Studying the role of Th17 cells in autoimmune diabetes and generation of a beta cell reporter mouse by lentiviral transgenesis

Lentivirale transgene Technologie zur Erforschung der Rolle von Th17 Zellen im autoimmunen Diabetes und zur Generierung einer Beta-Zell-Reportermaus

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AFFIDAVIT

Summary/ Zusammenfassung

English: Type 1 diabetes affects around 0.5% of the population in developed countries and the incidence rates have been rising over the years. The destruction of beta cells is irreversible and the current therapy available to patients only manages the symptoms and does not prevent the associated pathological manifestations. The patients need lifelong therapy and intensive research is being carried out to identify ways to eliminate autoimmune responses directed against pancreatic beta cells and to replace or regenerate beta cells. The work presented herein aimed at analyzing the role of the Th17 T cell subset, characterized by secretion of the pro- inflammatory cytokine IL-17A, in autoimmune diabetes and also at generating a beta cell reporter mouse line in the NOD background, the most widely- used mouse model for type 1 diabetes. We generated IL-17A knockdown (KD) NOD mice, using RNAi in combination with lentiviral transgenesis. We analyzed diabetes frequency in IL-17A deficient mice and found that the loss of IL-17A did not protect the transgenic mice from diabetes. Based on these observations, we believe that Th17 cells do not play a critical role in type 1 diabetes through the IL-17A pathway, though they might still be involved in the disease process through alternate pathways. We also generated NOD and NOD-SCID mice with a transgene that drives the beta cell specific expression of a luciferase reporter gene. We used a lentiviral construct, which combined a luciferase sequence and a short- hairpin RNA (shRNA) expression cassette, allowing gene- knockdown under the beta cell specific rat insulin promoter (RIP). These mice will be of use in studying beta cell phenotypes resulting from the knockdown of target genes, using non- invasive bioimaging. We believe that the generation of these reporter mouse lines for diabetes studies will prove valuable in future investigations. Furthermore, the demonstration that the loss of IL-17A does not alter susceptibility to type 1 diabetes should help clarify the controversial involvement of Th17 cells in this disease.

Deutsch: In Industrieländern erkranken etwa 0,5 % der Bevölkerung an Typ-1-Diabetes und die Krankheitsrate ist in den letzten Jahren angestiegen. Die dabei stattfindende

Zerstörung der insulinproduzierenden Beta-Zellen ist irreversibel und die derzeitig verfügbaren Therapien behandeln lediglich Symptome, verhindern die pathologischen Auswirkungen aber nicht. Patienten benötigen daher eine lebenslange Therapie und es wird intensiv daran gearbeitet, Wege zu identifizieren, die die autoimmune Antwort gegen pankreatische Beta-Zellen unterbinden, oder aber Beta-Zellen ersetzen, beziehungsweise regenerieren lassen.

Die vorliegende Arbeit hat zum Ziel, die Rolle der Th17 T-Zellen, welche durch die Sekretion des proinflammatorischen Zytokins IL-17A gekennzeichnet sind, in Typ-1-Diabetes zu analysieren. Zusätzlich wurden Reportermauslinien im NOD Hintergrund, dem am weitesten verbreiteten Mausmodell für Typ-1-Diabetes, generiert.

Durch RNAi, in Kombination mit lentiviraler transgener Technologie, wurden IL-17A *Knockdown* (KD) NOD Mäuse generiert. Die Diabeteshäufigkeit in IL-17A defizienten Mäusen wurde mit dem Ergebnis untersucht, dass der Verlust von IL-17A die transgenen Mäuse nicht vor Diabetes schützt. Von dieser Beobachtung ausgehend wurde gefolgert, dass Th17 Zellen zumindest über den IL-17A Signalweg keine entscheidende Rolle bei Typ-1-Diabetes spielen, diese allerdings durch alternative Signalwege durchaus im Krankheitsprozess beteiligt sein könnten.

Außerdem wurden NOD und NOD-SCID Mäuse mit einem Transgen, das die Beta-Zell spezifische Expression eines Luciferasereporters steuert, generiert. Hierbei wurde ein lentivirales Konstrukt genutzt, welches sowohl die Luciferase, als auch eine short-hairpin RNA (shRNA) Expressionssequenz beinhaltet, um einen Genknockdown unter Kontrolle des spezifischen Insulinpromotors der Ratte (RIP) zu erlauben. Diese Mäuse werden bei zukünftigen nicht-invasiven bildgebenden Untersuchungen des Beta-Zell Phänotyps, der aus dem *Knockdown* von Zielgenen resultiert, von großem Nutzen sein. In zukünftigen Untersuchungen wird sich die Generierung dieser Reportermauslininien für Diabetesstudien sicherlich als wertvoll erweisen. Des Weiteren sollte die Erkenntnis, dass der Verlust von IL-17A die Anfälligkeit für Typ-1-Diabetes nicht verändert, zu einem besseren Verständnis der kontrovers diskutierten Beteiligung der Th17 Zellen führen.

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Abbreviations

AGO2	Argonaute 2
AHR	Aryl hydrocarbon receptor
AMDCC	Animal Models of Diabetic Complications Consortium
APC	Antigen presenting cell
ATP	Adenosine tri phosphate
BB rat	Biobreeding rat
BLI	Bioluminescence imaging
bp	Base pair
B6	C57BL/6
Ca^{2+}	Calcium ion
CCD	Charge coupled device
CCR	Chemokine receptor
CDKs	Cyclin dependent kinases
cDNA	Complementary DNA
CFA	Complete Freund's antigen
CIA	Collagen induced arthritis
CKIs	Cyclin dependent kinase inhibitors
CMV	Cytomegalovirus
CNS	Central nervous system
CO2	Carbon dioxide
Cre	Cre recombinase
CT	X-ray computed tomography
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
Ctrl	Control
CY	Cyclophosphamide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide triphosphate
DTZ	Dithizone
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
ECL	Enzymatic chemiluminescence
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
Fig.	Figure
FoxO1	Forkhead box protein O1
FoxP3	Forkhead box P3
Fwd primer	Forward primer
GAD65	Glutamic acid decarboxylase 65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
G- CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GLP-1	Glucagon –like peptide- 1
Glut2	Glucose transporter 2
GM- CSF	Granulocyte macrophage colony stimulating factor
HCG	Human chorionic gonadotropin
HEK	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hluc	Firefly luciferase
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
IA-2	Insulinoma associated antigen 2
IBD	Inflammatory bowel disease

IFN	Interferon
IgE	Immunoglobulin E
IGF	Insulin like growth factor
IGRP	Islet- specific glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
IL-17R	Interleukin17 receptor
INK4	Inhibitors of CDK4
iNOS	Inducible nitric oxide synthase
IRF4	Interferon regulatory factor 4
I.P.	Intra peritoneal
IQGAP1	IQ motif containing GTPase activating protein 1
Kb	Kilo base
KD	Knockdown
KDa	Kilo Dalton
Km	Dissociation constant
LB	Lysogeny broth
LoxP	Locus of X- over P1
LPS	Lipopolysaccharide
LT	Lentiviral transgenesis
LTR	Long terminal repeats
MafA	V- maf musculoaponeurotic fibrosarcoma oncogenes homolog A (avian)
MafB	V- maf musculoaponeurotic fibrosarcoma oncogenes homolog B (avian)
MACS	Magnetic activated cell sorting
MAP	*Mycobacterium avium* subspecies paratuberculosis
MBP	Myelin basic protein
Men1	Multiple endocrine neoplasia type 1
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIF	Macrophage migration inhibitory factor
min/ mnt	Minutes
MIP	Mouse insulin promoter
miRNA	microRNA
MLL	Myeloid/lymphoid or mixed-lineage leukemia
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MS	Multiple sclerosis
N	Normal
NFAT	Nuclear factor of activated T cells
ng	nano gram
Ngn3	Neurog3
NKT	Natural killer T cell
nm	nano meter
NO	Nitric oxide
No:	Number
NOD	Non- obese diabetic mouse
NOD SCID	Non-obese diabetic severe combined immunodeficiency
nTreg	Naturally occurring regulatory T cell
O2	Oxygen
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated membrane pattern
PBS	Phosphate buffered saline
PC 1/3	Prohormone convertase PC 1/3
PCD	Programmed cell death
PCR	Polymerase chain reaction
Pdx1	Pancreatic and duodenal homeobox 1
PEC	Polyelectrolyte complex
PET	Positron emission tomography
PI	Propidium iodide

PL	Placental lactogen
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PMG	Pregnant mares serum
PNK	Polynucleotide kinase
pol	RNA polymerase
PP	Pancreatic polypeptide
PRL	Prolactin
PRR	Pattern recognition receptor
PS	Phosphatidyl serine
P/S	Penicillin / Streptomycin
PTPN22	Protein tyrosine phosphatase, non- receptor type 22
Puro	Puromycin
qPCR	Quantitative real time PCR
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa B ligand
RBC	Red blood cell
Rev primer	Reverse primer
RIPA buffer	Radioimmunoprecipitation assay buffer
RIP OVA	Rat insulin promoter- ovalbumin
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROR	Retinoic acid related orphan receptor
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institue-1640
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SCID	Severe combined immunodeficiency
shRNA	short hairpin RNA
siRNA	small interfering RNA

SPECT	Single photon emission computed tomography
SPF	Specific pathogen free
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
SV40	Simian vacuolating virus 40
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
TC	Tissue culture
TCR	T cell receptor
TF	Trifusion
Tg	Transgenic/ Transgene
TGF	Transforming growth factor
Th cell	T helper cell
Th1	T helper cell type 1
Th2	T helper cell type 2
Th9	T helper cell type 9
Th17	T helper cell type 17
TLR	Toll like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine substrate
TNF	Tumour necrosis factor
T1D	Type 1 diabetes
U	Unit
UV	Ultra violet
WT/wt	Wild type
ZnT8	Zinc transporter 8
2- ME	Beta mercaptoethanol
3’ UTR	3’ untranslated regions
°C	Degree Celsius
µg	Micro gram
µl	Micro liter

Chapter 1

INTRODUCTION

1.1 Immune system

The immune system is a complicated system of structures, which protects an organism against diseases. It does this by means of identifying and eliminating pathogens. To function properly, the immune system needs to correctly distinguish between harmful pathogens and the organism's own cells and tissues. In higher organisms, the immune system is highly evolved and includes a complex and dynamic system made up of countless different proteins, enzymes, cells and tissues that interact with each other[1, 2].

The pathogens when trying to invade the body first encounter the mechanical, chemical and biological barriers. These prevent the pathogens from entering an organism or in some cases, eject them from the body upon entry. The uninterrupted skin usually acts as a mechanical barrier preventing the entry of pathogens into the organism. Coughing and sneezing ejects pathogens from the body while the mucus in the respiratory system traps microbes. Examples of chemical barriers include the β-defensins secreted by skin and the respiratory system and the lysozymes and phospholipase A2 in the tears and saliva. These have antimicrobial properties. Gastric enzymes that protect against ingested pathogens are another example of chemical barriers. The commensal flora in the gut competes with harmful bacteria for food and space. They modify the gut environment and eliminate the pathogenic bacteria thus acting as a biological barrier[3].

The immune system is broadly divided into two separate but complementary parts: the innate immune system and the adaptive immune system[3]. Innate immune responses are non-specific while adaptive immunity is characterized by antigen specificity. Innate immune system is usually the most dominant defense system and the innate immune responses are not specific to distinct antigens[3]. The innate immune cells identify pathogens using the pattern recognition receptors (PRRs) that identify pathogen associated membrane patterns (PAMPs), which are associated with a broad range of microbes. This is turn lead to broad range specificity[4, 5]. The innate immune cells include mast cells, phagocytes, macrophages, dendritic cells, natural killer cells, neutrophils,

eosinophils, basophils and γδ T cells[4]. The pattern recognition receptors are broadly classified into secreted, transmembrane and cytosolic. The transmembrane PPRs include toll like receptors (TLR), which are able to activate several classes of adaptive immune responses including antibody, B and T cell responses[5].

The pathogens are eliminated by a variety of mechanisms including phagocytosis, respiratory burst and release of toxic molecules. The complement system includes a cascade of several plasma proteins and plays an important role in enhancing the removal of microbes. The complement system enhances the recruitment of inflammatory cells, causes the opsonization of pathogens and eliminates the neutralized antigen antibody complexes from the body. It also destroys the pathogen-infected cells by disrupting the cell membranes and causing subsequent cell death[3, 6]. Many pathogenic organisms have evolved methods to evade elimination by the innate immune system. These evasion mechanisms include intracellular replication, mimicking host cells, receptor modifications to avoid phagocytosis and even direct killing of phagocytes.

Innate immune responses are able to fight and clear a large number of infections. In cases where innate immunity is insufficient to do so, adaptive immune responses are elicited. The innate immune system plays an important role in activating the adaptive immune responses through antigen presentation. The adaptive immune responses are pathogen specific[3, 4]. The adaptive immune system is composed of highly specialized cells, which can recognize and remember specific pathogens[4]. The lymphocytes could either be naïve cells that have never encountered their antigen; effector cells that have encountered their cognate antigen and are involved in an active response or memory cells that retain the memory of already encountered antigens for future reference. The memory enables the adaptive immune system to mount increasingly efficient responses against previously encountered pathogens upon re-encounter[3, 4].

The adaptive responses are activated by the innate immune system and the major players are the B and T lymphocytes. The adaptive immune cells exhibit a wide range of specificities. This adaptability arises from the process of somatic recombination that

happens during the development of B and T cells in the central lymphoid organs. This recombination generates cells with unique antigen receptor specificity leading to a large number of cells capable of recognizing a large number of different antigens[4]. B cells play a major role in humoral immune responses owing to the production of antibodies. These are proteins that can bind to specific antigens and result in their elimination. T cells on the other hand are crucial for cell-mediated immune responses and they are able to cause direct cell death[7].

1.2 Autoimmune diseases:

Disorders of the immune system can lead to autoimmunity or immunodeficiency. Autoimmune diseases are caused by a breakdown of immunologic tolerance that ends up with the immune system attacking the body's own cells. Immune tolerance is the ability of the immune system to tolerate or ignore the body's own cells and non-pathogenic organisms and to react only to non- self. It is reported that approximately 3% of the populations in developed countries suffer from autoimmune diseases[8]. Immunologic tolerance has two different yet complementary arms, central tolerance and peripheral tolerance.

Central tolerance occurs in the thymus where the maturing lymphocytes are exposed to self-antigens in these organs either endogenously expressed or made available from peripheral sites[9]. The process includes two stages of lymphocyte selection. Positive selection leads to the selection of lymphocytes, which can recognize self-MHC molecules and these selected lymphocytes are further whittled down by the process of negative selection. During negative selection, the lymphocytes that react with high affinity to the self-antigens are deleted before they can mature and leave these organs so as to prevent auto immunity[9- 11]. Peripheral tolerance occurs after the T cells mature and reach the periphery. Many tissue specific antigens are not expressed in the thymus and hence some of the mature T cells exiting from the thymus could potentially be reactive to these tissue specific antigens in the periphery and lead to autoimmunity. Peripheral tolerance mechanisms are in place to prevent this possibility. The autoreactive T cells are usually

deleted or subjected to a state of unresponsiveness called anergy. Regulatory T cells also contribute to peripheral tolerance by suppressing autoreactive T cells[9, 12].

A defect in either central or peripheral tolerance mechanisms could lead to the survival of autoreactive T cell clones, which could potentially react to self-antigens thus leading to autoimmunity. Central tolerance weeds out the autoreactive T cells and prevent their entry into periphery. Autoreactive specificities that manage to escape this first line of elimination are actively controlled by the peripheral tolerance mechanisms. It is known that despite these tolerance mechanisms, autoreactive T cells still persist in the peripheral T cell repertoire indicating that the tolerance mechanisms are not absolute. Inefficient tolerance in the thymus by means of altered selection of T cell repertoire or impaired negative selection could contribute to autoimmunity as this might lead to the presence of additional autoreactive specificities in the periphery[12].

Once the T cells are in the periphery, several possibilities could contribute to autoimmunity. T cells require homeostatic signals for survival in the periphery. Increased survival of T cells and increased TCR signaling could lead to preferential stimulation of self-reactive T cells. Defects in the removal of self-reactive clones could lead to an accumulation of autoreactive cells causing autoimmunity[13]. There is also some data indicating that presentation of self- antigens by activated mature APCs could lead to activation of autoreactive T cells and not the induction of tolerance. As mentioned earlier, autoreactive specificities are found to be present in the peripheral T cells repertoire. Some pathogens have antigens similar to self- antigens and the pathogen activated T cells could cross react with self-antigens and cause autoimmunity. Regulatory T cells play a crucial role in peripheral tolerance. Any defect in this cell population also contributes to autoimmunity[12].

The breakage of immune tolerance leads to the immune system mounting a severe response against self-antigens and leading to autoimmune diseases. Some people are more susceptible to developing autoimmunity. This increased susceptibility is found to depend on several genetic and environmental factors. Genetic susceptibility to

autoimmune diseases is associated with multiple genes[7, 14]. The three main gene sets associated with disease are immunoglobulins, T cell receptors and the major histocompatibility complexes[15-19]. Gender is also thought to be important as more than 75% of people who suffer from autoimmunity are women[8, 20]. Pregnancy, hormonal changes and imbalanced X chromosome inactivation are some of the factors suggested to be responsible for the increased susceptibility of women to autoimmunity[21, 22]. Higher estrogen and lower testosterone levels in females are also believed to contribute to this increased incidence[23].

Several environmental factors are also found to be associated with autoimmunity. There is a lot of evidence suggesting a role for bacterial, viral and parasitic infections in autoimmunity. These include the observation of autoimmunity following infections and the detection of high titers of antibodies against infectious agents in patients with autoimmune diseases[7, 24]. A number of explanations have been put forward to explain how infections might trigger autoimmunity. A possible mechanism is molecular mimicry where the infectious agent displays structural similarity to some of the host proteins and the antibodies generated end up attacking the host proteins owing to their similarity to the pathogen[25]. It is also possible that the infectious agent could act as a superantigen thereby activating a large number of T cells in the absence o f appropriate costimultion[26]. The infectious agent may also be able to cause polyclonal activation where it acts as an adjuvant and exacerbates pre existing autoimmune reactions[27]. All these could lead to breakage of tolerance and result in autoimmunity. Both *Klebsiella pneumoniae* and *coxsackievirus B* have been found to be associated with Type 1 diabetes[28].

Autoimmune diseases are broadly classified into systemic disorders and localized disorders. Systemic disorders are the autoimmune disorders in which the immune responses are not directed towards tissue specific antigens and examples include systemic lupus erythematosus and rheumatoid arthritis. In localized disorders, immune responses are directed towards antigens that are tissue or organ specific and examples include type 1 diabetes, vitiligo and celiac disease[1]. Autoimmune diseases are usually diagnosed and monitored using autoantibody tests, symptoms and measurement of inflammation and

organ function. There is currently no cure for these diseases and the treatment usually varies from patient to patient. It is usually aimed at relieving the symptoms, minimizing the tissue damage and preserving the organ function.

1.3 Type 1 Diabetes:

Type 1 diabetes mellitus (T1D) is an autoimmune disorder, which involves the irreversible and selective destruction of the insulin producing β cells of the pancreas[29]. This causes the reduction and eventual loss of insulin production leading to increased glucagon secretion by the α- cells. Insulin insufficiency and excessive glucagon secretion finally result in hyperglycemia and ketoacidosis[30]. The disease accounts for about 10% of all diabetes cases while Type 2 Diabetes accounts for the remaining 90%. Type 1 Diabetes incidence varies geographically and it affects 0.5% of the total population in developed countries[230]. Gillespie[31] in her review observed that one study of incidence rates mentioned a yearly incidence increase of 3%[32] while another reported that the diabetes incidence was expected to increase by 40% in 2010 as compared to 1998[33]. Patterson et al.[34] in 2009 reported that the prevalence of cases in individuals younger than 15 years is expected to rise by 70%, suggesting a shift towards an earlier age [24].

As in the case of other autoimmune diseases, both T- cells and B-cells are believed to be responsible for the disease. Though both CD4+ and CD8+ T-cells are found to be required for development of T1D[35], the initial role is found to be played by CD8+ T cells [35-37]. Several mechanisms have been reported for the process of β- cell destruction by T-cells. These include direct killing by cytotoxicity, release of factors like proinflammatory cytokines (which can result in the production of inducible Nitric oxide synthase, iNOS) or by signaling through the programmed cell death (PCD) pathway[38-40]. CD4 cells mostly provide help to CD8+ T cells and B cells by providing cytokines like IL-21 thereby enhancing the autoimmune response[24]. The exact role played by B-cells and their autoantibodies is still not clear though most of the autoantigens in diabetes were identified from the presence of autoantibodies[41-43]. It is likely that B cells are active participants in the disease process owing to their ability to present antigen to diabetogenic

CD4 and CD8 T cells[24]. Several proinflammatory cytokines including MIF, IFN-γ, IL-1, IL-17, TNF-α and IL-21 have been implicated in the development of T1D and these cytokines are believed to play a role by the recruitment of effector cells to the pancreas and also by enabling the release of reactive oxygen and nitrogen species[44-46].

Autoimmune diseases are caused by improper immune responses against self-antigens. In case of type 1 diabetes, several autoantigens have been identified so far. The 4 principal autoantigens are believed to be glutamic acid decarboxylase (GAD65)[47], insulinoma-associated antigen- 2(IA-2)[48], insulin[49] and zinc transporter 8 (ZnT8)[50]. 90% of the people diagnosed with type 1 diabetes have autoantibodies to at least one of these antigens[31]. It has been found that as the disease progresses, the number of islet antigens recognized by T-cells and autoantibodies increases and this phenomenon is termed as epitope spreading. Recently, Krishnamurthy et al.[51] reported that eliminating the immune response to insulin in NOD mice can protect from the development of the disease but the same effect could not be observed on elimination of the immune response to islet-specific glucose-6- phosphatase catalytic subunit related protein (IGRP) indicating that there is a hierarchy of antigens and that insulin might be the primary autoantigen and that IGRP might be secondary thereby supporting the idea of epitope spreading.

Both genetic and environmental factors are believed to contribute to the development of type 1 diabetes. The role played by genetic factors is well supported by experimental studies but the environmental role is still not well elucidated. Some studies suggest a role for viruses including enteroviruses[52], rotavirus[53] and rubella[54]. There is a clear link between the bacterial composition of the intestine and type 1 diabetes[24]. *Mycobacterium avium* subspecies paratuberculosis (MAP) has also been shown to be a risk factor in diabetes. Other reported environmental triggers include cow's milk, wheat proteins and vitamin D[24]. Several genes have been identified to be associated with type 1 diabetes and the most important one among these is the HLA locus. Some HLA haplotypes like DR4-DQ8 and DR3-DQ2 are found to be associated with a high risk of disease[55] whereas another haplotype, DR15-DQ6 is found to be protective[56]. To date, several other

candidate genetic loci have been identified including insulin[57], CTLA-4 (cytotoxic T lymphocyte antigen 4), PTPN22[24] and KIAA0350 (Fig. 1 Todd et al.[58]).

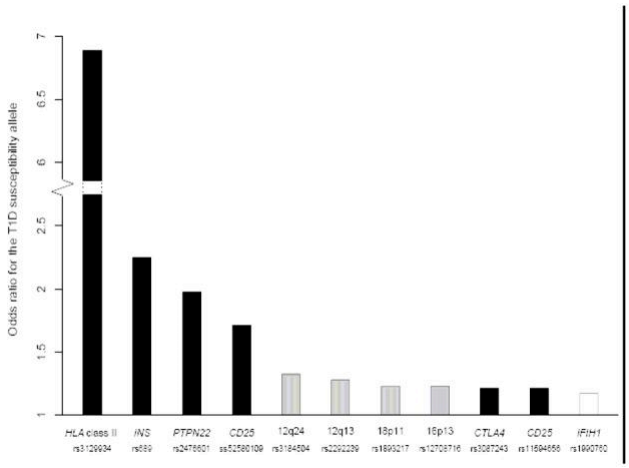


Fig. 1: Todd et al. *Nature Genetics* (2007). 39(7): 857-864.[58]

Type 1 Diabetes is divided into two phases. During the first phase called peri- insulitis, mononuclear infiltrates surround the islets and this stage is also called non destructive insulitis. The second stage is destructive insulitis when the infiltrates invade the islets and destroy them leading to overt diabetes when there is no longer sufficient insulin production to regulate blood glucose levels[230]. It is believed that the clinical manifestations are observed only after about 80- 90% of the β- cells is destroyed[24, 31].

1.4 Background and the relevance of current work:

To date, there is no cure for type 1diabetes and the only long-term therapy option available to patients is daily injections of insulin. Even in ideal cases, insulin replacement still shortens life expectancy by at least around 10 years[24]. In addition to being cumbersome, this approach is incapable of preventing the long term pathologies associated with type 1 diabetes including kidney and heart disease, neuropathy, and retinopathy. This has led to an intensive search for potential therapeutic strategies.

Current research is focused on 3 main objectives. These include islet cell transplantation, modulation of autoreactive immune cells and endogenous β- cell regeneration. Islet cell transplantation is an attractive option but the islets are obtained from cadavers and the patients have to subjected to life long immune suppressive regimen. If we could enable endogenous β- cell regeneration, it would eliminate the need for immune suppression and this would offer a better choice for the patients. Even if we could achieve successful islet function by transplantation or regeneration, the autoreactive immune cells are still present in the patients and they could destroy the new β- cells leaving all therapy fruitless in the absence of immune suppression. Hence it is quite critical that the role played by the immune system in the disease development be elucidated so that we could eliminate the autoreactive cells and the key immune factors and give the β- cells a chance to survive. This would be a task of epic proportions as the exact roles played by the various immune cells and the immune molecules are largely unknown and new factors are being identified with more research.

The promise of islet cell transplantation as a potential therapy was improved on significantly when Shapiro et al.[59, 60] established the protocol which later came to be known as the 'Edmonton protocol'. Though the transplanted islets can restore the insulin production, they are susceptible to the same autoimmune attack, which causes the initial destruction of the patient's β- cells. Hence the approach carries the disadvantage of the need for a lifelong immunosuppressive regimen. Even with severe immune suppression, long-term transplant survival is rarely achieved. Another major disadvantage is the scarcity of available donor islets for transplant. In addition, the current isolation procedures are not efficient enough and not all the isolation attempts produce transplant-ready islets, further complicating the matter. Han et al.[61] mention that islets are subjected to extreme conditions including mechanical shear, enzyme digestion, inflammatory cytokines, free radicals and osmotic shock during isolation and transplantation. They are also deprived of their trophic support. Upon transplantation into diabetic patients, the islets face additional unfavorable conditions like high serum lipid and glucose levels[61]. Islets have also been shown to become fully vascularised only 1-2 weeks after

transplantation and this results in lack of nutrients and oxygen[62] in the initial weeks after transplantation. All these fact ors contribute to significant islet loss and dysfunction after transplantation, thereby undermining the efficiency and purpose of the procedure.

The difficulties encountered with islet transplantation and the destruction of the transplanted islets by the immune system have clarified the need for identifying strategies to protect the β - cells from immune destruction or to enable endogenous β - cell regeneration. Even if immune tolerance can be induced, it might be critical to enable β-cell regeneration in cases where the disease is already apparent. Several studies have looked at the potential of generating β - cells. Seaberg et al.[63] identified a pancreatic precursor which can differentiate into cells which resemble β - cells. Recently Zhou et al.[64] reported that adult pancreatic exocrine cells can be reprogrammed to β - cells by expression of the transcription factors Ngn3, Pdx1 and MafA. Though much work needs to be done before the search for a cure can be realized, the greatest promise might lie with endogenous beta cell regeneration coupled with tolerance induction.

The two projects encompass these two separate yet complementary fields of research. The first project dealt with unraveling the role played by IL-17A, the key cytokine produced by one of the T helper subsets in type 1 diabetes. The second project involved generation of reporter mice in which the endogenous β- cell mass could be monitored in real time. We aimed at coupling the real time monitoring of beta cell mass with tissue specific knockdown of target beta cell modulator genes.

1.5 The NOD Mouse Model:

The development of autoimmune diabetes results from a combination of genetic susceptibility and environmental factors. A thorough understanding of the disease process is required for the development of a successful therapy. The difficulty of studying the disease in humans has necessitated the use of animal models, which ideally mimic the characteristics of the human disease closely. There are several diabetes models, the use of which offers different perspectives for understanding the disease. These include the

diabetes prone BB (biobreeding) rat, the NOD (non-obese diabetic) mouse, *in silico* models based on the NOD mouse, humanized mouse models, transgenic pseudo- self antigens expressed in the islets (RIP-OVA) and beta cell damage models like streptozotocin induced diabetes[65]. The NOD mouse is the most widely used model for studying autoimmune diabetes *in vivo*.

The NOD mouse provides a spontaneous model where aggressive insulitis is observed around 12 weeks of age and around 80% of the females develop hyperglycemia by 30 weeks of age[66]. NOD mice were first described in 1980[67, 68] and since then they have been widely used as an experimental model for type 1 diabetes. The mice develop spontaneous diabetes with the incidence rates being 60% - 80% in females and 20%- 30% in males[68, 69]. The pattern of diabetes occurrence is different between females and males in that diabetes onset occurs between 12 to 14 weeks of age in the female mice whereas in the case of males, it is slightly later. In their review, Anderson and Bluestone[70] report that the mouse strain is a good model for type 1 diabetes as it develops spontaneous autoimmune diabetes that is quite similar to the human T1D. In both cases, the disease is primarily mediated by autoreactive T cells (both CD4+ and CD8+), the disease is under complex polygenic control and there is an abundance of pancreas specific autoantibodies[70] .

The NOD mouse has become an invaluable model for unraveling the mystery of type 1 diabetes. Ever since its inception, the model has been used for a staggering number of investigations that have been aimed at improving our understanding of T1D i.e. the whole process of disease development, the role played by autoantigens and the immune cells and also aimed at developing potential therapeutic strategies. Numerous transgenics and knockout strains have been developed in the NOD background. All these efforts have helped enormously in the study of the disease pathology.

Though more than 170 approaches have been found to delay or prevent T1D onset in the NOD mice[71], very few have been found to be effective in human T1D[70]. In their review, Shoda et al.[72] analyzed the multitude of successful interventions in NOD mice and the

reasons for the difficulty in translating these to treat or cure human type 1 diabetes. They noted that out of the 463 agents evaluated, only 23 were initiated in diabetic NOD mice though 16 out of the 23 were considered successful. Anti- CD3 treatment has been found to be beneficial in both NOD mice and in recently diabetic patients[31, 70, 72, 73]. Though some of the other therapies including oral insulin and nasal insulin administration showed success in animal models, most have shown none or very little efficacy in treating human T1D. Shoda et al. [72] attribute this lack of success to discrepancies in timing, dosage and protocols. Roep[73] notes that some of the candidate therapies derived from animal models proved unsafe and that most animal studies end their assessments too early. The animal studies were done in prediabetic mice while the human studies included diabetic patients. The dosages used in human studies were also significantly lower than the ones found to be beneficial in animal studies. They recommend that careful consideration of dosage, timing, protocols and species-specific differences might permit better clinical translation.

1.6 Models of induced diabetes in research:

Spontaneous diabetes models like the NOD mouse need 4-5 months to develop the disease. This is a long duration, which in some cases is inconvenient. There are some models of induced and accelerated diabetes, which help overcome this problem. Diabetes can be induced by the injection of several substances including cyclophosphamide, streptozotocin and alloxan. Upon injection, the mice develop diabetes within a short duration ranging from 2-3 days to 2-3 weeks. This depends on the nature and the dosage of the chemical used. These induced models can be used to gain more insight into the pathogenic mechanisms involved in T1D and also to test novel therapeutic approaches.

Cyclophosphamide (CY) is a nitrogen mustard compound. It is a widely used chemotherapeutic agent and is able to cause DNA alkylation. High dose CY to pre diabetic NOD mice leads to synchronous onset of T1D 1-2 weeks after CY treatment. This is attributed to CY being detrimental to B cells and Treg cells. Several studies have reported the B cell cytotoxicity of CY[74, 75]. In their study, Brode et al.[76] observed that CY treatment could cause rapid changes in the various lymphoid subsets. They observed

major reduction in the number of B cells and minor decreases in the CD4+ and CD8+ T cell numbers. These changes were found to be transient with the numbers going back to pre treatment levels over time. They found a sharp decrease in the number of CD25+ Tregs with the reduction being most drastic in the pancreas. The reduced Treg numbers failed to go up over time. It was observed that CY treated Tregs had reduced suppressive potential and had increased apoptosis. All these data together suggests that in pre diabetic mice, CY could cause accelerated diabetes by taking away the regulatory compartment and thus enhancing inflammation.

Streptozotocin (STZ) is a cytotoxic glucose analogue. It is also used as a chemotherapeutic agent. STZ is transported into the cells via the Glut2 glucose transporter and preferentially accumulate in the pancreatic beta cells, which express high levels of Glut2. Hence it exhibits pancreas specific toxicity. On uptake, streptozotocin is split into glucose and a methylnitrosurea moiety. The methylnitrosurea moiety has alkylating properties and upon transfer from STZ to DNA, causes the fragmentation of DNA. This in turn destroys the beta cells. It is believed that in addition to DNA methylation, protein methylation might also play a small contributing role in the loss of beta cell function. Both NO and ROS are also reported to play minor roles in the destruction process. Thus streptozotocin can cause a state of insulin dependent diabetes via the necrosis of beta cells[77].

STZ at high doses causes non-immune toxic form of diabetes as described before. Multiple low doses of STZ on the other hand results in the expression of β-cell autoantigens (eg. GAD autoantigens)[78]. This triggers a β-cell specific inflammatory form of diabetes in susceptible rodent strains[79]. STZ is also able to cause damage in other organs expressing the Glut2 transporter, mostly kidney and liver[80, 81]. STZ is relatively stable at room temperature at least for up to an hour.

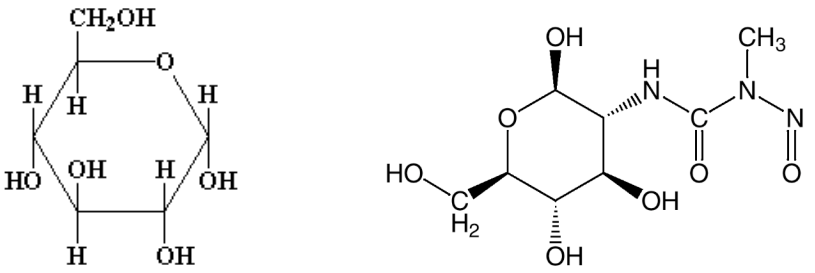


Fig.2: Chemical structures of glucose and streptozotocin respectively.

1.7 RNA interference (RNAi) as a tool for elucidation of gene function:

RNA interference or RNAi has emerged as a powerful research tool in functional genomics where it being used to analyze gene function and to ablate specific genes for therapeutic purposes[82, 83]. In RNA interference (RNAi), double stranded RNA introduced into cells silences the gene expression of the homologous[84]. RNA interference occurs in mammalian cells in response to small RNAs including small interfering RNAs (siRNAs) and microRNAs (miRNAs) and the process results in sequence specific gene silencing. These molecules direct sequence specific cleavage of perfectly complementary mRNAs and transcript degradation and translational repression of imperfectly complementary targets.

The RNAi pathway is initiated by the enzyme Dicer, which cleaves the long double stranded RNA molecules into 21-23 base pair (bp) fragments. In the case of siRNAs, the passenger strand/ sense strand is then cleaved by a process mediated by the endonuclease argonaute 2 (AGO2) and the antisense strand is incorporated into the RNA induced silencing complex (RISC). In the case of miRNAs the passenger strand is unwound and discarded by helicase activity instead of cleavage and the antisense strand is loaded onto RISC. The antisense strand then guides the complex to complementary sequences in target mRNAs, which are then cleaved by AGO2 resulting in silencing [82, 84- 86]. In cases

where there is imperfect matching with 3' UTRs, cleavage of target mRNA does not occur. Instead, translation of the mRNA is prevented leading to translational repression and subsequent reduction in protein levels and silencing[87].

RNAi with long double stranded RNA is of limited use in mammalian systems owing to their ability to elicit strong interferon responses. Both siRNAs and shRNAs are widely used to achieve the silencing of target genes in mammalian systems. A major disadvantage with siRNA is the lack of stable transfection. siRNAs once inside the cell, do not integrate into the host cell DNA and hence every time the transfected cell divides, siRNAs are diluted out[86]. This leads to a gradual loss of gene knockdown making them an ideal for transient gene silencing experiments. On the other hand, endogenously expressed shRNAs can be used to achieve long term and stable knockdown of their target transcripts[86]. The integration into host genomic DNA allows shRNAs to be expressed for long periods of time in a stable manner leading to a long term and stable silencing of target gene expression[82].

shRNAs could be used to genetically encode siRNAs in an organism and the siRNAs generated by cleavage do not activate immune responses due to their small size. Hence shRNAs could be used to induce long term stable silencing in mammalian systems. They are usually delivered into cells using vector molecules and once transcribed, resemble pre-microRNA, the endogenous substrate of Dicer. shRNA sequences are 21- 29 bp long and their expression is usually driven by polymerase III promoters. Once transcribed, they form hairpin structures and are then cleaved by the cellular machinery resulting in siRNA molecules. These are then loaded onto RISC and can result in gene silencing[84, 86].

Conventional shRNA vector systems have the shRNAs under the control of pol III promoters like U6 or H1. Pol III promoters are expressed in all cell types and hence this would lead to a ubiquitous and constitutive gene silencing pattern. The Pol III promoters are strong and generate large amounts of transcription products, which sometimes lead to toxicity. Hence conventional shRNA systems could be used to achieve systemic silencing of target genes in cases where it is desired. Though ubiquitous expression of shRNAs

could be used efficiently in a wide variety of experimental setups, some experimental setups demand a strict control on the timing, expression levels and sites of shRNA expression[84]. There are some cases where a systemic silencing is not required or is deleterious. Some genes are found to be critical for the organism's development and hence systemic silencing of these genes could be embryonically lethal eg. menin. In these cases, a tissue or cell specific silencing of the genes might be desirable and might be sufficient for the purpose of the study. Some genes are found to be important during the early developmental stages of an organism and hence it might be advisable to silence them at a later time point[83, 84]. All these different circumstances require shRNA expression systems, which can be regulated in accordance with the needs of the experiment setup. Conditional shRNA expression would allow the shRNAs to be expressed only when needed and only in the tissues / cells where needed[83].

