CHARACTERISATION OF THE IMMUNOMODULATORY ACTION OF A UNIQUE ANTI-CXCR3 MONOCLONAL ANTIBODY EXPLOITING A CONSERVED HUMAN AND MURINE EPITOPES FOR TRANSPLANTATION

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Abbreviations

αCXCR3-9C5 – anti-CXCR3 monoclonal antibody, clone 9C5
αCXCR3-173 – anti-CXCR3 monoclonal antibody, clone 173 (PerCpCy5.5 labelled)
γIRE-1 - IFN-response element
ADCC - antibody-dependent cellular cytotoxicity
AEC - Animal Ethics Committee
Akt - Protein kinase B
APC - antigen presenting cells
APC – Allophycocyanin
ARC - Animal Resource Centre
ASM – airway smooth muscle
ATG - anti-thymocyte globulin
BAL - bronchiolar lavage fluid
BGL - blood glucose levels
CDC - complement-dependent cytotoxicity
CFSE - Carboxyfluorescein succinimidyl ester
CITR - Collaborative Islet Transplant Registry
CNI - calcineurin inhibitor
CTLA-4 - cytotoxic T-lymphocyte-associated protein 4
CTL - cytotoxic T lymphocyte
CXCR3 – chemokine receptor 3
CXCL9/10/11 – chemokines of CXCR3
CXCL9 - monokine induced by gamma interferon (MIG or CXCL9)
CXCL10 - interferon-induced protein of 10kDa, (IP-10 or CXCL10)
CXCL11 - interferon-inducible T cell alpha chemoattractant (I-TAC or CXCL11)
DC - dendritic cell
DT - diphtheria toxin
dLN – distal lymph node
DRY - aspartate-arginine-tyrosine motif
DM - Diabetes mellitus
EAE - experimental allergic encephalomyelitis
Eff – effector
FACS - Fluorescence-activated cell sorting
Foxp3 - forkhead box P3 transcription factor
Gαi – G protein subunit
GADA - glutamate decarboxylase
GITR - glucocorticoid-induced TNFR-related protein
GP – glycoprotein
GPCR - G-protein coupled receptor
GVHD - graft versus host disease
H&E – haematoxylin and eosin
HLA - human leukocyte antigen
HO-1 - heme oxygenase 1
IAA - insulin islet antigens/insulin autoantibodies
IA-2A - protein tyrosine phosphatase-like protein IA-2
IBD - inflammatory bowel disease
ICAM-1 - Intercellular Adhesion Molecule 1
ICI - insulin containing islets
IDI - insulin deficient islets
IDDM1 – insulin dependent diabetes mellitus 1
IDDM2 – insulin dependent diabetes mellitus 2
IFN – interferon
IgG1 – immunoglobulin G1
IGRP - Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL – interleukin
IL2R – interleukin 2 receptor
IMPDH - Inosine Monophosphate Dehydrogenase
i.p. – Intraperitoneal
IRSE - IFN-stimulated response elements
i.v. – intravenous
JAK - c-Jun N-terminal kinase/ Janus kinase
kDa – kilodalton
LAT - Linker of Activated T cells
LCMV - Lymphocytic choriomeningitis
MØ – macrophages
M199 - Islet Isolation Media
mAb – monoclonal antibody
MACS – magnetic-activated cell sorting buffer
MAPK - Mitogen-activated protein kinase
Mem – memory
MFI - Mean fluorescence intensity
MHC - major histocompatibility complex
MLR – mixed lymphocyte reaction
mRNA – messenger RNA
MST - median survival time
mTOR - mammalian target of rapamycin
NFAT - Nuclear factor of activated T-cells
NFκB - p65 nuclear factor kappa-light-chain-enhancer of activated B cells
NK - Natural Killer
NKT - Natural killer T cells
NLRP3 - nucleotide-binding oligomerisation domain leucine rich repeat and pyrin domain-containing protein
NOD – non-obese diabetic mouse
PBS - Phosphate-buffered saline
PC61 – anti-CD25 antibody, clone PC61
PI3K - phosphatidylinositide 3-kinases
PLC - Phospholipase C
pLN – pancreatic lymph node
POD – post-operative days
PTX - pertussis toxin
Rapa – rapamycin
RBCL - red blood cell lysis buffer
RIP - rat insulin promoter
SEM – standard error of the mean
STAT - Signal Transducer and Activator of Transcription
STZ – streptozotocin
T1D – Type 1 diabetes
T2D – Type 2 diabetes
T-bet - T-box expressed in T cells
TCR - T cell receptor
TGF - Transforming growth factor
ttkcc – the Kinghorn Cancer Centre
Th – CD4+ T helper cell
TLR - toll-like receptor
TNF - tumour necrosis factor
Treg – regulatory T cells (CD4+ Foxp3+)
VCAM-1 - vascular cell adhesion molecule 1
ZAP-70 - Zeta-chain-associated protein kinase 70
Awards and Presentations arising from this Thesis

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- Mentee-Mentor Award, TSS, Melbourne, (2015)
- Young Investigator Award, Annual Scientific Meeting, TSANZ, Canberra, (2015)
- JDRF Top-up Scholarship (2014)

Presentations

Abstract

Transplantation of foreign tissues into genetically dissimilar recipients, called an allograft, results in an inflammatory immune response against the allograft, necessitating suppression of the rejection response. However, current immunosuppressive drugs broadly suppress the immune system and have number of associated side-effects. More selective, alternative therapies such as inhibition of T cell migration to sites of allograft are for this reason of great interest. CXCR3 is a chemokine receptor highly expressed on activated Th1 and CD8+ T cells, with elevated expression in patients undergoing allograft rejection and animal models of allograft rejection and autoimmune disease.

Due to the clinical application of islet transplantation for use in patients with diabetes we investigated the efficacy of a novel anti-CXCR3 mAb in models of islet transplant, autoimmune diabetes and islet transplant into autoimmune diabetic recipient mice. We hypothesised that transient blockade of CXCR3 signalling would delay islet allograft rejection and alter diabetes incidence.

As anticipated inhibition of CXCR3 signalling impaired chemotaxis of CXCR3 expressing cells and prolonged islet allograft survival in models of islet transplant. This was associated with reduced CD8+CD44 expression and reduced CD8+ effector and memory T cells. An increased frequency of Treg was also seen in islet allograft recipients. Further, Islet allograft survival was prolonged in autoimmune diabetic allograft recipients, a finding yet to be shown within the literature.

The role of CXCR3 in diabetes is ambiguous; with some prior studies indicating accelerated diabetes onset, while others show a delay. Of note treatment with our anti-CXCR3 antibody showed no change in diabetes incidence or pathology. In particular recruitment of Tregs from the pancreas to the pancreatic lymph node was unaffected, in contrast to previous literature showing impaired Treg migration and resultant accelerated diabetes onset.

Therefore transient antibody targeting of CXCR3 signalling with our antibody shows promise for use in allograft, in particular in islet allograft with safety in models of diabetes.
Experiments that were not the sole work of the author

All experiments were performed solely by the author at the Garvan Institute of Medical Research, with the exception of:

Figure 3.1c – experiment performed by Remy Robert

Figure 4.16 – experiment performed with Nathan Zammit

Figure 4.17a – experiment performed with Nathan Zammit

Figure 4.18 - experiment performed with Nathan Zammit

Figure 5.4 - experiment performed by Eliana Marino
Chapter 1. General Introduction

The role of CXCR3 in type 1 diabetes and islet transplantation
Overview

The immune system is highly mobile, this key element of its function is determined by the secretion and response to a large family of small proteins called “chemokines”, taken from the Greek –κinois or movement. The involvement of chemokines and their receptors in recruitment of immune cells to sites of inflammation is well established\(^8\). In particular recruitment of T cells to sites of inflammation under conditions of damage or infection is crucial for clearance of infection and resolution of inflammatory processes\(^9,10\). Under conditions of inflammation in the autoimmune as well as the allogeneic rejection response, recruitment of immune cells is unwanted and results in damage to either self-tissue or to allogeneic replacement tissues, respectively\(^11,12\). Additionally, chemokines play an important role in immune cell development and homeostasis, dictating the flow of immune cells from the bone marrow to peripheral sites for further development\(^13,14\).

The immune system plays a key role in many known pathologies, making targeting the immune system an appealing therapeutic strategy for intervention\(^15\). However cellular based therapies suffer numerous caveats. For instance current therapeutic approaches that target T cells by depletion or by impairing their activation result in systemic suppression of the immune system and are often not T cell specific resulting in toxicities in other tissues\(^16-20\). For these reasons other approaches need to be explored. Targeting chemokine signalling pathways, which are primarily involved in migration of activated T cells towards inflammation, is one appealing therapeutic strategy to intervene in these inflammatory responses\(^3,21-26\). In this thesis we will develop this concept further and explore in a series of \textit{in vitro} and \textit{in vivo} models, the potential for targeting one such chemokine ligand-receptor system, namely the CXCR3–CXCL9/10/11 family.
Type 1 Diabetes Mellitus

Classification and Epidemiology

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterised by hyperglycaemia, which can be subdivided into several distinct subtypes based on the pathogenic processes leading to this hyperglycaemia. Of these the two major subtypes include type 1 and type 2 diabetes mellitus. Type 2 diabetes mellitus (T2D) is a heterogeneous group of disorders characterised by varying degrees of insulin resistance, impaired insulin secretion and increased glucose production and accounts for approximately 85% of individuals with diabetes. Type 1 diabetes mellitus (T1D) is characterised by insulin deficiency, typically due to autoimmune beta cell destruction (type 1A) although a small proportion of individuals lack markers indicative of autoimmune disease (type 1B). Of all individuals with diabetes, individuals with T1D account approximately 10%\(^{27-30}\).

Diabetes mellitus currently affects an estimated 382 million people, 8.3% of adults, with estimates for 2035 predicting a rise to 592 million individuals affected by the disease globally\(^29\). Australia in particular, has an estimated diabetes prevalence of 7.4%, with approximately 7.7% of these individuals having T1D and one of the highest incidence rates of T1D in children globally (22.3 cases per 100,000 children)\(^{29,31,32}\).

Symptoms

In individuals with T1D the insulin-producing pancreatic beta cells, within the islets of Langerhans, are destroyed through an autoimmune process. Due to the importance of insulin secretion in glucose metabolism the loss of insulin results in a number of symptoms. Under physiological conditions the pancreatic islets produce both insulin and glucagon, which act in concert to regulate blood glucose levels. Insulin secretion triggers glucose uptake and storage as glycogen by insulin sensitive tissue such as the muscle and liver. In contrast glucagon mediates glycogenolysis, gluconeogenesis and ketone body formation by liver and lipolysis by adipocytes in order to breakdown glucose stores\(^{27,28,33}\). The lack of insulin produced in individuals with T1D results in impaired glucose uptake by liver and muscle and dysregulated, elevated glucagon levels. If left untreated this results in elevated blood glucose (hyperglycaemia) and
symptoms of polyuria, polydipsia and polyphagia, volume depletion, tachycardia, hypotension and metabolic acidosis. In extreme cases, if unchecked, hyperglycaemia can result in coma and be fatal. In addition to these acute symptoms, chronic hyperglycaemia results in an increased risk of severe micro and macro-vascular complications including; retinopathy, nephropathy, neuropathy, ischaemic heart disease, stroke and peripheral vascular disease.

**Genetic and Environmental Susceptibility**

Type 1 diabetes (T1D) results from the interaction of environmental factors within genetically susceptible individuals. A role for genetics in T1D is demonstrated by the high concordance rate seen within monozygotic twins, which are genetically identical, showing between 22.7 to 70% concordance, particularly where T1D develops early in life i.e. within the first 10 years. This rate is much higher than that seen for dizygotic twins (0-13%) indicating an important genetic component for early diabetes, but also highlighting an environmental component, as genes do not account for 100% of disease susceptibility. The increased risk of T1D in individuals with first degree relatives who have T1D provides further evidence for the role of genetics. Moreover individuals without diabetes who have first degree relatives affected by TID, have significantly greater levels of autoantibodies against islet antigens (insulin (IAA), glutamate decarboxylase (GADA), and the protein tyrosine phosphatase-like protein IA-2 (IA-2A)), with 31% found to have at least one positive autoantibody compared to none in controls who had no first degree relatives with T1D. Islet autoantibodies are strong predictors of T1D risk with higher autoantibody titer, presence of particular epitopes and IgG subclasses in patient serum correlating to increased risk of diabetes development. In addition these individuals have worse beta cell function and reduced insulin sensitivity showing heritability of the disease.

T1D is a polygenic disease with at least 50 loci associated with susceptibility to the disease, of these the largest contribution comes from the so called Insulin-Dependent Diabetes Mellitus (IDDM) 1 locus. Linkage of this region to the human leukocyte antigen (HLA) class II major histocompatibility complex (MHC) allele accounts for over 50% of the genetic risk of developing T1D. Many other non-HLA genes show strong genetic linkage associated with TID susceptibility, such as


IDDM2 a polymorphic locus near the human insulin gene and IDDM12 a polymorphism of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene\textsuperscript{41-43}.

In addition to the genetic component of T1D susceptibility a number of environmental factors have been suggested to contribute to disease development, though to date this aspect of T1D is not well understood. Of the suggested environmental factors birth route via caesarean birth has been shown to increase the incidence of T1D, possibly due lack of exposure to the vaginal microbiome of the mother, leading to an altered gut microbiota\textsuperscript{44}. Diet has also been suggested to contribute to T1D development with early exposure to cows milk leading to an increased incidence of T1D. Shorter duration of breastfeeding and earlier exposure to cow milk proteins are both thought to alter the gut microbiome which plays an important role in development of oral and peripheral tolerance\textsuperscript{45,46}. Further, exposure to some infectious agents, such as enteral viruses, has been linked to an increased diabetes incidence\textsuperscript{47}. However controversy surrounds many of these proposed environmental triggers due to a number of conflicting studies\textsuperscript{44-46,48-51}. As such more research is needed to determine with more certainty the roles of these environmental triggers in T1D development.

**Human Pathogenesis and Natural History**

T1D results from an autoimmune destruction of the insulin-producing pancreatic beta cells, found within the islets of Langerhans. In 1965 a pivotal article by Gepts \textit{et. al.}, noted the presence of a lymphocytic infiltrate, now termed insulitis, and significantly reduced number of insulin-secreting beta cells in the pancreatic islets upon autopsy of patients with T1D. However glucagon (alpha), somatostatin (delta) and pancreatic polypeptide secreting cells were unaffected in these individuals\textsuperscript{52,53}. This led Gepts and colleagues to propose that; “the islet inflammation may be secondary to a viral infection or represent an immunological autoimmune process”\textsuperscript{52}.

Much research has been since conducted to test the hypothesis that T1D is an autoimmune condition. Key studies that support this notion are those which have identified islet cell autoantibodies against insulin (IAA), glutamate decarboxylase (GADA) and the protein tyrosine phosphatase-like protein IA-2 (IA-2A) in people with T1D as well as murine models\textsuperscript{54-56}. In addition the identification of islet specific
T cell clones, including those against insulin and Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) further support the idea that T1D exhibits an autoimmune atiology\textsuperscript{57,58}. In line with these findings T cell suppression using prednisone and anti-thymocyte globulin (ATG) or cyclosporine were found to delay onset of T1D\textsuperscript{59,60}.

The major leukocytes found within the islet infiltrate are CD8 cytotoxic T cells (CTL) and B cells, with a small proportion of CD4 T cells and macrophages occasionally present\textsuperscript{11,61-63}. Within the affected pancreas all cells of the islet hyper-express MHC I and secrete interferon (IFN)-\(\alpha\) and this appears to precede insulitis, suggesting a role for the islets themselves in the initial activation of an immune response against the beta cells\textsuperscript{61-65}. Beta cells are also able to secrete chemokines (e.g. CXCL10) involved in recruitment of both CD8+ and CD4+ T to the islet, providing further evidence for a beta cell specific role in the immune response\textsuperscript{66}. Collectively these data indicate a role for T cells in the pathogenesis of the disease, but most significantly support the concept that T1D has an autoimmune aetiology\textsuperscript{54-60}.

Study of the natural history of the disease in humans has been limited by its reliance on specimens obtained from autopsy of deceased patients with T1D. However our current understanding of the pathogenesis of TID has evolved over time with initial models suggesting a linear decline in beta cell mass and onset of overt diabetes with only 10% residual beta cell mass. These models have now been expanded to encompass a relapsing-remitting decline in beta cell mass and onset of overt diabetes occurring with variable beta cell mass remaining (40-50% in some individuals)\textsuperscript{40,52,67-69}. The pancreatic pathology also suggests a heterogeneous and lobular distribution of affected islets with some islets heavily infiltrated while other neighbouring islets are unaffected. Beta cells may also be classified based on insulin content as insulin deficient islets (IDI) or insulin containing islets (ICI)\textsuperscript{53,65}. The medical significance of these patterns is unknown.
Animal Models

Due to the limited availability of human pancreas specimens our current understanding of T1D has been heavily reliant on experimental animal models, of which the non-obese diabetic (NOD) mouse has been extensively studied. The NOD mouse shares a number of important features with the human disease although a number of differences should also be noted (reviewed by 70-72). As for patients with T1D, NOD mice show destruction of the pancreatic beta cell with loss of insulin production and hyperglycaemia70 (Figure 1.1). There is a strong genetic component to diabetes in the NOD mouse, with a significant contribution by the MHC and other loci that overlap with human loci involved in T1D73,74. Further to this, there is also a strong environmental influence on disease susceptibility, which appears to relate to infection and diet. As an example of this environmental susceptibility, the ‘cleanliness’ with respect to viral load of the NOD colony is known to significantly affect disease penetrance in this model70,75. Increased viral load results in a decreased diabetes incidence in the NOD colony while specific-pathogen-free mice show a greater diabetes incidence75. As understood for T1D, NOD mice also show islet specific autoantibodies and T cell autoreactivity against beta cell specific antigens (insulin and IGRP) with disease driven by CD4+ and CD8+ T cell cytotoxicity, which in the later stages requires the presence of B cells76-80. NOD mice also show beta cell replication and a type 1 IFN signature early in disease progression, as in the human disease63,77,81-83.

The age of onset and staging of disease in the NOD mouse differs from that seen in individuals with T1D, with insulitis beginning at 4 weeks of age and overt diabetes developing from 12 to 30 weeks of age in 80% of female mice. In comparison, onset of overt TID in humans is variable and can present at any age starting from infancy into old age68,71. The NOD mouse also shows a clear female gender bias, with 80% of female but only 10-40% of males developing diabetes, another aspect of the NOD disease not seen in humans72,75. The inbred nature of the NOD strain also means study of this model is representative of a single case study of diabetes, rather than the heterogeneous human population68,71. Finally NOD mice are able to survive 1-2 months with hyperglycaemia without requirement of insulin for survival, which is not seen in humans and results from their resistance to ketoacidosis75.
Within the NOD model disease development can be divided into 4 major stages based on age and immune infiltrate. Initial infiltrate from 4-6 weeks of age consists primarily of antigen presenting cells (APC), as well as CD4+ T cells, found in 10-30% of islets and accompanied by a type 1 IFN signature. From around 8 weeks of age up-regulation of chemokines (e.g. CXCL9, CXCL10, CXCL11, CCL4, CCL5, CCL2, CCL22) and adhesion molecules (ICAM-1, VCAM-1), as well as a 7 fold increase in T cells, and CD11c+ cell infiltration occurs, with 50-60% of islets showing predominantly peri-insulitis\textsuperscript{77}. At 11-12 weeks, the point at which development of overt diabetes is first seen, a 3 fold increase in marginal zone B cells is evident\textsuperscript{1,77,81}. An important role for B cell presentation of beta cell antigen, in a MHC class 1-restricted manner, to cytotoxic CD8+ T cells for their activation and proliferation has been identified\textsuperscript{1}. Together this results in progressive beta cell loss.

\textbf{Figure 1.1: Comparison of Human T1D and NOD diabetes.}  
\textbf{Left;} H&E stained islets infiltrated with immune cells (blue) from a pre-diabetic NOD mouse and a person with T1D and pancreas transplant showing recurrent autoimmunity, with two neighbouring islets, one heavily infiltrated and one without infiltrate, courtesy of S. Pilgrim, Sydney (top) and G. Burke, Miami, (bottom)  
\textbf{Middle;} Insulin stained islets from a diabetic NOD mouse showing heavily infiltrated islets (blue) and residual insulin positive cells (brown) and from a healthy person showing an islet without infiltrate and insulin positive staining (brown) courtesy of S. Pilgrim, Sydney (top) and S. Bonner Weir, Cambridge, Massachusetts (bottom)  
\textbf{Right;} T cell infiltration (green) of an islet from a NOD mouse showing residual insulin staining (red) and T cell infiltration (αCD3, brown) of an islet from a person with T1Ds, courtesy of S. Grey, Sydney (top) and T. Kay, Melbourne (bottom)
and onset of overt diabetes. This time point is associated with a cytotoxic related gene profile\textsuperscript{77}. Development of diabetes in the NOD mouse is dependent on both CD4+ and CD8+ T cells and a breakdown of regulatory and tolerance mechanisms is thought to contribute to disease development in the NOD mouse\textsuperscript{72,84-88}.

A number of other models of diabetes exist, briefly these include: the BB rat, which develops insulitis and spontaneous diabetes, characterised by hypoinsulinaemia, hyperglycaemia, glycosuria, ketonaemia and ketosis. However unlike human or NOD diabetes the BB rat displays lymphopenia\textsuperscript{89,90}. Models of experimentally induced diabetes are also used, one such model is the RIP-LCMV model. In this model mice transgenically express the Lymphocytic choriomeningitis (LCMV) viral glycoprotein (GP) under the control of the rat insulin promoter (RIP). This does not itself result in diabetes but leads to beta cell specific expression of the LCMV GP and upon infection of mice with LCMV leads to generation of GP specific CD8+ T cell clones, which recognise and destroy GP expressing beta cells leading to hyperglycemia\textsuperscript{90-92}.

**Therapies**

Currently no cure for diabetes exists and the primary treatment involves administration of exogenous insulin typically via multiple daily injections or a pump system, which aims to maintain euglycaemia artificially\textsuperscript{93}. While most individuals with T1D are able to maintain blood glucose levels (BGL) with regular administration of insulin, it is an imperfect solution and requires constant monitoring to maintain euglycaemia, preventing hyper- or hypoglycaemia\textsuperscript{27,93}. The risk of complications in individuals with diabetes is significantly reduced through tight management of blood glucose, although fear of hypoglycaemia prevents many individuals from maintaining optimal glycaemic control\textsuperscript{27,93-96}.

Hypoglycaemia is a severe complication of insulin therapy, characterised by palpitations, trembling, sweating, hunger and confusion due to low blood glucose levels\textsuperscript{97}. Many individuals are able to recognise and respond appropriately to these symptoms. However individuals with hypoglycaemic unawareness lack the physiological response to hypoglycaemia and have an impaired ability to appropriately manage their blood glucose levels. In addition to those with
hypoglycaemic unawareness a small group of individuals with diabetes show significant variability in their blood glucose level, which is poorly managed by exogenous insulin, known as ‘brittle diabetes’. Individuals suffering these more extreme episodes make up approximately one third of individuals with T1D and suffer recurrent episodes of severe, disabling hypoglycaemia, increasing the risk of complications and mortality\textsuperscript{27,97-99}. These individuals with hypoglycaemic unawareness and brittle diabetes have a significantly reduced quality of life and diminished ability to carry out everyday tasks and it is in these individuals that islet transplant is advised\textsuperscript{27,98,100-102}.
Islet Transplantation

Transplantation

Transplantation is a medical procedure involving the transfer of organs from a donor into a recipient, primarily to replace damaged or absent organs. The evolution of modern transplantation began as early as the 2nd century B.C. with incomplete accounts of skin autograft performed by Sushruta followed in the 16th century A.D by the better documented works of Tagliacozzi. In the 1940s the role of the immune system in failure of transplants was recognized when skin grafts received by burns patients from unrelated donors underwent a process of inflammatory necrosis, while skin grafts from the patient themselves successfully engrafted. This anti-graft immune response was termed ‘rejection’ and was followed in the mid-1970s with drugs able to suppress the rejection response. These immunosuppressive drugs revolutionised the practice of transplantation, impairing the host immune response against the donor tissue and allowing successful engraftment of allogeneic tissue.

Today the use of transplantation is much more widespread, with significant improvements in immunosuppressants and surgical techniques leading to higher rates of graft and patient survival. A number of organs and tissues are transplantable today including: heart, liver, kidney, pancreas, lung, skin and pancreatic islets among others. There are however two major caveats to transplantation; firstly the availability of donor organs and secondly the immune response to foreign tissues.

Allograft Immune Response – A role for T cells

Transplant of tissues between genetically dissimilar individuals of the same species is termed an allograft, with foreign tissues recognised by the recipient immune system and rapidly destroyed, leading to organ/tissue rejection. This occurs due to the introduction of MHC antigens on the surface of donor cells, designated HLA in humans, which differ to those of the recipient. These are expressed on all cells of the body, with MHC class II molecules expressed by professional APC, primarily dendritic cells (DC), macrophages (MØ), monocytes and B cells and MHC I expressed ubiquitously on all nucleated cells. The recognition of these genetic
MHC variants, termed ‘mismatches’, between the donor and recipient result in a T cell mediated immune response against the donor tissue leading to acute allograft rejection responses\textsuperscript{110}.

**Antigen Presentation (Signal 1)**

Cell mediated immune reactions involve T cells, which can be subdivided into CD4+ and CD8+ T cells based on their expression of cluster of differentiation (CD)4 and CD8 surface glycoproteins. CD4 and CD8 T cells recognise antigen presented by either MHC II or MHC I, respectively, via their T cell receptor (TCR) (Signal 1)\textsuperscript{108,109}. In the transplant setting this interaction between the MHC molecules and TCR can occur through direct, indirect or semi-direct antigen presentation and represents the first signal of T cell activation. Indirect presentation requires uptake and processing of donor expressed MHC into peptides and presentation by recipient APCs. Direct presentation involves transfer of donor derived APCs within graft tissue, which migrate to the recipients draining lymph nodes and are recognised, via their MHC expression, as foreign by recipient T cells\textsuperscript{109-111,112}. A more recently proposed pathway, the semi-direct pathway of allorecognition, involves uptake of intact donor MHC by recipient APC and presentation of the intact allogeneic MHC on the surface of recipient APCs\textsuperscript{112,113}. The direct and semi-direct pathways of recognition are unique to the allograft setting, where foreign MHC II and MHC I are introduced with the graft tissue\textsuperscript{112-114}.

