliferation of primed T-cells to be less likely.

In order to test the *in vivo* properties of IFNγ-DC, a surrogate NOD-SCID chimeric model of islet transplantation was developed. Although there are several models of islet transplantation that exist with human islets, including transplantation of islets into the spleen and bone marrow [303, 304], none of these models have used donor-derived DC, which adds to the complexity of the model. Firstly, a large amount of blood (100ml) is required from the donor which is obtained

at the time organ procurement (with research consent from the families of the donor and overseen by the human ethics committee at the Royal Adelaide Hospital), to generate the donor-derived DC. From the time that the blood became available it was processed and DC were differentiated for 24 hr, during the time of islet isolation. The pancreas was shipped to isolating centres either in Sydney (Westmead Hospital) or Melbourne (St Vincent's Hospital), which form part of the Australian Islet consortium. The following day the islets were shipped back to Adelaide and transplanted as soon as possible into previously induced diabetic mice (note diabetic mice were stably diabetic and maintained using insulin pellets or injections), concurrently the donor-DC were matured for another 24hrs (with the approval of animal ethics committees of the University of Adelaide and the IMVS). Day post islet transplantation, PBMNC and DC were administered to transplanted mice. The procedure was logistically difficult to achieve and therefore was limited to the use of only South Australian donors. This NOD-SCID model was based on previously developed human-skin mouse transplant model, previously published by our laboratory, which also used donor-derived DC [305]. Nevertheless a total of 14 mice were transplanted for this study, 6 of which were treated with control DC (UT-DC), 6 with IFNγ-DC and 2 which were challenged with PBMNC alone no DC.

All treatment groups failed to demonstrate allograft rejection, as defined by the reversal of euglycaemia (Figure 4.3.5A and 4.3.5B). However mice spleen and the islet graft site demonstrated evidence of some PBMNC engraftment, with human CD3 protein expression (Figure 4.3.6A) and CD4, CD8 T–cell markers (Figure 4.3.6B) and Th1 proinflammatory gene expression (Figure 4.3.6C) in the spleen and graft site respectively. However, the human PBMNC engraftment was insufficient to cause complete islet graft rejection, a problem previously experienced in studies using the NOD-SCID humanised mouse model [306-308] [309]. Although anti-asialo 1 was used to inhibit the reported residual activity of NK activity in

the NOD-SCID mouse [310] [311], it may have not been sufficient to prevent NK cytotoxicity, thus limiting the potential of PBMNC and DC to engraft. Poor DC engraftment may have contributed to defective DC/T-cell interactions, resulting incomplete allograft rejection. It has been previous reported that human myeloid cells have a reduced capacity to engraft into immuno-compromised hosts, which limits the activation of T-cells by DC, rendering them anergic and ineffective in mediating graft rejection [310, 312, 313]. Secondly the DC to PBMNC ratio of 1:100 may have been insignificant to mediate a robust rejection response. Thus a 1:10 ratio may have given a better response, but the ratio in this study was limited by the number of DC generated from donor peripheral blood (approximately 6-7 million DC per 100ml of blood) Furthermore the dose of human islets of 3000IEQ is substantially larger than the constitutive islet mass found in mice, which also may have influenced incomplete graft rejection as seen at days 21 and 30. However incomplete rejection has also been reported in human skin transplant models, where rejection was detected post-facto by histology [306-308]. Therefore incomplete rejection, is more likely to be related to the strain of NOD-SCID mice, as described recently by King et al in 2008. In this study the NOD-SCID or otherwise known as the ND/LtSz-Prkdc^{scid} was compared to the NOD.cg-Prkdc^{scid}IL2rg^{tmWjl}/SzJ (NOD-SCID IL2ry^{null}). NOD-SCID IL2ry^{null} has a targeted mutation in the IL-2 receptor gamma chain, and in comparison to the common NOD-SCID the NOD-SCID IL2ry^{null} was shown to be a superior for the use in islet transplantation. This was evidenced by better PBMNC engraftment, directly related to the fact that these mice do not develop NK cells and do not spontaneously start producing T- and B-cells at an older age. Furthermore this model was able to demonstrate robust rejection of human islet, which is believed to be related to the more physiological CD4:CD8 T-cell ratio which was only achieved by the NOD-SCID IL2ry^{null}. Finally this study also demonstrated that I.V route of administration also improved PBMNC engraftment [303]. Moreover, studies have also shown that PBMNC, administered by I.P take 7-14 days to drain from the peritoneal cavity

and into circulation [314, 315]. Accordingly the I.P route of administration may also be a limiting factor, which affected complete islet allograft rejection. Another limiting factor associated with this model relates to the fact that a nephrectomy of the transplanted kidney was not performed. A nephrectomy or staining of the native pancreas of the transplanted mice was necessary to determine the source of insulin regulating the BGL, accordingly to exclude the regeneration of the native pancreas and thus production of mouse insulin. The re-generation of the native pancreas indeed may have affected BGL results in figure 4.3.5A where all mice exhibited normal BGL. However in figure 4.3.5B 3 mice had low detectable levels of c-peptide, which may have not been enough to regulate BGL at the levels seen in figure 4.3.5A.

In summary, the observations of IFN-γ mediated DC maturational arrest described in chapter 3 were further investigated in this chapter by using a DC protocol which generates DC in 48h. It was demonstrated that early exposure of $CD14^+$ monocytes to IFN-γ inhibits efficient phosphorylation of IL-4 activated STAT-6 inhibiting the expression of IRF4 and RelB expression. Subsequently, arresting DC maturation and promoting a tolerogenic phenotype that resulted in the generation of antigen-specific Tregs. Although it failed to show statistical significance, IFNγ-DC *in vivo* trends towards improved C-peptide levels compared to the other 2 control groups. However the model failed to demonstrate overt allograft rejection as evidenced by hyperglycaemia, which makes it difficult to be conclusive about the role of IFNγ-DC *in vivo*. IP route of administration and problems associated with human PBMNC engraftment in the NOD-SCID mouse, are confounding factors which have limited the model to demonstrate complete allograft rejection. Therefore in future, the more applicable NOD-SCID $IL2ry^{null}$ mouse should be used and PBMNC should be administered by I.V to improve human cell engraftment and prevent inter animal variability. Overall, the ability to generate Tol-DC in a

short timeframe is fundamental in the ability for translational DC cellular therapy to be transferred into the clinic for the treatment of allogeneic transplant rejection.

CHAPTER 5 - Adenoviral mediated expression of PD-L2 in human islets maintains stable *in vitro* function and promotes signalling through inhibitory PD-1 T-cell receptor

5.1 Introduction

Type 1 diabetes mellitus is a debilitating autoimmune disorder, which is characterised by the destruction of insulin producing pancreatic beta-cells, resulting in insulin deficiency and hyperglycaemia. T1D is a condition commonly diagnosed in children, but which can develop at any age. [316]. Insulin deficiency promotes increased gluconeogenesis and lipolysis resulting in elevated levels of blood fatty acids and ketone bodies, that can cause hyperglycaemic comas and death [317]. Prolonged episodes of hyperglycaemia have been associated with cardiovascular complications, renal disease, diabetic retinopathy, and peripheral neuropathy [318]. The current standard therapy for T1D involves the use of lifelong exogenous insulin replacement therapy, which is administered either multiple daily injections or by insulin pump [319]. However this approach is limited by the inability to achieve physiological blood glucose levels. Poor blood glucose control leaves patients vulnerable to the development of complications including hypo-glycaemia episodes, which are life threatening and severely compromise the life-style of patients [320]. The destruction of beta-cells is mediated by an array of cellular immune responses, which involves the coordinated interaction of CD4+ and CD8+ T-cells, B lymphocytes, dendritic cells and macrophages, which infiltrate islets [321-324]

CD8+ cytotoxic T-cells (CTL) are known to play a key role in the pathogenesis of T1D. CD8+ T-cells are the dominant cell type found infiltrating the pancreas of T1D patients and pancreatic grafts [325-330]. NOD mice have been instrumental in demonstrating the role of CD8+ T-cell mediated development of T1D. In particular mice lacking function of CD8+ T-cells fail to develop insulitis or diabetes [331-334]. Adoptive transfer studies have demonstrated that CD8+ T-cell clones isolated from NOD mice transfer T1D in the absence of CD4+ T-cells [335-337]. CD8+ T cells mediate the killing of their target cells by direct cell contact. They use cytolytic granules that contain membrane –disrupting proteins perforin and granzyme, a serine protease

family member [338, 339]. *In vitro* studies have demonstrated that granule-mediated cytotoxicity is the dominant mechanism of beta-cell destruction [340-342]. CD4+ T-cells also play a crucial part in T1D development. In mouse models the disruption of the CD4+ T-cell subset, MHC or MHC II transactivator, results in protection against insulitis and diabetes [331, 343, 344]. However the reduced insulitis seen in these mouse models suggests that in the absence of CD4+ T-cells, CD8 T-cells fail to become efficiently activated. This autoimmune response in T1D patients can go unnoticed for many years, as the beta-cell destruction is progressive and asymptomatic. When symptoms do arise, it's not uncommon to find more than 80% of beta-cells destroyed at diagnosis [4], resulting in the clinical manifestation of insulin deficiency. Islet transplantation is an emerging therapy, which aims to replace insulin producing beta-cells and ameliorate the need for daily insulin injections.

Islet transplantation is a potentially curative approach to treating T1D, as it may improve metabolic control of blood glucose levels to an extent, which cannot be achieved with insulin injections or pumps. However patients have to replace the use of insulin for the use of immunosuppressive agents, which are associated with severe side-effects (as discussed in chapter 1 section 1.2.3) including the toxicity of islets. With the adoption of the Edmonton protocol in 2000, the success of islet transplantation has dramatically improved, with several groups reporting insulin independence and normalisation of blood glucose levels [22, 23, 345-347]. However, long-term insulin-independence frequently fails to persist 5 years post transplantation [348, 349]. A variety of factors contribute to the short and long-term loss of islet graft function. Islet transplantation is a form of cell transplantation, which in comparison to solid whole organ transplantation, isolated islets have extended ischemia times. Furthermore, during the isolation process islets undergo mechanical and enzymatic digestion to separate them from surrounding tissue in the pancreas, which compromises their integrity [350, 351]. Islets are

infused into the portal vein of the liver, which is not the natural location of islet tissue, where as many as 50-70% if infused islets are lost immediately post-transplantation [352]. Subsequently remaining islets are extremely vulnerable to the alloimmune response and to the autoimmune processes that initiated the original disease [348].

Shortly after transplantation islets come into direct contact to blood and its elements including monocytes, macrophages, platelets and complement proteins, which promote a thrombotic / inflammatory reaction known as 'Instant blood-mediated inflammatory reaction (IBMIR). Accordingly IBMIR is characterized by the rapid (within 5 minutes) activation and binding of platelets, activation of the coagulation and complement systems, with the rapid infiltration of leukocytes cells into the islet graft [111, 112]. This reaction is primarily driven by the inflammatory factors that are endogenously produced by human islets such as tissue factor, IL1-β, IL-8, Macrophage Inflammatory Protein-2, Migration Inhibitory Factor and Monocyte Chemoattractant Protein-1[113, 114]. Long-term allograft rejection of islets involves the activation of the adaptive immune response. The specific cytotoxic mechanisms which cause islet loss are believed to be similar of those involved in autoimmune-immune mediated beta-cell destruction as described above. Accordingly antigen presenting cells transferred with islet graft also play a role in activating the adaptive immune response. In order to overcome this barrier of allograft rejection, islet transplant recipients are subjected to a lifetime of immunosuppressive drugs.