Several recently discovered vector systems allow for spatial and temporal regulation of shRNA expression. There are vector systems in existence, which allow conditional /inducible knockdown and tissue/ cell specific knockdown. Inducible knockdown systems generally use Pol II or Pol III promoters under the control of drug responsive repressors or transactivators. In this case, the expression of the shRNA can be turned on or off at will by the addition or withdrawal of the corresponding drug[83]. This technique provides an easy and economical way to regulate the timing of gene silencing with the added advantage that the knockdown is reversible. The cre/ loxP recombination method can be used to generate transgenic animals with conditional shRNA mediated gene knockdown for genes whose constitutive silencing could be embryonically lethal. In this method, two loxP sites flanking a stop signal that hinders constitutive shRNA expression are inserted within the promoter driving the shRNA expression to generate transgenic mice. These transgenic mice are then crossed with cell/ tissue specific cre expressing mice in which cre mediates the deletion of the stop signal and results in activation of the shRNA expression. Thus the cre/ loxP recombination method provides both spatial and temporal control over shRNA expression with the disadvantage being that once induced, the expression is irreversible[83].

It is now believed that the best way to induce effective gene silencing in a constitutive and tissue/ cell specific manner is by using the endogenous miRNA pathway to generate shRNAs[83]. The miRNA based shRNA system involves substitution of the miRNA stem with the desired shRNA to induce specific gene silencing. In the commonly used miR30-based vectors, the mature miR30 encoding region is replaced with shRNA sequences targeting the transcript of interest[84]. These artificial miRNAs resemble endogenous RNAi substrates and are more amenable to Pol-II transcription leading to tissue- specific expression of the shRNAs[82]. In their review, Sharma and Rao[88] suggested that efficient delivery is obtained when the hairpins are transcribed by RNA polymerase II. When using Pol II promoters, the transcript levels are similar to the low levels of endogenously expressed miRNAs. Though the level of shRNA produced with miRNA based system is much lower than that produced with overexpression in a conventional system, the shRNA works equally well. It is suggested that adopting a more physiologic way might render the shRNA functionally more competent and additionally, less toxic[83]. Usually molecular markers are integrated into these vectors and both the shRNA and the marker are transcribed as a bicistronic transcript. Thus only the shRNA expressing cells would express the molecular marker[84].

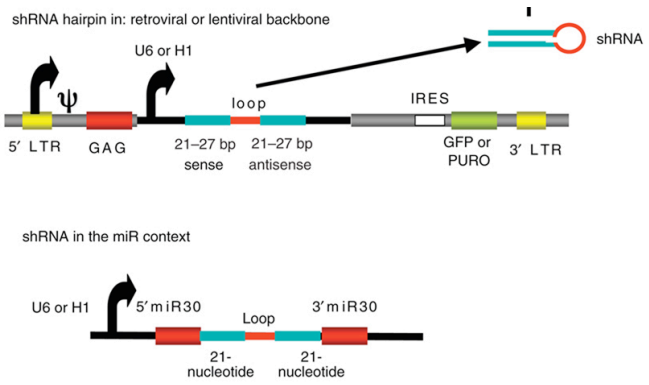


Fig.3: Sharma & Rao. *Nature Immunology* (2009). 10(8): 799-804.[88]

Both siRNAs and shRNA constructs are highly charged and do not cross hydrophobic cell membranes by diffusion. Therefore for delivery into *in vivo* systems, vehicles are needed which could help these molecules bypass the biological membranes[82]. The serum RNases degrade naked siRNAs rapidly and hence it is better to have these delivered to cells using appropriate carriers. A variety of methods have been used for the delivery of these molecules and these include the use of cholesterol, aptamers and nanoparticles for siRNAs and viral vectors including adenovirus and lentivirus in case of shRNAs. Nanoparticles are found to protect siRNA from vascular degradation and can deliver them into cells through endocytosis. Cholesterol formulations like polyelectrolyte complex (PEC) form micelles, which can efficiently deliver siRNA without eliciting interferon responses. Neutral nanoliposomes are extremely efficient in tissue delivery with no identifiable toxicity[85]. As opposed to siRNAs, shRNAs have to be delivered into the nucleus and hence the carriers have to be able to penetrate the nuclear envelope. Viruses are their derivatives are widely used as carriers for shRNA delivery[82].

1.8 Viral vectors and generation of transgenic animals:

For delivery of transgenes (foreign DNA) into mammalian systems, efficient delivery methods are needed. Viral vectors are now being actively researched and used for this purpose. These vectors are derived from viruses where the viral genes in the vector genome are replaced with foreign sequences. The packaging capacities of the viral vectors vary. Adenoviruses are able to infect both dividing and non dividing cells, show high levels of transgene expression and grow to very high titers *in vitro*. All of these features make them attractive for use as vectors. They are able to replicate inside the nucleus of mammalian cells but do not integrate efficiently into the host genome. Hence there is a need for repeated administration, which in turn could trigger strong immune responses and liver toxicity. Similar to adenoviral vectors, adeno- associated viral vectors can transduce both dividing and non-dividing cells and they are non-integrating with no reported toxicity. Being non- integrating, both adenoviral vectors and adeno-associated virus vectors are unsuitable for long-term therapy[82].

Retrovirus vectors on the other hand integrate their genome stably into the host cell DNA thereby allowing the long-term expression of the inserted transgene. The viral genome usually has several essential genes and at each end of the genome, there are long terminal repeats (LTRs), which contain promoter/ enhancer regions and sequences involved in integration. Lentiviruses are a subclass of retroviruses and include among others, the Human Immunodeficiency Virus (HIV). Since the native viruses can cause fatal disease, non- replicating viruses are used for transgene expression[82, 83].

Viral vectors containing either ubiquitous or tissue-specific promoters are used to ensure ubiquitous or when desired, tissue specific expression of the transgene. Owing to its integration into the host genome, the genomic backbone of lentivirus could be used as a means for the life long expression of the transgene. A continuous expression of the transgene without generation of the infectious virus is required. HIV based vectors are primarily used as the biology of HIV replication has been characterized extensively. To avoid the generation of replication competent virus particles, a number of precautionary steps are taken. These include removing the accessory genes from the viral genome and splitting the packaging genes between different expression cassettes[83].

In their review, Singer & Verma[89] note that third generation lentiviral vectors consists of four plasmids; the transfer vector, Gag-pol, Rev and an envelope protein. The transfer vector contains the transgene to be delivered in the viral backbone. Gag-pol codes for integrase, reverse transcriptase and structural proteins. Rev enhances the nuclear export of the viral genomic RNA thereby enhancing viral titers. The envelope protein is usually pseudotyped with VSV-G (Vesicular Stomatitis Virus protein G) and this broadens host tropism[82, 83, 89]. A deletion in the 3' LTR generates replication defective particles and this adds a level of safety to the entire process by preventing the replication of viral genomic RNA. These four plasmids are transfected into human embryonic kidney cells and the viral particles that accumulate in the supernatant can be collected and concentrated by ultracentrifugation thus giving high titer viral preparations[83, 89, 90]. These viral preparations are then injected into single celled embryos to generate transgenic animals.

One of the major concerns associated with this technique is the unknown integration pattern. The virus integrates randomly in the genome and hence could integrate within gene sequences and disrupt endogenous gene expression. Though this is an alarming concept, a study by Yang et al.[91] demonstrated that lentivirus integrated preferentially within intragenic regions and especially in the introns. Lentiviral vector mediated transgene expression can be maintained for long periods of time and the approach has been found to be effective in generating transgenic mice in strains which have been found to be difficult to manipulate with the other transgenic approaches. Though lentiviral transgenesis has many advantages compared to conventional methods, there are several problems that have to be overcome. Some of these include genetic mosaicism, variability of transgene expression, the limits on construct size, vector toxicity problems with insertional mutagenesis and finally biosafety concerns[90].

1.9 Combining RNAi and lentiviral transgenesis for analyzing gene function in transgenic mice:

As mentioned before, viral vectors provide a near perfect delivery method for shRNA containing plasmids. Lentiviruses are widely used as vectors for shRNA expression cassettes as they integrate stably into the host genome and hence are suitable for long-term gene silencing[82, 89]. In their review, Sliva and Schnierle give a schematic representation of gene knockdown using lentiviral transgenesis (Fig. 4). shRNAs are first cloned into plasmids and then these are used to generate high titer lentivirus. The lentivirus is then injected into single celled embryos to generate transgenic animals with reduced gene expression levels. Since lentiviral vectors integrate into the host genome, progeny from the transgenic animal usually inherit the provirus and express the transgene[89]. Hence progeny from the transgenic founder are usually found to inherit stable knockdown of gene expression.

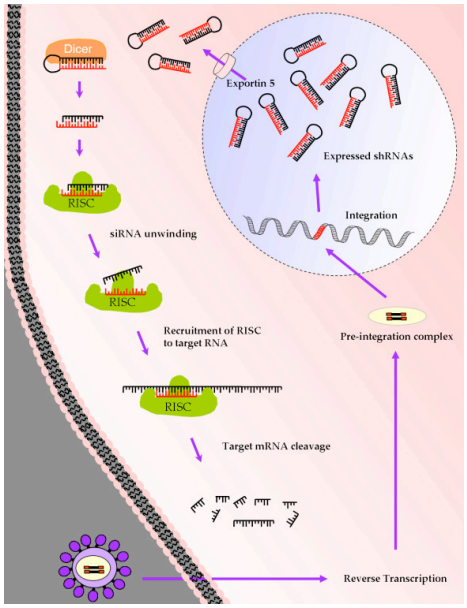


Fig.4: Sliva K, Schnierle BS. *Virol J.* (2010) 7:248.[82]

In the search for elucidating the function of over 30,000 genes in the human genome, one of the widely used techniques is the generation of gene knockout mice. This involves the generation of ES knockout lines and the injection of the knockout cells into a blastocyst[89]. The ES knockout line must first be generated, characterized and finally injected into a blastocyst. The resulting chimeric founder has to be then bred back to homozygosity[82, 89]. Hence though it is an extremely useful approach, it is time consuming, expensive and labor intensive. Traditional pronuclear injections result in multiple transgene copies integrated at a single locus, with not all copies being expressed. However this will result in all the copies being transmitted together to the future generations[89, 92].

Using lentiviral transgenesis and RNAi for generation of transgenics on the other hand, is time and cost effective and less labor intensive. While knockout of a gene ensures a complete lack of the target protein and gene function, RNAi results in the expression of the gene albeit to a significantly lower degree. However, *in vivo* inhibition of gene

products by RNAi have been found to yield phenotypes quite comparable to classical knock out animals[82]. A reduction in protein levels might better reflect a normal physiologic state than a complete lack of it. The technique has the added advantage that it allows quite a large degree of regulation and control over gene modulation as opposed to classical knockout models.

Lentiviral vectors can be used to generate large and small transgenic animals with high efficiencies via transduction of fertilized eggs[89, 90]. Though other retroviral vectors are also able integrate into host genome, they are unsuitable to generate transgenic animals as the provirus expression is silenced during embryogenesis[89]. Embryo viability is found to be very high following lentiviral transduction. Also, large number of founder animals can be generated with this approach[90]. Lentiviral transgenesis is found to result in several independent copies of integrated provirus that will segregate independently in the future generations. Thus though the founder may carry different transgene copies depending upon the developmental stage of the manipulated embryo, these copies are found to segregate independently in the subsequent generations as each provirus integrates independently. In many cases, injection cycles result in mosaic founders (F0) and once the transgenic F1 mice are generated and the different copies segregated, the transgene is usually transmitted stably into future generations. Once germline transmission is achieved, the knockdown phenotype generally stays unaltered[89].

Analyzing the role of IL-17A in type 1 diabetes

1.10 T cells:

T cells originate from the haematopoietic stem cells in the bone marrow. The early progenitors derived from the stem cells migrate to the thymus where they lose their potential to develop into B cells and natural killer cells. They then undergo rigorous cell division and expand to form a large population of immature thymocytes[93]. In the thymus, T cells develop and mature under the influence of thymic hormones and peptides[1]. The early thymocytes do not express either CD4 or CD8 and hence are referred to as double negative (CD4-CD8-). During the process of maturation, these double negative cells under TCR rearrangement and develop into a population of double positive cells expressing both CD4 and CD8[1, 93]. Finally they lose the expression of one of the transmembrane glycoproteins and become single positive cells (either CD4+CD8- or CD8+CD4-), which are then released from the thymus into the periphery[10, 11].

T cells play the central role in cell-mediated immune responses. The majority of T cells have a TCR composed of two glycoprotein chains called α chain and β chain and these T cells are referred to as αβ T cells[1, 93].There is a minor population of T cells where the TCR is composed of two different glycoprotein chains called γ chain and δ chain and these T cells called γδ T cells constitute about 2% of the total T cell pool. γδ T cells are found in high abundance in the gut mucosa. These cells are non-MHC restricted and they seem to be able to recognize whole proteins.

The entire process involves two stages of selection during which around 98% of the thymocytes that fail the selection processes are removed. The remaining 2% of thymocytes that survive leave the thymus for the periphery as mature albeit naïve T cells. Double positive thymocytes undergo positive selection in the thymic cortex where they are presented with self-antigens complexed with MHC molecules on the surface of thymic epithelial cells. All T cells are required to recognize self-antigens in order to

mount effective immune responses against foreign antigens. Hence, during the process of positive selection, only those thymocytes that can interact with self-antigens with optimum affinity are selected and given signals required for survival. Thymocytes with no affinity to self-antigens undergo apoptosis (death by neglect) and are removed by macrophages[10, 11].Thymocytes that are positively selected on MHC class I molecules downregulate the expression of CD4 and become CD8 single positive cells. Similarly, thymocytes that are selected on MHC class II molecules downregulate CD8 expression and become CD4 single positive cells[1, 10, 11, 94].

The positively selected single positive thymocytes migrate to the medulla where they are again presented with self- antigens complexed with MHC molecules on antigen presenting cells. Those cells that interact too strongly with self- antigens are removed by negative selection where they undergo TCR mediated apoptosis. The remaining cells leave the thymus as mature naïve T cells. The process of negative selection tries to prevent potentially autoreactive T cells from reaching the periphery. Together, the processes of positive and negative selection play an important role in the generation of central tolerance. Autoreactive T cells, which fail to be deleted in the thymus, are usually identified and suppressed in the periphery by means of peripheral tolerance mechanisms[1, 10, 11].

Activation of the T cell requires two distinct signals. The first signal is provided by the interaction between the TCR and specific antigenic peptide complexed with the MHC molecule present on the surface of an antigen-presenting cell. The second signal requires the interaction between a co- stimulatory molecule like CD28 present on the T cell and its partner protein on the antigen-presenting cell. CD28 is the most important co stimulatory receptor present on T cells. Its interaction partner is the B7 protein composed of CD80 and CD86. This second signal called co-stimulation is critical in T cell activation. In the absence of a co- stimulatory signal, the T cell becomes anergic.

Naïve T cells, which have never encountered their cognate antigen, are characterized by their surface expression of L-selectin (CD62L). They usually do not express the

activation markers like CD25 and CD69. Upon encountering its cognate antigen, the naïve T cell gets activated and downregulates CD62L expression. The various activation markers like CD25, CD44 and CD69 are upregulated. Some of these activated cells go on to acquire the memory phenotype. Memory T cells are further subdivided into central memory T cells (T_{CM}) or effector memory T cells (T_{EM}). Central memory T cells migrate to the lymph nodes and facilitate memory responses while effector memory T cells migrate to periphery and promote elimination of pathogens[3]. Central memory T cells express CD62L and CCR7 while effector memory T cells do not express these markers. Central memory cells secrete IL-2 but do not produce effector cytokines like IL-4 and IFNγ while effector memory T cells secrete both IL-4 and IFNγ.

There are several T cell subsets, each with a distinct function. T helper (Th) cells are CD4+ cells and they are activated by antigenic peptides complexed with MHC class II molecules expressed on the surface of antigen presenting cells. Once activated, Th cells undergo major expansion and secrete proteins called cytokines. Cytokines play an important role in regulating and assisting immune responses. Th cells also assist in the maturation of B cells into plasma cells and also in the activation of cytotoxic T cells and macrophages. Cytotoxic T cells or CTLs are CD8+ cells that attack and destroy virally infected cells and tumor cells[1]. They are activated by antigenic peptides complexed with class I MHC molecules which are present on the surface of almost all the cells in the body. Memory T cells are antigen specific T cells which persist in the body after infections. These cells expand rapidly after re- exposure to their cognate antigen and thereby provide the immune system with a memory of the infections it has already encountered. Regulatory T cells or suppressor T cells are responsible for shutting down T cell responses towards the end of an immune response and also for suppressing autoreactive T cells which may have escaped from the thymus[1]. Naturally occurring regulatory T cells (nTreg) arise in the thymus while adaptive Tregs are derived in the periphery. Tregs can be identified by the expression of their lineage specific transcription factor FoxP3.

1.11 T helper lineage:

T helper cells play an important role in regulating and enhancing immune responses to antigens. They are involved in B cell antibody class switching, activation of cytotoxic T cells and enhancement of the bactericidal property of macrophages. Mature T helper cells express CD4 and are called CD4+ T cells. They bind to MHC class II molecules and hence they interact with antigenic peptides bound to MHC class II molecules expressed on the surface of antigen presenting cells. Antigen presenting cells usually take up the antigen, processes it and these APCs then travel to the lymph nodes. Once there, the antigenic peptides are transported to the APC surface where it is displayed bound to MHC class II molecules for recognition by T cells with the specific TCR. Once the T helper cell encounters its cognate antigen in the presence of proper co-stimulation, it is activated and undergoes rapid proliferation. This is done by the secretion of a potent growth stimulatory factor i.e. IL-2. IL-2 can function in an autocrine manner and the proliferation is further enhanced by the fact that activated T cells can produce alpha subunit of the IL-2 receptor (CD25). The autocrine and paracrine action of IL-2 can lead to clonal activation.

1.12 Th1/Th2 Paradigm:

Naïve CD4+ T cells are activated and differentiate into different T helper subsets in the presence of their specific antigens. Mossman and Coffman proposed the existence of 2 different T helper subsets more than 20 years ago. Upon encountering an antigen presented by the antigen-presenting cell (APC), naïve CD4+ T cells can differentiate into either IFNγ producing Th1 cells or Th2 cells which produce IL-4, IL-5 and IL-13. Proper T helper responses play an essential role in the host defense against infections. Th1 responses enhance cellular responses against viruses and intracellular pathogens while Th2 responses enhance humoral immunity and control extracellular pathogens including helminthes. On the other hand, uncontrolled and persistent T effector responses can lead to deleterious conditions like allergy and autoimmunity. Uncontrolled Th2 responses have been found to lead to conditions like asthma[95]. Abnormal Th1 responses have been

implicated to play at least a partial role in the development of several autoimmune diseases including inflammatory bowel disease (IBD) and psoriasis[96, 97]. Over the years it has been noticed that several pathological conditions could not be simply explained by the Th1-Th2 paradigm. Efforts to resolve these questions have recently led to the discovery of several new T helper subsets like regulatory T cells (Treg), Th17 cells and Th9 cells.

The first clue suggesting the existence of distinct T cell populations mediating humoral and cell mediated responses was provided from the work of Parish and Lew[98]. Around mid 1980s, Tim Mossman's lab identified 2 distinct T cell types able to produce different growth factors. Th1 cells were found to produce IL-2 and IFNγ while Th2 cell produced something different. Meanwhile Bob Coffman's lab had developed an assay to test IgE production. Mossman and Coffman tested the supernatants from the two T cell subsets in the assay and found that supernatant from the Th1 clone containing IL-2 and IFNγ could not induce IgE. On the other hand, supernatant from the Th2 clone when added to LPS stimulated B cells could induce IgE responses. They also discovered that when both supernatants were added together, the IgE production was inhibited suggesting that a Th1 factor is able to block the Th2 induced IgE response. They were able to identify this Th1 factor as IFNγ by using neutralizing antibodies. The growth factor released by Th2 clones was identified as IL-4. These observations led to the development of what came to be later known as the Th1/ Th2 paradigm.

The Th1/Th2 paradigm was suggested on the basis of the observation that each CD4+ Th cell subset has distinct cytokine profile and fulfills different effector functions. Th1 cells secrete IL-2 and IFNγ and are crucial for cell- mediated immunity while Th2 cells secrete IL-4, IL-5 and IL-13 and mediate humoral immunity. Signal transducer and activator of transcription (STAT) proteins are essential for the Th subset differentiation. STAT-1 and STAT-4 are required for Th1 differentiation while STAT-6 is required for Th2 differentiation. T-bet is the lineage specific transcription factor for Th1 development while for the Th2 cells, it is GATA-3. IL-12 is also found to be important for the induction of Th1 cells. Activation of T cells in the presence of IFNg leads to the

activation of STAT-1 and the Th1 lineage specific transcription factor T-bet. Expression of T-bet induces IFNg secretion and IL-12Rβ2 expression which makes the cells responsive to IL-12. The engagement if IL-12R by IL-12 causes the activation of STAT-4 resulting in enhanced IFNg secretion by the cells. The increased secretion of IFNg in turn, causes a further upregulation of T-bet and strengthens Th1 commitment. Activation of T cells in the presence of IL-4 induces the activation of Th2 lineage specific transcription factor GATA-3. Upon engagement is IL-4R by IL-4, STAT-6 is activated which enhances the production of IL-4. This leads to a positive feedback loop resulting in enhanced Th2 differentiation[99]. The two T helper subsets were found to be able to cross regulate each other.

For quite some time after the introduction of the Th1/Th2 paradigm, it was believed that Th1 cells are pathogenic and are responsible for organ specific autoimmunity while a Th2 response is beneficial and non pathogenic. But this assumption was brought into question when data emerged from the EAE (Experimental Autoimmune Encephalomyelitis) mouse model contradicting it. EAE is the animal model for multiple sclerosis (MS). It was found that neutralizing IFNγ increased the disease severity while administration of IFNγ reduced the severity[100- 102]. It was observed that the disruption of genes encoding either IFNγ or IFNγR converted EAE resistant strains to susceptible phenotype[103, 104]. These data indicated a protective role for IFNγ in EAE. Also animals, which lack other Th1 molecules like STAT1 and IL-12Rβ2 developed more severe disease[105, 106]. All these data raised the possibility that other Th subsets may exist which mediate tissue damage and autoimmunity.

1.13 Discovery of IL-23 and Th17 Cells:

The discovery of IL-23 by Robert Kastelein was finally able to shed some light on the contradictory data obtained regarding the role of Th1 in autoimmune diseases. Sequence database searches with structure based alignment tools in the laboratory of Fernando Bazan and Robert Kastelein led to the discovery of a novel sequence, which they named p19. p19 was found to share an overall sequence identity of 40% with the p35 subunit of

IL-12 and they were unable to purify p19 from the supernatant of transiently transfected cells. Since it was known that IL-12p35 requires IL-12p40 for secretion, further experiments in the line revealed that secretion of p19 requires its partnering with IL-12p40[107]. This resulting cytokine was named IL-23[108]. IL-23 is found to be expressed mainly by activated dendritic cells and phagocytic cells.

IL-23 belongs to the IL-12 family of cytokines. IL-12 is a heterodimeric cytokine composed of 2 subunits p35 and p40. In mice, the transgenic expression of p19 induced elevated levels of pro inflammatory cytokines TNFα and IL-1 leading to fatal systemic inflammation due to massive T cell and macrophage infiltration[109]. The exact role of IL-23 in the development of autoimmunity (especially in EAE) was elucidated in a landmark paper by Daniel Cua and his colleagues. They studied the role of IL-12 and IL-23 in EAE development by inducing EAE in mice deficient for each of the subunits forming IL-12 and IL-23 i.e. IL-12p35, IL-12p40 and IL-23p19 deficient mice. They were able to show that animals deficient in IL-23 (p19-/-) and both IL-12 and IL-23 (p40-/-) were protected from EAE while IL-12 deficiency (p35-/-) did not have any effect on EAE induction. They were able to further show that the delivery of IL-23 gene transfer vectors into the central nervous system (CNS) could reconstitute EAE in the p19-/- and p40-/- animals while the gene transfer of IL-12 into the CNS did not reconstitute the disease in p40-/- mice[110]. Thus it was demonstrated that IL-23 rather than IL-12 might be the more important cytokine for autoimmune inflammation in this mouse model.

Daniel Cua's group did similar experiments in another autoimmune disease model; the collagen induced arthritis and discovered that IL-23 deficient mice were protected from the disease while the IL-12 deficient mice developed exacerbated disease. They also noticed that the disease resistance correlated with the absence of IL-17 producing CD4+ cells despite the normal induction of collagen specific Th1 cells[111]. Aggarwal et al.[112] had previously reported that IL-23 activated CD4+ T cells show elevated production of IL-17. Further studies showed that IL-23 is essential for the expansion of a distinct CD4+T cell population with a unique expression pattern of pro inflammatory cytokines. These cells

were found to produce IL-17A, IL-17F, IL-6 and tumor necrosis factor. Passive transfer studies demonstrated that these IL-23 dependent T cells are highly pathogenic[113].

Studies done by Harrington et al.[114] and Park et al.[115] showed that Th17 differentiation is independent of the transcription factors involved in Th1 (T-bet, Stat1 and Stat4) and in Th2 (Stat6 and c-Maf) differentiation. They also discovered that addition of anti IFN-γ and anti IL-4 to the Th17 cultures could increase IL-17 expression. This led to the suggestion that IFNγ and IL-4 negatively regulates Th17 cells and that IL-23 might induce the differentiation of naïve T cells into Th17 cells. This suggestion led to a conceptual problem as Aggarwal et al.[112] had already shown that unlike memory T cells, naïve T cells do not express IL-23R. Later studies demonstrated that IL-6 in combination with TGF-β was required for the differentiation of naïve T cells into Th17 cells[116- 118]. This was surprising as TGF-β was considered an immunosuppressive cytokine. It was also known that naïve T cells in the presence of TGF-β expressed FoxP3, the transcription factor that induces their differentiation to regulatory T cells. It was shown that IL-6 could suppress the TGF-β induced differentiation of regulatory T cells and redirect the differentiation program to the Th17 lineage.

1.14 Characteristics of Th17 cells:

Th17 cells are a sub population of CD4+ effector T cells. They have a high expression of the transcription factors RORγt and RORα while they express little T-bet or GATA-3. They secrete a variety of cytokines including IL-17 (IL-17A), IL-17F, IL-21, IL-22, IL-9, TNF and GM-CSF[119]. They also express the chemokine receptor CCR6. TH17 population is highly heterogeneous. In addition to IL-17A/ IL-17F double producers, populations that are only IL-17A or IL-17F positive are also reported[120]. Lee et al.[121] have reported that IL-17F is expressed earlier than IL-17A during Th17 development. In mice, these cells develop in the presence of TGF-β and IL-6[117]. Bettelli et al.[116] demonstrated that IL-6 is able to inhibit the FoxP3 expression induced by TGF-b and thus downregulate the Treg differentiation and enhance the Th17 pathway. IL-6 is required for IL-21 production, which then acts in an autocrine manner to induce more IL-21. This in turn

leads to IL-23R expression by the cells[122]. IL-23 signaling is critical for the survival of Th17 cells. Both IL-2 and IL-27 are found to suppress Th17 differentiation[123].

In humans, the Th17 differentiation happens in the presence of TGF-β and IL-1b along with IL-21 or IL-23[124- 126]. Human Th17 cells are found to develop from CD161+ precursors and virtually all of them express CD161 and are CCR4+CCR6+[127, 128]. They express the cytokines IL-17A, IL-17F, IL-22, IL-26 and IFN-γ. They also express RORγt and IL-23R just like murine Th17 cells[129]. Thus, the current view is that at least in murine cells, TGFβ along with IL-6 lead to the differentiation of naïve T cells into Th17 cells. Th17 cells secrete IL-21, which acts in an autocrine manner to bring about the amplification of the Th17 lineage. IL-23 is believed to be an important survival factor that is essential for the stabilization and expansion of Th17 cells. The Th17 pathway in humans seems to reflect this to an extent.

Murine Th17 cells express high levels of a master transcription factor, RORγt. They are also found to express a second orphan receptor, RORα the presence of which is found to have an additive effect on Th17 differentiation[130, 131]. STAT3 is also found to play a crucial role in Th17 differentiation[132], while STAT5 is found to negatively regulate the pathway[133]. IRF4 plays an important role in this pathway, as it is able to induce IL-21, which in turn leads to amplification of Th17 cells[134]. The presence of natural agonists for AHR in the medium is found to enhance the differentiation of Th17 cells *in vitro*[135].

1.15 IL-17 and its functions:

IL-17 was first cloned in 1993 by Rouvier et al.[136]. IL-17 is the founding member of the IL-17 family, which includes IL-17A, IL17-B, IL-17C, IL-17D and IL-17E (also called IL-25) and IL-17F. The others members of the family were identified as a result of bioinformatics strategies based on the IL-17A sequence[137]. Genes for IL-17A and IL-17F are located on the same chromosome in both mouse and humans. Genes for the other family members are located on different chromosomes. IL-17A and IL-17F share the highest sequence identity of around 50% while IL-25 is the most divergent (16%)[120, 138].

Murine IL-17A and human IL-17A share 62% homology. Hymovitz et al.[139] demonstrated that all family members except IL-17B exist as homodimers consisting of five highly conserved cystine residues forming a cystine knot structure. IL-17A is also able to form heterodimers with IL-17F. IL-17A homodimers are found to be the most potent with the heterodimers and IL-17F homodimers having decreasing potencies[140- 142.]

IL-17 family cytokines signal through IL-17 receptor family members. There are 5 different IL-17 receptors, IL-17RA to IL-17RE[143]. Functional receptors are believed to be composed of homodimers or heterodimers. IL-17RA is able to associate with IL-17RC and transduce signals from both IL-17A and IL-17F as well as IL-17A/ IL-17F heterodimers. IL-17RA is expressed primarily in immune cells while IL-17RC is expressed mainly in non-immune cells[144, 145]. IL-17A has a higher affinity for IL-17RA while IL-17F has a higher affinity for IL-17RC[139]. IL-17F is able to signal through IL-17RA but its affinity is almost 10 fold lower than that of IL-17A. Upon binding of IL-17A, IL-17RA recruits IL-17RC to form the IL-17RA/ IL-17RC complex.

IL-17A and IL-17F are pro inflammatory cytokines, which act on cells to induce the production of a variety of cytokines (IL-6, IL-8, GM-CSF, G-CSF), chemokines (CXCL1, CXCL10) and metalloproteinases[99, 108]. Both IL-17A and IL-17F are produced by a variety of cell types including CD4+ cells, CD8+ T cells, NKT cells, γδ T cells and neutrophils. NKT cells also found to secrete IL-17 under proper stimulation. During airway inflammation, invariant NKT cells that lack NK1.1 marker are found to secrete copious amounts of IL-17A[146]. γδ T cells are found to secrete large amounts of IL-17 during both *Mycobacterium tuberculosis* and *Escherichia coli* infections[147, 148].

IL-17 is involved in protective immune responses against bacterial and fungal infections. It does so by activating the production of pro-inflammatory cytokines and chemokines and by recruiting and activating neutrophils and macrophages. IL-17R deficient mice succumb to *K. pneumoniae* infections due to reduced chemokine production and neutrophil recruitment[149]. On the other hand, Overexpression of IL-17 in the lungs led to enhanced production of pro- inflammatory cytokines, increased neutrophils recruitment

and clearance of the infection[150]. This suggests a partial role for IL-17 in protection against *K. pneumoniae*. A complementary role for IL-17 is suggested in the protective immunity against *M. tuberculosis, B. pertussis, S. aureus and L. monocytogenes*[151- 154]. IL-17 deficient mice have increased mortality from *C. albicans* infections[155]. IL-17 is also found to be protective in other fungal infections including *Pneumocystis carinii* and *Aspergillis fumigatus*[156- 157].

1.16 Th17/ IL-17A in autoimmune diseases:

There is a plethora of evidence suggesting a role for Th17 cells and the various Th17 factors in a variety of autoimmune diseases. Despite this, much confusion exists regarding the extent to which these cells are pathogenic. Tzartos et al.[158] were able to identify IL-17+ CD4+ and CD8+ cells in active lesions in the brain from MS patients. There is evidence for the presence of IL-17 and IL-22 receptors on inflamed endothelium in MS lesions and these cytokines were found to enhance the migration of T cells across the blood brain barrier[159]. *In vitro* differentiated Th17 cells have been found to transfer EAE but the disease is significantly milder in comparison with Th1 cells. In this model, the authors were able to detect IFNγ much earlier than IL-17 in the CNS, which led them to conclude that Th17 cells could contribute to EAE but need Th1 cells to disrupt the blood brain barrier first[160]. IL-17A knockout and IL-17F knockout mice develop a milder form of EAE and only marginal differences in clinical EAE scores are observed indicating that neither vitally contributes to the disease[161, 162].

Fossiez et al.[163] found that IL-17 could induce the secretion of pro inflammatory cytokines including IL-6, IL-8 and G-CSF from stromal cells. Th17 cells found in arthritic synovium express RANKL (receptor activator of nuclear factor kappa B ligand), which is found to enhance osteoclast formation[164]. These data suggest a role for Th17 cells in rheumatoid arthritis. A number of studies point to a role for this Th lineage in psoriasis. Increased levels of RORγt, IL-17, IL-22 and IL-23 mRNA are reported in psoriatic lesions[129, 165- 167]. IL-17 deficient mice develop experimental autoimmune uveitis[168] thereby precluding a major role for this cytokine in EAU. But anti- IL-17 is

found to prevent or at least diminish the disease[169] indicating that IL-17 is able to contribute to this autoimmune condition.

As mentioned, before the discovery of Th17 cells, autoimmune pathologies were attributed solely to Th1 cells. Contradictions in the data led to questions and finally the characterization of Th17 subset. The autoimmune diseases were then attributed to Th17 cells and their effector cytokines. But it has been observed that both Th17 cells and Th1 cells are able to induce several autoimmune conditions like EAE and EAU with distinct disease pathologies. Hence, data to date indicate that though Th17 cells may play a role in some autoimmune diseases, this role is not exclusive and Th1 cells might still be pathogenic in autoimmune disorders with critical roles in induction or effector phases. Thus it seems that both Th17 cells and Th1 cells seem to be involved in the development of autoimmune pathologies.

1.17 Th17 / IL-17A in type 1 diabetes: pathogenic or not?

Preliminary evidence has suggested that in the NOD mouse during the course of T1D, IL-17 is expressed in the pancreas[170]. Jain et al.[171] observed that inhibiting Th17 cells with the induction of IFNγ inhibited IL-17 production and it could restore normoglycemia in the pre diabetic NOD mice. Two separate studies which used the adoptive transfer effects observed that transfer of islet specific Th17 cells could induce diabetes but only after the cells converted to IFNγ producing cells[172, 173]. Bradshaw et al.[174] observed that the monocytes from T1D patients spontaneously secrete pro inflammatory cytokines like IL-6 and IL-1β and these monocytes in turn could induce more IL-17 secreting cells from memory T cells. This led them to speculate that the activated innate immune system might drive the expansion of Th17 cells in T1D patients. Emamaullee et al.[175] analyzed the role of anti-IL-17 and recombinant IL-25 in type 1 diabetes using the NOD mouse model. They reported that these strategies failed to have any benefit in the young mice but could prevent diabetes when the mice were treated around 10 weeks of age. They found that both strategies reduced the number of peri islet infiltrates and GAD65 autoantibody levels and led to an increase in the number of T regulatory cells. This led

them to conclude that Th17 cells are involved in the development of autoimmune diabetes and that therapeutic strategies directed against Th17 cells may be beneficial. These data seem to suggest that though IL-17 might play a role in the development of insulitis, the progression to overt diabetes might depend on IFNγ[176]. Arif et al.[263] were able to observe increased IL-17 reactivity in type 1 diabetes patients in comparison with control subjects. They could detect IL-17A, IL-22 and RORC in the islets close to disease diagnosis, though the observation was from a single patient. They found that IL-17A on its own had no proapoptotic effect on cultured beta cells, but could exacerbate beta cell death induced by the combination of IL-1β and IFNγ or TNFα and IFNγ. All these observations led them to conclude that IL-17 could be used as a biomarker as well as a therapeutic target in autoimmune diabetes. Munegowda et al.[264] recently reported that both CD4+ Th17 cells and Th17 stimulated CD8+ CTLs play distinct roles in T1D and EAE. They found that Th17 stimulated CTLs but not CD4+ Th17 cells themselves were critical in type 1 diabetes development whereas CD4+ Th17 cells and not Th17 activated CTLs could induce EAE.

1.18 AIM:

As mentioned in the previous section, a lot of evidence point to a role for Th17 cells and their signature cytokine IL-17A in the development of type 1 diabetes. But so far, the results are conflicting and do not conclusively prove a pathogenic role for IL-17A in T1D. Hence, we aimed to identify the role, if any of IL-17A in autoimmune diabetes and to conclusively prove a positive or negative role for this cytokine in autoimmune diabetes. So far, studies have made use of adoptive transfer experiments of *in vitro* differentiated Th17 cells or anti IL-17 antibodies. Both these strategies do not seem to be optimal owing to the widely described plasticity observed with Th17 cells and the conflicting results obtained with antibodies.

We felt that the best way to study the role of this cytokine in the context of type 1 diabetes would be to generate NOD mice in which IL-17 is systemically silenced. As mentioned earlier, RNAi is an extremely useful tool to analyze gene functions and

lentiviral transgenesis could be used in conjunction with RNAi to generate transgenic animals in which desired genes are silenced. Hence we aimed to use lentiviral transgenesis and RNAi to generate transgenic NOD mice with reduced IL-17A levels. We then aimed to analyze diabetes incidence in the transgenic line to identify whether IL-17A plays a contributing role in diabetes. Diabetes incidence levels in these mice would better reflect the role of IL-17A, as this strategy does not involve or require additional manipulations.