**T cell Co-stimulation (Signal 2)**

Cell mediated immune reactions also require a second costimulatory signal for activation (Signal 2). This involves binding of T cell expressed CD28 to CD80/CD86 (B7.1/B7.2) expressed by APCs, in addition to a number of other costimulatory signals such as CD40-CD40L (CD154). Together with MHC interactions these signals activate T cells resulting in expansion and differentiation of antigen activated T cells\textsuperscript{115-118}

**Cytokine and Chemokine Milieu (Signal 3)**

In addition to TCR and co-stimulation, CD4+ T cells are driven to differentiate into effector lineages depending on the cytokine milieu and the cytokine receptors
induced during activation\textsuperscript{119}. Within the allograft immune response damage at the site of the allograft releases pro-inflammatory cytokines and chemokines that contribute to T cell activation, differentiation and migration of T cells (signal 3)\textsuperscript{8,16,120-124}. Some of the triggers leading to this damage include mechanical injury and infection. Ischemia reperfusion injury, which results from storage, transport, transplantation and reperfusion of the organ also results in release of danger associated molecules (e.g. DAMPs), damaging the organ\textsuperscript{125}.

The CD4+ T helper (Th) cell subsets involved in allograft rejection include; Th1, Th17 and CD4+ regulatory T cells, Foxp3+Treg\textsuperscript{126-128}.

These 3 signals result in activation of antigen specific T cell subsets, which are able to both directly damage or provide help to other immune cell subsets, mediating allograft rejection and offer attractive therapeutic targets for the prevention of allograft rejection.

\textit{T cell Effectors and Immune Regulation}

T cell subsets differentiate based on different environmental cues, signal 1-3 above and are characterised by their expression of different transcription factors, cytokines and chemokines. Differentiation of the Th1 subset is driven by interleukin (IL)-12, IFN-\gamma, activation of Signal Transducer and Activator of Transcription (STAT) 4 and the transcription factor T-bet\textsuperscript{123,129,130}. Activated Th1 highly express CXCR3 and IFN-\gamma and are associated with allograft rejection\textsuperscript{123,131}. Alternatively Th cells may become Th17 cells, which differentiate in response to Transforming growth factor (TGF)-\beta and IL-6 and release IL-17 and IL-6. Their exact role in allograft rejection remains uncertain, although the ratio of Th17 to Tregs appears to predict allograft dysfunction and production of IL-17 enhances local inflammation\textsuperscript{132-137}. Regulatory CD4+ T cells (Tregs) are characterised by expression of the transcription factor forkhead box P3 (Foxp3). These may be thymically derived or induced from naïve CD4+ T cells in the periphery upon exposure to TGF-\beta\textsuperscript{138,139}. Tregs are essential to peripheral tolerance and can control allograft responses. Indeed, in experimental models that allow allograft survival many have found expanded Tregs to be essential for this phenomenon. For instance, studies indicate an increased ratio of Tregs to CD3+ T cells positively correlates with allograft function. Treg transfer was also able
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to induce antigen-specific tolerance in allograft models\textsuperscript{119,140-144}. The suppressive activity of Tregs is attributed to multiple mechanisms but close proximity to the cell targeted for regulation appears to be key. Mechanisms of regulation include; impaired T cell activation and maturation via IL-10 and TGF-β secretion, inhibition of pro-inflammatory transcription factors, cytolysis and cell contact mediated inhibition of T cell co-stimulation via CTLA-4\textsuperscript{139,145-148}.

**Islet Allograft Success and Immunosuppressive Therapy**

The allograft immune response is a major factor hindering the application of islet transplantation. However recent improvements in islet isolation techniques as well as the introduction of the Edmonton Protocol of immunosuppressive therapy have significantly improved islet allograft success rates\textsuperscript{101,149,150} and from 1999 to 2012 a total of 864 allogeneic islet transplants were performed (figure 1.2)\textsuperscript{151}.

Although islet allograft success rates have improved from initial transplantation outcomes to those seen in 2010, discussed below, a number of issues including; the availability of HLA matched donor organs, the risk of surgical complications, particularly for pancreas transplant, and most importantly the need for life-long treatment with immunosuppressant drugs to prevent graft rejection exist\textsuperscript{16,100}. The current immunosuppressant regimens required to maintain allograft function broadly suppress immune function, resulting in a number of side-effects\textsuperscript{16,17}. These include; an increased susceptibility to infections, development of cancer\textsuperscript{18} and toxic effects of the drugs such as nephrotoxicity\textsuperscript{19} and impaired beta cell function (diabetogenic drugs)\textsuperscript{20}. These toxic side effects may also damage the replacement organ resulting in impaired function\textsuperscript{152}. In addition current immunosuppressive therapy appears to impede development of long-term immunological tolerance\textsuperscript{153}.

These issues limit pancreas and islet cell allograft to the most serious cases\textsuperscript{100,154}. Islet transplant is also not performed in children or those individuals with diabetes, where the benefits of transplant for the individual are outweighed by the requirement for life long immune suppression\textsuperscript{16,154}. However earlier transplantation in these individuals has the potential to improve glycaemic control and may minimise long-term complications.
The advent of the Edmonton protocol, by Shapiro et al., in 2000 revolutionised the course of islet transplant with 7 out of 7 patients achieving insulin-independence\textsuperscript{149}. This drug regimen was corticosteroid free, using an IL-2 receptor antagonist for induction together with the mammalian target of rapamycin (mTOR) inhibitor (sirolimus/rapamycin) and a calcineurin inhibitor (CNI) (tacrolimus) for maintenance\textsuperscript{149}. The Collaborative Islet Transplant Registry (CITR) indicates rates of 51% insulin-independence at 1 year, 36% at 2 years and 27% at 3 years for transplants performed from 1999-2002. Transplants performed from 2007-2010 showed significantly improved insulin-independence rates of 66%, 55% and 44% at 1, 2 and 3 years post-transplant, respectively. The period from 2007-2010 has also seen the use of a T-cell depleting antibody in addition to an antibody that blocks activity of the inflammatory cytokine tumour necrosis factor (TNF)-α (etanercept) and involves maintenance therapy with an mTOR inhibitor or an inosine monophosphate dehydrogenase inhibitor (mycophenolic acid) combined with a CNI\textsuperscript{149,150}.

![Figure 1.2: Islet Transplantation](image)

*Figure 1.2: Islet Transplantation*

Collection, isolation and infusion of pancreatic islets from a donor into the portal vein of an islet transplant recipient with T1D. Bright field image of human islets curtesy of S. Grey and islet transplant image adapted from\textsuperscript{7}.

Although a number of individuals are no longer insulin-independent by the 3\textsuperscript{rd} year post transplant, islet graft survival (C-peptide ≥0.3 ng/mL) remains in 83% of individuals and over 90% of transplanted individuals remain free of severe hypoglycaemic and life-threatening events 5 years post-transplant\textsuperscript{150}. While insulin-
independence is as yet suboptimal the improvements seen in blood glucose control and absence of hypoglycaemia in individuals who had unmanageable glucose prior to transplant, significantly improves their quality of life and manageability of their diabetes\textsuperscript{101,102,155,156}.

**Current Immunosuppressants**

Immunosuppressant therapies target the 3 main steps in T cell activation; TCR/MHC interaction (signal 1), co-stimulation (signal 2) and cytokine & chemokine signalling (signal 3), while others exert their function via the depletion of T cells\textsuperscript{15,119}.

Firstly, Tacrolimus and Cyclosporine A function through blockade of nuclear factor of activated T-cells (NFAT), impairing T cell activation via inhibition of calcineurin (signal 1). However these drugs, also known as CNIs, cause significant nephrotoxicity, hypertension and impaired beta cell function\textsuperscript{15,157,158}. Sirolimus (rapamycin) is an mTOR inhibitor, which also impairs cell cycle, proliferation and activation, although mediates this in a calcineurin independent manner\textsuperscript{159}. Co-stimulatory blockade (signal 2) is targeted by CTLA-4-Ig (Abatacept and Belatacept) and anti-CD40 (5C8), which inhibits interactions of CD80/CD86 with CD28 and CD40 with CD40L, respectively\textsuperscript{160-162}.

Cytokine signalling (signal 3) is also targeted by a number of drugs. Etanercept antagonises TNF signalling, reducing the pro-inflammatory effects of this cytokine on the immune system\textsuperscript{163}. Basiliximab & Daclizumab are directed against IL-2R\alpha and impair T cell proliferation and activation\textsuperscript{164,165}.

Finally drugs which deplete T cells include; OKT3, an anti-CD3 antibody and aletuzumab, a CD52 targeting antibody. Anti-thymocyte globulin (ATG), a polyclonal-immunoglobulin preparation with multiple antibody specificities is raised in rabbits or horses against human thymocytes for T cell depletion via complement-dependent cell lysis\textsuperscript{119,166-168}. Importantly use of T-cell depleting antibodies are associated with increased cancer risk\textsuperscript{167}.

Each of these drugs have adverse side effects that limit their use, as outlined briefly in table 1.1\textsuperscript{15}.  

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\textsuperscript{101,102,155,156}
In addition to inhibiting these stages of T cell activation, the targeting of immune cell migration is another attractive strategy. Recruitment of immune cells into sites of inflammation is essential in allograft and autoimmune destruction, with local production of chemokines causing migration of cells expressing their cognate receptors. Therapeutic strategies directed against chemokine receptors preferentially expressed by the alloreactive and autoreactive T cells mediating disease may prove useful in the design of novel anti-inflammatory therapies.

Table 1.1: Immunosuppressant Side-effects

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molecular Target (pathway/receptor)</th>
<th>Primary Target Cell Population</th>
<th>Side-effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>TCR activation/Calcineurin-dependent dephosphorylation of NFAT</td>
<td>T lymphocytes</td>
<td>Nephrotoxic, hypertension</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>TCR activation / Calcineurin-dependent dephosphorylation of NFAT</td>
<td>T lymphocytes</td>
<td>Nephrotoxic, diabetogenic, hypertension</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>mTOR/complexes with FKBP blocking mTOR activation</td>
<td>T lymphocytes</td>
<td>Hyperlipidaemia, peripheral oedema, myelosuppression</td>
</tr>
<tr>
<td>OKT3</td>
<td>Apoptosis of CD3 expressing cells via Ab binding, prevents T cell activation</td>
<td>T lymphocytes (mature)</td>
<td>Cytokine storm, pulmonary oedema, thrombocytopenia</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>Binds to CD52 causing antibody-dependent cellular-mediated lysis</td>
<td>(mature B &amp; T cells, NK cells, monocytes, dendritic cells)</td>
<td>Thrombocytopenia, fever, hypotension</td>
</tr>
<tr>
<td>Basiliximab</td>
<td>T cell differentiation/ proliferation/Binds IL-2 receptor α chain (CD25) and competes with IL-2 for binding</td>
<td>T lymphocytes (activated)</td>
<td>Fever and chills</td>
</tr>
</tbody>
</table>
Chemokines

Chemokines are a family of 8-12 kDa peptides, which induce directional migration of leukocytes in both physiological and pathological settings. These molecules are released by many different cells, both of immune and non-immune origin, in order to recruit immune cells into sites of inflammation. Chemokines can be subdivided into 4 different classes based on cysteine number and spacing. The largest group, cysteine-cysteine (CC) chemokines, have the first 2 cysteines in adjacent positions and cluster on chromosome 17q11 & q12. The second largest group, CXC chemokines, have the first 2 cysteines separated by an intervening amino acid, clustering on chromosome 4q12-4q13 and 4q21. Finally the last two groups, containing either two highly related or a single unique chemokine, are the C and CX3C chemokines respectively. These chemokines consist of either 2 cysteine residues (C) or two cysteine residues separated by 3 amino acids (CX3C).

Each of these chemokines also bind to G protein coupled chemokine receptors, which show redundancy with a single receptor able to bind multiple different chemokines, although this does seem to be restricted by subclass i.e. CC chemokines typically don’t bind CXC receptors. Chemokine receptors have 7 transmembrane spanning domains flanked by an acidic extracellular N-terminal and an intracellular C-terminal.

Within the chemokine-chemokine receptor system, in addition to redundancy and cross-over between chemokine-receptor targets there is also overlap in the functional targets of each chemokine with multiple chemokine-chemokine receptors responsible for migration of a single cell type e.g. T cells. Broadly CC chemokines target monocytes and granulocytes while CXC chemokines target neutrophils, however both families have particular chemokines responsible for directing T cell migration. This T cell effect can also be broken down into those chemokines targeting Th1 (CCR5 and CXCR3) and those targeting Th2 (CCR3 and CCR4) responses.

Mechanistically chemokines mediate migration of different cells expressing their cognate receptor along a concentration gradient. Upon inflammation, endothelial cells of blood vessels up-regulate cellular adhesion molecules (e.g. ICAM-1, VCAM-1), these adhesion molecules are able to bind to molecules on the leukocytes travelling within the circulatory system, slowing leukocytes, which roll across...
endothelial surfaces. In addition exposure to inflammatory stimuli results in endothelial cell expression of proteoglycan bound chemokines, causing activation of firm adhesion by integrins and high affinity binding of chemokine receptor expressing leukocytes. This synergistic binding both activates leukocytes and causes extravasation out of the circulatory system, where cells may then travel along a chemokine gradient towards the site of inflammation (figure 1.3)\textsuperscript{8,174-176}. In addition chemokines are important in spacial and temporal localisation of immune cells within lymphoid organs, allowing appropriate T and B cell activation\textsuperscript{13}. 

T cells are critical immune cells in the inflammatory reaction. Therefore studying the chemokines and receptors which govern their recruitment is important in understanding lymphocyte infiltration and also for the development of T cell targeted therapies. One of the chemokine receptors shown to be highly expressed in a number of cancers, autoimmune diseases and in allogeneic transplantation is CXCR3, a chemokine receptor primarily upregulated on activated CTL and Th1 cells\textsuperscript{21,177-182}. Manipulating the ability of cells to migrate into the islet allograft, or potentially the pancreas in pre-diabetic individuals via inhibition of this chemokine receptor is an appealing therapeutic concept.

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**Figure 1.3: Leukocyte Chemotaxis**

1) Endothelial cells and leukocytes within the circulation become activated by inflammation and chemokine exposure up-regulating adhesion molecules (e.g. ICAM-1) and their ligands (e.g. integrin)

2) Fast moving leukocytes are slowed by binding of adhesion molecules and roll along the endothelial surface

3) Slow moving leukocytes firmly adhere to the endothelial surface

4) Leukocytes transmigrate out of the circulation and migrate along chemokine gradients towards inflammation and injury.
CXCR3

CXCR3 is a G-protein coupled receptor (GPCR) composed of a 7 transmembrane domain flanked by an intracellular carboxyl (C)-terminal and an extracellular N-terminus both of which are involved in chemokine mediated receptor activation. CXCR3 is located on the X chromosome (Xq13). Up-regulation of CXCR3 expression occurs in the presence of inflammatory signals with T cell expression dependent on the presence of T-bet and IFN-γ signalling and non-persistent T cell receptor (TCR) triggering.

CXCR3 exists as 3 alternatively spliced isoforms in humans, the most studied CXCR3-A consists of a 368 amino acid sequence of 40kDa, which binds the 3 classic CXCR3 chemokines; monokine induced by gamma interferon (MIG or CXCL9), interferon-induced protein of 10kDa, (IP-10 or CXCL10) and interferon-inducible T cell alpha chemoattractant, (I-TAC or CXCL11). The CXCR3-B isoform has a longer C-terminal domain and in addition to binding the 3 classic chemokines, binds platelet factor 4 (CXCL4) with high affinity. The affinity of binding differs for the CXCR3-A and CXCR3-B isoforms. CXCL11 has the highest affinity for CXCR3-A with CXCL10 followed by CXCL9 having lower affinity. However CXCL4 has highest affinity for CXCR3-B followed by CXCL11, CXCL10 and CXCL9. Finally, the alternate isoform (CXCR3-alt) differs from CXCR3-A in its C-terminus, lacking several transmembrane spanning domains and binding only CXCL11.

CXCR3 Expression

These CXCR3 isoforms perform differential functions with CXCR3-A expression on immune cells important in recruitment to sites of inflammation, as well as for their activation and proliferation. However, interestingly CXCR3-B seems to be predominantly expressed on cells of the vascular endothelium facilitating angiostatic, apoptotic and anti-proliferative functions. CXCR3 is also implicated in cancer with higher expression of CXCR3-A and reduced expression of CXCR3-B thought to predict poor overall survival due to greater proliferative and migratory and reduced apoptotic and angiostatic action. In mice only the CXCR3-A isoform has been identified with high sequence homology (86%) to human CXCR3. It is responsible for facilitating chemotactic, proliferative, angiostatic and...
apoptotic functions of CXCR3, supporting an important biological role for this receptor.\(^5,199\)

CXCR3 is expressed on immune cells with a particularly high expression on activated and memory CD4+ Th1 and CD8+ CTL with expression also seen on Natural Killer (NK), Natural killer T cells (NKT), B cells and DC.\(^{21,177,200-202}\). This has also been shown in C57BL/6 mice using an anti-CXCR3 monoclonal antibody with 23% of CD3+CD4+, 43% of CD3+CD8+ and 34% of CD4+Foxp3+ T cells and on 59% of CD4+CD44+ T cells and 85% of CD8+CD44+ T cells.\(^{200}\).

**CXCR3 Chemokines**

The 3 characteristic CXCR3 chemokines are transcribed in response to IFN-\(\gamma\), IFN-\(\alpha\), IFN-\(\beta\) and TNF-\(\alpha\), triggering calcium mobilisation, and activation of signalling pathways involving; phosphatidylinositol 3-kinases (PI3K)/Protein kinase B (Akt) as well as p65 nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) or c-Jun N-terminal kinase/ Janus kinase (JAK)/STAT-1\(^{189,203-208}\).

Importantly the expression of these chemokines relies on different cytokines and signalling through different promoters, resulting in temporally and spatially different expression patterns for the 3 chemokines and accounting for the various roles of CXCR3. CXCL9 production is dependent only on IFN-\(\gamma\) mediated signalling, while CXCL10 and CXCL11 production are induced by IFN-\(\gamma\), IFN-\(\alpha\), IFN-\(\beta\) and TNF-\(\alpha\)\(^4\). All of the CXCR3 chemokines can be produced through the common promoter NF-κB. Additionally the CXCL9 promoter contains an IFN-response element (\(\gammaIRE-1\))\(^{208}\), the CXCL10 promoter contains an IFN-stimulated response element (IRSE)\(^{205}\) and the CXCL11 promoter contains an IFN-regulatory factor 1 (IRF1)\(^{209}\) each of which can also induce these CXCR3 chemokines.

While IFN-\(\gamma\) is responsible for the transcription of these chemokines it is also essential in up-regulation of CXCR3 expression\(^{185}\). This results in recruitment of CXCR3 expressing immune cells, CD4+ Th1, CD8+ cytotoxic T cells and NK cells, in response to CXCL9, CXCL10 and CXCL11 release. These cells in turn secrete further IFN-\(\gamma\) increasing chemokine secretion and immune cell recruitment resulting in an inflammatory amplification loop and exacerbation of inflammation\(^5\) (Figure 1.4).
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**Receptor Structure**

CXCR3 signalling and internalisation are activated by chemokine binding through alternate receptor domains. For this reason a number of studies have investigated the importance of these domains in activation and internalisation of the human CXCR3 receptor with absence resulting in impaired or abrogated ligand binding and consequently reduced downstream signalling, calcium mobilisation, actin polymerisation and chemotaxis as well as receptor internalisation\(^2,183\).

Briefly, using a pre-B cell leukaemia cell line transfected to express human CXCR3 a number of regions of the extracellular N-terminus have been shown to play an essential role in chemokine binding and receptor activation, while the intracellular C-
terminal domain and 3 cytoplasmic loops are involved in CXCR3 internalisation (figure 1.5, human CXCR3-A).

Figure 1.5: Snakeplot structure of Human CXCR3-A amino acid sequence
Including transmembrane conformation and residues of importance to receptor activation and signalling as well as internalisation:
Red: location of anti-CXCR3-9C5 binding, also contains Y27 and Y29, essential in chemokine binding and receptor activation
Green: S245 necessary for CXCL11 migration, actin polymerisation and integrin dependent adhesion, LLL motif necessary for CXCL11 receptor internalisation and migration
Blue: D112 & D278 for binding and activation by CXCL9, CXCL10 and CXCL11, D282 and E293 for binding and activation by CXCL9 and CXL10. Adapted from 6

CXCL9, CXCL10 and CXCL11 require conservation of N-terminal residue R216 of the second extracellular loop, tyrosine phosphorylation of Y29 and Y27 and charged residues D112 and D278 for optimal binding. Charged residues D282 and E293 are also required for CXCL9 and CXCL10, while the proximal 16 amino acids and residues R197 and R212 of the second extracellular loop are necessary for CXCL10 and CXCL11 binding but non-essential for CXCL9.
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The intracellular C-terminus of human CXCR3 is necessary for CXCL9 and CXCL10 internalisation, dependent on serine/threonine phosphorylation of the C-terminal and for CXCL11 internalisation, dependent on conservation of the third intracellular loop. A DRY (aspartate-arginine-tyrosine) motif in the third transmembrane domain is necessary for CXCL9, CXCL10 and CXCL11 induced chemotaxis and calcium mobilisation, while the C-terminal LLL motif is also necessary for chemotaxis in response to CXCL9, CXCL10 and CXCL11 but not calcium mobilisation or receptor internalisation.183

Signalling Pathways

Activation of signalling pathways by CXCR3 depends on the ligand as well as the receptor isoform, G-protein and cell type involved leading to primarily chemotactic/proliferative or apoptotic/angiostatic effects.187,203 Upon ligand binding, GPCRs signal through G proteins α, β and γ causing activation of the regulatory α subunit and dissociation of the catalytic β, γ subunits.210

Using a number of in vitro studies it has been identified that human CXCR3 signals primarily through the pertussis toxin (PTX) sensitive G-protein, Gαi. However the actions of CXCR3-B have been found in some cases to facilitate PTX insensitive activation, with proliferation of CXCR3-B transfected cells unaffected by PTX.187,203 Conversely the CXCR3-B selective chemokine CXCL4 was shown to mediate PTX dependent signalling in T cells.203 For this reason it is thought that signalling of the CXCR3-A isoform occurs through Gαi, while CXCR3-B generally signals through Gαs.187,194,203 Ligand driven human CXCR3 receptor activation stimulates a number of signalling pathways with all 4 CXCR3 ligands able to activate calcium mobilisation and signalling through Extracellular signal-regulated kinases (ERK)1/2 (Ras/Raf/Mek), Mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.194,203 Ligation with CXCL11 stimulates the most rapid and sustained response for CXCR3-A and similarly CXCL4 is the dominant stimulator of CXCR3-B.187,194,203 However CXCL4-CXCR3B signalling shows significantly lower calcium mobilisation and does not result in internalisation or chemotaxis.203

In human derived T cells it was found that although CXCL11-CXCR3 ligation induced MAPK and PI3K/Akt signalling neither pathway was responsible for
chemotaxis, instead this occurred through Phospholipase C (PLC) and F-actin polymerisation\textsuperscript{194}. It was also found that while ERK signalling was in part responsible for human CXCR3 mediated chemotaxis it also displayed a pattern of biphasic activation associated with mitogenic and proliferative activity\textsuperscript{196}. CXCR3-B mediates apoptotic and anti-proliferative functions though a number of pathways including inhibition of the anti-apoptotic gene heme-oxygenase 1 (HO-1), via MAPK, and blockade of ERK1/2 signalling. Increased survival and proliferation of human breast cancer cells was seen upon inhibition of CXCR3-B\textsuperscript{195}. Human CXCR3 ligation has also been shown to phosphorylate a number of molecules downstream of the TCR (ZAP-70, LAT and PLC\textgamma 1), which suggests cross-talk between these two molecules and possibly a co-stimulatory role of CXCR3 in T cell activation\textsuperscript{203,211}.

Studies conducted in mice have identified only the CXCR3-A isoform, which therefore must regulate the opposing functions seen for CXCR3-A and CXCR3-B in humans. \textit{In vivo} and \textit{in vivo} studies using C57BL/6 wild type (WT) and CXCR3-/- mice have shown CXCR3-B-like functions with CXCL9 found to inhibit VEGF induced angiogenesis, proliferation and migration. This occurred via reduced PLC\textgamma and ERK phosphorylation reducing angiogenesis and fibrosis in endothelial and stellate cells from fibrotic mouse livers\textsuperscript{212}. In contrast CXCL10 expression in cardiac allografts, of WT and CXCL10-/- B6/129 (C57BL/6x129Sv/J (H-2\textsuperscript{b})) mice, was essential for immune infiltration of allograft, mediating CXCR3-A-like functions\textsuperscript{213}.

Due to the varied roles of CXCR3 it has been identified as a potential therapeutic target in a number of disease processes, most importantly cancer, transplantation and autoimmunity\textsuperscript{178-182,214,215}. Due to the potential benefits of reduced immune cell trafficking to sites of inflammation in autoimmune and allogeneic conditions, such as Type 1 diabetes and islet transplantation, investigation of CXCR3 mediated immune cell migration is of great interest. A number of studies have investigated CXCR3 as a therapeutic target with deletion or use of antibodies against CXCR3 and its chemokines showing beneficial effects for some but not all studies\textsuperscript{216,217}. 


**Experimental Models**

**CXCR3 in Inflammation**

The role of CXCR3 in health and disease is highly complex, orchestrating a number of processes from recruitment of immune cells\(^2\(^2\)\) to wound healing\(^2\(^1\)\(^8\)\) and viral clearance\(^2\(^1\)\(^9\),\(^2\(^2\)\(^0\)\). Under normal immune conditions CXCR3 is important in the antiviral immune response. One *in vivo* study investigated this effect using vaginal infection of C57/BL6 WT and CXCR3\(-/-\) mice with HSV-2 revealing a role for CXCR3 in CD8\(^+\) T cell cytotoxicity, T-bet, perforin and granzyme B expression\(^2\(^1\)\(^9\)\). Further, *in vivo* models using OT-I TCR transgenic WT and CXCR3\(-/-\) mice on a STOCK Tg(CAGDsRed*MST)1Nagy/J background were infected with vaccinia virus revealing impaired ability of immune cell invasion and contact-dependent interactions with virally infected cells, leading to poor viral clearance in CXCR3\(-/-\) mice\(^2\(^2\)\(^0\)\).

