Role of MHC class I expression in the pathogenesis of Type 1 Diabetes

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Abstract:
Type 1 Diabetes (T1D) is a polygenic autoimmune disease that occurs as a result of the selective destruction of insulin producing beta cells of the pancreas by infiltrating mononuclear cells. The aetiology of T1D is not clearly understood, however numerous studies have shown that a combination of various factors, including genetic predisposition, viral and bacterial attack, drugs and other environmental factors have both causative and protective effects in disease development. T1D develops from the activation of $CD4^+$ and $CD8^+$ T cells of our immune system, which leads to a series of stochastic events resulting in the selective killing of beta cells of the pancreas through Major Histocompatibility complex (MHC) class I dependent process.
Autoantigens expressed onto beta cells are important for the development of disease, but they are not an absolute requirement for the early recruitment of immune infiltrates into the islets. This has been shown in genetically modified mice lacking MHC class I expression on beta cells, which presented immune cell infiltrates localized near the pancreatic ducts. Similar observations were also noted in mice lacking the expression of native insulin. These early ductal infiltrates suggest that in Non Obese Diabetic (NOD) mice, immune responses to ductal cell antigens may precede the immune response to beta cell antigens including proinsulin.
To understand the role of ductal cell self-antigens in the development of invasive insulitis, we developed a tissue specific knockout NOD mouse model, in which MHC class I was absent on Glial Fibrillary Acidic Protein (GFAP) expressing cell types including the ductal cells (Class I GFAP bald) of the pancreas. Invasive insulitis was significantly reduced in class I GFAP bald mice and we showed that the absence of class I mediated presentation of ductal cell antigens, other than insulin, significantly reduced the development of insulitis in NOD mice. By removing MHC class I from GFAP expressing cell types, we demonstrated the importance of autoantigens expressed in these cell types in the early development of insulitis in NOD mice.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 papers published in peer reviewed journals and 1 original manuscript. The core theme of the thesis is autoantigens, other than insulin, expressed in the GFAP expressing ductal tissue of the pancreas are important in the development of insulitis in NOD mice. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the department of Immunology and Pathology under the main supervision of Professor Robyn Slattery and co-supervison of Dr. Eliana Marino Moreno.

In the case of chapters my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name	Co-Author(s) Monash Student
1	Advances in our understanding of the pathophysiology of Type 1 diabetes: lessons from the NOD mouse.	Published	50% literature review and writing the manuscript	1) *K. P. Mansour*- Assited in finding literature and drafted one section 2) *Robyn Slattery*- supervisor	Yes No
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I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name:

Main Supervisor signature: Date:

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List of Abbreviations

AAb ..... Autoantibody

AAg ..... Autoantigen

ADP ..... Adenosine diphosphate

AIRE ..... Autoimmune regulator

AMP ..... Adenosine monophosphate

AMREP ..... Alfred Medical Research and Education Precinct

APC ..... Antigen-presenting cell

AS-ODN ..... Antisense oligodeoxyribonucleotides

ATG ..... Anti-thymocyte globulin

BCR ..... B cell receptor

BSA ..... Bovine serum albumin

CAII ..... Carbonic anhydrase II

CCR ..... Chemokine receptor

CD40L ..... CD40 ligand

CD62L ..... CD62 ligand

CRAMP ..... Cathelicidin-related antimicrobial peptide

cre ..... Causing recombinase

CTLA-4 ..... Cytotoxic T-lymphocyte associate antigen-4

CVB ..... Coxsackievirus B

DBA ..... Dolichos biflorus agglutinin

DC ..... Dendritic cell

Deaf1 ..... Deformed epidermal autoregulatory factor-1

DNA ..... Deoxyribonucleic acid

DPBS ..... Dulbecco's Phosphate-Buffered Saline

EC.....Endothelial cell

EDTA.....Ethylenediaminetetraacetic acid

EFA.....Essential fatty acid

EGC.....Enteric Glial cell

ENS.....Enteric nervous system

EYFP.....Enhanced Yellow Florescent Protein

FasL.....Fas ligand

FCS.....Fetal Calf Serum

FoxP3.....Forkhead box P3

FO.....Follicular

GAD.....Glutamic acid decarboxylase

GCSF.....Granulocyte-colony stimulating factor

GF.....Gluten free

GFAP.....Glial fibrillary acidic protein

GFP.....Green Fluorescent protein

GI.....Gastrointestinal

GM-CSF.....Granulocyte-Macrophage Colony-Stimulating Factor

GP.....Glycoprotein

GPR.....G-protein coupled receptor

GWAS.....Genome-Wide Association Study

H&E.....Hematoxylin and Eosin

HA.....Hyaluronan

HbA1C.....Glycated hemoglobin

HBSS.....Hanks balanced salt solution

HDACs.....Histone deacetylases

HLA..... Human leukocyte antigen

IA2..... Insulinoma associated antigen

IBD..... Inflammatory bowel disease

ICA69..... 69kDa islet cell autoantigen

ICAM-1..... Intercellular adhesion molecule-1

ICOS..... Inducible T cell co-stimulator

IDO..... Indoleamine 2,3 dioxygenase

IDDM..... Insulin-Dependent Diabetes Mellitus

IFA..... Incomplete Freund's adjuvant

IGRP..... Islet-specific glucose-6-phosphatase catalytic subunit-related protein

IL-22..... Interleukin 22

IFN..... Interferons

LADA..... Latent Autoimmune Disease of the Adults

LCMV..... Lymphocytic choriomeningitis virus

LN..... lymph node

LoxP..... Locus of X over P1

LPS..... Lipopolysaccharide

mAb..... Monoclonal antibody

MADCAM..... Mmucosal addressin cell adhesion molecule

MHC I..... Major Histocompatibility complex I

MLNs..... Mesenteric lymph node

MT-1 MTP..... Membrane type-1 matrix metalloproteinase

MZ..... Marginal zone

NF-κB..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NK..... Natural Killer

NKT.....Natural Killer T cells

NOD .....Non-Obese Diabetic

NOR .....Non-Obese Resistant

NP.....Nuclear protein

OCT.....optimum cutting temperature

PAC.....Precinct Animal Centre

PBS.....Phosphate buffered saline

PCR .....Polymerised Chain Reaction

PD-1 .....Programmed cell death 1

pDC .....Plasmacytoid DC

PFA .....Paraformaldehyde

pSCs .....Peri islet Schwann cells

RBC.....Red blood cell

ROS .....Reactive oxygen species

SCE .....Stem cell educator

SCID.....Severe combined immunodeficiency

SCFA.....Short Chain Fatty Acid

SDS .....Sodium Dodecyl Sulfate

SERT .....Serotonin-selective reuptake transporter

SGF .....Specific Germ Free

SNP .....Single nucleotide polymorphism

SPF .....Specific pathogen free

STZ.....Streptozotocin

TAE .....Tris-acetate EDTA

T1D .....Type 1 Diabetes

TLR ..... Toll like receptor

TNF ..... Tumor Necrosis Factor

Treg ..... Regulatory T cell

TRPV-1 ..... Transient Receptor Potential Vanilloid type 1

TSA ..... Tissue Specific Antigen

UV ..... Ultraviolet

VCAM-1 ..... Vascular cell adhesion molecule-1

VNTR ..... Variable number tandem repeat

VLA ..... Very Late Antigen

β2m ..... β2 microglobulin

5-HT ..... serotonin, 5-hydroxytryptamine

1. Introduction

Type 1 Diabetes (T1D) is a polygenic autoimmune disease, in which insulin secreting beta cells of the pancreas are selectively destroyed [1-3]. The disease usually develops at a younger age and is characterized by high blood glucose levels. The aetiology of T1D is not clearly understood but, a combination of genetic, environmental and immunological factors contributes to the development of disease. In genetically susceptible individuals the autoimmune response is triggered by one or more environmental factors and could asymptomatically progress over many months to years before the patient develops hyperglycaemia. Excess glucose in the blood should be controlled by administering exogenous insulin to avoid the development of micro and macro vascular complications [4].

T1D is also known as juvenile diabetes and is further classified into two subtypes based on the pathogenesis. Type 1A diabetes or the autoimmune diabetes is diagnosed by the presence of one or more self-reactive autoantibodies to islet antigens while, Type 1B or idiopathic diabetes shows no evidence of immunological and genetic contribution towards the pathogenesis and progression of the disease [5]. In certain cases, the pathogenesis of T1D progress very slowly and symptoms begin to appear at adult age. This subtype is known as the latent autoimmune disease of the adults (LADA) sometimes referred to as Type 1.5 diabetes [6, 7]. These adult cases of T1D are sometimes misdiagnosed as Type 2 diabetes. The work described in this thesis was performed to understand the mechanisms involved in the pathogenesis of the Type 1A or the autoimmune form of T1D. Throughout this thesis any reference to T1D implies the autoimmune subtype of T1D.

The incidence of T1D is increasing worldwide [8, 9] and a permanent cure for the disease is currently not available. Patients with T1D depend on exogenous insulin and extensive management of blood glucose levels to minimise the risk of developing micro and macro vascular complications.

Investigation into the genetics and immunopathological mechanisms that lead to initial progression and beta cell destruction in individuals at risk of developing T1D is difficult due to a protracted pre-diabetic phase, inaccessibility of islet tissue, and limitations in beta cell markers that reflect cell mass and functionality. Genome Wide Association Studies (GWAS) have identified more than 50 distinct susceptibility regions linked to T1D and the main genes predisposing to T1D fall within the Major Histocompatibility (MHC) region, also known as the Human leucocyte antigen (HLA) [10-12]. These highly polymorphic regions contribute for about 40-50% risk in the development of disease. Beyond HLA genes, the polymorphism within the VNTR (Variable Number Tandem Repeats) region of the insulin promotor and polymorphism of the CTLA-4 gene (cytotoxic T lymphocyte antigen-4) are also associated with T1D development but to a lesser extent when compared to HLA region [13]. The association studies may assist in the screening for those at risk of developing T1D, but a comprehensive understanding of the immunopathological mechanisms underlying the development of T1D is also required. The knowledge gained from such studies will help develop immunomodulatory strategies to prevent the onset of disease in genetically susceptible individuals. Understanding the pathogenesis of T1D in humans is limited due to the unavailability of pancreatic samples and due to the asymptomatic progression of the disease. Accordingly, our understanding of the genetics, etiology, and pathogenesis of T1D has heavily depended on animal models and among the different animal models of T1D, the Non Obese diabetic

(NOD) mouse is the most widely used model to study the pathogenesis of T1D [1, 14-16]. NOD mice develop the disease spontaneously and share genetic and immunopathogenic features with human T1D. The importance of NOD mice in T1D research is driven by more than the scarcity of human pancreatic tissue samples. Though, the mechanisms of human and NOD autoimmune diabetes are not identical, it is well documented that the role of MHC haplotype is similar, when equating the development disease in both NOD and in humans [17]. In addition to the similarities in MHC haplotype there are also other pathogenic similarities (Table 1) to prove that the NOD mouse is a gold standard model to study pathogenesis of human T1D [18].

1.1 NOD mouse model

1.1.1 Genetics

The inbred NOD mouse strain originated as a hyperglycaemic sub-strain of the cataract prone mouse (CTS) at Shionogi laboratories, Japan [19]. At the time of weaning NOD mice develop around pancreatic islets a mononuclear cell infiltrate (insulitis) that progresses at approximately 100 days of age to invasive insulitis and complete beta cell destruction [20]. Although NOD mice have an increased genetic susceptibility to T1D, the penetrance of disease can be modulated by various environmental factors. Hence, not all NOD mice in a colony will develop T1D. Importantly, a number of T1D susceptibility genes identified in NOD mice (designated *Idd)* have been found to contribute to T1D susceptibility in humans (designated *IDDM*). To date >40 *Idd* regions have been identified [21, 22] but only a small number of these regions have been localised to candidate genes, and these will be discussed below.

The Major histocompatibility complex (MHC) is the strongest susceptibility region and was the first identified in both humans (*IDDM1*) and in mice (*Idd1*). The

mouse MHC region is located on Chromosome 17 and contains a number of genes known to contribute to disease. These genes make up a haplotype that contains both MHC class I and class II susceptibility genes. In mice, MHC class I genes comprise of K^d and D^b alleles and MHC class II genes comprise of I-A and I-E alleles [23, 24]. Although NOD mice express the I-A heterodimer that is composed of I-Aα and I-Aβ chains, the I-E heterodimer is not expressed due to a deletion in the promoter region of the I-Eα gene. Furthermore, the replacement of histidine and serine with proline and aspartic acid at positions 56 and 57 respectively, within the I-Aβ chain prevents T1D in NOD mice [25-27].

The *Idd3* locus encodes the cytokines IL2 and IL21 and these are strong candidate genes for T1D susceptibility [28-30]. In NOD mice, IL2 expression levels are abnormally low, with low dose IL2 treatment reducing the severity of insulitis and inhibiting T1D onset [31, 32]. Further reduction of IL2 levels, in NOD mice heterozygous for a deletion of the IL2 gene, accelerated T1D development [33]. In contrast, IL21 is highly expressed in NOD mice and NOD mice deficient in the IL21 receptor are protected from T1D [30, 34].

The *Idd5.1* locus includes two candidate susceptibility genes encoding CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) and ICOS (inducible T-cell co-stimulator). CTLA-4, a receptor involved in inhibiting T-cell activation, is present in four distinct isoforms in NOD mice, one of which is the ligand-independent CTLA-4 isoform (liCTLA-4). It contains a single nucleotide polymorphism (SNP) in exon 2 that causes liCTLA-4 to be expressed at reduced levels, decreasing the activation threshold of T-cells and consequently disease susceptibility. Similarly, expression levels of the soluble CTLA-4 (sCTLA-4) isoform are reduced in T1D patients [35]. CTLA-4 therefore represents an attractive target for immunotherapeutic intervention. The ICOS gene of NOD mice has a SNP

encoding a non-conservative amino acid change in the leader sequence of exon-1. This change causes higher expression of ICOS, which heightens T-cell co-stimulation. Consistent with the idea that increased expression of ICOS contributes to susceptibility, $ICOS^{-/-}$ NOD mice are protected from T1D [36, 37].

The *Idd7* locus contains a gene, or several linked genes, thought to influence allelic exclusion of T-cell receptor (TCR) genes during T-cell development [38]. These studies were performed in TCR transgenic mice and may not represent the normal path of TCR rearrangement found in human T-cells. However, if such defects are present, this could lead to dual TCR expression and autoimmunity [39].

The *Idd9* locus contains three regions: *Idd9.1, Idd9.2* and *Idd9.3* [40]. Although the genes localised at *Idd9.1* are unknown, they are associated with increased B-cell pathogenic activity [41], low numbers of induced invariant natural killer T-cells (iNKT) and reduced Treg development and activity in NOD mice [42, 43]. Candidate susceptibility genes at *Idd9.2* and *Idd9.3* encode CD30, TNFR2 (Tumor Necrosis Factor Receptor 2), and CD137, respectively [40].

The *Idd13* locus contains multiple susceptibility genes, including the candidate genes β2-microglobulin (*β2m)*, *Cd93*, *Nkt2*, and *Bim* [44, 45]. In inbred mouse populations, there are three allelic variants of *β2m* that encode isoforms differing by a single amino acid at residue 85 [46, 47]. Wild-type NOD mice express the $\beta2m^a$ isoform, whereas NOD mice congenic for the NOR (Nonobese-resistant) *Idd13* region express the $\beta2m^b$ isoform and are protected from T1D. *β2m* was confirmed as a diabetes susceptibility gene in reconstitution experiments in which NOD mice lacking endogenous *β2m* and transgenic for $\beta2m^b$ were protected from the development of T1D compared with mice transgenic for $\beta2m^a$ [48]. The mechanism of protection conferred by different *β2m* isoforms has not been elucidated, but is proposed to relate to the expression level of MHC class I

(unpublished, Slattery). As such, although humans are non-polymorphic at the *β2m* loci, there may be related changes in the expression level of MHC class I that influence antigen presentation, thereby modulating thymic selection and/or peripheral activation of $CD8^+$ T-cells [44]. In NOD mice, the *Cd93* gene has a SNP that results in a conformational change in the CD93 protein [45]. Although the function of this protein is not yet well defined, its absence in C57Bl/6 (B6) $CD93^{-/-}$ mice results in a reduced number of iNKT-cells, which may promote T1D in NOD mice [45]. A third gene within the *Idd13* region that controls NKT-cell numbers has been mapped to the *Nkt2* gene. NOD mice congenic for the B6 *Nkt2* region had increased NKT-cell numbers and a reduced incidence of T1D [49]. The forth candidate susceptibility gene to be localized within the *Idd13* locus is *Bim* which encodes the proapoptotic protein BIM. The failure to induce BIM in thymocytes confers resistance to thymic deletion in NOD mice [50].

Of >40 susceptibility loci identified in NOD mice only a small number of these regions contain genes that have orthologues associated with human T1D. Nevertheless, a number of the non-orthologous candidate susceptibility genes in NOD mice have led to studies that provided valuable insights into the immunopathogenic mechanisms of T1D relevant to humans, and these are discussed later in the review.

1.1.2 Environment

1.1.2.1 Geography

Various environmental parameters have been associated with T1D susceptibility in humans. They include geographical location, dietary components, and infectious agents. Worldwide incidence data on T1D suggests that there is an inverse correlation between disease incidence and proximity to the equator that could be explained by a number of variables. Regions furthest from the equator have

reduced exposure to sunlight with a concomitant reduction in both UV radiation and temperature. In human T1D, it is difficult to segregate the roles of sunlight variables from the confounding co-variables of genetics and culture. However, genetically controlled studies utilizing inbred NOD mice have allowed the independent contribution of UV radiation and temperature on T1D to be assessed.

NOD mice maintained at a temperature of 23.7°C compared to 21°C had a lower incidence of T1D. This suggests that the inverse correlation between equatorial distance and incidence of T1D may in part be explained by temperature [51]. Since UV radiation is essential for epidermal vitamin D synthesis, and the active form of vitamin D, 1,25-Dihydroxyvitamin D_3 (1,25 D_3), influences the development of Treg cells, the effect of vitamin D on T1D has been studied in NOD mice. It is believed that Vitamin D has a role to play in the development of T1D [52, 53]. These studies revealed that early deficiency of vitamin D in NOD mice resulted in accelerated T1D development [54], whilst NOD mice administered supplementary vitamin D were protected from developing T1D and this was correlated with an increased frequency of Treg cells within the pancreatic lymph node (pLN) [55, 56]. It is thought that vitamin D also modulates the immune response through inhibition of the nuclear factor kappa beta (NFκB) pathway in DCs and macrophages. This results in decreased production of the pro-inflammatory cytokines IL12 and IFNγ that leads to decreased MHC class II expression on antigen-presenting cells (APCs) and MHC class I expression on beta cells [57].

Vitamin D supplementation at birth has been shown to significantly protect from T1D in humans [58, 59]. A recent study also suggests the beneficial side of having enough vitamin D concentration in children at risk of developing T1D [60]. On contrary a six months administration of vitamin D to children with T1D had not

effect on the residual beta cell function [61] and supplementation of vitamin D in patients with recent onset T1D failed to reduce loss of beta cell function [62, 63].

1.1.2.2 Diet

T1D is a multifactorial condition; diet and environment play an inevitable role in disease modulation [64, 65]. The earliest evidence for dietary influences in T1D susceptibility came from studies in NOD mice. A positive association was found between a high fat diet and T1D incidence in these mice [66]. An increase in T1D progression in NOD mice has also been correlated with wheat or corn enriched diets and this has been attributed to the wheat protein gluten [67]. It has been speculated that a mechanism by which dietary antigens (Ags) could influence susceptibility is by modulation of the mucosal immune system via the release of tolerogenic, allergenic, or pro-inflammatory cytokines [68]. NOD mice fed cereal/wheat based diets expressed significantly higher levels of IFNγ, TNFα, and iNOS that are known to drive the immune response towards T1D [68].

Essential fatty acids (EFA) have also been shown to protect from T1D. The offspring of mice fed a low omega-6 to omega-3 (*n-6/n-3*) EFA ratio diet showed a decrease in T1D incidence [69]. Similarly, omega-3 reduced the incidence of T1D in genetically susceptible children indicating its benefit as a supplement to assist in the prevention of human T1D [70]. The protective effect of omega-3 may partly explain why T1D incidence is lower in Japan where there is a high consumption of fish, compared to countries with westernised diets [71]. One mechanism by which omega-3 may protect from T1D is through direct binding to the G-protein-coupled receptor (GPR) 120 on macrophages, which exert a wide range of potent anti-inflammatory effects [72]. Additionally, omega-3 may act indirectly through the

anti-inflammatory influence of bioactive products resulting from its breakdown and enzymatic conversion, such as resolvins [73].

Diet and the use of antibiotics can also modulate the gut microbial diversity, which has recently been shown to regulate the immune system. NOD mice housed under 'specific germ free' (SGF) conditions, have a higher incidence of T1D than those housed in less stringent conditions. Furthermore, NOD mice treated with probiotics have elevated IL10 production and reduced T1D development [74]. The bacteria responsible for this protection are members of the *Bacteroidetes phyla*, capable of producing short chain fatty acids (SCFA)s. SCFAs can bind GPRs on immune cells and thereby mediate an anti-inflammatory response that protects from T1D [75].

Human and murine studies have demonstrated that defects in the induction of central and peripheral tolerance checkpoints [76] also correlate with an altered gut microbiota [77-81], which are notable contributors to T1D pathology. Building on previously extensive reviews on the gut microbiota topic, it is certain that an altered microbiota and SCFAs deficiency is a primary causal factor triggering T1D [82-85]. The gut microbiota through the production of dietary SCFAs plays a significant role in host defense by modulating the immune system and metabolism. Studies conducted by Eliana Marino's group has shown that the combination of a diet rich in acetate and butyrate protected 90% of the NOD mice against T1D, yielding exceptionally high levels of the corresponding SCFAs to the feces [77]. In this study, SCFAs-induced T1D protection happened via changes in gut/ immune regulation- expanding regulatory T (Treg) cells and reducing pathogenic B cells, $CD4^+$, and $CD8^+$ T cells. Diet rich in SCFA acetate and butyrate not only reduced the levels of serum LPS and pro-inflammatory interleukin 21 (IL-21) but also increased the level of serum IL-22, an important cytokine that maintains a healthy commensal microbiota, gut epithelial integrity, mucosal immunity and ameliorates

metabolic disease [86-89]. Alternatively, SCFAs also can reduce islet-specific immune responses by increasing the production of antimicrobial peptides (AMP) in the beta-cells [90]. As it has been shown, C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ), and defensins disrupt surface membranes of bacteria thus enabling a broad regulation of commensal and pathogenic bacteria in the gut [91-93]. Diana's group showed that insulin-secreting beta-cells produced the cathelicidin-related antimicrobial peptide (CRAMP), which was defective in NOD mice. Intraperitoneal administration of SCFA butyrate stimulates the production CRAMP on pancreatic beta-cells via G protein-coupled receptors (GPCRs), which also correlated with the conversion of inflammatory immune cells to a regulatory phenotype [90]. Likewise, another study has shown that microbial SCFAs contribute to the increasing concentrations of serum IL-22 [77] required for beta-cell regeneration by up-regulating the expression of Regenerating Reg1 and Reg2 genes in the islets [94].

Composition of the gut microbiota in T1D patients is different when compared with healthy controls. The ratios of Firmicutes to Bacteroidetes were lower in the children with T1D when compared to healthy controls. It is believed that the abundance of more diverse bacteria in the gut is beneficial to ferment the food and to produce more immunomodulatory products [95, 96]. Study conducted by Syed et.al showed that endogenously originating lipids, PAHSAs (Palmitic acid esters of hydroxyl steric acid) reduced the activation of islet reactive $CD4^+$ and $CD8^+$ Tcells, and delayed the onset of T1D in NOD mice [97].

Whether probiotics, or SCFA treatment, will prevent T1D in those at risk remains to be determined although probiotics are currently being trialled in at-risk children [98]. In contrast, some bacterial strains promote inflammation and thereby exacerbate the development of T1D. Gluten fed NOD mice contain greater

quantities of aerobic, micro-aerophilic, caecal bacteria and are more likely to develop T1D than those fed gluten free diets [99]. Furthermore, a positive correlation was observed between numbers of gram positive aerophilic and anaerobic bacteria found in the gut of NOD mice and incidence of T1D, regardless of diet [99]. It is thought that such bacterial strains promote a pro-inflammatory cytokine environment that drives T1D by inducing mucosal DCs to secrete Th1 type cytokines.

1.1.2.3 Viruses

The identification of enterovirus from the pancreatic islets in children with T1D suggests that viral attacks may have contributed to disease progression [100]. A number of viruses have been positively associated with T1D onset and several mechanisms have been proposed to explain this association [101]. Viral infection of the gastrointestinal mucosa with Rotavirus increases the intestinal permeability, creating a 'leaky gut'. Opportunistic gut bacteria that migrate through the leaky gut can induce inappropriate sub-mucosal immune responses that signal through Toll-like receptors (TLRs) and drive inflammation [102]. Other viruses, such as Coxsackie virus B (CVB), can directly infect islet tissue, or neighbouring neuro-endocrine cells, promoting inflammation in both islets and associated endothelial cells (ECs) [103]. At the time of insulitis development, NOD islet ECs develop an activated phenotype with upregulation of adhesion molecules, such as CD54 (ICAM-1), CD106 (VCAM-1), and of MHC class I and II molecules [103, 104]. Islet ECs from human histological samples taken during the peri-onset stage of T1D show a similar phenotype, displaying increased expression of adhesion and MHC molecules [105].

Another potential mechanism of virally induced T1D is molecular mimicry, whereby T-cells that are activated by specific viral antigens cross-react with

autoantigens (AAgs) that share structural similarities. This model has been investigated for its capacity to trigger T1D in transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) antigen nucleoprotein (NP), or glycoprotein (GP). Mice expressing these antigens on pancreatic beta cells remained tolerant and free from T1D in the absence of viral infection [106]. However, when challenged with LCMV, NP and GP specific T-cells were activated and beta cells were killed, causing T1D [106]. Blocking or depletion of pro-inflammatory cytokines has been found to reduce T1D incidence in NOD mice following viral infection [107].

1.1.3 Immunopathology

1.1.3.1 Disease initiation

The inaccessibility of professional APCs (pAPCs) has made it challenging to measure their role in human T1D. Researchers rely heavily on studies in NOD mice to understand how pAPCs may promote T1D, and importantly, how their central role in tolerance may be exploited in immunotherapeutic approaches. pAPCs are the first cells to accumulate marginally around the islets, thereby initiating a cascade of immunopathological events that culminate in beta cell destruction and T1D [108, 109]. The events triggering the accumulation of pAPCs in the islet milieu remain to be fully elucidated. It has been proposed that a wave of beta cell death occurs in NOD mice as part of tissue remodelling. This could occur either in response to viral infection or other metabolic changes around the time of weaning [110, 111]. The migration of CCR7 positive monocyte/macrophages and DCs to the islets is promoted by the elevated expression of lymphoid tissue-related chemokines, such as CCL19, found in NOD mice [112]. Macrophages produce the cytokines TNFα and IL1β that are directly beta cell toxic, and over-produce IL12, driving the further recruitment of DCs to the accumulated beta cell debris. This

exacerbates the inflammatory response and leads to the inappropriate activation of autoreactive T-cells in NOD mice [113]. Furthermore, due to increased prostaglandin (PGE_2) production, macrophages from NOD mice are impaired in their phagocytic ability and their ability to present self-antigens for the induction of tolerogenic immune responses [114-116]. Therefore, NOD mouse macrophages have an important role not only in the initiation of insulitis, but also in driving its progression towards beta cell destruction and T1D. Based on the important roles of IL-1β and TNFα in beta cell damage, children recently diagnosed with T1D have been treated with IL-1 receptor and TNF antagonists in a number of different trials. Both treatments promoted mild increases in insulin production within 4-5 months of treatment [117].

In NOD mice, multiple DC subsets are present including myeloid DCs (mDCs), lymphoid DCs, and plasmacytoid DCs (pDCs). mDCs have an important role in antigen processing and presentation to autoreactive T-cells leading to their activation. In NOD mice there is a 5-fold increase in the number of immature mDCs and fewer mature mDCs. The immature DCs underproduce IL10, have deficient responses to GM-CSF, decreased intracellular and surface expression of MHC class II, reduced co-stimulatory molecule expression, and lowered expression of the CD40 signalling molecule [118]. However, the mature mDCs in NOD mice express elevated NFκB in response to Ag, and this leads to increased secretion of IL12p70 and TNFα, and increased expression of co-stimulatory molecules. Increased IL12 leads to autocrine activation of DCs as well as increased activation of antigen specific $CD8^+$ T-cells [119]. This intrinsic defect of the mature mDC population therefore tips the balance towards a Th1 immune response that drives beta cell destruction and T1D. Likewise, mDCs from T1D patients display elevated NFκB activation and IL12 secretion [120]. In addition to defects

in the myeloid population there are also defects in the lymphoid DC population in NOD mice. Lymphoid DCs in NOD mice show reduced indoleamine 2,3-dioxygenase (IDO)-mediated catabolism of tryptophan in response to IFNγ, resulting in the increased proliferation of effector T-cells [121]. pDCs have an immature phenotype, are poor antigen presenters, and are therefore known to be important for the induction of tolerogenic immune responses. In NOD mice depletion of pDCs was associated with a reduction of IDO in the pancreas and this correlated directly with the development and severity of insulitis [122]. The importance of DCs in T1D has been exploited in on-going clinical trials. Autologous DCs modified ex-vivo to have reduced co-stimulatory ability have been used in T1D patients and at risk individuals to drive tolerogenic immune responses. While Safety has been established the efficacy of this approach is not yet known.

1.1.3.2 Natural killer (NK) cells

NK cells are normally associated with defence against viruses, intracellular pathogen infected cells, malignant cells, and foreign or transplanted cells. It is thought that NK cells may have an important early role in the immunopathogenesis of T1D since they are found to infiltrate pancreatic islets of NOD mice and have also been detected in the pancreatic islets of cadaveric T1D patients [123, 124]. Furthermore, NK cells within the insulitis lesion display an activated phenotype, expressing higher levels of killer cell lectin-like receptor group G 1 (KIRG1), programmed cell death 1 (PD1), IL2R (CD25), and CD69 than NK cells from the pLN or spleen and this correlates with increased beta cell destruction. The activated NK cells found in the insulitis lesion may directly induce beta cell apoptosis through perforin and granzyme mediated cytotoxic damage since they express CD107a which is a marker of granule exocytosis [125]. NK cells are able

to recognize NOD beta cells since these express RAE1 (retinoic acid early transcript 1) and NKp46 ligand, which are the ligands for NK cell receptors NKG2D and NKp46, respectively. While it has been shown that blockade of NKG2D and NKp46 reduces T1D incidence, it may be that the protection is due to inhibition of interactions other than with NK cells, such as with cytotoxic T-cells that also express NKG2D [126]. Alternatively, they may indirectly damage beta cells since they also express high levels of pro-inflammatory cytokines such as IFNγ [127]. Although NK cells undoubtedly have a role in the early insulitis lesion it is unlikely that they are essential in the immunopathogenic process that drives complete beta cell destruction since NOD beta cells, lacking MHC class I/*β2m*, yet retaining NK receptor ligands, are not killed by NK cells [128].

1.1.3.3 B Lymphocytes

Although macrophages and DCs are the primary pAPCs involved in the insulitis initiation, B lymphocytes also have a pivotal early role in the pathogenesis of T1D development. B-cells have been identified in the insulitic lesions of biopsies from human T1D patients, as well as in the pancreatic biopsies of diabetes prone mice and rats [129]. The significance of B-cells as important contributors to disease became clear when diabetes prone NOD mice lacking B-cells were generated. Although B-cell deficient NOD mice developed mild insulitis, suggesting that B-cells are not required for the initiation of disease, they were significantly protected from the development of diabetes, confirming their role in disease progression [130-133]. Similar results were obtained following antibody mediated depletion of B-cells [134].

Two main roles have been proposed for B-cells in the pathogenesis of T1D. Role 1: B-cells may contribute to disease via the production of autoantibodies (AAbs), known to correlate with early pathogenesis. A role for AAb in promoting T1D

development has also been supported by maternal AAb studies whereby inhibition of AAb transfer from mothers to NOD offspring in several models, was found to protect against T1D development in offspring [135, 136]. Additionally, there is indirect evidence that suggests that AAbs enhance the effector functions of DCs and NK cells since FcR$\gamma^{-/-}$ NOD mice are protected from disease [137]. These findings have led to the proposal that AAbs may augment beta cell destruction through binding AAg and promoting Fcγ receptor mediated antigen uptake by APCs or by promoting antibody dependent cellular cytotoxicity of beta cells. However, AAb are not requisite for T1D development since NOD transgenic mice expressing only membrane bound B-cell receptor (BCR), and without the capacity to secrete antibody, developed an increased incidence of insulitis and T1D compared to NOD B-cell deficient mice [138]. Role 2: B-cells may contribute to disease via the recognition, uptake, and processing of AAgs and presentation to diabetogenic T-cells. This antigen presenting role of B-cells must occur after the activation of T-cells involved in the initial immune phase of T1D, since many B-cell deficient mice develop non-invasive insulitis [130]. The progression from non-invasive to invasive insulitis is well documented, but a mechanistic understanding of this crucial switch is not well understood [139, 140]. It is likely that the highly proliferative nature of B-cells allows them to efficiently capture beta cell antigen for processing and presentation to activated diabetogenic $CD4^+$ T-cells and $CD8^+$ T-cells resulting in the rapid expansion of cells invading and killing the islet beta cells [141, 142].

Because of their known role in antigen presentation, and because they are expanded in NOD mice, the marginal zone (MZ) B-cell population has been implicated in the pathogenesis of T1D [143]. However, it is unlikely that the increase in MZ numbers observed in NOD mice is a primary defect promoting

T1D, since loss of MZ B-cells following complete splenectomy failed to protect NOD mice from T1D, whereas the removal of follicular (FO), and not MZ, B-cells in anti-CD20 treated NOD mice did protect from T1D [144].

Treatment of recently diagnosed T1D patients with anti-CD20 mAb transiently depleted B cells and resulted in transient preservation of beta cell mass. While B-cells clearly contribute to T1D pathogenesis in NOD mice and humans, it is also clear that T1D can develop via alternative pathways in both species. T-cells from B-cell deficient NOD mice were able to transfer T1D to NOD scid recipients, albeit at a reduced frequency compared with T-cells from B-cell sufficient donors [131]. In humans, T1D has likewise been reported in B-cell deficient patients [145]. Therefore, while B-cells present as an attractive therapeutic target in those at risk of developing T1D, it is unlikely that their blockade will provide protection in all patients.

1.1.3.4 T Lymphocytes

There is a large body of evidence implicating T-cells in the development and progression of T1D in humans and NOD mice. Both $CD4^+$ and $CD8^+$ T lymphocytes are pivotal during the early and late stages of disease in mice. Whole splenocytes or purified populations of both $CD4^+$ and $CD8^+$ T-cells can transfer T1D to young NOD and non-diabetes prone F1 mouse strains, whereas neither $CD4^+$ nor $CD8^+$ T-cells alone can transfer disease [146, 147]. Likewise, T-cell depletion of susceptible NOD mice inhibited disease progression and T1D, reinforcing the central role of T-cells in immunopathogenesis [148]. The early discovery that T-cells are essential in the immunopathogenesis of T1D has led to a major focus on these cells, from thymic development to understanding the key mediators of cytotoxic versus regulatory subset development and beta cell killing.

1.1.3.5 Development

The architecture of the NOD thymus is abnormal, comprising unusually large perivascular spaces, and disorganised thymic medulla. Additionally, NOD thymocytes have reduced expression of the integrin-type fibronectin receptors alpha4beta1 (VLA-4) and alpha5beta1 (VLA-5), that cause defects in cell migration. Since the thymocytes trapped within the giant perivascular spaces of the NOD thymus are consistently VLA-5 negative, their accumulation may be due to an impairment of normal thymocyte migration [149]. Although the overall number of T-cells emigrating from the NOD thymus is normal, there may be an increase in the proportion of T-cells that bear TCRs with autoreactive specificity. The unique MHC class II haplotype present in NOD mice, and in many Caucasoid T1D patients, influences the ability to bind to self-peptide and mediate negative selection. This deficiency was tested in transgenic NOD mice expressing non-autoimmune associated MHC class II haplotypes. These mice were protected from developing T1D, a protection that involved thymic deletion of autoreactive $CD4^+$ T-cells in TCR transgenic 4.1NOD mice expressing the MHC class II molecule I-E [150].

It has been proposed that NOD mice fail to express self Ags efficiently. This could be due to the lack of MHC class II molecule I-E, or alternatively, due to the inability of AIRE (autoimmune regulator) to induce expression of self Ags in medullary thymic epithelial cell (mTEC). NOD mice have reduced thymic expression of the AAg ICA69, and the gene encoding this protein carries a SNP in the promoter region important for AIRE binding. This could explain the reduced thymic expression of this self Ag and the potential for reduced deletion of thymocytes with specificity for it [151]. Although there is no direct evidence that the expression of proinsulin is similarly reduced in the NOD thymus, enhanced

expression of this important AAg in transgenic NOD mice protects from T1D [152]. In humans elevated thymic expression of proinsulin is also associated with protection from T1D [153].

NOD mice have a defect that limits allelic exclusion, identified by the increased heterogeneity of TCR alpha genes expressed on T-cells from transgenic NOD AI4 mice [154]. As a consequence of inefficient allelic exclusion two different TCRs can be expressed on the surface of developing thymocytes and this has been shown to allow escape from negative selection and development of autoimmunity in other models [39].

Once the process of thymic selection is complete, thymocytes upregulate receptors on their surface, such as CCR7, allowing them to respond to chemokines and emigrate from the thymus to the peripheral lymphoid organs. Interestingly, CCR7 is elevated on NOD T-cells and CCR7 deficient NOD mice are protected from T1D [155, 156]. However, there is to date no evidence that this polymorphism contributes to altered thymic emigration.

1.1.3.6 Activation

Since autoreactive T-cells are found in normal healthy people and mice, the escape of such cells from the NOD thymus cannot alone account for the development of T1D. Therefore, there must be defects in the peripheral regulation of autoreactive T-cells. Following selection within the thymus, naive $CD4^+$ and $CD8^+$ T-cells travel to lymph nodes (LNs) where they await activation upon MHC presentation of their complementary antigens by pAPCs and costimulatory signals. The activation of islet reactive T-cells occurs within the pLN as their early removal in NOD was found to prevent development of T1D, whereas early removal of spleen had no impact on T1D [157].

The unique MHC class II of NOD mice contributes not only to the loss of thymic tolerance but also to the loss of peripheral tolerance. Autoreactive $CD4^+$ T-cell activation is associated with the unique I-A^{g7} molecule. The lack of an acidic residue at position 57 of the β chain prevents formation of a salt bridge with arginine 76 in the α chain [158, 159]. As a consequence, I-A^{g7} is able to form salt bridges with bound peptides, enhancing peptide-MHC class II binding. A similar binding property has been found in the human MHC class II genotype HLA DQA1*0301, DQB1*0302 associated with T1D [160], suggesting that the homologous human HLA haplotype may lead to T1D development through the same mechanism.

The maintenance of tolerance to islet cell antigen requires their expression by stromal cells in the pLN, and this is regulated by the deformed epidermal auto regulatory factor 1 (Deaf1) transcription factor. Deaf1 is negatively regulated by its binding to a Deaf1 isoform called, Deaf1-VAR which prevents localization of Deaf1 to the nucleus. In the absence of Deaf1 in the nucleus the transcription of tissue specific antigens (TSAs) is reduced. In NOD mice undergoing destructive insulitis Deaf1-VAR is in excess and the expression of TSAs are consequently downregulated [161]. Reduced expression of Deaf1 was also found in T1D patients. However, in NOD mice islet reactive T-cells are activated as early as 3 weeks of age, well before any changes in Deaf1 expression are observed, as such this cannot be the primary peripheral tolerance defect that leads to the development of insulitis.

The activation of islet reactive T-cells requires signalling through costimulatory molecules expressed by pAPCs including CD40, CD80, and CD86. CD40 binds CD40L on T-cells leading to an upregulation of CD40 and TNF receptors on APCs. Early inhibition of CD40L in NOD mice caused a significant reduction in

the development of insulitis and T1D suggesting that autoreactive T-cells are dependent on this interaction for activation [162]. In addition to the importance of CD40/CD40L interaction for activation of T-cells, this interaction may also be important for the expansion of T-cells in the insulitis lesion. Recently CD40 was identified on the surface of islet invasive T-cells raising the possibility that pathogenic T-cells may cross-stimulate via CD40/CD40L interaction [163]. CD80/86 on APCs can ligate CD28 on T-cells and lead to their activation. This activation can be negatively regulated by the inhibitory molecule CTLA-4 on T-cells which also binds CD80 and CD86. CTLA-4 negative regulation promotes the maturation of DCs that express IDO and regulate effector T-cells. NOD mice express reduced levels of liCTLA-4 with concomitant reduction in the usual negative regulation of T-cell activation [35]. Consistent with this, deletion or blocking of CTLA-4 in NOD mice resulted in exacerbation of T1D [164]. Furthermore, NOD mice with induced overexpression of liCTLA-4 had a reduction in the incidence of T1D [165]. Although liCTLA-4 is not found in humans, the soluble isoform of CTLA-4 (sCTLA4) is reduced in T1D patients [35]. Based on these studies patients with recent onset T1D were treated with CTLA-4Ig, a fusion protein composed of the Fc region of IgG1 and extracellular domain of CTLA-4, that inhibits the co-stimulation of T cells. Although C peptide levels were initially higher after CTLA-4Ig treatment there was no significant preservation of beta cell mass after 2 years [166].

Another negative regulatory molecule belonging to the CD28 family and involved in T1D development in NOD mice is PD-1. PD-1 is expressed on activated T-cells, and its ligands, PD-L1 and PD-L2, are expressed on DCs. T-cell proliferation is inhibited when PD-1 binds to its ligands on DCs. Interestingly PD-L1 is also expressed on islet cells and blockade of PD-L1 in NOD mice, using monoclonal

antibodies (mAb), accelerated the rate of insulitis and T1D progression suggesting that the PD-1/PD-L1 pathway negatively regulates autoreactive T-cells [167]. Consistent with this, T1D patients have reduced expression of this important negative regulator of T-cell proliferation [168].

