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DIET, MICROBES AND GUT IMMUNE RESPONSES IN THE PATHOGENESIS OF EXPERIMENTAL TYPE 1 DIABETES

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If we knew what it was we were doing, it would not be called as research, would it?

Albert Einstein

ABSTRACT

Rohini Emani

Diet, microbes and gut immune responses in the pathogenesis of experimental type 1 diabetes

Type 1diabetes (T1D) is an autoimmune disease, which is influenced by a variety of environmental factors including diet and microbes. These factors affect the homeostasis and the immune system of the gut. This thesis explored the altered regulation of the immune system and the development of diabetes in non-obese diabetic (NOD) mice.

Inflammation in the entire intestine of diabetes-prone NOD mice was studied using a novel *ex-vivo* imaging system of reactive oxygen and nitrogen species (RONS), in relation to two feeding regimens. In parallel, gut barrier integrity and intestinal T-cell activation were assessed. Extra-intestinal manifestations of inflammation and decreased barrier integrity were sought for by studying peritoneal leukocytes. In addition, the role of pectin and xylan as dietary factors involved in diabetes development in NOD mice was explored.

NOD mice showed expression of RONS especially in the distal small intestine, which coincided with T-cell activation and increased permeability to macromolecules. The introduction of a casein hydrolysate (hydrolysed milk protein) diet reduced these phenomena, altered the gut microbiota and reduced the incidence of T1D. Extra-intestinally, macrophages appeared in large numbers in the peritoneum of NOD mice after weaning. Peritoneal macrophages (PM) expressed high levels of interleukin-1 receptor associated kinase M (IRAK-M), which was indicative of exposure to ligands of toll-like receptor 4 (TLR-4) such as bacterial lipopolysaccharide (LPS). Intraperitoneal LPS injections activated T cells in the pancreatic lymph nodes (PaLN) and thus, therefore potentially could activate islet-specific T cells. Addition of pectin and xylan to an otherwise diabetes-retarding semisynthetic diet affected microbial colonization of newly-weaned NOD mice, disturbed gut homeostasis and promoted diabetes development.

These results help us to understand how diet and microbiota impact the regulation of the gut immune system in a way that might promote T1D in NOD mice.

Keywords: type 1diabetes, nutritional factors, reactive oxygen species, non-obese diabetic (NOD) mouse, T-lymphocytes, macrophages, dietary fibres, gut microbiota

TIIVISTELMÄ

Rohini Emani

Ruokavalion ja mikrobiston vaikutus suoliston immuunijärjestelmään ja tyypin 1 diabeteksen kehittymiseen

Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto, Turku, Molekyylilääketieteen tohtoriohjelma (TuDMM)

Tyypin 1 diabetes on autoimmuunitauti, jonka patogeneesiin vaikuttavat monet ympäristötekijät, mukaan luettuna ruokavalio ja mikrobit. Nämä tekijät vaikuttavat suolen toiminnan tasapainoon ja suolen immuunijärjestelmään. Tämä väitöskirja käsittelee immuunijärjestelmän muuttunutta säätelyä ja diabeteksen kehittymistä diabetesaltiille Non-Obese Diabetic (NOD)-hiirille.

Väitöskirjassa on tutkittu reaktiivisten happi- ja typpiyhdisteiden (RONS) välittämää tulehdusta NOD-hiirten suolistossa sekä ravintokuitujen roolia suolen toiminnan tasapainossa, mikrobien kolonisaatiossa ja diabeteksen kehittymisessä NOD-hiirillä. Normaaliin ruokavalioon siirtyneet vieroitetut hiiret kärsivät suolen lisääntyneestä läpäisykyvystä, tulehduksen lisääntymisestä suolistossa ja tyypin 1 diabeteksesta. Lisäksi vieroittamisen ja ruokavaliomuutoksen jälkeen NOD-hiirten vatsaontelosta löydettiin suuria määriä makrofageja. Vatsaontelon makrofagit tuottivat enemmän interleukiini-1-reseptoriassosioitua kinaasia M (IRAK-M), mikä viittaa aiempaan altistukseen Toll:in kaltaisen reseptori neljän (TLR-4) ligandeille kuten bakteerien tuottamalle lipopolysakkaridille (LPS). Vatsakalvon alainen LPS-injektio johti T-solujen aktivoitumiseen haiman imusolmukkeissa. Saarekespesifisten T-solujen aktivoituminen haiman imusolmukkeissa on yksi keskeisimpiä tapahtumia tyypin 1 diabeteksen patogeneesissa NOD-hiirillä.

Pektiinin ja ksylaanin lisääminen muutoin diabetekselta suojaavaan ns. semisynteettiseen ruokavalioon vaikutti NOD-hiirten suolen kolonisoitumiseen mikrobistolla ja suolen tulehdusvasteiden tasapainoon ja lisäsi diabeteksen ilmaantumista. Tämän tutkimuksen perusteella mikrobistolla ja ruokavaliolla, etenkin ruokavalion sisältämillä ravintokuiduilla näyttäisi olevan merkitystä suoliston immuunijärjestelmän säätelyssä ja tyypin 1 diabeteksen ilmaantumisessa NOD-hiirillä.

Avainsanat: Tyypin 1 diabetes, ravintoaineet, reaktiiviset happiradikaalit, T-lymfosyytit, makrofagit, nonobese diabetic (NOD) hiiri, ravintokuidut, suolistomikrobisto

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ABBREVIATIONS

APC	antigen-presenting cells
Ab	antibody
BB	BioBreeding
cDNA	complementary deoxyribonucleic acid
CPM	counts per minute
CCR	chemokine receptor
CTL	cytotoxic lymphocytes
CTLA-4	cytotoxic T-lymphocyte antigen 4
CH-NOD	NOD mice maintained on casein hydrolysate
CLR	C-type leptin receptor
CCL	chemokine ligand
CE	NOD mice weaned to cellulose diet
CVB1	coxsackieviruses B1
DHE	dihydroethidium
DGGE	denaturing gradient gel electrophoresis
DC	dendritic cells
EDTA	ethylenediaminetetracetic acid
EAE	experimental encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal regulated kinases
FOXP3	forkhead/winged helix transcription factor box 3 protein
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FF	fermentable fibres

FITC-DEXTRAN	fluorescein isothiocyanate-dextran
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony stimulating factor
GALT	gut-associated lymphoid tissue
HLA	human leukocyte antigens
H_2O_2	hydrogen peroxide
IA-2A	islet antigen 2 antibodies
ICA	islet cell autoantibodies
ICAM-1	intracellular adhesion molecule-1
IVIS	in vivo-imaging system
IFN	interferon
IFN-γR	interferon gamma receptor
IRAK-M	interleukin receptor associated kinase M
IRF	interferon regulatory factor
iNOS	inducible nitric oxide synthase
IBD	inflammatory bowel disease
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
MLN	mesenteric lymph node
MDA5	melanoma differentiation-associated gene 5
MHC I, MHC II	major histocompatibility complex
MAMP	microbe-associated molecular pattern
MYD88	myeloid differentiation primary response protein 88
MAPK	mitogen-activated protein kinases
M-CSF	macrophage colony-stimulating factor
MS	multiple sclerosis

NAD	nicotinamide adenosine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NC	normal chow diet
NOD	non-obese diabetic
NLR	nod like receptor
NK	natural killer
NO	nitric oxide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
PM	peritoneal macrophages
PCR	polymerase chain reaction
PNOD	NOD mice weaned to ProSobee diet
PaLN	pancreatic lymph node
PX	Pectin and xylan containing diet
PRR	pattern recognition receptors
PD-1	programmed cell death protein-1
PFA	paraformaldehyde
PPAR- γ	peroxisome proliferator-activated receptor gamma
RA	rheumatoid arthritis
RFU	relative fluorescence unit
$REG_{3\gamma}$	regenerating islet-derived protein 3 gamma
RT-qPCR	reverse transcription quantitative polymerase chain reaction real time PCR
SCFA	short-chain fatty acid
STAT	signal transducer and activator of transcription
TLR	toll-like receptor
T1D	type 1 diabetes
TCR	T cell receptor

TNF-α	tumor necrosis factor alpha
TAM	tumour-associated macrophages
Tfh	T follicular helper ells
Th	T helper cells
VNTR	variable nucleotide tandem repeat
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III).

I.   Emani R, Asghar MN, Toivonen R, Lauren L, Söderström M, Toivola DM, van Tol EA, Hänninen A. 2013. Casein hydrolysate diet controls intestinal T cell activation, free radical production and microbial colonisation in NOD mice. *Diabetologia* 56: 1781-91.

II. Toivonen RK, Emani R, Munukka E, Rintala A, Laiho A, Pietilä S, Pursiheimo JP, Soidinsalo P, Linhala M, Eerola E, Huovinen P, Hänninen A. 2014. Fermentable fibres condition colon microbiota and promote diabetogenesis in NOD mice. *Diabetologia* 57:2183-92.

III. Emani R, Alam C, Pekkala S, Zafar S, Reddy EM, Hänninen A. Peritoneal cavity is a route for gut-derived microbial signals to promote autoimmunity in NOD mice. *Scandinavian journal of immunology* 10:1111/sji.12253.

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1 INTRODUCTION

T1D is a T cell-mediated autoimmune disease, which is caused by a destructive immune response towards the insulin-producing β-cells of the pancreas, which abolishes the production of insulin. Patients suffering from T1D need to receive insulin therapy to survive. To date, the origin of T1D is not clearly understood.

As Finland has the highest incidence of diabetes in the world, it is important to examine the factors underlying the cause of this autoimmune disorder. Several environmental factors contribute to the development of T1D, including, diet and microbes. Innate and adaptive immune responses in the gut appear to play a significant role in T1D. Diet and microbial factors responsible for changes in the regulation of mucosal immunity were investigated in this study. In particular, the effects of dietary fibres on microbial colonisation and the gut immune system were studied. An *ex vivo* imaging system was also used to evaluate inflammatory activity in different areas of the gut and the effect of an anti-diabetogenic diet on this activity.

PM in NOD mice were also analysed, since these macrophages may become activated when intestinal immune homeostasis is disturbed. Based on the results, PM phenotype appeared to reflect their *in vivo* exposure to bacterial LPS derived from the intestine, which in turn appeared to play a role in the activation of T and B cells in the pancreas-draining lymph nodes of NOD mice. Sudden exposure of the immature gut to large amounts of dietary fibres pectin and xylan increased diabetes development in NOD mice possibly through its effects on microbial colonisation and its consequences on gut immune homeostasis.

2 REVIEW OF THE LITERATURE

2.1 Overview of the immune system

The immune system protects our body against the invasion of different intra- and extracellular pathogens such as viruses, bacteria and fungi, as well as by eliminating defective cells of the host. The immune system is composed of an interactive network of lymphoid organs, cells, cell-bound and humoral factors and signalling molecules, which respond to invading pathogens. The immune system reacts to pathogens and foreign substances first by activating the host defence and then by boosting the response to eradicate the invading pathogens. The immune system needs to specifically recognise pathogens and discriminate between self and nonself. However, under abnormal conditions, the immune system makes mistakes in recognising antigens, and signals to attack the body's own cells or tissues. This can lead to autoimmune diseases, such as T1D, rheumatoid arthritis or multiple sclerosis (Alberts et al. 2002; Janeway et al. 2001). The immune system is divided into two parts, the innate immune system (non-specific) and the adaptive immune system (specific).

2.1.1 Innate immune system

The innate immune system is the defence mechanism that protects the host from daily microbial challenges. When it encounters a pathogen the innate immune system becomes activated non-specifically and protects the host by providing four kinds of defensive barriers. Firstly, the anatomic barrier against invading pathogens uses the epithelial surface such as skin or mucosal epithelium. The second barrier is a physiological barrier, which removes the pathogens mechanically by flushing the epithelium. The third barrier is a cellular barrier, which is activated, when a pathogen crosses the physiological barrier (Alberts et al. 2002). The cellular barrier utilizes a mechanism called phagocytosis (a form of endocytosis). The fourth barrier is protective mechanism by inflammation. In inflammation myeloid and other innate leukocytes respond to pathogens by passing intestinal barriers. Inflammation induces the production of many substances, including reactive oxygen and nitrogen species (RONS), which enables the leukocytes to kill invading microbes. Inflammation is one of the responses to an infection or irritation. Inflammation is regulated by chemical factors released from damaged tissue during formation of a wound or due to establishment of infection by foreign pathogens (Abbas et al. 2012).

Mast cells, eosinophils, basophils, dendritic cells, neutrophils, blood monocytes, macrophages and NK cells are the specialized cells involved in innate immune responses. Innate immune cells recognise microbe-associated molecular patterns (MAMPS), via their pattern recognition receptors (PRRS) (Alberts et al. 2002; Takeuchi and Akira 2010). Antigen presenting cells (APC), such as macrophages and dendritic cells, express these PRRS, which alert them to deal with factors that are foreign and potentially dangerous. Pathogens are engulfed by macrophages and dendritic cells, after which they are processed and presented to T lymphocytes, which release cytokines that are needed for the activation of the specific and long-term immunity, also known as the adaptive immunity. Both the innate and adaptive immune systems are needed to protect the host from pathogens (Janeway et al. 2001; Richard et al. 2007).

2.1.2 Macrophages

Macrophages were first discovered by Elie Metchnikoff in the 19th century, for which he was awarded the Nobel Prize in 1908 (Cavaillon 2011). Monocytes, macrophages and dendritic cells are immune cells that are derived from myeloid stem cells; they originate from the bone marrow and circulate in the bloodstream as monocytes. Macrophages mature once they have migrated to the infected tissue, where they then phagocytose foreign antigens (Hettinger et al. 2013). Besides macrophages, also monocytes, dendritic cells and neutrophils act as phagocytic cells (Murray and Wynn 2011).There are several markers for distinguishing macrophages and dendritic cells, to simplify based on the surface marker expression. Macrophages express higher levels of F4/80 in mouse, while dendritic cells express higher levels of CD11c, and also higher levels of MHC II (Guilliams et al. 2014).

Macrophages have roles in tissue remodelling, including wound healing and tissue repair in adults (Sutterwala et al. 1997). Furthermore, macrophages also perform tissue-specific functions. For example, the macrophages in the liver are known as Kupffer cells, which remove circulating toxins. The macrophages in the lungs are known as alveolar macrophages, and they eliminate inhaled antigens (Murray and Wynn 2011). Thus, macrophages are tissue-specific, but their primary functions are to act as sentinels. Macrophages are capable of identifying pathogens with PRRs. Macrophages recognise conserved motifs called MAMPs; pathogens are engulfed by phagocytosis in order to maintain tissue homeostasis. Upon phagocytosis, neutrophils and macrophages secrete nitric oxide (NO), hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-), generated by lysosomal NADPH oxidase and other enzymes as a process of respiratory bursts, which are lethal to bacteria.

Macrophages are efficient in recognising and clearing pathogens via the use of a plethora of receptors, such as NOD-like receptors (NLR), TLR, C-type leptin receptor (CLR), retinoic acid inducible-(RIG-I)-like receptors and the mannose and scavenger receptors. TLR signalling plays a key role in identifying the patterns of microbial components. Thus far, 10 TLRs have been identified in humans and 13 in mice. TLR signalling is an essential mechanism of the innate immune responses, as its activation results in the activation of pro-inflammatory cytokines, chemokines and co-stimulatory molecules, which then induce an antigen-specific adaptive immune response. TLR2 and TLR4 are activated upon bacterial stimuli (Takeuchi et al. 1999). While, the primary roles of TLR1 and TLR6 are to act as co-receptors for TLR2 in stimulating specific signalling events based on the binding of a particular pathogen (Takeuchi et al. 2002).