A model using adoptive transfer of P14 LCMV-specific T cells, comprised of WT & CXCR3\(-/-\) cells, into C57BL/6 recipient mice followed by infection with LCMV lead to reduced development of short-lived effector T cells when transfected with CXCR3\(-/-\) cells. Further, co-localisation of CD8\(^+\) virus specific T cells and antigen presenting cells in the splenic marginal zone of irradiated C57BL/6 mice was impaired when mice were adoptively transferred with C57BL/6 WT & Rag2\(-/-\)-CXCR3\(-/-\)-OT-I bone marrow and infection with vaccinia virus (VV)-OVA. Together these models suggest an important role for CXCR3 in T cell-APC co-localisation and subsequent T cell development\(^2\(^2\)\(^1\),\(^2\(^2\)\(^2\)\). In addition *in vitro* studies using OVA specific CD8\(^+\) T cells from IRF-9\(-/-\) C57BL/6 mice suggest that CXCR3 signalling via a type 1 IFN dependent pathway preferentially affects proliferation and activation (CD25 and CD69 expression) of naïve (CD44\(^{low}\)) CD8\(^+\) T cells\(^1\(^9\)\(^2\)\).

Activated Th1 and CD8\(^+\) CTL cells highly express CXCR3\(^2\(^2\)\), which is necessary for their recruitment to sites of infection but is also involved in deleterious effects seen under conditions of disease and uncontrolled inflammation, e.g. autoimmunity. For this reason the roles of CXCR3 signalling in numerous models of inflammatory disease have been investigated using either antibody blockade or deletion of CXCR3 signalling, via administration of anti-CXCR3 antibodies or gene deletion.
Inhibition of CXCR3 signalling reduces immune cell chemotaxis to inflamed sites, such as; the dermis, lymph nodes and prostate and is also involved in migration in response to renal ischemia reperfusion injury, arthritic pathology and experimental allergic encephalomyelitis (EAE)\(^3,22-25\).

Lymphocyte recruitment is reduced in rats following anti-CXCR3 antibody administration in a *Mycobacterium butyricum* induced arthritis model\(^3\) as well as in Ischemia reperfusion injury in Sprague–Dawley rats, using temporary left renal occlusion, following treatment with the CXCR3 antagonist TAK-779\(^24\). The antagonist TAK-779 also reduces immune cell infiltration of the prostate in a NOD/LtJ mouse model of Experimental autoimmune prostatitis, using immunization with a mixture of prostate antigens in CFA\(^23\). Further antagonism of CXCR3 using SCH546738 in experimental autoimmune encephalomyelitis (EAE), induced by immunization of C57BL/6 mice with pertussis toxin and myelin peptide (MOG 35-55) in CFA, lead to reduced disease severity and delayed onset\(^25\). Disease severity and immune cell recruitment to the colon are also reduced in an experimental IL-10-/- C57BL/6 mouse model of colitis following administration of anti-CXCL10 Ab\(^223\).

Finally, CXCR3/-/ C57BL/6 mice had reduced recruitment to sites of inflammation in models of collagen-induced arthritis, Complete Freund's adjuvant (CFA) induced LN inflammation and TLR agonist (poly I:C and LPS), Con A and OVA induced dermal inflammation\(^22\). In addition within this model of dermal inflammation over 90% of Th1 and CD8+ CTL expressed CXCR3\(^22\).

In contrast to the beneficial effect seen in these studies, a further study looking at the effects of an anti-CXCL10 antibody found no significant difference in recruitment or disease severity in multiple models of inflammatory disease. These models included; collagen induced arthritis induced in DBA/1 mice, EAE induced in SJL/J mice using immunization with a myelin protein (proteolipid protein) in incomplete Freund's adjuvant and inflammatory bowel disease (IBD) induced by CD4\(\beta\) CD45RB\(Hi\) T cell transfer from female CB6 F1 mice to female CB17 scid/scid mice. Of note however, initial *in vivo* studies on CD8+ T cell chemotaxis found inhibitory effects of the antibody occurred only at high doses (1000ug i.p.), while all further experimental studies used significantly lower doses (8-500ug i.p.)\(^224\).
In addition to expression on effector T cells, CXCR3 is expressed on a subset of Th1 Tregs. These Tregs are induced in response to Th1 conditions and upregulate CXCR3, under the control of T-bet, and regulatory markers (IL-10, TGF-β, Granzyme, CTLA-4, GITR, CD103). These Treg show superior ability to suppress Th1 mediated inflammatory disease\(^{225}\). In models of CXCR3 deficiency in both the NOD and C57BL/6 mouse CD25+Foxp3+Treg were found to accumulate in the spleen and draining lymph nodes with reduced ability to infiltrate the inflamed pancreas an liver, respectively\(^{217,226}\). However other studies have indicated no impact of CXCR3 blockade or deficiency on Treg migration to sites of inflammation with improved disease outcomes\(^{124,200,227}\). One study found impaired migration of Th1 and CTLs in CXCR3 deficient mice, while migration of Treg to the inflamed dermis was unaffected\(^{22}\).

Taken together the role of CXCR3 on regulatory T cells appears to be variable with an important role for migration of Th1 specific Treg and suggesting redundancy or use of alternative chemokine receptors for recruitment of other Treg subsets. Given the number of studies showing beneficial results for inhibition of CXCR3 in inflammatory diseases, it is likely that other subsets of Treg, which are less dependent on CXCR3, are involved in Th1 mediated diseases.
CXCR3 in Diabetes

A number of human studies of patients with T1D show increased expression of CXCR3 and its ligand CXCL10, with elevated expression correlating with recent onset of overt diabetes\textsuperscript{214,228-234}. Elevated expression of CXCR3 has also been suggested to be a good predictor of diabetes development in high risk first degree relatives\textsuperscript{178}.

CXCR3 and its’ ligands, particularly CXCL10 are involved in both immune cell recruitment to the pancreas and in impaired beta cell survival or proliferation\textsuperscript{66,124,235}. The beta cells, originally thought of as innocent bystanders in the immune mediated attack, are now known to express CXCR3 and CXCL10 following initial inflammatory insult. CXCL10 acts in an autocrine/paracrine manner through toll-like receptor (TLR)-4, rather than CXCR3, to enhance Akt driven apoptotic signals, impair insulin secretion and reduce beta cell viability resulting in decreased beta cell mass, independent of insulitis\textsuperscript{66,124,235}. CXCL9 is also expressed by islet vascular endothelial cells rather than beta cells and contributes to immune cell recruitment\textsuperscript{235}.

Investigation into the role of these chemokines in \textit{in vivo} models of diabetes using virally induced RIP-LCMV transgenic mice revealed a role for CXCR3 in the recruitment of T cells into the pancreas. Following infection with LCMV, development of LCMV specific CD8+ T cells occurs, which are recruited to the pancreas where LCMV-GP is expressed. In CXCR3 deficient C57BL/6 mice this recruitment of immune cells into the pancreas was significantly impaired and consequently diabetes onset was also significantly delayed in this model\textsuperscript{124}. However these results were not replicable in further studies in RIP-LCMV transgenic mice. Little to no delay in onset of diabetes or insulitis was evident in C57BL/6 RIP-LCMV mice deficient in CXCR3 (B6.129P2-Cxcr3tm1Dgen/J) or CXCL10 (B6.129S4-Cxcl10tm1Adl/J) or in C57BL/6 RIP-LCMV mice following treatment with an anti-CXCL10 mAb or CXCR3 antagonist NIBR2130\textsuperscript{236,237}.

Within the NOD mouse CD4+ T cells and CD4+Foxp3+ regulatory T cell migration are dependent on CXCR3 expression\textsuperscript{217}. In CXCR3 deficient NOD mice accumulation of CD4+Foxp3+ Treg in the pancreatic lymph node and loss of pancreatic infiltration were evident, resulting in enhanced diabetes incidence\textsuperscript{217}. Of note disease penetrance and diabetes incidence in this model was quite low.
Additional studies in the NOD mouse used deletion of the inflammasome component NLRP3 (nucleotide-binding oligomerisation domain leucine rich repeat and pyrin domain-containing protein), which is involved in Th1 cell activation, proliferation and migration into the pancreatic lymph node and pancreas. This study found that NLRP3 deficiency altered chemokine-chemokine receptor expression with increased CXCR3 and CCR5 in the spleen but reduced expression of the chemokine-receptors and their chemokines in the pancreas. Together this lead to impaired migration of activated Th1 cells into the pancreas, though no change in the migratory ability of Tregs was noted\textsuperscript{238}.

Overall, data on the effect of CXCR3 blockade or deletion within the diabetes literature is variable. Some studies show that reduced CXCR3 expression provides an inhibitory effect on autoreactive infiltrating T cells, reducing disease incidence. In contrast other studies show that CXCR3$^+$ Tregs are essential in prevention of the disease and loss of this chemokine receptor prevents their migration, increasing diabetes incidence upon CXCR3 gene deletion.
**CXCR3 in Allograft**

Within the context of allograft transplantation the chemokine receptor CXCR3 and its chemokines are upregulated in kidney, heart and lung donor organs and are also expressed by pancreatic islets. Xenograft models of kidney or islet transplant from pig to non-human primate also indicate an upregulation of CXCR3 and its chemokines during allograft rejection.

Using a model of BALB/c (H-2^d) murine cardiac allograft into C57BL/6 or C57BL/6x129Sv/J (H-2^b) mice expression profiles of CXCR3 and its ligands show upregulation of CXCR3 and CXCL10 by cardiac endothelial cells within 24hrs. Donor derived CXCL9 expression has also been shown to increase at 24 hours upon exposure to IFN-γ secreting memory CD8+ T cells following A/J (H-2^a) cardiac allograft into C57BL/6 (H-2^b) mice. This is followed by expression of CXCL9, CXCL10 and CXCL11 by the cardiac endothelium and infiltrating macrophages and lymphocytes at day 3 and persisting to day 7 when rejection typically occurs. mRNA expression of chemokines; CCL3, CCL4, CCL5, CXCL2 CXCL9, CXCL10 and CXCL11, as well as their receptors; CCR1, CCR2, CCR5, CCR4 and CXCR3 were all increased in wild type BALB/c (H-2^d) cardiac allografts into C57BL/6 or C57BL/6x129SvJ (H-2^b) recipients. For cardiac allograft from BALB/c (H-2^d) mice into C57BL/6x129SvJ (H-2^b) mice the number of CXCR3 expressing cells was shown to increase steadily over the course of allograft rejection, with NK cell infiltrate peaking at day 3 and CD3+ cell infiltrate progressively increasing until graft loss at day 7. Importantly models using cardiac allograft of WT BALB/c hearts into C57BL/6 CXCR3 deficient recipient mice or transplantation C57BL/6x129SvJ CXCL10 deficient donor hearts into WT BALB/c hosts show significant prolongation of graft survival (~60 and 40 days, respectively) and reduced immune cell infiltration of cardiac allografts. This indicates that in cardiac allografts the initial release of donor derived CXCL10 chemokine drives chemotaxis of recipient immune cells expressing CXCR3 to the graft. Monoclonal antibodies against CXCR3 or CXCL10 also prolonged BALB/c cardiac allograft survival when transplanted into C57BL/6 or C57BL/6x129Sv/J recipients. This was characterised by intact myocardium and vasculature and reduced CD4+ and CD8+ T cell infiltration, albeit to a lesser extent than seen in knockout models (~20 and 14 days, respectively).
In addition to cardiac allografts, skin allografts from C56BL/6 (H-2^b) donors were also shown to upregulate CXCR3 and its chemokines CXCL9, CXCL10 and CXCL11 when transplanted into MHC mismatched BALB/c (H-2^d) recipients, compared to isografts. Treatment of skin allograft recipients with an anti-CXCL11 antibody did not alter pro-inflammatory cytokine expression (INF-γ, TNF-α) or proliferation within the allograft. However, a reduction in immune cell infiltration of the graft and prolonged survival was seen in anti-CXCL11 treated compared to untreated mice. C57BL/6 CXCR3 deficient MHC mismatched skin allografted into BALB/c (H-2^d) recipients also resulted in prolonged allograft survival compared to wild type allograft recipients.

Biopsies from rejecting human lung allografts also showed high expression of CXCL10 by infiltrating and alveolar macrophages, as well as epithelial cells, which were obtained from patient bronchiolar lavage fluid (BAL) samples. BAL was highly chemotactic for CXCR3 expressing, IFN-γ+ T cells (Th1 and CTL), which was inhibited by anti-CXCR3 or anti-CXCL10 antibodies. A model of lung allograft rejection in mice, used transfer of a C57BL/6 CXCR3-/- or WT CD8 T cell clone specific for OVA antigen (OT-I), into C57BL/6 CC10-OVA mice expressing OVA antigens on the cells lining their airway. The mice transferred with CXCR3-/- CD8 T cells displayed significantly improved graft survival and reduced lung infiltration compared to those transferred with WT CD8 T cells. Interestingly, a significantly increased number of Tregs were recruited to the lung, in a CCR6 and CCR7 dependent but CXCR3 independent manner. The study proposed that under normal circumstances both CD8+ T cells and Tregs are recruited to the lung, however the ratio of Teff:Treg is insufficient to prevent rejection. The reduction in Teff cells recruited to the lung in this CXCR3-/- model, paired with increased Treg accumulation, appear sufficient for the prevention of rejection.

The role of CXCR3+ cells were also characterised in a model of graft versus host disease (GVHD) with CD8+ T cell transfer from C3H.SW (H-2D^b CD45.2+) mice into irradiated B6/SJL(H-2D^b CD45.1+) recipients. Disease was inducible by both CXCR3+ and CXCR3- cell transfer, although CXCR3- cells were found to upregulate CXCR3 upon APC stimulation to mediate this effect. These CXCR3+CD8+ T cells showed higher expression of cytotoxic markers (TNF-α, IFN-γ, Granzyme B, FasL and Annexin B) with an increased ability to lyse target cells.
and mediate GVHD, compared to CXCR3- CD8+ T cells. GVHD was prevented in this model upon anti-CXCR3 mAb administration for 21 days, but not when administered for 7 days. This is thought to be due to the ability of CXCR3-CD8+ T cells to upregulate CXCR3 at the earlier time point but not later in disease progression.\textsuperscript{248}

Although CXCR3 has been found to have an important role in allograft rejection, redundancy within the chemokine system exists and as such loss of CXCR3 does not confer complete protection on its own. However, in combination with inhibition of other chemokine receptors, such as CCR5, which is also responsible for T cell chemotaxis, improved allograft survival was evident. WT BALB/c (H-2\textsuperscript{d}) cardiac allograft into B6;129P-Cmkbr5tm1Kuz (H-2\textsuperscript{b}) CCR5-/- recipient mice or WT B6;129PF2/J recipient mice treated with an anti-CXCR3 mAb resulted in allograft survival of 29-34 days. However when CCR5-/- mice were treated in combination with an anti-CXCR3 mAb, 100% of WT BALB/c (H-2\textsuperscript{d}) cardiac allografts showed long term survival for over 100 days.\textsuperscript{249} Interestingly, while graft infiltration was significantly reduced, an increased frequency of graft infiltrating Tregs were present. These cells play a crucial role in long-term graft survival, as indicated by graft loss at post-operative day (POD) 22 upon depletion of these cells.\textsuperscript{249} Use of a small molecule antagonist, TAK-779, which targets CCR5 and CXCR3, was also shown to improve cardiac (C57BL/6 H-2\textsuperscript{b} > BALB/c H-2\textsuperscript{d}) and islet (C57BL/6 H-2\textsuperscript{b} > C3H/HeJ H-2\textsuperscript{k}) allograft survival, although mean survival times were increased only by ~5days compared to controls, rather than long-term survival seen with the previous study. Immune cell recruitment and expression of inflammatory chemokines (CCL3, CCL2 and CCL5) and cytokines (TNF-\(\alpha\), IFN-\(\gamma\), IL-4 and IL-10) were all reduced with preserved cardiac allograft morphology.\textsuperscript{250}

A role for islet allograft survival has also been shown in a number of studies. However, typically these studies focus on cardiac allograft, with minimal data on the islet allograft. It is important to note that although similarities within allograft rejection exist, tissue specific differences, such as for vascularised vs. non-vascularised grafts are important considerations. In support of this, differential functions of the chemokine receptor CCR2 have been shown, via MHC mismatched allograft from WT BALB/c (H-2\textsuperscript{d}) donors into WT or CCR2-/- C57BL/6 (H-2\textsuperscript{b}) recipients. Using these models CCR2 was shown to have an important early role in
islet allograft rejection but no significant role was seen for cardiac allograft rejection\textsuperscript{251}. Studies on BALB/c (H-2\textsuperscript{d}) or C3H/HeJ (H-2\textsuperscript{k}) donor islet allograft into C57BL/6 (H-2\textsuperscript{b}) recipients show promising results with administration of an anti-CXCR3 mAb or the TAK-779 antagonist, found to prolong mean islet allograft survival from 12.5 to 25 days or 22 to 28 days, respectively. Further, histology from treated mice indicates a reduced immune cell infiltration of the islet allograft\textsuperscript{200,250}. The study in 2003 by Baker \textit{et al.} on the role of CXCR3 and its ligand CXCL10 in islet allograft represents a more in depth investigation of the role of this signalling pathway in islet allograft. Using either CXCR3 deficient or anti-CXCL10 antibody treated C57BL/6 (H\textsuperscript{2b}) recipients of A/J strain (H\textsuperscript{2K\textsuperscript{a}}) islets grafted under the kidney capsule, Baker \textit{et al.} found a significant prolongation of graft survival for both treatment groups (~20 days) compared to controls (~12.7 days). Further investigation revealed that although graft chemokine expression (CCL5, CCL3 and CXCL10) was not different for treated compared to controls a significant reduction in CD8 T cells was evident (both mRNA and graft histology) as well as reduced markers of NK cells, CD4 T cells and macrophages (CD19, CD4, F4/80)\textsuperscript{252}.

Fully MHC-mismatched BALB/c (H-2d) to C57BL/6 (H-2b) cardiac allograft survival is also further enhanced by synergistic effects of CXCR3 inhibition and low dose immunosuppressive therapy (rapamycin or cyclosporine A) resulting in permanent engraftment of 100\% of cardiac and 80\% of islet allografts\textsuperscript{200,216}.

Taken together this indicates that although the role of CXCR3 in islet allograft rejection has not been extensively studied, its roles in immune cell migration into sites of allograft are well characterised. In particular inhibition of CXCR3 signalling has been shown to significantly prolong islet allograft survival.
Thesis Aims

Inflammation is a naturally occurring process, responsible for clearance of infections and dead or damaged tissues and initiation of tissue repair. However an inflammatory disease represents a condition where inflammation is not controlled, characterised by continuous recruitment of immune cells to target sites resulting in collateral damage to local tissues. In the context of autoimmune disease this inflammation is misdirected specifically at self-tissue, while in allogeneic transplantation inflammation occurs against non-self-tissue. In both circumstances T cells are recruited to sites of inflammation and play a critical and unwanted role in tissue damage. Chemokines and their receptors are necessary for recruitment of T cells, as well as other cells involved in the autoimmune and alloimmune inflammatory processes, making them an appealing therapeutic target to intervene in these inflammatory responses.

The current landscape of available immunosuppressants is limited by their broad action, impairing the response against auto- and allo-antigens, as well as those responses to viral infection and cancer. In addition many of these drugs display adverse side effects or toxicity towards tissues such as the kidney\textsuperscript{16-20}. For this reason much research is currently being conducted to identify new therapeutic targets as alternatives to current immunosuppressive therapy.

One such approach would be to impair lymphocyte migration to sites of inflammation via chemokine receptor blockade. To test this idea we have developed a novel anti-CXCR3 monoclonal antibody, targeting a conserved epitope found in mice, non-human primates and humans. Due to the long-term risks associated with current immunosuppressant regimens, which limit the use of islet transplantation to individuals with severe, unmanageable T1D, we determined the efficacy of this antibody in mouse models in the context of islet allograft and T1D, for use clinically in islet transplant into patients with type 1 diabetes.

In addition to a clinical application in islet transplantation we were interested in targeting CXCR3 broadly in inflammatory responses as a T cell targeting therapy\textsuperscript{3,23}. Historically these therapies directed at T cells, such as tacrolimus and ATG\textsuperscript{15}, have shown promise in the clinic and due to the importance of CXCR3 in regulating
proliferation, activation and migration of T cells we believe this will be another encouraging target. Mouse models of islet allograft transplantation and diabetes will also offer insight into the efficacy of our antibody broadly in inflammation. The conserved nature of our antibody in mice and humans also makes results generated in the mouse model more readily translatable for clinical application by humanising the antibody.

The chemokine receptor CXCR3 is highly expressed on Th1 and CTLs and has been shown to play an important role in recruitment of these cells into sites of inflammation. Previous literature has also shown that expression of CXCR3 correlates with both onset of diabetes and allograft rejection. CXCR3s’ involvement in allograft rejection is well established in mouse models. However the role of CXCR3 signalling in the development of diabetes is less clear. Importantly CXCR3 signalling has been found to promote allograft loss in a number of mouse models. In contrast the incidence of diabetes in the NOD mouse following deletion of CXCR3 was increased. This was attributed to an impaired ability of regulatory T cells to migrate into the diabetic NOD pancreas. As yet studies on temporal CXCR3 blockade in the NOD mouse have not been performed, although the possibility of differential effects for deletion versus antibody blockade have been highlighted in studies of other molecules. Finally, as islet transplantation is a treatment for patients with T1D, it is of interest to test the efficacy of targeting CXCR3 for islet transplantation in the NOD model. We note that no previous literature has investigated a role for CXCR3 in this context.

Therefore the aims of this thesis were:

- To characterise the *in vivo* effects of our novel anti-CXCR3 monoclonal antibody, particularly on T cell chemotaxis.
- To characterise the efficacy of this anti-CXCR3 antibody in a model of BALB/c to C57BL/6 MHC mismatched islet allograft.
- To characterise the efficacy of this anti-CXCR3 antibody in a model of autoimmune type 1 diabetes using the NOD mouse model.
- To characterise the efficacy of this anti-CXCR3 antibody in a model of mismatched islet allograft into NOD autoimmune diabetic recipients.
Chapter 2. Materials and Methods
## Materials

### Table 2.1. Buffers/Reagents

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td><strong>FACS buffer</strong></td>
<td>0.5% bovine serum albumin (BSA) 0.2% Sodium azide 1x Dulbecco's Phosphate Buffered Saline (DPBS) to 1L</td>
<td>Gibco BRL, Sigma-Aldrich</td>
</tr>
<tr>
<td><strong>MACS Buffer</strong></td>
<td>0.5% BSA, 2mM ethylenediamine tetra acetic acid (EDTA) DPBS to 1L</td>
<td>Gibco BRL, Sigma-Aldrich</td>
</tr>
<tr>
<td><strong>Red Blood Cell Lysis Buffer</strong></td>
<td>0.15M Ammonium chloride 10mM potassium bicarbonate 0.1mM Disodium EDTA Triple distilled water to 1L pH 7.2-7.4</td>
<td>Merck</td>
</tr>
<tr>
<td><strong>Chemotaxis buffer</strong></td>
<td>49% HBSS (phenol red) 49% RPMI (L-glut, Pen/strep) 2% FBS</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Serum Free Islet Isolation Media (M199-)</strong></td>
<td>10.6g Medium 199 (with Hanks salts and L-glutamine) 0.35g sodium bicarbonate dH2O to 1L</td>
<td>Sigma-Aldrich</td>
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<tr>
<td><strong>Islet isolation media (M199+)</strong></td>
<td>100mL heat inactivated bovine calf serum (BCS) M199- to 900mL</td>
<td>Gibco</td>
</tr>
<tr>
<td><strong>Liberase</strong></td>
<td>Liberase-Enzyme Blend-RI 30μL Liberase + 0.5μL Thermolysin Sterile M199- to 3mL per mouse</td>
<td>Roche T-Flex, Indianapolis, IN, USA</td>
</tr>
<tr>
<td><strong>Materials and Methods</strong></td>
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</table>
| **Avertin 2.5%** | 2-2-2 tribromoethanol  
2-methyl-2-butanol  
Saline 0.9% | Sigma-Aldrich  
Fisher  
Baxter |
| **Alloxan** | 0.04g alloxan tetrahydrate  
2mL sterile water for injection | Sigma-Aldrich |
| **Streptozotocin (STZ)** | 0.02g streptozotocin | |
| **0.1% TBS-T** | 0.12% Tris Base  
0.87% Sodium Chloride  
0.1% Tween20  
Millique Water to 1L | Ajax Finechem  
Ajax Finechem  
Ajax Finechem |
| **CFSE culture media** | 10% FCS, 1:100 L-glutamine  
1:100 Pen-strep  
1:2000 β-mercaptoethanol (@1000x 55mM)  
1:100 Sodium pyruvate (@100x 100mM)  
1:100 HEPES (@1M)  
RPMI-1640 to 500mL | Invitrogen  
Invitrogen  
GIBCO  
Invitrogen  
Invitrogen |
| **CFSE staining buffer** | 1% FCS  
DPBS | Invitrogen  
Invitrogen |
| **CFSE quenching buffer** | 20% FCS  
DPBS | Invitrogen  
Invitrogen |
| **Rapamycin Vehicle**  
(used with rapamycin stock in DMSO) | 0.2% Carboxymethyl cellulose  
0.25% Polysorbate 80 (tween)  
0.9% NaCl solution to 100ml | Sigma-Aldrich  
Sigma-Aldrich |
Methods

Mice

6-10 week old C57BL/6 mice and BALB/c mice were obtained from Australian BioResources (Moss Vale, NSW Australia) for use as allograft recipients and donors, respectively or for use in *in vivo* MLR experiments. C57BL/6 mice were also used for antibody characterisation experiments.