Generation of β- cell reporter mouse models and modulation of β- cell mass

1.19 Pancreas and β-cells:

Pancreatic β-cells, responsible for the synthesis and secretion of insulin in response to a glucose challenge, are located in the islets of Langerhans. Islets are comprised of a heterogeneous population of endocrine cells, including insulin-producing β-cells (approx. 65–90%), glucagon-secreting α -cells (15-20%), somatostatin-secreting δ -cells (3-10%) and polypeptide (PP)-secreting cells (1%)[177]. Recent studies have shown that pancreatic β- cells harbor immense powers of self-renewal. Systemic insulin demand is found to change and β- cell mass undergoes compensatory changes correspondingly. Some physiological and pathological states such as aging, pregnancy, insulin resistance and obesity increase the insulin demand and facultative β- cell proliferation is essential to meet the increasing demands for insulin[178]. Some studies have reported an increase of 40-50 fold[179, 180] and the failure to increase beta cell mass according to the demand may result in hyperglycemia and subsequent development of diabetes.

Though pancreatic development is first morphologically discernable in mice at embryonic day (e) 8.75-9.0, the differentiation of the five endocrine cell types i.e. β, α, δ, ε and pancreatic polypeptide (PP) cells starts only by e13.5[181]. Expression of Ngn3 in the pancreatic progenitors is crucial for the commitment to endocrine lineage[182]. Following differentiation, the endocrine cells in mice cluster to form functional islets from e16.5 onwards. Cell adhesion molecules and matrix metalloproteinases are believed to be involved in islet formation. The transcription factors MafA and MafB are crucial for terminal β- cell development. β- cells acquire glucose responsiveness during the early postnatal period and this requires the expression of Glut2 glucose transporter and PC1/3, which cleaves proinsulin to active insulin[181].

A number of different factors are found to be involved in regulating the β- cell mass postnatally and include nutrients, hormones, growth factors and cell cycle regulators among others. Several mitogenic factors including glucose, insulin, prolactin, placental lactogen, growth hormone, glucagon –like peptide- 1 (GLP-1), platelet derived growth factor, insulin like growth factor (IGF)-1 and IGF- 2 stimulate β- cell growth and proliferation. Moderate increases in glucose levels can enhance β- cell proliferation and survival. It is suggested that glucose can increase the number of cells entering the cell cycle and that it has antiapoptotic effects. On the other hand, consistent exposure of β- cells to increased glucose levels could lead to oxidative stress and β- cell apoptosis. Increased insulin / IGF signaling in β- cells is found to enhance proliferation. The increase in β- cell mass is found to be augmented on costimulation with glucose. Pregnancy causes an increased insulin demand owing to increased maternal body weight and insulin resistance. This facultative β- cell growth during pregnancy is controlled by several hormones including prolactin (PRL), placental lactogen (PL), estrogen and progesterone. All the pregnancy related adaptations in the maternal pancreas are reversed upon parturition and the β- cell mass goes back to pre partum levels. Upon pregnancy in rodents, β- cell mass is found to increase by about 150% and it is accompanied by increased insulin synthesis and secretion. Insufficient maternal β- cell adaptation could lead to gestational diabetes[178, 183].

Cell cycle regulators like cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CKIs) are found to play a major role in post natal β- cell proliferation. Many of the insulinotropic factors induce the expression of cyclins. Cyclin D2 is found to be crucial for normal postnatal β- cell expansion. Cyclin D1 is involved in β- cell proliferation though it is not required for β- cell development. Though cyclin D3 and E are also expressed in the islets, no role for these in β- cell proliferation has been reported so far. Both CDK4 and CDK2 are expressed in the islets. CDK4 is the best-characterized CDK in the pancreas and is found to be indispensable for β- cell proliferation and for maintaining normal β- cell mass *in vivo*. CKIs including Cip/Kip proteins and INK4 (inhibitors of CDK4) members are expressed in mature β- cells and play an essential role

in regulating β- cell growth. Two of the four INK proteins, $p18^{Ink4c}$ and $p15^{Ink4b}$ inhibit β- cell proliferation. There are three Cip/ Kip family members- $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. They are all expressed in islets, and play essential roles in limiting β- cell proliferation. All three are found to inhibit facultative β- cell proliferation and growth. Several transcription factors including E2F, Rb and p53 also regulate postnatal β- cell mass [178, 183].

Intracellular proteins and pathways that transduce the β- cell mitogenic signals are currently being elucidated and include several transcription factors like FoxO1, Pdx1, NFAT and menin. NFAT, STAT5 and Wnt signaling pathways are believed to be involved in cyclin and CDK expression in the islets[183]. FoxO1 is found to inhibit β- cell proliferation and it is partially mediated by enhanced expression of $p27^{Kip1}$. It is suggested that FoxO negatively regulates β- cell proliferation by controlling the expression and function of proliferation enhancer, Pdx1. Signaling through the insulin / IGF pathway promotes the exclusion of FoxO1 from the nucleus thereby enhancing the expression, nuclear localization and activity of Pdx1. β- cell mitogens enhance intracellular Ca^{2+} and Ca^{2+} signaling mediates β- cell proliferation. Calcineurin/ NFATc signaling is suggested to be involved in the regulation of β- cell proliferation and there is some evidence that mitogens like glucose and GLP-1 are able to stimulate NFAT activity at the insulin promoter. Menin is a tumor suppressor and loss of menin expression is found to be sufficient to trigger β- cell proliferation and subsequent islet hyperplasia. Menin regulates the expression of p18Ink4c and p27Kip thereby influencing β- cell growth and proliferation[178].

Though many of the factors and pathways involved in pancreatic development and β- cell growth and proliferation have been identified, most of them are yet to be fully characterized. Advances in understanding the factors which govern β- cell growth and maintenance would accelerate progress in generating successful therapies for a multitude of pathologies including type 1 diabetes. β- cell mass at any given time is governed by neogenesis, cell differentiation, cell proliferation, cell size and cell death[183]. Improved *in*

vivo imaging would allow the quantification of β- cell mass, growth and death in both normal physiological states and in pathological conditions and the data would significantly contribute to the search for therapy. As mentioned earlier, an ideal therapy for type 1 diabetes would require the ability to achieve β- cell neogenesis and regeneration in parallel with immune tolerization approaches and a thorough knowledge of the whole process of pancreatic development is crucial for this.

1.20 *in vitro* models for β-cell studies:

As mentioned before, isolation and purification of β- cells is a tedious process. Once in culture, primary β- cells do not proliferate easily and since all cells have a finite life span, they soon submit to senescence and die. It is also extremely hard to maintain the native characteristics of primary β- cells (eg. glucose responsiveness) in culture over time. Hence diabetes research has involved the use of β- cell tumour lines, which have an infinite life span. They are extremely useful in studying the molecular basis of β- cell function. They are also ideal tools for testing potential drugs for the development of therapeutic options. Cell culture offers research possibilities that are difficult to be performed *in vivo*. During cell culture, various parameters including culture conditions, cell growth and cell density can be kept controlled and the various secreted factors can be assayed from the culture media. A number of rodent, human and hamster β- cell lines are currently in use and most of them have been derived from insulinomas or neoplastic islets[184, 185].

β- cell lines have been generated using various transformation approaches including irradiation, viral infection and targeted expression of recombinant oncogenes. RIN and INS cell lines were derived from radiation treated rat cells. Mouse cell lines including NIT-1 and MIN6 were generated from insulinomas of transgenic mice expressing the large T antigen of SV40. Though they are exceedingly useful β- cell research tools, they do not recapitulate primary β- cell physiology perfectly. A major disadvantage with the cell lines is the induction of hexokinase in the cells while in culture. Normal β- cells express the high-Km glucokinase isotype and this enables them to secrete insulin only

when the glucose concentration exceeds the physiological threshold. But the induction of low-Km hexokinase in the cell lines makes them responsive to sub physiological glucose levels. Hence compared to normal β- cells, most cell lines usually have a hypersensitivity to glucose. They have defective secretory characteristics and do not respond to glucose in the physiological range[184, 185].

For the purpose of this thesis, I would like to briefly describe the rat insulinoma cell line INS-1E and the mouse insulinoma cell line NIT-1. INS-1E cell line is a rat β- cell line that was derived from the parental INS- 1 cell line. INS-1 cell line was isolated from a radiation induced rat insulinoma[186] and the cell line displays many important features of β- cells including high insulin content and glucose responsiveness within the physiological range[185]. But these cells were found to be heterogeneous and hence Janjic et al.[187] improved on the original cell line by generating Ins-1E cell line based on their insulin content and also their secretory response to glucose. Later they characterized the cell line over a period of 2 years and found that the cells could maintain β- cell phenotype over 116 passages and that the glucose-induced response was dose dependent[188]. Menin has been found to inhibit insulin production in INS-1 cells[189].

NIT-1 cell line is derived from NOD/ Lt mice which are transgenic for the SV40 large T antigen under the control of RIP promoter and which develop β- cell adenomas[184, 185]. SV40 or Simian vacuolating virus 40 is a circular DNA virus, which is a member of the polyomavirus family. It is able to infect eukaryotic cells and has the potential to cause tumours. It is believed to suppress the transcription of the tumour suppressor p53 and hence is able to transform a variety of cell lines[185]. NIT-1 cell line expresses high levels of insulin mRNA but the cells are not responsive to glucose in the physiological range and the insulin secretion is responsive to the glucose concentration in the media. The cells when cultured shed a mature ecotropic type C virus[190].

1.21 Relevance of a RIP- luciferase NOD mouse model:

For the development of an effective therapeutic intervention for T1D, it is imperative that we have a clear understanding of the disease process as a whole, the main culprits and the critical checkpoints that are overridden in disease. Conventionally, the investigations have used an approach where representative groups of animals are sacrificed at required time points for analyses and data collection. This approach carries some disadvantages in that the number of animals which are required for each experiment is high, which might put limitations on the scope of experiments as well as the frequency.

Type 1 diabetes is caused by the destruction of β- cells by the immune system and one of the most promising therapeutic strategies being intensively pursued is endogenous β- cell regeneration. Hence the ability to monitor β- cell mass either in the normal course of disease development or in the presence of therapeutic interventions is absolutely critical. The project proposes to address this issue by generating transgenic mice in the NOD background in which the β- cell mass can be imaged non- invasively using bioluminescence. *In vivo* imaging of luciferase expressing cells is already well established[191] and luciferase constructs have been used to transduce islets that were then monitored non-invasively post- transplantation [192, 193]. Also, transgenic mice which express mouse insulin promoter (MIP)/ RIP driven luciferase gene have been reported though the work has been done in mouse strains which are not T1D relevant[194, 195]. We propose to build on this concept by generating pancreas specific luciferase transgenic mice in the NOD background and combine it with pancreas specific silencing of target genes. The mice will express the luciferase gene in the pancreas under the control of rat insulin promoter (RIP). We anticipate that during the normal course of the disease development, beta cells are destroyed over time and this should lead to reduced luminescence intensity with time. This strategy will also be used to generate transgenic mice in which target genes are constitutively silenced exclusively in the beta cells using RNA interference (RNAi). The approach would facilitate the non-invasive monitoring of β- cell mass in NOD mice and simultaneously allow beta cell specific gene silencing.

1.22 Bioluminescence Imaging (BLI):

Bioluminescence is the process by which visible light is emitted by living organisms. Bioluminescence imaging (BLI) is widely used in the study of biological processes as it allows non- invasive imaging eliminating the need for killing the animal and as multiple measurements can be obtained from the same animal over time, this reduces the biological variations. A number of bioluminescent systems have been identified in nature so far and the most widely used luciferase is from the North American *firefly (Photinus pyralis*). The enzyme is usually expressed as a molecular reporter in vivo and the imaging includes the detection of visible light, which is produced when the enzyme causes the oxidation of its substrate[196]. In systems using firefly luciferase as the reporter, the substrate D- luciferin has to be injected into the animal before imaging. Lipshutz et al.[197] reported that firefly luciferase has a half-life of about 3 hours in vivo. The reaction has been found to require ATP and magnesium in addition to the substrate[198] and the light emission is found to peak at around 560 nm[199]. In the presence of magnesium and ATP, firefly luciferase causes the oxidation of luciferin and the reaction releases photons. In their review, Sadikot and Blackwell[196] explain that the emitted photons can be detected by a charge coupled device (CCD) camera, which can convert the emitted photons into electrons and then spatially encode the intensity of the photons into electrical charge patterns and thus generate an image.

Bioluminescence imaging is economical, non- invasive and easy to execute[196]. Also, it is completely non- toxic and hence it is an ideal approach for long term studies in living cells, tissues and animal models. Another important advantage is the high sensitivity due to the low background levels of bioluminescence in mammalian tissues. But the approach has several disadvantages as well. Bioluminescent reporters are dimmer than fluorescent reporters and hence the resolution is limited. The limited transmission of light through animal tissues and the loss of photon intensity with increasing tissue depth is another consideration to be overcome. And the sources which are closer to the surface appear brighter compared with sources which are located deeper[196, 200].

1.23 Menin as a modulator of β- cell growth and proliferation:

As mentioned earlier, menin is a transcriptional regulator, which is highly conserved across vertebrates and which is found to regulate β- cell proliferation. It shares little homology with other known proteins[201, 202]. In both mice and rats, the protein is broadly expressed and expression is observed in almost all tissues with the highest expression in neuroendocrine and haematopoietic cells[178, 203]. Both human menin and rat menin consist of 610 amino acids while mouse menin has 611 amino acids. Mouse menin shows an overall identity of 96.5% to the to the human protein while rat menin shows an overall identity of 97%. Four distinct conservational domains including domain A, domain B, domain C and domain D have been identified. Domains A and C- terminal domain D in mouse and are highly identical to their human counterparts while short domain C is quite divergent between humans and the two rodent species. Domain B shows a small sequence divergence in comparison with domain C. The presence of at least two different menin splice variants have been identified in all species and these include the long λ transcript (2.4 kb product) and the short σ transcript (2.0 kb product). Both are found to be co expressed in a variety of tissues[203].

Menin is believed to be critical for haematopoiesis, consistent with its high expression in haematopoietic cells. Novotny et al.[204] observed that menin deficient embryonic stem cell lines were unable to undergo proper differentiation into haematopoietic colonies and that the differentiation could be reinstated with the reexpression of menin or Hoxa9 leading them to conclude that Hoxa9 mediates menin's function. Maillard et al.[205] recently reported that menin has only modest effects on haematopoiesis in steady- state conditions and they noted a decrease in neutrophils, lymphocyte and platelet numbers on menin ablation. However they observed that despite normal initial homing of menin deficient progenitors to the bone marrow, there was a marked functional defect in the long-term haematopoietic stem cells (HSC) in the context of bone marrow transplantation. This suggested that menin is involved in regulating the molecular pathways, which are essential during the adaptive HSC response to stress.

Recent studies have identified a critical role for menin in endocrine pancreas. Yan et al.[206] reported that menin is able to interact with IQGAP1 (IQ motif containing GTPase activating protein 1) and enhance the intracellular adhesion of β- cells. Chen et al.[207] suggests that menin has an essential role in Wnt/ β- catenin signaling and that activation of the Wnt/ β- catenin signaling can inhibit islet cell proliferation. Multiple endocrine neoplasia type 1 (MEN1) is a tumor syndrome characterized by tumors in multiple endocrine organs including parathyroids, anterior pituitary and endocrine pancreas[208] (OMIM 131100). Thakker et al.[209] reported that pancreatic islet tumors occur in 30%-80% of MEN1 patients and can be a major cause of death in those patients. The condition arises from mutations in the gene Men1 that codes for menin. It has been reported that 80% of the mutations cause loss of protein function[210]. The remaining 20% is believed to involve missense mutations, which result in the mutant protein degradation[211].

Larsson et al.[212] reported loss of heterozygosity in germline and somatic Men1 mutations which gave the indication that Men1 gene is a tumor suppressor. Lin et al.[213] demonstrated that the loss of menin in primary human fibroblasts, when complemented with the expression of SV40 large and small T antigen and oncogenic ras, led to a transformed phenotype. They reported that menin acts as a direct repressor of hTERT, the protein component of telomerase enzyme. Supporting the role of menin as a tumor suppressor, homozygous loss of menin in mice causes the death of embryos between embryonic day 11.5 to 13.5 and the embryos exhibit several severe developmental defects[214]. Heterozygote Men1 knockout mice gestate normally but after 9-16 months, they develop endocrine tumors, which are similar to the ones observed in MEN1 disease[214, 215]. To further analyze the role of menin, conditional knockouts in which Men1 gene was disrupted specifically in the pancreatic islets were generated and these mice were reported to develop multiple insulinomas along with elevated serum insulin levels and reduced blood glucose levels[216, 217]. Scacheri et al.[218] generated mice with tissue-specific homozygous loss of menin in the liver but did not observe any tumor formation causing them to conclude that Men1 loss might be critical only in endocrine tissues. Scacheri et al.[219] recently analyzed the genome wide menin binding patterns and found

that menin could occupy the promoter regions of thousands of human genes suggesting a role for menin as a global regulator of transcription. They also found that the specific bias for endocrine tumour formation upon menin loss might be due to the dysregulation of distinct genes including HLXB9, that are targeted by menin only in the endocrine tissues.

Karnik et al.[220] showed that though the $Men1^{+/-}$ islets were phenotypically similar to the wild type islets, at around 7 months of age, they showed increased BrdU uptake and there was islet hyperplasia and hypoglycemia in the mice. It has been reported that menin regulates pancreatic islet growth by promoting the expression of cyclin dependent kinase inhibitors p27 and p18[220]. The ability of menin to bind and enhance the expression of caspase 8[221] has also been reported as a probable mechanism that prevents the transformation process. Menin has been shown to interact with a number of transcription factors and co- factors including JunD[222], Smad1, Smad3, Smad5 [223, 224] and MLL1 and MLL2[225] thereby suggesting a role for it in transcriptional regulation. Menin is reported to form a protein complex with the trithorax group members MLL and MLL2 and the complex is able to methylate histone H3 on lysine 4. The methylation is associated with transcriptional activation and it is believed that menin stimulates histone methyltransferase activity of trithorax group containing complexes and thereby inhibit endocrine cell growth[178, 220].

Schnepp et al.[226] reported that depletion of Men1 accelerates S phase entry and this was followed by increased cyclin-dependent kinase 2 (CDK2) activity and decreased expression of p27 and p18. They also reported that pancreatic islet cells display increased proliferation as early as 7 days following Men1 depletion. Recently menin was discovered to play a role in controlling the islet cell mass in pregnant mice. It was reported that during pregnancy stimulated proliferation of maternal pancreatic islets, menin and its targets are repressed by prolactin via STAT5 signaling. Transgenic expression of menin in maternal β-cells prevented the expansion process leading to hyperglycemia and gestational diabetes[227]. Recently Yang et al.[228] reported that men1 excision could prevent STZ induced hyperglycemia in mice by increasing the number of functional beta cells. They observed an increase in islet size, beta cell proliferation and

circulating insulin levels upon men1 excision. In a related study, they also observed that men1 excision could cure mice from pre existing hyperglycemia, whether it is spontaneous or induced[229].

In view of all this research, Men1 is a potential target gene, the knockdown of which can be used to study β- cell proliferation and regeneration in the bioluminescent NOD mouse. We hypothesized that reducing Men1 expression during T1D onset may prevent or at least delay hyperglycemia. Recently Fontanière et al.[208] reported that menin is essential for the proper development of pancreatic endocrine cells and that loss of menin led to failure of the endocrine cell development and altered pancreatic structure. Hence a complete disruption of menin may be deleterious due to the potential to cause islet dysfunction or malignancies. But the partial reduction, which can be achieved by RNAi, could have the benefit of achieving increased β-cell mass and insulin production while minimizing the risk of insulinoma development. The ability to monitor beta cell mass by BLI was expected to enable us to effectively study how the loss of this regulatory gene affects β-cell survival and regeneration in T1D.

1.24 AIM:

We aimed to generate a transgenic β-cell specific reporter mouse line in the NOD background. Several groups had already generated transgenic mouse lines with β-cell specific expression of luciferase. But none of these reporter mice have been generated in the diabetes susceptible NOD background. Hence we aimed to generate a NOD β-cell reporter mouse line using lentiviral transgenesis. In addition, we aimed to couple β-cell specific luciferase expression with β-cell specific knockdown of target genes. For this purpose, we used a construct that carried the luciferase gene and an shRNA expression cassette under the β-cell specific RIP promoter. Hence by using a single construct, we could ensure specific knockdown target genes in the pancreas and use bioimaging to look at the effects of gene knockdown over time. This strategy would allow us to non-invasively study β-cell modulation via RNAi using luciferase bioimaging. We decided to use menin as a target gene for RNAi to enhance β-cell proliferation and aimed to verify

the enhanced β-cell proliferation on menin knockdown in insulinoma cells *in vitro* as a first step and then aimed to generate menin knockdown mice in the pRLM background.

Chapter 2

MATERIALS

2.1 Chemicals:

CHEMICAL	SUPPLIER
30% acrylamide solution	BioRad
Agarose	Roth
Beta mercaptoethanol	Roth
Brefeldin A	Sigma
BSA	Sigma
$CaCl_2$	Roth
Chloroform	Roth
Cyclophosphamide monohydrate	Sigma
DMSO	AppliChem
D-luciferin	Gold Biotech
Dithizone	Sigma-Aldrich
Ethanol	Roth
Ethidium bromide	Roth
EDTA	Roth
Glycine	Roth
Glycerol	Roth
H_2SO_2	Roth
H^3-thymidine	Hartmann Analytic
Isofluran	cp-pharma
Isopropanol	Roth
Ionomycin	Sigma
KCl	Roth
Ketamine (Ketanest)	Pfizer
$KHCO_3$	Roth
Methanol	Roth

$MgCl_2$	Roth
NaCl	Roth
Na_2-EDTA	Roth
$NaHCO_3$	Sigma
$Na_2HPO_4x2H_2O$	Roth
$NaH_2PO_4xH_2O$	Roth
NaOH	Roth
NH_4Cl	Roth
PMA	Sigma
Salmon sperm DNA	Sigma
SDS	Roth
Sodium citrate	Sigma
Streptozotocin	Sigma
TEMED	BioRad
Tris	Roth
Tris-HCl	Roth
Tween20	Roth
Xylazine (Rompun 2%)	Bayer

2.2 Reagents:

2.2.1 Antibiotics and Hormones

REAGENT	SUPPLIER
Ampicillin	Roth
Penicillin streptomycin solution	Invitrogen
Puromycin	Roth
PMS	Sigma
HCG	Sigma

2.2.2 Bacterial strains and transfection reagents

REAGENT	SUPPLIER
DH5α™	Invitrogen
MAX Efficiency® DH5α™	Invitrogen
Novablue	Invitrogen/ Merck/ Novagen
One Shot® TOP10	Invitrogen
Fugene HD	Roche
Genejammer	Agilent (Stratagene)
Polybrene	Sigma
Polyfect	Qiagen

2.2.3 Cell culture reagents

REAGENT	SUPPLIER
D-MEM	GIBCO
D-PBS	GIBCO
Enzyme-free Hank's based cell dissociation buffer	GIBCO
FCS	Invitrogen
Glutamine	Invitrogen
Ham's F-12K	Sigma-Aldrich
HEPES	GIBCO
Histopaque	Sigma-Aldrich
Passive lysis buffer	Promega
RPMI-1640	GIBCO

Sodium pyruvate	GIBCO
Trypsin	GIBCO

2.2.4 Enzymes and PCR reagents

REAGENT	SUPPLIER
AgeI, AluI, BamHI, EcoRI, EcoRV, Hpa1, NheI, NotI, PmeI, PstI, SalI, XhoI	Fermentas
PNK	Fermentas
CIAP	Fermentas
T4 DNA ligase, 10X ligase buffer	Fermentas
Dreamtaq polymerase, Dreamtaq buffer	Fermentas
dNTP	Fermentas
$MgCl_2$	Fermentas
RNase A	Qiagen

2.2.5 General reagents

REAGENT	SUPPLIER
^{32}P-α-dCTP	Amersham GE Healthcare, UK
Bromophenol blue	Roth
Buffer G, Buffer O	Fermentas
CFA	Sigma
Collagenase P	Roche
Compensation beads	BD
DNA ladder (1Kb plus)	Fermentas
DNA ladder (low range)	Fermentas

Dual luciferase system	Promega
Dynabeads mouse T activator CD3/CD28	Invitrogen
ECL	Perkin Elmer
ELISA 96 well plate	Nunc
MicroAmp clear adhesive film	Applied Biosystems
MOG peptide	Biotrend
Pertussis toxin	Sigma
Primer-probe pair	Universal probe library, Roche
Protease inhibitor cocktail	Sigma
Protein ladder	BioRad
Reaction plate (384 well clear optical)	Applied Biosystems
RNAlater	Qiagen
TE buffer	Fluka Analytical
Trans-Blot transfer medium nitrocellulose membrane	BioRad
Hybond^{TM}-N^{+} membrane	Amersham GE Healthcare,UK
Absolute Blue SYBR Green ROX mix	Thermo Scientific
Trizol	Invitrogen
Universal probe master mix (ROX)	Roche
Whatman paper	VWR
X-ray film	Fujifilm

2.3 Antibodies:

2.3.1 Western blot

ANTIBODY	**SUPPLIER**
Rabbit anti menin	Bethyl Laboratories

Anti rabbit HRP	Santa Cruz Biotechnology

2.3.2 Cell culture and FACS

ANTIBODY	SUPPLIER	Concentration/ dilution
Anti CD3	eBioscience	5µg/ml
Anti CD28	eBioscience	1µg/ml
Anti IL-4	eBioscience	1µg/ml
Anti IFNg	eBioscience	1µg/ml
B220 PE	BD	1:300
B220 APC e780	eBioscience	1:400
CD4 APC Cy7/ V500	BD	1:800
CD4 APC	eBioscience	1:800
CD8 PE Cy7	BD	1:800
CD8 e450	eBioscience	1:1600
CD25 PerCp Cy5.5	eBioscience	1:300
CD25 PE Cy7	BD	1:300
CD44 PE Cy5.5	BD	1:300
CD45 eFluor450	eBioscience	1:800
CD62L PE	eBioscience	1:300
FoxP3 PE	eBioscience	1:50
IL-17A PE	eBioscience	1:50
IFNg APC	BD	1:50
TCR APC	BD	1:300
Anti CD16/32 (Fc block)	eBioscience	1:100
Purified anti mouse CD25	eBioscience	1µg/µl
Purified anti mouse CD62L	BioLegend	1µg/µl

2.4 Cytokines:

CYTOKINE	SUPPLIER
rmIL-1b	R&D
IL-2	R&D
IL-6	R&D
IL-12	eBioscience
IL-23	R&D
rhTGF-b	R&D

2.5 cDNA and cell lines:

REAGENT	SUPPLIER
IL-17A cDNA	OpenBiosystems
Mouse menin cDNA	OpenBiosystems
Rat menin cDNA	OpenBiosystems
293F	Invitrogen
INS-1E	Kindly provided by Prof. Wollheim CB
NIT-1	LGC Standards

2.6 Kits:

KIT	SUPPLIER
DNeasy blood and tissue kit	Qiagen
FoxP3 staining kit	eBioscience

QIAquick Gel Extraction kit	Qiagen
Intracellular cytokine staining kit	BD
MACS separation kit (CD62L)	Miltenyii Biotech
QIAfilter Plasmid Maxi kit	Qiagen
QIAquick PCR purification kit	Qiagen
RNeasy kit	Qiagen
Transcriptor first strand DNA synthesis kit	Roche
Rediprime II labeling kit	Amersham GE Healthcare UK
Probe Quant G-50 MicroColumns	Amersham GE Healthcare, UK

2.7 PCR primers and probes:

GAPDH: fwd: 5'- agcttgtcatcaacgggaag-3'
rev: 5'-tttgatgttagtggggtctcg-3'
Probe: #9

IL-17A: fwd: 5'-tgtgaaggtcaacctcaaagtct-3'
rev: 5'- gagggatatctatcagggtcttcat-3'
Probe: #50

IL-17F: fwd: 5'- caagaaatcctggtccttcg-3'
rev: 5'-gagcatcttctccaacctgaa-3'
Probe: #45

IL-21: fwd: 5'- tcagctccacaagatgtaaagg-3'
rev: 5'- gccttctgaaaacaggcaaa-3'
Probe: #100

IFNg: fwd: 5'- atctggaggaactggcaaaa-3'
rev: 5'- ttcaagacttcaaagagtctgaggta-3'
Probe: #21

RORgt: fwd: 5'- gcagaactgccccattga-3'
rev: 5'-gacattcggccaaacttga-3'
Probe: #21

Hluc primers: fwd: 5'-atggccgatgctaagaacat-3'
rev: 5'- ggatgatctggttgccgaaa-3'

SCID primers: fwd: 5'- ggaaaagaattggtatccac-3'
rev: 5'- agttataacagctgggttggc-3'

2.8 shRNA sequences:

IL-17 shRNA oligos (NM_010552)

#176

target – ccaaggacttcctccagaa

fwd- tccaaggacttcctccagaattcaagagattctggaggaagtccttggttttttc

rev- tcgagaaaaaaccaaggacttcctccagaatctcttgaattctggaggaagtccttgga

#188

target - tccagaatgtgaaggtcaa

fwd- ttccagaatgtgaaggtcaattcaagagattgaccttcacattctggatttttttc

rev- tcgagaaaaaatccagaatgtgaaggtcaatctcttgaattgaccttcacattctggaa

#203

target - tcaacctcaaagtctttaa

fwd- ttcaacctcaaagtctttaattcaagagattaaagactttgaggttgatttttttc

rev- tcgagaaaaaatcaacctcaaagtctttaatctcttgaattaaagactttgaggttgaa

#288

target - gactctccaccgcaatgaa

fwd-tgactctccaccgcaatgaattcaagagattcattgcggtggagagtctttttttc

rev- tcgagaaaaaagactctccaccgcaatgaatctcttgaattcattgcggtggagagtca

#303

target – tgaagaccctgatagatat

fwd- ttgaagaccctgatagatatttcaagagaatatctatcagggtcttcatttttc

rev- tcgagaaaaaatgaagaccctgatagatattctcttgaaatatctatcagggtcttcaa

#362

target - gtgtcaatgcggagggaaa

fwd- tgtgtcaatgcggagggaaattcaagagatttccctccgcattgacacttttttc

rev- tcgagaaaaaagtgtcaatgcggagggaaatctcttgaatttccctccgcattgacaca

#497

target - gcacctgcgtggcctcgat

fwd- tgcacctgcgtggcctcgatttcaagagaatcgaggccacgcaggtgctttttc

rev- tcgagaaaaaagcacctgcgtggcctcgattctcttgaaatcgaggccacgcaggtgca

Menin shRNA oligos (NM_008583.1)

Start position: **780**

22_mer: ggtctcggatgtcatatggaac

tgctgttgacagtgagcgagtctcggatgtcatatggaactagtgaagccacagatgtagttccatatgacatccgagacctgcct
actgcctcgga

Start position: **803**

22_mer: gcctcagccgctcctacttcaa

tgctgttgacagtgagcgacctcagccgctcctacttcaatagtgaagccacagatgtattgaagtaggagcggctgaggctgcc
tactgcctcgga

Start position: **1170**

22_mer: cactgactctttggaactgttg

tgctgttgacagtgagcgaactgactctttggaactgttgtagtgaagccacagatgtacaacagttccaaagagtcagtgtgcct
actgcctcgga

Start position: **1206**

22_mer: gctgctctggctgctgtatgac

tgctgttgacagtgagcgactgctctggctgctgtatgactagtgaagccacagatgtagtcatacagcagccagagcagctgcc
tactgcctcgga

Start position: **1447**

22_mer: gacactgccactgttatccaag

tgctgttgacagtgagcgaacactgccactgttatccaagtagtgaagccacagatgtacttggataacagtggcagtgtctgcct
actgcctcgga

Start position: **1449**

22_mer: cactgccactgttatccaagac

tgctgttgacagtgagcgaactgccactgttatccaagactagtgaagccacagatgtagtcttggataacagtggcagtgtgcct
actgcctcgga

Start position: **2079**

22_mer: tttccagagtgagaagatgaaa

tgctgttgacagtgagcgcttccagagtgagaagatgaaatagtgaagccacagatgtatttcatcttctcactctggaaatgccta
ctgcctcgga

Start position: **2213**

22_mer: actacacactctctttcctaaa

tgctgttgacagtgagcgcctacacactctctttcctaaatagtgaagccacagatgtatttaggaaagagagtgtgtagttgccta
ctgcctcgga

Start position: **2760**

22_mer: agctccgactcttatctgtgaa

tgctgttgacagtgagcgcgctccgactcttatctgtgaatagtgaagccacagatgtattcacagataagagtcggagcttgcct actgcctcgga

2.9 Linker:

Not1-Xho1 Linker sequence:

5’ GGCCGATCTCGAGATC 3’

2.10 Buffers:

ELISA:

Wash solution for ELISA (PBS.Tween20)

500 ml of 10X PBS is diluted with 4.5 L of distilled water to obtain 5L of 1X PBS. 2.5 ml Tween20 is added to PBS to obtain a 0.05% wash solution.

FACS &MACS:

ACK lysis buffer

Dissolve 8.29g ammonium chloride, 1.0g potassium bicarbonate and 37.3 mg Na_2EDTA to 800ml of distilled water and adjust the p^H to 7.2-7.4. Make up the volume to 1L with distilled water.

Annexin binding buffer

Make 1L binding buffer containing 10mM Hepes (2.38g/L), 150mM NaCl (8.766g/L), 5 mM KCl (0.372g/L), 1mM $MgCl_2$ (0.203g/L) and 1.8mM $CaCl_2$ (0.199g/L). Make up the volume with NaOH p^H-7.4.

MACS buffer

Prepare a solution containing PBS pH-7.2, 0.5% FCS and 2mM EDTA.
0.3722 gm EDTA is added to 500 ml PBS and left on a shaker at 50°C for 4-5 hours. 2.5 ml FCS is added to the buffer, which is then filtered and stored at 4°C.

PBS. EDTA for blood collection

0.3722 gm EDTA is dissolved in 500 ml PBS to make a 2mM solution and it is then stored at 4°C.

Southern Blot:

10XSSC

Make a solution of p^H 7.6: Sodium citrate 150 mM and NaCl 1.5 M.

Denaturation buffer

Make a solution of NaCl 1.5 M, NaOH 0.5 M.

Neutralization buffer

Make a solution of Tris-HCl 0.5M (p^H 7.2), NaCl 1.5M and EDTA 1mM and autoclave.

Sodium phosphate buffer 0.5 M, p^H7.2

Make a solution of $Na_2HPO_4x2H_2O$ (1M) 34.2%, $NaH_2PO_4xH_2O$ (1M) 15.8% and adjust p^H to 7.2.

Church buffer

Mix sodium phosphate buffer 0.25M, EDTA 1 mM, Bovine serum albumin (BSA) 1%, SDS 7% and salmon sperm DNA 0.1 mg/ml.

Church wash (5L)

Mix 200 ml 0.5M sodium phosphate buffer p^H7.2 and 250 ml 20% SDS.

Western Blot:

SDS PAGE Running buffer

To obtain a 10X solution, dissolve 10g SDS, 30.3g Tris and 144.1g glycine in 800ml distilled water. Adjust the volume to 1L with distilled water. Store at 4°C.

Laemmli Buffer

For a 2X solution, mix 10ml 1.5M Tris (pH-6.8), 6 ml 20% SDS, 30 ml glycerol, 15 ml β-mercaptoethanol and 1.8 mg bromophenol blue. Adjust the volume to 100ml with distilled water. Make 10ml aliquots and store at -20°C. Store the working solution at 4°C.

Transfer buffer for wet blots

For a 1X solution, dissolve 2.9 g glycine, 5.8 g Tris and 0.37 g SDS in 200 ml methanol. Adjust the volume to 1L with distilled water.

TBS.T

Dissolve 6.05 g Tris (50 mM) and 8.76 g NaCl (150mM) in 800 ml of distilled water and adjust the p^H to 7.5. To make 0.2% TBST, add 500 µl of Tween 20 to the solution and make up the volume to 1L with distilled water.

Blocking solution for western blot

Dissolve 5g of nonfat dried milk powder in 100ml of 0.2% TBST to obtain a 5% milk powder blocking solution.

GENERAL BUFFERS:

Na- citrate buffer

Dissolve 1.47 gm of Na- citrate in 50 ml distilled water and adjust the p^H to 4.5. Buffer should be made fresh with each round of streptozotocin injections.

Cell depletion buffer

Prepare a solution containing PBS pH-7.2, 0.1% FCS and 2mM EDTA.
0.074 gm EDTA is added to 100 ml PBS and left on a shaker at 50°C for 4-5 hours. 100 µl FCS is added to the buffer, which is then filtered and stored at 4°C.

Chapter 3

METHODS

3. Experimental Techniques:

3.1 Bacterial Culture Techniques:

3.1.1Transformation of bacteria

Transformation is done using competent bacterial cells. The cells are thawed on ice and 1 μl of the DNA (ligation) is added to the cell suspension. The suspension is left on ice for a minimum of 30 minutes. The cells are then subjected to heat shock at 42°C for 60 seconds and immediately cooled on ice.150 μl of LB media with ampicillin is added to the suspension and the suspension is plated onto LB-ampicillin plates and incubated at 37°C overnight in an incubator.

3.1.2 Miniprep preparation

Nova Blue™ or Top10™ competent cells are transformed with the desired construct and cultured overnight to obtain colonies. The colonies are picked randomly and cultured in LB-ampicillin media (8 hours in the day or 16 hours in the night). The cultures are then spun down and used to make minipreps.

