CHEMOKINE-MEDIATED MODULATION OF AUTOIMMUNITY IN TYPE 1 DIABETES

by

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ABSTRACT

Type 1 diabetes is an autoimmune disorder characterized by destruction of insulin-producing beta cells by autoreactive T cells. Despite management of type 1 diabetes with insulin therapy, affected individuals face devastating complications, such as blindness and kidney failure. Islet transplantation has emerged as a promising therapy to replace beta cells; unfortunately, it is still challenged by a high rate of graft failure due to allo- and recurrent auto-immune responses. The pathogenesis of type 1 diabetes results from a combination of genetic and environmental factors that cause an imbalance in regulatory mechanisms, including defects in T regulatory (Treg) and natural killer T (NKT) cells. We believe that enhancing the activity of these cells locally may have the desirable effect of modulating the immune response against beta cells. Interestingly, the chemokines CCL22 and CCL17 preferentially recruit Tregs. We hypothesized that expression of these chemokines in islets would prevent beta cell destruction by enhancing influx of immunoregulatory cells. We used viral vectors to induce expression of CCL22 or CCL17 in islets and tested this hypothesis in models of spontaneous and recurrent autoimmune diabetes in the non-obese diabetic mouse. We found that CCL22 expression in pancreatic islets prevented diabetes development and protected islet transplants from recurrent autoimmunity, although not indefinitely. CCL22-expressing islets recruited Tregs, invariant NKT and plasmacytoid dendritic cells, resulting in a tolerogenic milieu characterized by lower IFNγ levels and increased expression of indoleamine 2,3-dioxygenase. CCL22 induced expression of CTLA-4, ICOS and CD62L on Tregs and enhanced their ability to modulate dendritic cell function, indicatory of a superior suppressive function. Notably, depletion of Tregs abrogated CCL22's protective effect, suggesting that CCL22 modulatory properties are dependent on Tregs. When comparing CCL22 and CCL17, we found similar recruitment of Tregs and invariant NKT cells, but unlike CCL22, CCL17 did not impact Treg function. Nevertheless, CCL17 expression in islet grafts was equally protective from recurrent autoimmunity. Our findings suggest a novel strategy for protecting beta cells from immune attack, by using chemokines CCL22 or CCL17 to harness the natural regulatory properties of immune cells such as Tregs and invariant NKT cells.

PREFACE

Animal studies were reviewed and approved by the University of British Columbia Committee on Animal Care (protocols # A10-0371 and A11-0202). L. Bischoff was trained for animal ethics (#2565-07) and practical animal care (#RBH-273-07, RA-107-07, RSHX-78-07).

Dr P. Robbins (University of Pittsburgh, Pittsburgh, Pennsylvania, USA) provided the AAV8-RIP/GFP plasmid used for the AAV8-RIP/CCL22 construct. dsAAV8 vectors were generated at the Children's Hospital of Philadelphia. Ad-CCL22 vector was generated by J. Montane.

The overall hypothesis in this study was generated by B. Verchere, T. Kieffer, R. Tan and J. Dutz. In Chapter 3, L. Bischoff performed all studies in the islet transplant model of recurrent autoimmune diabetes, from experimental design to data acquisition and analysis. J. Montane conducted experiments in the model of spontaneous diabetes development and L. Bischoff contributed to characterization of immune infiltrate in the pancreas and pancreatic lymph nodes. A version of this chapter was published in Montane & Bischoff et al. J Clin Invest Aug;121(8):3024-8, where L. Bischoff is co-first author.

In Chapter 4, L. Bischoff generated the hypothesis, designed experiments, acquired and analyzed data. The novel findings presented in this chapter improve our knowledge about the impact of the chemokine CCL22 on T regulatory cell activation and have implications for targeting CCL22-producing tumors.

In Chapter 5, P. Orban and J. Courtade designed, produced and purified the Ad-CCL17 vector. Hypothesis and experimental design were generated by L. Bischoff. All studies except data presented in Figure 23 were performed by L. Bischoff. In vitro cell culture with CCL17 in Figure 23 was performed by S. Alvarez, who also contributed to mouse monitoring and CCL17 ELISA. Data from Chapters 4 and 5 are part of a manuscript in preparation (Bischoff et al.).

In all these studies, technical assistance with islet isolation, islet transplantation and injection in the pancreatic duct was provided by G. Soukhatcheva, D. Dai and M. Komba.

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LIST OF ABBREVIATIONS

αGalCer	α galactosylceramide
AAV	adeno-associated virus
Ad	adenovirus
BB rat	biobreeding rat
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CMV	cytomegalovirus
ConA	concanavalin A
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FoxP3	forkhead box P3
GAD	glutamic acid decarboxylase
HLA	human leucocyte antigen
ICOS	inducible T cell co-stimulatory
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IGRP	islet-specific glucose-6-phosphatase catalytic subunit related protein
IL	interleukin
INS	insulin
iTreg	induced T regulatory cells
MAPK	mitogen-activated protein
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
MOI	multiplicity of infection
MZ	marginal zone
LacZ	β galactosidase
lyDC	lymphoid dendritic cell
NK	natural killer cell
NKT	natural killer T cell
iNKT	invariant natural killer T cell

NOD	non-obese diabetic
PAMP	pathogen-associated molecular patterns
pDC	plasmacytoid dendritic cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
RIP	rat insulin promoter
RPMI	Roswell Park Memorial Institute media
Scid	severe combined immune deficiency
STZ	streptozotocin
TCR	T cell receptor
TLR	Toll-like receptor
TGF	transforming growth factor β
Th	$CD4^+$ T helper cell
TNFα	tumor necrosis factor α
Treg	T regulatory cell
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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DEDICATION

To my love Flo and my family

CHAPTER 1:

INTRODUCTION

1.1. TYPE 1 DIABETES

1.1.1. DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels, or hyperglycemia. The underlying cause of hyperglycemia is a defective production or response to insulin, a hormone produced by beta cells in the pancreatic islets of Langerhans that triggers cells throughout the body to uptake and use blood glucose as a source of energy. Diabetes is classified in 2 main categories: type 1 and type 2. Type 1 diabetes , also known as insulin-dependent or juvenile diabetes, is an autoimmune disorder that causes destruction of insulin-producing beta cells leading to insulin deficiency. Type 1 diabetes has an early onset in childhood, typically before the age of 20, and accounts for 10% of all cases of diabetes. Type 2 diabetes, formerly referred to as non-insulin dependent diabetes, is the most common type of diabetes and results from a combination of beta cell dysfunction and resistance to insulin action. Type 2 diabetes is closely linked to obesity and sedentary lifestyle and develops in adults although it is now also occurring at younger ages.

Diagnosis of diabetes is based on the following criteria: fasting blood glucose levels above 7 mM, or blood glucose levels higher than 11 mM two hours after a glucose challenge, as well as a glycosylated hemoglobin value higher than 6.5%. The classical symptoms of diabetes include excessive thirst, frequent urination and fatigue although these symptoms may be more subtle in type 2 diabetes as they appear more gradually. There is no cure for diabetes so far. However, since Drs. Frederick Banting and Charles Best successfully isolated insulin in 1922, patients with type 1 diabetes can be treated with insulin therapy. More recently, replacement of beta cells by islet transplantation has emerged as a promising therapy for type 1 diabetes. Management of type 2 diabetes initially focuses on the close monitoring of blood glucose levels, healthy diet and exercise, but patients may also require oral anti-diabetic medications and eventually insulin. Despite optimal management, chronic hyperglycemia damages the blood vessels and causes devastating long-term complications in both type 1 and type 2 diabetes, such as retinopathy, nephropathy, neuropathy and cardiovascular diseases.

The World Health Organization currently estimates that 346 million people are afflicted with this disease worldwide. Moreover, diabetes prevalence is rapidly increasing due to a number of

factors such as population growth and ageing, as well as a global shift towards sedentary lifestyle and unhealthy diet.

The morbidity and mortality associated to diabetes and its complications represent a major socio-economic burden. With more than 2 million diabetic patients in Canada, diabetes is predicted to cost the Canadian healthcare system an average of $16.9 billion per year by 2020.

1.1.2. ISLET TRANSPLANTATION

Replacement of beta cells by islet transplantation has emerged in recent years as a highly promising therapy of type 1 diabetes, enabling physiologic control of blood glucose. Islet transplantation consists of the isolation of islets from a donor's pancreas and then infusion into the portal vein of a diabetic recipient. Islets disperse within the liver and release insulin in response to glucose. It is a minimally invasive procedure compared to whole pancreas transplantation for which the major concerns are the surgical complications.

Islet transplantation has gained considerable attention since the publication in 2000 of the Edmonton protocol, which reported the successful restoration of insulin independence in recipients of islet grafts. This major breakthrough was based on an improved technique for islet isolation combined with an optimized immunosuppressive regimen [1] and was adopted by multiple centers for an international clinical trial. The main indications for islet transplantation are failure to control diabetes by insulin therapy and frequent hypoglycemic episodes [2]. Over 400 individuals with type 1 diabetes have received islet transplants since 1999 [3], generally in two separate islet infusions to obtain an adequate beta cell mass. The rate of success is 65% one year after transplantation in terms of insulin independence; however, islet graft function is gradually lost such that less than 20% of recipients remain insulin independent 5 years post-transplantation.

Several factors influence islet graft survival, including the quality of the islet preparation, engraftment and revascularization, immunosuppressive drugs and both the inflammatory and immune responses [3]. In order to improve the long-term clinical outcome of islet transplantation, effective strategies are required to counteract each of these factors. For example, further refinement of islet isolation techniques will help reduce the cellular stress induced by ischemic and mechanical damage. Unlike other solid organ transplants, the immune

destruction of islet grafts in type 1 diabetic recipients involves not only an alloimmune reaction directed against the foreign tissue, but also recurrence of the autoimmune response that initially destroyed the patient's beta cells [4-6]. Immunosuppressive drugs used to control the immune-mediated graft rejection adversely affect glycemic control, beta cell function and replication [7-9]. Therefore, alternative immunotherapies targeting both allorejection and recurrent autoimmunity need to be developed to improve beta cell survival.

1.1.3. PATHOGENESIS OF TYPE 1 DIABETES

Study of type 1 diabetes has been difficult due to limited availability of pancreas from diabetic patients; nonetheless, 150 specimens have been compiled over a century for histopathological analysis [10]. These studies reveal that infiltration of pancreatic islets by immune cells, so called insulitis, is present in 78% of patients with recent type 1 diabetes onset and tends to disappear with disease progression. Importantly, diabetic patients present with a 70-90% loss of their beta cell mass, while the remaining islets appear atrophic and consist mainly of alpha cells. The observation that only insulin-positive islets are infiltrated indicates that the immune reaction is specifically directed against beta cells. Destruction of insulin-producing cells may occur over a period of several years before the threshold of beta cell loss is reached and the first symptoms of diabetes appear.

The various levels of inflammation observed in the pancreas of diabetic patients indicate progressive stages of beta cell destruction. In the early phase, immune cells are visible in the islet periphery but the beta cell mass is relatively normal. At later stages, there is a more diffuse infiltrate in and around islets associated with pronounced beta cell loss. Eventually, insulin-producing cells are completely destroyed and the inflammatory process subsides. The major component of the immune infiltrate throughout insulitis progression is the $CD8^+$ T population while $CD4^+$ lymphocytes are the least abundant cell subset (Figure 1) [11, 12]. Significant numbers of macrophages and dendritic cells are seen at early stages of insulitis and their levels remain fairly constant. B cells seem to mainly infiltrate islets at later stages when the proportion of beta cells decline, but they do not actively secrete antibodies locally. However, T regulatory and natural killer cells are rarely detected within the islet infiltrate.

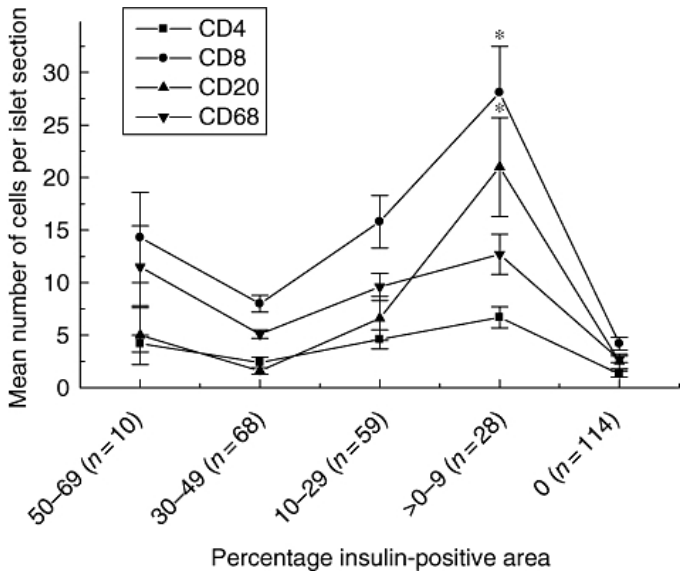

Figure 1: Relationship between immune infiltrate and beta cell mass in type 1 diabetes *(Willcox et al, 2009)*

Immune infiltrate in pancreatic islets was characterized in 29 patients with recent-onset of type 1 diabetes. $CD8^+$ and $CD4^+$ T cells, as well as macrophages ($CD68^+$) and B cells ($CD20^+$) were quantified by histochemistry.

Immune destruction of beta cells is recognized to be principally mediated by T cells. This idea is supported by the predominance of cytotoxic $CD8^+$ T cells in insulitis and the presence of $CD8^+$ cells reactive to islet antigens in the blood of patients with type 1 diabetes [13]. Moreover, the use of immunosuppressive drugs that specifically target T cells has been shown to delay diabetes progression [14, 15]. Additional evidence comes from a case report in which autoimmune diabetes was transferred after transplantation of bone marrow (not depleted for T cells) from a diabetic donor [16].

Dendritic cells are believed to be important in the initial phase of diabetes pathogenesis by activating and differentiating diabetogenic T cells. Both macrophages and dendritic cells contribute to the inflammatory process via cytokine and chemokine secretion, but they may also play a direct role in the destructive phase as they can cause beta cell death [17]. Islet-infiltrating macrophages likely also participate in the clearance of apoptotic cells.

The potential role of B cells in type 1 diabetes is supported by the presence of antibodies directed against beta cells in the blood of diabetic patients. These circulating autoantibodies

are used to predict diabetes onset, such that individuals with high titers of 2 or more autoantibodies have 89% risk of developing diabetes within 5 years [18]. However, this humoral response is not essential for the pathogenesis of type 1 diabetes, since depletion of antibodies by plasmapheresis or immunotherapy directed against the humoral response fail to prevent disease [19]. Furthermore, type 1 diabetes development has been reported in a patient with B cell deficiency [20], suggesting that B cells are not required for the autoimmune destruction of beta cells.

1.1.4. ETIOLOGY OF TYPE 1 DIABETES

1.1.4.1. Beta cell death as the initial trigger

Type 1 diabetes evolves as a multistep process with a defined temporal sequence of islet infiltration by immune cells, yet, little is known about the triggering event owing to the difficulty to pinpoint the beginning of the autoimmune response. One scenario considers that beta cell death is the primary event leading to immune sensitization. Whether beta cell apoptosis is part of a normal tissue turnover [21] or caused by a non-specific injury, beta cell antigens are exposed and may act as danger signals particularly in the presence of inflammatory mediators. Of note, islets themselves secrete cytotoxic cytokines, such as IL-1β or TNFα, and chemokines that recruit immune cells [12, 22], thereby contributing to their own demise. Antigen presenting cells, mostly dendritic cells, take up beta cell proteins in the pancreas and migrate to the pancreatic lymph node where they activate T cells and initiate the antigen-specific diabetogenic response.

Diabetogenic T cells recognize a number of epitopes derived from beta cell proteins, such as insulin and its precursors, glutamic acid decarboxylase (GAD), islet amyloid polypeptide and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [13, 23]. At the time of diagnosis, patients with type 1 diabetes have T cell reactivity to a wide spectrum of these beta cell antigens. Interestingly, studies in the preclinical phase reveal that T cells respond to increasing numbers of islet antigens [24], a pattern also observed in the humoral response against beta cells [18]. These findings suggest that the autoimmune response initially targets a single antigen and expands to other antigens as the disease progresses, a phenomenon called epitope spreading. Although insulin or its precursors are the most likely candidates, the identity of that primary beta cell antigen is still under debate.

1.1.4.2. Defective tolerance to self

A prerequisite for the autoimmune response against beta cells to occur is the presence of islet-reactive T cell clones. Such diabetogenic clones exist because of a failure in central tolerance, a process consisting of deletion of potentially autoreactive T cells during their maturation in the thymus. Several backup mechanisms are in place in the periphery to control autoreactive T cells that escape negative selection, so called peripheral tolerance. Indeed, tolerance can also be achieved by induction of anergy or cell death. In this regard, the main immune players are T regulatory (Treg), natural killer T (NKT) and dendritic cells (DC), which use negative regulators such as CTLA-4, PD-1 and anti-inflammatory cytokines [25]. Unfortunately, these regulatory pathways are also affected in type 1 diabetes owing to deficiencies in Treg or NKT as well as hyperactivation of DCs, which promote T cell activation instead of tolerance [25, 26]. Many of these immune defects are determined by genetic factors that predispose to diabetes. A number of studies have established a strong relationship between genetics, environmental factors and type 1 diabetes. Most likely type 1 diabetes is a combination of factors that concur to initiate an immune reaction to beta cell antigens.

1.1.4.3. Genetics of type 1 diabetes

Genetic predisposition to type 1 diabetes is evidenced by the higher prevalence in siblings of diabetic patients, with a risk increased by 15 times compared to the general population. The highest risk is observed in monozygotic twins, followed by first and second-degree relatives. Genome linkage studies have identified over 40 loci associated with type 1 diabetes; however, fine mapping of the specific genes involved is still underway for most of these loci [27]. Interestingly, many of these genetic determinants are also associated with other autoimmune diseases [28].

The first genomic determinant was found in the major histocompatibility complex (MHC) region, more particularly in the clusters of class I and class II molecules. These genes are highly polymorphic and encode surface proteins that present antigens to T lymphocytes. The strongest association with type 1 diabetes is conferred by MHC class II alleles; the most common haplotypes (DR3–DQ2 and DR4–DQ8) are carried by 90% of patients with type 1 diabetes [27]. MHC class I alleles, such as HLA-A2 and HLA-A24, also contribute to type 1 diabetes (albeit more weakly) by influencing the severity of disease [29-31]. Interestingly, the different predisposing MHC molecules have in common the presence of specific amino acid

residues in the peptide-binding site, which influences the affinity for peptides and the likelihood of presenting certain epitopes, for example beta cell auto-antigens [32].

A second susceptibility locus is in the regulatory region of the insulin gene, which influences pancreatic and thymic expression of insulin. Polymorphisms resulting in lower insulin expression in the thymus predisposes to autoimmune diabetes, likely by affecting central tolerance induction towards insulin antigens [27].

Several predisposing genes directly affect T cell function or activation. For instance, variants in regulators of T cell receptor signaling interfere with tolerance induction in T cells. Mutations of the IL-2 receptor leading to defective IL-2 signaling compromise the function of T regulatory cells. Polymorphisms of CTLA-4, an important negative regulator of T cell function, have also been associated to type 1 diabetes. Other genetic determinants alter the innate immune system by promoting inflammatory pathways, for example by eliciting interferon responses (polymorphisms in IFIH1 and EBI2) [27].

1.1.4.4. Environmental factors

The limited concordance in monozygotic twins suggests that type 1 diabetes cannot be entirely attributed to genetics and that progression to clinical disease in susceptible individuals is influenced by differential exposure to environmental factors. A role for the environment and lifestyle in the development of type 1 diabetes is further implied by the rising incidence of type 1 diabetes worldwide as well as the geographical differences despite similar genetic profiles of the population. Notably, groups that migrate from a low-incidence to a high-incidence area display an increase in the number of new cases [33]. Unlike genetics, environmental factors that trigger type 1 diabetes may be controlled. Therefore, their identification could provide potential means of intervention in order to prevent type 1 diabetes.

Diet in early life and childhood has been proposed to play a role in triggering type 1 diabetes. Breastfeeding is believed to protect from disease whereas early exposure to cow's milk may promote development of beta cell autoimmunity. Likewise, early introduction of cereals in a baby's diet is thought to increase the risk of diabetes in susceptible individuals. Several studies have addressed these questions but the conclusions remain conflicting.

Evidence pointing to the participation of viral infections comes from the seasonal pattern of beta cell autoimmunity emergence in children. Indeed, prospective studies have shown that the initial appearance of autoantibodies, usually in the fall and winter, coincides with enterovirus infections [33]. Moreover, enterovirus RNA is frequently detected in the circulation at onset of disease but also in the pancreas of diabetic patients, particularly Coxsackievirus B4 [34-36]. Several enteroviruses have a tropism for beta cells; they can cause beta cell death and induce inflammatory mediators [37], both of which may initiate an autoimmune reaction. The general inflammation in the pancreas caused by enteroviral infection may result in activation of pre-existing autoreactive T cells, so called bystander activation [37]. Another possibility is the presence of molecular mimicry between viral proteins and beta cell antigens. For example, there is sequence homology between the islet autoantigen GAD and a Coxsackie B4 protease that could lead to T cell cross-reactivity, but this theory has not been supported by functional assays [38].

1.1.5. RECURRENT AUTOIMMUNITY

Following beta cell destruction and diabetes development, insulitis resolves due to a contraction of the immune response, but a small population of autoreactive memory T cells subsists. When their specific islet antigens reappear, these memory cells are rapidly reactivated to destroy the new beta cells, a phenomenon called recurrent autoimmunity.

Such a situation is observed in type 1 diabetes recipients of islet and pancreas transplants, as evidenced by the re-emergence in the circulation of autoreactive $CD8^+$ T cells [6, 39] as well as autoantibodies [5, 40] shortly after transplantation. Analysis of pancreas explants after graft failure revealed a selective loss of beta cells and the presence of autoreactive $CD8^+$ T cells within islets [39, 41]. The remarkably higher success rate of islet transplantation in non-diabetic individuals or patients with type 2 diabetes suggests that recurrent autoimmunity is an important cause of islet graft failure [42]. Moreover, the presence of circulating autoreactive $CD8^+$ T cells in blood of type 1 diabetes recipients positively correlates with subsequent graft failure [6].

Beta cells have the ability to regenerate as part of normal homeostasis as well as following injury [43, 44]. These new beta cells may arise through self-replication [45] or originate from progenitor cells that derive from other islet cells or duct epithelium [43, 46]. Several reports

indicate that a repair mechanism is in place in patients with type 1 diabetes in an attempt to replenish the beta cell mass by proliferation [47, 48]. Unfortunately, beta cell regeneration provides another occasion for the immune system to be reexposed to autoantigens and be reactivated. It is believed that the clinical onset of type 1 diabetes arises when the rate of beta cell destruction exceeds their proliferative capacity. Inhibition of the recurrent autoimmune response would allow endogenous beta cells to recover, and thereby potentially reverse type 1 diabetes.

1.1.6. THE NON-OBESE DIABETIC MOUSE: MODEL OF TYPE 1 DIABETES

Discovered in 1974, the non-obese diabetic (NOD) mouse is an inbred strain that spontaneously develops autoimmune diabetes resulting in insulin dependence. NOD mice usually become overtly diabetic between 12 and 30 weeks of age, with an incidence reaching 80% in females but only 20% in males [49]. Both genders have islet infiltration; however, females display a more invasive and destructive insulitis, which progresses to diabetes more rapidly [50]. Of note, NOD mice are susceptible to a variety of autoimmune diseases as they have self-reactivity to several tissues such as the thyroid, colon and salivary glands [51].

Despite the gender bias, diabetes in NOD mice strongly resembles human type 1 diabetes, with the most striking similarity being the MHC class II predisposition. Of note, the diabetogenic MHC class II molecule in NOD mice ($I\text{-}A^{g7}$) has an alteration of a certain amino acid that is equivalent to the one found in the human orthologue DQ8 [27]. Additional predisposing genes with correspondence in NOD mice and humans include IL-2 and CTLA-4 [27, 51]. Viral infections in NOD mice also influence development of autoimmunity; however, studies have revealed that viruses may either prevent or promote diabetes depending on the virus or the timing of infection [52]. In NOD mice, Coxsackievirus B4 also correlates with diabetes as it accelerates disease onset [53].

As in human type 1 diabetes, beta cell death in NOD mice occurs following mass islet infiltration by dendritic cells, macrophages, $CD4^+$ and $CD8^+$ T cells, as well as B lymphocytes and natural killer (NK) cells [54-56]. Studies in mice have shown that $CD4^+$ and $CD8^+$ T cells from diabetic mice, alone or in combination, are able to transfer diabetes [57], indicating that it is primarily a T cell-mediated disease. Notably, final beta cell destruction is achieved by autoreactive $CD8^+$ T cells that target islet-specific autoantigens [6, 58]. The main target

peptides are the same in humans and mice, with T cell reactivity against insulin, islet-antigen 2 (IA-2), GAD and IGRP [59], as well as a humoral response against insulin [60]. Further, recurrent autoimmunity is observed in NOD mice after islet transplantation [61].

Accordingly, NOD mice are the prevalent animal model of type 1 diabetes and have proved to be a valuable tool in studying the progression and nature of the autoimmune response against beta cells. For instance, the fact that islets are first infiltrated by DCs and macrophages approximately at 5 weeks of age [55] following a wave of beta cell apoptosis [62], implies that these cells play a crucial role in the initiation of the autoimmune response. These cells transport islet antigens to the pancreatic lymph nodes and prime diabetogenic T cells, which subsequently invade pancreatic islets [63, 64]. Islet-infiltrating T cells produce inflammatory cytokines and further amplify the immune response by inducing the maturation and migration of resident DCs to the pancreatic lymph node, where they expand more diabetogenic T cells [65].

The NOD mouse model has informed us about the functional importance of certain immune defects associated with type 1 diabetes [27], the mechanisms of epitope spreading [66] as well as recurrent autoimmunity [61]. It has also improved our understanding of the role of viral infections in the induction or prevention of type 1 diabetes [67, 68]. It's important to recognize, however, that important differences exist between the NOD mouse and human immune systems and in disease pathogenesis that may complicate the interpretation of certain findings. Nevertheless, studies in NOD mice can provide proof of principle for the potential of immune-based interventions in type 1 diabetes. Several clinical trials are based on treatments with proven preclinical efficacy in NOD mice, for instance T cell depletion with CD3 antibodies or oral insulin prophylaxis [69].

The Bio-breeding (BB) rat is another model of spontaneous autoimmune diabetes with several characteristics of type 1 diabetes. However, unlike humans, BB rats are lymphopenic and severely deficient in $CD8^+$ T cells due to a mutation in a gene important for T cell survival [27]. This important distinction and the higher maintenance cost partly explain why BB rats are less commonly used for the study of type 1 diabetes than NOD mice.

1.2. THE IMMUNE SYSTEM

The immune system evolved to protect the organism from a variety of foreign pathogens, such as viruses, bacteria and parasites, but also to discriminate healthy from abnormal cells to eliminate potential malignancies. The immune system consists of physical barriers, like the skin and mucosal surfaces, and immune cells or leukocytes. All immune cells derive from pluripotent hematopoietic cells in the bone marrow that give rise to the myeloid and lymphoid lineages, which subsequently differentiate in the different immune subsets. Immune cells are broadly classified into 2 categories, innate or adaptive, based on the immediacy and specificity of their response as well as their ability to establish immunological memory. Advances in the immunology field may have revealed that the separation between innate and adaptive immunity is not as clear as previously believed. Inherited or acquired deficiencies in either innate or adaptive immunity exposes the host to the risk of infections and cancers, indicating that interactions between innate and adaptive immune cells are essential for mounting optimal immune responses.