C57BL/6 recipient mice selected for islet allograft were treated with Alloxan (110mg/kg) two days prior or Streptozotocin (STZ)(180mg/kg) five days prior to islet cell transplantation, to chemically induce diabetes, and blood glucose levels monitored for onset of hyperglycaemia (BGL>15mmol/L). Following transplant both C57BL/6 and NOD allograft recipients were monitored daily for elevated BGL for the first week followed by monitoring 2-3x/week. BGL >15mmol/L for 2 consecutive days indicated rejection of transplanted islet grafts and mice were euthanised.

6-8 week old female non-obese diabetic (NOD/Lt or NOD/ShiLtJ) mice were obtained from Australian BioResources (Moss Vale, NSW Australia) or Walter and Eliza Hall Institute (Parkville, Victoria Australia). Mice were monitored for weight and blood glucose level (BGL) using a Free Style Lite Blood Glucose Monitoring System, once per week until BGL reached above 8mmol/L at which point twice weekly monitoring was employed. Mice were monitored for onset of diabetes, classified as blood glucose level (BGL) >15mmol/L for 2 consecutive days, at which point mice were euthanised. For studies using pre-diabetic mice, NOD mice were euthanised at 12 weeks of age. NOD islet allograft experiments used NOD mice with BGL>8mmol/L as recipients of BALB/c islet allograft (4 donors:1 recipient).

RAG-/- mice were obtained from Animal Resource Centre (ARC, Perth Australia) for use in cell transfer experiments.

All animal experiments were performed with approval from Garvan/St Vincents Hospital Animal Ethics Committee (AEC) and mice housed in static micro-isolator cages.
**Islet Isolation and Transplantation**

**Islet Isolation**

Cannulations were performed by S. Walters or N. Zammit. Donor mice were injected with 400μL avertin solution and a midline incision performed to expose the peritoneal cavity (pancreas and liver) and heart cut to stop blood flow. Distension of the pancreas was performed by clamping the pancreatic/bile duct followed by careful insertion of a 30G needle into the bile duct and perfusion with 3mL Liberase-Enzyme Blend into the pancreas. Pancreas was carefully excised and put into prepared 50mL Falcon tubes on ice. This was repeated for each donor mouse to give a ratio of 3 donor mice per 1 recipient mouse for C57BL/6 and RAG-/- recipients or 4 donor mice per 1 recipient mouse for NOD recipients with 1 50mL Falcon tube per recipient mouse.

Pancreata were digested at 37°C in a water bath for 10 minutes. Tubes were then removed directly onto ice, resuspended in 30mL islet isolation media (M199+), shaken and vortexed to dislodge acinar tissue from islets followed by centrifugation at 1250rpm for 3min. The supernatant was discarded and cells washed twice more. Cell pellets were resuspended again, filtered through a 425 micron sieve (US standard sieve, A.S.T.E. E.11 specifications dial MFG, Co. Chicago, II, USA) and centrifuged as above. Supernatant was discarded and tubes containing pellet left upside down briefly to drain excess supernatant. Pellets were then resuspended in 10mL Ficoll-paque plus (Amersham) followed by addition of a further 10mL Ficoll. Ficoll was then overlayed with a further 10mL serum free islet isolation media (M199-) and centrifuged on a density gradient at 2830rpm for 22min with no acceleration or brake. Supernatant, containing separated islets, was then poured into clean tubes, divided in two and topped up to 50mL before centrifuging at 1250rpm for 3min. Here supernatant was discarded and cell pellets transferred to a single Falcon tube. The cell pellet was then divided between 1.5mL Eppendorf tubes depending on the number of recipients and pellet aspirated into a section of thin tubing in preparation for transplantation. All prior steps were done on ice at 4°C unless otherwise stated.
**Islet Transplantation**

S. Walters or N. Zammit conducted all islet transplants. Following isolation of islets recipient mice were prepared for transplantation and maintained under light isoflurane anaesthesia (1.5-2% isoflurane in O₂) for the duration of the transplant using a Bains paediatric circuit. Briefly mice were placed on a heat mat to maintain body temperature and the area over the left kidney shaved and sterilised using alcohol wipes. The tubing containing the islet pellet was attached to a Hamilton syringe and tubing trimmed leaving pellet close to the end of the tubing. A small incision was made over the kidney and kidney brought into the wound through gentle blunt dissection, allowing access to the capsule. A small nick was made at the inferior renal pole of the kidney capsule allowing the tubing to be inserted and pellet gently released towards the superior renal pole. Mice were administered 0.2mL ketoprofen analgesic (0.5mg/kg) and 0.5-1mL saline for rehydration into the surgical site. The inner and outer skin layers of the incision were then sutured, wound closed with surgical staples and betadine antiseptic applied. Mice were left to recover in a fresh cage with heat lamp and heat pad till fully awake. Blood glucose was monitored on days 1-7 and every second/third day thereafter until rejection occurred (BGL>15mmol/L).

**Antibody**

The anti-CXCR3 antibody (clone 9C5) was supplied by the Mackay Laboratory (Monash University), produced by Dr Remy Robert. Briefly the mouse IgG1 antibody was generated through immunisation of mice deficient for the CXCR3 receptor (CXCR3-/- mice) with a murine pre-B cell line (L1.2) transfected with the receptor of interest (CXCR3 transfected cells). Following immunisation, antibody clones were isolated and selected for strong reactivity against mouse CXCR3 (mCXCR3). The clone used in this study recognises a highly conserved region of the CXCR3 N-terminus and efficiently binds human, mouse and marmoset lymphocytes.

Fluorescent conjugation of αCXCR3-9C5 antibody was performed for use in antibody characterisation assays (internalisation and competitive binding). For this an Allophycocyanin (APC) Conjugation Kit (Abcam) was used and conjugation performed according to protocol provided. Briefly 6μL APC modifier reagent was added to 60μL αCXCR3-9C5 at 2μg/μL antibody and resuspended gently. This mix
was then transferred into the lyophilized APC conjugation mix, gently resuspended and left standing in the dark for 3hrs at room temperature (20-25°C). Finally APC conjugate was resuspended with 6μL APC Quencher reagent and stored at 4°C until required. This procedure was also performed for IgG Isotype control antibody.

**Antibody Treatments**

**Allograft**

C57BL/6 mice received 2mg/kg αCXCR3-9C5 monoclonal antibody intravenously (i.v.), unless otherwise stated. Allograft recipient mice, C57BL/6 or NOD, received 2mg/kg αCXCR3-9C5 once per week from the day of allograft for 9 weeks or until loss of allograft, respectively. Alternatively allograft recipient mice received 2mg/kg i.v. Isotype (IgG) control antibody once per week from the day of allograft until loss of allograft, as a control for αCXCR3-9C5 treated mice. Additional groups received 2mg/kg i.v. αCXCR3-9C5 or Isotype in combination with sub-therapeutic rapamycin, administered i.p. at 0.1mg/kg for 7 days in C57BL/6 allografts or 0.5mg/kg for 7 or 14 days in NOD allografts. An untreated control group was also used in NOD allograft experiments. 10x Rapamycin stock (LC laboratories, Woburn, MA, USA) (in DMSO) was diluted in vehicle solution for 50μL i.p.

**Type 1 Diabetes**

NOD mice were treated with 1mg/kg αCXCR3-9C5 monoclonal antibody i.v. unless otherwise stated. Mice received 1mg/kg i.v. αCXCR3-9C5 once every 4 days from 6 weeks of age (6W) or 8 weeks of age (8W) until study end point was reached, at 12weeks of age or onset of overt diabetes. An Isotype (IgG) antibody was administered to an additional control group using the same regimen or mice were untreated as controls.

**CD25 Depletion – PC61**

C57BL/6 islet allograft recipient mice, receiving 2mg/kg αCXCR3-9C5, were also treated with 200μg purified rat anti-mouse CD25 1gG mAb (clone PC61, Bioexpress) via i.v. tail vein injection. The antibody was confirmed via flow cytometry to deplete the CD4+CD25+ T cell compartment, containing the CD25+Foxp3+ regulatory T cell subset.
Cell Population Analysis

Lymphocyte Isolation

Lymphocytes were isolated from spleen, pooled lymph nodes (axillary and inguinal), pancreatic lymph node and blood. Splenocytes were homogenised in a 50x9mm petri dish using two frosted glass slides (Menzel-Glaser, Braunschweig, Germany) and centrifuged at 1250rpm for 3min. Supernatant was discarded and pellet resuspended in 2mL red blood cell lysis buffer (RCLB) for 2min to deplete erythrocytes. Lysis was quenched by addition of FACS buffer and cells washed twice. Blood samples were collected, through tail bleed in live mice or cardiac puncture in euthanised mice, in 200μL or 2mL Heparinised saline (10UI/mL Pfizer), respectively. Blood samples underwent red blood cell lysis to deplete erythrocytes leaving lymphocytes and washed as above. Lymph nodes and thymus were homogenised as above with no red blood cell lysis and cells washed twice to remove fat. All cell suspensions were filtered using a 70μm nylon mesh filter (BD), counted using trypan blue (0.4% Sigma) and a haemocytometer (Blood samples excluded) and resuspended at 1x10^7 cells/mL, unless otherwise stated. All steps were performed on ice at 4°C unless otherwise stated.

Splenocyte isolation for culture and antibody characterisation were performed as above in azide free MACS buffer rather than FACS buffer under aseptic conditions and used a 2mL syringe plunger to homogenise cells through a 70μm nylon mesh filter (BD).

Flow Cytometry

Isolated lymphocytes were resuspended at a concentration of 4x10^7 cells/mL and 2x10^6 cells per 50μL plated out per well in FACS buffer for each staining condition in a 96 well-V bottom plate (Greiner Bio-One)(Table 2.2). Antibody staining was performed at a final dilution of 1:200 unless otherwise specified (Table 2.3). Following incubation in the dark at 4°C for 30min the plate was centrifuged and supernatant removed. Cells stained for Natural killer and B cell markers as well as those from internalisation, competitive binding or CFSE assays were resuspended in 200μL FACS buffer and passed through a 70μm filter into 5mL microtubes (BD Biosciences). Cells stained for T cell markers were resuspended in
fixation/permeabilisation buffer (fixation/permeabilisation concentrate and diluent 1:3 dilution, eBioscience) for at least 2 hours followed by incubation with Foxp3 antibody resuspended in 1x permeabilisation buffer (eBioscience) in the dark at 4°C for 30min. Following the final antibody incubation T cells were washed and filtered as above into 5mL microtubes before flow cytometry was performed on a LSRII SORP flow cytometer with FACSDiva software (BD Bioscience). Data was analysed using FlowJo (TreeStar Inc, Ashland Oregon USA). Light scatter gating was performed on all samples to include live lymphocytes and exclude doublet cells, dead cells and debris unless otherwise specified.

**CFSE Proliferation Assay/Mixed Lymphocyte Reaction**

Total lymphocytes were isolated from the spleen as above in MACS buffer under sterile conditions and cells washed twice and resuspended at 1x10^7 cells/mL in warm CFSE staining buffer. The cell suspension was transferred to a fresh 15mL Falcon tube wrapped in aluminium foil. CFSE (carboxyfluorescein succinimidyl ester; Sigma-Aldrich) was prepared from stock (10μM) for *in vivo* (1μL of 10μM CFSE stock) studies. On a dry portion of a fresh 15mL tube 1μL CFSE per 1x10^7 cells/mL was placed and cells quickly inverted and vortexed to ensure even distribution of CFSE stain before incubation at 37°C for 10min. Staining was quenched by adding 5mL ice cold CFSE quenching buffer and incubating for 3 min. Cells were centrifuged and washed twice more with ice cold CFSE quenching buffer before performing a final cell count using trypan blue exclusion.

*In vivo* cell transfer experiments were performed using cells from C57BL/6 mice and transferred into sub-lethally irradiated BALB/c mice. BALB/c mice were irradiated with a single irradiation dose of 2.7Gy using an X-RAD 320 Biologic Irradiator (Precision X-ray). 48 hours later mice were intravenously injected with 2x10^7 CFSE labelled whole spleen cells. Mice also received either 2mg/kg anti-CXCR3-9C5 i.v. immediately following cells or were untreated as controls. Mice were euthanised and splenocytes isolated 72hrs later for flow cytometry.
Antibody Characterisation

Chemotaxis Assay

This was carried out by the Mackay Laboratory, briefly a murine pre-B cell line transfected with human CXCR3 (L1.2 hCXCR3) was incubated with a range of concentrations of αCXCR3-9C5, from 0μg/mL to 20μg/mL, for 10min at 37°C. Following incubation with αCXCR3-9C5, 500,000 cells of each antibody concentration were suspended in 75μL chemotaxis buffer and loaded into the top chamber of individual HTS transwell plates (Corning; 5μm pore size, 96 well #3387-8EA). Human chemokine; CXCL9, CXCL10 or CXCL11 (Prepotech) diluted to 30nM in 240μL chemotaxis buffer were added to the bottom chamber of each HTS transwell plate and incubated at 37°C for 4 hours. Following incubation 200μL of cells from the bottom chamber were resuspended in 100μL FACS buffer + 2%PFA and left overnight. Cells were read on the HTS plate reader on BD LSR flow cytometer and the degree to which αCXCR3-9C5 inhibited migration expressed as a percentage inhibition, compared to 0μg/mL αCXCR3-9C5, for each concentration of αCXCR3-9C5 for hCXCL9, hCXCL10 and hCXCL11.

Receptor Internalisation Assay

Following isolation of splenocytes in MACS buffer, cells were resuspended at 2x10⁶ cells/50μL in duplicate for each time point (0, 10, 20, 30, 60, 120 and 180 minutes) of the internalisation assay. Cells were resuspended with 1μl (2μg/μL) APC-conjugated αCXCR3-9C5 for each time point or APC-conjugated IgG Isotype in 50μL MACS buffer and incubated at 4°C for 30min to allow for antibody-receptor binding. Isotype treated cells and αCXCR3-9C5 treated 0 time point cells remained at 4°C for all further steps. For all other time points cells were incubated at 37°C for the allocated time (0, 10, 20, 30, 60, 120 and 180 minutes) followed by removal onto ice and quenching with 500μL ice cold PBS. Cells were washed twice followed by fixation in 1:1 PBS and 4% formaldehyde. Once all time points were collected cells were washed with cold PBS twice and stained for flow cytometry (table 2.2). All steps were performed in azide free conditions.
Competitive Binding Assay

Following isolation of splenocytes in MACS buffer, cells were resuspended at $2 \times 10^6$ cells/50μL in duplicate. Cells were incubated for 30 minutes at 4°C in azide free conditions with 0.2μg APC-conjugated αCXCR3-9C5, APC-conjugated Isotype, PerCpCy5.5-conjugated CXCR3-173 or PerCpCy5.5-conjugated Isotype antibody. Cells were then washed twice with MACS buffer followed by a further round of staining with 0.2μg antibody for 30 minutes at 4°C. Cells stained initially with APC-conjugated αCXCR3-9C5 or APC-conjugated Isotype were stained with PerCpCy5.5-conjugated CXCR3-173 or PerCpCy5.5-conjugated Isotype antibody. Cells stained initially with PerCpCy5.5-conjugated CXCR3-173 or PerCpCy5.5-conjugated Isotype were stained with APC-conjugated αCXCR3-9C5 or APC-conjugated Isotype antibody. Cells were then stained for flow cytometry (table 2.2).

Antibody Titrations

In order to determine frequency and dose of αCXCR3-9C5 appropriate for the NOD and C57BL/6 mouse strains antibody titration experiments were conducted. C57BL/6 mice were injected with a range of doses of αCXCR3 9C5 (0, 1mg/kg, 2mg/kg, 5mg/kg and 10mg/kg) on day 0 and followed for 10 days with tail vein blood collected throughout the 10 days. NOD mice were injected with 1mg/kg αCXCR3-9C5, 1mg/kg Isotype or untreated as controls on day 0 and followed for 6 days with blood taken throughout the 6 days. Blood samples were processed and analysed by flow cytometry to determine CXCR3 expression on T and B cells for each concentration and time point (table 2.2).

Adoptive Transfer

Adoptive transfer experiments were performed on RAG-/- recipients of BALB/c islet allografts 14 days post allograft. Transferred splenocytes were harvested from long term surviving (>100days) C57BL/6 recipients of BALB/c islet allografts, treated with a combination of αCXCR3-9C5 (2mg/kg i.v. 1/week for 9 weeks) and sub-therapeutic rapamycin (0.1mg/kg i.p for 7 days). Alternatively, spleens from untouched C57BL/6 mice were used as controls.

Splenocytes were isolated under aseptic conditions and total T cells isolated via magnetic separation using a Pan T cell kit (Miltenyi Biotec, Auburn, CA).
Subsequently CD25- effector T cells were isolated through positive depletion of CD25+ cells using a CD25 Microbead kit (Miltenyi Biotec, Auburn, CA) following manufacturers instructions. Magnetic separations were performed to a purity of >95% using AUTOMACS (Miltenyi Biotec, Auburn CA), assessed by flow cytometric analysis.

Whole T cells or CD25 depleted effector T cells (2x10^6 cells) were then adoptively transferred into Rag-/- recipient mice via tail vein i.v.

**Histology**

At designated end points mice were euthanized using CO₂ and tissues collected. Pancreata were collected from NOD mice. Kidneys were collected from long term surviving islet transplant recipient mice.

Tissues were collected into labelled cassettes stored in 10% formaldehyde for 2 days followed by transfer into 70% ethanol and paraffin embedding (performed by TKCC Histopathology Facility). Sections of 5μm were cut using a Microtome (Leica, Wentzler, Germany), mounted onto slides and allowed to air dry overnight. Kidney sections were mounted on 20 slides with sections 1-20 at the top of each slide followed by sections 21-40 at the base of each slide allowing better visualisation of islet graft site. Pancreas sections were mounted with sections on slides 1-8 contiguous, 9-16 contiguous and 17-24 contiguous (2-3 sections per slide) with a ~150μm section discarded between each set of 8 slides, to prevent recounting the same islet.

**H&E**

For kidney sections slides 1, 10 and 20 were stained using H&E to identify the optimum slide to visualise remaining islet grafts. Pancreas sections slides 1, 9 and 17 were stained for H&E to allow grading of insulitis. H&E was performed using an autostainer (Leica autostainer XL) and coverslipper (Leica CV5030) provided by TKCC Histopathology Facility.

Briefly insulitis in the NOD sections was graded on a 5 point scale using Leica microscope (Leica DC 200 camera, Leica Microsystems) and software to visualise each islet at 100-200x magnification. Degree of insulitis was graded as; 0; no
infiltration, 1; periductal/peri-insulitis, within the pancreas, localised around but not within the islet, 2; circumferential, <25% of the islet infiltrated, 3; invasive, 25%-50% of the islet infiltrated, 4; destructive, >75% of the islet infiltrated. A minimum of 70 islets were counted per mouse over the 3 histological levels and expressed for each grade as a percentage of total islets scored for each group.

**Insulin Staining**

In order to determine degree of insulin production by islets remaining within the islet allograft, slides contiguous with H&E stained sections were deparaffinised using the autostainer (Leica autostainer XL). Insulin staining was performed using the DAKO EnVision + System-HRP (DAKO Cytomation) with 100μL reagent per tissue section and incubations at room temperature in the dark maintaining sections under moist conditions.

Once deparaffinised, tissue sections were treated with 0.3% H$_2$O$_2$ (DAKO; Peroxidase block) for 5min and washed twice with 1x PBS. Tissue sections were incubated for 1 hour with insulin primary antibody (Promega #4590; 1:100 rabbit anti-mouse insulin antibody in DAKO antibody diluent; Cell signalling). Slides were washed twice with 1x PBS and incubated for 30min with secondary, polymer-HRP anti-rabbit antibody (DAKO). Slides were washed three times followed by development with DAB and substrate buffer (DAKO; 1 drop DAB/mL substrate) for 2min keeping timing consistent for all slides. Slides were washed thoroughly with dH$_2$O and counterstained (Leica autostainer XL) and cover slipped (Leica CV5030).

**Foxp3+ Staining**

In order to determine infiltration of pancreata and islet grafts by Foxp3+ regulatory T cells, Foxp3 staining was performed on sections contiguous with those used to stain for H&E and/or insulin. Slides were deparaffinised using the autostainer (Leica autostainer XL) followed by antigen retrieval using a pressure cooker (DAKO Cytomation, Pascal, California Inc. S2800). Briefly, slides were placed in an antigen retrieval box containing citrate buffer (DAKO; 10mM, pH 6, 1:10 with dH$_2$O) and heated in a pressure cooker (125°C for 30sec, 95°C for 10sec) followed by cooling the antigen retrieval box with cold running water. Following antigen retrieval tissue sections were washed with dH$_2$O, treated with 0.3% H$_2$O$_2$ (DAKO; Peroxidase
block) for 10min and washed twice with TBS-T. Tissue sections were treated with protein block (DAKO) and incubated for 1 hour. Protein block was flicked off and briefly washed with TBS-T followed by incubation for 2 hours with Foxp3 primary antibody (eBioscience; rat anti-mouse purified Foxp3, clone FJK-16s, 1:100 in DAKO antibody diluent; Cell signalling). Slides were washed three times with TBS-T and incubated for 30min with secondary, biotin anti-rat antibody (Jackson Laboratories; biotin SP-conjugated affinity purified F(ab’’) fragment goat anti-rat IgG, Fc with spacer, 1:1000 in DAKO antibody diluent; Cell signalling). Slides were washed three times with TBS-T and incubated for 30min with the ABC Vectastain kit (Elite PK6100) in PBS. Slides were washed thoroughly with TBS-T followed by development with DAB and substrate buffer (DAKO; 1 drop DAB/mL substrate) for 30sec keeping timing consistent for all slides. Slides were washed thoroughly with dH2O, counterstained (Leica autostainer XL) and cover slipped (Leica CV5030).

Statistics

Data was analysed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Kaplan-Meir survival curve analysis using a Log-rank (Mantel Cox) test was performed to determine changes in allograft survival or diabetes onset. Holm-Sidak multiple comparison t tests were performed followed by further analysis using unpaired t test (Mann-Whitney) or one-way ANOVA on select results. Results were considered statistically significant when the p value was less than 0.05

Table 2.2: Flow Cytometry Staining Conditions;

<table>
<thead>
<tr>
<th>Stains</th>
<th>Antibodies</th>
</tr>
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<tbody>
<tr>
<td>T cells (C57BL/6 and NOD)</td>
<td>CD4, CD8, CD25, Foxp3, CD44, CD62L, CXCR3</td>
</tr>
<tr>
<td>NK &amp; B cells (NOD only)</td>
<td>B220, CD21, CD23, CD3, NKp46, CXCR3</td>
</tr>
<tr>
<td>CFSE Staining</td>
<td>CD4, CD8, CD44, CD62L, CXCR3, CFSE (521nm)</td>
</tr>
<tr>
<td>Antibody Titration</td>
<td>B220, CD4, CD8, CD25, Foxp3, CXCR3</td>
</tr>
<tr>
<td>Internalisation Assay</td>
<td>CD4, CD8, APC-CXCR3-9C5/Isotype, PerCpCy5.5-CXCR3-173/Isotype</td>
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<tr>
<td>Competitive Binding Assay</td>
<td>CD4, CD8, APC-CXCR3-9C5/Isotype, PerCpCy5.5-CXCR3-173/Isotype</td>
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### Table 2.3: Antibodies for Flow Cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
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<td>7D4</td>
<td>FITC</td>
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</tr>
<tr>
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<td>MEL-14</td>
<td>PE</td>
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<tr>
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<td>FJK-16s</td>
<td>APC</td>
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<td>Biolegend</td>
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<tr>
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<td>APC</td>
<td>1:100</td>
<td>Abcam kit</td>
</tr>
<tr>
<td>IgG Isotype (for CXCR3-9C5)</td>
<td>IgG</td>
<td>APC</td>
<td>1:100</td>
<td>Abcam kit</td>
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Chapter 3. Characterising αCXCR3-9C5
Chapter 3. Characterising αCXCR3-9C5

The chemokine receptor CXCR3 is involved in the recruitment of immune cells to sites of inflammation\(^3,24,200,263\). Targeted blockade of immune cell recruitment to sites of inflammation has clinical potential for preventing conditions like autoimmunity and transplant rejection. For this thesis I will explore the efficacy of a novel monoclonal antibody against CXCR3 in animal models of autoimmunity and transplant rejection.

αCXCR3-9C5 Impairs Chemotaxis of CXCR3 transfected cells

This antibody was generated by Remy Robert in the Mackay lab. The CXCR3 antibody was raised in a CXCR3-/− mouse against a highly conserved epitope found in the mouse, marmoset and human CXCR3 sequence (figure 3.1a). The antibody clone, CXCR3-9C5, binds a region of the CXCR3 sequence critical for chemokine binding\(^2\), as shown for the human CXCR3-A sequence (Figure 3.1b). As a result it was expected that administration of the anti-CXCR3-9C5 (αCXCR3-9C5) antibody would inhibit chemotaxis of a murine pre-B cell line transfected with human CXCR3-A (L1.2 hCXCR3) to express the CXCR3 receptor. The efficacy of this antibody in inhibition of CXCR3 induced chemotaxis was determined by incubating CXCR3 transfected cells with αCXCR3-9C5 (0-100μg/mL) and measuring the change in the ability of these cells to migrate across a membrane towards human CXCR3 chemokines; hCXCL9, hCXCL10 and hCXCL11. A strong inhibitory effect on chemotaxis of transfected cells in response to increasing doses of αCXCR3-9C5 for human (h)CXCL9 was seen, with ~90% inhibition at a concentration of 20μg/mL. In addition chemotaxis in response to hCXCL10 and hCXCL11 showed 50-60% inhibition at a concentration of 20μg/mL αCXCR3-9C5 (figure 3.1c). These data show the efficacy of the mAb αCXCR3-9C5 in blockade of CXCR3 dependent chemotaxis.
Characterising αCXCR3-9C5

We next investigated CXCR3 receptor expression and internalisation following αCXCR3-9C5 treatment of target cells to determine the effects of αCXCR3-9C5 ligation on cell stability. For this we investigated if αCXCR3-9C5 ligation mediated neutralisation of the receptor or depletion of bound cells or internalisation of the chemokine receptor.