Also within the CD28 family is ICOS, a costimulatory molecule upregulated on activated T-cells and important in T1D. NOD mice have a higher expression level of ICOS than non-autoimmune strains. Both ICOS and ICOSL deficient NOD mice are protected from T1D and this is caused by the failure to activate beta cell reactive T-cells [36]. ICOS may therefore have an important role in maintaining the balance between the activation of effector and regulatory T-cells that control the development of T1D in NOD mice.

1.1.3.7 Homing to the islet

Activated islet specific T-cells in NOD mice show normal upregulation of CD44 and downregulation of CD62L, allowing them to migrate out of the LN via the efferent lymphatics. They then re-enter the circulation via the thoracic duct and migrate along the ECs associated with islet tissue. In prediabetic NOD mice, the beta cells, ECs, and mononuclear cells infiltrating the islets produce multiple chemokine proteins that facilitate the tissue specific homing of effector cells. The expression pattern of these chemokines is complex and it is unlikely that targeting of single chemokines will be of significant therapeutic benefit. However, beta cell expression of a chemokine decoy receptor that binds an array of chemokines completely prevented T1D in NOD mice [169].

The islet ECs normally protect beta cells from inappropriate infiltration by immune cells travelling in the blood. However, NOD islet ECs take on an activated phenotype at the time of insulitis development, expressing hyaluronan (HA), mucosal vascular addressin cell adhesion molecule 1 (MADCAM-1), ICAM-1, and

VCAM-1, important for adhesion of activated monocytes and T-cells [170]. T-cell expression of membrane type-1 matrix metalloproteinase (MT1-MMP) facilitates their transmigration into the islets by cleaving the CD44/HA interaction [171]. Activated ECs also upregulate MHC class I and II expression important for the presentation of Ag to $CD8^+$ and $CD4^+$ T-cells, respectively. The reasons for this upregulation are not known, but many viruses thought to be associated with T1D are known to infect microvasculature ECs and persistent infection of these cells has been associated with increased expression of adhesion molecules and MHC molecules [172]. The importance of ICAM-1 in the development of T1D has been demonstrated in anti-ICAM-1 treated NOD mice that were found to be protected from the development of T1D [173, 174]. Others have reported that an increase in MHC class I expression on islet ECs was associated with T1D in NOD mice [104] and human histological samples taken during the peri-onset stage of T1D show upregulation of MHC class I and II, as well as adhesion molecules, on the islet ECs [105]. Recently, a novel therapeutic approach was tested in NOD mice using a mAb that blocks MT1-MMP cleavage of CD44/HA and thereby prevents the transmigration of activated T-cells into the islet, resulting in protection from T1D [171].

1.1.3.8 Insulitis

Despite the many studies showing that both $CD4^+$ and $CD8^+$ T-cells are required for progression of disease to complete beta cell destruction and T1D, the precise role of $CD4^+$ and $CD8^+$ T-cells in the initiation phase of disease has been controversial. $\beta2M^{-/-}$ NOD mice, which lack MHC class I expression and $CD8^+$ T-cells, do not develop insulitis [175]. This work was interpreted to mean that $CD8^+$ T-cells are critical for the initiation of insulitis. However, an alternative explanation for the complete lack of insulitis is that protection was conferred by

other B6-derived *Idd13*-linked protective genes backcrossed to the NOD with the β2M deficiency [175-178]. NOD mice lacking β2M/MHC class I on APCs only, developed a mild peri-islet infiltrate consisting of $CD4^+$ T-cells that did not progress to invasive insulitis in the absence of $CD8^+$ T-cells [179]. This indicates that $CD4^+$ T-cells initiate peri-insulitis independently of $CD8^+$ T-cells and subsequently provide help to $CD8^+$ T-cells that can then respond to the earliest beta cell antigens.

It is thought that the earliest autoantigenic target of T-cells is insulin because a high frequency of both $CD4^+$ and $CD8^+$ T-cell clones isolated from the insulitic lesion of NOD mice react to peptides from insulin. In particular, early insulitic $CD4^+$ T-cells predominantly respond to insulin peptide B:9-23 [180], and $CD8^+$ T-cells respond to peptide B:15-23 [181]. Replacement of the NOD insulin gene with a mutated insulin gene, encoding alanine at residue 16, protected from T1D, but periductal insulitis persisted around some islets [182]. This suggests that although insulin is an important early AAg recognized by T-cells, there may be upstream AAgs that precede insulin. Whatever the upstream antigens are, clearly the induction of tolerance to insulin is important and blocks the progression of insulitis. NOD mice treated with oral porcine insulin or peptides were protected from T1D [183], and those administered with human insulin developed Treg cells that were also able to transfer this protection [184]. However, unfortunately in none of the clinical trials in which insulin was delivered systemically or orally, was there a delay in onset of T1D compared with untreated control subjects at risk of developing T1D [185, 186]. However, the problem with these trials may have been the route of administration since a recent pilot study using a single intra-muscular injection of insulin with Incomplete Freund's Adjuvant (IFA) in recent onset T1D

patients showed an increase in insulin specific Treg cells two years after treatment [187].

Once initiated by T-cells specific for insulin, islet cell damage ensues with concomitant release of AAgs that results in the activation of an increasingly heterogeneous autoreactive T-cell repertoire. A large number of these AAgs have been defined in NOD mice and have been reviewed extensively elsewhere [188]. It is more difficult to study the expanding heterogeneity of human T-cell responses in T1D due to the low T-cell frequency in peripheral blood and the inaccessibly of islets. However, if we are to design therapeutic intervention strategies that induce tolerance in T-cells of diabetic patients, it is imperative that we identify the human antigenic targets. Currently, there are only three targets, recognized by both $CD4^+$ and $CD8^+$ T-cells, that have been identified in the NOD mouse and that are also found in human T1D. These include two beta cell antigens, insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and a neuroendocrine antigen, glutamic acid decarboxylase (GAD) [189]. GAD-65 alum has been used to treat new onset T1D patients but failed to show significant improvement in C-peptide levels. Combination therapy with GAD-65 alum and Vitamin-D/ibuprofen are currently in phase -2 clinical trials [190].

1.1.4 Balance of immunopathogenesis and regulation

The insulitic lesion of NOD mice includes a number of $CD4^+$ effector T-cell subsets that have been categorised based to their cytokine production as Th1, Th2, Th17, Th40, Treg, and NKT-cells. On the basis of these subsets, T1D has been considered a Th1-mediated disease, because increased levels of IFNγ, and lower levels of IL4 were correlated with beta cell destructive insulitis [191]. Furthermore, induction of a Th2 bias by administration of IL4, and infection with various helminths, confers protection from T1D in NOD mice [192, 193]. However, this

simple categorization of T1D as a Th1-mediated disease was brought into question when IFNγ and IFNγR deficient NOD mice remained susceptible [194, 195]. It is now recognized that cytokines are involved in the crosstalk between a greater range of T-cell subsets that control the balance between effector and regulatory immune responses. Th17-cells were identified as a subset of effector T helper cells that differentiate in response to TGFβ, IL6, and require IL23 for population expansion. They produce mainly IL17A, IL17F, and IL22, which have broad actions on the immune system due to the distribution of the IL17 and IL22 receptors [196]. Th17 cells have been shown to be important for a number of autoimmune diseases, such as asthma and psoriasis, but their role in T1D has only been reported in a few studies and remains controversial. Serum IL17 is elevated in NOD mice and there are reports of increased numbers of Th17-cells in NOD islets [197]. Consistent with their proposed role in disease, the blockade of Th17-cells in NOD mice with mAb against IL17 resulted in reduced insulitis [198]. Conversely, IL17 deficient NOD mice were not protected from T1D [199]. Furthermore, when Th1 and Th17-cells from NOD BDC2.5 mice were independently transferred to NOD scid recipients, those receiving Th1-cells developed more severe disease than those receiving Th17-cells. Interestingly, recipients of Th17-cells were found to have converted to a Th1 phenotype as measured by their production of IFNγ suggesting that Th17-cells in NOD mice are not the primary cell type driving pathogenesis [200]. These studies also highlight the plasticity of the different $CD4^+$ T effector populations involved in a dynamic immune response.

Recently, a highly pathogenic subset of $CD4^+$ effector T-cells, termed Th40-cells, was identified in the insulitis lesion of NOD mice [201]. In addition to CD40L expressed on all activated T-cells, this subset expresses the CD40 protein itself and produces the proinflammatory cytokines IFNγ, TNFα, IL6, and sometimes IL17.

The percentage of Th40 cells in NOD mice correlates with increased insulitis and this population is essential for the transfer of disease to NOD scid recipients [202]. Further adding to the complexity of the dynamic interactions between the different $CD4^+$ effector T-cell populations, the production of IL6 by Th40 cells can convert Treg cells to Th17-cells in NOD mice. Importantly, Th40 cells have been identified in increased numbers in T1D patients and were found to be reactive to known islet autoantigens [203].

Treg cells are another important subset of effector T helper cells involved in resolution of immune responses and in preventing autoimmunity. Although NOD mice have normal numbers of Treg cells, their ability to regulate pathogenic T-cells is limited [204]. IL2, encoded within the *Idd3* susceptibility loci, is expressed at reduced levels in NOD mice compared to non-autoimmune prone strains and this is correlated with the development of T1D. Since IL2 has an important role in the induction and maintenance of FoxP3-expressing Tregs, it has been proposed that reduced IL2 promotes T1D through an imbalance between Tregs and pathogenic T-cells [205]. Indeed, it has been shown that islet infiltrating Tregs in NOD mice have reduced levels of IL2 receptor (CD25), FoxP3, and Bcl2 as a consequence of reduced IL2 signalling. Consistent with this, administration of low dose IL2 to susceptible NOD mice restored CD25 expression on islet infiltrating Tregs and were protected from T1D [206]. It was also possible to correct the functional defect in Tregs of NOD mice by co-culturing them with cord blood stem cells [207]. This enhancement of Treg function using stem cells has recently been tested in T1D patients and showed promising therapeutic potential [208].

Natural Killer T (NKT) cells are a rare but an important effector T-helper cell subset, expressing an invariant TCRα chain, Vα14-Jα 18 in mice and Vα24-Jα18 in humans, and are also important in regulating effector T-cells. In NOD mice, NKT-

cells are severely reduced in number and are functionally deficient with an impaired ability to produce cytokines upon stimulation [209, 210]. Although NKT-cells can usually produce large quantities of both IL4 and IFNγ, NKT-cells in NOD mice produce decreased levels of Th2 cytokines, particularly IL4 which may lead to a Th1 bias [211]. The reduced number of NKT-cells correlates with exacerbation of T1D, and reconstitution of NOD mice with normal numbers of NKT-cells prevents T1D [212]. Furthermore, it has been found that the impact of NKT-cells on T1D in NOD mice is not always attributable to cytokine production [211]. NKT-cells may also regulate T1D development through cell to cell contact with conventional T-cells since this has been found to inhibit differentiation and induce anergy of islet reactive T-cells in NOD mice [213]. These findings demonstrate that NKT-cells may influence T1D via multiple non-mutually exclusive mechanisms. However, the significance of NKT-cells in human T1D is yet to be definitively confirmed.

1.1.5 Killing of beta cells

Since the infiltration of macrophages within the inflamed islet precedes that of T-cells, it is thought that initial beta cell death may not be antigen specific, instead being mediated by cytokines produced by macrophages. These cytokines include TNFα, IFNγ, and IL1β that bind their respective receptors on beta cells and induce apoptosis of beta cells via STAT1 and NFκB pathways [214]. They can also induce the expression of reactive oxygen species (ROS) such as nitric oxide (NO) which initiates DNA strand breakage and activation of poly-ADP ribose polymerase (PARP), causing beta cell apoptosis. Although macrophages are important for initial beta cell damage, they do not kill sufficient numbers of beta cells to cause T1D, since NOD scid mice with functional macrophages, but lacking lymphocytes, do not develop T1D. β-cell abnormalities also contribute to the development of

disease progression. Islet cell overexpression of MHC class I [215], beta cell endoplasmic reticulum stress [216] and the abnormalities in the islet extracellular matrix [217] are among the major driving forces that precipitate T1D. A recent study identified that in T1D patients a rare population of cell type exists which expresses both T cell receptor (TCR) and B Cell Receptor (BCR) along with other lineage markers of both B and T cells [218]. These cell types known as DE (dual expressers) encode an antigen capable of activating autoreactive $CD4^+$ T cells and could be the source of idiotypic autoantigens in T1D.

In addition, Beta cell stress can cause changes in the post translational machinery and results in the formation of new peptides [219]. These neopeptides when presented in a genetically susceptible background has the potential to active the T cells to become pathogenic [220]. HIP or Hybrid Insulin Peptide is a post-translationally modified $CD4^+$ T cell epitope and HIP reactive T cells has been identified in NOD mice and humans [221]. The presentation of hybrid peptides containing non-genomically encoded amino acid sequences could trigger an immune response towards the organ that presents.

The events that cause the non-invasive insulitis lesion to become invasive are not understood. However, the mechanisms by which beta cells are killed once this switch occurs have been well defined using NOD mice deficient in beta cell MHC class I, Fas or FasL, perforin or granzyme. After a critical threshold of beta cell antigen has been released by nonspecific killing and presented to islet specific T-cells, they become activated, are recruited to and retained within the islet tissue where they proliferate and contribute to beta cell death [222]. Activated T-cells can kill beta cells through a Fas/FasL pathway. NOD mice deficient for either Fas or FasL are protected from T1D and transfer of wild-type NOD splenocytes into Fas deficient NOD scid mice resulted in delayed T1D [223].

Unlike $CD4^+$ T-cells which cannot kill beta cells in an antigen specific manner, $CD8^+$ T-cells recognize upregulated MHC class I on beta cells and can mediate antigen specific beta cell killing [224]. NOD mice lacking beta cell MHC class I expression are largely protected from the development of T1D. This demonstrates that a direct interaction between $CD8^+$ T-cells and beta cells is the primary mechanism for antigen-specific beta cell killing [128, 225]. Perforin-deficient NOD mice have a similar reduction in T1D incidence, suggesting that $CD8^+$ T-cells use the perforin/granzyme cytotoxicity pathway to kill beta cells [226]. Recently the role of $CD8^+$ T-cells in human T1D was also demonstrated. Islet autoreactive $CD8^+$ T-cells were identified in focal islet regions from cadaveric T1D donors and this was associated with beta cell destruction [227]. Furthermore, in humans it has been shown that the structural basis of $CD8^+$ mediated killing of human beta cells is different from other TCR-MHC class I interactions [228]. The TCR of a human autoreactive $CD8^+$ T-cell was highly peptide-centric in its recognition of MHC class I bearing proinsulin peptide, thus forming a very weak interaction with the MHC molecule itself. This interaction may explain why such autoreactive T-cells escape thymic selection. Based on these important studies implicating T cells in the development of T1D, there have been a number of trials using anti-CD3. Early studies using humanised anti CD3 were promising, with treated patients showing a reduced dependence on insulin over 4 years. However a more recent and large trial using anti–CD3 (Otelixizumab) showed no improvement in C peptide, insulin dependence or HbA1c levels [229].

1.1.6 Relevance of NOD mice in current T1D research

For the past 35 years, the NOD mice model has been extensively used to understand the pathogenesis and to test for therapeutic interventions of T1D [230]. The extensive use of this model in T1D research is because of its pathogenic

similarities that it shares with human T1D. The identified similarities in the pathogenesis of NOD and human T1D is summarised in Table 1

Table 1. Pathogenic similarities in NOD mice compared to Humans

Similarities	Human	NOD
Insulitis	Present	Present
Non- MHC genes linked	>50 [231, 232]	>40 [15]
Shared Autoantigens	Proinsulin, Insulin, Insulin hybrid peptides, GAD65, ZnT8, IGRP, Phogrin, Chromogranin A, ICA 69, Islet amyloid polypeptide [233]	Proinsulin, Insulin, Insulin hybrid peptides, GAD65, ZnT8, IGRP, Phogrin, Chromogranin A, ICA 69, Islet amyloid polypeptide [233]
Autoreactive $CD4^+$ T cells	Present [233]	Present [233, 234]
Autoreactive $CD8^+$ T cells	Present [233, 235]	Present [233]
B cells	Likely an important cell type in the development of disease because anti CD-20 therapy delayed the progression of disease [236]	Important in the development of disease [237]
Neutrophils	Circulating neutrophils are low in patients with T1D [238-240]	Low in numbers [241]
Onset	Diagnosed usually at young age	3-6 months
Autoimmunity	Spontaneous	Spontaneous
Insulin Autoantibodies	Present [242]	Present [243, 244]
Environmental influence on gene penetrance	Present	Present

Studies in NOD mice over the past few decades have contributed to numerous immunomodulatory therapies and many of them have been tested in humans. Despite the successful protection from T1D seen in NOD mice, there has been limited success with therapeutic interventions in individuals at-risk of T1D and patients. In NOD mice, non-antigen specific therapies including those that target the T-cells using anti-CD3 mAb, B cells using anti-CD20 mAb, and APCs using a soluble CTLA4-Ig showed some protection from T1D. Unfortunately, however, similar therapies showed limited efficacy in humans. Treatment of recently diagnosed T1D patients with Rituximab, a humanised anti-CD20 mAb, resulted in transient preservation of beta cell mass. Although improvement was transient, this therapy holds promise for those at-risk of T1D if administered earlier in the disease process. Genetic screening and earlier diagnosis will be important for opening an earlier therapeutic window of intervention [245, 246].

Antigen specific immune modulation trials in NOD mice, such as those involving insulin, were successful. However, none of the human trials in at-risk children have demonstrated significant efficacy. Explanations that have been proposed for the failure of these trials include the possibility that the route of administration rendered the insulin ineffective, the dose was inadequate to induce tolerance, or alternatively, such an approach may work only in those at-risk individuals that entered the trial prior to the development of activated T-cells and insulin AAbs [185, 186]. Preclinical studies in NOD mice using a combination of murine anti-thymocyte globulin and granulocyte-colony stimulating factor (ATG/GCSF) demonstrated that ATG has the potential to deplete pathogenic T cells and GCSF promotes Tregs [247]. Based on these observations in NOD mice, clinical trials conducted in new-onset T1D patients showed that administration of low dose ATG preserves C-Peptide, reduces HbA1c, and increases Tregs [248].

Combinational therapies (CTs) that suppress T-cell activation and enhance tolerance have also been successful in NOD mice. A recent pilot study using CT in T1D patients involved the use of Rapamycin to supress effector T-cell proliferation in combination with IL2 to induce the formation of Tregs. Although this approach did promote an increase in Tregs, unfortunately none of the treated patients showed an increase in preserved beta cell mass [249]. Using the knowledge gained from NOD, immune interventions have been trialled in humans and have successfully delayed the loss of insulin production in T1D patients [250]. These interventions using teplizumab an anti CD3 antibody, were not only useful in T1D patients, but also delayed progression to clinical diabetes in relatives at risk of developing the disease [251].

Another innovative therapeutic approach that has been successfully tested in NOD mice is the use of immature DCs to induce tolerance [252]. This approach involves the ex-vivo engineering of NOD DCs with antisense oligodeoxyribonucleotides (AS-ODN) to inhibit the expression of CD80/86. After transfer back into NOD mice these immature DCs migrated to the pLN and induced tolerance to beta cell Ags. Clinical trials using engineered DCs have now been initiated and support the on-going investigation of this approach for treatment of at risk individuals in further trials [253]. One of the most recent and exiting therapeutic approaches, currently in Phase I clinical trials, is known as stem cell educator (SCE) therapy. This approach originated from NOD mouse studies in which splenocytes were cultured with human cord blood stem cells. Tregs cultured in this way showed an increase in the $CD4^+$ $CD62L^+$ subpopulation and were able to suppress T1D in NOD recipients [254].

One of the major challenges faced when using NOD mice for experimental studies is the lack of non-invasive methods to directly visualise the initiation and

progression of islet attack. Since individual NOD mice within a cohort vary in the penetrance of disease development, a non-invasive method that helped to sort mice that should or should not go on to develop diabetes has been developed by Wenxian Fu et.al [255]. Using the principle of Magnetic Resonance Imaging of magnetic nanoparticles Wenxian Fu showed that it is possible to distinguish genetically and environmentally matched NOD mice that progress or do not progress to diabetes. This technique can now be extrapolated to predict the development of disease in humans at risk.

In summary, the NOD mouse has been extensively studied and has informed much of the current understanding of the immunopathogenesis of T1D. There are many similarities in the genetics and immunopathogenic mechanisms that lead to T1D in NOD mice and humans. Understanding these pathways has given us insight into a number of potential therapeutic avenues that are currently being trialled (Table 2).

Table 2 Summary of Clinical outcomes for T1D patients based on NOD mouse studies

Lessons from NOD mice	Potential relevance to human T1D	Clinical significance
Role of Environmental • Vitamin D protects from T1D in NOD [55, 56, 256].	• Reduced incidence of T1D in equatorial regions of high sunlight. • UV-B is essential for the synthesis of Vitamin D. • Vitamin D has anti-inflammatory effects.	• Vitamin D supplementation: at birth protected from T1D; in recent onset T1D had no significant protection [62, 63, 257].

• Omega 3 fatty acids protect from T1D in NOD [69].	• Reduced incidence of T1D in ethnic groups with a culture of increased fish consumption. • Omega 3 fatty acids are anti-inflammatory.	• Omega 3 supplementation: in genetically susceptible children reduced T1D incidence; in pregnant mothers and their HLA higher T1D risk babies efficacy not yet known [70]. Trials on-going [258].
• Probiotics protect from T1D in NOD [74].	• Higher T1D incidence associated with higher standard of living, hygiene and antibiotic use.	• Probiotic supplementation administered to at risk children (PRODIA study) established safety. Efficacy not yet known [98].

Role of Cytokine/Cytokine receptors • Macrophage production of TNF α and IL-1β are directly beta cell toxic in NOD mice [113].	• Macrophages present in pancreatic samples of cadaveric T1D patients [259].	• Recombinant IL-1 receptor antagonists (Anakinra & Kineret): in recent onset T1D children no change in proinflammatory cytokine gene expression, insulin secretory capacity, or HbA1c levels. Lower insulin requirements were reported up to 4 months post treatment [117]. • TNF antagonist (Etanerncept , Infliximab and Adalimumab): in recent onset T1D children reduced HbA1c and increased insulin production at 24 weeks post treatment [117].

Role of dendritic cells • Defective DC maturation in NOD [118]. • Increased NFκB activation in myeloid DCs of NOD [119, 260]. • Increased IL-12 production by NOD DCs [119].	• DCs control tolerance vs activation of T cells. • Increased NFκB activation in myeloid DCs of T1D patients [120].	• Autologous DCs, manipulated to reduce co-stimulatory ability and promote tolerance, given to T1D patients and at risk individuals. Safety established. Efficacy not yet known [253]. Trial on-going [261].
Role of co-stimulation • Reduced expression of co-stimulation inhibitory molecule	• Reduced expression of soluble CTLA-4	• CTLA-4 Ig fusion protein co-stimulation blockade (Abatacept): in recent onset T1D patients resulted in

liCTLA-4 in NOD [35]. • Over expression of liCTLA-4 reduced T1D incidence in NOD [165]. • Early treatment with CTLA-4 Ig protected from T1D [164]. • Reduced expression of inhibitory molecule PD-L1 [167]. • Higher expression of ICOS [262].	in human T1D [35]. • Reduced expression of PD-1 on T cells in human T1D [168].	initial improvement in C peptide levels. No preservation of beta cell mass after 2 years [166]. Currently in Phase II phase of the clinical trial. Expected completion date in 2026. Clinical trial number NCT03929601.

Role of beta cell antigens (pro)Insulin • Transgenic NOD mice overexpressing proinsulin in the thymus were protected from T1D suggesting the role of thymic expression of insulin in maintaining tolerance [152]. • Transgenic NOD mice expressing mutated proinsulin (lacking immunogenic peptide) were protected from T1D [182].	• Polymorphism in human insulin promoter associated with lower thymic proinsulin expression, loss of tolerance to insulin and T1D [153]. • Insulin autoantibodies present in human T1D[263].	• Attempts to induce tolerance to insulin with: • Intra-nasal delivery: In autoantibody positive individuals had some increase in antibody and decrease in T cell responses to insulin [264]. Clinical trial number NCT00336674. Expected to complete in December 2019. No results posted yet. • Oral delivery: in recent onset T1D patients had no improvement in C peptide secretion or IgG insulin antibodies, and accelerated beta cell loss in some treated patients [265-267]. • Intra muscular delivery with IFA adjuvant: in recent onset T1D patients had some increased insulin specific Treg cell at 2 years post treatment [187, 268].

• Insulin autoantibodies precede T1D in NOD [263]. • T cell responses against insulin present in NOD[263]. GAD65 • GAD65 autoantibodies present in NOD [263].	• T cell responses against insulin in human T1D[263]. • GAD65 autoantibodies present in human T1D.	• Attempts to induce tolerance to GAD65/Alum with: • <u>Subcutaneous delivery</u>: In recent onset T1D patients had no significant improvement in C peptide levels [269]. Combination therapy with Vitamin-D and ibuprofen currently in phase 2 trials. Another clinicai trial of intralymphatic administration of GAD alum in T1D patients is also progressing NCT02352974 [270].

Role of T cells • Autoreactive T cells present in insulitis lesion of NOD [129]. • Autoreactive T cells transfer disease to NOD scid [131]. • $CD8^+$ T cells are the primary mediators of beta cell killing in NOD [177]. • Intravenous treatment of anti CD3 protected from T1D in NOD [148].	• Autoreactive T cells present in T1D patients. • $CD8^+$ T cells present in islets of cadaveric T1D patients [259].	• Anti-CD3 monoclonal antibody in recent onset T1D patients and at risk individuals reduced dependence on insulin over 4 years [273]. However a recent large trial (Otelixizumab) showed no efficacy in terms of C-peptide, insulin dependence and HbA1c [274]. However, Teplizumab an anti-CD3 antibody delayed progression to clinical T1D in individuals that are at high-risk [275].

• Oral antiCD3 reversed T1D in NOD [271]. • Combined treatment of anti CD3 with IL-1RA causes synergistic reversal of T1D in NOD [272].		
<u>Role of B cells</u> • B cells present in insulitic lesion of NOD [129]. • Antibodies against beta cell antigens present in NOD [263].	• B cells present in pancreatic samples of cadaveric T1D patients [277]. • Antibodies against beta cell antigens present in human T1D.	• Anti-CD20 (Rituximab): in recent onset T1D patients had improved HbA1c and insulin dependence at 1 year. However depressed IgM levels indicate B cell immunosuppression [245, 278].

• B cell deficient NOD mice were protected from T1D [134]. • Anti CD20 depletion of B cells protected from T1D [276].		
<u>Role of Tregs</u> • Lower levels of IL-2 in NOD [279]. • Tregs have reduced levels of IL-2R and FoxP3 expression in NOD.	• Polymorphism in IL2RA causing diminished IL-2 response in Treg from human T1D [280].	• Stem Cell Educator (SCE) to promote Treg development. Autologous lymphocytes co-cultured with cord blood stem cells (CB-SC) given to T1D patients improved C-peptide, HbA1c and insulin dependence at 40 weeks post treatment [254, 281-283].

Though the exact pathogenesis of T1D is still not clear, it is well documented that immune mediated destruction of the islets is well-orchestrated by multiple players of the innate and adaptive arm of the immune system. Among the different types of immune cells that infiltrated the human islets, $CD8^+$ T cells were more predominant when compared to $CD4^+$ T cells, B cells and Dendritic cells [284, 285]. T cell autoreactive responses to diverse islet derived and non-islet antigens have been identified in humans with T1D [285]. In addition, the physiological condition of beta cells along with dietary components and microbes also play a crucial role in the development of disease [286, 287].

By using NOD, we learned a lot about the genetics and the pathogenesis of human T1D [288], but the failures in translating successful therapies in NOD to humans has challenged the relevance of using this model for therapeutic interventions. Observations in NOD mice may not be the same in humans. For instance, the presence of beta cell autoantibodies is a strong indicative of ongoing autoimmune response in NOD mice but similar presence in humans do not imply an ongoing autoimmune attack. Autoantigen specific T cells such as the Chromogranin A specific $CD4^+$ T cells, insulin and IGRP specific $CD8^+$ T cells are able to transfer diabetes in NOD SCID mice but circulating insulin antigen-specific autoreactive T cells alone is not sufficient to cause insulitis or T1D in humans [289]. Above all in NOD mice insulin is believed to be the initiating antigen but in humans there are several initiating antigens and the pattern of insulitis in NOD mice is more severe when compared to humans with T1D [290-292]. In humans since multiple antigens located in diverse tissues are involved in the development of disease, it is not possible to ascertain whether one immune response is more predominant and pathogenic than the other. This is not the case with NOD mice, as it has been shown that NOD mice lacking the native insulin gene but replaced with a mutated

insulin gene were completely protected from insulitis and disease development [293]. Using intervention therapies disease progression has been successfully delayed/ reversed in NOD mice but successful intervention therapies in NOD mice have failed to translate in clinical trials [294]. Despite these differences and unsuccessful clinical trials, there is no animal model, other than the NOD mice, that develops spontaneous autoimmunity and T1D in a similar way as in humans [230].

Because of the differences in humans and animals, successful therapeutic interventions in animals may not always translate efficiently in humans. Based on the outcome of unsuccessful clinical trials, we cannot rule out the use of animals from medical research. Animal studies are an absolute requirement prior to any clinical trial. This is because in human trials, multiple parameters cannot be controlled at the same time due to the heterogenicity of the population but, these parameters can be controlled in inbred mice strains and the results are often reproducible at different geographical locations. The major advantages of using animal models in medical research is summarised in Table 3.

Table 3. Importance of the use of animals is medical research

Study	Humans	NOD mice
Multiple intervention studies	Due to ethical reasons, multiple intervention studies cannot be performed	Can be performed
Lymphocyte study	Easy access to lymphocytes are limited to peripheral blood sample	Invasive studies can be performed and lymphocytes in tissues can be accessed

Access to pancreatic samples	Pancreatic organ donors/autopsy	Easy to obtain
Multi-centre study	Heterogenous population and therefore results obtained from different study centres may vary	NOD colonies are inbred and therefore variations in results can be controlled.
Genetic Mutation studies	Limited	Genetic studies can be explored using transgenic and knockout technologies
Diet and environmental studies	Cannot be controlled	Restricted diet and environmental parameters can be controlled by maintaining in SPF
Cost	Expensive	Relatively cheap
Detecting rare T1D susceptibility alleles	Require more affected patient samples and practically impossible to get the required sample numbers.	Though large numbers required, it is practically possible

To minimise the differences in results observed in mice and in human trails, we should mimic the human system in mice. This raises the need for the using humanised NOD to understand the pathogenesis of T1D [295, 296]. When Shulian Tan used humanised NOD mice with engineered $CD4^+$ T cells recognising insulin B:9-23, beta cell destruction was not observed until challenged with Streptozotocin (STZ) or by immunisation with InsB:9-23 peptide/CFA. This clearly shows that circulating insulin reactive T cells alone was not sufficient to cause disease in an environment that mimics humans. This also suggests that there may be other

autoantigens either upstream or along with insulin that are required to cause beta cell destruction and hyperglycaemia [297]. Using humanised NOD mice and by looking over the wall of pancreas to identify the initiating autoantigen/s should be our next approach to find therapies in T1D. Nevertheless, despite the failure of few clinical trials, NOD mice remains as the standout model for exploring the early and late stage pathogenesis of T1D [298].

Antigen and non-antigen specific immunomodulatory trials have attempted in both humans and in NOD mice. Despite several studies and clinical trials, there exists no single treatment either to halt the disease progression or prevent the disease development. Over the past 2 decades of T1D research, we have understood multiple factors contributing to the development of disease. This include genetic polymorphisms, defects in central and peripheral tolerance, beta cell phenotype, autoantigens, autoantibodies, environmental factors such as vitamin D and cow's milk, maternal antibodies, dietary gluten and much more [299]. We have extrapolated our knowledge we got from NOD mice to clinical trials in humans [300, 301]. Despite all our trails, we still have not achieved either a complete cure from T1D or an effective method in preventing the development of disease. Genetic vaccinations and gene therapy [301] are of possible hope to treat T1D but as prevention is always better than cure, identifying the initiating autoantigen in T1D is of prime importance. The work described here uses the NOD mouse as the animal model to understand the role of major histocompatibility complex (MHC) class I in the pathogenesis of T1D.

1.2 Rationale of study

Immune cells bearing receptors for self-antigens escape the central and peripheral tolerance mechanisms to become pathogenic and intrude the islets [302]. The chemokines, along with the beta cell antigens presented by $CD11c^+$ cells facilitate the initial entry of auto reactive T cells into the islet milieu [303]. The initial infiltration, followed by a series of stochastic events, results in the selective killing of beta cells of the pancreas through MHC class I dependent process [304]. Both $CD4^+$ and $CD8^+$ T cells play their respective role in beta cell destruction. Indeed, it has been shown that continuous support from $CD4^+$ T cells is required by the $CD8^+$ T cells to maintain their cytotoxic strength and to cause beta cell destruction and disease progression [305]. In the past, there were studies which show that diabetes can be induced on a NOD SCID background by using T cell receptor (TCR) transgenic mice (BDC2.5/NOD SCID) and without the help of $CD8^+$ T cells [306, 307]. These transgenic mice differ from traditional NOD and more than 90% of $CD4^+$ T cells in these mice recognise the β cell protein chromogranin A. The T cell repertoire is skewed in favour of the transgene-encoded specificity and more over beta cell apoptosis seen in TCR transgenic NOD SCID mice was induced by Fas/Fas-L or TNF-α/TNF-αR or by IL-1/inducible nitric oxide synthase pathways. When low dose $CD4^+$ T cells from BDC2.5 transgenic mice were adoptively transferred to adult NOD SCID mice they were unable to transfer diabetes or insulitis without the co-transfer of $CD8^+$ T cells. These findings demonstrate that on a NOD background, $CD8^+$ T cells are required for the final stages of beta cell damage but, in a TCR transgenic environment, where autoantigen specific T cells are present relatively in high abundance, beta cell death can occur by other alternative route that does not require the help of $CD8^+$ T cells will occur.

It has been thought that autoantigens expressed by beta cells are the initial targets for autoreactive T cells and by preventing the autoantigen presentation by beta cells; it may be possible to stop the immune infiltration into the islet milieu. A pioneering study conducted by the Slattery lab demonstrated that even in the absence of presentation of beta cell autoantigens, autoreactive cells continued to infiltrate into the islets [179, 308]. This entry of immune cells into pancreatic islets and associated tissue suggested that, autoantigens expressed by cells other than the pancreatic beta cells are also important in the development of insulitis in NOD mice. Careful analysis of pancreata from NOD mice lacking MHC class I on beta cells, revealed that, early immune infiltrates did not home towards the islets but instead they accumulated near the pancreatic ducts [179].

Pancreatic ducts are physiologically close to the beta cells and their main function is to deliver enzymes into the duodenum and also secrete bicarbonate to maintain the duodenal pH [309]. In addition to their role in supporting digestion, ductal cells are also identified as the progenitors for beta cells and as such they share similar antigens with islet beta cells [310, 311]. The presence of ductal infiltrates seen in NOD mice lacking MHC class I on beta cells, suggests that the early development of insulitis precedes the involvement of islet beta cells. It is likely that ductal cell autoantigens are chronologically ahead to beta cell autoantigens for the autoreactive attack. This is because pancreatic ductal cell infiltrates were also noted in genetically modified NOD mice lacking native insulin [312] and ductal cells are the progenitors for beta cells [313, 314].

Pancreatic ductal cells express numerous autoantigens including the cytoskeletal Glial Fibrillary Acidic Protein (GFAP). GFAP is the classical marker for the glial cells of the central and peripheral nervous system and autoreactivity to GFAP expressing peri-islet Schwann cells (pSCs) of the pancreas has been reported in

NOD mice as early as three weeks of age [315]. It may be quite surprising that, in addition to the beta cells of the pancreas, auto reactivity to antigens expressed in distal tissues, including salivary glands, thyroid and cells of the neuronal glia has also been reported in NOD mice [316-320]. The importance of autoantigens expressed in the tissues distal to islets is gaining interest and the broken tolerance to autoantigen/s expressed in anatomically distal sites to the pancreas may possibly culminate into beta cell autoreactivity.

The immune infiltrates accumulating near the pancreatic ducts may cause damage to these cell types resulting in the release of numerous antigens that are shared with beta cells. Though there is no direct evidence showing the destruction of GFAP expressing ductal cells during the pathogenesis of T1D, studies conducted by Michael Dosch et al. have shown that GFAP expressing glial cells that encapsulate the islets are killed even before the beta cells [315, 321]. GFAP is now an identified autoantigen in T1D and serum autoantibodies to GFAP is a novel biomarker for predicting T1D in humans [322]. It is fascinating that a cytoskeletal protein widely expressed in the glial cells of the central and peripheral nervous system is also an early autoantigen in T1D.

GFAP expressing cell types especially the pre-beta ductal cells may be the earliest target for autoreactive T cells and by ablating the self-antigens presented by the ductal cells, we may be able prevent the immune infiltration into the islets and associated tissue.

1.2.1 Hypothesis

MHC class I expression in GFAP positive cells of the pancreas is essential for the development of invasive insulitis in NOD mice.

1.2.2 Aim

To determine the role of MHC class I expression on $GFAP^{+}$ ductal cells of the pancreas in the development of invasive insulitis in NOD mice.

2. Materials

2.1 Buffers and Solutions

2.1.1 Tris(hydroxymethyl)aminomethane acetate (TAE) buffer (50X stock)

- 242g Tris Base
- 57mL Glacial Acid (100% acetic acid)
- 100mL Ethylenediaminetetraacetic acid (EDTA) 0.5M pH8
- Make up to 1L with Milli Q water

2.1.2 Phosphate Buffered Saline (PBS, 10X stock)

- 2g KCl
- 2g KH_2PO_4
- 80g NaCl
- 14.3g Na_2HPO_4
- Make up to 1L with Milli Q water

2.1.3 Ear Punch Buffer (1X)

- 50mM TrisHCl pH8 (12.5mL of 2M)
- 100mM NaCl (2.92g)
- 100mM EDTA (100mL of 0.5M)
- 1% SDS (5g)
- Make up with Milli Q water (total of 500mL)

2.1.4 HANKS + 2% fetal calf serum (FCS)

- 500mL HANKS buffer
- 10mL heat inactivated FCS

- Filter to sterilize

2.1.5 FACS buffer

- 500mL PBS
- 2.5g BSA
- 2mL EDTA (stock 0.5M)

2.2 Equipment

- Flow cytometer (BD LSRII, Fortessa and BD FACS Calibur)
- Fluorescent microscope (Olympus BX61)
- Cryostat (Leica)
- Centrifuge (Eppendorf)

2.3 Mice used

Table 4 Mice strains

Mice strain	Genotype	Description
NOD	Wild Type	Non Obese Diabetic mice. These mice spontaneously develop diabetes (T1D).
NOD EYFP	NOD EYFP$^{+/+}$	Mice have a lox P flanked stop sequence followed by the Enhanced Yellow Fluorescent Protein

		(EYFP) gene inserted into the ROSA 26 locus. When NOD EYFP mice are bred to mice expressing the cre recombinase protein, the stop sequence is deleted and EYFP expression is visible in tissues of double mutant offspring.
NOD β2M Knock out	NOD $\beta2M^{-/-}$	Mice lack MHC class I on all cell types and these mice do not develop diabetes.
NOD $\beta2M^{-/-}$flβ2M^{a}	NOD $\beta2M^{-/-}$ flβ2M$^{a+/-}$	The wild type β2M gene is replaced with a floxed β2M^{a} gene as described by Hamilton et al. [48]. These mice are MHC class I sufficient and develop spontaneous diabetes.
NOD GFAPcre	NOD GFAPcre+/-	Hemizygous GFAPcre mice express cre recombinase under the

		control of human GFAP promoter.
Class I GFAP bald	NOD GFAPcre $\beta 2M^{-/-}$ $fl\beta 2M^{a+/-}$	In these mice, wild type β2M gene is replaced with a floxed $\beta 2M^{a}$ gene and expression of cre is under the control of human GFAP promoter. Cre mediated recombination and thereby loss of MHC class I expression occurs in cells expressing GFAP.
Class I CAII bald	NOD CAIIcre $\beta 2M^{-/-}$ $fl\beta 2M^{a}$	In these mice, wild type β2M gene is replaced with a floxed $\beta 2M^{a}$ gene and expression of cre is under the control of human Carbonic anhydrase II promoter. Cre mediated recombination and thereby loss of MHC class I expression occurs

		in cells expressing CAII.
Reporter mice	NOD GFAPcre^{+} EYFP^{+}	Generated by crossing NOD EYFP mice to NOD GFAP mice. In these mutant mice, the cre is expressed in GFAP expressing cells. EYFP is detected in tissues where cre mediated recombination has removed the floxed stop codon from the ROSA 26 locus.

2.4 Methods

To understand the role of MHC class I on the ductal cells of the pancreas, Ductal tissue specific knockout mice were generated in two parallel ways. Cre–lox technology was used to generate knockouts in both the approaches [323, 324]. In the first approach, we purchased the desired mice, GFAPcre, which expresses cre in the ductal cells of pancreas, from commercially available sources. Commercially available GFAPcre mice were on FVB background and we backcrossed to NOD to generate NOD GFAPcre mice. In the second approach, we generated our NOD CAIIcre mice that express cre in ductal cells of the pancreas, by microinjection. To create ductal tissue specific MHC class I knockout mice, systematic mating pattern was followed for both mice strains that express cre (GFAPcre and CAIIcre).

Though the promotors which drive cre are different, it is well established that both carbonic anhydrase II (CAII) and GFAP are expressed in the ductal cells of the pancreas, therefore when crossed to a mice carrying floxed β2M gene, ductal tissue specific deletion of β2M occurs and MHC class I expression is restricted in those ductal tissues which express cre. The detailed descriptions on the making of both strains are mentioned below.

2.4.1 Generation of tissue specific knockout mice using cre-lox technology

2.4.1.1 Generation of NOD GFAPcre mice

Our aim was to develop a NOD mice that lack MHC class I expression on the ductal cells of the pancreas. Cre-lox recombination technology was used to generate tissue specific MHC class I knockout mice. Causing recombinase (cre) is a protein that recognises specific DNA sequences called loxP sites (Locus of X-over P1). Binding of the cre protein to specific lox P sites, causes the recombination of the DNA resulting in deletion of the gene of interest placed in between the lox P sequences. Two different strains of mice were used to create tissue specific knockouts. In the first mice strain the expression of cre recombinase was under the control of a tissue specific promoter. In our case, we used the GFAP promoter to drive the cre expression in ductal tissues. In the second mice strain, our gene of interest β2Ma, the essential molecule for MHC class I expression, was placed in between specific sequences called loxP sites (floxed β2Ma). F1 generation of the cross between the GFAPcre mice and the floxed β2Ma mice created our experimental mice in which cre mediated recombination occurred in GFAP expressing cell types. A schematic representation for the generation of tissue specific knockout mice is mentioned in Figure 1.