3.1.3 Maxiprep preparation

For maxipreps, the positive minipreps are diluted 1:100 and used to transform either DH5α™ or MAX Efficiency® DH5α™ cells and are plated onto LB-ampicillin plates and allowed to grow overnight. Colonies are picked and allowed to grow in LB- ampicillin media as above, following which 150 μl of the cultures are transferred to 2L flasks containing 150 ml of LB- ampicillin media and cultured overnight. The cultures are then spun down and used to make maxipreps with the Qiaquick maxi kit. The DNA is then resuspended in 200 μl distilled water or TE buffer. The concentration is checked using a spectrophotometer.

3.2 Molecular Biology Techniques:

3.2.1 Cloning

shRNA sequences targeting the protein of interest are designed using either primer3 program or using the RNAi central gateway. Forward and reverse oligos are obtained and then phosphorylated using PNK enzyme. The oligos are then annealed and cloned into a suitable vector. For this the vector is digested using suitable enzyme pairs and gel purified. The annealed oligos are purified using the PCR purification kit. The shRNA is then ligated with the digested vector using the ligase enzyme. The ligation is used to transform NovaBlue competent bacteria.

3.2.2 Verification of insert- vector ligation

The transformation of competent bacterial cells with the vector- insert ligations lead to the growth of bacterial colonies on the LB agar plates. These colonies have to be analyzed and the presence of the vector- insert sequence in the bacterial colonies verified. Before ligation, the vector sequence is cut with appropriate restriction enzymes and the phosphate group from the 5' terminus is removed using alkaline phosphatase. This prevents the digested vector sequence from recircularising without taking up the insert. In principle, all bacterial colonies that grow should have taken up a vector-insert construct. This is because only those cells are able to grow on ampicillin plates owing to the presence of an ampicillin resistance gene in the vector. Though the addition of alkaline phosphatase prevents the vector sequences from religating, there is a possibility that sometimes the vector sequences ligate without taking up the insert. The bacterial cells that take up these recircularised vectors do not contain the insert, but are still able to grow on ampicillin plates. Hence it is critical to verify the presence of the insert in the bacterial cells before starting minipreps.

This is done using 2 different methods. The first one is restriction digestion of the minipreps. Here, bacterial colonies are randomly selected and minipreps are made from them. The DNA is then digested with the same enzyme pair used during cloning. The DNA samples are run on a gel after digestion. Only those minipreps containing the insert would give 2 bands on the gel, one corresponding to the vector backbone and the other a smaller band corresponding to the insert. The minipreps containing the recircularised vector would give a single band corresponding to the vector backbone. Those minipreps, which give 2 bands, are sent for sequencing to further verify that the vector- insert sequences are in the correct orientation.

The second method is colony PCR. This method is more time saving and less labor intensive than restriction digestion. Here, small amounts of the bacterial colonies are used as template for a PCR reaction using primer sequences complementary to the appropriate vector. Only those colonies that have taken up the vector sequence would give an amplified product. The colonies with the vector- insert constructs would give a larger sized band compared to the colonies with recircularised vectors, owing to the presence of the insert. These colonies, which give the larger sized bands on gel, are then picked and used to make minipreps. Hence the additional work making minipreps from incorrect ligations can be avoided. The minipreps are then sent to sequencing for further validation.

3.2.3 Luciferase assay

For luciferase assay, 293F cells were plated out at a starting density of 2.5 X 10^5 on 24 well plates. The next day, cells were co transfected with psi-check 2 containing the target cDNA and the shRNA constructs. Psi-check 2 contains two luciferase sequences- a firefly luciferase (expressed constitutively and used for normalization purposes) and a renilla luciferase. The cDNA is cloned into the 3' UTR of the renilla luciferase gene. 100 ng of the reporter (psi- check 2) along with 300 ng of the shRNA construct is used per well along with the transfection

reagent polyfect (Qiagen). The plates are left in the incubator for 2 days after which the cells are washed once with PBS and lysed with a passive lysis buffer (Promega). 5 µl of each sample is then transferred to an opaque 96 well plate and the luciferase activity is measured with a Fluostar Optima luminometer. For this, the dual luciferase system from Promega is used. The values obtained for firefly luciferase are used to normalize the values obtained for renilla luciferase. The sequences that give the lowest values for renilla luciferase are selected as they are expected to have the highest knockdown efficiencies.

3.2.4 Tail DNA synthesis

Tail DNA synthesis is done using the DNeasy Blood and Tissue kit from Qiagen. Tails samples are obtained from mice and they are placed in eppendorf tubes. To each tube, 180µl of buffer ATL is added along with 20µl Proteinase K. The tubes are vortexed and the samples are digested overnight at 56°C in a water bath. The tubes are then vortexed and to each tube, 200µl buffer AL is added. The tubes are mixed thoroughly and 200µl ethanol (96-100%) is added. The mixture is then pipetted into a DNeasy mini spin column placed in a 2ml collection tube. Centrifuge at ≥8000 rpm for 1 minute. Discard the flow through and the collection tube. Place the column in a fresh 2 ml collection tube, add 500µl buffer AW1 and centrifuge at ≥ 8000 rpm for 1 minute. Discard flow through and collection tube. Place the column in a fresh collection tube, add 500µl buffer AW2 and centrifuge at 14000 rpm for 3 minutes. Discard flow through and collection tube. Place the column in a clean 1.5 ml eppendorf tube and add 200µl buffer AE directly onto the membrane. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at ≥8000 rpm to elute the DNA.

3.2.5 RNA extraction

Samples are usually resuspended in 1 ml trizol and frozen till needed. For RNA extraction, the samples are thawed and incubated at 15-30°C for 5 minutes. 0.2 ml

chloroform is added to each tube and the tubes are then shaken vigorously by hand for 15 seconds. The tubes are incubated at 15-30°C for 2-3 minutes and then centrifuged at 12000 G for 15 minutes at 2-8°C. The solution separates into a lower red phenol-chloroform phase, an interphase and an upper colorless aqueous phase, which contains the RNA. The aqueous phase is transferred into a fresh eppendorf tube. 0.5 ml isopropanol is added to the tubes and then incubated at 15-30°C for 10 minutes. The samples are then centrifuged at 12000 G for 10 minutes at 2-8°C. The supernatant is removed and the pellet is resuspended in 1 ml 75% ethanol. The tubes are vortexed and centrifuged at 7500 G for 5 minutes at 2-8°C. The supernatant is then removed and the pellet is air-dried. 100µl distilled water is added to the tubes and RNA is dissolved by incubating the tubes at 55-60°C on a thermal shaker for 10 minutes.

Alternatively, samples are saved frozen in 1ml of RNAlater RNA stabilization reagent from Qiagen. RNA is then extracted from these samples using the RNAeasy mini kit. The samples are first thawed and appropriate amounts are weighed out. 600µl buffer RLT is added to the samples which are then homogenized using a homogeniser. The lysates are then centrifuged at full speed for 3 minutes. The supernatants are transferred to fresh tubes. Equal volumes of 70% ethanol are added to the tubes and mixed by pipetting. ≤700µl of the liquid is then transferred to an RNAeasy spin column placed in a 2 ml collection tube. The column is then centrifuged for 15 seconds at ≥8000 G and the flow through is discarded. The column is placed in a new 2 ml collection tube and 700µl buffer RW1 is added to the column. The column is centrifuged for 15 seconds at ≥8000 G and the flow through is discarded. The column is placed in a new 2 ml collection tube and 500µl buffer RPE is added. The column is centrifuged for 15 seconds at ≥8000 G and the flow through is discarded. The column is placed in a new 2 ml collection tube and 500µl buffer RPE is added for a second time. The column is again centrifuged for 2 minutes at ≥8000 G and the flow through is discarded. The column is again placed in a new 2 ml collection tube and then

centrifuged for 1 minute at full speed. The collection tube is then discarded. The column is then placed in a fresh 1.5 ml eppendorf tube. 30-50µl RNAse free water is pipetted straight onto the membrane. The column is then centrifuged for 1 minute at ≥8000 G to elute the RNA.

3.2.6 cDNA Synthesis

cDNA is synthesized using the Transcriptor First Strand cDNA Synthesis kit from Roche. Up to 1µg of RNA is used per reaction. In each reaction tube, 1µg of RNA is mixed with 2.5µM Anchored oligo (dT) primer and 60µM random hexamer primer. The volume is made up to 13µl. The RNA is then denatured by heating the tube for 10 minutes at 65°C in a thermal block cycler. The tube is then immediately cooled on ice. To the tube, a master mix containing 4µl reaction buffer, 20 units RNase inhibitor, 2µl of a 1mM dNTP mix and 10 units of reverse transcriptase enzyme is added. The final volume of each reaction is 20µl. The tubes are then centrifuged and the final reaction is done on a thermal cycler. The cycle involves 10 minutes at 25°C, 30 minutes at 55°C followed by 5 minutes at 85°C. Placing the tubes on ice stops the reaction. The reaction mixture is then diluted 1:2 or 1:4. In cases where the mRNA expression levels are quite low, undiluted cDNA samples are used for qPCR.

3.2.7 PCR

Genomic PCR

Genomic PCR is done on tail DNA samples obtained from mice. Genomic DNA is extracted from tail samples and subjected to PCR. For genotyping purposes, PCR is carried out using specific primers for example GFP, luciferase, SCID etc.

qPCR (Real time quantitative PCR)

cDNA is synthesized using the first strand cDNA synthesis kit from Roche. cDNA is usually diluted 1:2 or 1:4 and used for qPCR. The reactions are done using the primer-probe pair systems from Roche Universal probe library and Universal probe master mix (ROX). PCR setup is done using QIAgility from Qiagen. The reactions are set up on a 384 well plate and real time PCR is carried out using the ABI 7900HT sequence detection system from Applied Biosciences. Primers used are *GAPDH* (fwd: 5'- agcttgtcatcaacgggaag-3', rev: 5'-tttgatgttagtggggtctcg-3', probe: #9); *IL-17A* (fwd: 5'-tgtgaaggtcaacctcaaagtct-3', rev: 5'-gagggatatctatcagggtcttcat, probe: #50); *IL-17F* (fwd: 5'-caagaaatcctggtccttcg-3', rev: 5'-gagcatcttctccaacctgaa-3', probe: #45); *IL-21* (fwd: 5'- tcagctccacaagatgtaaagg-3', rev: 5'- gccttctgaaaacaggcaaa-3', probe- #100); *IFNg* (fwd: 5'- atctggaggaactggcaaaa-3', rev: 5'-ttcaagacttcaaagagtctgaggta-3', probe- #21); *RORgt* (fwd: 5'-gcagaactgccccattga-3', rev: 5'-gacattcggccaaacttga-3', probe: #21).

For analyzing luciferase expression in mice organs, we use a slightly different protocol. Organs are taken out and immediately saved in 1.5 ml of RNAlater reagent. The organs are then stored at -20°. RNA is made from around 30 mg of dehydrated organs and cDNA is synthesized using the first strand cDNA synthesis kit. Undiluted cDNA is then subjected to real time PCR using the hluc primers and a SYBR green master mix instead of using specific probes. The reactions are set up on a 384 well plate and real time PCR is carried out using the ABI 7900HT sequence detection system from Applied Biosciences.

3.2.8 Western blot

The blot is done using the Protean 3 system from BioRad. Samples are run on a 12 % running gel. For preparation of the cell lysates, the cells are spun down and resuspended in a 1:10 mix of RIPA buffer (Sigma) with protease inhibitor cocktail, left on ice for 30 minutes with occasional shaking. The suspensions are then spun down; the supernatants are transferred to fresh tubes and frozen. The cell lysates are mixed with equal volumes of Laemmli buffer and boiled for 10 minutes at 95°C. The samples are then loaded onto the gel along with a protein marker and the gel is run in the running chamber using the running buffer. Once the samples have run enough, the gel is removed from the chamber. The samples are then transferred to a nylon membrane in a transfer chamber with the transfer buffer and this usually takes an hour and a half. Once the transfer is complete, the membrane is cut to size and blocked for 1-2 hours at room temperature with a 5% milk powder solution. The primary antibody diluted in 5% milk powder solution is added at the end and the membrane is left overnight. The next day, the primary antibody solution is removed and the membrane is washed 4 times for 20 minutes with a 0.2% TBST solution. The secondary antibody is diluted in the milk powder solution and is added to the membrane and incubated for 1.5- 2 hours. The membrane is again washed with 0.2% TBST solution. The membrane is then incubated in the ECL (enzymatic chemiluminescence) reagent for a minute. The membrane is then exposed to a film that is then developed in a developer. Alternatively, the membrane is exposed to a camera and the image captured directly.

3.2.9 Southern blot

Around 10μg of genomic DNA are digested overnight at 37°C with 25 U/sample of EcoRI restriction enzyme. Next day fragments are separated on a 1.0% agarose gel by electrophoresis for several hours and separation of the bands is documented together with a ruler on a UV imaging system (Herolab GmbH Laborgeraete,

Germany). The gel is left under UV for around 10 minutes to enhance DNA fragmentation and is then washed in denaturation solution 2 times for 20 minutes and finally in neutralization solution again 2 times for 20 minutes. DNA transfer is performed by capillarity in 20x SSC buffer on Hybond^{TM}-N^{+} membranes. Membranes are air-dried and the DNA is covalently bound to the membrane using a UV-linker (1200 J/m^2, UV Stratalinker®1800, Stratagene, California).

Radioactive labelling of the probe: GFP sequence is cut out from pLBM vector using appropriate restriction enzymes and is gel purified. 50-100 ng of GFP is used as the probe. It is denatured at 95°C for 5 min and labelled with radioactive ^{32}P-α-dCTP (50 μCi) using the Rediprime II labelling kit (Amersham GE Healthcare, UK) according to the manufacturer's instructions. To reduce unspecific background, unincorporated nucleotides are removed by gel filtration with Probe Quant G-50 MicroColumns. The probe is ready for hybridization reaction after denaturation at 95°C for 5 minutes.

Hybridization: Membranes containing the DNA samples are blocked for 2 hours in church buffer at 65°C in a water bath shaker and after 2 hours the labelled probe is added and hybridized for 16 hours under the same conditions. Next day membranes are washed twice for 20 minutes in church washing buffer. Signal is detected by exposure of the hybridised membrane to X-ray film for 5-7 days at -80°C.

3.2.10 ELISA

ELISA kits were obtained from eBioscience and the 96 well plates from nunc. The assay was carried out following the manufacturer's instructions. All the steps are followed by thorough wash steps and the incubation is done on a shaker. The plates are coated overnight with the capture antibody. For this, the antibody is resuspended in coating buffer and added to the wells and the plates are then left on a shaker at 4°C. The next day, the wells are washed thrice. Assay diluent is

added to the wells and left for an hour on the shaker. Top standard and its dilutions (7 dilutions of twofold starting from 1:2) are made with assay diluent along with sample dilutions when needed. The standards and the samples are added to the wells of the plate in replicates and left on the shaker for 2 hours. The plates are then washed. The detection antibody diluted in the assay diluent are added to the wells and left for an hour. Following this, the wells ware washed thoroughly. Diluted HRP antibody is added to the wells and left for 30 minutes. The wells are then thoroughly washed. TMB substrate is added to the wells and the plates are left on the shaker for the reaction to proceed. When suitable, the reaction is stopped by the addition of 2N sulfuric acid. The plates are then read in an ELISA reader.

3.3 RNAi and Lentiviral Transgenesis Techniques:

3.3.1 Lentivirus Production

The selected sequences are used to generate lentiviral vectors expressing the corresponding constructs. 293 F cells are plated on 15 cm plates and transfected the next day with the viral components and the shRNA construct. 3 µg pCMV-VSVg, 3 µg RSV-rev, 4 µg pMDL-gag/pol and 5 µg pAdvantage are added to 1 ml serum free media along with 20 µg of shRNA construct and 70 µl Fugene HD. The mixture is incubated at room temperature (RT) for 30 minutes and then pipetted onto the cells. Medium is replaced after 24 hours. Viral supernatant is collected, spun down and filtered both after 48 hours and 72 hours. Viral preparations are then ultra centrifuged and the supernatant is removed. Viral pellets are resuspended in 100 µl PBS by overnight incubation at 4°C. The virus samples are then aliquoted into eppendorf tubes, flash-frozen in liquid nitrogen and stored at -80°C. 1 µl of each virus sample is used for virus titrations.

3.3.2 Virus Titration

293 F cells are plated on 6 well plates at a starting density of 4 x 10^5 cells/ well. The plates are left in the incubator overnight. 1 µl of the virus sample is added to the first well while 1:10 and 1:100 dilutions of the virus are used to infect the second and third wells respectively. The plates are left in the incubator for 2-3 days after which, the percentage of infected cells is determined by FACS. The virus titers are calculated from the percentage of GFP+ cells.

Calculation of virus titer:

If 4 x 10^5 cells are plated out per well for infection, it is assumed that by the time of infection (addition of virus) next day, there would be around 8 x 10^5 cells per well. It is also assumed that one virus infects one cell and vice versa.

If 30% of cells are GFP+ in the sample infected with 1µl of virus suspension,

Number of virus particles in 1µl= 30 x 800000= 2.4 x 10^5
Number of viral particles / ml= (2.4 x 10^5) x 1000

Final virus titer= 2.4 x 10^8/ ml

3.3.3 Viral infection of cells

For infection with the virus, cells are plated out on 24 well plates at a starting density of 2.5 x 10^5 cells/well. The plates are left overnight in the incubator. Virus is added to the cell media with or without polybrene. Due to the really slow growth of insulinoma cells, they are cultured for 2-3 days before infection with lentivirus. Polybrene seems to be toxic for both Ins-1E and NIT-1 cells and hence they are infected just with the virus in the absence of any transfection reagent.

The cells are cultured and passaged when needed and are used for various analyses.

3.3.4 Generation of transgenic mice

The virus samples with titers above $1x10^8$ viral particles/ ml are used to inject embryos and generate transgenic mice. Female mice are injected I.P. with pregnant mares serum (PMG) and 48 hours later, with human chorionic gonadotropin (HCG). They are then mated with males overnight. The next day, oviducts are excised from the female mice and the embryos are collected under a microscope.

Viral particles are injected into the perivitelline space of single celled embryos. The embryos are then implanted into pseudopregnant female recipients, which then carry them to term.

3.4 Immunology Techniques:

3.4.1 Purification of CD4+CD62L+ T cells

Naïve T cells are isolated using the CD4+CD62L+ T cell isolation kit from Miltenyi biotech using the kit protocol. A single cell suspension is made from pooled lymph node and spleen cells. Up to 10^8 cells are resuspended in MACS buffer to which the 100μl CD4+ T cell biotin-antibody cocktail is added. The cells are incubated in the fridge from 10 minutes. To the suspension, 300 μl MACS buffer and 200μl anti-biotin micro beads are added. After an incubation of 15 minutes in the fridge, the cells are washed after the addition of 10 ml MACS buffer. The cells are then resuspended in 500 μl MACS buffer. Depletion of non-CD4+ cells is accomplished using a LS column. The LS column is placed in the magnetic filed of a MACS separator and the column is rinsed by the addition of 3ml of MACS buffer. The cell suspension is then applied onto the column. The

unlabeled cells that pass through the column are collected into a tube and the column is washed 3 times with 3 ml of buffer. This contains the CD4+ T cell fraction. The CD4+ cells collected are spun down and resuspended in 800 μl of buffer to which 200 μl of CD62L micro beads are added. The cell suspension is then refrigerated for 15 minutes. The cells are then spun down after the addition of 10ml of buffer. Positive selection of CD4+CD62L+ cells is achieved through a second cycle of magnetic separation using an MS column. The MS column is placed in the magnetic filed of a separator and rinsed by the addition of 500 μl of buffer. The cell suspension is then applied to the column and the column is then washed 3 times with the addition of 500μl of buffer. This contains the non-CD62L+ fraction. At the end of washing, the column is removed from the separator and placed on a falcon tube. 1 ml of buffer is pipetted into the column and pushing the plunger into the column flushes out the labeled CD4+CD62L+ T cells. The CD4+CD62L+ cells are resuspended in RPMI.

3.4.2 T helper differentiation *in vitro*

Lymph nodes and spleens are obtained from mice. The tissues are ground and the cells are then treated with an RBC lysis buffer to remove the red blood cells. The cells are then passed through a cell strainer to obtain a single cell suspension. The naïve CD4+CD62L+ cells obtained from MACS are resuspended in RPMI supplemented with 10% FCS, penicillin / streptomycin, glutamine, HEPES and sodium pyruvate. Differentiation is done on 24 well plates. 0.5-1 X 10^6 naïve T cells are plated out per well. The cells are then stimulated with 5μg/ ml anti CD3 and 1μg/ ml anti CD28 in the presence of cytokines. The cytokine concentrations used for are as follows: Th17: TGFβ- 2ng/ml, IL-6- 30 ng/ ml, IL-23- 15 ng/ ml, IL-1β- 10 ng/ml, aIFNg- 1μg/ ml; Th1: IL-2- 2 ng/ ml, IL-12- 10 ng/ml, aIL-4- 1μg/ ml. The cells are cultured for 5 days. On day 5, the cells are replated in restimulation media (RPMI supplemented with 10%FCS, penicillin/streptomycin, glutamine) and restimulated with ionomycin (750ng/ml) and phorbol- myristate-acetate (50ng/ml) for 4 hours in the presence or absence of Brefeldin-A (5 μg/

ml). The cells restimulated in the presence of Brefeldin-A are used for intracellular cytokine staining. The media from the cells restimulated without Brefeldin-A are used for ELISA and trizol is added to the cells and RNA synthesized from these samples is used for real time PCR.

3.4.3 aCD3/CD28 bead stimulation

A single cell suspension is made from the spleens of mice. Splenocytes are then plated out on 24 well plates with aCD3/CD28 beads in the 2:1 bead to cell ratio. The cells are cultured for 2 days. The cells are then separated from beads, spun down and the supernatants are used for ELISA.

3.4.4 MOG peptide stimulation

Splenocytes from MOG_{35-55} immunized mice are stimulated with MOG_{35-55} peptide (10μg/ ml). The cells are cultured for a period of 2 days. The supernatants are collected and used for ELISA.

3.4.5 Cell depletion and NOD-SCID transfer

6-week-old NOD-SCID mice are used for transfer studies. CD25 and $CD62L^{hi}$ depleted splenocytes are transferred from either wt or transgenic mice into the recipient NOD-SCID mice. Spleens are first obtained from groups of mice and are ground to make single cell suspensions. The cells are then pooled and counted after which they are spun down and resuspended in 1 ml depletion buffer. 10 μg each of CD25 antibody and CD62L antibody is then added to the cell suspension. The cells are then incubated on a roller for 20 minutes at 4°C. The cells are washed with 4 ml depletion buffer, spun down and resuspended in 1 ml depletion buffer.

Antibody bound cells are depleted from the cell suspension using sheep anti- rat IgG dynabeads. 50 µl dynabeads is used per 10^7 cells. Required volume of dynabeads is taken out and washed with depletion buffer. The beads are then resuspended in the initial volume of depletion buffer and required volume of the dynabeads is added to the cell suspension. It is then incubated on a roller for 30 minutes at 4°C. 1 ml of depletion buffer is added to the cell suspension and the tube is then placed in a magnet. The bead bound cells stick to the magnet while the unbound cells remain in the suspension and can be decanted. The beads are washed thoroughly several times to obtain all unbound cells. The cell suspension containing unbound cells is then spun down and this would contain the CD25/ $CD62L^{hi}$ double negative cells. Cells are then resuspended in PBS at the desired concentration and injected into the tail veins of NOD- SCID recipients (used $1x10^7$ cells per mouse).

3.4.6 Islet isolation and *in vitro* culture

Pancreata are digested using Collagenase P and the islets are isolated according to the protocol from Bluestone lab[230]. The islets are purified using histopaque. The purified islets are then resuspended in RPMI-10 and cultured using the basic protocol from DiLorezo lab[231]with modifications. The islets are resuspended in RPMI 10 with 50 units of IL-2/ml and cultured on 24 well plates overnight. The next day, the cell suspension is passed through a 40µm strainer and the filtrate is then spun down. The pelleted cells are resuspended in the restimulation media and restimulated for 4 hours with PMA (50ng/ml) and ionomycin (750ng/ml) with or without Brefeldin-A. The samples are then used for intracellular cytokine staining, ELISA or qPCR.

3.4.7 FACS analyses

Extracellular Staining: Single cell suspension is prepared from mouse organs. The cells are spun down and the cell number is counted. Required

numbers of cells are resuspended in PBS. The cells are blocked for 10 minutes at 4°C with an Fc blocking antibody to prevent excessive antibody binding. A mix of the desired antibodies for staining is made and added to the cell suspension. The cells are stained for 30 minutes at 4°C. The cells are then washed twice with PBS and analyzed using a FACS Canto.

Intracellular Staining: FoxP3 staining is done using the FoxP3 staining kit from eBioscience. The intracellular cytokine staining is done using the intracellular cytokine staining kit from BD.

3.5 Bioimaging Techniques:

3.5.1 Preparation of D-luciferin/ Anesthetic mixture

The anesthetic is prepared by mixing 10 ml of PBS with 8 ml Ketamine (25mg/ml) and 2 ml Xylazine (2%). It is recommended to use 5 μl of D-luciferin (dissolved in PBS at 30mg/ml) along with 10 μl of anesthetic per gram of body weight. For a mouse weighing 30 grams, 300 μl of anesthetic is mixed with 150 μl of luciferin. During our experiments, we found out that the anesthetic concentration was mostly high enough to be fatal and hence started using 4μl anesthetic instead of 10μl per gram of body weight. The luciferase signal from our mouse line was also very high and hence we modified the protocol to use 3μl luciferin per gram of body weight.

3.5.2 Imaging of cultured cells

Cells are cultured in 24 well plates and 2 μl of D-luciferin is added to each well just prior to imaging.

3.5.3 Imaging of whole mouse

The anesthetic/ D-luciferin mix is prepared as above. The appropriate amount of the mix to be injected is calculated and injected I.P. prior to imaging. As sometimes mice die during imaging, around 70-75% of the recommended dose of anesthetic- luciferin mix is injected to avoid unnecessary death. The time of injection is noted and the first image is taken at 10 minutes after injection. The earlier images were taken using Night Owl Molecular Light Imager from EG&G Berthold. With this machine, the images were acquired and processed using the WinLight 32 software. The first image was taken with a 20 second exposure time and the second image was taken immediately after with a 1-minute exposure time. Halfway through the project, we were able to obtain IVIS® Spectrum, a high end imaging system from Caliper Lifesciences. In this case, the images were acquired and processed using the Living Image® 4.1 software and we used a one second exposure time. We were able to pick up a strong signal from the thymi of luciferase positive mice with the IVIS system, which we were unable to do with the Night Owl imager.

3.6 Cell Culture Techniques:

3.6.1 Preparation of media

293F Media:

25 ml FCS is filtered into 500 ml DMEM. To the bottle, penicillin/streptomycin and glutamine are added. The media is stored at 4°C.

Ins-1E Media:

RPMI- 1640 media is supplemented with 10% FCS, P/S, glutamine, HEPES (5mM), sodium pyruvate (0.5mM) and 2- mercaptoethanol (25μM). The media is then stored at 4°C.

NIT-1 Media:

1 vial of nutrient mixture F-12 Ham Kaighn's modification (Ham's F-12K) powder is resuspended in 500 ml autoclaved water. 1.5 g of sodium bicarbonate is added to the solution and it is then filtered and split into 2 bottles each of 250 ml. To each bottle 25 ml of filtered FCS, penicillin/streptomycin and glutamine are added. The media is stored at 4°C.

3.6.2 Cell culture and passaging

293 F Cell Line:

The 293 F human embryonic kidney cell line is maintained on advanced DMEM supplemented with 5% FCS, penicillin/ streptomycin (P/S) and glutamine in 10 cm plates. The cells are passaged every 3 days. For this, the media is removed by aspiration; the cells are washed with PBS and trypsinized with 1 ml trypsin. Trypsin is then inactivated by addition of 9 ml media. 1 ml of cell suspension from the plate is transferred to a new plate along with 9 ml fresh media to obtain a 1:10 ratio. The plates are maintained in the incubator at 37°C and at 5% CO2 concentration.

INS-1E Cell Line:

Ins-1E cell line is maintained on RPMI- 1640 media supplemented with 10% FCS, P/S, glutamine, HEPES, sodium pyruvate and 2- mercaptoethanol. Media is renewed every 3 days. Since the cell line grows slower than HEK cells, the cells are passaged every 5-7 days depending on the confluency. Instead of using 1 ml, 2 ml of the cells are used to plate out fresh plates. The plates are maintained in the incubator at 37°C and at 5% CO2 concentration.

NIT-1 Cell Line:

NIT-1 cell line is maintained on Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5g/L sodium bicarbonate and heat-inactivated dialyzed fetal bovine serum, 10%. The cell line grows considerably slow; the cells are passaged every 7-8 days depending on confluency. Fresh media is added every 3 days. The cells are maintained in 25ml TC flasks in a volume of 5 ml. Cells are found to grow better in TC flasks compared to plates. For passaging, the cells are dissociated using an enzyme- free Hank's- based cell dissociation buffer (GIBCO) instead of trypsin. The cells are incubated with the buffer for 4-5 minutes; the flasks are tapped vigorously to dissociate the cells. The cells are resuspended in media, spun down and replated in fresh media. As with INS-1E cells, cells are plated out at a 1:5 ratio.

3.6.3 Puromycin selection

The cells are fist cultured on 24 well plates and infected with virus containing the puromycin construct. The media containing the virus is removed and the cells are maintained on normal media till they are really confluent. Once they are confluent, the normal media is replaced with media containing puromycin. The cells are maintained in the puromycin media for a while to ensure proper removal of puromycin susceptible non-infected cells. The cells are transferred to 6 well

plates and then to 10 cm plates or 5 ml TC flasks once they start getting confluent. Finally the puromycin media is removed and the selected cells are then maintained in normal media.

The puromycin concentrations to be used are determined by puromycin titrations of WT cells. The highest concentration where the cells are still surviving is selected. For Ins-1E cells, a concentration of 1μg/ ml is used; while for NIT-1 cells, a concentration of 0.5μg/ ml is used. For 293F cells, a concentration of 5μg/ ml is used.

3.7 General assay techniques:

3.7.1 PI/ AnnexinV Staining for apoptosis

The staining is done on serum-starved cells, as cell death is more dramatic compared to cells grown in the presence of serum. The cells are cultured in serum deficient media, are harvested over time and the apoptosis levels are analyzed by staining with PI and annexin. The cells are first manually dissociated and washed once with PBS followed by 1 ml of annexin binding buffer. The cells are then spun down and resuspended with 1:50 dilution of Annexin-PE antibody and stained for 15 minutes in the dark at room temperature. The cells are then washed once with PBS and stained with a 1:50 dilution of PI for 30 minutes in the dark at room temperature. The cells are then washed once with PBS and analyzed by FACS.

3.7.2 PI Staining for cell cycle analyses

The cells are washed with PBS and spun down. To the cells, 3 ml of ice cold 70% ethanol is added drop wise while vortexing at a slow speed. The cells are then incubated for 30 minutes on ice at the end of which they are washed once with PBS. A 1:50 dilution of PI is then added to the cells. 1μl RNase enzyme is also

added to ensure the degradation of RNA in the sample. Incubation is done for 30 minutes in the dark at room temperature. The cells are then washed once with PBS and analyzed by FACS.

3.7.3 EFluor670 Staining

0.5μM eFluor670 is resuspended in 1ml of PBS without FCS. 10^6 cells are then resuspended in the eFluor670 solution and stained for 10 minutes in the dark at 37°C. The cells are then washed twice with PBS containing 1%FCS, resuspended in media and plated out. Usually around $2X10^5$ cells are plated out per well of a 24 well plate.

3.7.4 Cyclophosphamide (CY) Injection

For CY induced diabetes studies, mice older than 6 weeks are used. A solution of 20mg/ml CY in PBS is prepared. The tube is left on a roller for 1-2 hours to ensure complete dissolution of CY in PBS. 2mg of CY per 10 gm of body weight has to be injected I.P. The mice are given a second injection of CY 14 days into the time course, if desired.

3.7.5 High dose Streptozotocin (STZ) Injection

High dose STZ injections were done according to the AMDCC protocol[232]. Mice are fasted for 4 hours prior to injection. Appropriate amount of STZ is weighed out and added immediately before injection to sodium citrate buffer to obtain a final concentration of 22.5 mg/ml. This is done as STZ degrades within 15-20 minutes in the solution. The mice are usually lightly anaesthetized with isoflurane and the appropriate amount of the STZ solution is injected I.P. The final dosage required is 150mg STZ per kg of body weight. The injection usually leads to a peak in insulin release and hence mice are provided with 10% sucrose water

overnight to prevent hypoglycemia. The mice are tested 2 days later for hyperglycemia.

Chapter 4

RESULTS

Analyzing the role of IL-17A in autoimmune diabetes

As mentioned in the introduction, Th17 cells are reported to be pathogenic in a variety of autoimmune diseases mainly by way of their primary effector cytokine IL-17A. IL-17A deficient mice are found to be partially protected from experimental models of MS and rheumatoid arthritis[242, 261]. This has led the scientific community to believe that IL-17A might be pathogenic in other autoimmune diseases as well. Though a number of studies have already suggested a pathogenic role for this cytokine in type 1 diabetes[171- 176], none of these results seem to show a direct link between IL-17A and autoimmune diabetes. The remarkable plasticity observed in Th17 cells seems to further complicate the scenario. Hence, we decided to look at the relationship between Th17 cells and type 1 diabetes in the context of IL-17A. We used the common model for type 1 diabetes, the NOD mouse. We felt that this would be an ideal system as these mice develop autoimmune diabetes spontaneously and the disease closely resembles type 1 diabetes in humans.

We resorted to RNAi to generate transgenic NOD mice with reduced IL-17A expression, as we believe that it resembles the normal physiological state much better than a complete knockout of the protein. The idea was to follow diabetes development in the transgenic mice to see whether reduction in IL-17A levels by RNAi conferred protection from diabetes to these mice. If IL-17A is indeed pathogenic in type 1 diabetes as suggested, loss of this protein albeit not complete should be able to at least offer partial protection from type 1 diabetes.

We first generated shRNA constructs targeting IL-17A and the knockdown efficiencies of these shRNAs were then verified *in vitro*. The construct that led to the highest knockdown was then used to generate lentivirus. The high titer lentivirus carrying the shRNA was then used to generate the transgenic mice. All these steps are detailed below.

4.1 Generation and validation of shRNA constructs

We decided to opt for a ubiquitous expression of the shRNA. Since no study has reported a role for IL-17A in the haematopoietic system or in lymphocyte development, we didn't see the need to opt for cell specific knockdown or inducible knockdown of IL-17A. Towards this purpose, shRNA was cloned into the pLBUG vector where it is under the control of a ubiquitous promoter leading to systemic expression of the shRNA and hence systemic knockdown of the protein.

4.1.1 Generation of IL-17A shRNA construct:

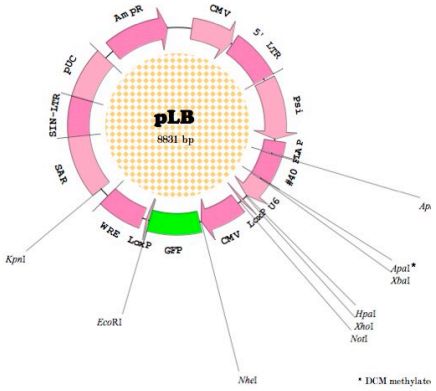
Several shRNA sequences directed against IL-17A coding sequence were designed using the algorithm available at RNAi Central[244] and ordered from Sigma. The oligos were phosphorylated using PNK and then the forward and reverse oligos were annealed to obtain double stranded sequence. The shRNAs were then cloned into the pLBUG vector, obtained by replacing the CMV promoter upstream of GFP in pLB vector with a Ubiquitin promoter (Fig.1A). For cloning in the shRNAs, pLBUG was first digested with Hpa1/ Xho1 and the digests were run on a gel and the digested vector band was cut out and gel purified. shRNAs were then ligated with the digested vector at a 1:4 vector to insert ratio to generate pLBUG constructs containing the desired shRNA sequence. In the pLBUG-shRNA construct, the shRNA is under the control of the U6 promoter, which leads to systemic expression of the shRNA. The construct also has a GFP sequence under the control of another ubiquitous promoter, the ubiquitin promoter and hence GFP expression is used as a marker for shRNA expression (Fig.1B).

Once the shRNA constructs are generated, the knockdown efficiencies of the shRNAs have to be tested. This is done using an *in vitro* assay for which the cDNA of interest has to be cloned into a reporter construct called psi-check2 (Fig.1C). The setup and the principle behind the assay are detailed below. IL-17A cDNA was cloned into the psi-check2 vector to generate IL-17 psi-check.

IL-17 cDNA was obtained from Open Biosystems as a vector construct (pCR4-TOPO). The cDNA had to be cloned into the psi-check2 vector between Xho1/ Pme1 restriction sites. Unfortunately, pCR4-TOPO containing IL-17 cDNA did not have an Xho1 restriction site. To overcome this problem, we decided to cut pCR4-TOPO at the Not1 site and insert a Not1- Xho1 linker into the construct. Not1-Xho1 linker was designed and obtained from Sigma. pCR4-TOPO was then digested with Not1 and ligated with the Not1-Xho1 linker. The ligations were used to transform bacteria and minipreps were made. pCR4-TOPO containing the linker was then digested with Xho1/Pme1, the 600bp IL-17 sequence was cut out and gel purified. Psi-check2 vector was simultaneously cut with Xho1/Pme1 and ligated with the IL-17 cDNA to generate the IL-17 psi-check (Fig.1D).

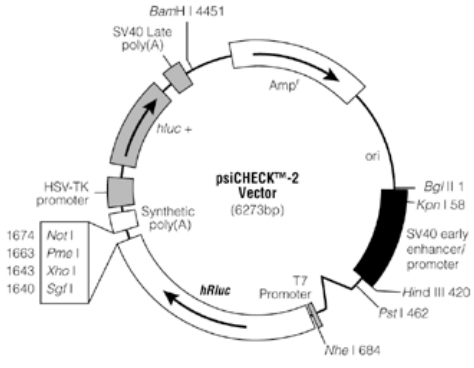
Fig.1

A.



B.

C.



D.