Current protocols of immunosuppressive therapies for islet transplantation include a glucocorticoid-free regimen that consist of sirolimus, tacrolimus and daclizumab [22]. Although, 'islet friendly' when compared to other immunosuppressive regimens, these drugs are still cytotoxic to transplanted islets [353]. *In vitro* studies have demonstrated that treatment of islets

with tacrolimus and sirolimus inhibits the insulin secretory capacity of islets and increases the mitochondrial release of pro-apoptotic factors [354]. The University of Miami is currently running a phase II trial using a steroid and calcinerine free protocol to try and minimise the side effects associated with the use of sirolimus (www.citregistry.org – NCT 00315627). Studies have also demonstrated that that in addition to the Edmonton protocol the use of etanercept (TNF-α inhibitor) induction in combination with extenatide (a glucagon-like peptide 1 analogue), reduce the number of islets required for transplantation [355]. An approach, which is also being used in phase III clinical trials at the University of Illinois (www.citregistry.org – NCT00679042). However, the systemic effect and long-term use of these drugs is associated with increased susceptibility of recipients to opportunistic infections, development of malignancies and cardiac complications [356]. Accordingly, there is a need to develop novel therapies that target the local environment of the graft to induce allograft acceptance without the use immunosuppressive regimens, which also targets the persistent autoimmunity of T1D.

Gene therapy is a novel therapeutic approach, which involves the introduction of therapeutic molecules to cells and tissues to specifically target diseases, such as allograft rejection [159]. There are 4 major species of viral vectors that are commonly used in gene therapy, which include adenoviruses (AdV), adeno-associated viruses, herpes simplex virus and retroviruses (including lenti-viruses). AdV-based vectors are among the most commonly used vectors studied in clinical trials, where its efficacy as a vector has been extensively examined [357-360]. AdV vectors originate from the human serotype 2 and 5 AdV species. In humans the wild-type AdV infection manifests itself as a mild respiratory disease, which is otherwise known as ‘the common cold’ [361]. Modifications to the virus via the removal of replication genes (E1-, E3- deleted), has enabled it to be applicable as a gene therapy vector. AdV vectors are known to transduce both dividing and non dividing cells (including islets) with high efficiency for

transient gene expression [362, 363]. Recombinant vectors containing the gene of interest can be produced in high titres and the vector itself does not integrate into the host's chromosomes, avoiding the risk of insertional mutagenesis [364]. AdV vectors require the presence coxsackie/adenovirus receptor (CAR), heparin sulphate, $\alpha_V\beta_5$ integin and adherin to enter the target cell [365, 366]. *Ex vivo* modification of human islets with AdV vectors, has been shown to be effective in promoting transgene expression without affecting their viability or function, when compared to others vectors [367-370].

In this chapter, an AdV vector was used to over-express the immunomodulatory molecule programmed cell death ligand 2 (PD-L2). PD-L2 is one of two ligands that bind to programmed cell death 1 (PD-1). PD-1 is an inhibitory receptor expressed by activated T-cells and B-cells and forms part of the immunoglobulin superfamily. As a monomeric protein, it recruits SHP-1 and SHP-2 tyrosine phosphatases that bind to its cytoplasmic immunoreceptor tyrosine-based switch motif to mediate its inhibitory function. Binding of PD-1 during TCR signalling blocks T-cell proliferation, cytokine production and cytolytic T-cell function and also impairs T-cell survival as described in chapter 1 (section 1.5). PD-L1 is the other known ligand for PD-1, it's widely expressed by hematopoietic and non-hematopoietic cells, including islets. Unlike PD-L1, PDL-2 is not expressed by islets and is highly restricted to DC, macrophages, B-cells and bone-marrow derived mast cells. PD-L2 also has a 3-fold higher affinity for PD-1 compared to PD-L1 and *in vitro* PD-L2 appears to be the dominant inhibitory ligand in humans.

Therefore, it was hypothesised that the AdV mediated expression of human PD-L2 by human islets may promote signalling through inhibitory PD-1 pathway inhibiting T-cell activation and thus T-cell mediated immune responses. In order to test this hypothesis two recombinant AdV vectors were generated – one encoding a full length transmembrane isoform (AdV-PDL2-FL)

and another encoding a soluble isoform (AdV-PDL2-SOL). This chapter demonstrates that AdV transduced islets maintain stable *in vitro* function and are efficiently able to express PD-L2. However the main finding of this chapter suggests that soluble PD-L2 may potently suppress allogeneic and autoimmune T-cell function, as evidenced by its ability to potently induce PD-1 T-cell signalling.

5.2. METHODS AND MATERIALS

5.2.1 Generation of Adenoviral constructs containing PD-L2 isoforms

PD-L2 isoforms were amplified using primers designed to incorporate *Eco*R1 and *Bam*H1 (New England Biolabs, USA) restriction sites in the forward and reverse primers respectively (for primer sequences and PCR conditions refer to appendix A). PD-L2 gene isoforms were amplified from cDNA reversed transcribed from DC RNA (see sections 8.1, 8.2 and 9.1 for detailed methods). Purified PCR products and pEGFP plasmid were digested with *Eco*R1 and *Bam*H1 to allow directional cloning of the gene of interest into the plasmid, allowing correct orientation and fusion to EGFP. Digestion products underwent a ligation and ligated products were then transformed into competent TG1-α *E.coli*. Positive colonies were screened by PCR. PD-L2-EGFP isoforms from pEGFP-N1 recombinant plasmids were cloned into CMV-pShuttle vector. Recombinant CMV-pshuttle vector underwent homologous recombination with pADEasy-1 Vector in BJ5183 *E.coli*. Basic Local Alignment Search Tool was used align the PDL2-FL and PDL2-SOL sequences with all highly similar nucleotides sequence on the NCBI database. Please refer to section 11 in chapter 2 for detailed methods on the generation of adenoviral constructs.

5.2.2 Transduction of human islets with adenoviral constructs

Human source of islets was obtained from the Australian Islet Consortium from deceased organ donors. Islets were isolated at isolating centres St Vincent's Institute in Melbourne, Victoria and Westmead Hospital, Sydney and shipped to Adelaide. Islets were washed with serum free CMRL media and then resuspended in serum free CMRL containing the desired viral dilution. Infection volumes for a 24 well and 6 well plate were 0.2ml and 0.5ml respectively.

Islets were transduced for 3-4h under serum free conditions at 37°C and 5% atmospheric CO_2. Then, an equal volume of complete CMRL media containing 20% Albumex 20 (Australian Red Cross, Australia) was added per well and incubated for 48h (37°C and 5% CO_2), with media exchange performed daily.

5.2.3 FACS analysis

Islets were dissociated as per described in chapter 2 section 2.13.2. Viability of islet cells was established by staining with propidium iodide (PI) as per manufacturer's instructions (Invitrogen, USA). Briefly, islet cells were resuspended with 100µl per tube of islet FACS wash (5% albumex 20 in PBS, Azide free) and stained with 2µl of PI per tube (20µg/ml)and placed on ice. Cells were analysed on FACS CANTO II (BD Bioscience, USA) within 1 hour of staining. Cells were kept on ice at 4°C until analysed. To detect the expression of PD-L2, islet cells were stained with PD-L2 primary mAb (eBioscience, USA) and detected with Goat anti-mouse IgG-FITC as per described in chapter 2 sections 2.3.2.

5.2.4. Western Blot

Islets were transduced as described in 5.2.2 and cultured at 37°C and 5% CO_2. Islets were harvested at days 3, 5 and 7 days post viral infection. At each time point islets were washed with PBS to minimise the presence of FCS proteins and then lysed with western blot lysis buffer (refer to chapter 2 section 2.15). Samples were collected and stored at -80°C. Samples for each experiment were thawed at RT and then mixed with 5µl of loading buffer and 2µl of reducing agent and run through Invitrogen pre-cast gels as per manufacturer's instructions (Invitrogen, USA) at 200mV for approximately 45min. A chemiluminescence kit by invitrogen (Invitrogen, USA) was used to visualise blot, in brief membrane was incubated with 1:1000 anti-

mouse horseradish peroxidise (HRP) for 1h at RT and then visualised with kit as per manufacturer's instructions.

5.2.5. Static insulin release assay

Islets were harvested and washed in low glucose RPMI (2.8mM glucose). Approximately 50IEQ of islets were resuspended in 0.6ml low glucose solution (2.8mM glucose). Half of islet suspension (0.3ml) was transferred into 1.3ml of either low glucose solution (2.8mM glucose) or high glucose (25mM) solution in 5ml tubes. Islet were mixed well with the glucose solutions and incubated with loose lids at 37°C / 5% CO_2 incubator for 2 hours. Supernatants were then harvested and stored at -80°C prior to further analysis. Collected samples were analysed by human insulin ELISA as per manufacturer's instructions (Mercodia, Sweden).

5.2.6. Immunohistochemistry

Islets were spun onto slides using a Shandon cytospin II (Thermo Scientific, USA) and fixed with cold acetone for 5min. Acetone fixing was used to quench the EGFP signal. Slides were blocked with 3% goat serum in PBS for 30min at RT. Slides were incubated with primary mouse-anti PD-L2 (12.5µg/ml) (eBioscience, USA) and guinea pig-anti insulin (1:100 dilution) (Millipore, USA) for 2h at RT prior to washing with PBS for 5min. Primary antibodies were detected with anti-mouse FITC IgG (1:100 dilution) and anti-guinea pig rhodamine IgG (1:100 dilution) (Jackson Immuno Research, USA). Slides were incubated with detection secondary antibodies for 1h at RT. Slides were washed with PBS for 5 min prior to mounting with DAKO mounting media (DAKO, Denmark).

5.2.7. IL-2 ELISA

Human islets were transduced at optimal viral concentrations for 48h. Approximately 10 IEQ was co-cultured with hPD-1/mCD28 chimera T cell murine hybridoma ($2x10^4$) (kindly supplied by Prof. Simon Davis and Dr Sara Morgan - The Weatherall Institute of Molecular Medicine, Oxford, UK, http://www.imm.ox.ac.uk/wimm-research/mrc-human-immunology-unit/simon-davis), per well in a 96 well plate, thus at an approximate 1:1 islet cell to T-cell ratio. Supernatants were harvested after 3 days of culture in 37˚C / CO_2. Samples were stored at -80°C prior to analysis. A mouse IL-2 ELISA (as per manufacturer's instructions) (eBioscience, USA), was used to measure the ability of transduced islets to induce signalling though to see how this assay works refer to appendix D.

5.3 RESULTS

5.3.1 Cloning of PD-L2 membrane-bound and soluble forms into adenoviral vectors

Bioinformatic tools (Primer3) were used to design primers to amplify either the full length truncated gene or a truncated isoform of PD-L2. Primers were designed for soluble PD-L2 to incorporate a stop codon within the transmembrane section of the gene sequence. Directional cloning was used to insert the gene of interest into the pEGFP-N1 vector. EcoR1 and BamH1 restriction sites were incorporated into the forward and reverse primers respectively for each isoform. As shown in figure 5.3.1B both the PD-L2-FL and PDL2-SOL were successfully amplified from human DC. These PCR products were digested and ligated into the pEGFP-N1 plasmid as shown in figure 5.3.1A to allow fusion EGFP sequence. Recombinant plasmids were amplified and selected on the basis of kanamycin-neomycin resistance. Positive colonies were then screened by PCR for PDL2 expression (figure 5.3.1B). Amplified recombinant pEGFP-N1 plasmids were digested and ligated into CMV p-Shuttle vector as depicted in figure 5.3.1C. Recombinant p-Shuttle vectors were transferred into competent TG1-α *E. coli*. Positive colonies were selected based on their kanamycin resistance. These colonies were also screened by PCR for PDL2 expression (Figure 5.3.1C). Purified recombinant p-Shuttle vectors were electroporated into BJ5183 containing pADEasy-1 vector. This allowed for homologous recombination between the p-Shuttle and pAdEasy vector (Figure 5.3.1D). Recombinant pShuttle vectors were DNA sequenced to confirm the correct sequence of PD-L2, prior to homologous recombination. Basic Local Alignment Search Tool enabled the alignment of PDL2-FL and PDL2-SOL sequences with all highly similar sequence on the NCBI database (Figure 5.3.1D). This search found that the gene cloned into the pShuttle had greater than 98% homology with the human PD-L2 molecule (**NM_025239.3**), this was evident for both PD-L2 isoforms.