1.2.1. INNATE IMMUNE CELLS

Innate immune cells constitute the first line of defense as they respond within hours of an infection or injury. These cells express a limited repertoire of receptors that recognize components conserved among micro-organisms, collectively referred to as pathogen-associated molecular patterns (PAMP). These receptors, which include Toll-like receptors (TLR), are able to detect pathogens or components of damaged cells in the extracellular space or in the cytoplasm and trigger a cascade of inflammatory mediators to contain the infection. The nature of this immune response is not specific for a particular antigen and therefore does not improve after re-challenge with the pathogen. The cells belonging to the innate immune system are macrophages, dendritic cells, granulocytes and natural killer cells.

1.2.1.1. Macrophages

Macrophages derive from circulating monocytes and reside within lymphoid and peripheral tissues, where they constantly survey for signs of tissue damage and invading organisms. These specialized phagocytic cells engulf pathogens and apoptotic cells in a mechanism triggered by a variety of receptors, such as scavenger and Fc receptors [70]. Macrophages also play a role in priming T cell responses by presenting antigens to T cells through MHC molecules and providing co-stimulatory signals [71]. The functional phenotype of macrophages is flexible and depends on micro-environmental signals [72]. For example, macrophages activated by TLR ligands and IFNγ become polarized M1 macrophages that produce chemokines and antimicrobial molecules such as nitric oxide, reactive oxygen species, IL-1 and TNFα [73]. Conversely, in the presence of IL-4 and IL-13, macrophages develop an M2 anti-inflammatory phenotype and promote tissue repair through secretion of TGFβ and other growth factors.

1.2.1.2. Dendritic cells

Like macrophages, dendritic cells (DCs) originate from monocytes, hence they share several cell surface markers and have some overlapping functions. The hallmark of DCs is their role as antigen presenting cells, which is central for initiating adaptive immune responses. When they detect PAMP or other danger signals, DCs mature by upregulating MHC and co-stimulatory CD80/86 molecules, and migrate to regional lymph nodes where they activate T cells [74]. Under steady state conditions, DCs remain immature and induce tolerance towards processed antigens [75]. DCs shape the immune response using a variety of effector molecules that polarize $CD4^+$ cells, activate other leukocytes or suppress T cells. The tolerogenic mechanisms of DCs include the secretion of TGFβ and IL-10 to induce regulatory T cells [76], but also the production of an enzyme called indoleamine 2,3-dioxygenase (IDO), which suppresses T cell proliferation by degrading the essential amino acid tryptophan [77].

Both human and murine DCs can be subdivided into three major populations identified by their localization, migratory pattern, cytokine profile and surface markers, including CD8α, CD11b and PDCA-1 (see Table 1) [78]. All DC subsets present antigens to $CD4^+$ T cells, but lymphoid DC (lyDC) have the ability to present antigens to $CD8^+$ T cells as well [79]. Plasmacytoid DC (pDC) specialize in fighting viral infections by producing large amounts of type I IFNs (α and β) following activation of the viral sensors TLR7 and TLR9 [80].

	Lymphoid DC	Myeloid DC	Plasmacytoid DC
Phenotype	CD11c ^{high} CD8α $^{+}$ CD11b $^{-}$	CD11c ^{high} CD11b $^{+}$ CD8α $^{-}$	CD11c ^{med} PDCA-1^{+} CD8α $^{-}$ CD11b $^{-}$
Localization	Lymphoid tissues	Lymphoid and peripheral tissues	Lymphoid tissues Circulation
Cytokines	IL-12 +++ IL-10 +	IL-10+++ IL-12 +	IFN α, β +++ IL-6+
T helper response	Th1	Th2	Th1 or Treg

Table 1: Major dendritic cell subsets in humans and mice.
The main characteristics of each subset are summarized here. Of note, the cell surface markers indicated are for murine DCs.

1.2.1.3. Granulocytes

As suggested by their name, granulocytes are characterized by an abundance of granules in their cytoplasm but also by multi-lobulated nuclei. These cells are further subdivided into neutrophils, basophils and eosinophils based on the staining properties of their granules. Granulocytes are short-lived cells that release the content of their granules upon activation. Neutrophils are the most frequent granulocytes, mostly found in the circulation and inflamed tissues. Their role is essential in acute bacterial infections as phagocytes but also as producers of powerful antimicrobial molecules, such as myeloperoxidase and proteases [81]. Eosinophils mostly reside in peripheral tissues and constitute an important defense against parasitic infections. Additionally, eosinophils have the ability to act as antigen presenting cells and induce Th2 responses via cytokine secretion [82]. The least common granulocytes, namely basophils, contain histamine granules and secrete large amounts of IL-4 and IL-13 to control parasitic infections [83]. Both eosinophils and basophils are closely associated with the pathogenesis of allergic reactions, particularly in the respiratory tract.

1.2.1.4. Natural killer cells

Natural killer (NK) cells are present in blood, lymphoid organs and virtually all tissues, generally identified by the markers CD56 in humans and CD49b in mice. NK cells are important for detecting and eliminating viral infections and tumors. Their effector function is finely tuned by activating and inhibitory receptors, which determine the net balance of the signal [84]. To

maintain self-tolerance, NK cells are predominantly under inhibitory control, which can be overcome by receptor-mediated or cytokine stimulation. Inhibitory receptors include the family of killer cell immunoglobulin-like receptor (KIR) and lectin-like NKG2A. A larger number of activating receptors are expressed on NK cells, the major ones being NKG2D and natural cytotoxicity receptors NKp44 and NKp46. Both host and pathogen-encoded ligands bind to these receptors [85]. NK cells are notably responsive to changes in expression of MHC class I molecules, which are ubiquitously expressed on healthy cells, but downregulated in virus-infected cells as well as malignant cells. Reduced MHC class I ligation on KIR inhibitory receptors unleashes NK cell effector function on the target cell.

Cytotoxicity is the major function of NK cells and is mediated by several mechanisms. Activated NK cells release granules containing perforin and granzyme; perforins form pores in cell membranes allowing granzymes to enter and activate the caspase apoptosis cascade [86]. Another mechanism used by NK cells to induce apoptosis is through ligation of death receptors on target cells, such as FAS [87]. In addition, NK cells produce cytokines, mainly IFNγ and TNFα, but also IL-5, IL-10 and GM-CSF growth factor [88]. Recently, NK cells have been reported to develop immunological memory to a specific pathogen [89], a property typically associated with adaptive immunity, suggesting that the classical definition of innate immune cells does not apply to NK cells anymore.

1.2.2. ADAPTIVE IMMUNE CELLS

The adaptive immune system principally consists of the highly specialized T and B lymphocytes, which develop in the thymus and bone marrow, respectively. These populations possess an incredibly diverse repertoire of receptors capable of recognizing all potential pathogens. Such diversity is generated by somatic recombinations of an array of gene segments used to form the different chains of T or B cell receptors [90]. T and B cells are then subjected to clonal selection based on their receptor‘s ability to recognized antigens, including self-antigens [91]. Clones with high affinity for self-antigens are deleted, a process called central tolerance that is crucial to avoid autoimmunity. Selected lymphocytes traffic to secondary lymphoid organs, such as the spleen and lymph nodes, in a naïve state until they encounter their specific antigen and acquire their effector function. Although slower than innate immunity, the adaptive immune response is highly specific for the responsible pathogen and therefore more efficient in eradicating the pathogen. Additionally, antigen-experienced

lymphocytes have the ability to develop long-term immunological memory, providing a rapid response to the pathogen upon re-challenge.

1.2.2.1. B lymphocytes

The B cell receptor consists of an immunoglobulin (Ig) attached to the cell surface, which is formed by two light chains and two heavy chains. B cells are able to recognize intact extracellular antigens or pathogens and initiate a humoral response by producing specific antibodies. These antibodies bind to surface antigens to signal phagocytes or other effector cells to destroy the antigen. Upon activation, B cells become plasma cells that secrete low affinity IgM antibodies or establish germinal centers in lymphoid tissues for further maturation of their affinity [92]. This process, termed class switching, occurs through somatic hypermutation resulting in the production of high affinity IgG, IgA and IgE antibodies. The infectious context and cytokine environment influence the type of antibody produced. IgG is the main subclass and is able to cross the placental barrier transferring passive immunity to the fetus. IgA play an important role at mucosal surfaces, particularly in the gut and respiratory tract. IgE cooperates with basophils in parasitic infections and also participate in allergic reactions. In addition to the humoral response, B cells influence T cell responses in their capacity of antigen presenting cells [93].

1.2.2.2. T lymphocytes

The T cell receptor (TCR) is formed by a variable portion that binds to the ligand and a non-variable portion that transduces the signal, namely the CD3 complex [94]. In classical T cells, the variable portion is a heterodimer composed of an alpha and a beta chain. The TCR recognizes small portions of antigens or epitopes on MHC molecules and this interaction is stabilized by co-receptors. Two main T cell populations are defined according to the co-receptor used: $CD8^+$ and $CD4^+$ T cells. Co-receptors determine which MHC molecule the TCR binds, such that $CD4^+$ cells recognize peptides on class II while $CD8^+$ cells recognize peptides on class I molecules. To acquire effector function, T cells require co-stimulatory signals in addition to antigen recognition. In this regard, the most important receptors are the CD28 family, which bind the co-stimulatory molecules CD80/86 on antigen presenting cells [95].

1.2.2.2.1. Cytotoxic $CD8^+$ cells

Following activation in lymphoid tissues, rapid and massive expansion of $CD8^+$ T occurs with the help of cytokines such as IL-12 and type I IFN, as well as pro-survival signals [96]. $CD8^+$ T cells undergo their terminal differentiation into effector cells under the influence of IL-2, IL-12, IL-21 and IL-27. Also called cytotoxic T lymphocytes (CTL), $CD8^+$ cells use similar effector molecules as NK cells to destroy target cells, including FAS, perforin and granzyme B, as well as IFNγ and TNFα [86, 96]. Primed $CD8^+$ T cells target intracellular pathogens or transformed cells through recognition of their specific antigen on MHC class I molecules. More recently, CTLs were shown to prevent excessive tissue injury during infections by secreting the immunosuppressive cytokine IL-10 [97, 98].

1.2.2.2.2. $CD4^+$ T cells

$CD4^+$ T cells orchestrate panoply of immune responses by helping other immune cells, earning them the name of T helper cells. Originally, two main subsets were identified: IFNγ-producing Th1 cells and IL-4-producing Th2 cells. The current knowledge is that $CD4^+$ T cells may differentiate into four different subsets depending on the pattern of signals they receive during their priming, namely Th1, Th2, Th17 and induced T regulatory cells (iTreg) [99]. These 4 populations are distinguished by their cytokine profile and their transcription factors, which act as master regulators of their function (see Figure 2). Cytokines produced by each subset provide positive feedback for that population while inhibiting the other subsets, thereby amplifying one particular pathway. Notably, commitment to one lineage is reversible owing to the flexible genetic program of T helper cells, particularly iTregs and Th17 cells [100].

1.2.2.2.2.1. Th1, Th2 and Th17 cells

Th1 cells mediate host defense against intracellular pathogens. They promote activation of macrophages through IFNγ and development of $CD4^+$ and $CD8^+$ T cell memory via IL-2 [99]. Th1 cells have been associated with a variety of autoimmune disorders, notably due to their production of lymphotoxin-α (LTα) [101, 102]. Th2 cells play an essential role in combatting extracellular parasites in collaboration with basophils and eosinophils, and likewise have been implicated in allergic reactions. In addition, cytokines secreted by Th2 cells influence the immunoglobulin class switch in plasma cells. The balance between Th1 and Th2 cells was

regarded to be an important determinant of the outcome of an immune response; however, the identification of new players makes the interpretation more complex. Th17 cells were first described for their pathological role in several autoimmune diseases, including rheumatoid arthritis and multiple sclerosis [103]. Nevertheless, these cells offer protection from extracellular bacteria and fungi and participate to homeostasis of the intestinal mucosa [104]. Th1, Th2 and Th17 cells are collectively designated as $CD4^+$ effector cells.

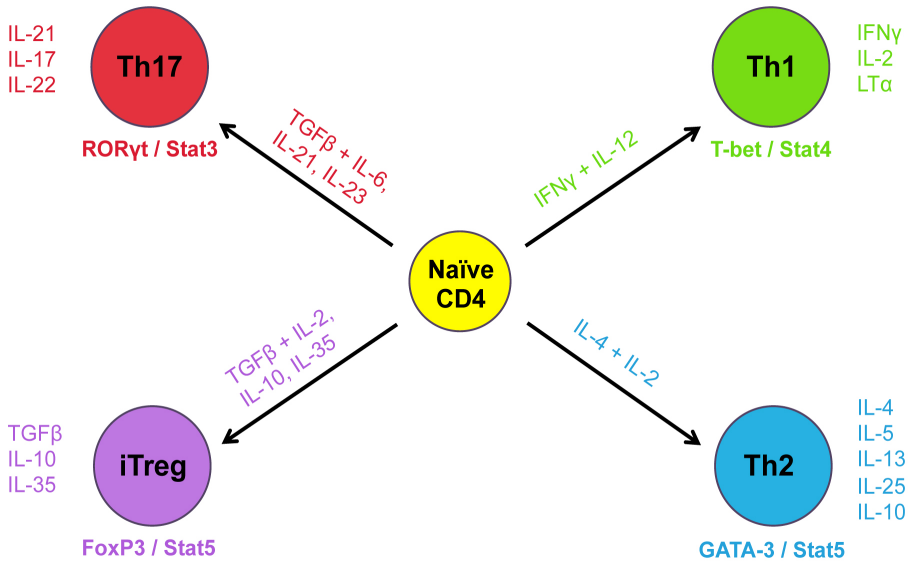

Figure 2: Differentiation of naïve $CD4^+$ cells into the different T helper cells
Each subset is characterized by specific transcription factors and cytokines produced. The signals that induce each pathway are shown on the arrows.

1.2.2.2.2.2. Tregs

Induced Tregs (iTreg) are generated from naïve $CD4^+$ T cells under various conditions such as chronic inflammation or organ transplantation [105, 106], or in the presence of the immunosuppressive cytokines TGFβ, IL-10 and IL-35 [107-109]. Induced Tregs share many features with natural Tregs, which arise as a separate $CD4^+$ subset from the thymus. Natural Tregs were selected based on their specificity for self-antigens, whereas induced Tregs recognize foreign antigens, but both play a critical role in immune homeostasis and self-tolerance. On the other hand, Tregs represent a major barrier in vaccination and cancer eradication given their suppressive function. The transcription factor FoxP3 acts as a master regulator of Treg function, as ectopic expression of FoxP3 in naïve $CD4^+$ cells induces expression of Treg-signature molecules and confers suppressive function [110, 111].

Importantly, both in humans and mice, mutations of FoxP3 cause Treg deficiency, leading to multi-organ autoimmunity [112]. Another similarity between induced and natural Tregs is their dependence on IL-2 for fitness and survival, explaining their expression of the IL-2 high affinity receptor CD25 [113, 114]. Therefore, FoxP3 along with CD25 are excellent markers for Tregs in mice, but not as specific in humans as they may be expressed by naïve $CD4^+$ cells [115].

The wide range of suppressive action mediated by Tregs resides in their ability to directly control both innate and adaptive immunity, by targeting DC, NK cells, B cells, $CD4^+$ and $CD8^+$ T cells [116, 117]. Tregs utilize four main suppressive mechanisms, which may operate in synergy or independently depending on the biological setting (Figure 3). One of these mechanisms is the secretion of anti-inflammatory cytokines, notably IL-10, TGFβ and IL-35. Cytolysis is another mechanism used by Tregs to modulate immune responses, and is specifically used to suppress NK cells, cytotoxic T lymphocytes and B cells [118]. Tregs can also induce metabolic disruption either by consuming local IL-2 required for T cell survival [119], or by increasing intracellular levels of cAMP, a second messenger important for inhibition [120]. Additionally, Tregs modulate the maturation and function of DCs, which are essential for T cell priming and differentiation. This process involves Treg surface expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3).

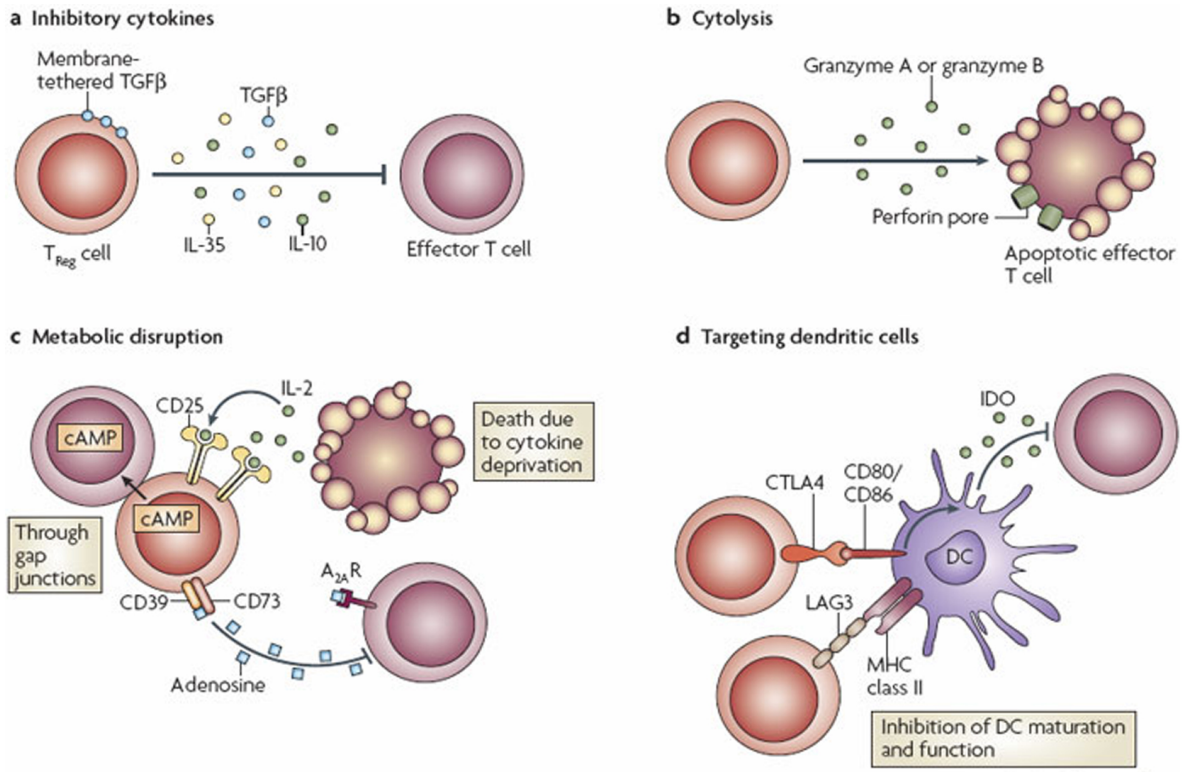

Figure 3: Major suppressive mechanisms of Tregs *(Vignali et al, 2008)*
Tregs use four basic modes of action to suppress other immune cells: secretion of inhibitory cytokines (a), cytolysis (b), metabolic disruption (c) and targeting DCs (d).

1.2.3. BRIDGING INNATE AND ADAPTIVE IMMUNITY

More recently, a new category has been established for immune cells that have features of both innate and adaptive immunity. These cells express antigen-specific receptors but quickly unleash effector functions without priming and co-stimulation, as they display a constitutive activated phenotype. Their receptors have limited diversity and recognize glycolipids or carbohydrates, often of self-origin, instead of peptides [121]. Innate-like lymphocytes preferentially reside in peripheral tissues, where they form specialized niches. These intermediate lymphocytes include natural killer T cells, gamma delta (γδ) T cells, CD8αα cells, marginal zone and B1 B cells. It is possible that this category will further expand with improvements in the characterization of leukocytes and advances in the immunology field.

1.2.3.1. Natural killer T cells

Originating from the T cell lineage and displaying features of NK cells, natural killer T (NKT) cells stand at the crossroads of adaptive and innate immunity. Like conventional T cells, NKT cells express an αβ TCR, but their receptor binds to a non-classical antigen presenting molecule called CD1d, which presents lipid antigens instead of peptides. CD1d is widely expressed in tissues, notably on immune cells, such as DCs and lymphocytes, as well as epithelial and stromal cells [122]. Two subsets of NKT cells have been described based on their TCR repertoire: type I and type II NKT cells. Type I NKT cells are also called invariant NKT (iNKT) cells because their TCR is constant, using the rearrangement Vα24-Jα18 in humans, or the homologue Vα24-Jα18 in mice. Type II or non-invariant (ni) NKT cells express a more diverse TCR repertoire using several sets of rearrangements.

Both types of NKT cells are believed to react to self and foreign antigens. For instance, iNKT cells recognize normal endogenous lipid antigens, such as phosphatidylinositol or the lysosomal glycosphingolipid called iGb3, as well as tumor-derived lipids [123]. Type II NKT cells were shown to react with sulfatide, which is abundantly expressed in the myelin sheath of axons [124], of relevance for the pathogenesis of multiple sclerosis. A number of exogenous ligands have also been identified for iNKT cells, the most potent being α galactosylceramide (αGalCer), which is ubiquitously found in the environment and likely mimics the endogenous ligand of iNKT cells.

	Type I NKT cells (invariant)	**Type II NKT cells (non-invariant)**
TCR αβ-chain	Vα24-Jα18 + Vβ11 (humans) Vα14-Jα18 + Vβ2, 7, 8 (mice)	diverse
Lipid activation	αGalCer, iGb3, phosphatidylinositol	sulfatide
Markers for identification (mice)	CD49b, CD3, CD1d-tetramer loaded with αGalCer	CD49b, CD3
Cytokine profile	IFNγ, IL-4	IFNγ, IL-4, IL-13

Table 2: Characteristics of type I and II NKT cells

Invariant NKT cells preferentially localize in the liver and thymus, whereas niNKT cells are more abundant in the spleen and bone marrow. Both types of NKT cells exhibit NK cell markers, such as CD49b in mice, display a memory phenotype and most of them are either $CD4^+$ or CD4 and CD8 double negative. NKT cells are characterized by vigorous secretion of cytokines, mainly IL-4, IFNγ and IL-13, as well as various chemokines upon activation [125]. Like NK cells, NKT cells possess cytotoxic functions triggering apoptosis via similar mechanisms [126]. Another important aspect of NKT cell function is their crosstalk with other immune cells. Indeed, NKT were shown to modulate DC function through CD40L and PD-1 pathways [68, 127], and control the activity of NK cells [128, 129]. Interactions with Tregs were also described in different settings [130]. Moreover, NKT cells have the ability to induce differentiation of Th2 cells [131] and expansion of Tregs [68, 130].

NKT cell activity is critical against bacterial, viral and parasitic infections [132], and profoundly influences malignant and autoimmune disease outcomes [122]. Importantly, NKT cell subsets were shown to play opposite roles in some *in vivo* models. In the context of malignancies, iNKT cells provide anti-tumor immunity, whereas type II NKT cells promote tumor growth [133]. In a model of autoimmune hepatitis, type II NKT cells were protective while iNKT cells were pathogenic. *In vitro* analysis revealed that iNKT and type II NKT cells may counter-regulate each other by inducing anergy [134, 135]. However, both subsets were protective in several experimental models of autoimmune diseases, including type 1 diabetes, multiple sclerosis and rheumatoid arthritis [122, 133]. Additionally, iNKT were shown to promote tolerance to organ transplants [136]. The powerful immune properties of iNKT cells could be exploited in vaccines, transplantation and certain autoimmune disorders. Currently, therapeutic applications of iNKT cells are investigated for cancers [137].

1.2.3.2. Other innate-like lymphocytes

γδ T cells express an alternative TCR composed of the γ and δ chains instead of the conventional αβ combination. They are the first T cells to arise from the thymus and preferentially localize in the skin and lungs. γδ T cells possess a broad range of activities from antigen presentation, regulation and protection against tumors and infections such as Mycobacterium tuberculosis [138]. CD8αα T cells express a homodimer of CD8 alpha chains, which similarly binds to MHC class I molecules. Exclusively found in intestinal epithelium, CD8αα T cells play a regulatory role using inhibitory molecules such as TGFβ and CTLA-4, and provide protection from colitis [139]. B1 B cells are mainly found in the pleural and peritoneal

cavities and are known to secrete large amounts of natural antibodies in the absence of infection [140]. These antibodies have low affinity but broad specificities to protect against various pathogens; conversely, they also contribute to ischemic injury [140]. Marginal zone (MZ) B cells are found in the spleen, where they detect and protect from blood-borne pathogens, in particular encapsulated bacteria [141]. Both B1 and MZ B cells participate in immune regulation by inducing apoptosis of classical lymphocytes [142].

1.2.4. CONTROL OF LEUKOCYTE TRAFFICKING BY CHEMOKINES

Chemokines are small molecules from the cytokine family that control leukocyte trafficking in homeostasis and immune responses. To date, over 50 chemokines have been described and categorized in 4 groups depending on the sequence of conserved cysteine residues at the N-terminus. The CC group has 2 adjacent cysteines, the XC group has only one cysteine, the CXC group has 2 cysteines separated by another amino acid and in the CX3C group, cysteines are separated by 3 residues. The majority of chemokines belong to the CC and CXC subgroups. Although some chemokines can be anchored on the cell membrane, chemokines are usually secreted and form soluble chemotactic gradients. The chemotactic network may appear redundant as several chemokines act on the same receptor. Yet, these chemokines often differ in their cellular or tissue sources, receptor affinities and biological activities.

Chemokine receptors are seven-transmembrane spanning molecules coupled to G proteins for signal transduction. Upon receptor engagement, the subunits of the G protein dissociate and activate the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. These pathways collectively trigger rapid activation of cell adhesion molecules called integrins, cell polarization and directional migration [143, 144]. Interestingly, both PI3K and MAPK are involved in other important process such as proliferation, survival and differentiation [145, 146], indicating that chemokines may also influence these cellular activities.

The chemokine network is regulated by several mechanisms, the most important one being through the expression of chemokine receptors. The number of a given receptor at the cell surface determines the sensitivity for its ligand, hence cell migration may be controlled by modulation of chemokine receptor levels. Moreover, some receptors are differentially expressed on leukocytes, thereby allowing the preferential recruitment of particular cell

populations. Another strategy consists in the use of decoy receptors, called DARC and D6, which bind a broad range of inflammatory chemokines without transducing the signal. Mainly expressed by endothelial cells in venules and lymphatics, these decoy receptors contribute to the clearance of chemotactic gradients during inflammatory responses [147]. Finally, several tissue enzymes, such as elastases and metalloproteinases, have the ability to inactivate chemokines by cleavage [148].

Chemokines play a central role in the immune system by orchestrating leukocyte migration. The lymphoid system is controlled by homing chemokines CCL19 and CCL21, which drive leukocytes via their receptor CCR7 to the draining lymph nodes [148]. Once there, cell subsets are organized into specialized areas by other chemokines, for instance CXCL13 for B cell zones. This process is essential for priming adaptive immune cells by allowing interactions of lymphocytes with antigen presenting cells. Chemokines may also deliver co-stimulatory signals, enhancing T cell proliferation and cytokine production [149]. Additionally, chemotactic gradients direct effector cells to sites of inflammation by inducing their arrest on endothelial surfaces through integrins and triggering their extravasation [150]. Inflammatory chemokines are released within a tissue by epithelial, stromal or resident immune cells upon detection of danger signals and PAMPs to recruit innate and adaptive immune cells. The importance of chemokines and their receptors in host defense against infections has been demonstrated in deficient mouse models, for instance the targeting of CCR1, CCR2, CCR5 and their ligands [147].

Selective migration to certain tissues under steady state conditions is regulated by homing chemokines, such as CCL27 and CCL17 in the skin as well as CCL25 in the intestinal tract [151]. CXCL16 expression in the liver contributes to the homeostasis of iNKT cells [152]; likewise, neutrophil function is regulated by CXCL12 in the bone marrow [153]. Additionally, chemokines play a role in angiogenesis (CXCL1 and CXCL5) and wound healing (CCL2, CXCL8) by promoting mobilization and proliferation of endothelial and epithelial cells [147]. Their participation in organogenesis has also been described in the central nervous system, where CXCL1 coordinates oligodendrocyte function [154].