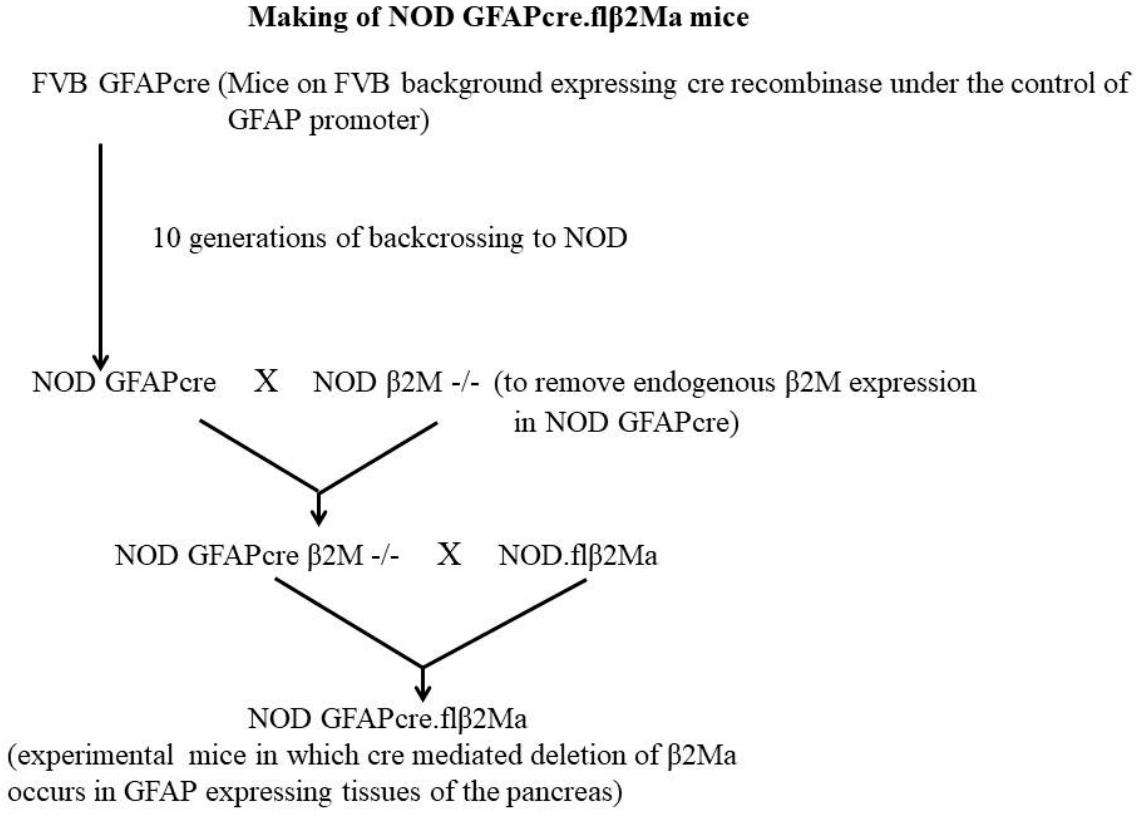


Figure 1 *Showing the breeding pattern for generating NOD mice lacking MHC class I on GFAP expressing cells of the pancreas (Class I GFAP bald mice).*

We used FVB-Tg(GFAP-cre)25Mes/J (Stock number 004600) from the Jackson Laboratory (Bar Harbor, ME, US) and backcrossed onto NOD/Lt for at least 10 generations. The generated NOD GFAPcre mice express cre recombinase under the control of human GFAP promoter. Hemizygous transgenic mice were identified by Polymerised Chain Reaction (PCR) based strategy using primers oIMR 1900 and oIMR 1901 as mentioned in Table 5 [325].

2.4.1.2 Generation of NOD GFAPcre.flβ2Ma mice (Class I GFAP bald mice)

NOD GFAPcre mice had the wild type β2M in their genomic DNA. To remove the endogenous β2M expression and to replace it with a floxed β2Ma gene we crossed

the NOD GFAPcre mice to NOD β2M knockout mice and generated the NOD GFAPcre^{+}β2M$^{-/-}$ mice. Later NOD GFAPcre^{+}β2M$^{-/-}$ mice were then crossed to NOD.flβ2Ma mice carrying a floxed β2Ma transgene [326, 327]. The offspring of the cross between NOD GFAPcre mice on a β2M knockout background (NOD GFAPcre^{+}β2M$^{-/-}$) and NOD.flβ2Ma generated the tissue specific class I GFAP bald knockout mice (Figure 1). Class I GFAP bald mice were expected to have normal MHC class I expression in all the cell types except in cells that express GFAP. Hemizygous mice were identified by PCR based strategy using primers specific for cre and floxed β2Ma as mentioned in Table 5.

2.4.1.3 Generation of NOD CAIIcre mice

The background of the mice strain plays a significant role in the development of insulitis. Since our NOD GFAPcre mice originated from FVB background, it is possible that some of the FVB-derived genomic interval may still be present in the NOD GFAPcre mice. These hitchhiking FVB elements may contribute to an undesired phenotype that may prevent the development of insulitis. As a backup plan we also created another NOD mice strain in which the cre recombinase was driven by a ductal cell specific promotor carbonic anhydrase II (CAII) [328].

CAIIcre mice were generated before my commencement of my PhD candidature. CAIIcre transgene as described in Figure 2 were microinjected into NOD zygotes and two transgene positive founder animals were screened by PCR. Hemizygous transgenic mice were identified by PCR based strategy using primers specific for cre and CAII as mentioned in Table 5. In the beginning of my PhD candidature the NOD CAIIcre founder 1 were at generation 3 and NOD CAIIcre founder 2 were at generation 2 respectively.

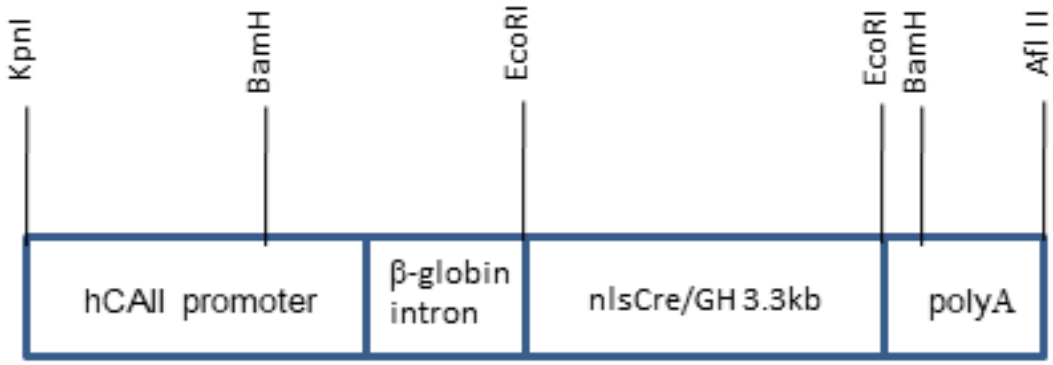


Figure 2 *CAIIcre transgene construct- cre sequence is driven under the control of human carbonic anhydrase II promoter***.**

2.4.1.4 NOD CAIIcre mice breeding strategies

At different generations, both CAIIcre transgenic founders were bred to NOD EYFP (Enhanced Yellow Fluorescent Protein) mice to determine the pattern of cre expression. This was important to understand the penetrance of cre. Simultaneously the NOD CAIIcre mice were also crossed to NOD $\beta2M^{-/-}$ mice to make the transgene positive NOD mice on a β2M knockout background (NOD $CAIIcre^{+}\beta2M^{-/-}$). The complete breeding strategy for founder1 and founder 2 is depicted in Figure 3 and Figure 4.

Figure 3 *Genogram showing the breeding pattern for CAIIcre founder 1*

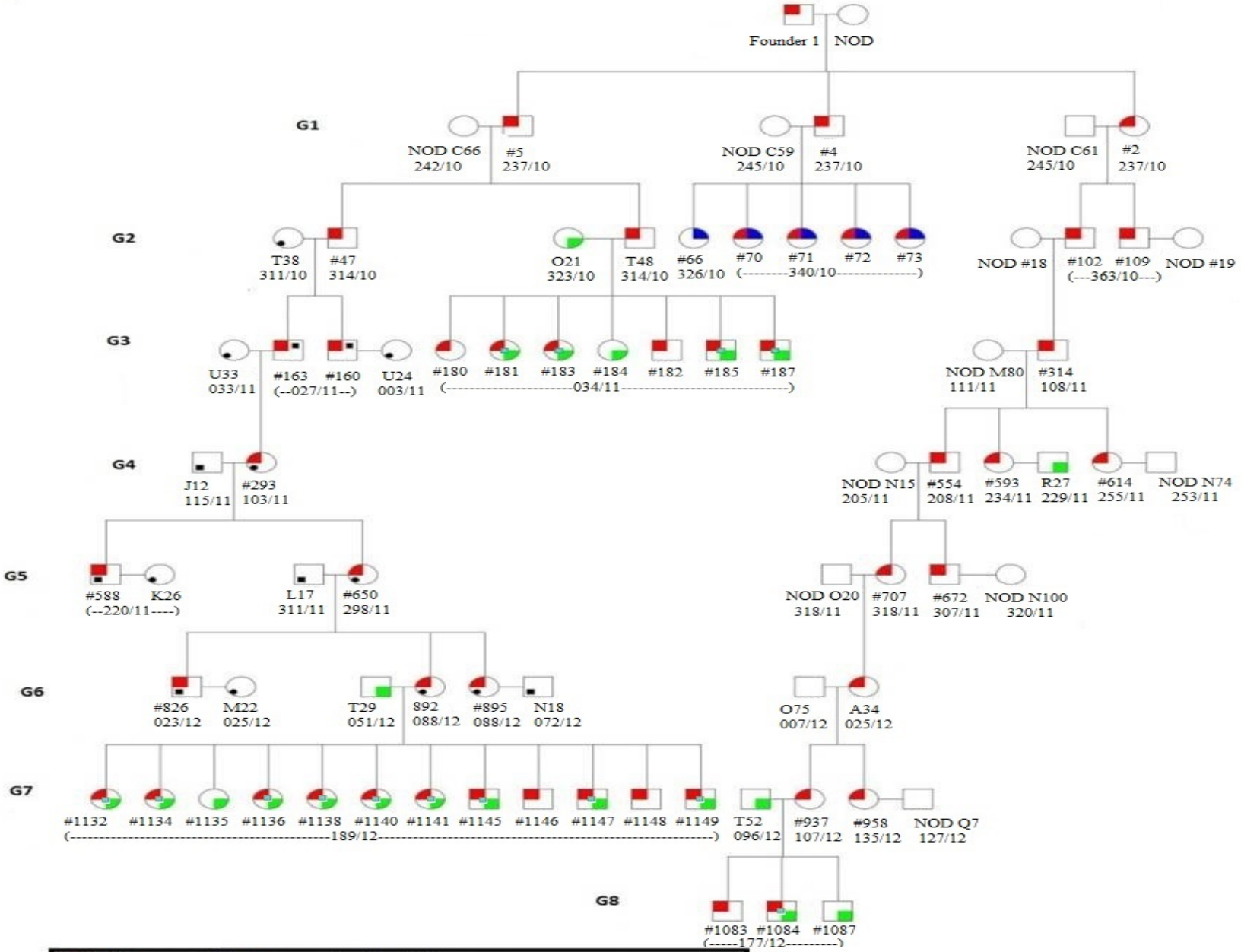
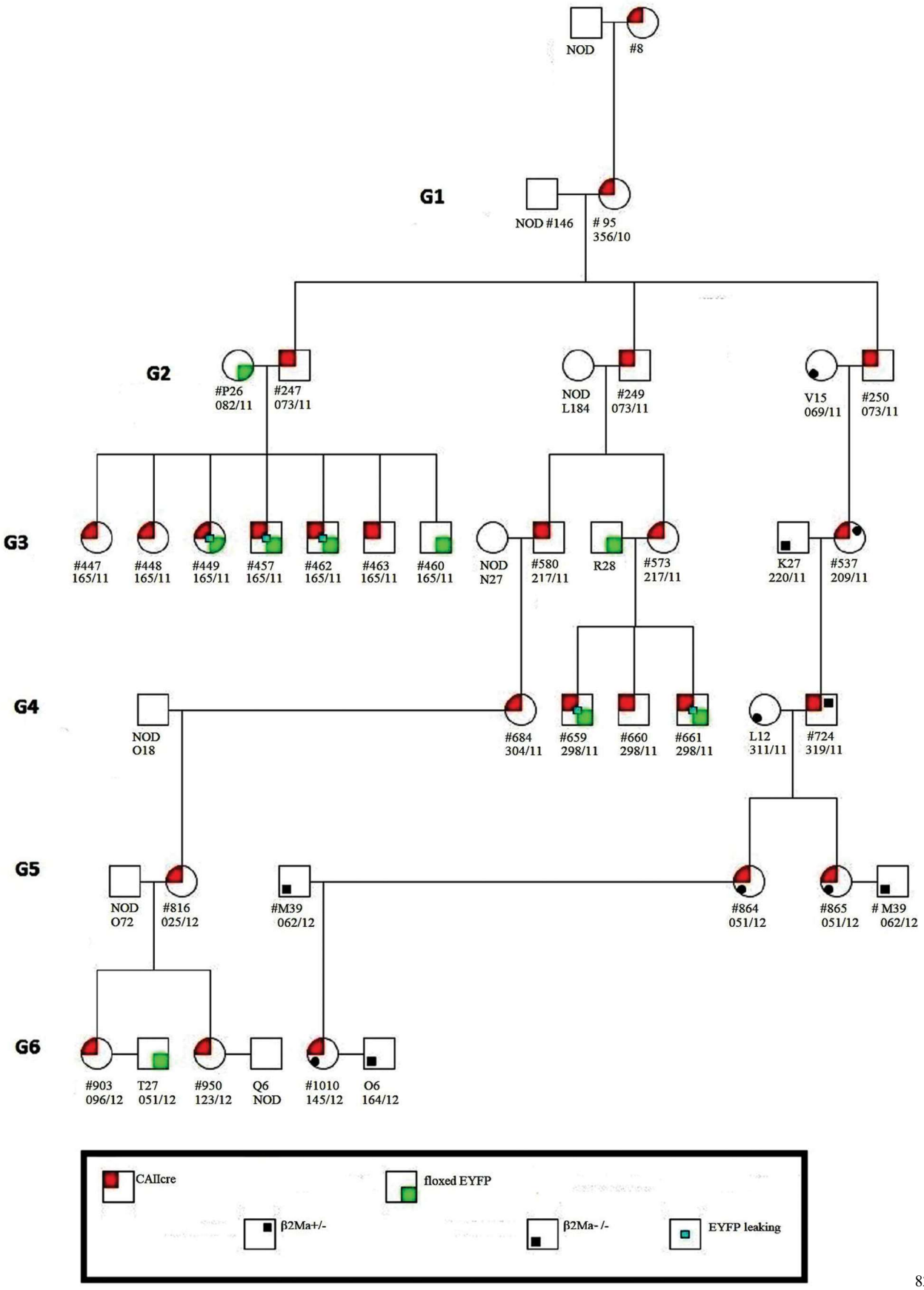


Figure 4 *Genogram showing the breeding pattern for CAIIcre founder 2*



2.4.1.5 Generation of NOD CAIIcre.flβ2Ma mice (Class I CAII bald mice)

NOD CAIIcre^{+}β2M$^{-/-}$ mice were later crossed to NOD.flβ2Ma mice carrying a floxed β2Ma transgene to generate NOD CAIIcre^{+} β2M$^{-/-}$ flβ2Ma^{+} mice (Class I CAII bald). Hemizygous mice were identified by PCR based strategy using primers specific for cre and floxed β2Ma as mentioned in Table 5.

2.4.2 Generation of reporter mice (NOD GFAPcre^{+} EYFP^{+}/ NOD CAIIcre^{+} EYFP^{+})

To determine the pattern of cre expression in both GFAP driven cre and in CAIIcre transgenic mice, these mice were crossed to NOD EYFP mice to generate the NOD GFAPcre^{+}EYFP^{+}/NODCAIIcre^{+}EYFP^{+} reporter mice.

C57BL/6Gt(ROSA)26Sor.EYFP mice (gift by Dr. David Izon at St Vincent's Institute for Medical Research, Melbourne, Australia) carries a loxP-flanked STOP sequence followed by the Enhanced Yellow Fluorescent Protein (EYFP) gene [329]. C57BL/6.Gt(ROSA)26Sor.EYFP mice were backcrossed to NOD/Lt for at least 10 generations. Crossing the mutant NOD EYFP mice to NOD GFAPcre mice generated NOD GFAPcre^{+}EYFP^{+} mice, and by crossing with NOD CAIIcre mice generated NOD CAIIcre^{+}EYFP^{+} mice. Both these reporter mice were used to check the pattern of cre expression in NOD GFAPcre and NOD CAIIcre mice respectively. The presence of EYFP transgene was identified by PCR based strategy using primers ROSA 1, ROSA 2 and ROSA 3 as mentioned in Table 5. ROSA 1 and ROSA 3 primer sets generate 600 bp product identified as wild type mice while ROSA 1 and ROSA 2 primer sets generate 300 bp product identified as mutant mice.

2.5 Mice Genotyping

Mice strains used in this project were genotyped three weeks after birth by PCR. Trained technicians at the Monash animal facility and at the Precinct Animal Centre (PAC) provided mouse ear punches for DNA extraction. All animal experiments performed throughout this project were approved by the animal ethics committee of AMREP.

2.6 Isolation of DNA from ear punch

2-3 millimetres length of ear punches were immersed in a 20µl proteinase K solution and kept for overnight incubation in a water bath at 55^0C. After overnight incubation, the samples were vortexed and later diluted with 380µl of Milli Q water and stored at 4^0C until used for the PCR

2.7 Preparing the Master Mix for PCR

Reagents kept at -20^0C were thawed and vortexed before they were used to make the master mix. The concentrations of the components of the master mix for different types of PCR are mentioned in Table 7 to Table 11. PCR cycle steps for different types of PCR are mentioned in Table 6.

2.8 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate the products of PCR amplification. Gels were made by dissolving molecular biology grade agarose in 100 ml of 1X TAE buffer by microwaving. Following microwaving the molten TAE buffer was cooled until it was warm and 1µl of ethidium bromide (10mg/ml) was added before pouring onto the gel casting unit. The number of combs inserted varied according to sample number and usually range from 30-60 samples per strain. The gel along with the inserted combs was allowed to sit until the gel

solidifies. Following solidification the gel was transferred to electrophoresis unit filled with 1X TAE buffer. Combs were carefully taken out and samples to be analysed were mixed with 2µl loading dye and loaded onto wells. Electrophoresis was performed at 100 volts for 50 minutes. For visualising the products in the gel, the gel was removed from the tank and viewed under UV light in a gel documenting unit (Bio-Rad).

Table 5 List of primer sequences

cre PCR: F primer Ins90	5’ CGA GCT CGA GCC TGC CTA TCT TTC
cre PCR: R primer Ins91	5’ CGG GAT CCT AGT TGC AGT AGT TCT
cre PCR: F primer creS1	5’ TGC CAA GAA CCT GAT GGA CA 3’
cre PCR: R primer creAS4	5’ TGC TAA CCA GCA TTT TCG TTC TGC
CAII PCR CAIIS1	5’ AGA GAC CAC AGA ACC GAA CTC CTT
CAII PCR CAIIAS1	5’ GCC AGC CAG CAT TAT GAC ATT TG
β_2M PCR: F1 primer # 3 β2M intron A	5’ AGG GGT AAT TGC TCA GCT CTC 3’
β_2M PCR: F2 primer Neo Forward	5’ TCT GGA CGA AGA GCA TCA GGG 3’
β_2M PCR: R primer β_2M exon	5’ CAG TAG ACG GTC TTG GGC TC 3’
oIMR1900 F primer	5’ ACT CCT TCA TAA AGC CCT 3’
oIMR1901 R primer	5’ ATC ACT CGT TGC ATC GAC CG 3’
ROSA 1	5’ AAA GTC GCT CTG AGT TGT TAT 3’
ROSA 2	5’ GCG AAG AGT TTG TCC TCA ACC 3’
ROSA 3	5’ GGA GCG GGA GAAA TGG ATA TG 3’

Table 6 PCR cycles (Minutes (m), Seconds (s))

CAII PCR	Cre PCR	β2m PCR	EYFP PCR	GFAPcre PCR
94^0C for 2 m	94^0C for 3 m	94^0C for 3 m	94^0C for 6 m	94^0C for 2 m
94^0C for 30 s	94^0C for 30 s	94^0C for 15 s	94^0C for 30 s	94^0C for 30 s
61^0C for 30 s	62^0C for 30 s	62^0C for 30 s	56^0C for 50 s	52^0C for 50 s
72^0C for 30 s	72^0C for 45 s	72^0C for 30 s	72^0C for 2 m	72^0C for 40 s
cycles 36	cycles 39	cycles 35	cycles 34	cycles 36
72^0C for 8 m	72^0C for 5 m	72^0C for 5 m	72^0C for 5 m	72^0C for 5 m
Hold at 4^0C	Hold at 4^0C	Hold at 4^0C	Hold at 4^0C	Hold at 4^0C

Table 7 CAII PCR Master Mix (20µl)/sample

Master Mix	(µl)
H_2O	14.5
Taq buffer (10X)	2
dNTPs (20mM)	0.2
F primer CAIIS1 (50µM)	0.1
R primer CAIIAS1 (50µM)	0.1
Taq Pol	0.1
Sample	3

Table 8

GFAPcre PCR Master Mix (20µl)/sample

Master Mix	(µl)
H_2O	14.3
Taq buffer (10X)	2
dNTPs (20mM)	0.2
F primer oIMR1900 (50µM)	0.2
R primer oIMR1901 (50µM)	0.2
Taq Pol	0.1
Sample	3

Table 9

Cre PCR Master Mix (20µl)/sample

Master Mix	(µl)
H_2O	14.3
Taq buffer (10X)	2
dNTPs (20mM)	0.2
F primer creS1 (50µM)	0.1
R primer creAS4 (50µM)	0.1
Ins 90 (50µM)	0.1
Ins 91 (50µM)	0.1
Taq Pol	0.1
Sample	3

Table 10 EYFP PCR Master Mix (20µl)/sample

Master Mix	(µl)
H_2O	14.2
Taq buffer (10X)	2
dNTPs (20mM)	0.2
Common primer ROSA1 (50µM)	0.3
Knock in primer ROSA2 (50µM)	0.2
Wild type Primer ROSA3 (50µM)	0.2
Taq	0.1
Sample	3

Table 11 β2M PCR Master(20µl)/sample

Master Mix	(µl)
H_2O	14.3
Taq buffer (10X)	2
dNTPs (20mM)	0.2
F primer #3 intron A (50µM)	0.1
F primer Neoforward (50µM)	0.1
R primer β2M exon2 (50µM)	0.2
Taq Pol	0.1
Sample	3

2.9 Tissue harvesting

Mice were identified using the ear IDs and euthanized using CO_2 chamber. Using sterile dissection tools the mice were carefully dissected and spleen, lymph nodes and other tissue were extracted and collected into 15ml falcon tubes containing Dulbecco's phosphate buffered saline (DPBS) + 2% FCS. For histological analysis, the organs were cut into two halves and the first half was fixed in 4% PFA overnight. Following PFA fixation the sections were then sucrose embedded and cryopreserved in optimal cutting temperature (OCT) compound (ProSciTech) by snap freezing in dry ice and hexane. The frozen blocks were kept at -80^{0}C until sectioning using cryostat. The second half of the organs were fixed in 10% formalin solution (Invitrogen) overnight and given for wax embedding at Monash histology platform.

2.10 Single cell preparation from harvested tissues

For analysing the immune cells by flow cytometry, single cell suspension was required. Spleen and lymph nodes were mashed through a 70µm strainer (BD) using 1ml syringe plungers into Petri dishes. Spleen filtrate from the Petri dish was collected into 15 ml tubes and centrifuged at 1500 rpm for 5 minutes at 4^{0}C. Supernatant was carefully decanted and 1 ml of RBC lysis solution was added to the pellet, mixed by pipetting and incubated for 1 minute at room temperature. Following a minute incubation, 10 ml of FACS buffer was added to stop the reaction and centrifuged as before. After decanting the supernatant, the obtained pellet was resuspended in 1 ml FACS buffer and kept on ice. Lymph nodes were also mashed in the similar method and an RBC lysis step is not necessary for the filtrate obtained from mashing the lymph nodes.

2.11 Pancreatic Ductal cell isolation

The pancreas was dissected and single cell suspensions were prepared as described by Maximilian Reichert et al [330]. Briefly, dissected pancreas was cut into small pieces in a petri dish containing Hanks Balanced Salt Solution (HBSS). Manual pipetting was done to dissociate the tissue. The semi dissociated tissue in petri dish were later transferred to 50ml falcon tube containing collagenase (1mg/ml) solution and incubated at 37^0C with intermittent shaking for 15 minutes. The reaction was stopped by adding ice cold hanks solution and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and 1 ml of trypsin EDTA (Thermofisher scientific) was added to the pellet and incubated at room temperature for 5 minutes. The reaction was stopped by adding 5ml of 10% FCS in HBSS. Before centrifuging at 1500 rpm the tube was topped up with 40-45ml of HBSS. The supernatant was discarded and the pellet was resuspended in 1ml HBSS.

2.12 Cell counting

Cell count was performed using the Z2 coulter counter (Beckman Coulter). 10µl of the single cell suspension sample was added to 10ml DPBS in coulter counter cups. Coulter counter was set to give the output reading as number of cells/ml. Post counting one million cells were plated onto 96 well (BD) for staining before proceeding to analysis by flow cytometry.

2.13 Immunophenotyping by flow cytometry

Single cell suspension containing one million cells were added to 96 well plates and centrifuged at 1500 rpm for 3 minutes at 4^oC. After centrifuging, the supernatant was discarded and the pellet was resuspended in 50µl of primary antibody cocktail and incubated for 20-30 minutes at 4^0C. After incubation, 100µl

of FACS buffer was added to all wells and the plate was then centrifuged at 1500 rpm for 3 minutes. The supernatant discarded and the pellet was resuspended in secondary antibody and incubated for 20-30 minutes at 4^0C. Following incubation, the wash step to remove the unbound antibody was repeated and centrifuged as before. The pellet was resuspended in 100 µl of FACS buffer and transferred to FACS tubes for analysis by the flow cytometer (BD LSRII).

Table 12 Antibodies used in flow cytometry

Antibodies used in flowcytometry	Clone	Conjugate	Concentration	Dilution	Make
Rat anti mouse CD4	RM4-5	BV 605	0.2mg/ml	1:400	BD
Rat anti mouse CD4	RM4-5	Pacific blue	0.2mg/ml	1:400	BD
Rat Anti-Mouse CD8a	53-6.7	PerCP	0.2mg/ml	1:400	BD
Rat Anti-Mouse CD19	1D3	Pe-Cy7	0.2mg/ml	1:200	BD
Hamster Anti mouse CD11c	HL3	APC-Cy7/ biotin	0.2mg/ml	1:200	BD
Rat Anti-mouse CD11b	M1/70	PE	0.2mg/ml	1:600	BD
DBA		Biotin	5mg/ml	1:100	Vector
Guinea pig anti-insulin	Polyclonal	Purified	50µg/ml	1:100	DAKO
CD45.1	A20	APC	0.2mg/ml	1:400	BD
Streptavidin		PerCP-CY5.5	0.2mg/ml	1:400	BD

2.14 Histology

2.14.1 Formalin fixed tissue processing for Hematoxylin and Eosin (H&E) staining

The pancreata and salivary gland kept in formalin solution were sent to the Monash histology platform for processing and embedded in paraffin wax. The paraffin embedded blocks were later cut at 5µm thickness at three levels 100µm apart. Two sections per level were mounted onto poly-L-lysine coated (Sigma) slides (Menzel-Glaser). The sections were then stained with H&E.

Table 13 Dewaxing and H&E protocol

Place slides in the rack to Xylene solution #1 for dewaxing	2 minutes
Place slides in the rack to Xylene solution #2 for dewaxing	2 minutes
Place slides in the rack to Xylene solution #3 for dewaxing	2 minutes
Place slides in Absolute Ethanol solution #1	2 minutes
Place slides in Absolute Ethanol solution #2	2 minutes
Place slides in 70% ethanol	2 minutes
Place slides in tap water	30 seconds
Place slides in Harris's Haematoxylin	5 minutes
Place slides in running tap water	30 seconds
Place slides in acid alcohol	Quick dip
Place slides in running tap water	30 seconds

Place slides in Schott tap water	3-10 seconds
Place slides in running tap water	30 seconds
Place slides in Eosin	5 minutes
Place slides into 70% ethanol	2 minutes
Place slides into Absolute Ethanol solution #2	2 minutes
Place slides into Absolute Ethanol solution #1	2 minutes
Dehydrate in Xylene X 3	2 minutes
Coverslip using mounting media	

2.14.2 Fixed Frozen tissue processing for immunofluorescence

Harvested tissues were fixed in 4% PFA overnight and the following day the fixed tissues were cryoprotected by immersion in a beaker of 20% sucrose/Phosphate Buffered Saline (PBS) solution maintained at 4^{o}C. After approximately 8 hours, when the tissue had sunk to the bottom of the beaker, the cryoprotected tissue was embedded in OCT by snap freezing in -20°C chilled isopentane. Cryoprotected OCT embedded tissue were kept at -80°C until sectioning using the cryostat (Leica). OCT blocks were cut at 5µm, and the sections were then blocked in solution containing 3% Bovine Serum Albumin (BSA) and 5% milk powder in PBS. The blocked sections were then stained with polyclonal guinea pig antibodies against insulin (DAKO) and detected using Texas red conjugated polyclonal rabbit antibodies against guinea pig IgG (Abcam). EYFP was detected by staining the sections with Alexa Fluor 555 conjugated polyclonal rabbit antibodies against Green Fluorescent Protein (GFP) (Invitrogen). For staining the ductal cells of the

pancreas biotinylated Dolichos biflorus Agglutinin (DBA) lectin against α-linked N acetyl galactosamine was used and detected using streptavidin conjugated Dylight 405. The stained sections were mounted in fluoromount (Sigma) and observed under the fluorescent microscope (Olympus BX 61) and images were acquired using Olympus F-view II camera. The methods for immunofluorescence detection of EYFP in tissue required optimization and formed the basis of the manuscript (unpublished- Appendix A).

Table 14 Antibodies used in immunofluorescence

Antibody	Clone	Conjugate	Dilution	Manufacturer
Guinea pig anti-insulin	Polyclonal	Purified	1:100	DAKO
Anti-guinea pig IgG	Polyclonal	Texas red	1:600	Invitrogen
Dolichos biflorus agglutinin (DBA) lectin		Biotin	1:100	Vector laboratories
Streptavidin		Dylight 405	1:600	Invitrogen
Anti-GFP	Polyclonal	Alexa flour 555	1:2000	Invitrogen

2.14.3 Insulitis Scoring

Using a light microscope (Olympus BX51), the degree of insulitis in pancreatic sections was graded from 0-4. A healthy islet with no immune infiltrates as seen in the non-diabetic mice pancreata is categorised as grade 0 insulitis. The presence of periductal infiltrates comprising macrophages and dendritic cells was categorised as grade 1 insulitis. Grade 2 insulitis were identified by the presence of circumferential insulitis around the entire islets. Invasive insulitis with less than 50% of the islets been infiltrated were categorised as grade 3 and islets with more than 50% infiltrates were scored as grade 4. The scoring of insulitis was blinded to avoid making biased inferences. Images were acquired using the Olympus DP70 camera.

The above-mentioned methods section described the making of tissue specific knockout mice and the complete techniques used to assess the pathogenesis of T1D in NOD mice. Once the mice were generated, the main objective was to confirm the pattern of cre expression. This was extremely important because in addition to the desired expression, cre recombinase may be expressed at ectopic sites [331]. Ectopic expression of cre has been reported in many transgenic knockout approaches [332] and therefore we wished to validate our mice model before we assessed the development of insulitis. We validated our model using the reporter mice and the results of validation are mentioned in chapter 3.

CHAPTER 3

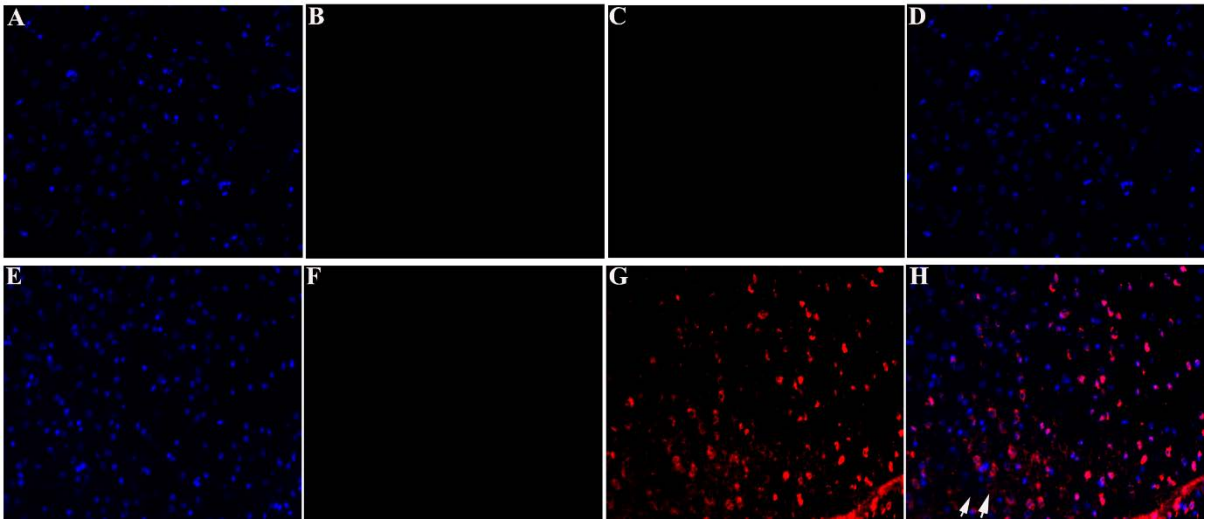
3. Determining the pattern of cre expression in GFAPcre and CAIIcre mice models

3.1 Determining the pattern of cre expression in GFAPcre mice

3.1.1 EYFP expression was detected in pancreatic ductal and glial cells in GFAPcre reporter mice

To check the penetrance of cre, the GFAPcre mouse were crossed to NOD EYFP mice to generate the reporter NOD GFAPcre^{+}EYFP^{+} mice (GFAPcre reporter mice from now on). Since cre recombinase was driven by the GFAP promoter, we expected the expression in all GFAP expressing cell types, which included the ductal cells of the pancreas and the glial compartment of the central and peripheral nervous system. This was confirmed by the analysing brain and pancreatic tissue sections cut from GFAPcre reporter mice. We were not able to detect the natural fluorescence of EYFP in the brain and pancreatic sections but when we probed for EYFP expressing cells using anti-GFP antibody we identified cre expression in brain (Figure 5) and pancreatic (Figure 6) sections from GFAPcre reporter mice. The expression of cre in desired tissue types confirmed the true specificity of the GFAP promoter and before proceeding to generate ductal specific MHC class I knockout mice, we also confirmed that cre was not expressed ectopically in undesired tissue types which included the lymphoid and myeloid cell types (Figure 7)

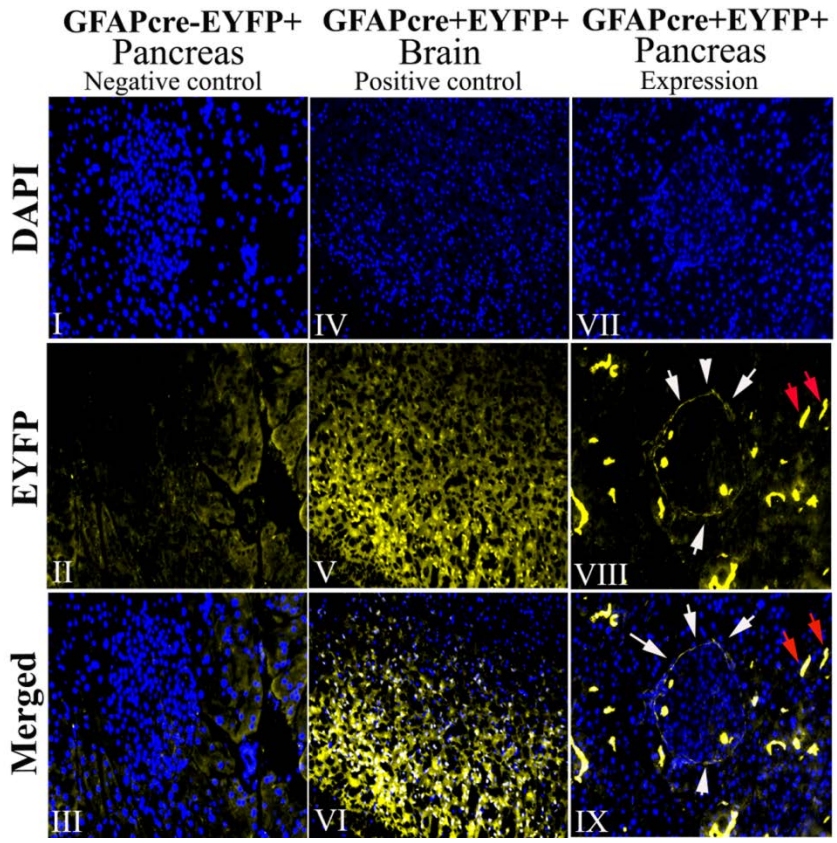
Figure 5 Brain sections from GFAPcre reporter mice showing cre expression



Top Panel: Brain sections of GFAPcre^{-}EYFP^{+}mice. (A) Brain sections stained with DAPI (blue). (B) Natural EYFP fluorescence not detected in the EYFP channel. (C) Alexa fluor 555 conjugated anti-GFP antibody could not detect EYFP expressing cells in cre negative control mice. (D) All three channels merged.

Bottom Panel: Brain sections of GFAPcre^{+}EYFP^{+}mice. (E) Brain sections stained with DAPI (blue). (F) Natural EYFP fluorescence not detected in the EYFP channel. (G) EYFP expressing cells detected using Alexa fluor 555 conjugated anti-GFP antibody (Red). (H) All the three channels merged and DAPI stained cells expressing EYFP is seen in purple.

Figure 6 Pancreatic sections from GFAPcre reporter mice showing cre expression



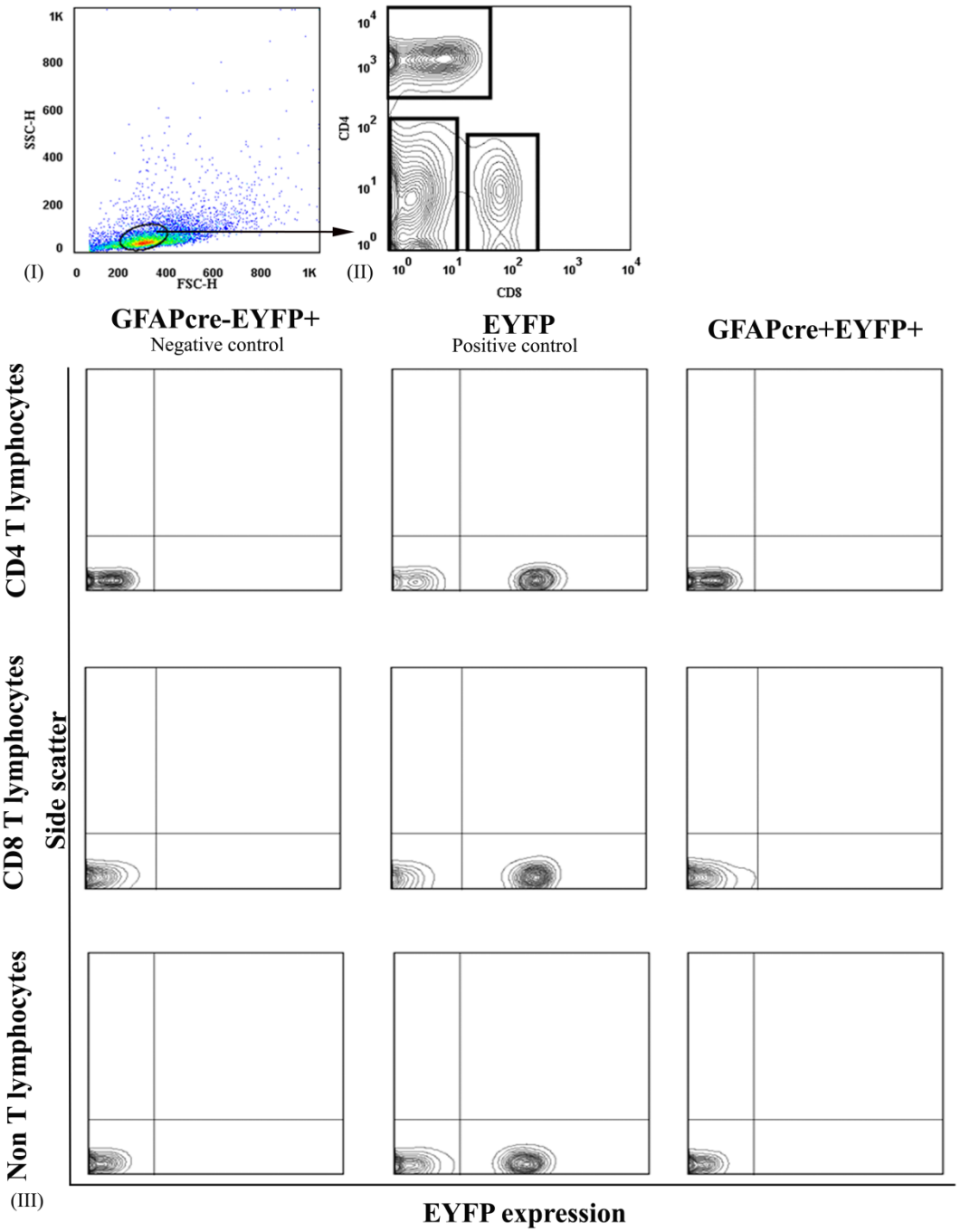
Detection of EYFP in pancreatic neuroendocrine and ductal cells indicates cre expression. Fluorescent microscopic analysis showing cre expression in morphologically identified pancreatic neuroendocrine pSC and ductal cells of the reporter mice (n=3). Pancreatic tissue sections from reporter (VII, VIII, IX) and control mice (I, II, III) were stained using a rabbit antibody against GFP conjugated to Alexa Fluor 555 and nuclear stained with DAPI. White arrows (VIII and IX) point to EYFP expressing pSC and red arrows point to EYFP expressing ductal cells. Stained brain sections from reporter mice (IV, V, and VI) were used as a positive control for EYFP signal.

3.1.2 EYFP expression was not detected in the immune cells of GFAPcre reporter mice

Ectopic expression of cre has variably been reported with the use of cre-lox systems. The specificity of the promoter dictates the fidelity of cre mediated recombination and EYFP expression. It was important to assess the pattern of cre expression in GFAPcre reporter mice. This is because, in experimental class I GFAP bald mice, any non-specific expression of cre in immune cell types would have resulted in deletion of $\beta 2M^{a}$ and loss of MHC class I molecules from these cell types. This would have confounded the interpretation of results.

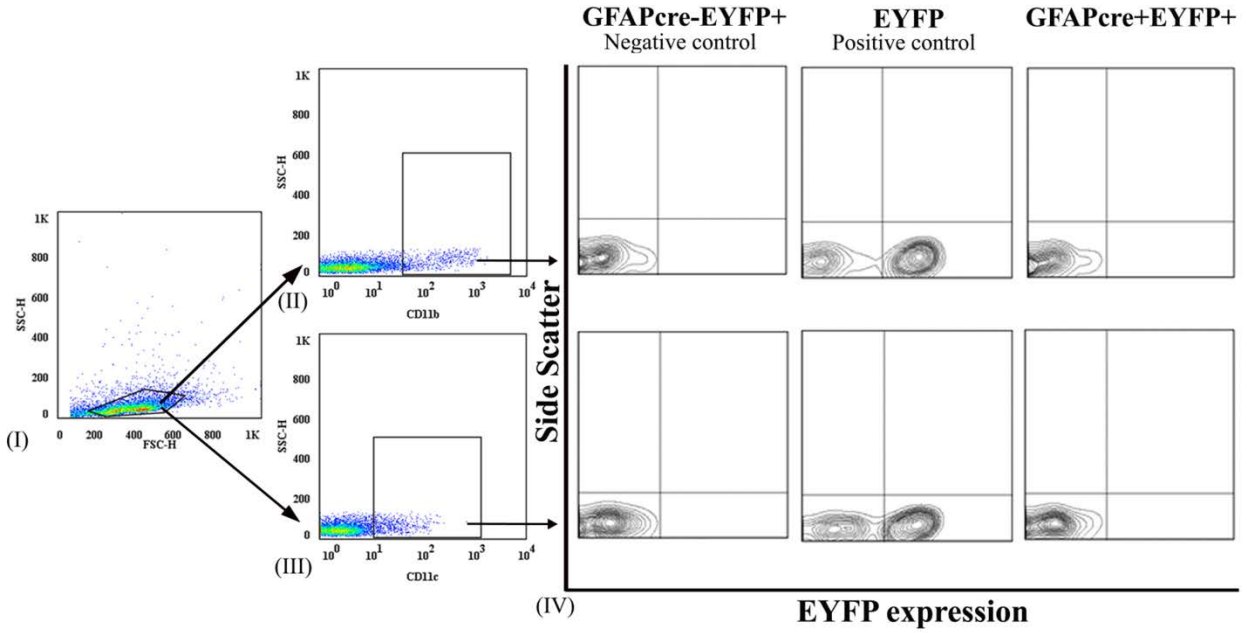
EYFP expression was not detected in the lymphoid (Figure 7) and myeloid cells (Figure 8) isolated from GFAPcre reporter mice (n=5). The absence of ectopic expression of EYFP in immune cell types indicated that GFAPcre mice were an appropriate model to study the role of MHC class I expression on pSCs and ductal cells in the initiation of insulitis.

Figure 7 **Absence of EYFP expression in T cells and non T cells confirmed the true specificity of GFAPcre promotor in GFAPcre reporter mice**.



Flow cytometric analyses of splenocytes from reporter mice were unable to detect lymphocytes expressing EYFP (n=5). Dot plots showing live gating of lymphocytes in forward and side scatter (I). Sequential gating on live lymphocytes and contour plots showing $CD4^+$ T cells, $CD8^+$ T cells and Non T lymphocytes (II). EYFP expression was not detected in $CD4^+$ T cell, $CD8^+$ T cell and non T lymphocytes in reporter and control mice (III). Splenocytes from mice known to express EYFP in immune cells were used as a positive control.

Figure 8 **EYFP expression was not detected in macrophages and dendritic cells of GFAPcre reporter mice.**



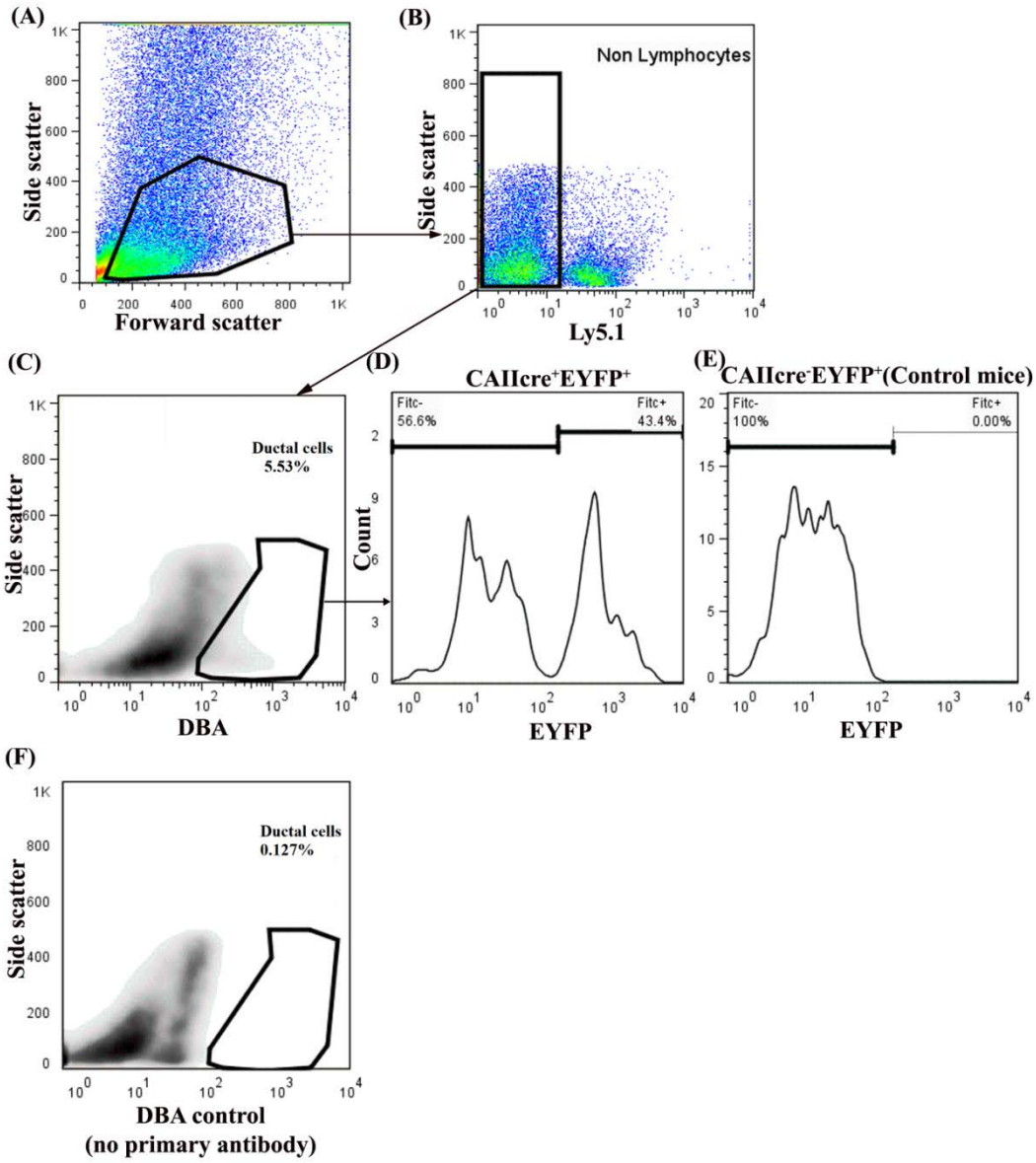
Dot plots showing live splenocytes gated in forward and side scatter (I). Sequential gating on live splenocytes and dot plots showing macrophages gated using CD11b expression (II) and dendritic cells gated using CD11c expression (III). EYFP expression was not detected in macrophages and dendritic cells in reporter and control mice (IV). Splenocytes from mice known to express EYFP were used as a positive control

3.2 **Determining the pattern of cre expression in CAIIcre mice**

3.2.1 Expression of cre recombinase in pancreatic ductal cells in CAIIcre reporter mice.

To check the penetrance of cre expression, CAIIcre^{+} mice were crossed to NOD EYFP^{+} mice to generate CAIIcre^{+}EYFP^{+} reporter mice (CAIIcre reporter mice). Flow cytometric analysis of pancreatic single cells isolated from CAIIcre reporter mice (n=4) showed ductal cells expressing EYFP (Figure 9). The expression of cre in a subset of ductal cells validated CAIIcre mice as a model suitable to study the role of MHC class I expression on ductal cells of the pancreas in the pathogenesis of T1D.

Figure 9 **Pancreatic ductal cells from CAIIcre reporter mice showing cre expression**

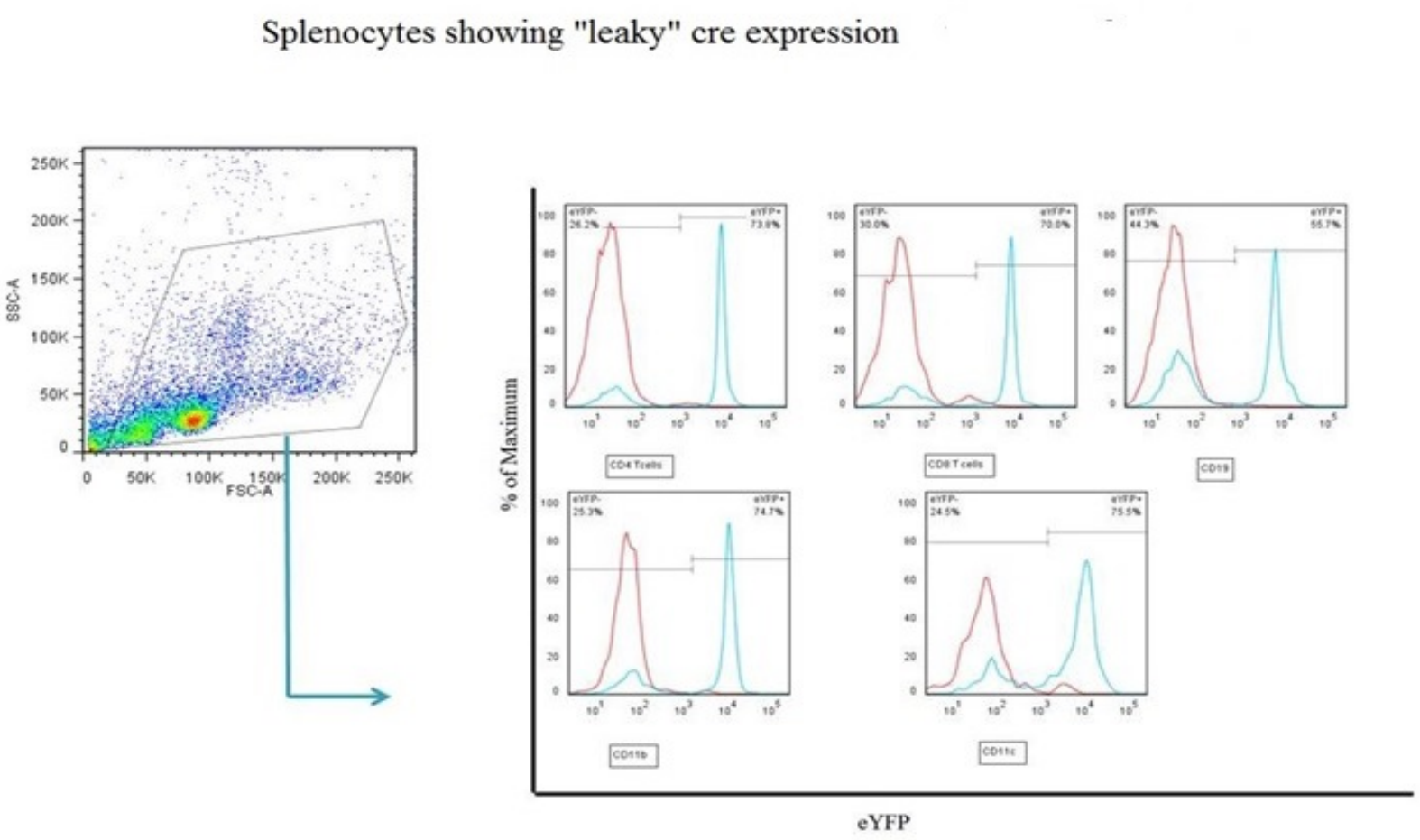


Pancreatic single cell suspensions were gated based on the low side scatter properties of ductal cells and selected on non-lymphocytes cells using the pan leucocyte marker Ly5.1 (B). BA positive ductal cells were selected (C) and EYFP expression was observed in the FITC channel (D). Control mice was negative for EYFP expression (E). Control for DBA (F).

3.2.2 Ectopic expression of cre was detected in the immune cell compartments in CAIIcre mice

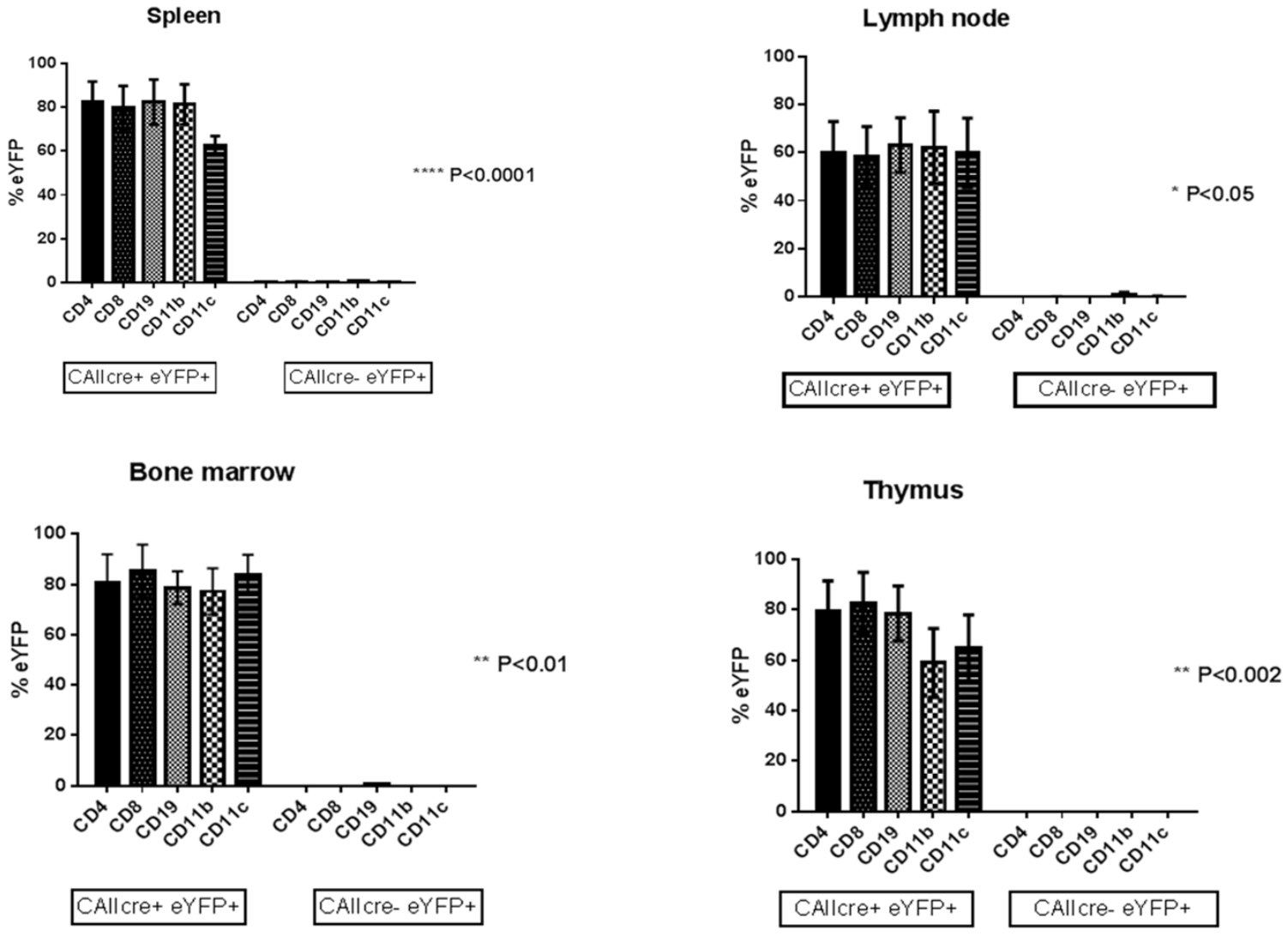
In CAIIcre reporter mice, cre expression was confirmed in the ductal cells of the pancreas, but unexpectedly we also detected EYFP expression in lymphoid and myeloid cells at generation 3 (Figure 10). The leakiness of cre in transgenic mice has been reported in the literature [331] and Slattery lab had experienced this non-specific expression of cre in other mice models. It was expected that with cre-lox technology, continued breeding will allow the epigenetic modifications to settle and expression of cre will become true to its promoter. To make the cre expression specific to CAII+ pre-beta ductal cells, at generation 3, we crossed the transgene via the female germline. Even after crossing the transgene via female and the male germ line, expression of cre was found to leak at generation 10. This was unexpected and despite breeding for 10 generations and making the CAIIcre mice on a β2M knock out background, we concluded that NOD CAIIcre was not an appropriate model to use for insulitis studies. This is because leakiness of cre in the lymphoid and myeloid population will lead to the loss of MHC class I on Antigen Presenting Cells (APCs) in Class I CAII bald mice and it is known that absence of class I mediated presentation by APC will protect the mice from developing insulitis [179]. If we proceed to use this leaky model for assessing insulitis, though MHC class I will be deleted from ductal cells, it will also be deleted from APCs, thus we will not be able to say that the protection from insulitis seen in Class I CAII bald mice is due to the absence of autoantigen presentation by ductal cells.

Figure 10 **Ectopic expression of cre identified in splenocytes isolated from CAIIcre reporter mice**



Phenotyping of CAIIcre reporter mice (G10). Splenocytes stained with appropriate antibodies for T cells, B cells, Macrophages and Dendritic cells showing EYFP expression; Negative control ($CAIIcre^{-}$ $EYFP^{+}$) indicated in red; Sequential gating: Spleen single cells / Live/ EYFP.

Figure 11 **Percentage of EYFP expressing lymphoid and myeloid cell types in CAIIcre reporter mice**



The bar graph shown represents % EYFP expression seen in lymphoid and myeloid population of CAIIcre^{+} EYFP+ (n=4) and CAIIcre- EYFP^{+} mice. All data is representative of +/- SEM. A two-way ANOVA with Bonferroni's multiple comparison test was used to compare the groups.

3.3 Discussion

Pattern of cre expression was checked in both cre lines (NOD GFAPcre and NOD CAIIcre). The fidelity of cre expression was specific when cre was driven by the GFAP promoter as we were unable to detect any nonspecific expression of cre especially in the lymphoid and myeloid cell types. Unlike the GFAP promoter, cre was ectopically expressed in the lymphoid and myeloid cells when it driven under the control of the CAII promoter. The absence of any leakiness of cre in the immune compartment confirmed that GFAPcre was the appropriate model to study the role of MHC class I on GFAP expressing cells, in particular the ductal and pSCs of the pancreas. Identifying the pattern of cre expression was extremely important because any cre mediated loss of MHC class I from immune cells would have confounded the interpretation of results [333]. NOD GFAPcre mice were then bred on to a β2M knockout background and later crossed to floxed β2Ma mice to generate class I GFAP bald mice. Generated class I GFAP bald mice were used to study the development of insulitis.

It is well documented that autoantigens play a major role in the development of insulitis in NOD mice and humans, but what is unknown is autoantigen presented by which tissue type initiates the immune responses towards beta cells. When presentation of beta cell autoantigens were ablated by removing the MHC class I expression on beta cells, we were able to prevent the accumulation of immune infiltrates into the islets but careful examination of the pancreata revealed that immune infiltrates were still present near the pancreatic ducts. Was it the pancreatic duct or is it any other tissue which share similar antigens to pancreatic ducts, the prime accused, in the initiation of immune response towards beta cells. We investigated this by generating a NOD mice that lack MHC class I expression on GFAP expressing cell types including the ductal cells of the pancreas. The

limitation of this model was that since GFAP is expressed in multiple cell types all the GFAP expressing cells will lose MHC class I and therefore we are ablating the presentation of autoantigens expressed in multiple GFAP expressing tissue types including the ductal and the neuronal cells of the pancreas. Ductal cells are the progenitors for beta cells and therefore early upstream autoantigen/s expressed in ductal cells may have a crucial role in the development of insulitis. In class I GFAP bald mice, though multiple GFAP positive cell types lose MHC class I expression, it is of high importance to study the presentation of autoantigens in cell types closely related to beta cells.

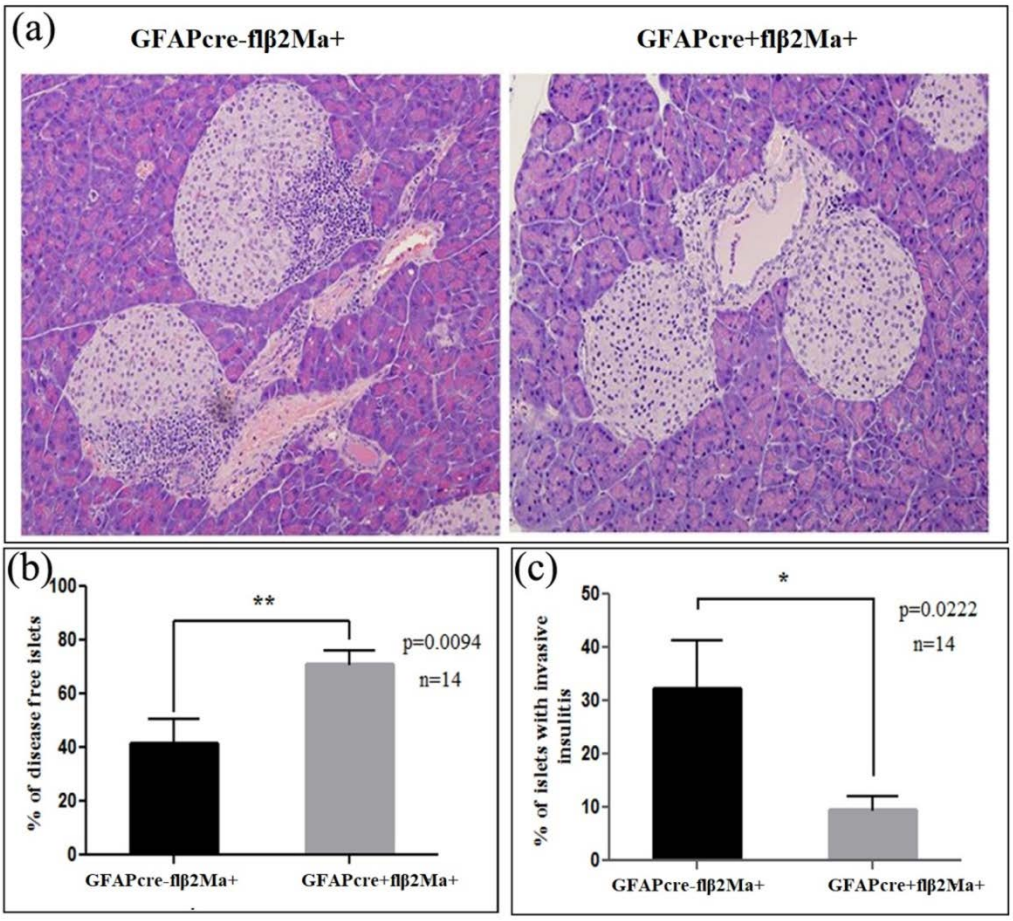
CHAPTER 4

4 Assessment of pathogenesis in class I GFAP bald mice

4.1 Severity of insulitis is significantly reduced in the absence of MHC class I on GFAP expressing cell types

Class I GFAP bald mice were generated by crossing the NOD GFAPcre mice to NOD.flβ2ma mice (Figure 1). These mice were used to assess whether MHC class I presented self-antigens, expressed by GFAP positive cells, were necessary for the initiation and progression of insulitis. Histological analyses of pancreatic sections showed insulitis was significantly reduced in mice lacking MHC class I molecules on GFAP expressing cells compared with class I sufficient age and sex matched control mice (Figure 12a). Class I GFAP bald mice had more disease free islets (70 ± 5%) compared with class I sufficient controls (41 ± 8%) (Figure 12b). Conversely the percentage of islets with invasive insulitis was also significantly reduced in class I GFAP bald mice. Class I GFAP bald mice had 9.5 ± 2% compared with MHC class I sufficient controls (32 ± 9%) (Figure 12c). These data suggest that the loss of MHC class I from GFAP expressing cells somehow protected the mice from insulitis. Since a subset of GFAP positive cells expresses proinsulin, we therefore wanted to test whether the significant reduction of invasive insulitis seen in these mice is due to the absence of MHC class I mediated presentation of the autoantigen proinsulin.

Figure 12 **Class I GFAP bald mice had more disease free islets**



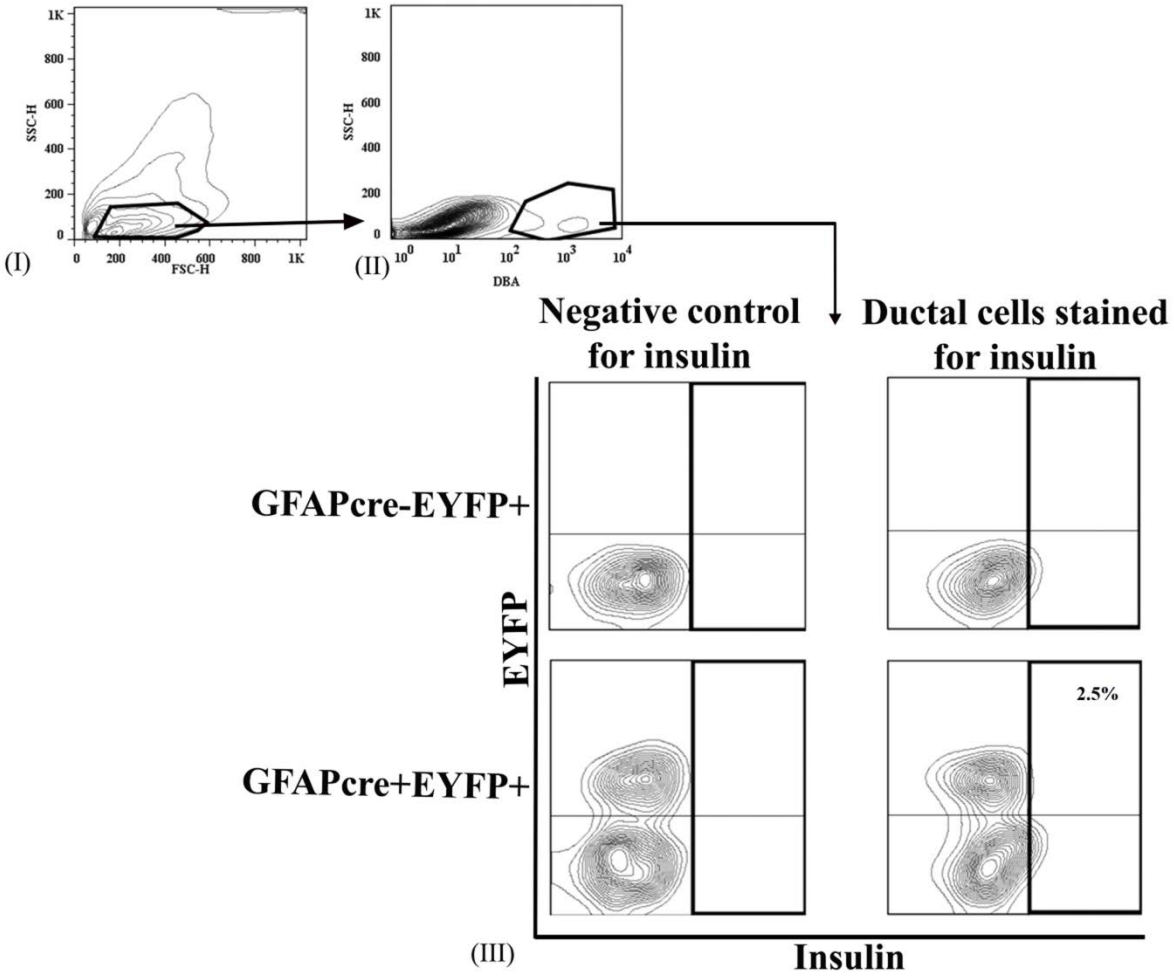
The pancreatic sections from NOD GFAP bald mice at 100-160 days of age were analyzed by haematoxylin and eosin staining. Microphotography shows mononuclear infiltrated islets in control and disease free islets in GFAP bald mice (a). Statistical data showing the percentage of disease free islets (b) and islets with invasive insulitis in GFAP bald and control mice (c).

4.2 GFAP positive ductal cells do not express proinsulin

Our data from reporter mice show that ductal cells are made up of GFAP positive and GFAP negative cell types (Figure 13(iii)). We detected proinsulin expression in 2.5 ± 0.6% of the ductal cells and identified that 90% of the proinsulin expressing ductal cells were GFAP negative. Since GFAP positive cells were found not to co-express proinsulin, the reduction of invasive insulitis seen in class I

GFAP bald mice cannot be due to the loss of expression of proinsulin. It must be due to the loss of the presentation of another autoantigen present in the GFAP expressing ductal cells. There are a number of other autoantigens that ductal cells share with beta cells. It is possible therefore that one of these autoantigens is an early autoantigen that can initiate insulitis.

Figure 13 **Insulin positive pancreatic ductal cells do not express EYFP**



Contour plots showing pancreatic single cells live gated for low side scatter (I). DBA stained pancreatic ductal cells (II) from reporter and control mice (n=4). DBA stained ductal cells showing insulin and EYFP expression (III). Out of the 2.5% of insulin expressing ductal cells, more than 90% of the cells did not express EYFP.

4.3 Discussion

Utilizing Class I GFAP bald NOD mice model we found that the expression of MHC class I on GFAP positive cells was important for the development of insulitis. Not only did the mice lacking MHC class I on GFAP expressing cells have more disease free islets, they were also significantly protected from developing invasive insulitis. This suggests that MHC class I expression on GFAP positive cells is important *early* in the disease process. Because proinsulin is widely held to be the earliest autoantigen, we wondered whether the reduction in insulitis in class I GFAP bald mice may have been due to the loss of MHC class I mediated presentation of proinsulin peptides expressed by ductal cells of the pancreas.

The decrease in the presence of islets with insulitis, even low level periductal insulitis in class I GFAP bald mice suggests there has been a loss of immune response to ductal and/or pSC antigens. However, it is unlikely that the protection from insulitis observed in class I GFAP bald mice was due to the inability of ductal cells to present proinsulin peptides to $CD8^+$ T cells. This is because a proportion of ductal cells retained class I expression in GFAP bald mice and also expressed proinsulin. Thus, a subset of ductal cells would have been able to present proinsulin peptides to $CD8^+$ T cells capable of initiating disease. This begs the question as to which antigen/s expressed by GFAP positive ductal and/or pSCs may be important for the initiation of insulitis.

Ductal cells are beta cell progenitors and as such they share a number of autoantigens with beta cells, including proinsulin, GAD65, ICA69 and HIP/PAP [334-338]. Thus, it is plausible that the initiation of an immune response to ductal cell antigens may lead to cross reactive attack of beta cells expressing shared

antigen/s. Although not widely discussed, the argument that ductal cell antigens may have an important role in the initiation of periductal insulitis has credence. Peri ductal insulitis persisted in mice lacking native proinsulin indicating that the immune response to autoantigens expressed by ductal cells can occur independently of the immune response to proinsulin [339]. The observed reduction of insulitis in class I GFAP bald mice is published in the *journal of Neuroimmunology* (Appendix A).

While we are focussing only on beta cells and associated tissues, where we see massive immune mediated destruction, it is of critical importance to identify the root cause of the initiation of this autoimmune response. In addition to beta cells, immune infiltrations have been identified in multiple tissue types. Although beta cell destruction is the most pronounced and heavily manifested, we need to consider the immune mediated destruction occurring in other tissue types which are anatomically distal to pancreas. This is because; in genetically susceptible individual an autoreactive response to antigen/s expressed in distal tissues can lead to epitope spreading and culminate in targeting the heavily stressed and vulnerable beta cell [340].

In class I GFAP bald mice GFAP is expressed in multiple glial cells including the enteric glia. The reduction of insulitis can also be due to the absence of presentation of gut derived autoantigens by the enteric glial cells. Further studies need to be conducted to understand the role of enteric glia and gut derived autoantigens in the pathogenesis of T1D.

5. Final Discussion and Conclusion

The experiments conducted in class I GFAP bald mice identified that autoantigens other than insulin, expressed by the ductal cells of the pancreas are important in the development of invasive insulitis in NOD mice. Our findings are of importance because it is widely believed that proinsulin is the initiating autoantigen in T1D and autoreactivity to proinsulin precede the autoreactivity to all the other identified autoantigens in T1D. The reduction of invasive insulitis seen in class I GFAP bald mice is due to the absence of autoantigen presentation by multiple cells types that express GFAP. The reduction but not total absence of infiltration is due lack of presentation of autoantigens other than insulin by GFAP expressing cells and antigen/s derived from ductal cells of the pancreas is one of the critical requirement in orchestrating the immune responses to beta cell antigens.

Among the many different cell types that expresses GFAP, few of the cell types that are well connected to beta cells include, the ductal cells of the pancreas, pSC of the pancreas and the enteric glial cells. Therefore, in class I GFAP bald mice, cre mediated deletion of MHC class I, occurs not only in the ductal and Schwann cells but, also occurs in the enteric glial cells. Due to the limited availability of resources, we only focussed on understanding the importance of MHC class I expression on pancreatic ductal cells. It is likely that the reduction of insulitis seen in class I GFAP bald mice is also due to the absence of presentation of antigen/s by the enteric glial cells, which is an important cell type in maintaining the integrity of the gut.

The chronological order of the immune response to autoantigens implicated in T1D is not completely understood. There has long been an assumption that proinsulin is the primary autoantigen, however, in addition to the findings presented here, it has also been shown that the immune response to antigens expressed by pSCs precede

the immune response and attack of beta cells [315]. Our data, and the findings of Michael Dosch et al [315], suggests there may be several alternative autoantigens that could initiate an immune response which culminates in beta cell auto reactivity [341].

While it is not known what causes the inflammation around the ducts of the pancreas, there are a number of hypotheses which have been proposed. The pro inflammatory milieu of the islets could be driven by chemicals [342], microbial infections [343, 344], food allergens [52, 345] or the defective clearance of apoptotic bodies by macrophages [346]. Inflammation is associated with tissue damage that can result in the release of otherwise sequestered self-antigens. Amongst many of the mechanisms involved in the gut-beta cell-immune crosstalk, GFAP expressing cells are critical players in the development of invasive insulitis. Our findings indicate that self-antigens expressed by GFAP positive cells are important in the development of insulitis in NOD mice.

6. Future Directions

As quoted by Hippocrates "*All disease begins in the gut*", and several studies has shown the presence of leaky gut in NOD mice and in humans with T1D [1, 347]. It is likely that antigens originating in the gut could possibly trigger T1D, as such changes in the gut, including the changes in the diet and gut microbiota can cause both protective and destructive effects on beta cells. Studies have shown that gut microbiota through the production of dietary Short Chain Fatty Acids (SCFA) protected 90% of the NOD mice against T1D development [348]. The protection from T1D was mediated by the increase in number of Tregs and by the reduction in number of pathogenic B cells, $CD4^+$ and $CD8^+$ T cells. Diet rich in SCFAs especially acetate and butyrate increased the level of serum IL-22, an important

cytokine that helps to maintain the integrity of the gut epithelial cells and is also required for beta cell regeneration [349].

How antigen expression in other distal tissues to beta-cells control the invasive infiltration of immune cells into the pancreas? Is the gut an important compartment as a source of antigens that trigger T1D? Is the gut microbiota influencing T cell priming against beta-cells? The microenvironment regulates beta-cell function and maturity, in particular close interaction with endocrine cells, neuronal, immune, and vascular cells [350, 351]. Pancreatic ducts are physiologically neighbouring to the beta-cells, and their primary function is to deliver enzymes or pancreatic juices provided from the exocrine pancreas into the duodenum to help digestion. As such, the pancreatic beta-cells can be influenced by the gut, which is intimately connected not only through the pancreatic ducts but also by lymph ducts [352]. Beyond the pancreas, there is hardly any tissue in the body that has not been somehow in contact with gut microbial SCFAs. From food fermentation, bacteria in the large bowel produce many metabolites that are used by the epithelial cells in the gut. The remaining amount is transported to the liver where they are metabolised and then release to systemic circulation. As such, SCFAs have a broad spectrum of remarkable beneficial properties that impact on many systems, in particular under inflammatory conditions, regulate metabolic and immune responses [82, 84, 85].

6.1 GFAP- not the usual suspect!

It has been shown in the pancreas; GFAP-expressing pSC can attract and recruit autoreactive cells, which precede the attack on beta-cells [315, 321]. The early T cell attack on GFAP-expressing cells progressively results in the release of glial cell antigens, GFAP and insulin [315, 353]. GFAP epitopes for autoreactive T and

B cells have now been identified in Non-Obese Diabetic (NOD) mice and humans with T1D. Serum GFAP antibodies are now used as a predictive marker for the development of T1D and it has been shown that administration of GFAP as a vaccine delayed the progression of T1D by regulating T cell differentiation [322, 354]. GFAP expressing glial cells of the peripheral nervous system requires TRPV1 (Transient Receptor Potential Vanilloid type 1) expression for their proper maturation. TRPV1 is a non-selective cationic channel which when activated can alter the intracellular ion concentration. Studies have shown that depleting TRPV1 expressing cells reduced the development of insulitis in NOD mice [355, 356]. It is fascinating that a cytoskeletal protein widely expressed in pancreatic ductal cells and in pancreatic glial cells of the central and peripheral nervous system may work as an early autoantigen in T1D.

The fine-tuned gut-neuro-immune network may be profoundly affected by SCFAs deficiency related to dysbiosis and diet alterations at very early stages of the initiation of the disease. Thus, dampening the initial immune response or preventing the perpetuation of the immune response by maintaining the integrity of the gut is among alternative approaches to prevent T1D.

6.2 The SCFAs: modulator of gut inflammation and auto-reactivity

After many years of efforts and studies focusing on the destruction of the beta-cells in the pancreas, still there is no cure or method of prevention for T1D. So, make us wonder whether we have been losing the battle only because we are not looking beyond the walls of the pancreas. T1D can be viewed as an orchestrated autoimmune response originated in the gut. This is evident from the observation that in many autoimmune diseases including T1D, the integrity of the epithelial barrier is compromised leading to a phenomenon termed as “leaky gut” [357, 358].

Pathogens, microbial products and food-derived antigens find the leaky gut as a route to encounter the resident immune cells. For example, Gram-negative bacteria produce lipopolysaccharides (LPS), an identified endotoxin that can induce immune responses via the toll like receptor 4 (TLR-4) expressed on monocytes [359]. Given the gut connects to the pancreas through pancreatic lymph nodes (PLN) and mesenteric lymph nodes (MLN), bacterial and food products can hyper activate resident T and B autoreactive cells in the gut or the gut-associated MLN [360]. Alternatively, it has been shown that gut microbial products can reach PLN and locally modify the presentation of pancreatic self-antigens [361]. Therefore, excess of food, chemicals, and microbial antigens can skew the intestinal immune system toward a perpetually pro-inflammatory state that may trigger T1D. Newly-diagnosed children with T1D present autoantibodies to GAD, a pancreatic beta-cells autoantigen that also is produced by many bacterial species [362]. For instance, T1D patients present antibodies against a heat shock protein from the *Mycobacterium avium subspecies paratuberculosis*, MAP Hsp65, which has a high degree of homology with human GAD65, suggesting that cross-reactivity between MAP Hsp65 and GAD65 potentially could be a mechanism of triggering TID [363]. Strong homology has been found between the islet-antigen IGRP and several gut- and oral-derived microbial peptides. These peptide sequences encode for magnesium transporter (Mgt), for hypothetical protein IEM_00289 and NAD synthetase, respectively, which activate NY8.3 $CD8^+$ T cells with comparable potency to IGRP native peptide[364]. Thus, molecular mimicry between excess of gut microbial antigens and islet cell autoantigens may be a mechanism by which gut dysbiosis leads to T1D development.

There are pieces of evidence of compromised gut integrity, dysbiosis and associated inflammation of the gastrointestinal tract (GI) in NOD mice and patients

with T1D [365-370], similar to have been shown in other inflammatory or autoimmune gut diseases (i.e. infection, celiac disease, IBD). The gut microbiota and the ENS play a critical role in diabetic gastrointestinal motility disorders, as individuals with diabetes suffer symptoms such as nausea, heartburn, vomiting, diarrhoea, abdominal pain, and constipation [371, 372]. For example, it is known that slow motility of the GI leads to alterations of the gut microbiota that favours pathogenic bacterial overgrowth and subsequently diarrhoea [373, 374]. As such, the abundance and diversity of bacteria needed to maintain the integrity of the gut was significantly lower in children with T1D compared to healthy controls [375]. On the other hand, animal studies have suggested that accelerated colonic transit time, relative to constipation, could be cause by autonomic neuropathy and diabetes-induced denervation of sympathetic nerve terminals [371, 376]. Diet and/or deficiency of dietary SCFAs also can modulate the intestinal motility and survival of enteric neurons by miRNAs, which are involved in energy homeostasis, lipid metabolism and in proliferation and development of GI smooth muscles. miRNAs have been vastly studied in organ damage caused by diabetes, and one study has shown in mice that high-fat diets delay the GI transit, partly by inducing apoptosis in enteric neuronal cells, and this effect was shown to be mediated by Mir375 associated with reduced levels of Pdk [377]. There is still too much to understand about the intrinsic mechanisms underlying the connection between the gut microbiota and the ENS and how impact the course of T1D. Particularly high fibre or specialized acylated starch diets that boost the microbial production of SCFAs are effective in the control of gut infections and diarrhoea, as it has been shown to promote commensal acetate-producing bacteria [378].

6.3 IL-22 and ENS take control of T1D

Activation of IL-22 through microbial SCFAs contributes to the maintenance of gut homeostasis by the close connection between the intestinal-resident innate lymphoid cell 3 (ILC3) and EGCs. IL-22 is expressed by ILC3, which lie close to EGCs [379], but its role in T1D is still elusive [380]. ILCs sustain appropriate immune responses to commensals and pathogens at mucosal barriers by potentiating adaptive immunity and regulating tissue inflammation [381, 382]. Likewise, EGCs have critical roles in maintaining gut homeostasis, as they can sense the pathogenic bacteria through toll-like receptors (TLRs). EGCs surround neurons and also connect with blood vessels and lymphatics [383], which allowed EGC-derived signalling molecules modulate mucosal immunity. As such, EGCs sense environmental stimuli and extend their stellate projections into the ILC3 aggregates within the crypto-patches of the intestinal lamina propria and release neurotrophic factors that stimulate IL-22 secretion from ILC3s [384]. The notion that gut microbiota impact on the development and maturation of EGCs was shown in germ-free (GF) mice, which present a defective influx of EGCs into the intestinal mucosa [385]. This occurs via expression of the neuroregulatory receptor (RET), as ablation of RET in ILC3 leads to reduced IL-22 production and compromised epithelial protection in colon inflammation mouse models [385].

Align with this idea; does the early auto-reactivity to GFAP observed during insulitis originate in the gut? This is possible to the connections between the pancreas, the ENS, and the gut. The fine-tuned neuro-beta-cell cross-talk is more likely to be broken by the pathological changes occurring in a perturbed gut. Alterations of the gut microbiota, referred to as dysbiosis, decrease epithelial permeability causing inflammation and associated tissue damage that exposes numerous self-antigens harboured in the gut and associated enteric neuronal

tissues. Gut microbial products can also sense enteric neurons and EGCs partly by pattern recognition receptors, such as TLRs. Indeed, pathogenic and commensal SCFA-producing bacteria up-regulate differentially toll-like receptor 2 (TLR2) expression on human EGCs [386]. Expression of TLR2 on enteric neurons and EGCs controls $nNOS^{+}$ neurons and acetylcholine-esterase–stained fibers in the myenteric ganglia. For example, *E. coli* promoted expression of MHC II molecules on EGCs and significantly induced S100B protein overexpression and nitric oxide (NO) release from EGC, which was counteracted by pre-treatment with TLR and S100B inhibitors [386]. As such, the myenteric plexus of TLR2Ko mice presented reduced expression of glial markers, GFAP, and S100B. Overexpression of GFAP has been observed correlating with inflammatory responses in the gut [387]. S100B is considered as a neurotrophin, due to its either tropic or toxic effects depending on the concentration in the extracellular milieu. Excess amount of S100B acts on RAGE (receptor for advanced glycation end-products) leading to the phosphorylation of mitogen-activated protein kinases (MAPK) and subsequent activation of the nuclear factor κB (NF-κB) and the associated release of Nitric oxide (NO). Excess nitric oxide causes damage to the tissue resulting in inflammation and reduced integrity of the gut [388, 389]. The protective role of EGCs in the maintenance of the gut epithelial integrity has been demonstrated in mice lacking GFAP positive (+) glia that presented fatal hemorrhagic jejuno-ileitis [390].

During chronic tissue inflammation, it has been shown significantly increased expression of GFAP on glial cells after stimulation with LPS and pro-inflammatory cytokines [391], similar to what has seen in Crohn's disease (CD) and necrotising enterocolitis (NEC). On the other hand, the presence of MHC class II expression on activated EGCs suggests that these cell types can present antigens [392, 393]

derived from multiples sources, including microbes and host. EGCs, with the help of their stellate projections, sample microbial antigens crossing the epithelial barrier and activate diabetogenic T cells. This is given under dysbiosis, predominant in T1D and many autoimmune diseases, the release of microbial antigens such as LPS may break the tolerance of EGCs leading to overexpression of glial cell markers GFAP and S100B. Thus, GFAP expressing glial cells may have a protective role in maintaining the integrity of the gut, but under uncontrolled inflammatory conditions, it may lead to the autoreactivity. As such, glial cell-derived protein GFAP is now an identified autoantigen in T1D and autoantibodies to GFAP has been detected in NOD mice and humans with T1D [322], thus showing the relevance of the microbiota-EGCs pathways in T1D.

One study has shown that SCFA butyrate can induce increasing excitatory choline acetyltransferase (ChAT) neurons through the butyrate transporter monocarboxylate transporter (MCT), which is expressed by enteric neurons [394]. However, it is still unknown what factors control neuronal MCT2 expression. Further studies will be necessary to determine how SCFAs regulate MCT2 expression and controls the activity of intestinal neural circuits. SCFAs exert their function through two mechanisms, via metabolite sensing GPCRs or inhibition of histone deacetylase activity [77, 85, 395, 396]. There are three receptors for SCFA acetate, butyrate and propionate, namely GPR43 (FFA2), GPR41 (FFA3), and GPR109a. GPR43 is activated by SCFAs with varying potency – acetate > propionate > butyrate. GPR43 is expressed on gut epithelial cells and certain immune cells [397]. GPR109a is expressed on a variety of immune cells, as well as adipocytes, hepatocytes, gut and retinal epithelium, vascular endothelium, and neuronal tissue [398]. GPR109a is primarily activated by both niacin and butyrate ligands. While under normal physiological conditions niacin levels are not high

enough to activate the receptor; levels of butyrate, obtained from the gut environment, and its oxidised form β-hydroxybutyrate are sufficient to stimulate a response [398]. Similarly, GPR41 has been reported to be express on EGCs and enteric neurons [399] [400, 401]. GPR41 also binds the three major SCFA, but with differing affinities [402].

SCFAs also can modulate gut motility by the production of serotonin by epithelial enterochromaffin cells (ECs) [403, 404]. For instance, GF mice present gut dysmotility that was reversed by inoculation with SCFAs-producing bacteria. Tested in human-derived EC cell lines, SCFAs increased serotonin (5-hydroxytryptamine [5-HT]) by up-regulating the expression of tryptophan hydroxylase 1 (Tph1) [404] and by the serotonin-selective reuptake transporter (SERT), which is expressed by intestinal epithelial cells [405]. Another critical role of SCFAs on the ENS is evidenced by the conversion of primary bile acids synthesized de novo into secondary bile acids in the liver [406]. Aside from their role in dietary fat absorption, secondary bile acids can activate several GPCRs and nuclear hormone receptors, including the G-protein–coupled bile acid receptor 1 (TGR5) and farnesoid X receptor (FXR), highly expressed in enteric neurons and enteroendocrine L cells that improved intestinal inflammation and glucose tolerance in HFD-fed mice [407]. TGR5 also affect peristalsis that is mediated partly by serotonin 5-HT [408], implicating its potential for the treatment of constipation and diarrhoea. Altogether suggest the relevance of the gut-neuro-immune axis in T1D

Similar to the effects exert through the GPCR's, SCFAs can influence the function and development of immune cells directly through epigenetic regulation of gene expression such as inhibition of histone deacetylases (HDACs) [85, 409]. HDACs allows the conversion of repressive chromatin structures, which takes place on

lysine residues on N-terminal tails of histones 3 and 4, to increase gene transcription. HDACs are a group of 18 known enzymes that remove acetyl groups from the histones tails that bind DNA [410]. Although little is known about the effects of SCFAs on EGCs through epigenetic modifications, it has been shown that butyrate treatment increases acetylation of the H3K9 in primary enteric neurons and the EGC *in vitro* [411].

Overall, an immune response to antigens presented by GFAP-expressing pSCs in the pancreas but also by GFAP expressing enteric glial cells in the gut is novel findings involved in the initiation of the autoimmune process. Could trigger antigen-experienced autoreactive cells move up the gut and reach the ductal and beta-cells, and break the GFAP expressing neuronal mantle of the islets? This is an unexplored field and requires further research. Given the close location and connection between the gut and the pancreas and their intrinsic dependence from the nervous system, this fine-tuned immuno-neuro-islet cross-talk may be profoundly affected by perturbed gut homeostasis at very early stages of the initiation of the T1D. Dampening the initial immune response or preventing the perpetuation of the islet-specific immune response by maintaining the integrity of the gut is among the possible therapeutic approaches to reprogram T1D [380, 412]. Thus, any hope for a cure may lie in methods that can halt immune-mediated beta-cell damage by maintaining or improving gut-immune tolerance. To win the battle against T1D, let us look beyond the walls of pancreas and focus on the gut-neuro-immune axis of T1D.

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Appendix A – Papers and Manuscripts written duing the candidature

1) Advances in our understanding of the pathophysiology of Type 1 diabetes: lessons from the NOD mouse. (*Journal of Clinical Science)*
2) Pancreatic ductal cell antigens are important in the development of invasive insulitis in Non-Obese Diabetic mice *(Journal of Neuroimmunology)*
3) Dietary SCFAs, IL-22, and GFAP: The Three Musketeers in the Gut–Neuro–Immune Network in Type 1 Diabetes *(Frontiers in immunology)*
4) A simple method to improve the visualization of EYFP in paraformaldehyde fixed frozen tissue sections (unpublished)

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Review Article

Advances in our understanding of the pathophysiology of Type 1 diabetes: lessons from the NOD mouse

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Abstract

T1D (Type 1 diabetes) is an autoimmune disease caused by the immune-mediated destruction of pancreatic β-cells. Studies in T1D patients have been limited by the availability of pancreatic samples, a protracted pre-diabetic phase and limitations in markers that reflect β-cell mass and function. The NOD (non-obese diabetic) mouse is currently the best available animal model of T1D, since it develops disease spontaneously and shares many genetic and immunopathogenic features with human T1D. Consequently, the NOD mouse has been extensively studied and has made a tremendous contribution to our understanding of human T1D. The present review summarizes the key lessons from NOD mouse studies concerning the genetic susceptibility, aetiology and immunopathogenic mechanisms that contribute to autoimmune destruction of β-cells. Finally, we summarize the potential and limitations of immunotherapeutic strategies, successful in NOD mice, now being trialled in T1D patients and individuals at risk of developing T1D.

Key words: autoimmunity, immunopathogenesis, insulitis, non-obese diabetic mouse (NOD mouse), Type 1 diabetes (T1D)

INTRODUCTION

Experiments using NOD (non-obese diabetic) mice have paved the way for significant advances in the current understanding of T1D (Type 1 diabetes). T1D is an autoimmune disorder in which the insulin-producing β-cells of the pancreas are selectively destroyed. In this condition, inflammatory infiltrates, mainly comprising DCs (dendritic cells), macrophages and B- and T-lymphocytes, invade the islets. The progressive loss of β-cells ultimately causes insulin deficiency and consequent hyperglycaemia. Despite exogenous insulin administration, many patients develop debilitating microvascular and macrovascular complications, leading to increased morbidity and mortality.

Investigation into the genetics and immunopathological mechanisms that lead to initial progression and β-cell destruction in individuals at risk of developing T1D is difficult due to a protracted pre-diabetic phase, inaccessibility of islet tissue and limitations in β-cell markers that reflect cell mass and functionality. Although GWAS (genome-wide association studies) have identified more than 40 distinct susceptibility regions linked to T1D that may assist in the screening for those at risk of developing T1D, a comprehensive understanding of the immunopathological mechanisms underlying the development of T1D is also required. The knowledge gained from such studies will help develop immunomodulatory strategies to prevent the onset of disease in genetically susceptible individuals. Accordingly, our understanding of the genetics, aetiology and pathogenesis of T1D has heavily depended on studies using the NOD mouse strain that spontaneously develops T1D. The present review will discuss the lessons learned from NOD mice in determining the mechanisms underlying T1D susceptibility and pathogenesis, as well as the value and limitations of this model.

Abbreviations: AAb, autoantibody; AAg, autoantigen; AIRE, autoimmune regulator; APC, antigen-presenting cell; β2m (*B2m*), β_2-microglobulin; CCR, CC chemokine receptor; CD40L, CD40 ligand; CD62L, CD62 ligand; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; DC, dendritic cell; Deaf1, deformed epidermal autoregulatory factor 1; EC, endothelial cell; EFA, essential fatty acid; FasL, Fas ligand; FcRγ, Fc receptor γ; FoxP3, forkhead box P3; GAD, glutamic acid decarboxylase; GP, glycoprotein; GPR, G-protein-coupled receptor; HA, hyaluronan; HbA_{1c}, glycated haemoglobin; ICAM-1, intercellular adhesion molecule-1; ICOS, inducible T-cell co-stimulator; IDO, indoleamine 2,3-dioxygenase; IFA, Incomplete Freund's Adjuvant; IFNγ, interferon γ; IL, interleukin; IL-1RA, IL-1 receptor antagonist; iNKT cell, invariant natural killer T-cell; iNOS, inducible NO synthase; liCTLA-4, ligand-independent CTLA-4; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; mAb, monoclonal antibody; mDC, myeloid DC; MT-1-MTP, membrane type-1 matrix metalloproteinase; MZ, marginal zone; NF-κB, nuclear factor κB; NK, natural killer; NKG2D, natural killer group 2D; NKT cell, natural killer T-cell; NOD, non-obese diabetic; NP, nucleoprotein; pAPC, professional APC; PD-1, programmed cell death 1; pDC, plasmacytoid DC; PD-L1, PD-1 ligand; pLN, pancreatic lymph node; ROS, reactive oxygen species; SCFA, short-chain fatty acid; sCTLA-4, soluble CTLA-4; SNP, single nucleotide polymorphism; T1D, Type 1 diabetes; TCR, T-cell receptor; TNF, tumour necrosis factor; TNFR2, TNF receptor 2; T_{reg} cell, regulatory T-cell; TSA, tissue-specific antigen; VCAM-1, vascular cell adhesion molecule-1.

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THE NOD MOUSE MODEL

Genetics

The inbred NOD mouse strain originated as a hyperglycaemic sub-strain of the CTS (cataract-prone mouse) at the Shionogi Laboratories, Fukushima-ku, Japan [1]. At the time of weaning, NOD mice develop around pancreatic islets a mononuclear cell infiltrate (insulitis) that progresses at approximately 100 days of age to invasive insulitis and complete β-cell destruction [2]. Although NOD mice have an increased genetic susceptibility to T1D, the penetrance of disease can be modulated by various environmental factors. Hence not all NOD mice in a colony will develop T1D. Importantly, a number of T1D-susceptibility genes identified in NOD mice (designated *Idd)* have been found to contribute to T1D susceptibility in humans (designated *IDDM*). To date >40 *Idd* regions have been identified [3,4], but only a small number of these regions have been localized to candidate genes, and these will be discussed below.

The MHC is the strongest susceptibility region and was the first identified in both humans (*IDDM1*) and in mice (*Idd1*). The mouse MHC region is located on chromosome 17 and contains a number of genes known to contribute to disease. These genes make up a haplotype that contains both MHC class I- and class II-susceptibility genes. In mice, MHC class I genes comprise K^d and D^b alleles and MHC class II genes comprise I-A^{g7} and I-E^b alleles [5,6]. Although NOD mice express the I-A heterodimer that is composed of I-Aα and I-Aβ chains, the I-E heterodimer is not expressed due to a deletion in the promoter region of the I-Eα gene. Furthermore, replacement of histidine and serine residues with proline and aspartic acid residues at positions 56 and 57 respectively within the I-Aβ chain prevents T1D in NOD mice [7–9].

The *Idd3* locus encodes the cytokines IL (interleukin)-2 and IL-21, and these are strong candidate genes for T1D susceptibility [10–12]. In NOD mice, IL-2 expression levels are abnormally low, with low-dose IL-2 treatment reducing the severity of insulitis and inhibiting T1D onset [13,14]. Further reduction in IL-2 levels in NOD mice heterozygous for a deletion of the IL-2 gene accelerated T1D development [15]. In contrast, IL-21 is highly expressed in NOD mice and NOD mice deficient in the IL-21 receptor are protected from T1D [12,16].

The *Idd5.1* locus includes two candidate susceptibility genes encoding CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) and ICOS (inducible T-cell co-stimulator). CTLA-4, a receptor involved in inhibiting T-cell activation, is present in four distinct isoforms in NOD mice, one of which is the ligand-independent CTLA-4 isoform (liCTLA-4). It contains a SNP (single nucleotide polymorphism) in exon 2 that causes liCTLA-4 to be expressed at reduced levels, decreasing the activation threshold of T-cells and consequently increasing disease susceptibility. Similarly, expression levels of the sCTLA-4 (soluble CTLA-4) isoform are reduced in T1D patients [17]. CTLA-4 therefore represents an attractive target for immunotherapeutic intervention. The ICOS gene in NOD mice has a SNP encoding a non-conservative amino acid change in the leader sequence of exon 1. This change causes higher expression of ICOS, which heightens T-cell co-stimulation. Consistent with the idea that increased expression of ICOS contributes to susceptibility, $ICOS^{-/-}$ NOD mice are protected from T1D [18,19].

The *Idd7* locus contains a gene, or several linked genes, thought to influence allelic exclusion of TCR (T-cell receptor) genes during T-cell development [20]. These studies were performed in TCR transgenic mice and may not represent the normal path of TCR rearrangement found in human T-cells. However, if such defects are present, this could lead to dual TCR expression and autoimmunity [21].

The *Idd9* locus contains three regions: *Idd9.1*, *Idd9.2* and *Idd9.3* [22]. Although the genes localized at *Idd9.1* are unknown, they are associated with increased B-cell pathogenic activity [23], low numbers of induced iNKT cells [invariant NKT cells (natural killer T-cells)] and reduced T_{reg} cell (regulatory T-cell) development and activity in NOD mice [24,25]. Candidate susceptibility genes at *Idd9.2* and *Idd9.3* encode CD30, TNFR2 (tumour necrosis factor receptor 2) and CD137 respectively [22].

The *Idd13* locus contains multiple susceptibility genes, including the candidate genes β_2-microglobulin (*B2m)*, *Cd93*, *Nkt2* and *Bim* [26,27,30–32]. In inbred mouse populations, there are three allelic variants of *B2m* that encode isoforms differing by a single amino acid at residue 85 [28,29]. Wild-type NOD mice express the $B2m^a$ isoform, whereas NOD mice congenic for the NOR (non-obese-resistant) *Idd13* region express the $B2m^b$ isoform and are protected from T1D. *B2m* was confirmed as a diabetes-susceptibility gene in reconstitution experiments in which NOD mice lacking endogenous *B2m* and transgenic for $B2m^b$ were protected from the development of T1D compared with mice transgenic for $B2m^a$ [30]. The mechanism of protection conferred by different *B2m* isoforms has not been elucidated, but is proposed to relate to the expression level of MHC class I (R.M. Slattery, unpublished work). As such, although humans are non-polymorphic at the *B2m* loci, there may be related changes in the expression level of MHC class I that influence antigen presentation, thereby modulating thymic selection and/or peripheral activation of $CD8^+$ T-cells [26]. In NOD mice, the *Cd93* gene has a SNP that results in a conformational change in the CD93 protein [27]. Although the function of this protein is not yet well defined, its absence in C57Bl/6 (B6) $CD93^{-/-}$ mice results in a reduced number of iNKT cells, which may promote T1D in NOD mice [27]. A third gene within the *Idd13* region that controls NKT cell numbers has been mapped to the *Nkt2* gene. NOD mice congenic for the B6 *Nkt2* region had increased NKT cell numbers and a reduced incidence of T1D [31]. The fourth candidate susceptibility gene to be localized within the *Idd13* locus is *Bim*, which encodes the pro-apoptotic protein BIM. The failure to induce BIM in thymocytes confers resistance to thymic deletion in NOD mice [32].

Of the >40 susceptibility loci identified in NOD mice only a small number of these regions contain genes that have orthologues associated with human T1D. Nevertheless, a number of the non-orthologous candidate susceptibility genes in NOD mice have led to studies that have provided valuable insights into the immunopathogenic mechanisms of T1D relevant to humans, and these are discussed later in the review.

Environment

Geography

Various environmental parameters have been associated with T1D susceptibility in humans. They include geographical location, dietary components and infectious agents. Worldwide incidence data on T1D suggests that there is an inverse correlation between disease incidence and proximity to the equator that could be explained by a number of variables. Regions furthest from the equator have reduced exposure to sunlight with a concomitant reduction in both UV radiation and temperature. In human T1D it is difficult to segregate the roles of sunlight variables from the confounding co-variables of genetics and culture. However, genetically controlled studies utilizing inbred NOD mice have allowed the independent contribution of UV radiation and temperature on T1D to be assessed.

NOD mice maintained at a temperature of 23.7 °C compared with 21 °C had a lower incidence of T1D. This suggests that the inverse correlation between equatorial distance and incidence of T1D may in part be explained by temperature [33]. Since UV radiation is essential for epidermal vitamin D synthesis, and the active form of vitamin D 1,25 D_3 (1,25-dihydroxyvitamin D_3) influences the development of T_{reg} cells, the effect of vitamin D on T1D has been studied in NOD mice. These studies revealed that early deficiency of vitamin D in NOD mice resulted in accelerated T1D development [34], where NOD mice administered supplementary vitamin D were protected from developing T1D, and this was correlated with an increased frequency of T_{reg} cells within the pLN [pancreatic LN (lymph node)] [35,36]. It is thought that vitamin D also modulates the immune response through inhibition of the NF-κB (nuclear factor κB) pathway in DCs and macrophages. This results in decreased production of the pro-inflammatory cytokines IL-12 and IFNγ (interferon γ) that leads to decreased MHC class II expression on APCs (antigen-presenting cells) and MHC class I expression on β-cells [37]. Vitamin D supplementation at birth has been shown to significantly protect against T1D in humans [38]. However, supplementation of vitamin D in patients with recent-onset T1D failed to reduce the loss of β-cell function [39].

Diet

The earliest evidence for dietary influences in T1D susceptibility came from studies in NOD mice. A positive association was found between a high-fat diet and T1D incidence in these mice [40]. An increase in T1D progression in NOD mice has also been correlated with wheat- or corn-enriched diets and this has been attributed to the wheat protein gluten [41]. It has been speculated that a mechanism by which dietary antigens could influence susceptibility is by modulation of the mucosal immune system via the release of tolerogenic, allergenic or pro-inflammatory cytokines [42]. NOD mice fed on cereal/wheat-based diets expressed significantly higher levels of IFNγ, TNFα (tumour necrosis factor α) and iNOS (inducible NO synthase) that are known to drive the immune response towards T1D [42].

Conversely, EFAs (essential fatty acids) have been shown to protect from T1D. The offspring of mice fed on a low omega-6/omega-3 ($n-6/n-3$) EFA ratio diet showed a decrease in T1D incidence [43]. Similarly, omega-3 reduced the incidence of T1D in genetically susceptible children, indicating its benefit as a supplement to assist in the prevention of human T1D [44]. The protective effect of omega-3 may partly explain why T1D incidence is lower in Japan, where there is a high consumption of fish compared with countries with westernised diets [45]. One mechanism by which omega-3 may protect from T1D is through direct binding to GPR120 (G-protein-coupled receptor 120) on macrophages, which exerts a wide range of potent anti-inflammatory effects [46]. Additionally, omega-3 may act indirectly through the anti-inflammatory influence of bioactive products resulting from its breakdown and enzymatic conversion, such as resolvins [47].

Diet and the use of antibiotics can also modulate the gut microbial diversity, which has recently been shown to regulate the immune system. NOD mice housed under 'specific germ-free' (SGF) conditions have a higher incidence of T1D than those housed in a non-germ-free environment. Furthermore, NOD mice treated with probiotics have elevated IL-10 production and reduced T1D development [48]. The bacteria responsible for this protection are members of the *Bacteroidetes phyla*, which are capable of producing SCFAs (short-chain fatty acids). SCFAs can bind GPRs on immune cells and thereby mediate an anti-inflammatory response that protects from T1D [49]. Whether probiotics, or SCFA treatment, will prevent T1D in those at risk remains to be determined, although probiotics are currently being trialled in at-risk children [50]. In contrast, some bacterial strains promote inflammation and thereby exacerbate the development of T1D. Gluten-fed NOD mice contain greater quantities of aerobic, micro-aerophilic and caecal bacteria and are more likely to develop T1D than those fed on gluten-free diets [51]. Furthermore, a positive correlation was observed between the numbers of Gram-positive aerophilic and anaerobic bacteria found in the gut of NOD mice and the incidence of T1D, regardless of diet [51]. It is thought that such bacterial strains promote a pro-inflammatory cytokine environment that drives T1D by inducing mucosal DCs to secrete Th1-type cytokines.

Viruses

A number of viruses have been positively associated with T1D onset and several mechanisms have been proposed to explain this association [52]. Viral infection of the gastrointestinal mucosa with rotavirus increases the intestinal permeability, creating a 'leaky gut'. Opportunistic gut bacteria that migrate through the leaky gut can induce inappropriate sub-mucosal immune responses that signal through TLRs (Toll-like receptors) and drive inflammation [53]. Other viruses, such as CVB (Coxsackie virus B), can directly infect islet tissue or neighbouring neuro-endocrine cells, promoting inflammation in both islets and associated ECs (endothelial cells) [54]. At the time of insulitis development, NOD mouse islet ECs develop an activated phenotype with up-regulation of adhesion molecules, such as CD54 [ICAM-1 (intercellular adhesion molecule-1)], CD106 [VCAM-1 (vascular cell adhesion molecule-1)] and of MHC class I and II molecules [54,55,55a]. Islet ECs from human histological samples taken during the peri-onset stage of T1D show a similar phenotype, displaying increased expression of adhesion and MHC molecules [56].

Another potential mechanism of virally induced T1D is molecular mimicry, whereby T-cells that are activated by specific viral antigens cross-react with AAgs (autoantigens) that share structural similarities. This model has been investigated for its capacity to trigger T1D in transgenic mice expressing the LCMV (lymphocytic choriomeningitis virus) antigen NP (nucleoprotein) or GP (glycoprotein). Mice expressing these antigens on pancreatic β-cells remained tolerant and free from T1D in the absence of viral infection [57]. However, when challenged with LCMV, NP- and GP-specific T-cells were activated and β-cells were killed, causing T1D [57]. Blocking or depletion of pro-inflammatory cytokines has been found to reduce T1D incidence in NOD mice following viral infection [58].

IMMUNOPATHOLOGY

Disease initiation

The inaccessibility of pAPCs (professional APCs) has made it challenging to measure their role in human T1D. Researchers rely heavily on studies in NOD mice to understand how pAPCs may promote T1D and, importantly, how their central role in tolerance may be exploited in immunotherapeutic approaches (Figure 1). pAPCs are the first cells to accumulate marginally around the islets, thereby initiating a cascade of immunopathological events that culminate in β-cell destruction and T1D [59,60]. The events triggering the accumulation of pAPCs in the islet milieu remain to be fully elucidated. It has been proposed that a wave of β-cell death occurs in NOD mice as part of tissue remodelling. This could occur either in response to viral infection or other metabolic changes around the time of weaning [61,62]. The migration of CCR7 (CC chemokine receptor 7)-positive monocyte/macrophages and DCs to the islets is promoted by the elevated expression of lymphoid-tissue-related chemokines, such as CCL19 (CC chemokine ligand 19), found in NOD mice [63]. Macrophages produce the cytokines TNFα and IL-1β that are directly β-cell toxic and over-produce IL-12, driving the further recruitment of DCs to the accumulated β-cell debris. This exacerbates the inflammatory response and leads to the inappropriate activation of autoreactive T-cells in NOD mice [64]. Furthermore, due to increased prostaglandin (PGE_2) production, macrophages from NOD mice are impaired in their phagocytic ability and their ability to present self-antigens for the induction of tolerogenic immune responses [65–67]. Therefore NOD mouse macrophages have an important role not only in the initiation of insulitis, but also in driving its progression towards β-cell destruction and T1D. On the basis of the important roles of IL-1β and TNFα in β-cell damage, children recently diagnosed with T1D have been treated with IL-1RAs (IL-1 receptor antagonists) and TNF antagonists in a number of different trials. Both treatments promoted small increases in insulin production within 4–5 months of treatment [68].

In NOD mice multiple DC subsets are present including mDCs (myeloid DCs), lymphoid DCs and pDCs (plasmacytoid DCs). mDCs have an important role in antigen processing and presentation to autoreactive T-cells leading to their activation. In NOD mice there is a 5-fold increase in the number of immature mDCs and fewer mature mDCs. The immature DCs underproduce IL-10, have deficient responses to GM-CSF (granulocyte/macrophage colony-stimulating factor), decreased intracellular and surface expression of MHC class II, reduced co-stimulatory molecule expression and lowered expression of the CD40 signalling molecule [69]. However, the mature mDCs in NOD mice express elevated NF-κB in response to antigen, and this leads to increased secretion of IL-12p70 and TNFα, and increased expression of co-stimulatory molecules. Increased IL-12 leads to autocrine activation of DCs, as well as increased activation of antigen-specific $CD8^+$ T-cells [70]. This intrinsic defect of the mature mDC population therefore tips the balance towards a Th1 immune response that drives β-cell destruction and T1D. Likewise, mDCs from T1D patients display elevated NF-κB activation and IL-12 secretion [71]. In addition to defects in the myeloid population there are also defects in the lymphoid DC population in NOD mice. Lymphoid DCs in NOD mice show reduced IDO (indoleamine 2,3-dioxygenase)-mediated catabolism of tryptophan in response to IFNγ, resulting in the increased proliferation of effector T-cells [72]. pDCs have an immature phenotype, are poor antigen presenters and are therefore known to be important for the induction of tolerogenic immune responses. In NOD mice depletion of pDCs was associated with a reduction in IDO in the pancreas and this correlated directly with the development and severity of insulitis [73]. The importance of DCs in T1D has been exploited in on-going clinical trials. Autologous DCs modified *ex vivo* to have reduced co-stimulatory ability have been used in T1D patients and at risk individuals to drive tolerogenic immune responses [179]. Although safety has been established, the efficacy of this approach is not yet known.

NK (natural killer) cells

NK cells are normally associated with defence against viruses, intracellular pathogen infected cells, malignant cells and foreign or transplanted cells. It is thought that NK cells may have an important early role in the immunopathogenesis of T1D since they are found to infiltrate pancreatic islets of NOD mice and have also been detected in the pancreatic islets of cadaveric T1D patients [74,75]. Furthermore, NK cells within the insulitis lesion display an activated phenotype, expressing higher levels of KIRG1 (killer cell lectin-like receptor group G1), PD-1 (programmed cell death-1), IL-2R (IL-2 receptor; CD25) and CD69 than NK cells from the pLN or spleen, and this correlates with increased β-cell destruction. The activated NK cells found in the insulitis lesion may directly induce β-cell apoptosis through perforin- and granzyme-mediated cytotoxic damage, as they express CD107a which is a marker of granule exocytosis [76]. NK cells are able to recognize NOD β-cells as these express RAE1 (retinoic acid early transcript 1) and NKp46 ligand, which are the ligands for NK cell receptors NKG2D (natural killer group 2D) and NKp46 respectively. Although it has been shown that blockade of NKG2D and NKp46 reduces T1D incidence, it may be that the protection is due to inhibition of interactions other than with NK cells, such as with cytotoxic T-cells that also express NKG2D [77]. Alternatively, they may indirectly damage β-cells since they also express high levels of pro-inflammatory

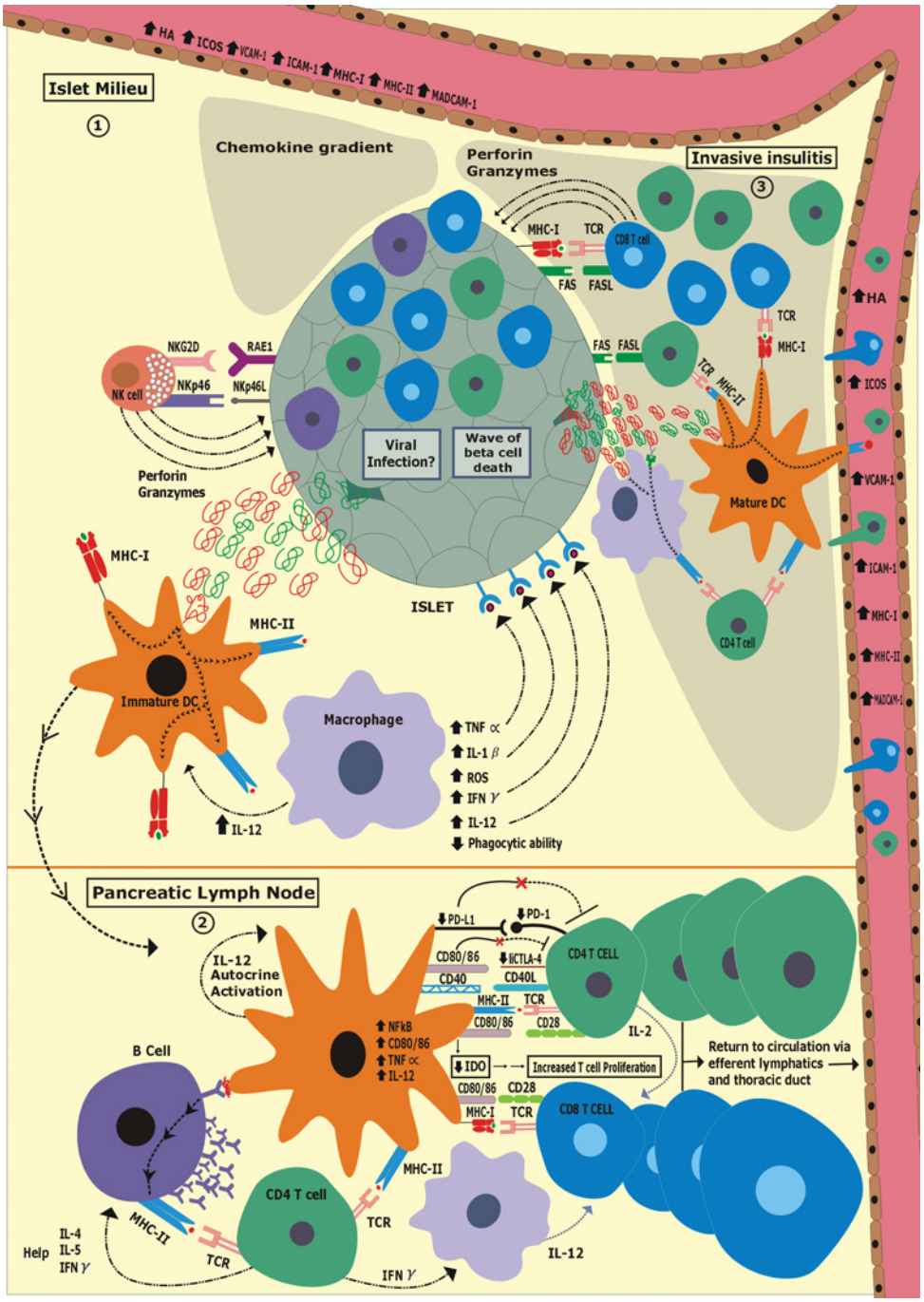

Figure 1 **Key immunopathogenic features of β-cell destruction in NOD mice**

(**1**) The initial trigger that causes the release of β-cell antigens is unknown, but it has been proposed that viral infection and/or a wave of β-cell death may promote the initial recruitment of NK cells, DCs and macrophages to the islet milieu. NOD mouse macrophages have a reduced phagocytic ability and secrete increased levels of ROS, TNFα and IL-1β that promote β-cell death, as well as increased levels of the pro-inflammatory cytokines IFNγ and IL-12 that increase local MHC expression and promote DC activation. (**2**) Activated DCs migrate to the pLN, where they deliver β-cell antigen to resident APCs and T-cells. NOD mouse DCs have a reduced tolerogenic capacity due to decreased expression of PD-L1 and IDO, as well as increased expression of NF-κB, CD80/CD86, TNFα and IL-12, leading to activation of autoreactive CD4 and CD8 T-cells. Activated T-cells are expanded further by encounter with activated B-cells presenting β-cell antigen. (**3**) Activated lymphocytes migrate across a chemokine gradient from the inflamed islet associated vascular endothelium, expressing up-regulated HA, ICOS, VCAM-1, ICAM-1, MADCAM-1, MHC class I and MHC class II, into the islet tissue. Local activated APCs in the islet milieu continue to take up, process and present antigen, promoting the retention and expansion of CD8 and CD4 T-cells that kill β-cells via perforin/granzyme and Fas/FasL mechanisms respectively. RAE1, retinoic acid early transcript 1.

cytokines such as IFNγ [78]. Although NK cells undoubtedly have a role in the early insulitis lesion it is unlikely that they are essential in the immunopathogenic process that drives complete β-cell destruction as NOD β-cells lacking MHC class I/*B2m* yet retaining NK receptor ligands are not killed by NK cells [79].

B-lymphocytes

Although macrophages and DCs are the primary pAPCs involved in the insulitis initiation, B-lymphocytes also have a pivotal early role in the pathogenesis of T1D development. B-cells have been identified in the insulitic lesions of biopsies from human T1D patients, as well as in the pancreatic biopsies of diabetes-prone mice and rats [80]. The significance of B-cells as important contributors to disease became clear when diabetes-prone NOD mice lacking B-cells were generated. Although B-cell-deficient NOD mice developed mild insulitis, suggesting that B-cells are not required for the initiation of disease, they were significantly protected from the development of diabetes, confirming their role in disease progression [81–84]. Similar results were obtained following antibody-mediated depletion of B-cells [85].

Two main roles have been proposed for B-cells in the pathogenesis of T1D. (i) B-cells may contribute to disease via the production of AAbs (autoantibodies), known to correlate with early pathogenesis. A role for AAbs in promoting T1D development has also been supported by maternal AAb studies whereby inhibition of AAb transfer from mothers to NOD offspring in several models was found to protect against T1D development in the offspring [86,87]. Additionally, there is indirect evidence that suggests that AAbs enhance the effector functions of DCs and NK cells, as FcRγ (Fc receptor γ)$^{-/-}$ NOD mice are protected from disease [88]. These findings have led to the proposal that AAbs may augment β-cell destruction through binding AAgs and promoting FcRγ-mediated antigen uptake by APCs or by promoting antibody-dependent cellular cytotoxicity of β-cells. However, AAbs are not requisite for T1D development as NOD transgenic mice expressing only membrane-bound BCRs (B-cell receptors) and without the capacity to secrete antibody developed an increased incidence of insulitis and T1D compared with NOD B-cell-deficient mice [89]. (ii) B-cells may contribute to disease via the recognition, uptake and processing of AAgs and presentation to diabetogenic T-cells. This antigen-presenting role of B-cells must occur after the activation of T-cells involved in the initial immune phase of T1D, as many B-cell-deficient mice develop non-invasive insulitis [81]. The progression from non-invasive to invasive insulitis is well documented, but a mechanistic understanding of this crucial switch is not well understood [90,91]. It is likely that the highly proliferative nature of B-cells allows them to efficiently capture β-cell antigen for processing and presentation to activated diabetogenic CD4$^+$ T-cells and CD8$^+$ T-cells, resulting in the rapid expansion of cells invading and killing the islet β-cells [92,93].

Because of their known role in antigen presentation, and because they are expanded in NOD mice, the MZ (marginal zone) B-cell population has been implicated in the pathogenesis of T1D [94]. However, it is unlikely that the increase in MZ numbers observed in NOD mice is a primary defect promoting T1D, since loss of MZ B-cells following complete splenectomy failed to protect NOD mice from T1D, whereas the removal of follicular (FO), and not MZ, B-cells in anti-CD20-treated NOD mice did protect from T1D [95].

Treatment of recently diagnosed T1D patients with anti-CD20 mAb (monoclonal antibody) transiently depleted B-cells and resulted in transient preservation of β-cell mass. Although B-cells clearly contribute to T1D pathogenesis in NOD mice and humans, it is also clear that T1D can develop via alternative pathways in both species. T-cells from B-cell-deficient NOD mice were able to transfer T1D to NODscid recipients, albeit at a reduced frequency compared with T-cells from B-cell-sufficient donors [82]. In humans, T1D has likewise been reported in B-cell-deficient patients [96]. Therefore, although B-cells present as an attractive therapeutic target in those at risk of developing T1D, it is unlikely that their blockade will provide protection in all patients.

T-lymphocytes

There is a large body of evidence implicating T-cells in the development and progression of T1D in humans and NOD mice. Both CD4$^+$ and CD8$^+$ T-lymphocytes are pivotal during the early and late stages of disease in mice. Whole splenocytes or purified populations of both CD4$^+$ and CD8$^+$ T-cells can transfer T1D to young NOD and non-diabetes-prone F1 mouse strains, whereas neither CD4$^+$ nor CD8$^+$ T-cells alone can transfer disease [97,98]. Likewise, T-cell-depletion of susceptible NOD mice inhibited disease progression and T1D, reinforcing the central role of T-cells in immunopathogenesis [99]. The early discovery that T-cells are essential in the immunopathogenesis of T1D has led to a major focus on these cells, from thymic development to understanding the key mediators of cytotoxic versus regulatory subset development and β-cell killing.

Development

The architecture of the NOD mouse thymus is abnormal, comprising unusually large perivascular spaces and disorganized thymic medulla. Additionally, NOD mouse thymocytes have reduced expression of the integrin-type fibronectin receptors $\alpha4\beta1$ (VLA-4) and $\alpha5\beta1$ (VLA-5) that cause defects in cell migration. Since the thymocytes trapped within the giant perivascular spaces of the NOD mouse thymus are consistently VLA-5-negative, their accumulation may be due to an impairment of normal thymocyte migration [100,100a]. Although the overall number of T-cells emigrating from the NOD mouse thymus is normal, there may be an increase in the proportion of T-cells that bear TCRs with autoreactive specificity. The unique MHC class II haplotype present in NOD mice, and in many Caucasoid T1D patients, influences the ability to bind to self-peptide and mediate negative selection. This deficiency was tested in transgenic NOD mice expressing non-autoimmune-associated MHC class II haplotypes. These mice were protected from developing T1D, a protection that involved thymic deletion of autoreactive CD4$^+$ T-cells in TCR transgenic 4.1NOD mice expressing the MHC class II molecule I-E [101].

It has been proposed that NOD mice fail to express self-antigens efficiently. This could be due to the lack of the MHC class II molecule I-E or, alternatively, due to the inability of AIREs (autoimmune regulators) to induce expression of

self-antigens in mTECs (medullary thymic epithelial cells). NOD mice have reduced thymic expression of the AAg ICA69 (islet cell autoantigen of 69 kDa), and the gene encoding this protein carries a SNP in the promoter region important for AIRE binding [102]. This could explain the reduced thymic expression of this self-antigen and the potential for reduced deletion of thymocytes with specificity for it. Although there is no direct evidence that the expression of proinsulin is similarly reduced in the NOD thymus, enhanced expression of this important AAg in transgenic NOD mice protects from T1D [103]. In humans elevated thymic expression of proinsulin is also associated with protection from T1D [104].

NOD mice have a defect that limits allelic exclusion, which is identified by the increased heterogeneity of TCRα genes expressed on T-cells from transgenic NOD AI4 mice [105]. As a consequence of inefficient allelic exclusion, two different TCRs can be expressed on the surface of developing thymocytes and this has been shown to allow escape from negative selection and development of autoimmunity in other models [21].

Once the process of thymic selection is complete, thymocytes up-regulate receptors on their surface, such as CCR7, allowing them to respond to chemokines and emigrate from the thymus to the peripheral lymphoid organs. Interestingly, CCR7 is elevated on NOD mouse T-cells and CCR7-deficient NOD mice are protected from T1D [106,107]. However, there is to date no evidence that this polymorphism contributes to altered thymic emigration.

Activation

Since autoreactive T-cells are found in normal healthy people and mice, the escape of such cells from the NOD thymus cannot alone account for the development of T1D. Therefore there must be defects in the peripheral regulation of autoreactive T-cells. Following selection within the thymus, naive $CD4^+$ and $CD8^+$ T-cells travel to LNs, where they await activation upon MHC presentation of their complementary antigens by pAPCs and co-stimulatory signals. The activation of islet-reactive T-cells occurs within the pLN as their early removal in NOD mice was found to prevent development of T1D, whereas early removal of spleen had no impact on T1D [108].

The unique MHC class II of NOD mice contributes not only to the loss of thymic tolerance, but also to the loss of peripheral tolerance. Autoreactive $CD4^+$ T-cell activation is associated with the unique I-A^{g7} molecule. The lack of an acidic residue at position 57 of the β chain prevents formation of a salt bridge with Arg^{76} in the α chain [109–111]. As a consequence, I-A^{g7} is able to form salt bridges with bound peptides, enhancing peptide–MHC class II binding. A similar binding property has been found in the human MHC class II genotype HLA DQA1*0301, DQB1*0302 associated with T1D [112], suggesting that the homologous human HLA haplotype may lead to T1D development through the same mechanism.

The maintenance of tolerance to islet cell antigen requires their expression by stromal cells in the pLN, and this is regulated by the Deaf1 (deformed epidermal autoregulatory factor 1) transcription factor. Deaf1 is negatively regulated by its binding to a Deaf1 isoform called Deaf1-VAR, which prevents localization of Deaf1 to the nucleus. In the absence of Deaf1 in the nucleus, the transcription of TSAs (tissue-specific antigens) is reduced. In NOD mice undergoing destructive insulitis, Deaf1-VAR is in excess and the expression of TSAs are consequently down-regulated [113]. Reduced expression of Deaf1 was also found in T1D patients. However, in NOD mice, islet-reactive T-cells are activated as early as 3 weeks of age, well before any changes in Deaf1 expression are observed, and as such this cannot be the primary peripheral tolerance defect that leads to the development of insulitis.

The activation of islet-reactive T-cells requires signalling through co-stimulatory molecules expressed by pAPCs, including CD40, CD80 and CD86. CD40 binds CD40L (CD40 ligand) on T-cells, leading to an up-regulation of CD40 and TNFRs on APCs. Early inhibition of CD40L in NOD mice caused a significant reduction in the development of insulitis and T1D, suggesting that autoreactive T-cells are dependent on this interaction for activation [114]. In addition to the importance of the CD40/CD40L interaction for activation of T-cells, this interaction may also be important for the expansion of T-cells in the insulitis lesion. CD40 has been identified on the surface of islet-invasive T-cells, raising the possibility that pathogenic T-cells may cross-stimulate via CD40/CD40L interaction [115]. CD80/CD86 on APCs can ligate CD28 on T-cells and lead to their activation. This activation can be negatively regulated by the inhibitory molecule CTLA-4 on T-cells, which also binds CD80 and CD86. CTLA-4 negative regulation promotes the maturation of DCs that express IDO and regulate effector T-cells. NOD mice express reduced levels of liCTLA-4 with a concomitant reduction in the usual negative regulation of T-cell activation [17]. Consistent with this, deletion or blocking of CTLA-4 in NOD mice resulted in exacerbation of T1D [116]. Furthermore, NOD mice with induced overexpression of liCTLA-4 had a reduction in the incidence of T1D [117]. Although liCTLA-4 is not found in humans, sCTLA-4 is reduced in T1D patients [17]. On the basis of these studies, patients with recent-onset T1D were treated with CTLA-4Ig, a fusion protein composed of the Fc region of IgG1 and extracellular domain of CTLA-4, which inhibits the co-stimulation of T cells. Although C-peptide levels were initially higher after CTLA-4Ig treatment, there was no significant preservation of β-cell mass after 2 years [118].

Another negative regulatory molecule belonging to the CD28 family and involved in T1D development in NOD mice is PD-1. PD-1 is expressed on activated T-cells and its ligands, PD-L1 and PD-L2, are expressed on DCs. T-cell proliferation is inhibited when PD-1 binds to its ligands on DCs. Interestingly PD-L1 is also expressed on islet cells and blockade of PD-L1 in NOD mice, using mAbs, accelerated the rate of insulitis and T1D progression, suggesting that the PD-1/PD-L1 pathway negatively regulates autoreactive T-cells [119]. Consistent with this, T1D patients have reduced expression of this important negative regulator of T-cell proliferation [120].

In addition, within the CD28 family is ICOS, a co-stimulatory molecule up-regulated on activated T-cells and important in T1D. NOD mice have a higher expression level of ICOS than non-autoimmune strains. Both ICOS- and ICOSL (ICOS ligand)-deficient NOD mice are protected from T1D and this is caused by the failure to activate β-cell-reactive T-cells [18]. ICOS may

therefore have an important role in maintaining the balance between the activation of effector and regulatory T-cells that control the development of T1D in NOD mice.

Homing to the islet

Activated islet-specific T-cells in NOD mice show normal up-regulation of CD44 and down-regulation of CD62L (CD62 ligand), allowing them to migrate out of the LNs via the efferent lymphatics. They then re-enter the circulation via the thoracic duct and migrate along the ECs associated with islet tissue. In pre-diabetic NOD mice, the β-cells, ECs and mononuclear cells infiltrating the islets produce multiple chemokine proteins that facilitate the tissue-specific homing of effector cells. The expression pattern of these chemokines is complex and it is unlikely that targeting of single chemokines will be of significant therapeutic benefit. However, β-cell expression of a chemokine decoy receptor that binds an array of chemokines completely prevented T1D in NOD mice [121].

The islet ECs normally protect β-cells from inappropriate infiltration by immune cells travelling in the blood. However, NOD mouse islet ECs take on an activated phenotype at the time of insulitis development, expressing HA (hyaluronan), MADCAM-1 (mucosal vascular addressin cell adhesion molecule-1), and ICAM-1 and VCAM-1, which are important for adhesion of activated monocytes and T-cells [122]. T-cell expression of MT1-MMP (membrane type-1 matrix metalloproteinase) facilitates their transmigration into the islets by cleaving the CD44/HA interaction [123]. Activated ECs also up-regulate MHC class I and II expression important for the presentation of antigens to $CD8^+$ and $CD4^+$ T-cells respectively. The reasons for this up-regulation are not known, but many viruses thought to be associated with T1D are known to infect microvasculature ECs and persistent infection of these cells has been associated with increased expression of adhesion molecules and MHC molecules [54–56,124]. The importance of ICAM-1 in the development of T1D has been demonstrated in anti-ICAM-1-treated NOD mice that were found to be protected from the development of T1D [125,126]. Others have reported that an increase in MHC class I expression on islet ECs was associated with T1D in NOD mice [55], and human histological samples taken during the peri-onset stage of T1D show up-regulation of MHC class I and II, as well as adhesion molecules, on the islet ECs [56]. A novel therapeutic approach has been tested in NOD mice using a mAb that blocks MT1-MMP cleavage of CD44/HA and thereby prevents the transmigration of activated T-cells into the islet, resulting in protection from T1D [123].

Insulitis

Despite the many studies showing that both $CD4^+$ and $CD8^+$ T-cells are required for progression of disease to complete β-cell destruction and T1D, the precise role of $CD4^+$ and $CD8^+$ T-cells in the initiation phase of disease has been controversial. $\beta 2M^{-/-}$ NOD mice, which lack MHC class I expression and $CD8^+$ T-cells, do not develop insulitis [127]. This work was interpreted to mean that $CD8^+$ T-cells are critical for the initiation of insulitis. However, an alternative explanation for the complete lack of insulitis is that protection was conferred by other B6-derived *Idd13*-linked protective genes back-crossed to the NOD with the β2M deficiency [127–130]. NOD mice lacking β2M/MHC class I on APCs only developed a mild peri-islet infiltrate consisting of $CD4^+$ T-cells that did not progress to invasive insulitis in the absence of $CD8^+$ T-cells [131]. This indicates that $CD4^+$ T-cells initiate peri-insulitis independently of $CD8^+$ T-cells and subsequently provide help to $CD8^+$ T-cells that can then respond to the earliest β-cell antigens.

It is thought that the earliest autoantigenic target of T-cells is insulin because a high frequency of both $CD4^+$ and $CD8^+$ T-cell clones isolated from the insulitic lesion of NOD mice react to peptides from insulin. In particular, early insulitic $CD4^+$ T-cells predominantly respond to insulin peptide B-(9–23) [132], and $CD8^+$ T-cells respond to peptide B-(15–23) [133]. Replacement of the NOD mouse insulin gene with a mutated insulin gene, encoding Al^{16}, protected from T1D, but periductal insulitis persisted around some islets [134]. This suggests that, although insulin is an important early AAg recognized by T-cells, there may be upstream AAgs that precede insulin. Whatever the upstream antigens are, clearly the induction of tolerance to insulin is important and blocks the progression of insulitis. NOD mice treated with oral porcine insulin or peptides were protected from T1D [135], and those administered with human insulin developed T_{reg} cells that were also able to transfer this protection [136]. However, unfortunately, in none of the clinical trials in which insulin was delivered systemically or orally was there a delay in onset of T1D compared with untreated control subjects at risk of developing T1D [137,138]. However, the problem with these trials may have been the route of administration as a pilot study using a single intramuscular injection of insulin with IFA (Incomplete Freund's Adjuvant) in recent-onset T1D patients showed an increase in insulin-specific T_{reg} cells 2 years after treatment [139].

Once initiated by T-cells specific for insulin, islet cell damage ensues with concomitant release of AAgs that results in the activation of an increasingly heterogeneous autoreactive T-cell repertoire. A large number of these AAgs have been defined in NOD mice and have been reviewed extensively elsewhere [140]. It is more difficult to study the expanding heterogeneity of human T-cell responses in T1D due to the low T-cell frequency in peripheral blood and the inaccessibility of islets. However, if we are to design therapeutic intervention strategies that induce tolerance in T-cells of diabetic patients, it is imperative that we identify the human antigenic targets. Currently, there are only three targets recognized by both $CD4^+$ and $CD8^+$ T-cells that have been identified in the NOD mouse and that are also found in human T1D patients. These include two β-cell antigens, insulin and IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein), and a neuroendocrine antigen, GAD (glutamic acid decarboxylase) [141]. GAD-65 alum has been used to treat new-onset T1D patients, but failed to show significant improvement in C-peptide levels. Combination therapy with GAD-65 alum and vitamin D/ibuprofen are currently in Phase II clinical trials [142].

Balance of immunopathogenesis and regulation

The insulitic lesion of NOD mice includes a number of $CD4^+$ effector T-cell subsets that have been categorized based on their

cytokine production as Th1, Th2, Th17, Th40, T_{reg} cells and NKT cells. On the basis of these subsets, T1D has been considered a Th1-mediated disease, because increased levels of IFNγ and lower levels of IL-4 were correlated with β-cell destructive insulitis [143]. Furthermore, induction of a Th2 bias by administration of IL-4, and infection with various helminths, confers protection from T1D in NOD mice [144,145]. However, this simple categorization of T1D as a Th1-mediated disease was brought into question when IFNγ- and IFNγR (IFNγ receptor)-deficient NOD mice remained susceptible [146,147]. It is now recognized that cytokines are involved in the cross-talk between a greater range of T-cell subsets that control the balance between effector and regulatory immune responses. Th17-cells were identified as a subset of effector T-helper cells that differentiate in response to TGF-β (transforming growth factor-β) and IL-6, and require IL-23 for population expansion. They produce mainly IL-17A, IL-17F and IL-22, which have broad actions on the immune system due to the distribution of the IL-17 and IL-22 receptors [148]. Th17-cells have been shown to be important for a number of autoimmune diseases, such as asthma and psoriasis, but their role in T1D has only been reported in a few studies and remains controversial. Serum IL-17 is elevated in NOD mice and there are reports of increased numbers of Th17-cells in NOD mouse islets [149]. Consistent with the proposed role for Th17 cells in disease, the blockade of IL-17 in NOD mice with a mAb resulted in reduced insulitis [150]. Conversely, IL-17-deficient NOD mice were not protected from T1D [151]. Furthermore, when Th1- and Th17-cells from NOD BDC2.5 mice were independently transferred to NODscid recipients, those receiving Th1-cells developed more severe disease than those receiving Th17-cells. Interestingly, recipients of Th17-cells were found to have converted into a Th1 phenotype as measured by their production of IFNγ, suggesting that Th17-cells in NOD mice are not the primary cell type driving pathogenesis [152]. These studies also highlight the plasticity of the different $CD4^+$ T-cell effector populations involved in a dynamic immune response.

A highly pathogenic subset of $CD4^+$ effector T-cells, termed Th40-cells, has been identified in the insulitis lesion of NOD mice [153]. In addition to CD40L expressed on all activated T-cells, this subset expresses the CD40 protein itself and produces the pro-inflammatory cytokines IFNγ, TNFα, IL-6 and, sometimes, IL-17. The percentage of Th40-cells in NOD mice correlates with increased insulitis, and this population is essential for the transfer of disease to NODscid recipients [154]. Further adding to the complexity of the dynamic interactions between the different $CD4^+$ effector T-cell populations, the production of IL-6 by Th40-cells can convert T_{reg} cells into Th17-cells in mice [154a]. Importantly, Th40-cells have been identified in increased numbers in T1D patients and were found to be reactive to known islet autoantigens [155].

T_{reg} cells are another important subset of effector T-helper cells involved in resolution of immune responses and in preventing autoimmunity. Although NOD mice have normal numbers of T_{reg} cells, their ability to regulate pathogenic T-cells is limited [156]. IL-2, encoded within the *Idd3* susceptibility loci, is expressed at reduced levels in NOD mice compared with non-autoimmune-prone strains, and this is correlated with the development of T1D. Since IL-2 has an important role in the induction and maintenance of FoxP3 (forkhead box P3)-expressing T_{reg} cells, it has been proposed that reduced IL-2 promotes T1D by causing an imbalance between T_{reg} cells and pathogenic T-cells [157]. Indeed, it has been shown that islet-infiltrating T_{reg} cells in NOD mice have reduced levels of IL-2R (CD25), FoxP3 and Bcl2 as a consequence of reduced IL-2 signalling [157a]. Consistent with this, administration of low-dose IL-2 to susceptible NOD mice restored CD25 expression on islet-infiltrating T_{reg} cells and protected from T1D [158]. It was also possible to correct the functional defect in T_{reg} cells of NOD mice by co-culturing them with cord blood stem cells [159]. This enhancement of T_{reg} cell function using stem cells has recently been tested in T1D patients and showed promising therapeutic potential [160].

NKT cells are a rare but an important effector T-helper cell subset, expressing an invariant TCRα chain Vα14-Jα18 in mice and Vα24-Jα18 in humans, and are also important in regulating effector T-cells. In NOD mice, NKT cells are severely reduced in number and are functionally deficient with an impaired ability to produce cytokines upon stimulation [161,162]. Although NKT cells can usually produce large quantities of both IL-4 and IFNγ, NKT cells in NOD mice produce decreased levels of Th2 cytokines, particularly IL-4, which may lead to a Th1 bias [163]. The reduced number of NKT cells correlates with exacerbation of T1D, and reconstitution of NOD mice with normal numbers of NKT cells prevents T1D [164]. Furthermore, it has been found that the impact of NKT cells on T1D in NOD mice is not always attributable to cytokine production [163]. NKT cells may also regulate T1D development through cell–cell contact with conventional T-cells, since this has been found to inhibit differentiation and induce anergy of islet-reactive T-cells in NOD mice [165]. These findings demonstrate that NKT cells may influence T1D via multiple non-mutually exclusive mechanisms. However, the significance of NKT cells in human T1D is yet to be definitively confirmed.

Killing of β-cells

As the infiltration of macrophages within the inflamed islet precedes that of T-cells, it is thought that initial β-cell death may not be antigen-specific, but instead be mediated by cytokines produced by macrophages. These cytokines include TNFα, IFNγ and IL-1β that bind their respective receptors on β-cells and induce apoptosis of β-cells via STAT1 (signal transducer and activator of transcription 1) and NF-κB pathways [166]. They can also induce the expression of ROS (reactive oxygen species) such as NO, which initiates DNA strand breakage and activation of PARP [poly(ADP ribose) polymerase], causing β-cell apoptosis. Although macrophages are important for initial β-cell damage, they do not kill sufficient numbers of β-cells to cause T1D, since NODscid mice with functional macrophages, but lacking lymphocytes, do not develop T1D.

The events that cause the non-invasive insulitis lesion to become invasive are not understood. However, the mechanisms by which β-cells are killed once this switch occurs have been well defined using NOD mice deficient in β-cell MHC class I, Fas or FasL (Fas ligand), perforin or granzyme. After a critical threshold

Table 1 Summary of clinical outcomes for T1D patients based on NOD mouse studies

Lessons from NOD mice	Potential relevance to human T1D	Clinical significance
Role of environment		
Vitamin D protects from T1D in NOD mice [35,36]	Reduced incidence of T1D in equatorial regions of high sunlight UV-B is essential for the synthesis of vitamin D Vitamin D has anti-inflammatory effects	Vitamin D supplementation: at birth protected from T1D; recent-onset T1D had no significant protection [38,39]
Omega-3 fatty acids protect from T1D in NOD mice [43]	Reduced incidence of T1D in ethnic groups with a culture of increased fish consumption Omega 3 fatty acids are anti-inflammatory	Omega-3 supplementation: in genetically susceptible children, T1D incidence was reduced; in pregnant mothers and their HLA higher T1D risk babies efficacy not yet known [44]; trials is on-going
Probiotics protect from T1D in NOD mice [48]	Higher T1D incidence associated with higher standard of living, hygiene and antibiotic use	Probiotic supplementation administered to at-risk children (PRODIA study) established safety; efficacy is not yet known [50]
Role of cytokine/cytokine receptors		
Macrophage production of TNFα and IL-1β are directly β-cell toxic in NOD mice [64]	Macrophages present in pancreatic samples of cadaveric T1D patients [181]	Recombinant IL-1RA (anakinra/kineret): in recent-onset T1D children, no change in pro-inflammatory cytokine gene expression, insulin secretory capacity or HbA_{1c} levels was observed; lower insulin requirements were reported up to 4 months post-treatment [68] TNF antagonist (etanercept, infliximab and adalimumab): in recent-onset T1D children, HbA_{1c} was reduced and insulin production was increased at 24 weeks post-treatment [68]
Role of DCs		
Defective DC maturation in NOD mice [69]	DCs control tolerance compared with activation of T-cells	Autologous DCs, manipulated to reduce co-stimulatory ability and promote tolerance, were given to T1D patients and at-risk individuals; safety was established, but efficacy is not yet known [179]; trial is on-going
Increased NF-κB activation in myeloid DCs of NOD mice [70]	Increased NF-κB activation in myeloid DCs of T1D patients [71]	
Increased IL-12 production by NOD mice DCs [70]		
Role of co-stimulation		
Reduced expression of co-stimulation inhibitory molecule liCTLA-4 in NOD mice [17]	Reduced expression of soluble CTLA-4 in human T1D patients [17]	CTLA-4Ig fusion protein co-stimulation blockade (abatacept): in recent-onset T1D patients, treatment resulted in an initial improvement in C-peptide levels; no preservation of β-cell mass was observed after 2 years [118]
Overexpression of liCTLA-4 reduced T1D incidence in NOD mice [117]		
Early treatment with CTLA-4Ig protected from T1D in NOD mice [116]		
Reduced expression of inhibitory molecule PD-L1 [119]	Reduced expression of PD-1 on T-cells in human T1D patients [120]	
Higher expression of ICOS [182]		
Role of β-cell antigens		
Proinsulin		
Transgenic NOD mice overexpressing proinsulin in the thymus were protected from T1D, suggesting the role of thymic expression of insulin in maintaining tolerance [103]	Polymorphism in human insulin promoter is associated with lower thymic proinsulin expression, loss of tolerance to insulin and T1D [104]	Induction of tolerance to insulin was trialled with: (i) Intra-nasal delivery: autoantibody-positive individuals had some increase in antibody and decrease in T-cell responses to insulin [184]
Transgenic NOD mice expressing mutated proinsulin (lacking immunogenic peptide) were protected from T1D [134]	T-cell responses against insulin in human T1D patients [183] Insulin autoantibodies present in human T1D patients [183]	(ii) Oral delivery: recent-onset T1D patients showed no improvement in C-peptide secretion or IgG insulin antibodies; accelerated β-cell loss in some treated patients was detected [185,186]

Table 1 Continued

Lessons from NOD mice	Potential relevance to human T1D	Clinical significance
Insulin autoantibodies precede T1D in NOD mice [183]		(iii) Intramuscular delivery with IFA: recent-onset T1D patients showed some increased insulin-specific T_{reg} cells at 2 years post-treatment [139]
T-cell responses against insulin present in NOD mice [183]		
GAD65		Induction of tolerance to GAD65/alum was trialled with:
GAD65 autoantibodies present in NOD mice [183]	GAD65 autoantibodies present in human T1D patients [183a]	(i) Subcutaneous delivery: recent-onset T1D patients had no significant improvement in C-peptide levels [187]; combination therapy with vitamin D and ibuprofen is currently in Phase II trials
Role of T-cells		
Autoreactive T-cells present in insulitis lesion of NOD mice [80]	Autoreactive T-cells present in T1D patients [187a]	Anti-CD3 mAb in recent-onset T1D patients and at risk individuals reduced the dependence on insulin over 4 years [190]; however, a recent large trial (otelixizumab) showed no efficacy in terms of C-peptide, insulin-dependence and HbA_{1c} [191]; trials are on-going
Autoreactive T-cells transfer disease to NODscid mice [82]	CD8 T-cells present in islets of cadaveric T1D patients [181]	
CD8 T-cells are the primary mediators of β-cell killing in NOD mice [129]		
Intravenous treatment of anti-CD3 protected from T1D in NOD mice [99]		
Oral anti-CD3 reversed T1D in NOD mice [188]		
Combined treatment of anti-CD3 with IL-1RA caused synergistic reversal of T1D in NOD mice [189]		
Role of B-cells		
B-cells present in insulitic lesion of NOD mice [80]	B-cells present in pancreatic samples of cadaveric T1D patients [193]	Anti-CD20 (rituximab): recent-onset T1D patients had improved HbA_{1c} and insulin-dependence at 1 year; however, depressed IgM levels indicated B-cell immunosuppression [175]
Antibodies against β-cell antigens present in NOD mice [183]	Antibodies against β-cell antigens present in human T1D patients [195]	
B-cell-deficient NOD mice were protected from T1D [85]		
Anti-CD20 depletion of B-cells protected from T1D in NOD mice [192]		
Role of T_{reg} cells		
Lower levels of IL-2 in NOD mice [194]	Polymorphism in IL-2RA causing diminished IL-2 response in T_{reg} cells from human T1D patients [196]	Stem cell educator (SCE) to promote T_{reg} cell development; autologous lymphocytes co-cultured with cord blood stem cells (CB-SC) given to T1D patients improved C-peptide, HbA_{1c} and insulin-dependence at 40 weeks post-treatment [180]
T_{reg} cells have reduced levels of IL-2R and FoxP3 expression in NOD mice		

of β-cell antigen has been released by non-specific killing and presented to islet-specific T-cells, they become activated, and are recruited to and retained within the islet tissue where they proliferate and contribute to β-cell death [167]. Activated T-cells can kill β-cells through a Fas/FasL pathway. NOD mice deficient for either Fas or FasL are protected from T1D and transfer of wild-type NOD splenocytes into Fas-deficient NODscid mice resulted in delayed T1D [168].

Unlike $CD4^+$ T-cells, which cannot kill β-cells in an antigen-specific manner, $CD8^+$ T-cells recognize up-regulated MHC class I on β-cells and can mediate antigen-specific β-cell killing [169]. NOD mice lacking β-cell MHC class I expression are

largely protected from the development of T1D. This demonstrates that a direct interaction between $CD8^+$ T-cells and β-cells is the primary mechanism for antigen-specific β-cell killing [79,170]. Perforin-deficient NOD mice have a similar reduction in T1D incidence, suggesting that $CD8^+$ T-cells use the perforin/granzyme cytotoxicity pathway to kill β-cells [171]. Recently, the role of $CD8^+$ T-cells in human T1D was also demonstrated. Islet autoreactive $CD8^+$ T-cells were identified in focal islet regions from cadaveric T1D donors and this was associated with β-cell destruction [172]. Furthermore, in humans, it has been shown that the structural basis of $CD8^+$-mediated killing of human β-cells is different from other TCR–MHC class I interactions [173]. The TCR of a human autoreactive $CD8^+$ T-cell was highly peptide-centric in its recognition of MHC class I bearing proinsulin peptide, thus forming a very weak interaction with the MHC molecule itself. This interaction may explain why such autoreactive T-cells escape thymic selection. On the basis of these important studies implicating T-cells in the development of T1D, there have been a number of trials using anti-CD3. Early studies using humanized anti-CD3 were promising, with treated patients showing a reduced dependence on insulin over 4 years. However a more recent and large trial using anti-CD3 (otelixizumab) showed no improvement in C peptide, insulin dependence or HbA_{1c} (glycated haemoglobin) levels [174].

SUMMARY

Studies in NOD mice over the past few decades have contributed to numerous immunomodulatory therapies and many of them have been tested in humans. Despite the successful protection from T1D seen in NOD mice, there has been limited success with therapeutic interventions in individuals at risk of T1D and patients. In NOD mice, non-antigen-specific therapies, including those that target the T-cells using anti-CD3 mAbs, B-cells using anti-CD20 mAbs, and APCs using a sCTLA4Ig, have shown some protection from T1D. Unfortunately, however, similar therapies showed limited efficacy in humans. Treatment of recently diagnosed T1D patients with rituximab, a humanized anti-CD20 mAb, resulted in transient preservation of β-cell mass. Although improvement was transient, this therapy holds promise for those at risk of T1D if administered earlier in the disease process. Genetic screening and earlier diagnosis will be important for opening an earlier therapeutic window of intervention [175,176].

Antigen-specific immune modulation trials in NOD mice, such as those involving insulin, were successful. However, none of the human trials in at-risk children have demonstrated significant efficacy. Explanations that have been proposed for the failure of these trials include the possibility that the route of administration rendered the insulin ineffective, the dose was inadequate to induce tolerance or, alternatively, such an approach may work only in those at-risk individuals that entered the trial prior to the development of activated T-cells and insulin AAbs [137,138].

Combinational therapies that suppress T-cell activation and enhance tolerance have also been successful in NOD mice. A recent pilot study [177] using combinational therapy in T1D patients involved the use of rapamycin to supress effector T-cell proliferation in combination with IL-2 to induce the formation of T_{reg} cells. Although this approach did promote an increase in T_{reg} cells, unfortunately none of the treated patients showed an increase in preserved β-cell mass [177].

Another innovative therapeutic approach that has been successfully tested in NOD mice is the use of immature DCs to induce tolerance [178]. This approach involves the *ex vivo* engineering of NOD DCs with AS-ODNs (antisense oligodeoxyribonucleotides) to inhibit the expression of CD80/CD86. After transfer back into NOD mice, these immature DCs migrated to the pLN and induced tolerance to β-cell antigens. Clinical trials using engineered DCs have now been initiated and support the on-going investigation of this approach for treatment of at-risk individuals in further trials [179]. One of the most recent and exciting therapeutic approaches, currently in Phase I clinical trials, is known as SCE (stem cell educator) therapy. This approach originated from NOD mouse studies in which splenocytes were cultured with human cord blood stem cells. T_{reg} cells cultured in this way showed an increase in the $CD4^+CD62L^+$ sub-population and were able to suppress T1D in NOD recipients [180].

In summary, the NOD mouse has been studied extensively and has informed much of the current understanding of the immunopathogenesis of T1D. There are many similarities in the genetics and immunopathogenic mechanisms that lead to T1D in NOD mice and humans. Understanding these pathways has given us insight into a number of potential therapeutic avenues that are currently being trialled (Table 1).

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Pancreatic ductal cell antigens are important in the development of invasive insulitis in Non-Obese Diabetic mice

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ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune disease in which insulin producing beta cells of the pancreas are selectively destroyed. Glial Fibrillary Acidic Protein (GFAP) expressed in peri-islet Schwann cells (pSCs) and in the ductal cells of the pancreas is one of the candidate autoantigens for T1D. Immune responses to GFAP expressing cell types precede the islet autoimmunity in Non-Obese Diabetic (NOD) mice. By removing MHC class I from GFAP expressing cell types, we tested the role of autoantigens presented by these cell types in the development of invasive insulitis. Our findings indicate that antigens expressed by pancreatic ductal cells are important in the development of invasive insulitis in NOD mice.

1. Introduction

The immune mediated destruction of beta cells in T1D results in the progressive loss of beta cells, causing insulin deficiency and consequent hyperglycemia (Jayasimhan et al., 2014; van Belle et al., 2011). The presence of multiple auto antibodies and evidence of T cell reactivity to autoantigens indicates multiple targets are involved in disease pathogenesis in both humans and in NOD mice (Han et al., 2013; Roep and Peakman, 2012; Pearson et al., 2016).

Investigations in NOD mice have identified numerous autoantigens and many of these autoantigens including Glutamic Acid Decarboxylase 65 (GAD65), Insulinoma Associated antigen 2 (IA2) and Islet Cell Autoantigen 69 (ICA69) are found not only in beta cells, but also in the cells of the neuroendocrine system (Mallone et al., 2011; Mauvais et al., 2016; Lieberman and DiLorenzo, 2003). It is important to identify the initiating autoantigen to design immunoregulatory strategies to treat the disease in genetically susceptible individuals. To date Proinsulin is the earliest autoantigen that has been identified to drive islet infiltration (Nakayama et al., 2005; Wong, 2005; Wegmann and Eisenbarth, 2000). Mice lacking native proinsulin, and carrying a mutated non-antigenic proinsulin, were protected from developing infiltrates into the islets. However, these transgenic mice had infiltrates into the islet associated pancreatic ductal tissue. This suggests that there are antigenic targets of an autoimmune attack on the islet associated ductal tissue of the pancreas, which precedes islet autoimmunity (Nakayama, 2011).

The possibility that an initiating antigen is expressed in *peri* islet tissues is further supported by the finding that, immune responses to autoantigens expressed in pSCs precede the immune response to beta cells (Winer et al., 2003; Tsui et al., 2008). The early infiltrates seen around the pancreatic ducts and the attack on GFAP expressing pSCs implicates these cells in the autoimmune process. Studies conducted by Razavi et.al also support the involvement of pancreatic sensory neurons in controlling islet inflammation (Razavi et al., 2006). This raises the question of whether an autoantigen present in GFAP positive cells may not trigger an autoimmune response that precedes the immune responses to beta cell antigens (Pang et al., 2017; Kaufman, 2003).

GFAP expressing ductal cells and pSCs of the neuroendocrine pancreas share with islet beta cells a number of autoantigens implicated in T1D (Bouwens and Pipeleers, 1998; Mally et al., 1996a; Gurr et al., 2002; Lieberman and DiLorenzo, 2003). An early immune response against autoantigens expressed in GFAP positive cell types may result in the subsequent cross reactive attack of beta cells (Kaufman, 2003). To understand the role of early upstream antigens expressed in pSCs and in

Abbreviations: DBA, *Dolichos biflorus* Agglutinin; EYFP, Enhanced Yellow Fluorescent Protein; GFAP, Glial Fibrillary Acidic Protein; GFP, Green Fluorescent Protein; H&E, Haematoxylin and Eosin; HBSS, Hanks Balanced Salt Solution; IA2, Insulinoma Associated Antigen 2; ICA-69, 69 kDa Islet cell Autoantigen; MHC, Major Histocompatibility Complex; NOD, Non-Obese Diabetic; OCT, Optimum Cutting Temperature; PBS, Phosphate Buffered Saline; PFA, Paraformaldehyde; pSCs, Peri-Islet Schwann Cells; RBC, Red blood cell; T1D, Type 1 Diabetes; β2M, Beta 2 Microglobulin

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ductal cell types of the pancreas, our lab generated NOD mice lacking MHC class I expression on GFAP expressing cells and assessed the development of insulitis.

2. Materials and methods

2.1. Animals

All animals were maintained under specific pathogen free (SPF) conditions at the Precinct, Animal Centre at the Alfred Hospital (PAC, Melbourne, Australia). All procedures involving mice were performed in compliance with the Animal Ethics guidelines of Monash University and were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP).

2.1.1. Generation of transgenic NOD GFAPcre mice

FVB-Tg(GFAP-cre)25Mes/J (Stock number 004600) were purchased from the Jackson Laboratory (Bar Harbor, ME, US) and backcrossed onto NOD/Lt for at least 10 generations. The generated NOD GFAPcre mice express cre recombinase under the control of human GFAP promoter. Hemizygous transgenic mice were identified by PCR based strategy using primers ACT CCT TCA TAA AGC CCT and ATC ACT CGT TGC ATC GAC CG specific for the GFAP and cre gene respectively (Zhuo et al., 2001). Even after 10 generations of backcrossing to NOD, there is a possibility of having a sizeable FVB derived genomic interval surrounding the GFAPcre transgene in NOD GFAPcre mice.

To rule out the possibility of any alterations in disease incidence by this hitchhiking FVB genomic interval, we compared the diabetic incidence in transgene positive NOD mice to conventional NOD mice. Since the pattern of incidence in transgene positive NOD mice and in conventional NOD mice was similar, we did not attempt to sequence the surrounding genomic intervals around the GFAPcre transgene. Endogenous β2M expression in GFAPcre transgene positive mice was removed by crossing to NOD $\beta2M^{-/-}$ mice (Serreze et al., 1994). Homozygous β2M knockout and GFAPcre transgene positive mice were known as NOD GFAPcre mice.

2.1.2. Generation of NOD GFAPcre.flβ2Ma mice (class I GFAP bald mice)

NOD.flβ2Ma mice carrying a floxed β2Ma transgene (Hamilton-Williams et al., 2003; Hamilton-Williams et al., 2001) on an endogenous β2M knockout background were crossed with NOD GFAPcre mice to generate NOD GFAPcre.flβ2Ma mice. The NOD $GFAPcre^{+}$ $\beta2M^{-/-}$ $fl\beta2Ma^{+}$ (class I GFAP bald mice) exhibit ductal cell and neuronal cell specific deletion of MHC class I whereas NOD $GFAPcre^{-}\beta2M^{-/-}fl\beta2Ma^{+}$ (Control mice) are class I GFAP normal mice respectively. 100–160 day old female NOD class I GFAP bald and NOD class I GFAP normal mice were used for insulitis studies.

2.1.3. Generation of Reporter mice (NOD $GFAPcre^{+}EYFP^{+}$)

C57BL/6.Gt(ROSA)26Sor.EYFP mice (gift by Dr. David Izon at St Vincent's Institute for Medical Research, Melbourne, Australia) carries a loxP-flanked STOP sequence followed the Enhanced Yellow Fluorescent Protein (EYFP) gene (Srinivas et al., 2001). C57BL/6.Gt(ROSA)26Sor.-EYFP mice were backcrossed to NOD/Lt for at least 10 generations. Crossing the mutant NOD EYFP mice to NOD GFAPcre mice generated the reporter mice (NOD $GFAPcre^{+}EYFP^{+}$), exhibiting EYFP expression in GFAP driven cre expressing tissues. To analyze the pattern of cre expression, tissues extracted from reporter and control (NOD $GFAP\text{-}cre^{-}EYFP^{+}$) mice were analyzed via immunohistochemistry and flow cytometry.

2.2. Flow cytometry

2.2.1. Pancreatic ductal cell isolation and staining

The pancreas from the reporter and control mice was dissected and single cell suspensions were prepared as described by Maximilian Reichert et al. (Reichert et al., 2013). The pancreatic single cell suspensions were incubated with biotinylated *Dolichos biflorus* Agglutinin (DBA) (Vector Labs, Burlingame, CA, US) (Xiao et al., 2013), washed and labelled with streptavidin conjugated to PerCP (BD Biosciences, USA). Following a second wash, cells were fixed in 4% paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS), permeabilized using 0.1% saponin and stained with polyclonal guinea pig anti-insulin antibodies (Dako, Carpinteria, CA) and secondary goat anti guinea pig Alexa Fluor 647 antibody (Thermo Fisher scientific, USA). The stained cells were acquired on FACS Calibur (BD Biosciences) and the data were analyzed using FlowJo software (Version 7.6.5). Establishment of the gate was based on the staining profiles of the negative control. We also attempted to stain for MHC class I molecules in pancreatic ductal cells. Despite multiple attempts, we were unable to view the MHC class I expression on ductal cells of the pancreas. We believe that the prolonged enzymatic digestion method used in isolating ductal cells, may have damaged the surface proteins which annihilated the detection of MHC class I molecules using antibody dependent flow cytometric methods.

2.2.2. Splenocytes isolation and staining

Red Blood Cell (RBC) lysed spleen single cell suspensions from the reporter and control mice were stained for CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70) and CD11c (HL3) with anti mouse monoclonal antibodies from BD Biosciences. The stained cells were acquired on a FACS Calibur and data was analyzed using FlowJo software. Establishment of the gate was based on the staining profiles of the negative control.

2.2.3. Brain cell isolation

The brain was dissected from reporter and control mice and collected in Hanks Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}). The brain hemispheres were mechanically dissociated and passed through a 70 μm cell strainer. The suspension was centrifuged at 250*g* for one minute and the collected pellet resuspended in HBSS containing 0.5% trypsin EDTA (Gibco, USA). Following 15 min incubation at 37 °C with intermittent shaking, the cell suspension was centrifuged, washed in HBSS and passed through a 70 μm strainer for analysis via flow cytometry.

2.3. Histology

2.3.1. Immunofluorescence

Pancreatic tissue from the reporter and control mice was fixed overnight in 4% PFA, immersed in 20% sucrose solution for 8 h and then snap frozen in Optimum Cutting Temperature (OCT) compound (Tissue-Tek). 5 μm tissue sections were cut and stained using Alexa Fluor 555 conjugated rabbit antibody against Green Fluorescent Protein (GFP) (Invitrogen, Carlsbad, CA, USA). Stained sections were visualized using a fluorescent microscope (Olympus BX61) at 20× magnification. Images were captured in AnalySIS 5.0 software and analyzed using Image J.

2.3.2. Histochemistry

Paraffin embedded pancreatic tissue from 100 to 160 day old class I GFAP Bald and GFAP normal mice was cut at three levels (100 μm apart) and each 5 μm section stained with Haematoxylin and Eosin (H&E). The degree of mononuclear cell infiltration was determined in 10–60 islets from each pancreas, in a blinded fashion. Insulitis severity was scored on a scale of 0–4 as previously described (Hamilton-Williams et al., 2003). The proportion of islets exhibiting invasive insulitis or disease free was determined and expressed as a percentage of total islets scored. H&E stained sections were visualized using the Olympus BX51 microscope at 20× magnification.

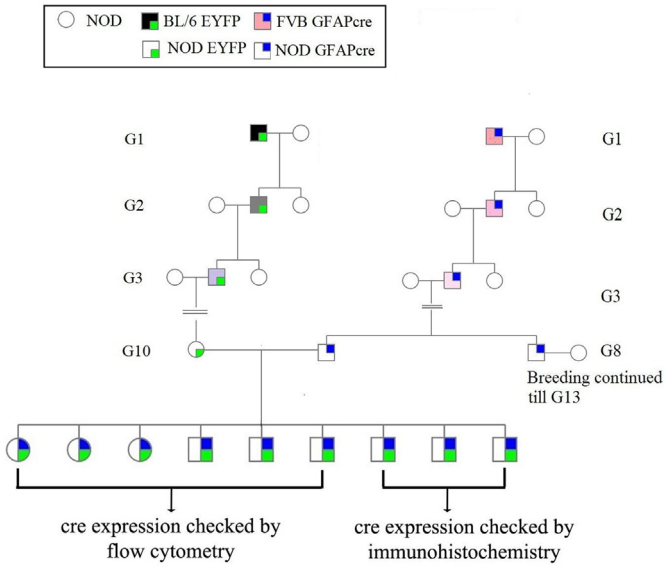


Fig. 1. Genogram shows the breeding strategy for NOD GFAPcre and reporter mice. FVB.GFAPcre mice were crossed to NOD mice for at least 10 generations to obtain NOD GFAPcre mice. At generation eight the GFAPcre mice were crossed to NOD EYFP mice to generate reporter mice. The pattern of cre expression in reporter mice was determined by flow cytometry and immunohistochemistry.

2.4. Statistical analysis

All data were represented as means ± SEM. Statistical difference was determined by using the unpaired *t*-test for column comparisons. Significance was accepted at two tailed *p* values ≤ .05 and analyzed with Graphpad prism software (version 7.02).

3. Results

3.1. Characterization of EYFP expression

3.1.1. Detection of EYFP expression in pSCs and ductal cells of reporter mice

NOD GFAPcre mice were generated by backcrossing the FVB GFAPcre mice to NOD mice for 10 generations (Fig. 1). The FVB X NOD GFAPcre mice at generation eight were crossed to NOD EYFP mice that carried a stop codon flanked by lox sites. The resultant NOD GFAP-cre$^+$EYFP$^+$ reporter mice lost the stop codon and thus expressed EYFP. The expression of EYFP indicating cre expression was determined by flow cytometry (Fig. 2a) and immunohistochemistry (Fig. 2b). Flow cytometric analyses of brain cells isolated from reporter mice showed EYFP expression in 28 ± 1.6% of the cells. EYFP was also detected in fixed pancreatic cryosections using fluorescence microscopy. The pattern of fluorescence was consistent with the morphology of pancreatic pSCs (Fig. 2b (VIII) white arrows) and ductal cells (Fig. 2b (VIII) red arrows) of the pancreas.

3.1.2. EYFP expression was not detected in immune cells of reporter mice

Ectopic expression of cre in off-target tissue types has variably been

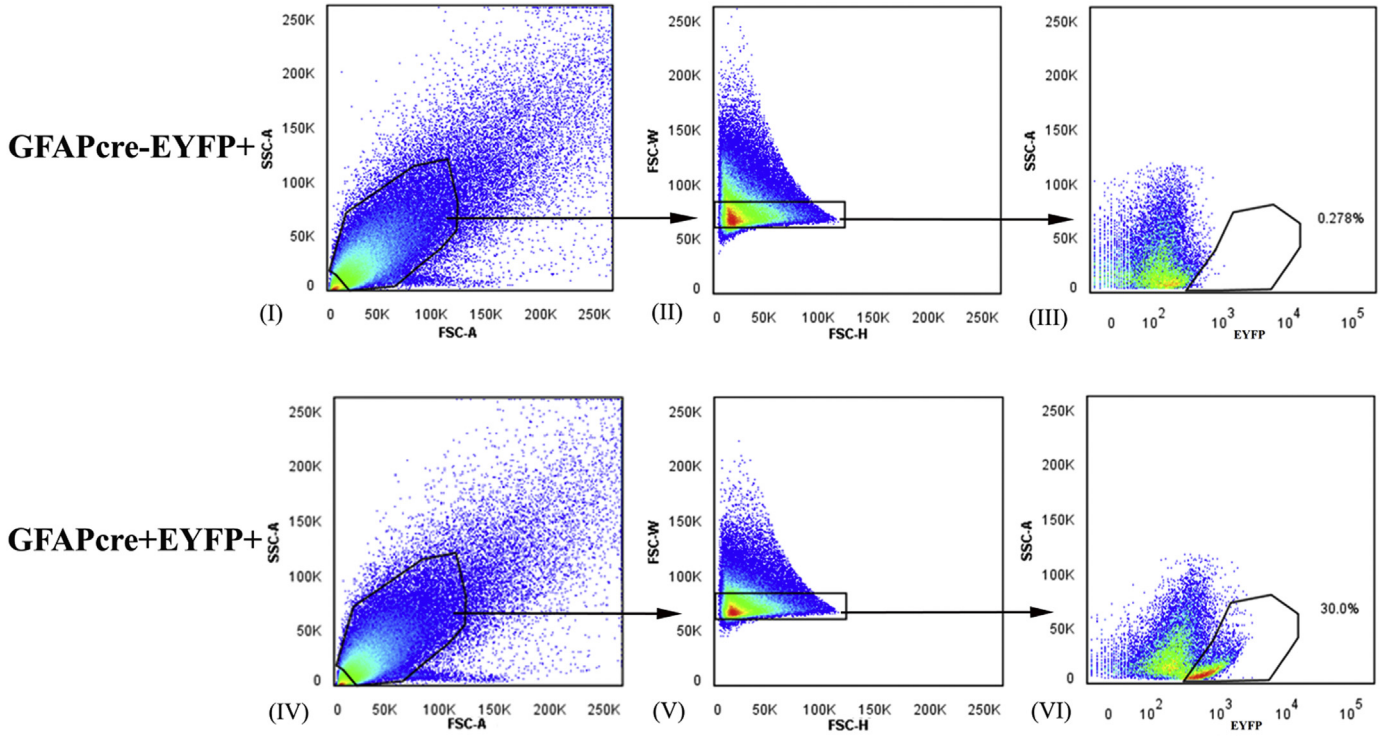


Fig. 2. a. Detection of EYFP in brain cells indicates cre expression. Flow cytometric analysis of brain cells isolated from control (top panel) and reporter mice (n = 3) showing gating strategy used and EYFP expression. Dot plot shows brain single cell suspension gated in forward and side scatter. First doublets were excluded from live gate (I, IV) based on FSC-H and FSC-W (II, V) then EYFP expressing cells were gated (x axis) vs side scatter (y axis) (III, VI). Brain cells in reporter mice express EYFP (VI) and the absence of expression in control mice (III). b Detection of EYFP in pancreatic neuroendocrine and ductal cells indicates cre expression. Fluorescent microscopic analysis showing cre expression in morphologically identified pancreatic neuroendocrine pSC and ductal cells of the reporter mice (n = 3). Pancreatic tissue sections from reporter (VII, VIII, IX) and control mice (I, II, III) were stained using a rabbit antibody against GFP conjugated to Alexa Fluor 555 and nuclear stained with DAPI. White arrows (VIII and IX) point to EYFP expressing pSC and red arrows point to EYFP expressing ductal cells. Stained brain sections from reporter mice (IV, V, and VI) were used as a positive control for EYFP signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

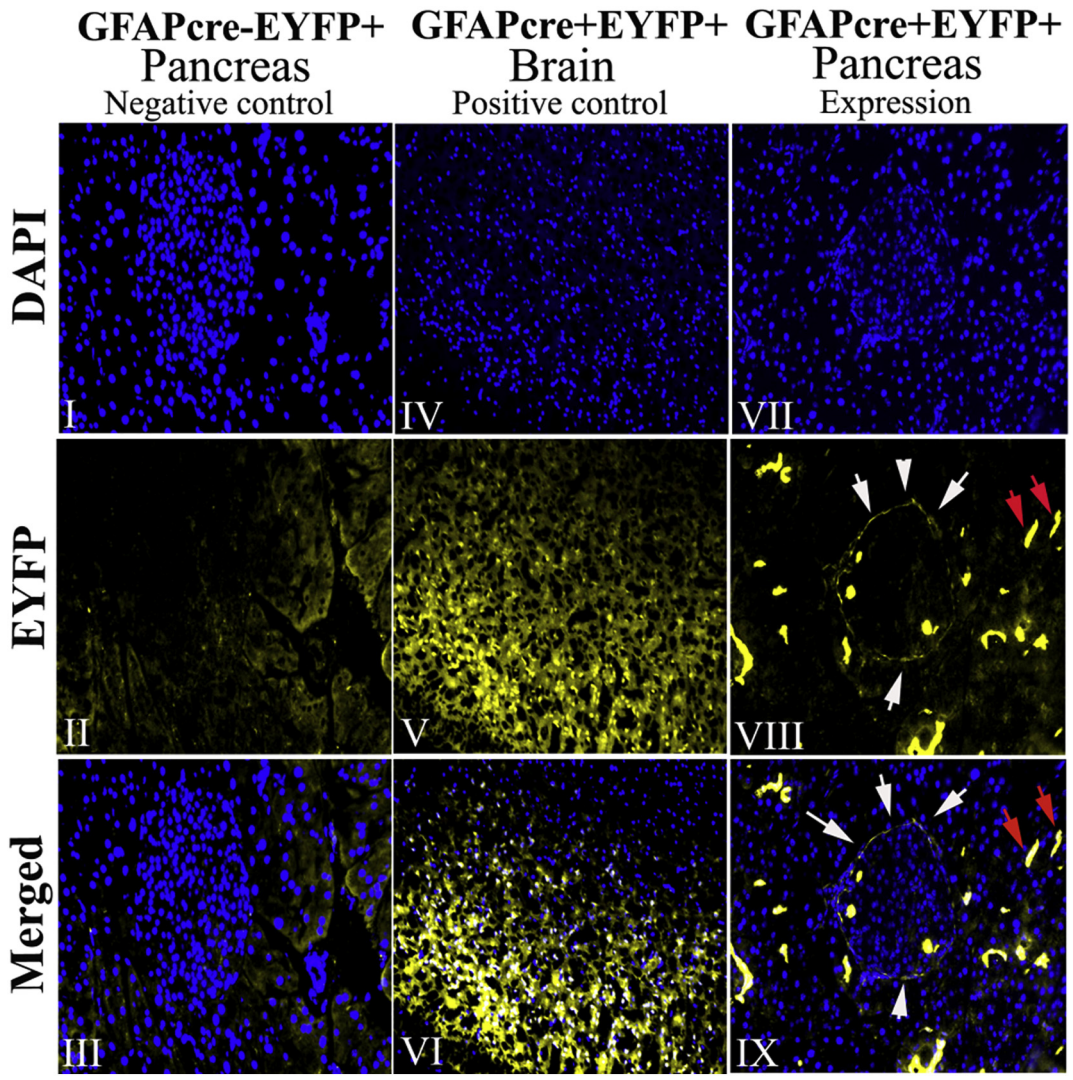


Fig. 2. (*continued*)

reported with the use of cre-lox systems (Smith, 2011; Harno et al., 2013). The specificity of the promoter dictates the fidelity of its expression and therefore the pattern of cre mediated recombination and EYFP expression. It was important to assess the pattern of cre expression in GFAPcre mice. This is because, in class I GFAP bald mice any non-specific expression of cre in immune cell types, would have resulted in deletion of β2Ma and loss of MHC class I molecules from these cell types. This would have confounded the interpretation of results.

EYFP expression was not detected in CD4 T cells, CD8 T cells (Fig. 3a) dendritic cells or macrophages (Fig. 3b) isolated from reporter mice ($n = 5$). The absence of EYFP expression in the entire splenocyte population (Supplementary Fig. 1A) indicates that GFAPcre mice are an appropriate model in which to study the role of MHC class I expression on pSCs and ductal cells in the initiation of insulitis.

3.2. Severity of insulitis is significantly reduced in the absence of MHC class I on GFAP expressing cell types

Class I GFAP bald mice were generated by crossing the NOD GFAPcre mice to NOD.flβ2ma mice (Fig. 4). These mice were used to assess whether MHC class I presented self-antigens, expressed by GFAP positive cells, were necessary for the initiation and progression of insulitis. Histological analyses of pancreatic sections showed insulitis was significantly reduced in mice lacking MHC class I molecules on GFAP expressing cells compared with class I sufficient age and sex matched control mice (Fig. 5a). Class I GFAP bald mice had more disease free islets (70 ± 5%) compared with class I sufficient controls (41 ± 8%) (Fig. 5b). Conversely the percentage of islets with invasive insulitis was also significantly reduced in class I GFAP bald mice. Class I GFAP bald mice had 9.5 ± 2% compared with MHC class I sufficient controls (32 ± 9%) (Fig. 5c). These data suggest that the loss of MHC class I from GFAP expressing cells somehow protected the mice from insulitis. Since a subset of GFAP positive cells expresses insulin, we therefore wanted to test whether the significant reduction of invasive insulitis seen in these mice is due to the absence of MHC class I mediated presentation of the autoantigen insulin.

3.3. GFAP positive ductal cells do not express insulin

Our data from reporter mice show that ductal cells are made up of GFAP positive and GFAP negative cell types (Fig. 6(III)). We detected insulin expression in 2.5 ± 0.6% of the ductal cells and identified that 90% of the insulin expressing ductal cells were GFAP negative. Since GFAP positive cells were found not to co-express insulin, the reduction of invasive insulitis seen in class I GFAP bald mice cannot be due to the loss of expression of insulin. It must be due to the loss of the presentation of another autoantigen present in the GFAP expressing ductal cells. There are a number of other autoantigens that ductal cells share with beta cells. It is possible therefore that one of these autoantigens is an early autoantigen that can initiate insulitis.

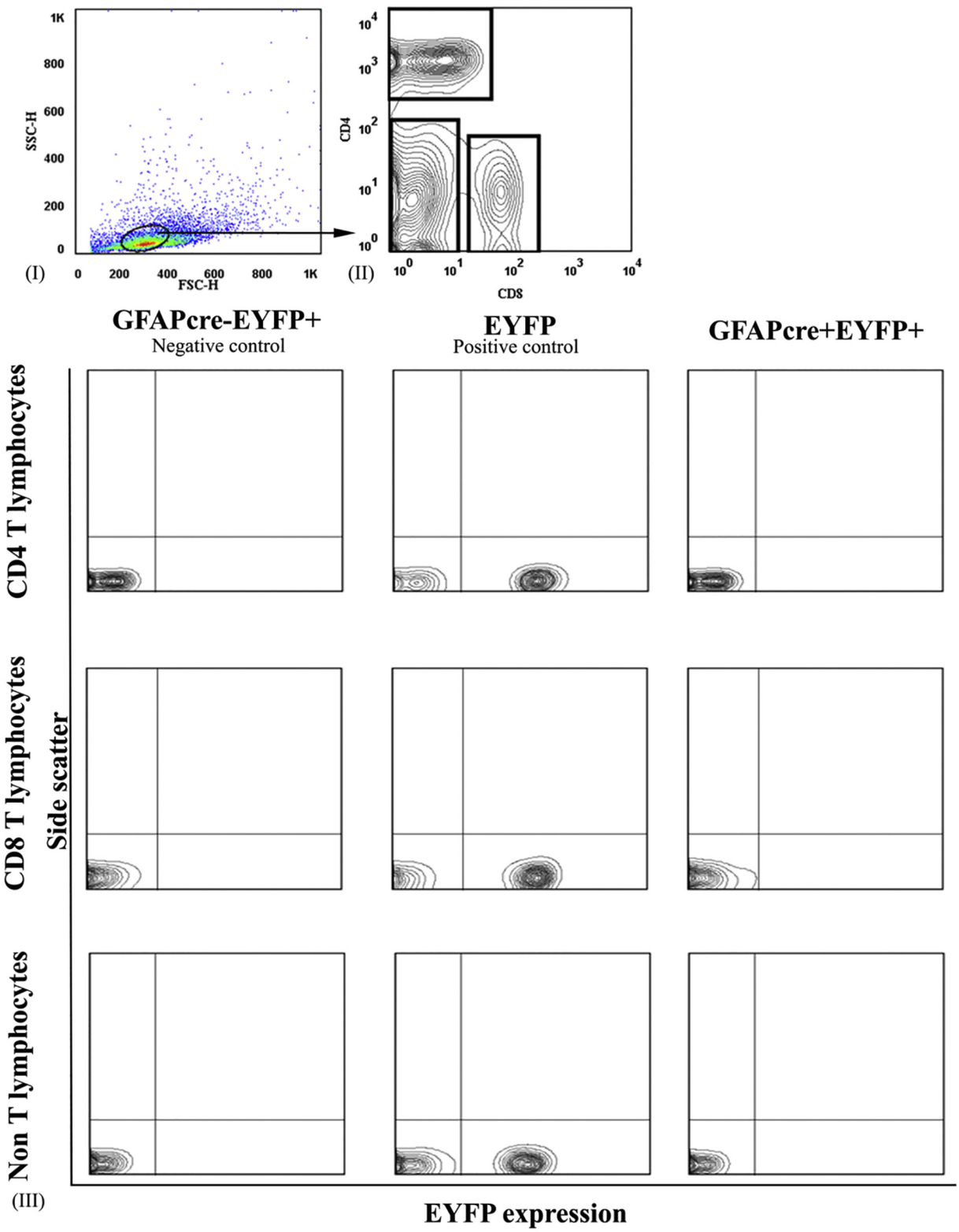
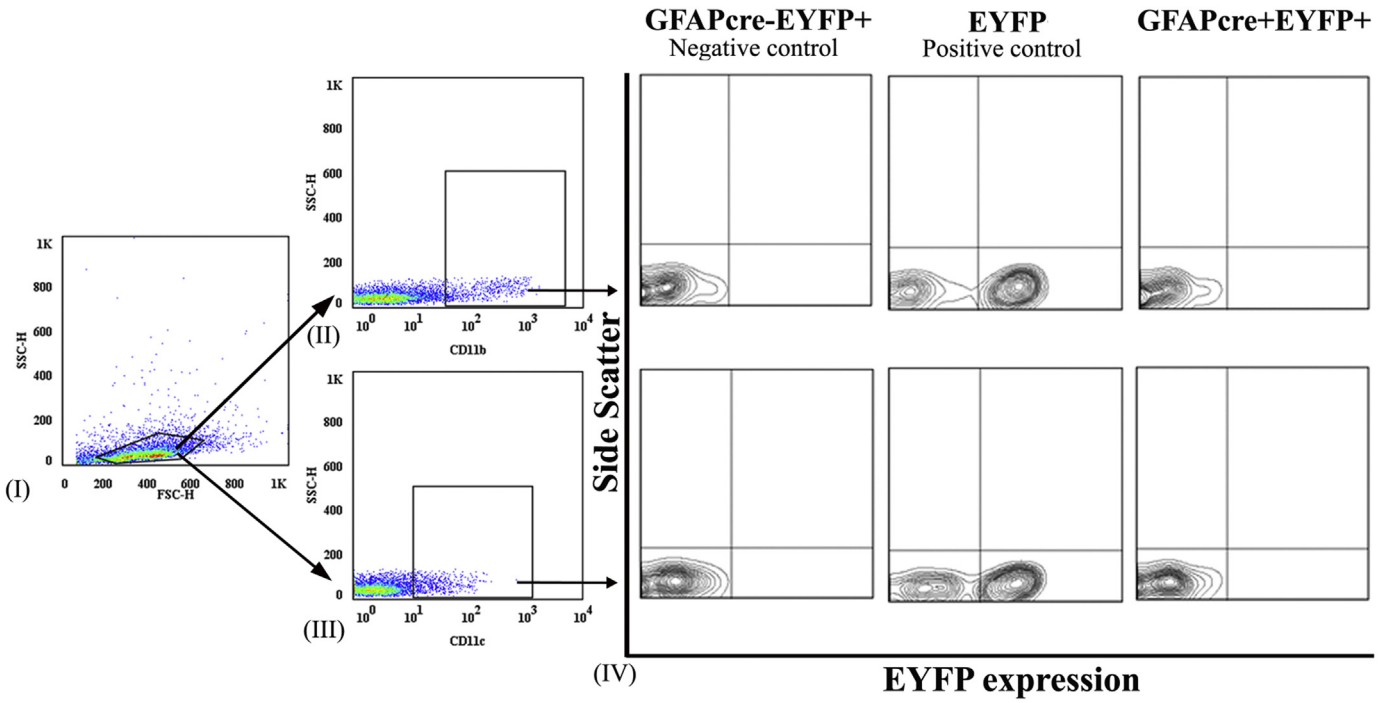


Fig. 3. a. EYFP expression was not detected in immune cells of reporter mice. Flow cytometric analyses of splenocytes from reporter mice were unable to detect lymphocytes expressing EYFP (n = 5). Dot plots showing live gating of lymphocytes in forward and side scatter (I). Sequential gating on live lymphocytes and contour plots showing CD4 T cells, CD8 T cells and Non T lymphocytes (II). EYFP expression was not detected in CD4 T cell, CD8 T cell and non T lymphocytes in reporter and control mice (III). Spleenocytes from mice known to express EYFP in immune cells were used as a positive control. b. EYFP expression was not detected in macrophages and dendritic cells of reporter mice (n = 5). Dot plots showing live splenocytes gated in forward and side scatter (I). Sequential gating on live splenocytes and dot plots showing macrophages gated using CD11b expression (II) and dendritic cells gated using CD11c expression (III). EYFP expression was not detected in macrophages and dendritic cells in reporter and control mice (IV). Splenocytes from mice known to express EYFP were used as a positive control.



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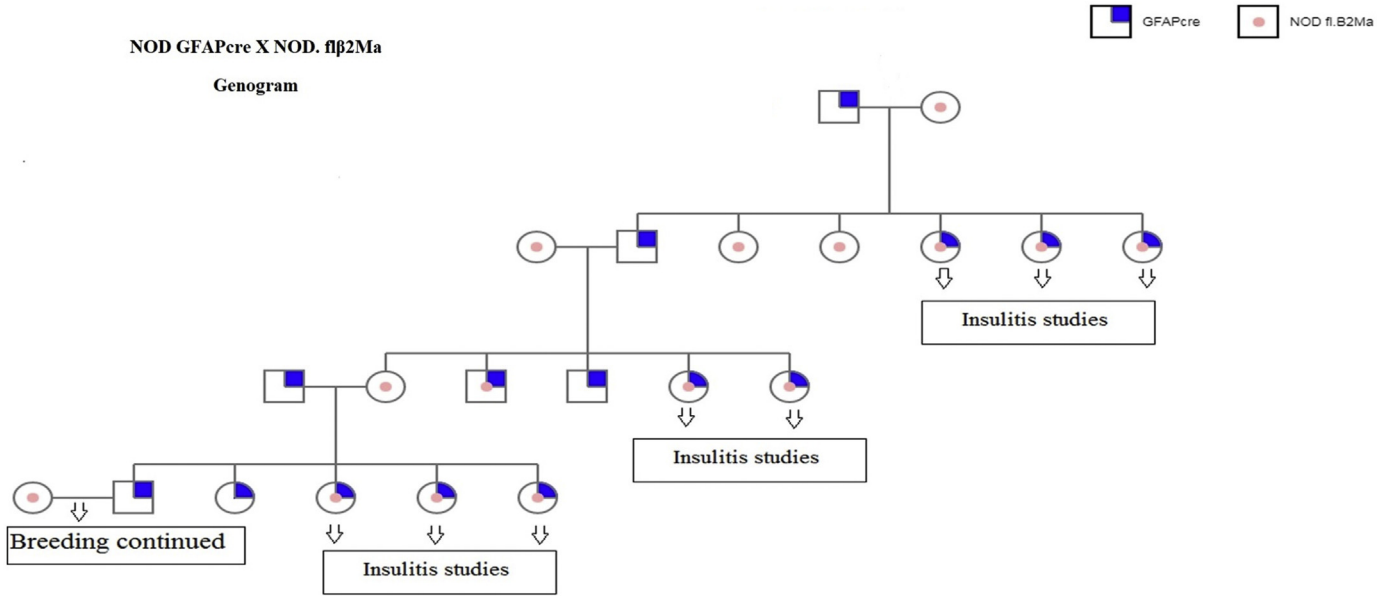

Fig. 4. Genogram shows the breeding strategy in generating female NOD GFAP bald mice. NOD GFAPcre mice on β2M knockout background were crossed with NOD.flβ2Ma mice and the female GFAP bald littermates at 100–160 days of age were chosen for insulitis studies.

4. Discussion

We aimed to understand the role of MHC class I on GFAP expressing cells in the initiation of disease in NOD mice. Cre-lox technology was used to generate tissue specific knockout mice. Cre was expressed under the control of the GFAP promoter and thus cre mediated loss of MHC class I occurred in GFAP expressing cells including ductal and pSC types. Transgenic expression of cre has occasionally been reported to occur in off-target tissues. We therefore wanted to confirm that cre was not leaking in the immune compartment because any cre mediated loss of MHC class I from immune cells would have confounded the interpretation of results (de Jersey et al., 2007). The absence of any leakiness of cre in the immune compartment confirmed that the model was appropriate to study the role of MHC class I on GFAP expressing cells, in particular the ductal and pSCs of the pancreas.

Utilizing this model we found that the expression of MHC class I on GFAP positive cells was important for the development of insulitis. Not only did the mice lacking MHC class I on GFAP expressing cells have more disease free islets, they were also significantly protected from developing invasive insulitis. This suggests that MHC class I expression on GFAP positive cells is important *early* in the disease process. Because proinsulin is widely held to be the earliest autoantigen, we wondered whether the reduction in insulitis in class I GFAP bald mice may have been due to the loss of MHC class I mediated presentation of insulin peptides expressed by ductal cells of the pancreas.

The decrease in the presence of islets with insulitis, even low level

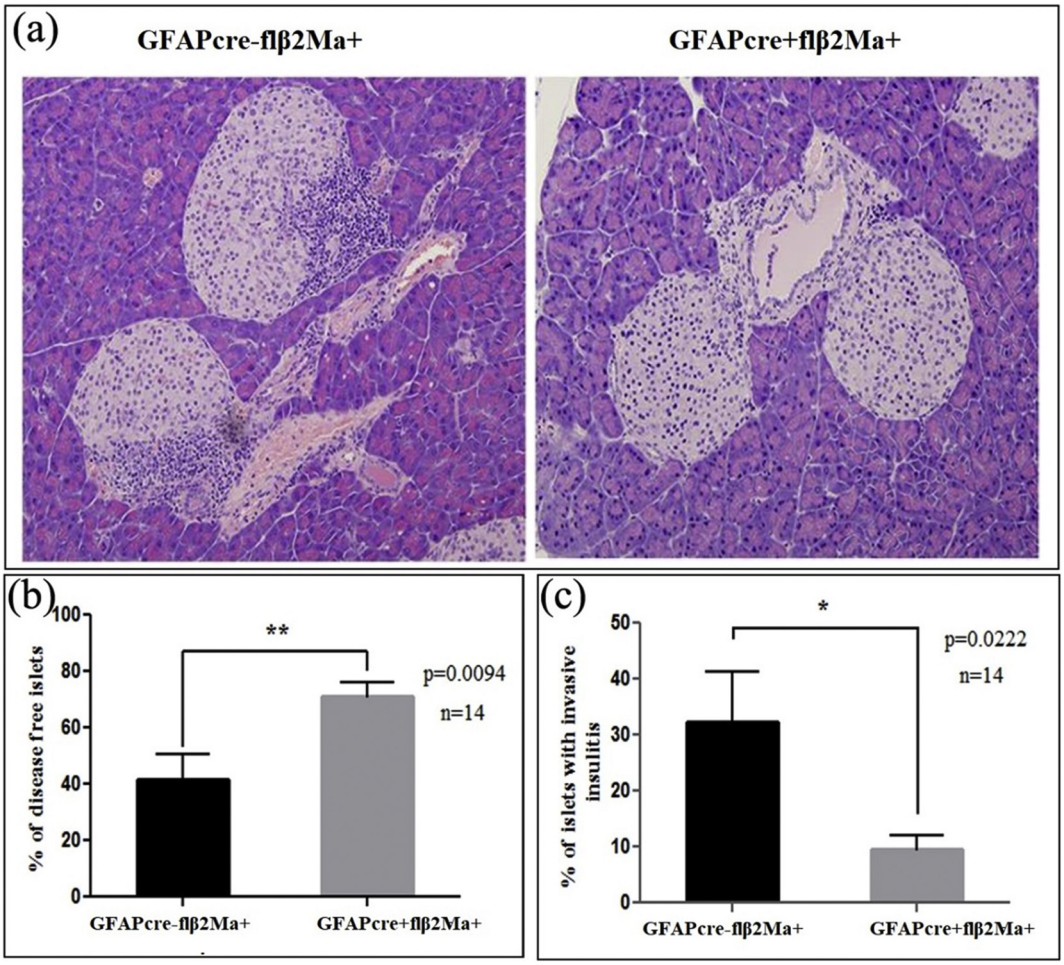

Fig. 5. GFAP bald mice had more disease free islets. The pancreatic sections from NOD GFAP bald mice at 100–160 days of age were analyzed by haematoxylin and eosin staining. Microphotography shows mononuclear infiltrated islets in control and disease free islets in GFAP bald mice (a). Statistical data showing the percentage of disease free islets (b) and islets with invasive insulitis in GFAP bald and control mice (c). 236 islets were scored for GFAPcre-flβ2Ma + group and 265 islets were scored for GFAPcre + flβ2Ma + group respectively.

periductal insulitis in class I GFAP bald mice suggests there has been a loss of immune response to ductal and/or pSC antigens. However, it is unlikely that the protection from insulitis observed in class I GFAP bald mice was due to the inability of ductal cells to present insulin peptides to CD8 T cells. This is because a proportion of ductal cells retained class I expression in GFAP bald mice and also expressed insulin. Thus a subset of ductal cells would have been able to present insulin peptides to CD8 T cells capable of initiating the disease. This begs the question as to which antigen/s GFAP positive ductal and/or pSCs express that may be important for the initiation of insulitis.

Ductal cells are beta cell progenitors and as such they share a number of autoantigens with beta cells, including proinsulin, GAD65, ICA69 and HIP/PAP (Inada et al., 2008; Bouwens and Pipeleers, 1998; Gurr et al., 2002; Mally et al., 1996a; Mally et al., 1996b). Thus, it is plausible that the initiation of an immune response to ductal cell antigens may lead to cross reactive attack of beta cells expressing shared antigen/s. Although not widely discussed, the argument that ductal cell antigens may have an important role in the initiation of periductal insulitis has credence. Peri ductal insulitis persisted in mice lacking native proinsulin indicating that the immune response to autoantigens expressed by ductal cells can occur independently of the immune response to proinsulin (Moriyama et al., 2003).

The chronological order of the immune response to autoantigens implicated in T1D is not completely understood. There has long been an assumption that proinsulin is the primary autoantigen, however, in addition to the findings presented here, it has also been shown that the immune response to antigens expressed by pSCs precede the immune response and attack of beta cells (Winer et al., 2003). Our data, and the findings of Michael Dosch et al. (Winer et al., 2003), suggests there may be several alternative autoantigens that could initiate an immune response which culminates in beta cell auto reactivity (Dilorenzo, 2011).

While it is not known what causes the inflammation around the ducts of the pancreas, there are a number of hypotheses which have been proposed. The pro inflammatory milieu of the islets could be driven by chemicals (Bodin et al., 2015), microbial infections (Kondrashova and Hyöty, 2014; de Beeck and Eizirik, 2016), food allergens (Virtanen, 2016; Rewers and Ludvigsson, 2016) or the defective clearance of apoptotic bodies by macrophages (O'Brien et al., 2002). Inflammation is associated with tissue damage that can result in the release of otherwise sequestered self-antigens. Our findings indicate that self-antigens expressed by GFAP positive cells are important in the development of insulitis in NOD mice. Further studies using the Class I GFAP bald NOD mice will help researchers to understand the importance of ductal cell antigens in insulitis progression and diabetes development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.01.001.

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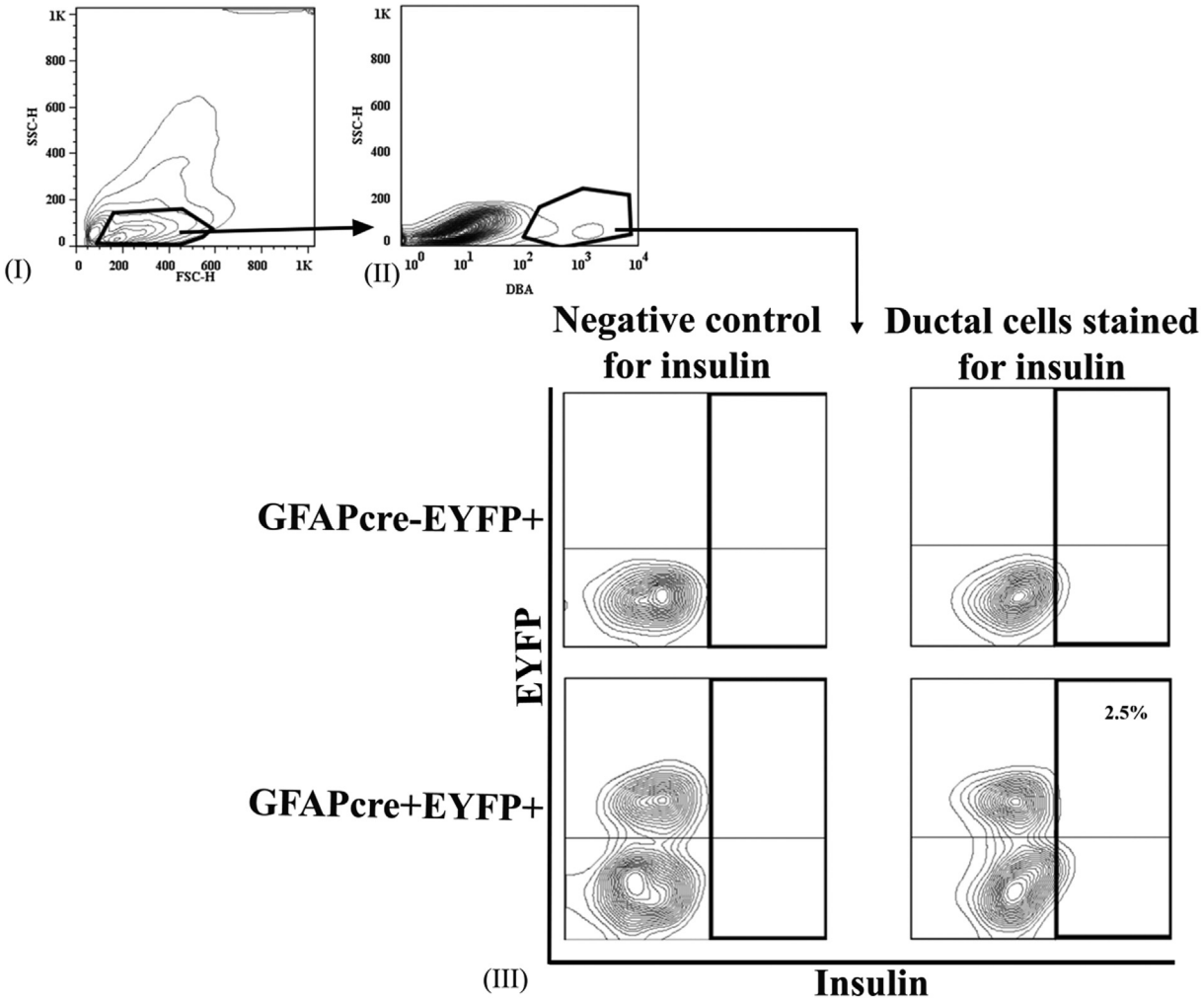

Fig. 6. Insulin positive pancreatic ductal cells do not express EYFP. Contour plots showing pancreatic single cells live gated for low side scatter (I). DBA stained pancreatic ductal cells (II) from reporter and control mice (n = 4). DBA stained ductal cells showing insulin and EYFP expression (III). Out of the 2.5% of insulin expressing ductal cells, > 90% of the cells did not express EYFP.

Competing interest

The authors declare they have no competing interest associated with this manuscript.

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Dietary SCFAs, IL-22, and GFAP: The Three Musketeers in the Gut–Neuro–Immune Network in Type 1 Diabetes

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Microbial metabolites have a profound effect on the development of type 1 diabetes (T1D). The cross-talk between the gut microbiota, the nervous system, and immune system is necessary to establish and maintain immune and gut tolerance. As quoted by Hippocrates, "All disease begins in the gut." Although this has been recognized for 2,000 years, the connection between the gut and autoimmune T1D is not yet well-understood. Here, we outline new advances supported by our research and others that have contributed to elucidate the impact of microbial metabolites on the physiology of the pancreas and the gut through their remarkable effect on the immune and nervous system. Among many of the mechanisms involved in the gut–beta-cell–immune cross-talk, glial fibrillary acidic protein (GFAP)-expressing cells are critical players in the development of invasive insulitis. Besides, this review reveals a novel mechanism for microbial metabolites by stimulating IL-22, an essential cytokine for gut homeostasis and beta-cell survival. The close connections between the gut and the pancreas are highlighted through our review as microbial metabolites recirculate through the whole body and intimately react with the nervous system, which controls essential disorders associated with diabetes. As such, we discuss the mechanisms of action of microbial metabolites or short-chain fatty acids (SCFAs), IL-22, and GFAP on beta-cells, gut epithelial cells, neurons, and glial cells via metabolite sensing receptors or through epigenetic effects. The fine-tuned gut–neuro–immune network may be profoundly affected by SCFA deficiency related to dysbiosis and diet alterations at very early stages of the initiation of the disease. Thus, dampening the initial immune response or preventing the perpetuation of the immune response by maintaining the integrity of the gut is among the alternative approaches to prevent T1D.

Keywords: SCFA (short chain fatty acids), GFAP—glial fibrillary acidic protein, gut microbiota, glial cell, interleukin 22 (IL-22), ILC3s, beta cells, diabetes

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease in which T cells destroy the insulin-producing beta-cells of the pancreas (1–3). The beta-cell's attack happens when T cells recognize autoantigens such as glutamic acid decarboxylase (GAD), islet cell autoantigen 69 (ICA69), insulinoma-associated antigen 2 (IA2), islet-specific glucose-6-phosphatase catalytic

subunit–related protein (IGRP), and proinsulin, which are widely accepted as the initiating autoantigens in T1D (4, 5). Antigenic targets for T cell priming are not solely expressed in beta-cells, but also in multiple tissues distal to islets, and they can be found in food like insulin or produced by bacteria like GAD (6–8). This all leads to many questions. How antigen expression in other distal tissues to beta-cells control the invasive infiltration of immune cells into the pancreas? Is the gut an important compartment as a source of antigens that trigger T1D? Is the gut microbiota influencing T cell priming against beta-cells? The microenvironment regulates beta-cell function and maturity, in particular close interaction with endocrine cells, neuronal, immune, and vascular cells (9, 10). Pancreatic ducts are physiologically neighboring to the beta-cells, and their primary function is to deliver enzymes or pancreatic juices provided from the exocrine pancreas into the duodenum to help digestion. As such, the pancreatic beta-cells can be influenced by the gut, which is intimately connected not only through the pancreatic ducts but also by lymph ducts (11). Beyond the pancreas, there is hardly any tissue in the body that has not been somehow in contact with gut microbial SCFAs. From food fermentation, bacteria in the large bowel produce many metabolites that are used by the epithelial cells in the gut. The remaining amount is transported to the liver where they are metabolized and then released to systemic circulation. As such, SCFAs have a broad spectrum of remarkable beneficial properties that affect many systems, in particular under inflammatory conditions, regulating metabolic, and immune responses (12–14).

One example is the nervous system, which is also critical for the pancreas to function (15). Both the endocrine and the exocrine part of the pancreas are innervated by the sympathetic and the parasympathetic nervous system, as such pancreatic sensory neurons have been shown to play a critical role in controlling islet inflammation (16). Similarly, the enteric nervous system (ENS) via the enteric glial cells (EGCs) is vital to maintain gut and immune homeostasis (17, 18), given that diabetic animals and patients presented gastrointestinal motility disorders (19). In this review, we will discuss the gut–neuro–immune axis in T1D and its effect on beta-cell priming. In particular, we will focus on the role of GFAP-positive cell types as critical players in T1D and on the impact of the gut microbiota, SCFAs, and their mechanisms of action through interleukin 22 (IL-22).

GFAP—NOT THE USUAL SUSPECT!

Beta-cells are involved in late T cell priming, suggesting that they are not required during the induction of T1D (20, 21). So, a critical consideration is—what might be driving the initiation of T1D? It has been shown in the pancreas that GFAP-expressing peri-islet Schwann cells (pSC or glial cells) can attract and recruit autoreactive cells, which precedes the attack on beta-cells. Two studies support the finding that immune responses to autoantigens expressed in pSCs precede the immune response to beta-cells (6, 7). In particular, they showed that early T cell attack on GFAP-expressing pSCs progressively results in the release of glial cell antigens, GFAP, and insulin (6, 7). GFAP epitopes for autoreactive T and B cells have now been identified in non-obese diabetic (NOD) mice and humans with T1D. Serum GFAP antibodies are now used as a predictive marker for the development of T1D, and it has been shown that administration of GFAP as a vaccine delayed the progression of T1D by regulating T cell differentiation (22, 23). GFAP-expressing glial cells of the peripheral nervous system require TRPV1 expression for their proper maturation, and studies have shown that depleting TRPV1-expressing cells reduced the development of insulitis in NOD mice (16, 24). It is fascinating that a cytoskeletal protein widely expressed in pancreatic ductal cells and also in pancreatic glial cells of the central and peripheral nervous system may work as an early autoantigen in T1D.

Exploring further this idea, Slattery's group has recently shown that ablation of autoantigen presentation in GFAP-expressing cell types reduced the development of invasive insulitis in NOD mice (25). We can speculate that reduction, but not total elimination of invasive infiltration, may be due to the absence of presentation of autoantigens other than insulin by GFAP-expressing cells, suggesting that Ag-derived ductal cell is one of the critical requirements in orchestrating the initiation of autoimmune responses to beta-cell antigens.

THE SCFAS: MODULATOR OF GUT INFLAMMATION AND AUTOREACTIVITY

After many years of efforts and studies focusing on the destruction of the beta-cells in the pancreas, there is still no cure or method of prevention for T1D. So, it makes us wonder whether we have been losing the battle only because we are not looking beyond the walls of the pancreas. T1D can be viewed as an orchestrated autoimmune response originated in the gut. This is evident from the observation that in many autoimmune diseases including T1D, the integrity of the epithelial barrier is compromised, leading to a phenomenon termed as "leaky gut" (26, 27). Pathogens, microbial products, and food-derived antigens find the leaky gut as a route to encounter the resident immune cells. For example, Gram-negative bacteria produce lipopolysaccharides (LPS), an identified endotoxin that can induce immune responses via the toll-like receptor 4 (TLR-4) expressed on monocytes (28). Given the gut connects to the pancreas through pancreatic lymph nodes (PLNs) and mesenteric lymph nodes (MLNs), bacterial and food products can hyperactivate resident T and B autoreactive cells in the gut or the gut-associated MLNs (29). Alternatively, it has been shown that gut microbial products can reach PLNs and locally modify the presentation of pancreatic self-antigens (30). Therefore, excess of food, chemicals, and microbial antigens can skew the intestinal immune system toward a perpetually pro-inflammatory state that may trigger T1D. Newly-diagnosed children with T1D present autoantibodies to GAD, a pancreatic beta-cell autoantigen that is also produced by many bacterial species (31). For instance, T1D patients present antibodies against a heat shock protein from the *Mycobacterium avium* subspecies *paratuberculosis*, MAP Hsp65, which has a high

degree of homology with human GAD65, suggesting that cross-reactivity between MAP Hsp65 and GAD65 potentially could be a mechanism of triggering TID (32). Strong homology has been found between the islet-antigen IGRP and several gut- and oral-derived microbial peptides. These peptide sequences encode for magnesium transporter (Mgt), for hypothetical protein IEM_00289 and NAD synthetase, respectively, which activate NY8.3 $CD8^+$ T cells with comparable potency to IGRP native peptide (33). Thus, molecular mimicry between excess of gut microbial antigens and islet cell autoantigens may be a mechanism by which gut dysbiosis leads to T1D development.

T1D is a multifactorial condition; diet and environment play an inevitable role in disease modulation (1, 13). Human and murine studies have demonstrated that defects in the induction of central and peripheral tolerance checkpoints (34) also correlate with an altered gut microbiota (35–39), which are notable contributors to T1D pathology. Building on previously extensive reviews on the gut microbiota topic, we have firmly discussed that an altered microbiota and SCFA deficiency are primary causal factors triggering T1D (12–14, 40). The gut microbiota through the production of dietary SCFAs plays a significant role in host defense by modulating the immune system and metabolism. Studies conducted by our group have shown that the combination of a diet rich in acetate and butyrate protected 90% of the NOD mice against T1D, yielding exceptionally high levels of the corresponding SCFAs to the feces (35). In this study, SCFA-induced T1D protection happened via changes in gut/immune regulation-expanding regulatory T (Treg) cells and reducing pathogenic B cells, $CD4^+$, and $CD8^+$ T cells. Diet rich in SCFA acetate and butyrate not only reduced the levels of serum LPS and pro-inflammatory interleukin 21 (IL-21) but also increased the level of serum IL-22, an important cytokine that maintains a healthy commensal microbiota, gut epithelial integrity, and mucosal immunity and ameliorates metabolic disease (41–44). Alternatively, SCFAs can also reduce islet-specific immune responses by increasing the production of antimicrobial peptides (AMPs) in the beta-cells (45). As it has been shown, C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) and defensins disrupt surface membranes of bacteria, thus enabling a broad regulation of commensal and pathogenic bacteria in the gut (46–48). Diana's group showed that insulin-secreting beta-cells produced the cathelicidin-related antimicrobial peptide (CRAMP), which was defective in NOD mice. Intraperitoneal administration of SCFA butyrate stimulates the production of CRAMP on pancreatic beta-cells via G protein-coupled receptors (GPCRs), which also correlated with the conversion of inflammatory immune cells to a regulatory phenotype (45). Likewise, another study has shown that microbial SCFAs contribute to the increasing concentrations of serum IL-22 (35) required for beta-cell regeneration by up-regulating the expression of Regenerating Reg1 and Reg2 genes in the islets (49).

There are pieces of evidence of compromised gut integrity, dysbiosis, and associated inflammation of the gastrointestinal tract (GI) in NOD mice and patients with T1D (50–55), similar to what has been shown in other inflammatory or autoimmune gut diseases (i.e., infection, celiac disease, IBD). The gut microbiota and the ENS play a critical role in diabetic gastrointestinal motility disorders, as individuals with diabetes suffer from symptoms such as nausea, heartburn, vomiting, diarrhea, abdominal pain, and constipation (56, 57). For example, it is known that slow motility of the GI leads to alterations of the gut microbiota that favors pathogenic bacterial overgrowth and subsequently diarrhea (58, 59). As such, the abundance and diversity of bacteria needed to maintain the integrity of the gut were significantly lower in children with T1D compared to healthy controls (60). On the other hand, animal studies have suggested that accelerated colonic transit time, relative to constipation, could be caused by autonomic neuropathy and diabetes-induced denervation of sympathetic nerve terminals (56, 61). Diet and/or deficiency of dietary SCFAs can also modulate the intestinal motility and survival of enteric neurons by miRNAs, which are involved in energy homeostasis, lipid metabolism, and proliferation and development of GI smooth muscles. miRNAs have been vastly studied in organ damage caused by diabetes, and one study has shown in mice that high-fat diets delay the GI transit, partly by inducing apoptosis in enteric neuronal cells, and this effect was shown to be mediated by Mir375 associated with reduced levels of Pdk (62). There is still too much to understand about the intrinsic mechanisms underlying the connection between the gut microbiota and the ENS and how this affects the course of T1D. Particularly high-fiber or specialized acylated starch diets that boost the microbial production of SCFAs are effective in the control of gut infections and diarrhea, as it has been shown to promote commensal acetate-producing bacteria (63).

IL-22 AND ENS TAKE CONTROL OF T1D

Activation of IL-22 through microbial SCFAs contribute to the maintenance of gut homeostasis by the close connection between the intestinal-resident innate lymphoid cell 3 (ILC3) and EGCs. IL-22 is expressed by ILC3, which lies close to EGCs (64), but its role in T1D is still elusive (14). ILCs sustain appropriate immune responses to commensals and pathogens at mucosal barriers by potentiating adaptive immunity and regulating tissue inflammation (65, 66). Likewise, EGCs have critical roles in maintaining gut homeostasis, as they can sense the pathogenic bacteria through toll-like receptors (TLRs). EGCs surround neurons and also connect with blood vessels and lymphatics (67), which allowed EGC-derived signaling molecules to modulate mucosal immunity. As such, EGCs sense environmental stimuli and extend their stellate projections into the ILC3 aggregates within the crypto-patches of the intestinal lamina propria and release neurotrophic factors that stimulate IL-22 secretion from ILC3s (68). The notion that gut microbiota affects the development and maturation of EGCs was shown in germ-free (GF) mice, which present a defective influx of EGCs into the intestinal mucosa (69). This occurs via expression of the neuroregulatory receptor (RET), as ablation of RET in ILC3 leads to reduced IL-22 production and compromised epithelial protection in colon inflammation mouse models (69).

Aligned with this idea, does the early autoreactivity to GFAP observed during insulitis originate in the gut? This is possible to the connections between the pancreas, the ENS, and the gut. The fine-tuned neuro–beta-cell cross-talk is more likely to be broken by the pathological changes occurring in a perturbed gut. Alterations of the gut microbiota, referred to as dysbiosis, decrease epithelial permeability, causing inflammation, and associated tissue damage that exposes numerous self-antigens harbored in the gut and associated enteric neuronal tissues. Gut microbial products can also sense enteric neurons and EGCs partly by pattern recognition receptors, such as TLRs. Indeed, pathogenic and commensal SCFA-producing bacteria up-regulate differentially toll-like receptor 2 (TLR2) expression on human EGCs (70). Expression of TLR2 on enteric neurons and EGCs controls $nNOS^+$ neurons and acetylcholine-esterase-stained fibers in the myenteric ganglia. For example, *Escherichia coli* promoted expression of MHC II molecules on EGCs and significantly induced S100B protein overexpression and nitric oxide (NO) release from EGC, which was counteracted by pre-treatment with TLR and S100B inhibitors (70). As such, the myenteric plexus of TLR2Ko mice presented reduced expression of glial markers, GFAP, and S100B. Overexpression of GFAP has been observed to correlate with inflammatory responses in the gut (71). S100B is considered as a neurotrophin, due to its either tropic or toxic effects depending on the concentration in the extracellular milieu. Excess amount of S100B acts on RAGE (receptor for advanced glycation end-products), leading to the phosphorylation of mitogen-activated protein kinases (MAPK) and subsequent activation of the nuclear factor κB (NF-κB) and the associated release of NO. Excess NO causes damage to the tissue, resulting in inflammation and reduced integrity of the guts (72, 73). The protective role of EGCs in the maintenance of the gut epithelial integrity has been demonstrated in mice lacking GFAP-positive (+) glia that presented fatal hemorrhagic jejuno-ileitis (74).

During chronic tissue inflammation, significantly increased expression of GFAP on glial cells after stimulation with LPS and pro-inflammatory cytokines has been shown (75), similar to what has been seen in Crohn's disease (CD) and necrotizing enterocolitis (NEC). On the other hand, the presence of MHC class II expression on activated EGCs suggests that these cell types can present antigens (76, 77) derived from multiples sources, including microbes and host. EGCs, with the help of their stellate projections, sample microbial antigens crossing the epithelial barrier and activate diabetogenic T cells. This is given under dysbiosis, predominant in T1D and many autoimmune diseases, and the release of microbial antigens such as LPS may break the tolerance of EGCs leading to overexpression of glial cell markers GFAP and S100B. Thus, GFAP-expressing glial cells may have a protective role in maintaining the integrity of the gut, but under uncontrolled inflammatory conditions, it may lead to autoreactivity. As such, glial cell-derived protein GFAP is now an identified autoantigen in T1D and autoantibodies to GFAP has been detected in NOD mice and humans with T1D (23), thus showing the relevance of the microbiota–EGC pathways in T1D.

One study has shown that SCFA butyrate can induce increasing excitatory choline acetyltransferase (ChAT) neurons through the butyrate transporter monocarboxylate transporter (MCT), which is expressed by enteric neurons (78). However, it is still unknown what factors control neuronal MCT2 expression. Further studies will be necessary to determine how SCFAs regulate MCT2 expression and control the activity of intestinal neural circuits. SCFAs exert their function through two mechanisms, via metabolite sensing GPCRs or inhibition of histone deacetylase (HDAC) activity (13, 35, 79, 80). There are three receptors for SCFA acetate, butyrate, and propionate, namely GPR43 (FFA2), GPR41 (FFA3), and GPR109a. GPR43 is activated by SCFAs with varying potency—acetate > propionate > butyrate. GPR43 is expressed on gut epithelial cells and certain immune cells (81). GPR109a is expressed on a variety of immune cells, as well as adipocytes, hepatocytes, gut and retinal epithelium, vascular endothelium, and neuronal tissue (82). GPR109a is primarily activated by both niacin and butyrate ligands. While niacin levels are not high enough to activate the receptor under normal physiological conditions, levels of butyrate, obtained from the gut environment, and its oxidized form, β-hydroxybutyrate, are sufficient to stimulate a response (82). Similarly, GPR41 has been reported to be expressed on EGCs and enteric neurons (83, 84). GPR41 also binds the three major SCFAs, but with differing affinities (85).

Similar to the effects exerted through the GPCRs, SCFAs can influence the function and development of immune cells directly through epigenetic regulation of gene expression such as inhibition of HDACs (13, 86). HDACs allow the conversion of repressive chromatin structures, which takes place on lysine residues on N-terminal tails of histones 3 and 4, to increase gene transcription. HDACs are a group of 18 known enzymes that remove acetyl groups from the histones tails that bind DNA (87). Although little is known about the effects of SCFAs on EGCs through epigenetic modifications, it has been shown that butyrate treatment increases acetylation of the H3K9 in primary enteric neurons and the EGC *in vitro* (84).

SCFAs can also modulate gut motility by the production of serotonin by epithelial enterochromaffin cells (ECs) (88, 89). For instance, GF mice present gut dysmotility that was reversed by inoculation with SCFA-producing bacteria. Tested in human-derived EC cell lines, SCFAs increased serotonin (5-hydroxytryptamine [5-HT]) by up-regulating THER expression of tryptophan hydroxylase 1 (Tph1) (89) and by the serotonin-selective reuptake transporter (SERT), which is expressed by intestinal epithelial cells (90). Another critical role of SCFAs on the ENS is evidenced by the conversion of primary bile acids synthesized *de novo* into secondary bile acids in the liver (91). Aside from their role in dietary fat absorption, secondary bile acids can activate several GPCRs and nuclear hormone receptors, including the G-protein-coupled bile acid receptor 1 (TGR5) and farnesoid X receptor (FXR), highly expressed in enteric neurons and enteroendocrine L cells that improved intestinal inflammation and glucose tolerance in HFD-fed mice (92). TGR5 also affect peristalsis that is mediated partly by serotonin 5-HT (93), implicating its potential for the treatment of constipation and diarrhea. Altogether, this suggests the relevance of the gut–neuro–immune axis in T1D (**Figure 1**).

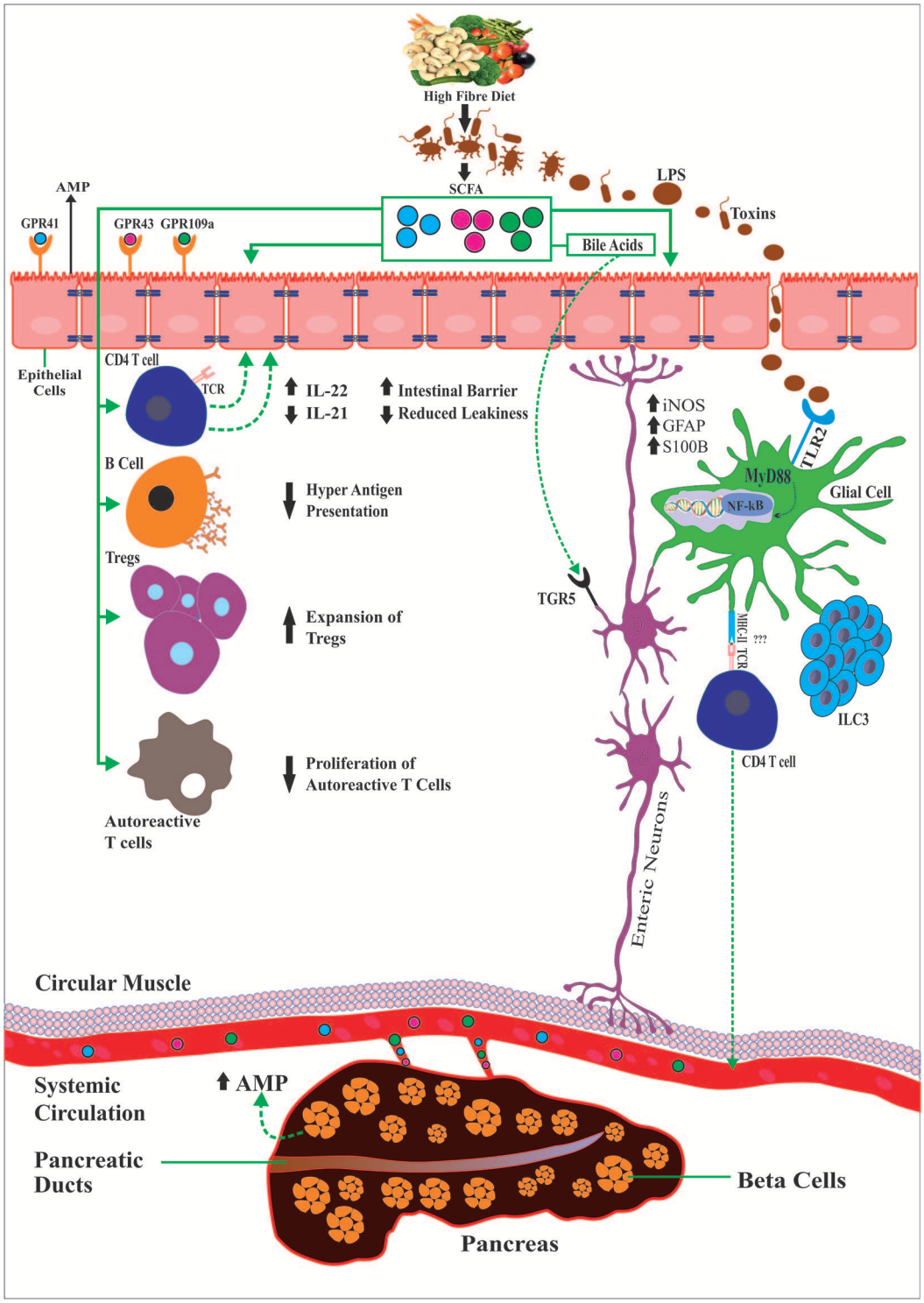

FIGURE 1 | Diet and gut microbiota through the production of dietary SCFAs exert anti-inflammatory effects by controlling the activity of multiple immune cell types, outside or locally in the intestinal mucosa, the enteric glial cells and neurons but also glial cells in the pancreatic islets and the beta-cells. As such SCFAs promote IL-22 production $CD4^+$ T cells or by supporting ILC3 cells, the major producers of IL-22. Also SCFAs can reduce production of pro-inflammatory cytokines IL-21, LPS, induce beta cell regeneration via AMPS, regulate GFAP in the gut and beta-cells, modulate the expansion of regulatory T and reduction of autoreactive CD8 T cells and reducing B cell hyperactive antigen presentation capacity. Activation of GPRCs (GPR41 and GPR43) on enteroendocrine cells of the intestinal epithelium and TLR signaling (e.g., TLR2 and TLR4) maintains subsets of enteric neurons resulting changes in gut motility, conversion of primary bile acids into secondary bile acids, which activate TGRS expressed by enteroendocrine cells and enteric neurons among many others.

CONCLUDING REMARKS

Among the described effects that SCFAs have on modulating the immune system, beta-cell biology, and gut homeostasis, we have uncovered a novel role for SCFAs by modulating the ENS in the gut, central for the control and prevention of T1D. Overall, an immune response to antigens presented not only by GFAP-expressing pSCs in the pancreas but also by GFAP-expressing EGCs in the gut is a novel finding involved in the initiation of the autoimmune process. Could it trigger antigen-experienced autoreactive cells to move up the gut and reach the ductal and beta-cells, and break the GFAP-expressing neuronal mantle of the islets? This is an unexplored field and requires further research. Given the close location and connection between the gut and the pancreas and their intrinsic dependence from the nervous system, this fine-tuned immuno–neuro-islet cross-talk may be profoundly affected by perturbed gut homeostasis at very early stages of the initiation of the T1D. Dampening the initial immune response or preventing the perpetuation of the islet-specific immune response by maintaining the integrity of the gut is among the possible therapeutic approaches to reprogram T1D (12, 14). Thus, any hope for a cure may lie in methods that can halt immune-mediated beta-cell damage by maintaining or improving gut–immune tolerance.

AUTHOR CONTRIBUTIONS

EM developed the conceptual idea, wrote and edited the manuscript. AJ wrote and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

A simple method to improve the visualization of EYFP in paraformaldehyde fixed frozen tissue sections

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Abstract:

Background

Fluorescent proteins are widely used in a variety of applications to study the organization and function of living systems. Enhanced Yellow Fluorescent Protein (EYFP) is among the most popular versions of fluorescent proteins used in lineage tracing and protein expression studies in genetically engineered mice. The expression of EYFP in live cells can be visualized using flow cytometric and fluorescence microscopy techniques. However, imaging the natural fluorescence of EYFP in frozen tissue sections has been challenging.

Methods

Pancreatic and brain sections dissected from Non Obese Diabetic (NOD) mice that express EYFP either in the glial cells of the neurons or in the ductal cells of the pancreas were used to image EYFP expression.

Results

We were able to identify EYFP expression in sections cut from the fixed frozen tissue, but the natural fluorescence of EYFP was undetected in unfixed frozen tissue sections imaged immediately after dissection. We also found that fixing the frozen sections post sectioning were suboptimal for imaging the natural fluorescence of EYFP.

Conclusion

We demonstrate that tissue fixation using crosslinking fixative, prior to sectioning, is a mandatory requirement for the better visualization of EYFP in brain and pancreatic sections of EYFP transgenic mice.

Keywords: EYFP, cre, tissue sections, fluorescence microscopy, anti-GFP.

Abbreviations

BSA	Bovine Serum Albumin
CAII	Carbonic Anhydrase II
DAPI	4, 6-diamidino-2-phenylindole
DBA	Dolichos Biflorus Agglutinin
EYFP	Enhanced Yellow Fluorescent Protein
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
NOD	Non Obese Diabetic
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde

1. Introduction:

The green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* was one of the first fluorescent proteins used for gene expression and protein localization studies [1-4]. By using protein engineering approaches, Roger Tsien and co-workers mutated wildtype GFP and generated a longer wavelength emitting variant protein, having an emission in the yellow region of the visible spectrum. This variant protein, the Enhanced Yellow Fluorescent Protein (EYFP) is a modified GFP with a targeted substitution of the threonine (T) residue for tyrosine (Y) at position 203 (T203Y) and is now a widely used fluorescent protein [5].

The expression of EYFP in live cells can be easily visualized using a flow cytometer equipped with 488 nm laser [6]. However, imaging the highly soluble EYFP in frozen tissue sections is problematic due to poor photostability, loss of cell membrane integrity during sectioning and subsequent leaching of the fluorochrome [6-9]. Though the targeted substitution of T203Y has changed the spectral profile, the commercial polyclonal anti-GFP antibody is able to bind EYFP, as its structural conformation was not much altered in the enhanced version [5]. Here we describe an efficient fixing method that causes retention of the EYFP protein within the cellular architecture, thus allowing better visualization of intracellular EYFP expression in frozen brain and pancreatic tissue sections of EYFP transgenic mice.

2. Materials and Methods

2.1 Mice

Mice were maintained under specific pathogen free (SPF) conditions at the Precinct, Animal Centre at the Alfred Hospital (PAC, Melbourne, Australia). All procedures involving mice were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP). NOD EYFP mice, that express EYFP conditionally [10], were crossed with either GFAPcre mice (cre expressed under the Glial Fibrillary Acidic Protein (GFAP) promoter) [11] or with CAIIcre mice (cre expressed under the Carbonic Anhydrase II (CAII) promoter) [12]. In double transgenic offspring cre mediated recombination resulted in the expression of EYFP in GFAP expressing neuronal cells of GFAPcre^{+}EYFP^{+} mice and in CAII expressing ductal cells of the pancreas of CAIIcre^{+}EYFP^{+} mice respectively (Fig-1).

2.2 Tissue optimization

2.2.1 Frozen Tissue (FT)

Freshly dissected brain tissue from GFAPcre^{+}EYFP^{+} mice and pancreas tissue from CAIIcre^{+}EYFP^{+} mice was harvested and embedded in Tissue Tek O.C.T compound (ProSciTech, Australia) by snap freezing in -20°C chilled isopentane. In parallel, control tissue, from GFAPcre^{-}EYFP^{+} and CAIIcre^{-}EYFP^{+} mice were embedded in OCT. Later the same day, cryostat sections of 5µm were cut from the embedded tissue, mounted on superfrost plus slides (Thermo Fisher scientific, Massachusetts, USA) and observed for fluorescence using the Olympus BX61 microscope.

2.2.2 Fixed Frozen Tissue (Fi-FT)

Brain tissue from GFAPcre^{+}EYFP^{+} and control GFAPcre^{-}EYFP^{+} mice, and pancreas from CAIIcre^{+}EYFP^{+} and control CAIIcre^{-}EYFP^{+} mice, were fixed overnight at 4^{o}C in fresh 4% paraformaldehyde (PFA). The following day the fixed tissues were cryoprotected by immersion in a beaker of 20% sucrose/Phosphate Buffered Saline (PBS) solution maintained at 4^{o}C. After approximately 8 hours, when the tissue had sunk to the bottom of the beaker, the cryoprotected tissue was embedded in OCT by snap freezing in -20°C chilled isopentane. Cryostat sections were cut at 5µm, mounted on slides and fluorescence visualized using an Olympus BX61 microscope.

2.2.3 Staining of Fixed Frozen Tissue

Fixed frozen brain tissue from GFAPcre^{+}EYFP^{+} and control GFAPcre^{-}EYFP^{+} mice, and pancreas tissue from CAIIcre^{+}EYFP^{+} and control CAIIcre^{-}EYFP^{+} mice, was cut at 5µm and mounted on slides and incubated in a blocking solution containing 3% Bovine Serum Albumin (BSA) and 5% milk powder in PBS at room temperature for 20 minutes. The brain sections from

GFAPcre^{+}EYFP^{+} and control GFAPcre^{-}EYFP^{+} mice were then stained with Alexa Fluor 555 conjugated rabbit anti-GFP polyclonal antibody that cross-reacts with YFP (Invitrogen, Carlsbad, CA, USA), henceforth called AF-anti-YFP. The sections were counter stained with 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) before mounting with fluoromount (Sigma). Pancreatic sections from CAIIcre^{+}EYFP^{+} and control mice were stained with polyclonal guinea pig antibodies against insulin (Dako, Carpinteria, CA, USA), AF-anti-YFP and biotinylated Dolichos Biflorus Agglutinin (DBA) lectin (Vector labs, Peterborough, UK) to label ductal cells [13]. Following a wash in PBS, anti-insulin antibodies were detected using Texas red conjugated rabbit antibody against guinea pig (Abcam, Cambridge, MA, USA) and DBA labelled ductal cells by streptavidin conjugated to Dylight 405 (Abcam). The stained slides were observed for fluorescence using the Olympus BX61 microscope.

3. **Results**

3.1 Frozen Tissue sections failed to preserve EYFP

EYFP is a highly soluble protein and fluorescence is dependent on its structural conformation. Our initial efforts to preserve and visualize the natural fluorescence of EYFP in sectioned tissue were unsuccessful, despite successful imaging of EYFP in single cells using flow cytometric techniques (Fig-2). We were unable to detect fluorescence in any of the frozen brain sections from GFAPcre^{+}EYFP^{+} or pancreatic sections from CAIIcre^{+}EYFP^{+} mice even when the AF-anti-YFP antibody was used to enhance the signal. We concluded that because EYFP is highly soluble it may have diffused out of the cells in tissue sections. We therefore sought to develop a fixation method to resolve this problem.

3.2 Fixing post-sectioning did not preserve EYFP in frozen tissue sections

To retain the EYFP protein in frozen tissue sections, a fixation method was required to anchor the protein to the cellular architecture. The first choice of fixative was ice cold acetone however, EYFP was not detected in tissue sections fixed with ice cold acetone and stained with AF-anti-YFP antibody, because during the process of fixing, organic solvents like acetone precipitate the EYFP protein by replacing water, resulting in a change in the conformational structure of EYFP and thereby losing the of natural fluorescence [14]. The second choice of fixative was freshly made 4% PFA solution. Since aldehyde fixatives act by crosslinking the protein by forming methylene bridges, the structural conformation of the EYFP is not affected during the process of fixation [15]. Despite using 4% PFA as fixative and staining with AF-anti-YFP, we were unable to detect EYFP in either brain or pancreatic tissue sections. The absence of EYFP in tissue sections fixed with PFA suggested that the protein needs to be anchored to the cellular architecture even before the tissue is subjected to sectioning.

3.3 EYFP is retained in Fi-FT sections

In order to retain EYFP inside the cell, the tissue should be fixed prior to sectioning. We used the Fi-FT approach where we pre-fixed the whole tissue in 4% PFA, followed by sucrose impregnation as described in methods section 2.2.2. Fi-FT sections of brain and pancreas retained the EYFP protein within the cell and we were able to detect the natural fluorescence of EYFP on cut sections albeit at very low intensity.

3.4 Imaging EYFP florescence in Fi-FT sections using anti-YFP antibody

To enhance the signal and to image the cells that express the EYFP protein we stained the Fi-FT sections with AF-anti-YFP antibody. Stained brain sections from GFAPcre^{+}EYFP^{+} identified EYFP expressing neuronal cells (Fig-3) and

stained pancreatic sections from CAIIcre^{+}EYFP^{+} mice co-stained with DBA identified EYFP expressing ductal cells (Fig-4).

4. Discussion

Whilst visualizing the natural fluorescence of EYFP in live cells was successful using confocal microscopy and flow cytometry, imaging the natural fluorescence of EYFP in tissue sections was problematic [8]. We tried to image the natural fluorescence of EYFP in frozen tissues isolated from transgenic mice known to express the protein. To visualize the natural fluorescence of EYFP, we used frozen pancreatic tissue sections from CAIIcre^{+}EYFP^{+} mice and brain sections from GFAPcre^{+}EYFP^{+} mice.

We were unable to detect the natural fluorescence of EYFP in frozen sections cut from either tissue type. The absence of natural fluorescence in frozen tissue sections prompted us to opt for a fixation method that would preserve the EYFP within the tissue sections. The principle behind using any fixative is to anchor the protein to the cellular architecture [16]. We used ice cold acetone to fix the EYFP, so that it could be observed using fluorescence microscopy. Despite using acetone as the fixative, we were unable to visualize the natural fluorescence of EYFP. We suspected that the absence of natural fluorescence in acetone fixed sections is due to a conformational change in EYFP. Organic solvents like acetone dehydrate proteins which can cause a change in the conformational structure [14]. The natural fluorescence of EYFP is dependent on its conformation and therefore, acetone fixation likely compromised the conformation resulting in the loss of fluorescence [17, 18]. Although still widely used acetone is not an ideal fixative for use when studying florescent proteins.

Though monoclonal antibodies are exquisitely dependent on the confirmation of the target epitope, polyclonal antibodies are not [19]. In order to detect the

acetone dehydrated EYFP, we therefore used polyclonal AF-anti-YFP. However, we were unable to detect any EYFP signal in the sections probed with AF-anti-YFP antibody. Since we still were unable to detect EYFP using polyclonal AF-anti-YFP, we changed the fixative to one that is known not to cause any conformational change in proteins [20]. Tissue sections were thus fixed with 4% PFA and probed with AF-anti-YFP antibody. However EYFP remained undetectable with this method. We concluded that highly soluble nature of EYFP allowed it to easily leach out of membrane compromised cells from tissue sections [7].

In order to prevent leaching of EYFP from cryostat sections of cells with compromised membranes, we fixed the whole tissue in 4% PFA then cryoprotected the tissue in sucrose prior to embedding in OCT. The pre-fixing and sucrose embedding of tissue prior to sectioning resulted in the preservation of the natural conformation of EYFP and the cellular morphology respectively. Polyclonal AF-anti-YFP antibodies were then successfully used to amplify the EYFP signal for imaging. In summary, our results demonstrate that fixing the tissue using cross linking fixatives prior to sectioning is a mandatory requirement to retain the soluble EYFP protein within the cellular architecture.

Conclusion

We demonstrate that tissue fixation using crosslinking fixative, prior to sectioning, is a mandatory requirement for the better visualization of EYFP in brain and pancreatic sections of EYFP transgenic mice.

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Competing Interests

The authors declare they have no competing interests.

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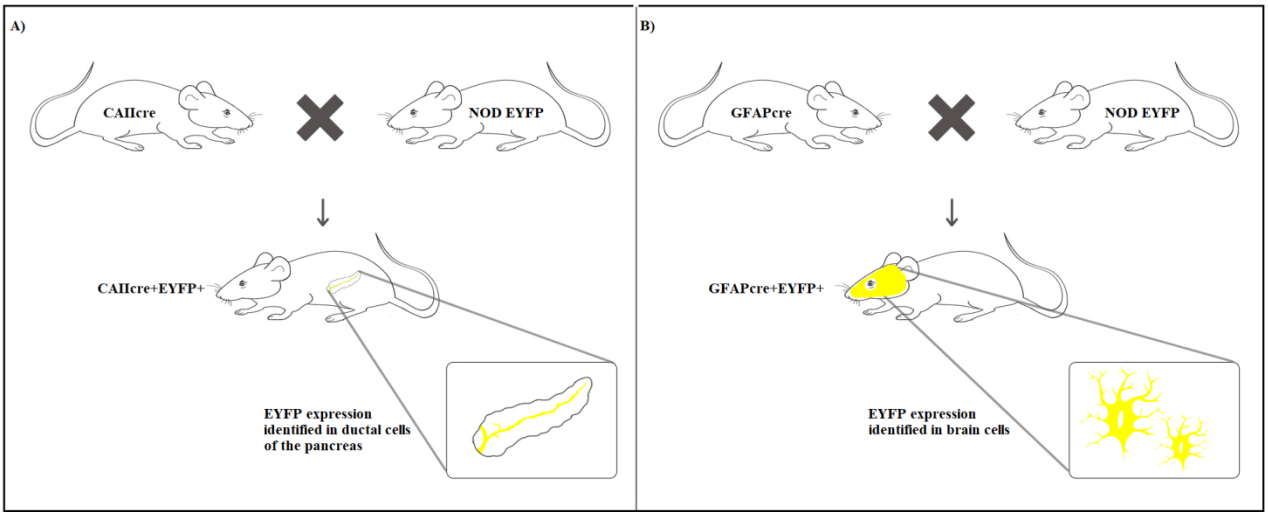
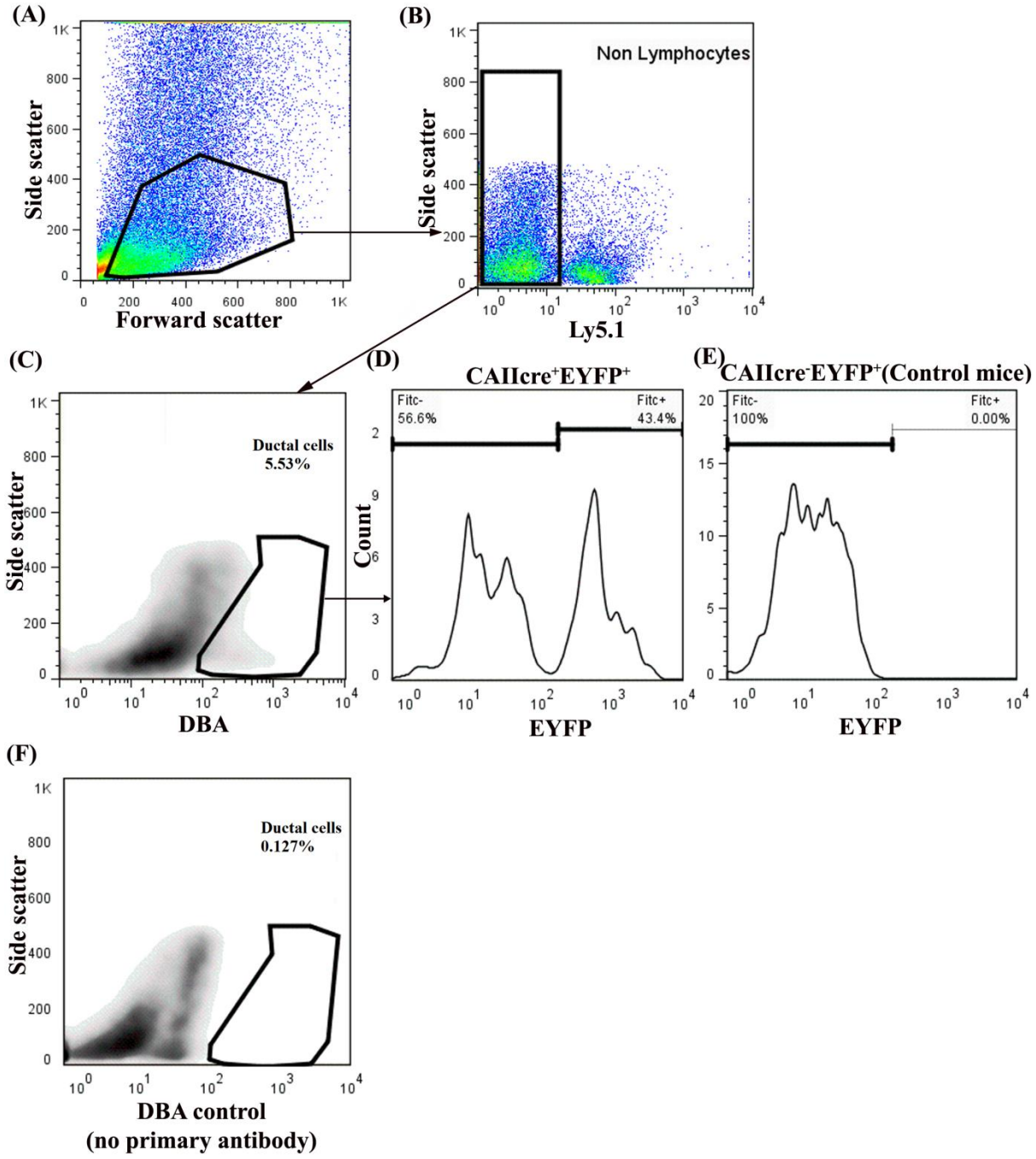
Figures and legends

Fig 1



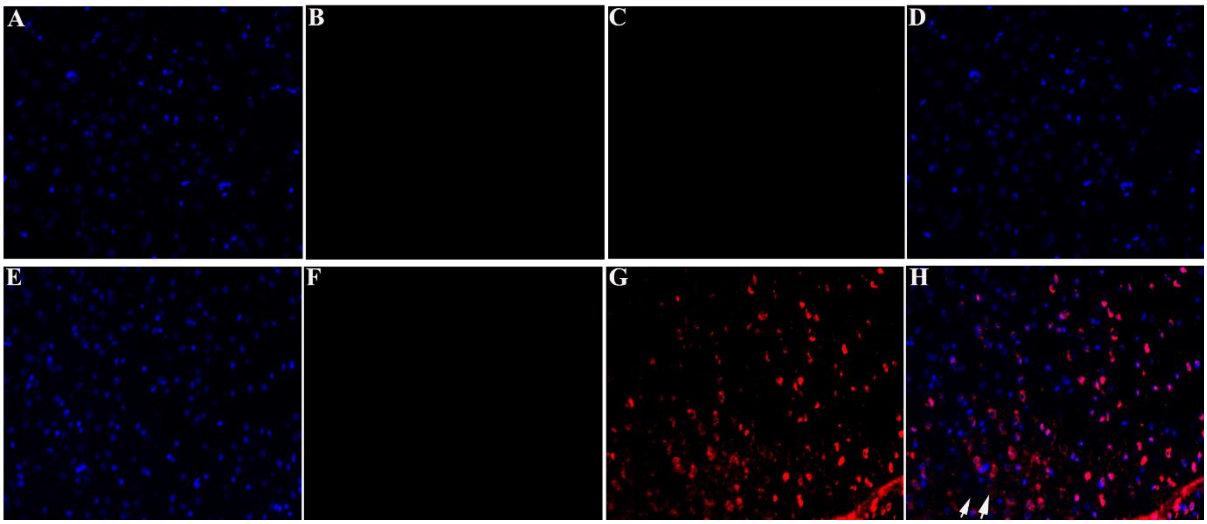
Generation of EYFP transgenic mice. A) CAIIcre mice were crossed to NOD EYFP mice to generate double transgenic CAIIcre^{+}EYFP^{+} mice. EYFP expression is identified in pancreatic ductal cells. B) GFAPcre mice were crossed to NOD EYFP mice to generate double transgenic GFAPcre^{+}EYFP^{+} mice. EYFP expression is identified in brain cells.

Fig 2



Pancreatic single cell suspensions were gated based on the low side scatter properties of ductal cells and selected on non-lymphocyes cells using the pan leucocyte marker Ly5.1 (B). DBA positive ductal cells were selected (C) and EYFP expression was observed in the FITC channel (D).Control mice was negative for EYFP expression (E). Control for DBA (F).

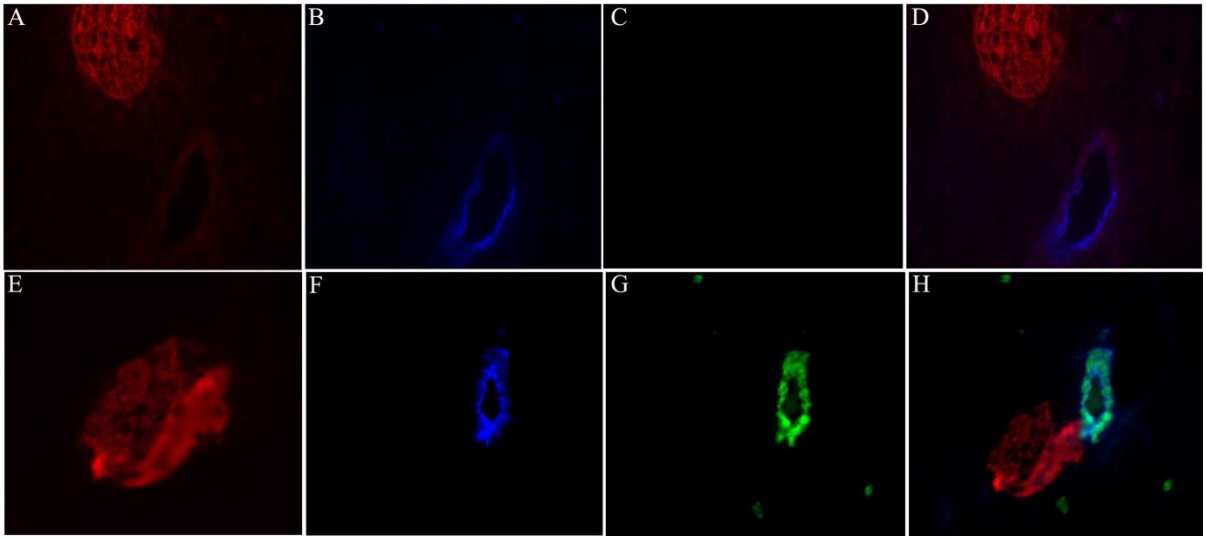
Fig 3



Top Panel: Fi-FT brain sections of GFAPcre^{-}EYFP^{+}mice. (A) Brain sections stained with DAPI (blue). (B) Natural EYFP fluorescence not detected in the EYFP channel. (C) Alexa flour 555 conjugated anti-GFP antibody could not detect EYFP expressing cells in cre negative control mice. (D) All three channels merged.

Bottom Panel: Fi-FT brain sections of GFAPcre^{+}EYFP^{+}mice. (E) Brain sections stained with DAPI (blue). (F) Natural EYFP fluorescence not detected in the EYFP channel. (G) EYFP expressing cells detected using Alexa fluor 555 conjugated anti-GFP antibody (Red). (H) All the three channels merged and DAPI stained cells expressing EYFP is seen in purple.

Fig 4



Top Panel: Fi-FT pancreatic sections of CAIIcre^{-}EYFP^{+}mice. (A) Beta cell stained using guinea pig antibody against insulin and detected by Texas red conjugated rabbit antibody against guinea pig IgG (red). (B) Ductal cells stained using biotin conjugated DBA and detected using streptavidin conjugated Dylight 405 (blue). (C) Alexa fluor 555 conjugated anti-GFP antibody could not detect EYFP expressing cells in cre negative control mice. (D) All three channels merged.

Bottom panel: Fi-FT Pancreatic sections of CAIIcre^{+}EYFP^{+} mice. (E) Beta cell stained using guinea pig antibody against insulin and detected by Texas red conjugated rabbit antibody against guinea pig IgG (red). (F) Ductal cells stained using biotin conjugated DBA detected using streptavidin conjugated Dylight 405 (blue). (G) EYFP expressing cells detected using Alexa fluor 555 conjugated anti-GFP antibody (green). (H) All three channels merged.