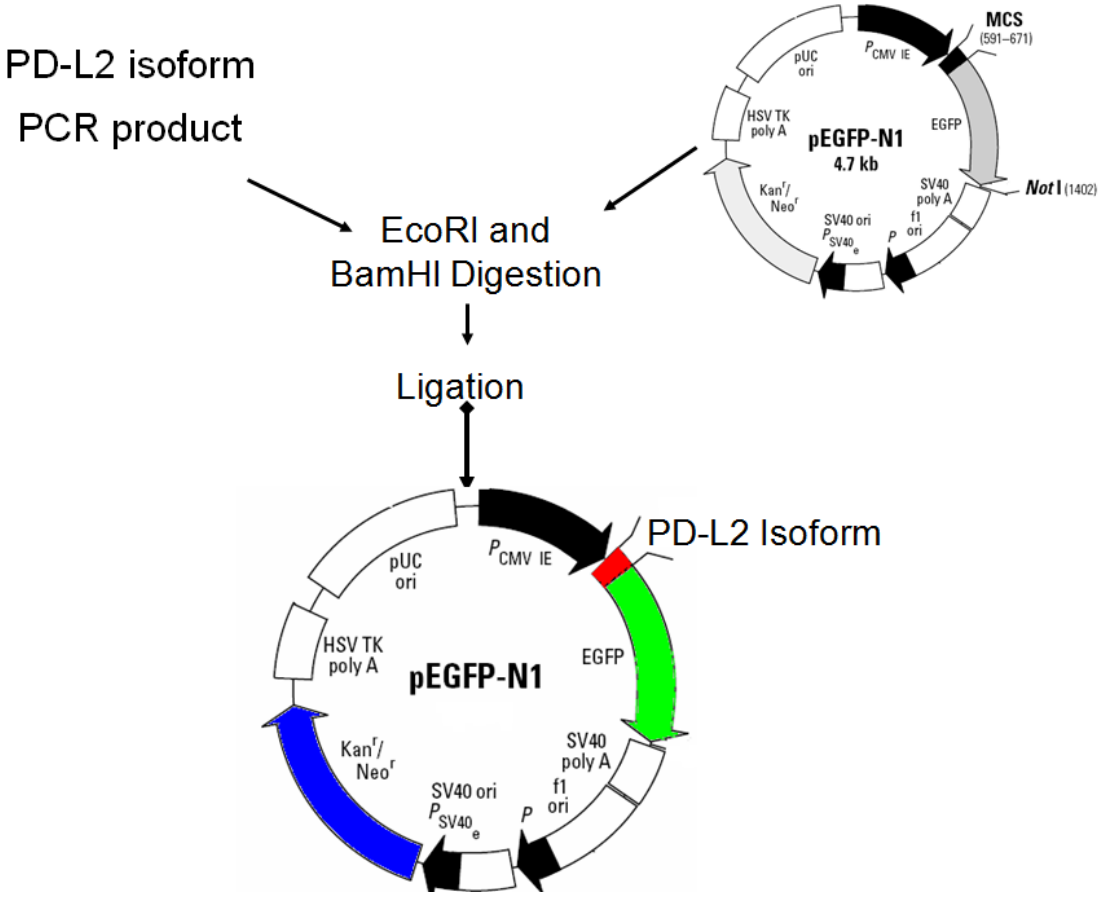


Figure 5.3.1A: Pictorial representation of cloning strategy into pEGFP-N1 plasmid, purified PCR products and pEGFP plasmid was digested with EcoR1 and BamH1 to allow forced cloning of gene of interest into the plasmid, allowing correct orientation and fusion to EGFP. Digestion products were ligated and transformed into competent TG1-α Ecoli.

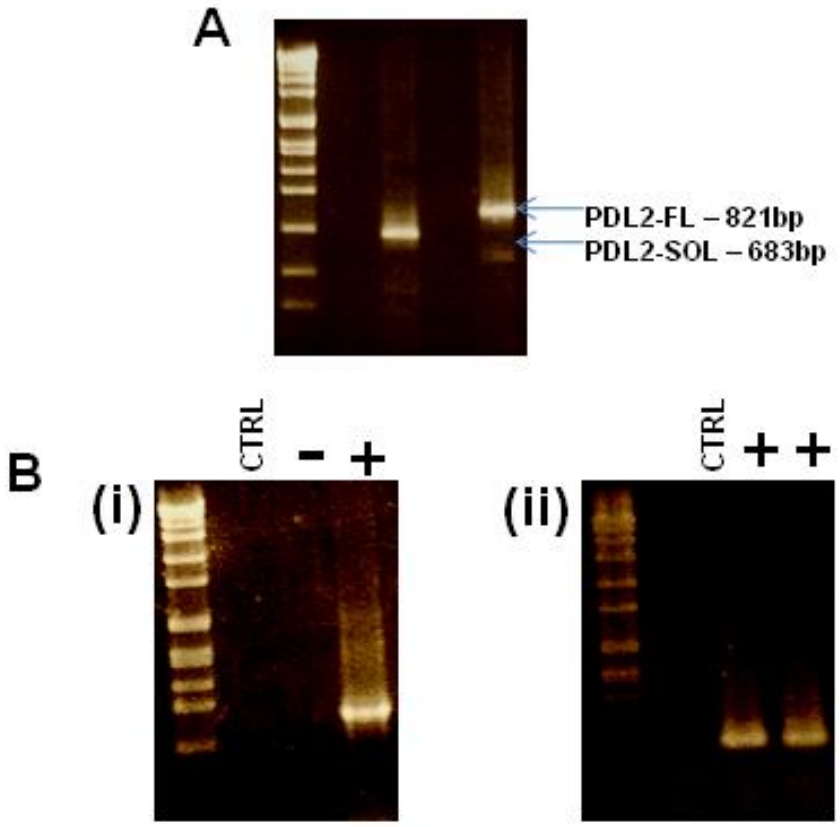


Figure 5.3.1B: PD-L2 isoforms was PCR amplified from DC cDNA and cloned into pEGFP-N1 vector. (A) RNA was extracted from DC and reverse transcribed into cDNA. PD-L2 isoforms were amplified using primers designed to incorporate EcoR1 and BamH1 restriction sites in the forward and reverse primers respectively. (B) Positive colonies were screened by PCR (i) showing PDL2-FL and (ii) PDL2-SOL.

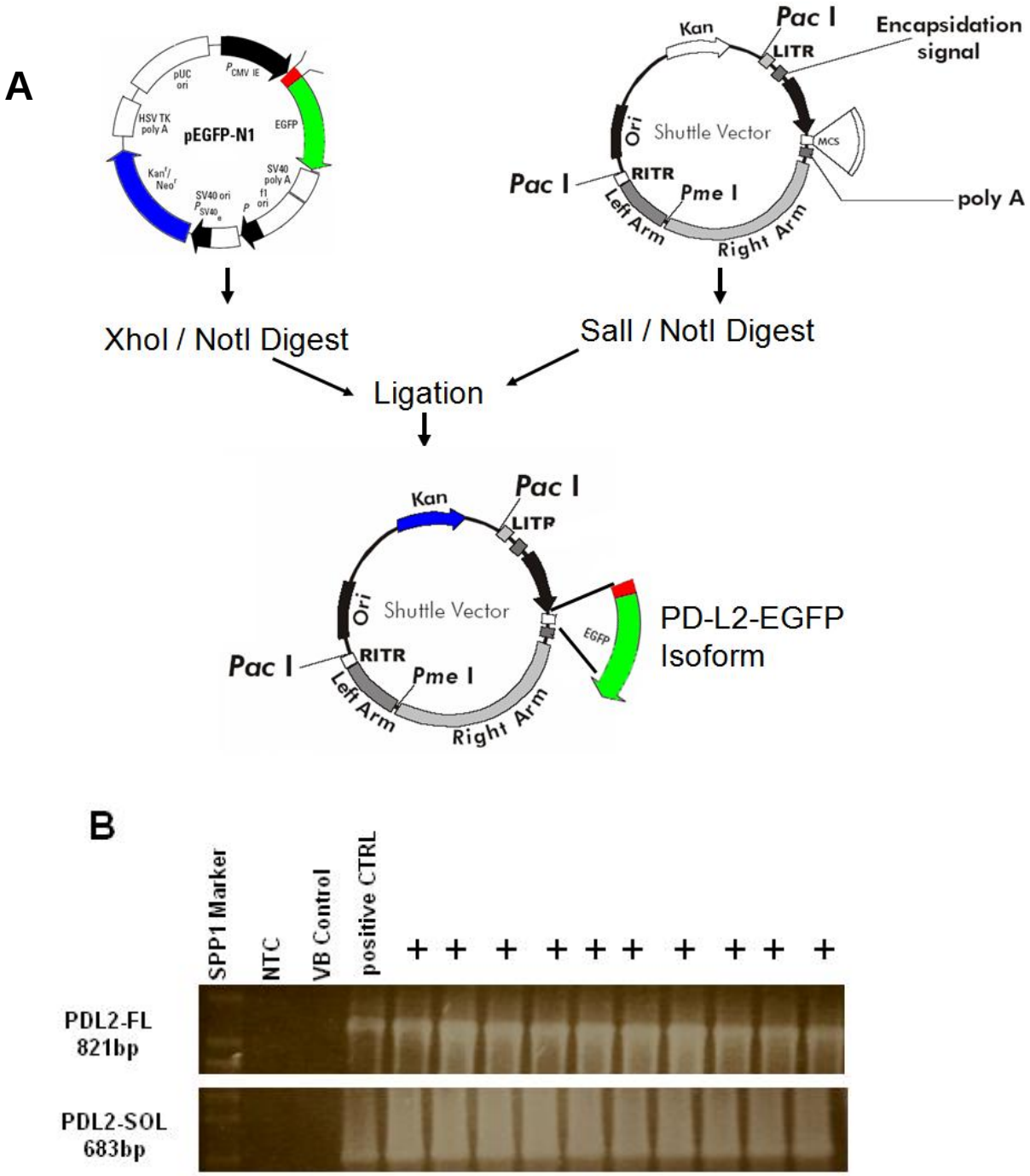
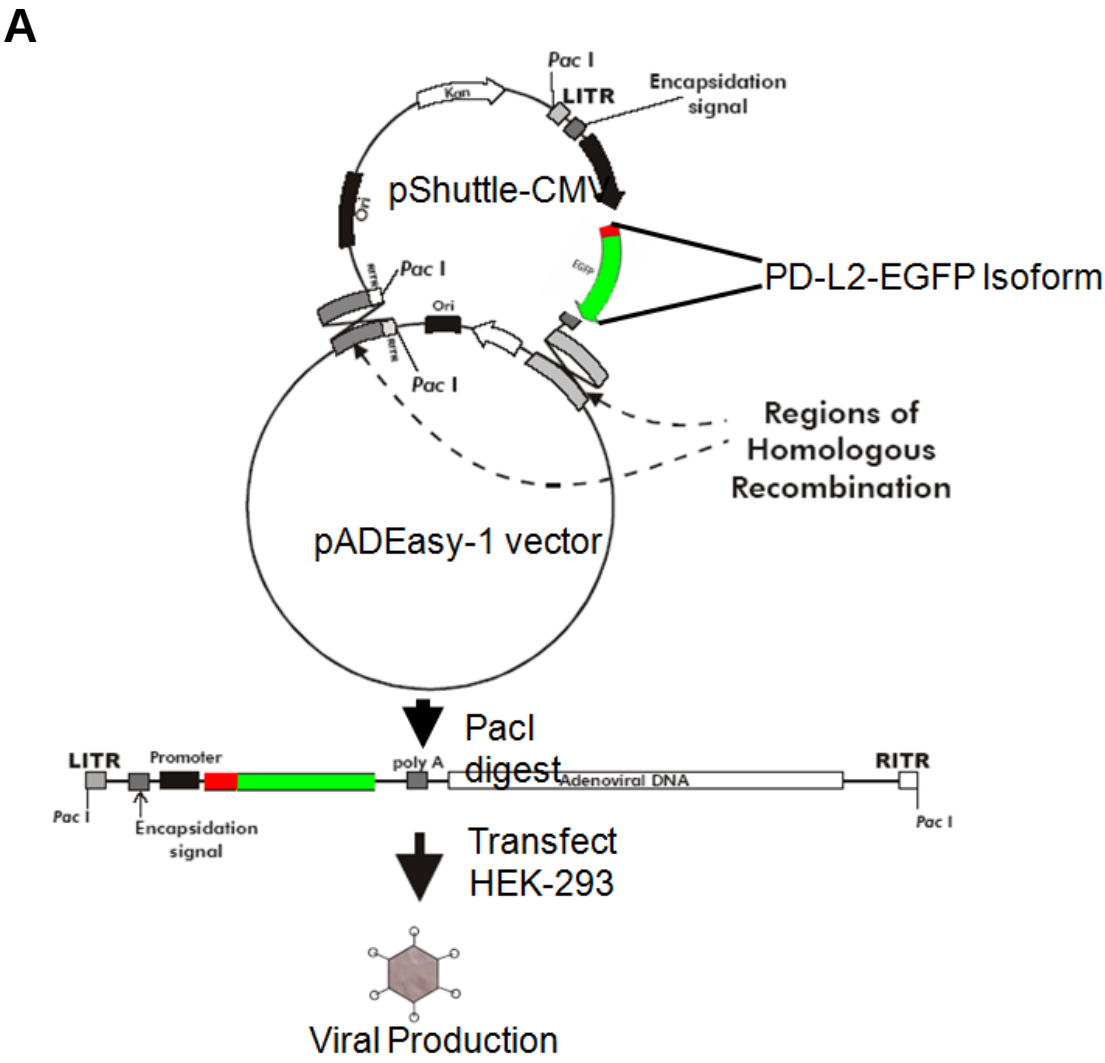