Aberrant regulation of chemokines has been implicated in a number of inflammatory diseases, including asthma, atherosclerosis, and lupus erythematosus, by excessive recruitment of leukocytes [155]. Pathogens such as the human immunodeficiency virus [156] and malaria [157] have been shown to manipulate the chemotactic system for dissemination or to evade immune response by inactivating chemokines as observed with parasitic worms [158]. Tumors

take advantage of the chemotactic network to dampen immune responses, for instance by producing CCL22 that attract immunosuppressive cells [159]. Moreover, tumors may utilize homing receptors to metastasize to certain tissues, for example CCR7 to migrate to lymph nodes or CCR9 for intestinal metastasis [156]. Accordingly, the chemokine system represents a potential target for immunotherapies in various diseases.

1.3. THE IMMUNOLOGY OF TYPE 1 DIABETES

1.3.1. DEFECTIVE IMMUNE REGULATION IN TYPE 1 DIABETES

As with many autoimmune disorders, type 1 diabetes is characterized by a number of anomalies in different compartments of the immune system. An imbalance between Th1 and Th2 cytokines has been observed in both patients with type 1 diabetes and NOD mice, evidenced by higher levels of IFNγ and a corresponding decline in IL-4 in the circulation [160]. This Th1 shift was further demonstrated by the predominance of IFNγ in pancreatic islets of NOD females, favoring activation of antigen presenting cells and enhancing cytotoxic $CD8^+$ responses. Notably, the low levels of IL-4 in autoimmune diabetes are linked to deficiencies in the NKT population, which is a major source of IL-4. Indeed, in patients with type 1 diabetes, NKT cells isolated from blood and pancreatic lymph nodes were shown to produce less IL-4 [161, 162]. Additionally, autoimmune diabetes is associated with numerical deficiencies of NKT cells both in humans and mice [161, 163]. NKT cells are potent suppressor of autoimmune reactions, thus anomalies in their numbers or function may allow self-reactive cells to expand.

Both dendritic cells and macrophages display a persistent hyperactivated phenotype with secretion of inflammatory cytokines TNFα, IL-12 and IL-1 in NOD mice [22, 164]. Likewise, human DCs within insulitic lesions produce more TNFα and IL-1β [165]. Moreover, the antigen presenting ability of DCs is impaired in patients with type 1 diabetes [166, 167]. In view of the central role of DCs in priming adaptive immune cells, such alterations in their function may have profound implications; they likely contribute to the pathogenesis of type 1 diabetes through insufficient induction of Tregs and abnormal activation of autoreactive T cells.

Additionally, significant anomalies of Tregs likely underlie autoimmune diabetes. Tregs from patients with type 1 diabetes exhibit reduced suppressive function *in vitro* [168, 169]. Studies in NOD mice have confirmed this observation [170] and have revealed a progressive decline of Treg function with age [171, 172]. Tang and colleagues described the defective survival of Tregs within pancreatic islets secondary to IL-2 insufficiency, perhaps accounting for the relative loss of Tregs in diabetes. Of note, the decline in Tregs in islets preceded massive immune infiltration and resulted in expansion and cytokine production by effector cells.

In summary, the pathogenesis of type 1 diabetes involves multiple defects affecting important immune regulators such as DCs, Tregs and NKT cells. Each of these cell subsets represent key safeguards from autoimmunity by keeping self-reactive cells in check and the combined anomalies at every checkpoint precludes possible compensation by the other regulators. Several reports indicate that these defects in immunoregulation are localized to the pancreas and its draining lymph node, thereby enhancing the risk of autoimmunity in the pancreas. In this regard, the interference of viral infections may further determine the nature of the autoimmune reaction by drawing the attention of leucocytes to beta cells.

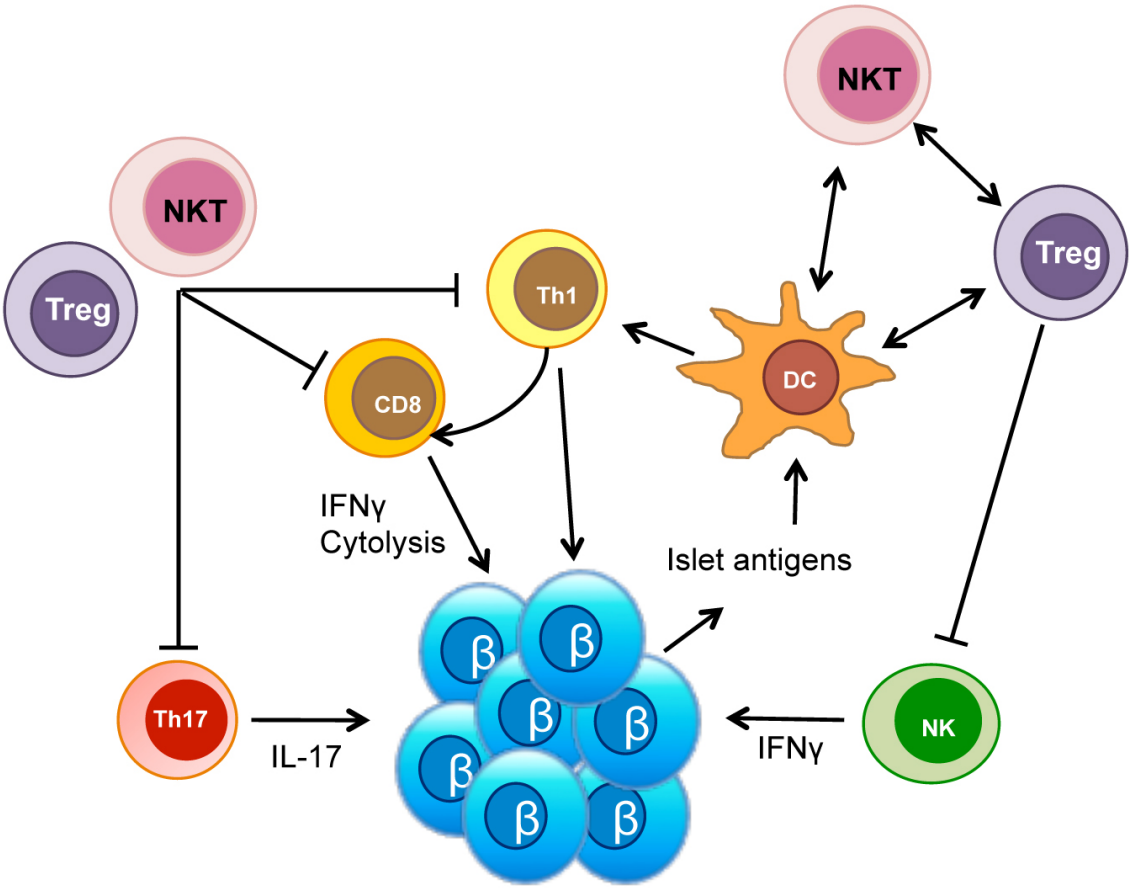

Figure 4: Autoimmune mechanisms and regulatory checkpoints in type 1 diabetes

1.3.2. LESSONS FROM ANIMAL MODELS

To determine the relevance for diabetes development of these anomalies in key immune players, a number of mechanistic studies have been undertaken in mouse models. The protective role of Tregs in diabetes was first suggested by the phenotype observed in patients and mice with a loss-of-function mutation in the FoxP3 gene [173]. Further studies demonstrated the correlation between diabetes development and Treg depletion using different approaches [174-176]. Conversely, infusion of Tregs protects from spontaneous disease and diabetes induced by adoptive transfer of diabetogenic T cells [177, 178]. In this regard, antigen-specific Tregs are superior to polyclonal Tregs, however *in vitro* expansion is required to obtain sufficient numbers of Tregs [179]. Diabetes could also be prevented in NOD mice by systemic Treg expansion induced by IL-10 therapy [180] or rescue of Treg survival with low doses of IL-2 [172], suggesting that endogenous Tregs retain functional activity in an adequate environment.

Several reports have demonstrated a causative link between DC dysfunction and autoimmune diabetes, incriminating their failure to induce Tregs by inappropriate co-stimulation [164, 175, 181]. Typically, immunogenic DCs express IL-12 and efficiently activate T effector cells, while tolerogenic DC exhibit a semi-mature phenotype and produce IL-10. Induction of tolerogenic DCs *in vivo* through various approaches, including vitamin D3 and retinoic acid, resulted in protection from autoimmune diabetes via expansion of Tregs [182-184]. Moreover, adoptive transfer of DCs conditioned *in vitro* to display a tolerogenic phenotype prevented diabetes in NOD mice [185, 186]. A recent report showed that the various DC subsets played different roles in autoimmune diabetes using a model of DC ablation and transfer of selected DC populations [187]. Saxena and colleagues demonstrated that mDC were pathogenic whereas pDC protected from diabetes. Interestingly, pDC regulatory mechanisms involved production of IDO and interaction with NKT cells.

The protective role of NKT cells in diabetes was suggested by studies in mice deficient for CD1d and therefore lacking NKT cells. CD1d knockout mice presented an accelerated development of diabetes and transfer of diabetes to immunocompromised mice was aggravated in the absence of NKT cells [188, 189]. On the other hand, expansion of iNKT cells via stimulation with αGalCer or transgenic expression of Vα14-Jα18 TCR was associated with protection from diabetes [190, 191]. Similarly, transgenic overexpression of type II NKT cells prevents diabetes onset in NOD mice [192], indicating that both subsets of NKT cells regulate the autoimmune response against beta cells. Immune modulation by NKT cells in the context of

diabetes involves important interactions with other immune players, such as Tregs, pDC and NK cells [68, 129, 193].

1.3.3. CURRENT IMMUNE THERAPIES FOR TYPE 1 DIABETES

The first attempts of immune therapies in type 1 diabetes were based on targeting T cells. Clinical trials with cyclosporine or anti-CD3 antibodies have shown some promise in delaying diabetes progression [14, 194, 195]; however, the generalized immunosuppression exposed patients to opportunistic infections [69]. In a multi-center trial, insulin was administered orally in order to desensitize the immune system to insulin antigens; unfortunately, this strategy failed to delay or prevent type 1 diabetes [196]. Similar approaches were used with GAD and heat shock protein, two additional auto-antigens implicated in type 1 diabetes, but their efficacy and benefits were poor [197]. While the development of antigen-based vaccines is an attractive therapeutic option, in practice it would be difficult due to the extent of discovered and undiscovered autoantigens [59].

Cellular therapies are now widely considered for the treatment of a variety of immune disorders, with the idea of harnessing the regulatory properties of certain immune cells, the favourite candidate being the Treg subset. The protocol for Treg-based therapy is to use autologous cells from a patient, expand them *in vitro* and reinfuse large numbers of Tregs to the patient. The efficiency of tolerogenic DC to prevent autoimmune diabetes in mice motivated the clinical application of DC-based therapies. A current method consists in expanding and conditioning DCs *ex vivo* to generate immature DCs that are reinfused in the patient. Clinical trials using cell-based therapies are still underway to demonstrate the potential of this approach in preventing or protecting from autoimmune diabetes. Some concerns with these strategies include the non-specific nature of the immune regulation and the difficulties to generate sufficient amounts of pure populations. Moreover, induced Tregs may become pathogenic effector cells owing to their plasticity.

So far, immune therapies have proven disappointing in preventing or ameliorating type 1 diabetes due to unspecific targeting and occurrence of adverse effects. The ideal intervention to prevent beta cell destruction would provide immune tolerance specifically to islet antigens without impacting the ability to mount normal immune responses. Continued research on the

autoimmune and regulatory mechanisms in type 1 diabetes will help design more specific strategies to protect beta cells from immune destruction.

1.4. THESIS HYPOTHESIS AND SPECIFIC AIMS

Insulin therapy is life saving but only delays complications of type 1 diabetes and requires daily insulin injections and blood glucose monitoring. Current treatments do not cure or prevent diabetes because they do not tackle the cause: autoimmunity. Therapies consisting of the reintroduction of insulin-producing beta cells, either by transplantation of pancreatic islets or reprogrammed stem cells, are promising but they similarly face the problem of recurrent autoimmunity. There is a great demand for immunotherapies with limited side effects to prevent or reverse disease, as current therapies are either ineffective or unspecific. An immune therapy localized to the pancreatic islet would allow specific modulation of the immune response against beta cells.

Studies in NOD mice have pointed to a local immunoregulatory defect in pancreatic islets, evidenced by defects in Treg numbers, hyperactive DCs and Th1 bias secondary to NKT cell deficiency. Notably, similar defects were reported in patients with type 1 diabetes, at the level of circulating cells as well as in the proximity of pancreatic islets. The protective roles of Tregs, NKT cells and some subsets of DCs were demonstrated in NOD mice using approaches of systemic expansion; however, these interventions may have a generalized impact on the immune system. We believe that enhancing the activity of these cells locally may have the desirable effect of modulating the immune response against beta cells without perturbing normal immune processes. Given the importance of chemokines in guiding leukocytes, we reason that immune cells could be directed to sites of interest by taking advantage of their trafficking machinery. Hence, expression of selected chemokines in pancreatic islets or islet transplants should induce recruitment of the immune subsets that express their corresponding receptor. We hypothesize that chemokine-mediated influx of immunoregulatory cells in the islet proximity will correct the local immune defects and protect beta cells.

The overall objective of this study is to develop a strategy to modulate the immune response against beta cells via recruitment of Tregs. For this purpose, we selected the chemokines CCL22 and CCL17 that act via CCR4, a receptor that is highly expressed on Tregs. Notably, these chemokines are used by various human tumors to foster immune privilege and evade the immune response. We hypothesize that expression of CCL22 or CCL17 in islets would recruit Tregs, which would in turn prevent beta cell loss by inhibiting the autoimmune response.

To address this hypothesis, we proposed the following specific aims:

1. To determine the ability of CCL22 to protect beta cells from autoimmune destruction in models of diabetes development and recurrent autoimmunity against islet transplants.

2. To study the mechanisms of CCL22 modulatory properties in the context of recurrent autoimmune diabetes.

3. To examine CCL17's ability to prevent beta cell loss and compare its immunoregulatory properties to CCL22.

CHAPTER 2:

MATERIALS AND METHODS

2.1. ANIMALS

Animals were housed in the Children and Family Research Institute animal facility and cared for according to the guidelines of the Canadian Council on Animal Care and regulations of the University of British Columbia. NOD / ShiLtJ and NOD.scid mice were purchased from the Jackson Laboratory and bred in our facility to expand the colonies. CD1d-deficient NOD mice were donated by Dr. David Serreze at the Jackson Laboratory. Blood glucose was monitored in wild type and CD1d-deficient NOD females once per week from 8 weeks of age. The time of diabetes onset was determined as two consecutive measures above 20mM of blood glucose. Islet transplantation was performed within 10 days of diabetes onset and to maintain normoglycemia in the meantime, mice received a subcutaneous insulin implant (LinShin). For some experiments, diabetes was induced in NOD.scid females using streptozotocin (STZ), a widely used drug that is specifically toxic to beta cells. NOD.scid females received a single intraperitoneal injection of 200 mg of STZ per gram of body weight and were transplanted 4 days later if blood glucose levels were above 20mM.

2.2. ISLET ISOLATION AND TRANSPLANTATION

For syngeneic islet transplantation in wildtype or CD1d-deficient NOD mice, pancreatic islets were isolated from NOD.scid donors by collagenase (Sigma) injection in the pancreatic duct, oscillating digestion and purification by filtration through 400-600 µm filter as described previously [198]. Islets were hand-picked and cultured in complete RPMI made with RPMI 1640 (Gibco), 1% glutamax (Gibco), 1% 100 units/ml penicillin (Sigma) and 100µg/ml streptomycin (Sigma) and 10% fetal bovine serum (FBS, from Gibco). On the following day, 500 islets were transplanted into the renal subcapsular space of diabetic recipients under isofluorane anaesthesia. The time of graft failure was determined by measurement of blood glucose >20mM on two consecutive days.

2.3. GENERATION OF VIRUSES

Recombinant double strand adeno-associated viruses of serotype 8 (AAV8) were generated at the Children's Hospital of Philadelphia by triple transfection protocol as previously described [199]. We selected the rat insulin promoter (RIP) because its small size allows the use of double-stranded AAV8, which are more efficient than single-stranded AAV8 for pancreatic transduction [200]; moreover, RIP has been used successfully in a number of studies to target beta cells in mice. The AAV8-RIP/GFP plasmid was provided by Paul Robbins (University of Pittsburgh, Pittsburgh, Pennsylvania, USA). The AAV8-RIP/CCL22 construct was made by replacing GFP with murine CCL22 cDNA obtained from Invitrogen.
To generate adenoviral vectors Ad-CCL22 and Ad-CCL17, CCL22 and CCL17 cDNAs were cloned in pShuttle$^{+/+}$ plasmid downstream of the cytomegalovirus (CMV) promoter. Adenoviruses were constructed by cotransfection of HEK 293 cells with CCL22 or CCL17 vectors and the Adenovirus helper plasmid (ABM Good). Adenoviruses were purified with Vivapure Adenopack 100 Kit (VivaScience) and titrated by the end-point dilution method of tissue culture infective dose. A control vector containing the β-galactosidase gene (Ad-LacZ) was kindly provided by Dr. Timothy Kieffer (University of British Columbia).

2.4. ADMINISTRATION OF VIRAL VECTORS

AAV8 vectors were infused into the pancreatic duct as described previously [200]. Briefly, one microclamp was applied to the sphincter of Oddi to avoid leakage of the vector into the duodenum, and another to the proximal part of the common bile duct to prevent vector reflux into the liver or gall bladder. Then, a 30-gauge catheter was inserted into the pancreatic duct and 100 µl of AAV8 vector or phosphate buffered saline (PBS) were slowly injected. A drop of tissue adhesive was applied to the site of injection to seal it and prevent leakage in the peritoneal cavity. Prediabetic NOD females received 1.5 x 10^{11} viral particles of AAV8-RIP/CCL22 or AAV8-RIP/GFP, or PBS alone at 8 weeks of age.
Adenoviruses were administered in the culture of isolated islets and left for overnight transduction at 37°C. The multiplicity of infection used for adenoviruses were calculated assuming that one islet contains an average of 1000 cells. Islets were washed twice with complete RPMI prior to transplantation.

2.5. ASSESSMENT OF ISLET FUNCTION

In vitro function of transduced islets was assessed by glucose stimulated insulin secretion 20 hours post-infection. Islets were pre-incubated in triplicates for 1 hour in Krebs-Ringer Buffer (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1mM MgCl2, and 2.5 mM CaCl2, pH 7.4) with 0.1% BSA and 1.67 mM glucose at 37°C. Islets were then incubated with low (1.67mM) or high (16.7mM) glucose concentration for 1 hour. The supernatant was recovered and islets were lyzed in glacial acetic acid with 1% BSA. Insulin levels were determined with Mercodia Mouse Insulin ELISA kit.

In vivo islet function after AAV8 administration or islet transplantation was assessed by intraperitoneal glucose tolerance test. Mice were fasted for 5 hours and injected with 2g per kg of body weight D50 glucose (Baxter Healthcare), and blood glucose was measured at 0, 15, 30, 60, 90, and 120 min.

Islet viability after transduction with Ad-CCL22 was determined with the Molecular Probes cytotoxicity kit according to the manufacturer's protocol. The fluorescent red dye Ethidium homodimer-1 was used to detect damaged cells. For experiments with Ad-CCL17, we used the Invitrogen TUNEL assay to determine cell death after overnight infection. Following fixation and permeabilization, fragmented DNA was labeled with fluorescein-dUTP, thus apoptotic cells appeared green. Islets were visualized with an Olympus 1x71 microscope and Delta-Vision system camera.

2.6. ADMINISTRATION OF CD25 ANTIBODY *IN VIVO*

For depletion of Tregs *in vivo*, CD25 neutralizing antibody (PC61) and an isotype control (IgG) were obtained from AbLab (University of British Columbia). Mice were injected intraperitoneally with 100 µg of PC61 or IgG every week following treatment with AAV8-RIP/CCL22 or after islet transplantation. We observed durable and significant reduction (~90%) of Tregs from the circulation and tissues in mice treated with PC61, despite the persistence of a small proportion of Tregs with low levels or no CD25, as described previously [201].

2.7. DETERMINATION OF CHEMOKINE PROTEIN LEVELS

Chemokine expression after viral transduction was determined in islets infected with Ad-CCL22 or Ad-CCL17, as well as islets isolated from AAV-RIP/CCL22 treated mice 1 month post-injection. CCL22 and CCL17 were quantified in the supernatant of islets cultured overnight using Quantikine ELISA kits from R&D Systems.
CCL22 expression in islet grafts was also determined by Western blot. Ten micrograms of protein from islet lysates were resolved on a 20% SDS-polyacrylamide gel at 100 volts. After transfer to Immobilon-P membranes (Millipore) at 100 volts for 1 hour using the Trans-Blot System (Bio-Rad), immunoblot analysis was performed using polyclonal anti-CCL22 antibody (R&D Systems) and detected by chemiluminescence (Amersham).

2.8. HISTOLOGICAL ANALYSIS

Following excision, tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin and cut in sections of 5 µm. Tissue sections were deparaffinized in xylene, rehydrated in graded ethanol and washed in PBS. Antigen retrieval was necessary prior to immunostaining for CD45 and FoxP3 (10 mM citrate buffer; pH 6.0). Sections were incubated for 1 hour with blocking buffer containing 5% goat serum followed by incubation with primary antibodies for 1 hour at room temperature or overnight at 4°C. Sections were washed and incubated with respective secondary antibodies for 1 hour at room temperature. The primary antibodies used were guinea pig anti-insulin (Dako), rabbit anti-glucagon (Dako), rat anti-mouse CD45 (BD Pharmingen) and rat anti-mouse FoxP3 (Ebioscience). The secondary antibodies used for immunofluorescence included Alexa Fluor 594 anti-guinea pig or rabbit (Molecular Probes) and Alexa Fluor 488 anti-rat (Molecular Probes). For FoxP3 immunohistochemistry, we applied biotinylated anti-rat (Biolegend) then Streptavidin/HRP (Dako), followed by DAB substrate (Biogenex) and hematoxylin counterstaining (Invitrogen). All antibodies were diluted in PBS with 1% bovine serum albumin (BSA). Immunostained slides were washed, dehydrated and mounted with Cytoseal (Thermo Scientific). Images were captured using an Olympus Bx61 microscope and *In Vivo* or DP software.

2.9. IMMUNE CELL ISOLATION FROM TISSUES

Pancreata were excised and digested with 1,000 U/ml of collagenase (Sigma) in Hank's buffer solution (Life Technologies) for 20 minutes at 37°C. The resulting cell homogenate was filtered through a 40 µm filter basket and washed in PBS. Immune cells were further isolated on a Ficoll gradient (GE Healthcare) by centrifugation during 30 minutes at 2000rpm. Harvested grafts, lymph nodes and spleens were strained through a 40 µm mesh, washed and resuspended in PBS. The spleen suspension was further treated with 0.155M NH_4Cl (Sigma) + 10mM $KHCO_3$ (Fisher) + 10uM Na_2EDTA (Fisher) for 2 minutes to lyse red blood cells and then washed with PBS.

For some *in vitro* experiments, we further isolated immune populations with EasySep kits from Stemcell, which use a magnetic bead system to select the populations of interest. Cells populations can be isolated from any type of tissue and used for downstream applications such as tissue culture or flow cytometry. Using the kits for CD4 pre-enrichment and CD25 positive selection, we obtained a purity of 90% for the $CD4^+$ $CD25^{high}$ population (Tregs), as well as $CD4^+$ $CD25^-$ (conventional $CD4^+$) cells at 93% pure. Dendritic cells were isolated with the CD11c positive selection kit with an average purity of 85%.

Viable cells were counted using a hemacytometer (Baxter Scientific) and Trypan Blue exclusion dye (Stemcell).

2.10. FLOW CYTOMETRY, ANTIBODIES AND TETRAMERS

For immunostaining, cells were resuspended in PBS with 1mM EDTA (Fisher) and 10% FBS (FACS buffer). The antibodies used in these studies are listed in Table 1. Cells were incubated with antibodies for surface markers during 30 minutes at 4°C and washed with FACS buffer. To detect antigen-specific populations, cells were stained with tetramers for 45 minutes at 4°C. For intracellular staining of FoxP3, we used the Cytofix/Cytoperm kit from BD Bioscicences to fix, permeabilize and stain the cells. Fluorescent counting beads (Invitrogen) were used to determine the absolute number of cells in samples. Briefly, a certain amount of beads was added to samples and data acquisition was set to stop when a minimum of 5000 beads were counted. The absolute number of cells was calculated with the following formula: number of events in desired gate / number of beads counted x number of beads added. Data were

acquired with FACSCalibur and a LSR II (BD Biosciences) cytometers and CellQuest software. Data were then analyzed using FlowJo7.6 software.

Antibody/Reagent	Clone	Fluorochromes	Manufacturer
CCR4	2G12	PE, APC, PE-Cy7	Biolegend
CD3	17A2	APC-Alexa Fluor 780	Ebioscience
CD4	RM4-5	Alexa Fluor 700, PerCP-Cy5.5, PE-Cy5	BD, Ebioscience
CD8	53-6.7	APC, PE-Cy7	BD, Ebioscience
CD11b	M1/70	Alexa Fluor 700	Ebioscience
CD11c	N418	PE, PerCP-Cy5.5	Ebioscience
CD19	eBio1D3	Alexa Fluor 700	Ebioscience
CD25	PC61	APC	BD
CD44	IM7	Alexa Fluor 488	Biolegend
CD49b	HMa2	FITC	Ebioscience
CD62L	MEL-14	PerCP-Cy5.5	Biolegend
CD69	H1.2F3	FITC, PerCP-Cy5.5	Ebioscience, Biolegend
CD86	GL1	PE-Cy5	Ebioscience
CTLA-4	UC10-4B9	PE, APC, Brilliant Violet 421	Ebioscience, Biolegend
FoxP3	FJK-16s, MF23	Alexa Fluor 488, 647	Ebioscience, BD
ICOS	C398.4a	FITC	Ebioscience
PDCA-1	eBio927	APC	Ebioscience
Insulin H-2kd tetramer		PE	Core facility at CFRI, UBC
IGRP H-2kd tetramer		PE	Core facility at CFRI, UBC
BDC2.5 MHC class II tetramer		PE	NIH tetramer facility
αGalCer CD1d tetramer		PE, APC	NIH tetramer facility

Table 3: Antibodies for flow cytometry

2.11. CELL CULTURE AND CHEMOKINE TREATMENT

To study the impact of chemokines on cell activation and function, whole splenocytes or isolated cells populations were resuspended in RPMI with 1% glutamax, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were seeded at a density of 0.5-1 x 10^6 cells/ml in 96-well plates (BD Biosciences) and treated with chemokines for 6, 24 or 48 hours. Recombinant CCL22 and CCL17 were purchased from R&D Systems and reconstituted in PBS with 0.1% BSA. Chemokines were used at concentrations ranging from 100 to 3000 pg/ml.
For co-culture experiments, Tregs ($CD4^+$ $CD25^{high}$) and conventional CD4 ($CD4^+$ $CD25^-$) were isolated and pretreated with 500 pg/ml of CCL22. After 48h, DCs were freshly isolated and

added to the culture at a ratio of 1:2. Cells were harvested 2 days later for analysis by flow cytometry or real time PCR.