Additional TLRs such as TLR3, TLR9 and TLR7/TLR8 are activated by viral double-stranded DNA, single-stranded RNA and bacterial CpG (Alexopoulou et al. 2001; Diebold et al. 2004; Hemmi et al. 2000). TLR signalling results in the activation of NF-kB and MAP kinase to promote regulatory reactions. Moreover, the activation of TLR3 mediates the activation of interferon regulatory factor-3 (IRF3) by the TIR-domain-containing adapter-inducing interferon-β (TRIF) -dependent pathway to induce type-I interferon and inflammatory cytokines (Kawai and Akira 2010). TLR7/TLR8 activates IRF7, this ultimately promotes IFN-α stimulation by the MYD88-dependent pathway upon activation of NF-kB and activator protein-1 (AP-1) (Akira and Takeda 2004). TLR signalling is mediated by an interaction between the ligand and the TIR domain, which consists of cytosolic adaptor proteins. Once activated, myeloid differentiation primary response protein 88 (MYD88), recruits IL-1 receptor-associated kinase (IRAK). The activated IRAK phosphorylates and then associates with TNF receptor

associated factor TRAF6. This finally leads to the activation of the JNK and NF-kB signalling pathways (Kawai and Akira 2006).

2.1.3 Macrophage activation

Macrophages are activated by various stimuli, as well as by the disruption of tissue homeostasis, either by infection or inflammation. Macrophages are highly versatile in changing their phenotype and function based on the signal received from the tissue environment. Activated macrophages produce reactive oxygen and nitrogen intermediates, and pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, which are the key cytokines involved in the initiation of inflammation. These cytokines commits towards Th1 responses as well as mediate protection against tumour and intracellular pathogens (Mantovani et al. 2013). Macrophages are classified into different types based on their activation phenotypes: classically activated macrophages, alternatively activated macrophages, regulatory macrophages, tissue-regenerative macrophages, and tumour-associated macrophages.

2.1.4 Classically activated macrophages

Classically activated macrophages, known as M1 macrophages, are formed during cell-mediated immune responses. They are activated by two signals, first by priming with interferon gamma (INF-γ) and later with endogenous or exogenous TNF-α. Macrophages express TLRs and other PRRs, and when they phagocytose bacteria they produce pro-inflammatory cytokines and chemokines, such as IL-1, IL-6, TNF-α, IL-12 and IL-23. This results in the recruitment of neutrophils, basophils and eosinophil's and T lymphocytes, such as Th1 or Th17 cells, which are known to maintain a state of inflammation (Bettelli et al. 2006; Langrish et al. 2005; Veldhoen et al. 2006). Classically activated macrophages also produce inducible NO; NO damages DNA and is required to clear bacteria within macrophages. M1 macrophages produce superoxide anions and oxygen radicals via the expression of NADPH oxidase (Adams et al. 1997). In mouse models, lack of this oxidase results in the growth of huge abscesses that are filled with enteric commensal bacteria (Gyurko et al. 2003; Shiloh et al. 1999). This suggests that the elimination of ingested bacteria by macrophages requires NO. Hence, classically activated macrophages are specialised in destroying intracellular microorganisms. Indeed, classically activated macrophages are associated with many pathologies, including chronic inflammatory autoimmune and inflammatory bowel diseases (Abraham and Medzhitov 2011; Mosser and Edwards 2008; Woollard and Geissmann 2010).
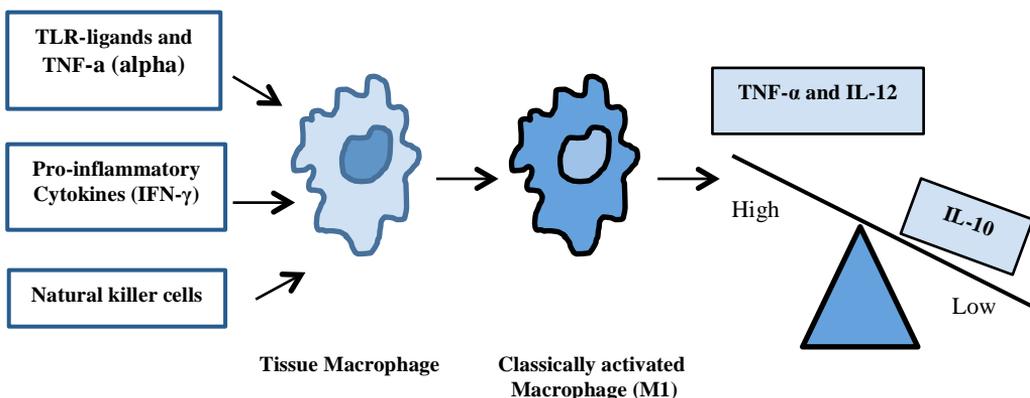
Figure 1: Classically activated macrophages: Signals via TLRs, IFN-γ and NK cells induce classically activated macrophages. Activated macrophages promote the Th1 response for effective clearing of intracellular microbes and effective removal of tumour cells. Modified from (Mosser and Edwards 2008).

2.1.5 Alternative activation of macrophages

Alternatively activated macrophages are known as M2, or wound healing, macrophages. These macrophages are activated in response to innate and adaptive immune signals. Th2 type cytokines, such as IL-4, IL-13 and IL-33, are responsible for the polarisation of macrophages into this phenotype via an alternative process of gene expression. It is activated by the transcription factors IRF4, Signal transducer and activator of transcription 6 (STAT6) and Peroxisome proliferator-activated receptor gamma (PPAR-γ) (Satoh et al. 2010). These macrophages are primarily found during infections, especially after tissue damage and during the healing phase. M2 macrophages are poor producers of RON/S and unlike M1 macrophages, they do not express inducible nitric oxide synthase (iNOS) (Mosser and Edwards 2008). M2 macrophages produce greater amounts of immunomodulatory cytokines, including IL-10, TGF-β and Vascular endothelial growth factor (VEGF). M2 macrophages express chemokine ligand 17 (CCL17), CCL22 and CCL24 chemokines, which are the ligands for the chemokine receptor 3 (CCR3) and CCR4 chemokine receptors that are known to be involved in polarised Th2 immune responses (Katakura et al. 2004; Watanabe et al. 2002). Thus, IL-10 produced by M2 macrophages acts as an antagonist of IL-1, and reduces inflammation. Hence, M2 macrophages are known to be anti-inflammatory.

Tissue Macrophage   **Alternatively activated Macrophage (M2)**
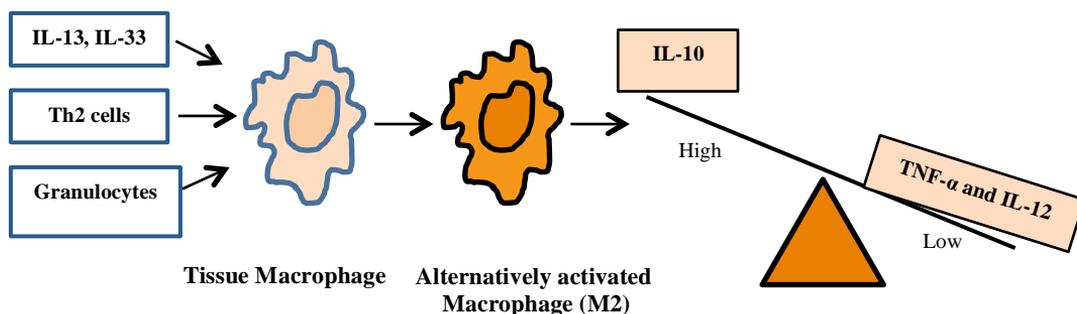
Figure 2: Alternatively activated macrophages: IL-4, IL-33, IL-13 and granulocytes (eosinophils, mast cells and basophils) induce alternatively activated macrophages. They activate Th2 immune responses for effective clearance of parasites, and help in tissue repair and would healing. Modified from (Mosser and Edwards 2008).

2.1.6 Regulatory macrophages

Macrophages known as regulatory macrophages possess some of the characteristics of M1 and M2 macrophages. These macrophages express CD206 and CD163 and express high amounts of IL-10 and low levels of TNF-α and IL-12, like M2 macrophages (Biswas and Mantovani 2010). Regulatory macrophages are highly phagocytic and express major histocompatibility class II (MHC-II) and co-stimulatory molecules. These macrophages can emerge during the innate and

adaptive immune response; regulatory macrophages can also arise at a later stage of adaptive immunity (Mosser and Edwards 2008). However, the exact mechanisms behind polarisation of macrophages towards regulatory macrophages still need to be elucidated. Mitogen-activated protein kinases (MAPK) and extracellular-signal regulated kinases (ERK) might play a role in this process (Mosser and Edwards 2008).
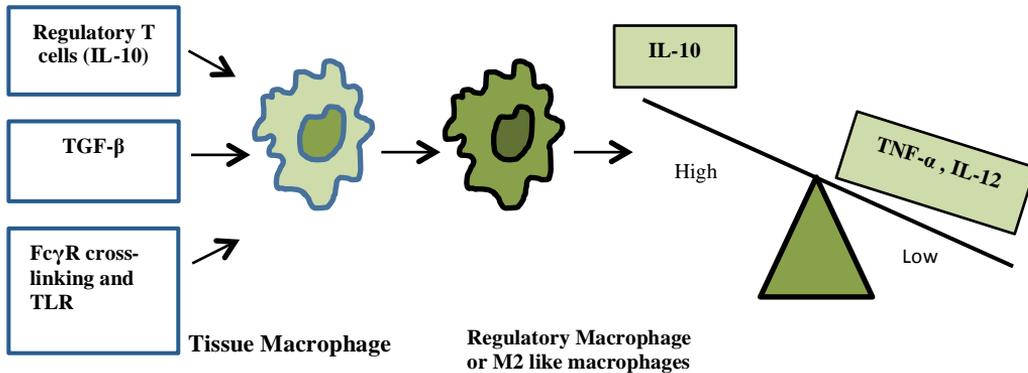
Figure 3: Regulatory macrophages: Immune complexes, apoptotic cells and IL-10 induce regulatory macrophages. Activated regulatory macrophages help in tumour immunoregulation. Modified from (Mosser and Edwards 2008).

2.1.7 Tissue macrophages

Tissue macrophages play a crucial role in tissue metabolism, immune responses, tissue repair and homeostasis (Davies et al. 2011). Thus, macrophages are multifunctional and have an important role in diseases, such as cardiovascular, chronic inflammatory, autoimmune, cancer and metabolic diseases. However, these tissue macrophages likely originate from blood monocytes (Epelman et al. 2014). Moreover, data has shown that tissue macrophages are capable of self-renewal, especially during an infection (Hashimoto et al. 2013; Jenkins et al. 2011). Tissues macrophages are of embryonic origin and are exclusive of the monocytes input (Ginhoux and Jung 2014; Gomez Perdiguero and Geissmann 2013) and are derived from embryonic progenitors as demonstrated in lineage-tracking experiments. They were present in the yolk sac at an early age of 7.5 days of embryonic development, and were independent of blood monocytes (Chorro et al. 2009; Ginhoux et al. 2010; Yona et al. 2013). Liver Kupffer cells, the alveolar macrophages of lungs, the microglia of the central nervous system and splenic and PM are derived from non-haematopoietic precursors and persist in adults independent of blood monocytes through a process called self-renewal (Ajami et al. 2007; Alliot et al. 1991; Hashimoto, et al. 2013; Merad et al. 2002; Tarling et al. 1987; Yona, et al. 2013). However, it remains uncertain whether these macrophages are present in parenchymal tissues. Moreover, these macrophages safeguard tissues during an infection or tissue damage by releasing mediators that ultimately initiate innate immune responses.

2.1.8 Tumour-associated macrophages (TAMs)

Macrophages display remarkable plasticity towards environmental stimuli. Contradictory to the role of macrophages in the host defence, TAMs promote tumour progression by suppressing

adaptive immunity against tumour cells. Often this leads to lack of adaptive immune cells in tumours, and their presence in tumour tissue is often associated with a negative prognosis in colorectal cancers. Fifty percent of cells types found in solid tumours belong to macrophage populations (Gordon and Taylor 2005).

TAMs are recruited from blood monocytes that are attracted to the site of the tumour by several factors such as macrophage colony-stimulating factor (M-CSF), interleukine-3, granulocyte macrophage colony stimulating factor (GM-CSF) and chemokine ligand CCL2. The production of these compounds by malignant tissues promotes the further growth of tumours by suppressing the adaptive immunity (Mantovani et al. 2004; Qian and Pollard 2010). Macrophages that are recruited to tumours differentiate into the M2-like phenotype. This leads to a deficiency in M1 anti-inflammatory signals, and further secretion of the IL-10 cytokine. TAMs enhance the differentiation of monocytes into macrophages instead of dendritic cells (Sica et al. 2000). TAMs show low levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation, reduced production of inflammatory cytokines, and reduced levels of NO as a result of low expression levels of iNOS and RON (Klimp et al. 2001). Genetic profiling of TAMs has revealed the up-regulation of many M2-associated genes in these cells, supporting the notion that TAMs act as M2 macrophages in promoting further tumour progression (Biswas et al. 2006).

2.1.9 Adaptive immune system

The adaptive immunity is specifically targeted towards a particular foreign antigen, and it provides more specificity and diversity than the innate immunity, as well as a immunological memory of foreign antigens. Unlike the immediate reactions of the innate immunity it takes several days to mount a specific adaptive immune response. Adaptive immunity is mediated by humoral (antibody) and cell-mediated immune responses. B cells and T cells carry out the humoral and cell-mediated immune responses through antigen specificity.

The humoral response is arbitrated by B-lymphocytes that mature in the bone marrow. B-lymphocytes are the antibodies, or immunoglobulin, producing immune cells. When naïve B cells encounter foreign antigens, they differentiate into plasma cells and produce secreted antibodies. B cells also produce cytokines for immune regulatory functions (Carsetti et al. 2004).

T lymphocytes are the primary type of cells involved in cell-mediated immune responses. T lymphocytes develop from hematopoietic stem cells in the bone marrow, although they mature in the thymus. They express antigen-binding receptors on their surface, which are known as T cell receptors. Antigen presenting cells, (APCs) display antigenic peptides to T cells via MHC receptors. Co-stimulatory molecules expressed on the surface of APCs, and the cytokines they secrete induce T cells to differentiate into Th1, Th2, Th17 and T regulatory cells (Treg). Cytokines play a crucial role in stimulating and mediating signal to other cells that are involved in the immune response. Thus, cytokines also play an important role in the adaptive immune system (Janeway et al. 2001).

T lymphocytes consist of CD4+ T helper (Th) cells, which were restricted by MHC-II, which means, they are capable to recognise the antigen presented by the MHC-II, but not by MHC-I. While CD8+ cytotoxic killer T (Tc) cells are able to recognise the antigen presented by the MHC-I. T lymphocytes are activated and differentiate in response to stimuli to become lineage-

committed and produce specific cytokines. The inappropriate activation of Th and Tc lymphocytes would lead to inflammation (Kindt 2006).

2.1.10 CD4+ T cell subsets

T cells are the primary components of the adaptive immune system. Upon antigen stimulation, naïve CD4+ T cells become activated and differentiate into functional effector T cells (Abbas et al. 1996). Different subsets of effector T-helper (Th) cells protect the host against various infections, as well as from inflammatory and autoimmune diseases (Bettelli et al. 2007; Nicholson and Kuchroo 1996; Umetsu and DeKruyff 1997). Initially, CD4+ T cells were divided into Th1 and Th2 cells. However, later studies have recognized other subsets of CD4+ T cells, including Th17 cells, regulatory T cells (Tregs), Th9, Th22 and follicular helper T cells (Crotty 2011; Korn et al. 2009). In terms of their function, distinct Th cells subsets are characterized by the production of specific cytokine signatures. The specific differentiated states of the subsets are dependent on transcriptional regulators and cytokines that program subset-specific transcriptomes. This programming amplifies the differentiation of the Th-cells and concomitantly, opposes alternative fates (Kara et al. 2014; Zhu and Paul 2010).