Figure 5.3.1C: PD-L2-EGFP isoforms from pEGFP-N1 recombinant plasmids were cloned into CMV-pShuttle vector. (A) Illustrates schematically the digestion of pEGFP-N1 PDL2 recombinant plasmid and the CMV-pShuttle vector with compatible RE enzymes to allow forced cloning of gene of interest into pShuttle vector. (B) PCR screening of positive colonies for PD-L2 expression (+ = positive colonies for PD-L2).



B

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PDL2-FL SEQ   GTTGAGCCTGG-ATTGCAGCTTCACCAGATAGCAGCTTTATTCACAGTGACAGTCCCTAA
              ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
NM_025239.3   GTTGAGCCTGGAATTGCAGCTTCACCAGATAGCAGCTTTATTCACAGTGACAGTCCCTAA

PDL2-SOL SEQ  GTTGAGCCTGGAATTGCAGCTTCACCAGATAGCAGCTTTATTCACAGTGACAGTCCCTMM
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_025239.3   GTTGAGCCTGGAATTGCAGCTTCACCAGATAGCAGCTTTATTCACAGTGACAGTCCCTAA
```

Figure 5.3.1D: CMV-pShuttle vector underwent homologous recombination with pADEasy-1 Vector. (A) Pictorial of cloning strategy, briefly BJ5183 E.*coli* containing pAdEasy-1 vector was electroporated to allow entry of PD-L2 recombinant pShuttle vector. Prior to homologous recombination, PD-L2 recombinant adenoviral constructs were sequenced. (B) Using Basic Local Alignment Search Tool the PDL2-FL and PDL2-SOL sequences were aligned with all highly similar sequence on the NCBI database of human origin.

5.3.2 AdV transduction of human islets does not affect their viability

Isolated human islets were transduced with purified AdV particles with a range of viral titres from 10^3 to $3x10^3$ MOI, based on the assumption that each islet cluster has approximately 1000 cells. Islets were transduced with AdV for 3-4h in serum free conditions. EGFP was expressed by all islet clusters transduced with either the AdV-PDL2-FL construct or AdV Vector blank (AdV-VB) 24h post infection (figure 5.3.2 (A). AdV-VB transduced islets were considerably brighter under fluorescence compared to the AdV-PDL2-FL. Figure 5.3.2 (B) depicts FACS analysis data, which summarises the calculated viabilities of islets cells 48h post transduction. Transduction of islets at the highest tested MOI did not affect the viability of islets cells when compared to the untreated islets. Accordingly $3x10^3$ was chosen as an optimal dose for islet transduction, to achieve maximal transduction efficiency.

5.3.3 Transduction of human islets with AdV does not affect their function

Human islets were analysed for function 48h post transduction at the optimal MOI of $3x10^3$, by treating transduced islets with low and high concentrations of glucose in the static insulin release assay. Supernatants were analysed by ELISA for insulin concentration. The stimulation index was calculated as per appendix E. As shown in figure 5.3.3 the ability of transduced islets to produce insulin in response to high glucose is not affected by the transduction with adenovirus, as the stimulation index was not significantly different from the untreated group compared to the AdV treated islets.

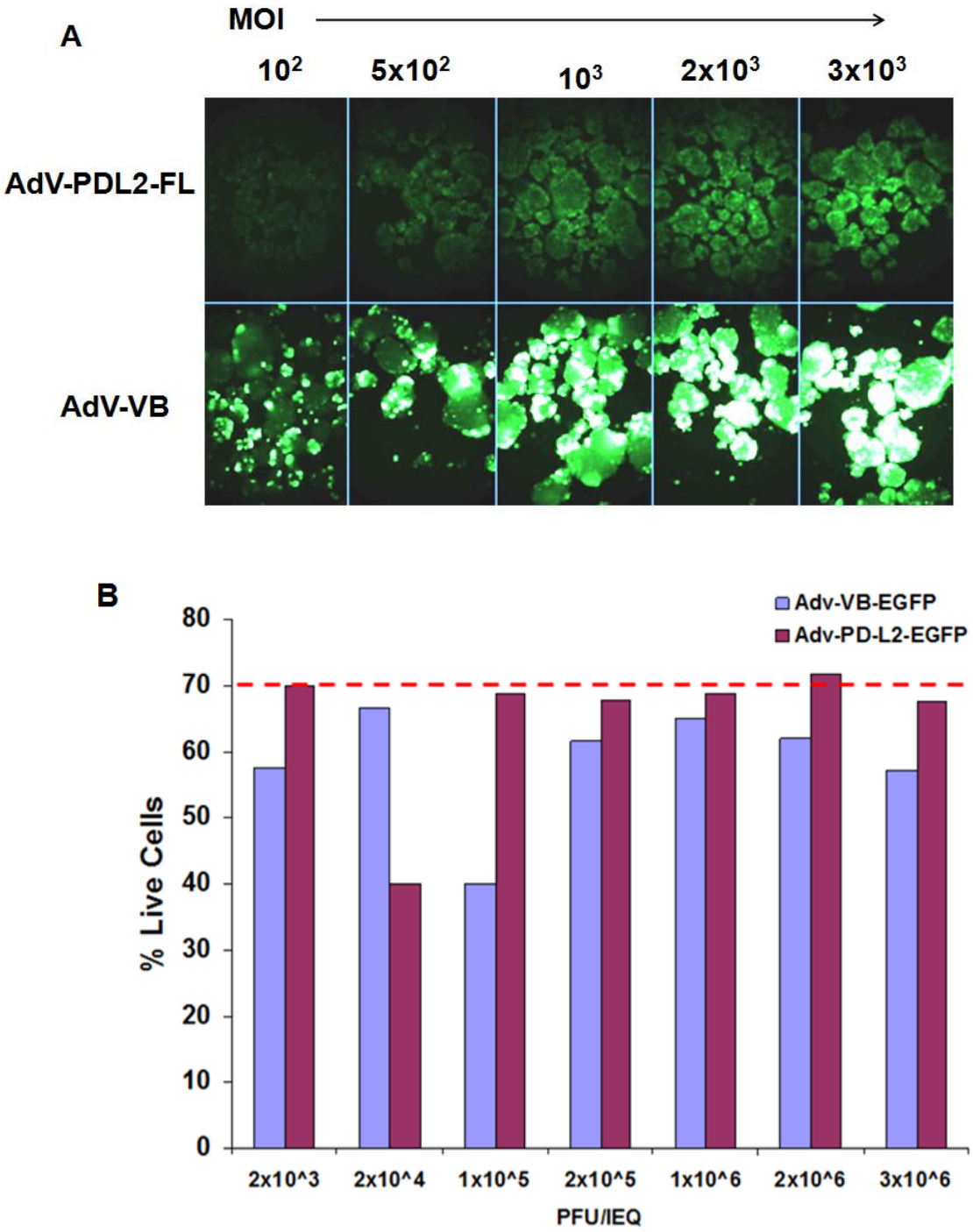

Figure 5.3.2: Human islets were successfully transduced with AdV vectors without significantly affecting viability. (A) Human islets were transduced with varying MOI ranging from 10^2 - $3x10^3$ and assessed by fluorescent microscopy after 48h (B) Transduced islets were harvested 48h post transfection and disaggregated with accutase and stained with PI to distinguish live cells from necrotic islet cells. Red dash line defines the viability of untreated islet cells.

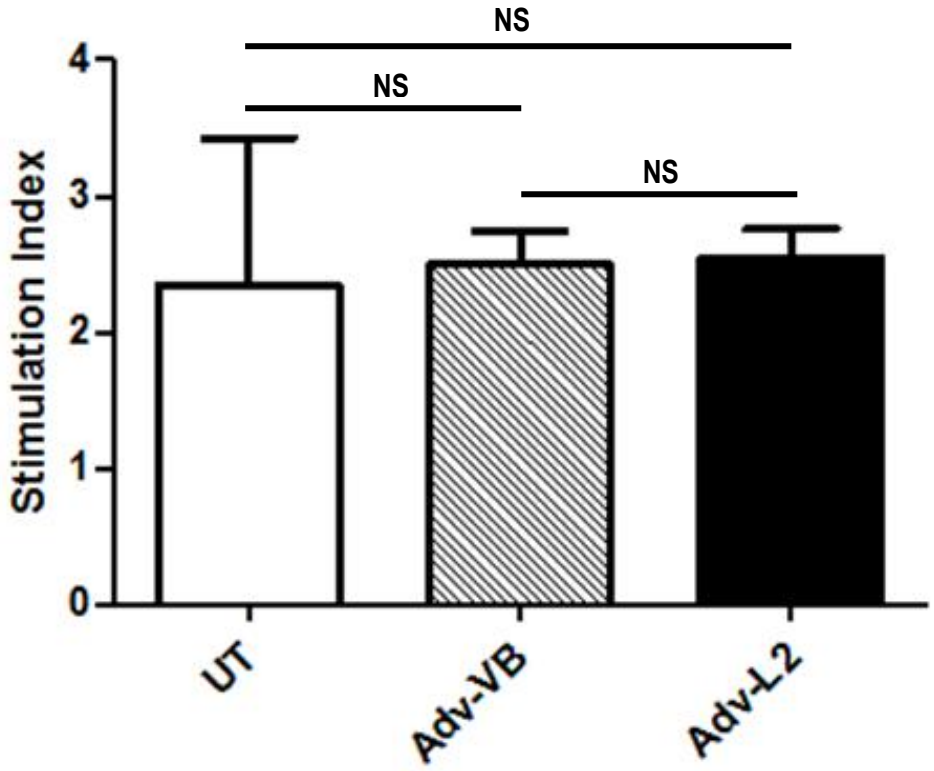

Figure 5.3.3: AdV transduction of human islets does not affect their function to produce insulin in response to a high glucose environment. Human islets were transduced at optimal MOI ($3x10^3$) and harvested 48h post transfection and used in a static insulin release assay and compared to islets that were not infected with an AdV. Mean ± SD, NS= No Significance.

5.3.4 Islets transduced with AdV-PDL2-FL induces surface PD-L2 expression for at least 7days

Human islets were dissociated 48h post transduction and analysed by FACS analysis. AdV-PDL2-FL induced PD-L2 protein surface expression in 17-19% of islet cells (Figure 5.3.4A), which was significantly higher than the expression seen in untreated and AdV-VB transduced islets ($p<0.03$) (Figure 5.3.4A). Immunohistological analysis of transduced islets (Figure 5.3.4B), showed that AdV-PDL2-FL transduced islets co-expressed insulin and PD-L2. To determine the time course of expression of PD-L2 by transduced islets, PD-L2 protein content was examined by western blot 3, 5, and 7 days post initial transduction. AdV-VB was used as a transduction control. As shown in figure 5.3.4C, PD-L2 protein expression was seen at all time points tested, thus expression was sustained for at least 7 days post transduction.

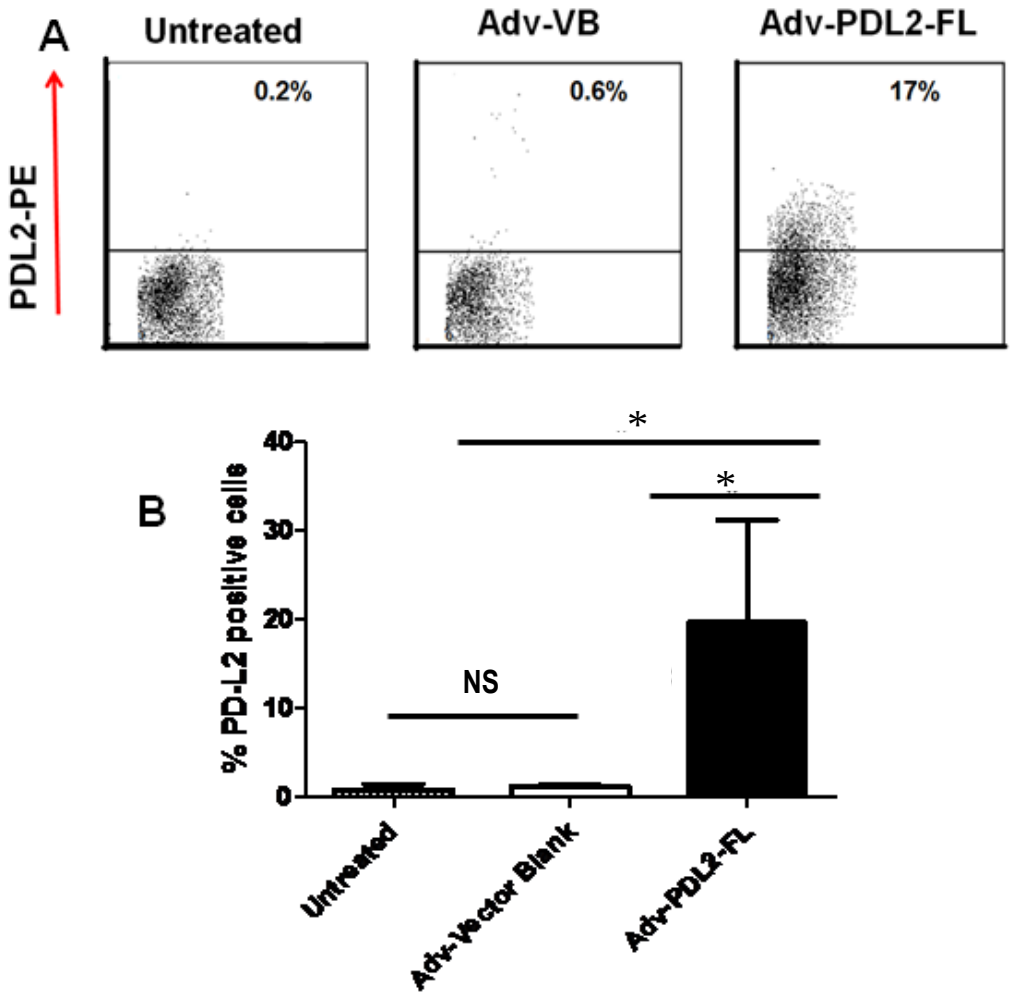

Figure 5.3.4A: AdV-PDL2-FL transducted human islets specifically upregulate the expression of transmembrane PD-L2. (A) Human islets were transduced at optimal MOI ($3x10^3$) and harvested 48h post transfection and disaggregated and stained with mAb mouse anti-human PD-L2. This primary mAb was detected using anti-mouse IgG-PE. **(B)** Percentage of cells expressing PD-L2 combined from 4 independent experiments. Mean ± SD *=$p<0.05$, NS= No Significance.

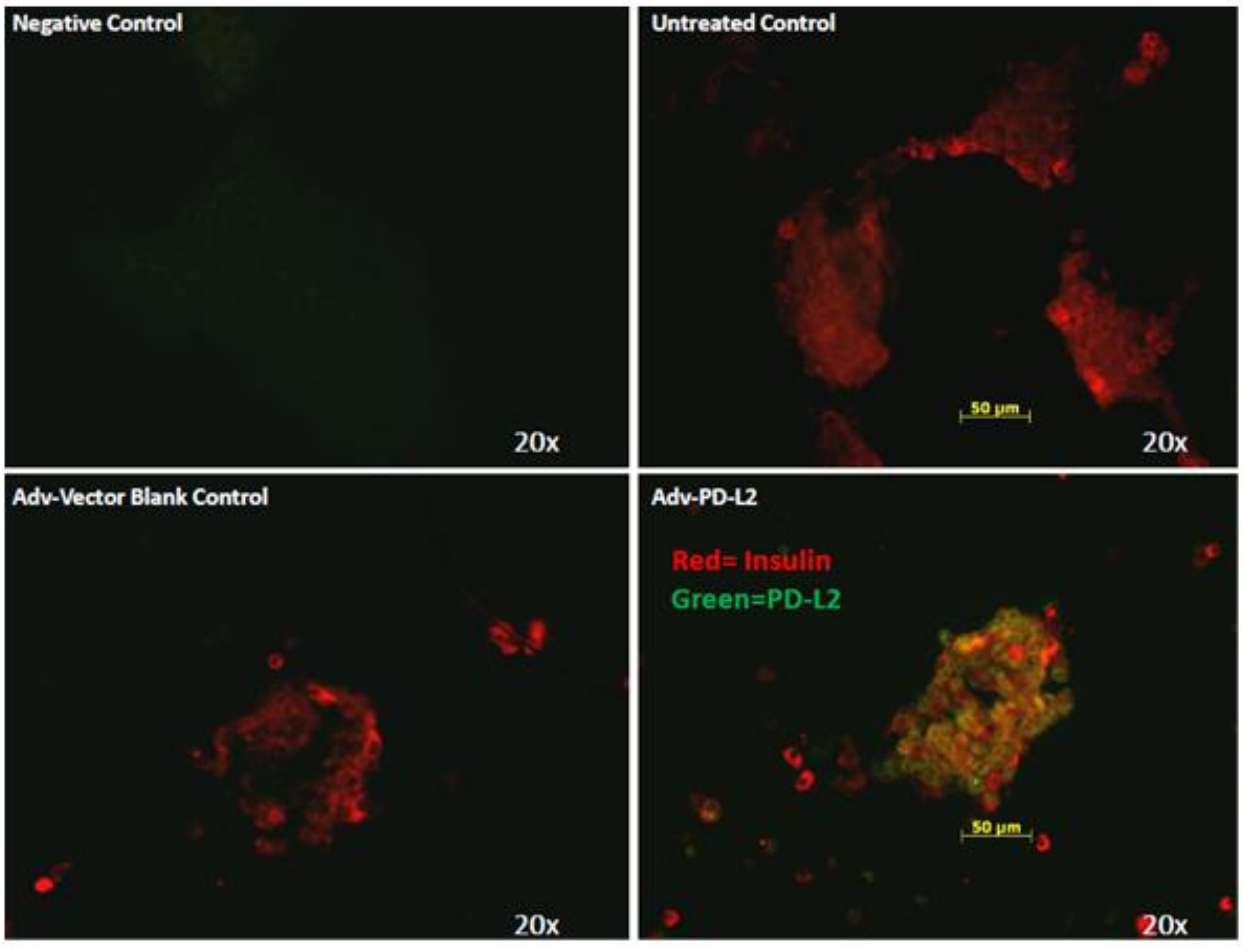

Figure 5.3.4B: AdV-PDL2-FL transducted human islets co-express insulin and PD-L2. Islets were transduced at optimal MOI ($3x10^3$) and harvested 48h post transfection. Islets were transferred on to slides and stained with insulin (RED) and PDL2 (GREEN). 200x objective.

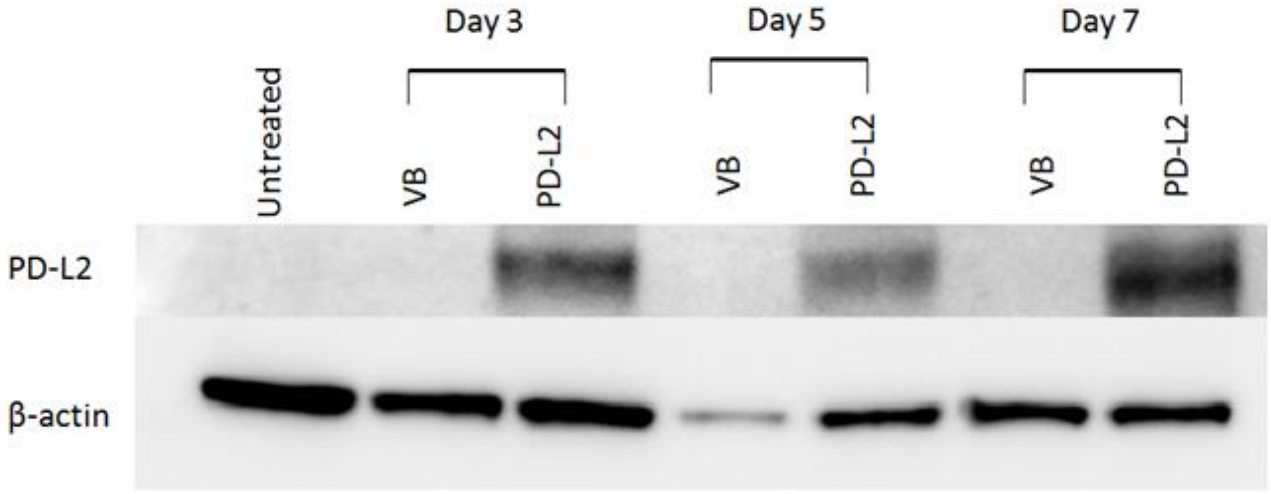

Figure 5.3.4C: AdV-PDL2-FL transducted human islets sustain expression of transmembrane PD-L2 up to 7 days post transfection in vitro. Optimally transduced islets (MOI $3x10^3$) were harvested 3, 5 and 7 days post transduction. Protein lysates were assessed by western blot and visualised using chemiluminescence.

5.3.5 PD-L2 proteins expressed by human islets are able to bind PD-1 and induce signalling