2.12. CHEMOTAXIS ASSAYS

Chemotaxis assays were performed using 5µm pores Transwell plates from Millipore. The media used in these experiments was RPMI with 1% glutamax, 100 units/ml penicillin and 100µg/ml streptomycin. The chemotactic stimuli or media alone was added in the bottom well and the upper chamber was seeded with 10^5 cells. As chemotactic stimuli, we used recombinant CCL22 or CCL17 at various concentrations, as well as 100 islets transduced overnight with Ad-CCL22 or Ad-CCL17. Cells were left to migrate at 37°C for 4 hours, except for a time course analyzed at 1, 2 and 4 hours. The upper wells were aspirated and 25 mM of EDTA was added for 5 minutes to detach migratory cells from the membrane. The migratory fraction was recovered and resuspended in FACS buffer for cell identification and quantification. Migration index was calculated as the number of cells migrating toward the chemotactic stimuli divided by the number of cells migrating toward medium only.

2.13. REAL TIME PCR

Tissues or cultured cells were placed in RNAlater stabilization reagent (Qiagen) until RNA isolation could be performed. Total RNA was extracted using the RNeasy micro kit from Qiagen. Complementary DNA was prepared using the SuperScript III Reverse Transcriptase (Invitrogen) and random primers (Invitrogen). The Applied Biosystems 7500 System was used to monitor real time amplification of cDNA with the EvaGreen Mastermix (ABM). The primers used for real time PCR and listed in table 2 were designed with OligoPerfect designer from Invitrogen. Relative quantification of gene expression was performed using the housekeeping genes gapdh and 36b4 as a controls. The comparative Ct (cycle threshold) method was used for relative quantification of mRNAs of interest. The Ct value is defined as the cycle number in which the detected fluorescence exceeds the threshold value. The fold difference was calculated as follows:

$$\text{Fold difference} = 2^{-\left(Ct_1(\text{gene of interest}) - Ct_1(36b4)\right) - \left(Ct_2(\text{gene of interest}) - Ct_2(36b4)\right)}$$

Where Ct_1 (gene of interest) and Ct_1 (36b4) represent the Ct values for the treated samples; Ct_2 (gene of interest) and Ct_2 (36b4) represent the Ct values for the untreated or control samples.

Gene	Forward primer	Reverse primer
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC
36B4	ACTGGTCTAGGACCCGAGAAG	TCCCACCTTGTCTCCAGTCT
IFNγ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTGTGCAGGATTTTCATG
TGFβ	CACCGGAGAGCCCTGGATA	TGTACAGCTGCCGCACACA
IL-10	CAGAGCCACATGCTCCTAGA	GTCCAGCTGGTCCTTTGTTT
IL-17	CAGGGAGAGCTTCATCTGTGT	GCTGAGCTTTGAGGGATGAT
Granzyme B	GCTGCTCACTGTGAAGGAAGT	TGGGGAATGCATTTTACCAT
IDO	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
CD11c	CTGGATAGCCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA

Table 4: Primers for real time PCR

2.14. STATISTICAL ANALYSIS

Data in graphs are represented as means +/- standard error of the mean (SEM). Survival curves were generated using Kaplan-Meier life-table analysis and compared using the log-rank test. Student's t-test was used to compare between two groups and one-way ANOVA to compare more than two groups, with the Dunnett post-test for comparison to a control column. All statistical analysis were performed with the Graph Pad software and differences were considered significant when $p<0.05$.

CHAPTER 3:

CCL22 EXPRESSION IN ISLETS PROTECTS FROM DIABETES AND RECURRENT AUTOIMMUNITY

3.1. INTRODUCTION

Defects in Treg numbers and/or function underlie development of a number of autoimmune disorders, including type 1 diabetes. Correction of these deficiencies by systemic infusion of Tregs or promotion of their function has been demonstrated to prevent both diabetes development and islet graft rejection in NOD mice [180, 202]. Although successful, these strategies are nonspecific, and increase the risk of malignancies and infections due to the suppression of normal immune responses. The ideal Treg-based therapy would be specific for the tissue undergoing autoimmune attack. One way of achieving specific tolerance is to direct Tregs to the pancreas or islet graft, thereby compensating for the local Treg deficit [172] and suppressing the immune response against beta cells.

Chemokines control leukocyte trafficking to peripheral tissues and lymphoid organs, and coordinate immune responses through the differential expression of chemokine receptors. Like other immune cells, Tregs express several chemokine receptors involved in homing to lymph nodes and sites of inflammation, such as CCR2, CCR5 and CCR7 [203]. Unlike most other immune cells, Tregs are characterized by constitutive high levels of CCR4 and preferentially migrate to the CCR4 ligand CCL22, which is secreted by mature DCs and macrophages under steady-state conditions [204]. Originally described as monocyte-derived chemokine (MDC), CCL22 may also be produced by NK, B and T cells during inflammatory processes [205, 206]. CCL22 allows antigen-presenting cells to interact with $CCR4^+$ T cells [207], especially Tregs given their high levels of CCR4 expression, and appears to promote Th2 immune responses [208]. Interestingly, CCL22 expression has been implicated in the recruitment of Tregs towards a variety of tumors, such as Hodgkin lymphomas, ovarian and gastric cancers [159, 209, 210]. Tregs recruited by the tumor create an immunosuppressive microenvironment that allows the tumor to evade the immune response and leads to an adverse clinical outcome. We hypothesized that CCL22 expression in islets would likewise foster immune privilege to beta cells and islet grafts by attracting Tregs locally. We proposed to determine whether CCL22 expression in islets is able to protect from diabetes and recurrent autoimmunity.

Recurrent autoimmunity is commonly studied in the well-established model of syngeneic islet transplantation in NOD mice. Following spontaneous onset of diabetes, NOD females receive islets from MHC-compatible NOD.scid donors. Accordingly, the alloimmune response is absent and islet grafts are primarily destroyed by recurrent autoimmunity against beta cells [61, 211,

212]. To induce the expression of CCL22 in pancreatic beta cells and islet transplants, we chose two viral vectors with distinct applications. The first vector is an adenovirus, which efficiently transduces many cell types (including beta cells) *in vitro* [213]. Moreover, our laboratory has previously used adenoviral vectors to successfully overexpress genes of interest in islet grafts [214]. The second vector is an adeno-associated virus of serotype 8 (AAV8), which has the ability to infect islets *in vivo*, and therefore is suitable for CCL22 delivery to the pancreas of NOD females [200].

3.2. RESULTS

3.2.1. CCL22 EXPRESSION IN ISLETS AFTER VIRAL TRANSDUCTION

To induce CCL22 expression in cultured islets, we generated an adenoviral vector (Ad-CCL22) containing a mouse CCL22 cDNA downstream of the cytomegalovirus (CMV) promoter to drive constitutive gene expression. A similar construct expressing β-galactosidase (LacZ) was used as a control vector. Different multiplicities of infection (MOI) were tested to determine the appropriate dose of virus to obtain significant CCL22 production by islets without altering their viability or function. Following infection, islets produced CCL22 in a dose-dependent manner; however, islet toxicity was observed with the highest dose of Ad-CCL22 (Figure 5A, B). Islets transduced overnight with an MOI of 10 secreted levels of CCL22 (~4.6 pg/ml per islet) similar to what DC produce in culture [215], while retaining a normal insulin response to glucose stimulation (Figure 5C). From these data it would be predicted that a graft of 500 islets would produce CCL22 amounts comparable to those found in the tumor microenvironment [159, 216].

For gene delivery to pancreatic islets *in vivo*, we used a recombinant double-stranded AAV8 vector carrying the CCL22 gene under the control of the rat insulin promoter (RIP) to induce CCL22 expression specifically in beta cells. By injection of 1.5×10^{11} viral particles into the pancreatic duct, AAV8 enables local delivery and long-term gene expression within islets throughout the pancreas [200], thus avoiding unnecessary exposure of the entire body to virus. To assess CCL22 expression in islets and the impact on islet function, NOD.scid mice were injected with AAV8-RIP/CCL22 or the control vector containing GFP. One month post-injection, high levels of CCL22 were detected in isolated islets (Figure 6A). As previously reported,

expression of the gene delivered by AAV8 gradually increased until 2 weeks post-injection and persisted over 6 months [200]. Treated animals displayed normal glucose tolerance 2 and 4 months post-injection, indicating that CCL22 expression does not alter islet function (Figure 6B).

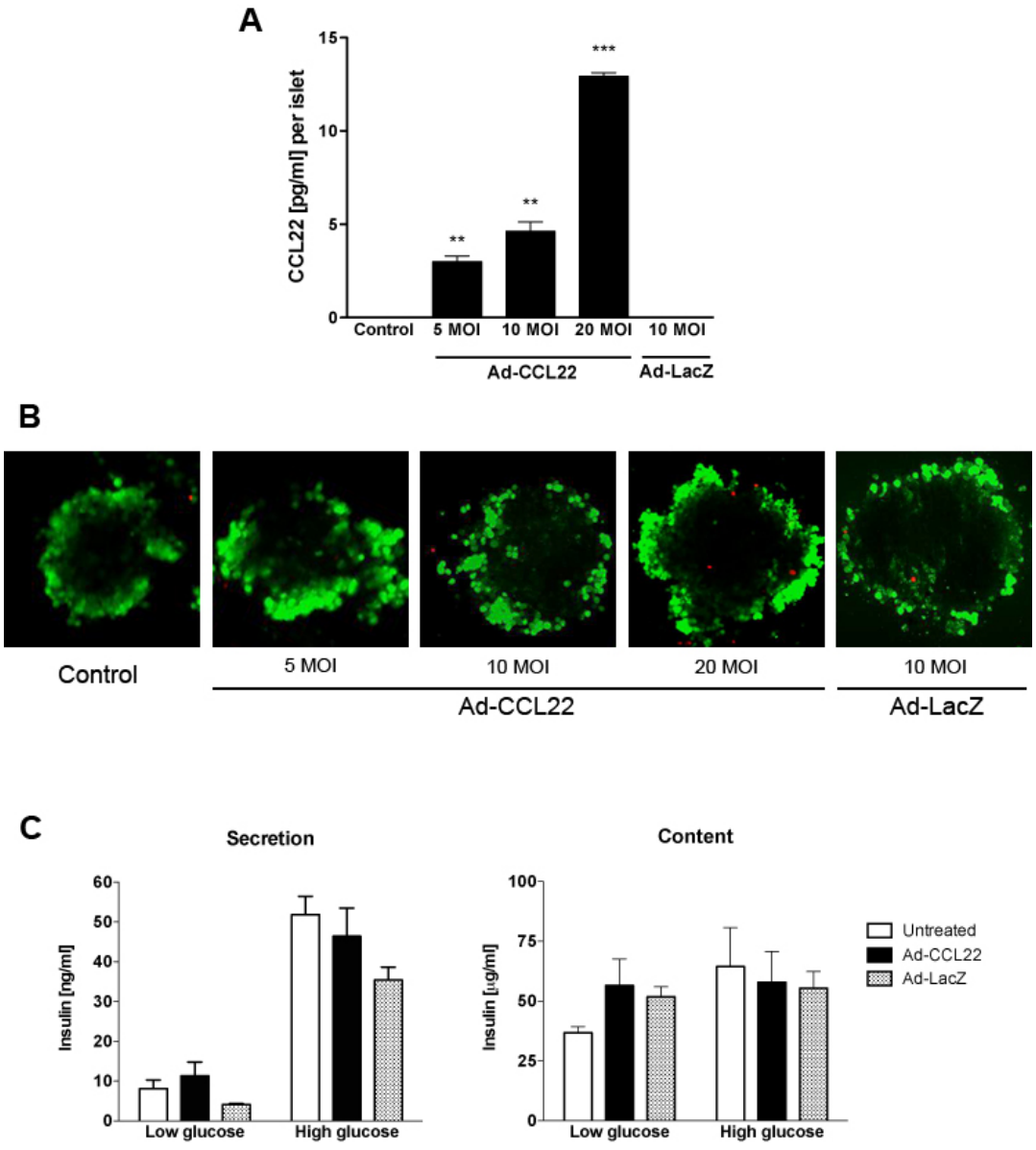

Figure 5: Gene delivery to cultured islets with Ad-CCL22

A. Islet secretion of CCL22 after transduction with Ad-CCL22. CCL22 was produced in a dose-dependent manner in islets infected with increasing MOI of Ad-CCL22, but was absent in cultures of untreated (control) and Ad-LacZ transduced islets. **p<0.01 and ***p<0.001 versus control. **B.** Islet viability following infection was determined by live/dead cell assay. Toxicity was observed in islets transduced with 20 MOI of Ad-CCL22 as shown by the higher number of dead islet cells (red). Representative pictures are shown for the different conditions. **C.** *In vitro* islet function after transduction with 10 MOI of Ad-CCL22 or Ad-LacZ was assessed by glucose-stimulated insulin release at low (1.67mM) and high (16.7mM) glucose concentrations. Islets transduced with Ad-CCL22 exhibit normal insulin secretion comparable to untreated islets. Insulin levels in the supernatant and islet lysates were determined by ELISA. p=NS.

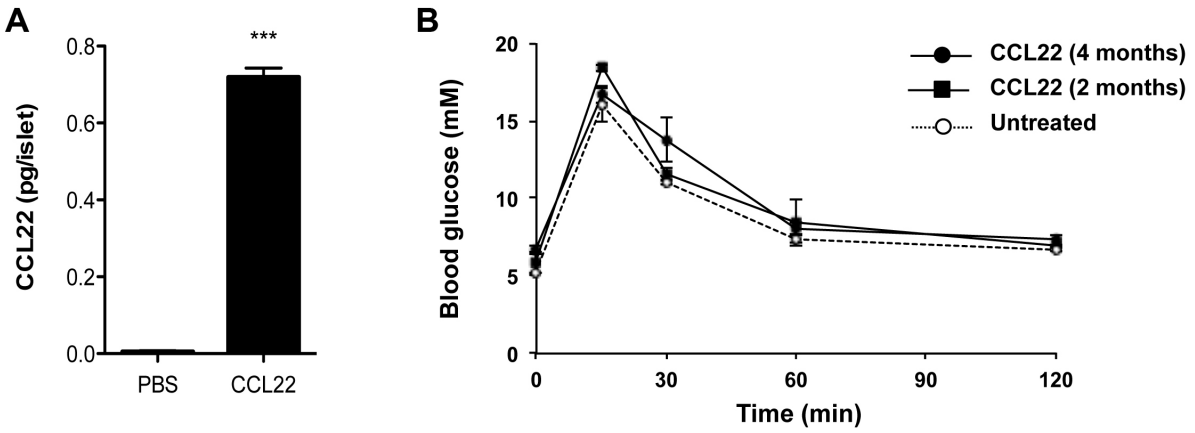

Figure 6: Gene delivery *in vivo* with AAV8-RIP/CCL22

A. CCL22 expression in pancreatic islets of NOD.scid mice was measured 1 month after injection of AAV8-RIP/CCL22 or PBS in the pancreatic duct. N=4 for both PBS and CCL22 treated NOD.scid mice. ****p*<0.001 versus PBS. **B.** *In vivo* islet function was determined 2 and 4 months after CCL22 gene transfer in NOD.scid mice by intraperitoneal glucose tolerance test. No significant difference in glucose tolerance was observed after intraductal injection and CCL22 expression. Results are expressed as mean ± SEM. N=4 for each group.

3.2.2. CCL22 PROTECTS BETA CELLS FROM RECURRENT AUTOIMMUNITY

Syngeneic islet transplantation in NOD mice is a very stringent model of recurrent autoimmune diabetes characterized by rapid graft loss and resistance to tolerance [217]. To determine the ability of CCL22 to protect islet grafts from recurrent autoimmunity, diabetic NOD females were transplanted with 500 NOD.*scid* islets that were transduced *ex vivo* with either Ad-CCL22, Ad-LacZ or left untreated.

Survival of syngeneic islet grafts in diabetic NOD recipients was markedly improved in mice transplanted with islets expressing CCL22 compared to Ad-LacZ transduced and untreated islets (Figure 7A). Approximately half of the grafts expressing CCL22 maintained normoglycemia at 6 weeks post-transplant, whereas all control grafts had failed within 2 weeks. Protection of CCL22-expressing grafts from recurrent autoimmunity was not permanent, as hyperglycemia returned in all recipients of CCL22-expressing grafts by 60 days post-transplantation. One possible explanation for the observed transient protection of syngeneic islet transplants expressing Ad-CCL22 is gradual loss of adenovirus and islet CCL22 expression [218, 219]. Indeed, NOD.scid islets transduced with Ad-CCL22 and transplanted into NOD.scid recipients with streptozotocin (STZ)-induced diabetes showed a gradual loss of

CCL22 expression over the first 40 days post-transplantation (Figure 7B), suggesting that sustained chemokine secretion may be required for long-term protection.

Histological analysis of islet grafts 10 days post-transplantation revealed many insulin-producing beta cells in CCL22-expressing grafts, but virtually none in controls (Figure 7C). We also noted a decrease in glucagon-producing alpha cells in grafts transduced with Ad-LacZ or left untreated, in accordance with a recent report [220]. Comparable immune infiltration was found in all groups of recipients, as shown by CD45 immunostaining. These observations suggest that islet CCL22 expression protects beta cells from recurrent autoimmunity and alpha cells from bystander injury, without blocking immune infiltration.

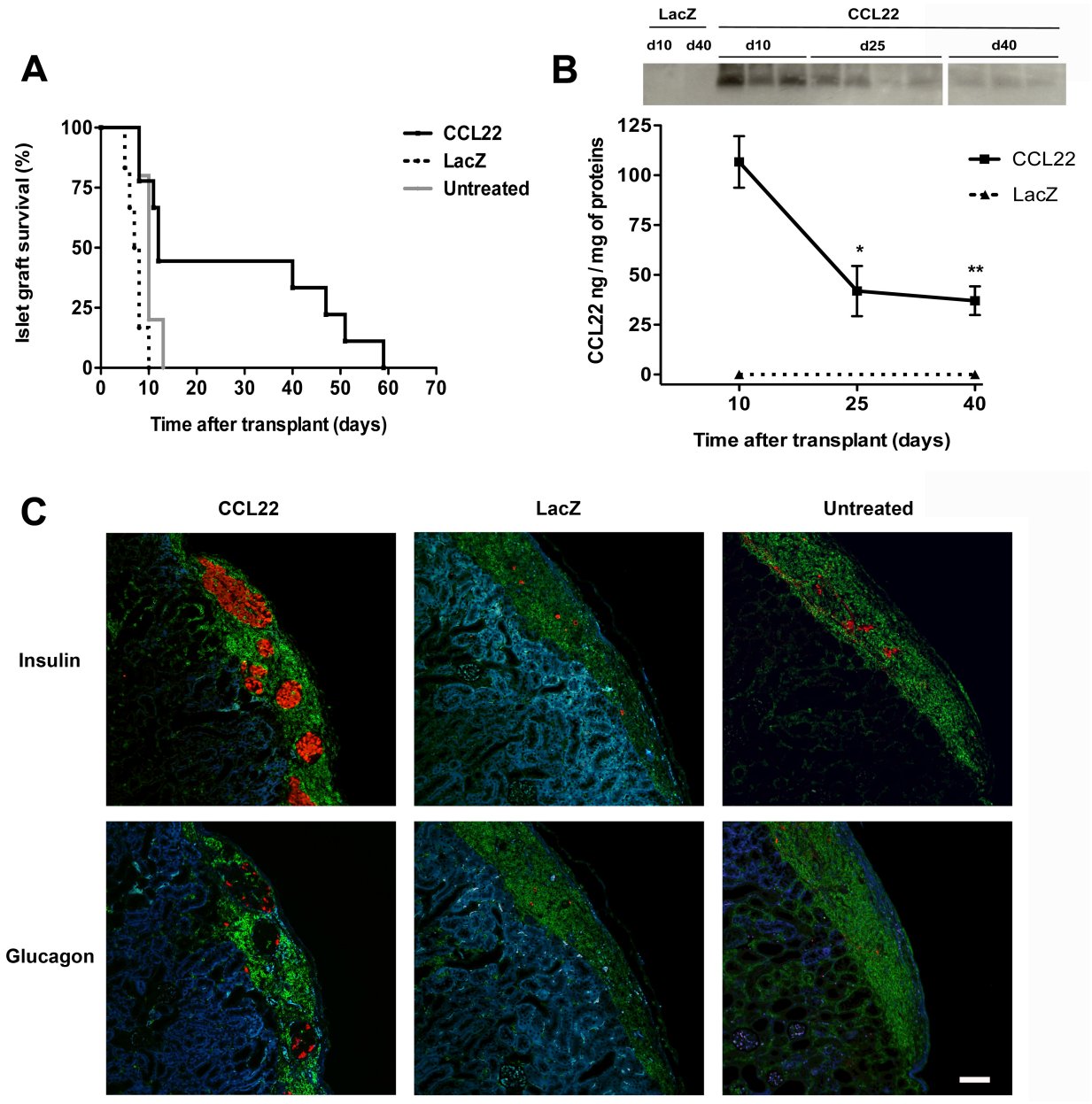

Figure 7: CCL22 expression in islet grafts protects beta cells from recurrent autoimmunity

A. Diabetic NOD mice were transplanted with 500 islets transduced with 10 MOI of Ad-CCL22 (n=9) or Ad-LacZ (n=6), or left untreated (n=5). Survival curves were compared using the log-rank test. $p<0.01$ versus untreated and LacZ. **B.** CCL22 expression in the islet graft over time was determined by western blot (upper panel) and ELISA (lower panel) in NOD.scid recipients of islets transduced with Ad-CCL22 or Ad-LacZ. We observed a ~70% decline in CCL22 levels by day 40. Results are expressed as mean ± SEM. *$p<0.05$ and **$p<0.01$ versus CCL22 at day 10. **C.** Immunostaining of sections of islet grafts 10 days post-transplantation for insulin- or glucagon-positive cells (red), and $CD45^+$ cells (green). Pictures are representative of all grafts examined from each treatment group. Scale bar is 100 µm.

3.2.3. CCL22 PROTECTS FROM AUTOIMMUNE DIABETES

To determine whether beta cell specific expression of CCL22 can delay or prevent diabetes, prediabetic 8-week old NOD females were injected with AAV8-RIP/CCL22, AAV8-RIP/GFP or PBS via the pancreatic duct. A prerequisite for our approach to succeed is a sufficient number of beta cells at the time of gene delivery. In 8-week old NOD females, insulitis is already present but at least 70% of the beta cell mass remains [221, 222]. NOD mice transduced with AAV8-RIP/CCL22 (CCL22-NOD) were protected from diabetes, with 9 of 11 mice remaining normoglycemic up to 160 days, whereas all GFP-NOD and PBS-NOD mice developed diabetes within the first 100 days (Figure 8A). To compare islet infiltration and beta cell survival in CCL22-NOD and PBS-NOD mice, pancreas sections were analyzed by histology at different times. Despite extensive insulitis in both groups, CCL22 expression was associated with preservation of insulin-positive cells at 15 days, 1 month, and 3 months after intraductal injection (Figure 8B). These data indicate that CCL22 expression in pancreatic islets protects beta cells from autoimmune attack and prevents diabetes in NOD mice.

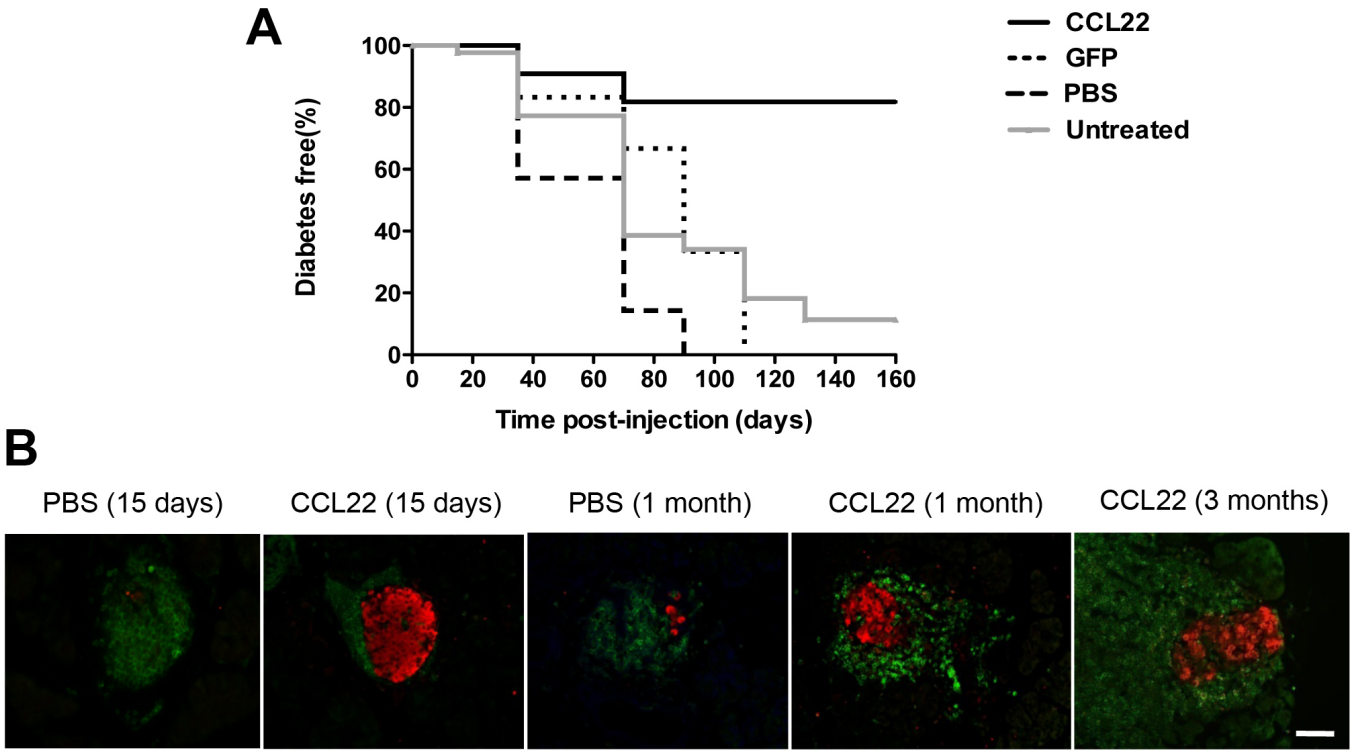

Figure 8: CCL22 expression in pancreatic islets protects from diabetes development
A. NOD females were injected at 8 weeks of age with 1.5 x 10^{11} vp of AAV8-RIP/CCL22 or AAV8-RIP/GFP, or PBS (as control) via the pancreatic duct. Kaplan-Meier survival curves were derived from blood glucose data and compared using the log-rank test. p=0.001 versus untreated NOD, p<0.001 CCL22 versus PBS; p<0.01 versus GFP. **B.** Immunostaining for insulin-positive beta cells (red) and islet-infiltrating leukocytes by CD45 staining (green) of PBS- and CCL22-NOD mice at indicated time points. Scale bar is 100 µm.

3.2.4. CCL22 ENHANCES INFLUX OF TREGS TO ISLETS

To verify that CCL22 expression recruited Tregs in our models of recurrent autoimmunity and diabetes, we examined the presence of Tregs in islet grafts and pancreata. Immunostaining of islet graft sections showed greater numbers of FoxP3$^+$ cells surrounding CCL22-expressing islets compared to controls (Figure 9A, B). Analysis by flow cytometry confirmed this observation, demonstrating a 7-fold increase in the frequency of Tregs in the islet graft but not in the draining lymph node (Figure 9C). Interestingly, Tregs present in CCL22-expressing grafts expressed higher levels of CCR4 than Tregs in controls (Figure 9D), suggesting that CCL22

recruits the cells with highest expression of its receptor, or induces upregulation of CCR4 on Tregs.

Likewise, pancreata from CCL22-NOD mice appeared to contain more $FoxP3^+$ cells than PBS-NOD animals at 15 days, 1 month, and 3 months after injection (Figure 10A). The frequency of $CD4^+$ $FoxP3^+$ cells in the pancreas of CCL22-NOD mice (but not in the pancreatic lymph node) was significantly elevated by 1 month after injection and more markedly elevated at 3 months, as determined by flow cytometry (Figure 10B). Accordingly, the absolute number of Tregs was elevated in CCL22-expressing pancreata 1 month post-injection (Figure 10C).