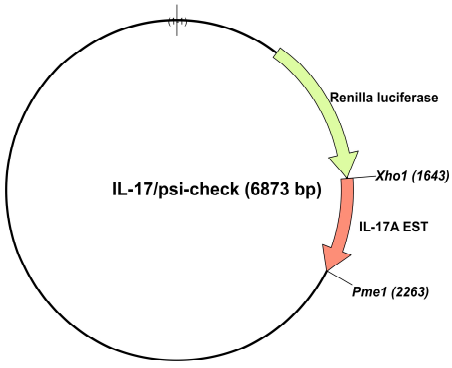


Fig1. ***A & B***. Schematic diagram of pLB. pLBUG was generated by replacing the CMV upstream of GFP with Ubiquitin promoter. ***C***. Schematic diagram of psi-check2. ***D***. Schematic diagram of IL-17/ psi-check.

4.1.2 Verification of shRNA silencing efficiency and specificity in vitro by luciferase assay:

To verify the knockdown efficiencies of cloned in shRNAs, luciferase assay is used. Psi-check2 is a reporter construct having 2 luciferase sequences, a firefly luciferase sequence and a renilla luciferase sequence. Once mammalian cells are co- transfected with the shRNA construct and the psicheck-2 containing cDNA of interest, renilla luciferase expression is used as a marker to calculate the knockdown efficiency of the shRNA.

The cDNA of interest is cloned into the 3'UTR of the renilla luciferase sequence in psi-check2. 293 cells are then transfected with the psi-check2 reporter along with the different shRNA constructs. The cells are allowed to grow in culture over a period of ≥ 48 hours. During this time, transcription results in the formation of mRNAs. Firefly luciferase is constitutively expressed. But since the cDNA is cloned into the 3' UTR of renilla luciferase sequence, a fusion mRNA is formed. The shRNAs that are formed after transcription from the shRNA constructs can then bind to this fusion mRNA due to their complemetarity with the cDNA sequence. This results in cleavage of the fusion mRNA and this depends on the knockdown efficiency of the shRNA. The expression of renilla luciferase is inversely proportional to the knockdown efficiency of the shRNA tested. Firefly luciferase expression levels are constitutive and are used to compensate for the difference in transfection efficiencies. The sequences that can cause the lowest renilla expression levels are selected as these are expected to have the highest knockdown efficiencies.

293cells were transfected with the different shRNA constructs along with the IL-17 psi-check reporter. The cells were lysed after a period of 48 hours and the luminescence intensities were analyzed. All the sequences were found to have good knockdown efficiencies (Fig. 2A). #176 was found to have the highest knockdown efficiency consistently and was used to generate high titer lentivirus.

One criticism that is usually directed against RNAi is that the effects of the shRNA could be non-specific. To address this, we decided to test the knockdown efficiency of sequence #176 against IL-17A psi-check and also against a completely different gene, PTPN22. For the experiment, we used #176 and an shRNA already found to be effective against PTPN22 cDNA, named P4.

We transfected 293 cells with either #176 or P4 in conjunction with either IL-17 psi-check or PTPN22 psi-check. Hence we had 4 different experiment conditions.

1. #176 + IL-17 psi-check
2. #176 + PTPN22 psi-check
3. P4 + IL-17 psi-check
4. P4 + PTPN22 psi-check

PTPN22 cDNA had been cloned into psi-check2 to generate the PTPN22 psi-check. shRNA directed against PTPN22 (P4) had been cloned into the pLBUG vector. Hence only the cDNA and the shRNA differ in the 4 setups and everything else including the rest of the parts of the constructs, cells and experiment conditions are identical between the 4 different experimental setups. Hence if we see any knockdown, it should be specific to the shRNA used. If the #176 shRNA knockdown is specific, we should see a significantly reduced luciferase signal only when #176 is transfected in conjunction with IL-17 psi-check (option 1) and not when #176 is transfected in conjunction with PTPN22 psi-check (option 2).

As expected, #176 caused a reduced luciferase signal only against IL-17A psi-check and this knockdown effect was not observed against PTPN22. P4 was able to cause a reduced signal only against PTPN22 psi-check (Fig. 2B). The results showed that the knockdown observed with #176 was specific to IL-17A and that this shRNA could not knockdown the expression of a random gene. These data show that the knockdown efficiency measured in this reporter assay reflect the specific targeting of IL-17A cDNA by the shRNA#176.

Fig.2

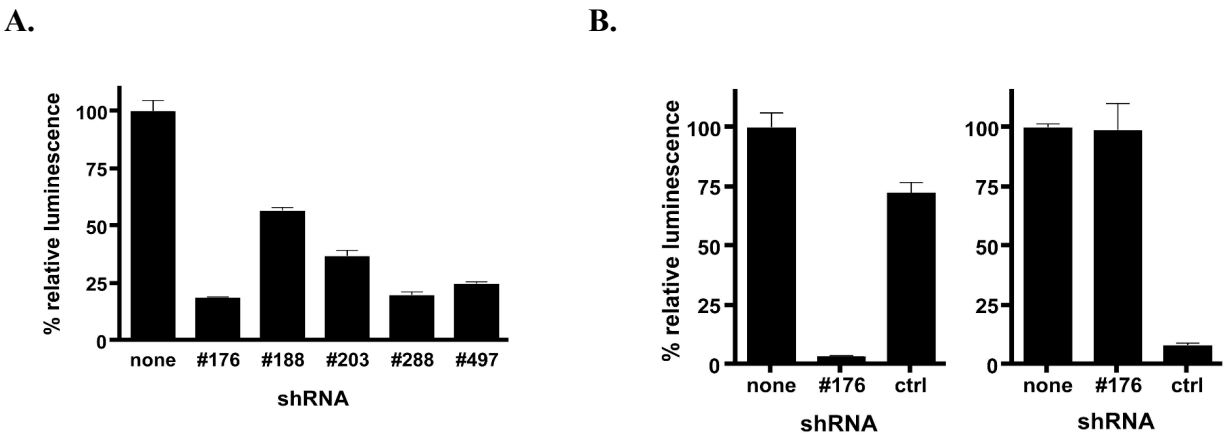


Fig.2. ***A***. Validation of Il-17a shRNA silencing efficiency by dual luciferase assay. Data are representative of several independent experiments. ***B***. Validation of Il-17a shRNA silencing specificity. Knockdown efficiencies of #176 and P4 (ctrl) against IL-17/ psi-check (left) and PTPN22/ psi-check (right) are shown. Data are representative of two experiments and mean and SEM of triplicate values are shown.

4.2 Generation and lineage of IL-17A KD transgenic mice

Once we identified an shRNA that could significantly reduce IL-17A expression, the next step was to generate a NOD mouse line with this vector construct. We used lentiviral transgenesis to achieve this. The various steps are detailed below.

4.2.1 Generation of lentivirus and IL-17A KD transgenic line:

To generate transgenic mice, we inject lentivirus carrying the desired transgene into single- cell embryos, which then give rise to the transgenic mice. To generate lentivirus, 293 cells are co- transfected with the shRNA- construct along with the various viral components. Inside the cells, the viruses are packaged and the packaged viral particles containing the shRNA-construct are released from the cell into the cell media. The viral

particles can be isolated by ultra centrifugation of the cell supernatant. The virus is then titrated and a high titer virus (≥1 x 10^8 particles / ml) is used for embryo injections.

shRNA#176 was co- transfected into 293 cells along with the various viral components and a transfection reagent that facilitates the uptake of the constructs. The cells were allowed to grow in culture. The transfection efficiency could be tracked by GFP expression as all infected cells should be GFP + owing to the GFP sequence in #176-pLBUG construct. After a period of 48 hours, the cell media was collected, centrifuged down to remove cell debris and was saved. The media collection was repeated at 72 hours and the infected cells were then terminated. The cell supernatants were subjected to ultracentrifugation and the virus pellet was resuspended in PBS.

To check the virus titers, 293 cells were infected with 1.0 μl, 0.1 μl or 0.01 μl of the virus suspension. The cells were allowed to grow in culture for 3 days and GFP expression was analyzed under the microscope. At the end of the experiment, cells were taken out and the % of GFP+ (infected) cells was calculated by FACS. We were able to obtain a virus suspension that could infect around 25% of the cells at 1.0 μl concentration and this came out to a virus titer of $2x10^8$ virus particles/ml (Fig.3A). Hence we used this virus suspension for generating the transgenic mice.

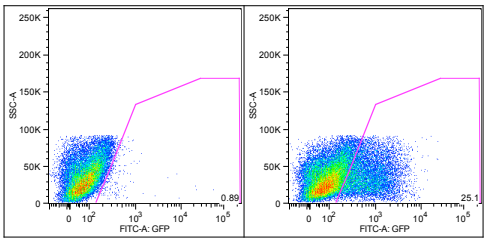
Female NOD mice were super ovulated by injections of PMG and HCG to induce the production of multiple eggs. The super- ovulated females were then mated with males and the embryos were collected from the oviducts of the fertilized females. These single-cell embryos were used for injections. Lentivirus generated with #176 was injected into the perivitelline space of single- cell embryos that were then transplanted into pseudo-pregnant females. We used CD1 females as recipients since they make excellent mothers. The recipient females carried the embryos to term. 6 pups were obtained from the first cycle of injection.

We took out blood from these pups and analyzed GFP expression by FACS. Out of the 6 pups, 5 were GFP negative as none of their cells expressed GFP. But the 6^{th} pup had

almost all cells express high levels of GFP (Fig. 3B). This mouse was used as the founder for the transgenic line.

Fig.3

A.



B.

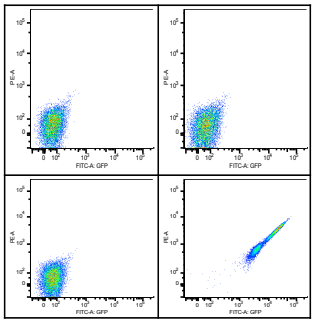


Fig.3. ***A***. Flow cytometry data showing around 25% of virus infected cells. ***B***. GFP expression in blood samples from the positive founder in comparison with three negative mice.

4.2.2 GFP expression levels and lineage studies:

We observed 2 distinct GFP+ populations in the founder mouse. They were designated as 'High' and 'Low' based on their higher and lower mean fluorescence intensities (Fig.4A). This difference in expression levels could have been due to several reasons. One could have been that some cell subsets expressed GFP much strongly than others. But we found the 2 GFP populations in all cell subsets indicating that this might not have been the reason. Another possibility was that there might have been 2 different viral integrants in our founder mouse, which could have led to the 2 expression patterns observed. A third possibility was our founder having been a chimera. To get a clearer picture, we bred the founder mouse and analyzed the N1 and N2 generations over time.

We had 3 litters of pups from the founder. We took out blood from all the pups and looked at the GFP expression. To our surprise, we found that in all the pups we analyzed, we could observe only a single GFP expression pattern. Some of the mice had all their cells with the high expression and some had all their cells with the low expression (Fig.4B). None of the N1 pups were found to have the 2 cell populations together. This

observation precluded the possibility of 2 virus particles having been infected the single-cell embryo. This observation strongly pointed to the possibility of a chimera.

Fig.4

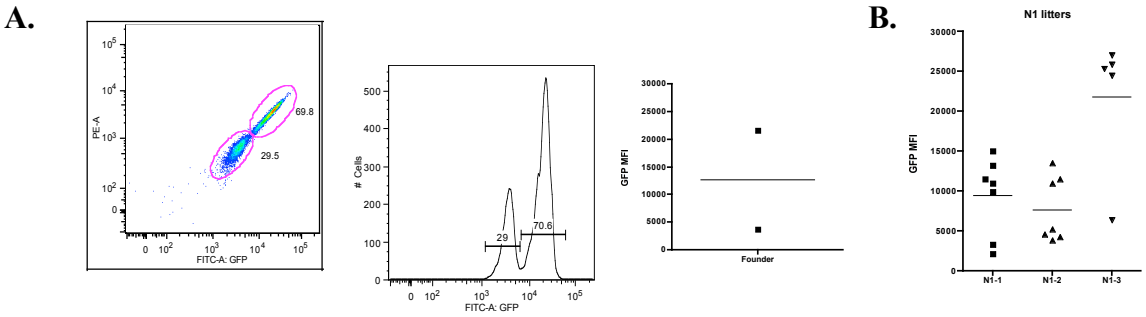


Fig.4. ***A***. Differential expression of GFP in the founder mouse as measured by flow cytometry. ***B.*** GFP expression patterns (MFI) in the N1 litters.

4.2.3 High & Low Transgenic lines:

Mice in N1 were found to have either the high population or the low population and never a mixture. Next, we mated the high mice and the low mice separately as 2 different lines. We named them 'High line' and 'Low line'. It should be noted that 'high' and 'low' refer to the mean fluorescence intensities of GFP expression and that it has nothing to do with IL-17A knockdown efficiencies. For the purpose of this project, mice from 'High line' were used for most experiments unless stated otherwise. Both lines are still being maintained separately.

We took out blood from the N2 mice and analyzed the GFP expression levels. Again to our surprise, all the pups from a high parent were found to express only the high GFP population and all the pups from a low parent expressed only the low GFP population (Fig.5A). This segregation was found to hold true in all future generations (Fig.5B).

Fig.5

A. **B.**

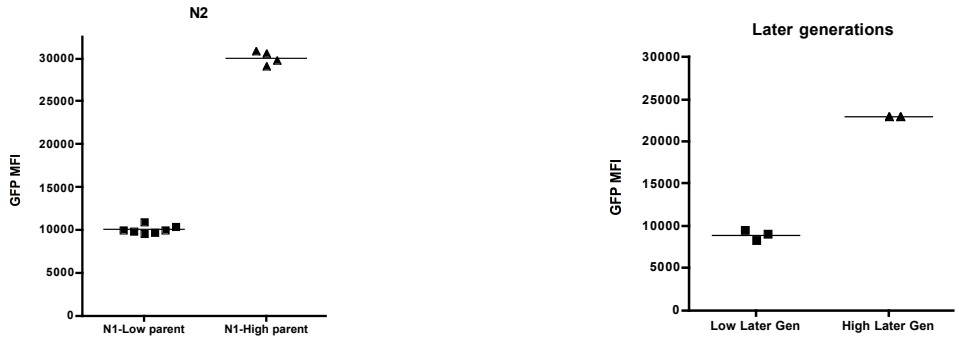


Fig.5. ***A & B***. Segregation of high and low cell subsets in N2 (A) and in later generations (B). MFI of GFP expressing cell populations in the mice are shown.

This pattern led us to believe that the founder was a chimera and that it had 2 viral copies. It seems that at the time of infection, the embryo was in fact 2 celled instead of being single celled and each cell was infected with a distinct lentivirus particle. This made our founder mouse a chimera, with some cells expressing one construct and the others the other construct. During meiosis, some germ cells carried the high GFP expression copy and others carried the low GFP expression copy. This led to segregation of the 2 expression patterns in the subsequent generations.

4.2.4 Southern blot analysis:

To verify this belief, we decided to look for the viral integrants by Southern blot. Each mouse line was believed to carry a distinct viral integrant. The integration site is random and should be distinct in each transgenic line. Since the viral particle carried the GFP sequence, it could be used as a probe to detect the integration site. Unfortunately we did not have DNA from the founder mouse. Hence we had to do the assay with the high and the low GFP expressing pups. We used EcoR1 to cut the genomic DNA. It cuts the DNA

randomly and since we didn't know where the integration site was, we couldn't predict the size of the fragment we were looking for. But if the high and the low transgenic lines have a single distinct viral integrant as we suspected, they should have different integration sites and hence we should ideally detect GFP bands at 2 different sites on the gel- one site for all the low mice and another site for all the high mice. It would have been really rare if the viral integrants integrated at the same site or if they integrated at different sites but gave similar sized bands upon restriction digestion with EcoR1.

DNA samples from 3 each of low and high mice were obtained and analyzed by Southern blot using a GFP probe. We observed a single band in both groups of mice but at different sizes (Fig.6). This showed that there was a single distinct viral integrant in each transgenic line and that the viral integration site was distinct in each line. This further validated our belief that the founder embryo was 2 celled at the time of the injection and that each cell got infected with a distinct viral copy and each copy integrated at a distinct genomic site.

Fig.6

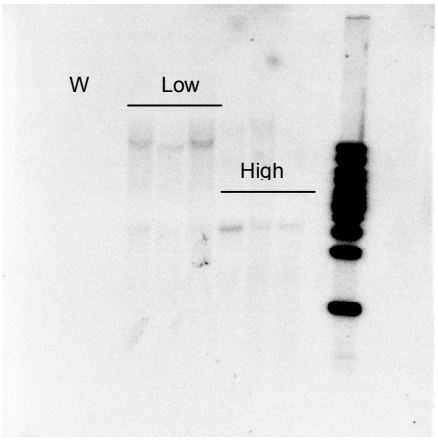


Fig.6. Southern blot analysis of low and high transgenic mice. W represents the wt sample.

4.3 Phenotypic characterization of lymphocyte compartments in transgenic mice

We found that the transgenic mice were viable and fertile and they did not present any gross phenotypic defects. Though IL-17A has not been reported to play any significant role in either the haematopoietic or the lymphoid system, it was still critical to ensure that the lymphoid populations in the transgenic mice were normal. Hence we decided to characterize the T cell subsets in the transgenic mice and also look at the activation status of the transgenic T cells.

Cells were isolated from thymus, spleen and lymph nodes of both WT and transgenic mice and stained with a variety of antibodies against T cell markers. Transgenic mice showed no defects in any of the lymphoid compartments indicating that loss of IL-17 did not compromise lymphoid development.

4.3.1 Characterization of T cell subsets:

T cells undergo maturation in the thymus. There the double positive CD4+CD8+ T cells switch off one of the markers and commit themselves to a single lineage, either CD4 or CD8. We decided to look at the single and double positive populations in the thymus to ensure that the selection processes and overall development of T cells in the thymus is not compromised by the transgene. For this, we isolated T cells from the thymi of mice and stained them for CD4 and CD8. We then analyzed the results by FACS. The percentages of CD4/CD8 double positive cells and CD4+ or CD8+ single positive cells were quite similar in the thymi from both WT and transgenic mice (Fig.7A). This indicated that the transgene did not alter T cell development in the thymus.

Once the T cells mature, they migrate out from the thymus into the periphery. To make sure that the transgene did not interfere with the T cell populations in the peripheral lymphoid organs, we isolated T cells from spleens and lymph nodes and looked at the CD4+ and CD8+ populations. We observed similar percentages of CD4+ and CD8+ cells

in the lymph nodes and spleens from both groups of mice (Fig. 7B) indicating that peripheral T cell subsets were normal in the transgenic mice.

Fig.7

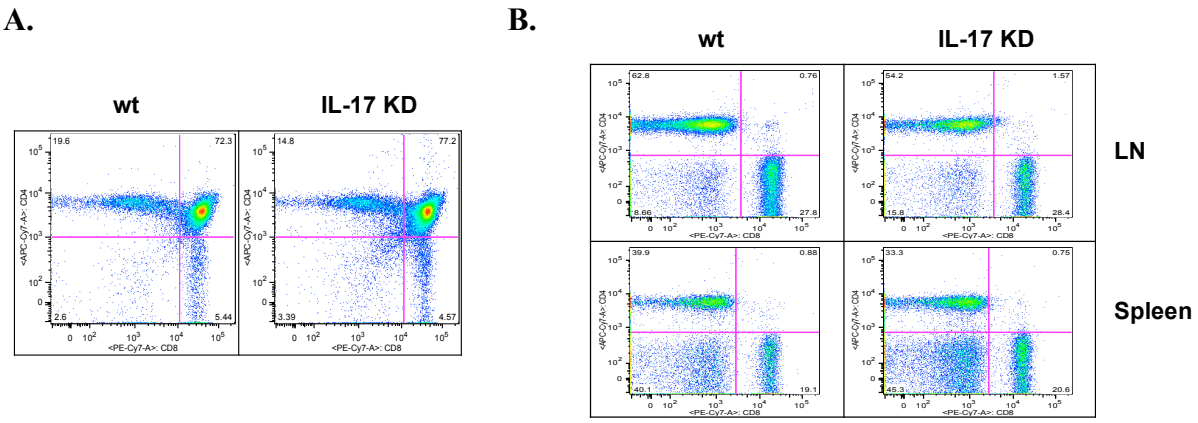


Fig.7. ***A.*** CD4/ CD8 expression in the thymi of wt and KD mice as measured by flow cytometry. ***B.*** CD4/ CD8 expression in the lymph nodes and spleens of wt and KD mice.

4.3.2 Characterization of T cell activation status:

Once T cells are mature, they leave the thymus and enter the periphery. These mature cells are still to encounter their cognate antigen and hence are referred to as naïve T cells. CD62L is commonly used as a marker to identify these cells as naïve T cells express high levels of this marker. Once the naïve T cells encounter their antigen, CD62L expression is downregulated. CD44 can be used as a marker to identify these cells, which have already encountered their antigen and have now become effector memory T cells. Hence, CD62L expression and CD44 expression could be used to analyze the activation status of T cells. To ensure that the transgene did not affect the activation status of T cells in the periphery, we looked at the CD62L and CD44 expression by transgenic T cells.

T cells from spleen and lymph nodes were isolated and were stained for T cell receptor and the activation markers, CD62L and CD44. We then analyzed the T cell populations by FACS. Similar percentages of TCR+, CD4+ and CD8+ cells were observed in the organs from both WT and transgenic mice (7A, 7B, 8A). The % of CD62L+ and CD44+ cells were also quite similar between the 2 groups of mice indicating that the transgene did not have any effect on the activation status of peripheral T cells (Fig. 8B).

Fig.8

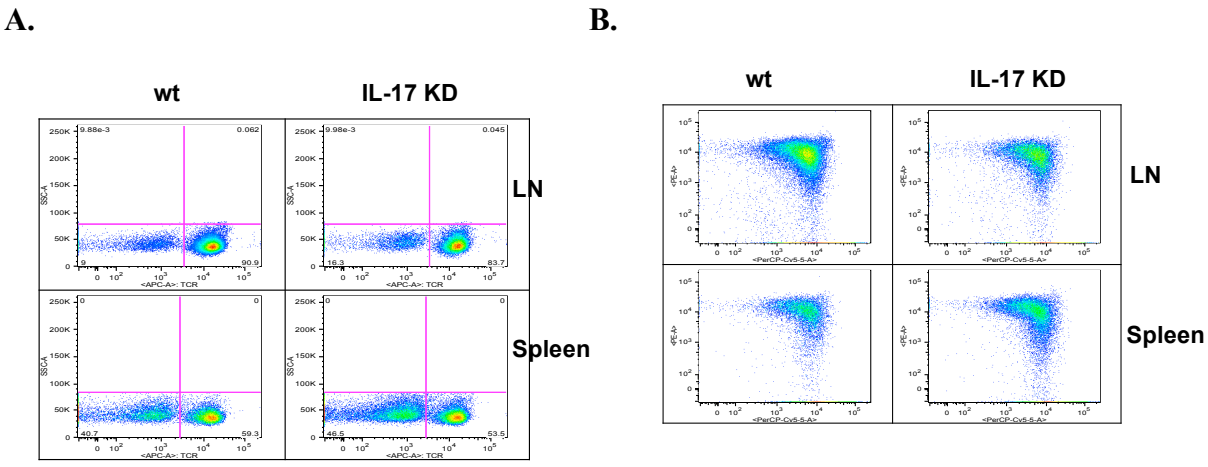


Fig.8. ***A***. TCR expression in lymph nodes and spleens from mice. ***B.*** CD62L / CD44 expression (CD62L: PE, CD44: PE Cy5.5) in lymph nodes and spleens from wt and KD mice.

4.3.3 Characterization of regulatory T cell compartment:

A number of studies have suggested an inverse relationship between Th17 cells and regulatory T cells. The current belief is TGFb regulates Th17 and Treg differentiation pathways in a concentration dependent manner. At lower concentrations, TGFb enhances Th17 differentiation in synergy with IL-6. But in higher concentrations, TGFb downregulates Th17 pathway and instead enhances Treg differentiation[233].

Since the transgene was expected to downregulate IL-17A expression, this in turn could have impaired Th17 differentiation pathway in the transgenic mice. Current reasoning then suggested that impairment in Th17 differentiation could have possibly led to an enhanced Treg differentiation. To investigate this possibility, we isolated T cells from the peripheral lymphoid organs and then looked at the FoxP3+ population. Similar percentages of FoxP3+ cells were observed in the spleens and lymph nodes from both WT and transgenic mice indicating that the presence of the transgene did not alter peripheral Treg compartment in the transgenic mice (Fig.9).

Fig.9

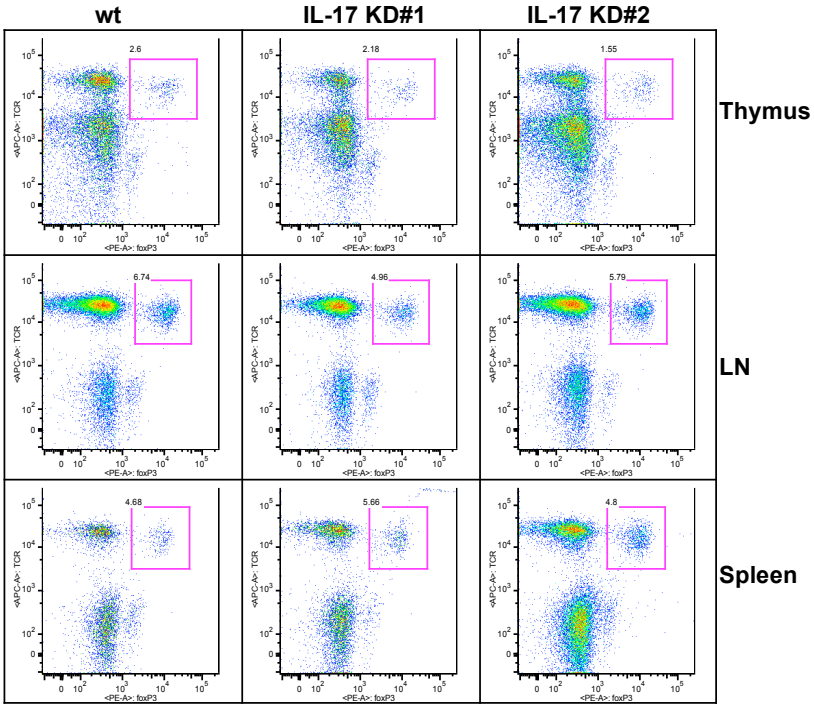


Fig.9. Treg populations in the lymphoid organs of KD mice in comparison with a wt mouse.

4.4 Verification of RNAi and reduced IL-17A levels in transgenic mice

We had already verified the knockdown efficiency of the shRNA *in vitro* by luciferase assay. Once we had the transgenic mice, the next step was to verify that the shRNA was effective in the transgenic mice and that the IL-17A levels in these mice were reduced. IL-17A expression is quite low *in vivo* under normal conditions and hence it is quite difficult to pick up a reduction when the normal levels are already low. Hence we decided to differentiate naïve T cells *in vitro* into Th17 cells and then look at the IL-17A levels. Once differentiated, Th17 cells produce significant amounts of IL-17A and reduction in the cytokine levels could be picked up much better against the high standard expression by differentiated cells.

First we isolated naïve T cells from WT, 'high' or 'low' mice and differentiated them *in vitro* into Th17 cells. We used several different Th17 polarizing cytokine mixes.

1. aCD3
2. aCD3+ TGFb
3. aCD3+ TGFb+ IL-6
4. aCD3+ TGFb+ IL-6+ IL-23
5. aCD3+ TGFb+ IL-6+ IL-23+ IL-1b

Cells were put in culture along with one of these cytokine mixes and allowed to grow for 5 days. They were then restimulated with PMA and ionomycin for a period of 5 hours. The supernatants were then collected and the amount of IL-17A in the supernatants was estimated by ELISA. We found that under minimal activation i.e. aCD3, cells produced some IL-17A. This minimal production was abolished in the presence of TGFb. Cells started to produce significant amounts of IL-17A when IL-6 was added to the mix and the addition of IL-23 and IL-1b was found to have an additive effect on IL-17A production as reported previously (10A).

But most importantly, we found a significant reduction in the amounts of IL-17A secreted by the transgenic cells under all the conditions tested. Th17 cells from both the high and low transgenic lines showed similar knockdown of IL-17A (Fig. 10A). The results indicated that the shRNA works effectively in cells from transgenic mice and that consequently, transgenic cells produce significantly reduced amounts of IL-17A even under maximal stimulation. The similar knockdown observed in the cells from the 2 transgenic lines indicated that both lines show strong IL-17A knockdown regardless of their GFP expression pattern and that the knockdown of IL-17A does not seem to be related to the strength of the GFP signal.

Since we saw a similar knockdown in both transgenic lines, we used mice from High line for all our experiments from now on. Low mice are being bred in our facility and the line is being maintained for future use if need arises.

Though we could show a significant KD *in vitro* in differentiated T cells, it was possible that *in vivo* this was not the case. It has already been suggested that all the requirements for full effector Th17 differentiation are not met *in vitro* and that in addition to the cytokine mix, there might be several other requirements, which seem to be satisfied only when the differentiation happens in an *in vivo* environment. Hence it was quite critical to show that reduction in IL-17A levels that we observed *in vitro* could be shown *in vivo* as well. As the % of Th17 cells in the body is quite low and as the IL-17A levels *in vivo* is almost undetectable, this was a really difficult challenge.

To try and overcome this challenge, we decided to use a 2-pronged approach. We designated groups of mice either WT or KD and analyzed IL-17A levels in *in vitro* differentiated T cells and in *ex vivo* pancreatic islets. We took out islets from the mice and looked at the IL-17A levels in the islets. We also took out naïve T cells, differentiated them *in vitro* and looked at the IL-17A production by differentiated Th17 cells. We analyzed IL-17A expression at both the mRNA and protein levels. We hoped to show a tight correlation between the knockdown observed in both *in vitro* differentiated cells and diseased islets from the mice.

4.4.1 in vitro differentiated cells:

We isolated naïve T cells were from either WT or KD mice. These were then differentiated *in vitro* into Th17 cells by the addition of aCD3, TGF-b, IL-6, IL-23 and IL-1b. We also added anti IL-4 and anti IFNg to avoid differentiation of naïve T cells into Th2 or Th1 lineages. The cells were grown in culture over a period of 5 days. The cells were then restimulated *in vitro* in the presence of PMA and ionomycin for 4 hours. The media was collected and the amount of IL-17A secreted was analyzed by ELISA. mRNA was isolated from the differentiated cells and the level of IL-17A mRNA expression was determined by qPCR. Compared to the WT mice, KD mice had significantly lower levels of IL-17A, both at the mRNA and protein levels (Fig.10B and C). We found similar knockdown of IL-17A at both the protein and mRNA levels.

4.4.2 ex vivo pancreatic islets & pancreatic lymph nodes:

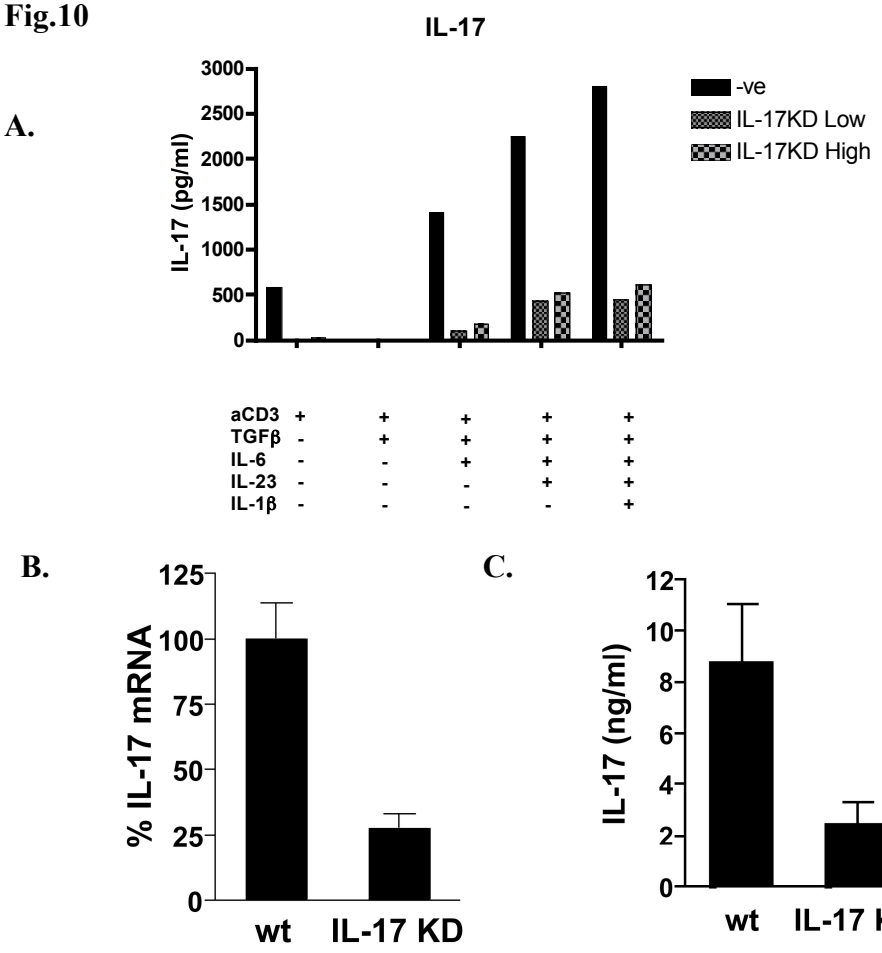
We had used mice that were over 5 months old and hence were already suffering from insulitis and in some cases, overt diabetes. We assumed that since these mice were already in the disease process, we might be able to detect IL-17A in the diseased islets. There are data already describing a method to get infiltrating T cells out from the pancreatic islets using IL-2[231]. We decided to modify this method to suit our purposes. Pancreatic islets were isolated from WT or KD mice and were put in culture overnight with IL-2 to facilitate the emigration of infiltrating T cells. The next day, the cells were resuspended in media and were strained through a filter to remove islets. The cell suspension containing the T cells was spun down. The cells were then put back in culture and restimulated with PMA and ionomycin for 4 hours. Since previous experiments had already shown that the islets upon restimulation hardly produce detectable levels of cytokines (data not shown), media was not saved for ELISA.

RNA was extracted from the cells following restimulation and IL-17A levels were analyzed by qPCR. IL-17A levels were detectable only in a fraction of the mice tested

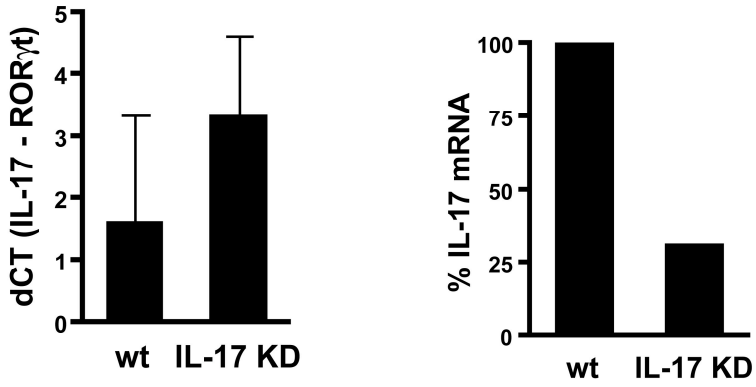
and was found to be highly variable between mice. To compensate for this variability, IL-17A levels were compared relative to RORgt expression in mice. This was done as we had seen that RORgt levels were quite comparable between WT and KD mice (data shown later). Once normalized to RORgt expression levels, KD islets were found to have greatly reduced levels of IL-17A mRNA compared to WT mice (Fig.10D). We also looked at IL-17A levels in the pancreatic lymph nodes of wt and KD mice and observed similarly reduced IL-17A expression in the lymph nodes of KD mice (Fig.10E). Though the observations are based on a limited number of data points, we saw a strong similarity between the level of knockdown observed in *in vitro* differentiated cells and in pancreatic islets and pancreatic lymph nodes, which seems to strengthen the case.

All these observations seem to confirm that shRNA expression and function are extremely efficient in the transgenic mice leading to significant reduction in IL-17A levels.

Fig.10



D.



E.

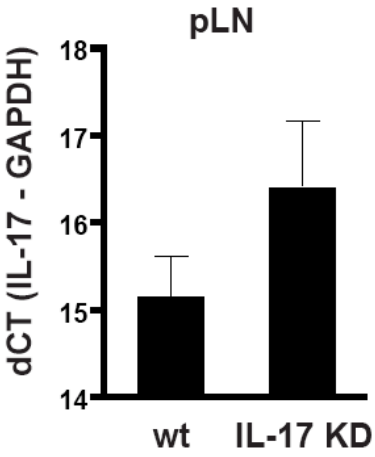


Fig.10. ***A.*** IL-17A protein expression levels in low and high transgenic mice. Representative of at least two independent experiments. ***B & C***. IL-17 mRNA and protein expression levels in *in vitro* differentiated Th17 cells from wt and KD mice (P< 0.0001). Results are representative of three independent experiments. ***D***. IL-17 expression in the pancreatic islets normalized to RORgt expression. ***E.*** IL-17 expression in the pancreatic lymph nodes.

4.5 Characterization of *in vitro* differentiated Th17 cells from KD mice

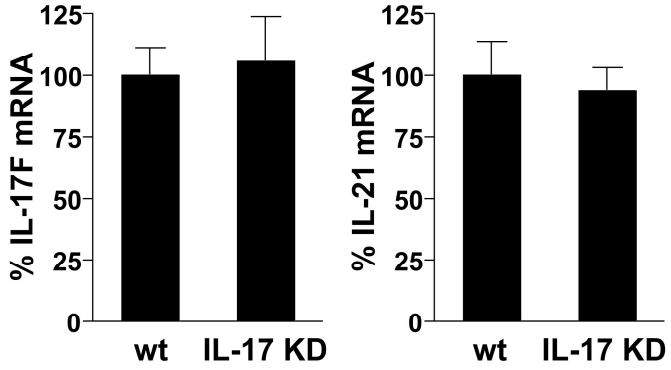
Th17 cells secrete a variety of cytokines including IL-17F, IL-21 and IL-22 in addition to IL-17A. Although none of these cytokines have been reported to cross regulate each other, such a possibility still existed. Since we saw a significant reduction in IL-17A levels in the transgenic mice, it was crucial to verify that none of the other Th17 effector cytokines were affected by the presence of the transgene. Since IL-22 is believed to be a

minor cytokine compared to IL-17F and IL-21, we did not look at IL-22 levels. Th17 cells are also characterized by their expression of a lineage specific transcription factor, RORgt. We analyzed the expression levels of RORgt in the transgenic mice to ensure that loss of IL-17A did not interfere with Th17 lineage in these mice.