The function of the transgenic PD-L2 protein was tested to eliminate any problems associated with protein misfolding post transcription. Firstly, PDL2-FL expressed by HEK-293 cells was tested by flow cytometry for its ability to bind to a recombinant PD-1Fc protein. PD-L2-FL protein induced by AdV-PD-L2 in HEK-293 effectively bound to its receptor PD-1 (Figure 5.3.5), with high efficiency, untransduced and AdV-VB transduced controls did not express PD-L2 and thus did not bind to PD-1Fc (See APPENDIX H). To test the efficiency of both PD-L2 isoforms to induce signalling after binding, a murine T-cell hybridoma cell line, engineered to express the human PD-1 extra-cellular portion fused with murine CD28 was used (kindly supplied by Prof. Simon Davis and Dr Sara Morgan - The Weatherall Institute of Molecular Medicine, Oxford, UK) Accordingly binding of PD-1 promotes signalling and produces murine IL-2 as a response. Transduced islets were co-cultured with PD-1/CD28 T cell hybridoma and supernatants were collected for IL-2 analysis by ELISA. Figure 5.3.5B demonstrates that signalling was specifically induced by both PD-L2 isoforms. Full length PD-L2 induced signalling by 20 fold compared to untreated or AdV-VB transduced islets. However this was significantly less than soluble PD-L2, which induced signalling by 1000 fold. Blocking PD-L2 function with a neutralising mAb inhibited the ability of soluble PD-L2 to bind and signal through PD-1, demonstrating that signalling was specifically provided by soluble PD-L2.

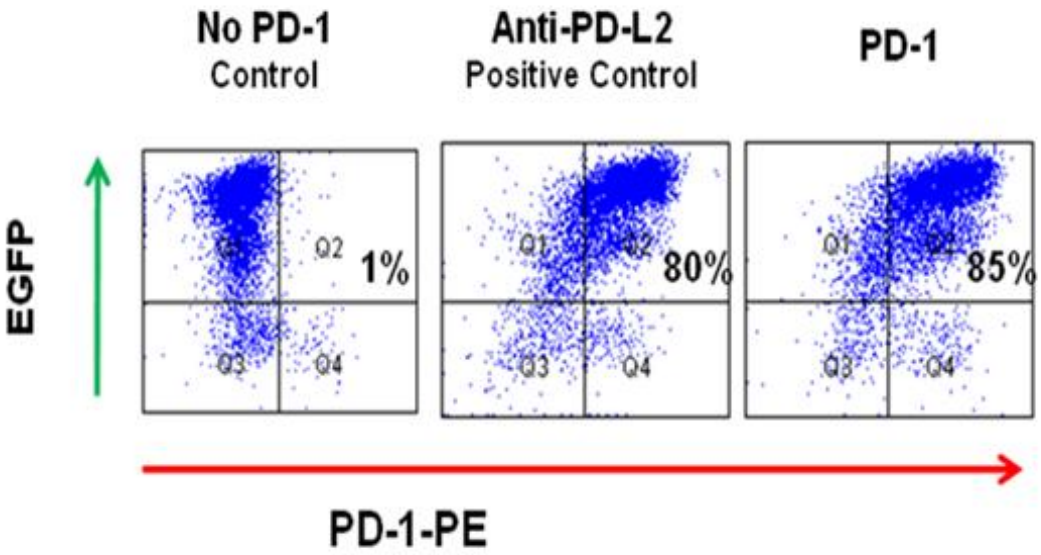

Figure 5.3.5A: PD-L2 molecules induced by AdV-PDL2 are able to physically bind to PD-1 fc recombinant protein. Optimally transduced HEK-293 were harvested 24h post transduction and were tested for their ability to bind to recombinant PD-1fc by flow cytometry. Representative of 3 independent experiments

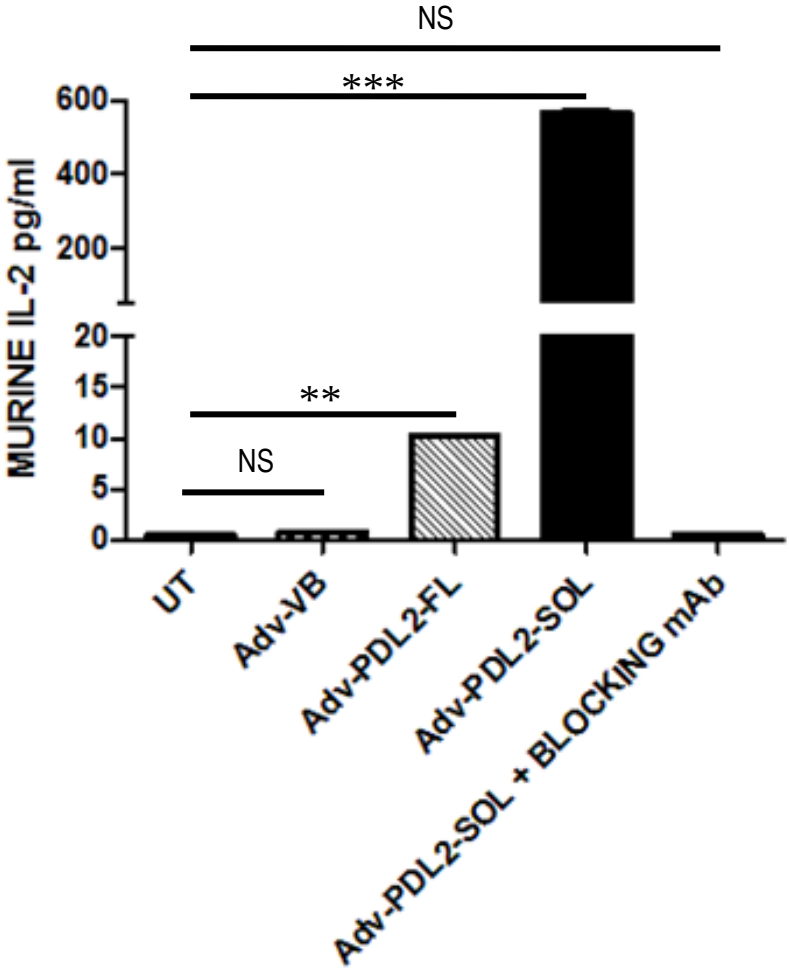

Figure 5.3.5B: PD-L2 molecules expressed by AdV-PDL2 transduced islets specifically promote PD-1 signalling. Optimally transduced human islets (MOI $3x10^3$) were co-cultured with murine T cell hybridoma expressing hPD-1/mCD28 chimera for 3 days. Supernatants were collected and concentration of IL-2 was used as a measure of PD-1 signalling. IL-2 concentration was determined by ELISA. Neutralizing antibody targeting PD-L2 was used to determine the specificity of PD-1 signalling. Mean ± SD of triplicates of sample, **=p<0.01, ***=p<0.001, NS= No Significance. Representative of 4 independent experiments.

5.4 DISCUSSION

Disruption to the PD-1 pathway in mouse model, results in the spontaneous development of autoimmune diseases such as lupus-like proliferative arthritis, glomerulonephritis, autoimmune dilated cardiomyopathy and diabetes [174, 175, 371]. These studies demonstrate that PD-1 plays a key role in the induction and maintenance of tolerance, providing inhibitory signals that regulate both the central and peripheral tolerance. PD-L2 was a logical candidate molecule to investigate in the context of islet allo-immunity, given its strong affinity for PD-1 and evidence in human studies, which suggest PD-L2 is the potent ligand out of the two [184, 195]. This chapter aimed to investigate the induction of PD-L2 in human islets, which naturally do not express this inhibitory ligand. It was hypothesized that the expression of PD-L2 by human islet may promote PD-1 signaling, which may affect the proliferation of allo- and also auto-reactive T-cells which express the PD-1 receptor.

In order to investigate this hypothesis recombinant AdV constructs were generated, which either expressed the soluble or full transmembrane isoform of PD-L2. Bioinformatic tools confirmed that the sequence used to generate the vectors, were homologous to the native sequence of human PD-L2. Proper protein folding was evidenced by the ability of PD-L2-FL to bind to PD-1 Fc recombinant proteins despite some discrepancy in some bases in the sequencing analysis (<2%). This discrepancy may be related to the fact that only forward and reverse primers were used to determine the sequence of the inserted PD-L2 isotypes. Accordingly the efficiency of the sequencing itself may have not been optimal resulting in this small difference in bases. It would have therefore been beneficial to do the sequencing with internal primers to increase the efficiency of the sequencing. However given the ability of the produced molecules to bind to PD-1 and detectable with relevant anti-bodies, it demonstrated that the protein was folding properly and functional. The transduction of human islets with

either AdV-PDL2-FL or with the control vector (AdV-VB) did not demonstrate deleterious effects on the viability or functional characteristics of the islets. Similarly, other studies have also shown efficacy of using AdV as a gene therapy vector for islet transplantation [118, 120, 121, 372, 373]. However AdV vectors are limited by their poor capacity to transduce the inner mass of islets [363]. For this reason the expression of AdV induced PD-L2 expression was limited to only 17-19% of islet cells. Despite this low transduction efficiency of islets, immunoflorescence microscopy demonstrated that the AdV-PDL2-FL vector was also able to transduce beta-cells. This provided further evidence that AdV transduction of human beta-cells does not affect their ability to express insulin. A primary part of maintaining islet graft function is associated with the survival of beta-cells. T-cells in particular play a crucial part in beta-cell destruction both in allo-immune and auto-immune processes. In the context of alloimmunity, TCR signaling by donor and recipient APC promotes T-cell activation (as discussed in chapter 1 section 1.3) and the externalization of the PD-1 molecule [169, 170], alloreactive T-cells infiltrate the graft to carry out their cytolytic functions to mediate islet mass destruction. PD-L2 expression may therefore directly protect beta-cells from the cytolytic function of T-cells, as the PD-L2 will bind to PD-1 receptor on these activated T-cells, to prevent their cytolytic activity. Similarly, the underlying problem of auto-reactive T-cells may also be managed in this manner as all activated T-cells express the PD-1 receptor. Moreover, the PD-1 pathway is naturally involved in the homeostasis of peripherial tolerance and the interruption of this pathway is believed to play an important role in the mediation of diabetes. Thus expression of PD-L2 may help in re-establishing this homeostasis, which is interrupted in T1D patients.