In order to examine receptor expression on splenocytes the αCXCR3-9C5 antibody was fluorescently conjugated followed by incubation with isolated splenocytes to determine CXCR3 expression by flow cytometry. A second fluorochrome conjugated
Characterising αCXCR3-9C5

CXCR3 antibody sold commercially (CXCR3-173) was used to validate CXCR3 expression. Upon treatment of splenocytes with αCXCR3-9C5 or CXCR3-173, binding to the CXCR3 receptor on CD4 and CD8 T cells was found to be comparable for both antibodies (figure 3.2a). This CXCR3-173 monoclonal antibody was raised against a similar epitope of CXCR3 as αCXCR3-9C5 (figure 3.2b)\textsuperscript{200}.

Receptor internalisation and competitive binding assays

Receptor internalisation and competitive binding assays were then performed (see figure 3.3 for schematic representation) in order to determine the mechanism by which αCXCR3-9C5 elicits its inhibitory effect on ligand binding and chemotaxis.

Receptor internalisation was examined through incubation of splenocytes with αCXCR3-9C5 antibody for varying times (0, 10, 20, 30, 60, 120, 180 minutes) at 37°C, in azide free conditions, followed by fixation and staining for flow cytometry (figure 3.4a). In comparison to isotype treated cells, those incubated with αCXCR3-9C5 showed binding for both CD4+ and CD8+ T cells indicating specificity of binding to CXCR3. No significant difference in the degree of binding was seen following incubation with αCXCR3-9C5 at any time, suggesting the αCXCR3-9C5 antibody clone was not affecting basal CXCR3 expression on these cells and was not

Figure 3.2: Equivalent Binding of CXCR3+ T cells by αCXCR3-9C5 and commercially available CXCR3-173 antibody.

(a) Lymphocytes incubated with either CXCR3-173 (commercially available) or αCXCR3-9C5 at 4°C were found to bind comparable frequencies of CXCR3+CD4+ and CXCR3+CD8+ T cells, by flow cytometry. Mean+/−SEM data from 2 replicate experiments performed in duplicate. (b) Both antibodies were raised against the extracellular N-terminal domain of the CXCR3 receptor, targeting similar and overlapping epitopes of the CXCR3 receptor.
Characterising αCXCR3-9C5

Inducing receptor internalisation. Due to the use of fixation however, the possibility of detecting internalised receptor poses a potential caveat.

Following incubation with αCXCR3-9C5 at 37°C, cells were also treated with the alternate CXCR3-173 antibody to validate this result. However CXCR3-173 was unable to bind those cells pre-treated with αCXCR3-9C5 (figure 3.4b). Binding of CXCR3-173 to cells pre-treated with isotype rather than αCXCR3-9C5 showed a similar frequency of binding to CD4+ and CD8+ T cells compared to binding by αCXCR3-9C5. This result, combined with the overlapping peptides against which the two antibodies were raised, suggested competitive binding between the antibodies for a similar epitope on the CXCR3 receptor and as such competitive binding was investigated further.

![Diagram of internalisation and competitive binding assay](image)

**Figure 3.3: Internalisation and competitive binding assay.**

Schematic representation; order of antibody application for each in vitro assay. Splenocytes, represented by blue cells, αCXCR3-9C5 fluorescently labelled (red) and CXCR3-173 fluorescently labelled (grey).

In order to clarify the ability of CXCR3 to be bound by the two antibodies splenocytes were incubated at 4°C in azide free conditions with either antibody alone (αCXCR3-9C5 or CXCR3-173 alone) or sequential incubation of cells with αCXCR3-9C5 followed by CXCR3-173 or incubation with CXCR3-173 followed by αCXCR3-9C5. Firstly this did not affect the frequency of CD4+ or CD8+ T cells.
detected through flow cytometry (figure 3.4c) suggesting that the antibody is not causing T cell depletion. Incubation with each antibody alone showed binding of significantly more CXCR3 expressing cells compared to isotype, as expected, with a slightly lower frequency of CD4+ T cells bound by CXCR3-173 than αCXCR3-9C5 (figure 3.4d).

**Figure 3.4:** αCXCR3-9C5 competitively binds CXCR3 receptor but does not cause internalisation or lymphocyte depletion.

**Internalisation Assay:** Incubation of splenocytes at 37°C, azide free, with CXCR3-9C5 antibody for 0, 10, 20, 30, 60, 120 and 180 minutes or isotype control antibody and analysis of CXCR3+CD4+ and CXCR3+CD8+ T cell binding by flow cytometry for (a) CXCR3-9C5 binding (b) or CXCR3-173 binding post incubation with CXCR3-9C5.

**Competitive binding assay:** Incubation of splenocytes with αCXCR3-9C5 or CXCR3-173 alone or sequentially with CXCR3-173 staining prior to or followed by αCXCR3-9C5 staining, or isotype control expression. (c) The frequency of CD4+ and CD8+ T cells. (d) Expression of CXCR3 for isotype, αCXCR3-9C5 and CXCR3-173 treated CD4+ and CD8+ T cells, (e) binding of αCXCR3-9C5 or (f) binding of CXCR3-173 to CD4+ and CD8+ T cells treated with either antibody alone or sequentially.

Mean+/-SEM p<0.05*, p<0.01**, p<0.001***, data from 2-3 replicate experiments performed in duplicate.
Incubation of cells with CXCR3-173 did not affect binding of cells by αCXCR3-9C5, regardless of whether CXCR3-173 was applied prior to or post αCXCR3-9C5 (figure 3.4e). However binding of cells by CXCR3-173 was impaired when cells were incubated with αCXCR3-9C5 following initial treatment with CXCR3-173 and to an even greater extent when stained with αCXCR3-9C5 prior to incubation with CXCR3-173 (figure 3.4f).
Summary & Conclusions

CXCR3 is an important chemokine receptor, particularly for activated CD4+ and CD8+ T cells and natural killer cells\textsuperscript{21,22,188,200,201}. Here we characterise a novel αCXCR3-9C5 antibody raised against a conserved epitope of CXCR3 found in a number of species including mice and humans. This monoclonal antibody targets an epitope containing residues critical for chemokine binding and as a result impairs chemotaxis of CXCR3 transfected cells in a concentration dependent manner to the three CXCR3 ligands, CXCL9, CXCL10 and CXCL11\textsuperscript{2}. In addition ligands with higher affinity to CXCR3, CXCL10 and CXCL11, showed a lower level of chemotactic inhibition compared to the lower affinity chemokine CXCL9\textsuperscript{189,190}. These results show no change in CXCR3 expression in internalisation assays with αCXCR3-9C5, suggesting that αCXCR3-9C5 does not induce receptor internalisation. However, due to the fixation of cells following αCXCR3-9C5 treatment this data may represent surface and internal CXCR3 expression. Future experiments using biotinylated αCXCR3-9C5 followed by staining post incubation with fluorochrome conjugated streptavidin and with no fixation would be expected to clarify this.

Results also did not indicate depletion of T cells with no change in the frequency of CD4+ of CD8+ T cells following αCXCR3-9C5 treatment compared to isotype control, as well as compared to CXCR3-173, known to neutralise but not deplete CXCR3 expressing cells\textsuperscript{200}. This result is in agreement with previous literature showing minimal effector function of mouse IgG1 antibodies for both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)\textsuperscript{264}. However, further in vivo investigation will be required to clarify cell depletion through these pathways. This can be achieved briefly by incubating effector cells with the αCXCR3-9C5 mAb followed by addition of complement (CDC) or NK92 (a cell line with characteristics of NK cells)(ADCC) which lyse the target effector cells. Lysis can then be measured using absorbance to determine lactate dehydrogenase release.

When looking at our αCXCR3-9C5 antibody in relation to the commercially available CXCR3-173 we found that the degree of chemotactic inhibition for CXCL10 found with αCXCR3-9C5 showed a similar efficacy, ~50-60% inhibition at
20μg/mL, as the CXCR3-173 antibody. In contrast, CXCR3-173 showed only minor (~25%) inhibition for CXCL11 and no effect on chemotaxis in response to CXCL9, while αCXCR3-9C5 resulted in a higher level of chemotaxis inhibition for CXCL11 (~50%) and CXCL9 (~90%) at concentrations of 20μg/mL. Both antibodies were found to neutralise rather than deplete T cells upon binding.

Studies of the CXCR3 receptor structure by Colvin et al. revealed that phosphorylation of residues Y29 and Y27 is essential for chemokine binding by CXCL9, CXCL10 and CXCL11. The region targeted by αCXCR3-9C5 encompasses these residues (figure 3.1 & 3.2). Further the proximal 16 amino acids of the CXCR3 N-terminal, which are targeted by CXCR3-173 but not αCXCR3-9C5, are important for CXCL10 and CXCL11 but not CXCL9 binding, this difference in binding sites may account for the distinct effects of the two antibodies.

Finally these results strongly suggest competitive binding between the αCXCR3-9C5 and CXCR3-173 antibodies. These antibodies were raised against overlapping peptides of the CXCR3 receptor with binding assays showing impaired binding of CXCR3-173 in the presence of our αCXCR3-9C5 antibody. The ability of αCXCR3-9C5 to bind to T cells was not significantly affected by the presence of CXCR3-173, either administered prior to or post incubation with αCXCR3-9C5. In contrast the ability of CXCR3-173 to bind T cells, both pre-incubated with αCXCR3-9C5 or where αCXCR3-9C5 was administered following CXCR3-173 was significantly impaired. This suggests that CXCR3-173 was displaced by αCXCR3-9C5, due to steric hindrance or a higher binding affinity for this shared epitope, resulting in competitive binding. Due to the competitive binding of αCXCR3-9C5 and CXCR3-173 for this shared epitope of the CXCR3 receptor, fluorescently labelled CXCR3-173 expression represents CXCR3 receptor not bound by αCXCR3-9C5. Thus comparisons between αCXCR3-9C5 treated and untreated cells for expression of CXCR3-173 could be used to determine the frequency of cells bound by αCXCR3-9C5. This also allows the expression of free, functionally available CXCR3 to be determined and was used in further experiments as a measure of αCXCR3-9C5 binding and free CXCR3 expression.
Chapter 4. αCXCR3-9C5 in the Islet Allograft Model
Chapter 4. αCXCR3-9C5 in the Islet Allograft Model

The chemokine receptor CXCR3 has been shown to be highly upregulated in a number of inflammatory conditions in humans including; cancer, autoimmunity and allograft rejection\textsuperscript{178,179,181,182,239}. Due to its known role in recruitment of lymphocytes we sought to investigate the role of CXCR3 in a mouse model of islet allograft transplantation. Islet allograft rejection is both a well-established and well-studied model for human transplant rejection\textsuperscript{267-269}. This model has also gained further importance due to the emergence of clinical islet transplantation as a treatment for individuals with brittle diabetes and hypoglycaemic unawareness\textsuperscript{102,149,155}.

A number of studies have investigated the effects of CXCR3 blockade and deletion in mouse models of allograft transplantation (skin, cardiac, GVHD and also in islets) showing improved allograft survival due to impaired recruitment of immune cells to the foreign tissue\textsuperscript{44,200,216,246,252}. This prompted us to investigate the role of αCXCR3-9C5 antibody in a BALB/c (H2\textsuperscript{d}) to C57BL/6 (H2\textsuperscript{b}) MHC mismatched islet allograft model allowing us to determine the efficacy of this new antibody in an established model.

Peripheral Immune cell characterisation of CXCR3 Expression

Initial studies of the efficacy of different doses of αCXCR3-9C5 antibody in C57BL/6 mice were conducted to determine the effect of αCXCR3-9C5 treatment on the peripheral lymphocyte populations. Using this information we also determined the ideal dose and timing regimen of αCXCR3-9C5 treatment for mice receiving an islet allograft transplant or for untouched mice.

These experiments were conducted on 10 week old C57BL/6 mice, intravenously (i.v.) administered 1mg/kg, 2mg/kg, 5mg/kg or 10mg/kg doses of αCXCR3-9C5 on day 0 followed by tail bleed on days 2, 4, 8 and 10 post i.v. Untouched C57BL/6 mice were used as controls. The peripheral blood frequency of B cells and CD4+, CD4+Foxp3+ (Treg) and CD8+ T cells and their respective binding by CXCR3-173, as an indication of free CXCR3 unbound by αCXCR3-9C5, were determined via flow cytometry. No significant changes in peripheral lymphocyte (B or T cell)
frequencies were evident with administration of αCXCR3-9C5 regardless of dose or time post administration (figure 4.1 & 4.2).

The frequency of CXCR3-173 binding to CXCR3 was significantly decreased in mice treated with αCXCR3-9C5 with a similar reduction in binding seen for all doses of αCXCR3-9C5 (1mg/kg-10mg/kg) administered. For this reason statistical comparisons were performed between the lowest dose (1mg/kg) of αCXCR3-9C5 and controls, unless otherwise stated. Administration of CXCR3-173 showed minimal binding to B cells regardless of αCXCR3-9C5 administration for all doses and time points examined, indicating a very low expression of CXCR3 on B cells, unaffected by αCXCR3-9C5 (figure 4.1a). CXCR3 expression on T cell subsets showed reduced binding by CXCR3-173 compared to control expression for CXCR3+CD4 (figure 4.1b), CXCR3+CD4Foxp3+ (figure 4.2a) and CXCR3+CD8 (figure 4.2b) T cells, This indicates a reduced frequency of free CXCR3, unbound by αCXCR3-9C5.

A significant reduction in CXCR3-173 binding was evident for CD4+ T cells at day 2 (figure 4.1bi) and day 8 (figure 4.1biii) between control and all αCXCR3-9C5 doses. However due to variability at day 4 only control and the 2mg/kg αCXCR3-9C5 dose were found to differ significantly (figure 4.1bii). On day 10 no significant difference was found between control and 1mg/kg αCXCR3-9C5 treated peripheral blood CD4+ T cell CXCR3-173 binding, indicating a loss of efficacy at this time point (figure 4.1biv).

Analysis of CXCR3-173 binding for CD4+Foxp3+ (figure 4.2a) and CD8+ T cells (figure 4.2b) revealed significant reductions at all time points compared to controls.

This lead us to consider all doses equally effective at blocking CXCR3 function in vivo for up to 8 days, with loss of efficacy for CD4+ T cells at this point. For this reason administration of multiple doses of αCXCR3-9C5 antibody at 2mg/kg on a weekly basis (every 7 days) to consistently block the CXCR3 receptor was considered optimal for further experiments.
Figure 4.1: \(\alpha\text{CXCR3}-9\text{C5} \) Antibody Titrations – C57BL/6

Analysis of peripheral blood lymphocyte expression of CXCR3, determined using CXCR3-173 by flow cytometry. Mice were treated with 1mg/kg, 2mg/kg, 5mg/kg or 10mg/kg \(\alpha\text{CXCR3}-9\text{C5} \) i.v. on day 0 or untouched as controls for (a) B cells and (b) CD4+ T cells on day (i) 2, (ii) 4, (iii) 8 and (iv) 10. Expressed as percent lymphocytes \((n=3 \text{ mice/group})\). Mean+/−SEM \(p<0.05^*, p<0.001^{***}\).
Figure 4.2: αCXCR3-9C5 Antibody Titrations – C57BL/6
Analysis of peripheral blood lymphocyte expression of CXCR3, determined using CXCR3-173 by flow cytometry. Mice were treated with 1mg/kg, 2mg/kg, 5mg/kg or 10mg/kg αCXCR3-9C5 i.v. on day 0 or untouched as controls for (a) CD4+Foxp3+ Tregs and (b) CD8+ T cells on day (i) 2, (ii) 4, (iii) 8 and (iv) 10. Expressed as percent lymphocytes (n=3 mice/group). Mean⁺⁻⁻⁻SEM p<0.05*, p<0.01**, p<0.001***, p<0.0001****
αCXCR3-9C5 in the Islet Allograft Model

αCXCR3-9C5 in C57BL/6 Islet Allografts

Once an appropriate timing and dosage had been determined in C57BL/6 mice we investigated the expression of CXCR3 in the spleens of untouched control C57BL/6 and control C57BL/6 recipients of an islet allograft.

Islet allograft survival follows a typical pattern of initially elevated blood glucose, due to chemical treatment with alloxan/STZ to induce diabetes. This is followed by a sharp decline in blood glucose back to euglycaemia upon islet allograft. Mice were monitored for an initial reduction in blood glucose, back to euglycaemic levels, followed by a rise in blood glucose i.e. hyperglycaemia. This model is able to physiologically mimic what would happen clinically. In the islet allograft model euglycaemia indicates that the islet allograft is functionally able to produce insulin and restore blood glucose. This is then followed by a gradual loss of function, measured by a rise in blood glucose level (BGL), over the course of immune mediated destruction and allograft loss (see figure 4.3d). Islet allograft survival was determined as the number of post-operative days maintaining euglycaemia before onset of hyperglycaemia (BGL>15mmol/L) indicating loss of allograft.

Islet allograft was performed using diabetic C57BL/6 (H2b) recipient mice, chemically induced using alloxan (110mg/kg) or STZ (180mg/kg), transplanted with MHC mismatched BALB/c (H2d) islets under the kidney capsule (figure 4.3). These mice were then either left untreated as controls or administered 2mg/kg i.v. αCXCR3-9C5. An additional group of C57BL/6 mice, which did not receive islet allograft transplant, remained untouched, with the exception of αCXCR3-9C5 administration (2mg/kg i.v) as indicated.

Initial experiments aimed to characterise the expression of CXCR3 and effects of administration of 2mg/kg i.v. αCXCR3-9C5 on levels of free CXCR3 and changes in the frequency of T cell populations. These experiments used either αCXCR3-9C5 treated C57BL/6 mice (αCXCR3-9C5 treated untouched recipients) which were untouched or islet allograft recipients (αCXCR3-9C5 treated islet allograft recipients) compared to untreated control mice (untouched controls or control islet allograft recipients).
Further investigation of the effects of αCXCR3-9C5 on islet allograft survival employed 4 treatment regimens from the day of transplant. These included; once weekly i.v. injections of 2mg/kg Isotype control antibody or αCXCR3-9C5 antibody with no other treatment or Isotype or αCXCR3-9C5 antibody in combination with sub-therapeutic doses of the immunosuppressant rapamycin, at 0.1mg/kg, i.p., every day for the first week following transplant (see figure 4.3c).

Figure 4.3: Allogeneic Experimental Model
Overview of C57BL/6 experimental model including a) BALB/c to C57BL/6 Islet allograft schematic, b) timing of treatments and endpoints, chemical induction of diabetes with alloxan 48 hours prior to first treatment dose with isotype or αCXCR3-9C5 mAb with or without rapamycin and receipt of MHC mismatched BALB/c islet allograft c) treatments received by Islet allograft recipients for determination of efficacy of αCXCR3-9C5 mAb on graft survival, d) schematic of typical BGL over the course of rejection.

Basal and Islet Allograft CXCR3 Expression

In order to determine the proportion of cells expressing CXCR3 in untouched and islet allograft recipient mice, flow cytometry was performed on isolated splenic T cells.
Basal expression of CXCR3 on T cell subsets within the spleens of control untouched mice (unfilled black) was determined based on isotype antibody expression (grey line with gating based on grey dashed line) (figure 4.4). The median of the values for proportion of CXCR3 positive cells were determined for untouched control mice with 8.5% of CD4+, 21.9% of Treg and 24.7% of CD8+ T cells found to express CXCR3. The median of the values for proportion of CXCR3 positive cells for control islet allograft recipient mice (unfilled black) was also determined at day 4 post-transplant compared to isotype antibody expression (grey line with gating based on grey dashed line) (figure 4.4). This revealed upregulation of CXCR3 expression on T cells isolated from the spleens of control islet allograft recipients compared to untouched control mice with 15.5% of CD4+, 33.4% of CD4+Foxp3+ and 28.1% of CD8+ T cells expressing CXCR3. CXCR3 expression for CD4+, Tregs and CD8+ T cells were also determined for αCXCR3-9C5 treated untouched and islet allograft recipient mice on POD 4, determined via CXCR3-173 binding. CXCR3 expression was detected on each T cell subset following treatment with 2mg/kg αCXCR3-9C5 (grey shaded, dashed line) at a reduced level compared to control treated mice. Mean, median and range values are given for each group in Table 4.1.

Figure 4.4: C57BL/6 CXCR3 Expression Profiles
Representative histograms of C57BL/6 splenic T cell subsets (CD4+, CD4+Foxp3+Treg and CD8+) showing median for proportion of CXCR3+ staining for untouched C57BL/6 and islet allograft recipient C57BL/6 mice at 4 days post-transplant. Isotype mAb (grey line) compared to untreated control (unfilled black) or αCXCR3-9C5 treated (grey shaded, dashed line) CXCR3 expression (n=2-6 mice/group).
αCXCR3-9C5 in the Islet Allograft Model

The proportion of cells expressing free unbound CXCR3 on CD4+, Treg and CD8+ T cell subsets was next determined for C57BL/6 mice, which received 2mg/kg, IV, αCXCR3-9C5 or were left untreated as controls, at day 4 and day 8 post treatment. This was also performed for an additional group of mice which received an islet allograft transplant followed by treatment with 2mg/kg, IV, αCXCR3-9C5 or left untreated as controls and expression determined at day 4 and day 8 post treatment.

Initial analysis revealed little change in raw cell counts at day 4 or between untouched groups. Initial raw counts of the total number of splenocytes in control transplant recipients showed a large but non-significant decrease at day 8 (figure 4.5). Of note these mice were treated with STZ to chemically induce diabetes prior to receipt of an allograft (see summary & conclusions). Although this change was non-significant compared to αCXCR3-9C5 treated islet allograft recipients it did impact upon determination of changes in cell populations by flow cytometry. As a result both total number and frequency were assessed for allograft recipients (figure 4.9-4.12), while only total splenocyte number was analysed for untouched mice (figure 4.6-4.9).

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n= 4 | 6 | 3 | 2

**Table 4.1: C57BL/6 CXCR3 Expression Profiles**
Mean and median values as well as range of C57BL/6 splenic T cell subsets (CD4+, CD4+Foxp3+ Treg and CD8+) proportion of CXCR3+ staining for untouched C57BL/6 and islet allograft recipient C57BL/6 mice at 4 days post-transplant (n=2-6 mice/group).
αCXCR3-9C5 in the Islet Allograft Model

The total number of CD4+, CD4+Foxp3+ or CD8+ T cells found in untouched C57BL/6 mice either treated with αCXCR3-9C5 or untreated controls was not different between groups at either day 4 or day 8 post-treatment (figure 4.6a, 4.7a, 4.8a).

Figure 4.5: Untouched and transplant recipient mice total splenocyte counts
C57BL/6 mice were treated i.v. with 2mg/kg αCXCR3-9C5 or untreated as controls, of these a cohort of mice were received a BALB/c islet allograft under the kidney capsule (transplant) while another group did not (untreated). Mice were then assessed for total splenic lymphocyte counts at day 4 and 8 post i.v. (n=2-6 mice/group)

Figure 4.6: αCXCR3-9C5 treatment in untouched C57BL/6 mice – CD4+ T cells
Untouched C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5, were assessed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. Splenocytes were expressed as cell number for (a) CD4+ T cells and (b) CD4+CXCR3+ showing binding of free CXCR3, determined via CXCR3-173 binding. Mean+/− SEM p<0.05* (n=2-6 mice/group).

Analysis of the number of cells expressing free unbound CXCR3, via CXCR3-173 binding, for each T cell subset suggested a trend toward reduced expression for
CD4+, CD4+Foxp3+ and CD8+ T cells at day 4 compared to untreated controls, although small group sizes prevent statistical analysis (figure 4.6b, 4.7b, 4.8b). When the number of cells expressing free CXCR3 was determined at day 8 post αCXCR3-9C5 administration a significant decrease was evident for CD8+ T cells but not CD4+ or CD4+Foxp3+ T cells, compared to untreated controls (figure 4.8b).

Figure 4.7: αCXCR3-9C5 treatment in untouched C57BL/6 mice – CD4+ Foxp3+ T cells
Untouched C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5, were assessed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. Splenocytes were expressed as cell number for (a) CD4+Foxp3+ T cells and (b) CD4+Foxp3+CXCR3+ showing binding of free CXCR3, determined via CXCR3-173 binding. Mean+/− SEM p<0.01** (n=2-6 mice/group).

Figure 4.8: αCXCR3-9C5 treatment in untouched C57BL/6 mice – CD8+ T cells
Untouched C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5, were assessed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. Splenocytes were expressed as cell number for (a) CD8+ T cells and (b) CD8+CXCR3+ T cells showing binding of free CXCR3, determined via CXCR3-173 binding. Mean+/− SEM p<0.05* (n=2-6 mice/group).
αCXCR3-9C5 in the Islet Allograft Model

The number of cells expressing free unbound CXCR3 was also found to differ significantly for CD4+, CD4+Foxp3+ and CD8+ T cell subsets at day 8 post αCXCR3-9C5 administration compared to day 4 post treatment. The number of cells expressing free CXCR3 was found to be significantly decreased at day 4 compared to day 8 post treatment, suggesting loss of αCXCR3-9C5 binding and recovery of free unbound CXCR3 at 8 days post treatment (figure 4.6b, 4.7b, 4.8b).

Analysis of CD4+ and CD8+ effector (CD44<sup>high</sup>, CD62L<sup>low</sup>), memory (CD44<sup>high</sup>, CD62L<sup>high</sup>) and naïve (CD44<sup>low</sup>, CD62L<sup>high</sup>) T cell subsets at day 8 following treatment with αCXCR3-9C5 was also performed compared to untreated controls (figure 4.9a & b). No difference was seen between groups for CD4+ T cells. Of note, changes in CD4+CXCR3+ frequency seen at day 4 were not maintained to day 8 post-treatment (figure 4.6b) and as such although no change in CD4+ T cell subsets are evident at day 8 controls (figure 4.9a), changes may still exist at day 4 post-treatment. At day 8 following treatment with αCXCR3-9C5 a decrease in the number of CD8+ memory T cells was evident compared to untreated controls, although this was found to be non-significant. Of note analysis of percentage CD8+ memory T cells did show a significant decrease in CD8+ memory T cells for αCXCR3-9C5 compared to controls.