These results indicate that CCL22 expression in islet grafts and pancreatic islets enhances the influx of Tregs, consistent with observations from tumors that produce the same chemokine. Tregs are primarily found in the vicinity of CCL22-expressing islets while their frequency remains unchanged in the draining lymph nodes hence their accumulation appears to be specific for the site of CCL22 expression.

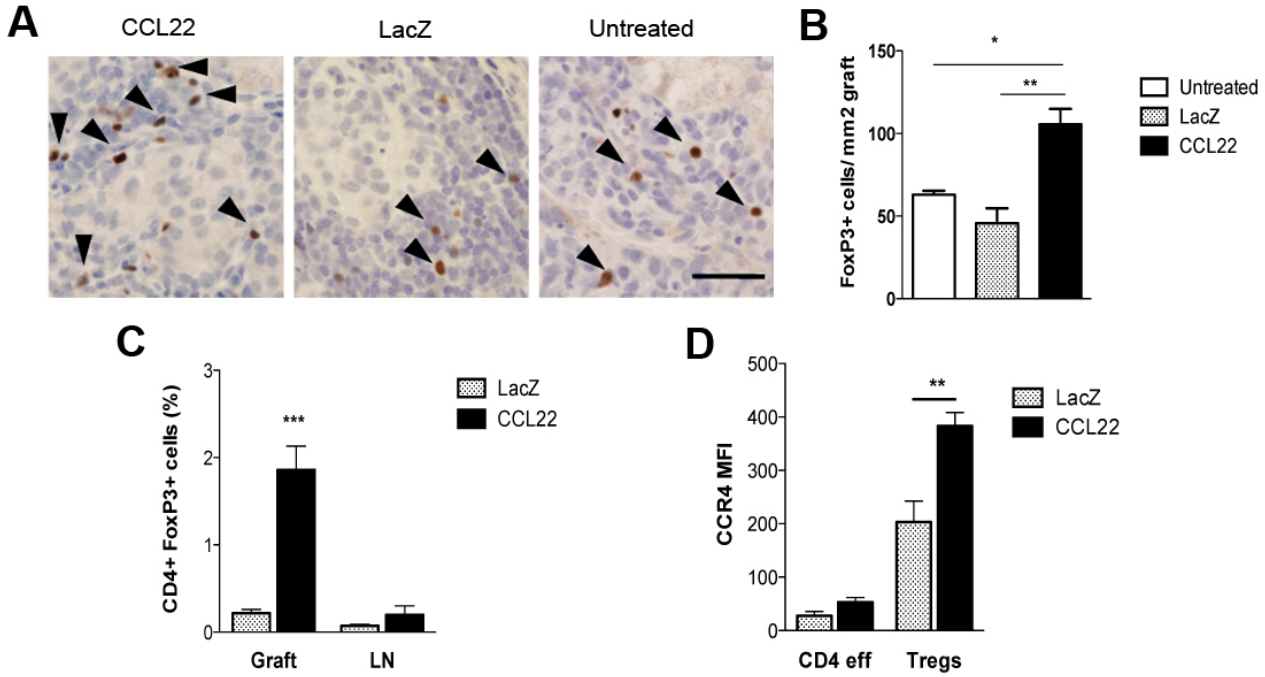

Figure 9: Tregs are recruited by CCL22-expressing islet grafts

A. Islet grafts were immunostained for FoxP3$^+$ cells (arrows) 10 days post-transplantation. Images are representative of all grafts examined from each treatment group. Scale bar is 100 µm. **B.** The number of FoxP3$^+$ cells was determined as a proportion of infiltrated graft area on sections immunostained for FoxP3. One representative section from each graft was analyzed (CCL22: n=4; LacZ n=3; control: n=2). *p<0.05 and **p<0.01. **C.** The frequency of Tregs in islet grafts and draining lymph nodes (LN) of CCL22 and LacZ recipients were quantified by flow cytometry 10 days post-transplantation. ***p<0.001 versus LacZ. **D.** CCR4 expression by CD4$^+$ FoxP3$^-$ effector cells and CD4$^+$ FoxP3$^+$ Tregs was examined in the islet graft 10 days post-transplantation. Tregs in CCL22 grafts (n=7) express higher levels of CCR4 than in LacZ grafts (n=3). Data shown as mean fluorescence intensity (MFI) of CCR4 in indicated cell populations. **p=0.01.

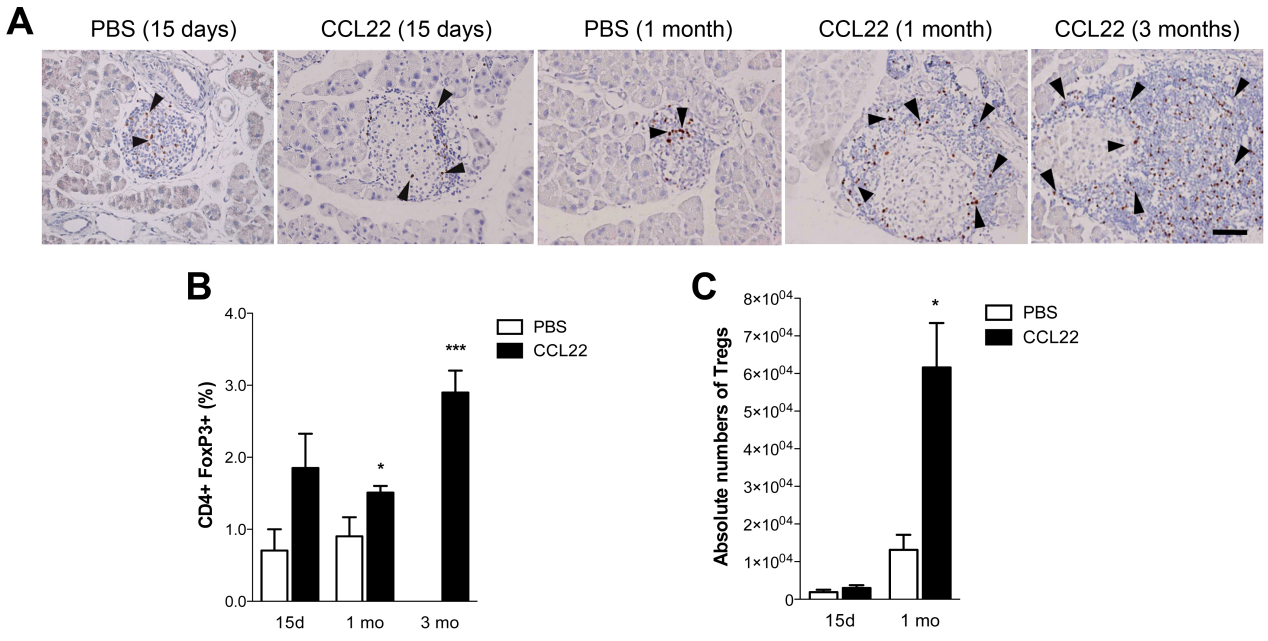

Figure 10: CCL22 expression in pancreatic islets attracts Tregs

A. Pancreas sections of PBS- and CCL22-NOD mice were immunostained for FoxP3$^+$ cells at indicated times. We observed the presence of FoxP3$^+$ cells (arrows) surrounding islets. Scale bar is 100 µm. **B.** The frequency of Tregs in the pancreas of PBS- and CCL22-NOD mice was analyzed by flow cytometry 15 days, 1 month and 3 months post-injection. *p<0.05 and ***p<0.001 versus PBS at 1 month. **C.** The absolute number of Tregs in the pancreas was determined at different times after injection of PBS or CCL22 viral vector. At 1 month post-injection, the number of Tregs was 3 times higher in the pancreas of CCL22-NOD compared to PBS-NOD (*p<0.05).

3.2.5. TREGS ARE REQUIRED FOR CCL22 PROTECTION FROM AUTOIMMUNITY

To determine if CCL22 mediates protection from diabetes and recurrent autoimmunity via Tregs, we administered CD25 depleting antibodies (PC61) or isotype controls to mice throughout the duration of the experiment. Several groups have used this method to efficiently deplete Tregs *in vivo* [223, 224], since CD25 is highly expressed on Tregs and is required for their survival [176]. Administration of CD25 antibody caused depletion of CD4$^+$ CD25^{high} cells from the circulation and tissues of treated mice. Importantly, this treatment reversed the protective effect of CCL22 in both the recurrent autoimmunity and diabetes models, indicating that it is dependent on Tregs. Indeed, islet transplants rapidly failed in recipients treated with PC61, whereas mice treated with isotype control had comparable islet graft survival to untreated recipients of CCL22-expressing grafts (Figure 11A). In NOD mice, administration of

this antibody does not accelerate diabetes, however NOD-CCL22 females are no longer protected from autoimmune diabetes (Figure 11B).

Treatment with CD25 antibody may not exclusively deplete Tregs as other cells have been shown to express CD25, mainly effector T cells, but also B and T cell precursors [225, 226]. CD25 is transiently expressed by effector T cells upon activation albeit at lower levels than Tregs, and is absent on naïve and memory T cells [227]. We could argue that if CD25 depletion had any effect on effector cells, we would see protection from autoimmunity when treating animals with PC61. Hence the loss of protection observed is likely due to depletion of Tregs. These results support our hypothesis that CCL22 expression in islets protects from diabetes and recurrent autoimmunity by recruiting Tregs.

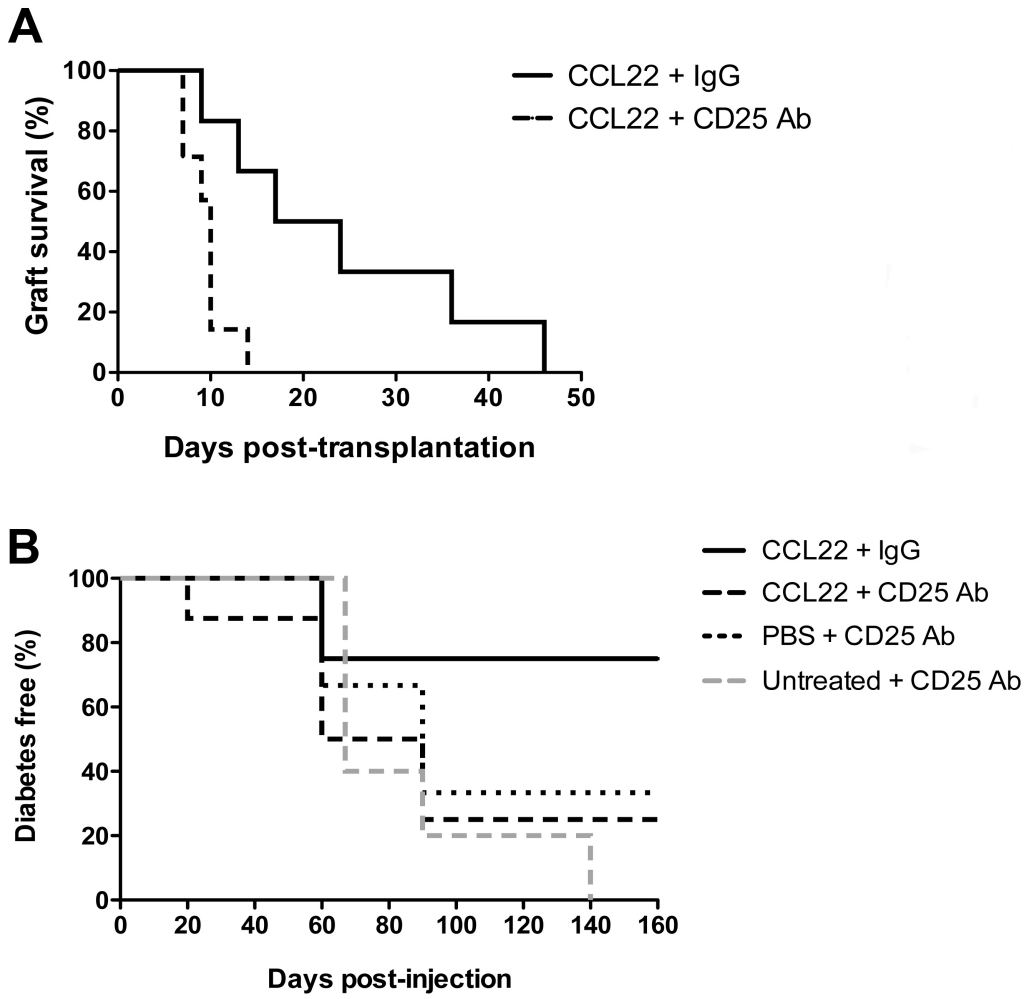

Figure 11: The protective effect of CCL22 is lost after depletion of Tregs

A. Recipients of CCL22-expressing islet grafts were treated with CD25 antibody (CD25 Ab, n=7) or an isotype control (IgG, n=6). Survival curves were compared using the log-rank test. *p*=0.01. **B.** Diabetes development was monitored in CCL22, PBS and untreated NOD mice after administration of CD25 Ab or IgG. Treatment with CD25 Ab in CCL22-NOD mice resulted in more rapid and frequent diabetes onset than injection with IgG (*p*=0.01) and was not significantly different from that in PBS and untreated mice.

3.2.6. IMPACT OF CCL22 ISLET EXPRESSION ON AUTOREACTIVE T CELLS

The autoimmune response against beta cells in NOD mice is mediated by cytotoxic $CD8^+$ T cells that react to insulin and IGRP epitopes [61, 228], as well as $CD4^+$ T cells that recognize epitopes derived from chromogranin A, first identified as the BDC2.5 clones [229, 230]. To detect these cells in the circulation and tissues of mice, we used MHC class I and II tetramers that are specific for these beta cell self-antigens. In transplant recipients, the frequencies of

islet-specific $CD8^+$ and $CD4^+$ cells in recipients of CCL22-expressing grafts were similar to controls, both within the graft and draining lymph node (Figure 12A). This finding indicates that CCL22 expression does not prevent migration of autoreactive T cells to the graft.

The presence of autoreactive $CD8^+$ T cells in blood predicts the onset of diabetes [228], and deletion of these cells can prevent diabetes in NOD mice [231]. Therefore, we sought to determine whether these diabetogenic cells were affected by CCL22 expression in pancreatic islets. We found that the frequency of IGRP- and insulin-reactive T cells in peripheral blood was decreased in CCL22-NOD mice compared with that in controls (Figure 12B), consistent with the protection from diabetes development. The proportion of IGRP-specific $CD8^+$ cells also appeared to be lower in the pancreas and was significantly reduced in the pancreatic lymph node of CCL22-NOD mice (Figure 12C). Moreover, BDC2.5-reactive $CD4^+$ cells were found in lower proportions in CCL22-expressing pancreata. In this model, CCL22 expression in pancreatic islets seems to limit expansion of autoreactive T cells and their accumulation in the pancreas.

The absence of impact of CCL22 on the frequency of autoreactive T cells in transplant recipients may be related to the different timing. Indeed, tissues from islet graft recipients were examined 10 days post-transplantation, whereas pancreata and pancreatic lymph nodes were harvested from CCL22-NOD mice one month post-injection. In fact, analysis in transplant recipients at a later time would be impossible because control recipients would not be available. However, one can speculate that modulation of autoreactive cells takes more time and that a reduction in the frequency of autoreactive T cells occurs later. Another possibility is that intervention at an earlier time in the pathogenesis of diabetes (prediabetic versus overtly diabetic mice) may have a stronger effect on recently primed autoreactive cells, as opposed to autoreactive memory cells [232].

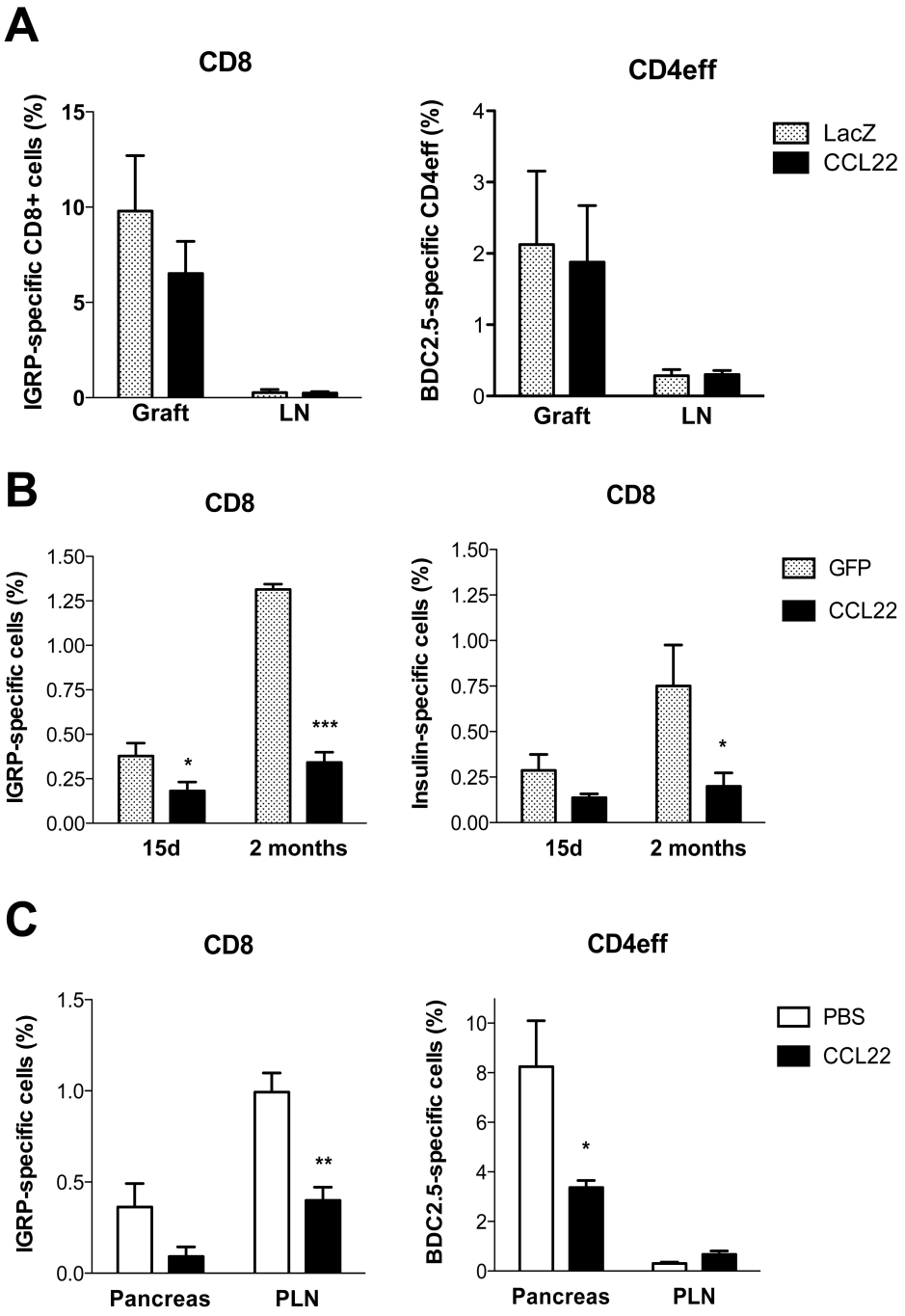

Figure 12: Impact of CCL22 expression in islets on autoreactive T cells

A. Islet-specific T cells were measured in the islet graft and draining lymph node (LN) from CCL22 and LacZ recipients 10 days post-transplantation. The frequency of IGRP-specific $CD8^+$ cells and BDC2.5-specific CD4 effector cells was comparable between the 2 groups of recipients (p=NS). **B.** The presence of IGRP- and insulin-specific $CD8^+$ cells in the circulation of GFP- and CCL22-NOD mice was examined at 15 days and 2 months after gene delivery. We observe a reduction of both populations in CCL22-NOD mice (*p<0.05; ***p<0.001). **C.** Islet-specific T cells were detected in the pancreas and pancreatic lymph node (PLN) 1 month after injection of PBS or CCL22 vector. p<0.05; **p<0.01.

3.3. DISCUSSION

The chemokine CCL22 has been shown to modulate the anti-tumor immune response and thus promote tumor growth. Here, we report the successful harnessing of CCL22 properties to protect beta cells from autoimmunity in NOD mice using a gene delivery approach. Our findings indicate that CCL22 protects from spontaneous diabetes when expressed in pancreatic beta cells, as well as from recurrent autoimmunity when present in islet transplants. In both models, CCL22 expression was associated with preservation of beta cells and accumulation of Tregs locally. The fact that depletion of $CD4^+$ $CD25^{high}$ cells abrogated the effectiveness of CCL22 suggests that Tregs are required to mediate CCL22 protection. Of note, NOD mice transduced with AAV8-CCL22 display reduced frequencies of islet-specific T cells in the circulation, pancreas and pancreatic lymph nodes, in correlation with diabetes protection. Kim and colleagues reported that transgenic expression of CCL22 in islets accelerates diabetes onset in NOD mice due to enhanced recruitment of activated T cells as well as antigen presenting cells [233]. Expression of CCL22 in the pancreas from birth is likely detrimental to islets because it draws the attention of a number of immune cells to islets, even before the homeostatic wave of beta cell apoptosis. However, we demonstrate here that AAV8-mediated expression of CCL22 starting from 8 weeks of age protects beta cells from autoimmune destruction, underlining the importance of the time of intervention.

Our approach represents a novel strategy to increase Treg numbers in the pancreas and islet graft by manipulating their migration. Current Treg-based therapies consist of the infusion of large quantities of Tregs into patients with diabetes and other pathologies [234]. However, given that Tregs represent less than 1% of peripheral blood mononuclear cells, they need to be expanded *in vitro* prior to clinical application and this task is difficult owing to their hyporesponsive phenotype. Moreover, there is a risk of co-infusing effector T cells because of the lack of proper isolation methods of homogenous populations of human Tregs [235]. Recruitment of endogenous Tregs to the site of inflammation may circumvent all of these problems. In addition, controlling Treg trafficking by gene- or peptide-delivery approaches has a therapeutic potential for many autoimmune diseases and transplant settings.

To advance towards clinical application, a better understanding of the mechanisms underlying CCL22 protection is necessary. As Curiel and colleagues reported, Tregs recruited by ovarian cancers that produce CCL22 inhibited proliferation and cytotoxicity of tumor-specific T cells

[159]. Although insulitis clearly persists in the face of CCL22-mediated protection from diabetes, we hypothesize that diabetogenic T cells are suppressed by recruited Tregs. CCL22 expression in islets may not only increase the proportions of Tregs in the pancreas or islet graft, but may also enhance Treg interactions with effector cells by positioning Tregs at the site of inflammation. It is possible that CCL22 functionally activates Tregs as suggested by a study in which Tregs infiltrating breast tumors display a higher activation status [236]. Our observation that Tregs in CCL22-procuding grafts express higher levels of CCR4 supports the idea of a more activated phenotype in recruited Tregs, since CCR4 is upregulated in Th2 cells upon T cell receptor stimulation [237]. Furthermore, other cells expressing CCR4, albeit at lower levels than Tregs, may also be recruited and play a role in the protective effect of CCL22. Tregs may suppress autoimmunity through inhibitory mediators or through the interplay with other immune cells. These hypotheses will be explored in chapter 4.

CHAPTER 4:

MECHANISMS OF CCL22 PROTECTION FROM AUTOIMMUNITY

4.1. INTRODUCTION

Despite numerous reports on the effect of CCL22 expression in tumors, there is still little research on the detailed mechanism of immune suppression induced by CCL22. We demonstrated the ability of CCL22 to protect beta cells from autoimmune diabetes; however, mechanistic studies are essential for the clinical translation of this potential therapy.

Expression of CCL22 in several pathologies, including malignancies [159, 209, 216, 236] and chronic infections [238, 239], is associated with the accumulation of Tregs. These observations parallel our findings in models of diabetes development and recurrent autoimmunity in NOD mice, in which we induced islet expression of CCL22. In most studies of CCL22-producing cancers, immunosuppression in the tumor microenvironment is attributed to the activity of recruited Tregs. However, the receptor for CCL22 is expressed by cells other than Tregs, although at lower levels, and these cell populations may also be attracted by CCL22 and contribute to the immune modulation. Indeed, CCR4 is present on a variety of immune cells both in humans and mice. In the T cell compartment, CCR4 is found on conventional $CD4^+$ cells upon activation [237, 240], particularly in the Th2 subset, and memory $CD8^+$ cells [241]. Additionally, DCs [242, 243], NK [244] and NKT cells [241] have all been shown to express CCR4 among other chemokine receptors and migrate towards CCL22 *in vitro*. Notably, NKT cells and DCs possess important immunoregulatory properties and are able to inhibit T cell responses. Therefore the immune modulation observed in the presence of CCL22 may result from the combined activities of different cell subsets.

Chemokine receptors, including CCR4, activate the phosphoinositide 3-kinase pathway (PI3K), which is responsible for directional cell migration, but is also involved in cell proliferation, survival and differentiation [143, 144]. Hence, besides their role in controlling leukocyte trafficking, chemokines may also play a role in the activation and function of immune cells. For instance, CCL2 and CCL3 were shown to impact Th1 and Th2 cytokine production by monocytes and T cells and therefore influence $CD4^+$ cell differentiation [245-247]. Molon and colleagues demonstrated that engagement of a chemokine receptor by its ligand may act as a co-stimulatory signal in T cells [149]. Such a phenomenon has been observed upon CXCR4 and CCR5 triggering [248]. It has also been reported that CCR4 and its ligands are able to modulate innate immune responses, such as cytokine secretion in macrophages [249], response to TLR9 activation [250] and eosinophil degranulation [251]. A potential role for

CCL22 in Treg function has been suggested by the observation that in breast tumors producing CCL22, infiltrating Tregs are characterized by higher activation status and proliferation rate [236]. Another study suggested that CCL22 promoted survival of human Tregs *in vitro* [252]. Altogether, these observations support a potential role for CCL22 in cell activation and/or function in addition to chemotaxis.

We speculated that immune cells recruited by CCL22 create a tolerogenic milieu inhibiting autoreactive T cells and thereby protecting beta cells. We believe that different cell populations attracted and perhaps activated by CCL22 collaborate to achieve local immunosuppression. These hypotheses were addressed in a number of analyses *in vitro* and *in vivo* to clarify the mechanisms by which CCL22 modulates the immune response. The consistency of our findings between the NOD models of diabetes development and recurrent autoimmunity suggests that the effect of CCL22 on the autoimmune response is likely similar in both models. Thus, *in vivo* studies were performed in the model of recurrent autoimmunity as the shorter experiment duration allows for more rapid phenotyping.

4.2. RESULTS

4.2.1. CCL22 PREFERENTIALLY ATTRACTS TREGS, INKT CELLS AND PDCS

To compare the ability of CCR4-expressing cells to respond to CCL22, we examined CCR4 expression in DC and T cell subsets, as well as NK and NKT cells with or without activation by concanavalin A (ConA). The lectin ConA is a widely used to activate T cells *in vitro* [253] and it has been shown to stimulate monocytes and NKT cells as well [254, 255]. Our results confirm that CCR4 is present on a large proportion of Tregs under steady state conditions and even more after treatment with ConA (Figure 13A). We observed constitutive expression of the CCL22 receptor in approximately 20% of DCs and 45% of NKT cells. As expected, conventional $CD4^+$ and $CD8^+$ cells expressed CCR4 only upon activation. The converse expression profile was seen on NK cells, which loose CCR4 expression after stimulation with ConA. Analysis of expression levels in $CCR4^+$ cells revealed that all populations examined express lower amounts of CCR4 than Tregs except for NKT cells, which displayed significantly

higher levels of the CCL22 receptor (Figure 13B). This interesting finding suggests that NKT cells may be even more sensitive to CCL22 gradients than Tregs.