Functionally, PD-L2 expressed by transduced human islets was able to bind to the PD-1 receptor. More importantly both isoforms of PD-L2 were able to induce PD-1 signaling. Soluble PD-L2 was able to induce signaling via PD-1 by a surprisingly 1000 fold when compared to the

transmembrane isoform. This interesting fact, to the best of our knowledge has not been previously shown in the literature, although the relative expression of both isoforms was not determined in this study. Until now the inhibitory function of human PD-L2 has commonly been investigated using soluble PD-L2Ig [195, 201, 374]. Rodent studies in which PD-L2 is blocked or knocked out, have failed to demonstrate a potent inhibitory function of PD-L2, which may be related to species differences. However it may also be a reflection of the restricted expression of PD-L2 as a transmembrane molecule. Thus low bioavailability of PD-L2 to the PD-1 receptor reduces its potential to efficiently induce signaling. This is supported by evidence, which has shown that the concentration of PD-1 molecules at the immunological synapses is determined by the affinity and availability of its ligand, in particular PD-L2 more so than PD-L1 is important in recruiting PD-1 [375]. In addition, gene splice variants of PD-L2 in humans do exists which suggests that humans may naturally produce soluble PD-L2 [376]. In light of the data presented in this chapter, investigations into the function and bioavailability of naturally occurring human soluble PD-L2, warrants further investigation. Furthermore the ability of soluble PD-L2 to potently activate PD-1 signaling is of special relevance to islet cell protection against immunological attack, as soluble PD-L2 may also protect those islet cells which remain untransduced, an obvious advantage over the use of the transmembrane isoform. However, there is evidence to suggest that AdV vectors can trigger an innate immune response through the induction of type 1 interferons [377, 378].

The direct administration of AdV *in vivo* has been of concern, with studies demonstrating that patients may suffer from complications [379, 380]. Other studies have shown that the local delivery of low and intermediate doses of AdV vectors in humans are well tolerated up to the dose of 10^{11} virus particles [381]. However the immunogenicity of AdV particles is not significant in the context of this study, because the strategy of gene transfer involves *ex vivo*

modification of islets. Hence islets are transfected in *in vitro* and would subsequently be washed to remove virus particles prior to transplantation. In the setting of islet transplantation, the advantages of using AdV as a gene therapy vector outweigh the reported disadvantages. In particular, they accommodate large or multiple transgene expression, they are replication deficient, non-oncogenic and transduce both dividing and non dividing cells. AdV vectors provide high short-term transient expression of genes and high viral titers can be easily generated. Furthermore, a recent study using CTLA-4Ig AdV mediated expression in mouse islets, demonstrated that local immunosuppression can be achieved with soluble secreted factors, without disturbing other immune responses [382]. The use of non-viral vectors to induce transgenic expression in islets have also been used, but with poor success mainly due to the toxicity of transduction reagents, methods and inefficiency [383].

In conclusion, in this chapter the immunomodulatory molecule PD-L2 was successfully cloned into an AdV vector to encode either a soluble or full length transmembrane isoform. Transduced human islets maintained stable *in vitro* function. The potent capacity of soluble PD-L2 to induce PD-1 signaling in comparison to its transmembrane counterpart was clearly highlighted in this chapter. Overall the secretion of soluble PDL2 by islets has the potential to protect the islet allograft from activated allo and auto reactive T-cells, given its signaling capacity and warrants further investigation in a chimeric NOD-SCID islet transplant model. Studies investigating the inhibitory role of human PD-L2 thus far have been limited. Only one study in the past year has looked that the role of human PD-L2 in the context of xenotransplantation, which demonstrated promising results in its role to negatively regulate human T-cell responses [204]. In light of this study and the data presented in this chapter human PD-L2 in the setting of allo- and auto- immunity warrants further investigation, as it may have great potential as a gene therapy molecule.

CHAPTER 6- Conclusions

Islet transplantation is a developing therapy for patients affected by T1D, which has been limited by problems associated with hypoxia, poor revascularisation and allograft rejection. Immunosuppressive agents used to prevent allograft rejection are associated with severe side effects including islet toxicity, increased susceptibility of recipient to the potential development of malignancies, opportunistic infections and cardio-vascular problems. Currently, islet transplantation is only available to patients with poor blood glucose control that experience severe episodes of hypo-glycaemia unawareness. In order for islet transplantation to be used widely as a potentially curative treatment for T1D there is a need to develop novel therapies to treat allograft rejection without the use of immunosuppressive agents. Dendritic cells (DC) play an important role in promoting allograft rejection, especially those derived from the donor tissue. However, under certain conditions DC can be modulated to promote acceptance of an allograft and induce tolerance. Thus, this thesis has explored strategies to promote immature hyporesponsiveness by manipulating monocyte-derived DC function.

In chapter 3, the immunomodulatory effects of IFN-γ on human monocyte-derived DC were investigated. IFN-γ, a cytokine known for its pro-inflammatory role in allograft rejection, also has a paradoxical role in supporting allograft tolerance. Using a standard 7-day *in vitro* DC propagation protocol it was demonstrated that IFN-γ exerts its immunomodulatory function on monocytes early during DC differentiation (IFNγ-DC_{D0}), resulting in an immature DC (iDC) phenotype with reduced expression of maturation marker CD83 and gene expression of NF-κB transcription factor RelB. IFNγ-DC_{D0} induced a state of T-cell hyporesponsiveness in MLR, which was independent of the generation of total Foxp3+ T-cells. The T-cell assay used to determine the promotion of Foxp3 Tregs, limited this chapter to demonstrate conclusively the difference in the promotion of Foxp3+ Tregs between UT-DC and IFNγ-DC_{D0}. Accordingly the use of total Foxp3+ population is not suitable in the context of these *in vitro* assays. As there