Professional antigen-presenting cells, such as dendritic cells, play a crucial role in the induction of transcriptional programs via PAMPs and PRRs. This helps to activate the immune system pathogen-specifically (Joffre et al. 2009). Cytokines secreted by Th1 cells provide immunity against intracellular pathogens, such as *Mycobacterium tuberculosis, Listeria monocytogenes*, and *Leishmania*, by a cell-mediated immunity against them (Adorini 1999; Harris et al. 2009; Hovav et al. 2003; Jankovic et al. 2007). STAT4 and TBX21 (T-bet) are the key transcriptional factors in Th1 cells and promote the secretion of the proinflammatory cytokines IFN-γ and tumour necrosis factor-beta (TNF-β), (Coffman and Mosmann 1991; Liberman et al. 2003; Placek et al. 2009). Th2 cells mediate immune responses against extracellular pathogens, such as helminths. STAT6 and GATA3 are transcription factors expressed by Th2 cells. They induce the production of multiple Th2-specific cytokines including IL-4, IL-5, IL-13 and IL-25 (Elo et al. 2010; Kanhere et al. 2012; Liberman, et al. 2003; Shulman et al. 2013; Zhu 2010). Cytokines secreted by Th2 cells, such as IL-4 and IL-13, play a significant role in the proliferation and activation of B-cells. IL-5 helps to recruit eosinophils and basophils and promotes their proliferation and activity by binding to its receptor. This in turn favors the production of histamine and leukotrienes, the mediators of acute inflammation (Bosnjak et al. 2011; Caraballo and Zakzuk 2012). However, enhanced Th2 responses also promote the pathogenesis of allergic reactions, such as asthma (Bosnjak, et al. 2011).

IL17 protects the host from extracellular pathogens, such as bacteria and fungi, via IL-17-mediated mobilization of the neutrophil response (Curtis and Way 2009; van de Veerdonk et al. 2009). Th17 cells expressing transcriptional factors, such as STAT3, BATF, RORa and RORc, produce IL-17A, IL-17F, IL-21, IL-22, and IL-9 cytokines (Durant et al. 2010). In general, the controlled regulation of cell differentiation is necessary during the host's immune defense, because the inappropriate regulation of these mechanisms leads to inflammatory and autoimmune diseases, such as T1D, rheumatoid arthritis (RA), multiple sclerosis (MS) and Crohn's disease (CD), where an increase in the response of Th17 cells, and perhaps Th1 cells, has been observed (Damsker et al. 2010; El-behi et al. 2010; Hemdan et al. 2010; Jager et al. 2009; Marwaha et al. 2012).

Follicular helper T (Tfh) cells express BCL6 as their key transcription factor. This increases the transcription of *Il21* and *Cxcr5* resulting in the production of IL-21 and CXCR5 (Baumjohann et al. 2011; Kroenke et al. 2012). In secondary lymphoid organs, these Tfh cells promote class-switch recombination the activation and survival of B cells, and the differentiation of memory B cells (Crotty 2011). Th9 and Th22 cells have been studied less and are more poorly characterized than the other Th subsets. Th9 and Th22 cell are named based on their cytokines they secrete, i.e. IL-9 and IL-22, respectively (Blom et al. 2011; Perumal and Kaplan 2011). Th9 cells, together with Th2 cells, take part in the elimination of extracellular parasites. Th9 cells provide immunity against infections via the amplification of the chemotactic potential and by the activation of mast cells and basophils (Jager, et al. 2009). Studies have shown that Th9 cells to be linked to allergies (Soroosh and Doherty 2009). Studies have also shown that IL-22-mediated epithelial innate immune responses play a role in the pathogenesis of inflammatory skin diseases, HIV-associated mucosal enteropathogenesis and also in autoimmune diseases (Kagami et al. 2010; Xu et al. 2013; Zhao et al. 2013).

Tregs cells are crucial for maintaining peripheral tolerance and are specialized in immune suppression (Fontenot et al. 2003). Tregs plays a vital role in preventing chronic inflammatory diseases and in limiting antitumor immunity (Vignali et al. 2008). They express FOXP3 as the primary transcription factor and secrete immune regulatory cytokines such as IL-10 and TGFβ (Goodman et al. 2012). Thymus-derived natural Tregs (nTreg) mature in the thymus, and they express the IL2rα chain (CD25) and FOXP3, which are essential for their development and for immunosuppression (Fontenot, et al. 2003). Extra-thymically derived induced regulatory T cells (iTreg) are produced from CD4+ T cells upon antigenic stimuli under the tolerogenic condition and mount the antigen-specific immunosuppressive response (Haribhai et al. 2011). Thus, nTreg and iTreg cells play a significant role in sustaining of immunological homeostasis (Bilate and Lafaille 2012). Moreover, iTreg cells are known to be induced in various models of inflammatory, autoimmune and allergic diseases, such as colitis, arthritis, diabetes, experimental autoimmune encephalomyelitis (EAE) and asthma (Piccirillo 2008; Yadav et al. 2013).

2.1.11 The gut immune system

The gut immune system plays a vital role in maintaining gut homeostasis when the intestinal tract is constantly exposed to dietary factors containing inorganic materials as well as microbes. The epithelial cells of the gut are responsible for the uptake of vital nutrients from ingested food. Hence, they have to provide an excellent permeability, while at the same time ensure that commensal microbes do not cross this barrier. Because of the constant exposure to antigens, the initial stage of intestinal immunity is dominated by innate immune responses. Dendritic cells, macrophages and innate lymphoid cells are the critical cells of the innate immune system. Dendritic cells, macrophages and certain B cells (expressing B-cell receptor) play an essential role in antigen presentation and activate the adaptive immune system, comprising T and B cells, in the gut tissue. (Hooper and Macpherson 2010; Tomasello and Bedoui 2013). The epithelial barrier, cross-talk between innate immune cells and commensal bacteria, and the adaptive immune system are the major players in the gut immune system, and their dysfunction can lead to disease.

2.1.12 The role of diet and microbes in shaping gut immunity

Gut microbial colonisation depends on dietary factors, and it is known that diet and microbes play a central role in the regulation of health, disease and the shaping of the immune system, as the gut harbours trillions of bacteria (Eckburg et al. 2005; Proctor 2011; Round and Mazmanian 2009). The gut epithelium plays an essential role in the regulation and production of antimicrobial peptides in the presence of commensal microbiota (Brown et al. 2013). According to the hygiene hypothesis, diminished exposure to environmental microbes in early childhood might be the reason for the increased incidence of asthma, allergies and T1D in Western countries (Strachan 1989; 2000). This may limit the opportunity for infections by pathogens at an early age. A lack of immune tolerance would then result in abnormal hyperactive immune responses towards weak antigens at a later stage in life (Noverr and Huffnagel 2004; Wills-Karp et al. 2001).

Several factors influence the maturation of immune system in infants, who are more susceptible to infections than adults. Lymphoid organs, such as Peyer's patches and mesenteric lymph nodes (MLN), form after birth. During the infant's first year of life, microbes colonise the gut, and this is influenced by several external factors (Tapiainen et al. 2006). Importantly, vaginal delivery exposes the baby to the mother's vaginal and intestinal flora. This may offer protection from T1D, as the incidence of T1D was higher in children born by caesarean section than in children born by vaginal delivery, who were colonised by a variety of different microbes (Gronlund et al. 1999; Penders et al. 2013). The immunologically underdeveloped infant's intestine also exhibits greater permeability, thereby providing an opportunity for antigens and microbes to gain access into the systemic circulation (Koenig et al. 2011; Palmer et al. 2007). Gut permeability is reduced by the increased formation of tight junctions during the maturation of an individual, which is assisted by breast milk proteins and colostrum, while early exposure to dietary antigens can delay this phenomenon (Catassi et al. 1995). Several studies have shown that increased gut permeability is associated with T1D development in both human and animal studies (Fasano and Shea-Donohue 2005; Kuitunen et al. 2002; Watts et al. 2005).

The gut harbours different subsets of T lymphocytes, such asTh1, Th2, Th17 cells and Tregs (Atarashi et al. 2011; Neurath et al. 2002). Th17 cells aid in safeguarding against infections with pathogenic microbes, but can also enhance harmful auto-inflammatory functions, while Tregs in turn perform crucial roles in immune suppression. As a result, under healthy circumstances, co-occurrence of Th17 cells and Tregs is required to maintain an immune balance (Ivanov and Honda 2012). Microbes are important in regulating the Th17 balance. It was shown that even one microbial strain can be imperative for the development of Th17 cells in the intestine of mice (Ivanov et al. 2009). Therefore, commensal microbial colonisation is important, as the presence of different bacteria stimulates the induction and development of Tregs and oral tolerance towards specific antigens (Bilate and Lafaille 2012; Hrncir et al. 2008; Ostman et al. 2006).

The interaction between gut microbes and the host immune system is a very complex process. Homeostasis in the gut requires the production of short-chain fatty acids, which is needed to maintain a healthy mucosa (Wong et al. 2006). The hygiene hypothesis proposes that, due to a lack of exposure to pathogens in childhood, reduced microbial diversity due to the use of antibiotics, and a lack of oral tolerance leads to an imbalance in immune regulation (Abrahamsson et al. 2012). Infants with a reduced bacterial diversity were more prone to develop eczema later in life than were individuals who harboured a more complex microbial

flora already in their childhood (Abrahamsson, et al. 2012; Forno et al. 2008; Ismail et al. 2012). The colonisation of commensal bacteria into the gut mucosa led to the development of a normal healthy gut immunity (Cerf-Bensussan and Gaboriau-Routhiau 2010; Sekirov et al. 2010). Deliberate changes in the intestinal microbiota, as a result of treatments with chemicals or antibiotics, have resulted in an increase in intestinal inflammation and a microbial imbalance in animal models (Bhinder et al. 2013). Disturbances in the balance between commensal and pathogenic bacteria might be the cause of inflammatory bowel disease, in addition to many other autoimmune diseases, including T1D (Mazmanian et al. 2008), while a reduced number of bacterial phyla in the gut might be the reason for disturbances in gut homeostasis in T1D. Segmented filamentous bacteria (SFB), which reside in the small intestine, promote IL-17 production by Th17 cells (Gaboriau-Routhiau et al. 2009; Ivanov, et al. 2009). An analysis of faecal microbes revealed elevated levels of probiotic bacteria in biobreeding diabetic resistant rats, while Bacteroidetes and Ruminococcus were more abundant in biobreeding diabetes prone rats (Roesch et al. 2009). In humans gut microbiota comparison studies using human intestinal tract chip (HITChip) in diabetic children from the ages of 1 to 5 years (de Goffau et al. 2014), in comparison with age matched controls, have shown an increased number of Bacteroidetes in diabetic children, while healthy controls have a more balanced microbiota and an increased amount of Clostridium cluster 1V and X1Va (de Goffau, et al. 2014). Also, studies conducted in 16 Caucasian children with T1D compared to healthy control children have shown a decline in the number of bacteria essential for maintaining the integrity of the gut, such as *Blautia coccoides/Eubacterium rectale* group, *Prevotella* responsible for mucin degradation, lactic acid-producing bacteria, such as *Lactobacillus* and *Bifidobacterium*. In addition, polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (RT-PCR) has also shown similar results. Further studies are required to understand the role of microbes in human T1D (Murri et al. 2013). A reduction in bacterial diversity might lead to an increase in inflammation and permeability, and ultimately disturbances in gut homeostasis (Hooper et al. 2012; Vaarala 2008; Zipris 2013). Hence, the presence of the right microbes is essential for a healthy gut immune system.

Studies in germ-free animal models have demonstrated the importance of commensal microbes in the development of intestinal immune regulation. Germ-free mice, which have an immature immune system, due to a lack of microbial stimuli, were shown to have a defect in the development of CD4+ T cells and IgA-producing plasma cells, as well as smaller lymph nodes and payer's patches than control mice. (Hrncir, et al. 2008; Moreau et al. 1978; Tlaskalova-Hogenova et al. 1983). Many morphological changes were observed in germ-free mice compared with conventionally raised mice. These include an enlarged caecum, due to the presence of unmodified mucus glycoproteins that are formed by epithelial cells (Donowitz and Binder 1979). Additionally, germ-free mice exhibit elevated levels of serum IgE (Cahenzli et al. 2013). During early life, the development of proper intestinal microbial stimuli is crucial for immune regulatory functions, and deficiencies in these stimuli result in many autoimmune diseases (Hrncir, et al. 2008). Under germ-free conditions NOD mice have an imbalance in their Th1, Th17 and Treg cell populations in the intestine, which leads to accelerated insulitis (Alam et al. 2011).

Comparative studies of the fecal microbiota of European and rural African children have shown lack of cellulose and xylan-degrading bacteria and reduced short-chain fatty acids (SCFA) in European children (De Filippo et al. 2010). This difference was due to the diets of the subjects. African children have a diet low in animal protein and fat, diet rich in starch, fibre polysaccharide diet (fibre-rich), mainly the vegetarian diet (nutrient rich diet). The diet of

European children consists primarily of processed foods, containing predominantly animal protein and fat, starch, sugar and low in fibre (energy-dense diet). A polysaccharide-rich diet also confers protection against inflammation and non-colonic diseases (De Filippo, et al. 2010). Changes in the quality and quantity of carbohydrates in one's diet can affect microbial colonisation of the intestine. Diets rich in carbohydrates give rise to production of short chain fatty acids, which have immunoregulatory effect. (Walker et al. 2011). People who eat a diet rich in fats and devoid of vegetables and fruits are prone to asthma, while people who consume a Mediterranean diet, which is rich in fibre and protein, are less likely to suffer from cardiovascular disease and asthma (Thorburn et al. 2014; Wood et al. 2012). A recent study in mice has also demonstrated a role for dietary emulsifiers in the microbial changes in that they promote low-level inflammation and metabolic syndrome (Chassaing et al. 2015). These dietary and microbial factors play a role in maintaining gut homeostasis by reducing inflammation of the intestinal epithelium and moderating intestinal permeability in many diseases, including T1D (Brown et al. 2012).

2.1.13 Diet and microbial metabolites mediate immune regulation

Metabolites produced by commensal bacteria play an important role in gut maturation and immune homeostasis. Symbiotic bacteria that digest dietary fibres produce many metabolites and certain vitamins, which are beneficial for epithelial regeneration (De Filippo, et al. 2010). SCFAs are an important group of metabolites produced by commensal bacteria, and their main function is to maintain gut homeostasis and systemic immunoregulatory effects (Smith et al. 2013). SCFAs are primarily produced in the distal colon following the fermentation of polysaccharides. That results in the production of acetate, propionate and butyrate by commensal bacteria (Tan et al. 2014). SCFAs, particularly acetate, are found in the systemic circulation, and exacerbated inflammatory reactions were reported in the absence of SCFAs in the tissues and guts of germfree mouse models (Herbst et al. 2011; Maslowski et al. 2009). Recent studies also showed that propionate influences the activation of macrophages and dendritic cells (DC) in the bone marrow, and Th2 cells in the airways of the lungs (Trompette et al. 2014). Thus, low-level inflammation and poor gut homeostasis due to deprived dietary intake of fibre might lead to a leaky gut and autoimmune diseases (Brown, et al. 2012; De Filippo, et al. 2010; Zimmer et al. 2012). Individuals with a low bacterial diversity are susceptible to adiposity, insulin resistance and inflammatory phenotypes. These symptoms were observed in 23% of the Danish population, and they correlated with a low bacterial diversity (Le Chatelier et al. 2013). Studies have shown that decreased bacterial diversity is also associated with inflammatory bowel disease (IBD) (Lepage et al. 2011; Manichanh et al. 2006). Diets rich in polysaccharides provide a niche for *Prevotella* and *Xylanibacter* colonisation in African children. These bacteria produce butyrate, which is a SCFA that mainly promotes gut homeostasis and reduces inflammation, and they were entirely absent in the feces of European children (Thorburn, et al. 2014).