The ability of these immune cells to migrate towards CCL22 was explored in chemotaxis assays. We examined cell recruitment by 100-1000 pg/ml of recombinant CCL22 and observed significant migration of Tregs, NKT cells and plasmacytoid DCs (pDC), regardless of the concentration used (Figure 14A). Surprisingly, pDC recruitment was comparable to that of Tregs and NKT cells, despite the lower levels of CCR4. We questioned whether migrating Tregs and NKT cells enhanced the recruitment of pDCs, and performed a time course to determine the migration kinetics of these three populations. Migration of pDC started after 2 hours and continued for the rest of the experiment, whereas Tregs and NKT cells were already recruited at the 1-hour time point (Figure 14B). We speculate that pDCs follow Tregs and NKT cells during their recruitment by CCL22. We next examined the ability of CCL22-expressing islets to attract immune cells. Likewise, we found selective recruitment of Tregs, NKT cells and pDCs compared to control islets (Figure 14C). Of note, control islets also recruited several cell subsets, more particularly NKT cells which were highly recruited both by CCL22-expressing and untreated islets. One possible explanation is that isolated islets produced various cytokines and chemokines, as reported previously [256, 257]. These chemokines likely participate to cell recruitment in our assay and perhaps synergize with CCL22 [258].

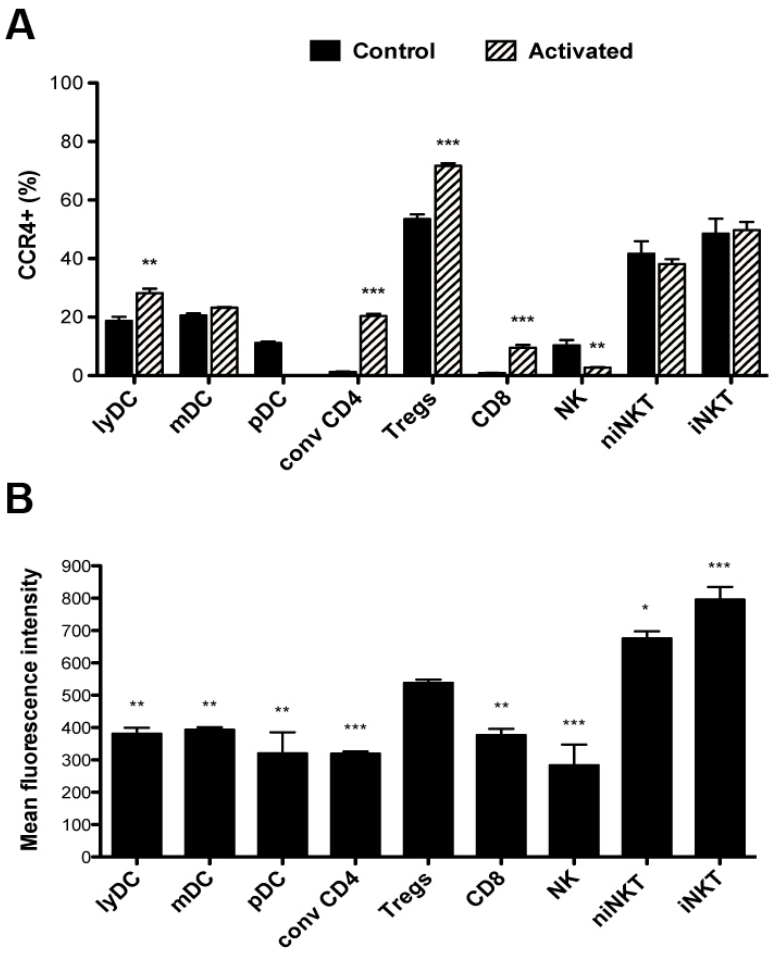

Figure 13: CCR4 expression profile on immune subsets

A. Expression of CCR4 on leukocytes in steady state conditions or after activation with ConA (2.5µg/ml). Activation induced CCR4 expression on higher frequencies of lymphoid DCs, conventional $CD4^+$ cells, Tregs and $CD8^+$ cells. **p<0.01 and ***p<0.001 versus control. Compared to Tregs, CCR4 is expressed by similar frequencies of NKT cells but lower frequencies of all other subsets regardless of activation (p<0.001). **B.** Levels of CCR4 expression on $CCR4^+$cells in steady state conditions. Of note, cellular levels of CCR4 assessed by mean fluorescence intensity were similar regardless of activation. **p<0.01 and ***p<0.001 versus Tregs. N=6 for both analyses.

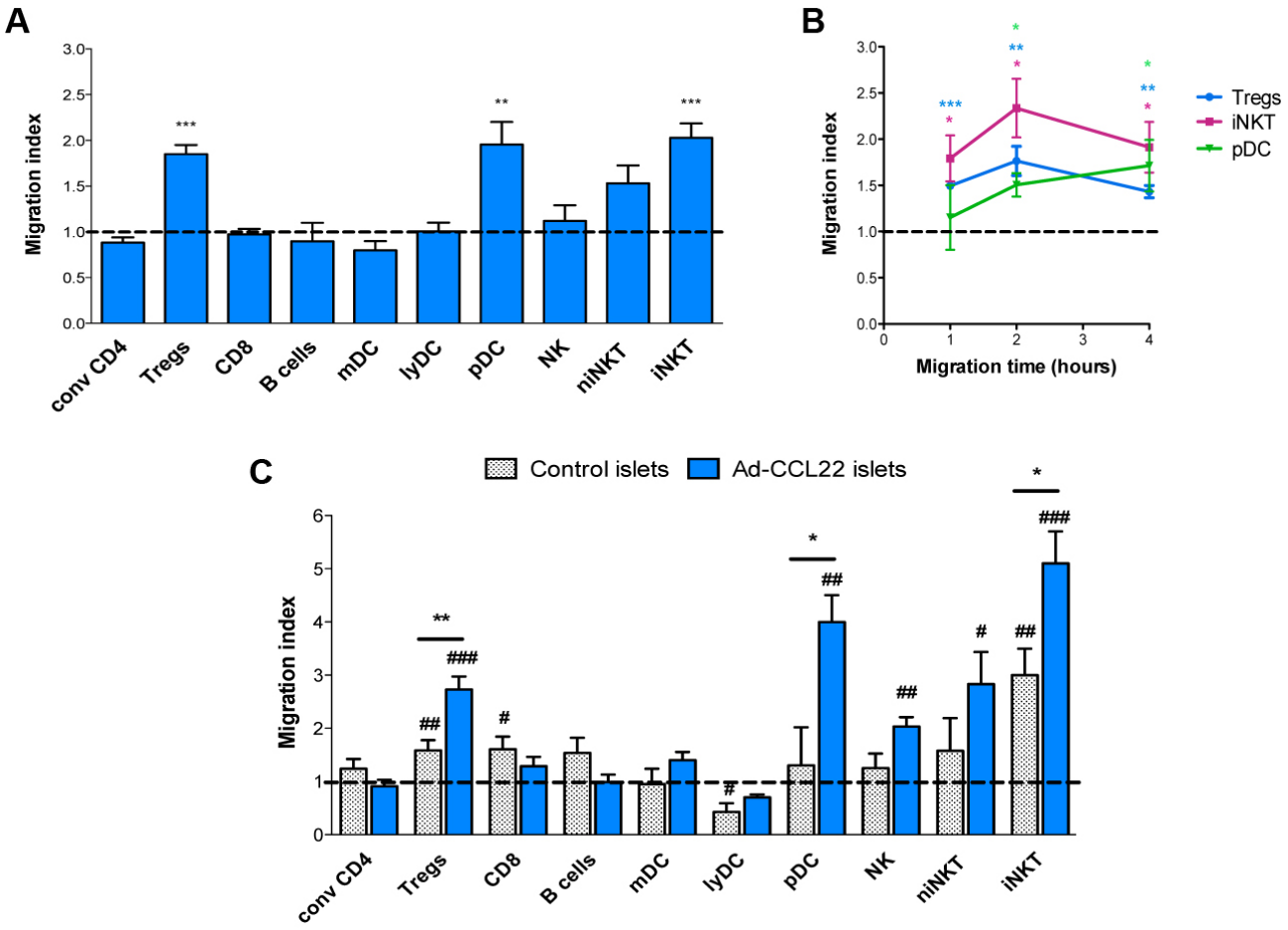

Figure 14: Migration of Tregs, iNKT cells and pDC towards CCL22

A. Chemotaxis assay to examine migration of splenocytes towards 500pg/ml of recombinant CCL22 over a 4-hour period. Specific migration to CCL22 is expressed as an index after normalization to media alone. **p<0.01 and ***p<0.001 versus media alone. **B.** Time course analysis for the migration of Tregs, iNKT cells and pDCs. *p<0.05 and **p<0.01 versus media alone. C. Migration of splenocytes towards CCL22-expressing islets and control islets. #p<0.05, ##p<0.01 and ###p<0.001 versus media alone; *p<0.05 and **p<0.01 versus control islets.

4.2.2. CCL22 ACTIVATES TREGS *IN VITRO*

To determine whether CCL22 has an effect on leukocyte activation, we incubated whole splenocytes or isolated cell subsets with recombinant CCL22 and analyzed the presence of activation markers by flow cytometry after 24 and 48 hours. Expression of CD86 and MHC class II on dendritic cells, as well as CD44 and CD69 on $CD8^+$ and conventional $CD4^+$ cells remained the same after treatment with CCL22, suggesting that CCL22 does not impact the activation status of these cell populations. However, we observed the selective activation of Tregs as shown by expression of CTLA-4, ICOS, CD62L, CCR4 and CD69 on higher proportions of Tregs (Figure 15A); in addition, levels of ICOS and CCR4 were upregulated after 24 hours and 48 hours of incubation, respectively, and CD69 tended to be upregulated on CCL22-treated Tregs (Figure 15B).

ICOS is a member of the CD28 co-stimulatory family that is induced on T cells following activation and enhances proliferation of effector cells [259, 260]. In Tregs, ICOS promotes survival [261] and production of the anti-inflammatory cytokine IL-10 [259]. CTLA-4 is another member of the CD28 family present on Tregs and activated T cells. CTLA-4 binds to CD80 and CD86 with a higher affinity than CD28 and provides inhibitory signals instead of co-stimulation, resulting in dampening of immune responses [259]. Its expression on Tregs is essential for tolerance induction, since $CTLA\text{-}4^+$ Tregs limit activation of conventional T cells [262] through modulation of DC function [117, 263]. The integrin CD62L is an important lymphoid homing molecule expressed mainly on naïve T cells and downregulated after activation. However, a subset of Tregs retains expression of CD62L allowing them to inhibit effector T cells in lymph nodes more efficiently [264]. Interestingly, expression of either of these markers on Tregs confers them with superior suppressive activity and ability to protect from autoimmunity, particularly in the context of autoimmune diabetes [261, 265-267].

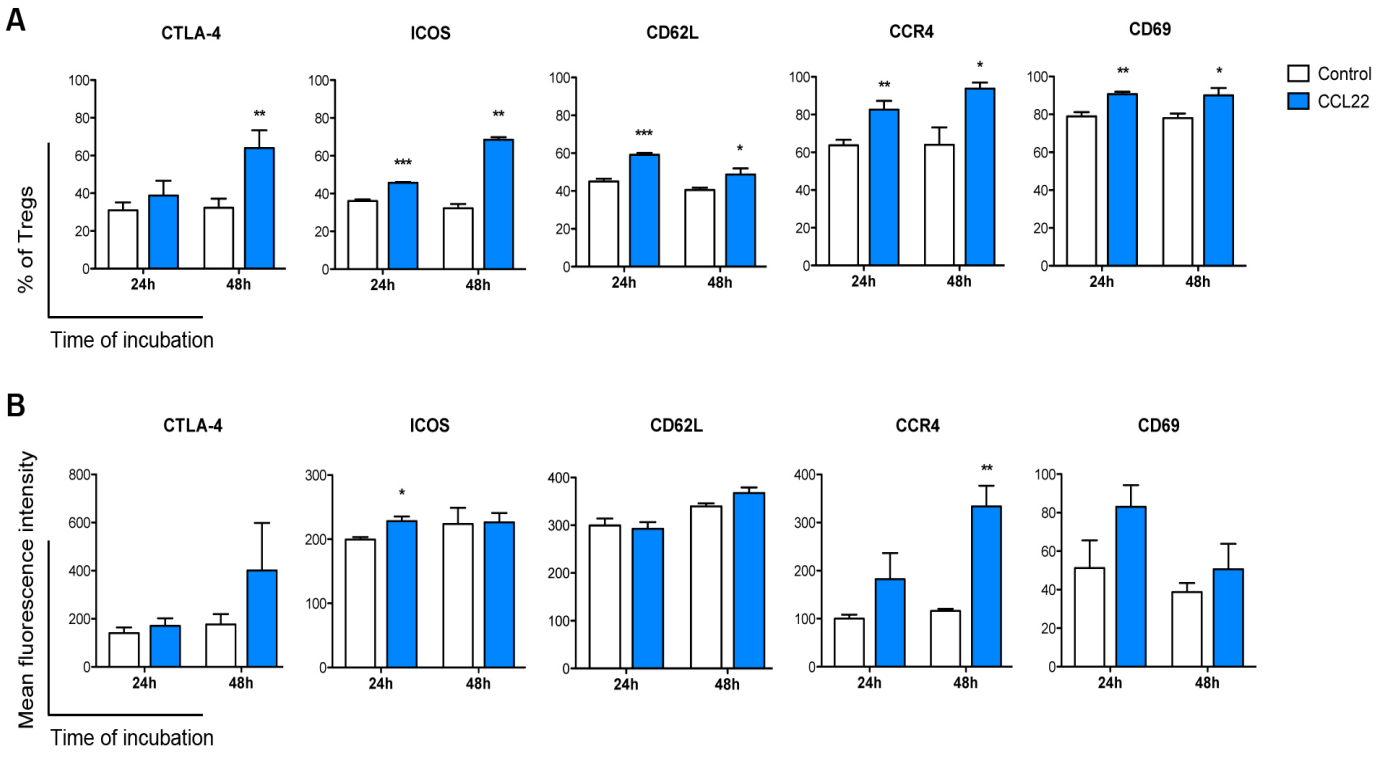

Figure 15: CCL22 activates Tregs *in vitro*

The presence of CTLA-4, ICOS, CD62L, CCR4 and CD69 was analyzed on isolated Tregs after incubation with 500pg/ml of CCL22 or media alone. **A.** Higher proportions of Tregs express all of these activation markers after treatment with CCL22. **B.** Levels of ICOS and CCR4 were upregulated on Tregs after treatment with CCL22. *p<0.05 and **p<0.01 versus control.

4.2.3. CCL22 ENHANCES THE FUNCTION OF TREGS

Since ICOS is involved in Treg survival, we tested the functional consequence of ICOS upregulation on CCL22-treated Tregs in a viability assay in the presence or absence of the pro-survival cytokine IL-2. In the presence of IL-2, viability was comparable between control and CCL22-treated Tregs over a 24 hour-period. As expected, in the absence of IL-2, Treg viability declined but CCL22 treatment restored Treg viability (Figure 16A). This finding indicates that CCL22 promotes Treg survival, consistent with a previous study on human Tregs [252]. In contrast to a report showing Treg proliferation in CCL22-expressing tumors [236], we found no direct impact of CCL22 on the proliferation rate of Tregs. We next examined gene expression of several Treg effector molecules after incubation with CCL22. Although modest, we noted

changes at 24 hours in IL-17 expression, which was reduced, while IFNγ expression was increased (Figure 16B). Additionally, IL-10 was first downregulated at 24 hours and then upregulated at 48 hours, corresponding with the increased frequency of $ICOS^+$ Tregs.

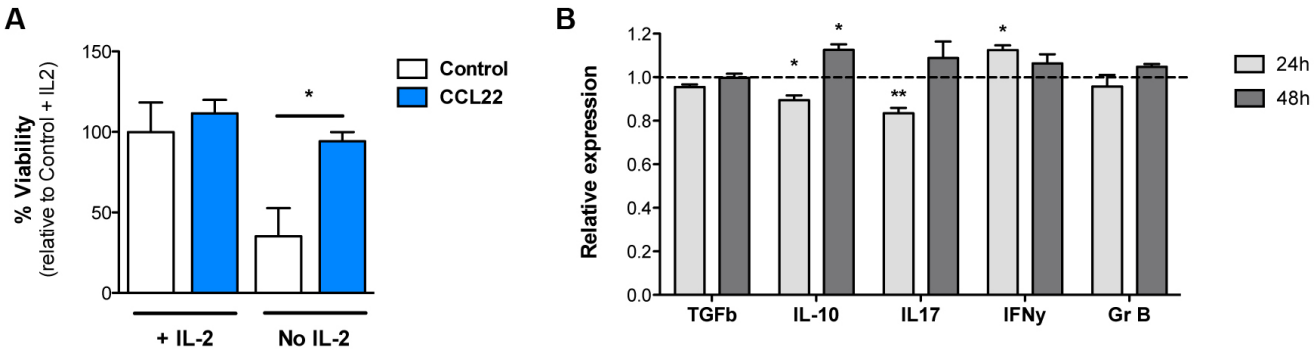

Figure 16: CCL22 enhances Treg function

A. The impact of CCL22 on Treg viability was examined by flow cytometry using annexin-V staining. Isolated Tregs were cultured with 500pg/ml of CCL22 for 24 hours in the presence or absence of IL-2 (100U/ml). *p<0.05. **B.** Gene expression of TGFβ, IL-10, IL-17, IFNγ and granzyme B (Gr B) was analyzed in isolated Tregs after incubation with 500 pg/ml of CCL22. Data are shown as gene expression relative to untreated Tregs. *p<0.05 and **p<0.01 versus untreated Tregs at the same time point.

One of the mechanisms used by Tregs to suppress immune responses is to modulate DC activation and function, which is achieved through interactions between CTLA-4 and CD80/CD86 molecules on DCs [117]. Tregs may reduce the capacity of DC to activate effector cells by downregulating co-stimulatory molecules on DC [268]. In addition, Tregs were shown to condition DCs to express the potent regulatory enzyme indoleamine 2,3-dioxygenase (IDO) in a CTLA-4 dependent manner [269]. IDO is able to inhibit T cell proliferation by degrading the essential amino acid tryptophan [77]; additionally, the accumulation of kynurenine catabolites triggers apoptosis in T cells [270]. As CCL22 doubles the expression of CTLA-4 on Tregs, we tested the ability of CCL22-treated Tregs to modulate DC function in co-culture experiments. After 48 hours, DC activation status was modestly downregulated by CCL22-treated Tregs (Figure 17A). However, IDO expression was markedly induced in DCs cultured with CCL22-treated Tregs (Figure 17B). These findings indicate that CCL22 improves the ability of Tregs to modulate DC function.

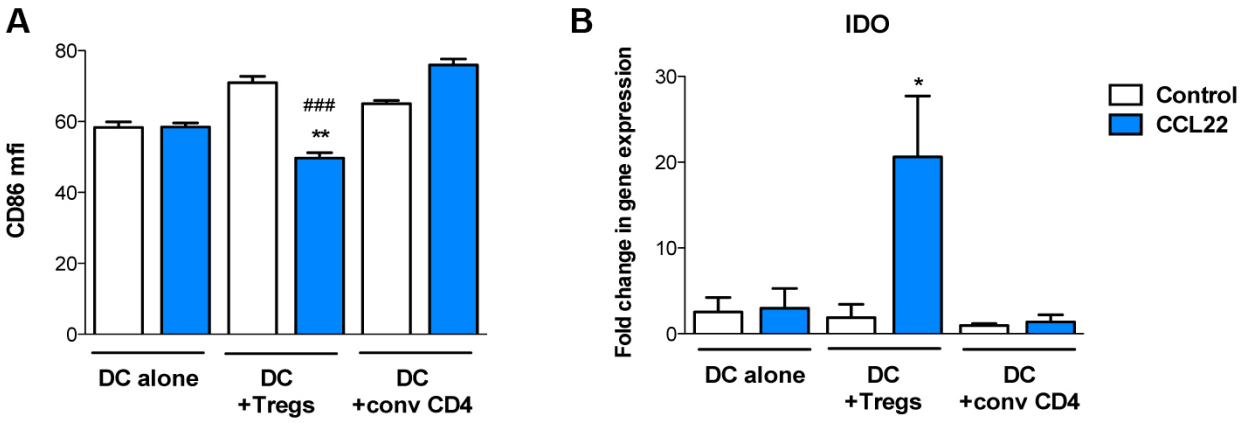

Figure 17: CCL22 improves Treg's ability to modulate DC function

The ability of Tregs to modulate DC function was analyzed in co-culture experiments. Isolated Tregs or conventional $CD4^+$ cells were pre-treated for 48 hours with 500pg/ml of CCL22 then isolated DCs were added to the culture at a ratio of 1:2. **A.** DCs co-cultured with CCL22-treated Tregs expressed lower levels of the co-stimulatory CD86 at 48 hours, as determined by flow cytometry. $^{**}p<0.01$ versus DC alone, $^{###}p<0.001$ versus all other conditions. **B.** CCL22-treated Tregs induced gene expression of IDO in DCs, as analyzed by real time PCR. Data was analyzed using the $2^{-\Delta\Delta CT}$ method and represent the fold change compared to untreated DCs. $^{*}p<0.05$ versus all other conditions.

4.2.4. CCL22 EXPRESSION IN ISLET GRAFTS REGULATES IMMUNE RESPONSES

To understand the mechanisms of CCL22 protection from autoimmune diabetes, we sought to characterize the immune infiltrate in CCL22-expressing islet grafts at different times post-transplantation. We observed a progressive graft infiltration by $CD8^+$ and conventional $CD4^+$ cells comparable between LacZ and CCL22 groups (Figure 18). Consistent with previous findings, recruitment of Tregs was more evident in CCL22-expressing grafts, with a significant increase in Treg numbers 10 days post-transplantation. Interestingly, iNKT cells markedly infiltrated CCL22-expressing grafts 5 days post-transplantation and the increased number of iNKT cells in the graft persisted over time. Both NK and type II NKT cells were retained in CCL22-expressing 10 days post-transplantation, contrary to LacZ-expressing grafts, in which these cell populations declined with time. Infiltration by myeloid and lymphoid DCs was similar in both groups, but the number of pDC was higher in CCL22-expressing grafts 10 days post-transplantation.

These findings confirm our observations in chemotaxis assays showing the selective recruitment of Tregs, iNKT cells and pDCs. The kinetics of graft infiltration demonstrate that iNKT cells are the first population to be recruited by CCL22-expressing grafts, and that they remain within the graft thereafter. These results support a previously unsuspected role for iNKT cells in the immunomodulation induced by CCL22. It is likely that iNKT cells and Tregs recruited by CCL22 interact or combine their suppressive activities to mediate CCL22 protection from autoimmune diabetes, potentially with the help of pDC and NK cells, as previously described [68, 129, 193].

To determine the molecular mechanism of CCL22-induced protection, we performed real-time PCR analysis of the graft and draining lymph nodes of LacZ and CCL22 transplant recipients. We observed no change in the gene expression of IL-10, TGFβ or granzyme B, all of which are members of Treg's panoply of effector molecules. In the draining lymph nodes, the expression of all genes examined was comparable between LacZ and CCL22 groups. However, IDO mRNA levels were increased by 8-fold in CCL22-expressing grafts 10 days post-transplantation (Figure 19). At the same time point, we also noted downregulation of IFNγ in CCL22-expressing grafts compared to controls. These findings point to the likely participation of DCs, as they are a major source of IDO [271]. Additionally, they correlate with our observation that CCL22 enhances the ability of Tregs to induce IDO expression in DCs. Lower levels of IFNγ in CCL22-expressing grafts are likely due to inhibition of IFNγ-producing effector T cells. Altogether, these findings suggest that CCL22 expression in islets produces a local tolerogenic milieu.

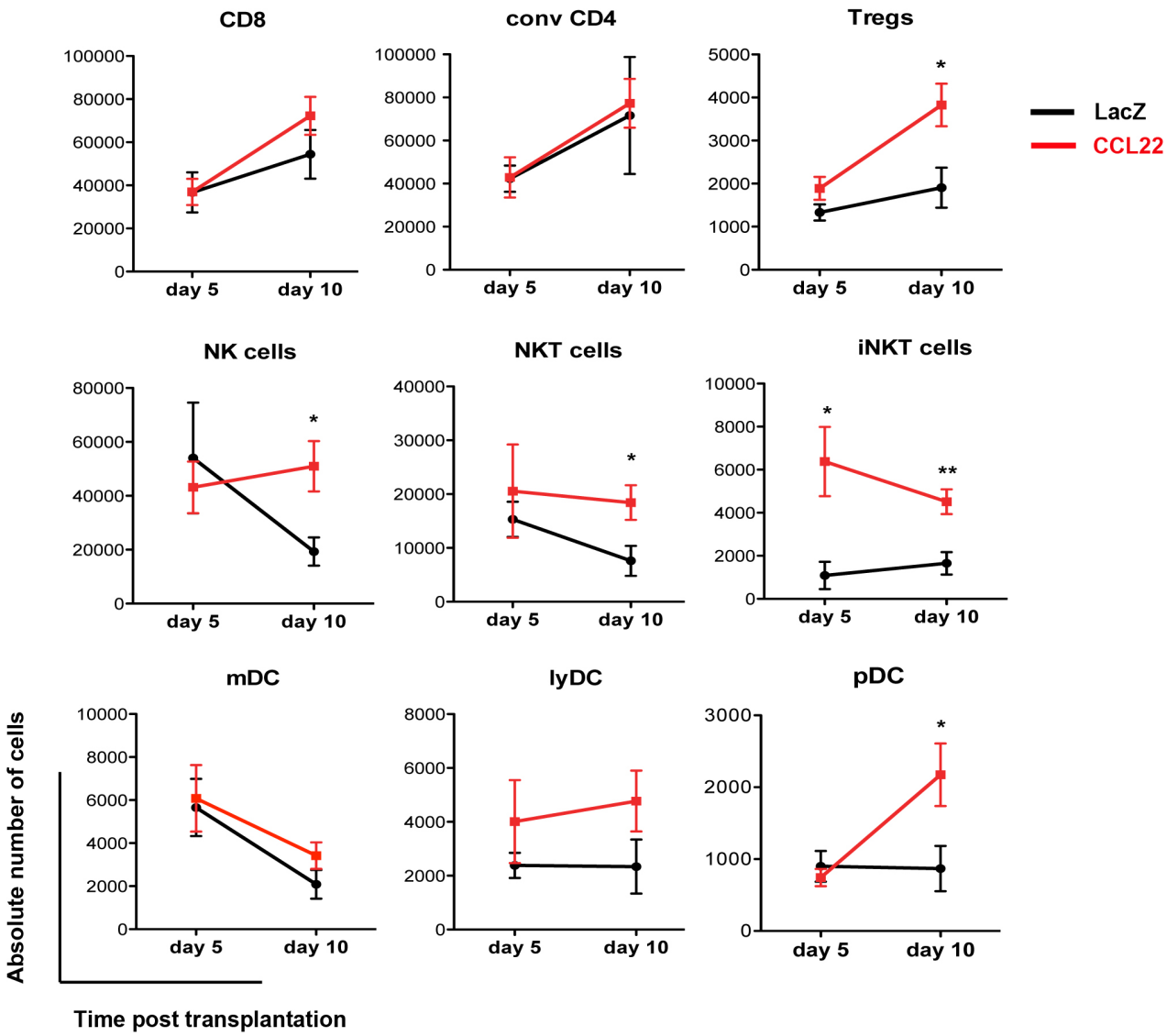

Figure 18: Characterization of immune infiltrate in CCL22-expressing islet grafts

Immune infiltration in islet grafts was examined 5 and 10 days post-transplantation in CCL22 (n=6) and LacZ (n=5) recipients. The absolute number of cells was determined by flow cytometry using counting beads. We observed a marked influx of iNKT cells at day 5 and subsequent recruitment of Tregs and pDCs, as well as retention of NK and type II NKT cells in CCL22 expressing grafts. *p<0.05 and **p<0.01 versus LacZ at the same time point.