are inherent problems associated with human activated T-cell effectors that are known to express Foxp3 [271]. It is therefore necessary to use other markers such as CD25, Foxp3^{HI} and CD127 low, which better reflect the presence of suppressive Tregs. Therefore in hindsight the reduced capacity of IFNγ-DC_{D0} to promote Foxp3 Tregs, may have reflected poor stimulatory capacity of these cells to activate T-cells. IFN-γ treatment, on the other hand, at day 5 (IFNγ-DC_{D5}) did not modulate the stimulatory capacity of DC compared to untreated DC. Accordingly this chapter highlighted that the effect of IFN-γ to act as either a 'pro-inflammatory' or 'anti-inflammatory' cytokine, is dependent on the timing of IFN-γ exposure during DC maturation or differentiation respectively. Thus IFN-γ is only effective in producing maturation arrested DC, when administered during DC differentiation. This effect however is not exclusive to IFN-γ as IL-10 has also been reported to mediate its DC modulatory effects early during DC differentiation, having minimal effects on already differentiated DC. More importantly this chapter demonstrated that these IFN-γ modulated cells are of DC lineage, which has not been previously demonstrated by other groups. Overall chapter 3 demonstrated that the early application of IFN-γ during DC differentiation produced maturation arrested DC, which could potentially promote tolerogenic immune responses. In chapter 4 this concept was further explored by using a rapid / fast DC protocol, making the use the IFN-γ modulated DC more relevant to translational DC Therapy.

In chapter 4 a FAST-DC protocol was used to generate IFNγ-DC in 48h, which featured similar iDC phenotype seen in chapter 3. The mechanism used by IFN-γ to modulate DC was also further examined. It was demonstrated at the molecular level that IFN-γ treatment reduced the phosphorylation of IL-4 activated STAT-6, which in turn affected the downstream gene expression of Interferon regulatory factor 4 (IRF4). Rapidly generated IFNγ-DC were shown to be terminally differentiated and poor stimulators of allogeneic T-cells causing significant T-cell

hyporesponsiveness and promoting a higher frequency of $CD4^{+}CD25^{+}$ $Foxp3^{HI}$ T-regulatory cells. This however was significantly different to observations in chapter 3, where IFNγ-DC_{D0} failed to promote a larger Treg population compared to UT-DC. It is believed that the difference in response is directly related to the different strategies used to define the Treg population in each chapter. As briefly discussed above not all T-cells expressing Foxp3 are suppressive in function, it has been previously reported that in humans CD4+CD25+$Foxp3^{HI}$ T-cell population is a better indicator of the suppressive function of human Tregs [271]. Therefore based on these findings the strategy of identifying suppressive Foxp3 population was modified in chapter 4, which may then reflect the difference in the Treg results between chapters. However the suppressive activity of the Tregs generated by IFNγ-DC was confirmed in suppression assays. This then warranted further investigation of IFNγ-DC in an *in vivo* of islet transplantation.

A surrogate chimeric NOD-SCID model of islet transplantation was then developed in chapter 4. This model was different to that of previously published models of islet transplantation, as it involved the use of donor-derived DC, hence donor peripheral blood was required by this model, which considerably limited the potential of this model. As only South Australian donors could be used and only a few DC could be generated each time, thus limiting the ratio of DC:PBMNC used to reconstitute the transplanted NOD-SCID mice. Accordingly, the model achieved poor levels of human cell engraftment, which affected its ability to promote overt allograft rejection, as evidenced by hyperglycaemia. Poor cell engraftment may be attributable to the use of IP route of administration and the strain of NOD-SCID mice. It is therefore recommended as discussed in chapter 4 that a NOD-SCID $IL2ry^{null}$ mouse be used in future, as it has superior human cell engraftment capability and approximately 20 times less human PBMNC are required to mediate a robust allograft rejection. This would make the model developed in this chapter far more feasible, as less DC would be need to be propagated from

donor blood monocytes. Accordingly, due to the limitations encountered the potential of IFNγ-DC to be tolerogenic *in vivo* still remains elusive, although the c-peptide levels in IFNγ-DC treatment were promising (Figure 4.3.5B). Further investigations into the genes modulated by IFN-γ in DC is required, an extensive assessment by micro gene array of IFNγ-DC is necessary, as IFN-γ is known to affect the expression of over 200 known genes. This will help to further characterise the mechanism used by IFN-γ to modulate DC function. In addition, it of interest to establish whether this phenomenon of controlling DC function mediated by IFN-γ occurs naturally *in vivo* as a homeostatic mechanism of controlling immune responses. Never the less this strategy of generating donor-derived tolerogenic DC in just 2 days is both novel and clinically applicable, allowing the *in vivo* targeting of the direct pathway of recognition to promote allograft tolerance in humans a possible reality.

In chapter 5, human islets were genetically modified with programmed cell death ligand 2 (PD-L2), as an alternative approach to protecting the allograft from the recipient's immune system. PD-L2 is a ligand for programmed death 1 (PD-1), an inhibitory molecule that limits the expansion of allo- and auto-reactive T-cells. Islets do not express PD-L2, thus it was hypothesised that the induced expression of PD-L2 would confer protection to islets. In this chapter, two recombinant adenovirus constructs were generated - one encoding a soluble isoform and the other expressing a full transmembrane molecule. It was demonstrated that AdV transduction did not affect the viability or insulin producing capacity of islets. Induced PD-L2 was able to bind and induce signalling through PD-1. However soluble PD-L2 was more efficient at inducing signalling by 1000 fold, than the transmembrane isoform, a phenomenon which has not been previously described before in humans. These results are promising and warrant further investigation in a chimeric model of islet transplantation, to determine the protective effects of PD-L2 in a transplantation setting. The expression of soluble PD-L2 by