2.2 Overview of T1D

2.2.1 Natural History of T1D

T1D is a T-cell mediated disease that results in the selective destruction of the insulin-producing β cells of the pancreas. This leads to increased glucose levels in the blood. Patients

with T1D are dependent on insulin therapy for their entire life (Atkinson 2012; Sherry et al. 2005).

Individuals with an increased genetic susceptibility progress towards diabetes in response to various environmental factors. These links have been studied extensively, but the extent of association between any particular factor and the disease progression has not been determined. However, an environmental factor is regarded as a necessary trigger for autoimmunity leading to the activation of humoral and cellular immune reactions against islet cells. This process is evidenced by the appearance of autoantibodies related to T1D (Atkinson and Eisenbarth 2001). Children with a genetic susceptibility, who develop IAA antibodies in their early childhood, may eventually become diabetic without developing other autoantibodies (Achenbach et al. 2008).

Preclinical diabetes is detected in individuals based on their serum islet autoantibodies. These include insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), insulinoma-associated protein 2 autoantibodies (IA-2A) and the zinc transporter 8 antibodies (ZnT8A) (Bonifacio and Ziegler 2010; Nokoff and Rewers 2013). Later stages of preclinical diabetes are characterized by diminished insulin secretion and the loss of glucose tolerance. This occurs months to even decades after multiple islet autoantibodies are identified. Decreased levels of C-peptide and the loss of the first phase insulin response (FPIR) at this stage indicate metabolic changes before the onset of T1D. During this phase, individuals depend on exogenous insulin. Based on histological studies of the pancreas, the β-cell mass in individuals with new-onset of type 1 diabetes is reduced 80-90% compared to healthy individuals (Patterson et al. 2009).
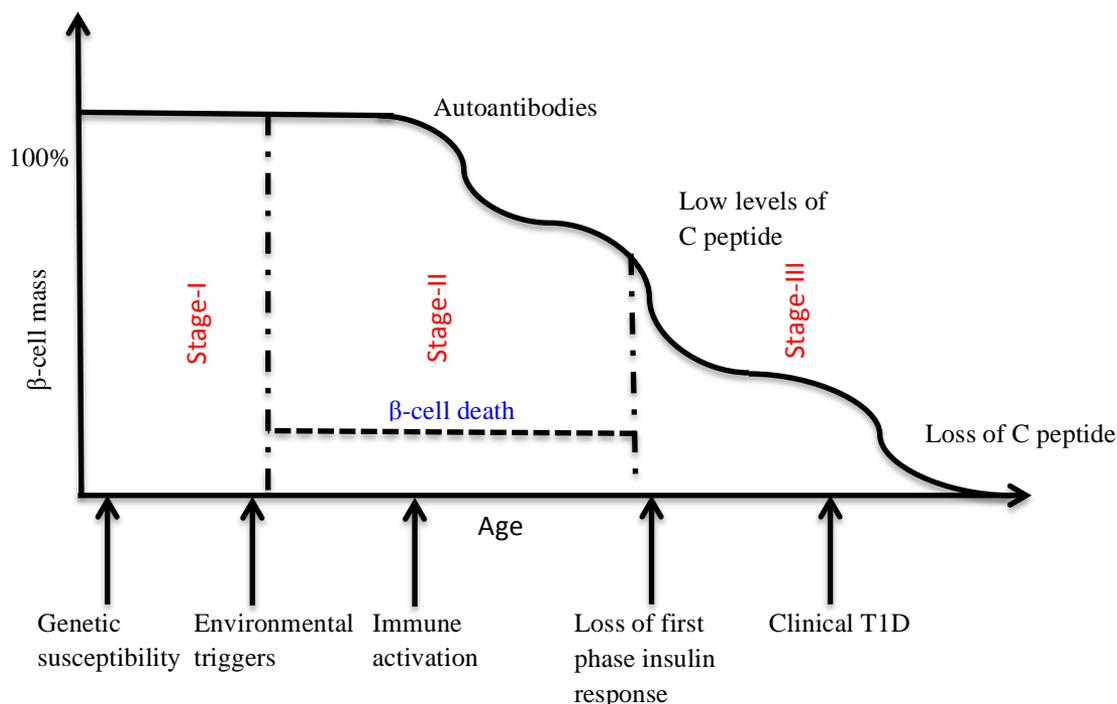
Figure 4: Natural history of T1D development. The figure represents stages of T1D development, where genetic and environmental factors trigger immune activation and gradual loss of beta cells in the islets of the pancreas. This leads gradually in the development of T1D, Modified from (Eisenbarth 1986)

2.2.2 Epidemiology of T1D

T1D is caused by a variety of genetic and environmental factors. Interestingly, over the last two decades, there has been a noticeable increase in the incidence of T1D in children under the age of 0-14 years in Finland (Harjutsalo et al. 2008) Recent reports have shown that the incidence has risen to 57.4 cases/100,000 per year (Gale 2014). Several research studies have been conducted to estimate the incidence of T1D. Studies conducted by the World Health Organization (WHO) have shown that 347 million people worldwide suffer from diabetes. This includes both type 1 and type 2 diabetes. (Gore et al. 2011; Rathmann and Giani 2004; Wild et al. 2004).

Approximately, 3-8% of the population in Western countries suffer from autoimmune diseases (Kunz and Ibrahim 2009). Studies by the EURODIAB centre have shown a remarkable increase in the frequency of T1D. From 1999-2008, the incidence of T1D increased by 3.3% annually, while the incidence of T1D in Finland increased at an annual rate of 3.6% from 1998 to 2005. A study of non-Hispanic white (NHW) youths in the USA showed that the incidence of T1D increased from 24.4 per 100, 000 in 2002 to 27.4 per 100,000 in 2009 (Dabelea et al. 2014). Studies on 84 million children in 57 countries conducted by the DIAMON project group also showed that the incidence of diabetes increased by 2.8% in 2006 (Group 2006). The highest incidences of T1D were observed in Finland and Sardinia (40 and 37.8 per 100,000, respectively). This trend was not only evident in Finland and Sardinia, but also in the USA, Canada, Australia and New Zealand the incidence of diabetes has increased. However, lower rates of T1D were found in southern parts of Europe, Venezuela (0.1 per 100,000), China (0.1-4.5 per 100,000) and other Asian countries (Group 2006; Harjutsalo, et al. 2008). T1D may reduce the life span of the patients and it is therefore important to investigate the underlying causes of T1D (Katz and Laffel 2015; Livingstone et al. 2015).

2.2.3 Role of genetic factors in T1D

Human Leukocyte Antigens (HLAs): Nearly four decades ago, the HLA region was recognised as a genetic region associated with T1D (Atkinson and Eisenbarth 2001). Subsequently, HLA genotyping has led to the identification of particular HLA genes associated for T1D, and other genetic loci susceptible for T1D (Bluestone et al. 2010). Out of all genetic regions associated with the risk of acquiring T1D the HLA locus on chromosome 6p21.31 is the most important. This genetic region includes more than 200 genes that encode HLA class I and class II antigens, of which the class II antigens appear to be the foremost genetic contributors to the development of diabetes. HLA contributes approximately 50% of the genetic risk of acquiring T1D (Erlich et al. 2008; Noble and Erlich 2012). Polymorphisms of class II HLA gene encoding DR and DQ are the major genetic determinant of the disease. DR-DQ haplotypes HLA-DRB1*03:01, DQA1*05:01-DQB1*02:01 (abbreviated as DR3) and HLA-DRB1*04, DQA1*03:01-DQB1*03:02 (abbreviated as DR4) are primarily known to associate with T1D (Ilonen et al. 2002; Lambert et al. 2004; Pugliese 2014). Hence, this strong association of

particular HLA molecules and T1D susceptibility is commonly used for HLA screening to detect children at risk of developing T1D (Pociot et al. 2010).

The insulin gene (INS): The insulin gene located on the chromosome 11p15.5 is responsible for 10% of the genetic susceptibility to T1D. Variable number tandem repeats (VNTRs) are located in the insulin promoter regions, and these regions have been separated into three categories: I, II and III (Bennett et al. 1997). Inadequate insulin expression in the thymus is known to be associated with the VNTR1 allele, and it is linked with T1D (Bennett and Todd 1996; Pugliese et al. 2001). VNTR III alleles in turn, and an increased expression of insulin messenger mRNA in the thymus, are known to have a protective effect against autoimmune T1D (Mehers and Gillespie 2008). Studies in NOD mice have clearly implicated insulin as an important autoantigen (Steck and Rewers 2011).

Protein tyrosine phosphatase non-receptor type 22 (PTPN22): *PTPN22* genes are involved in preventing spontaneous T cell activation and are located on chromosome 1p13. A lymphoid specific protein phosphatase is encoded by *PTPN22* (Bottini et al. 2004). Studies in animal models with a dysfunction in PEP (Dai et al. 2013), which is a homologous to human lymphoid specific protein phosphatase, have shown an increase in antigen-free T cell proliferation and cytokine production compared to controls (Cloutier and Veillette 1999).

The *PTPN22* gene has many polymorphic variants; polymorphisms in this gene are known to be linked with many autoimmune diseases, including T1D (Begovich et al. 2004; Kyogoku et al. 2004; Velaga et al. 2004). Several studies have reported a T1D association with an amino acid alteration due to a nucleotide conversion (C to T) at position 1858 in *PTPN22* (Douroudis et al. 2008; Fedetz et al. 2006).Various reports have shown that 15% of the Finnish population carry the 1858T allele of the *PTPN22* gene, while this allele is found in 10% of other Scandinavians, in 7-8% of the Western populations and in 2-3% of the Italian and Sardinian populations (Zoledziewska et al. 2008).

Interferon induced helicase C domain 1 (IFIH1): The association of IF1H1 with T1D was first reported by Smyth in 2006 (Smyth et al. 2006). IF1H1, which is also known as MDA5, is an intracellular protein that facilitates the production interferon in response to viral RNA (Randall and Goodbourn 2008; Rotondi et al. 2007). The association of IF1H1 with T1D was linked with gene regions on chromosome 2 by genome wide studies (Winkler et al. 2011). Additionally, the functional role of IF1H1 in T1D was demonstrated in studies conducted by Qu in 2008 (Qu et al. 2008). Studies have also shown that IFIH1 plays a significant role in the induction of inflammation and apoptosis to clear virus infected cells, suggesting that viruses might contribute to the initiation of T1D (Yeung et al. 2011).

Cytotoxic lymphocytes associated 4 (CTLA-4) genes: CTLA-4 is the third major gene region that is known to be associated with T1D (Donner et al. 1997). CTLA-4 acts as a negative regulator of T cell stimulation and plays a role in the induction of peripheral tolerance. CTLA-4 is primarily expressed by CD4+ and CD8+ T cells upon T cell receptor (TCR) mediated activation. However, CTLA-4 polymorphisms are known to be associated with T1D in humans (Ahmadi et al. 2013; Donner, et al. 1997; Jin et al. 2014).

All of the factors mentioned above have either direct or indirect roles in the development of T1D. However, the exact mechanisms underlying the pathogenesis of T1D are not yet known

(Steck et al. 2005). The increasing incidence of T1D cannot simply depend on genetic factors. Thus, environmental factors play a significant role in T1D.

2.2.4 Environmental factors in the pathogenesis of T1D.

The role of viruses in the pathogenesis of T1D

The aetiology of T1D is very complex. Several viruses are known to associate with T1D, but their pathogenesis is not clearly understood (Gale 2012). Many viruses such as mumps, rubella, cytomegalovirus, enteroviruses and retroviruses, are known to play a role in virally induced β cell autoimmunity, and they can cause a cytolytic infection in pancreatic β cells. Early studies have shown that in 20% of affected individuals, a rubella virus infection led to an infection of the fetal pancreas (Ginsberg-Fellner et al. 1985). Occasional studies have shown associations between T1D and other viruses, such as cytomegalovirus, Epstein-Barr virus and retroviruses (Conrad et al. 1997; Jun and Yoon 2003). Evidence for enterovirus-mediated T1D was obtained from an early study conducted in 1969, which showed that higher serum neutralising antibody titers to coxsackie viruses, particularly of the B4 strain, were observed in newly diagnosed T1D patients, compared with healthy controls (Gamble et al. 1969). Moreover, the Finnish diabetes prediction and prevention (FDPP) study, carried out in the Finnish population, has also shown that enterovirus infections are associated with the pathogenesis of T1D (Sadeharju et al. 2001). Antibodies against enteroviruses were found in individuals who also carried T1D related autoantibodies. This suggests that enteroviral infections can cause T1D (Alidjinou et al. 2014; Hober and Sauter 2010; Yeung, et al. 2011). T1D patients with cellular immune responses against coxsackieviruses were also reported (Juhela et al. 2000). The significance of enteroviruses in the European population was evaluated by a study conducted in 249 children. Here, coxsackieviruses B1 (CVB1) antibodies were much more frequent in children with diabetes compared to healthy controls (Oikarinen et al. 2014). Several early studies also found antibodies against coxsackievirus in the blood samples of children suffering from and susceptible to T1D (Andreoletti et al. 1997; Chehadeh et al. 2000; Lonnrot et al. 2000; Yin et al. 2002). A study showed that coxsackievirus B4 (CVB4) induced the disease by exploiting molecular mimicry. A protein sequence of the virus was found to be similar to that of the human glutamic acid decarboxylase (GAD) protein in the islets and could therefore activate, autoreactive T cells (Vreugdenhil et al. 1998). However, no consistent data on a particular viral mechanism during the onset of diabetes was found (Filippi and von Herrath 2008). Hence, understanding the particular role of the virus in the pathogenesis of T1D would require extensive studies.

Bacteria

Although the importance of microbial factors as key modulators of T1D was accepted long ago, this aspect has recently received a lot of attention. The well-known hygiene hypothesis states that early exposure to microbes stimulates immune-regulatory mechanisms, which prevent autoimmune reactions and allergies (Bach and Chatenoud 2012). However, the underlying mechanism has not yet been explicitly evaluated. Intestinal bacteria have significant effects on T cells, macrophages and dendritic cells. In animal models, *Bacteroides fragilis* is known to control the expression of Interleukin-10 (IL-10) in T cells in addition to diminishing Th17-mediated immune regulation in the lamina propria (Round and Mazmanian 2010). Finnish children with a genetic risk for T1D were followed from birth until 2.2 years, and when their stool samples were analysed by 16s rRNA sequencing an increased ratio of *Bacteroides* to

Firmicutes were observed before seroconvertion. The children with a high abundance of *Bacteroides* developed T1D later in their lives (Davis-Richardson et al. 2014). Also, studies by others pointed to a difference in the microbial composition of children with T1D compared to controls (de Goffau, et al. 2014). Studies have also shown that the gut microbiota affects both the innate and adaptive immunity (Purchiaroni et al. 2013; Slack et al. 2009). Alterations in intestinal bacteria and disturbed homeostasis may lead to intestinal inflammation, which might lead to the destruction of β cells and, thus, autoimmune T1D (Vaarala 2013).

Dietary factors

Several studies have demonstrated the importance of diet and its effects on the microbiota in the gut. Recent research has shown a relationship between diet and microbial changes in the development of T1D, which is reviewed in Munyaka (Munyaka et al. 2014). Dietary alterations have reduced diabetes in animal models. When NOD mice were weaned to ProSobee, a soy-based protein, they exhibited a significantly reduced incidence of T1D (Flohe et al. 2003), and alterations in the gut microbiota of ProSobee fed NOD mice were also reported (Alam et al. 2010). Similar results have been achieved by feeding diabetes-prone bio-breeding (BB) rats and NOD mice with casein hydrolysate, or infant formula (Beales et al. 2002; Graham et al. 2004; Lefebvre et al. 2006).

Studies have shown that early exposure to cow's milk proteins is associated with an increased risk of T1D (Luopajarvi et al. 2008). Increased levels of IgG and IgA antibodies were detected towards cow's milk protein in children diagnosed with early T1D (Dahlquist et al. 1992; Vaarala 2004; Virtanen et al. 1994). Children fed with highly hydrolysed milk have less GAD antibodies, compared to children fed with conventional milk proteins (Knip et al. 2010). However, the follow-up of these studies suggested that there was no significant difference in the incidence of T1D between subjects fed with conventional milk proteins compared with subjects fed with hydrolysed milk proteins (Knip et al. 2014).