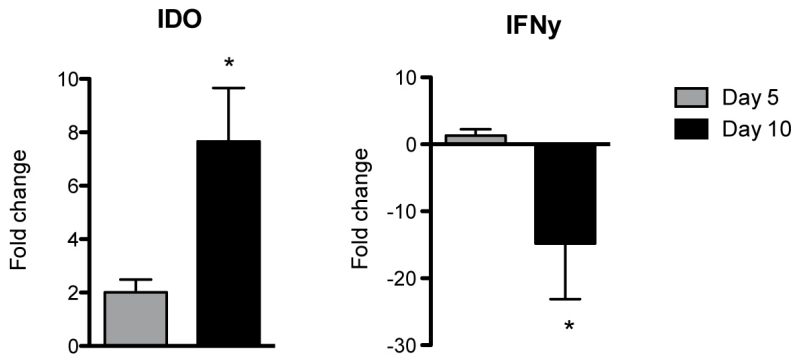

Figure 19: CCL22 induces a tolerogenic milieu in the islet graft

Real-time PCR analysis of islet grafts 5 and 10 days post transplantation revealed an increase of IDO expression and decrease in IFNγ mRNA levels at day 10. *p<0.05 versus LacZ. The fold change in gene expression compared to LacZ grafts was determined with the $2^{-\Delta\Delta CT}$ method.

4.2.5. TREGS IN CCL22-EXPRESSING GRAFTS DISPLAY AN ACTIVATED PHENOTYPE

The finding that CCL22 activates Tregs *in vitro* led us to hypothesize that Tregs within CCL22-expressing islet grafts presented a similar phenotype. We previously observed that graft-infiltrating Tregs express higher levels of CCR4 in CCL22 recipients (Figure 5D). We further characterized the expression of activation markers on Tregs in the islet graft and found that CTLA-4, ICOS and CD62L were present on a higher proportion of Tregs in CCL22-expressing grafts (Figure 20A). However, Treg populations in the draining lymph node did not differ between transplant groups (Figure 20B). Gobert and colleagues reported a similar finding in the context of human breast tumors, where most Tregs expressed CTLA-4, ICOS and CCR4 [236]. Several studies have shown that antigen-specific Tregs are more potent in suppressing immune responses than polyclonal Tregs [272, 273]. More particularly, islet-specific BDC2.5 Tregs are more effective at preventing diabetes in NOD mice [266]. To determine whether graft-infiltrating Tregs are specific for islet antigens we used BDC2.5 MHC class II tetramers. The frequency of BDC2.5-specific Tregs was similar between the two groups of recipients both in the graft and the draining lymph node (Figure 20C). This observation suggests that CCL22 did not preferentially recruit islet-specific Tregs, nor did it promote their expansion. Overall, our data shows that Tregs in CCL22-expressing grafts exhibit a higher activation status with a phenotype associated with superior regulatory function.

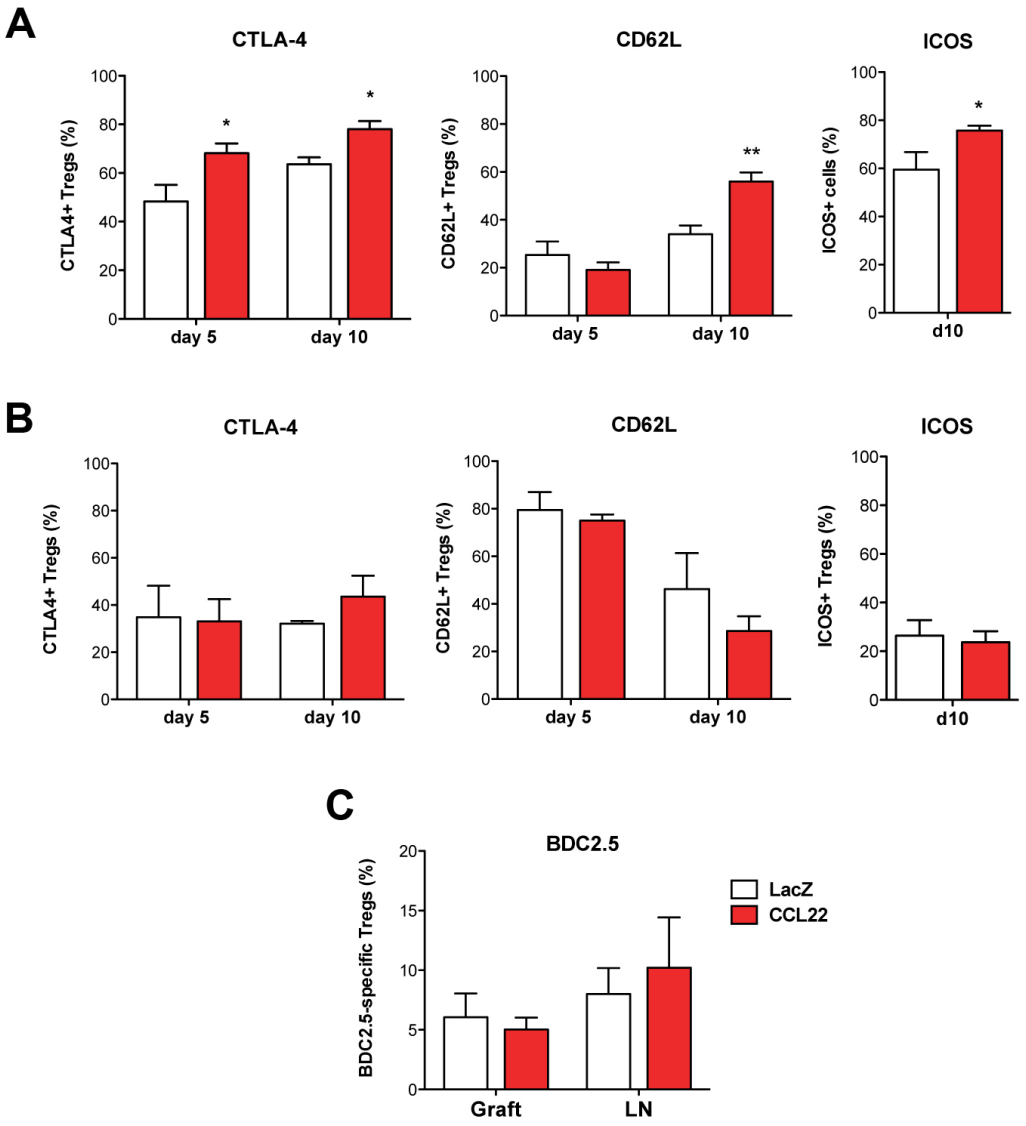

Figure 20: Tregs in CCL22-expressing grafts display an activated phenotype

Treg phenotype in the graft and draining LN of CCL22 and LacZ recipients was characterized 5 and 10 days post-transplantation by flow cytometry. **A.** Higher proportions of Tregs expressed CTLA-4, CD62L and ICOS in the CCL22-expressing grafts. *p<0.05 and **p<0.01 versus LacZ. **B.** In the draining LN, there was no difference in Treg phenotype between CCL22 and LacZ recipients. **C.** The frequency of islet-specific Tregs was similar between groups of recipients both in the graft and draining LN.

4.3. DISCUSSION

Several studies in the oncology field have demonstrated the capacity of CCL22 to suppress the immune response, a property that has been ascribed to Tregs recruited by CCL22. By engineering the expression of CCL22 in islets, we demonstrated that CCL22 protects beta cells from autoimmune destruction. In this study, we sought to improve our understanding of the mechanisms underlying CCL22 effectiveness in preventing autoimmune diabetes. We confirmed that expression of CCL22 in islets enhances influx of Tregs to the transplant site. Importantly, we now provide evidence that besides recruiting Tregs, CCL22 also selectively activates Tregs and promotes their suppressive function. Indeed, our data show that CCL22-conditioned Tregs are characterized by higher expression of CTLA-4, ICOS and CD62L, all of which are associated with superior Treg suppressive function. Notably, a similar phenotype is observed on Tregs that infiltrate CCL22-expressing grafts. Tregs treated with CCL22 display an improved survival in stringent conditions, and enhance the production of IDO by DCs. In parallel, IDO levels are significantly higher in CCL22-expressing islet grafts, suggesting that Tregs similarly interact with DCs *in vivo* to induce production of this tolerogenic enzyme. Interestingly, Onodera and colleagues described the importance of the CCL22-CTLA-4 axis in the induction of IDO during physiologic conditions [274]. They showed that DCs in mesenteric lymph nodes secreted large amounts of CCL22 and thereby attracted Tregs, which induced IDO expression in these DCs in a CTLA-4 dependent mechanism. Our findings suggest that we recreated this tolerogenic axis by inducing CCL22 expression in islet transplants.

CCR4 is mostly known as a receptor essential for Treg trafficking, although its expression is not exclusive to Tregs. We report here that approximately 45% of NKT cells express CCR4 constitutively, and more importantly, that CCR4 levels are higher on NKT cells, especially iNKT cells, compared to Tregs, suggesting a higher sensitivity to CCL22 gradients. In concordance with this idea, we demonstrated that iNKT cells are the first leukocyte subset to be recruited by CCL22-expressing islet grafts, whereas influx of Tregs and pDC is only observed 10 days post-transplantation. Intriguingly, all subsets of DCs express low levels of CCR4, and yet, increased numbers of pDC are found in CCL22-expressing grafts. The time course analysis of CCL22 chemotaxis shows that pDC migration follows that of iNKT cells and Tregs, raising the possibility that pDC migration is induced by CCL22-recruited cells rather than directly by CCL22. For instance, NKT cells are known to recruit immune cells, including DCs [275], through the secretion of chemokines, such as MIP-1 and RANTES [125, 276]. Moreover, iNKT

cells were shown to modulate immune responses through interactions with pDC [68], thus it is possible that in our transplant model, iNKT cells induce migration of pDC to the islet graft to exploit their activity. The fact that NK and type II NKT cells are retained within CCL22-expressing grafts indicates that these subsets also participate in crosstalk and potentially contribute to immunomodulation.

NKT cells possess the ability to influence a variety of immune cells, including conventional T cells, Tregs, DCs and NK cells either by cell-to-cell contact or via cytokines [277]. A protective role has been attributed to both types of NKT cells in the context of autoimmune diabetes, as demonstrated by transgenic overexpression or adoptive transfer of these cells [192, 278]. The remarkable recruitment of iNKT cells by CCL22-expressing grafts at an early time point highly suggests that iNKT cells are important for CCL22 effectiveness. It is possible that Tregs and iNKT cells cooperate to mediate CCL22 protection of beta cells. Such interactions have been implicated in the prevention of autoimmune diabetes [68, 193], rejection of heart transplants [279] and graft versus host disease [280] in rodent models. CD1d-deficient NOD mice will allow us to examine the role of NKT cells in CCL22-mediated protection from recurrent autoimmunity. Thymic expression of CD1d is required for NKT cell development, thus CD1d-deficient mice lack these cells [189]. In the NOD background, CD1d deficiency is associated with accelerated diabetes onset, consistent with a protective role of NKT cells [188].

Overall, our data suggest that CCL22-induced suppression of effector T cells is achieved through the interplay of several immune players. CCL22 induces a tolerogenic environment within the graft, consisting of larger populations of cells with immunomodulatory properties, in particular iNKT cells and Tregs, which are critical in preventing autoimmune responses. The interplay between these subsets results in elevated expression of the inhibitory enzyme IDO and lower levels of IFNγ. The frequency or number of conventional $CD4^+$ and $CD8^+$ cells, including islet-specific T cells, did not decline over time suggesting that immunosuppression did not occur through induction of effector T cell apoptosis. Instead, effector cells may be blocked in an anergic state, preventing them from proliferating or producing cytokines [281]. This possibility is consistent with the reduction of IFNγ in CCL22-expressing grafts. Notably, IDO activity renders effector T cells hyporesponsive, but other mechanisms of anergy induction include IL-2 deprivation and adenosine accumulation, both of which are caused by Tregs. To validate this hypothesis, a closer analysis of effector T cells is necessary, such as the evaluation of their ability to proliferate or produce cytokines and other effector molecules.

In conclusion, CCL22 protection from autoimmune diabetes involves several immune subsets, which appear to cooperate to induce tolerance towards beta cells. The principal mediators of CCL22's effect are Tregs and NKT cells, which are selectively recruited owing to their high expression of CCR4. The other ligand for CCR4, namely CCL17, has also been described in human tumors that attract Tregs [209, 216, 236]. Hence, it is possible that CCL17 will similarly offer protection from autoimmune diabetes. As suggested by a previous study [215], CCL22 and CCL17 may act in synergy and may thus have value as an adjunct therapy to protect beta cells from immune destruction. In Chapter 5, we assessed the ability of CCL17 to modulate autoimmune diabetes.

CHAPTER 5:

CCL17 EXPRESSION IN ISLETS PROTECTS BETA CELLS FROM RECURRENT AUTOIMMUNITY

5.1. INTRODUCTION

In addition to CCL22, CCR4 has another high-affinity ligand called CCL17 or thymus- and activation-regulated chemokine (TARC). Like CCL22, CCL17 is constitutively expressed in the thymus and is produced by activated macrophages and dendritic cells, as well as T cells stimulated by IL-4 [205, 282]. Unlike CCL22, CCL17 is expressed by bronchial epithelium [283], vascular endothelium [284], keratinocytes [285], fibroblasts and smooth muscle [286]. Moreover, CCL17 exhibits binding affinity for CCR8 [287], another chemokine receptor that is found on Tregs [204] and activated Th2 cells [237, 288]. Both in humans and mice, CCL17 efficiently recruits not only Tregs [209, 289] and NKT cells [241, 290], but also Th2 and memory $CD8^+$ T cells [291, 292].

CCL17 is considered a homing chemokine for the skin and lungs and its localization on endothelium enhances leukocyte extravasation to the tissues during inflammatory conditions [293]. A pathogenic role has been attributed to CCL17 in colitis [294, 295], atherosclerosis [296] and atopic dermatitis [285], through the recruitment of effector T cells. Despite this apparent pro-inflammatory role, CCL17 activity correlates with immunomodulation. Indeed, CCL17 has also been described in the environment of various tumors that efficiently escape immune control [297, 298]. Interestingly, CCL17 expression in tumors often coincides with that of CCL22, although at lower levels [209, 216, 236], perhaps as a strategy to combine the regulatory properties of both chemokines. In a humanized mouse model of breast cancer, siRNA-mediated knockdown of CCL17 and CCL22 in DCs reduces Treg recruitment while increasing infiltration of $CD8^+$ T cells [289], suggesting that their combined activities *in vivo* attract Tregs and block effector T cells. Neutralization studies *in vivo* revealed that CCL17 reduces the expression of both Th1 and Th2 cytokines in the lung, whereas CCL22 selectively inhibits IFNγ [286], implying different modulatory properties.

Although CCL17 shares many features with CCL22, it also exhibits diverse activities. For instance, in mouse cardiac allograft models, CCL17 promotes allorejection, while CCL22 is associated with long-term tolerance [299, 300]. One study revealed that CCL17 limited the expansion and promoted apoptosis of Tregs [296], in contrast to the effect of CCL22 on Tregs that others reported [236, 252] and that we demonstrated. Differences between these two chemokines may originate from distinct effects on their receptor CCR4. In a comparative study, CCL22 but not CCL17 induced CCR4 desensitization and internalization on Th2 cells upon

engagement [301]. Moreover, CCL22 was shown to be a more powerful agonist of CCR4 than CCL17 and to induce a more robust activation of integrins on $CCR4^+$ cells, resulting in more efficient leukocyte migration [302]. Another possible reason for their different activities is that CCL17 binds to a second receptor, namely CCR8, which might trigger different responses in immune cells.

Together, these findings support the possibility of non-redundant roles for CCL22 and CCL17 in immune system homeostasis and in pathological settings, with diverse abilities to modulate immune responses depending on the context. Given the utilization of CCL17 by tumors to efficiently evade immune destruction, we predict that CCL17 will similarly protect beta cells from autoimmunity. Recruitment of Tregs and NKT cells by CCL17 may have the same desirable effect observed with CCL22 in prevention of diabetes and recurrent autoimmunity. In this chapter, we examined CCL17's ability to activate immune cells *in vitro* and to modulate recurrent autoimmunity against transplanted islets.

5.2. RESULTS

5.2.1. CCL17 GENE DELIVERY TO ISLETS *IN VITRO*

As for CCL22, we generated an adenoviral vector (Ad-CCL17) containing a mouse CCL17 cDNA downstream of the CMV promoter to induce constitutive expression of CCL17 in cultured islets. Islets were transduced with different MOIs of virus to determine the optimal dose of Ad-CCL17 that does not adversely affect islet function. Following infection, CCL17 was expressed in islets in a dose-dependent manner as determined by mRNA and protein levels (Figure 21A, B). To determine whether any cell toxicity was caused by Ad-CCL17 infection, we examined islet viability and function after transduction with different MOIs of Ad-CCL17 in comparison with 10 MOI of Ad-CCL22. Glucose-stimulated insulin release in islets transduced with 10 or 20 MOI of Ad-CCL17 was similar to that observed in CCL22-expressing islets (Figure 21C), indicative of a normal islet function. However, by TUNEL assay, there was an apparent increase in islet cell death upon treatment with 20 MOI of Ad-CCL17 (Figure 21D). We therefore selected the dose of 10 MOI to transduce islets with Ad-CCL17 for subsequent experiments, as for Ad-CCL22. At this dose of adenovirus, we induced expression of 3.5 pg/ml

of CCL17 per islet, levels that are comparable to what DCs produce in culture [300]. The estimated concentration of CCL17 produced by islet transplants should therefore be similar to that of CCL17-expressing tumors [216, 236, 298], and thus suitable for our studies *in vivo*.

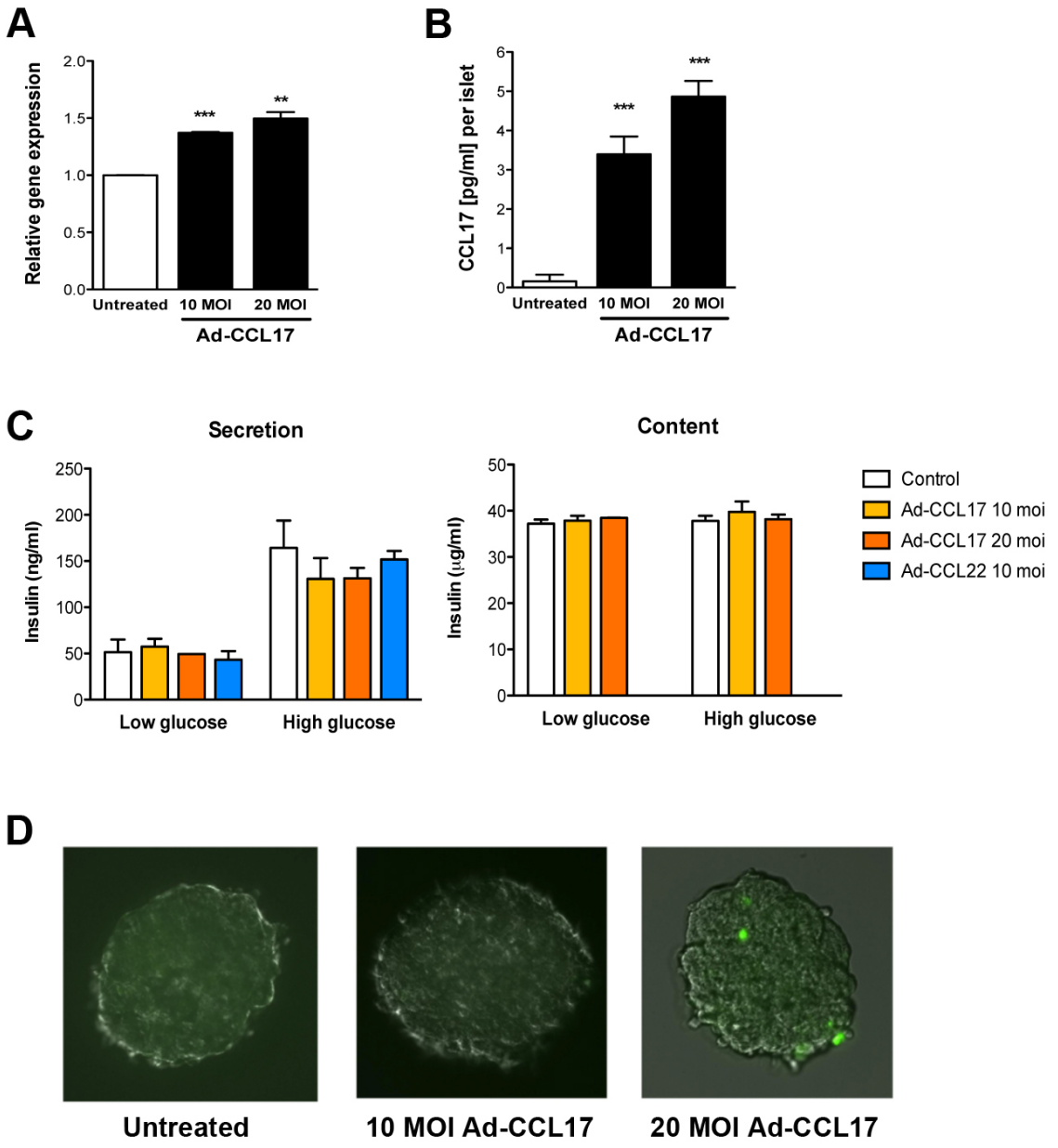

Figure 21: Islet transduction with Ad-CCL17

A. Real-time PCR analysis of CCL17 mRNA levels after overnight transduction with 10 and 20 MOI of Ad-CCL17. **B.** Islet secretion of CCL17 after transduction with Ad-CCL17 was quantified by ELISA. Results are representative of two separate experiments. ****p*<0.001 versus untreated. **C.** *In vitro* islet function after transduction with 10 or 20 MOI of Ad-CCL17 was assessed by glucose-stimulated insulin release at low (1.67mM) and high (16.7mM) glucose concentrations and compared to Ad-CCL22 (MOI 10). Insulin levels in the supernatant and islet lysates were determined by ELISA. *p*=NS. **D.** Islet viability following adenoviral infection was assessed by TUNEL assay. Increased cell toxicity was apparent in islets transduced with 20 MOI of Ad-CCL17 as shown by the higher number of dead islet cells (green). Representative pictures are shown for the different conditions.

5.2.2. CCL17 ALSO RECRUITS TREGS AND INKT CELLS

The chemotactic capacities of CCL17 and CCL22 were compared in migration assays using recombinant proteins. We observed similar recruitment profiles for both chemokines, with significant migration of Tregs and iNKT cells towards CCL17 and CCL22 (Figure 22A). Neither conventional $CD4^+$, $CD8^+$ nor NK cells migrated towards recombinant CCL17 or CCL22. Preliminary analysis of the whole DC population showed no recruitment by CCL17; however, migration of the different DC subsets remains to be assessed. It is possible that CCL17 induces selective migration of one DC subpopulation as we observed for CCL22.

We next performed chemotaxis assays with islets transduced with Ad-CCL17 or Ad-CCL22, using an MOI of 10 for both viral vectors. CCL17-expressing islets recruited more iNKT cells than control islets, similar to islets infected with Ad-CCL22 (Figure 22B). In contrast, CCL17-expressing islets did not have the same ability to recruit Tregs, whose migration was comparable to control islets. This finding is surprising given the recruitment of Tregs by recombinant CCL17 over the same period of time. Since islets infected with 10 MOI of Ad-CCL17 produced ~3.5 pg/ml per islet, a chemotaxis assay performed with 100 islets should have triggered migration of Tregs. Additionally, numerous reports described the ability of CCL17 to efficiently recruit Tregs both *in vitro* and *in vivo* [204, 296]. One possible explanation is that islets produced a mediator that interfered with CCL17's chemotactic effect on Tregs. Future migration assays with CCL17-expressing islets will determine if this phenomenon is reproducible and examine the recruitment of DC sub-populations.

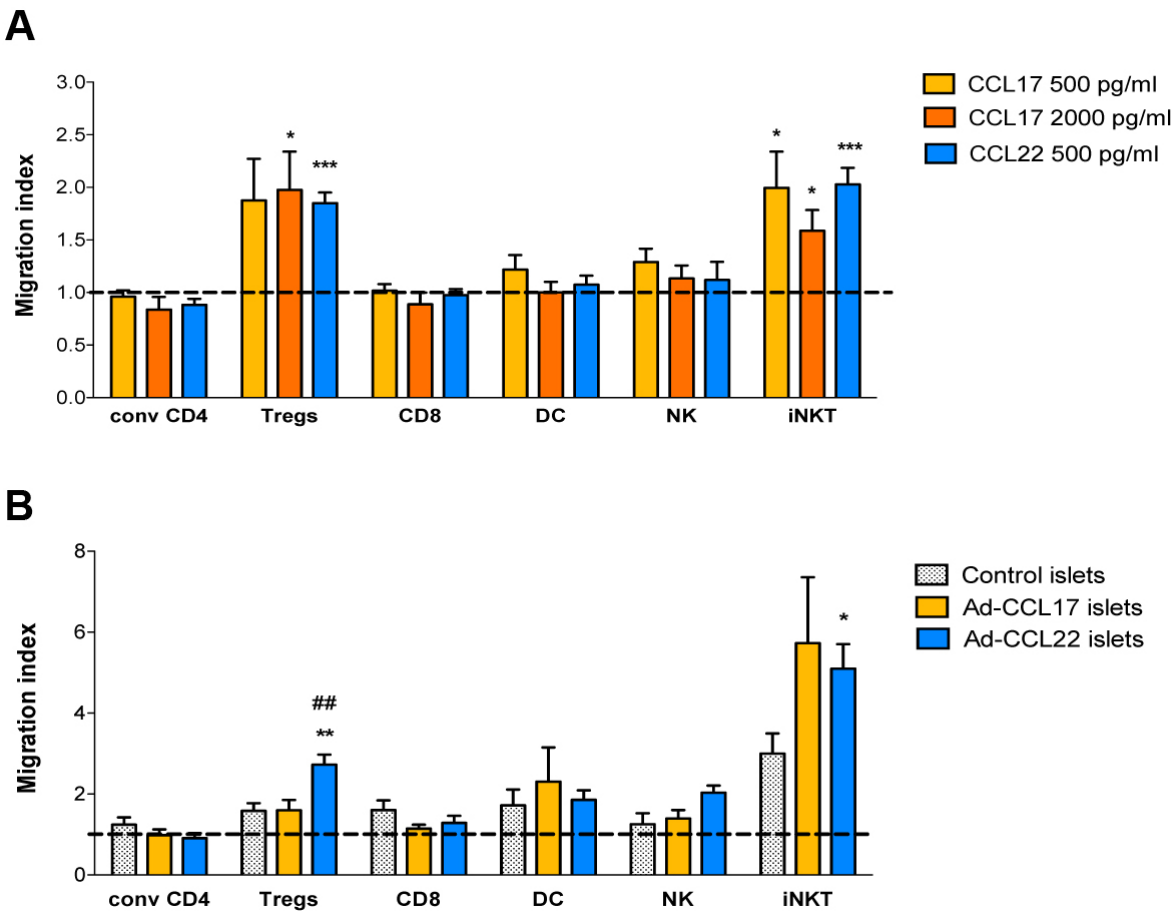

Figure 22: Chemotactic properties of CCL17

A. Migration of splenocytes over 4 hours towards different concentrations of recombinant CCL17 and CCL22. CCL17 data represent results from one experiment. *p<0.05 and ***p<0.001 versus media alone; p=NS between CCL17 and CCL22. **B.** Chemotaxis assay using islets transduced with 10 MOI of Ad-CCL17 or Ad-CCL22 or untreated islets. Splenocytes were left to migrate for 4 hours. The experiment was performed once with Ad-CCL17 islets. *p<0.05 and **p<0.01 versus control islets. ##p<0.01 versus Ad-CCL17.