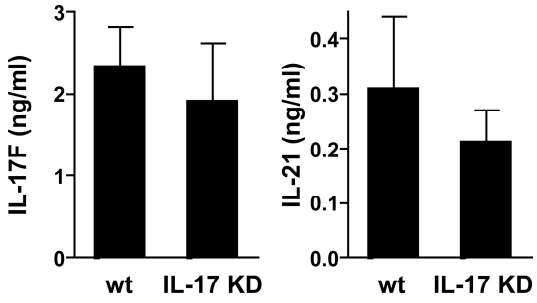
To rule out any perturbation in the levels of IL-17F and IL-21, we differentiated Th17 cells *in vitro* and looked at the levels of these cytokines in parallel with IL-17A. No significant difference was observed in the levels of IL-17F and IL-21 in the KD mice compared to WT mice, either at the mRNA or protein level (Fig.11A and 11B). RORgt expression levels were also found to be comparable between the KD and WT mice (Fig.11C). This indicated that the KD was specific to IL-17A and did not affect other TH17 effector cytokines. The comparable levels of RORgt suggested that loss of IL-17A did not compromise Th17 differentiation pathway in the transgenic mice.

Fig.11

A.



B.



C.

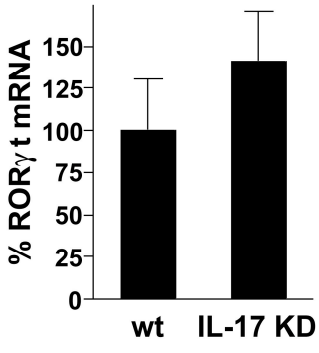


Fig.11. ***A & B.*** Protein and mRNA expression levels of IL-17F and IL-21 in *in vitro* differentiated Th17 cells from wt and KD mice. Data shown are the combined results from two identical experiments with a total of 5 mice in each group ***C***. RORgt expression in *in vitro* differentiated Th17 cells. Data shown are the combined results from two identical experiments with a total of 5 mice in each group.

4.6 Characterization of *in vitro* differentiated Th1 cells from KD mice

As mentioned in the introduction, Th1 and Th17 cells seem to share a complicated relationship. Before the discovery of Th17 cells, many of the Th17 dependent diseases were solely attributed to Th1 cells and their signature cytokine IFNg. Both Th17 cells and Th1 cells are believed to be involved in the diabetes disease process. It was possible that a reduction in IL-17A could somehow result in an enhanced Th1 differentiation and increased IFNg levels. IL-17A deficiency has already been reported to cause higher expression of Th1 associated molecules including IFNg in a model of colitis[234]. Since we were trying to analyze the effect of IL-17A KD on diabetes, it was important to verify that the transgene had no effect on Th1 differentiation or IFNg levels in our mice. This would have enabled us to further restrict any effects observed, specifically to the IL-17 KD and not to an alteration in the Th1 pathway.

For this purpose, we looked at IFNg production as a measure of Th1 differentiation. We took out naïve T cells from both WT and KD mice. These were then differentiated *in vitro* into Th1 cells by the addition of IL-2, IL-12 and anti IL-4. The cells were cultured for a period of 5 days and then were restimulated for 4 hours in the presence of PMA and ionomycin. The amount of IFNg secreted by the cells was determined by ELISA. RNA was extracted from the cells and used for qPCR. We failed to observe any significant difference in IFNg production between the WT and KD cells (Fig.12A&B). This data showed that the transgene did not impair the ability of the KD cells to undergo Th1 differentiation.

Fig.12

A. **B.**

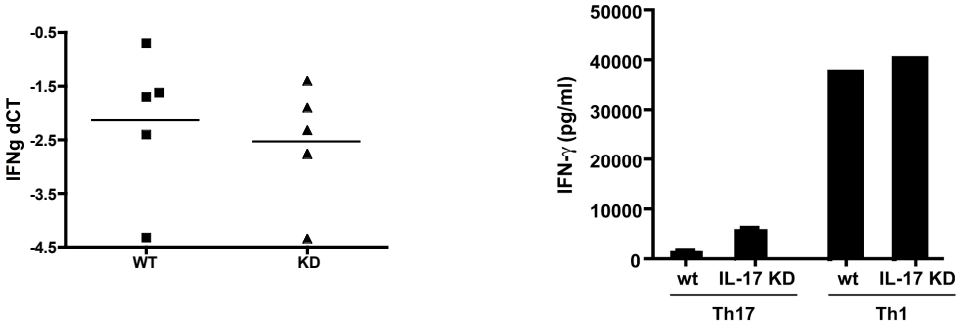


Fig.12. ***A.*** IFNg expression (mRNA) by *in vitro* differentiated Th1 cells from both wt and IL-17KD mice. ***B***. IFNg protein levels in *in vitro* differentiated T cells from wt and IL-17KD mice. Data is representative of three independent experiments.

4.7 Cytokine expression profiles in KD pancreatic islets

Though we had looked at the various Th17 and Th1 factors *in vitro*, we decided to do the same with *ex vivo* pancreatic islets. This was because *in vitro* data might not correctly reflect the *in vivo* status and we felt that *ex vivo* islets from diseased mice might provide us with a clearer picture. As mentioned earlier, we wanted to ensure that none of the Th17 factors like IL-17F, IL- 21 or RORgt were affected by the transgene and that any effect could be traced back only to reduced IL-17A levels. We also decided to look at IFNg levels. This in fact was due to 2 reasons. We wanted to ensure that IFNg levels were similar between WT and KD islets. Though we could see no difference *in vitro*, reduced IL-17 levels could lead to increased IFNg production by Th1 cells *in vivo*. Also Th17 cells have been described to be extremely plastic and the reduction in IL-17A could have made them much more susceptible to Th1 conversion.

Though Th17 and Th1 cells are found to play significant roles in several autoimmune diseases, delineating these two cell populations and their individual roles in the disease

state has been quite difficult. This is confounded by the fact that Th17 cells exhibit a significant degree of plasticity and have been reported to take on a Th1 phenotype over time. Th17 cells were able to transfer diabetes but the authors found that adoptively transferred Th17 cells had somehow converted to IFNg producing cells[172, 173]. A number of studies have demonstrated the presence of cells that co express both IL-17 and IFNg[235-237]. Annunziato et al.[238] observed that both IL-17+IFNg+ cells and IL-17+ IFNg- cells express receptors for IL-12 and IL-23 and that stimulation with IL-12 can lead to T-bet expression and subsequent IFNg secretion. Hirota et al.[239] recently showed that *in vivo*, Th17 cells are able to switch to IFNg production and this depends on the disease condition. All the data leads to the idea that Th17 cells are able to downregulate Th17 related factors and upregulate Th1 factors and thus convert to a Th1 phenotype and that the process might involve a double producer phenotype.

We took out islets from 5 month old mice and put them in culture overnight with IL-2. The cells were then strained through a filter and were restimulated *in vitro*. RNA was isolated from the restimulated cells and we did qPCR for the different targets. mRNA was detected for all the targets though at different levels. We were able to detect IFNg at higher levels compared to the other cytokines implying a role for this cytokine in the disease process. We detected comparable amounts of IL-17F, IL-21 and RORgt in the islets from WT and KD mice (Fig.13A). This indicated that all the Th17 factors except IL-17A are unaffected. We also saw similar levels of IFNg in the islets from WT and KD mice indicating that loss of IL-17 did not enhance Th1 differentiation or Th17 conversion (Fig.13B). All the data from *ex vivo* pancreatic islets concurred with the *in vitro* data indicating that the integration of the transgene didn't have any off target effect as far as TH17/ Th1 pathways were concerned.

Fig.13

A. **B.**

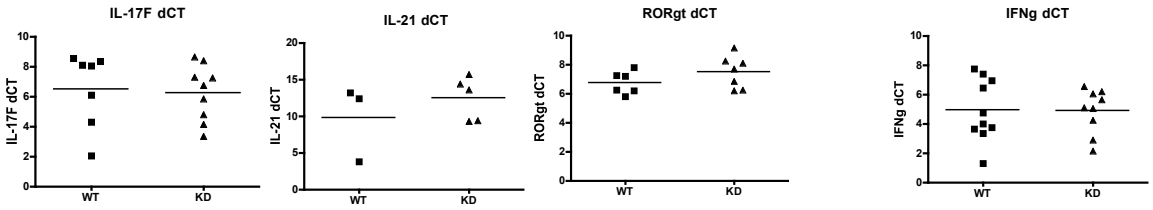


Fig.13. ***A***. mRNA expression levels of IL-17F, IL-21 and RORgt in the pancreatic islets of wt and KD mice. ***B***. IFNg mRNA levels in the pancreatic islets of wt and KD mice.

4.8 Analysis of diabetes in IL-17A KD mice

Once we had verified efficient knockdown of IL-17A both *in vitro* and *ex vivo*, the next step was to study diabetes incidence in the transgenic mice. IL-17A deficient mice are found to be protected, at least partially from several autoimmune conditions. This shows that IL-17A is at least partially responsible for these autoimmune pathologies. These observations have led to the idea that this cytokine might be pathogenic in all similar diseases including type 1 diabetes. As mentioned in the introduction, several studies also suggest a role for IL-17A in type 1 diabetes, though the results are not really conclusive. Hence we felt that our transgenic mice might give us a clearer picture as to the role of this cytokine in Type 1 diabetes.

Towards this purpose, we used 3 different models of diabetes: spontaneous diabetes, cyclophosphamide induced diabetes and adoptive transfer of diabetogenic splenocytes into immunodeficient recipients. Each of this is detailed below.

4.8.1 Spontaneous diabetes:

As mentioned earlier, NOD mice develop autoimmune diabetes spontaneously. Females are found to be more susceptible to the disease than males with around 70-80% developing the disease with time. Hence they form a valuable model for studying this disease. We established large cohorts of female KD or WT mice and maintained them in the SPF facility. The mice were maintained for a period of over 6 months during which time they developed the disease. The mice were tested every 2 weeks and every 1-week towards the later half of the observation period. For testing, urine blood sugar levels were checked with diastix. We observed no difference in the disease incidence level between the 2 groups of mice. Transgenic mice developed diabetes just like their WT counterparts and both the frequency and kinetics of the disease were found to be similar (Fig.14A).

4.8.2 Cyclophosphamide induced diabetes:

Spontaneous diabetes studies take a long time, as we have to wait for the mice to get sick spontaneously. This leads to time restrictions and induced diabetes models are used to overcome this problem. Cyclophosphamide is a chemotherapeutic drug, which has been found to induce diabetes in susceptible subjects. Injection of cyclophosphamide accelerates the diabetes process leading to the development of synchronous diabetes in all the mice within 2-3 weeks. This is believed to be due to depletion of regulatory T cells and B cells from the injected mice.

We had failed to observe protection from diabetes in transgenic mice using spontaneous diabetes model. To verify the results obtained with spontaneous diabetes, we decided to use cyclophosphamide-induced diabetes. We injected small cohorts of WT or KD mice with cyclophosphamide on day 0 followed by a second injection on day 14. Mice were tested every 2 days for diabetes. We did this experiment twice and both times we failed to observe any difference between the 2 groups of mice. The disease kinetic was found to be indistinguishable between both groups of mice. The combined data are shown in the fig. (Fig.14B).

4.8.3 Adoptive transfer of splenocytes into NOD SCID recipients:

Another widely used model is the adoptive transfer of diabetogenic splenocytes[78, 240]. This is also an induced model of diabetes. Here, CD25 depleted or CD25/ CD62L double depleted donor T cells are transferred to immunodeficient recipient mice[241]. These recipient mice lack a functional immune system and hence they act as an empty vessel for the adoptively transferred cells. When donor T cells are transferred after depletion of regulatory T cells, these cells undergo homeostatic proliferation in the recipients and autoimmune specificities can undergo unchecked proliferation resulting in the recipients developing severe pathologies. This is a useful model as any effect observed can be specifically restricted to donor cells as the recipients lack a functional counterpart.

As a third alternative, we used adoptive transfer. We took out T cells from WT and KD mice. We then depleted CD25 from these cells and injected them into NOD-SCID recipients. WT CD25 depleted cells were injected into a group of NOD SCID recipients while transgenic CD25 depleted cells were injected into a separate group of mice. We then tested the mice regularly for diabetes. The experiment did not work properly as very few mice became sick. None of the WT mice developed diabetes during the entire period of the study. But 3 of the NOD SCID mice that had received transgenic cells developed the disease (Fig.14C). Though these data are not entirely conclusive, it suggested to us that transgenic cells though had reduced IL-17A, could effectively transfer the disease. This at the least showed that loss of IL-17A did not seem to protect the mice.

To improve on the model, we isolated CD25/ $CD62L^{hi}$ double depleted splenocytes from groups of wt or KD mice and transferred them into cohorts of NOD SCID recipient mice. The recipient mice were then tested for diabetes development by glycosuria measurements every 2-3 days. We found that cells from the transgenic mice were able to transfer disease susceptibility similar to wt cells, confirming the diabetogenicity of KD splenocytes, despite the reduced IL-17A levels present (Fig.14D).

To analyze the role of IL-17A in type 1 diabetes, we used 3 different but equally useful models. Data from all the three models failed to show any protection that is associated with reduced IL-17A levels. KD mice were as susceptible to developing diabetes as the WT mice. All the data conclusively suggested that loss of IL-17A did not offer any protection from autoimmune diabetes to the transgenic mice and hence IL-17A might not have a crucial role in the development of this autoimmune disease.

Fig.14

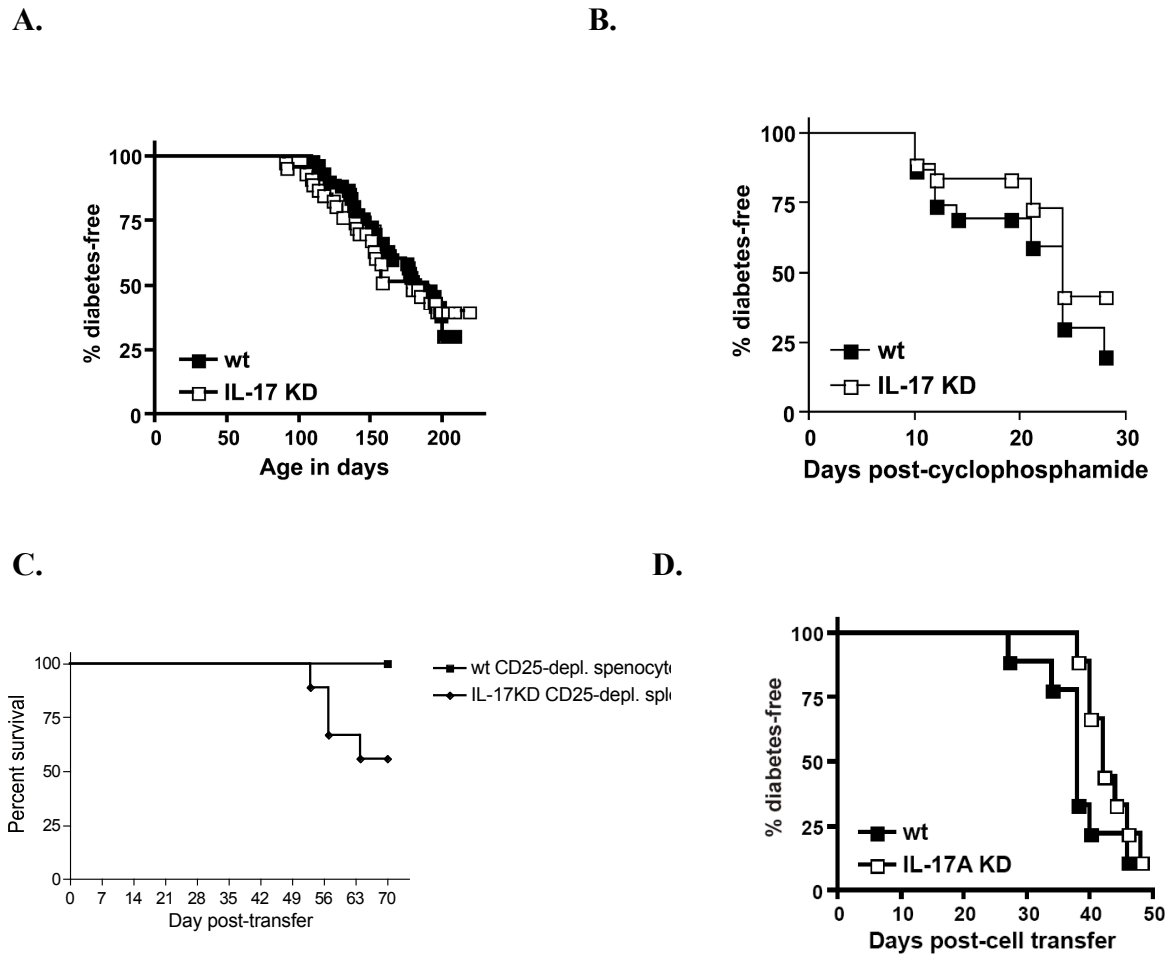


Fig.14. ***A***. Comparison of spontaneous diabetes frequency of wt (n=63) and KD (n=47) mice (P=0.76). ***B***. Comparison of cyclophosphamide induced diabetes frequency of wt (n=23) and KD (n=18) mice (P=0.24). Data show the combined results of two similar experiments. ***C***. Comparison of diabetes frequency in NOD

SCID mice injected with wt or KD $CD25^-$ splenocytes. ***D***. Comparison of diabetes frequency in NOD SCID mice injected with either wt (n=9) or KD (n=9) $CD62L^{hi-}CD25^-$ splenocytes (P=0.23).

4.9 Verification of systemic gene silencing *in vivo*:

We failed to observe any protection from type 1 diabetes despite reduced IL-17A levels, in contrast to the expectation. IL-17A KD transgenic mice still retain residual amounts of IL-17A as we use RNAi and not a complete knockout of IL-17A. As shown in previous experiments, we detected around 70% reduction in IL-17A levels meaning that the transgenic mice were still able to retain around 30% of IL-17A. This led us to question whether the lack of protection is related to the residual IL-17A present in the transgenic mice. There might be a dose effect where, IL-17A when present even at low doses could still contribute to the disease. A protection from diabetes might be observed only with a complete removal of IL-17A.

To address this question, we decided to test the systemic efficiency of the knockdown in the context of a second autoimmune disease, previously shown to be IL-17A dependent. IL-17A has already shown to play a significant pathogenic role in EAE, the experimental model for human autoimmune disease, multiple sclerosis (MS). IL-17A knockout mice are found have reduced frequency as well as severity of the disease[242]. We postulated that if the reduced IL-17A levels in our transgenic mice are sufficient to offer partial protection from EAE, they should by reason, be sufficient to deter type 1 diabetes as long as the disease is IL-17A dependent and follows the same disease parameters. This means that if our transgenic mice showed protection from EAE due to reduced IL-17A levels, then it should mean that autoimmune diabetes is not dependent on IL-17A.

For this we decided to induce EAE in transgenic IL-17A KD mice. EAE is a model for brain inflammation and can be induced in susceptible strains by the injection of CNS antigens including MOG, MBP, PLP etc. Depending on the antigen and the genetic makeup of the strain used, the animals can exhibit a monophasic form of the disease, a relapsing-remitting form of the disease or chronic EAE. The disease manifested is

mediated by T helper cells and is characterized by central nervous system perivascular infiltration of lymphocytes and subsequent destruction of myelin sheath, which results in paralysis in mice[243]. The study that showed partial protection from EAE was done in C57BL/6 mice and showed a monophasic form of EAE. C57BL/6 mice are ideal for EAE studies as they are more susceptible to the disease while NOD mice are not. The disease in NOD mice is less severe. We have previously found the NOD mice in our colony to develop a relapsing remitting form of the disease. We find that the relapse is much more severe compared to the first acute phase of the disease.

We set up small cohorts of WT and KD mice. These were injected with MOG peptide emulsified in CFA along with a shot of pertussis toxin. This was followed by another shot of pertussis toxin 2 days later. The mice were checked regularly for signs of the disease. At the end of the study, the mice were sacrificed and used for cytokine analyses. This part of the study was carried out in collaboration with Prof. Heinz Wiendl's lab and was done by Stefan Bittner.

4.9.1 Incidence and disease severity after EAE induction:

We found that in the first acute phase, transgenic mice showed significant protection from the disease. This protection was abolished in the relapsing phase (Fig.15A). When we analyzed the cumulative disease burden, we found that the KD mice had significantly lower disease burden compared to the WT mice (Fig.15B). These observations indicated that the transgenic IL-17A KD mice were partially protected from EAE. This led us to believe that systemic IL-17A silencing should have been sufficient to impair autoimmune diabetes, had it been IL-17A dependent.

4.9.2 Cytokine analyses of T cells from diseased mice upon recall response:

To further investigate the possible reason for this partial protection, we took out cells from the diseased mice and analyzed them. We took out diseased splenocytes and restimulated them *in vitro* with CD3/CD28 dynabeads. The cells were cultured along

with the beads for a period of 2 days and the IL-17A levels in the supernatants were measured by ELISA. We found that under maximal stimulation, KD cells were able to produce IL-17A, though at significantly lower levels than WT cells (Fig.15C). These data concurred with previous results.

We also did a MOG recall response where we restimulated the cells in presence of MOG peptide and looked at the cytokine levels by ELISA. Upon MOG specific stimulation, both WT and KD MOG reactive cells produced comparable amounts of IFNγ (Fig.15D). While MOG reactive WT cells produced significant amounts of IL-17A, it was almost undetectable in cell supernatants from KD mice (Fig.15E). These data suggested that the partial protection from EAE, which we observed, seem to arise from the restricted ability of KD cells to produce IL-17A.

Fig.15

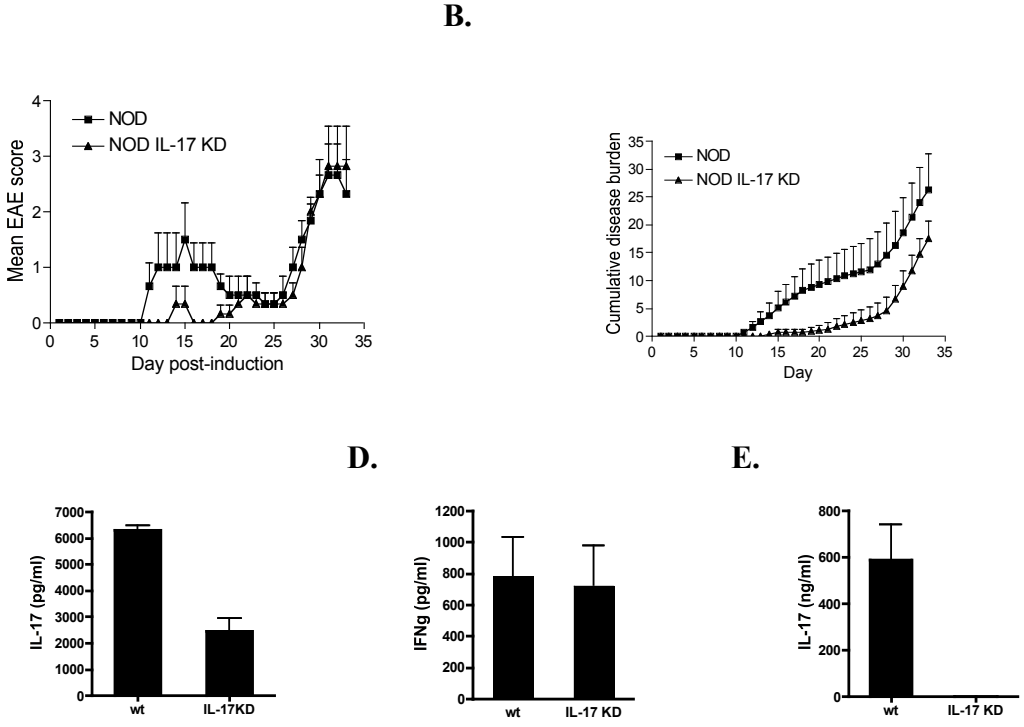


Fig. 15 ***A***. Mean EAE scores of wt (n=6) and KD (n=6) mice. P=0.0014 ***B***. Cumulative disease burden (individual disease scores were integrated for each mouse, group averages are shown with SEM, P<0.0001). ***C***. IL-17 expression by the cells on stimulation with antibody- coated beads (P<0.0001). ***D***. IFNg expression on restimulation of splenocytes from diseased mice with MOG_{35-55} peptide. ***E***. IL-17 expression on restimulation of splenocytes from diseased mice with MOG_{35-55} peptide.

The results from MOG recall response supported the observations from the EAE experiment that systemic silencing of IL-17A was sufficient to impair IL-17A dependent autoimmunity. Since the KD mice were partially protected from EAE, the residual IL-17A in the KD mice was not enough to contribute to EAE development. Following the same principle, the lack of any protection from diabetes that we observed could not be attributed to the residual IL-17A in the mice. All these observations lead to the conclusion that autoimmune diabetes is independent of IL-17A in autoimmune diabetes in the NOD mouse model.

Generation of β- cell reporter mouse models & modulation of β- cell mass

Generation of RIP-hluc mouse models

Type 1 diabetes is caused by the autoimmune destruction of the beta cells, which produce insulin. As mentioned before, the disease is incurable and the symptoms usually manifest only after about 80% of the beta cell mass is already destroyed. There are several models available for type 1 diabetes including the NOD mouse that we use. The disease is usually followed by measuring the glucose in the blood or urine of the animals. Though this gives a clear idea of the disease incidence, glucose measurements do not give any idea regarding the extent of beta cell destruction. It is impossible to know the extent of autoimmunity unless we sacrifice the animal. Also none of this would allow us to accurately follow the autoimmune destruction over time relative to the disease symptoms.

All these challenges paved way for beta cell imaging via one of the several imaging modalities currently available. There are several studies that have tried using MRI, PET, SPECT, CT and ultrasound imaging for non invasively assessing beta cell mass. A more relevant approach is bioluminescence imaging of beta cells. Several research groups have already generated beta cell specific bioluminescent reporter mice. These reports have indicated that this is a valid approach for following the disease *in vivo* in a non-invasive manner. Though there are already reporter mice expressing luciferase specifically in the pancreatic beta cells, none have been generated in any of the spontaneously diabetes susceptible models of type 1 diabetes. Hence we aimed to generate a beta cell reporter mouse model in the NOD background. In addition, we planned to integrate this with beta cell specific RNA interference to achieve modulation of beta cell mass. This will allow us to generate a NOD mouse model in which beta cell mass could be non-invasively imaged over time to study the process of autoimmune destruction. The simultaneous integration

of RNAi would also allow us to study changes in beta cell mass resulting from the pancreas specific knockdown of candidate genes.

We aimed to achieve this by using a construct in which the expression of luciferase and shRNA are under the control of a single beta cell specific promoter. We used RIP promoter as it has already been shown to drive beta cell specific expression of target genes. The construct also has a mir30 motif to facilitate the tissue specific expression of the shRNA. Hence we end up with beta cell specific luciferase expression coupled with the knockdown of the target gene. When we use shRNAs directed against known beta cell modulators, these shRNAs could cause the knockdown of modulators and the luciferase expression pattern over time could be used to identify the resulting changes in beta cell mass. We named the construct pRLM: R stands for the RIP promoter, l for luciferase, m for mir30 motif.

4.10 Generation of pRLM construct:

To generate the pRLM construct, we started with the pLBM construct. pLBM has a CMV promoter that drives the expression of GFP (Fig.1). To generate pRLM, we switched CMV and GFP for RIP and luciferase. RIP was cut out from a vector with Xba1. Hluc was cut out using Nhe1/Not1. CMV was cut out from pLBM using Xba1/ Nhe1 and GFP was cut out using Nhe1/ Not1. RIP was then cloned into pLBM between Xba1 and Nhe1 (as Xba1 has compatible ends with Nhe1) while hluc was cloned between Nhe1 and Not1 (Fig.1). This gave rise to pRLM vector with CMV/GFP replaced with RIP/hluc.

Fig.1

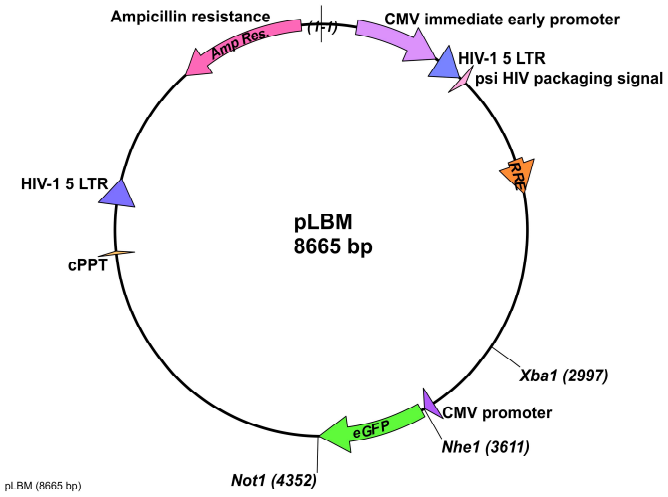


Fig.1. A schematic representation of pLBM vector. CMV/ GFP in pLBM was replaced with RIP/ hluc to obtain pRLM construct.

4.11 Verification of specificity of pRLM construct *in vitro*:

Once we generated the construct, the next step was to ensure that it contained the RIP and hluc sequences. The presence of the cloned in RIP-hluc sequence in the construct was verified by sequencing. The next step was to ensure the tissue specific activity of the promoter. We had to make sure that RIP promoter was active only in insulin expressing cells, i.e. primarily in the beta cells of the pancreas. For this we decided to use an *in vitro* approach involving rat insulinoma cell line INS-1E. These cells are beta cell tumor cells and are used as *in vitro* models for pancreatic beta cells.

We decided to test the expression of RIP in 2 different cell lines- human embryonic kidney cells line 293F and INS-1E. If RIP activity is tissue specific, we should be able to see gene expression only in the INS-1E cell line and not in the 293F cell line. We used GFP signal or luciferase signal as markers for gene expression.

To verify the tissue specificity of RIP-luciferase expression, virus samples were made with both RIP- GFP construct and a mixture of pLBM/ pRLM. RIP-GFP infected cells expressed GFP and hence the infected cells could be identified under a microscope or by FACS. But pRLM infected cells could not be identified under a microscope or by FACS due to their lack of GFP expression. This makes the estimation of infection efficiency impossible. To overcome this challenge, we used a mixture of pLBM/ pRLM to infect the cells. When using the mixture, half the cells would be infected with pLBM and the other half with pRLM. The pLBM-infected cells could be detected under a microscope and by FACS, owing to their GFP expression. This in turn could be used to determine the infection efficiency of the virus sample.

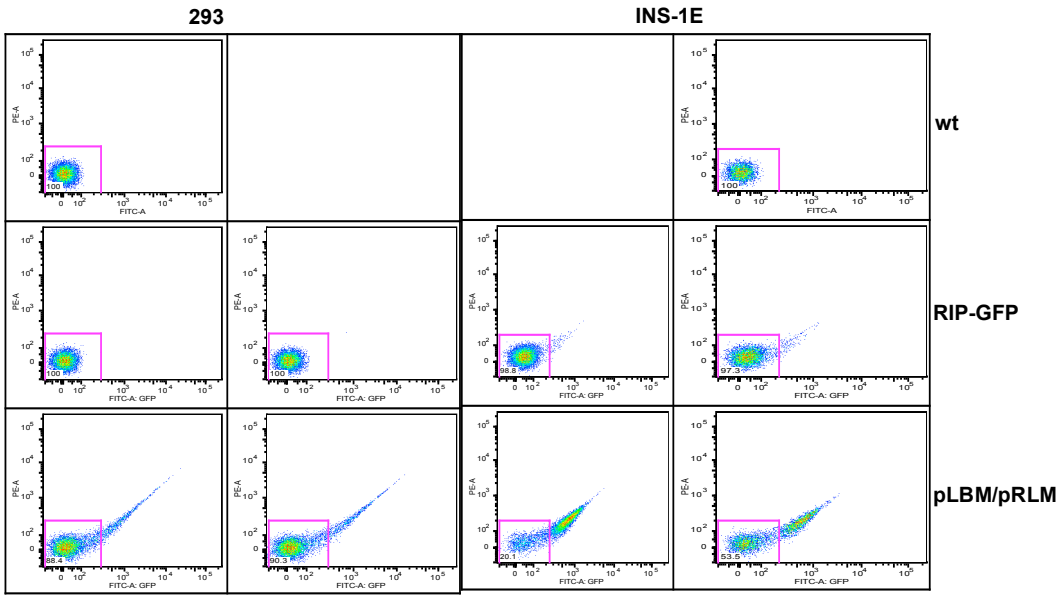
293 cells and INS-1E cells were infected with either the RIP-GFP virus or the pLBM/pRLM virus mixture. Cells were cultured over time and the GFP expression was analyzed under the microscope and also by FACS. Luciferase expression by the cells was analyzed by luciferase assay at the end of the culture period after cell lysis.

GFP expression was observed only in the RIP- GFP infected INS-1E cells and not in 293F cells. No green cells were observed in RIP-GFP infected 293F cells under the microscope. Both 293F and INS-1E cells infected with the pLBM-pRLM virus mixture were green under the microscope (Data not shown). To verify the GFP expression patterns observed under the microscope, infected cells were further analyzed by FACS. As before, GFP expression was observed only in the RIP- GFP infected INS-1E cells. But both cell lines showed significant GFP expression when infected with the pLBM/ pRLM mixture, though INS-1E cells seem to be better infected as more cells are GFP+ (Fig.2A).

Next we analyzed luciferase expression by the virus-infected cells. After passive lysis, luciferase activity in the lysates was measured using Fluostar Optima. Only the cells infected with the pLBM/pRLM virus mixture showed detectable luciferase activity and the infected INS-1E cells showed a significantly higher expression compared to the 293F cells (Fig.2B). The difference could not be attributed wholly to a better infection, as it was more than a 17-fold increase.

Fig.2

A.



B.

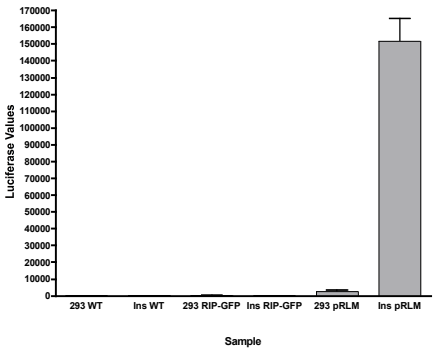


Fig.2. ***A***. GFP expression levels in virus infected cells. ***B***. Luciferase expression by the virus infected cells.

The expression patterns of both GFP and luciferase observed above indicated that RIP promoter activity was switched on only in beta cells while it remained inactive in the human embryonic kidney cells. The data showed that RIP promoter is tissue specific and could drive luciferase expression only in insulin expressing cells.

4.12 Generation and genotyping of pRLM mice:

Once we had the appropriate construct, the next step was to generate NOD mice with beta cell specific expression of luciferase. We generated lentivirus using the pRLM construct, as described before. The high titer pRLM virus was then injected into NOD embryos, which were then carried to term by pseudopregnant recipient mice. We were able to generate several founders. All of these were found to be viable and fertile and exhibited no gross phenotypic defects.

pRLM mice could be genotyped by bioimaging but this was a labor-intensive process. Due to technical reasons, genotyping was done mostly by genomic PCR for hluc. We used 3 different primer sets and all of these were able to detect luciferase sequences in genomic DNA samples from mice. The different primer sets gave different sized products and we mainly used primer set 3 for genotyping.

Tails were obtained from pups and tail DNA was isolated. Around 100 ng of tail DNA was used for genotyping. The PCR setup and program are detailed below.

PCR setup:

100 ng DNA
0.5 μl fwd primer
0.5 μl rev primer
2.5 μl Dreamtaq buffer
0.5 μl Dreamtaq polymerase
1.0 μl dNTP
Made up to a final volume of 25 μl with distilled water

PCR Cycle:

Temperature	Time	No: of cycles
94°C	5 minutes	1
94°C	30 seconds	
50°C	30 seconds	5
72°C	1 minute	
94°C	30 seconds	
58°C	30 seconds	35
72°C	1 minute	
72°C	10 minutes	1

A representative gel from genomic PCR is shown in Fig.3.

Fig.3

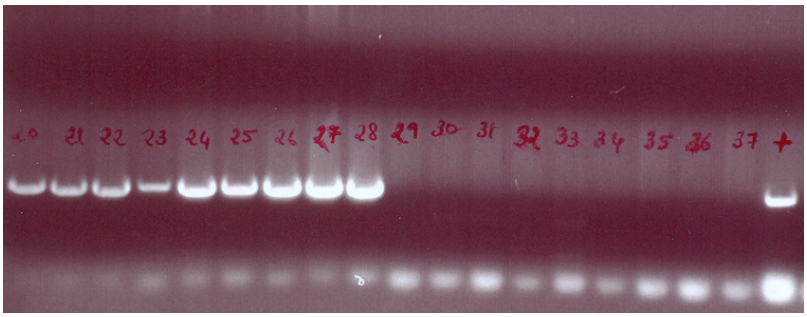


Fig.3. Representative gel from luciferase genotyping. Positive control is in the far right lane. #20- #28 are pRLM+.

4.13 Bioimaging of non-diabetic and diabetic pRLM mice:

Once we had the pRLM mice, we decided to test the luciferase expression via luciferase bioimaging. For this we used pRLM+ mice identified from genotyping PCR. We selected a random pRLM+ mouse and imaged it along with a negative control. We injected the

mice with the anesthetic along with the recommended amount of D-luciferin. The mice were then placed inside the machine and images were taken using the CCD camera. We found a robust signal from the + mouse while no signal was observed in the negative mouse.