Another well-studied dietary factor is wheat gluten, and several studies have shown that it is associated with T1D (Funda et al. 2008; Maurano et al. 2005; Mueller et al. 2009). Studies have shown that NOD mice and BB rats fed with cereal diet have a higher rate of T1D, while mice fed with a gluten free diet had reduced the rate of T1D (Adlercreutz et al. 2014; Marietta et al. 2013). Studies have also shown that when mice were kept on a diet consisting of 30% wheat proteins, compared with 10%, they exhibited a lower incidence of T1D (Funda, et al. 2008; Mueller, et al. 2009). Similar findings were observed in human T1D, when wheat protein was introduced with a delay after weaning (Schmid et al. 2004). A study by the German BABYDIAB and The American DAISY study have shown an increased risk of developing T1D upon exposure to a diet containing cereals at 3 months compared to 7 months of age (Antvorskov et al. 2014; Norris et al. 2003). However, reports from the BABYDIAB studies have indicated that exposure to gluten, either early or delayed, has no effect on delaying or reducing the risk of autoimmune related T1D (Beyerlein et al. 2014).

Vitamin D is an essential nutrient. Upon exposure to sunlight, the skin produces vitamin D endogenously; northern areas, such as Canada and most of Europe, have very little exposure to the sun during winter months (Webb et al. 1988). Therefore, diets in these regions are often supplemented with vitamin D. Condensed amounts of vitamin D in the supplements resulted in an increase in the incidence of T1D (Zipitis and Akobeng 2008). The intake of vitamin D during pregnancy through vitamin D rich foods reduced islet autoimmune antibodies in children

after 4 years (Fronczak et al. 2003). Studies have shown that the intake of vitamin D supplements reduces T1D, while inadequate intake in infants results in an increase in the risk for T1D (Hypponen et al. 2001). However, children with T1D and multiple islet autoantibodies have lower levels of vitamin D in plasma than do children who are negative for islet autoantibodies. Still, vitamin D was not related to a faster progression of T1D (Raab et al. 2014). Contradictory studies have shown that vitamin D is not necessary in preventing T1D in Finnish and Estonian populations (Reinert-Hartwall et al. 2014). Larger studies are required to understand the importance of vitamin D for T1D.

2.2.5 Gut immune regulation and a microbial link to T1D

The gut-associated lymphoid tissue (GALT) is an important part of the immune system. The intestine encounters antigens from food and microorganisms, and then adjusts and maintains a delicate balance between a tolerance towards non-self and self-antigens in the presence of commensal microbiota (Wu and Wu 2012).

This tolerance mechanism, however, is disturbed during T1D when the insulin-producing β cells of the pancreas are destroyed by the host's immune system. Studies in animal models have shown that the onset of T1D is associated with gut enteropathies (Bosi et al. 2006; Maurano, et al. 2005; Vaarala 2013; Westerholm-Ormio et al. 2003). Increased intestinal permeability was observed in pre-diabetic patients (Bosi, et al. 2006) and in obese and non-obese mice (Cani et al. 2009; Maurano, et al. 2005). Alterations in the activation of dendritic cells in the gut innate immune system have been reported during the onset of T1D (Badami et al. 2011). Signs of immune activation have been detected in biopsies of the small intestine from T1D-affected children (Westerholm-Ormio, et al. 2003). Moreover, signs of increased antibody responses and T cell responses towards food antigens were also detected in T1D patients (Barbeau et al. 2014).

According to reports by the Trial to Reduce insulin-dependent diabetes mellitus (IDDM) in the Genetically at Risk (TRIGR), or the Finnish Dietary Intervention trial for prevention of (FINDIA), the microbial composition in the gut is altered and less diverse in children who produce autoantibodies against β cells, while healthy controls maintained a stable microbiota (de Goffau et al. 2013). Studies associated with the human microbiome project have suggested that healthy adults have a large gut microbial diversity and that these populations of microbes are stable. Conversely, reduced microbial diversity was shown to be associated with T1D and inflammatory bowel diseases (Turnbaugh et al. 2009; Vaarala 2013). Furthermore, increased numbers of *Bacteroides* in the gut were also observed in BB rats compared with diabetes resistant BB rats (Brugman et al. 2006; Roesch, et al. 2009). Moreover, male NOD mice have more *Firmicutes* than *Bacteroides* in their gut, and a lower diabetic rate compared with female NOD mice (Markle et al. 2013). Increased gut permeability in human and animal studies might be due to imbalances in bacterial colonisation (Vaarala 2008).
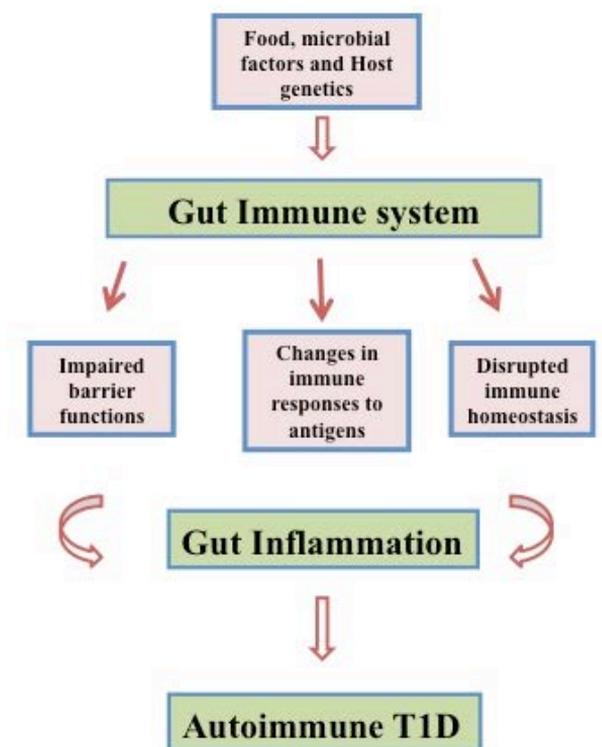
Figure 5. Proposed factors important in the regulation of gut immune responses: Dietary mediated microbial colonisation and its influence on gut immune regulation. An improper diet leads to gut permeability, inflammation and disturbed homeostasis, which might trigger inflammation and as a result lead to autoimmune T1D. The data was compiled from different review articles.

2.2.5 Pathogenic role of macrophages in T1D

Macrophages belong to immune cells that safeguard the host from infections and alert the immune system to defend the host. During autoimmune T1D, macrophages were detected in the islet infiltrate of young NOD mice (Gordon 2003). Preventing the entry of macrophages by inhibiting their adhesion promoting receptor, either by pharmacologically blocking it or by deleting its gene, reduced the rate of T1D (Hutchings and Cooke 1990). Furthermore, studies in mouse models suggest that T cells in macrophage-depleted environments are unable to differentiate into cytotoxic CD8+ T cells (Jun et al. 1999). However, the up-regulation of IL-4, which mediates the Th2-type immune response and the down-regulation of IFN-γ, which mediates Th1-type immune responses, resulted in T cells losing their ability to differentiate into effector T cells. Hence, in the absence of macrophages, T cells lose their ability to kill β cells (Jun, et al. 1999).

Classically activated macrophages that produce pro-inflammatory cytokines, induce NO and ROS. The expression of these inflammatory molecules might lead to destruction of insulin-producing β cells in the pancreas during T1D (Espinoza-Jimenez et al. 2012; Gordon 2003; Szablewski 2014). Insulin-producing β cells are very sensitive to free radicals, due to their diminished ability to scavenge free radicals (Asayama et al. 1986). ROS produced by macrophages can damage β cells by cleaving DNA. That results in the induction of the DNA repair enzyme poly (ADP) ribose dinucleotide (PARP). This depletes nicotinamide adenosine dinucleotide (NAD) leading to β cell death by necrosis (Burkart et al. 1999; Jun, et al. 1999). Alternatively activated macrophages have an opposite effect, as they reduce inflammation in the pancreas, decrease hyperglycemia and reduce β cell death. During helminthic infections and allergic responses, immune cells mediate Th2 responses by secreting IL-4 and IL-13, thereby resulting in the induction of alternatively activated macrophages (Stein and Keshav 1992; Szablewski 2014). These are controlled by the transcription factor STAT6 (Robinson et al. 2008; Roy et al. 2002). Protection against helminth antigens occurs via the Th2-mediated response. (Reyes and Terrazas 2007; Stempin et al. 2010). IL-10 and TGF-β are secreted by alternatively activated macrophages, which also express higher levels of the programmed cell death 1 (PD-1) molecule, thereby inhibiting the proliferation of T cells (Terrazas et al. 2005). Instead, activated macrophages induced by helminth antigens decrease T1D-triggered inflammation and glucose tolerance during obesity (Liu et al. 2009). CD4+ and CD8+ T cells and macrophages are involved in the destruction of β cells of the pancreas, which results in T1D (Turley et al. 2003). Other immune cells are also present in the pancreatic islets and PaLNs, such as NKT cells and B cells. Together with T cells and dendritic cells, all these contribute to the destruction of the β cells of the pancreas (Bluestone, et al. 2010).

3. AIMS OF THE STUDY

The aim of my study was to investigate the role of the gut immune system and its two most-important environmental modifiers, the gut microbiota and dietary components in the pathogenesis of T1D using a non-obese diabetic (NOD) mouse model. Diet and microbial colonisation-dependent inflammatory phenomena in the intestine and their potential extra-intestinal manifestations were addressed.

The specific aims of my thesis are as follows

1) To evaluate inflammatory activity in the whole gut of NOD mice, using ex vivo imaging.
2) To study whether dietary fibres have a pathogenic role in diabetes.
3) To characterize the extra-intestinal manifestations of impaired gut barrier function in the NOD mouse and their potential relevance to islet-autoimmunity.

4. MATERIALS AND METHODS

4.1 Mice used for the experiments *(Publication I, II and III)*

NOD mice are widely used animals for studying T1D. Hence, we used NOD mice and the age-matched healthy BALB/c and C57BL/6 mice as the control strains originally purchased from Jackson laboratories (Bar Harbor, ME, USA). All animals were raised and bread in the central animal laboratory of Turku University. All animal research work was carried out according to the guidelines of National Board of Animal Experimentation in Finland, performed under the license number (ESAVI/3210/ 04.10.03/2011). NOD, BALB/c and C57BL/6 were grown under specific pathogen-free conditions, and they were fed either with casein hydrolysate (hereafter referred as CH-NOD) or ProSobee (hereafter referred as PNOD). Casein hydrolysate and ProSobee were infant formulas from Mead Johnson Nutritionals. Mice were weaned either PNOD or CH-NOD and continued during these studies. No significant differences in the growth or development were observed in the mouse group placed on a regular chow diet compared to NOD mice fed with infant formula.

Mice from several litters were allocated into 3 different groups and were fed with either cellulose or PX or normal chow diets from weaning. For complete semisynthetic diets, Basal mix 80% ™ (Harlan Laboratories, Madison, WI, USA) was used for the base, which was supplemented with 20% whey, or casein or fibres (pectin and xylan or cellulose alone) as indicated.

From age 12 to 34 weeks blood glucose was monitored weekly by puncturing the tail vein. Blood glucose exceeding 14mM for two consecutive measurements was considered as an indication of diabetes.

4.2 Isolation of immune cells *(Publication I, II and III)*

4.2.1 Peritoneal lavage cells

 Peritoneal cells were isolated by flushing the peritoneum with 5ml ice-cold RPMI-1640 medium (Gibco, Garland island, NY, USA) and drawn back into the same syringe. The total number of cells retrieved was determined by multiplying the concentration of cells/ml in the lavage with the volume of medium injected (5 ml).

4.2.2 Lamina propria lymphocytes

Lymphocytes were isolated from the small intestine of 6-week-old NOD, CH-NOD and BALB/c mice by collagenase (0.5mg/ml, type VIII, Sigma-Aldrich, St Louis, MO, USA) digestion followed by mechanical disruption. Enrichment of mononuclear cells was performed by gradient centrifugation at RT for 20min at 1,200g with Lympholyte (Cedarlane, Burlington, ON, Canada).

4.3 In-vivo Imaging System (IVIS) Imaging *(Publication I)*

In-vivo and ex-vivo imaging was performed by anaesthetizing the mice with Isoflurane (Baxter, Deerfield, IL, USA) followed by intraperitoneal injection of L-012 at 50mg/kg of the body weight in saline. L-012 is a chemiluminescent probe (Wako Chemicals, Richmond, VA, USA),

a luminol derivative, and produces light when modified by RONS species. Anaesthetized mice were imaged under the IVIS-100 (Xenogen, Alameda, CA, USA) camera and a series of images were taken for 30min. Ex-vivo imaging was performed by dissecting the whole gut and placing it onto a petri-dish.

4.4 Gut permeability (*Publication I*)

The permeability of gut was analysed by giving an intragastric bolus of fluorescent isothiocyanate (FITC-Dextran 4, Sigma-Aldrich, St Louis, MO, USA; 40 kDa) to the mice by using a feeding needle. After 4hours, mice were euthanized and blood was collected by cardiac perforation. Fluorescence was measured from duplicate serum samples at 485nm excitation (VictorPerkin Elmer, Waltham, MA, USA).

4.5 Laser capture microscopy and isolation of islet RNA for cytokine profiling (*Publication I*)

Pancreases were obtained from six-week old NOD and CH-NOD mice. They were frozen at -80°C. Frozen pancreas sections were cut to 16 µm thick and placed on nuclease-free PEN glasses (Zeiss, Oberkochen, Germany). Hematoxylin and eosin (Sigma-Aldrich, St Louis, MO, USA) staining was performed to visualize pancreatic islets. Using the laser capture microscopy (Zeiss), islets were cut with a UV laser and automatically captured into an adhesive cap. 30-50 sections were obtained for each sample and processed for RNA isolation using Array pure nanoscale RNA purification kit (Epicentre, Madison, WI, USA) and for quantitative PCR.

4.6 Microbiota profiling

4.6.1 Analysis of gut microbial short-chain fatty acids (*Publication I and II*)

Total DNA was isolated from the pooled caecum and colon content of six-week old NOD and CH-NOD mice and of seven-week old PX, CE, and NC-fed nod mice to analyse intestinal microbiota composition and fatty acid profiles by Alimetrics Ltd (Espoo, Finland). Additional microbial profiling was performed by stratifying the chromosomal DNA based on the guanine and cytosine (G+C) nucleotide content. In % G+C profiling, every DNA sample was fractionated by 72-h CsCl equilibrium density gradient ultracentrifugation (100,000x g) and chromosomes with distinct G+C contents were parted. Subsequently, the gradients formed by ultracentrifugation were pumped through a flow UV absorbance detector at 280 nm. A gas chromatography was used to measure short-chain fatty acids by using pivalic acid as an internal standard (Dicksved et al. 2008).

4.6.2 DNA extraction for the Next Generation Sequencing analysis (*Publication II*)

Whole DNA was isolated from 100mg of frozen, pooled colon and caecum samples, using GenoXtract Stool Extraction Kit VER 2.0 (Hain Lifescience, Nehren, Germany). Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) was used to measure DNA for its purity and concentration (Publication II).

4.6.3 Next Generation Sequencing analysis for microbiota samples (*Publication II*)

Illumina MiSeq system (Illumina, San Diego, CA, USA) was used to perform Next Generation Sequencing (NGS). Samples were analysed for the V4 region of 16S rRNA gene (250bp) and

then amplified using Kapa HiFi PCR kit (Kapa Biosystems, Wilmington, MA, USA) and Veriti thermal cycler (Thermo Fisher, Waltham, MA, USA). (Aglient, Santa Clara, MA, USA) was used to measure the quality of the libraries Bioanalyzer 2100. QIIME pipeline was used for analysing the dataset (Caporaso et al. 2011). Data filtering was done as defined previously (Bokulich and Mills 2013).