5.2.3. CCL17 DOES NOT IMPACT ACTIVATION OF IMMUNE CELLS

We demonstrated for the first time that CCL22 has immunoregulatory properties beyond chemotaxis, as CCL22 induces functional activation of Tregs. We sought to determine whether CCL17 had a similar influence on Tregs or other immune subsets. Mixed populations of cells were treated with various concentrations of recombinant CCL17 ranging from 100 to 3000 pg/ml for different periods of time. Expression of CCR4 and the activation marker CD69 was unchanged on DCs, Tregs, iNKT, $CD8^+$ and conventional $CD4^+$ cells after incubation with CCL17, regardless of the concentration. Likewise, isolated Tregs cultured with CCL17 for 24 hours were comparable to untreated Tregs with respect to CTLA-4, CD62L and ICOS markers, indicating the absence of activation by CCL17 (Figure 23). These preliminary results point to differences between the two CCR4 ligands in terms of biological activities, since Treg function appears to be enhanced by CCL22 but not CCL17. Although both chemokines are known to recruit $CCR4^+$ cells, previous reports showed their diverse effect on their receptor, suggesting differential signalling upon binding to CCR4 [301, 302].

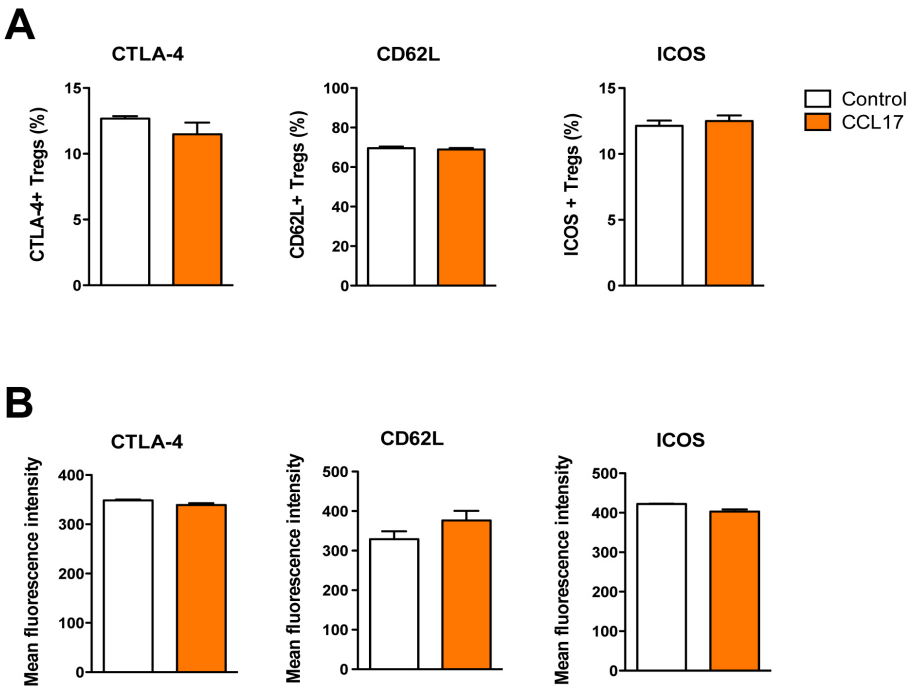

Figure 23: CCL17 does not activate Tregs

The expression of CTLA-4, ICOS, CD62L was assessed on isolated Tregs after 24 hours incubation with 500pg/ml of CCL17 or media alone. **A.** The proportion of Tregs expressing these activation markers remained unchanged after treatment with CCL17. **B.** Levels of CTLA-4, CD62L and ICOS were comparable between CCL17-treated and untreated Tregs.

5.2.4. CCL17 PROTECTS FROM RECURRENT AUTOIMMUNE DIABETES

The link between CCL17 and immunosuppression of anti-tumor responses motivated the study of this other CCR4 ligand in the setting of autoimmune diabetes. To determine whether CCL17 properties can be harnessed to modulate recurrent autoimmunity against beta cells, we transplanted diabetic NOD females with CCL17-expressing islets. We observed prolonged survival of transplants expressing CCL17 (Figure 24), remarkably resembling the pattern of islet grafts transduced with Ad-CCL22. Indeed, about 50% of CCL17-expressing grafts are protected from recurrent autoimmunity; however, these grafts eventually start failing approximately 40 days post-transplantation. We showed that adenoviral expression of CCL22 decreased significantly after such a period of time; this is likely the case for Ad-CCL17 as well, indicating again that prolonged expression of the chemokine may be required for long term protection from recurrent autoimmunity. Interestingly, CCL17 appears to be as effective as

CCL22 in protecting syngeneic transplants despite the apparent absence of Treg activation. Hence, the immunomodulatory properties of CCL17 appear to be primarily due to its chemotactic effect on Tregs and iNKT cells as described by others and confirmed by us.

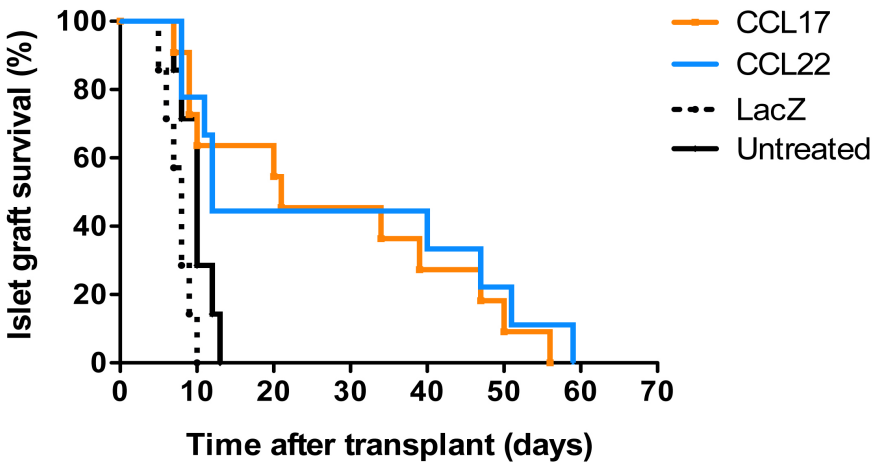

Figure 24: CCL17 protects syngeneic islet grafts from recurrent autoimmunity
After spontaneous onset of diabetes, NOD females were transplanted with 500 islets transduced with 10 MOI of Ad-CCL17 or Ad-LacZ or control islets. CCL17-expressing grafts (n=10) display significantly longer survival than untreated grafts (p<0.01, n=6) and LacZ (p<0.01, n=6) grafts, comparable to CCL22-expressing grafts (p=NS, n=9).

5.3. Discussion

This initial assessment of CCL17 properties in the context of autoimmune diabetes confirms that CCL17 expression in islets has the desirable effect of dampening the immune response against beta cells. Our results from chemotaxis assays showed that iNKT cells migrate to recombinant CCL17 and islets expressing CCL17. In contrast, recruitment of Tregs was not consistent in these two different settings, as only recombinant CCL17 attracted Tregs. In the literature, murine Tregs were shown to migrate towards CCL17 from a recombinant source or produced by DCs, as efficiently as towards CCL22 [204, 289]. The absence of Treg recruitment by CCL17-expressing islets may result from an interfering factor produced by islets, however, the reasons remain unclear and the experiment requires repetition. Should this observation be confirmed, the data would point to a preferential recruitment of iNKT cells by CCL17-expressing islets, which would be beneficial given the protective role of iNKT autoimmune diabetes.

In cell culture experiments, treatment with recombinant CCL17 did not modify the activation status of the different populations examined, including DCs, Tregs, conventional $CD4^+$, $CD8^+$ and iNKT cells. This finding reveals an important distinction between CCL17 and CCL22 in their influence on Treg activation and function. Further, it supports the idea that these two chemokines trigger different responses through their receptor CCR4, as described previously [301, 302]. In this regard, it would be of interest to investigate the differential signalling of CCR4 upon engagement of CCL17 and CCL22.

Despite these differences with CCL22, CCL17 expression in islet grafts resulted in significant protection from recurrent autoimmunity, almost identically to CCL22. Our results indicate that effectiveness of CCL17 principally relies on the recruitment of immunoregulatory cells such as iNKT cells and perhaps Tregs. We could argue that the improved Treg function induced by CCL22 plays little role in the mechanisms of protection from recurrent autoimmunity, since CCL17 is equally protective without enhancing Treg activity. By extension, it is possible that the ability of both chemokines to modulate the autoimmune response against beta cells is dependent on iNKT cells. Similar experiments to the ones performed to understand CCL22's mechanism of action will help us understand how CCL17 mediates protection of beta cells from immune destruction.

Among the human malignancies associated with expression of CCL17 and CCL22, the vast majority produce these chemokines conjointly [209, 216, 236, 303, 304]. The relative contribution of each chemokine to the immunosuppressive microenvironment in these various cancers has not been determined. However, our current knowledge of CCL17 and CCL22 suggests that these chemokines have non-redundant roles *in vivo*; hence, tumors may take advantage of their complementary or synergistic activities to foster immune privilege even more efficiently. The ability of chemokines to synergize has been described in both humans and mice [305-307]; this synergy may occur either at the level of receptor binding or intracellular signalling. More particularly, a broad range of chemokines have been shown to form complexes with CCL22 and increase CCL22-mediated chemotaxis *in vitro* [258]. Further investigations *in vitro* and in animal models will help determine whether such synergy occurs with CCL17 as well. Since both CCL22 and CCL17 were able to prevent autoimmune destruction of beta cells, we believe that in their combination they would prove to be at least as effective as each chemokine alone, and possibly more potent therapeutically.

CHAPTER 6:

CONCLUSION

6.1. SUMMARY

Immune homeostasis relies on a continual balance among the different immune cells in order to defend the organism against infections and tumors while preventing injury to self by excessive inflammation or autoimmunity. In autoimmune disorders, this balance is shifted toward the aberrant activation of deleterious cell subsets that target self-antigens resulting in immune destruction of normal tissues. Such situations arise because the mechanisms of self-tolerance are defective, in particular Tregs, which are crucial to inhibit autoimmune responses in the periphery. Type 1 diabetes, both in humans and mice, is associated with multiple immune defects, including reduced activity of Tregs [26, 168-170] and iNKT cells [162, 308, 309], as well as an activated phenotype in DCs [164, 310]. Numerous studies in NOD mice have established the protective roles of Tregs and iNKT cells in diabetes, either by enhancement of their function or increasing their numbers systemically [172, 175, 190, 191, 311]. However, these interventions are nonspecific and carry the risk of side effects owing to the generalized boost of immunosuppressive cells; therefore, effort is currently concentrated on developing immunotherapies specific for the particular tissue undergoing immune attack, such as beta cells in the case of diabetes.

In this study, we sought to direct endogenous Tregs and iNKT cells to pancreatic islets or islet transplants in order to harness their modulatory properties locally. Our strategy consisted of manipulating the trafficking of these cells by overexpressing the chemokines CCL22 and CCL17 in islets to preferentially recruit Tregs. The most compelling argument for our choice comes from human tumors, which foster immune privilege in their microenvironment by secreting CCL22 and CCL17. To determine the ability of CCL22 and CCL17 to modulate the autoimmune response against beta cells, we engineered the production of these chemokines in pancreatic islets or islet grafts of NOD mice using a gene therapy approach.

CCL22 gene delivery to pancreatic beta cells in prediabetic NOD females resulted in prevention of diabetes onset in 80% of mice, which was associated with lower frequencies of autoreactive T cell populations. CCL22 and CCL17 were also protective against recurrent autoimmunity, since their expression in syngeneic islet transplants in diabetic NOD recipients significantly delayed graft rejection. Protection from recurrent autoimmunity was not indefinite, however, and did not correlate with a reduction of islet-specific T cells. The discrepancy between these two models of autoimmunity against beta cells may be due to the different viral vectors used or

the timing of the intervention. To transduce pancreatic islets *in vivo*, we used an AAV vector that allows long-term expression of the encoded gene with low immunogenicity; conversely, transplanted islets were transduced with adenoviral vectors, which induce inflammatory responses and only provide short-term expression of the transgene [312, 313], as confirmed by our data. Adenovirus immunogenicity may cause additional damage to islet grafts thereby further promoting graft failure. Sustained expression of CCL22 or CCL17 in islet transplants using a less immunogenic viral vector may provide greater and long-term protection. On the other hand, the immune attack in the NOD model of recurrent autoimmunity may be more aggressive than that in prediabetic NOD mice, in which the autoimmune process is not yet fully developed at the time of viral gene transfer, and therefore harder to counteract. Several strategies in NOD mice have proven successful to prevent diabetes but were completely ineffective against recurrent disease in islet transplantation [314]. Accordingly, the effectiveness of CCL22 and CCL17 against the strong recurrent autoimmune response points to the great therapeutic potential of these chemokines in diabetes.

The immunomodulatory properties of CCL22 and CCL17 have been attributed to their ability to recruit Tregs. In agreement with this notion, we found enhanced recruitment of Tregs to pancreatic islets and islet grafts expressing CCL22. Moreover, depletion of Tregs completely abrogated the protective effect of CCL22 from diabetes development and recurrent autoimmunity, indicating that Tregs are required to mediate CCL22 protection. In the studies of CCL22-expressing tumors, no other cell population has been examined; however, other leukocytes are known to express CCR4 and migrate towards its ligands. The analysis of CCR4 expression profile on different cell subsets revealed that a significant proportion of iNKT cells exhibits high levels of CCR4 expression, even higher than Tregs. In chemotaxis assays, Tregs and iNKT cells similarly migrated towards recombinant CCL22 and islets expressing CCL22. Additionally, we found a remarkable influx of iNKT cells in CCL22-expressing grafts preceding any other immune subset, including Tregs. These findings strongly point to a previously unsuspected role of iNKT cells in the immunomodulation induced by CCL22. To address the importance of iNKT cells in our model, NOD mice lacking NKT cells will be transplanted with CCL22-expressing islets.

Another cell population, namely pDCs, emerged as a potential player in CCL22-mediated protection. Indeed, pDC recruitment was observed in CCL22 chemotaxis assays as well as in CCL22-expressing grafts. The weak expression of CCR4 on pDCs can hardly account for their migration towards CCL22, which is similar to Tregs and iNKT cells. Our data suggest that the influx of pDCs sequentially follows that of iNKT cells; hence, it is likely that pDCs are attracted

by iNKT cells rather than directly by CCL22. Interestingly, Diana and colleagues showed that iNKT cells promoted the accumulation of pDCs in the pancreas and thereby prevented autoimmune diabetes in mice [68]. The mechanism of pDC recruitment was not addressed in this report; yet, it seems very likely that it occurred through chemokines secreted by iNKT cells. Further, NK and type II NKT cells appeared to be retained in CCL22-expressing islet grafts whereas they withdrew from control grafts. We demonstrated *in vitro* that type II NKT cells have the ability migrate to CCL22, but not NK cells. With regard to type II NKT cells, we observed *in vivo* a relative retention (rather than recruitment) of these cells by CCL22, as their numbers were comparable between CCL22 and control grafts at an early time point. Our findings suggest that NK and type II NKT cells also participate in the control of the autoimmune response. Their efflux from the graft may be inhibited to allow suppression of their activity by Tregs and iNKT cells [133, 174], or cooperation as previously described for iNKT and NK cells [129].

This cellular crosstalk induced by CCL22 resulted in a tolerogenic environment within the islet graft characterized by lower levels of IFNγ and upregulation of IDO. IFNγ is a pleiotropic inflammatory cytokine produced by a broad range of leucocytes [315]. Of note, IFNγ is also an important effector molecule of cytotoxic $CD8^+$ cells implicated in beta cell death [316], thus, reduction of IFNγ in the CCL22-expressing graft may indicate inhibition of islet-specific $CD8^+$ cells. The activity of IDO is known to suppress T cell function and is associated with tolerance induction in many settings, including transplantation and autoimmune diabetes [317]. Interestingly, IDO is expressed both constitutively as well as inducibly in pDCs [318], and diabetes protection by IDO is tightly related to the presence of pDCs [187]. In our model of recurrent autoimmune diabetes, higher levels of IDO in CCL22-expressing grafts are concomitant with the local accumulation of pDC, suggesting that pDC participate in the protective effect of CCL22 by producing IDO. In culture experiments, we demonstrated that CCL22 activates Tregs by promoting expression of CTLA-4, ICOS and CD62L; notably CCL22 enhanced the ability of Tregs to induce IDO expression in DCs. Graft-infiltrating Tregs displayed a similar activated phenotype in the presence of CCL22, with higher proportions of $CTLA\text{-}4^+$ cells. As Tregs utilize CTLA-4 to induce IDO expression in DCs [117], we speculate that IDO expression in CCL22-expressing grafts is the result of Treg interaction with pDCs.

Similar *in vitro* experiments with CCL17 revealed that CCL17 had no influence on Treg activation, however, the capacity of CCL17-treated Tregs to modulate DC function remains to be tested. Nevertheless, CCL17 equally protected islet grafts from recurrent autoimmunity, which suggests that Treg activation is not essential for the immune modulation induced by

these chemokines. Alternatively, CCL17 may protect beta cells from autoimmune destruction through mechanisms distinct from those of CCL22. Further investigations will be necessary to understand the immune processes caused by CCL17 and identify the differences from CCL22. Still, our data point to a common denominator for CCL22 and CCL17 immunomodulatory properties, that is to say the recruitment of iNKT cells.

In conclusion, we report here the successful implementation of a strategy to harness the immunomodulatory properties of CCL22 and CCL17 to modulate the immune response against beta cells, thereby providing protection from primary and recurrent autoimmunity. Mechanistic studies highlight iNKT cells and Tregs as the key players mediating CCL22's protective effect. Our data suggest that iNKT cells and Treg cooperate to induce tolerance towards beta cells through modulation of pDCs. Our working model for the cellular and molecular mechanisms of CCL22-mediated protection from diabetes is illustrated in Figure 25.

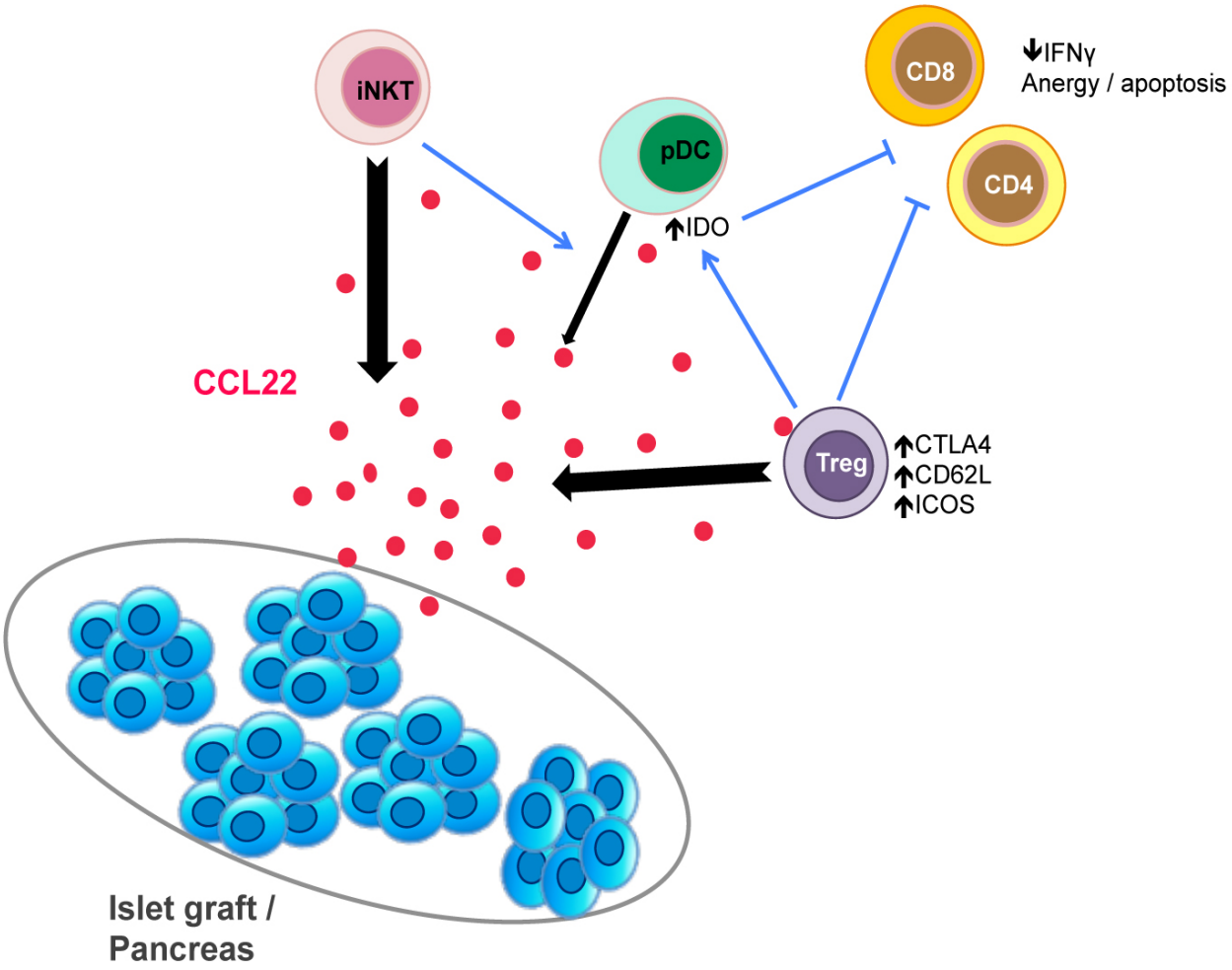

Figure 25: Working model for CCL22-mediated protection of beta cells from autoimmunity
The major mediators of CCL22's immunomodulatory effect include iNKT cells, Tregs and pDCs represented in the microenvironment of CCL22-expressing islets. Autoreactive $CD4^+$ and $CD8^+$ cells are likely inhibited by IDO activity as well as by different Treg suppressive mechanisms. Red circles represent CCL22 secretion by islets. Black arrows designate cell recruitment by CCL22-expressing islets. Blue lines indicate the influence of a particular cell or molecule on the indicated target.

6.2. SIGNIFICANCE

Type 1 diabetes remains an incurable disease with devastating complications despite insulin therapy. Beta cell replacement by islet transplantation has a great potential for treating patients with type 1 diabetes, but recurrent autoimmunity continues to be a major obstacle. Clinical trials are currently underway to test different immunotherapies, including the use of CD3 antibody to deplete T cells and infusion of Tregs or DCs engineered to remain immature to modulate the immune response [69]. The non-specific and systemic nature of these approaches may expose patients to risk of infections or tumors due to general immunosuppression. We need new therapies for type 1 diabetes that can protect beta cells from autoimmune destruction in affected individuals and following islet transplantation but that avoid the adverse effects of immunosuppression.

Our findings suggest a novel strategy for protecting beta cells from immune attack, by using the chemokines CCL22 or CCL17 to harness the natural regulatory properties of immune cells such as Tregs and iNKT cells. We found that expression of CCL22 recruits iNKT cells and Tregs to the islet and protects beta cells from both primary immune destruction and recurrent autoimmunity. Also known to recruit iNKT cells and Tregs, CCL17 was similarly effective in the islet transplant setting. These studies provide proof of principle that manipulation of endogenous Treg or iNKT cell trafficking has tremendous therapeutic potential for preventing beta cell loss in type 1 diabetes. To move this approach closer to clinical translation, we need to find ways to safely deliver these chemokines specifically to beta cells in humans. For this reason, it seems most likely that CCL22 or a similar approach would first be used in islet transplants, since the chemokine could be administered with the transplant on a scaffold or other slow-release mechanism. In the longer-term, this therapeutic approach might be used to attenuate autoimmune responses in islets prior to the development of autoimmunity, as a tool to prevent type 1 diabetes.

These studies provide significant insight into the immunomodulatory mechanisms of CCL22, a chemokine that is often expressed by human cancers to evade immune destruction. We show for the first time that CCL22 expression *in vivo* highly recruits iNKT cells in addition to Tregs. Previously unnoticed for their role in the growth of CCL22-expressing tumors, iNKT cells may now be identified as a new target for future therapies to destroy these tumors.

Both iNKT cells and Tregs are known to protect from autoimmunity [319, 320] and induce tolerance to allografts [136, 321, 322]; hence, CCL22 therapy could be beneficial in other autoimmune disorders and organ transplantation. Moreover, a similar strategy with different chemokines could be used to manipulate trafficking of cells of interest in various settings, for instance driving effector T cells to the site of tumors or chronic infections.

6.3. FUTURE DIRECTIONS

We demonstrate the successful utilization of chemokines to regulate immune responses locally in the context of autoimmune diabetes. Our data shows that CCL22 protects beta cells from the immune attack via recruitment of iNKT cells and Tregs. CCL22's mechanisms of action also include the participation of pDCs, which are likely responsible for the greater production of the tolerogenic enzyme IDO. Yet some outstanding questions remain to be answered. For instance, NK and type II NKT cells are retained within CCL22-expressing grafts, but their role is still unclear. These different immune players are likely to interact within the islet graft thus it would be interesting to examine the cellular crosstalk induced by CCL22. Additionally, characterization of autoreactive T cells will help determine the end result of this approach. Similar studies could be performed to verify whether CCL17 protects beta cells from autoimmunity using the same mechanisms.

As with any other type of transplanted tissue, islet grafts face the alloimmune response of the host, which hinders graft survival. For our approach to be relevant in islet transplantation, we also must understand its impact on allorejection. Therefore, our laboratory has examined the ability of CCL22 to protect islet transplants in an allogeneic mouse model. Importantly, CCL22 expression in islet grafts provides long-term protection from the alloimmune response and potentially induces donor-specific tolerance (Montane et al, unpublished data). Together our findings indicate that CCL22 is a potential therapy to enhance islet transplant survival.

Human and murine immune systems slightly differ in their development and activation both in the innate and adaptive responses [323]. However, iNKT cells and Tregs possess similar suppressive functions [115, 324] and respond to CCR4 ligands [241, 325] in both species. Hence, we anticipate that CCL22 therapy will be efficient in humans as well. Our approach could be tested in a humanized mouse model using immune-deficient mice that support

engraftment of human tissue and hematopoietic cells, allowing the study of human islet transplantation in the presence of a functioning human immune system [326].

At present, clinical translation of our approach depends on the use of viral vectors for chemokine expression in islets. We demonstrate that our approach is feasible both with adenoviral and AAV vectors, but preference should be given to AAV vectors owing to their lower immunogenicity. Notably, the efficacy of AAV has also been demonstrated in numerous preclinical studies and is being increasingly applied in human clinical trials [327, 328]. An alternative to viral vectors would be to use a slow-release vehicle that could be directed to the vicinity of the islet. Examples of such vehicles include biodegradable and biocompatible polymers used to encapsulate therapeutic agents, including drugs and proteins, for prolonged release [329, 330]. Chemokines like CCL22 and CCL17 are ideal compounds for nanomedicine approaches: they need not be expressed within cells; only localized high protein levels are required for effectiveness.

In the search for new immunotherapies for type 1 diabetes, we might consider testing other chemokines that preferentially recruit cells with immunosuppressive properties. A recent report highlights CCL28, the ligand for CCR10, in Treg-related escape of tumors from immune destruction [331], making CCL28 a candidate chemokine for immunomodulation in type 1 diabetes. Further, several chemokines could be combined to take advantage of their respective properties or their synergistic effects. Our strategy may also be associated with Treg-based therapies to improve their efficacy by directing Tregs to the pancreas or other site of interest.

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