![Graph showing changes in CD4+ and CD8+ T cell subsets](image_url)

**Figure 4.9: αCXCR3-9C5 treatment in untouched C57BL/6 mice – T cell activation**

Untouched C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5, were assessed by flow cytometry for splenic lymphocyte populations at day 8 post i.v. Splenocytes were expressed as cell number for (a) CD4+ and (b) CD8+ effector (CD44<sup>high</sup>CD62L<sup>low</sup>), memory (CD44<sup>high</sup>CD62L<sup>high</sup>), and naïve (CD44<sup>low</sup>CD62L<sup>high</sup>) subsets and their respective levels of free CXCR3 determined via CXCR3-173 binding. Mean+/− SEM p<0.05*, p<0.01**, (n=5-6 mice/group).
αCXCR3-9C5 in the Islet Allograft Model

Analysis of the number of cells expressing free unbound CXCR3, via CXCR3-173 binding for effector and memory CD8+ T cells showed significantly reduced free CXCR3 following αCXCR3-9C5 treatment compared to controls (figure 4.9b).

These same analyses were then performed in islet allograft recipients treated with αCXCR3-9C5 compared to control islet allograft transplant recipient mice. Splenocytes were examined for changes in both number and frequency of CD4+, CD4+Foxp3+ and CD8+ T cells and their respective number and frequency of cells expressing free CXCR3 at day 4 and 8 post injection and transplant in these mice (figure 4.10-4.13).

As no change in raw cell counts was evident for islet allograft recipient splenocytes collected at day 4 post-transplant and αCXCR3-9C5 treatment, changes in number and frequency of T cell populations and their respective CXCR3 expression were consistent. No change in the number or frequency of CD4+, CD4+Foxp3+ or CD8+ T cells was evident at day 4 following αCXCR3-9C5 treatment compared to control islet allograft recipients (figure 4.10a, 4.11a and 4.12a).

The proportion of cells expressing free unbound CXCR3 were reduced for both number and frequency of CD4+, CD4+Foxp3+ and CD8+ T cells at day 4 post αCXCR3-9C5 administration compared to controls, although due to small group size this was non-significant (figure 4.10c & d, 4.11c & d, 4.12c & d).

Differences in 8 day raw cell counts for islet allograft recipients (figure 4.5) resulted in inconsistent changes between groups for number and frequency of splenocyte T cell subsets. Due to the decreased raw cell counts for control islet allograft recipients the number of CD4+, CD4+Foxp3+ and CD8+ T cells were decreased compared to αCXCR3-9C5 treated islet allograft recipients (figure 4.10-4.12a). In comparison the frequency of CD4+, CD4+Foxp3+ and CD8+ T cells were not different (figure 4.10-4.12b) for αCXCR3-9C5 treated islet allograft recipients compared to controls.
This difference in raw cell counts also obscured any change in the proportion of cells compared to controls. While analysis of cell number for CD4+, CD4+Foxp3+ and CD8+ T cells showed little change in the number of cells expressing free CXCR3 (figure 4.10c, 4.11c, 4.12c) a decreased frequency of cells expressing free CXCR3 is evident for αCXCR3-9C5 treated islet allograft recipients compared to controls for each subset (figure 4.10d, 4.11d, 4.12d). Due to small group size these changes were non-significant.

Control islet allograft recipient mice showed an upregulation in the frequency of cells expressing free CXCR3 from day 4 to day 8 post-transplant for CD4+, CD4+Foxp3+ and CD8+ T cell frequency, as seen in Figure 4.4 (figure 4.10d, 4.11d, 4.12d). This same trend was evident for αCXCR3-9C5 treated transplant recipients from day 4 to day 8 with increased free unbound CXCR3 for CD4+, CD4+Foxp3+ and CD8+ T cells for both number and frequency, compared to controls (figure 4.10c & d, 4.11c & d, 4.12c & d). This trend was similar to that seen in naïve mice (figure 4.1, 4.2, 4.6, 4.7 & 4.8), suggesting a partial loss of αCXCR3-9C5 binding to CXCR3 by day 8 post αCXCR3-9C5 administration.

**Figure 4.10: αCXCR3-9C5 treatment in transplanted C57BL/6 mice – CD4+ T cells**

C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5 received a BALB/c islet allograft under the kidney capsule followed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. and allograft. Controls did not receive αCXCR3-9C5 treatment. Splenocytes were expressed as cell number (a & c) or frequency (b & d) for CD4+ T cells (a & b) and showing their respective binding of free CXCR3 (c & d), determined via CXCR3-173 binding. Mean+/-SEM (n=2-4 mice/group)
αCXCR3-9C5 in the Islet Allograft Model

Figure 4.11: αCXCR3-9C5 treatment in transplanted C57BL/6 mice – CD4+Foxp3+ T cells
C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5 received a BALB/c islet allograft under the kidney capsule followed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. and allograft. Controls did not receive αCXCR3-9C5 treatment. Splenocytes were expressed as cell number (a & c) or frequency (b & d) for CD4+Foxp3+ T cells (a & b) and showing their respective binding of free CXCR3 (c & d), determined via CXCR3-173 binding. Mean+/-SEM (n=2-4 mice/group)

Figure 4.12: αCXCR3-9C5 treatment in transplanted C57BL/6 mice – CD8+ T cells
C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5 received a BALB/c islet allograft under the kidney capsule followed by flow cytometry for splenic lymphocyte populations at day 4 and 8 post i.v. and allograft. Controls did not receive αCXCR3-9C5 treatment. Splenocytes were expressed as cell number (a & c) or frequency (b & d) for CD8+ T cells (a & b) and showing their respective binding of free CXCR3 (c & d), determined via CXCR3-173 binding. Mean+/-SEM (n=2-4 mice/group)

CD4+ and CD8+ effector (CD44^{high}, CD62L^{low}), memory (CD44^{high}, CD62L^{high}) and naïve (CD44^{low}, CD62L^{high}) T cell subsets were also analysed at day 8 following islet transplant and treatment with αCXCR3-9C5 compared to control islet transplant.
αCXCR3-9C5 in the Islet Allograft Model

recipients (figure 4.13). A reduced number of CD4 and CD8 effector, memory and naïve cell subsets were evident due to reduced absolute raw cell counts in untreated 8 day allograft transplant recipients (figure 4.13a & c). However, these changes were not reflected in cell frequencies, where a non-significant reduction in total CD8 effector and memory T cells was evident in αCXCR3-9C5 treated mice compared to control islet allograft recipients (figure 4.13b & d). In addition following αCXCR3-9C5 treatment a trend towards a reduced frequency of free CXCR3 on CD4 effector and memory and CD8 effector, memory and naïve T cells was also evident, compared to control expression (figure 4.13b & d). Due to small group size these changes were non-significant.

Figure 4.13: αCXCR3-9C5 treatment in transplanted C57BL/6 mice – T cell activation

C57BL/6 mice, treated i.v. with 2mg/kg αCXCR3-9C5 received a BALB/c islet allograft under the kidney capsule followed by flow cytometry for splenic lymphocyte populations at day 8 post i.v. and allograft. Splenocytes were expressed as cell number (a & c) or frequency (b & d) for CD4+ or CD8+ effector (CD44highCD62Llow), memory (CD44highCD62Lhigh), and naïve (CD44lowCD62Llow) T cell subsets and showing their respective binding of free CXCR3, determined via CXCR3-173 binding. Mean+/− SEM (n=2-4 mice/group)
αCXCR3-9C5 in the Islet Allograft Model

Effector and memory T cell subsets are important mediators of inflammation and allograft rejection\textsuperscript{21,270-272}. Due to the reductions in CD8+ effector and memory splenocyte populations of untouched and allograft recipients at POD 8 we assessed changes in markers of T cell activation, CD44 and CD62L. Representative FACS plots of CD8+ T cells from the spleens of untouched or islet allograft transplant recipients (figure 4.14ai & bi) at POD 8 show increased memory and effector subsets for control mice compared to mice treated with 2mg/kg αCXCR3-9C5 (figure 4.14aii & bii).

Representative histograms of the activation markers CD44 (figure 4.14a & biii) and CD62L (figure 4.14a & b iv) are also shown for control mice (black unfilled) or those treated with αCXCR3-9C5 (grey shaded). The addition of αCXCR3-9C5 resulted in no change in CD62L expression, however a substantial decrease in the expression of CD44\textsuperscript{high} CD8+ T cells compared to untreated controls was observed for both untouched and islet allograft recipient mice.

![Representative FACS plots of CD8+ effector (CD44\textsuperscript{high}CD62L\textsuperscript{low}), memory (CD44\textsuperscript{high}CD62L\textsuperscript{high}), and naive (CD44\textsuperscript{low}CD62L\textsuperscript{low}) subsets from either (a) untouched/untransplanted or (b) islet allograft recipient mice for (i) untreated controls or (ii) αCXCR3-9C5 treated (2mg/kg i.v.) mice at 8 days post-transplant and/or αCXCR3-9C5. Representative histograms of (iii) CD44 and (iv) CD62L are also shown for mice treated with αCXCR3-9C5 (grey shaded) or untreated controls (unfilled black) (n=2-4mice/group)](image)

**Figure 4.14: Altered activation of naïve and allogeneic C57BL/6 splenocytes**

Representative FACS plots of CD8+ effector (CD44\textsuperscript{high}CD62L\textsuperscript{low}), memory (CD44\textsuperscript{high}CD62L\textsuperscript{high}), and naive (CD44\textsuperscript{low}CD62L\textsuperscript{low}) subsets from either (a) untouched/untransplanted or (b) islet allograft recipient mice for (i) untreated controls or (ii) αCXCR3-9C5 treated (2mg/kg i.v.) mice at 8 days post-transplant and/or αCXCR3-9C5. Representative histograms of (iii) CD44 and (iv) CD62L are also shown for mice treated with αCXCR3-9C5 (grey shaded) or untreated controls (unfilled black) (n=2-4mice/group).
The decreased CD8+CD44<sup>high</sup> expression found in these mice resulted in a reduction in CD44+CD8+ T cells at day 8 for both untouched and islet allograft recipient mice following treatment with αCXCR3-9C5 when compared to untreated controls (figure 4.15b). This change was non-significant due to small group size. No change was seen for CD4+ CD44 expression. When frequency of CD8+ T cells were analysed a slight decrease was evident for both untouched and allograft recipients treated with αCXCR3-9C5 compared to controls (figure 4.15a).

Figure 4.15: Altered CD44 expression of untouched and transplanted C57BL/6 splenocytes
Untouched or islet allograft recipient mice were treated with 2mg/kg αCXCR3-9C5 i.v. or untreated as controls and analysed by flow cytometry at day 8 post-transplant/i.v. for frequency of (a) CD8+ T cells or (b) expression of CD44<sup>high</sup> CD4+ and CD8+ T cells. Mean+/− SEM (n=2-4 mice/group)

**αCXCR3-9C5 Prolongs C57BL/6 Islet Allograft Survival**

Using this initial characterisation it was determined that regular administration of αCXCR3-9C5 once per 7 days at a concentration of 2mg/mL would maintain expression of free unbound CXCR3 at a low level. We then further investigated the effect this antagonism of the CXCR3 receptor had on islet allograft survival.

Transplantation of BALB/c to C57BL/6 islet allografts were performed and mice were treated with one of 4 regimens. These were either; 2mg/kg i.v. Isotype control antibody or 2mg/kg i.v. αCXCR3-9C5 antibody once per week or a combination of Isotype or αCXCR3-9C5 antibody with sub-therapeutic doses of the immunosuppressant rapamycin, at 0.1mg/kg, i.p., every day for the first week.
following transplant\(^2\) (see figure 4.3). Mice were then monitored for the onset of hyperglycaemia, indicative of allograft rejection.

Rejection of islet allografts occurred in all recipients treated with isotype control antibody by POD 24 with a median survival time (MST) of 22 days. However, treatment of mice with \(\alpha\)CXCR3-9C5 resulted in a significant prolongation of allograft survival with MST of 31 days and permanent engraftment (>100 days) in 1 out of 7 mice. When rapamycin was given in combination with \(\alpha\)CXCR3-9C5 50% of mice showed long term islet allograft survival with a MST of 81.5 days (figure 4.16). In contrast to those mice treated with a combination of \(\alpha\)CXCR3-9C5 and rapamycin, treatment of mice with isotype and rapamycin resulted in a MST of 40 days and did not show any long-term allograft survival. Although treatment with rapamycin improved allograft survival compared to mice treated with isotype alone this change was not significant.

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**Figure 4.16: Allogeneic C57BL/6 Islet Transplant**

Kaplan-Meier survival curve of C57BL/6 (H2\(^b\)) recipient mice, transplanted under the kidney capsule with BALB/c (H2\(^d\)) islet allografts. Mice received either; isotype antibody (1/week, 2mg/kg, i.v. for 9 weeks)(●), \(\alpha\)CXCR3-9C5 mAb (1/week, 2mg/kg, i.v. for 9 weeks)(■), or combined Isotype + rapamycin (1x/day, 0.1mg/kg, i.p. for 7 days)(▼) or \(\alpha\)CXCR3-9C5 + rapamycin (1x/day, 0.1mg/kg, i.p. for 7 days)(♦) from the morning of allograft. Mice were monitored a loss of euglycaemia indicating loss of allograft. Mean+/-SEM p<0.05* (n=4-7 mice/group).

Isotype vs. \(\alpha\)CXCR3 p= 0.0291*
Isotype+Rapa vs. \(\alpha\)CXCR3+Rapa p=0.087
\(\alpha\)CXCR3 vs. \(\alpha\)CXCR3+Rapa p=0.3309
**αCXCR3-9C5 and Rapamycin Therapy Preserve Islet Allograft Architecture**

Examination of islet allograft pathology taken from long term (>POD 100) surviving grafts from mice treated with a combination of αCXCR3-9C5 and rapamycin, was then performed. Staining for H&E and insulin revealed a preserved islet architecture and strong insulin labelling with isolated pockets of mononuclear cell infiltration restricted to the edges of the graft (figure 4.17a). Due to the role of regulatory T cells in allograft survival\textsuperscript{144,273-275}, characterised by their expression of the Foxp3 transcription factor, we next examined Foxp3+ T cells within the graft. Consequently we found robust staining (brown) within the observed pockets of immune infiltrate at the edge of the long term surviving islet allografts (figure 4.17b).

![Figure 4.17: Long-term surviving allograft pathology](image)

**Figure 4.17: Long-term surviving allograft pathology**

Representative histology of long-term surviving (POD>100) islet allografts from mice treated with 2mg/kg αCXCR3-9C5 mAb (i.v. 1x/week for 9 weeks) + 0.1mg/kg rapamycin (i.p. 1x/day for 7 days) POD>100. (a) Hematoxylin & Eosin staining (H&E), Insulin staining (INS) (brown) and (b) Foxp3+ cell staining (brown). Flow cytometric analysis of CD4+Foxp3+ T cells as % of total lymphocytes for untouched controls (○), αCXCR3-9C5 + rapamycin (as above, POD>100) (♦) and αCXCR3-9C5 alone (POD>100) (■) measured in spleen, thymus and blood. Individual values of each mouse represented. p<0.05 *
In addition to the presence of Foxp3+ cells at the graft site, we found an increased frequency of CD4+Foxp3+ Tregs within the spleens of long term surviving islet allograft recipients (Figure 4.17c). The frequency of Foxp3+ regulatory T cells isolated from the spleens of long-term surviving mice treated with αCXCR3-9C5 and rapamycin was significantly greater, ~1.05% of total lymphocytes, than that seen for untreated control C57BL/6 mice, which had not received an allograft, ~0.625% of total lymphocytes. The individual long-term surviving αCXCR3-9C5 treated mouse showed a similarly elevated regulatory T cell population as that seen in αCXCR3-9C5 and rapamycin treated mice. No change was seen in the thymus or blood of these mice compared to controls (figure 4.17c).

A role for Regulatory T cells in αCXCR3-9C5 and Rapamycin treated Islet Allograft survival

Next to establish whether regulatory T cells played a role in promoting allograft survival in this model we employed strategies to deplete T cells from our allograft models. Firstly we performed islet allografts, as previously described, followed by weekly treatments with isotype or αCXCR3-9C5 antibody. In addition one group received αCXCR3-9C5 in combination with an anti-CD25 depleting antibody (PC61). This effectively removes the CD4+CD25+Foxp3+ T cell fraction by starving them of IL-2, necessary for Treg survival261. As previously described mice treated with isotype had a MST of 22 days, while αCXCR3-9C5 treatment resulted in a MST 31 days. However, in mice treated with PC61 the beneficial effects of αCXCR3-9C5 on graft survival were lost, resulting in a MST of 26 days (figure 4.18a).

Next, to further elucidate this role of Tregs, an in vivo cell transfer model was used (figure 4.18b). RAG-/ recipient mice firstly received a BALB/c islet allograft followed by transfer of T cells from either untreated control mice or long term surviving islet allograft recipient mice (treated with αCXCR3-9C5 and rapamycin). Transferred T cells were either whole T cells (figure 4.18c) or CD25 depleted T cells (CD25-), lacking CD4+CD25+Foxp3+ T cells (figure 4.18d). In support of previous findings the transfer of whole T cells from long-term surviving mice resulted in prolongation of allograft survival in RAG-/ mice compared to T cells from controls. These results were non-significant due to small group size (figure 4.18c). Transfer of
CD25 depleted cells on the other hand resulted in a complete loss of this prolongation compared to control T cell transfer (figure 4.18d). This supports a role for CD4+CD25+Foxp3+ Tregs in the maintenance of αCXCR3-9C5 induced long term allograft survival.

Figure 4.18: A role for Regulatory T cells in αCXCR3-9C5 allograft survival
(a) Kaplan-Meier survival curve of BALB/c islet (H2d) allografts transplanted under the kidney capsule of C57BL/6 (H2b) recipient mice treated i.v. with 2mg/kg Isotype mAb (●), 2mg/kg αCXCR3-9C5 mAb (■)(1x/week for 9 weeks) or 2mg/kg αCXCR3-9C5 mAb in combination with the αCD25 mAb PC61 (depletes CD25+ cells) (□). (n>3 mice/group)
(b) Schematic of T cell transfer experiments for RAG-/ mice which received BALB/c islet allograft followed by adoptive T cell transfer from long-term surviving αCXCR3-9C5 and rapamycin treated mice or untouched controls. Transfer experiment mice received T cell transfer of either (c) whole T cells or (d) CD25- T cells. (n=2-3 mice/group)
αCXCR3-9C5 in the Islet Allograft Model

Summary & Conclusions

The chemokine receptor CXCR3 has a well-established role in mediating chemotaxis of T cells to sites of inflammation and as such has been shown to undergo upregulation under conditions of inflammation. Here we show that in agreement with previous literature, upon receipt of an islet allograft, stimulating an inflammatory response, the proportion of cells expressing CXCR3 was upregulated from basal levels on CD4+, Treg and CD8+ T cell subsets. In addition administration of our novel αCXCR3-9C5 antibody resulted in a reduced ability of CXCR3-173 mAb to bind to the CXCR3 receptor. We interpret this to indicate receptor occupancy by αCXCR3-9C5, acting as a receptor antagonist, and possibly affecting the ability of chemokines, CXCL9, CXCL10 and CXCL11 to bind CXCR3 and mediate biological functions e.g. chemotaxis.

Doses of αCXCR3-9C5 between 1-10mg/kg were found to be equally efficacious at blocking CXCR3-173 binding for up to 8 days for CD4+ T cells and for over 10 days for CD4+Foxp3+ Treg and CD8+ T cells. From this, a once weekly 2mg/kg αCXCR3-9C5 dose was chosen for use in further experiments. In the spleens of both un-transplanted and allograft recipient mice the proportion of cells expressing free CXCR3 on T cell subsets was reduced at day 4 following treatment with αCXCR3-9C5 with partial or complete recovery of free, unbound CXCR3 by day 8, validating findings in peripheral blood.

Importantly a reduction in absolute number of whole splenocytes for islet allograft recipient mice at day 8 was noted, which affected determination of real changes in the lymphocyte populations in the spleens. For this reason both cell number and frequencies were displayed for islet allograft splenocyte analyses. A number of possible explanations for this exist, such as immune cell migration from the spleen to the graft site. This experiment was performed using STZ to induce diabetes with a previous paper showing lymphopenia post STZ administration. The difference between groups then may result from slightly longer time from STZ administration to transplant or differential effects between mice and for this reason and due to small group size future studies will involve
repetition of this experiment using alloxan rather than STZ to eliminate this possibility.

Investigation of the effect of αCXCR3-9C5 on activation of T cells revealed that both the proportion of CD4+ and CD8+ naïve, memory and effector T cells expressing free CXCR3 were reduced. Although only CD8+ T cells showed a marked decrease in the total memory and effector T cell populations compared to untreated mice (figure 4.9 & 4.13). This reduction, seen in both un-transplanted and allograft recipients, resulted from a decrease in CD44^{high} CD8+ T cells (figure 4.14). No significant change in the frequency of CD8+ T cells between αCXCR3-9C5 treated and control groups were evident (figure 4.15). It is difficult to determine if in this case depletion is occurring as this population accounts for only a 1-1.5% change in total CD8+ T cells as a frequency of lymphocytes with variability and a small group size. This may indicate depletion or natural variability and as such repetition with larger group number may assist in determination if the change in CD44^{high} CD8+ T cells was a result of depletion of memory/effector subsets, or a reduction in expression of the activation marker CD44.

The role of CXCR3 in allograft rejection is well-established in humans, xenografts and in mouse models $^{180,182,200,216,239-243,252}$. Here we provide further evidence of the essential role of CXCR3 in allograft rejection and for the efficacy of αCXCR3-9C5 treatment of mice. Treatment of mice with αCXCR3-9C5 alone or in combination with low doses of the immunosuppressant rapamycin resulted in a significant prolongation of functioning islet allograft survival (figure 4.16). In addition 15% of αCXCR3-9C5 treated and 50% of αCXCR3-9C5 and rapamycin treated mice exhibited functional islet allografts for over 100 days. Islet allograft pathology also displayed preserved islet morphology with no lymphocytes found within the graft site and concurrently showed a robust staining with insulin, indicating well-functioning grafts (figure 4.17). The limited immune infiltrate present at these grafts was encircled by Foxp3+ regulatory T cells. These Treg were found in significant frequencies in the spleens of long term surviving mice and were found to be partially responsible for the prolonged allograft survival in αCXCR3-9C5 treated mice, as determined by PC61 treatment and T cell transfer experiments (figure 4.18a). For controls tissue from younger 6-8 week old un-manipulated C57BL/6 mice were
αCXCR3-9C5 in the Islet Allograft Model

compared to treated long-term surviving allograft recipient mice (figure 4.17c). These controls were chosen due to the logistical and ethical issues involving use of aged controls. Of note previous literature indicates that an age related increase in Treg frequency occurs between 16 and 40 weeks of age in C57BL/6 mice, which may account for this change\textsuperscript{277,278}. However, while literature suggests that this increase can be seen throughout the periphery, our data shows a significant increase in Treg frequency only in the spleens of treated mice and no significant change within the blood. In order to rule out this age related increase in Treg as a potential confounding factor, it would be interesting to repeat this experiment using age matched controls.

Interestingly a similar pattern of infiltration was shown in a recent article focusing on the role of CXCR3 in viral infection. This paper indicated that while CXCR3 knockout CD8+ T cells were able to migrate to sites of infection they were less likely to penetrate regions of heavy infection, hesitating at the perimeter of the infected area. CXCR3 knockout CD8+ T cells were also significantly more motile with an impaired ability to maintain contact with infected cells\textsuperscript{220}. It is possible that αCXCR3-9C5 treatment may also impair contact dependent interactions resulting in reduced allograft destruction and greater peripheral lymphocyte localisation, restricted to small pockets at the periphery of the graft but not within the allograft. From this we conclude that αCXCR3-9C5 is able to antagonise the CXCR3 receptor resulting in reduced free CXCR3 chemokine receptor and a reduction in activation marker CD44. Reduced frequencies of CD44\textsuperscript{high} effector and memory CD8+ T cell subsets were also evident as a result of altered CD44 expression. In addition αCXCR3-9C5 appears to work synergistically with rapamycin to enhance islet allograft survival, likely through promoting regulatory and inhibiting effector T cells\textsuperscript{279}. 

αCXCR3-9C5 in the Islet Allograft Model
Chapter 5. αCXCR3-9C5 in the NOD Autoimmune Diabetic Model
Chapter 5. αCXCR3-9C5 in the NOD Autoimmune Diabetic Model

Type 1 diabetes is an autoimmune disease characterised by progressive loss of islet beta cells until onset of overt diabetes. For a number of individuals maintenance of euglycaemia with insulin administration is inadequate to prevent hyper- and hypoglycaemic episodes. In these individuals with variable blood glucose or with hypoglycaemic unawareness islet or pancreas transplant may be recommended98,100-102. For this reason we next investigated the role of αCXCR3-9C5 mAb in models of type 1 diabetes.

To do this we determined the effect of αCXCR3-9C5 in the NOD mouse model of diabetes. The NOD mouse is a model of spontaneous diabetes caused by the development of self-directed B and T cells that target and destroy insulin producing beta cells. The NOD mouse is a useful model due to the natural development of autoimmunity, pancreatic islet infiltration and loss of euglycaemia, similar to that seen in the human disease. However it differs from human T1D as discussed previously within the introduction61,71,72,77.

We were interested in this model for a number of reasons; firstly no previous literature looks at both the islet allograft and NOD model in the context of chemokine therapies within a single study. This will be advantageous if we wish to pursue αCXCR3-9C5 in a clinical context for islet transplantation in individuals with type 1 diabetes. Moreover such a study may highlight a clinically relevant role of αCXCR3-9C5 for use in human islet transplantation. Additionally other models looking at CXCR3 in the context of diabetes have given variable results with studies in the RIP-LCMV virally induced model showing reduced immune infiltration and delayed diabetes onset, while CXCR3−/− NOD mice were shown to have accelerated diabetes onset resulting from impaired Treg trafficking into the pancreas124,217. Of particular interest to us was the result of Yamada et al. who found accelerated development of diabetes in CXCR3 deficient NOD mice217. A role for CXCR3 in the migration of Treg cells into sites of inflammation has been shown for Th1 type Tregs, which express CXCR3 under the control of the T-bet transcription factor in a number of autoimmune conditions217,225,226. Acceleration of diabetes driven by our
antibody would make further studies into the efficacy of our antibody in a clinical, type 1 diabetes transplantation context unfounded.