4.7 Western blotting (*Publication II and III*)

4.7.1 Protein expression analysis of colon epithelial cells

Colon epithelial cells from seven-week old NC, PX and CE fed NOD mice were isolated by rinsing the emptied colon into PBS. Then, they were cut and opened longitudinally. Colons were disrupted by agitation, using 1mmol/1 EDTA-HBSS solution at 37°C for 30 min. The cell lysates were centrifuged for 30min at 1400g (4°C) to remove cell debris. Protein concentration was estimated using the Bradford assay (Bio-Rad, Hercules, CA, USA). 10% SDS gels were used to separate the proteins, which were then transferred onto the PVDF membrane (Millipore, Darmstadt, Germany). Membranes were incubated with monoclonal antibodies to IL-18 (at a concentration of 1:3000) and actin (at a concentration of 1:10,000) (anti-mouse IL-18, clone 39-3F, MBL, Japan; anti-mouse actin, AC-40, Sigma-Aldrich) followed by the second-step peroxidase-labelled anti-rat IgG (Cell Signalling Technology, Beverly, MA, USA). The ECL developing solution (Thermo Fisher, Waltham, MA, USA) was used for detection. The density of IL-18 bands were compared to actin band in the same membrane using the EX photometer (Thermo Fisher).

4.7.2 Protein expression analysis of IRAK-M from peritoneal macrophages

Peritoneal macrophages were isolated and sorted from 3 and 5-week old NOD mice. Sorted macrophages were lysed using lysis buffer (50mM tris-HCL, pH 7.5, 150mM NaCl, 0.5% TX - 100, 5% glycerol, 1% SDS, 1mM Na_3V0_4, 10mM NaF, and 1mM PMSF). Protein concentrations were measured using the DC Protein Assay (Bio-Rad, Hercules, CA, USA), and 6x sample loading buffer (0.5M Tris-HCl pH 6.8, 28% glycerol, 9% SDS, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) was added. 10% SDS-PAGE gels were used to separate the proteins, which were then transferred to PVDF membrane. The membrane was incubated overnight at +4°C with a primary antibody raised in rabbit specific for IRAK-M (Upstate, Merk Millipore, Billercia, MA, USA), followed by HRP-conjugated goat anti-rabbit (HyTest Ltd, Turku, Finland) secondary antibody. Pierce developing solution (Thermo Fisher, Waltham, MA, USA) was used for detection.

4.8 Flow cytometry

4.8.1 Activation marker analysis from peritoneal macrophages   (*Publication III*)

The peritoneal cells were counted and analysed by flow cytometry using FACS Calibur (BD biosciences, Franklin Lakes, NJ, USA) after incubating the peritoneal cells with anti-mouse allophycocyanin- (APC) conjugated F4/80 (Bio Legend, San Diego, CA, USA) and anti-mouse FITC-conjugated CD11b (Immunotools, Friesoythe, Germany) antibodies. Surface expression of activation markers was determined by incubating the cells with anti-mouse FITC-conjugated CD40 (Biolegend, San Diego, CA, USA) or anti-mouse CD80 (eBioscience, San Diego, CA, USA) and anti-mouse phycoerythrin- (PE) conjugated CD86 (eBioscience, San Diego, CA,

USA) antibodies. Expression of the adhesion molecule α4 integrin on peritoneal macrophages was analysed using the anti-mouse FITC-conjugated CD11b antibody (Immunotools, Friesoythe, Germany), allophycocyanin-conjugated anti-mouse B220 antibody (Caltag Laboratories, Buckingham, Buckinghamshire, UK) (to exclude CD11b+ B cells from the analysis) and the PE-conjugated anti-integrin α4 antibody (AbD Serotec, Oxford, UK).

4.8.2 LPS induced T and B cell activation from pancreatic lymph nodes (*Publication III*)

CD69 activation was analysed from the T and B cells of pancreatic lymph nodes by incubating the cells with FITC-conjugated anti-mouse CD4 (eBioscience, San Diego CA, USA) or anti-mouse CD8 (Immunotools, Friesoythe, Germany) or APC-conjugated anti-mouse B220 (Biolegend, San Diego CA, USA) antibodies along with the PE-conjugated anti-mouse CD69 (BD Bioscience). Cells were run for flow cytometry using FACS Calibur (BD biosciences, Franklin Lakes, NJ, USA) and analyzed using cell Quest (BD Bioscience) software.

4.8.3 Gut T cell analysis (*Publication I*)

Small intestinal lymphocytes were stained with phycoerythrin-conjugated anti-CD69 antibodies (BD Bioscience, Franklin Lakes, NJ, USA) and FITC-conjugated anti-CD4 (Immunotools, Friesoythe, Germany). Samples were run on Facs Calibur flow cytometer and analysed with cell Quest software (BD Biosciences).

4.8.4 T-cell cytokine profiles in gut-draining lymph nodes (*Publication I*)

Mesenteric lymph nodes of five-week old NOD, CH-NOD and BALB/c mice were isolated and 1.0 million lymph node cells were placed into a flat-bottomed 96-well plate. The plates were pre-coated with anti-CD3 antibody (eBioscience, San Diego CA, USA) in complete medium (DMEM with 10% FBS, L-glutamine and penicillin-streptomycin). Cytokine analysis was performed using the multiplex cytokine bead array (FlowCytomix, eBioscience) according to the protocol provided by the manufacturer from the supernatants collected after 48 hours. The FACS calibur flow cytometer (BD biosciences, Franklin Lakes, NJ, USA) was used to run the beads. The FlowCytomix pro 3.0 software was used for analysing the results.

4.8.5 In vitro LPS stimulation of peritoneal macrophages (*Publication III*)

Peritoneal macrophages were sorted by gating the FITC-conjugated anti-mouse F4/80 positive macrophage population. B cells were excluded out by gating the events positive for PE-conjugated anti-mouse B220. Cellular debris and dead cells were excluded by gating low forward scatter (FSC) and high side scatter (SSC). The purity of the sorted macrophages was ≥96%. They were plated onto 96-well flat-bottomed plates (Nunc) at 200,000 cells/well in complete medium (RPMI 1640 plus 10% FCS, L-glutamine 2mmol/l, 100 U/ml penicillin plus streptomycin). Cells were stimulated with 0.2, and 5.0 µg/ml LPS from Escherichia coli (Sigma-Aldrich, St Louis, MO, USA). Cell culture supernatants were collected after 48 hours and analysed for TNF-α using the Luminex platform, and for IL-10 and IL-12 using a flow-cytometric bead assay (BD Biosciences, Franklin Lakes, NJ, USA) kit. Beads were sorted using the BD Accuri (BD Biosciences, Franklin Lakes, NJ, USA) and the results were analysed using the BD C6 Accuri software.

4.8.6 In vivo responses to LPS stimulation (*Publication III*)

To determine the responsiveness of peritoneal macrophages to LPS stimulation, age- and sex-matched NOD, BALB/c and C57BL/6 mice were injected i.p. with 10µg LPS from Escherichia coli (Sigma-Aldrich, St Louis, MO, USA) in PBS buffer. After 6 hours, animals were sacrificed, and PMs were collected by peritoneal lavage, as described above. They were analysed for α4 integrin expression with anti-mouse PE-conjugated antibody (AbD Serotec, Oxford, UK). Peritoneal LPS in activating pancreatic lymph node Th1 cells was evaluated by injecting 30µg of LPS i.p. After 24 hours or five days, animals were sacrificed, and PaLN cells were analysed with anti-mouse CD69 (BD biosciences, Franklin Lakes, NJ, USA) and intracellular anti-mouse IFN-γ respectively (Biolegend, San Diego CA, USA).

4.8.7 Intracellular cytokine staining (*Publication III*)

Intracellular IFN-γ cytokine staining was performed by isolating lymphocytes from PaLNs of NOD mice with and without in-vitro LPS stimulation. Single cells suspension of lymph node cells were incubated in complete medium (RPMI 1640 plus 10% FCS, L-glutamine 2mmol/l, 100 U/ml penicillin plus streptomycin) and incubated with a cell activation cocktail (phorbol-12-myristate 13-acetate (40.5µM), inomycin (669.3µM) and brefeldin A (2.5mg/ml); BioLegend, San Diego CA, USA) for 4 hr at 37 ºC in 5% CO2. Stimulated cells were washed and surface-stained for 15 min in the dark at 4 ºC with FITC-conjugated anti-mouse CD4 (eBioscience, San Diego CA, USA) and APC-conjugated anti-mouse CD8 (Immunotools, Friesoythe, Germany) in FACS buffer. Rat serum was used to block non-specific binding. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) and permeabilized with 0.5% saponin (Merck, Millipore, Billercia, MA, USA). PE-conjugated anti-IFNγ and its isotype control (BioLegend, San Diego CA, USA) were used for the intracellular staining. The BD Accuri C6 flow cytometer software was used for the analysis of results.

4.9 Endotoxin measurements from mouse serum samples (*Publication III*)

Blood samples were taken aseptically by cardiac puncture and collected into sterile tubes (Sarstedt, Karl-Schiller-Str, Germany) and centrifuged at 1500 RMP for 10 min on a bench-top centrifuge (Thermo Fisher, Waltham, MA, USA). Serum samples collected into sterile Eppendorf tubes were stored at -80ºC until analysis. Serum samples and standards were incubated along with Limulus amebocyte lysate (LAL, chromogenic Endpoint assay kit, Hycult biotech, Uden, the Netherlands) reagents in duplicates according to the instructions of the manufacturer.

4.10 Histology

4.10.1 Small intestine and colon histology (*Publication I, II and III*)

For conventional histology, pieces of the ileum and colon were fixed in 10% buffered formalin and embedded in paraffin, while pancreases were snap-frozen in Optimal Cutting Temperature (OCT) (Sakura Finetek, CA, USA) compound. Colon, pancreas and ileum sections were stained with the standard haematoxylin and eosin (H&E; Sigma-Aldrich, St Louis, MO, USA) staining. H&E-stained ileum samples were measured for signs of inflammation and leukocytes aggregation under light microscopy (Olympus, BX51, Berlin, Germany). Colon samples were measured for hyperplasia, evaluated for goblet cells for their size, mucus content and acidity.

Insulitis scores were performed by assessing the degree of lymphocyte infiltration and categorised as follows: 0, no insulitis; 1, peri-insulitis with or without minimal infiltration in islets; 2, insulitis with <50% infiltration of islets; 3, invasive insulitis with >50% infiltration of islets. Each NOD mouse group was counted for 60 islets per pancreas.

4.11 Immunofluorescence

4.11.1 Myeloperoxidase detection (*Publication I*)

For the detection of myeloperoxidase-containing cells, ileum was fixed, snap-frozen and embedded in the optimal cutting temperature (OCT) compound. 5-7 µm thick cryosections were cut and fixed, in -20°C acetone and stained using anti-rabbit myeloperoxidase antibody (Thermo Fisher, Waltham, MA, USA) and Alexa488-conjugated donkey-anti-rabbit immunoglobulin (Invitrogen, Carlsbad, CA, USA). Sections were imaged using confocal microscopy (Leica TCS SP5 Matric, Wetzlar, Germany).

4.11.2 Detection of RONS in small intestine (*Publication I*)

Epithelial cell RONS production was detected by the in-vivo dihydroethidium (DHE) (Invitrogen, Carlsbad, CA, USA) staining. Using Ketalar (Pfizer, New York City, NY, USA) and Rompun (OrionPharma, Espoo, Finland) mice were anesthetized, and their abdomen, the peritoneal cavity was cut open via a small incision and DHE in PBS (200 µl at 5µM concentration) was injected into the lumen of the ileum. 20 min later mice were euthanized, and ileum samples were collected, and snap-frozen and further processed for histochemistry, counterstained with DAPI (Invitrogen, Carlsbad, CA, USA) and images were taken under UV microscope (Olympus, BX51, Berlin, Germany).

4.11.3 Insulitis detection (*Publication II*)

Pancreas tissue from 7-week-old PX, CE and NC mice were snap-frozen and embedded into OCT. 6-7µm thick cryosections were cut and fixed in -20°C acetone and subsequently stained with anti-rabbit Insulin H-86 (Santa Cruz Biotechnology) followed by donkey anti-rabbit alexa fluor 647 (Invitrogen, Carlsbad, CA, USA). The nuclei were stained with DAPI (4, 6-diamidino-2'-phenylindole, dihydrochloride; Invitrogen, Carlsbad, CA, USA) and viewed under UV microscope (Olympus, BX51, Berlin, Germany).

4.12 Quantitative real-time polymerase chain reaction

4.12.1 RNA isolation (*Publication I, II and III*)

Ileum, colon, and lymph node samples were collected into RNA*later* (Qiagen, Holden, Germany) immediately after dissection. Subsequently, RNA isolation was performed by homogenizing samples with power LyserTM (MoBio, Carlsbad, CA, USA) and using the Power LyserTM ultra clean tissue and cells RNA isolation kit. RNA from peritoneal macrophages was isolated using the RNEasy Mini Kit (Qiagen, Holden, Germany). Using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA), RNA purity was determined. cDNA synthesis was performed from RNA using oligo-Dt primers (DyNAmo CDNA synthesis Kit, Finnzymes, Finland).

4.12.2 Cytokine analysis by quantitative real time-PCR for small-intestinal tissue samples (*Publication I*).

Levels of cytokine expression in the small intestinal tissue of individual mice were analysed with quantitative real-time PCR using the Maxima SYBR Green qPCR master mix (Fermentas, St Leon-Rot, Germany) with Light Cycler 480 (Roche, Basel, Switzerland). Standards were run along with the control gene GAPDH to calculate the absolute copy numbers of RNA (molecules/µg). Table 1 provides information on the primers used.

Table 1. Primer sequences used for qPCR by SYBR green method

	Gene	Forward sequences	Reverse sequences
1	*Gapdh*	GGGTGTGAACCACGAGAAAT	ATCCACAGTCTTCTGGGTGG
2	*Foxp3*	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
3	*Rorγt*	GCTGCTGGCTGCAAAGA	TGCAGGAGTAGGCCACATTAC
4	*Il1β*	ACGGACCCCAAAAGATGAAG	CACGGGAAAGACACAGGTAG
5	*Il6*	CCGGAGAGGAGACTTCACAG	AAGTGCATCATCGTTGTTCATACA
6	*Il10*	AGGGCCCTTTGCTATGGTGT	TGGCCACAGTTTTCAGGGAT
7	*Il12p40*	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
8	*Il17*	TCCCTCTGTGATCTGGGAAG	CTCGACCCTGAAAGTGAAGG
9	*Il18*	TGGAGACCTGGAATCAGACA	CTGGGGGTTCACTGGCACTT
10	*Tgfβ*	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
11	*Il23p19*	TGGCATCGAGAAACTGTGAGA	TCAGTTCGTATTGGTAGTCCTGTTA
12	*β-actin*	ATCTGGCACCACACCTTCTACAAT	CCGTCACCGGAGTCCATCA

4.12.3 Gene expression analysis by quantitative real time-PCR for colon tissue samples (*Publication II*)

cDNA was synthesized with using the MaximaTM reverse transcriptase (Fermentas, St Leon-Rot, Germany) and oligo (dT) primers (Thermo Fisher, Waltham, MA, USA). Relative cytokine expression was determined by quantitative real-time PCR using Taqman probes (Roche Applied Sciences, Basel, Switzerland). All cytokine signals were normalized to β-actin expression. P-values were calculated from dCt values (Ct (target)-Ct (reference)). Table 2 provides information on the primers used.

Table 2. Primer sequences and the Universal Probe Library (Roche) probe numbers used for the Taqman qPCR approach.