transplanted human islets could potentially also inhibit the underlying cytolytic activity of auto-reactive T-cells present in T1D patients. The ability to target both the problem of alloimmunity and autoimmunity with one inhibitory molecule makes PD-L2 all the more attractive as gene therapy treatment for T1D.

Experiments to investigate the islet protective properties of PD-L2 *in vivo* were largely limited in this study by the availability of human islet tissue. This could be overcome by the use of a transgenic line of NOD-SCID mice, which express human HLA-A2.1. It has been demonstrated that NOD-SCID-HLA-A2 positive islets transplanted into diabetic NOD-Rag^{null}Prfl^{null} mice, are readily rejected by HLA-A2.1-negative human PBMNC, which is comparable to the rejection of transplanted human islets in NOD-SCID mice [309]. Thus NOD-SCID-HLA-A2 positive islets could potentially be transduced with AdV-PDL2 and used *in vivo* to further investigate the immunoregulatory role of human PD-L2 to protect islet survival and function. In addition this model would also address the inherent variability observed between donor preparations following islet isolation. Alternatively, human PD-L2 could also be characterised in a xenogeneic pig islet transplant model. Where human PD-L2 transgenic pig islets could be transplanted into NOD-SCID IL2ry^{null} and reconstituted with human PBMNC. The potential for human PD-L2 to promote human tolerogenic responses in a xenogeneic setting has been previously demonstrated, through the expression of human PD-L2 by pig APC. These finding further highlight the potential of human PD-L2 to protect pig islets in a xenogeneic model and warrants further investigation. The ability to use pig islets for the treatment of T1D, could serve as a great benefit as it could potentially overcome the shortage of organ donation and make this treatment more widely available.

Overall this thesis has contributed new knowledge in firstly the field of DC therapy. It has demonstrated that early application of IFN-γ during DC differentiation is crucial in altering the allo-fate of DC - producing hypo-responsive T-cells (Chapter 3). This has clinical applicability with the modification of the FAST-DC protocol, which allowed the propagation of IFN-γ tolerogenic DC in 48 hours (Chapter 4), making tolerogenic DC therapy more clinically applicable to deceased donor transplantation. Secondly this thesis has demonstrated soluble PD-L2 expression by human islet may have potential to protect against both allo- and auto-reactive T-cell mediated immune responses. In addition, the ability of soluble PD-L2 to act more potently then the transmembrane isoform to induce PD-1 signalling is an observation which has not been previously documented in the literature before.

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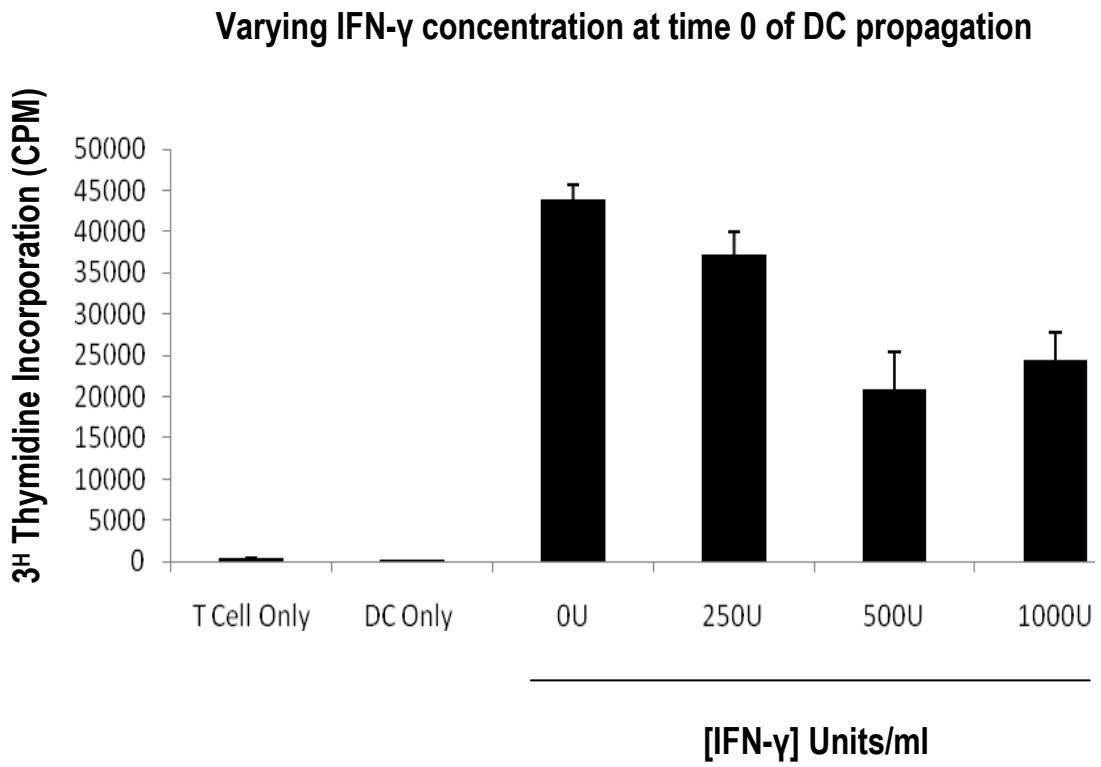
APPENDIX A- Table of primer sequences and conditions

Gene	Forward primer	Reverse Primer	Cycling Conditions
RelB	5'TTT TAA CAA CCT GGG CAT CC	5' CGC AGC TCT GAT GTG TTT GT	25s @ 95°C 25s@55°C; 25s@72°C for 50 cycles
IL-12	5' TTT GGA GAT GCT GGG CAG TAC A	5'GAT GAT GTC CCT GAT GAA GAA GC	25s @ 95°C; 25s@ 60°C; 25s@72°C for 50 cycles
IDO	5'GGC AAA GGT CAT GGA GAT GT,	5' CTG CAG TAT CCA TCA CGA AA,	25s @ 95°C; 25s@ 55°C; 25s@72°C for 50 cycles
HLA-G	5'AAG GCC CAC GCA CAG ACT GAC AGA ATG AAC	AGG TCG CAG CCA ATC ATC CA	25s @ 94°C; 25s@ 55°C; 25s@72°C for 50 cycles
IRF4	5'AGT CCT GAG CGA AAA CAG GA	5'AAA GCC AAG AGG TGC GAG TA	25s @ 95°C; 25s@ 55°C; 25s@72°C for 35cycles)
GAPDH	5'ATC ACT GCC ACC CAG AAG ACT	5'CAT GCC AGT GAG CTT CCC GTT	25s @ 95°C; 25s@ 55°C; 25s@72°C for 35cycles).
PD-L2-FL	5'CGG AAT TCA TGA TCT TCC TCC TGC TA	5'CGG GAT CCG ATA GCA CTG TTC ACT TC	1min @94°C 30sec@58°C 30s@72°C Note: final conc of $MgCl_2$ 3.5mM
PD-L2-SOL	5'CGG AAT TCA TGA TCT TCC TCC TGC TA	5'CGG GAT CCG ATG AAA ATG TGA AGC AG	1min @94°C 30sec@58°C 30s@72°C Note: final conc of $MgCl_2$ 3.5mM

APPENDIX B- Varying IFN-γ concentration at time 0 of DC propagation



APPENDIX C - STZ dose batch testing results

Dose (mg/kg)	mice n=	% Diabetic	Mortality
170	5	40%	0%
180	12	66%	8%
190-210	11	82%	27%

Note doses 190-210 were grouped together because they cause high degree of morbidity and mortality and were considered as highly toxic. 180mg/kg was chosen over 170 as it improved the rate of diabetes induction by approximately 30%, with low degree of morbidly observed.

APPENDIX D - Diagram of hPD-1/mCD28 chimeric assay

NOTE:
This diagram is included on page 220 of the print copy of the thesis held in the University of Adelaide Library.

The above diagram illustrates how signalling of human PD-1 is measured by murine IL-2 ELISA. In brief, when PD-L2 binds to the human PD-1 which forms the extracellular portion of the chimeric protein, which is fused to murine CD28. Thus signalling of PD-1 induces the production of murine IL-2 as the hPD-1/mCD28 chimeric protein is expressed in a murine T-cell hybridoma cell line.

APPENDIX E _calculation of stimulation index

$$\text{Stimulation Index (SI)} = \frac{\text{Insulin concentration after high glucose concentration stimulation}}{\text{Insulin concentration after low glucose concentration stimulation}}$$

APPENDIX F – Copy of publication in which data from chapter 3 was presented

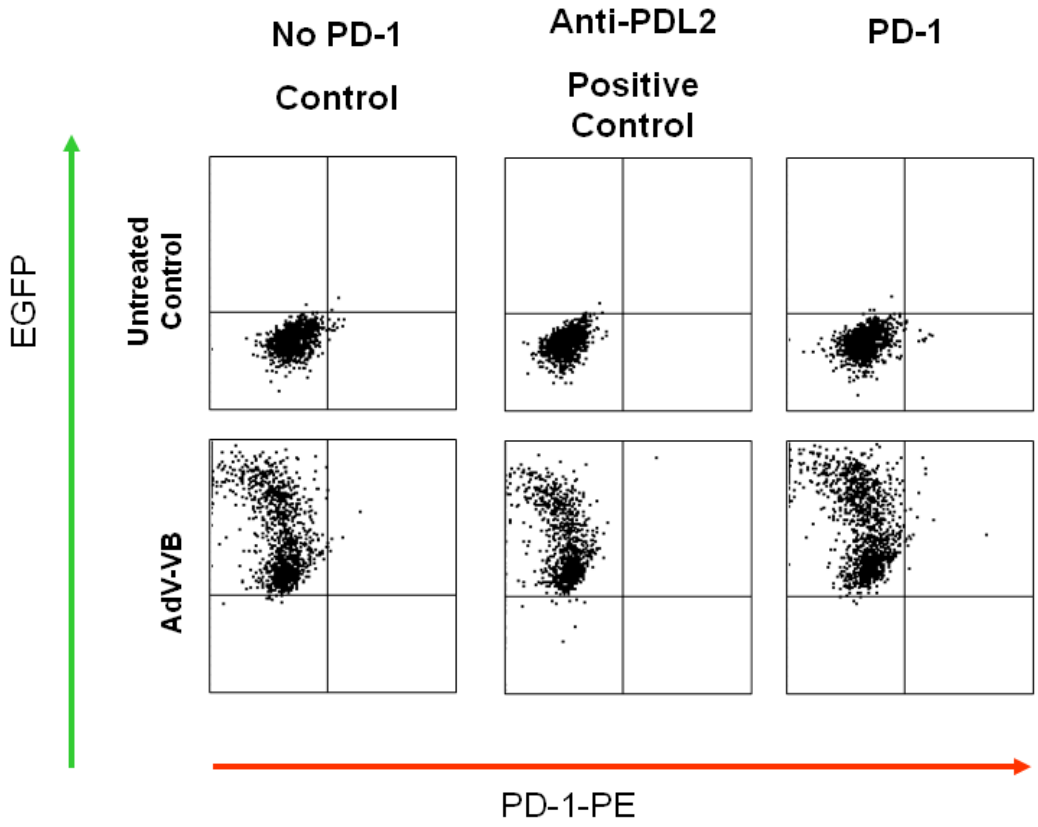
Available Online

Rojas D, Krishnan R. IFN-gamma generates maturation-arrested dendritic cells that induce T cell hyporesponsiveness independent of Foxp3(+) T-regulatory cell generation. Immunology letters; **132**:31-7.

APPENDIX G – Copy of publication of review

Available Online
Hughes A, Jessup C, Drogemuller C, Mohanasundaram D, Milner C, **Rojas D**, Russ GR, Coates PT. Gene therapy to improve pancreatic islet transplantation for Type 1 diabetes mellitus. Curr Diabetes Rev; **6**:274-84.

APPENDIX H – HEK-293 Transduction Controls



Both Untreated and AdV-VB transduced HEK-293 controls do not express PD-L2 and thus do not bind to PD-1Fc.