As such it is important to determine whether αCXCR3-9C5 confers protection, with delayed diabetes incidence/onset or prolonged islet allograft survival, in the NOD mouse model of diabetes. Deleterious effects of αCXCR3-9C5 treatment, such as impaired pancreatic Treg infiltration were also investigated.

**Peripheral Immune cell characterisation of CXCR3 Expression**

In order to determine the appropriate dosing regimen in the NOD mouse model initial antibody titration experiments were performed, as in C57BL/6 mice. Due to the previous finding in chapter 4 that doses of 1-10mg/kg were equally efficacious we performed further NOD experiments with 1mg/kg αCXCR3-9C5. NOD mice were administered a single i.v. of αCXCR3-9C5 or isotype at 8 weeks of age or untouched as controls, followed by FACS analysis of peripheral blood post injection on days 2, 4 and 6 to determine changes in peripheral lymphocytes and their respective frequency of free CXCR3.

Flow cytometry revealed minimal binding of CXCR3 by CXCR3-173 on B cells, as seen in C57BL/6 samples (figure 4.1a), indicating very low levels of CXCR3 expression which was not affected by treatment with αCXCR3-9C5(figure 5.1a). No difference in the frequency of total CD4+, Tregs and CD8+ T cells was evident between αCXCR3-9C5 treated and isotype or control groups for all time points (figure 5.1). However administration of αCXCR3-9C5 was found to significantly reduce the frequency of cells expressing free unbound CXCR3, as determined by CXCR3-173 binding, for all T cell subsets at day 2 post injection. Reduced CXCR3-173 binding of CD8+ T cells was also evident at day 4 for αCXCR3-9C5 treated, compared to isotype treated T cells. This reduction in the frequency of cells expressing free unbound CXCR3 was absent for all T cell subsets by day 6, suggesting loss of αCXCR3-9C5 binding (figure 5.1b-d).
Figure 5.1: αCXCR3-9C5 Antibody Titrations - NOD
Analysis of peripheral blood lymphocyte expression of CXCR3, determined using CXCR3-173 by flow cytometry. Mice were treated with 1mg/kg αCXCR3-9C5 or Isotype antibody i.v. on day 0 or untouched as controls for total (a) B cells, (b) CD4+ T cells, (c) Treg and (d) CD8+ T cells and on day (i) 2 (n=5-10 mice/group) (ii) 4 (n=5-10 mice/group), and (iii) 6 (n=2-3 mice/group). Expressed as percent of lymphocytes. Mean+/−SEM p<0.05*, p<0.001***


**Basal CXCR3 Expression**

Analysis of baseline frequency of free unbound CXCR3 in the spleens of untreated NOD mice (figure 5.2; black histogram) compared to isotype stained cells (grey histogram) was also performed by flow cytometry for CD4+, CD4+Foxp3+ and CD8+ T cells as well as for NK cells (figure 5.2).

This was performed at 3 different ages: 6 weeks (figure 5.2a), initial onset of mild pancreatic infiltration; 12 weeks (figure 5.2b) at which point a significant increase in islet infiltration occurs and at 15-25 weeks of age, onset of overt diabetes for females, with heavily infiltrated islets and loss of insulin producing beta cells (figure 5.2c).

![Figure 5.2: NOD CXCR3 Expression Profiles](image)

*Figure 5.2: NOD CXCR3 Expression Profiles*

Representative histograms of NOD splenic T (CD4+, CD4+Foxp3+ and CD8+) and NK (CD3-NKP46+) cell subsets, showing median for proportion of CXCR3+ staining (%) for NOD mice at (a) 6 and (b) 12 weeks of age and (c) at onset of overt diabetes. Isotype (grey) compared to Control (black) CXCR3 expression (n=3-7 mice/group).
The median value for the proportion of cells positively staining for CXCR3 on T cells, including CD4+, CD4+Foxp3+ Treg and CD8+ T cells, increased from 6 weeks to 12 weeks of age, peaking at 12 weeks where they either stabilised or decreased until the onset of diabetes at 20-25 weeks of age. In contrast proportion of cells positively staining for CXCR3 on NK cells was highest at 6 weeks of age with reduced expression at 12 weeks and at onset of diabetes at 20-25 weeks of age (figure 5.2). This profile would agree with an early innate response by NK cells at 6 weeks, followed by a more acquired, T cell, response at 12 weeks of age. Mean, median and range values are provided in Table 5.1.

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<td>14.1-15.8</td>
</tr>
<tr>
<td>CD8+CXCR3+</td>
<td>Median</td>
<td>10.5</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>10.31667</td>
<td>13.75</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>8.65-11.8</td>
<td>12.1-16.43</td>
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<tr>
<td>NK+CXCR3+</td>
<td>Median</td>
<td>51.2</td>
<td>40.85</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>51.83333</td>
<td>39.15</td>
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<tr>
<td></td>
<td>Range</td>
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<td>26.8-48.1</td>
</tr>
<tr>
<td>n=</td>
<td>3</td>
<td>4</td>
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</tbody>
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Table 5.1: NOD CXCR3 Expression Profiles
Mean and median values as well as range of NOD splenic T (CD4+, CD4+Foxp3+ and CD8+) and NK (CD3-NKp46+) cell subsets, showing median for proportion of CXCR3+ staining (%) for NOD mice at (a) 6 and (b) 12 weeks of age and (c) at onset of overt diabetes (n=3-7 mice/group).

NOD Insulitis Scoring

Baseline insulitis was determined in the NOD pancreas using formalin fixed, H&E stained sections from 4-6, 9 and 15-16 (diabetic BGL >15mmol/L) week old mice. In order to do this we employed an established 0-4 grading system, where grade 0 islets have no infiltrate, grade 1 peri-insulitis, grade 2 <25% infiltrate, grade 3 >25% and
grade 4 >75% infiltrate. Quantification of insulitis for each age expressed as a percent of total islets scored for each group, revealed a progressive increase in severity of insulitis as mice aged, as expected from previous literature (figure 5.3).

**Figure 5.3: NOD Insulitis Grading**
Representative histological images of H&E stained NOD pancreata, grade 0-4 insulitis scoring; grade 0 no infiltrate, grade 1 peri-insulitis, grade 2 <25% infiltrate, grade 3 >25% infiltrate and grade 4 >75%/complete infiltration.
Assessment of baseline insulitis in 4-6 week old, 9 week old and 15-16 week old diabetic (diabetic BGL >15mmol/L) NOD mice using H&E stained histological sections, expressed as percentage of islets scored per grade (>50 Islets scored/group n=3 mice/group).

**NOD Diabetes Incidence**
Cumulative diabetes incidence data for our laboratory was also obtained to validate NOD diabetes incidence data generated within these studies (figure 5.4). This is necessary as the incidence of diabetes within NOD colonies is strongly influenced by environmental factors such as diet and microbial infections and is known to vary between facilities. Figure 5.4 shows the fraction of normoglycaemic NOD female mice over time from birth to 50 weeks of age. Diabetes incidence rates of 60% at 25 weeks have been previously documented by our facility.
αCXCR3-9C5 in the NOD Islet Allograft Model

αCXCR3-9C5 does not alter Diabetes Incidence in the NOD mouse

Following determination of basal expression of CXCR3 (figure 5.2), degree of insulitis at different ages (figure 5.3) and the efficacy of a 1mg/kg dose of αCXCR3-9C5 to bind CXCR3 expressed by NOD peripheral blood lymphocytes (figure 5.1), we investigated the effect of long term treatment with αCXCR3-9C5 in the NOD mouse.

For this we established a model involving treatment of mice every 4 days with 1mg/kg i.v. αCXCR3-9C5 antibody or isotype control antibody, alternatively mice were untreated as controls. Mice began treatments from either; 6 weeks of age, where 6 weeks of age is early in the initial phase of immune infiltration or 8 weeks of age, the age at which a 7 fold increase in infiltration of leukocytes occurs in the NOD mouse (figure 5.3). Treatments were continued until euthanasia at 12 weeks of age for analysis of pancreatic pathology and changes in lymphocyte populations. Alternatively treatment was continued until onset of overt diabetes or 25 weeks of age, allowing incidence of diabetes in our model to be determined. Diabetes was determined as a blood glucose level (BGL) >15mmol/L on 2 consecutive days (figure 5.5).

Figure 5.4: NOD Diabetes incidence

Cumulative diabetes incidence data was obtained showing diabetes incidence for NOD colonies housed at our facility monitored for BGL>15mmol/L for 2 consecutive days which was considered diabetic. Graph shows fraction of mice maintaining euglycaemia at each age in weeks. Courtesy of S. T. Grey and E. Marino.

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Mice were treated until onset of overt diabetes, with initial onset for each group at approximately 15 weeks of age. Mice treated from 6 weeks of age with αCXCR3-9C5 (6W start) were the exception to this trend with a single mouse becoming diabetic at 10 weeks of age. A similar incidence of diabetes was also seen for all groups with 50-60% of mice diabetic at 25 weeks of age. This revealed no significant effect of 1mg/kg αCXCR3-9C5 on the kinetic of diabetes development in the NOD mice, regardless of age of initial treatment (figure 5.6).

To ensure the lack of effect on diabetes incidence was not due to the lower 1mg/kg αCXCR3-9C5 dose, compared to that used in allograft experiments, we also determined the efficacy of 2mg/kg αCXCR3-9C5 given from 6 weeks of age until onset of overt diabetes. No difference in diabetes incidence between mice treated with 1mg/kg or 2mg/kg was evident (figure 5.7).

**Figure 5.5: Autoimmune NOD Experimental Model**

Overview of experimental model including initial age of treatment (6 or 8 weeks of age) with isotype or αCXCR3-9C5 mAb administered 1mg/kg, every 4 days. Mice were monitored for onset of hyperglycaemia/diabetes (Incidence Study) or euthanised at 12 weeks of age to examine pathology (Pathology Study).
\( \alpha \text{CXCR3-9C5 in the NOD Islet Allograft Model} \)

**Figure 5.6: \( \alpha \text{CXCR3-9C5 does not alter NOD Diabetes incidence} \)

NOD mice were treated from 6 weeks with 1mg/kg Isotype (bite) or \( \alpha \text{CXCR3-9C5 antibody} \) (\( \uparrow \)) or from 8 weeks with Isotype (\( \square \)) or \( \alpha \text{CXCR3-9C5 antibody} \) (\( \blacksquare \)) every 4 days or untouched as controls (\( \circ \)). Blood glucose levels were monitored for a loss of euglycaemia and onset of diabetes, designated as BGL>15mmol/L for 2 consecutive days. Rates of diabetes in each group were monitored until 25 weeks of age and expressed as percent of normoglycaemic mice per group from 6-25 weeks of age. (n=7-18 mice/group)

**Figure 5.7: Equivalent diabetes incidence for 1mg/kg or 2mg/kg \( \alpha \text{CXCR3-9C5} \)

NOD mice were treated every 4 days from 6 weeks with 1mg/kg (\( \uparrow \)) or 2mg/kg (\( \Delta \)) \( \alpha \text{CXCR3-9C5 antibody} \). Blood glucose levels were monitored for a loss of euglycaemia and onset of diabetes, designated as BGL>15mmol/L for 2 consecutive days. Rates of diabetes in each group were monitored until 25 weeks of age and expressed as percent of normoglycaemic mice per group from 6-25 weeks of age. (n=5-13 mice/group)
αCXCR3-9C5 does not alter Insulitis or Regulatory T cell Migration in the NOD mouse

Although little difference was evident in diabetes incidence for αCXCR3-9C5 treated mice, markers of pathology including insulitis and changes in immune cell populations were examined due to the role of αCXCR3-9C5 in inhibiting chemotaxis.

In mice treated with 1mg/kg αCXCR3-9C5 from 6 or 8 weeks until 12 weeks of age (pre-diabetic) (figure 5.8a), no significant difference in insulitis was seen compared to isotype treated mice. This was determined by insulitis grading as shown previously (figure 5.3), statistical analysis was performed by comparing the percentage of islets with grade 3 and 4 insulitis for each group. Although severity of insulitis in pre-diabetic mice was reduced for untreated controls compared to all other groups, this change was not significant when comparing severe insulitis (combined percent grade 3 and 4) between isotype or αCXCR3-9C5 treated and control groups (figure 5.8a).

**Figure 5.8: αCXCR3-9C5 does not affect NOD pancreatic insulitis**

Insulitis grading of H&E stained pancreata from NOD mice treated from 6 weeks or 8 weeks of age with 1mg/kg αCXCR3-9C5 or isotype every 4 days or untreated as controls. (a) Insulitis at 12 weeks of age or (b) in diabetic mice graded as; grade 0 no infiltrate, grade 1 peri-insulitis, grade 2 <25% infiltrate, grade 3 >25% and grade 4 >75% infiltrate, expressed as percentage of islets scored per grade (>40 islets/group, n=3-7 mice/group). Significance determined for % grade 3+4 insulitis for αCXCR3-9C5 vs. isotype for each age matched group was found to be non-significant (paired t-Test). 6-12 W = 0.5490, 8-12W = 0.0894, 6-diabetic = 0.2823, 8-diabetic = 0.6792.
Further, mice treated with 1mg/kg αCXCR3-9C5 from either 6 or 8 weeks of age until onset of overt diabetes showed no significant difference in insulitis compared to isotype treated or control mice (figure 5.8b).

Regulatory T cells play an important role in restraining progression of effector cell mediated destruction in the NOD pancreas\textsuperscript{281}. Due to the previously described role of CXCR3 in recruitment of Foxp3+ Treg into the pancreas during diabetes in the NOD model\textsuperscript{217} we examined Treg infiltration into the pre-diabetic NOD pancreas.

Foxp3 positively stained cells within the NOD pancreas were enumerated from histological sections of pancreata harvested from mice treated with 1mg/kg αCXCR3-9C5 or isotype antibody from 6 or 8 weeks until 12 weeks of age or untreated controls. Representative histological images of pre-diabetic NOD islets, grade 3, are shown with Foxp3+ stained cells in brown for Isotype and αCXCR3-9C5 treated mice (figure 5.9a).

Few Foxp3+ T cells were found within the exocrine tissue and as such only Foxp3 positively stained cells associated with islet infiltrate were counted. Cells were enumerated for 3 non-contiguous sections of pancreas and the average number of Foxp3+ cells counted per islet for each mouse were expressed as individual data points (figure 5.9b). Infiltration of Foxp3 positively stained cells did not show significant differences when comparing αCXCR3-9C5 and respective isotype antibody treated groups or untreated controls for mice treated either from 6 or 8 weeks of age. The frequency of Foxp3+ staining was also found to generally be higher for islets with more severe insulitis. However, considerable variation in the average number of Foxp3+ cells within mice from each group exists.
αCXCR3-9C5 in the NOD Islet Allograft Model

Due to the well characterised role of CXCR3 in mediating immune cell migration, lymphocyte populations in these NOD mice were also investigated\textsuperscript{3,22,23}. Assessment of the frequency of cells expressing free CXCR3, through binding of CXCR3-173 antibody, and changes in overall cell populations within the spleen, distal lymph node (dLN) and pancreatic LN (pLN) were performed using flow cytometry. Prediabetic NOD mice treated from 6 or 8 weeks of age with 1mg/kg αCXCR3-9C5 or

\textbf{Figure 5.9: αCXCR3-9C5 does not affect NOD pancreatic regulatory T cell Infiltrate}

(a) Representative Immunohistochemistry of Foxp3\(^{+}\) stained sections of Isotype and αCXCR3-9C5 treated pre-diabetic (12 week old) NOD pancreas. Foxp3\(^{+}\) stained cells, shown in brown.

(b) Foxp3\(^{+}\) regulatory T cell quantification as average Foxp3\(^{+}\) cells per islet for 3 non-contiguous sections of pancreas for each NOD mouse, treated from 6 weeks or 8 weeks of age with 1mg/kg αCXCR3-9C5 or isotype every 4 days until 12 weeks of age or untreated as controls. Each data point represents average number of Foxp3\(^{+}\) cells per islet for each mouse. (n=4-5 mice/group)
Isotype every 4 days, until 12 weeks of age or untreated as controls were euthanised and tissues harvested for flow cytometry.

Lymphocytes were analysed for T cell subsets; CD4+, CD4+CD25+Foxp3+, CD4+CD25-Foxp3+ or CD8+ T cells and for NK cells. No difference in the total number of any cell population for mice treated with αCXCR3-9C5 was evident in the spleen, dLN and pLN compared to controls (figure 5.10 & 5.11).

Pre-diabetic mice treated with αCXCR3-9C5 from 6 weeks of age showed no apparent difference in expression of free CXCR3 for CD4+, CD4+CD25+Foxp3+ (Treg) T cells or for NK cells in any of the tissues examined, compared to isotype controls. Differences in free unbound CXCR3 were evident in the spleen and pLN for CD4+CD25-Foxp3+ Treg (figure 5.10b & f) and in the spleen for CD8+ T cells (figure 5.11a) for mice treated from 6 weeks with αCXCR3-9C5 compared to isotype controls although due to low group number these were not significant.
**αCXCR3-9C5 in the NOD Islet Allograft Model**

**Figure 5.10: Pre-diabetic NOD αCXCR3 Treatment – CD4+ T cell & CD4+Foxp3+ T cell analysis**

NOD mice treated from 6 or 8 weeks of age with 1mg/kg Isotype (grey triangles 6weeks or squares 8 weeks) or αCXCR3-9C5 antibody (black triangles 6weeks or squares 8 weeks) every 4 days or untouched as controls (white circles) until 12 weeks of age. CD4 T cells and their respective CXCR3 expression, determined via CXCR3-173 binding, were determined in the (a) spleen, (b) distal LN and (c) pancreatic LN. Treg (Foxp3+CD25+ and Foxp3+CD25-) cells and their respective CXCR3 expression, determined via CXCR3-173 binding, was assessed in the (d) spleen, (e) distal LN and (f) pancreatic LN. Results are expressed as total number of lymphocytes (n=2-4mice/group)
αCXCR3-9C5 in the NOD Islet Allograft Model

Figure 5.11: Pre-diabetic NOD αCXCR3 Treatment – CD8+ T cell & NK cell analysis

NOD mice treated from 6 or 8 weeks of age with 1mg/kg grey triangles 6weeks or squares 8 weeks) or αCXCR3-9C5 antibody (black triangles 6weeks or squares 8 weeks) every 4 days or untouched as controls (white circles) until 12 weeks of age. CD8 T cells and their respective CXCR3 expression, determined via CXCR3-173 binding, were determined in the (a) spleen, (b) distal LN and (c) pancreatic LN. NK cells and their respective CXCR3 expression, determined via CXCR3-173 binding, was determined in the (d) spleen, (e) distal LN and (f) pancreatic LN. Results are expressed as total number of lymphocytes (n=2-4mice/group)
Mice treated from 8 weeks of age with αCXCR3-9C5 showed reduced free CXCR3 on CD4+ and CD4+CD25+Foxp3+ cells in the spleen (figure 5.10a & b) and for CD4+CD25-Foxp3+ cells in the spleen and dLN (figure 5.10b & d), compared to respective isotype controls. In addition, free, unbound CXCR3 was reduced for CD8+ T cells in the spleen (figure 5.11a) and for NK cells in the spleen and dLN (figure 5.11d & e) for mice treated from 8 weeks of age with αCXCR3-9C5 compared to isotype controls. These changes were non-significant due to small group size.

Finally, due to changes in CD44 expression seen in C57BL/6 naïve mice and BALB/c to C57BL/6 islet allograft model, we tested if αCXCR3-9C5 treatment in the NOD model would produce a similar change. Analysis of CD44 expression and expression of free CXCR3 in the spleens of NOD mice treated with 1mg/kg αCXCR3-9C5 (grey shaded, figure 5.12) from 6 weeks of age was compared to splenocytes from both isotype and control (black figure 5.12) treated mice. As shown previously (chapter 4, figure 4.4) a reduction in CXCR3-173 binding was evident for αCXCR3-9C5 treated mice compared to control or isotype treated mice, however their respective CD44 expression was not different. This indicates a differential effect of αCXCR3-9C5 in the C57BL/6 allograft model compared to that seen in the NOD model and may offer an explanation as to the beneficial effects seen in the allograft but not the autoimmune NOD model.

**Figure 5.12: Unaltered activation of NOD splenocytes**
Representative histograms of CD4+ and CD8+ T cell CXCR3 and CD44 expression for pre-diabetic NOD mice treated from 6 weeks of age with 1mg/kg αCXCR3-9C5 i.v. (grey shaded) or isotype controls (black).
Summary & Conclusions

Here we studied the effect of αCXCR3-9C5 in a mouse model of autoimmune diabetes due to the role of CXCR3 in migration of immune cells to sites of inflammation\(^3,22\). CXCR3 and its ligands are also upregulated within the serum of individuals with type 1 diabetes\(^{214,228-231,233,282}\). Finally we were interested in the effect of our anti-CXCR3 antibody in this model due to the variable effects of inhibiting CXCR3 in previous literature\(^{124,217}\).

In NOD mice validation of diabetes incidence rates is important as diabetes incidence is strongly influenced by environment (diet and microbial infections) and thus diabetes incidence in the NOD is known to vary between facilities\(^75\). Diabetes incidence in our NOD colony was consistent with previous literature with 50-60% of our NOD cohort showing overt diabetes by 25 weeks of age. Incidence rates of 60% at 25 weeks have been previously documented by our facility\(^280\).

Here we found that treatment with αCXCR3-9C5 had no ameliorating or detrimental effect on the incidence or onset of diabetes in NOD mice. This is in contrast to previous studies in the NOD mouse showing an accelerated onset of diabetes following genetic deletion of CXCR3.

Within the NOD mouse we have shown that αCXCR3-9C5 does not alter disease outcomes with respect to the ability of cells to migrate into the pancreas, pancreatic LN, distal LN or spleen of treated compared to control mice. This was evident through unaltered insulitis for pre-diabetic and diabetic NOD mice, as well as a lack of changes in T and NK cell populations within primary and secondary lymphoid tissues. In particular no change in the ability of Foxp3+ regulatory T cells to infiltrate the pancreas was evident. This is in stark contrast to results of Yamada et al., which showed the CXCR3/-/- NOD mice had impaired pancreatic infiltration by Foxp3+ regulatory T cells. This difference in Foxp3+ cell accumulation may explain the differential effects seen between our study and results for CXCR3/-/- NOD mice\(^{217}\).

Of note, studies by Yamada et al. showed a very low diabetes incidence of 20-25% at 25 weeks of age indicating a low disease penetrance within these transgenic mice. This may indicate that the breeding of this transgenic line altered the genetics of the NOD mice which predispose to diabetes development and as such may not be a good
model of diabetes. Together our results show treatment with αCXCR3-9C5 is safe for use in the autoimmune NOD mouse model of type 1 diabetes.

Interestingly although basal expression of free CXCR3 in T cell subsets were seen to increase from 6 to 12 weeks of age, expression remained below that seen in C57BL/6 islet allograft recipient mice. These results, as well as the lack of effect of αCXCR3-9C5 administration on immune cell infiltration of the pancreas or CD44 expression suggest that CXCR3 may be dispensable for development of autoimmune diabetes in the NOD mouse model. Additionally these results may indicate involvement of other chemokine signalling pathways in the pathogenesis of diabetes in the NOD model, such as CCR2 and CCR5\cite{283,284}. 
Chapter 6. αCXCR3-9C5 in the NOD Islet Allograft Model
Chapter 6. αCXCR3-9C5 in the NOD Islet Allograft Model

Unlike results found in a previous study showing an acceleration of diabetes in a CXCR3 knockout NOD mouse\textsuperscript{217}, we showed no deleterious or beneficial effect of our αCXCR3-9C5 antibody in the autoimmune NOD mouse model. However we have shown a clear prolongation of MHC mismatched islet allograft survival upon treatment with our αCXCR3-9C5 antibody. For this reason we proceeded to test the efficacy of the antibody in a model of MHC mismatched islet allograft (BALB/c, H\textsuperscript{2}\textsuperscript{d}) into diabetic NOD mice (H\textsuperscript{2}\textsuperscript{gq}, BGL 8-19mmol/L). Islet allograft is used clinically to treat patients with unmanageable autoimmune diabetes, which involves a combined autoimmune and allogeneic immune response, both of which are present in this mouse model.

Figure 6.1: NOD Allogeneic Experimental Model
Overview of experimental model including monitoring of diabetic NOD mice for BGL>7mmol/L followed by receipt of a MHC mismatched BALB/c islet allograft and treatment with isotype or αCXCR3-9C5 mAb +/- rapamycin. Mice were monitored for onset of hyperglycaemia indicating loss of graft function, see graph of typical BGL over the course of rejection.
Mice were treated with one of 5 regimens including; 2mg/kg isotype (2) or αCXCR3-9C5 (3) antibody once per week, sub-therapeutic Rapamycin (0.5mg/kg)(4) once per day for the first 7 days of transplant or a combination of 2mg/kg αCXCR3-9C5 (1/week) with sub-therapeutic Rapamycin (0.5mg/kg, 1/day for 7 days)(5). An untreated control group (1) was also included. Rapamycin was administered i.p. and αCXCR3-9C5 or isotype antibody was administered i.v. (figure 6.1).

**αCXCR3-9C5 Prolongs autoimmune NOD Islet Allograft Survival**

Following transplantation of diabetic NOD mice, untreated control and isotype treated mice showed rapid islet allograft rejection, with a MST of 4 and 5 days respectively. In comparison allograft survival for mice treated with 2mg/kg αCXCR3-9C5 was significantly prolonged with a MST of 8 days and one mouse surviving to day 28 post-transplant (figure 6.2).

Mice treated with rapamycin also showed prolonged allograft survival with a MST of 11 days compared to control mice. However when low dose rapamycin and αCXCR3-9C5 antibody treatments were combined, similarly to that seen for the C57BL/6 allografts (chapter 4, figure 4.15), graft survival was substantially improved. Mice receiving once weekly αCXCR3-9C5 combined with rapamycin once per day for the first week following transplant showed a MST of 35 days with long-term survival in 1 out of 5 mice, surviving to day 90 post-transplant (figure 6.2).

Allograft survival was determined as the number of post-operative days maintaining euglycaemia before onset of hyperglycaemia (BGL>15mmol/L for 2 consecutive days) indicating loss of the islet allograft (figure 6.3).