Gene		Forward sequence	Reverse Sequences	Probe
1	*Il4*	CATCGGCATTTTGAACGAG	CGAGCTCACTCTCTGTGGTG	2
2	*Il6*	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA	6
3	*Il17*	CAGGGAGAGCTTCATCTGTGT	GCTGAGCTTTGAGGGATGAT	74
4	*Il18*	CAAACCTTCCAAATCACTTCCT	TCCTTGAAGTTGACGCAAGA	46
5	*Il22*	TGACGACCAGAACATCCAGA	AATCGCCTTGATCTCTCCAC	62
6	*Tgfβ1*	GCAACATGTGGAACTCTACCAG	CAGCCACTCAGGCGTATCA	66
7	*Rorα*	GAGATTGAAAACTACCAGAACAAGC	AATCCATCAATGCGTTTGG	22
8	*Tnfα*	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG	25
9	*Ifnγ*	TGCAGGAATCTCCCCTGA	CACAGGGGCTGTGTTTCTTC	21
10	*Xbp1*	CTGACGAGGTTCCAGAGGTG	GCAGAGGTGCACATAGTCTGAG	49
11	*Chop*	CCACCACACCTGAAAGCAG	TCCTCATACCAGGCTTCCA	33
12	*Bip*	ATAAACCCCGATGAGGCTGT	CATCAAGCAGTACCAGATCACC	64
13	*β-actin*	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA	64

4.12.4 Intestinal microbiota analysis (*Publication I and II*)

The 16S rRNA bacterial PCR analysis was performed using the primers specific for sequences of six different clusters of bacteria to analyse intestinal microbiota of *Bacteroides* spp, *Bifidobacterium* spp, *Clostridial clusters IV* and XIVa, *Lactobacillus* spp and Coriobacteriaceae family.

For the quantitative analysis of eubacterial number analysis, primers targeting the conserved region of 16S rRNA gene were applied. The bacterial numbers were calculated as the 16s RNA gene copy number per weight of intestinal content (Alimetrics, Espoo, Finland; ww.alimetrics.com). Colonisation of segmented filamentous (SFB) bacteria was determined by PCR after DNA was isolated from three consecutive stool samples using the FastDNA Spin kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and MagNA Lyzer homogenizer (Roche, Basel, Switzerland) according to the instructions of the manufacturers.

5. RESULTS

5.1 Diabetes, inflammation, immune activation and changes in gut microbes (*Publication I*)

5.1.1 T1D incidence in NOD mice fed with different diets.

In these experiments, NOD mice were used for diabetes follow-up studies, and the incidence of diabetes was 100% at 40 weeks of age (*Publication I-Figure 1F*). Insulitis scores were determined from the pancreatic islets of Langerhans at the age of six weeks for laboratory chow-fed NOD mice (*Publication I-Figure 7B*).

NOD mice on a casein hydrolysate (CH) diet had lower incidence of diabetes than did NOD mice fed with standard chow. Only sixty percent of the mice became diabetic by 30 weeks of age when they were given a CH diet, as compared with one hundred percent diabetic NOD mice fed with normal chow diet. Nearly similar results were obtained in a crossover study (mice were weaned to CH and their diet was changed to normal chow when they were 12 weeks old) (*Publication I-Figure 1F)*.

The CH diet efficiently reduced intestinal inflammation compared with NOD mice fed with standard chow as was determined using an in vivo and ex vivo imaging system (IVIS), shown in Figure 6 (*Publication I-Figure 1G, Figure 1H*).
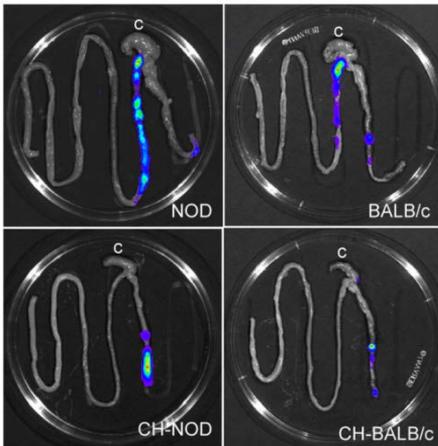

Figure 6. Representative image of ex-vivo imagingof the entire intestine of NOD mice at the age of five weeks. Whole gut imaging of NOD (upper left), BALB/c (upper right), CH-NOD (bottom left) and CH-BALB/c (bottom right) strains. The small intestine is towards the left and the cecum is marked as c. *(Publication 1).*

In vivo imaging showed a high production of RONS in 5-week-old NOD mice compared with the BALB/c control strain. Ex vivo imaging of the entire intestine revealed high signal intensity levels of RONS in the distal small intestine of the NOD mice, and this signal intensity was reduced in NOD mice that received a CH diet (*Publication I-Figure 2*).

5.1.2 Intestinal inflammation and permeability in young NOD mice

Several studies suggest that an increase in intestinal inflammation and permeability are associated with T1D (Alam, et al. 2010; Vaarala 2008). However, it is not very clear which part of the intestine is responsible for the inflammation, and the role of microbes in mediating inflammation and mucosal immune activation remains to be investigated.

Inflammation in the intestine was detected by in vivo imaging using the chemiluminescence probe L012, where RONS upon contact with the probe, emit luminescence, which is captured by the IVIS system. The results (*Publication I - Figure 1A and G*) show that the detection of RONS is always restricted to the small intestine. This signal mediated by RONS was seen soon after the mice were weaned to a normal chow diet, and it was reduced in NOD fed with the CH diet and in the control strain (*Publication I - Figure B-E and G*).

Therefore, we decided to determine whether the increase in intestinal inflammation would lead to an increase in the permeability of the gut. Hence, we measured the intestinal barrier function by intra-gastric gavage of fluorescein isothiocyanate dextran (FITC-dextran). BALB/c mice displayed low or undetectable absorption of FITC-dextran in their systemic circulation from five weeks of age onwards, while NOD mice had measurable FITC-dextran serum levels at six to eight weeks of age, which persisted until the mice were 10 to 12 weeks old (Figure 7). Remarkably, NOD mice weaned to a CH diet had lower FITC-dextran absorption compared with NOD mice weaned to a normal chow diet, and this was statistically significant at 10 to 12 weeks of age.
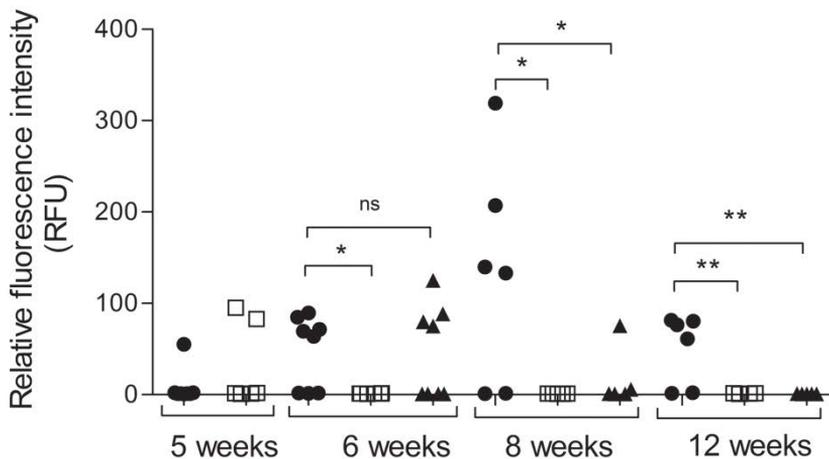

Figure 7. Concentrations of FITC-dextran in serum samples. Data represents NOD (black circles), CH diet fed NOD (black triangles) and BALB/c mice (white squares). Each data point represents an individual mouse. Relative fluorescence units (RFU) were calculated as the rate of emitted light at 485-535 nm per s) in the samples compared to the signal given by normal serum; *p<0.05 and **p<0.01 (*Publication 1-Figure 3A*).

5.1.3 Diet controls free radical production, T-cell activation and microbial colonisation

The production of RONS was evaluated from histological samples of the small intestine, in which leukocyte recruitment was occasionally observed in NOD mice fed with standard chow at 5 weeks of age (*Publication I-Figure 4A*). This might further link to leukocyte recruitment in the lamina propria of the small intestine. Accordingly, an increase in the numbers of neutrophils as well as an increase in the expression of the co-stimulatory molecule CD69 on T cells, were seen in the small intestine of NOD mice fed with standard chow compared with control BALB/c and CH-fed NOD mice (*Publication I-Figure 4B,C and D*). While few or undetectable levels of neutrophils were observed in the LP, there was a decrease in the levels of co-stimulatory molecules in CH-fed NOD mice compared to NOD mice fed with a normal chow diet (*Publication I-Figure 4B and D*).

The role of epithelial cells in the generation of RONS in the small intestine was evaluated by an in vivo dihydroethidium (DHE) staining. While NOD mice that were weaned to standard chow exhibited elevated levels of DHE staining in the cytoplasm of their intestinal villi, such staining was lower in CH-fed NOD mice and control BALB/c mice (Figure 8). Increased epithelial RONS were followed by the enlarged expression of *RegIIIγ* in the small intestine (*Publication I-Figure 5F*). *RegIIIγ* was highly expressed only in NOD mice that were fed a regular chow diet, and this was associated with higher IL-17 production in the small intestine and mesenteric lymph nodes, while CH-fed NOD mice exhibited reduced microbial colonisation (*Publication I-Figure 6A-E*) and a reduced Th17 type immune response in the small intestine (*Publication I-Figure 5E*).
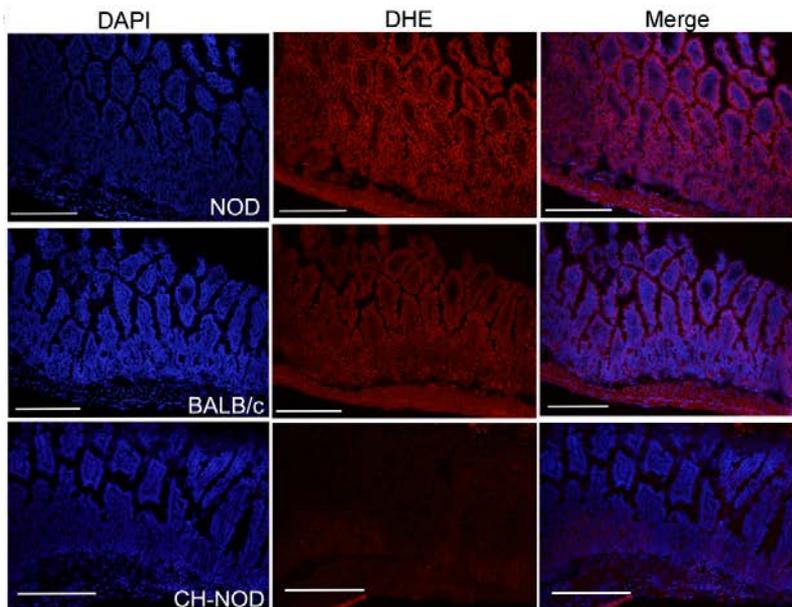

Figure 8: Representative picture of an in-vivo DHE staining for epithelial RONS. The small intestine of the NOD mice was stained in vivo for DHE (red) indicating the cytoplasmic reactivity in epithelial cells of the intestinal villi, and were counterstained with DAPI for nuclei (Blue) (*Publication I*).

5.2 Role of fermentable fibres in T1D (*Publication II*)

5.2.1 T1D incidence of NOD mice fed with different fibres

The role of fermentable fibres in T1D was evaluated in NOD mice. NOD mice fed with normal chow had a diabetes incidence of 95.7% (*Publication II-Figure 1A*) while NOD mice fed with pectin and xylan (PX) (consisting of 80% of Basal mix supplemented with 20% of whey and casein as proteins, and pectin and xylan as additive fibres) had a diabetes incidence of 53%.

76.5% of NOD mice fed on the cellulose diet (consisting of Basal Mix 80%, and supplemented with 20% of whey and casein as proteins and cellulose as additive fibre) remained diabetes-free (*Publication II-Figure 1A*). Cellulose is a non-fermentable fibre and is not readily hydrolysed. Microbial changes were determined by next-generation sequencing, and SCFAs were monitored by gas chromatography (*Publication II-Figure 5A-G*). The results show that fermentable fibres not only increased the incidence of T1D but also changed the gut micro-flora and the SCFAs (*Publication II-Figure 6A-E*). Thus, these findings demonstrate that changes in the gut microbial colonisation are dependent on diet.

NOD mice fed with a conventional chow diet had the most diverse microbiota and most diverse production of SCFAs, as the normal chow diet contains most of the common fermentable dietary fibres (*Publication II-Figure 6A- E*). PX-fed mice had intermediate levels, and cellulose-fed NOD mice had the lowest levels of SCFAs (*Publication II-Figure 6A- E*). The differences in the SCFA profiles are indicative of an alteration in the abundance of bacterial species in the gut of NOD mice (*Publication II-Figure 5A- G*).

5.2.2 Role of fibres in gut immune regulation

The mRNA levels of IL-18 and IL-1β in the large intestine were higher in NOD mice that were fed on a normal chow (NC) diet compared with cellulose and PX-fed NOD mice at the age of four weeks (*Publication II-Figure 2A and B*). NOD mice weaned to semisynthetic diets had undetectable levels of IL-1β and IL-18 in their colons. Epithelial IL-18 protein levels were then determined to evaluate the pro-inflammatory condition in the colon. NOD mice that were fed a normal chow diet had elevated IL-18 levels compared to PX and cellulose-fed NOD mice. This clearly suggests that semisynthetic diets have an anti-diabetic effect (*Publication II-Figure 4B and D*).

Il22 mRNA levels were elevated in the colon of PX and cellulose-fed mice, compared with normal chow-fed NOD mice, whose mRNA levels were barely detectable at 4 weeks of age (*Publication II-Figure 2C*). Interestingly, increased levels of *Foxp*3 were found in PX and cellulose-fed mice, while these levels were reduced in normal chow-fed NOD mice (*Publication II-Figure 2D*). This suggests that the number of Treg cells was increased in the colons of PX and cellulose-fed NOD mice. Levels of retinoic acid receptor-related orphan receptors gamma t (*Rorγt*) were lower in PX and cellulose-fed mice at 4 weeks of age compared with mice fed with normal chow, and this was statistically significant when the mice were 7 weeks old (*Publication II-Figure 2G*). Additionally, crypt hyperplasia was seen in mice fed with normal chow, but not in mice fed with a cellulose diet (*Publication II-Figure 4A* and *C*). Also the absence of crypt hyperplasia was observed in contrast to NOD mice fed on PX and normal chow (*Publication II-Figure 4A and C*).

5.2.3 Fermentable fibres and microbial colonisation

NOD mice fed with normal chow have a higher *Bacteroides* to *Firmicutes* species ratio than do NOD mice on a cellulose diet (*Publication II-Figure 5C*). A high *Bacteroides* to *Firmicutes* species ratio is also seen, when pectin and xylan were added to semisynthetic diets (*Publication II-Figure 5C*). NOD mice weaned to a normal chow diet and a PX diet had a significantly increased number of *Bacteroides* species and deeper crypts than did mice weaned to a cellulose diet (*Publication II-Figure 4 A and C, Figure 5C*). In addition, increased amounts of pro-inflammatory and stress-associated transcripts (*Publication II-Figure 2A-H*) and the expression of epithelial IL-18 (*Publication II-Figure 4B and D*) in the colon were detected in the mice fed with normal and PX chow. The mice weaned to a cellulose diet had, in turn, less signs of stress and inflammation in their colon (*Publication II-Figure 2 E and F*). Likewise, human studies have shown increased amounts of *Bacteroides* to be associated with islet autoimmunity (de Goffau, et al. 2014).