To our surprise, the signal we observed did not seem to pancreas specific. It was dispersed all over the abdomen especially under high signal intensity setting. At lower intensities, it appeared in kidney shapes on both sides though the signal was stronger on the pancreas side (Fig.4A). To try to localize the signal, we took out spleen and pancreas from the pRLM+ mouse after sacrificing it. We were able to observe luminescence from the spleen in addition to the pancreas. But the signal from the spleen was much lower than that from the pancreas (Fig.4B).

This pattern of expression was observed in most pRLM+ mice (figures in following sections). The signal is not localized to the pancreas but is dispersed over the abdominal area and this is more noticeable under the high signal intensity setting. When the mice are imaged under low signal intensity setting, the signal is more localized though still not restricted to the pancreas. We also observed a signal from the thymus in the pRLM+ mice.

All these observations made us question the specificity of luciferase expression. Hence we found it convenient when we had pRLM+ mice that had developed diabetes. We decided to image these diabetic mice to see if we could detect a signal even after the destruction of beta cells. We took the diabetic mice and imaged them alongside a non-diabetic pRLM+ mouse. We observed very little signal from the diabetic mice even after a long exposure time under maximum intensity conditions (Fig. 4C). The difference was really significant in comparison with the non-diabetic + mouse. This observation gave us the first indication that although the signal we see in pRLM+ mouse did not seem to be localized to the pancreas; the signal might still be coming from the beta cells. Since we observed only a faint signal in the diabetic mice, it meant that luciferase expression was reduced over the course of the disease leading to an absence of signal once the mice were

diagnosed as diabetic. Once the beta cells were destroyed, the signal went down supporting the notion of beta cell specific expression of luciferase.

Fig.4

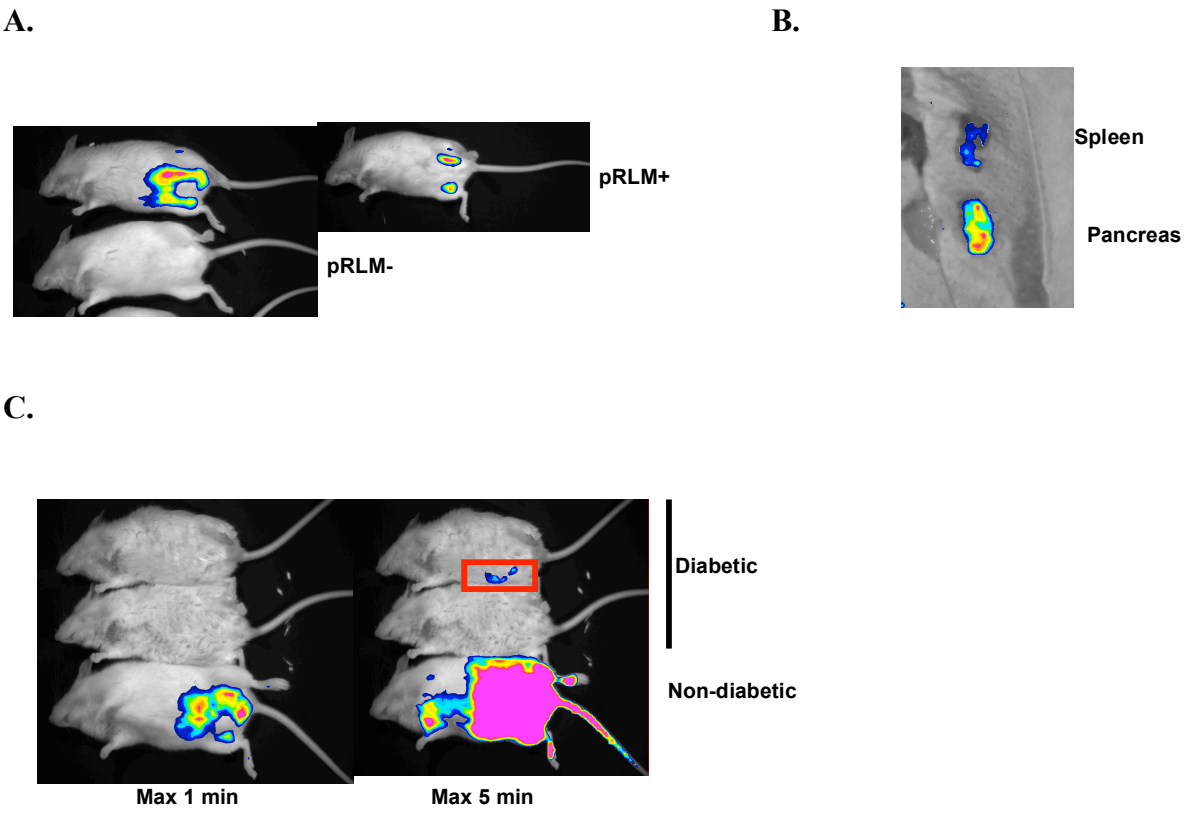


Fig.4. ***A***. An image from a pRLM+ mouse in comparison with a negative mouse. ***B***. Luciferase signal from the pancreas and spleen dissected out of a pRLM+ mouse. ***C***. Images from 2 pRLM+ diabetic mice in comparison with a non-diabetic pRLM+ mouse, under different signal intensities.

4.14 Specificity of luciferase expression:

To further investigate the pattern of luciferase expression in the pRLM mice, we made RNA from the pancreata, spleens, kidneys, livers and thymi from 2 wt and 2 pRLM+ mice. We then did real time PCR to analyze the expression of luciferase in these organs. We found high expression of luciferase in the pancreata from the pRLM+ mice.

Luciferase expression was also detected albeit at much lower levels in the thymi of the pRLM+ mice. No luciferase expression could be picked up from any of the other organs from the pRLM+ mice. None of the organs from the wt mice showed luciferase expression (Fig.5). The data indicated that in the pRLM+ mice, luciferase expression was limited to the pancreas with a limited expression in the thymus. None of the other organs situated in the abdominal area expressed luciferase indicating that the broad abdominal signal exhibited by the mice comes only from the pancreas and that the diffuse signal might be due to the really strong expression of luciferase by the beta cells.

Fig.5

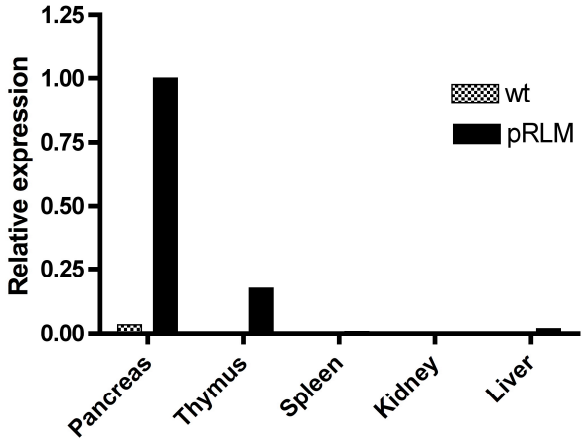


Fig.5. Luciferase expression in the organs from wt and pRLM+ mice.

4.15 Phenotype of pRLM mice:

pRLM mice were found to be viable and fertile and exhibited no gross phenotypic defects. We had used a transgene to generate these mice with pancreas specific expression of luciferase. We were able to ensure that they express luciferase in the pancreas. Hence it was crucial to ensure that the transgene integration and luciferase expression did not interfere with the normal metabolic processes in the transgenic mice. Hence we took groups of age matched pRLM+ and negative mice and compared their weights. We didn't observe any difference between the two groups. We also looked at the

fasting glucose levels of the mice. Again, the pRLM+ mice were found to have comparable fasting blood glucose levels to their WT counterparts (Fig.6 A&B). The data indicated that pRLM mice had a normal phenotype.

Fig.6

A. **B.**

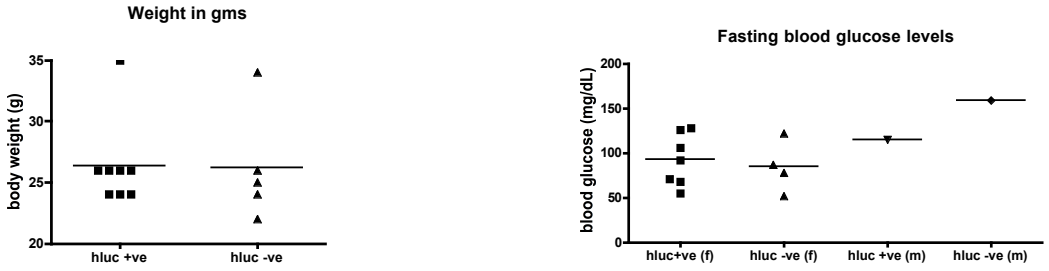


Fig. 6. ***A***. Weight in grams of pRLM+ mice in comparison with wt mice. ***B***. Fasting blood glucose levels of pRLM+ mice in comparison with wt mice.

4.16 Streptozotocin induced diabetes:

To further verify the specificity of the luciferase signal, we induced rapid immune independent diabetes in pRLM mice with streptozotocin. The mice were injected with high dose streptozotocin, which results in non-immune destruction of beta cells and development of diabetes within 2 days. The mice were imaged before the injections and regularly after the injections.

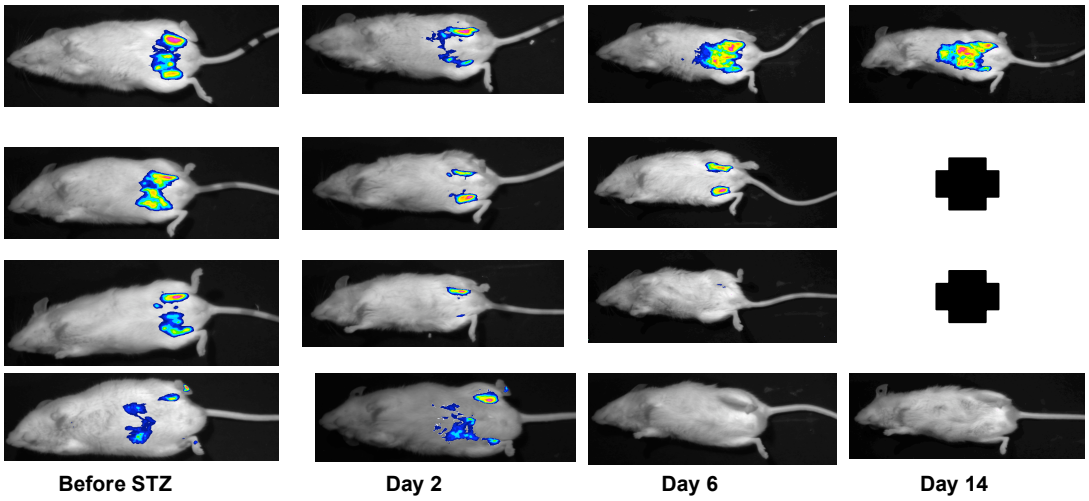
We observed robust signals from the mice before the start of the experiment. After injection, most mice developed diabetes and the signal went down rapidly over time (Fig.7A). We also observed that a really small percentage of mice gave robust signals despite developing severe diabetes (Fig. 7A). The loss of signal in the majority of the mice upon development of diabetes further indicated that luciferase expression was beta

cell specific. We could not find any reason for the persistence of signal in the minor population of mice that were severely diabetic.

We also looked at the phenotype of the mice during the course of STZ induced diabetes. Once the mice get diabetic, they lose significant amount of body weight. The blood glucose levels also go up owing to the severe destruction of beta cells leading to the reduction of insulin. Hence we decided to look at the weight loss and blood glucose levels in the sick mice. We found that both pRLM+ and negative mice lost comparable amounts of body weight upon developing the disease. The increase in blood glucose level was also comparable between the two groups of mice (Fig.7B). This further demonstrated that pRLM+ mice had a normal phenotype.

Fig.7

A.



B.

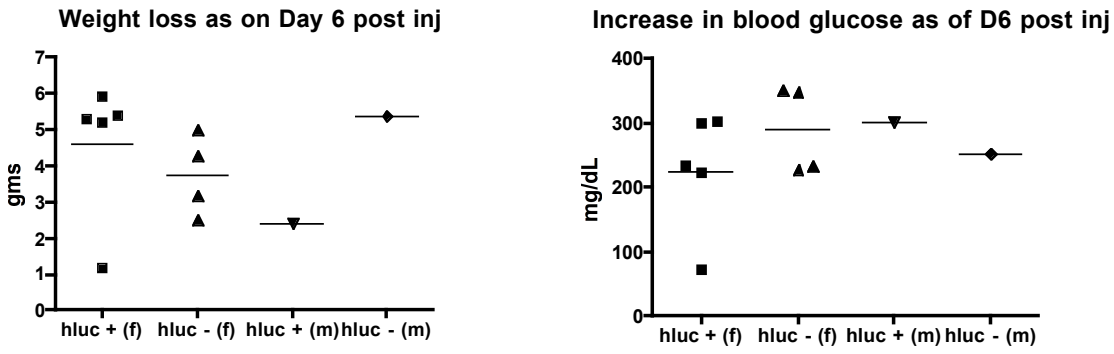


Fig.7. ***A***. Luciferase signal in pRLM+ mice over the course of STZ induced diabetes. ***B***. Weight loss and blood glucose levels in pRLM+ and wt mice upon disease development. F denotes female mice while m denotes male mice in the experiment.

4.17 Cyclophosphamide induced diabetes:

Since we could see a loss of signal upon STZ induced diabetes, we decided to use another model, the cyclophosphamide induced diabetes. We injected both wt and pRLM+ mice with cyclophosphamide and imaged them regularly. As mentioned before, cyclophosphamide injections cause the mice to develop diabetes over a period of 2-3 weeks. Since diabetes development after STZ treatment is immediate, CY system could be a better alternative to study the kinetics of the disease. We felt that the slower course of the disease with CY might help us see a gradual reduction of the signal over a longer time period. Data in Fig.8 show luciferase expression in one mouse, which developed diabetes 10 days after CY injection. We were testing and imaging the mice once a week and the diabetic mouse had shown a robust signal the week before and the signal reduced significantly the next week and hence we couldn't obtain a clearer picture of the disease / signal kinetics. However, the experiment provided further evidence for the pancreas specific expression of luciferase.

Fig.8

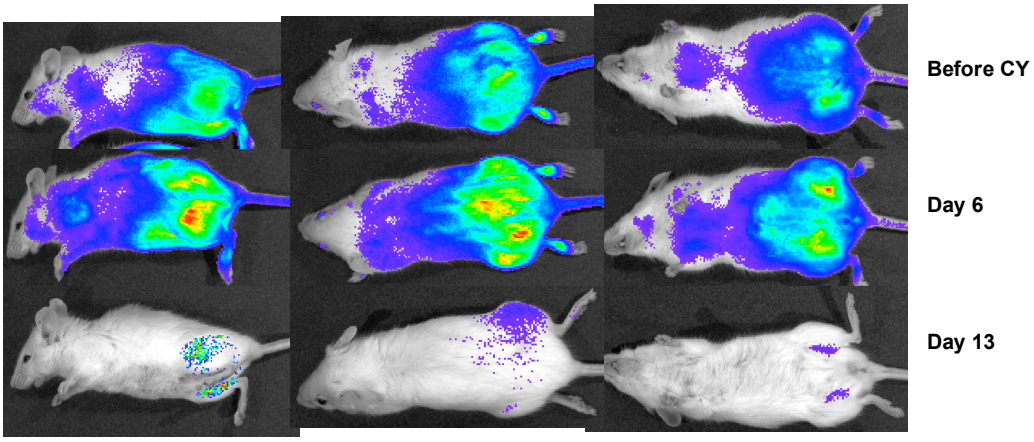


Fig.8. Luciferase signal over the course of cyclophosphamide induced diabetes.

4.18 Generation and genotyping of NOD-SCID pRLM mice:

Once we had the NOD pRLM line, we felt that it would be useful to generate a NOD-SCID pRLM line. We regularly do adoptive transfer studies using NOD-SCID recipients and felt that a NOD-SCID line expressing luciferase in the pancreas could be a really useful model. They could be used as a reporter mouse line to correlate beta cell destruction with specific immune cell subsets after adoptive transfer. The loss of luciferase expression could give a clearer picture regarding the effect of immune cell subsets on diabetes transfer, compared to urine glucose levels.

To generate NOD-SCID pRLM mice, we crossed NOD pRLM mice with NOD- SCID mice. The F0 pups were NOD/ NOD-SCID heterozygotes. Brother-sister matings of pRLM+ F0 mice gave rise to mice that were NOD homozygotes, NOD-SCID homozygotes or NOD/NOD-SCID heterozygotes. pRLM+ NOD-SCID homozygotes were identified by genomic PCR and were back crossed into NOD-SCID WT mice to generate and maintain the NOD-SCID pRLM+ transgenic line.

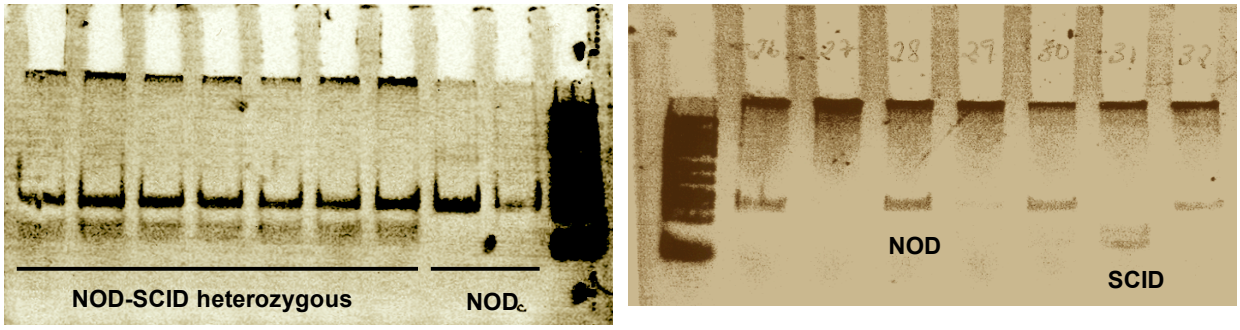
To identify NOD-SCID pRLM+ mice, we used two different genotyping PCR techniques. The hluc expression was identified using the genomic PCR described before. To distinguish between NOD and NOD-SCID strains from the F1 generation, we used a second genomic PCR followed by Alu1 digestion of the products. Upon digestion, NOD samples give 2 bands; one at 68bp and the other at 11bp while the NOD- SCID homozygous samples give 3 bands; at 38bp, 28bp and 11bp. Hence homozygous NOD samples gave only 2 bands while homozygous NOD-SCID samples gave 3 bands on the gel. Heterozygous samples gave all the 4 bands, making them easy to identify.

We used FACS to verify the results from the genomic PCR. NOD mice have T and B cells while these are absent or present at very low levels in NOD- SCID mice. Hence TCR and B220 were used as markers for NOD samples. NOD mice had TCR+ and B220+ populations while these were noticeably absent in the NOD-SCID mice samples. Representative PAGE gels from NOD-SCID genotyping showing NOD SCID

heterozygous mice, NOD homozygous mice and NOD-SCID homozygous mice are given below (Fig.9A). A representative FACS plot showing both NOD pRLM and NOD-SCID pRLM mice is also shown below (Fig.9B).

Fig.9

A.



B.

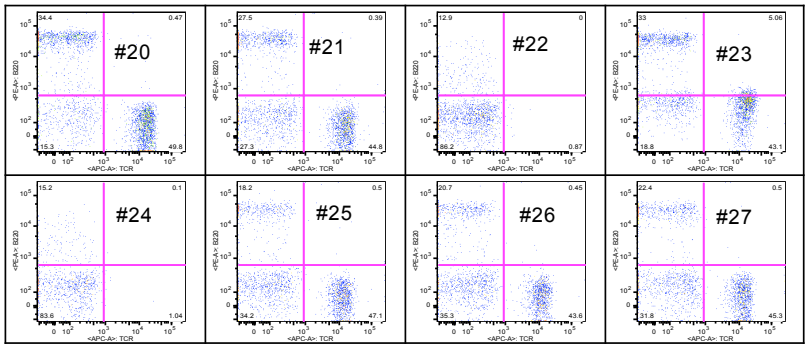


Fig.9. ***A***. Representative page gels showing NOD-SCID heterozygous mice (top left panel) and NOD and NOD-SCID homozygous mice (top right panel). ***B***. T and B cell poluations in mice, as measured by flow cytometry (bottom panel). TCR staining is on X- axis and B220 staining on Y-axis. #22 and #24 are NOD-SCID homozygous.

Modulation of β- cell mass

Type 1 diabetes is caused by the autoimmune destruction of beta cells. As mentioned in the introduction, an efficient therapy would have to combine immune cell modulation with beta cell regeneration. There are several genes which have been reported to modulate beta cell mass. Some of them are found to enhance beta cell division while some others are negative regulators of beta cell proliferation. Knockdown of these negative regulators could take away the inhibitory effect and result in increased beta cell proliferation. This enhanced proliferation might be able to counter act the destruction of beta cells by the immune system. Hence this is a viable area of research in the search for a cure for type 1 diabetes.

Following this line of thought, we wanted to be able to induce pancreas specific knockdown of negative regulators, the effects of which could then be monitored via luciferase expression. Menin is a transcription factor, which is found to act as a negative regulator of beta cell proliferation. Menin depletion has been shown to increase pancreatic islet cell proliferation[226]. Disruption of Men1 gene in pancreas has been reported to lead to the development of insulinomas along with increased serum insulin levels[216, 217]. Hence we chose menin as a target gene for beta cell mass modulation. We felt that the knockdown of menin could prevent or at least delay the development of diabetes. A partial reduction of menin, which can be achieved via RNAi, was a favorable strategy as a complete loss of menin has already been reported to be deleterious[208]. We could track the changes in beta cell mass resulting from menin KD via the expression of luciferase. Hence our plan was to generate menin- pRLM mice. These mice would have a pancreas specific expression of menin shRNA leading to a pancreas specific menin KD. The knockdown of menin should lead to enhanced beta cell proliferation which in turn should lead to enhanced luciferase signals. This increased beta cell proliferation might be able to protect the mice from diabetes development. The ideology is described in Fig.10.

Fig.10

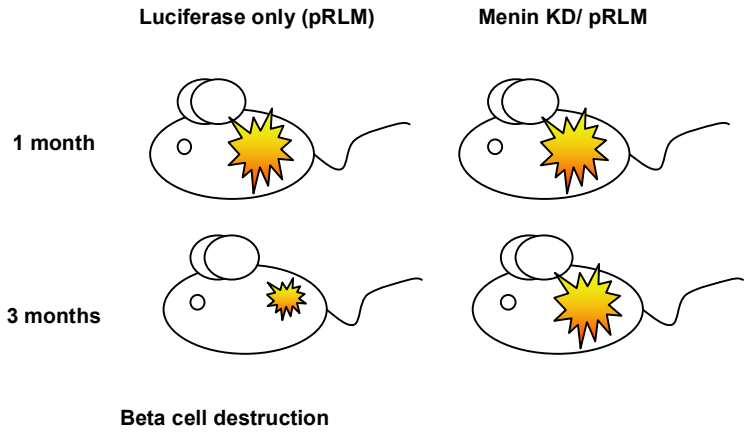


Fig.10. Schematic representation of the technique. pRLM mice develop diabetes over time and hence the luciferase signal goes down relative to the beta cell destruction. However in menin KD/ pRLM mice, knockdown of menin results in increased beta cell mass, which might lead to a stable signal over time.

4.19 Generation and validation of Menin constructs:

shRNAs were designed against menin cDNA and were ordered from Sigma. shRNAs were first cloned into the pLBM vector to test their knockdown efficiencies. They were then subcloned into other vectors for *in vitro* studies. We decided to validate menin knockdown *in vitro* before generating a menin- pRLM mouse line. For this purpose, we used two insulinoma cell lines. These are tumor cell lines that are derived from insulinomas. Hence they can be used as *in vitro* models of pancreatic beta cells. Some of the experiments were done in the rat insulinoma cell line INS-1E while others were done in the mouse insulinoma cell line NIT-1.

We didn't have the mouse insulinoma cell line in the beginning of the project and hence we did the preliminary experiments in the rat insulinoma cell line. Since we had 2 different models, i.e. the *in vitro* INS-1E cell line from rat and the *in vivo* NOD mouse model, we selected shRNAs which were expected to be active against both rat and mouse menin cDNA. These shRNAs could ideally knockdown menin in rat insulinoma cells as

well as in mouse pancreas. We were later able to obtain mouse insulinoma cell line NIT-1 and were able to select shRNAs active only against mouse menin cDNA. The generation of the constructs is detailed below.

4.19.1 shRNA-pLBM Construct generation:

shRNA sequences were cloned into the pLBM vector first. The knockdown efficiencies of the shRNAs had to be tested and for this shRNA-pLBM constructs were used. shRNA sequences were designed using the algorithm available at http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA[244] . Most sequences were complementary to both rat and mouse menin sequences as *in vitro* experiments were being carried out in a rat and a mouse cell line and the *in vivo* model was a mouse strain. #2760 was not active against rat menin cDNA. The corresponding synthetic DNA oligomers were ordered from Sigma-Aldrich. The sequences were amplified by PCR to generate complementary strands and purified by agarose gel electrophoresis followed by gel extraction. Purified sequences were then digested with Xho I / Eco RI and then ligated with pLBM vector (Fig.1), cut with the same enzyme pair, which contains an ampicillin resistance gene.

4.19.2 Subcloning:

Menin shRNA sequences were sub cloned from pLBM (expressing GFP) into 2 different vectors. For each round of sub cloning, the menin shRNA sequence was cut out from pLBM using different enzyme sets and then cloned into the new vector. The constructs were then used to transform NovaBlue or DH5 cells and minipreps were made which were validated by sequencing. The minipreps were then used to make maxipreps.

shRNA- Puro Subcloning:

As mentioned earlier, our plan was to validate menin knockdown *in vitro* in insulinoma cell lines first. For this we had to infect cells with menin shRNA, select the infected cells

and analyze their characteristics. It was important to restrict the analyses to shRNA-infected cells as they represented the cells with menin KD. We decided to use puromycin selection to identify the shRNA-infected cells and use them for further analyses. Hence we had to clone in the shRNAs into a construct containing a puromycin resistance gene. The cells infected with this shRNA-puro constructs could be selected on puromycin ensuring that only menin KD cells survived and were used for *in vitro* studies.

pLBM-puro contains a puromycin resistance sequence in place of the GFP sequence and so the cells containing the construct can be selected using puromycin. Hence the shRNAs were cloned into the pLBM-puro construct. The pLBM- menin shRNA constructs were digested with Nhe I/ Not I (to cut out GFP) and pLBM-puro construct was digested with the same enzyme pair to cut out puro sequence. The digested samples were run on gel, the menin backbones and the puro band were cut out from the gel and gel purified. The backbones were then ligated with the puro sequence to generate shRNA-puro constructs.

shRNA- pRLM (RIP-hluc) Subcloning:

To generate the menin-pRLM mouse line, shRNAs had to be cloned into the pRLM construct. Hence shRNAs were cut out from the pLBM vector and cloned into pRLM construct. pRLM contains a luciferase sequence in place of the GFP sequence under the control of RIP (Rat insulin promoter) promoter. The reporter is expressed only pancreatic cell lines and the cells can be imaged under the CCD camera. pRLM vector as well as pLBM- menin shRNA constructs were digested with Xba I/ Not I. The digested samples were run on gel and the menin shRNA backbones and RIP-hluc sequences were cut out, gel purified and ligated to generate shRNA-pRLM constructs.

4.19.3 Luciferase Assay:

The knockdown efficiencies of the different menin shRNA sequences were tested by luciferase assay. Since we had to test the menin KD in a rat cell line and a mouse cell

line, we had to ensure that the shRNAs could cause the degradation of both rat and mouse menin mRNA. Hence we had to test menin shRNA sequences against both rat and mouse menin cDNAs and for this both the rat and mouse menin cDNAs had to be cloned into psi-check2 construct.

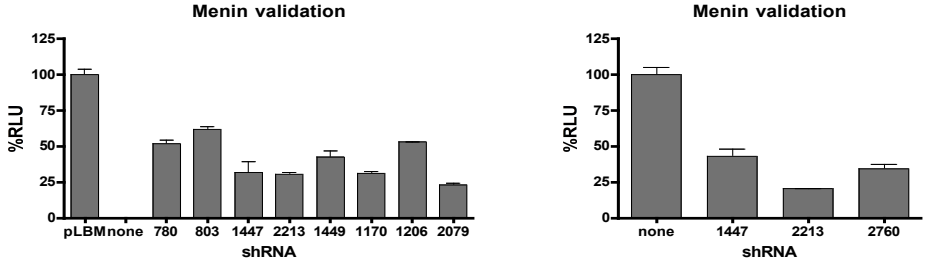
4.19.4 Menin psi-check generation:

Rat and mouse cDNA for menin were obtained from Openbiosystems. The bacterial samples containing the cDNA were streaked onto LB-ampicillin plates; colonies were picked and used to make minipreps. Minipreps were pooled and then digested with either Sal I / Not I (mouse menin cDNA) or EcoRV / Not I (rat menin cDNA). The digested samples were run on gel and the bands corresponding to the cDNA was cut out and gel purified. The psi-check 2 vector (Fig.4) was cut with either Xho I/ Not I (for cloning in mouse menin cDNA) or Pme I/ Not I (for cloning in rat menin sequence) and PCR purified. The cDNA sequences were then ligated with the digested psi-check 2 vector to generate mouse menin psi-check and rat menin psi-check.

9 different shRNA sequences were designed and cloned into the pLBM construct. All sequences except #2760 were selected based on their complementarity to both rat and mouse menin sequences. Hence most of the sequences were tested against both rat and mouse psi-check2 constructs. Most of the shRNAs were found to knock down menin expression significantly (Fig. 11A). We found that most shRNAs showed similar knockdown against both rat and mouse cDNA sequences indicating that they could cause similar menin KD in rat and mouse cells (Fig.11B).

Fig.11

A.



B.

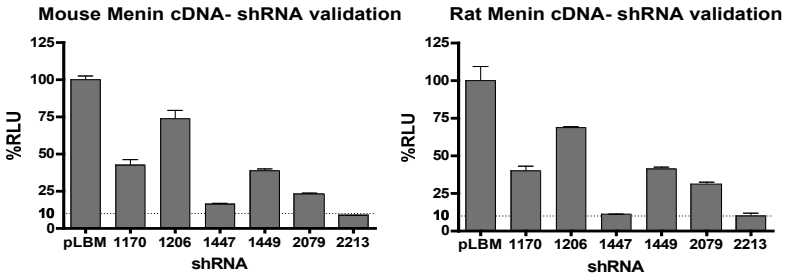


Fig.11. ***A***. Validation of menin shRNA silencing efficiencies by dual luciferase assay. Assay was done in triplicates against mouse menin cDNA. Results are representative of at least three independent experiments. ***B***. Validation of menin shRNA silencing efficiencies against mouse (left) and rat (right) menin cDNAs. Assay was done in triplicates.

4.20 *in-vitro* analyses using Ins-1E and NIT-1 Cells:

As mentioned earlier, we wanted to validate menin KD *in vitro* in addition to the luciferase assay. For this, first we subcloned shRNAs into pLBM-puro construct. We used these shRNA-puro constructs to generate lentivirus. pLBM-puro was used as the control. INS-1E or NIT-1 cells were then infected with the different shRNA-puro virus samples or pLBM-puro and were selected on puromycin. shRNA infected cells which survived were then grown and the knockdown of menin was verified by western blot. Once we verified the knockdown of menin in the NIT-1 cells by #2760, we looked at the phenotypic features of the menin KD cells. All these are detailed below.

4.20.1 Viral infection of cells:

To investigate menin KD *in vitro*, we generated lentivirus carrying the either pLBM-puro or the different shRNA-puro constructs. INS-1E cells or NIT-1 cells were cultured on 24 well plates and were infected with the virus. Since the cells had already been found to be really sensitive to transfection reagents, we added the virus suspension to the media in the

absence of any transfection reagent. The cells were allowed to grow for around 3-4 days after which the media was replaced with media containing the optimum concentration of puromycin. This was already determined by titration as described in the method section. The cells were allowed to grow in puromycin media for 2 days. The media was removed and replaced with fresh puromycin media. Only cells, which got infected with the shRNA-puro virus, would survive. These cells were then allowed to grow confluent in the 24 well plates. The cells were then grown out into 6 well plates and finally to 10 cm plates or 25 ml TC flasks.

The potency of the puromycin media was checked regularly by growing WT cells in the media and ensuring that they failed to survive in it. After selection of the cells, the puromycin media was then replaced with normal media and the cells were allowed to grow over time and they were used for the various analyses. Once the cells have been grown out into 6 well plates, they were grown in media lacking puromycin. This was to facilitate cell growth and division. The cells are then reselected on puromycin once in a while. This was done as both the cell lines used grow extremely slowly. INS-1E cells take at least 5-6 days to be confluent while NIT-1 cells take almost 9-10 days to become confluent.

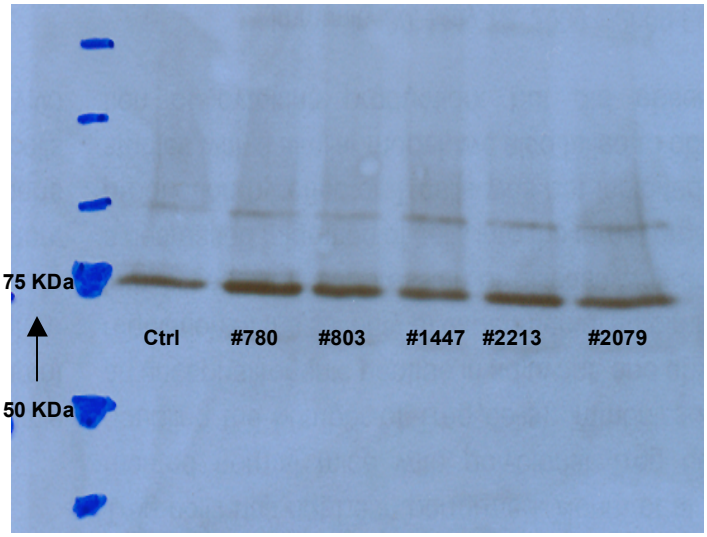
4.20.2 Analysis of menin knockdown:

To verify the KD of menin in the shRNA-virus infected cells, we used western blot. Cells were infected with the various shRNA-puro constructs and were then selected on puromycin media. Cells were grown out into 10 cm plates and were grown until confluent. Cells were then lysed and resuspended in RIPA lysis buffer. The lysates were then subjected to western blot. We used a rabbit anti menin antibody from Bethyl laboratories. We found that this antibody could pick up menin signals from both rat and mouse cell lines as opposed to the menin antibody from Santa- Cruz Biotech. The blots were much clearer when using NIT-1 cells in comparison with INS-1E cells. Although most sequences showed good KD in the luciferase assay, they failed to cause any reduction in the protein levels as detected by western blot (Fig.12A). The only sequence

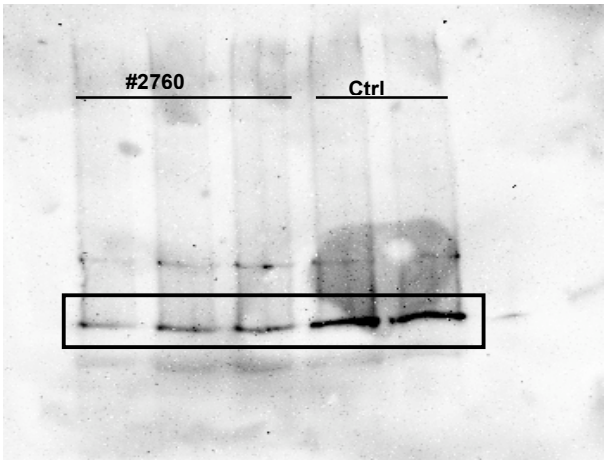
that seemed to be able to knockdown the protein in NIT-1 cells compared to the control (pLBM-puro infected cells) was the 9th sequence tested, #2760 (Fig.12B). We also looked at the menin mRNA levels in the KD cells. To our surprise we found that 2760 infected cells seemed to have almost double the amount of menin mRNA compared to the control cells (Fig.12C). The difference was significant and consistent.

Fig.12

A.



B.



C.

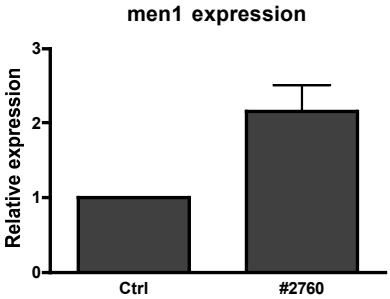


Fig.12. ***A***. Menin protein expression levels in shRNA infected NIT-1 cells in comparison with control cells. ***B & C***. Menin protein and men1 mRNA expression levels in #2760 infected NIT-1 cells in comparison with control cells.

Western blot indicated that none of the sequences except #2760 could knockdown menin at the protein level in the insulinoma cells. This was quite surprising as most of them were found to be quite efficient in luciferase assay. The fact that menin KD cells had significantly higher levels of menin mRNA further complicates the picture.

4.20.3 Analysis of phenotype of menin KD cells:

Once we detected that #2760 could knock down menin *in vitro*, we looked at the phenotype of the KD cells. For this we infected NIT-1 cells either with pLBM-puro or 2760-puro virus, cultured the puromycin selected cells and used them periodically for the various analyses.

Menin KD has already been shown to result in an increased proliferation and reduced apoptosis. Hence we decided to look at the proliferation and apoptosis in the 2760-puro infected cells in comparison with pLBM-puro infected cells.

