2013

THE ROLE OF MAF TRANSCRIPTION FACTORS IN TYPE 1 DIABETES

Tania Singh

CONTENTS

TITLE PAGE ---------- 4

ABSTRACT ---------- 5

REVIEW OF THE LITERATURE ---------- 6

1. DIABETES MELLITUS ---------- 6

1.1 Overview ---------- 6

1.2 Symptoms ---------- 6

1.3 Major forms of diabetes ---------- 7

Type 1 Diabetes (T1D) ---------- 7

Type 2 Diabetes (T2D) ---------- 7

2. TYPE 1 DIABETES: BROADER ASPECTS ---------- 8

2.1 Endocrine pancreas ---------- 8

2.2 Insulin: Manager of Glucose Homeostasis ---------- 9

2.3 Aetiology of T1D ---------- 10

Genetic factors linked with T1D ---------- 10

Environmental factors associated with T1D ---------- 10

2.4 Role of immunity in T1D ---------- 11

Introduction to the immune system ---------- 11

Innate Immunity ---------- 11

Adaptive Immunity ---------- 12

2.5 Autoimmunity in T1D ---------- 12

Immune cells implicated in T1D ---------- 12

Direct role of T cells in T1D progression ---------- 13

Thymus: School of thymocyte education ---------- 14

Major autoantigens predicted in T1D ---------- 15

INTRODUCTION ---------- 16

3. PROJECT DESCRIPTION ---------- 16

3.1 Project1: Analyzing the specific role of beta cells and the immune system in the Type 1 Diabetes symptoms observed in Maf deficient animals. ---------- 17

3.2 Project 2: Regulation of intra-thymic autoantigens expression by Maf transcription factors ----- 19

RESEARCH DESIGN AND METHODS ---------- 21

PROJECT 1 ---------- 21

Mice ---------- 21

Immunohistochemistry analysis ---------- 21

PROJECT 2 ---------- 24

Mice ---------- 24

Thymus collection ---------- 24

Genomic DNA extraction from mice embryo tails ---------- 24

Genotyping assay ---------- 24

RNA extraction ---------- 25

RNA quality assessment ---------- 25

cDNA synthesis ---------- 25

Quantitative PCR (Q-PCR) ---------- 25

RESULTS & DISCUSSION ---------- 26

Project1: Analyzing the specific role of beta cells and the immune system in the Type 1 Diabetes symptoms observed in Maf deficient animals ---------- 26

$MafA^{KOhp}$MafB+/- Vav-Cre animals develop symptoms of autoimmune response to β cells --------- 26

Development of peri-insulitis: Filtration of CD3+T cells ---------- 26

Loss of insulin+ cells in $MafA^{KOhp}$MafB+/- Vav-Cre animals ---------- 30

No signs of T cell infiltration in RIP-CRE model system ---------- 30

Project 2: Regulation of intra-thymic autoantigens expression by Maf transcription factors ------------ 31

Ins2 mRNA levels were not affected by loss of MafA and MafB expression ---------- 31

MafB does not regulate other autoantigens involved in the susceptibility of T1D ---------- 34

Autoimmune regulators are not regulated by Maf transcription factors ---------- 36

CONCLUSION ---------- 38

REFERENCES ---------- 39

ACKNOWLEDGMENTS ---------- 43

SUPPLEMENTARY DATA ---------- 44

Supplementary Tables ---------- 44

Total number of islets per pancreata sample analysed (Project1) (S.Table 1). ---------- 44

Total number of E18.5 embryos analysed under each genotype category (Project2) (S.Table 2) ---- 44

Primer list for genotyping (Project2) (S.Table 3) ---------- 45

Genotyping PCR programmes (Project2) (S.Table 4) ---------- 45

Genotype determination (Project2) (S.Table 5) ---------- 46

Q-PCR PCR programme (Project2) (S.Table 5) ---------- 46

Q-PCR Primer sequences (Project2) (S.Table 5) ---------- 47

Other major protocols followed in Project 2 ---------- 48

DNA extraction protocol ---------- 48

RNA extraction protocol ---------- 48

THE ROLE OF MAF TRANSCRIPTION FACTORS
IN
TYPE 1 DIABETES

Project 1
Analyzing the specific role of beta cells and the immune system in the Type 1 Diabetes symptoms observed in Maf deficient animals.

Project 2
Regulation of intra-thymic autoantigens expression by Maf transcription factors.

BY

TANIA SINGH

MASTERS THESIS

Submitted in partial fulfillment of the requirements for the degree of Masters of Science in Molecular Biology, Molecular Genetics and Biotechnology in the Faculty of Science, Department of Biology at Lund University, 2013

Lund, Sweden

Supervisor: Isabella Artner, PhD
Associate Professor
Endocrine Cell Differentiation and Function
Faculty of Medicine
Lund University

Opponent: Ewa Sitnicka, PhD
Associate Professor
Hematopoietic Stem Cell Laboratory
Faculty of Medicine
Lund University

ABSTRACT

Type 1 Diabetes is an autoimmune disease characterized by chronic hyperglycemia that results from a selective loss of insulin secreting pancreatic beta cells. The aetiology of T1D often involves both genetic and environmental factors like viral infections. In T1D, insulin is regarded as primary autoantigen that becomes one of the targets of autoimmune attack and is found to be expressed in both pancreatic islets and the thymus. Mechanisms behind the induction of central tolerance in governed by the thymus as it expresses tissue specific self-antigens. Here, we study the role of Maf transcription factors in T1D susceptibility due to its widespread role in transcriptional regulation of insulin and other beta cell factors that also act as islet autoantigens. Recent data suggest that variations in the Maf levels may cause susceptibility to viral infections that potentially trigger autoimmune response to self-tissues. Our previous studies have shown that deficiency of Maf transcription factors leads to the development of inflammation adjacent to islets. Infiltration of immune cells can be triggered by defects in the systemic immune system and/or in beta cells. The first project was designed to distinguish between these possibilities and evaluate the effect of changes in Maf levels on the development of autoimmune reaction. Here we examine if the T1D symptoms observed in the Maf mutant mice are caused by a systemic or local defect in beta cells. The second project focuses on defining the specific roles of Maf in intrathymic regulation of major autoantigens at the E18.5.

REVIEW OF THE LITERATURE

1. DIABETES MELLITUS

1.1 Overview

Diabetes mellitus is a life-long disease characterized by high blood sugar levels in the affected individuals as a result of insulin deficiency and/or resistance/insensitivity. This condition is referred to as chronic hyperglycemic state which causes disturbances in the metabolism of carbohydrates, fats and proteins due to the combined or individual defects in insulin secretion (abnormal cyclicity, diminished pulse frequency), insulin action and dysfunctional response to glucose levels [1],(1). This impairment in glucose homeostasis is one of the primary causes of morbidity and mortality in humans.

In healthy individuals, glucose uptake from the bloodstream and storage in the liver and peripheral tissues is controlled by insulin. Depending on the body energy requirements, glycogen becomes metabolized by gluconeogenesis and glycolysis. Thus insulin acts like a key for the cells that facilitates the movement/absorption of glucose by opening gated channels (2). Disturbances in the process of insulin secretion, lack of insulin producing beta (β) cells and abnormality in insulin sensitivity are broadly the reasons for the development of diabetes.

1.2 Symptoms

Classic diabetes symptoms are Hyperglycemia (high blood sugar levels), polydipsia (increased thirst), polyuria (excess urine production), polyphagia (hunger), unexplained weight loss, fatigue, blurry vision, recurrent infections etc. Serious health problems can arise in long term, if left untreated, including atherosclerosis, retinopathy, neuropathy, kidney failure, cardiovascular and

gastrointestinal defects. Diabetes management or maintenance of good glycemic levels becomes highly necessary for avoiding the above listed complications of long-term diabetes. With a tight diet regulation, administration of insulin or hyperglycemic agents and controlled physical activity, metabolic imbalances can be kept in check in diabetic patients for years.

1.3 Major forms of diabetes

Type 1 Diabetes (T1D)

T1D is the most severe form of diabetes, characterized by absolute insulin deficiency. It is a chronic cell-mediated immune disease characterized by the selective loss of insulin secreting pancreatic β cells, which most likely results from a slowly acting autoimmune process, depending on the presence of both genetic and environmental factors [2-4]. Manifestation of this disease often commences in early life beginning from infants and the development of full form clinical T1D can take from a few months to several years, with the incidence of new T1D cases doubling in children younger than 5 years according to current figures and a possible 70% increase in the number of cases in children younger than 15 years between 2005-2020 [5]. A more than fivefold rise in the T1D incidence rate has been recorded in Finland over the last 50 years [6]. In T1D patients, it is vital to compensate for the loss of endogenous insulin by daily monitored insulin injections.

Type 2 Diabetes (T2D)

This is the most prevalent form of diabetes and accounts for about 90 % of all diabetes cases. It is also known as noninsulin-dependent diabetes or adult-onset diabetes. It is caused by impaired β cell function and peripheral insulin resistance i.e. the body cannot effectively respond to the insulin that is produced [2]. Treatment involves diet, exercise and oral drug administration and

rarely requires external insulin injections for survival. T2D progresses slowly and shows milder forms of hyperglycemia. In adults its usually develops after the age of forty and is associated with inactive life style and obesity.

2. TYPE 1 DIABETES: BROADER ASPECTS

2.1 Endocrine pancreas:

The pancreas plays an essential role in secreting and synthesizing hormones and enzymes that maintain the metabolic and nutritional homeostasis [7]. Different tissue components of this organ include the acinar, ductal, and endocrine portion. Endocrine cells form a close and compact, circular structure of cluster of cells termed islet of Langerhans after the name of the German scientist, Paul Langerhans, who identified them in pancreatic tissue in 1869 (3). Endocrine tissue forms the smallest volume in the entire organ accounting for the total of 1-2%. About 1 million islets are dispersed throughout the human endocrine pancreas. Islets numbers varies from dozens to hundreds in murine animal models and is easily distinguishable from other portions of similar looking tissues within the pancreas due to its ovoid and compact structure. Islet clusters are specialized micro-organs comprised of five cell sub-types called alpha (α), beta (β), delta (δ), epsilon (ε) and PP cells. Insulin secreting β cells account for 70-80% of islet cells whereas α cells which produces the insulin antagonist glucagon, occupies mostly the islet periphery and comprise 20-25% of islet cells. δ (~5%), ε (< 1%) and PP (~1%) cells secrete somatostatin, ghrelin and pancreatic polypeptide, respectively.

2.2 Insulin: Manager of Glucose Homeostasis

The mysterious factor controlling the blood glucose level was discovered in the early 1920's by the collective efforts of Dr. Frederick Banting and Prof. John Macleod at Toronto University, who isolated insulin, for which they received the Nobel Prize in Medicine in 1923 (3).

Insulin is a globular protein belonging to a peptide hormone family, consisting of two chains, A and B which are 21 and 30 residues respectively [8]. Preproinsulin is the precursor of insulin which results from transcription of the insulin gene (*INS*) that lies on human chromosome 11. Preproinsulin contains a leader sequence marked by a 24-amino acid (a.a.) N-terminus and is cleaved immediately to generate proinsulin on translocation into the endoplasmic reticulum from the nucleus [8]. It is further processed in the endoplasmic reticulum to yield proinsulin polypeptide composed of A and B chains and a connecting C-peptide. During transport into Golgi apparatus, further proinsulin becomes converted into insulin and C-peptide (31 a.a.) via proteolytic cleavage [8]. Finally both C-peptide and insulin are stored in secretory granules/vesicles [8]. Insulin and C-peptide are released to the portal circulation as a reaction to the rise in the blood glucose level whereby insulin dissociates into active insulin monomers and C- peptide that are released in equimolar amounts. Thus C-peptide can be used as a marker of endogenous insulin secretion and β cell function [8].

Insulin plays a central role in glucose metabolism through its actions in the liver and is predominantly expressed by the pancreatic β cells and in low copy number in the thymus. In the mouse, two nonallelic *INS* gene copies, *Insulin 1* (*Ins1*) and *Insulin 2* (*Ins2*) on chromosome 19 and 7, respectively are present and transcripts from both genes are found in the pancreas whereas only *Ins2* is expressed in the thymus. Its role in the thymus is discussed later. Preproinsulin and

proinsulin precursors including the final insulin product have been identified to be a key antigen in the autoimmune destruction of β cells by immune cells and autoantibodies [9-11].

2.3 Aetiology of T1D

Both genetic and environmental factors have been attributed to play a role in initiation and progression of T1D.

Genetic factors linked with T1D

In T1D, strong clustering of susceptibility genes is found particularly in the human leukocyte antigen (HLA) region on Chromosome 6p21, specifically in the HLA-DRB1-DQB1 haplotypes. Other genetic loci associated with T1D are variable number of tandem repeats near the insulin gene (INS-VNTR); Cytotoxic T lymphocyte-associated antigen-4 (CTLA4); and Protein tyrosine phosphatase non-receptor-type 22 (PTPN22) [12]. Protein products of these genes play a crucial role in defining the major interactions between antigen presenting cells (APCs) and T cells and presumably effect the development and function of autoreactive T cells. Hereditary disease development is 6% in T1D patient sibling and in monozygotic twins concordance rate is found to be between 30-50% [13].

Environmental factors associated with T1D

The role of environmental factors is highlighted by the increase in the number of cases in the past few years. This is faster than what can be accounted for by genetic change alone. Viral infections (Encephalomyocarditis [14], Coxsackie B [15, 16], Rubella [17] and Parvovirus [18]), toxins, chemicals and dietary factors like vitamin D deficiency, exposure to milk protein casein and wheat protein gluten [19] may enhance the risk of T1D development/progression by either direct effects on β cells or by modulation of intermediate molecules and cells .

2.4 Role of immunity in T1D

Research in the past few years has asserted that autoimmune abnormalities are involved in cases of T1D. However it’s still not clear how exactly these autoimmune abnormalities are associated with pancreatic β cell damage. If these are the sole causative factor, a reason in part, the end result or just a marker, it is still to be known. Thus understanding every aspect of the autoimmune response in T1D becomes necessary and calls for extensive research in finding key factors involved in T1D susceptibility.

Introduction to the immune system

The immune system guards our body from foreign pathogenic agents like bacteria, parasites, fungi, toxins etc. It involves a highly complex network of cells, tissues and organs working in dynamic and corporate manner to generate cascades of immune responses against invading microbes, infected cells and tumors while ignoring the self-healthy tissues. This phenomenon eradicates the majority of infectious threats and provides a healthy internal body environment. Recognition of self-tissues (self-antigens) leads to the development of autoimmune diseases like T1D, Grave’s disease, Rheumatoid arthritis etc.

Innate Immunity

Innate immunity provides a first line of defence by monocytes and granulocytes including neutrophils that are activated in the initial phase of inflammation. Monocytes later differentiate into macrophages which enhances the infection signal. Macrophages act on the microorganisms by either engulfing or by releasing chemotactic cytokines. These act like messengers to attract other immune cells that bear receptors for specific chemokines/cytokines and initiate a cascade of immune subjected inflammatory process. Another major player in enhancing the immune reactivity against an infection are dendritic cells, that are the main APCs, as their primary

function is to present antigens and play a crucial role in the induction/activation of adaptive immune responses (T lymphocytes). Innate immunity lacks the capability of establishing long term memory and lots of pathogens adapt and gain the ability to surpass these innate immune mechanisms. This enables the second part of immune response to take the initiative i.e. adaptive immune response which fights back pathogenesis.

Adaptive Immunity

Components of the adaptive immune system are lymphocytes including T and B cells. These have the ability to specialize and recognize foreign or non self-antigens specifically and neglecting other self-antigens (own body cells). T cells are responsible for cell-mediated immunity and B cells act by producing antigen-specific antibodies. They also have the ability to develop long term memory cells that can initiate quick response on similar or same repeated infections in the future.

2.5 Autoimmunity in T1D

Immune cells implicated in T1D

A number of immune cells are known to populate the pancreatic infiltrate and draining lymph node of T1D patients and mouse models like Macrophages, Natural killer cells (NK), NK-T cells, B cells and Dendritic cells. Dendritic cells are the major APCs that may process and present β cell specific self-proteins to cytotoxic T cells. Its capability of activating T cells and production of cytokines have been shown to have a pathogenic role in enhancement of autoimmune reaction [20]. In addition several studies have identified activated NK cells in the T1D patients, suggesting a role in β cell destruction [21]. Functionally NK cells are cytotoxic and secrets cytokines like interferon γ, tumor necrosis factor etc. that induces cell death in

infected cells and tumors. Furthermore it has been reported that the killing of virus-infected β cells is NK cell-mediated and independent of T cells [22]. Role of B cells in the disease progression have also been studied due to the presence of autoantibodies in T1D susceptible individuals. Studies have shown that B cell depletion in NOD (non obese diabetic) mice prevents autoreactive T cell interaction with self–antigens and reduction of autoantibodies, suggesting their extra role as APCs besides autoantibody production [23-25]. All of these immune cells individually and/or collectively contribute to destruction of β cells in T1D. In the current project, our preliminary focus was on the T cell infiltration in pancreatic tissue.

Direct role of T cells in T1D progression

Interactions between infectious agents and immune cells can influence the activation of T cells and enhance tolerance. But this process is obstructed in individuals that carry mutations in T1D susceptibility alleles.

All T cells express the surface antigen CD3 (cluster of differentiation) that is a member of the immunoglobulin superfamily, composed of five polypeptide chains (4). T cells can be further classified into various subclasses depending on their expression of CD4 and CD8. T helper (Th) cells and regulatory T cells (Treg) develop from CD4+ T cells whereas cytotoxic T cells are the differentiated product of CD8+ T cells.

In T1D, β cells might be destroyed by islet antigen-specific T cells via their interaction through processed antigens on APCs through major histocompatibility complex (MHC) class I molecules [26]. Production of pro-inflammatory cytokines (interferon-γ, interleukin-1β, tumor necrosis factor etc.) can further elevate immune responses by activating macrophages. Also, these cytokines can induce reactive oxygen species that can mediate β cell apoptosis [20].

With the above mentioned pathogenicity of T cells, there is evidence of presence of T cells that might have a protective role in controlling the onset of T1D. These are the CD4+ regulatory T (T_{reg}) cells that are known to maintain the immune tolerance and help to protect the self from an unwanted unnatural autoimmune destruction [27].

Thymus: School of thymocyte education

The thymus is often referred to as a primary or central lymphoid organ due to its capability to express many tissue-specific self-antigens [28]. Self/non-self discrimination by the immune system is a prerequisite for survival of multicellular organisms [28, 29], as it directs varied and powerful defense mechanisms against foreign antigens/pathogens while maintaining tolerance/nonresponsiveness towards self-antigens [28]. The thymus plays a pivotal role in the maturation of T-cells from hematopoietic precursors [30]. Large receptor diversity of about 25 million specificities [31] are established in the T-cell repertoire through random differential gene rearrangements including the generation of self-reactive T-cells [32]. Self-reactive T cells pose an immediate threat of autoimmunity [33]. T cells that carry receptors specific for autoantigens are eliminated (negative selection) by an intense screening procedure (clonal deletion; clonal anergy) [28, 30] and only those developing T cells that show allowable (low) affinity between T cell receptors and self-peptide-MHC (major histocompatibility complex) are selected (positive selection) [28]. Those cells which survive screening procedures mature and migrate from the thymus to the peripheral lymphatic organs, where they can be activated in response to recognition of peptides through interaction with MHC-peptide complexes on APCs [28, 30]. Less appropriate amount of relevant autoantigen in the thymus may affect the elimination procedure of self-reactive T cells in the thymus. Similarly in T1D, low expression of

autoantigens like insulin in the thymus may let self-reactive T cells bypass stringent selection mechanisms and pose a direct risk to initiate autoimmune damage.

Major autoantigens predicted in T1D

Immune reactivity against self-antigens makes them fall in the category of autoantigens and a possible threat of autoimmunity. The phenomenon of epitope spreading may result in the recognition of multiple antigens presented on islet cells in the overall progression of immune reactivity in T1D [19]. Many of these have been listed in the pathogenesis of T1D. Presence of autoantibodies marks the first detectable signs of autoimmunity against β cells in clinical T1D. Studies have shown five main disease related autoantibodies, that are ICA (islet cell antibody), IAA (insulin autoantibodies), GAD65 (65kD isoform of glutamic acid decarboxylase), IA-2 (protein tyrosine phosphatase-related islet antigen-2 molecule) and IGRP/G6pc2 (islet specific G6Pase catalytic subunit-related protein) [19]. Recently ZnT8 (zinc transporter Slc30A8) has been recognized as a T1D autoantigen [34]. Presence of more than one autoantibody increases the possibility T1D development. Molecular mimicry mechanisms have also been thought to induce autoreactivity against initial self-antigen epitopes, where self-antigens can mistakenly be confused with processed foreign antigens (like bacterial or viral proteins) due to structural similarities and cause confusion between self and non-self that leads to cross-reactivity and autoimmune destruction of self tissues [19].

INTRODUCTION

3. PROJECT DESCRIPTION

Disease progression leading to the development of T1D is highly affected by the selective autoimmune destruction of β cells by autoreactive T cells. More than 80% of β cells are destroyed by the time T1D is detected clinically. Today the role of innate and adaptive immune cells involvement in the autoimmune destruction of β cells is clearly known. Thymocyte education to distinguish between self and non-self in the thymus is also very important for the generation of central tolerance and the very fact that impairment in this selection procedure may lead to the escape of autoreactive T cells in the peripheral lymphatic system, poses an immediate threat in generating an autoimmune response against β cells, thereby leading to T1D.

To be able to develop or design possible diabetes treatment strategies like generating β cell sources through de novo β cell differentiation, it becomes important to understand the molecular pathways underlying β cell development and function, as defects in its function will ultimately affect individuals to maintain healthy glycemic control. Understanding of the immune responses against β cells in both T1D and health status of isolated islets used for transplantation and characterization of islet cells enriched transcription factors involved in β cell development, maturation and function will be of high value. There are many key transcription factors / regulators like Pdx1, Nkx6.1, NeuroD1, Ngn3, Pax6 etc., which are important for pancreatic islet cell formation during development and activity in the adult [35]. Maf (musculoaponeurotic fibrosarcoma) transcription factors (TFs), do not only control the maturation of islet pancreatic cells but are also needed for their effective functional maintenance like glucose induced insulin

transcription [36]. Their embryonic expression has significant effect in guiding the differentiation of embryonic islet cell types and function.

The large Maf proteins are basic leucine zipper transcription factors with a transactivation domain and a DNA binding domain that recognises Maf-recognition DNA element (MARE) and target genes like *INS* [37, 38]. They are known to regulate diverse biological events like lens differentiation, segmentation of hind brain and hematopoiesis [39] including macrophage development [40] , T-cell development [41], and pancreatic alpha (α) and beta (β) cell development [42-46].

In our study we focus on the two Maf TFs MafA and MafB, due to their significance in islet cell maturation and function. These factors regulate key islet cell genes like Glut2, Pdx1, Nkx6.1, Pax6 etc. in a sequential and cooperative manner [44].

Recent data suggest that elevated large Maf protein levels in β cells cause susceptibility to viral infection and subsequent inflammation and T1D [47]. Also deficiency in large Maf proteins is likely to alter the composition of the immune system which may lead to an increase in local and/or systemic autoimmune inflammations and can lead to a gradual loss of β cells [47]. This is the scientific foundation for design of project 1, where we study the role of Maf factors in development of inflammatory symptoms in T1D susceptible mouse models.

Project 1: Analyzing the specific role of beta cells and the immune system in the Type 1 Diabetes symptoms observed in Maf deficient animals.

Results from previous studies in our group showed that 6 month old MafA-/-MafB+/- mice (systemic model) developed peri-insulitis, with inflammation detected around the islets (7% of all islets). These inflammatory cells were found to be condensed and expressed CD3, a T cell

marker. This model represented a study system with very low Maf levels as MafA from the entire genetic set up was completely deleted and only one copy of MafB was present. Occurrence of inflammation in this model may be caused by the absence of Maf proteins in the immune system or alternatively in β cells or a combination of defects in both may lead to autoimmune reactions against β cells. To distinguish between these possibilities project 1 was designed where loss of MafA and MafB expression in β cells and the immune system were studied further. To evaluate the role of Mafs in the development of T1D like symptoms in the systemic model two other model systems were designed namely a hematopoietic cell-specific system and a β cell-specific system, where MafA was conditionally [48] removed from the entire hematopoietic cell lineage ($MafA^{KOhp}$) or β cells ($MafA^{KO\beta}$), respectively. Mouse models for this project were generated by crossing $MafA^{fl/fl}$ mice [42] with transgenic mice expressing Cre recombinase under the control of regulatory regions from VAV (guanine nucleotide exchange factor) and RIP (rat insulin promoter) promoters, respectively. The *Vav* gene is almost exclusively restricted to hematopoietic cells, whereas RIP expression is restricted to β cells only. Thus use of VAV- and RIP-CRE transgenic mice [49, 50] results in conditional deletion of the *MafA* gene in hematopoietic cell lineage (entire immune system) or β cells, respectively.

To determine if a deficiency in Maf TFs alters the leukocyte cell population and leads to the infiltration of T cells in pancreatic tissue, pancreata of six month old Vav-Cre $MafA^{KOhp}$MafB+/+, $MafA^{KOhp}$MafB+/- and Rip-Cre $MafA^{KO\beta}$MafB+/+, $MafA^{KO\beta}$MafB+/- mutant mice with their respective wild-type (WT) controls were collected and stained with antibody specific for the T cell marker CD3, insulin and a nuclear marker DAPI using immunohistochemistry. In total seventeen pancreata were studied with three mice in each genotype category except two in Rip-Cre controls (S.Table 1).

With these two model systems we were able to study the role of Maf deficiency specifically in the lymphocytes and in the pancreatic β cells. In this study, TID symptoms were observed in two out of three MafA^{KOhp}MafB+/- Vav-Cre mice, showing condensed clusters of T cells around the islets (peri-insulitis) and in the islet vicinity. This highlights the importance of Maf TFs to the cells of immune system. The absence of T cell infiltration in the RIP-CRE model system suggests that low Maf levels in β cells are not sufficient to induce the development of T1D symptoms.

3.2 Project 2: Regulation of intra-thymic autoantigens expression by Maf transcription factors

Mechanisms involved in the interplay between the central and the peripheral tolerance provide the remarkable ability to protect against pathogenic invaders and autoimmunity. Defects in either or both of the tolerance mechanisms may cause development of autoimmune diseases like T1D.

Previous studies have shown that impaired thymic expression of autoantigens results in susceptibility to autoimmune diseases [28, 51]. Lack of tolerance or autoreactivity can be attributed to the low levels of antigens [30, 33, 52]. Thymic levels of the autoantigen insulin may be one of the crucial factors affecting the development of T1D as it has already been shown that insulin is expressed in the human thymic medulla [51].

Insulin is the primary T1D antigen that is expressed in both, pancreatic β cells and in rare cells of the thymic medulla [53, 54]. MafA and MafB are known to regulate *Insulin* expression in β-cells [37, 43] and previous studies suggest that MafA regulates *Ins2* expression in the thymus [51]. It has been hypothesized that loss or reduced MafA function can lead to impaired deletion of insulin specific autoreactive T cells and increased susceptibility to T1D [51]. In the developing β

cell MafB expression precedes MafA and MafB has a more significant role in *Insulin* transcription [36, 42, 44].

Here we evaluate if MafB has a similar role in the regulation of thymic *Ins2* transcription and also if we can reproduce results presented by Noso et al, which suggest that MafA regulates *Ins2* expression in the thymus [51]. Analyses of other autoantigens like Glut-2, G6pc2, IA-2 and Slc30A8 indicated in T1D susceptibility were also studied to assess a possible role of Maf TFs in their regulation in the thymus. As controls, AIRE and thyroglobulin gene expression were studied.

Here, Thymi from MafA and MafB mutant mouse embryos at embryonic day 18.5 (E18.5) were isolated and analyzed through quantitative-polymerase chain reaction (Q-PCR) to detect mRNA expression of *MafA, MafB, Ins2, AIRE, IA-2, Slc30A8, G6pc2, Glut2* and *Thyroglobulin*. E18.5 was chosen since MafB deficient mice die at birth due to central apnea and/or renal failure [44].

Data obtained from this current study suggests that Maf expression in E18.5 thymus is not required for the expression of *Ins2*. *Ins2* expression is maintained even in the absence of MafA and MafB. However, expressions of other autoantigens are affected by the alterations in MafA levels but not by MafB. Suggesting the role of Maf proteins specifically MafA in an intra-thymic regulation of other autoantigens at E18.5.

RESEARCH DESIGN AND METHODS

PROJECT 1

Mice

Vav-Cre and Rip-Cre mice strains including wild-type (WT) controls, with the following genotype were used in the study respectively: $MafA^{KOhp}$MafB+/+, $MafA^{KOhp}$MafB+/-; $MafA^{KO\beta}$MafB+/+ and $MafA^{KO\beta}$MafB+/-. Animals were housed and bred in accordance with regulations for the protection of laboratory animals, after approval from a local ethical committee.

Immunohistochemistry analysis

Pancreata from 6 month old mice with the above mentioned genotypes were collected. Serial sections throughout the entire pancreas were made and immunostained at 120µm section depth (interval). Sections were analysed microscopically and the total numbers of islets with- and without- inflammation were recorded.

Fixation and Paraffin imbedding:

Pancreata were fixed overnight in 4 % paraformaldehyde (SIGMA) at 4°C. Successively pancreata were washed in phosphate buffered saline (PBS, GIBCO), dehydrated in 70% ethanol (3*10 min), 100% ethanol (1*60 and 2*30 min) (Kemetyl), tissue clear (2*30 min) (Histolab) and paraffin (SIGMA) (2*60 min). Fixed and processed pancreata were embedded in paraffin block.

Sectioning via microtome:

6µm sections were cut using a microtome (MICROM, HM355S), mounted on glass slides (Thermo SCIENTIFIC) and kept overnight on a glass warmer (LABLINE) at 37°C to fix the tissues to the slides. Sections were stored at 4°C until further usage.

Paraffin removal:

Paraffin sections were de-waxed in the following solutions, tissue clear (2*5 min); 100% ethanol (2*5 min); 75% ethanol (1*4 min); 50% ethanol (1*4 min) and distilled water (1*5 min).

Antigen retrieval treatment:

De-waxed and dehydrated tissue sections on slides were heated in 10mM TEG buffer (Trizmabase, EGTA (SIGMA); pH-9.0) for 7.5 min at 90 volts in a microwave (Whirlpool). Slides were kept at room temperature for 25-30 minutes for cooling.

Immunostainings:

Slides were washed in PBS (3*5min) and sections were covered with a blocking solution (5% donkey serum in 1 % bovine serum albumin (BSA) (Jackson ImmunoResearch Laboratories) in PBS) in a humidified slide holder for 60 minutes.

Primary antibodies:

Rabbit anti-CD3 (Abcam) and guinea pig anti-insulin (Linco) at dilutions of 1:200 and 1:2,000 respectively were diluted in 5% donkey serum in 1 % BSA in PBS. 80µl of primary antibodies mixture was used to cover each section. Slides were incubated overnight at room temperature (RT) in a humidified slide holder.

Secondary antibodies:

After washing with PBS, secondary antibodies with Cy3- and Cy2-conjugated anti-rabbit and anti-guinea pig antibodies (Jackson ImmunoResearch Laboratories) were used respectively at a dilution of 1:500. 80µl of secondary antibody mixture was used to cover each section. Sections were incubated for 120 minutes at RT in a humidified slide holder.

Nucleus staining:

DAPI (Invitrogen) in 1 % BSA in PBS at dilution of 1:6000 was applied on the PBS washed sections for 10 min at RT.

Mounting sections:

Finally sections were covered with fluorescent mounting medium (DAKO) and a glass cover slip. Immunostained slides were kept in light protective cardboard holders.

Microscopy and Data analysis:

The number of islets in each section was counted and recorded. Immunofluorescence images of all sections with CD3+ cells around or in vicinity of islet cell were collected with Zeiss Axioplan 2 imaging (Zeiss, Germany) at a magnification of 20X in AxioVision Rel 4.9 software and saved. Total number of CD3+ cells were counted and used for final calculations and result interpretations.

PROJECT 2

Mice

MafA+/-MafB+/- mice were bred to generate total WT, MafA-/-MafB+/+; MafA-/-MafB+/- and MafA+/+MafB-/- littermates.

Thymus collection

Thymi were dissected from E18.5 embryos in ice cold PBS (GIBCO) and stored in RNA stabilizing solution (RNA later, Ambion) at -20°C.

Genomic DNA extraction from mice embryo tails

Genomic DNA was extracted from embryo tail tissue for genotyping (S. Data 1).

Genotyping assay

a. Genomic DNA was amplified by PCR using red taq DNA polymerase (SIGMA-ALDRICH), 2X bufferD (Epicentre) with four sets of primers (PCR primers and programmes are listed in S. Table 3-4).

b. PCR products were separated by agarose gel electrophoresis. 1.5% agarose gels (Ultra Pure) were prepared in TBE buffer (1M Tris/0.9M Boric acid/0.01M EDTA; PH8.0; Sigma). Gel red nucleic acid stain (Biotium) was used to stain the gel. To assess the DNA band size 100bp and 1kb DNA ladders (Thermo scientific; Fermentas life sciences resp.) were used.

c. Gels were visualized by an ultraviolet illuminator (Kodak) and the gel pictures were captured by a Kodak camera. DNA band assessments in all the samples lead to the detection of specific genotypes (S. Table 5).

RNA extraction

Total RNA from E18.5 mouse thymus was prepared using the RNeasy mini kit (Qiagen) and treated with RNase free DNaseI set (Qiagen). Eluted RNA was dissolved in RNase free water and stored at -80°C (S. Data 2).

RNA quality assessment

RNA was analysed with an Agilent 2100 bioanalyzer. Only RNA samples with RIN (RNA integrity number) higher than 7 were used for cDNA synthesis. RNA concentrations were measured with a Nano Drop ND-1000 spectrophotometer, and RNA concentrations were equalised for cDNA synthesis.

cDNA synthesis

cDNA was prepared with superscript III reverse transcriptase (RT), oligo (dT) primer, 5X first strand buffer, 0.1M DTT (Invitrogen), RNase OUT ribonuclease inhibitor (Recombinant). Controls were prepared without superscript III RT (RT negative control).

Quantitative PCR (Q-PCR)

Q-PCR measurements were performed using a Step One Plus real-time PCR system (Applied Biosystems). 20µl reaction mixture containing 10.5µl Fast SYBR Green master mix (Applied Biosystems), 2µl of 10nM forward-reverse primer mix, 5µl milli-Q water and 2.5µl cDNA template were used. (Q-PCR programme and Primer sequences are listed in the S. Table 6-7). For each run, RT negative control, Q-PCR negative control (blank) and a reference sample (HPRT) were included. Each sample was measured in triplicates and average C_T values were used for gene expression analysis. Gene expression data were normalized against HPRT expression. Q-PCR data were analysed by the comparative C_t method (ΔC_T method).

RESULTS & DISCUSSION

Project 1: Analyzing the specific role of beta cells and the immune system in the Type 1 Diabetes symptoms observed in Maf deficient animals.

Immunohistochemical analysis of CD3+ T cells to detect inflammation adjacent to islets (peri-insulitis) and/or inside the islet (insulitis) in six month old Maf mutant pancreata (Vav-Cre and Rip-Cre) were performed. In total 17 pancreata samples were analyzed including mutants with their respective controls (S.Table 1). In all pancreata sections analyzed, lymph nodes for the assurance of CD3 staining were checked and no sign of T cell infiltration was observed in control samples (Fig 1). Islets with more than twenty CD3+ T cells infiltration (inflammation symptoms) were analyzed and further categorized into two groups i.e. condensed cluster of CD3+ T cells **in contact** with an islet; and condensed cluster of CD3+ T cells **not in contact** with an islet (Fig 2.1-2).

MafA^{KOhp}MafB+/- Vav-Cre animals develop symptoms of an autoimmune response to β cells

Development of peri-insulitis: Filtration of CD3+T cells

The presence of activated β cell specific T cells in pancreatic tissue is well evident in T1D autoimmunity. Exact mechanisms and factors leading to this invasive reaction by the T cells remains unexplained. Our focus in this project was to determine how the loss of Maf transcription factors affects β cells as well as the immune system in generating T1D symptoms

(T cell infiltration), as reduction in Maf TFs, particularly in MafA may produce defects in β cells and the immune system or both.

Controls: No sign of CD3+ T cells infiltration in wild-type islets whereas CD3+ T cells in the lymph node were present

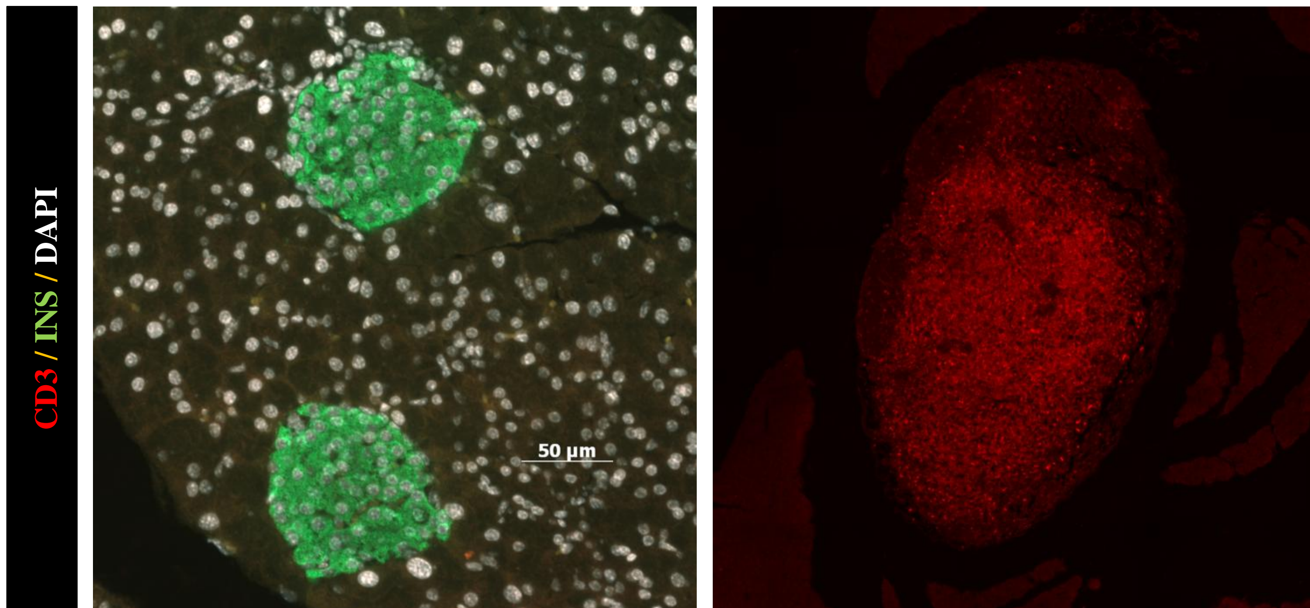


Figure 1: Immunohistochemical detection of islet (wild-type) with insulin staining (green) and nucleus (white) with DAPI staining (left), shows no sign of inflammation. CD3+ T cells with CD3 staining (red) in lymph node (right).

The VAV-CRE model system, where MafA was conditionally removed from the entire hematopoietic system, showed signs of CD3+T cells infiltration in $MafA^{KOhp}$MafB+/- mutants (Fig 2.1-2). This was observed in two out of the three Vav-Cre mutant samples with 2.4% of islets with peri-islet inflammation (Fig 2.3). Nonoccurrence of this phenotype in the third mutant may be due the possibility of inefficient conditional deletion of MafA from hematopoietic cells. Penetrance efficiency of Vav-Cre in these models will be checked in the future by analysis of β-galactosidase activity from a R26R reporter allele [55, 56] .

During the microscopic analysis of immunostained sections, CD3+ T cell infiltration was clearly seen in association with an islet, suggesting the importance of autoantigens of islet cells in

CD3+ T cells infiltration in MafA^{KOhp}MafB+/- islets (VAV-CRE model system)

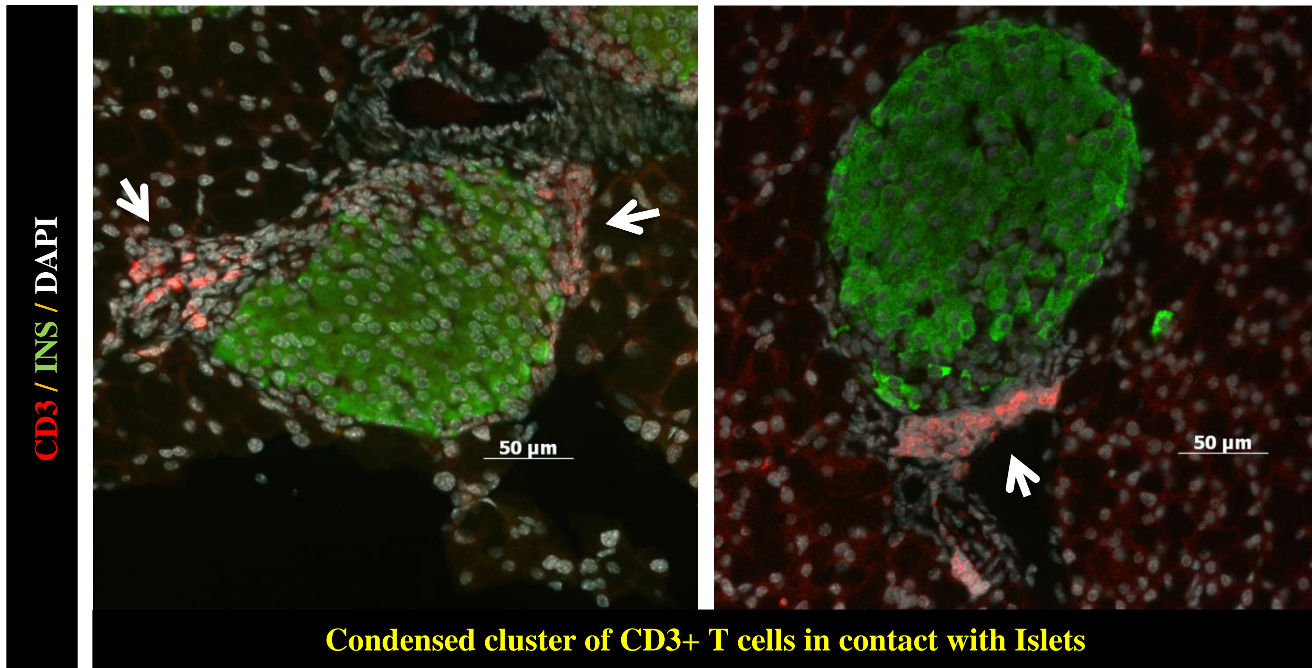


Figure 2.1 Immunohistochemical detection of mutant islet with insulin (green), T cells with CD3 (red) and nucleus (white) with DAPI staining (Arrows point to the condensed cluster of CD3+ T cells in contact with islets).

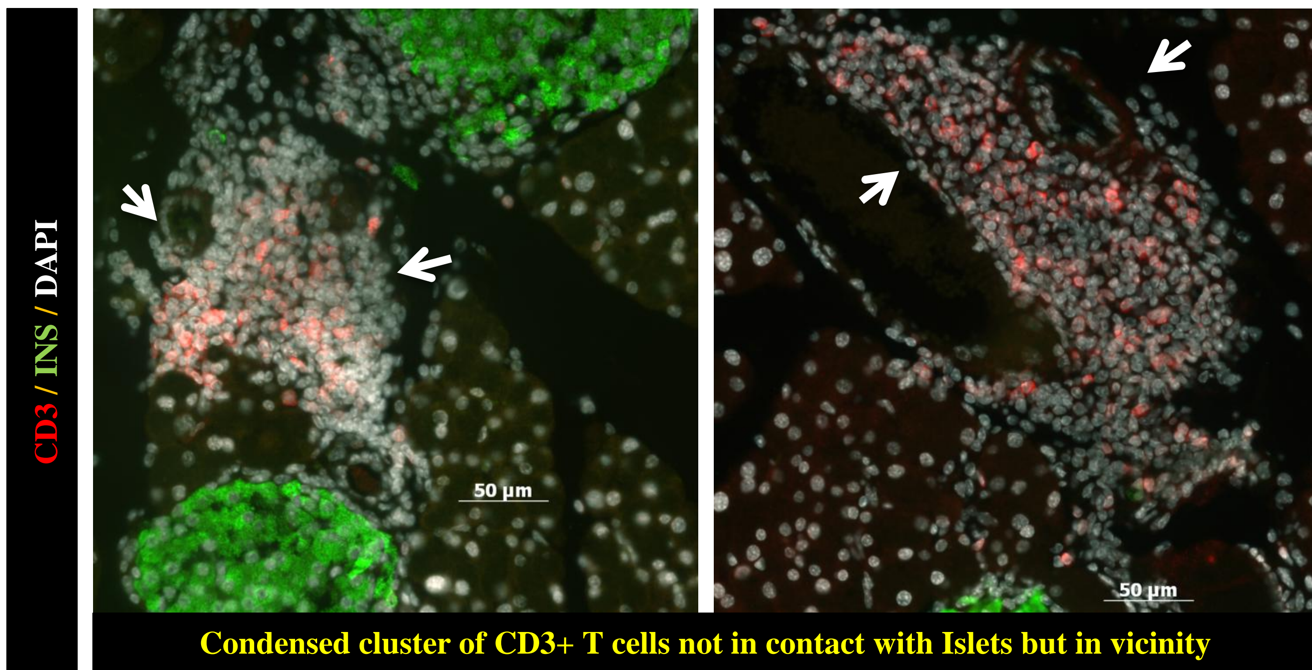


Figure 2.2: Immunohistochemical detection of mutant islet with insulin (green), T cells with CD3 (red) and nucleus (white) with DAPI staining (Arrows point to the condensed cluster of CD3+ T cells in vicinity of islets).

regulating the autoimmune response by T cells whereas, the non-islet pancreatic tissue region looked normal without any sign of T cell infiltration.

Inflammation in the vicinity of islets also reflects the importance of Mafs in affecting the repertoire of autoreactive T cells (Fig 2.2). Reduction in Maf levels in the hematopoietic cell lineage may affect the T cell development and its maturation process in the thymus and/or its activation pace in the peripheral lymphatic pool upon recognition of an autoantigen. Thus, Mafs deficiency in immune system may have the potential to alter it and may be one of the causative factors in generating T1D symptoms in Vav-Cre mutants.

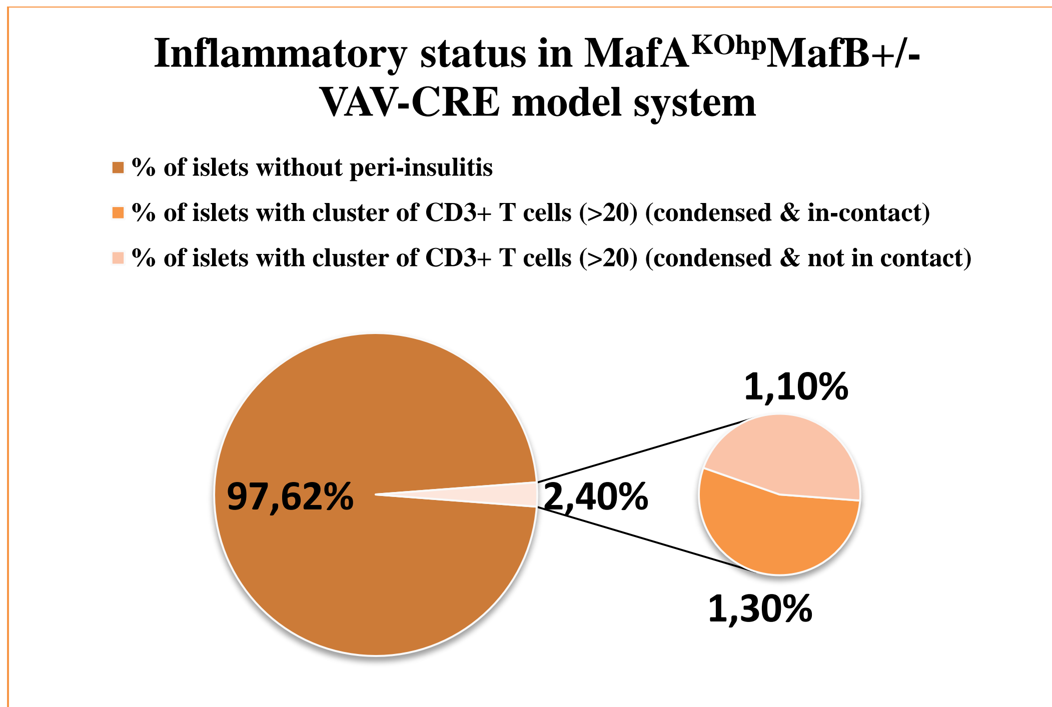


Figure 2.3: Total number of islets and islets with more than twenty CD3+ T cells around or in vicinity were counted. Data represent number of islets with inflammation among the total number of non affected islets.

Loss of insulin+ cells in MafA^{KOhp}MafB+/- Vav-Cre animals

T1D results from the loss of insulin producing β cells. The same phenomenon we observed in our Vav-Cre mutants where islets with CD3+T cell infiltration showed signs of loss of insulin+ cells/ β cell mass (Fig 2.4). This observation suggests the role of autoreactive T cells in autoimmune destruction of β cells.

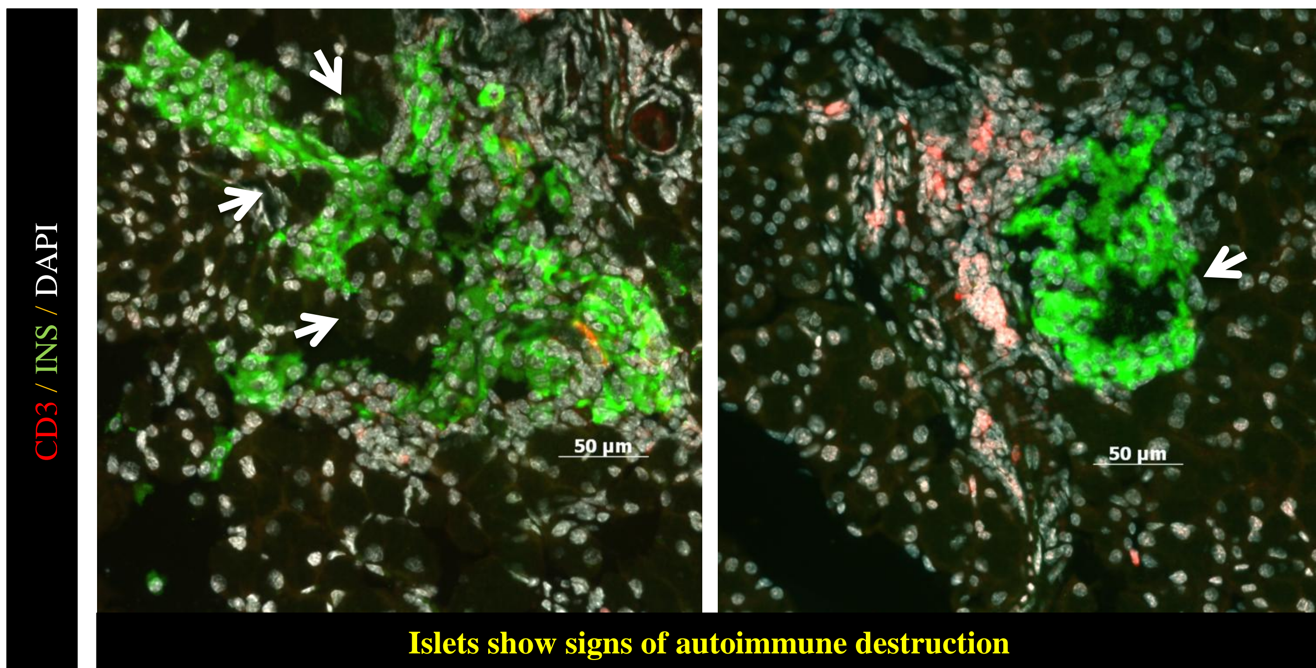


Figure 2.4: Signs of islet destruction that may correspond to autoimmune attack (Arrows point to the site of damage within islets).

No signs of T cell infiltration in RIP-CRE model system

In MafA$^{KO\beta}$MafB+/+ and MafA$^{KO\beta}$MafB+/- Rip-Cre mice, none of the six samples analyzed displayed signs of T cell infiltration, reflecting no role of Maf TFs in producing β cells that are defective. This indicates that Maf reduction in β cells is not sufficient to induce autoimmune attack/reaction.

Therefore, the knowledge gained from the current analysis suggests that the development of peri-insulitis in MafA-/-MafB+/- systemic model system is most likely a result of defects in the immune system and not in the β cells. Thus, Maf deficiency in the leukocyte cell population alone may be responsible for the activation of autoimmune response against islet antigens that may eventually lead to the development of T1D.

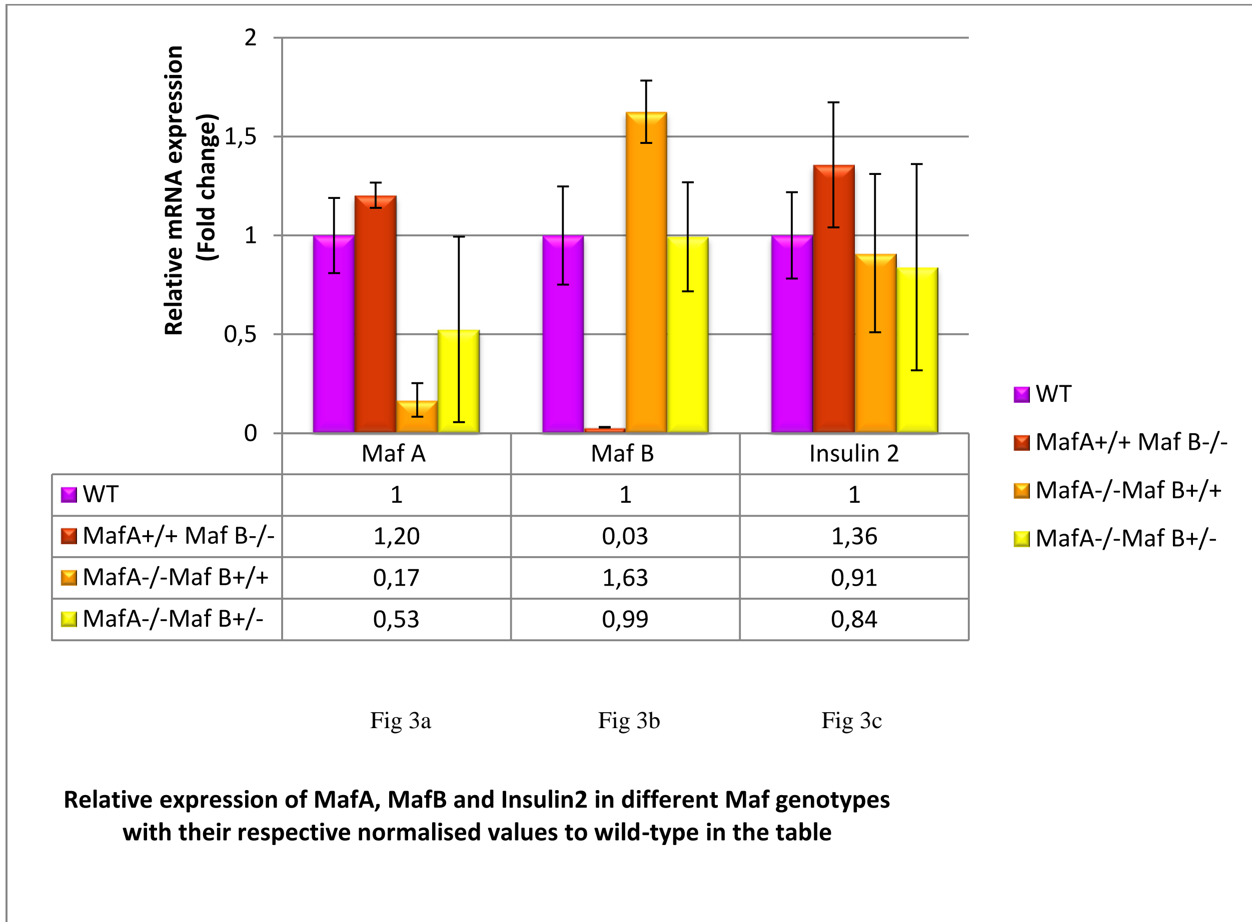
Project 2: Regulation of intra-thymic autoantigens expression by Maf transcription factors

Q-PCR analysis of *MafA, MafB, Insulin2, AIRE, IA-2, Slc30A8, G6pc2, Glut2* and *Thyroglobulin* was performed on thymic cDNA of E18.5 Maf mutants and wild-types.

Ins2 mRNA levels were not affected by loss of MafA and MafB expression

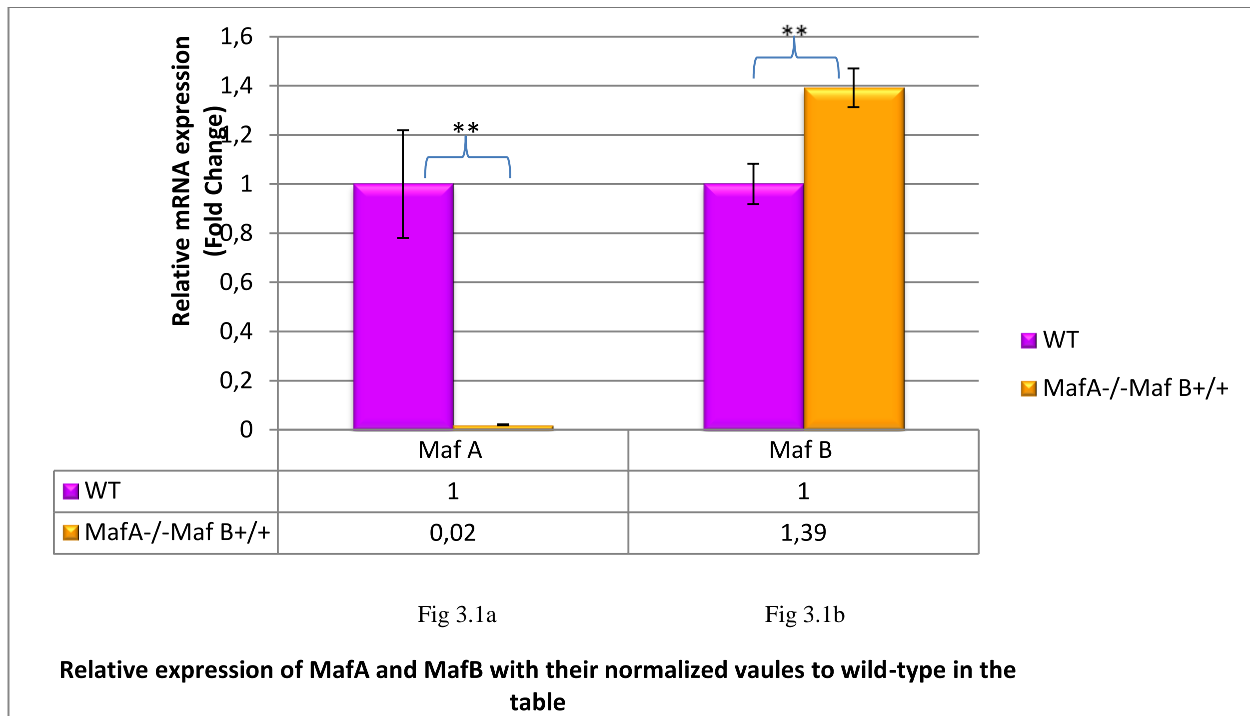
To study if loss of MafA and/or MafB affects Ins2 expression in the thymus, the transcription of MafA, MafB and Ins2 were studied in MafA and MafB single knockout mice (Fig 3). As expected MafA expression was much reduced in the homozygous knock-out mice (MafA-/-) compared to the wild-type mice, nevertheless small levels of MafA expression were still observed in the MafA-/- mutants (Fig 3a). For ruling out the possibility of genomic DNA contamination and/or MafA primer efficacy in this case, control samples with no reverse transcriptase (RT negative control) were analyzed. C_T values from control samples appeared very late with more than 5-6 cycle number difference with reference to C_T values from MafA expression in Maf mutants, which rules out the possibility of false positive detection due to non-specific amplification signal. MafA primer sequence, its binding efficiency to the target sequence and probability of primer dimer formation were also checked via bioinformatics tools, analysis of amplification plot and melt curve of amplified products in wild-type and mutant samples respectively. These analyses inferred no problems with the MafA primer design. Another reason

could have been due to sample (with different genotypes) contamination via aerosol transfer during the 96 well plate pipetting procedure and this was checked by repeating the Q-PCR experiment with similar genotype run at the same time, this resulted in a complete loss of MafA amplification in MafA-/- mutants (Fig 3.1a). Thus this problem was resolved.



	Maf A	Maf B	Insulin 2
WT	1	1	1
MafA+/+ Maf B-/-	1,20	0,03	1,36
MafA-/-Maf B+/+	0,17	1,63	0,91
MafA-/-Maf B+/-	0,53	0,99	0,84

Figure 3: *Ins2* transcription is unaffected by the loss of MafA or MafB in E18.5 mice embryos. Q-PCR analysis of *MafA* (3a), *MafB* (3b) *and Insulin2* (3c) transcription in the thymus of Wild-type and Maf mutant cDNA samples according to their relative gene expression levels normalized to HPRT and wild-types that was set as 1, are depicted here. Data in chart is expressed with means ± standard error.



	Maf A	Maf B
WT	1	1
MafA-/-Maf B+/+	0,02	1,39

Figure 3.1: *MafA* downregulation in MafA-/-MafB+/+ mutant E18.5 embryos. Q-PCR analysis of expression of *MafA* (3.1a) and *MafB* (3.1b) in the thymus of Wild-type and Maf cDNA samples according to their relative gene expression levels normalized to HPRT and wild-types that was set as 1, are depicted here. Data in chart is expressed with means ± standard error. ($P<0.05$)

MafB expression was maintained in MafA-/- mice and was completely lost in the MafB-/- sample (Fig 3b). *Ins2* expression in all the genotypes was complementary to each other. Strikingly *Ins2* expression was not significantly reduced in MafA-/- mutant, as previously shown by Noso et al (Fig 3c). This may be due to different age of the samples tested, since the time point of sample collection was not provided in this study [51]. No significant alterations in Ins2 levels in different mutant cDNA samples, suggest that Maf expression at this time point (E18.5) is not required for *Ins2* transcription in the thymus in contrast to the described roles in β cell function and maturation in both developing and adult animals.

Larger error bars/variation in the normalized Q-PCR data might be the result of variations in the age of the different litters or due to variability of RNA templates or difference in the number of

gene expressing cells (APCs). Also the lack of statistical significance of our results may be due to the low sample size (S.Table 2). Nevertheless, C_T and final normalized values generated for each gene under each mutant category was highly consistent which suggests that the assay design, methodology and data analysis were appropriate. To further evaluate a possible role of Maf factors in *Ins2* transcription in the thymus, postnatal (P) mRNA expression analysis will be performed on one week (P7) and/or two week (P14) old Maf mutant mice.

MafB does not regulate other autoantigens involved in the susceptibility of T1D.

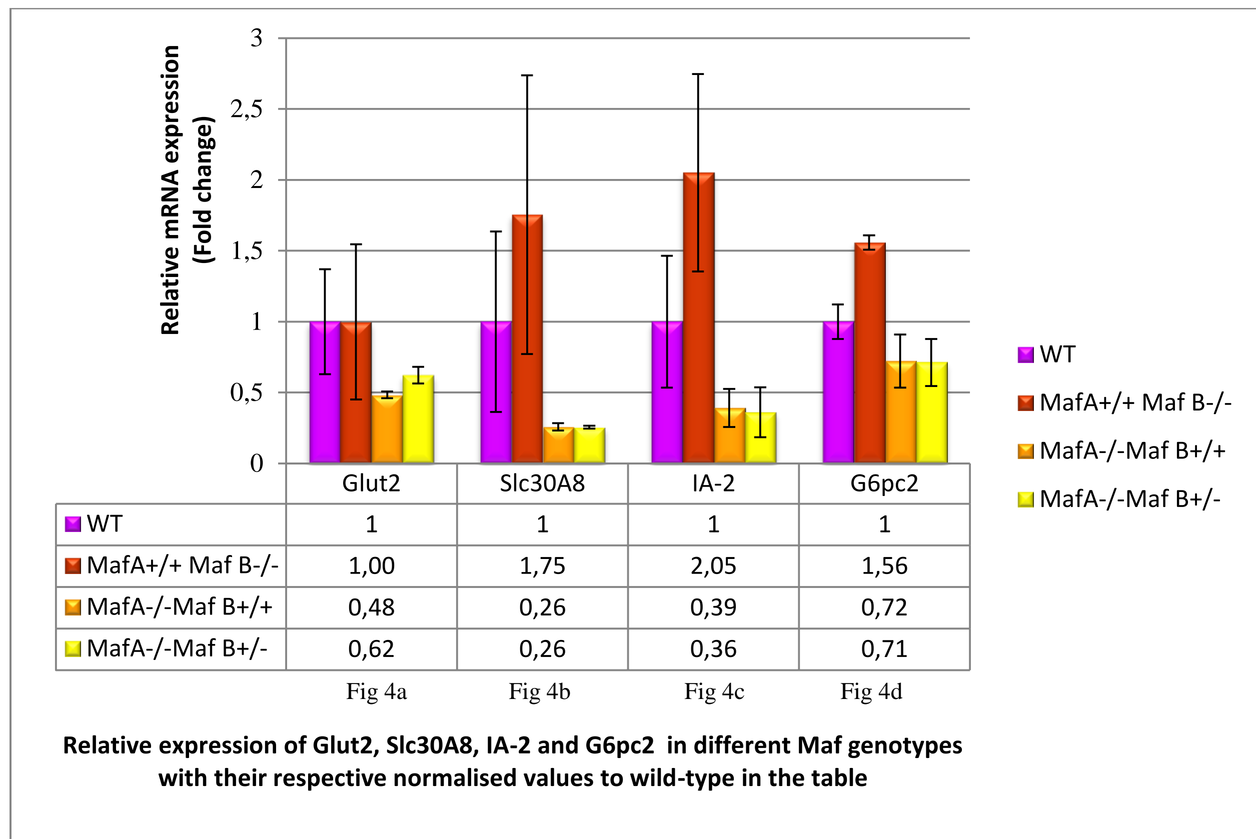
Expression of *IA-2*, *Slc30A8*, *G6pc2* and *Glut-2* genes were examined by Q-PCR to test if Maf TFs are important for the transcription/regulation of these T1D autoantigens (Fig 4). These genes have been examined here since they all represent key β-cell genes involved in normal maturation and functioning of islet cells and because of their primary role as autoantigens in T1D [57]. Previous studies have shown that MafB regulates Glut-2, Slc30A8 and G6pc2 expression in the pancreas [42, 44, 45]. Here we examined if Maf TFs are also important for their transcription in the thymus.

Glut-2 (Glucose transporter) enables passive glucose movement across the cell membrane and is mainly expressed in the liver and β-cells. Previous studies have shown that Glut-2 transcription is depended on MafB since loss of MafB results in low Glut-2 expression in β cells [10, 12].

IA-2 (Islet Antigen) is regarded as a primary autoantigen in T1D [27] and its deletion results in impaired glucose induced insulin secretion. Thus it acts as a positive regulator of insulin secretion. Similarly another major component of T1D autoimmunity is Slc30A8 (Zinc transporter) which is important for insulin storage and secretion in β cells [58]. Both these genes

appeared in the Q-PCR analysis between cycle numbers 26-28 approximately in all wildtype samples but appeared 3-4 cycle numbers late in MafA-/- mutants.

G6pc2 (Glucose-6-phosphatase) regulates the glucose sensing mechanism within pancreatic islet cells by converting glucose-6-phosphate back to glucose. Studies have reported that it is also a specific target of cell-mediated autoimmunity in T1D [10].



	Glut2	Slc30A8	IA-2	G6pc2
WT	1	1	1	1
MafA+/+ Maf B-/-	1,00	1,75	2,05	1,56
MafA-/-Maf B+/+	0,48	0,26	0,39	0,72
MafA-/-Maf B+/-	0,62	0,26	0,36	0,71

Figure 4: Gene expression profile of other important autoantigens. Q-PCR analysis of expression of *Glut2* (4a), *Slc30A8* (4b), *IA-2* (4c) and *G6pc2* (4d) *in* the thymus of Wild-type and Maf mutant cDNA samples according to their relative gene expression levels normalized to HPRT and wild-types that was set as 1, are depicted here. Data in chart is expressed with means ± standard error .

Results here strongly indicate that in the presence of MafA (Wildtype and MafA+/+MafB-/-), expression of all autoantigens is maintained whereas in MafA-/- mutants, more than 50% reduction in Slc30A8 and IA-2 levels were observed (Fig 4b-c). This implies that expression of

these autoantigens might be dependent on MafA. Also reduced Glut-2 and G6pc2 expression in MafA deficient mutant highlights the importance of MafA in their regulation in both β cells as well as in the thymus (Fig 4a, 4c).

In contrast to the role of MafB in the pancreas, MafB doesn't have a specific role in regulation of these autoantigens at E18.5 stage, as its absence does not affect their expression in MafB-/- mutants. Instead MafA demonstrates its specific role in regulating their expression in the thymus at E18.5. Variation in the gene expression from different genotype samples is not significant and this may be caused by low sample size and/or age variation between different litters.

Autoimmune regulators are not regulated by Maf transcription factors.

AIRE (Autoimmune regulator) acts as a transcriptional co-regulator and is required for the intra-thymic expression of numerous tissue-restricted self-antigens expressed in medullary thymic epithelial cells (mTEC) [59]. Here AIRE was used as a marker to represent the number of APCs. Therefore, AIRE expression was assessed to determine if changes in the insulin and other autoantigens expression level were caused by alterations in the number of thymic epithelial cells. AIRE expression was not affected in any of the genotypes considered (Fig 5a); suggesting the reduction of other autoantigens levels in MafA deficient mice was not due to a loss of AIRE expression, indicating the presence of similar numbers of APCs in Maf mutant thymus.

Thyroglobulin is an autoantigen associated with thyroiditis, another organ specific autoimmune disease [60]. In the present study it was used as a positive control to show that the expression of other/non related self-antigens were not affected by the loss of Maf transcription factors. Thyroglobulin expression was observed to be consistent and unaffected in all genotypes except in MafB-/- mutant embryos (Fig 5b). Thyroglobulin was observed very late between cycle

numbers 31-33 which reflects its lower expression in the thymus. This may lead to an expression pattern that is more susceptible to variations and may be the reason for much lower thyroglobulin expression in MafB-/- mutant.

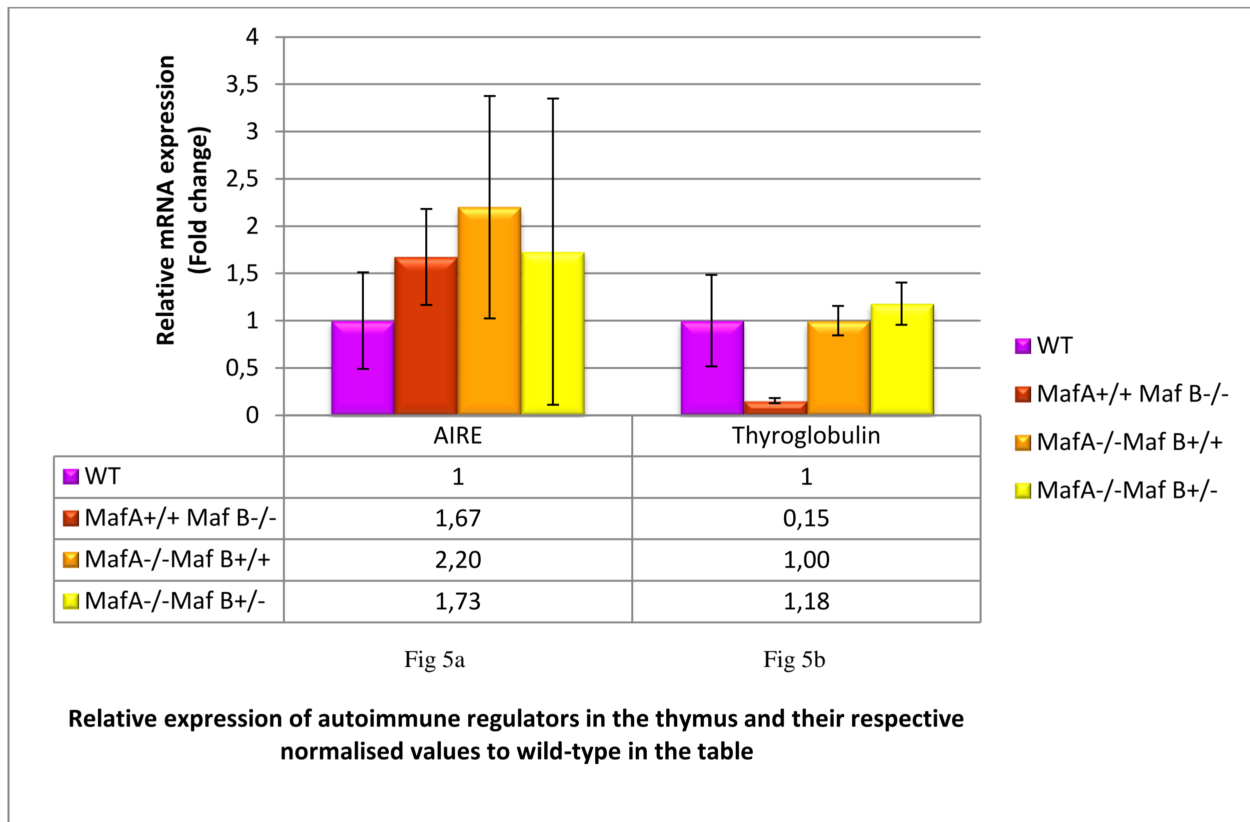


Figure 5: Gene expression profile of autoimmune regulators in the thymus. Q-PCR analysis of expression of *AIRE* (5a) and *Thyroglobulin* (5b) in the thymus of Wild-type and Maf cDNA samples according to their relative gene expression levels normalized to HPRT and wild-types that was set as 1, are depicted here. Data in chart is expressed with means ± standard error.

CONCLUSION

Multidisciplinary research at different levels has to be focused for the understanding of molecular pathogenesis and designing of preventive therapies for T1D. The ultimate goal/challenge will be to generate β cells in-vitro for the transplantation purposes in T1D patients. Identifying the functional role of factors involved in embryonic and adult β cells development and the disease-related factors in protecting or provoking the autoimmune response will be of key importance in reviving natural and newly transplanted β cells from the autoimmune destruction and controlling the pathogenicity of immune cells.

In Project 1, we observed that the absence of Maf factors in the β cell-specific system was not sufficient to induce T1D like symptoms as none of the mice studied showed signs of T cell infiltration. Whereas in the hematopoietic cell-specific system, condensed cluster of T cells were observed around and in vicinity of islets, suggesting the potential of Maf TFs in altering the leucocyte cell population and the risk of T1D development. This deficiency in Maf proteins may alter the composition of the immune system through their function in hematopoiesis, particularly in development and maturation of T cells, NK cells, macrophages and other antigen presenting cells. Additionally, role of Maf TFs as autoantigen regulators in the thymus may also play an important role in the induction of central tolerance, as observed in Project 2 by MafA in affecting the expression of major autoantigens marked in T1D susceptibility.

Overall data from both the projects suggest that alterations in the expression of Maf TFs in the pancreas and the organs of immune system may increase the susceptibility for T1D development by an autoimmune destruction of islet β cells.

REFERENCES

1. *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2008. **31 Suppl 1**: p. S55-60.
2. Zimmet, P., K.G. Alberti, and J. Shaw, *Global and societal implications of the diabetes epidemic.* Nature, 2001. **414**(6865): p. 782-7.
3. Atkinson, M.A. and G.S. Eisenbarth, *Type 1 diabetes: new perspectives on disease pathogenesis and treatment.* Lancet, 2001. **358**(9277): p. 221-9.
4. Devendra, D., E. Liu, and G.S. Eisenbarth, *Type 1 diabetes: recent developments.* BMJ, 2004. **328**(7442): p. 750-4.
5. Patterson, C.C., et al., *Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study.* Lancet, 2009. **373**(9680): p. 2027-33.
6. Knip, M., *Environmental triggers and determinants of beta-cell autoimmunity and type 1 diabetes.* Rev Endocr Metab Disord, 2003. **4**(3): p. 213-23.
7. Kordowich, S., A. Mansouri, and P. Collombat, *Reprogramming into pancreatic endocrine cells based on developmental cues.* Molecular and Cellular Endocrinology, 2010. **323**(1): p. 62-69.
8. Culina, S., V. Brezar, and R. Mallone, *Insulin and type 1 diabetes: immune connections.* Eur J Endocrinol, 2013. **168**(2): p. R19-31.
9. Jasinski, J.M. and G.S. Eisenbarth, *Insulin as a primary autoantigen for type 1A diabetes.* Clin Dev Immunol, 2005. **12**(3): p. 181-6.
10. You, S. and L. Chatenoud, *Proinsulin: a unique autoantigen triggering autoimmune diabetes.* J Clin Invest, 2006. **116**(12): p. 3108-10.
11. Baker, C., et al., *Human CD8 responses to a complete epitope set from preproinsulin: implications for approaches to epitope discovery.* J Clin Immunol, 2008. **28**(4): p. 350-60.
12. Gillespie, K.M., *Type 1 diabetes: pathogenesis and prevention.* CMAJ, 2006. **175**(2): p. 165-70.
13. Anaya, J.M., et al., *Familial clustering of autoimmune diseases in patients with type 1 diabetes mellitus.* J Autoimmun, 2006. **26**(3): p. 208-14.
14. Lee, Y.S., et al., *Role of nitric oxide in the pathogenesis of encephalomyocarditis virus-induced diabetes in mice.* J Virol, 2009. **83**(16): p. 8004-11.
15. Atkinson, M.A., et al., *Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes.* J Clin Invest, 1994. **94**(5): p. 2125-9.
16. Horwitz, M.S., et al., *Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry.* Nat Med, 1998. **4**(7): p. 781-5.
17. Ou, D., et al., *Cross-reactive rubella virus and glutamic acid decarboxylase (65 and 67) protein determinants recognised by T cells of patients with type I diabetes mellitus.* Diabetologia, 2000. **43**(6): p. 750-62.

18. Tirabassi, R.S., et al., *Infection with viruses from several families triggers autoimmune diabetes in LEW*1WR1 rats: prevention of diabetes by maternal immunization.* Diabetes, 2010. **59**(1): p. 110-8.
19. Thrower, S.L. and P.J. Bingley, *What is type 1 diabetes?* Medicine, 2010. **38**(11): p. 592-596.
20. Lehuen, A., et al., *Immune cell crosstalk in type 1 diabetes.* Nat Rev Immunol, 2010. **10**(7): p. 501-13.
21. Rodacki, M., et al., *Altered natural killer cells in type 1 diabetic patients.* Diabetes, 2007. **56**(1): p. 177-85.
22. Brauner, H., et al., *Distinct phenotype and function of NK cells in the pancreas of nonobese diabetic mice.* J Immunol, 2010. **184**(5): p. 2272-80.
23. Bour-Jordan, H., et al., *Constitutive expression of B7-1 on B cells uncovers autoimmunity toward the B cell compartment in the nonobese diabetic mouse.* J Immunol, 2007. **179**(2): p. 1004-12.
24. Silveira, P.A., et al., *The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors.* Eur J Immunol, 2002. **32**(12): p. 3657-66.
25. Serreze, D.V. and P.A. Silveira, *The role of B lymphocytes as key antigen-presenting cells in the development of T cell-mediated autoimmune type 1 diabetes.* Curr Dir Autoimmun, 2003. **6**: p. 212-27.
26. Thomas, H.E., et al., *Beta cell apoptosis in diabetes.* Apoptosis, 2009. **14**(12): p. 1389-404.
27. Juedes, A.E. and M.G. von Herrath, *Regulatory T-cells in type 1 diabetes.* Diabetes Metab Res Rev, 2004. **20**(6): p. 446-51.
28. Hanahan, D., *Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity.* Curr Opin Immunol, 1998. **10**(6): p. 656-62.
29. Kyewski, B. and J. Derbinski, *Self-representation in the thymus: an extended view.* Nat Rev Immunol, 2004. **4**(9): p. 688-98.
30. Heath, V.L., et al., *Intrathymic expression of genes involved in organ specific autoimmune disease.* J Autoimmun, 1998. **11**(4): p. 309-18.
31. Arstila, T.P., et al., *A direct estimate of the human alphabeta T cell receptor diversity.* Science, 1999. **286**(5441): p. 958-61.
32. Goodnow, C.C., et al., *Cellular and genetic mechanisms of self tolerance and autoimmunity.* Nature, 2005. **435**(7042): p. 590-7.
33. Kronenberg, M. and A. Rudensky, *Regulation of immunity by self-reactive T cells.* Nature, 2005. **435**(7042): p. 598-604.
34. Wenzlau, J.M., et al., *The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17040-5.
35. Cerf, M.E., *Transcription factors regulating beta-cell function.* Eur J Endocrinol, 2006. **155**(5): p. 671-9.
36. Matsuoka, T.A., et al., *Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells.* Mol Cell Biol, 2003. **23**(17): p. 6049-62.
37. Hang, Y. and R. Stein, *MafA and MafB activity in pancreatic beta cells.* Trends Endocrinol Metab, 2011. **22**(9): p. 364-73.

38. Kataoka, K., *Multiple mechanisms and functions of maf transcription factors in the regulation of tissue-specific genes.* J Biochem, 2007. **141**(6): p. 775-81.
39. Nishimura, W., et al., *A switch from MafB to MafA expression accompanies differentiation to pancreatic Î²-cells.* Developmental Biology, 2006. **293**(2): p. 526-539.
40. Hegde, S.P., et al., *c-Maf induces monocytic differentiation and apoptosis in bipotent myeloid progenitors.* Blood, 1999. **94**(5): p. 1578-89.
41. Ho, I.C., D. Lo, and L.H. Glimcher, *c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms.* J Exp Med, 1998. **188**(10): p. 1859-66.
42. Artner, I., et al., *MafA and MafB regulate genes critical to beta-cells in a unique temporal manner.* Diabetes, 2010. **59**(10): p. 2530-9.
43. Zhang, C., et al., *MafA is a key regulator of glucose-stimulated insulin secretion.* Mol Cell Biol, 2005. **25**(12): p. 4969-76.
44. Artner, I., et al., *MafB is required for islet beta cell maturation.* Proc Natl Acad Sci U S A, 2007. **104**(10): p. 3853-8.
45. Artner, I., et al., *MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells.* Diabetes, 2006. **55**(2): p. 297-304.
46. Artner, I., et al., *MafA is a dedicated activator of the insulin gene in vivo.* J Endocrinol, 2008. **198**(2): p. 271-9.
47. Kim, H. and B. Seed, *The transcription factor MafB antagonizes antiviral responses by blocking recruitment of coactivators to the transcription factor IRF3.* Nat Immunol, 2010. **11**(8): p. 743-50.
48. Rajewsky, K., et al., *Conditional gene targeting.* J Clin Invest, 1996. **98**(3): p. 600-3.
49. Georgiades, P., et al., *VavCre transgenic mice: a tool for mutagenesis in hematopoietic and endothelial lineages.* Genesis, 2002. **34**(4): p. 251-6.
50. Lee, J.Y., et al., *RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function.* J Biol Chem, 2006. **281**(5): p. 2649-53.
51. Noso, S., et al., *Insulin transactivator MafA regulates intrathymic expression of insulin and affects susceptibility to type 1 diabetes.* Diabetes, 2010. **59**(10): p. 2579-87.
52. Derbinski, J., et al., *Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels.* J Exp Med, 2005. **202**(1): p. 33-45.
53. Kent, S.C., et al., *Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope.* Nature, 2005. **435**(7039): p. 224-8.
54. Nakayama, M., et al., *Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice.* Nature, 2005. **435**(7039): p. 220-3.
55. Mao, X., Y. Fujiwara, and S.H. Orkin, *Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice.* Proc Natl Acad Sci U S A, 1999. **96**(9): p. 5037-42.
56. Soriano, P., *Generalized lacZ expression with the ROSA26 Cre reporter strain.* Nat Genet, 1999. **21**(1): p. 70-1.
57. Boitard, C., *Pancreatic islet autoimmunity.* Presse Med, 2012. **41**(12 P 2): p. e636-50.
58. Wijesekara, N., et al., *Beta cell-specific Znt8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion.* Diabetologia, 2010. **53**(8): p. 1656-68.
59. Zumer, K., K. Saksela, and B.M. Peterlin, *The Mechanism of Tissue-Restricted Antigen Gene Expression by AIRE.* J Immunol, 2013. **190**(6): p. 2479-82.

60. Caputo, M., et al., *Analysis of thyroglobulin gene polymorphisms in patients with autoimmune thyroiditis.* Endocrine, 2010. **37**(3): p. 389-95.

Internet source: (Written in ( ) parenthesis in the report)

1. http://www.who.int/diabetes/action_online/basics/en/index.html 20022013

2. http://iamyourpancreas.blogspot.se/p/what-is-type-1-diabetes.html 20022013

3. http://www.nobelprize.org/educational/medicine/insulin/ 05102012

4. http://link.springer.com/chapter/10.1007%2F978-3-211-79280-3_183 25022013

ACKNOWLEDGMENTS

This study is supported by the Juvenile Diabetes Research Foundation International, Swedish Research Council and Barndiabetesfonden.

I thank Isabella Artner for designing the study and giving me the opportunity to work under her supervision; Magdalena Mazur, Elvira Ganic for supporting me throughout the project and for providing friendly and motivational work environment in the lab; Jesper Karup, Marie Magnusson for helping me in handling the mice.

SUPPLEMENTARY DATA

Supplementary Tables

Total number of islets per pancreata sample analysed (Project 1) (S.Table 1).

	Vav-Cre Mice (MafA^{KOhp})						Non Vav-Cre Mice		
	MafA^{KOhp}MafB+/-			MafA^{KOhp}MafB+/+			CONTROL		
Sample No.	V:1	V:2	V:3	V:4	V:6	V:7	C:V:1	C:V:2	C:V:3
Total islets	342	322	517	491	610	397	958	449	428
Total number of islets with CD3+ T cell infiltration	12	4	0	0	0	0	0	0	0
Total number of CD3+ T cells	443	213	0	0	0	0	0	0	0
	Rip-Cre Mice (MafA$^{KO\beta}$)						Non Rip-Cre Mice		
	MafA$^{KO\beta}$MafB+/-			MafA$^{KO\beta}$MafB+/+			CONTROL		
Sample No.	R:1	R:2	R:3	R:4	R:5	R:6	C:R:1	C:R:2	C:R:3
Total islets	319	326	321	277	278	171	405	380	-NA-
Total number of islets with CD3+ T cell infiltration	0	0	0	0	0	0	0	0	-NA-

Total number of E18.5 embryos analysed under each genotype category (Project 2) (S.Table 2)

Genotype	Total number of samples
MafA+/+ MafB+/+ (WT)	5
MafA+/+ Maf B-/-	6
MafA-/- Maf B+/+	3
MafA-/- Maf B+/-	2

Primer list for genotyping (Project 2) (S.Table 3)

Genotype selection based on the product amplified from following primers	Forward & Reverse primer sequence
MafA -/-	5'-AGC AAG GCET CCT CCA AAC CCC-3' 5'-CAG AAC TGC GCT CCA CGT CTC-3'
MafA Wildtype (Floxed)	5'-AGC AAG GCT CCT CCA AAC CGC CCT-3' 5'-GTA CTC CTT CGG TGT CTC AGA TCC-3'
MafB +/+	5'-CTG GCC CAG ACT CCC TAT TC-3' 5'-TTA CTC CCG GAC CTC GCA C-3'
MafB-GFP	5'-GGA GAG GGT GAA GGA GAT GCT-3' 5'-GAC AGG GCC ATC GCC AAT TGG-3'

PCR programmes used for genotyping (Project 2) (S.Table 4)

Genotype	PCR programme
MafA -/-	**1)** 94°C -5min., **2)** 94°C - 30sec., **3)** 62°C -30sec., **4)** 68°C - 45sec., **5)** Repeat step 2-4 34 times, **6)** 70°C -10min., **7)** 8°C - ∞
MafA Floxed	**1)** 94°C -5min., **2)** 94°C - 30sec., **3)** 62°C -30sec., **4)** 68°C - 45sec., **5)** Repeat step 2-4 34 times, **6)** 70°C -10min., **7)** 8°C - ∞
MafB +/+	**1)** 95°C -5min., **2)** 95°C - 30sec., **3)** 65°C -30sec., **4)** 68°C - 45sec., **5)** Repeat step 2-4 30 times, **6)** 68°C -10min., **7)** 8°C - ∞
MafB-GFP	**1)** 94°C -5min., **2)** 94°C - 30sec., **3)** 60°C -30sec., **4)** 68°C - 45sec., **5)** Repeat step 2-4 34 times, **6)** 68°C -10min., **7)** 8°C - ∞

Genotype determination based on bands retrieved on agarose gel. Broad black lines represent bands corresponding to genotypes marked in first column (Project 2) (S.Table 5)

Genotype	MafA-/-	MafA Floxed	MafB-GFP	MafB+/+
MafA WT		▬		
MafA -/+	▬	▬		
MafA -/-	▬			
MafB WT				▬
MafB +/-			▬	▬
MafB -/-			▬	

PCR programme used in Q-PCR analysis (Project 2) (S.Table 6)

Holding stage: 50°C – 2min., 95°C – 2min. Extension stage: 95°C – 15sec., 60°C – 25sec.,73°C – 30sec. (45 cycles) Melt curve stage: 95°C – 15sec., 70°C – 15sec., 98°C – 15sec.

Primer sequences used in Q-PCR (Project 2) (S.Table 7)

Gene product	Name of the gene	Forward & Reverse primer sequence
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A	5'-GAGGAGGTCATCCGACTGAAA-3' 5'-GCACTTCTCGCTCTCCAGAAT-3'
MafB	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B	5'-GGCAACTAACGCTGCAACTCT-3' 5'-CAACGGAAGGGACTTGAACAC-3'
Insulin2	Insulin 2 RT	5'-GGCTTCTTCTACACACCCAT-3' 5'-CCAAGGTCTGAAGGTCACCT-3'
Glut-2	Glucose transporter 2	5'-CTCCAGGAAGGGTGCTAAACC-3' 5'-TGCTCCCTATCCGTTCTTCAA-3'
IA-2	Islet autoantigen	5'-GTGGCAAGATGACTATACCCAGC-3' 5'-ATGGTCTATCCTAGAGTGTGCAT-3'
Slc30A8	Zinc transporter	5'-CAGAGAACTTCGACAGAAGCC-3' 5'-CTTGCTTGCTCGACCTGTT-3'
G6pc2	Beta-cell specific protein islet glucose-6-phosphatase catalytic subunit-related protein	5'-AGGTGACCCTAAGCCGGAC-3' 5'-TCT TTGGGTAGAAGACCATCCC-3'
AIRE (Control)	Autoimmune regulator	5'-CAGCAACTCTGGCCTCAAAG-3' 5'-CTTCGAACTTGTTGGGTGTATAA-3'
Thyroglobulin (Control)	Thyroid antigen	5'-TCAGGAAGGCACTGCTTATGG-3' 5'-GCCCTCTCTGGGCTGATAATT-3'
HPRT (Reference gene)	Hypoxanthine phosphoribosyl transferase	5'-AGCCCCAAAATGGTTAAGGT-3' 5'-CAAGGGCATATCCAACAACA-3'

Other major protocols followed in Project 2

DNA extraction protocol (S. Data 1)

1. Add 300µl of tail buffer (100mM Tris-HCl pH 8.0, 200mM NaCl, 5mM EDTA, 0.2% SDS) with 3µl of Proteinase K (10µl of Proteinase K per 1ml of the tail buffer)
2. Incubate at 56°C overnight.
3. Add 100 µl of Potassium acetate, shake gently.
4. Spin for 20 min at 14,000 rpm at 4°C.
5. Keep supernatant, add 500 µl of Isopropanol, shake gently.
6. Spin for 20 min at 14,000 rpm.
7. Throw away the supernatant, keep the pellet.
8. Add 200 µl of 70% Ethanol.
9. Spin for 5 minutes at 14,000 rpm.
10. Throw away the supernatant, keep the pellet.
11. Air dry the pellet for 20-30 minutes.
12. Dissolve pellet in 50-100 µl of water or 10mM Tris.
13. Incubate at 37°C overnight.

RNA extraction protocol (adapted from RNeasy Mini QIAGEN guide book) (S. Data 2)

1. Put the thymus sample in a mixture of 600µl of RLT buffer (lysis buffer) and 6µl of β-mercaptoethanol.
2. Disrupt the tissue with needle and a syringe. Homogenize by passing the lysate at least 5 times through a blunt-gauge needle fitted to an RNase-free syringe.
3. Pipette the lysate onto Qia shedder column.
4. Centrifuge the lysate for 3 minutes at full speed.
5. Transfer the supernatant to a new microcentrifuge tube.
6. Add one volume of 70% ethanol to the cleared lysate and mix immediately by pipetting.
7. Transfer upto 700µl of the lysate to RNeasy spin column placed in a 2 ml collection tube.
8. Centrifuge for 15 seconds (s) at full speed.
9. Repeat step 7 & 8 if sample volume of lysate exceeds 700µl. Discard the flow through.
10. Reuse the collection tube in next step.
11. Add 300µl buffer RW1 (washing buffer) to the RNeasy spin column.
12. Centrifuge for 15s at 10,000 rpm and discard the flow through.
13. Add 80µl DnaseI incubation mix(10µl DnaseI stock solution + 70µl buffer RDD) directly to the RNeasy spin column.
14. Place the column on the benchtop (20-30°C) for 15 min.
15. Add 350µl buffer RW1 to the RNeasy spin column and centrifuge for 15s at 10,000 rpm.
16. Discard the flow through.
17. Add 500µl buffer RPE to the RNeasy spin column.
18. Centrifuge for 15s at 10,000 rpm. Discard the flow through.
19. Add 500µl buffer RPE to the RNeasy spin column.
20. Centrifuge for 2 min. at 10,000 rpm to wash the spin column membrane.
21. Place the Rneasy spin column in a new 1.5 ml collection tube and add 40µl Rnase free water.
22. Centrifuge at full speed to elude the RNA and store RNA at -80°C until further usage.