5.3 Role of peritoneal macrophages in T cell activation and T1D (*Publication III*)

5.3.1 Increased numbers of peritoneal macrophages and amplified expression of co-stimulatory molecules

Increased numbers of PM at 4-5 weeks and at 3 weeks were observed in NOD mice compared with the control strains, BALB/c, C57BL/6 and PNOD mice (*Publication III-Figure 1 and Figure 2 A-F*). Macrophage influx in the peritoneum was reduced in NOD mice weaned to a ProSobee diet. At the same time, macrophages from NOD mice exhibited increased levels of the co-stimulatory molecules CD40 and CD80 at three weeks of age, whereas the levels of CD40 and CD80 were reduced at the age of five weeks in NOD mice that were fed a regular chow diet compared to control strains (*Publication III-Figure 2 A, B,D and E*). Also, the levels of LPS in the serum of NOD mice at five weeks of age were higher than in the control strain (*Publication III-Figure 5*).

These results suggest that the increased gut permeability of NOD mice might have facilitated the translocation of gut microbes into the peritoneum, thereby triggering the activation of immune cells. A continuous exposure of peritoneum to gut microbes might be the reason for the increase in macrophage numbers and the expression of stimulatory molecules.

5.3.2 NOD mouse macrophages express higher levels of IRAK-M, which is suggestive of LPS tolerance

The role of PM in LPS tolerance was evaluated by analysing the protein expression of IRAK-M, a negative regulator for TLR signalling (Kobayashi et al. 2002). IRAK-M was not detected in the PM of NOD mice or control strains at the age of three weeks (Figure 9). However, elevated levels of IRAK-M were detected in the PM of NOD mice at the age of 5 weeks, while the levels of IRAK-M were lower or undetectable in the control strains (Figure 9). Furthermore, the PM of NOD mice showed lower levels of TNF-α and higher levels of IL-10 expression upon LPS stimulation in vitro at the age of 5 weeks, while 3-week-old NOD PM expressed higher levels of TNF-α, lower levels of IL-10, and undetectable levels of IL-12 upon in vitro LPS stimulation (*Publication III-Figure 3 B and C*). Similarly, an earlier study showed that the re-stimulation of macrophages resulted in lower TNF-α and IL-12 cytokine levels (Cole et al. 2012).
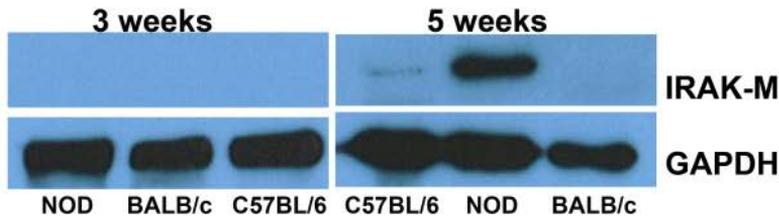
Figure 9: PM of NOD mice display characteristics of LPS-tolerance at 5 weeks of age. A protein analysis of PM from NOD mice reveals higher levels of IRAK-M at 5 weeks compared to 3 weeks of age and when compared to the control BALB/c and C57BL/6 mice. (*Publication III)*

5.3.3 Intraperitoneal inflammation induces the expression of CD69 and IFN-γ in pancreatic lymph node T cells

The role of LPS in the activation of PaLN T and B cells was evaluated. Upon an intraperitoneal LPS injection, NOD mice expressed statistically higher levels of CD69 T cells in PaLN cells compared to mice without LPS injected (*Publication III-Figure 4 A-C*). Surprisingly, there was an inherent difference in the CD69 expression levels between NOD mice weaned to normal chow or ProSobee diet, as the CD69 expression was higher on both CD4 and CD8 T cells in NOD mice fed normal chow (*Publication III-Figure 4 A and B*). An analysis of the intracellular expression of IFN-γ in CD4+ and CD8+ T cells showed that IFN-γ was clearly up-regulated upon LPS treatment in the NOD mice fed with normal chow diet (*Publication III-Figure 4 D and E*).

These results emphasize the idea that a prolonged increase in gut permeability leads to exposure of the peritoneal cavity to microbial LPS and that this leads to the activation of T and B cells in the PaLN. The fact that CD69 expression was reduced in ProSobee-fed NOD mice suggests that diet-dependent gut immune activation and T cell activation might promote islet-autoimmunity in NOD mice.

6. Discussion

Studies in non-obese diabetic mice and prospective studies in children genetically at risk of developing diabetes indicate that the development of diabetes-related autoimmunity associates with an impairment in the function of the epithelial barrier and inflammatory changes in the small intestine (Bosi, et al. 2006; Mooradian et al. 1986; Sapone et al. 2006; Secondulfo et al. 2004). On the other hand, also the large intestine and inflammatory changes in the large intestine have been implicated in diabetes-related autoimmunity (Alam, et al. 2011; Alam, et al. 2010; Toivonen et al. 2015; Turley et al. 2005). Therefore, an *ex-vivo* imaging system was used to visualise inflammation in the whole intestine in non-obese diabetic mice, and to evaluate its localisation in the intestine. Due to the fact that localised inflammation was indeed observed in parts of the gut, and since current dietary intervention strategies have failed to identify any protein or other component as diabetogenic, the second and third aspects of this thesis were to study the effects of fermentable fibers on gut homeostasis and diabetes-related autoimmunity, and to investigate the extra-intestinal effect of gut inflammation with potential relevance to diabetes-related autoimmunity. In the following chapter, these aspects are discussed in the above-mentioned succession.

6.1 Diet-dependent inflammatory changes in the gut (*Publication I*)

Many studies have suggested the presence of inflammation in the intestine of NOD mice during the onset of T1D, and it has been proposed that inflammation leads to the disruption of gut homeostasis (Alam, et al. 2010; Hadjiyanni et al. 2009; Lee et al. 2010; Maurano, et al. 2005; Sapone, et al. 2006). In this thesis we have shown, for the first time, an increase in the levels of RONS-mediated signals in the small intestine of NOD mice. Of note, this was observed soon after weaning, which corresponds to the time when the first inflammatory cells invade in the pancreatic islets. Increased levels of inflammation in the small intestine have also been reported to be associated with human T1D (Westerholm-Ormio, et al. 2003), although to a varying degree (Oikarinen et al. 2012). In our NOD mice, we found that these changes were reduced when the mice were placed on an anti-diabetogenic casein hydrolysate diet at weaning. Along with RONS-mediated inflammatory changes, gut permeability was consistently reduced when the mice were placed on a casein hydrolysate diet. In agreement with this, an earlier study showed that a casein hydrolysate diet corrects the impaired barrier function in diabetes-prone BB rats (Visser et al. 2010).

Not only did the anti-diabetogenic diet associate with reduced RONS-signals in the gut, but also microbial changes in the gut were observed. The levels of total bacteria were reduced, and with respect to the composition of the microbiota, the representation of *Bacteroides* genus was decreased and the representation of Lactobacilli and Bifidobacteria was increased. This suggests that the level of inflammation as determined by RONS-signals and associated T-cell activation depends on the amount and composition of microbiota in the gut. As these changes were induced by a dietary intervention, it is possible that the protective effects of the casein hydrolysate diet, as least in part, are secondary to its effects on the microbial colonisation of the gut.

Using specific staining techniques the increased RONS production in the small intestine was localized to intestinal epithelial cells rather than to phagocytic leukocytes. This observation was consistent with the finding of prominent *RegIIIγ* in the gut of NOD mice compared to the non-

diabetes prone BALB/c mice. RegIIIγ is an antibacterial peptide formed in a Toll-like receptor (TLR) dependent manner by epithelial cells in response to bacterial colonization (Cash et al. 2006). Thus, it can be speculated that epithelial cells in the intestine of NOD mice are sensitive to TLR-mediated signals, which occur when the mice are weaned to a normal diet and the gut is colonised with typical bacteria. A murine bacterial pathogens, *C.rodentium* causes further leakiness of the gut barrier in NOD mice, and increases the proliferation of islet-specific T cells in pancreatic lymph nodes (Lee, et al. 2010). The situation where NOD mice are weaned to a regular diet and the intestine is colonized with typical bacteria may therefore represent a form of microbial dysbiosis, which perturbs gut homeostasis and gives rise to inflammatory immune reactions.

6.2 Fermentable fibers and their association with the microbial colonization of the gut and immune regulation in the gut *(Publication II)*

 After the discovery that the composition of gut microbiota varies between individuals (Turnbaugh et al. 2007), evidence started to accumulate suggesting that disturbances in the composition of different microbial species within an individual's microbiota associate with inflammatory conditions both within and outside of the gut (Hooper, et al. 2012). Investigations were thereafter soon launched to study the role of the composition of the microbiota in children genetically predisposed to type 1diabetes. Although many of the findings of the early studies (de Goffau, et al. 2013; Murri, et al. 2013) were not confirmed later, the more recent studies (Endesfelder et al. 2014; Kemppainen et al. 2015; Kostic et al. 2015) have suggested that a reduction in the diversity of the microbiota (Kostic, et al. 2015) or its functional networks (Endesfelder, et al. 2014) may predispose to diabetes-related autoimmunity. Due to the fact that the amount of dietary fibers an individual consumes affects the composition of his or her gut microbiota, this thesis addressed the question of whether an early exposure to two fermentable fibers common in the diet plays a role in the development of diabetes.

Evidence for the influence of fermentable fibres on the microbial colonisation of the gut and in the development of T1D was indeed provided in paper II of this thesis. This evidence was obtained by placing NOD mice on a semisynthetic diet, in which the fibre composition could be modified without modifying other components of the diet. Semisynthetic diets are well known for their diabetes retarding effect in NOD mice and BB rat models (Beales, et al. 2002). Semisynthetic diets supplemented with defined components of interest provide a useful tool for studying the effect of various dietary components on the development of diabetes immune regulation in the gut. We found that the addition of two fermentable fibers, pectin and xylan, to a semisynthetic diet led to a greatly enhanced colonisation of the gut by bacteria belonging to the phylum *Bacteroidetes.*

As compared to a semisynthetic diet free from pectin and xylan, a diet containing pectin and xylan also correlated with an environment of increased pro-inflammatory phenomena in the colon, as evidenced by increased crypt hyperplasia and higher levels of IL-18 expression in epithelial cells. Increased levels of inflammatory cytokines have also been observed in the intestine of diabetic patients (Westerholm-Ormio, et al. 2003) as well as in NOD mice (Maurano, et al. 2005). The effect of the diet on gut-draining lymph nodes was also evaluated. Higher levels of FOXP3 and TGF-β were found in NOD mice weaned to a semisynthetic diet containing cellulose as the only fibre, as compared to levels in NOD mice weaned to the diet supplemented with pectin and xylan. The addition of pectin and xylan also promoted the development of diabetes in NOD mice.

Increased colonisation of the gut with microbes belonging to the Bacteroidetes family suggests that the effect of fibres on the histology and immunology of the colon of NOD mice might be due to differential microbial colonisation. Recent studies in individuals with a genetic predisposition towards developing type 1diabetes have shown that a microbiota rich in *Bacteroides* leads to islet autoimmunity (Brown et al. 2011; de Goffau, et al. 2013; Giongo et al. 2011; Roesch, et al. 2009). Numerous other studies have emphasized the effects of specific microbial colonisation in the immune homeostasis of the gut (Atarashi, et al. 2011; Gaboriau-Routhiau, et al. 2009; Ivanov et al. 2008; Mazmanian, et al. 2008; Wu and Wu 2012). Thus, human and animal studies suggest that a specific microbial colonisation could be responsible for T1D (Brown, et al. 2012; Murri, et al. 2013). However, results in all the above mentioned studies await conformation, and additional research is required to elucidate the role of these particular microbes in association with T1D.

6.3 Gut-derived microbial signals in the activation of immune cells in NOD mice.
(*Publication III*)

Independently of the observations made in paper I of this thesis, the research group has made an earlier observation of an abrupt and exaggerated increase in the number of peritoneal macrophages in NOD mice weaned to regular laboratory chow. Therefore, paper III of this thesis focused on investigating these peritoneal macrophages in NOD mice as sentinels of potential extra-intestinal manifestations of increased gut permeability to microbial signals in NOD mice.

The expression levels of CD40 and CD80 on the surface of peritoneal macrophages dropped at five weeks of age, which corresponded to the age of high RONS expression in the small intestine and a relatively leaky gut barrier (paper I). At five weeks of age NOD macrophages expressed an elevated level of intercellular IRAK-M kinase, which was absent at the time of weaning. IRAK-M is a negative regulator of TLR signalling (Kobayashi, et al. 2002). Upon continuous exposure to bacterial endotoxins in vitro or in vivo, IRAK-M is upregulated in macrophages. This leads to a decrease in the production of pro-inflammatory cytokines, especially TNF- α (Henricson et al. 1990; Kobayashi, et al. 2002). NOD peritoneal macrophages produced lower levels of TNF-α and increased levels of IL-10 at five weeks of age compared to 3 weeks of age. This indicated the desensitization and adaptation of macrophages to sustained exposure to LPS. Sepsis is a condition in which disseminated bacteremia leads to the activation of macrophages by bacterial components such as endotoxin. In sepsis patients, immune cells often become paralyzed, i.e desensitized to microbial stimuli after a sustained exposure. Interestingly, the production of cytokines from macrophages was also found to be lower or aberrant in sepsis patients and in animal models of septic infections compared to controls (Ayala et al. 1996; Cavaillon and Adib-Conquy 2006; Medvedev et al. 2000). Increased LPS was also observed in the plasma of NOD mice in paper III. This is consistent with the notion that the gut becomes more permeable to bacterial products in NOD mice. As observed in paper I and earlier by others, (Alam, et al. 2010; Maurano, et al. 2005; Newton et al. 2004), gut permeability appears to be increased in NOD mice. Furthermore, α4 integrin levels were declined in NOD peritoneal macrophages in contrast to Balb/c and C57BL/6 mice. Integrins play a central role in cell adhesion and migration and help in macrophage clearance after inflammation. (Bellingan et al. 2002; Da Gama et al. 2004). Lower expression of these integrins results in the poor or altered removal of peritoneal macrophages (Bellingan et al. 2002). Furthermore, injection of LPS into the peritoneum up-regulated the expression of CD69 on CD4 and CD8 T cells and to some extent on B cells in pancreatic lymph

nodes. It also increased IFNγ on CD4 and CD8 T cells of the pancreatic lymph node. Thus, if increased gut permeability allows the leakage of bacterial products into the peritoneum, it is possible that this in turn activates autoreactive Th1 and Tc1 cells in pancreatic lymph nodes, and might promote beta cell destruction in the pathogenesis of type 1diabetes.

7. CONCLUSIONS

In conclusion, the data presented in this thesis suggest the presence of diet-dependent inflammatory reactions in the gut, both in the distal small intestine and in the colon. Continuous inflammation, which alters the immune regulation and barrier function of the gut, can lead to further activation of an islet-destructive T cell response in the pancreas and islet-draining lymph nodes soon after weaning.

A further role for the fermentable fibres pectin and xylan in diet-dependent inflammatory reactions and diabetic autoimmunity was identified. The major sources of pectin and xylan are roots, berries and vegetables, as well as some products used by the modern food industry. Pectin and xylan had a substantial effect on gut microbes and in conditioning the colon in a way similar to that seen in NOD mice that were fed the diabetes-promoting normal chow diet. This suggests that autoimmune type 1diabetes can be influenced by the intake of fibres and by the type of microbes in the gut, which the fibres promote.

The increased permeability of the gut allowed the amount of peritoneal macrophages to increase, and the continuous exposure of the peritoneum to bacterial products led to macrophage tolerance towards bacterial endotoxins. Due to increased gut permeability and its extra-intestinal manifestations, T-cell activation and their differentiation into Th1 and Tc1 increased in PaLN. Increased Th1 and Tc1 cells are thought to be relevant for islet autoimmunity. The rate of diabetes was lower in NOD mice fed with an anti-diabetogenic diet compared to those on a regular diet.

Hence, the study demonstrates the importance of diet and associated changes in microbial colonisation and the immune regulation in the gut. Further studies are required to understand in more details, how diet associates with microbial colonisation and how it influence the pathogenesis of type 1 diabetes.

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