4.20.3.1 Analysis of proliferation:

Menin deficiency has been reported to enhance the proliferation of beta cells in mice. This enhancement is a really slow and long process that takes 5-6 moths to manifest and hence might not be recapitulated *in vitro* during the short-term study. The short duration might not be enough to pick up the slight increases in beta cell proliferation. Hence we decided to apply several complementary approaches to look at beta cell proliferation.

eFluor-670 Staining:

Equal numbers ($1x10^6$) of pLBM-puro (control) or 2760-puro infected cells were stained with eFluor670 proliferation dye and were plated out in culture at $1x10^5$ per well. Staining of the cells was analyzed by FACS on days 0, 3 and 7. No difference was observed between the 2 samples on any of the time points analyzed (Fig. 13A). This might be because the increase in proliferation might not have been large enough to be picked up by eFluor staining.

PI staining for DNA content:

We felt that a minor difference in proliferation might be picked up better if we look at the cell cycles using PI. We plated out equivalent number of pLBM-puro and shRNA-puro infected cells in 24 well plates. The cells were grown over time. Cell cycle analysis was done using PI on several time points including days 2, 5, 8, 12 and 15. PI intercalates with the DNA and the signal is proportional to the amount of DNA in the cells. This technique could be used to distinguish between cells in G0/ G1 phase or G2/ M phase. The taller curve represents non-dividing cells while the shorter curve represents the proliferating cells (Fig.13B). No significant difference was observed between the control and shRNA infected cells on any of the time points analyzed. Similar percentages of proliferating cells were observed in both sample sets over the course of the study (Fig.13B). Both menin KD and control samples had similar percentages of proliferating cells.

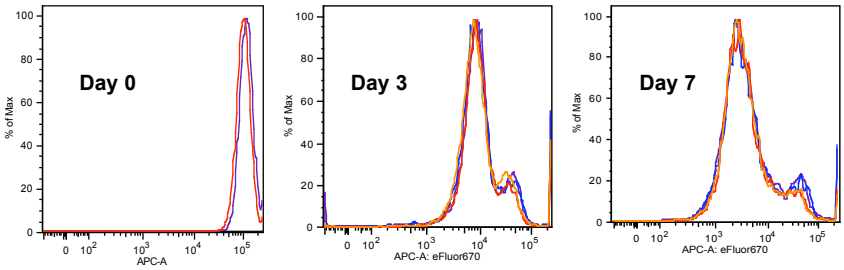
Cell Counts:

As an alternative measure, equal number of both control and shRNA infected cells were plated out and put in culture. Cell counting was done using FACS just to ensure that equal starting numbers were used. The cells were split after a few days and the cell numbers were counted again using FACS. There were 9 replicates for each sample. #2760-puro infected cells were found have an increased number of cells in comparison

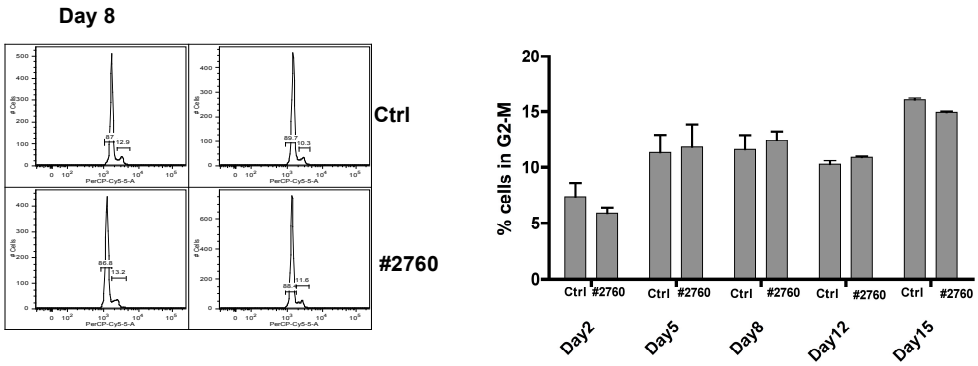
with the control (Fig.13C). This indicated that menin KD cells had an increased proliferation rate in comparison with the control cells. This is in line with previously published results.

Fig.13

A.



B.



C.

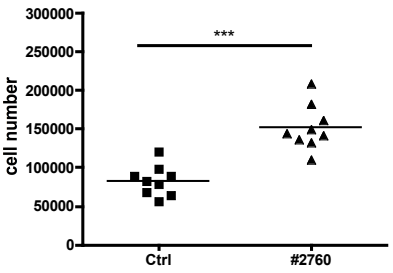


Fig.13. ***A***. eFluor-670 staining of cells on days 0, 3 and 7 post plating. The blue and violet lines represent two control samples while the orange and red lines represent two #2760 infected samples. ***B***. Representative data showing PI staining of cells on day 8 (left panel). The taller curve represents resting cells in G0/ G1 while the smaller curve on the right represents the proliferating cells in G2/ M. Percentages of proliferating cells in the samples over the course of the study (right panel). ***C***. Total cell numbers in samples on day 7 post plating. 9 replicates of each sample were plated out and analyzed.

Though we used 3 different techniques, we are not sure about the suitability of these to study the proliferation rates in beta cells. The techniques might not be appropriate to analyze proliferation rates when the differences are minor and occur over a long duration. Still the increased number of cells we observed in menin KD samples indicated that knockdown of menin could enhance beta cell division and even slight changes that accumulate over time could have a large cumulative effect in the end.

4.20.3.2 Analysis of apoptosis:

Overexpression of menin has been reported to increase apoptosis (70). Menin is believed to inhibit insulinoma formation by activation of caspase 8 involved in apoptosis (57). Hence it was quite interesting to see if menin KD cells showed a reduced level of apoptosis.

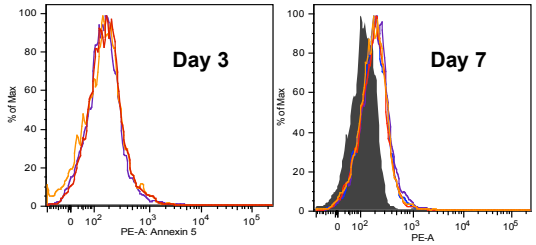
Annexin/PI staining of serum- starved cells:

Apoptosis was first analyzed in cells cultured in normal media. Cells were plated out at equal starting numbers and were allowed to grow in culture. They were taken out at different time points and stained with annexin PE. AnnexinV can bind to phosphatidylserine (PS) on the surface of cell membranes. PS is usually located on the inner surface of the cell membrane. But cells undergoing apoptosis translocate PS to the outer surface. This is an early apoptotic event and hence AnnexinV can be used to identify the cells, which start to undergo apoptosis. We failed to observe any difference between the 2 samples at any of the time points analyzed (Fig.14A).

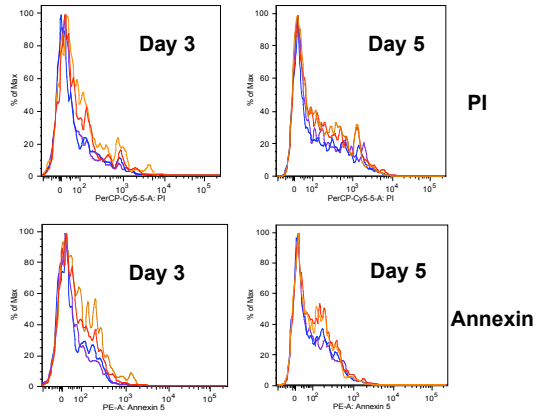
Since we felt that we might be able to pick up minor differences better by analyzing the apoptosis in FCS starved cells, we cultured cells in starvation media lacking FCS and then analyzed the apoptosis using PI and annexin PE. In contrast to AnnexinV, PI can be used to detect late apoptotic events as it stains dead cells. We observed increased apoptosis in shRNA-puro infected cells compared to the control cells at all time points analyzed. This was in contrast to previous reports (Fig.14B).

Fig.14

A.



B.



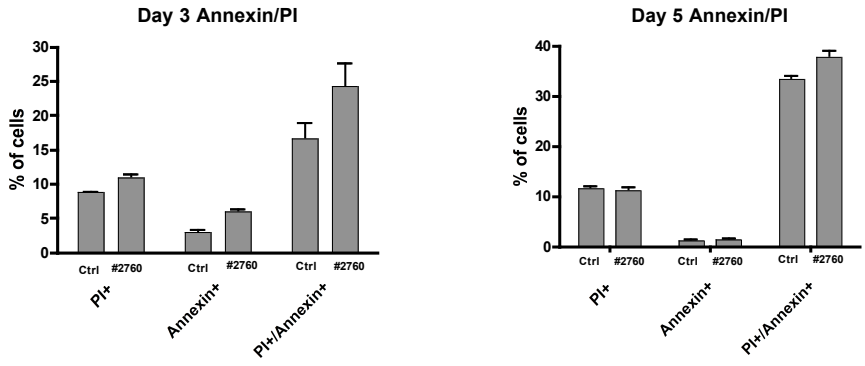


Fig.14. ***A***. AnnexinV staining of control and #2760 infected cells. The blue and violet lines represent control samples while the red and orange lines represent #2760 infected samples. ***B***. AnnexinV/ PI staining of control and #2760 infected cells on days 3 and 5 (top panel). The blue and violet lines represent control samples while the red and orange lines represent #2760 infected samples. Percentages of AnnexinV+, PI+ and double positive cells in control and #2760 infected samples on days 3 and 5 (bottom panel).

4.21 Generation of virus for embryo injection:

Our *in vitro* results indicated that #2760 could induce the knockdown of menin and that menin KD cells exhibited slightly increased proliferation. Hence we decided to use this sequence to generate menin KD mice in the pRLM background. High titer virus was generated using the 2760-pRLM construct and will be used to generate Menin KD mice in the pRLM background.

Chapter 5

DISCUSSION

IL-17A silencing does not protect NOD mice from autoimmune diabetes

Ever since their discovery, Th17 cells have been intensely studied. The discovery that IL-23 and not IL-12 is critical for autoimmunity[110] led to the idea that Th17 cells are responsible for autoimmune diseases as opposed to Th1 cells. A number of studies verified this idea as the cytokines secreted by this group of cells were discovered to be pathogenic in several autoimmune diseases. IL-21 is found to be pathogenic in several autoimmune disease models including EAE and CIA[245]. IL-21 is also found to be critical for the development of type 1 diabetes[246, 247]. IL-17A is found to be pathogenic in a variety of autoimmune conditions including EAE, CIA and chronic colitis[242, 245, 261]. The role played by the other Th17 effector cytokines is less well established. All these data strongly point to Th17 cells being extremely pathogenic. But they are also found to play critical roles in defense against extracellular bacteria and fungi. The absence of Th17 subset seriously compromises the immune system with the organism being severely susceptible to bacterial and fungal infections[149-157]. Th17 cells are mainly characterized by their signature cytokine, IL-17A. Currently, most of the effects associated with Th17 cells can be traced back directly to this effector cytokine.

IL-17A deficient mice are found to be partially protected from both CIA and EAE. This has led to the belief that this cytokine might play a pathogenic role in other autoimmune diseases including type 1 diabetes. Though several studies have hinted at a pathogenic role for IL-17A in autoimmune diabetes[171-176], the findings are not really conclusive. We felt that an IL-17A KD transgenic line could provide valuable insight into the role played by this cytokine in the context of autoimmune diabetes. A partial reduction of the protein better reflects a normal physiological state. We used a construct containing GFP along with the shRNA and used GFP expression as a marker for the KD.

We generated an IL-17A KD transgenic mouse, which was found to express two distinct populations of GFP+ cells. One population was found to have a lower MFI than the other. They were designated as 'low' and 'high' respectively. It has already been shown that in some transgenic mouse lines, different cell subsets express different levels of GFP[248]. But this didn't seem to be the case with our founder as all cell lineages had almost 100% of the cells expressing GFP but all exhibited the 2 cell populations. Once we bred the founder, we saw that the pups had only one GFP population, either the high or the low. We further bred the 'high' and 'low' mice as two separate transgenic lines and found that all pups from a 'high mouse' had cells with high GFP expression while all pups from a 'low mouse' had cells with low GFP expression. This was found to be true in all future generations. When we looked at the integration using Southern blot, we found that both the 'high' and 'low' mice had a single integrant but at different sites in the genome. All these data pointed to the possibility that the IL-17A KD founder was a chimera and that the 2 virus copies segregated in F0.

Since both the 'high' and 'low' mice differed in their GFP expression intensity, there was a possibility that they had different levels of KD. But the analysis of IL-17A levels in differentiated Th17 cells showed that both lines exhibited similar IL-17A reduction, despite the difference in the GFP expression. Hence the only difference between the 2 lines seems to the integration site and the GFP expression pattern. Hence the terms 'high' and 'low' refer only to the GFP expression and not to the level of IL-17A KD in these mice.

We were able to see significant KD of IL-17A *in vitro* and *ex vivo*. *In vitro* differentiated transgenic Th17 cells produced vastly reduced amounts of IL-17A. Both IL-17A protein and mRNA levels were significantly reduced. We were able to see a tight correlation between the reduction in both mRNA and protein levels. Though we could find a *KD in vitro*, this might not have been the case *in vivo*. It has already been suggested that all the conditions required for a full effector Th17 differentiation might not be present *in vitro*. This is suggested to the responsible for the incredible plasticity observed with *in vitro* differentiated Th17 cells[239]. Hence it was possible that though transgenic Th17 cells

differentiated *in vitro* could not produce significant amounts of IL-17A, under *in vivo* conditions, these cells could achieve full effector function with IL-17A secretion to the full capacity.

To investigate this possibility, we decided to look at the IL-17A levels in diseased pancreas *ex vivo*. We took out islets from the pancreas of diseased mice and analyzed the cytokine levels by qPCR. We could detect IFNg at quite high levels indicating a role for this cytokine in the disease process. On the other hand, we could detect IL-17A only at very low levels in a minor proportion of the mice and the expression was found to be quite variable. We feel that had this cytokine played a critical role in autoimmune diabetes, we should have been able to pick up IL-17A at higher levels. We found a high and comparable expression of RORgt in both WT and KD mice. Since RORgt expression was already found to be unaffected by the integration of the transgene, we looked at the islet IL-17A levels relative to the RORgt expression in the islets. We found that compared to WT beta cells, we could detect vastly reduced amount of IL-17A in the islets from KD mice. The degree of reduction was found to be quite comparable to the reduction observed in in vitro differentiated transgenic Th17 cells. This observation led to believe that the *in vitro* KD we observed seemed to reflect the knockdown *in vivo*.

Th17 cells produce a number of effector cytokines, in addition to IL-17A. These include IL-17F, IL-21 and IL-22[119]. Th17 cells are also characterized by their lineage specific transcription factor, RORγt[130, 131]. For the purpose of the study, it was critical to ensure that the transgene did not disrupt the Th17 pathway or the expression of the other cytokines. For this we analyzed the levels of RORgt, IL-17F and IL-21 in both *in vitro* differentiated Th17 cells and *ex vivo* islets. We found comparable amounts of both cytokines in both WT and KD mice at both mRNA and protein levels. This was the case with both differentiated Th17 cells and the islets. RORgt levels were also quite similar between the WT and KD samples. We did not look at IL-22 as this is considered to be a minor Th17 cytokine. All these data suggested that the integration of the transgene and the knockdown of IL-17A was specific and did not disrupt Th17 pathway in the transgenic mice.

For the purpose of the study, we had to ensure that Th1 differentiation in the transgenic mice was unaffected. Th17 and Th1 pathways are both believed to be involved in autoimmune pathology and it has been quite difficult to specify their distinct roles in autoimmune diseases. Th17 cells are also found to quite plastic with the ability undergo conversion to alternative phenotypes and are reported to acquire Th1 phenotype over time. There is some evidence indicating that *in vitro* differentiated and purified Th17 cells can undergo conversion into Th1 like cells once they are transferred into immunodeficient recipients[173]. Hirota et al.[239] recently showed that Th17 cells are characterized by the differentiation origin rather than the production of IL-17A. They discovered that Th17 cells can undergo alternative states depending upon the disease environment. Most Th17 studies have observed the development of IL-17A/ IFNg double positive and IFNg single positive cells in purified Th17 cell populations. Hence for the purpose of this study, we had to ensure that loss of IL-17A did not enhance Th1 pathway or Th17 conversion. For this we looked at IFNg levels in both *in vitro* differentiated Th1 cells and *ex vivo* pancreatic islets. We found that KD cells had similar levels of IFNg compared to WT cells indicating that reduced IL-17A levels did not lead to increased IFNg levels. This suggested the absence of Th17 conversion or enhancement of Th1 pathway.

Once we had mice in which at least 70% of IL-17A is eliminated, we analyzed the diabetes incidence in these mice. We used 3 different models of diabetes. This was done to foolproof the observations. We observed that KD mice developed spontaneous diabetes similarly to WT mice and that the disease frequency and kinetics were quite similar. To double-check the results, we used cyclophosphamide-induced diabetes. Cyclophosphamide can accelerate diabetes development in insulitic mice and cause a synchronous disease around 14 days after the injection. We did the experiment twice and both times, we observed that the transgenic mice were as susceptible to disease development as WT mice. All these data led us to believe that reduced IL-17A does not protect the mice from autoimmune disease. To check whether transgenic splenocytes could transfer diabetes, we transferred splenocytes from diabetic NOD mice into NOD-

SCID recipients. None of the mice that got WT splenocytes got sick while 3 of the mice that received transgenic cells developed diabetes. Though the general lack of disease development suggested that the experimental setup didn't work properly, the fact that 3 of the mice that got transgenic cells developed the disease indicated that transgenic cells could transfer diabetes despite reduced IL-17A production. The data from the diabetes incidence studies together suggested that reduction of IL-17A does not protect NOD mice from autoimmune diabetes. This observation indicated that IL-17A might not be critical in the development of type 1 diabetes.

One of the major concerns raised regarding our experimental system is the presence of residual amounts of the protein in the transgenic mice. Since we use knockdown mice as opposed to knockout mice, the protein is not removed completely. The KD cells are capable of IL-17A production, though at reduced levels. As shown before, there is around 30% of IL-17A still left in the transgenic mice. One of the major questions was whether this residual amount is capable of contributing to diabetes development. To investigate this possibility, we used another model of IL-17A dependent autoimmune disease, EAE. It has already been shown that IL-17A deficient mice are partially protected from EAE with the disease being less severe and occurring with less frequency[242]. We postulated that if the KD in the transgenic mice is sufficient to protect them from EAE at least partially, then it should also be sufficient to offer at least partial protection from autoimmune diabetes. This depended on one assumption that both EAE and T1D are controlled by the same parameters.

We induced diabetes in the IL-17A KD mice by the injection of MOG peptide emulsified in CFA. We have previously found mice in our colony to develop a relapsing remitting form of the disease. We found that the transgenic mice showed significant protection from EAE during the fist phase of the disease but this protection was absent in the relapse. The transgenic mice had a significantly lower cumulative disease burden compared to the WT NOD mice. In our experimental setup, the overall disease severity in the mice was low. In the previous study[242], the mice developed a severe form of EAE. We believe that this can be attributed to the strain differences. Iwakura et al. had done the

EAE study in C57BL/6 mice. These mice develop a monophasic disease and are much more susceptible to EAE compared to NOD mice.

Although the disease severity was low, we observed a clear, albeit partial protection in the IL-17A KD mice in comparison with the WT mice. This indicated that the KD observed in the transgenic mice is sufficient to impair IL-17A dependent autoimmunity. Though the mice still retained around 30% of IL-17A they were still partially protected from the disease, consistent with results from the IL-17A deficient mice. Hence, had type 1 diabetes been IL-17A dependent, we would have expected to see at least a partial protection from the disease in the transgenic mice. The lack of protection from diabetes we observed hence suggested that type 1 diabetes is not dependent on IL-17A and thus the removal of it has no effect on the development of the disease.

To investigate the reason for the partial protection from EAE we observed, we took out diseased splenocytes from mice and did an *in vitro* recall response. We stimulated them *in vitro* either non-specifically with beads or specifically with MOG peptide. We found that KD cells could produce IL-17A under maximal stimulation though at reduced levels compared to WT cells. Upon MOG specific stimulation, both WT and KD cells produced comparable amounts of IFNg indicating that the partial protection was not related to levels of this cytokine. But KD cells failed to produce any IL-17A in response to MOG stimulation while WT cells produced significant amounts of this cytokine. This observation suggested that the partial protection seen might have been due to the inability of KD cells to produce IL-17A.

We generated a mouse model with vastly reduced amounts of IL-17A. Th17 lineage in the mouse was uncompromised. Though the transgenic mice produced significantly lower amounts of IL-17A, they were not protected from autoimmune diabetes. The reduction in IL-17A was sufficient to protect the mice from EAE, an IL-17A dependent autoimmune disorder; indicating that systemic silencing was quantitatively sufficient to impair autoimmunity in an IL-17A- dependent context. Our results led us to conclude that autoimmune diabetes in the NOD mouse is independent of IL-17A. This does not

preclude the possibility that Th17 cells contribute to type 1 diabetes development. Th17 cells have been found to transfer diabetes into NOD-SCID mice but only after they converted into a Th1 like phenotype. anti-IL-17 was found to be unsuccessful in this model especially in young mice[172, 175]. Though Arif et al.[263] recently reported a crucial role for IL-17 in autoimmune diabetes, the scope of their results in clarifying the role of IL-17A in autoimmune diabetes is limited. The observation of Th17 associated factors in the islets was obtained from a single case report and they failed to observe any proapoptotic effect for IL-17A on its own. Though they were able to observe increased IL-17A reactivity (54% as opposed to 8%) in type 1 diabetes patients, they observed an increased IFNγ reactivity (62% as opposed to 13%) as well. The pattern of IL-17 reactivity was quite similar to the IFNγ response and the number of IL-17+ autoreactive cells was found to be fewer than that of IFNγ+ cells. There is the added problem that their assay was unable to distinguish dual secreting cells from single secretors which meant the IL-17 reactivity they observed could have been from double producers i.e. cells which produce IL-17 and IFNγ.

Munegowda et al.[264] on the other hand observed that Th17 cells on their own could not cause diabetes and that they stimulated CD8+ CTLs which could then kill beta cells through the perforin pathway and lead to diabetes development. This was in contrast to EAE where CD4+ Th17 cells induced EAE and Th17 activated CTLs could not. They concluded that T1D is directly mediated by Th17 stimulated CTLs whereas CD4+ Th17 cells play a crucial role in EAE pathogenesis by Th17 cytokine mediated tissue inflammation. The results from this study could be reconciled with ours in that a classical Th17 response involving IL-17 does not seem to be crucial for type 1 diabetes development. IL-21 has been reported to be indispensable for the development of diabetes in the NOD mouse model[246, 247]. This cytokine is found to play a crucial role in Th17 proliferation and is one of the effector cytokines produced by this subset. Hence Th17 cells might still play a role in diabetes development through effector mechanisms distinct from IL-17A production. But our results demonstrate that a characteristic Th17 response that involves IL-17A is not critical in all autoimmune diseases, at least not in autoimmune diabetes.

So far most studies have seen an extremely close relationship between Th17 cells and IL-17A that all effects observed with Th17 cells have been attributed to this cytokine. IL-17A has come to define Th17 lineage and its functions. Our study seems to show that an IL-17A dependent response seemed to be involved in other autoimmune diseases is not important in type 1 diabetes. Recently it was shown that Th17 cells adopt alternate expression profiles in different disease states[239]. Th17 cells were found to switch off IL-17 production and switch on IFNg production in chronic inflammatory conditions like EAE. But in an example of acute infection i.e. *C. albicans*, Th17 cells failed to take up alternate fates although they switched off IL-17 expression. These observations raise the idea that the functional fate of Th17 cells is not invariably linked to IL-17 expression by these cells. Hence it seems that the role played by IL-17A might be overestimated. Rather than concentrating on an anti-IL-17A therapy, we should follow a broader approach, as this might be better applicable to a wider range of disease conditions.

Further work needs to be done to determine whether Th17 cells play a contributing role in autoimmune diabetes via any of the alternate effector mechanisms. It would be interesting to analyze the role of IL-23 in the disease process. This was a line of research that we started on but unfortunately could not follow up. IL-23 is found to be pathogenic in several autoimmune diseases and this pathogenecity has been linked to Th17 cells. IL-23 has been found to be critical for the long-term survival and maintenance of Th17 subset. It is believed to impart pathogenic potential to Th17 cells. Recently two groups[249, 250]separately put forward the idea that IL-23 imparts pathogenic potential to Th17 cells via GM-CSF. They found that IL-23 could induce the production of GM-CSF in T cells and that GM- CSF was critical for the ability of Th17 cells to drive inflammation in the CNS in EAE. *In vitro* activated T cells required GM-CSF to passively transfer disease and GM-CSF neutralization upon onset of disease symptoms could block disease progression[251]. Hence it would be interesting to see whether loss of IL-23/ GM-CSF can protect mice from diabetes. It would also be interesting to see whether loss of the Th17 transcription factor RORgt could have an effect on the disease process. These are all

several avenues that can be pursued to further unravel the role, if any, of Th17 cells in the development of type 1 diabetes.

Generation β- cell reporter mouse models and modulation of β-cell mass

An efficient therapy for type 1 diabetes would have to involve beta cell regeneration. It would also have to involve the elimination of autoreactive immune cell specificities to ensure the long-term survival of implanted or regenerated beta cells. One of the main disadvantages of studying the disease in humans is the non-accessibility to the diseased organ, the pancreas. Hence the disease is intensively researched in animal models in which the diseased pancreas can be obtained upon sacrificing the animals. Even then, it is hard to follow the course of the disease and this challenge has given way to the search for non-invasive imaging techniques for analyses of pancreatic beta cell mass. This would allow the estimation of beta cell mass and autoreactive destruction in the same animal over the course of the disease. This strategy might be able to provide vital clues regarding the dynamics of beta cell mass during various stages of the disease. This in turn could facilitate the development of a successful cure or therapy option.

A number of studies have tried several beta cell imaging modalities PET, CAT, bioimaging etc. Jirák et al.[252] labeled beta cells with super paramagnetic iron oxide particles (SPIOs) and they found that the labeled islets could be detected and followed in vivo in a rodent model using MRI. Specific radiolabeled tracer molecules have been used to image transplanted islets by PET and SPECT[253]. But all these techniques have their own advantages and disadvantages. We chose luciferase bioimaging due to several reasons. It is non-toxic to the cells making it ideal for long-term studies. Using luciferase imaging to follow beta cell death during diabetes is more desirable than using fluorescent reporters like GFP. This is because the luciferase reaction requires ATP, which can be

present only in living cells and hence the signal better represents the living beta cell mass. On the other hand, GFP signal could be observed even after the cells are dead and hence the total signal would represent the whole beta cell mass including the dead cells. Hence using luciferase imaging to assess and follow beta cell mass presents a valuable research tool.

Luciferase imaging has already been used to assess beta cell mass in mouse models. Lu Y et al.[254] successfully used bioluminescent imaging to monitor islet graft survival over time. Several research groups have already generated transgenic mice expressing luciferase in the beta cells and these studies show a clear correlation between the signal intensity and the beta cell mass[255- 257]. Park et al.[256] generated transgenic mice expressing firefly luciferase under the control of mouse insulin 1 promoter in the CD1 background. They found that the mice had normal islet architecture and that the luciferase expressing beta cells could be readily imaged even after transplantation into NOD-SCID mice. These studies suggest that luciferase expression via viral transduction of reporter genes has no deleterious effect on beta cell development and maintenance. We felt that it would be quite useful to generate beta cell reporter mice in the context of a spontaneous diabetes model like the NOD mouse. These reporter mice would enable us to follow beta cell mass changes over the course of diabetes development. We also generated luciferase reporter mice in the NOD-SCID background for use in adoptive immune cell transfer experiments.

We decided to use the RIP promoter as it has been better validated than the mouse insulin promoter. We found the RIP-hluc construct to be specific and used this to generate the reporter mice. We found our mice to express luciferase, though this expression was dispersed around the abdominal area. We found an additional signal in the thymus, which could be explained owing to insulin expression in the thymus[262]. When we took out organs from the luciferin injected mice, we observed a signal albeit slight, from the spleen in addition to pancreas. The luciferase signal from the spleen was quite puzzling, as rat insulin promoter should restrict the expression of hluc to insulin expressing cells. We observed this disperse signal pattern in most of our pRLM mice even at the lowest

signal intensities and shortest exposure times. The pRLM+ mice exhibited no gross defects and behaved similar to wt mice under both normal and experimental conditions.

The first indication that despite its disperse nature, the signal could be specific to beta cells came when we imaged severely diabetic pRLM+ mice. Luciferase signals were barely detectable in the diabetic mice indicating that the signal was beta cell specific. To further clarify the origin of the signal, we used 2 different models of induced diabetes. We injected mice with STZ or CY and found that in both cases, mice lost their luciferase signal upon developing diabetes. Hence despite the strong abdominal signal before the induction of diabetes, the signal was significantly reduced / lost upon the loss of beta cells. These data strongly supported the beta cell specific expression pattern of luciferase. We could detect significant levels of luciferase mRNA only in the pancreas of the pRLM+ mice. Smaller luciferase levels were detected in the thymus while no expression was observed in any of the other organs tested. This further proved that luciferase expression was pancreas specific. In light of all the data, we believe that in our mice, luciferase expression is limited to insulin producing cells in the pancreas and the thymus. We believe that the broad abdominal signal we observe is due to a really strong luciferase expression. This strong signal even at the lowest signal intensity setting and shortest exposure means our imaging machine is severely limited.

Recently, Yong et al.[258] reported the generation of MIP-TF transgenic mice in the C57BL/6 background that have a MIP driving the expression a trifusion (TF) protein of three imaging reporters including a luciferase. They were able to observe a progressive reduction in bioluminescence preceding hyperglycemia, upon low dose STZ treatment. In their discussion, they reported that they are currently breeding the TF gene into the NOD mouse background. This approach is less ideal compared to our approach as crossing back a gene from the C57BL/6 background into the NOD background would always introduce linkage associated genes in addition to the desired gene from the C57BL/6 background into the NOD background. Hence we feel that our mice are better suited as a reporter model of spontaneous diabetes. As part of the project, we also generated a NOD-SCID pRLM mouse line. Towards this purpose, we crossed a NOD pRLM+ mouse with

NOD-SCID wt mice. We then mated the heterozygous pups to obtain NOD-SCID homozygous pRLM+ mice. We then selectively mated the homozygous pRLM+ mice back into NOD-SCID background to obtain and maintain the NOD-SCID pRLM line. We believe these mice could prove useful in the field of adoptive transfer research studies.

Originally we had planned to generate menin KD mice in the pRLM background. Menin is a tumour suppressor, the inactivation of which has been described to cause insulinomas. Hence a pRLM-menin knockdown mouse line would be an ideal model to study beta cell modulation using bioimaging. We aimed to generate a pRLM construct carrying an efficient menin shRNA and use the construct to generate a transgenic mouse line. Towards this purpose, we decided to validate the shRNA sequences *in vitro* in insulinoma cell lines first before proceeding to transgenic mice generation. We cloned in a total of 9 different shRNAs, most of them active against both rat and mouse menin. This was done intentionally as the *in vitro* experiments were done in both rat and mouse cell lines. During the early days of the project, we didn't have the mouse cell line NIT-1 and hence had to choose shRNA sequences which could knockdown menin in the rat insulinoma cell line INS-1 and which could also knockdown menin in the NOD mouse line, should we generate one.

All the shRNAs showed considerable knockdown efficiency in a luciferase reporter assay. These constructs were then used to infect insulinoma cells and the infected cells were subjected to western blot to analyze the knockdown of menin. This was a difficult premise as menin expression is markedly reduced in insulinoma cells[221]. However we were able to detect a band at around 70KDa, the reported size of menin. To our surprise, none of the first 8 shRNAs tested could knockdown menin *in vitro*. None of the cells infected with these shRNAs showed a reduction in menin levels. This was quite surprising as most of them showed very good knockdown efficiencies in the luciferase assay. Karges et al.[203] have reported the existence of 2 different isoforms of menin; the long λ transcript (2.4 kb product) and the short σ transcript (2.0 kb product). One of the possible reasons for the contradiction we observed might be that the shRNA sequences could have led to the degradation of only one of the isoforms and the other one might

have compensated for the loss. Still it does not fully explain how 8 different sequences could not knockdown menin sufficiently.

We tested #2079, our 9th sequence as Yokoyama et al.[259] found it effective in knocking down menin. Though our design algorithms had recommended this sequence before, we hadn't tested it, as it is not active against rat menin. As all the shRNA sequences tested, it showed good knockdown efficiency in the luciferase assay and was used to infect mouse insulinoma cells. #2760 infected cells showed a considerable reduction in menin protein expression in western blot. We isolated RNA from the infected cells and analyzed men1 mRNA levels by qPCR. We were amazed to see that #2760 infected cells had almost double the amount of men1 mRNA compared to control cells. This could be explained when the shRNA causes translational repression, not mRNA degradation and when the target protein is regulated by a feedback loop. In this scenario, the protein at sufficient levels prevents translation and transcription of its gene and when the shRNA prevents translation of the gene product, the feedback loop leads to increased gene transcription to compensate for reduced protein levels and leads to enhanced mRNA levels of the gene. Menin has already been reported to be regulated via a feedback loop[260] and hence fits the system perfectly.

We then looked at the proliferation and apoptosis levels in the menin knockdown cells. We couldn't pick up any difference in the proliferation levels using eFluor 670 staining. When we stained for cell cycle using propidium iodide, there was no significant difference between menin KD cells and control cells in any of the time points studied. However once we started with equal cell numbers, we could see an increased number of cells in the menin knockdown sample compared to the control sample, after few days in culture. Though we analyzed proliferation rates using 3 different techniques the results were contradictory. We believe that this might be due to the limited suitability of the techniques in the context of our experiment. Menin knockdown is found to cause insulinomas in mice but only over a very long period of time and hence the enhancement of proliferation is slight and cumulative over time. The experimental techniques we used might not be sensitive enough to pick up minor differences. The outcome is also

complicated by the fact that the knockdown cells were analyzed over a maximum period of 15 days, which might not be long enough for the differences to be apparent. However the difference in cell numbers we observed gives us the indication that there is a trend for increased cell numbers upon menin knockdown.

We analyzed the apoptosis levels in the knockdown cells using annexin/ PI double staining. Annexin would be able to pick up the early apoptotic cells while PI identifies late apoptotic cells. We couldn't see any difference between the samples when we stained them. We then decided to use serum starved cells for further experiments as we felt that the increased apoptosis in serum starved cells might better magnify slight differences difficult to be noticed. Contrary to previous studies, we observed an increased level of apoptosis in our menin knockdown cells. Overexpression of menin in insulinoma cells has been reported to lead to an increased rate of apoptosis[189]. Hence logically, knockdown of menin should lead to a decreased rate of apoptosis. However, we observed an enhanced apoptosis in menin knockdown cells that were serum starved and this effect was not noticeable in cells grown in serum containing media. Similar to proliferation, the cells were grown over a period of 12 days and analyzed at regular intervals.

Sayo et al.[189] were able to observe significant enhancement of apoptosis and reduction of proliferation in insulinoma cells transfected with menin overexpression constructs. However the transfection of cells with menin constructs would lead to a stronger effect compared to infection with virus containing menin constructs. Transfection of cells usually results in the uptake of several constructs by the same cell leading to a magnified effect. During infection of cells with virus containing the construct, each cell is infected with a single virus and hence a single construct, thereby eliminating the possibility of a magnified effect. This inherent contraction in the methods applied could to some extent explain the lack of or minor effects we observed in contrast to Sayo et al.[189] The other contributing factor as mentioned before could be the lack of sensitivity of the experiments employed as well as the inability to pick up minor changes, which could have a cumulative effect over time.

To summarize, we generated two beta cell reporter mouse lines; one in the NOD background and the other in the NOD- SCID background. We believe that these would be extremely useful in T1D research. These mouse lines would allow the beta cell specific knockdown of target beta cell modulators and bioimaging would enable the follow- up of disease in the transgenic mice in a non- invasive manner. We also identified an shRNA that could knockdown the beta cell modulator menin and hope to generate a menin knockdown mouse line in the pRLM background to study the effects of beta cell modulation in the development of spontaneous diabetes.

In conclusion, type 1 diabetes affects around 0.5% of the population in developed countries and the incidence rates have been going up over the years. The destruction of beta cells is irreversible and the current therapy available to patients only manages the symptoms and does not prevent the associated pathological manifestations. The patients need lifelong therapy and intensive research is being carried out to identify ways to eliminate autoimmune responses directed against pancreatic beta cells and to replace or regenerate beta cells. Our work aimed at analyzing the role of a pro-inflammatory cytokine IL-17A in type 1 diabetes development and also in generating a beta cell reporter mouse line in the NOD background. We generated IL-17A KD mice in the NOD background using RNAi and lentiviral transgenesis. We analyzed diabetes incidence in the IL-17A deficient mice and found that the loss of IL-17A did not protect the transgenic mice from diabetes. Based on our observations, we believe that Th17 cells might not play a critical role in type 1 diabetes through the IL-17A pathway, though they might still be involved in the disease process through alternate pathways. We also generated NOD and NOD-SCID mice, which exhibit beta cell specific luciferase expression combined with the ability to knockdown specific genes in beta cells. We used a single construct, which combined a luciferase sequence and an shRNA expression cassette under the beta cell specific RIP promoter and used lentiviral transgenesis to generate the transgenic mice. These mice will be of use in studying beta cell phenotype resulting from the knockdown of target genes, non- invasively using bioimaging. We characterized an shRNA active against men1 as a model for beta cell regeneration in the context of type 1 diabetes and hope to generate and characterize a menin KD mouse line in the pRLM background. We

believe our work has contributed to diabetes research in generating two reporter mouse lines for diabetes studies and also in demonstrating the lack of a crucial role for IL-17A in type 1 diabetes.

6. Bibliography

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LIST OF PUBLICATIONS

1. Joseph J, Bittner S, Kaiser FMP, Wiendl H, Kissler S. IL-17 silencing does not protect NOD mice from autoimmune diabetes (Acceptable for publication in *Journal of Immunology* with minor revision i.e. addition of an already created figure in the supplementary data)

AFFIDAVIT (Eidesstattliche Erklärung)

I hereby confirm that my thesis entitled: **"Studying the role of Th17 cells in autoimmune diabetes and generation of a beta cell reporter mouse by lentiviral transgenesis"** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature