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University of Turku

Faculty of Medicine
Department of Biomedicine
Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine (TuDMM)

Supervised by

Docent Arno Hänninen, MD, PhD
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor, Ilkka Junttila, MD, PhD
Department of Clinical Microbiology
University of Oulu
Oulu, Finland

Docent, Päivi Saavalainen, PhD
Folkhälsan Research Center
Helsinki, Finland

Opponent

Professor, Tuure Kinnunen, MD, PhD
Department of Clinical Microbiology
University of Eastern Finland
Kuopio, Finland

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To my family

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ABSTRACT

The gut microbiota has been suggested to be an important factor in the development of autoimmune diseases such as type 1 diabetes (T1D). Priming of islet specific T cells in the pancreatic lymph nodes (PaLN) and their migration to the pancreatic islets are critical steps in the destruction of insulin producing β-cells that leads the development of autoimmune diabetes. Perturbation of gut homeostasis by pathogenic microbes or imbalance of the gut microbiota during dysbiosis may provoke the autoimmune reactions that are associated with T1D. However, the mechanisms by which altered gut microbiota and its interaction with the immune system affect autoimmune diabetes development are unclear. In this thesis, we investigated how gut microbiota composition influences the progression of autoimmune diabetes in nonobese (NOD) mice and whether dysbiosis aggravates the immunological events associated with T1D pathogenesis.

This thesis will show how compromised intestinal barrier integrity and dysbiosis promote islet-specific T cell activation and their attraction to pancreatic islets. First, we show that healthy microbiota is associated with enhanced intestinal integrity and has a beneficial effect on the progression of autoimmune diabetes. We show that induction of dysbiosis with pathogenic microbes impairs intestinal barrier function, where among other factors plasmacytoid dendritic cells (pDCs) have an important regulatory role, and promote the autoimmune responses associated with the β-cell destruction. We describe a novel shared lymphatic drainage between the gut and pancreas that allows dendritic cell and bacterial migration to PaLNs during dysbiosis. Dysbiosis enhances the activation of islet-specific T cells and their priming with chemokine receptor CXCR3 in PaLNs. Additionally, we show that dysbiosis, followed by elevated endotoxin levels, enhances CXCL10 production within the pancreatic islets, which leads to enhanced attraction of CXCR3+ lymphocytes into pancreatic islets. Our results indicate that dysbiosis may be the initial factor that promotes lymphocyte infiltration into healthy pancreatic islets.

This thesis will give new insight into the effects of gut microbiota on the development of autoimmune diabetes and the role of dysbiosis in actuating the autoimmune responses associated with T1D pathogenesis.

KEYWORDS: autoimmune diabetes, islet-specific T cell, pancreatic islet, pancreatic lymph node, gut, dysbiosis

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TIIVISTELMÄ

Suolistomikrobiston uskotaan olevan tärkeä tekijä autoimmuunisairauksien, kuten tyypin 1 diabeteksen (T1D) kehittymisessä. Tyypillisiä vaiheita autoimmuunidiabeteksen kehittymisessä ovat saareke-spesifisten T-solujen aktivoituminen haiman imusolmukkeissa ja niiden siirtyminen haiman saarekkeisiin, johtaen insuliinia tuottavien β-solujen tuhoutumiseen. Patogeenisten mikrobien tai mikrobiston epätasapainon eli dysbioosin aiheuttama suoliston homeostaasin häiriintyminen voi edesauttaa näitä T1D:ssä havaittuja autoimmuunivasteita. On kuitenkin vielä epäselvää mitkä ovat ne mekanismit, joilla epätasapainoinen suolistomikrobisto ja sen vuorovaikutus immuunijärjestelmän kanssa vaikuttavat autoimmuunidiabeteksen kehittymiseen. Tämän tutkimuksen tarkoituksena oli selvittää, miten suolistomikrobiston koostumus vaikuttaa autoimmuunidiabeteksen kehittymiseen ja kuinka dysbioosi voi pahentaa T1D-patogeneesiin liittyviä immunologisia vasteita.

Tämä väitöskirja osoittaa, kuinka heikentynyt suolen seinämän eheys ja dysbioosi edistävät saareke-spesifisten T-solujen aktivaatiota ja niiden houkuttelua haiman saarekkeisiin. Osoitamme, että terveellä mikrobistolla ja hyvällä suolen seinämän eheydellä on hidastava vaikutus autoimmuunidiabeteksen kehittymisessä. Vastaavasti osoitamme, että dysbioosi edistää β-solujen tuhoutumiseen johtavia autoimmuunivasteita heikentämällä suoliston seinämän eheyttä, jossa muiden tekijöiden ohella plasmasytoidi dendriittisoluilla (pDC) on tärkeä säätelevä tehtävä. Osoitamme ennen kuvaamattoman imutieyhteyden suoliston ja haiman välillä, joka mahdollistaa dendriittisolujen ja bakteerien siirtymisen haiman imusolmukkeisiin dysbioosin aikana. Dysbioosi tehostaa saareke-spesifisten T-solujen aktivaatiota ja niiden kemokiinireseptori CXCR3 ilmentymistä. Lisäksi näytämme, kuinka dysbioosi ja siitä johtuva kohonnut endotoksiinitaso indusoi CXCL10 tuotantoa haiman saarekkeissa, mikä lisää CXCR3+ lymfosyyttien houkuttelua haiman saarekkeisiin. Tuloksemme osoittavat, että dysbioosi voi olla yksi ensimmäisistä tekijöistä lymfosyyttien tunkeutumisessa terveisiin haiman saarekkeisiin.

Tämä väitöskirja tuottaa uutta tietoa suolistomikrobiston vaikutuksista autoimmuunidiabeteksen kehittymisessä ja siitä, kuinka dysbioosi voi olla merkittävä laukaiseva tekijä T1D patogeneesille tyypillisissä autoimmuunivasteissa.

AVAINSANAT: autoimmuuni diabetes, saareke-spesifinen T solu, haiman saareke, haiman imusolmuke, suolisto, dysbioosi

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Abbreviations

APC	Antigen presenting cell
BLN	Brachial lymph node
cDC	Conventional dendritic cell
coMLN	Colon mesenteric draining lymph node
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C Motif Chemokine Receptor 3
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Dx	Dextran
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GI	Gastrointestinal
H&E	Haematoxylin and eosin
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFN	Interferon
IFNα	Interferon alpha
IFNAR	Interferon alpha/beta receptor
IFNγ	Interferon gamma
IFNGR	Interferon gamma receptor
IgA	Immunoglobulin A
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
KO	Knock-out
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II

MLN	Mesenteric lymph node
NOD	Nonobese diabetic
OVA	Ovalbumin
PaLN	Pancreatic lymph node
pDC	Plasmacytoid dendritic cell
qPCR	Quantitative polymerase chain reaction
RAG1	Recombination activating gene 1
RNA	Ribonucleic acid
SCFA	Short-chain fatty acid
siMLN	Small intestine mesenteric lymph node
T1D	Type 1 diabetes
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
Treg	Regulatory T cell
WT	Wild type
XCR1	X-C motif chemokine receptor 1

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Arno Hänninen, Raine Toivonen, Sakari Pöysti, Clara Belzer, Hubert Plovier, Janneke P Ouwerkerk, Rohini Emani, Patrice D Cani, Willem M De Vos. *Akkermansia muciniphila* induces gut microbiota remodelling and controls islet autoimmunity in NOD mice. *Gut.* 2018; 67:1445–1453

II. Sakari Pöysti, Satu Silojärvi, Raine Toivonen, Arno Hänninen. Plasmacytoid dendritic cells regulate host immune response to *Citrobacter rodentium* induced colitis in colon-draining lymph nodes. *European Journal of Immunology.* 2021; 51: 620–625

III. Sakari Pöysti, Raine Toivonen, Akira Takeda, Satu Silojärvi, Emrah Yatkin, Masayuki Miyasaka, Arno Hänninen. Infection with the enteric pathogen *C. rodentium* promotes islet-specific autoimmunity by activating a lymphatic route from the gut to pancreatic lymph node. *Mucosal Immunology.* 2022; 15:471–479

IV. Sakari Pöysti*, Satu Silojärvi*, Thomas C. Brodnicki, Tara Catterall, Xin Liu, Leanne Mackin, Andrew D. Luster, Thomas H.W. Kay, Urs Christen, Helen Thomas, Arno Hänninen. Gut dysbiosis and induction of CXCL10/CXCR3 in attraction of T-cells in islets during early islet-autoimmunity. *Manuscript*

* These authors contributed equally to this article.

1 Introduction

Type 1 diabetes (T1D) is a serious chronic disease characterized by the destruction of insulin secreting β-cells in the pancreatic islets. Due to the loss of insulin production, individuals with T1D require a life-long insulin treatment. The incidence of T1D has been steadily increasing throughout the world and it is now one of the most common chronic diseases in children, especially in Finland where the incidence rate is higher than in any other country. Even though β-cell destruction is known to be a consequence of autoimmune reactions, the mechanisms that underpin this process are poorly understood. Both genetic and environmental factors contribute to the development of T1D. While several genetic predispositions for T1D have been described, the defining role of environmental factors is uncertain.

The composition of gut microbiota has been put forward as a factor that is associated with T1D development, and this could partly explain the increased prevalence of diabetes in developed countries. It is known that gut microbiota composition influences diabetes incidence in NOD mice, but thus far no specific microbial species have been convincingly shown to predispose an individual to autoimmune diabetes. Altered intestinal homeostasis can potentially enhance the activation of autoreactive T cells which are responsible for β-cell destruction. The aim of this thesis is to identify the mechanisms relating to how altered gut microbiota promote autoimmune responses associated with T1D pathogenesis.

2 Review of the Literature

2.1 Type 1 diabetes

2.1.1 Incidence and prevalence

Diabetes-like symptoms were first described thousands of years ago, however the pancreas was not known to be the origin of the disease until 1889 (Luft, 1989). The term ‘insulitis’ was introduced by Swiss pathologist von Meyenberg in 1940, describing the inflammatory infiltration of leukocytes into islets of Langerhans in diabetes patients. Finally, between 1974 and 1976 the idea of autoimmune-mediated diabetes was introduced, and diabetes was separated into two types (type 1 and type 2). Since then, there has been extensive effort to identify the mechanism behind the pathogenesis of type 1 diabetes. However, despite intensive research, we still only partially understand the process.

T1D usually develops during childhood before the age of 14, but it can also present later during adulthood. T1D incidence worldwide has increased significantly since World War II, however the incidence varies substantially between different countries (Onkamo et al., 1999). A report from the DIAMOND Study Group showed that T1D incidence during 1990-1994 was highest in Finland (36.5/100,000 per year) and Sardinia (36.8/100,000 per year), while the lowest incidence occurred in China and Venezuela (0.1/100,000 per year) (Karvonen, 2006; Karvonen et al., 2000). Since then, the incidence has increased steadily worldwide and in 2006 T1D incidence in Finland reached its plateau of 64.2/100,000 per year (Harjutsalo et al., 2013). Even though there have been indications that the incidence of T1D in Finnish children has slightly decreased from the year 2011, there are estimated to be 50 000 active type 1 diabetes patients in Finland (Parviainen et al., 2020).

2.1.2 Genetic and environmental factors

Both genetic susceptibility and environmental factors contribute to the development of T1D. While the effects of environmental factors are still not properly defined, the genetic factors associated to T1D are relatively well recognized. Polymorphisms within the HLA II region, especially in HLA-DR and HLA-DQ loci, are known to

be the major genetic factor in T1D (Concannon et al., 2005). Haplotypes of DR3-DQ2 or DR4-DQ8 are most frequently present in T1D patients, while haplotypes DRB1*04:03 and DRB1*04:02 are associated with protection against T1D development (Aly et al., 2005; Erlich et al., 2008). Other non-HLA genetic risk factors, albeit to a lesser degree, are also associated with T1D such as polymorphisms in cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), the insulin gene (*INS*), a variant of the lymphoid- specific protein tyrosine phosphatise (*PTPN22*), Interleukin-2 receptor alpha (*IL2RA*) and Interferon induced with helicase C domain 1 (*IFIH1)* (reviewed in Ilonen et al., 2019). However, polymorphisms in these genes do not automatically lead to T1D development, and most children with both of the highest risk HLA haplotypes (DR3-DQ2 or DR4-DQ8) do not even develop T1D (Aly et al., 2005). In addition, the report from DIABIMMUNE study showed that the prevalence of T1D differs between regions despite the similar HLA-conferred susceptibility (Vatanen et al., 2016). This suggests that genetic predisposition only partially determines the risk of developing T1D. Therefore, it is not a surprise that there has been extensive effort to identify the potential environmental factors that contribute to the pathogenesis of T1D.

Several different environmental candidates have been proposed to "trigger" T1D pathogenesis, such as enterovirus and rotavirus infections (Clements et al., 1995; Coppieters et al., 2012; Honeyman et al., 2000; Oikarinen et al., 2012; Wang et al., 2021). The hygiene hypothesis, which posits that autoimmune disorders may arise as a consequence of reduced stimulation of the immune system by microbial exposure (Gale, 2014), has also been proposed as a candidate explanation for the increased T1D prevalence in developed countries.

2.1.3 NOD mouse model

Most of our knowledge concerning T1D pathogenesis relies on animal models, of which the nonobese diabetes (NOD) mouse model has been essential. The NOD mouse strain was established in the 1980s and are descended from the Cataract Shionogi (CTS) strain (Makino et al., 1980). Even though wild type (WT) NOD mice are not genetically modified, they develop spontaneous disease closely resembling human T1D, including progressive loss of insulin producing β-cells and hyperglycaemia. NOD mice develop autoantibodies and have circulating autoreactive T cells similar to human T1D (Melanitou et al., 2004; You et al., 2005). As already mentioned, genetics play a key role in autoimmune diabetes. As in humans, the most important genetic factor in developing diabetes in NOD mice is the presence of polymorphisms in the MHC II molecule (Driver et al., 2012). NOD mice express MHC II molecules I–Ag7, where the change of amino acids at position 57 resembles the high-risk allele DQ8 in humans (Latek et al., 2000; Lee et al., 2001).

Overall, NOD mice share over 50 diabetes risk-associated loci with humans (Driver et al., 2012).

Female NOD mice are more prone to develop diabetes than male mice, and in females T1D onset occurs after the age of 12 weeks while in males onset is slightly later (Anderson & Bluestone, 2004; Mathews et al., 2015). However, environmental factors such as diet, infections and gut microbiota affect diabetes incidence (Hansen et al., 2012; Marietta et al., 2013; Schmid et al., 2004; Zaccone et al., 2004). It should also be noted that housing conditions in different institutions can affect the age of diabetes onset. Leukocyte infiltration into pancreatic islets in NOD mice starts much earlier than disease onset and histological observations show that most of the female NOD mice demonstrate signs of insulitis at the age of 4-5 weeks and by 10 weeks of age infiltrating leukocytes are prominent in most of the islets (Anderson & Bluestone, 2004; Zakharov et al., 2020). Autoimmune diabetes in NOD mice is dependent on islet-specific cells and WT mice bear both autoreactive CD4 and CD8 T cells specific to islet antigens (Bach, 1994; Haskins & Wegmann, 1996; Wicker et al., 1986; Wong et al., 1996). Genetically modified NOD mice such as BDC2.5 and NOD8.3 mice have also allowed deeper investigation of the role of autoreactive T cells in autoimmune diabetes (Katz et al., 1993; Verdaguer et al., 1997). Due to the spontaneous lymphocyte infiltration and insulitis, NOD mice have been an important tool for investigating the pathogenesis of T1D.

2.1.4 T1D autoimmunity

T1D is characterised by autoimmune mediated infiltration of CD4 and CD8 T cells, B cells and macrophages into islets leading to β-cell destruction (Campbell-Thompson et al., 2016; Willcox et al., 2009). However, why the autoimmune reaction in T1D is specifically targeted against the body's own β-cells remains unclear. In T1D, immunological tolerance fails when certain genetic polymorphisms allow development of autoreactive lymphocytes that recognize β-cell autoantigens. However, environmental factors disrupt the process that suppresses autoreactivity in the periphery and can trigger autoreactive lymphocytes to recognize the autoantigens presented by HLA molecules.

2.1.4.1 Autoantibodies

The first detectable sign of β-cell autoimmunity is the appearance of autoantibodies. In T1D patients, the most common autoantibodies are islet cell antibodies (ICA), insulin autoantibodies (IAA), and antibodies specific against glutamic acid decarboxylase 65 (GADA), IA-2 molecule (IA-2A), and zinc transporter 8 (ZnT8A) (Lampasona & Liberati, 2016). Antibodies associated with T1D are usually found in

the serum of the patients before clinical disease manifests, in some cases even at birth, and remain detectable during the different stages of disease progression (Lundgren et al., 2015). Measurement of autoantibodies thus provides important predictive and diagnostic value in monitoring T1D progression. Even though autoantibodies associate with the risk of developing T1D, their role in β-cell destruction itself seems to be minor (Martin, Wolf -Eichbaum, et al., 2001). Studies evaluating the risk of T1D in children with diabetic parents show that although autoantibodies are transferred from T1D mothers to fetuses during pregnancy, they do not damage β-cells in the fetuses (Harjutsalo et al., 2006).

2.1.4.2 Autoreactive T cells

The generally accepted concept of β-cell destruction is that it is mediated through cellular immunity by T cells. Insulitis with infiltrating CD4 and CD8 T cells is one of the hallmarks of β-cell destruction supporting this concept (Campbell-Thompson et al., 2016; Willcox et al., 2009). While detection of autoantibodies has proven to be an effective way of predicting T1D development, detection of islet specific T cells is more challenging due to their low frequency in blood circulation. Production of autoantibodies by B cells is largely dependent on follicular Tfh cells and it has been shown that circulating $CXCR5^{+}PD\text{-}1^{+}ICOS^{+}$ Tfh cells are increased in children with newly diagnosed T1D and in autoantibody positive children (Viisanen et al., 2017). By measuring reactivity to islet antigens and by using specific HLA tetramers, T cells specific for different islet antigens have been identified from T1D patients (Roep & Peakman, 2012). T cells specific for major islet antigens including proinsulin, GADA and IA-2 molecule has been shown to be highly associated with T1D (Atkinson et al., 1992; Hawkes et al., 2000; Ihantola et al., 2020; Roep & Peakman, 2012). Since CD8 T cells are believed to be the main cells attacking β-cells, it has been informative to identify these islet specific CD8 T cells from T1D patients and evaluate how they are associated with the progression of the disease. It has been shown that particularly autoreactive CD8 T cells that are reactive to insulin peptide correlate with β-cell destruction (Pinkse et al., 2005). By using combinations of HLA tetramers, it has also been established that together with insulin, IGRP-specific CD8 T cells are commonly present in patients who have been recently diagnosed with the disease (Velthuis et al., 2010). Even though the presence of autoreactive T cells is associated with T1D, they can be found from healthy individuals as well (Bender et al., 2020; Culina et al., 2018; Velthuis et al., 2010), indicating that the failure of central tolerance and the presence of islet specific T cells in periphery do not automatically lead to autoimmune diabetes.

For islet specific T cell to be activated, there must be presentation of antigens by MHC molecules (HLA). The widely accepted model of initial T cell activation is that antigen presenting cells (APCs) take up β-cell antigens and present them to self-reactive T cells. As well as natural physiological β-cell turnover, β-cells are particularly sensitive to stressful conditions, and environmental inducers such as infections can potentially contribute to β-cell destruction and antigen release (Dahlquist, 2006). Excessive stress can also induce β-cells to produce neoantigens that are not present during thymic T cell education, which can be recognised by immune cells (Gonzalez-Duque et al., 2018; McLaughlin et al., 2016; Piganelli et al., 2021). Even in healthy individuals pancreatic islets contain resident APCs: macrophages and dendritic cells (DCs), which can sense and take up antigens from β-cells (Calderon et al., 2014). Islet APCs act as a link between β-cells and T cells, where DCs transport antigens to T cells and macrophages present antigens to arriving T cells within islets. Depletion of islet resident APCs results in the disappearance of infiltrating lymphocytes, which supports this proposed link (Hutchings et al., 1990; Nikolic et al., 2005). It is well characterized that in the NOD mouse model, autoreactive T cells are activated in pancreatic lymph nodes (PaLN) (Höglund et al., 1999; Jaakkola et al., 2003). This indicates that islet resident DCs deliver β-cell antigens from islets to PaLN and present them to T cells (**Figure 1**) (Clare-Salzler & Mullen, 1992; S. Turley et al., 2003). Furthermore, surgical removal of PaLN from NOD mice has been shown to prevent mice from developing diabetes, demonstrating that PaLNs are crucial location for autoreactive T cell priming (Gagnerault et al., 2002; Levisetti et al., 2004).

DCs mainly present autoantigens to CD4 T cells via MHC II, but a small subset of resident DCs can also cross-present to directly prime CD8 T cells via MHC I (Unanue et al., 2016). However, in steady state conditions DCs promote tolerance and do not express efficient costimulatory molecules to activate T cells, therefore the exact mechanism by which autoreactive T cells are activated is yet to be completely defined. It has been suggested that environmental factors such as infections can “break” this tolerance and allow complete activation signals. This is also explained by molecular mimicry, where environmental agents, for example from viruses, share similar properties or amino acid sequencies with β-cells, and can cause full activation of autoreactive T cells (Coppieters et al., 2012). After activation, autoreactive T cells migrate to the pancreas through blood stream and infiltrate pancreatic islets.

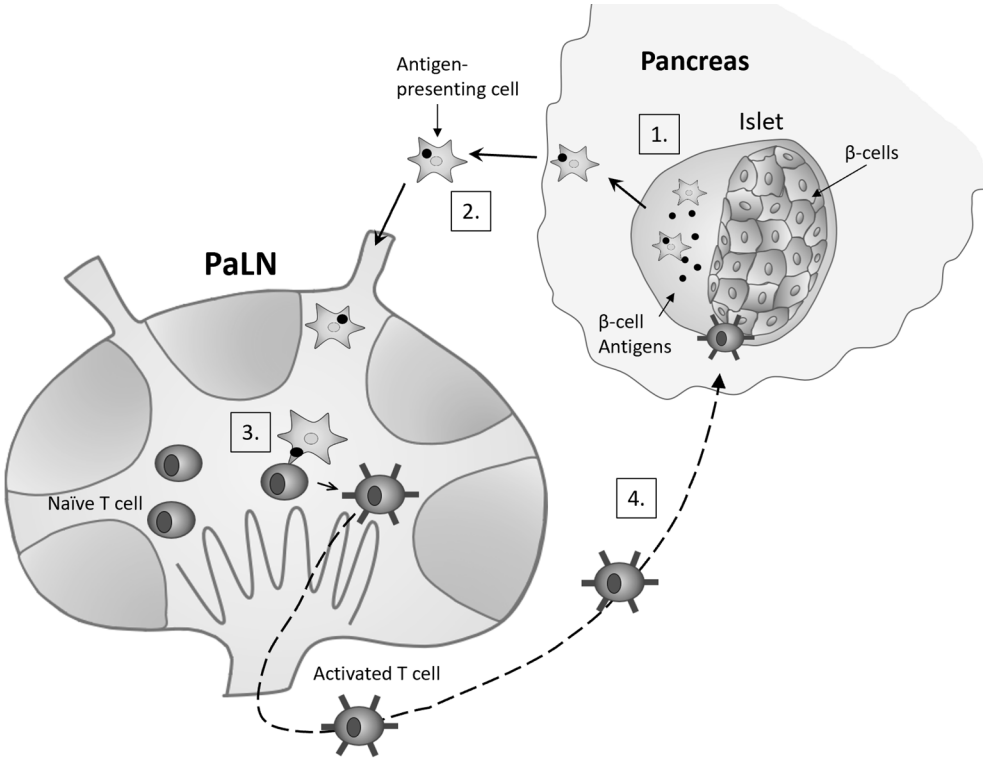

Figure 1. Illustration of how APCs deliver β-cell antigens to a PaLN and prime autoreactive T cells. (1.) β-cell antigens are processed by resident APCs in pancreatic islets and (2.) delivered to pancreatic lymph nodes. (3.) APCs prime autoreactive T cells by presenting β-cell antigens via MHC molecules. (4.) After activation, islet specific T cells egress from the PaLN and traffic to pancreatic islets. Modified from Mathis et al., 2001

2.1.4.3 Lymphocyte attraction to islets

Lymphocytes are normally attracted to the target tissues by chemokine signalling. Several chemokines are expressed in islets and their expression is driven by inflammatory cytokines such as Interferons (IFNs) and tumor necrosis factors (TNFs) (Christen & Kimmel, 2020). One of the important chemokines is CXCL10, which is elevated in the sera of recent-onset T1D patients (Nicoletti et al., 2002; Shimada et al., 2001). β-cells are known to express CXCL10 within islets of T1D patients and its expression is driven by IFNγ (Christen et al., 2003; Roep et al., 2010; Sarkar et al., 2012). Its receptor, CXCR3, is expressed on Th1 CD4 and effector CD8 T cells where CXCL10 expression promotes trafficking to target tissues (Groom & Luster, 2011). The CXCL10/CXCR3 axis is considered to be one of the most important factors in lymphocyte migration to pancreatic islets (Christen et al., 2003; Frigerio et al., 2002). The chemokine gradient attracts lymphocytes and, by a process that is mediated by vascular adhesion molecules, lymphocytes can infiltrate islets

from the blood circulation. Most of the infiltrating T cells express lymphocyte function-associated antigen 1 (LFA-1) (αLβ2), integrin alpha 4 beta 7 (LPAM-1) (α4β7) or very late antigen 4 (VLA-4) (α4β1) (Hänninen et al., 2007; Sandor et al., 2019). The integrin ligands VCAM-1, MAdCAM1, ICAM-1 are associated with the pathogenesis of T1D and they are expressed in the vasculature of the pre-insulitis islets (Baron et al., 1994; Calderon et al., 2011; Glowinska et al., 2005; Hänninen et al., 1993). Inflammation and local cytokine production can promote the expression of these ligands, thereby enhancing leukocyte infiltration into the pancreas. Deficiency or blockage of T1D-associated integrin ligands have been shown to delay leukocyte infiltration and T1D development (Baron et al., 1994; Hänninen et al., 1998; Martin, Van Den Engel, et al., 2001). Leukocytes in the insulitic lesions increase inflammation and hence promote infiltration of more leukocytes into islets.

2.1.4.4 Immunopathogenesis of beta-cell destruction

Before infiltrating leukocytes, healthy islets contain only resident APCs of which macrophages are dominant immune cell type (Calderon et al., 2015; Zakharov et al., 2020). It has been shown in NOD mice that T and B cells become the most abundant immune cell types already from the early phases of insulitis (Magnuson et al., 2015). While in NOD mice the ratios of CD4, CD8 and B cells are relatively even, in human T1D patients CD8 T cells have been shown to be the dominant population of infiltrating cells (Willcox et al., 2009). Even though the proportion of immune cells made up by macrophages decreases, their total number continues to rise within islets (Magnuson et al., 2015). Overall, many cell types can be found in the infiltrated islets including CD4 and CD8 T cells, B cells, DCs and macrophages. β-cell destruction is mediated mainly by T cells, especially CD8 T cells, where they attack β-cells based on recognized autoantigens. The role of B cells in infiltrated islets and β-cell destruction is uncertain. It has been hypothesized that since B cells are a common cell type to infiltrate islets, they are in close contact with CD8 T cells and can thus enhance CD8 T cell activity and thereby contribute to β-cell destruction (Leete & Morgan, 2021). Macrophages are believed to be important in lymphocyte attraction and together with DCs they present antigens to arriving lymphocytes (Unanue et al., 2016; Vomund et al., 2015). Since APCs are present in healthy islets, it has been proposed that they have a key role in creating the initial inflammatory conditions within islets that lead to β-cell antigen recognition and lymphocyte attraction (Cosentino & Regazzi, 2021).

It has been shown that MHC molecules are hyper-expressed in insulitic islets (Bottazzo et al., 1985; Foulis et al., 1987; Hanninen et al., 1992). Especially MHC class I molecules are highly expressed in β-cells, which supports the concept of CD8 T cell-mediated β-cell destruction. In addition, it has been shown that MHC I

deficient NOD mice are resistant to insulitis and diabetes (Katz et al., 1993; Serreze et al., 1994; Wicker et al., 1994). The strong diabetogenic effect of CD8 T cells have been demonstrated in transgenic 8.3NOD mice with autoreactive CD8 T cells. These 8.3NOD mice have extensive lymphocyte infiltration in islets and they develop diabetes at a very early age (Verdaguer et al., 1997). Autoreactive CD8 T cells can kill recognized β-cells in a contact-dependent manner via granzyme and perforin molecules (Trivedi et al., 2016). Even though CD8 T cells are considered to be essential in T1D pathogenesis, they need help from CD4 T cells. It has been even shown that the ability of IGRP-specific CD8 T cells to cause insulitis and diabetes is significantly reduced without the help of autoreactive proinsulin specific CD4 T cells (Krishnamurthy et al., 2008). Both CD4 and CD8 T cells can produce proinflammatory cytokines such as IFNγ and TNF which are directly toxic to β-cells (Calderon et al., 2008; Suk et al., 2001).

While β-cells themselves express MHC I molecules, it is thought that expression of MHC II in β-cells is limited *in vivo*, and that MHC II is mainly expressed in islet APCs (In 't Veld & Pipeleers, 1988; McInerney et al., 1991; Pavlovic et al., 1997). While islet-specific CD4 T cells are mainly activated in PaLNs, the possibility of direct antigen presentation by β-cells to CD4 T cells via MHC II has also been investigated. Russel et al. used ribonucleic acid (RNA) sequencing to show that 35,2% of the T1D patients express MHC II genes in their β-cells compared to only 7,8% of the healthy donors (Russell et al., 2019). Together with previous studies they also identified that MHC II expression in non-diabetic β-cells can be induced in the presence of IFNγ, IL1β and TNF (Pujol-Borrell et al., 1987; Russell et al., 2019). Considering the induced MHC II expression, it has been proposed that the initial autoreactive activation of CD4 T cells can also occur within islets. However, since the activation of T cells needs costimulatory molecules, it is debatable whether or not MHC II+ β-cells can effectively present antigens to autoreactive CD4 T cells. There are indications that stressed β-cells can function in a similar manner to APC cells to their MHC expression and production of neoantigens (Li et al., 2021; Piganelli et al., 2021). However, more studies are needed to determine their role in the initiation of autoreactive T cell activation. While the role of MHC II expression within islets in β-cell destruction itself is yet to be determined, overall MHC II-mediated antigen recognition by CD4 T cells is an essential part of diabetes development (Ostrov et al., 2018).

Regulatory Tregs are a key factor in maintaining peripheral tolerance. FoxP3 positive Tregs are known to control the progression of autoimmune diabetes, and one of the therapeutic approaches to treat diabetes has been to promote Treg functions (Kuhn et al., 2017; Mbongue et al., 2019). The number of Tregs correlate with the progression of T1D, where Tregs increase in blood circulation after diabetes onset (Viisanen et al., 2019). Tregs seem to control the progression of autoimmune

diabetes, rather than the initiation of the autoimmune reaction. Leukocyte infiltration to pancreatic islets is drastically increased in FoxP3-deficient mice and the rate at which they develop diabetes is accelerated (Chen et al., 2005; Feuerer et al., 2009). However, FoxP3-deficiency did not impact the initial autoreactive T cell activation in PaLN, indicating that Tregs mainly regulate autoimmunity locally in islets. Even though Tregs do not regulate the initial T cell autoimmune reaction, in islets they can control cytokine production and inflammation, which is a key factor in initial β-cell stress and antigen release (Feuerer et al., 2009; Herman et al., 2004).

Autoimmunity in islets is multifactorial and β-cell destruction is caused by cooperation of several immune cell types. The common assumption is that environmental triggers such as infections cause local inflammation and antigen presentation by APCs to lymphocytes via MHC II eventually leading to CD8 mediated β-cell destruction via MHC I. In addition to the antigen recognition based cellular immune response against β-cells, islet endocrine cells are sensitive to surrounding cytokines released from mononuclear cells which can have a direct cytotoxic effect on β-cells (Eizirik & Mandrup-Poulsen, 2001; Mandrup-Poulsen et al., 1985). At the same time Tregs fail to dampen the immune response against β-cells.

2.2 Gut microbiota

2.2.1 Healthy microbiota

The human gastrointestinal track (GI) contains trillions of microorganisms, including bacteria, viruses, archaea, and unicellular eukaryotes. Together they form a complex microbial ecosystem referred as gut microbiota (Lynch & Pedersen, 2016; Sekirov et al., 2010). The human microbiome consists of up to 10^{14} bacterial cells, and the densest microbial colonization can be found in the colon which contains approximately 70% of all the microbes in the human body (Ley et al., 2006; Whitman et al., 1998). Bacteroides and Firmicutes are the two phyla dominating the human gut microbiota and together with Verrucomicrobia, Proteobacteria and Actinobacteria they account for more than 90% of the total community (Bäckhed et al., 2005; Eckburg et al., 2005; Magne et al., 2020). The gut microbiota is also referred to as an “organ within an organ” and it plays a critical role in several functions (Lynch & Pedersen, 2016), including maturation and homeostasis of the immune system (Fulde & Hornef, 2014), protection against pathogen overgrowth (Kamada, Chen, et al., 2013) and influencing host-cell proliferation (Ijssennagger et al., 2015). This wide variety of functions has provoked gut microbiota to be the focus of research for multiple different chronic diseases such as autoimmune disorders, inflammatory diseases, cancer and neurologic disorders.

There are several factors that can influence the composition of an individual's microbiota and it is process that starts at birth. Exposure to external microbes at birth and early life are critical steps in the development of infant's gut microbiota. Studies have shown that mode of delivery influences postnatal microbiota, where vaginally delivered infants, when compared to infants delivered through caesarean section, harbour more diverse microbial community which resembles the maternal gut (Bäckhed et al., 2015; Dominguez-Bello et al., 2010; Huurre et al., 2008). During the first year of life microbial community expands rapidly and variation is high between individuals. However, after the one year of life gut microbial composition starts to stabilize and mature to resemble the microbiota of a young adult, where cessation of breast feeding and introduction of solid foods play an essential role (Bäckhed et al., 2015). These first steps dictate the guidelines for the shaping of gut microbiota through adulthood.

Even though, adult gut microbiota is thought to be a relatively stable community, it is still affected by multiple factors causing it to face some dynamic shaping. Studies have shown that major influencers on microbial composition of the GI track are environmental factors such as diet and obesity, infections, use of antibiotics and other drugs, and exposure to environmental microbes (De Filippo et al., 2010; Dethlefsen et al., 2008; Fujimura et al., 2014; Lupp et al., 2007; Xu & Knight, 2015). Some host-related factors including sex, and age are also factors which have been shown to have an effect on gut microbiota composition (Yatsunenko et al., 2012). Even though genetic factors are suggested to play a role in how the microbial community in GI track is shaped (Goodrich et al., 2014), its direct contribution to overall microbiota formation seems to be negligible and is overshadowed by environmental influences (Carmody et al., 2015; Jackson et al., 2018).

2.2.2 Dysbiosis

In a healthy individual, commensal microbes in the gut microbiota are harmless and even beneficial for maintaining healthy homeostasis in the GI track (Bäckhed et al., 2005; Tremaroli & Bäckhed, 2012). Healthy intestinal microbiota can be characterized by its diversity, stability and resilience, referring to richness of the microbial ecosystem, adaptation to perturbations and ability to recover back to a normal state if disturbed (Lozupone et al., 2012). However, several external triggers, such as diet, infections, and the use of antibiotics or other drugs can challenge this resilience and unbalance the microbial ecosystem leading to a condition called dysbiosis (Lozupone et al., 2012). A common phenomenon in dysbiosis is that the overall diversity of the microbiota is reduced where commensal symbiotic species decrease and the proportion of opportunist pathogenic species increase. Reduction of symbiotic species, such as microbes from Firmicutes phyla, which produce short

chain fatty acids (SFCA), mainly acetate, propionate, and butyrate, have a drastic effect on gut metabolism. SFCAs are important in host energy metabolism and are known to promote health by enhancing nutrient utilization, promoting lipogenesis and gluconeogenesis, producing anti-inflammatory functions and promoting epithelial barrier function (Bäckhed et al., 2004; Corrêa-Oliveira et al., 2016; Donohoe et al., 2011). At the same time, loss of microbial diversity and symbiotic species can open an opportunity for opportunistic microbes to expand in the changing environment. Some of these microbes are called pathobionts and they can have a pathogenic potential in certain circumstances, even though they are harmless in normal diverse microbial ecosystem (Chow et al., 2011).

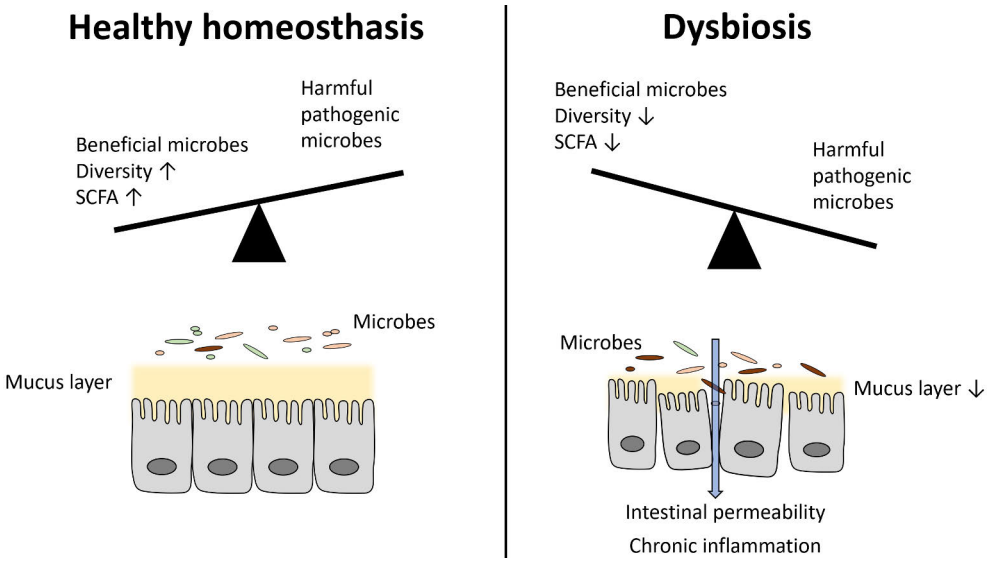

Figure 2. In healthy gut microbiota, a diverse microbial community with symbiotic microbes promote mucus production in the intestine and enhance intestinal barrier function. During dysbiosis microbial diversity is reduced and levels of more harmful potentially pathogenic microbes have increased. Altered microbial balance has an effect on the mucus layer and can lead to increased intestinal permeability and chronic inflammation. Based on (Chow et al., 2011; Kamada, Seo, et al., 2013; Kinashi & Hase, 2021)

One of the main characteristics of dysbiotic gut microbiota is altered intestinal barrier function. Commensal microbes play a significant role in maintaining an effective barrier between unwanted microbes and the intestinal epithelial layer. First of all, symbiotic microbes limit the colonization of pathogens directly by enhancing mucus production in the mucus layer, thereby outcompeting unwanted microbes for space and nutrients, and producing SCFAs and other antiviral metabolites such as fucose (Kamada, Seo, et al., 2013; Momose et al., 2008; Pacheco et al., 2012). The gut microbiota is in direct connection with the immune system and thus commensal

microbes can also indirectly inhibit pathogen colonization. When recognized by the immune cells in the gut, commensal microbes promote the production of antimicrobial peptides such as Reg3γ, and induce B cells and plasma cells to produce immunoglobulin A (IgA) into the lumen (Kamada, Seo, et al., 2013; Peterson et al., 2007; Vaishnava et al., 2011). Reduction of symbiotic commensal microbes, which occurs in dysbiosis, drastically effects gut homeostasis and barrier function and thus increases the permeability of the gut and can lead to chronic inflammatory conditions (Kamada, Seo, et al., 2013; Kinashi & Hase, 2021) (**Figure 2**).

In dysbiosis, the lack of diversity and colonization of nonsymbiotic species leads to disturbances between the gut microbiota and immune system (Levy et al., 2017; Meng et al., 2020). This imbalance of the microbiota is linked to several diseases such as inflammatory bowel disease (IBD) (Frank et al., 2007; Tamboli et al., 2004) and autoimmune diseases (Shahi et al., 2017; Yurkovetskiy et al., 2015). For example it has been shown that the expansion of pathogenic adherent-invasive Escherichia coli (AIEC) strains are associated with the pathogenesis of IBD (Darfeuille-Michaud et al., 2004; Sasaki et al., 2007). In addition, it has been suggested that mycobiome influences homeostasis in the gut and it has been shown that in Crohn's disease also fungi *Candida tropicalis* and *Serratia marcescens* are elevated when compared to healthy individuals (Hoarau et al., 2016). It should be stated that due to large variety in microbial diversity between individuals, it is difficult to define single microbial profile that typifies dysbiosis.

2.2.3 *Akkermansia muciniphila*

Akkermansia muciniphila is an anaerobic gram-negative bacterium from the phylum Verrucomicrobia and it was first discovered in 2004 while searching for new mucin-degrading microbes from human feces (Derrien et al., 2004). It was first described as strictly anaerobic (Derrien et al., 2004), but further studies have shown it to tolerate low levels of oxygen (Reunanen et al., 2015). *A. muciniphila* is an intestinal symbiont colonizing the mucosal layer mainly in the colon where it uses mucin as a source of carbon and nitrogen (Derrien et al., 2004). As a part of the normal microbial community, it represents 1-4% of the gut microbiota and can stably colonize human GI track during the first year after birth (Collado et al., 2007; Derrien et al., 2008).

As *A. muciniphila* locates in the mucus layer of the intestine, it is considered to have various beneficial properties that promote the homeostasis of the gut. A study by Everard et al showed that the number of the *A. muciniphila* is lowered in obese mice and correlates with a thinning of the mucus layer and impairment of mucosal barrier function (Everard et al., 2013). Furthermore, treatment with *A. muciniphila* counteracted this phenomenon and was able to restore mucosal barrier function. Other studies have shown similar results where *A. muciniphila* promotes intestinal

barrier function by strengthening the intestinal epithelial layer (Chelakkot et al., 2018; Reunanen et al., 2015). *A. muciniphila* is also known to regulate the immune system by promoting secretion of the antimicrobial peptide Reg3γ and by stimulating regulatory T cells in mice (Everard et al., 2013; Shin et al., 2014). Due to their beneficial properties, low abundance of *A. muciniphila* in gut microbiota has been linked to several chronic diseases and disorders such as metabolic disorders, obesity, diabetes, and IBD (Karlsson et al., 2012; Rajilić-Stojanović et al., 2013; Schneeberger et al., 2015; Zhang et al., 2013).

2.2.4 *Citrobacter rodentium*

Citrobacter rodentium is a gram-negative bacterium which is a mouse-specific intestinal pathogen and it shares several pathogenic properties with clinically important human enteropathogenic and enterohemorrhagic *E.coli* (EPEC/EHEC) (Koroleva et al., 2015). *C. rodentium* colonizes the intestinal mucosa via the formation of attaching and effacing lesions (A/E) in a similar manner to EPEC and EHEC. This A/E lesion formation is major factor that distinguishes EPEC and EHEC from other *E. coli* strains, and it can cause severe intestinal diseases that are linked to high morbidity and mortality worldwide (Nataro & Kaper, 1998). *C. rodentium* is an excellent tool to study A/E pathogens in mouse models. Additionally *C. rodentium* can be used to investigate other intestinal diseases such as inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (Higgins, Frankel, Douce, et al., 1999), dysbiosis (Collins et al., 2014) and tumorigenesis (Chandrakesan et al., 2014). *C. rodentium* can be used as a colitis model; it causes mild symptoms in immunocompetent mice but in certain strains and genetically modified immunodeficient mice, *C. rodentium* can cause severe colitis-like symptoms and mortality (Papapietro et al., 2013; Vallance et al., 2003)

When orally inoculated into mice, *C. rodentium* colonization starts from the caecum 24h after infection and proceeds to the colon in 2 to 3 days. Peak colonization can be observed between the days 8 and 10 (Lupp et al., 2007; Wiles et al., 2004). When *C. rodentium* colonization increases it alters gut microbiota composition and induces dysbiosis which can be seen in the reduced diversity of commensal microbes (Collins et al., 2014; Lupp et al., 2007). Deep sequencing from mice microbiota have shown that *C. rodentium* infection leads to alterations in the abundance of Proteobacteria, Deferribacteres, Clostridia, and Lactobacillaceae (Hoffmann et al., 2009). At the same time, an increase in Enterobacteriaceae lineages has been observed, which is mainly explained by the increase of *C. rodentium* itself (Hoffmann et al., 2009; Lupp et al., 2007). The colonization of *C. rodentium* is clearly detectable until day 14 post-infection when the bacterial burden starts to fall,

while complete *C. rodentium* clearance is usually observed between 21 and 28 days post-infection (Hoffmann et al., 2009; Lupp et al., 2007; Wiles et al., 2004).

C. rodentium colonization and attachment to epithelial cells is mediated by translocated intimin receptor (Tir), which leads to A/E lesion formation (Collins et al., 2014; Hartland et al., 1999). *C. rodentium* is recognized by epithelial and myeloid cells via toll-like receptor 2 and 4 (TLR), which signal through myeloid differentiation primary-response protein 88 (MYD88) (Gibson et al., 2008; Khan et al., 2006), and by nucleotide-binding oligomerization domain (NOD) within epithelial cells (Geddes et al., 2011). Both the innate and adaptive immune systems contribute to the response against *C. rodentium* infection. A wide range of T cell effector functions contribute to the protection and clearance of the pathogen, including production of IFNγ by Th1 cells (Shiomi et al., 2010). Th17 and Th22 also play a role in immune defence against *C. rodentium* where IL-22, secreted by Th22 cells, promotes secretion of antimicrobial peptide Reg3γ (Ota et al., 2011), and IL-17A, secreted by Th17 cells, is a potent inducer of neutrophils (Geddes et al., 2011). Macrophages and dendritic cells (DCs) also respond to the infection by producing pro-inflammatory cytokines such as IL1β, IL18 and TNF (Collins et al., 2014).

2.2.5 *Ruminococcus gnavus*

Ruminococcus gnavus is an anaerobic gram-positive bacterium, belonging to the Lachnospiraceae family in the Firmicutes phylum. Taxonomically *R. gnavus* was previously described to be part of *Ruminococcus* genus, but was recently reassigned to the genus *Blautia* (Lawson & Finegold, 2015; Liu et al., 2008). *R. gnavus* is a prevalent symbiont of the human gut and colonizes the mucus layer of the gut microbiota early in life (Sagheddu et al., 2016). In the mucus layer *R. gnavus* acts as mucin-degrading bacteria (Crost et al., 2013), which have been considered to be essential in maintaining homeostasis in the gut (Tailford et al 2015). Even though, it has a relatively low abundance (0,1%) in the gut microbiota, *R. gnavus* can be found in more than 90% of individuals (Henke et al., 2019). Regardless of the symbiotic nature, altered abundance of the *R. gnavus* has been linked to dysbiotic microbiota and several chronic diseases such as IBD and systemic lupus erythematosus (SLE) (Azzouz et al., 2019; Hall et al., 2017; Henke et al., 2019). To date, there have been case reports showing that *R. gnavus* can even cause bacteremia. In these cases, bloodstream infection led to life-threatening conditions for the patients (Hansen et al., 2013; Kim et al., 2017).

R. gnavus produces inflammatory polysaccharides and glucorhamnan (short glucose sidechains), which are recognized by the immune system through TLR4 signalling (Henke et al., 2019). It has been shown that colonization of germ-free mice with *R. gnavus* lowers the number of IL-22 expressing Th22 cells and innate

lymphoid cells in the GI track (Geva-Zatorsky et al., 2017). In the mucus layer, *R. gnavus* is coated by IgA which is known to inhibit bacterial binding to the epithelial cell layer and penetrating into the lamina propria (Peterson et al., 2007). It has been shown that *R. gnavus* binds to IgA in a "superantigen" mode which can be recognized by T cells and B cells leading to nonspecific activation (Bunker et al., 2019). As mentioned, *R. gnavus* is considered to be a part of symbiotic commensal microbiota in healthy gut but expansion of the *R. gnavus* is linked to several diseases. However, the exact mechanisms surrounding this are yet to be determined.

2.2.6 The role of gut microbiota in T1D

Current understanding of the role of gut microbiota in T1D mainly relies on studies made in NOD mice models. Even though mouse models cannot answer all the questions, their physiology and the composition of gut microbiota share similarities with humans. NOD mice develop diabetes at a high frequency and NOD mice have similar genetic HLA susceptibility and β-cell specific CD4 and CD8 T cells as seen in humans.

Several studies in NOD mice have linked gut microbiota to the development of autoimmune diabetes. It has been proposed that gut microbiota acts as regulator of inflammation in pancreatic islets and it has been shown that in germ free (GF) NOD mice, development of insulitis is accelerated when compared to WT NOD mice (Alam et al., 2011; Greiner et al., 2014). Alam et al. also showed that GF NOD mice had elevated numbers of IFNγ producing CD4 T cells and reduced Tregs in mesenteric and pancreatic lymph nodes. The immunomodulatory function of the intestinal microbiota is also demonstrated in NOD mice lacking MyD88, which is an essential modulator in recognizing microbial stimuli via TLR signalling. MyD88-deficient NOD mice are protected from diabetes in specific pathogen free (SPF) conditions (J. Peng et al., 2014; Wen et al., 2008). Interestingly, this protection however depends on the microbiota, since in GF conditions MyD88-deficient mice develop robust diabetes. Also, the composition of the microbiota in MyD88-deficient mice differs from WT NOD mice and transfer of the gut microbiota from MyD88 knock-out NOD mice prevents diabetes development in WT NOD mice.

One phenomenon associated with T1D is lack of diversity in microbiota and dysbiosis (Brown et al., 2011; Giongo et al., 2011; Kostic et al., 2015). Even though the data from human microbiota in T1D is relatively limited, there are reports showing that the microbiota in T1D children is less stable and less diverse when compared to healthy individuals. The common observation in the microbiota of seropositive children and newly diagnosed T1D patients is the increased abundance of Bacteroides species and increased Bacteroides to Firmicutes ratio (Davis-Richardson et al., 2014; De Goffau et al., 2013; Giongo et al., 2011; Mejía-León et

al., 2014). Associated with the alteration of this ratio, T1D patients are reported to have reduced abundance of butyrate producing bacteria, even though other SFCA producing species may even be elevated (Brown et al., 2011; De Goffau et al., 2014). It has been shown in NOD mice that high bacterial release of SFCA acetate and butyrate, resulting from a specific diet, prevent mice from developing diabetes almost completely (Mariño et al., 2017). It has also been suggested that altered abundance of certain fungi can be observed during the dysbiosis associated with the development of T1D in children (Honkanen et al., 2020).

Alterations of gut microbiota in T1D can potentially be associated with intestinal inflammation and increased intestinal permeability. However, whether or not altered microbiota modulates T1D progression or vice versa is debatable (Knip & Siljander, 2016). Butyrate is known to be important in maintaining intestinal homeostasis and intestinal barrier function by promoting mucus secretion and reinforcing epithelial tight junctions (Li et al., 2010; Peng et al., 2009). In addition, other mucin promoting bacteria such as *A. muciniphila* have been associated with protection against diabetes in NOD mice (Hansen et al., 2012). Furthermore, it has been shown that when intestinal barrier function is compromised, antigens from the gut are able to activate autoreactive T cells in PaLNs (Lee et al., 2010; Turley et al., 2005).

2.3 The immune system in the gut

Since the human gut harbours an extensive and complex microbial community, the intestine is in continuous exposure to antigen stimuli. Additionally, food antigens and potential pathogens entering the body through GI track challenge the intestinal immune system. To cope with the extensive antigen stimulus, the intestine is full of immune cells making one of the largest compartments of the immune system (Mowat, 2003). The GI tract contains specialized and concentrated immune cell areas referred as gut associated lymphoid tissues (GALTs) (Mörbe et al., 2021). In addition to the intestine, especially the lamina propria, is filled with immune cells such as CD4 and CD8 T cells, B cells, macrophages and DCs. Initial priming of the lymphocytes takes place in GALTs or mesenteric lymph nodes (MLN) (Brandtzaeg et al., 2007; Mowat & Agace, 2014).

2.3.1 Immune cells and structure of the gut immune system

The lining of intestinal epithelial cells (IECs), covered with a mucus layer, form a physical barrier between the microbes in the lumen and the host (Peterson & Artis, 2014). IECs have multiple immunological roles and they can sense microbial antigens directly from the lumen via pattern-recognition receptors including TLRs and NOD-like receptors (NLRs) (Goto, 2019). Specialized IECs, called M cells, can

also transport antigens through the epithelial layer to DCs in lamina propria (Jang et al., 2004). APCs, mainly DCs, in the lamina propria sense and take up antigens derived from the intestinal lumen and can prime the adaptive immune system in secondary lymphoid organs (Mann & Li, 2014) (antigen uptake by DCs is discussed in more detail in the next section). Macrophages are essential in intestinal homeostasis with several different functions. First of all they can phagocytose microorganisms and dead cells, and promote tissue renewal (Mowat & Agace, 2014). After specific stimuli, such as via TLR signalling, they produce proinflammatory cytokines and can also promote FoxP3 Treg functions in the mucosa (Denning et al., 2007; Turovskaya et al., 2009; Ueda et al., 2010). Other important innate immune cells in intestinal immune protection and promotion of barrier function are innate lymphoid cells (ILCs). They do not express specific antigen receptors like T and B cells, but they mainly respond to surrounding cytokines produced by other cells (Geremia & Arancibia-Cárcamo, 2017). ILCs can be divided into IFNγ producing ILC1s; IL-5 and IL-13 producing ILC2s; and IL-17 and IL-22 producing ILC3s (Fan et al., 2019; Geremia & Arancibia-Cárcamo, 2017). All three have their own role in intestinal immune protection, where for example ILC3s promote intestinal barrier integrity by inducing secretion of antimicrobial peptides such as β-defensins and Reg3γ (Killig et al., 2014). By cytokine secretion, innate immune cells regulate lymphocyte responses and mediate their differentiation.

Most of the lymphocytes are located in the lamina propria where they promote tolerance and homeostasis under normal conditions. CD4 T cells comprise a diverse population of IL-2, IL-17 and IFNγ producing cells and Tregs (Ivanov et al., 2006; Sathaliyawala et al., 2013; Veenbergen & Samsom, 2012). The distribution of different CD4 cells varies slightly along the intestine, where it is reported that the number of Th17 cells decreases progressively from the small intestine towards the colon, while FoxP3+ Tregs are most frequently presented in the colon (Denning et al., 2011; Maynard et al., 2007; Veenbergen & Samsom, 2012). Lymphocytes can also be found within the epithelial layer and these intraepithelial lymphocytes (IELs) provide protective immunity in the epithelium (Cheroutre et al., 2011; Sheridan & Lefrançois, 2010). However, it is important to note that expansion of IELs has also been associated with intestinal inflammatory diseases, indicating their possible negative functions (Giacomelli et al., 2008; Kanazawa et al., 2001; Yeung et al., 2000). The intestinal lamina propria is filled with IgA producing plasmablasts, which are essential for maintaining homeostasis and barrier function in the gut by preventing microbial adhesion and penetration through the intestinal barrier (Pietrzak et al., 2020). The production of secretory IgA (SIgA) is highly dependent on microbiota and studies in GF mice show that SIgA production is almost completely non-existent without microbiota stimulus, and that by IgA production

can be rescued by colonizing GF mice with microbiota (Hapfelmeier et al., 2010; Li et al., 2020).

The intestine has compacted lymphocyte areas in close contact with the epithelial layer which form GALTs (Buettner & Lochner, 2016; Mörbe et al., 2021). The small intestine and colon differ slightly in their GALT formation; Payer's patches (PP) can only be found in small intestine. PPs contain separate B-cell follicles and distinct T cell areas (Pabst et al., 2005; Spencer et al., 1986). The epithelium covering the PPs has numerous M cells, which can transport antigens to DCs allowing a rapid lymphocyte response already in the intestine (Owen, 1999). Even though the colon does not have PPs, it does have similar lymphocyte areas called isolated lymphoid follicles (ILF), which can be found in both the small intestine and colon (Buettner & Lochner, 2016; Mörbe et al., 2021). Similar to PPs, ILFs are in close contact with the intestinal epithelium which contains antigen transporting M cells. Murine ILFs mainly contain B cells with few surrounding T cells, but in human ILFs, T cells are more dominant (Fenton et al., 2020; Pabst et al., 2005). Overall GALTs provide a key site for antigen recognition and induction of adaptive responses within the intestine.

The gut immune system is in continuous crosstalk with intestinal microbes. Commensal microbes are essential in maintaining immunological homeostasis and tolerance, and they even contribute to the development of the whole immune system in the intestine. Germ-free animals show significant defects in their intestinal immune system where they have fewer and smaller GALTs and MLNs, and reduced numbers of T cells and IgA producing B cells in lamina propria when compared to SPF mice (Bouskra et al., 2008; Falk et al., 1998; Macpherson & Harris, 2004; Umesaki et al., 1993). Crosstalk between commensal microbes and immune system shape each other to promote healthy homeostasis in the gut.

2.3.2 Antigen recognition by dendritic cells

Dendritic cells (DCs) are responsible for recognizing antigens and presenting them to lymphocytes in mesenteric lymph nodes. It is well established that most of the intestinal conventional (also referred as classical) DCs (cDC) express CD103 marker and they can be further divided into CD11b- (cDC1) and CD11b+ (cDC2) cells. All CD103+ cDCs express high levels of chemokine receptor CCR7 which is the main regulator of DC migration to lymph nodes through lymphatic vessels (Worbs et al., 2006). However, a population of CD103- DCs, with migratory ability, in lamina propria have also been described, but they remain poorly characterized (Cerovic et al., 2013). DCs in the lamina propria are known to acquire antigens from the lumen via multiple mechanisms. Intestinal DCs can directly sample luminal bacteria through the epithelial layer by extended DC-derived dendrites or take up antigens

which are transported from the lumen by forming goblet cell-associated antigen passages (GAPs) (Farache et al., 2013; McDole et al., 2012). DCs can also acquire luminal antigens indirectly via tissue resident macrophages and M cells within the epithelial layer (Jang et al., 2004; Mazzini et al., 2014). After DCs in the lamina propria have taken up the antigens derived from the lumen, they migrate to mesenteric draining lymph nodes (MLN) to present them to lymphocytes and prime the adaptive immune system. Steady state trafficking of DCs is crucially dependent on this CCR7 signalling and it is significantly enhanced by the TLR pathway and inflammatory cytokines.

The functions of different cDC populations in the intestine is not completely understood. CD103+ DCs from lamina propria and MLN have shown to promote tolerance by promoting naïve T cell differentiation into Tregs via a TGF-β and retinoic acid-dependent mechanism (Coombes et al., 2007; Sun et al., 2007). However, CD103- DCs in MLN fail to successfully promote Tregs even in the presence of TGF-β (Coombes et al., 2007). A distinct DC population, plasmacytoid dendritic cells (pDCs), also contribute to oral tolerance and immune regulatory effects in the lamina propria (Goubier et al., 2008; Mizuno et al., 2012). In the mesenteric lymph nodes, intestinal DCs prime the adaptive immune response by presenting antigens through MHC molecules. Intestinal CD103+ cDCs have the ability to induce the expression of gut-homing receptors $\alpha_4\beta_7$-integrin and CCR9 in CD4 and CD8 T cells, thus promoting their migration to intestinal tissues (Johansson-Lindbom et al., 2005).

2.3.3 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are generated in bone marrow from hematopoietic stem cells via myeloid and lymphoid pathways where signalling through Fms-like tyrosine kinase ligand (Flt3L) and its receptor is essential (Chen et al., 2004). After maturation, pDCs use blood circulation and high endothelial venules (HEVs) to reach lymph nodes, rather than using lymphatic circulation (Sozzani et al., 2010). pDCs also traffic to peripheral tissues, such as mucosal tissues, through blood circulation. In mouse, pDCs can be defined by their expression of CD11c, B220, bone marrow stromal antigen 2 (BST2, also known as PDCA-1) and sialic acid-binding immunoglobulin-like lectin H (Siglec-H) (Nakano et al., 2001).

pDCs are a heterogenous population and their functions are multifaceted and can have immunogenic and tolerogenic properties (Swiecki & Colonna, 2015). pDCs recognize DNA and RNA from viruses through TLR7 and TLR9 receptors which leads to IFN I production (Bode et al., 2016). When first described, pDCs were mainly known by their effective production of type I IFN, and their ability to present antigens was thought to be a secondary feature (Grouard et al., 1997). However,

further studies have shown that not all pDCs produce IFN α. In the GI tract, pDCs in Payer patches do not produce IFN α and after TLR recognition they stimulate Treg and Th17 cells (Contractor et al., 2007; Dasgupta et al., 2014; Li et al., 2011). pDCs are therefore important in promoting tolerance against harmless antigens, such as commensal microbes, and thus maintaining homeostasis in the gut. Tolerogenic pDCs can promote tolerance by expressing indoleamine 2,3-dioxygenase (IDO), programmed cell death protein 1 ligand 1 (PDL1) and T cell co-stimulator ligand (ICOSL) (Diana et al., 2011; Ito et al., 2007; Pallotta et al., 2011). pDCs can also promote Tregs by producing transforming growth factor-β (TGFβ) and IL-6, which contribute to Th17 cell generation as well, respectively. Although it seems that pDCs in the lamina propria do not migrate to MLNs even after external antigen stimuli (Yrlid et al., 2006), pDCs are extensively activated in colon draining lymph nodes during *C. rodentium* infection (Toivonen et al., 2016). This suggest that pDCs have potential priming abilities as well in intestinal infections. As already mentioned, pDCs are a heterogenous population and different phenotypes may have distinct effects on different immune responses and their role in the mucosal immune system requires further investigation.

2.3.4 Lymphatic structure of the intestine

The mammalian GI tract contains an extensive lymphatic network, which forms a large part of the immune system of the gut. The lymphatic vasculature offers a drainage route for the numerous immune cells in the intestine to the lymph nodes. Additionally, they contribute to the transportation of antigens, absorption of dietary substances and maintaining fluid balance. The lamina propria and submucosa are covered with lymphatic capillaries which drain through mesenteric draining lymph nodes to the thoracic duct and finally into the blood circulation. In the small intestine the lymphatic vasculature, referred to as lacteals, locate within the villus structures and the length of these lacteals is relative to villus length along the small intestine. When arriving to the colon, where there are no villus-like structures, the lymphatic vasculature is more regular along the lamina propria and submucosa. The wall of lymphatic capillaries are formed by lymphatic endothelial cells (LECs) which are connected to each other by discontinuous cell–cell button junctions allowing one-way entrance for lymph and cells (Baluk et al., 2007). The intestine itself contains only lymphatic capillaries which are connected to lymphatic collecting vessels in the mesentery (Unthank & Bohlen, 1988). In collecting lymphatic vessels LECs are connected with tight and continuous zipper junctions leading to much lower permeability of the vessels. Smooth muscle cells SMC, covering the collecting lymphatic vessels, pump the lymph forward and at the same time intraluminal lymphatic valves inside the vessels prevent lymph backflow (Scallan et al., 2016;

Schulte-Merker et al., 2011). The importance of the lymphatic vasculature is demonstrated in mice by depleting intestinal and lymph node lymphatics. The loss of lymphatic structure in the intestine leads to severe inflammation of the gut and mortality as soon as 60 hours after depletion (Jang et al., 2013).

Epithelial cells in the intestine can directly absorb long-chain fatty acids, cholesterol and fat-soluble vitamins by packing them into chylomicrons which can then enter into lymphatic capillaries through flap valves formed by button junctions (Dash et al., 2015; Mansbach & Siddiqi, 2012). Interestingly, bacterial lipopolysaccharides (LPS) from gram-negative bacteria are also absorbed by enterocytes in the same manner (Hornef et al., 2002; Vreugdenhil et al., 2003). Blocking of chylomicron secretion has been shown to decrease the blood LPS levels in mice (Ghoshal et al., 2009). As previously mentioned, goblet cells can transport luminal antigens and enable whole bacteria to translocate through the epithelium where they are then taken up by APCs (McDole et al., 2012). Like chylomicrons, antigens and DCs can enter lymphatic capillaries through flap valves. Dendritic cells migrate through the lymphatic vasculature to MLNs by following the gradient of LEC-derived CCL21 chemokine, a ligand for CCR7 (Pflicke & Sixt, 2009; Weber et al., 2013). Even though it has been suggested that bacteria are transported to MLNs intracellularly by phagocytic cells, reports showing living bacteria in MLNs indicate that bacteria can enter lymphatic capillaries and travel with the lymphatic flow.

Lymphatics from the intestinal lamina propria flow to specific compartmentalized draining MLNs (**Figure 3**), a key site for induction of adaptive immune responses (Macpherson & Smith, 2006). When first described, MLN was thought to drain from both small intestine and colon (Carter & Collins, 1974; Tilney, 1971). Anatomically this MLN forms a chain of lymph nodes located in the mesentery. Even though earlier studies have already indicated that different parts of the small intestine drain to different LNs in the MLN chain (Gautreaux et al., 1994; Van den Broeck et al., 2006), the specific draining MLNs for the colon, coMLNs, were not described until 2015-2016 (Houston et al., 2016; Toivonen et al., 2016). Since the physiology and immune environment change along the intestine, it is beneficial that different LNs are specialized for different regions of the intestine. The small intestine is specialized for food absorption and thus immune tolerance against food antigens, while immune tolerance in the colon is more directed to the large community of commensal microbes. Also, diseases, such as celiac disease, colitis ulcerosa and Crohn's disease, usually affect specific regions of the intestine, supporting the concept of specialized LNs. Similarly, a recent study by Esterházy et al. showed that even different small intestine MLNs (siMLNs) and coMLNs have different functions in intestinal immune responses, where immune response is shifted towards either tolerization or immune induction depending on the site of lymphatic draining (Esterházy et al., 2019).

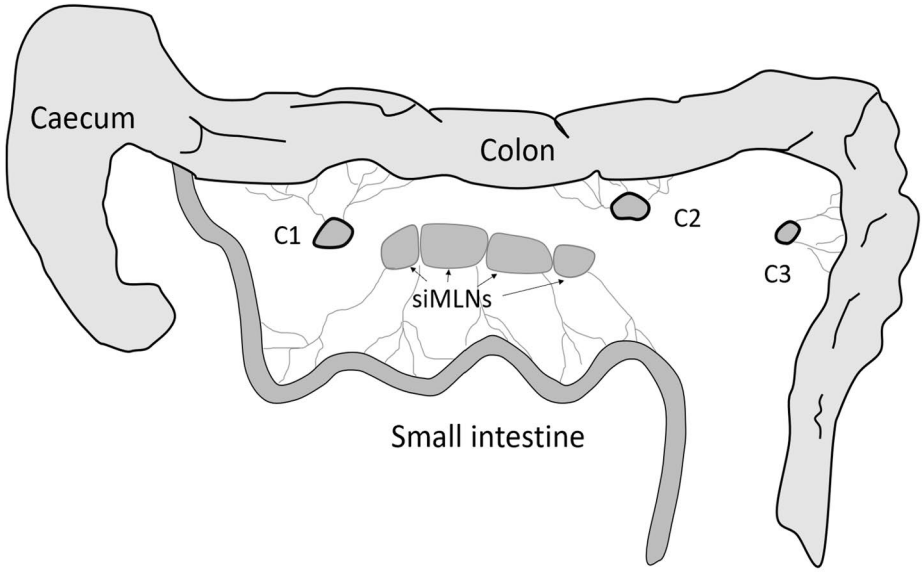

Figure 3. Illustration of the draining lymph nodes of the mouse intestine. The small intestine and the colon have specific compartmentalized draining lymph nodes. Three separate draining lymph nodes (C1-C3) enable the lymphatic draining from the colon. siMLN = small intestine mesenteric lymph node, C1-C3 = colon draining mesenteric lymph nodes. Based on Houston et al., 2016; Toivonen et al., 2016; Esterházy et al., 2019.

3 Aims

The composition of gut microbiota is associated with type 1 diabetes, but the underlying mechanisms are still poorly characterized. In this thesis we explore this topic with following aims:

1. To evaluate the role of gut microbiota in autoimmune diabetes in two nonobese diabetic (NOD) mouse colonies with either high or low diabetes incidence and determine whether we can identify specific microbes that have a significant effect on diabetes development.
2. To evaluate the role of plasmacytoid dendritic cells in maintaining gut integrity.
3. To visualize colon lymphatics and its draining lymph nodes with a particular focus on pancreatic lymph nodes and identify how dysbiosis promotes islet specific T cell activation in pancreatic lymph nodes.
4. To determine if dysbiosis-related low-grade endotoxemia affects autoimmune destruction of beta-cells.

4 Materials and Methods

4.1 Animals (I-IV)

All animal experiments were approved by the National Project Authorization Board of Finland (licenses: ESAVI/6082/04.10.07/2014 ESAVI/6608/04.10.07/2017; ESAVI/19866/2019, ESAVI/479/04.10.07/2016) in accordance with the EU Directive (2010/63/EU) or in Australia (St Vincent's Hospital Animal Ethics Committee number 017/17). Mice were bred and maintained in specific pathogen-free conditions with food and water ad libitum. All mouse strains used in studies I-IV are listed in the table 1.

Table 1. Mouse strains used in studies I-IV.

Strain	Provider	Characteristics	Studies
NOD/ShiLtJ	The Jackson Laboratory	Spontaneous diabetes development	Studies I, III, IV
NOD/MrkTac	Taconic	Spontaneous diabetes development	Study I
NOD.BDC2.5	The Jackson Laboratory	Islet specific CD4 cells	Study III
NOD.8.3	The Jackson Laboratory	Islet specific CD8 cells	Studies III, IV
BDCA2-DTR	The Jackson Laboratory	Conditional pDC depletion	Study I
C57BL/6	UTUCAL	Wild type controls	Studies II, III, IV
Tlr4 KO	The Jackson Laboratory	TLR4 knock-out	Study IV
KikGR	UTUCAL	Photoconvertible cells	Study III
NOD-Rag1^{null}	The Jackson Laboratory	No mature T cells and B cells	Study IV
NOD.*Ifnar1*	Monash University/ Australia	IFN-α KO	Study IV
NOD.*Ifngr1*	Monash University/ Australia	IFN-γ KO	Study IV
NOD.*Ifnlr1*	Monash University/ Australia	IFN-λ KO	Study IV
NOD.*Ifnar1/Ifngr1/Ifnlr1*	Monash University/ Australia	Triple IFN KO	Study IV

In Study I WT NOD colonies from two different providers were used. NOD/ShiltJ (NOD/Jax in the article) were provided by The Jackson Laboratory and NOD/MrkTac mice were provided by Taconic. Both NOD mice colonies were kept in separate units to avoid contamination. Female mice at the age of 6-8 weeks were used except in the diabetes development assessment experiment where NOD mice were monitored up to 30 weeks.

Plasmacytoid dendritic cells were studied by using the BDCA2-DTR (C57BL/6-Tg(CLEC4C-HBEGF)956Cln/J) mouse strain with a simian diphtheria toxin receptor (DTR) under a human C-type lectin domain family 4, member C promoter (*CLEC4C* or *BDCA2)*. Injection of diphtheria toxin (DT) to BDCA2-DTR mice leads to pDC depletion through the DT receptor. In study II, both male and female mice were used at the age of 6-8 weeks and BDCA2-DTR mice with conditionally depleted pDCs were compared to WT C57BL/6 mice.

KikGR (also known as Kikume) mice were used to observe cell trafficking from the colon wall to surrounding lymph nodes. KikGR mice were generated at UTUCAL via embryonic transfer using embryos received from Osaka University. KikGR mice express photoconvertible fluorescent protein Kikume Green-Red in all cells which fluoresces green until exposure to UV light after which they fluoresce red. WT NOD and C57BL/6 mice were used to evaluate bacterial antigen dislocation from the gut to lymph nodes.

The autoimmune effects of the dysbiotic gut were studied with WT NOD mice and transgenic NOD mice. More precisely NOD.BDC2.5 (NOD.Cg-Tg(TcraBDC2.5, TcrbBDC2.5)1Doi/ DoiJ) and NOD.8.3 (NOD.Cg-Tg(TcraTcrb NY8.3)1Pesa /DvsJ) mice were used to study islet-specific autoreactive T cells. NOD.BDC2.5 transgenic mice are characterized by their autoreactive CD4 T cells which express rearranged *Tcra* and *Tcrb* genes from the diabetogenic H2-A^{g7} restricted BDC2.5 Cd4+ T cell clone. NOD.8.3 transgenic mice are essential for studying islet-specific CD8 T cells since they express rearranged *Tcra* and *Tcrb* genes derived from the pancreatic beta cell cytotoxic CD8+ T cell clone NY8.3. These autoreactive CD8 T cells recognized islet β-cell antigens including islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP). While WT NOD mice develop diabetes spontaneously in 20 weeks on average, transgenic mice NOD.BDC2.5 and NOD.8.3 mice can develop diabetes at the age of 4-5 weeks. TLR4-KO (B6(Cg)-Tlr4tm1.2Karp/J) mice were used to evaluate the role of TLR4 signalling in induction of CXCL10 in pancreatic islets. IFN-α (IFNAR), IFN-γ (IFNGR), IFN-λ (IFNLR) and triple IFN knock-out NOD mice were generated in the Australian Phenomics Facility (Monash University, Clayton, Australia) with CRISPR/Cas9 gene editing by targeting *Ifnar1, Ifngr1* and *Ifnlr1*. Rag1-KO (NOD.Cg-$Rag1^{tm1/Mom}$/J) and different IFN-KO NOD mice were used to study CXCL10 production in pancreatic islets and were maintained at St Vincent's Institute (Australia).

4.2 Microbiota analysis (I, III)

Gut microbiota profiling was performed by 16S rRNA parallel sequencing. Bacterial DNA was isolated from stool or colon contents by using a QIAamp DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions. 16S rRNA sequencing was performed from amplicons of V4–V5 or V3–V4 variable regions by Illumina MiSeq system (Illumina, USA) as described previously (Toivonen et al., 2014). Data were analysed using QIIME software.

4.3 Oral transfer of microbiota and cohousing (I)

To study if gut microbiota from low diabetes incidence NOD mice (NOD/MrkTac) can rescue high diabetes incidence NOD mice (NOD/Jax) from developing early diabetes, microbiota was transferred either directly by oral gavage or by cohousing. For oral transfer of microbiota, caecal and colon contents of female NOD/MrkTac or NOD/Jax mice were dissolved and pooled in anaerobic PBS. Total bacteria were collected from the supernatant after centrifugation and $1x10^{10}$ (330µl) of bacterial suspension was administered orally by gavaging female NOD/Jax pups twice with a 3-day interval between. Pups from several different litters were allocated into two groups where one group received microbiota from NOD/MrkTac and the other group from NOD/Jax. For cohousing, pregnant NOD/Jax mice were kept in the same cage with NOD/MrkTac female mice for 14 days. Mice were separated before NOD/Jax dams gave birth.

4.4 Bacterial cultures and oral feeding (I-IV)

4.4.1 *Akkermansia muciniphila*

In study I the *A. muciniphila* strain MucT (CIP 107961T) was cultured and obtained from Willem de Vos laboratory (Wageningen University, Netherlands). Culturing was performed in strictly anaerobic conditions. Cultured *A. muciniphila* was washed with PBS and frozen in PBS containing 25% glycerol. Frozen bacterial stocks were shipped to the University of Turku where they were thawed and resuspended in anaerobic PBS prior to administration to mice. Immediately after resuspension, 200 µl of $2x10^8$ cfu *A. muciniphila* was administered orally to mice by gavaging.

4.4.2 *Citrobacter rodentium*

C. rodentium (strain ICC168, originally from G. Frankel, Imperial College, London, Obtained from F. Powrie, University of Oxford, UK) was cultured in Luria Broth

(LB) medium with Nalidixic acid (50 mg/L, Sigma) overnight (37°C). The bacterial culture was centrifuged at 800 g for 10 minutes (10°C) and concentrated 10 times. $1x10^9$ (200 µl) of concentrated *C. rodentium* solution was orally administered to mice.

In study III, GFP-expressing *C. rodentium* was used to identify bacterial matter movement from gut to lymph nodes. GFP-*C. rodentium* was created by transfecting GFP plasmid (pAIDkiGFP4) to *C. rodentium* by heat shock. For heat shock transfection *C. rodentium* was cultured overnight as described above. 1 ml of bacterial culture was centrifuged 800 g for 10 minutes (10°C) and resuspended in 500µl of $CaCl_2$ solution. The bacterial suspension was kept on ice for 20 min before adding GFP-plasmid (4000 ng/µl) to the bacterial suspension. The bacterial suspension was kept on ice again for 20 min before performing heat shock at 42 °C for 90 sec. After heat shock the bacterial suspension was kept on ice for 2 min. 2 ml of LB medium was added to the suspension and incubated at 37°C for 1 hour. GFP transfected *C. rodentium* were identified by plating 10µl of bacteria solution onto ampicillin LA plates and incubating overnight at 37°C. GFP transfection was confirmed with flow cytometry and fluorescence microscopy. GFP-*C. rodentium* was cultured and prepared for oral feeding as described above.

4.4.3 *Ruminococcus gnavus*

A clinical isolate of *R. gnavus* was obtained from Turku University Hospital and identified by MALDI-TOF. *R. gnavus* was cultured in fastidious anaerobe (FAB) broth (+ 5g/L glucose) in strictly anaerobic conditions at 37 °C for 48 hrs. Anaerobic conditions were achieved by using an anaerobic cylinder and anaerobic gas generating sachets (Oxoid™ AnaeroGen™, Thermo Scientific, USA). After 48h the bacterial culture was centrifuged at 3000 g for 10 minutes and the supernatant was discarded. The bacteria were resuspended in a volume of 200 µl (1×10^7 bacteria). Oral feeding was performed immediately by gavage as described above to limit the time bacteria were exposed to oxygen. To induce dysbiosis with *R. gnavus*, mice were first treated with a cocktail of broad-spectrum antibiotics (ampicillin 100mg/kg, vancomycin 50mg/kg, neomycin 100mg/kg and metronidazole 100mg/kg) by oral gavage five (5) days prior to infection with *R. gnavus*.

4.5 Determination of diabetes and insulitis (I, IV)

In follow-up experiments diabetes development was monitored by measuring blood glucose levels weekly from week 10 to 30 weeks. Blood glucose was measured from blood samples drawn from the tail vein and measured by a quick blood glucose meter (Contour, Germany). Mice with values of 12.0 mmol/L or higher were considered as

diabetic. Two consecutive readings on the following days were performed to confirm this. After mice were diagnosed with diabetes, they were sacrificed immediately.

Insulitis development was also used to assess development of diabetes before disease presentation. B-cell destruction was analysed by collecting the pancreas from sacrificed mice and quickly freezing them in liquid nitrogen. Cryosections were prepared from frozen pancreas samples and after fixation with ice-cold acetone, sections were stained with Haematoxylin and eosin (H&E) staining. Stained sections were analysed under a microscope and insulitis scores were evaluated from each pancreas. Scores from 0-3 were given to each islet based on the area of leukocyte infiltration. 50-70 islets were analysed per pancreas.

4.6 Gut permeability and serum endotoxin measurement (I, II, IV)

Serum samples were prepared from blood aliquots collected via cardiac puncture after CO_2 euthanasia of the mice. Intestinal permeability was analysed by measuring haptoglobin levels from the serum samples. A Haptoglobin ELISA Kit (ABCAM, ab272472, UK) was used to measure Haptoglobin according to the manufacturer's instructions using 1:10 000 and 1:100 dilutions.

Serum endotoxin levels were evaluated by measuring lipopolysaccharide (LPS) or lipopolysaccharide binding protein (LBP) from the serum samples. Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, USA) was used to measure LPS (study I) according to the manufacturer's instructions. A Mouse LBP ELISA Kit (ABCAM, ab213876, UK) was used to measure LBP (studies II and IV).

4.7 Immunohistochemistry and In situ hybridization from tissue samples (I-IV)

Tissue samples for immunohistochemistry analysis were snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT before sectioning. Frozen tissues were sliced into 6-8µm cryosections and placed onto Superfrost plus slides (Thermo Scientific, USA). Sections were fixed with ice-cold acetone at -20°C for 5 min before staining with selected antibodies. Sections were covered with ProLong Diamond Antifade Mountant with or without DAPI. Stained sections were imaged with Nikon Eclipse Ti-2 microscope (Japan) and photographed with Hamamatsu sCMOS Orca-Flash4.0 camera (Japan). Imaged sections were analysed with ImageJ software.

In situ hybridization was used to identify and image bacterial DNA from colon and lymph node samples. Tissues were collected to formalin and fixed for 24h before transferring them to 70% EtOH. Fixed tissues were casted into paraffin blocks and 5 µm sections were cut onto silane coated glasses. Deparaffination was performed

by first heating sections in 60°C for 10 min followed by incubation in 60°C xylene for 10 min. Sections were moved into fresh xylene in RT for 10 min and then to 99,5% EtOH for 5 min. Sections were dried before hybridization. To identify bacterial DNA, Alexa Fluor 647-conjugated EUB-338 probe (5′-GCTGCCTCCCGTAGGAGT-3′; 10 µg/ml, Invitrogen, USA) was applied onto the sections in hybridization buffer (20mM Tris-HCl, 0.9M NaCl, 0.1% SDS, 20% formamid, pH 7.4), and then the sections were covered with a coverglass and incubated at 50°C overnight. After incubation sections were washed 3 x 10 min in PBS before adding blocking solution (PBS, 5% FBS) for 30 min (RT)

Table 2. Antibodies used in immunohistochemistry.

DIRECT CONJUGATED ANTIBODIES

Antibody	Label	Clone	Provider	Study
CD4	Alexa Fluor 488	GK1.5	BioLegend	I, II, IV
CD8	APC	53–6.7	BioLegend	II, IV
CD11B	FITC	M1/70	BioLegend	II
CD19	Brilliant Violet 421	6D5	BioLegend	IV
CXCR3	Brilliant Violet 421	CXCR3-173	BioLegend	IV
F4/80	APC	BM8	BioLegend	II, III
FOXP3	eFluor615	XMG1.2	eBioScience	I
FOXP3	PE	XMG1.2	BioLegend	II
TCRB	FITC	H57-597	BioLegend	IV

PRIMARY-SECONDARY ANTIBODIES

Primary antibody	Provider	Secondary antibody	Provider	Study
RABBIT ANTI-TLR2	ThermoFisher	Anti-Rabbit AlexaFluor 488	Invitrogen	I
RABBIT ANTI-TLR2	Invitrogen	Anti-Rabbit AlexaFluor 647	Invitrogen	IV
RABBIT ANTI-TLR4	Abcam	Anti-Rabbit AlexaFluor 647	Invitrogen	I, IV
RABBIT ANTI-MUC2	Santa Cruz Biotechnology	Anti-Rabbit AlexaFluor 488	Abcam	I
RABBIT H-300 (MUC2)	Santa Cruz Biotechnology	Anti-Rabbit AlexaFluor 488	Abcam	II
RAT ANTI-CD45	BioLegend	Anti-Rat AlexaFluor 647	BioLegend	IV
RABBIT ANTI-CCL2	Novus Biologicals	Anti-Rabbit AlexaFluor 647	Invitrogen	IV
GOAT ANTI-CCL4	R&D Biosystems	Anti-Goat AlexaFluor 555	Invitrogen	IV
GOAT ANTI-CCL5	R&D Biosystems	Anti-Goat AlexaFluor 555	Invitrogen	IV
GOAT ANTI-CXCL10	R&D Biosystems	Anti-Goat AlexaFluor 555	Invitrogen	IV
RABBIT ANTI-CXCL12	CellSciences	Anti-Rabbit AlexaFluor 647	Invitrogen	IV

4.8 Flow Cytometry (II-IV)

For flow cytometry analysis of tissues (lymph nodes and spleen), tissues were collected into RPMI. Single cell suspensions were prepared after collagenase digestion (100µg/ml, 10min, 37°C, Sigma-Aldrich, USA) by pressing tissues through metal mesh. Cells were washed with FACS I buffer (PBS, 2% FBS, 0,01% NaN3). Osmotic blood cell lysis was performed for spleen samples by adding 1,6% NaCl (10 sec) and quickly adding 0,2% NaCl. Cell counts were calculated from 1:1 Trypan Blue cell solution with an automated TC20 cell counter (Bio-Rad, USA). 1-2 million cells were stained with selected fluorochrome conjugated antibodies in FACS I buffer. Zombie Red or Aqua (Biolegend, USA) were used to stain dead cells. In experiments with intracellular stainings, cells were permeabilized and fixed with a Transcription Factor Buffer set according to the manufacturer's protocol prior to antibody staining. In studies II and III to measure IFNγ production, cells were stimulated with Cell Activation mixture (Cat#423304, BD Bioscienses, USA) in DMEM 10% FBS for 4h (37°C) prior to cell staining. To identify islet antigen recognizing T cells in study IV, APC-conjugated Insulin B15-23 (VYLKTNVFL) and IGRP 206-214 (LYLVCGERG) in complex with H2K(d) MHC tetramers were used together with an irrelevant control peptide PE-conjugated LO91-99 tetramer at a final concentration of 1:500. Tetramers were obtained from the NIH Tetramer Core Facility (Emory University, USA). Stained cells were washed with FACS I and FACS II buffer (PBS, 0,01% NaN3) and stained cells were analysed with either LSRFortessa (BD Bioscienses, USA) or NovoCyte (Agilent, USA) flow cytometers. Flow cytometry data were analysed with FlowJo and NovoExperss (Agilent, USA) software.

Table 3. Antibodies used in flow cytometry.

Antibody	Label	Clone	Provider	Study
B220	APC	RA3-6B2	BioLegend	II
CD3	PE-Dazzle594	17A2	BioLegend	II, III
CD3	BV421	17A2	BioLegend	II, III
CD4	FITC	RM4-5	BioLegend	II, IV
CD4	AlexaFluor 488	GK1.5	BioLegend	III
CD4	PE-Cy5	GK1.5	BioLegend	III
CD8	APC/Cy7	53-6.7	BioLegend	II, IV
CD8	APC	53-6.7	BioLegend	II, III
CD11b	AlexaFluor 700	M1/70	BioLegend	III
CD11b	FITC	M1/70	BioLegend	III
CD11c	FITC	N418	BioLegend	II
CD11c	APC-Fire750	N418	BioLegend	III
CD11c	Brilliant violet 605	N418	BioLegend	III
CD19	PE-Dazzle594	6D5	BioLegend	II, III
CD19	Brillian violet 510	6D5	BioLegend	III
CD25	APC/Cy7	PC61	BioLegend	II
CD44	PerCP-Cy5.5	IM7	BioLegend	III, IV
CD45	Brilliant violet 785	30-F11	BioLegend	II, III, IV
CD64	PE-Cy7	X54-5/7.1	BioLegend	III
CD69	PE	H1.2F3	BioLegend	III, IV
CD80	APC	16-10A1	BioLegend	II, III
CD86	APC-Fire750	GL-1	BioLegend	II, III
CD103	Brilliant violet 421	2E7	BioLegend	III
CD103	Brilliant violet 510	2E7	BioLegend	II, III
CD103	APC	2E7	BioLegend	III
CXCR3	Brilliant violet 421	CXCR3-173	BioLegend	IV
IFNγ	PE	XMG1.2	BioLegend	II, III
PDCA-1	BV650	927	BioLegend	II
MHCII*	PE	OX-6	BioLegend	III
MHCII	Brilliant violet 421	M5/114.15.2	BioLegend	II, III
TCRβ	AlexaFluor 700	H57-597	BioLegend	III
TCRβ	Brillian violet 510	H57-597	BioLegend	II, III, IV
XCR1	Brilliant violet 650	ZET	BioLegend	III
XCR1	Brillian violet 421	ZET	BioLegend	III

4.9 RNA isolation and quantitative PCR (I-IV)

For RT qPCR analysis tissues were collected into RNA later (Qiagen, USA) and RNA was isolated with a PowerLyzer® UltraClean® Tissue & Cells RNA Isolation Kit (MoBio, USA) according to the manufacturer's instructions. A DNase Max Kit (Qiagen, USA) was used to remove genomic DNA according to the manufacturer's instructions. First strand cDNA synthesis was performed by using 1000ng of RNA in reaction with Maxima Reverse Transcriptase and oligo-dT primers. A 1:10 dilution of cDNA and LightCycler® 480 SYBR Green I Master (Roche, Switzerland) solution was used for qPCR, and the amplified product was detected using a LightCycler 480 (Roche, Switzerland). Ct values were normalized to β-actin expression and the relative expression of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

4.10 Visualization of lymphatics and antigen flow from gut to lymph nodes (III)

To study if an anatomic route from the gut to pancreatic lymph nodes exists, we injected FITC-labelled dextran subserosally into the intestinal wall. NOD mice were anesthetized using a 150 mg/kg ketamine (Ketalar 50mg/ml, Pfizer, USA) and 10 mg/kg xylazine (Rompun vet 20mg/ml, Bayer, Germany) mixture i.p. A small incision was made to gently reveal the caecum, small intestine and colon. 70kD FITC-Dx was injected into the wall of small intestine or colon. The incision was closed and mice were kept anesthetized for 30 min. Mice were kept on top of the heating pad to ensure sufficient fluidic circulation. After 30 min mice were sacrificed and a tissue block including caecum, small intestine and colon was prepared on a petri dish. To allow for better visualisation of the lymphatic vasculature, the small intestine was excluded from the tissue block when the colon was the only section that was injected. Tissue blocks were washed with PBS and fresh PBS was added to the petri dish. The FITC signal was visualized and analysed with a stereo microscope (Zeiss AxioZoom.V16, Carl Zeiss, Germany). After the lymphatics were imaged, pancreatic LNs were separated from other tissues to visualize FITC-Dx loading into subcapsular sinus of the pancreatic LNs.

The flow of antigen from the gut to lymph nodes and the uptake of antigen by APCs were studied by injecting Alexa Fluor 647 labelled ovalbumin (OVA-A647) into the colon wall of anesthetized NOD mice as described above. After 1 hour, mice were sacrificed and LNs were collected and single cell suspensions were prepared for flow cytometry analysis. The OVA-A647 signal was measured from $MHCII^+/CD11c^+$ dendritic cells and $MHCII^+/CD64^+$ macrophages.

4.11 Cell migration from colon to lymph nodes using KikGR mouse model (III)

Mice constitutively expressing the photoconvertible Kikume-protein ubiquitously in all cells (KikGR mice) were anesthetized with isoflurane, shaved, and their skin was antiseptically treated with iodine. A 20 mm skin incision was made anteriorly at the midline just below the costal margin. The peritoneal membrane was similarly incised. The cecum was identified and gently externalized for localization of the large intestine. Sterile foil with a narrow opening in the middle was placed over the trunk of the mouse, and the opening was placed such that a 15 mm long segment of the proximal colon could be visualized through the opening.

A Silver LED 415 (Prizmatix), set to maximum intensity, with a high numerical aperture polymer optical fiber (core diameter, 1.5 mm) light guide and fiber collimator, was used as a 415 nm violet light source. The proximal colon was exposed to light for 3 min and kept wet with sterile NaCl solution during the procedure while care was taken not to illuminate other parts of the intestine or the lymph nodes in mesenterium. The abdominal cavity and the skin were closed with 5-0 absorbable sutures (Vicryl™, Ethicon) in two layers. Buprenorpohine (0,3 mg/ml Temgesic) was administered s.c. 30 min before the operation and twice daily after the operation. Tissues were collected 24h after the procedure.

4.12 Adoptive transfer of BDC2.5 cells (III)

Lymph nodes were collected from female 5-week-old BDC2.5 NOD mice to RPMI medium. Single cell suspensions were prepared by pressing tissues through metal mesh as described earlier (without collagenase digestion). A CellTrace CFSE Cell Proliferation Kit (Thermo Scientific, USA) was used to stain lymph node cells. 10^7 cells were stained in 1 ml staining solution (1:1000 CFSE stain in PBS) at 37°C for 10 min. Cells were washed with 5 times volume of RPMI 10%FBS and centrifuged at 600g for 10 min. The supernatant was discarded and cells were resuspended in fresh RPMI at a concentration of 5 x 10^6 cells / ml. 200µl of stained cells (1 x 10^6) were injected into the tail vein of recipient female NOD mice. 20 µg of FTY720 (Cayman Chemical Company, USA) was injected to mice daily to prevent lymphocyte egress from lymph nodes. Lymph nodes from recipient NOD mice were collected 5 days after adoptive transfer and BDC2.5 proliferation was analyzed with flow cytometry.

4.13 *In vivo* induction of CXCL10 in pancreatic islets with LPS (IV)

To evaluate if direct LPS injections induce CXCL10 expression in islets *in vivo*, a 30 µg dose of LPS from *E. coli* (Sigma-Aldrich, USA) was injected to NOD and C57BL/6 mice. Different genetically modified NOD mice (described above) were used to evaluate the necessity of IFN-signalling for CXCL10 production *in vivo*. After 48 hours, pancreases were snap frozen and CXCL10 expression was analysed with immunohistochemistry (described above).

4.14 TLR4 inhibition *in vivo* (IV)

The effect of TLR4 inhibition on CXCL10 production in dysbiotic NOD mice was assessed by using daily intraperitoneal injections of TAK-242 (3mg/kg in PBS; Calbiochem, USA). Dysbiosis was induced in 5-week-old female NOD mice with *C. rodentium* as described earlier. CXCL10 expression from pancreatic islets was analysed by immunohistochemistry 7 days after inducing dysbiosis.

4.15 CXCL10 production *ex vivo* from stimulated islets (IV)

To evaluate CXCL10 production by islet cells, islets from 6-8 week old male NOD mice and knock-out NOD mice were isolated with collagenase P (Roche, Basel, Switzerland) and Histopaque-1077 (Sigma-Aldrich, USA) density gradient centrifugation as described previously (Graham et al., 2016). Handpicked islets (50 islets/well) were cultured in complete CMRL medium (10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamin) and stimulated with 100 U/ml IFNγ (Biolegend) and with LPS (LPS-EB Ultrapure, Jomar Life Research) in concentrations of 2, 100 or 500 ng/ml for 2-4 days. 100µl of supernatant was used to measure CXCL10 production with mouse CXCL10 ELISA kit (R&D Systems, USA) according to the manufacturer's instructions.

4.16 RNA sequencing of islets (IV)

RNA sequencing from LPS-stimulated 8 week old male NOD islets was performed with Illumina at Australian Genome Research Facility. Before sequencing, stimulated islets were stained with an anti-CD45 antibody and sorted into CD45+ cell and β-cell populations. Only β-cells were used for sequencing. FastQC (version 0.11.9) was used for quality control of 150Bp paired ends. Sliding window trimming method was used to trim low quality reads with Trimmomatic (version 0.40). Ensembl mouse genome (Mus_muscuslus.GRCm39) was used to build a STAR

genome index. The processed reads were aligned by STAR (version 2.6.1a) and quantified by HTSeq-count (version 2.0.2). DESeq2 (version 1.36.0) was used to determine if there was any difference in terms of gene expression levels. Differentially expressed genes were extracted before performing gene ontology and pathway enrichment analysis with gprofiler2 (version 0.2.1)

5 Results

5.1 *Akkermansia muciniphila* controls autoimmune diabetes in NOD mice

5.1.1 Reduced diversity of gut microbiota is associated with high diabetes incidence

The development of diabetes differed between colonies that were from different providers. 50 % of the NOD/Jax mice developed diabetes by the age of 18 weeks while in NOD/MrkTac 50% of the mice did not develop diabetes until the age of 25 weeks (I, Figure 1). 16S rRNA sequencing and quantitative PCR showed significantly lower alpha diversity and Bacteroides-Firmicutes ratio in NOD/Jax mice when compared to NOD/MrkTac mice.

To study if transfer of microbiota from NOD/MrkTac mice to NOD/Jax mice could rescue mice from early diabetes we transferred gut microbiota by direct oral gavage or by cohousing. Both methods were sufficient to transfer NOD/MrkTac microbiota and increase the diversity in NOD/Jax mice. However, transfer of microbiota did not affect early diabetes development in NOD/Jax mice (I, Figure 2). When we analysed the microbiota after the transfer, we saw that not all taxa were found in NOD/Jax offspring after the transfer. *Akkermansia* was one of the few taxa that was present at low abundancy in NOD/MrkTac microbiota but was not found in the NOD/Jax microbiota (I, Figure 3). This suggest that these members of microbiota may be important in lowering the rate of diabetes development in NOD/MrkTac mice.

5.1.2 *Akkermansia muciniphila* delays diabetes development by promoting microbiota remodelling

Since the *Akkermansia* taxon was one of the few taxa which did not transfer from NOD/MrkTac to NOD/Jax mice, we were interested to evaluate if transferring *A. muciniphila* directly by oral gavage could delay diabetes development in NOD/Jax mice. We orally gavaged NOD/Jax pups three times a week from weaning to 10 weeks of age with 2×10^8 cfu of *A. muciniphila* and saw that *A. muciniphila*

significantly delayed diabetes when compared to vehicle treated mice (I, Figure 4A). At the age of 15 weeks only one NOD/Jax mice gavaged with *A. muciniphila* developed diabetes while in vehicle group 11 mice had developed diabetes. *A. muciniphila* transfer to NOD/Jax mice was determined to be successful by 16S rRNA sequencing and no *A. muciniphila* was found from vehicle treated mice (I, Figure 4B). At the same time *A. muciniphila* treatment promoted microbiota remodelling by shifting the Bacteroides-to-Firmicutes ratio in a similar manner to what was seen in NOD/MrkTac mice. At species level, *A. muciniphila* also reduced significantly *Ruminococcus torques* abundance in NOD/Jax mice.

PAS staining of colon sections showed that *A. muciniphila* promoted mucus production by goblet cells and reduced crypt length in NOD/Jax mice (I, Figure 4C-F). Additionally, *A. muciniphila* promoted anti-inflammatory properties in the colon and reduced stress-related genes in intestinal epithelial cells (I, Figure 4G). These favourable homeostatic conditions were reflected in the endotoxin levels in the serum which were significantly lower in NOD/Jax mice treated with *A. muciniphila* (I, Figure 4H).

To observe how improved barrier function and immune regulation in the colon affected diabetes pathogenesis in pancreatic islets, we analysed frozen sections from pancreatic islets after *A. muciniphila* treatment. In line with the reduced LPS level in the serum, TLR2 and TLR4 expression in islets were significantly reduced in *A. muciniphila*-gavaged NOD/Jax mice when compared to vehicle treated mice (I, Figure 5A-B). We also found that FoxP3+ Tregs were increased in islets indicating that *A. muciniphila* promotes immune regulation in pancreatic islets (I, Figure 5C-G). Furthermore, *A. muciniphila* treatment reduced overall leukocyte infiltration and increased anti-inflammatory cytokine expression in pancreatic islets.

5.2 Plasmacytoid dendritic cells regulate immune response against *Citrobacter rodentium* infection

5.2.1 pDCs control colitis development and bacterial invasion into colon mucosa

It has already been shown that pDC depletion in *C. rodentium* infection causes severe colitis (Rahman et al., 2019). To study the role of pDCs in bacterial infection more precisely, we infected pDC-deficient BDCA2-DTR and WT C57BL/6 mice by oral gavage with *C. rodentium*. pDC-deficient mice developed severe colitis already 5 days after infection while no significant signs of colitis were observed in WT mice. BDCA2-DTR mice lacking pDCs lose 20% of their body weight (II, Figure 1A) and had severe colitis-like symptoms in the colon, including shortening of the colon,

lengthened crypts and loss of goblet cells in the colon epithelium (II, Supp. Figure 1). In addition, stress-related gene expression was upregulated which was associated with increased intestinal permeability (II, Supp. Figure 2). Impairment of gut barrier function was reflected in the penetration of bacteria into the colon epithelium. *In situ* hybridization showed that bacteria had invaded into the epithelial layer and between epithelial cells suggesting that pDCs contribute to controlling intestinal barrier integrity (II, Figure 1B-C).

5.2.2 pDC-depletion leads to impairment of immune regulation in the colon and coMLNs

Since *C. rodentium* induced severe inflammation and colitis in pDC-depleted mice, we wanted to study more precisely how pDC-deficiency during *C. rodentium* infection affects immune responses. Quantitative PCR showed that the expression of proinflammatory cytokines such as *Il-1β* and *Tnf* are increased in pDC-depleted BDCA2-DTR mice (II, Figure 2A) and when we analysed colon sections with immunohistochemistry, we also found that the number of macrophages is increased in the colon epithelium (II, Figure 2B-C). This showed that the lack of pDC in the context of *C. rodentium* infection results in an enhanced inflammatory response locally in the colon epithelium. At the same time when we analysed T cells in the colon, we found that regulatory FoxP3 positive T cells were reduced in BDCA2-DTR mice indicating a reduction in immune regulation (II, Figure 2D-F).

To evaluate whether the altered immune regulation in the colon is observed in colon draining lymph nodes, we performed flow cytometry analysis from previously identified lymph nodes (coMLN1 and coMLN2) (II, Figure 3). It has previously been shown that during *C. rodentium* infection pDCs are activated in coMLNs and more genes are upregulated in pDCs when compared to cDCs (Toivonen et al., 2016). When we depleted pDCs and analysed coMLNs after *C. rodentium* infection, we found that the lack of pDCs significantly increased the activation of cDCs (II, Figure 3A-B) suggesting that pDCs counteract pathways of full cDC activation in coMLNs. Since cDCs present antigens directly to T cells we wanted to evaluate how pDC depletion affects T cell responses during *C. rodentium* infection. When we analysed CD4 and CD8 cells we saw an increase in IFNγ production by both cell types (II, Figure 3CE-F), indicating an augmented Th1 cell and effector T cell response. In contrast, regulatory $FoxP3^{+}/CD25^{+}$ T cells were reduced in pDC -depleted mice (II, Figure 3C). This suggests that pDCs are important for activation of Tregs and for controlling the T cell-mediated immune response against *C. rodentium* infection in colon draining lymph nodes.

5.3 *Citrobacter rodentium* infection activates lymphatic route from gut to pancreatic lymph nodes

5.3.1 Lymphatic route from colon to pancreatic lymph nodes

It has been previously reported that DCs from the colon migrate into specific colon-draining lymph nodes (Houston et al., 2016; Toivonen et al., 2016). However, inflammation in the colon or alteration in gut microbiota have been suggested to increase activation of islet specific T cells in PaLNs. We wanted to address the possibility that a direct lymphatic route from the colon to PaLNs may exist. We injected fluorescent dextran (FITC-Dx) into the colon wall and visualized the connection of the colon and PaLNs. After injecting FITC-Dx into two different spots of the proximal and distal colon of NOD mice we observed that FITC-Dx accumulated into colon-draining lymph nodes as described earlier. However, we were able to see accumulation of FITC-Dx under the capsule of the PaLNs as well (III, Figure 1). Further experimentation with injections of AlexaFluor647 conjugated ovalbumin (OVA-A647) showed that OVA from the colon wall reaches PaLNs and is taken up by macrophages and DCs (Original publication III, Figure 2). At the same time no OVA signal was observed in brachial lymph nodes (BLN) indicating that most likely OVA flows directly into PaLNs rather than via systemic circulation. These results suggest that there is a direct lymphatic route between the colon and PaLNs.

5.3.2 *Citrobacter*-induced dysbiosis promotes DC and bacterial migration from the colon to pancreatic lymph nodes

Since we already found that there is a connection between the colon and PaLNs, we wanted to explore if DCs can migrate from the colon to PaLNs as well. We used the Kikume mouse model expressing photoconvertible Kikume-protein to observe DC migration from the colon in steady state and after *C. rodentium* infection (III, Figure 3). In steady state we found that DCs only migrate from the colon to colon-draining lymph nodes and not to PaLNs 24h after carefully exposing the colon wall to UV light (III, Figure 3A). However, when we infected the mice 3 days before exposing the colon wall to UV light, we were able to detect photoconverted DCs in the PaLNs as well, but not in BLNs (III, Figure 3B-D). Photoconverted cells in PaLNs after *C. rodentium* infection indicated that infection induces DC migration from the colon to PaLNs. Since no migrating cells were observed in BLNs, DCs most likely migrated through the lymphatic system between the colon and PaLNs.

We also wanted to study if bacteria are directed to PaLNs following infection in the colon. We infected NOD mice with *C. rodentium* and used *in situ* hybridization to detect 16S-RNA gene from lymph nodes seven days after infection. Bacterial DNA-derived fluorescence was significantly higher in both the coMLNs and PaLNs of infected mice when compared to non-infected mice (III, Figure 4A-B). Bacterial DNA was mainly located in the inner cortex of the lymph nodes while B-cell follicles were almost free of bacterial DNA. To study if bacteria from colon translocate to PaLNs in more detail, we infected NOD mice with GFP-expressing *C. rodentium.* Immunostaining of the lymph node sections with macrophage marker F4/80 showed that GFP signal was increased in the PaLNs of infected mice and that it co-localized with F4/80 in the medullary sinus of the lymph node (III, Figure 4D-E). This suggest that lymph itself can transport GFP-*C. rodentium*, or parts of it, to PaLNs where it is taken up by macrophages. Flow cytometry analysis of lymph node cells showed that dendritic cells in PaLNs also express GFP-*C. rodentium*-derived fluorescence (III, Figure 4F-G). GFP signal in CD103+ DCs indicate that some of the DCs can transport bacterial matter from the gut to PaLNs. Interestingly, resident CD103-XCR1+ DCs, cells that are capable of antigen cross-presentation, also expressed GFP in PaLNs.

5.3.3 Dysbiosis increases immune responses and autoreactive T cell activation in PaLNs

Since *C. rodentium* infection increased DC migration and bacterial translocation to PaLN, we wanted to evaluate how *C. rodentium* infection affects the immune response in PaLNs. Flow cytometry analysis of PaLN cells showed increased expression of co-stimulatory molecules CD80 and CD86 in DCs (III, Figure 5B-C), indicating increased activation due to *C. rodentium* infection. At the same time, IFNγ production by CD4 cells was significantly higher in infected mice (III, Figure 5D). To evaluate if *C. rodentium* infection promotes islet-specific T cell activation in more detail, we transferred islet specific T cells from BDC2.5 mice to WT NOD mice. BDC2.5 CD4 cells express TCR genes from diabetogenic T cell clones and proliferate spontaneously in the PaLNs of NOD mice. When recipient NOD mice were pre-treated with *C. rodentium,* proliferation of BDC2.5 CD4 cell was significantly enhanced when compared to non-infected mice (III, Figure 5E-F). In CD8 T cells, a similar increase in IFNγ production was seen in *C. rodentium*-infected mice (III, Figure 5G). To analyse if *C. rodentium* enhances islet-specific CD8 T cell activation, we infected 4-week-old transgenic NOD8.3 mice with *C. rodentium*. In NOD8.3 mice most of the CD8 T cells express T cell receptors recognizing islet-antigens. Flow cytometry analysis revealed a significant increase in CD44

expression after *C. rodentium* infection due to enhanced activation of islet-reactive CD8 T cells (III, Figure 5H).

5.4 Dysbiosis-induced CXCL10 targets pancreatic islets for autoimmunity

5.4.1 Dysbiosis accelerates diabetes and islet-autoimmunity in NOD mice by inducing CXCL10 expression

Dysbiosis induced by *C. rodentium* or *R. gnavus* did not have a significant visible effect on the health or well-being of NOD mice, but it led to impairment of the gut barrier function and increased LPS levels in the serum (IV, Figure 1A-B). The effects of dysbiosis in pancreatic islets were seen already 7 days after inducing dysbiosis. When analysing chemokine expression in the islets, CXCL10 was significantly increased after both *C. rodentium* and *R. gnavus* exposure (IV, Figure 1C-D). Since dysbiosis and elevated LPS levels also enhanced TLR2 and TLR4 expression in pancreatic islets, we wanted to investigate if CXCL10 expression is mediated by bacterial cell-wall components of gram-negative bacteria or by LPS via TLR4 recognition. TLR4 blocking by TAK-242 in NOD mice inhibited dysbiosis-induced CXCL10 expression in NOD mice (IV, Figure 3). Similarly, CXCL10 expression was lower in TLR4-KO mice during dysbiosis when compared to WT C57BL/6 mice.

We next analysed if the development of insulitis is affected by dysbiosis. At the day 14 of dysbiosis, insulitis was significantly accelerated in dysbiotic NOD mice when compared to control NOD mice (IV, Figure 1E-F). Accelerated insulitis development finally led to earlier onset of diabetes in dysbiotic NOD mice (IV, Figure 1G). By blocking free CXCL10 signalling with an anti-CXCL10 monoclonal antibody, insulitis development was inhibited by 40% in dysbiotic NOD mice, indicating that CXCL10 signalling is important for the progression of islet autoimmunity (IV, Figure 5).

5.4.2 CXCL10 induction during dysbiosis is mediated via LPS

Since LPS levels were increased in dysbiotic NOD mice, we wanted to study if systemically administered LPS can induce CXCL10 expression in islets. A single dose of 30µg LPS injection (I.P.) raised the expression of CXCL10 in NOD islets after 24 hours, an CXCL10 expression was even further accentuated in the following 24 hours (IV, Figure 2A). We also found that CXCL10 is more heavily induced by LPS in NOD mice when compared to C57BL/6 mice (IV, Figure 2B). Even without

induction, CXCL10 is more intense in NOD mice due to possible basal level dysbiosis that is characteristic of NOD mice. We wanted to study if LPS is a possible inductor of immune pathogenesis in islets, so we analysed CXCL10 expression *in vitro* from NOD islets. By stimulating islets with LPS, we found that 50ng/ml of LPS can increase the production of CXCL10 and when we costimulated islets with LPS and a low dose of IFNγ (100 U/ml), a concentration of 10ng/ml of LPS was enough to enhance the induction of CXCL10 (IV, Figure 2C).

Since T cells and resident macrophages are potent IFNγ producers and possibly contribute to CXCL10 secretion, we used different setups of *in vitro* stimulation of islet cells. LPS was able to induce CXCL10 secretion in T cell-deficient RAG1-KO NOD mice at the same level as in WT NOD mice, indicating that LPS induction of CXCL10 does not require T cells (IV, Figure 6A). Similarly, in IFNGR-KO NOD islets, LPS induced CXCL10 production and as expected, IFNγ was not able to induce or enhance CXCL10 production. After depleting macrophages with clodronate from NOD islets, LPS was unable to induce CXCL10 expression (IV, Figure 6B). This suggest that resident macrophages, also found from healthy islets, can potentially contribute to the initial induction of CXCL10 expression.

RNA sequencing from LPS-stimulated NOD islets also showed upregulation of CXCL10 in β-cells. Since these NOD islets contain resident macrophages, and taking into account that macrophages are needed for LPS-induced CXCL10 production in β-cells, LPS most likely induces resident macrophages to produce interferons and TNF, which consequently leads to CXCL10 promotion in β-cells. Our RNA sequencing data supports these mechanisms since both IFNγ and TNF signalling pathways are upregulated in β-cells (IV, Figure 7).

5.4.3 Dysbiosis enhances autoreactive T cell migration into pancreatic islets

Since autoreactive T cells are initially activated in PaLNs, we determined if dysbiosis has an effect on the ability of T cells to recognise β-cell antigens (IV, Figure 4). By using MHC-tetramers we studied insulin B15-23 or IGRP 206-214 specific T cells from the PaLNs of NOD mice. Even though we did not see any alteration in insulin-specific CD8 T cells during dysbiosis, IGRP-specific CD8 T cell levels were significantly increased in dysbiotic mice when compared to control NOD mice (IV, Figure 4A-B). Up to 50% of these IGRP-specific cells expressed the chemokine receptor CXCR3 (IV, Figure 4C-D), indicating their ability to migrate towards a gradient of CXCL10, observed in pancreatic islets during dysbiosis. By using transgenic 8.3NOD mice, in which over 90% of the CD8 T cells are IGRP-specific, we evaluated more of the dynamics of CXCR3 imprinting in PaLNs. First of all, *C. rodentium*-induced dysbiosis accelerated the activation of CD8 T cells in 4-week-

old male 8.3NOD mice (IV, Figure 4E-F). Based on the expressions of CD44 and CD69 we analysed the imprint of CXCR3 in different CD8 T cell activation phases (IV, Figure 4G-H). CXCR3 expression increased moderately during the transition to next activation phase and it was highest in the last activation phase (CD44+/CD69-). This is consistent with the CD69 expression, which is mediating the egress of T cells from the lymph nodes. Since a high proportion of IGRP-specific CD8 T cells were imprinted with CXCR3, we stained CD4, CD8 T cells and CD19 B cells from the anti-CXCL10 monoclonal antibody-treated dysbiotic mice and found that both CD4 and CD8 T cells are reduced in relation to B cells when compared to isotype-control-treated mice (IV, Figure 5).

6 Discussion

The role of gut microbiota in type 1 diabetes development has drawn more and more attention during the past decade. Even though there are strong associations between the composition of microbiota and diabetes, the exact mechanism by which microbiota influences pathogenesis is still unclear. Here we show that differences in gut microbiota are associated with diabetes incidence in NOD mice and that diabetes development can be affected by manipulating the gut microbiota. *A. muciniphila* is considered to be a beneficial symbiotic microbe and part of the normal human microbiota, promoting healthy homeostasis of the intestine. We show that transfer of *A. muciniphila* to NOD mice delays insulitis and overall diabetes development, and that this is mediated by its ability to promote intestinal integrity. Conversely, by inducing dysbiosis to NOD mice, intestinal barrier function is altered leading to accelerated diabetes development. We show how dysbiosis promotes islet autoimmunity and islet-specific T cell activation in PaLNs by utilising a direct lymphatic route from the gut.

6.1 Gut microbiota influences intestinal integrity

Dysbiosis drastically affects gut barrier integrity and the composition of gut microbiota is known to contribute to mucus layer formation and overall homeostasis of the intestine. We showed that by treating NOD mice with symbiotic *A. muciniphila*, intestinal barrier function can be enhanced while a completely opposite effect is seen when dysbiosis is induced in NOD mice. Studies have shown that *A. muciniphila* promotes mucus secretion in the intestine and the production of the antimicrobial peptide Reg3γ (Chelakkot et al., 2018; Everard et al., 2013; Reunanen et al., 2015; Shin et al., 2014). This is in line with our observations with NOD mice where *A. muciniphila* treatment thickened the mucus layer and resulted in decreased stress and increased Reg3γ expression within the intestinal epithelial layer. *A. muciniphila* also inhibits the colonization of pathobionts such as *Ruminococcus torques*, whose colonization is shown to be expanded in patients with Crohn's disease and ulcerative colitis (Png et al., 2010). The competitive nature of these two microbes was identified in a dietary intervention trial in Crohn's disease patients (Halmos et al., 2016), which resembles our observations in NOD mice following *A.*

muciniphila treatment. *A. muciniphila* can outcompete *R. torques* most likely via increased Reg3γ secretion or simply by competing from the same niche. In contrast, when we modelled dysbiosis in NOD mice by infecting the mice with *C. rodentium*, *A. muciniphila* was almost completely diminished. Metagenomic analysis of gut microbiota from several different patients has indicated that GI disorders are associated with a low abundance of *A. muciniphila* and thus it has been proposed that this low abundance of *A. muciniphila* could be considered as one of the main features in dysbiosis (Lopetuso et al., 2020).

Increased intestinal permeability is closely associated with intestinal dysbiosis in chronic intestinal inflammatory disorders. We modelled dysbiosis in mice using *C. rodentium* and with *R. gnavus*, which is part of normal human gut microbiota but considered to be a pathobiont (Hall et al., 2017; Henke et al., 2019). Even though inducing dysbiosis with these microbes did not cause clear symptomatic infection in our WT NOD colonies, the intestinal permeability was significantly increased. This led to increased LPS levels and endotoxemia in dysbiotic mice. In contrast, transfer of *A. muciniphila* to WT NOD mice lowered LPS levels showing its protective effect on intestinal barrier function. The thickness of the mucus layer is known to be essential factor in functional barrier function. For example, in colitis mouse models where, increased intestinal permeability is a common feature, the mucus layer thickness is decreased, allowing bacteria to penetrate into the epithelial layer and thereby exacerbating the disease (Johansson et al., 2008; Petersson et al., 2011). Similarly, mice with altered mucus layer development are more susceptible to colitis (An et al., 2007; Fu et al., 2011; Park et al., 2009). Mucus promoting microbes are decreased also during dysbiosis allowing microbes to be in closer contact with the host and its immune system (Levy et al., 2017; Meng et al., 2020). Inflammatory responses to penetrating bacteria can exacerbate the intestinal permeability further. Increased intestinal permeability has been associated with diabetes and increased haptoglobin/zonulin levels have been reported in individuals prior to clinical T1D diagnosis (Bosi et al., 2006; Sapone et al., 2006).

6.1.1 pDCs regulate inflammation in acute *Citrobacter rodentium* infection

As stated earlier, acute *C. rodentium* infection induces relatively mild and non-invasive inflammation of the colonic mucosa in immunocompetent WT mice. However, in our immunocompromised pDC-deficient mouse model, *C. rodentium* led to severe infection and inflammation in the colon. Plasmacytoid dendritic cells (pDC) are a unique set of dendritic cells with a particular capacity to produce type 1 IFNs and they play an important role in antiviral immunity (Cella et al., 1999). Apart

from this role, pDCs present antigens, produce chemokines for recruitment of other immune cells, promote NK-cell activation and augment Th1, Th17 and Treg responses, and thus, may be considered as an important type of DC both in innate and adaptive immune responses (Swiecki & Colonna, 2015). GALT represents an unique environment for pDCs, as pDCs in Peyer's patches (Contractor et al., 2007) and in colon-draining mesenteric lymph nodes (Toivonen et al., 2016) are conditioned to not produce type 1 IFNs. In response to signals such as IDO (Pallotta et al., 2011) or TGFβ and IL-6, pDCs activate Tregs or Th17 cells (Bonnefoy et al., 2011), respectively. Even though pDCs are able to promote Tregs and Th17 cells, which contribute to maintenance of intestinal homeostasis, the role of pDCs in the immune response to bacterial infection in the colon has received relatively little attention. The role of pDCs in controlling immune responses and inflammation in the intestine has been puzzling, as pDCs do not control colitis induced by DSS but in fact, aggravate it (Arimura et al., 2017). However, a study made by Rahman et al., and supported by our observations, highlights a protective role for pDCs in *C. rodentium* -induced colitis, where they control inflammation in the colon (Rahman et al., 2019).

Although there are multiple local responses to pathogen, all of which are important (Collins et al., 2014), infiltration of the intestinal mucosa by macrophages and other inflammatory cells appears to be unnecessary for controlling invasive *C. rodentium* infection (Khan et al., 2006). Studies in TLR-4-deficient mice have suggested that when inflammatory cells infiltrate the mucosa, this may even promote *C. rodentium* colonization (Khan et al., 2006). Imaging of bacterial DNA by in situ hybridization and imaging of inflammatory macrophages by immunohistochemistry suggested that pDCs moderate part of the host-response by limiting accumulation of inflammatory macrophages and perhaps secondary to that, impeding bacterial inoculation in colon mucosa. An increase in cytokine transcripts in the colon suggested that this feature of the host-response is also involved in producing inflammatory mediators including TNF and IL-1β locally in the colon mucosa. Although a Th1-response is necessary to clear *C. rodentium* infection (Higgins et al., 1999), tempering IFNγ-production in effector T cells may help control macrophage activation and their cytokine production once they infiltrate the colon mucosa. In pDC-deficient mice, there is excessive activation due to reduced immune regulation by FoxP3+ Tregs in the mucosa. Thus, increased Th1 activity and an impaired barrier function in pDC-depleted mice suggest that tempering Th1 activity may be one of the mechanisms by which pDCs moderate a host's antimicrobial response to avoid inflammation becoming detrimental. Regulation of inflammation by pDCs seems to be an important factor in controlling intestinal permeability as proinflammatory cytokines such as TNF and IFNγ can impair the tight junctions between intestinal epithelial cells (Bruewer et al., 2005; Ye et al., 2006), while anti-inflammatory

cytokines IL-10 and TGFβ can reinforce tight junctions (Howe et al., 2005; Madsen et al., 1997).

6.2 The lymphatic route from the gut to PaLNs promotes autoreactive T cell activation during dysbiosis

Autoreactive T cells and their activation are key factors in T1D pathogenesis. Activation of these islet specific T cells takes place in PaLNs (Clare-Salzler & Mullen, 1992; Höglund et al., 1999; Jaakkola et al., 2003). Impaired intestinal barrier function has been suggested to influence the progression of T1D (Li & Atkinson, 2015) and it is known that activation of islet specific T cells in PaLNs is enhanced during *C. rodentium*- or DSS-induced inflammation of the colon (Lee et al., 2010; Turley et al., 2005). It has also been shown in a streptozotocin-induced diabetes model that gut microbiota can translocate to PaLNs and contribute to T1D pathogenesis (Costa et al., 2016). However, how T cell-promoting bacteria or DCs reach PaLNs from the gut has not been described. The lymphatic structure of the colon is relatively well described and comprises specialised compartmentalized draining lymph nodes (Esterházy et al., 2019; Houston et al., 2016; Toivonen et al., 2016) but here we show that PaLNs are also connected to this lymphatic network and that during dysbiosis bacteria and DCs can be transported from the colon to PaLNs via a lymphatic route. We also show that the increased antigen stimuli in PaLNs activates islet-specific T cells and their potential homing to pancreatic islets.

In our studies, accumulation of FITC-labelled dextran and A647-labelled ovalbumin in PaLNs after injections in the colon wall suggested that a direct lymphatic route exists between the gut and PaLNs. Although by injections to the colon wall we cannot mimic natural lymphatic flow completely, it shows that lymphatic structures can potentially be used by DCs and bacteria to reach PaLNs. Recently, Esterházy et al used 3-D imaging of the lymphatic structures along the intestine to show the existence of lymph nodes, described as pancreatic–duodenal lymph nodes, which drain from the duodenum and from the ascending and transverse colon. This supports the possibility of a lymphatic connection between the gut and PaLNs (Esterházy et al., 2019). While under steady state conditions DCs from the colon migrate only to their designated draining lymph nodes (Houston et al., 2016; Toivonen et al., 2016), we saw that inflammation and dysbiosis induced by *C. rodentium* disturbs this homeostasis and in doing so enables DCs and bacteria to migrate to PaLNs. This indicates that dysbiosis and inflammatory conditions in the colon can alter the normal compartmentalized

lymph flow to specific draining lymph nodes and generate a route for DCs and bacteria to reach PaLNs (**Figure 4**).

Previous studies have already documented the presence of bacteria in PaLNs but their localization within the lymph node has not been described (Costa et al., 2016; Lee et al., 2010). By imaging bacterial DNA with *in situ* hybridization, bacteria were shown to accumulate in the medullary sinus and with GFP-*C. rodentium* we saw that bacteria co-localize with F4/80+ macrophages. GFP-*C. rodentium* was also detectable from migratory CD103+ DCs and resident DCs expressing XCR1. This indicates that bacteria can either be transported to PaLNs by DCs or in soluble form in the lymph flow. This increased bacterial stimulus could be a reason behind the increased T cell activation in PaLNs described previously (Lee et al., 2010; Turley et al., 2005). It is possible that increased inflammatory conditions promote islet-specific T cell activation in a bystander manner (Pane & Coulson, 2015). In steady state conditions most of the DCs presenting β-cell antigens are in an immature state and do not provide costimulatory signals for sufficient T cell activation and thus they are more likely to promote tolerogenic functions (Lutz & Kurts, 2009; Steinman et al., 2003). However, the surrounding inflammatory conditions and recognition of LPS via TLR could potentially activate DCs and promote antigen presentation by DCs, thereby leading to priming of islet-specific T cell activation (Bonifaz et al., 2002; Guerder et al., 2013; Sparwasser et al., 2000). We also saw that XCR1+ DCs with cross-presenting abilities (Kroczek & Henn, 2012) take up antigens originating from the gut, which indicates that dysbiosis can directly induce DCs in PaLNs to elicit not only CD4 responses but also CD8 responses.

As shown previously (Lee et al., 2010; Turley et al., 2005), perturbation of colon homeostasis can enhance islet-specific CD4 T cell proliferation in PaLNs when transferred from transgenic BDC2.5 NOD mice to WT NOD mice. We also showed that induction of dysbiosis in NOD8.3 mice promote IGRP specific CD8 T cell proliferation and activation in PaLNs in dysbiotic WT NOD mice. Our results suggest that the CD8 T cell response during dysbiosis is enhanced most likely due to increased activation of resident DCs and that their propensity to cross-present tissue-derived antigens from pancreatic islets increases if microbial structures reach pancreatic lymph nodes. Overall, it seems that immune tolerance to islet antigens is particularly sensitive to dysbiosis in the colon.

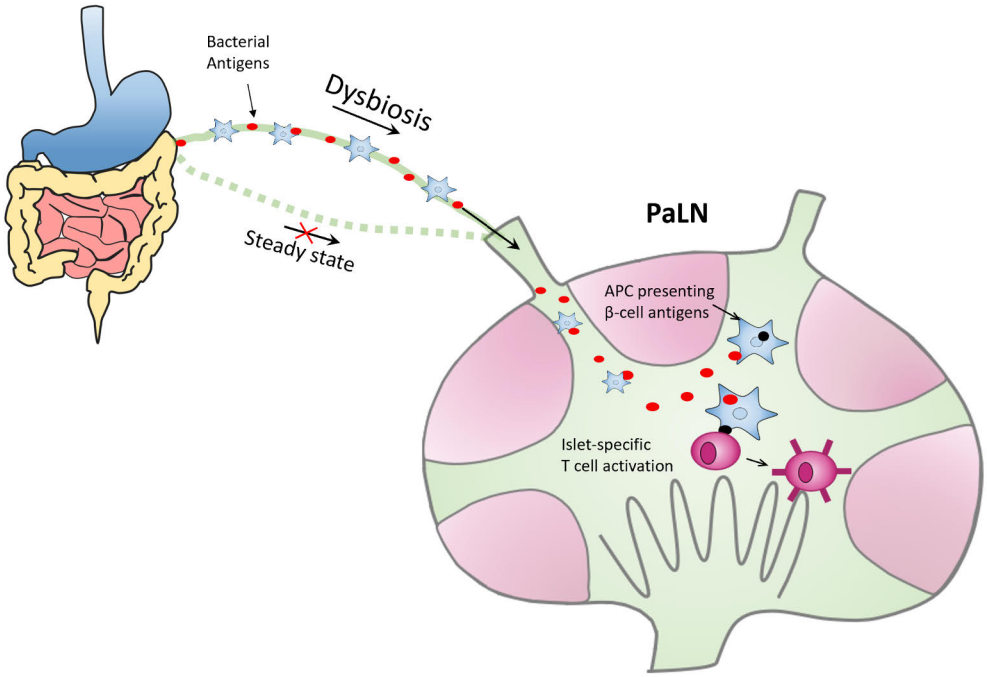

Figure 4. Dysbiosis initiates a lymphatic route from the gut to pancreatic lymph nodes (PaLN). The lymphatic route from the colon to PaLNs is initiated during dysbiosis which can then be used by DCs and bacteria to reach PaLNs. In the PaLNs, increased bacterial stimuli and activated DCs promote priming of islet-specific T cells. Modified from Mathis et al., 2001

6.3 Gut microbiota has an impact on islet chemokine production and T cell attraction

After initial activation in PaLNs, islet specific T cells egress from lymph nodes ready to home to their target tissue. We showed that during activation, IGRP-specific CD8 T cells start to express CXCR3, typically expressed on Th1 cells as well, which is the receptor for IFNγ-inducible CXCL9, CXCL10 and CXCL11 chemokines (Groom & Luster, 2011). It has been shown that while pancreatic islets of healthy individuals show very minimal CXCL10 expression, in individuals with recent onset of T1D it is possible to detect elevated CXCL10 expression in their pancreatic islets (Christen & Kimmel, 2020; Roep et al., 2010; Sarkar et al., 2012; Uno et al., 2010). Similar expression patterns have also been documented in mouse models (Carrero et al., 2013; Christen et al., 2003; Frigerio et al., 2002; Sarkar et al., 2012). Even though CXCL10 is highly associated with T1D, it is unclear what initiates the attraction of the first diabetogenic T cells into the islets prior to inflammation. We show that dysbiosis and elevated LPS levels potentially provide the initial stimuli for CXCL10 production in pancreatic islets and that the process is mediated by TLR signalling.

When we modelled dysbiosis with *C. rodentium* and *R. gnavus,* altered gut permeability and increased LPS levels were associated with enhanced CXCL10 production in pancreatic islets. It was initially shown that, in the RIP-LCMV model, the CXCR3-CXCL10 axis is crucial for T cells to infiltrate islets and for the development of autoimmune diabetes (Christen et al., 2003; Frigerio et al., 2002). On top of the CXCL10, its receptor CXCR3 has also been documented to be elevated within the islets of T1D patients (Roep et al., 2010; Tanaka et al., 2009; Uno et al., 2010). However more recent studies challenged this concept by showing that the use of neutralizing anti-CXCL10 treatment only partially delayed diabetes development and promoted β-cell regeneration rather than inhibiting lymphocyte infiltration (Coppieters et al., 2013; Morimoto et al., 2004). Similarly, varying results have been reported with regards to how CXCR3-deficieny affects autoimmune diabetes, with some reporting that CXCR3-deficiency delays disease development (Frigerio et al., 2002) and others reporting accelerated disease (Yamada et al., 2012). Even though reports on the consequences of blocking CXCR3-CXCL10 have been inconsistent, when combined with an anti-CD3 antibody, anti-CXCL10 treatment has been shown to be an effective therapeutic approach for preventing the progression of autoimmune diabetes (Lasch et al., 2015). We were able to demonstrate the therapeutic potential of anti-CXCL10 antibody treatment in our study when we showed that anti-CXCL10 antibody treatment in dysbiotic NOD mice inhibited insulitis and T cell infiltration into islets.

By using TLR4-KO mice and TLR4 blocking in NOD mice we saw that CXCL10 upregulation is dependent on TLR4 signalling. Transferring different microbes, also had an impact on the expression of TLRs within pancreatic islets. Resident macrophages and β-cells themselves have the ability to express toll like receptors, TLR2 and TLR4 (Garay-Malpartida et al., 2011; Nackiewicz et al., 2014; Vives-Pi et al., 2003). TLR4 is a well-known receptor for LPS and thus is an important factor in the recognition of gram-negative bacteria, whereas TLR2 is involved in the recognition of membrane proteins and components such as lipoteichoic acids from gram-positive bacteria (Mukherjee et al., 2016). As a gram-positive bacterium, *A. muciniphila* interacts with the host via TLR2 which is known to be one of the mechanisms by which it promotes intestinal barrier integrity (Plovier et al., 2016). We observed that that TLR2 and TLR4 expression are reduced in the pancreatic islets of *A muciniphila* treated NOD mice and this is most likely due to enhanced intestinal barrier function and lower LPS levels. Furthermore, we saw that both TLR2 and TLR4 are overexpressed in the pancreatic islets of dysbiotic mice and, as documented earlier (Wen et al., 2004), we also saw that TLRs in islets are upregulated in response to direct LPS injections (IV supp. fig 2). Islets are surrounded by an extensive capillary network and up to 20% of the total blood flow in the pancreas is concentrated in the islets (Muratore et al.,

2021), which potentially allow resident macrophages and β-cells to respond to microbial stimuli and LPS from the blood stream via TLR interaction (Ferris et al., 2017).

Since environmental factors and infections are thought to be potential triggers for autoimmune reactions and lymphocyte infiltration in T1D, we hypothesized that LPS could be the initial trigger for CXCL10 production and consequently the attraction of CXCR3 expressing diabetogenic lymphocytes. First, by stimulating islets from *Rag1-/-* NOD with LPS, we showed that CXCL10 production by islet cells is not dependent on T cells. CXCL10 was clearly detectable from islets even without T cells. Similarly, with IFN knock-out NOD mice we showed that overall IFN signalling is not necessary for LPS-induced CXCL10 production within islets. Our results indicate that LPS can promote CXCL10 within islets without IFN by any cell type. However, when we depleted macrophages from NOD islets before LPS stimulation, no CXCL10 was detectable, indicating that the effect of LPS in islets requires resident macrophages. Resident macrophages are the only leukocyte present in healthy islets (Calderon et al., 2015; Zakharov et al., 2020) and our results indicate that these resident macrophages are essential for the initial response to LPS and for the consequent CXCL10 production. Earlier studies suggest that islet endocrine cells are capable of producing CXCL10 by themselves (Antonelli et al., 2014; Frigerio et al., 2002; Nigi et al., 2020; Roep et al., 2010) but previously it was unclear whether LPS can directly promote this CXCL10 production. Our RNA sequencing results from LPS-stimulated islets show for the first time that β-cells also produce CXCL10 in response to LPS. This direct induction of CXCL10 seems to be mediated via an increase in TLR4 expression as it has been shown by us and others that TLR4 expression is increased in islet endocrine cells after LPS exposure (Wen et al., 2004). However, in β-cells both the IFN and TNF signalling pathways were significantly upregulated, indicating that the LPS response is more likely to be mediated through resident macrophages which are known to produce these proinflammatory cytokines in response to LPS (Beutler & Rietschel, 2003; Vaure & Liu, 2014). Our observations indicate that elevated LPS levels during dysbiosis may be the initial inducer of CXCL10-mediated lymphocyte attraction (**Figure 5**).

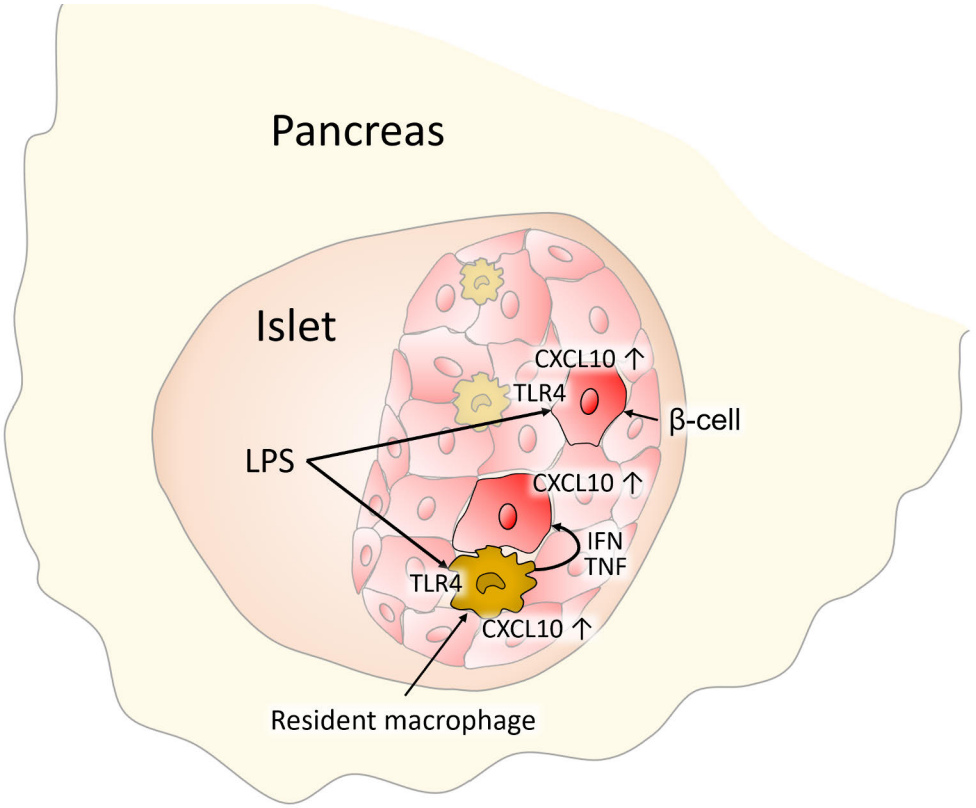

Figure 5. LPS-mediated CXCL10 production in pancreatic islets. Dysbiosis elevates systemic LPS levels which has a direct impact on pancreatic islet inflammation. LPS recognized by resident macrophages via TLR4 leads to IFN and TNF-mediated induction of CXCL10 in β-cells. In addition, β-cells express TLR4 and can directly recognize LPS and produce CXCL10.

7 Conclusions

The association between gut microbiota and T1D has been intensively studied for decades. However, thus far no specific microbial species or gut microbial profile has been identified that conclusively predisposes an individual to autoimmune diabetes. It seems that, rather than abundance of specific bacterial species, overall interaction between intestinal microbes and the immune system influence homeostasis in the gut. When interaction is disrupted, as is the case in dysbiosis, it can lead to chronic inflammatory conditions and even to the development of autoimmune diseases. Even though it has been suggested that dysbiosis can promote the development of autoimmune diseases, it has not been convincingly demonstrated how it influences the development of T1D. Resolving the mechanisms by which dysbiotic conditions in the gut influence autoimmune reactions will be extremely important in the future for understanding its role in the pathogenesis of T1D. In this thesis we studied at tissue and cellular level, mechanisms that link dysbiosis to the immunological events seen in T1D pathogenesis.

In this study we focus on two main factors. Firstly, we evaluate how dysbiosis can disrupt immunological tolerance against β-cell antigens and activate islet-specific T cells in pancreatic lymph nodes. Secondly, we determined how autoreactive lymphocytes are initially attracted to pancreatic islets to destroy β-cells and whether dysbiosis influences this phenomenon. We show how healthy homeostasis in the gut has a beneficial effect on diabetes progression by promoting intestinal integrity. We also show that dysbiosis in the gut leads to impairment of intestinal barrier function, where among other factors plasmacytoid dendritic cells have an important regulatory role, and enhances the autoimmune responses seen in type 1 diabetes. We identified a previously undescribed lymphatic route from the gut to pancreatic lymph nodes and during dysbiosis this route allows dendritic cell and bacteria migration to PaLNs. This leads to enhanced activation of islet specific T cells and their imprinting with CXCR3, a receptor for CXCL10. Similarly, we show that dysbiosis and increased endotoxemia can be the initial trigger for islet inflammation and lymphocyte attraction by inducing CXCL10 production in pancreatic islets. This study provides new insight into how microbiota can be a direct trigger for the activation of autoimmune reactions in type 1 diabetes. Although it is

challenging to definitively determine which microbial profiles predispose to these amplified autoimmune responses, our findings highlight the importance of a healthy microbiota and gut dysbiosis may be a potential target for the prevention of type 1 diabetes.

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