An additional group of pre-diabetic NOD mice received a variation of the combined αCXCR3-9C5 and rapamycin therapy to determine if this altered regimen could improve graft survival. These mice received a BALB/c islet allograft, as above, and treatment from the day of allograft with 2mg/kg αCXCR3-9C5 1/week and 0.5mg/kg rapamycin, 1 per day for 1 week followed by treatment every 2nd day for 1 week (2 weeks total).
Islet allografts were lost, as determined by onset of hyperglycaemia, at day 6, 7 and 17 for these mice (figure 6.4, black diamonds). However two mice treated with this regimen survived >75 days, but upon nephrectomy failed to become hyperglycaemic (figure 6.4, white diamonds). This indicates that the graft was not the source of insulin in these mice suggesting maintenance of pancreatic beta cell function. Mice were followed for 10 days post nephrectomy to confirm this result followed by euthanasia and collection of tissues for histology and flow cytometry to discern the mechanism behind this allograft independent long-term survival. Lack of residual islet mass in the kidney was confirmed by H&E staining with only mild infiltrate and no islet structures remaining (figure 6.5).
Figure 6.3: αCXCR3-9C5 NOD Islet Allograft
Blood glucose levels of NOD recipients of BALB/c (H2d) islet allograft from the day of transplant until loss of allograft (BGL > 15mmol/L) for mice treated with (a) untreated control, (b) Isotype (2mg/kg, 1/week, i.v.), (c) αCXCR3-9C5 antibody (2mg/kg, 1/week, i.v.), (d) rapamycin (0.5mg/kg for 7 days i.p.) (e) αCXCR3-9C5 (2mg/kg, 1/week) + rapamycin (0.5mg/kg, 1/day, 7 days) (n=3-5 mice/group)
Figure 6.4: αCXCR3-9C5 NOD Islet Allograft Long-term survival
Blood glucose levels of NOD recipients of BALB/c (H2d) islet allograft from the day of transplant for mice treated with αCXCR3-9C5 (2mg/kg, 1/week) + rapamycin (0.5mg/kg, 1/day, 7days + 1 every 2 days for 7days). Islet allograft recipients monitored until loss of allograft (BGL > 15mmol/L)(black) or maintained euglycaemia and received nephrectomy at day >75 (white) (n=5mice)

Figure 6.5. αCXCR3-9C5 NOD Islet Allograft Long-term survival shows minimal islet insulin staining
Kidney and pancreas histology from 2 NOD recipients of BALB/c (H2d) islet allograft, treated with αCXCR3-9C5 (2mg/kg, 1/week) + rapamycin (0.5mg/kg, 1/day, 7days + 1 every 2 days for 7days) which maintained euglycaemia following nephrectomy. H&E stained sections of kidney, H&E and Insulin stained sections of pancreatic islets and insulin staining of acinar tissue containing insulin positively stained cells.
αCXCR3-9C5 in the NOD Islet Allograft Model

Curiously no significant change in the proportions of T cell or NK cell subsets were detected through flow cytometry in the spleen, dLN or pLN (data not shown). In addition, although islets were found within the pancreas of these mice showing minor if any infiltrate, islets showed very little insulin staining (figure 6.5). However we did identify regions of acinar tissue, which stained positive for insulin and appeared to have residual beta cells post islet destruction. This pattern of recovery of euglycaemia in NOD mice post combined immunomodulation and islet allograft has been shown previously and may offer an explanation as to these results, see summary & conclusions²⁸⁵-²⁸⁸.
Summary & Conclusions

Previous literature looking at the role of CXCR3 in models of islet allograft rejection have yet to examine the effects of deletion or antibody blockade in a combined model of islet allograft transplant into diabetic NOD mice. As a result of the efficacy and safety shown for our αCXCR3-9C5 mAb in islet allograft (chapter 4) and diabetes models (chapter 5), respectively, we examined the effect of our antibody in a model of islet allograft into diabetic NOD mice.

Here we show for the first time efficacy of an anti-CXCR3 antibody therapy in a mouse model of diabetic islet allograft. Similarly to that seen in the C57BL/6 islet allograft recipients (chapter 4, figure 4.15), NOD mice showed prolonged graft survival upon treatment with αCXCR3-9C5 with a synergistic effect observed when treated in combination with low-dose rapamycin. Further both αCXCR3-9C5 treatments, alone or in combination with low-dose rapamycin, lead to a small proportion of islet allograft recipients exhibiting significantly longer survival. Together we show the potential of αCXCR3-9C5 treatment for use as part of a combination immunosuppressive therapy for use in islet allograft recipients with type 1 diabetes.

Of note we have also shown recovery of euglycaemia for two NOD islet allograft recipients, which received a combination of αCXCR3-9C5 and rapamycin. Although BGLs of these mice were below 15mmol/L at transplant, NOD mice would be expected to proceed to onset of overt diabetes due to autoimmune destruction of residual pancreatic beta cell mass. However here we show that these mice, upon receipt of an islet allograft, maintained euglycaemia for over 75 days but did not become hyperglycaemic upon islet graft removal via nephrectomy. Previous papers showing immunomodulation with complete Freund’s adjuvant (CFA) and islet allograft transplantation of diabetic NOD recipients have reported a similar phenomenon. These papers attributed euglycaemia post nephrectomy to regeneration or maintenance of residual beta cell mass with one paper suggesting CFA and islet allograft transplant skewed the balance of beta cell autoimmune destruction and beta cell replication toward replication resulting in recovery of euglycaemia. Further as with results found in our mice Suri et al. indicated that although mice maintained
euglycaemia, few islets were present (6% or less) and only some were insulin positive\textsuperscript{286}.

This phenomenon has also been found to occur in humans. In a single case study of an individual with type 1 diabetes who had received a whole pancreas transplant, islets were found to be insulin negative, however single insulin positive cells were found within the native pancreas suggestive of beta cell regeneration\textsuperscript{289}. Further a previous study by Liu \textit{et al.} investigated the role of immunosuppression combined with islet or whole pancreas transplant in patients with type 1 diabetes, showing a small but detectable amount of C-peptide was produced by the native pancreas a number of years after transplant\textsuperscript{290}.

Although this occurred in only a small subset of mice in our study it suggests that in addition to prolongation of survival of islet allografts, combined immunomodulation with low dose rapamycin and $\alpha$CXCR3-9C5 antibody may promote survival and replication of residual beta cell mass allowing maintenance of euglycaemia and this may also be applicable in humans although further investigation of this result is required\textsuperscript{285-288}.
Chapter 7. Clarifying the Role of αCXCR3-9C5
Finally of interest to us was the mechanism behind the effects of αCXCR3-9C5 in models of islet transplant. Previous studies on CXCR3 mAbs have focused primarily on disease outcomes with little examination of molecular mechanism. In vivo mixed lymphocyte reactions (MLR) were performed using transfer of CFSE labelled whole splenocytes from naïve C57BL/6 mice into sub-lethally irradiated BALB/c mice. Mice were also administered 2mg/kg i.v. αCXCR3-9C5 or untreated as controls. The spleens of these mice were then harvested 72 hours later and analysed by flow cytometry for changes in proliferation and activation markers, particularly for CD44 expression. CD44 is a marker of T cell development and activation with a high expression on effector and memory T cell subsets. CD44 is also involved in cell-cell interactions, adhesion and migration of activated cells.

Firstly proliferation of both CD4 and CD8 T cells was evident from CFSE dilution with the right-most peak showing un-proliferated cells and successive peaks showing generation of proliferated cells (figure 7.1a & 7.2a). Activation of proliferated cells, indicated by up-regulation of CD44 expression and downregulation of CD62L expression in proliferated compared to un-proliferated cells, was also evident. No difference was evident in CD4+ or CD8+ T cell proliferation or expression of CXCR3 or CD62L for both proliferated and non-proliferated cells for αCXCR3-9C5 treated (grey shaded histogram) compared to cells from control mice (black unfilled histogram) (figure 7.1a & 7.2a). Expression of CD4+CD44, CD44 MFI and CD4+ T cell subsets (CD44-CD62L+, CD44+CD62L+, CD44+CD62L-) was also not different for αCXCR3-9C5 treated cells compared to controls (black unfilled histogram), for proliferated and non-proliferated T cells (figure 7.1a, b, c).

In contrast CD8+ T cells treated with αCXCR3-9C5 (grey shaded histogram, figure 7.2a), showed a significantly reduced expression of CD44, similar to that seen in the C57BL/6 allograft model (chapter 4, figure 4.13), compared to untreated controls (black unfilled histogram, figure 7.2a). This was particularly evident in non-proliferated cells with reduced CD44 MFI compared to controls (figure 7.2b). Further when the frequency of CD8+ memory (CD44+CD62L+) T cells was examined following αCXCR3-9C5 treatment, a reduced frequency of non-
proliferated memory CD8+ T cells was evident compared to untreated control cells (figure 7.2d).

Figure 7.1: αCXCR3-9C5 treatment does not alter CD4+ T cell proliferation or activation
Figure 7.1: αCXCR3-9C5 treatment does not alter CD4+ T cell proliferation or activation
Analysis of CD4+ T cell proliferation and activation from sublethally irradiated BALB/c recipients of C57BL/6 CFSE labelled splenocytes, treated with 2mg/kg αCXCR3-9C5 or controls at 72hours post cell transfer. a) Representative histograms of CFSE proliferation and activation markers (CXCR3, CD62L and CD44) and b) Mean fluorescence intensity (MFI) for CD44 expression for proliferated and non-proliferated cells: αCXCR3-9C5 treated (grey shaded histogram) or control mice (black, unfilled histogram). c) Representative FACS plots of mean frequencies for CD4+ T cell subsets (CD44-CD62L+, CD44+CD62L+, CD44+CD62L-) for non-proliferated and proliferated αCXCR3-9C5 treated and control T cells and d) frequencies of CD4+ T cell subsets from c) (n=4/group for 2 repeat experiments).

Figure 7.2: αCXCR3-9C5 treatment alters expression of CD8+ CD44^{high} T cells
Analysis of CD8+ T cell proliferation and activation from sublethally irradiated BALB/c recipients of C57BL/6 CFSE labelled splenocytes, treated with 2mg/kg αCXCR3-9C5 or controls at 72hours post cell transfer. a) Representative histograms of CFSE proliferation and activation markers (CXCR3, CD62L and CD44) and b) Mean fluorescence intensity (MFI) for CD44 expression for proliferated and non-proliferated cells from αCXCR3-9C5 treated (grey shaded) or control mice (black, unfilled). c) Representative FACS plots displaying mean frequencies for CD8+ T cell subsets (CD44-CD62L+, CD44+CD62L+, CD44+CD62L-) for non-proliferated and proliferated αCXCR3-9C5 treated and control T cells and d) frequencies of CD8+ T cell subsets from c) (n=4/group for 2 repeat experiments).
Figure 7.2: αCXCR3-9C5 treatment alters expression of CD8+ CD44high T cells
Summary & Conclusions

Here we show a role of CXCR3 in regulating expression of CD44 on CD8+ but not CD4+ T cells. Use of in vivo MLR revealed reduced CD44 expression without affecting CD62L expression or the ability of these cells to proliferate following αCXCR3-9C5 treatment. Interestingly mice administered with αCXCR3-9C5 antibody showed no difference in CXCR3 expression at 72 hours post i.v. compared to untreated control expression. This may result from a high turnover of the CXCR3 receptor under conditions of alloantigen stimulation, which involves receptor degradation and de novo synthesis of new receptor for surface expression292.

While a greater proportion of cells which have proliferated express CD44, compared to those cells which are undivided, the effect of blockade of CXCR3 via αCXCR3-9C5 treatment appears to preferentially affect CD44 expression on non-proliferated CD8+ T cells (figure 7.2a). These untreated, undivided CD44 high CD8+ T cells may represent a subset of undivided, naive cells, which are becoming activated and will go on to proliferate and differentiate into memory and effector T cell subsets.

The effect on CD8+ T cell CD44 expression appears to particularly affect non-proliferated CD8+CD44+CD62L+ memory T cells. The reduction in non-proliferated CD44 high CD8+ T cells also appears to cause a non-significant decrease in the frequency of CD8+ memory (CD44+CD62L+) cells. This may result through a reduced frequency of activated parent cells, which subsequently divide into this memory subset. In the context of allograft immune response this reduction in CD44 expression resulted in decreased frequencies of effector and memory CD8+ T cells. CD44 expression also plays an important role in T cell migration to sites of infection or injury and reduced expression may impede migration and activation of these cells. Together this reduction in CD8+CD44+ T cells, presumably memory or effector CD8 T cells, likely contributed to the prolonged islet allograft survival seen upon treatment with αCXCR3-9C5 (figure 4.16).

This may highlight an important clinical application for αCXCR3-9C5 antibody targeting CD8+ memory T cells, which are poorly targeted by current immunosuppressive therapies244,293-296. These cells are instrumental in the allograft rejection response with CD8+ memory T cells found to infiltrate and produce IFN-γ
within the first 24 hours following transplant. IFN-γ then stimulates donor chemokine release, CXCL9 and CXCL10, recruiting effector T cells to the allograft and promoting rejection\textsuperscript{213,244,272}. Although this memory subset plays a critical role in allograft rejection, these cells display unique cell surface markers and costimulatory requirements, as well as distinct trafficking and activation profiles\textsuperscript{297}. As such naïve T cells are effectively depleted using ATG or aletuzumab and their activation and proliferation blocked by co-stimulation blockade therapies, such as anti-CTLA4, anti-CD40L and anti-CD154 antibodies, while memory T cells are resistant to these immunosuppressive therapies\textsuperscript{244,293-296}. Additionally, non-allograft specific, viral memory responses have been found to impede allograft tolerance induction, which were also co-stimulation blockade resistant\textsuperscript{298}.

Interestingly a marker common to CD8+ effector, central memory and effector memory T cells is the up-regulated expression of CXCR3, which presents an appealing therapeutic target for each of these cell types involved in mediating the allograft rejection response\textsuperscript{299-301}. 
Chapter 8. Discussion
Chapter 8. Discussion

Overview

The major aim of our studies was to delineate the function of a novel anti-CXCR3 monoclonal antibody, which targets a conserved epitope of CXCR3 found in a number of species including mice and humans. Due to prior studies showing efficacy of CXCR3 deletion and blockade in models of allograft transplantation\cite{200,216}, we investigated the role of our $\alpha$CXCR3-9C5 mAb in a MHC mismatched islet transplant. In addition islet allograft transplantation is used clinically in individuals with type 1 diabetes. This application warranted further investigation into the efficacy of $\alpha$CXCR3-9C5 as a rejection prophylaxis in mouse models of islet allograft transplantation into diabetic autoimmune NOD recipients, as well as in a model of spontaneous autoimmune diabetes alone.

This thesis explores the efficacy of $\alpha$CXCR3-9C5 mediated blockade of CXCR3 signalling for the prolongation of allograft survival in both autoimmune and non-autoimmune mouse models. However within the NOD model of autoimmune type 1 diabetes, we found no effect of antagonism of CXCR3 using our $\alpha$CXCR3-9C5 mAb suggesting the effect is specific to the allogeneic, but not the autoimmune response.

Allograft Transplantation

Several studies have highlighted a strong correlation between increased expression of CXCR3 and its chemokine ligands with processes of human allograft rejection, as well as T1D\cite{178-180,182,214,228,229,302}. In support of those findings our data also highlight an important role for CXCR3 mediated signalling in the allograft rejection response. This has been show previously in heart, lung, skin and islet allograft models. In all those studies, including ours, blockade of CXCR3 reduced the recruitment of immune cells to the graft site, resulting in a significant delay in allograft loss (figure 4.16, 4.17)\cite{200,213,216,245-247,252}.

Here we show that similarly to other islet allograft studies antagonism of CXCR3 signalling resulted in a prolonged islet allograft survival\cite{216,252}. We also found that within long-term surviving islet allografts, which showed preserved islet architecture and strong insulin staining, only minimal immune infiltrate was present. This
infiltrate was restricted to the graft periphery, suggesting impaired immune cell infiltration. Previous literature has highlighted the importance of CXCR3 signalling in immune cell migration, with inhibition of this signalling pathway by antibody blockade or gene silencing, decreasing infiltration and inflammation within the graft and resulting in enhanced allograft survival\textsuperscript{200,213,216}. Further investigation of the ability of immune cells to infiltrate the graft in the acute response following treatment with αCXCR3-9C5 will provide further evidence to confirm a role for αCXCR3-9C5 in impaired immune cell recruitment to the graft.

**T cell Activation & Regulatory T Cells**

While the majority of previous studies exclusively investigated the impact of CXCR3 blockade on chemotaxis\textsuperscript{200,216,252}, we have also examined a possible role for altered T cell activation or regulatory T cell accumulation in the enhanced survival of islet allografts. Interestingly, although a role for CXCR3 mediated T cell activation has been described previously it has yet to be investigated within models of allograft rejection\textsuperscript{192,219,299}. CXCR3 was previously shown to be involved in immune cell activation and proliferation in a model of type 1 IFN induced T cell stimulation. CXCR3 mediated signals induced the up-regulation of activation markers CD25 and CD69 on CD8 T cells, as well as promoting CD8 T cell proliferation, particularly for CD44\textsuperscript{low} CD8+ T cells\textsuperscript{192}.

We show here that αCXCR3-9C5 mediated antagonism of CXCR3 signalling in un-transplanted and islet allograft recipients as well as under conditions of in vivo MLR, resulted in a decreased proportion of CD8+ T cells with high CD44 expression. This resulted in a significantly reduced number of CD8 effector and memory T cells, both important contributors to the allograft rejection response\textsuperscript{244,270-272}. Although a role for CXCR3 in CD44 expression has not yet been shown it is possible that CXCR3 signalling may directly or indirectly affect T cell activation and CD44 expression. Moreover other studies have shown a critical role for CXCR3 in the co-localisation of T cells and DC within the splenic marginal zone, as well as the ability to form long-lasting cell-cell interactions with virally infected cells\textsuperscript{220,303}. Antagonism of CXCR3 may therefore result in altered T cell activation that could account for the decreased CD44 expression seen. This co-localisation and activation has been shown to favour CD8+ T cell expression of cytotoxic markers, perforin and granzyme
B\textsuperscript{219,221}. Although we have yet to investigate the phenotype or function of our αCXCR3-9C5 treated CD8+ T cells it is possible that they may also show reduced cytotoxicity, contributing to graft survival.

In addition to a role in T cell activation our results suggest that the improved survival of αCXCR3-9C5 treated islet allograft recipient mice is also dependent on CD4+CD25+Foxp3+ regulatory T cells. These regulatory T cells have been well characterised within the allograft immune responses to confer protection and limit graft destruction\textsuperscript{144,273,275}, and as such altered function of these cells was of interest. We found high numbers of Foxp3+ cells within the spleen and graft site of long-term surviving allograft recipients treated with αCXCR3-9C5 alone or in combination with low dose rapamycin. The involvement of CD25+Foxp3+ T cells is further supported by the loss of prolonged islet allograft survival in αCXCR3-9C5 treated mice upon co-treatment with a CD25 depleting antibody (PC61). Finally, cell transfer experiments from long-term surviving mice show improved islet allograft survival in the presence of CD25+ T cells compared to those mice transferred CD25 depleted T cells.

Use of the CD25 depleting antibody (PC61) has been previously shown to effectively deplete the CD4+CD25+Foxp3+ population of regulatory T cells, with up to 90% depletion for 2-3 weeks post-injection\textsuperscript{261}. This allows the protective role of Treg in the allograft immune response to be determined. A limitation to this method however, is the elimination of CD25+ cells, which are not Tregs and likely to play roles within the allograft immune response. This is particularly true for recently activated T cells expressing CD25\textsuperscript{304}. An alternative method of eliminating Treg cells uses the transgenic DEREG mouse (depletion of regulatory T cells), expressing the diphtheria toxin (DT) receptor under the control of the Foxp3 locus, and providing selective depletion of Foxp3+ Treg\textsuperscript{305,306}. Future studies may further clarify the roles of Tregs in this model using a more specific approach, such as the DREG mouse.

Of note the clinically used immunosuppressive agent used in these studies, rapamycin, has been shown to differentially affect effector and regulatory T cells. Rapamycin has been shown to promote the induction of regulatory T cells while
blocking activation and proliferation of effector T cells\textsuperscript{279,307}. As a result, we propose that the effects of rapamycin synergise with the impaired chemotaxis and activation of T cells driven by $\alpha$CXCR3-9C5, leading to enhanced regulatory and impaired effector and memory T cell responses; resulting in enhanced islet allograft survival. We also show that treatment of mice with $\alpha$CXCR3-9C5 alone is able to significantly prolong islet allograft survival and cause long-term survival in a small proportion of these mice, with a notable increase in regulatory T cells. This represents a novel mechanism by which CXCR3 may affect activation of the allograft immune response and will be investigated further in future experiments.

**Allograft transplantation in the NOD mouse**

The role of CXCR3 blockade in the clinically applicable model of islet allograft transplantation into a recipient with pre-established autoimmune T1D has not yet be characterised in the literature. To do this we employed islet allograft into the NOD mouse model of type 1 diabetes. Due to the involvement of both autoimmune and alloimmune response within this model the kinetic of rejection in isotype or control treated mice was significantly faster than that of the non-autoimmune allograft model. Upon investigation of the efficacy of our novel $\alpha$CXCR3-9C5 antibody within this model, a single antibody administration was found to significantly prolong survival of the allograft. Additionally, as seen in the non-autoimmune allograft model, a synergistic effect was seen when $\alpha$CXCR3-9C5 was administered in combination with low dose rapamycin, further enhancing islet allograft survival. Together this shows promise for clinical application of $\alpha$CXCR3-9C5 within the context of islet allograft transplantation into autoimmune recipients with T1D.

**The NOD Mouse**

Given the combined autoimmune and alloimmune nature of this NOD islet allograft transplant model and previous literature on accelerated disease in CXCR3-/- NOD mice, the effect of $\alpha$CXCR3-9C5 within an autoimmune diabetes model alone was of interest. Within autoimmune models of type 1 diabetes the role of CXCR3 is less well characterised. Virally induced models using the RIP-LCMV system (Chapter 1, animal models) initially showed a substantial role of CXCR3 for the recruitment of
immune cells into the pancreas. However, further characterisation of this model using both deletion and antibody blockade of CXCR3 and its chemokine CXCL10 though suggest that the CXCR3 signalling axis contributes only minimally to viral induced diabetes and suggests redundancy within the chemokine system may account for this difference. The spontaneous onset model of diabetes using the NOD mouse has also shown differential effects when targeting the CXCR3 signalling pathway. Firstly CXCL10, which is produced by the islets themselves, has been shown to inhibit beta cell proliferation through CXCR3 independent auto/paracrine signalling. As such, inhibition of CXCL10 was found to reduce diabetes incidence. However, upon CXCR3 deletion in a NOD mouse model, diabetes onset was found to be accelerated. This was attributed to an inability of CD4+Foxp3+ regulatory T cells to traffic from the pancreatic lymph node into the pancreas.

Investigation using antibody blockade rather than complete deletion of the CXCR3 receptor has not previously been examined. We show here for the first time that use of antibody mediated blockade of CXCR3 signalling within the NOD mouse model has no effect on the onset or incidence of diabetes in our model. Furthermore no change in the frequency of immune cells, including Foxp3+ Treg cells were seen in the spleen, distal or pancreatic lymph nodes; in αCXCR3-9C5 treated mice compared to controls. Nor was the ability of immune cells or Foxp3+ T cells to infiltrate the pancreas different.

Results seen for Yamada et al. using CXCR3 deficient NOD mice differ significantly from those found using our αCXCR3-9C5 antibody. This differential effect of transient antibody blockade compared to complete deletion is not unique, as seen in studies on CTLA-4, a critical negative regulator of T cell activation. Mouse studies using genetic deletion of the CTLA-4 gene showed high levels of lymphocyte accumulation within lymphoid and non-lymphoid tissues including the heart, which showed myocardial lesions. These mice died at 3-4weeks of age, likely due to this myocarditis and uncontrolled T cell activation. In contrast use of CTLA-4-Ig antibody blockade has shown considerable benefit for the treatment of tumours and is now used in human trials. CTLA-4 antibodies (e.g. ipilimumab) used in cancer, function through loss of the CTLA-4 co-inhibitory signal, enhancing co-stimulatory signalling of T cells and the resulting anti-tumour response. This antibody mediated
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blockade has relatively minor symptoms compared to those seen for CTLA-4 knockout mice. From this it is evident that although previous literature shows a deleterious role for CXCR3 deletion in the NOD mouse this does not preclude the therapeutic potential of an anti-CXCR3 antibody. In this case we show safety of αCXCR3-9C5 in a mouse model of diabetes with potential for use in an islet transplant context.

Conclusion

In conclusion we have characterised a novel αCXCR3-9C5 monoclonal antibody within models of islet allograft and autoimmune type 1 diabetes. Our findings support a role for CXCR3 signalling within the allograft immune response as previously described. We also establish that CXCR3 signalling in this model participates in additional, previously uncharacterised, functions involved in T cell activation and regulatory T cell activity. We have also shown safety and efficacy for use of αCXCR3-9C5 in models of autoimmune diabetes and islet allograft, respectively, in the NOD mouse model. This is important due to inconsistency and potentially detrimental effects seen within previous literature as to the effects of CXCR3 inhibition within this model of T1D. Further, prior to this study investigation of the role of CXCR3 blockade in a combined autoimmune islet allograft model had not been performed.

We propose that αCXCR3-9C5 may be a viable option for use clinically in islet transplantation into individuals with type 1 diabetes. Following islet allograft, in both the non-autoimmune C57BL/6 and in the autoimmune NOD mouse, αCXCR3-9C5 treatment shows a considerable benefit in prolonging allograft survival. Further, in contrast to previous studies, blockade of CXCR3 signalling using this antibody does not aggravate the autoimmune pathophysiology of the NOD mouse. Additionally due to the conserved nature of the epitope of CXCR3 which αCXCR3-9C5 targets, there is potential for efficient translation of the antibody into the clinic, by humanising the antibody. This study supports further investigation into the use of αCXCR3-9C5 as a more targeted therapeutic strategy, with potentially reduced toxicity or side effects than conventional immunosuppression, for use as part of a combination therapy in clinical islet allograft.
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Chapter 9. References


References


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