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THE INFLUENCE OF DIET AND MICROBES ON COLONIC IMMUNE REGULATION AND THEIR IMPLICATIONS ON TYPE 1 DIABETES

by

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In loving memory of my father

ABSTRACT

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The influence of diet and microbes on colonic immune regulation and their implications on type 1 diabetes

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Dietary and microbial factors are thought to contribute to the rapidly increasing prevalence of T1D in many countries worldwide. The impact of these factors on immune regulation and diabetes development in non-obese diabetic (NOD) mice are investigated in this thesis.

Diabetes can be prevented in NOD mice through dietary manipulation. Diet affects the composition of intestinal microbiota, which may subsequently influence intestinal immune homeostasis. However, the specific effects of anti-diabetogenic diets on gut immunity and the explicit associations between intestinal immune disruption and type 1 diabetes onset remain unclear.

The research presented herein demonstrates that newly weaned NOD mice suffer from a mild level of colitis, which shifts the colonic immune cell balance towards a proinflammatory status. Several aberrations can also be observed in the peritoneal B cells of NOD mice; an increase in activation marker expression, increased trafficking to the pancreatic lymph nodes and significantly higher antigen presenting cell (APC) efficiency towards insulin-specific T cells. A shift towards inflammation is likewise observed in the colon of germ-free NOD mice, but signs of peritoneal B cell activation are lacking in these mice. Remarkably, most of the abnormalities in the colon, peritoneal macrophages and the peritoneal B cell APC activity of NOD mice are abrogated when NOD mice are maintained on a diabetes-preventive, soy-based diet (ProSobee) from the time of weaning.

Dietary and microbial factors hence have a significant impact on colonic immune regulation and peritoneal B cell activation and it is suggested that these factors influence diabetes development in NOD mice.

Keywords: Type 1 diabetes, non-obese diabetic (NOD) mouse, diet, microbes, colon, immune regulation, B cells, germ-free

TIIVISTELMÄ

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Ruokavalion ja mikrobiston vaikutus paksusuolen immunisäätelyyn ja tyypin 1 diabeteksen kehitykseen

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Tyypin 1 diabeteksen (T1D) nopean yleistymisen uskotaan liittyvän osittain ravintotekijöihin ja suoliston mikrobistoon. Tämän väitöskirjatutkimuksen tarkoituksena on selvittää näiden tekijöiden vaikutusta suolen immuunisäätelyyn ja diabeteksen kehitykseen diabetes-alttiissa, nonobese diabetic (NOD) hiirissä.

NOD hiirissä diabeteksen voi ehkäistä ruokavaliomuutoksella. Ruokavalio vaikuttaa suolistomikrobiston koostumukseen ja siten myös suoliston immuunijärjestelmän tasapainoon. Diabetekselta suojaavan ruokavalion vaikutusta suolen immunisäätelyyn, sekä suoliston immuunijärjestelmän häiriöiden ja tyypin 1 diabeteksen välistä yhteyttä ei kuitenkaan tarkkaan tiedetä.

Tässä väitöskirjassa esiteltävät tutkimustulokset osoittavat, että juuri vieroitetuilla NOD hiirillä on lievän koliitin oireita, mikä vaikuttaa paksusuolen immuunijärjestelmän solujen aktivaatioon. Poikkeavuuksia havaittiin myös NOD hiiren peritoneaalisissa B-soluissa aktivaatiomarkkereiden ekspression nousuna, solumigraation lisääntymisenä haiman imusolmukkeisiin ja huomattavasti tehokkaampana antigeeniesittelynä insuliini-spesifisille T-soluille. Tulehduksellisia muutoksia esiintyi myös mikrobivapaiden (germ-free) NOD hiirten paksusuolessa, mutta merkkejä peritoneaalisten B-solujen aktivaatiosta ei havaittu näissä hiirissä. Suurin osa sekä paksusuolen että peritoneaalisten makrofagien ja B-solujen poikkeavuuksista hävisi kun NOD hiiret siirrettiin diabetekselta suojaavalle soijapohjaiselle ruokavaliolle (ProSobee) vieroitusiästä alkaen. Ruokavaliomuutos vaikutti myös olennaisesti NOD hiirten suoliston mikroflooran koostumukseen.

Näiden tulosten perusteella voidaan todeta, että ruokavaliolla ja mikrobisten tekijöiden läsnäololla on merkittävä vaikutus paksusuolen immuunisäätelyyn ja peritoneaalisten B-solujen aktivaatioon ja diabeteksen kehittymiseen NOD hiirillä.

Avainsanat: Tyypin 1 diabetes, non-obese diabetic (NOD) hiiri, ruokavalio, mikrobisto, paksusuoli, immunisäätely, B solu, mikrobivapaa.

CONTENTS

ABBREVIATIONS

APC	antigen presenting cell
Apc	allophycocyanin
BB	BioBreeding
BCR	B cell receptor
BSA	bovine serum albumin
BsA	bacterial sonicate from autologous intestine
BsH	bacterial sonicate from heterologous intestine
cDNA	complementary deoxyribonucleic acid
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
cpm	counts per minute
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DiI	1,1' - Dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine iodide
DSS	dextran sodium sulphate
EDG1	endothelial differentiation gene 1
EDG3	endothelial differentiation gene 3
EDTA	ethylenediaminetetracetic acid
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FO	follicular
Foxp3	forkhead/ winged helix transcription factor box 3 protein
FTY720	fingolimod, a synthetic S1P analogue
GAD65	glutamic acid decarboxylase 65
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GALT	gut-associated lymphoid tissue
GF	germ-free
GLC	gas-liquid chromatographic
HSP60	heat shock protein 60
HLA	human leucocyte antigen
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL2RA	interleukin-2 receptor alpha
i.p.	intra peritoneal
LP	lamina propria
LPL	lamina propria lymphocyte

LPS	lipopolysaccharide
MadCAM-1	mucosal addressin cell adhesion molecule-1
MFI	mean fluorescence intensity
MLN	mesenteric lymph node
mRNA	messenger ribonucleic acid
myD88	myeloid differentiation primary response gene
MZ	marginal zone
NOD	non-obese diabetic
PaLN	pancreatic lymph node
PBS	phosphate buffered saline
PE	phycoerythrine
PLN	peripheral lymph node
PNOD	NOD mouse maintained on ProSobee diet
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
PTPN22	variant of the lymphoid-specific protein tyrosine phosphatise
rtPCR	real-time PCR
SDS	sodium dodecyl sulfate
SPF	specific pathogen free
S1P	sphingosine 1-phosphate
Th	T helper cell
TLR	toll-like receptor
Treg	T regulatory cell
T1D	type 1 diabetes
UV	ultraviolet
VPC23019	(R)-phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester (S1P receptor 1/3 antagonist)

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publication, which are referred to with the roman numerals I-III in the thesis.

I Alam C, Valkonen S, Ohls S, Törnqvist K, Hänninen A. 2010. Enhanced trafficking to the pancreatic lymph nodes and auto-antigen presentation capacity distinguishes peritoneal B lymphocytes in non-obese diabetic mice. *Diabetologia.* 53(2):346-355.

II Alam C, Valkonen S, Palagani V, Jalava J, Eerola E, Hänninen A. 2010. Inflammatory tendencies and overproduction of IL-17 in the colon of young NOD mice are counteracted with diet change. *Diabetes*. 59(9):2237-2246.

III Alam C, Bittoun E, Bhagwat D, Valkonen S, Saari A, Jaakkola U, Eerola E, Huovinen P, Hänninen A. 2011. Effects of a germ-free environment on gut immune regulation and diabetes progression in non-obese diabetic mice. *Diabetologia*. 54(6):1398-1406.

1 INTRODUCTION

Type 1 Diabetes (T1D) is a disease that results from a faulty autoimmune response targeted against the insulin-producing β-cells of the pancreas. Patients suffering from T1D rely on continuous insulin replacement therapy and diet management. Despite great efforts, no efficient methods to prevent or to cure this disease exist to date.

The incidence of T1D is exceptionally high in Finland compared to other countries. Moreover, the incidence of the disease has increased five-fold in Finland over the last sixty years. The reasons underlying the high and increasing incidence of T1D are still largely unclear.

It is nevertheless widely accepted that dietary and microbial factors may be partly responsible for the increase in T1D incidence. The intestinal mucosa is constantly exposed to these factors and intestinal immune regulation is crucial for maintaining a balance between tolerance and inflammatory responses in the body.

Diet affects the composition of intestinal microbiota, which may subsequently influence intestinal immune homeostasis. Though it is known that diet manipulation can prevent diabetes in animal models, the specific effects of these anti-diabetogenic diets on gut immunity and the explicit associations between intestinal immune disruption and type 1 diabetes onset remain unclear. The mucosal immune regulation of the colon in non-obese diabetic (NOD) mice on a conventional diet and on a diabetes-preventing diet and in germ-free NOD mice is investigated in this thesis. The peritoneal B cell population, which is in close relationship with the intestinal immune cells are analysed since it may constitute a critical factor for linking a disruption in intestinal immunity to subsequent autoimmune responses in the pancreas. On the basis of the results presented in this work, a colon-peritoneum-pancreas axis is hypothesised as a route by which environmental factors may influence the autoimmune process in T1D in NOD mice.

2 REVIEW OF THE LITERATURE

2.1 Aetiology and occurrence of T1D

2.1.1 History of diabetes research and the discovery of type 1 diabetes

The symptoms of diabetes have been recognised since ancient times and an accurate description of the disease as well as some of its complications can be found in the Canon of Medicine written by Ibn Sina (lat. Avicenna) a thousand years ago. The pancreatic origin of the disease, however, was not discovered until the late 19th century, when Oskar Minkowsky and Joseph von Mering found that total pancreactomy leads to diabetes mellitus in experimental animals (Luft 1889). From this discovery emerged the hypothesis of an 'internal solution' of the pancreas controlling carbohydrate metabolism. A protocol for isolating the 'internal solution' which came to be known as 'insulin' was developed by the young researchers Frederick Banting, Charles Best and JB Collip in the laboratory of Professor John Macleod. John Macleod and Frederick Banting were subsequently awarded a Noble Prize in Physiology or Medicine in 1923 for the discovery of insulin. This discovery was indubitably one of the most important breakthroughs in diabetes research.

Long before the discovery of autoimmunity, physicians were able to differentiate between two different forms of diabetes. A distinction was made between 'fat' and 'thin' diabetes. This referred to the frequently different prognosis and treatment requirements for younger, slimmer patients and older overweight patients. Indeed, the terms 'type 1 diabetes' and 'type 2 diabetes', that we have become accustomed to use for insulin insensitive and autoimmune diabetes today, originate from this morphological distinction between patients. Following the discovery of autoimmune diseases in the 1950s, evidence slowly accumulated to identify diabetes as an autoimmune disease. In 1976 the autoimmune background of diabetes was certified, and the classifications of 'type 1 diabetes' and 'type 2 diabetes' came to be used for autoimmune and insulin insensitive diabetes respectively (reviewed in (Gale 2001)).

Though our understanding of the disease has improved dramatically since the 1920's, insulin therapy, which to this day is critical in type 1 diabetes care, relies on the concepts that became known through the work of Banting and colleagues almost a hundred years ago. Questions pertaining to the underlying causes, to prevention or cure of T1D are still largely unanswered despite extensive investigation.

2.1.2 T1D incidence

The incidence of T1D varies extensively in different countries. Wide-ranging studies have been carried out by the World Health Organisation to estimate T1D incidence worldwide. Their data, based on 75 million children from 50 different countries

show that the lowest incidence in 1990-1994 was found in China and Venezuela (0.1/ 100 000 per year) and the highest incidence is in Finland and Sardinia (36.5 and 36.8 / 100 000 per year). The difference in T1D incidence was thus greater than 350-fold in this study (Karvonen et al. 2000). What is more, in many countries, the incidence has been increasing at a remarkably fast rate over the last decades. In Finland, for instance, the incidence in 2005 had increased to 64.2 / 100000 (Harjutsalo et al. 2008) from the incidence of 36.5 / 100 000 observed in 1990-1994 (Karvonen et al. 2000). The average annual increase between 1990-1998 has been calculated to be 2.8% according to a report by the DIAMOND Project Group (DIAMOND 2006) that includes 57 different countries and 84 million children. An increase was seen across the world in countries of Asia, Europe and North America, with the exceptions of Central America and the West Indies. Seasonal variations have also been noted both in the birth patterns of children who later develop T1D as well as in the month of diagnosis. The seasonal variation is seen primarily in northern countries, where a disproportionally large fraction of patients are diagnosed and/ or born between November and February (reviewed in (Maahs et al. 2010)). Finland has the highest incidence of T1D in the world. Moreover, the incidence has increased more five-fold in Finland over the last sixty years; from 12/100000 in 1953, to 64.2/100000 in 2005 (Somersalo 1954; Harjutsalo et al. 2008). Given these observations with regard to T1D incidence in Finland and elsewhere, it is of little surprise that there is now considerable debate concerning the underlying causes for T1D.

An individual's genetic makeup is known to influence the likelihood for developing diabetes. Indeed, a multitude of different regions in the human genome has been linked to an increased risk for T1D. The study of genetics of T1D is moreover moving forward at a fast pace, due to methodological advances as well as significant resources allocated to this field of research. It is nevertheless evident, that regardless of the multiple loci associated to T1D, the impact of genetics on T1D aetiology is limited. In fact a family history of T1D only serves to increase the risk of T1D, and even in monozygotic twins, the proband-wise concordance for T1D is as low as fifty per cent (Kyvik et al. 1995). It is also argued, that the increase in magnitude in T1D incidence over a relatively short space of time cannot be attributed solely to enhanced genetic disease susceptibility.

Amongst the genetic loci that have been associated with increased susceptibility for T1D in humans, the far most important one is the *HLA* region on chromosome 6p21. This genetic region includes more than 200 genes, out of which 40% are associated with immune response functions. It is estimated that the *HLA* loci confers 40-50% of the genetic T1D risk (Santamaria 2010). This strong association between certain *HLA* molecules and T1D susceptibility is widely used for *HLA* screening to identify children at risk for developing T1D (Pociot et al. 2010).

In addition to the *HLA* region, a significant number of other genes have also been identified which confer an increased risk of T1D, albeit to a lesser degree. The insulin gene, cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), a variant of the lymphoid-

specific protein tyrosine phosphatise (*PTPN22*) and *IL2RA* are amongst the best known non-*HLA* associated genes that influence the risk for T1D (reviewed in (Concannon et al. 2009)). Studies in genetics frequently employ 'odds ratio' calculations to evaluate whether a given single nucleotide polymorphism is associated with a disease. An odds ratio of 1.00 would mean that a given polymorphism is equally likely to occur both in the patients and in the control group. The *HLA* region has an estimated odds ratio of 6.8, whereas non-*HLA* loci typically have estimated odds ratios of 1.3 or lower. Unless numerous single nucleotide polymorphisms are yet to be discovered, the relatively low odds ratio even for the *HLA* region suggests that genetic predisposition only partially determines the risk of developing T1D (Concannon et al. 2009).

It is hence clear, that the pathogenesis of human T1D is under genetic as well as environmental control, and it is plausible that environmental factors play a notable role in increased disease prevalence. It is of little surprise therefore, that considerable research efforts aim to identify the environmental causes that underlie an increasingly high T1D incidence in Finland and elsewhere.

2.2 Diet and microbial factors in the regulation of immune responses

It is widely accepted that dietary and microbial factors may be partly responsible for the increase in T1D incidence. These two factors are closely related, since the diet has a direct effect on microbial species prevalence in the intestine. A dietary change from a mixed to a vegan diet causes a significant qualitative, albeit not quantitative, change in the microflora within weeks (Peltonen et al. 1992). The same phenomenon is observed if diets differing in the quality and quantity of carbohydrates are administered to adults: particular diets cause a rapid growth of specific bacteria. This effect is reversed when the diet is altered again (Walker et al. 2011). Changes in specific indigestible dietary carbohydrates can also alter the bacterial species composition in the colon. For example, oligofructose and inulin stimulate the growth of Bifidobacterium (Gibson et al. 1995) and are therefore often added to probiotic dairy products. Even limited changes to the diet therefore can have considerable effects on the microflora.

The non-pathogenic microorganisms which constitute the commensal microflora that reside in our intestine under normal circumstances are critical for the development and maturation of the mucosal immune system (Calder et al. 2006). This in turn strongly affects the immune regulatory responses in the gut. Dietary and microbial factors can therefore play a central role in protecting from, or predisposing to, various immune disorders related to the gut. The microflora and the signalling between microbes and host have essential consequences both for the maintenance of health as well as for disease onset. Important roles for bacterial signalling have been observed in at least, the physiological processes and diseases outlined in Table 1. With regard to colorectal cancers, as little as 25% of cases are related to a family history of the disease, whilst the

majority of cases are sporadic occurrences in which dietary and microbial factors play an important role (Gill and Rowland 2002; Davis and Milner 2009). In many diseases, including T1D, the reasons behind microbial-dependent disorders seem to come down to an induction of inflammation in the gut epithelium and/ or increased permeability of the intestinal wall.

Table 1. Different physiological parameters and diseases affected by microflora or innate immune responses to bacteria.

Health	Disease
Digestion (Hooper et al. 2001)	Diabetes (Brugman et al. 2006; Wen et al. 2008)
Epithelial homeostasis (Hooper et al. 2001)	IBD (Manichanh et al. 2006)
Immune cell homeostasis (Macpherson and Harris 2004)	Cancer (Moore and Moore 1995)
Resistance to infections (Hooper et al. 2001)	Allergy (Penders et al. 2007)
Angiogenesis (Hooper et al. 2001)	Asthma (Penders et al. 2007)
Enteric nerve function (Hooper et al. 2001)	Obesity (Penders et al. 2007)

2.2.1 Role of diet and microbes in the development of T1D in animal models

Diet is known to modify the development of autoimmune diabetes in animal models (Table 2). Several different dietary adjustments have proven successful in reducing type 1 diabetes incidence in rats and mice. It has long been known that hypoallergenic diets, such as ProSobee, significantly reduce diabetes incidence in NOD mice (Elliott et al. 1988; Flohe et al. 2003). ProsoBee is an infant formula where soy constitutes the sole protein source. This formula is moreover both wheat- and milk free. Similarly, hydrolysed casein-based diets decreases disease incidence both in BioBreeding (BB) diabetes prone rats as well as in NOD mice (Lefebvre et al. 2006). The exact mechanisms by which these diets protect from T1D are still under investigation, but it is known that anti-diabetogenic diets change the microflora in the animals (Hansen et al. 2006), and may improve the barrier properties of the intestine (Visser et al. 2010).

The effect of cow milk protein on T1D incidence in diabetes-prone animals has been debated, as the experimental data available is inconsistent. A study by Daneman and colleagues (Daneman et al. 1987) report a significantly higher diabetes incidence in BioBreeding diabetes prone rats that receive cow milk protein, whereas a later study by Malkani an co-workers state that the effects of a diabetes-protecting diet is not abrogated by cow milk protein (Malkani et al. 1997). Wheat-based diets have in most animal studies proven diabetogenic and the effect is thought to be caused

by wheat gluten (Lefebvre et al. 2006). However, somewhat contradicting results, suggesting that both a gluten-free as well as a diet enriched with gluten decreases the risk of diabetes in NOD mice (Funda et al. 2008). Similar results have been presented by Mueller and co-workers (Mueller et al. 2009), where a hypoallergenic diet supplemented with 5% wheat increased diabetes incidence, whereas a wheat supplement of 30% did not. The authors of this study hypothesise that high wheat concentrations may have tolerogenic effects, or that wheat may contain components that are anti-diabetogenic (such as vitamin E or unsaturated fatty acids). Furthermore, a significant delay and reduction in diabetes development has been observed by delaying the introduction of wheat and barley proteins into the diet of NOD mice until they reach 10 weeks of age (Schmid et al. 2004). This suggests that exposure to dietary components at an early age may be more influential than a later introduction of the same components.

Microbial factors, including viruses, bacterial toxins and the gut microflora are also thought to significantly affect T1D susceptibility (Lefebvre et al. 2006). Viral infections have long been suspected to work as triggers for T1D. The underlying mechanisms may involve virally-induced β-cell specific autoimmunity, or direct cytolytic infection and destruction of pancreatic β-cells. A number of different viruses including coxsackie virus B, cytomegalovirus, retrovirus, reovirus and foot-and-mouth disease virus infections have been associated with an increased risk of T1D. Moreover, some viruses may protect from T1D in animals. These viruses include lymphocytic choriomeningitis virus and mouse hepatitis virus (Jun and Yoon 2003).

Streptomyces, which is a soil-borne pathogenic bacterium, is known to produce toxins that are cytolytic to β-cells. Toxins from *Streptomyces*, such as streptozotocin and bafilomycin A1 impair glucose tolerance and reduce β-cell mass in mice (Myers et al. 2003). Another agent that is known to cause destruction of β-cells in animals is alloxan, which is a substance produced through the oxidation of uric acid. Both streptozotocin and alloxan are widely used experimentally to induce diabetes in various rodent and nonhuman primate laboratory animal models (Tal et al. 2004; Lenzen 2008).

The importance of the composition of the gut microflora for maintaining the balance between immune cell activation and immune tolerance has been a subject of substantial research in recent years (Moore and Moore 1995; Ouwehand et al. 2002; Schiffrin and Blum 2002). Colonisation of germ-free animals with defined gut bacteria suggests that the normal microflora contains certain microbes which can elicit inflammatory responses, and others that induce tolerogenic immune responses. An alteration in the composition of the microflora hence may cause the pro-inflammatory responses in the gut that are associated with various inflammatory disorders (Round and Mazmanian 2009).

Table 2. Dietary modifications that influence diabetes development in animal models.

Dietary agent	Effect
Soy-based infant formula (ProSobee)	Significantly reduced T1D risk in NOD mice (Elliott et al. 1988; Flohe et al. 2003)
Hydrolysed casein-based infant formula (e.g. Nutramigen)	Significantly reduced T1D risk in BB diabetes prone rats and NOD mice (Lefebvre et al. 2006)
Wheat, wheat gluten	May increases risk or protect from diabetes in NOD mice, possibly depending on concentration (Funda et al. 2008; Mueller et al. 2009)
Barley	Delayed exposure to barley reduces diabetes risk in NOD mice (Schmid et al. 2004)
Vitamin D supplement	Protects from diabetes in NOD mice (Zella et al. 2003)
Vitamin E supplement	Delays diabetes onset in NOD mice (Beales et al. 1994)
Nicotinamide (vitamin B3) supplement	Prevents diabetes in NOD mice (Yamada et al. 1982)
Cow milk protein	Contradicting data from studies on BB diabetes prone rats; some reports state cow milk protein increase diabetes risk, other studies negate this postulation. (Daneman et al. 1987; Malkani et al. 1997)

2.2.2 Dietary and microbial factors that may influence T1D in humans

Studies relating to T1D in humans have not been entirely successful in pinpointing dietary risk factors unambiguously. Some studies have indicated that early exposure to cow milk and only brief breastfeeding results in an increased risk of TID (Vaarala et al. 1999). Other studies, however, have failed to make this association between breastfeeding/ cow milk exposure and T1D (Ziegler et al. 2003; Savilahti and Saarinen 2009). The role of breastfeeding in reducing the risk of T1D, therefore, remains inconclusive (Knip et al. 2010).

Other dietary elements that have been associated with an increased risk for T1D in humans include wheat gluten or cereal in general. An increased risk of developing T1D was associated with very early (< 3 months) exposure to cereal both in an American study (Norris et al. 2003) and in the German BABYDIAB survey (Ziegler et al. 2003). The former study further showed an increased T1D risk if cereal was introduced after 7 months of age (Norris et al. 2003). Similarly, an increase in T1D incidence has been suggested if berries and root fruits are given to infants under 3 months of age (Virtanen et al. 2006).

The role of viral infections as a trigger for T1D in humans has been studied extensively. Amongst the viruses that have been suggested to confer an increased T1D

risk are enteroviruses (in particular coxsackie virus B), rubella virus, mumps virus, rota virus, retrovirus, cytomegalovirus and Epstein-Barr virus (Jun and Yoon 2003; Filippi and von Herrath 2005). *In utero* rubella infections have a particularly strong association with T1D, as almost 20% of patients with congenital rubella infections develop T1D (Menser et al. 1978). A connection between Coxsackie virus B infection and T1D has been advocated since 1969, when Gamble and colleagues published that antibodies against this enterovirus are found more frequently in T1D patients than in control subjects (Gamble et al. 1969). The coxsackie B virus and enterovirus-specific antibodies have later been isolated from pancreases of diabetic patients (Oikarinen et al. 2008; Jaidane et al. 2010). Moreover, enteroviral RNA is more frequently found in serum from T1D patients than in control sera (Oikarinen et al. 2011). The correlation between enteroviral infections and T1D incidence has, however, not been eminent in all studies and it has also been argued that enterovirus infections are not likely to be the cause of the increase in T1D incidence, since these infections are much less common in Finland compared with Russia, despite the significantly higher T1D incidence in Finland (Honeyman 2005). Rubella infections, likewise, are almost eliminated in most developed countries today, due to efficient immunisation regimes (Lefebvre et al. 2006).

The effects of the normal gut microflora (commensal microflora) on T1D development have not been extensively examined. A recent study by Giongo and colleagues exists nonetheless which shows that the microflora of T1D children differs significantly from the microflora of healthy children. T1D patients developed, moreover, a less diverse and less stable microbiome (Giongo et al. 2011). These results suggest that the composition of the gut microflora plays a role in the development of T1D in humans as well.

2.2.3 Risks related to the immature gut immune system in infants

The yet immature gut immune system in infants may be particularly vulnerable to antigen-induced inflammation. The intestinal immune system is functionally and immunologically underdeveloped at birth in several aspects. The permeability of the intestinal wall is higher in newborns, making it possible for ingested antigens and bacteria to enter the lymphatics and bloodstream. Tight junctions, which in mature individuals seal the intestinal wall, develop in the postnatal period during the first few months of life in humans (Wagner et al. 2008). This developmental process, sometimes referred to as ‘gut closure’ is aided by breast milk and particularly colostrum, and delayed by early exposure to dietary antigens (Catassi et al. 1995). Gut barrier property irregularities may play a role in diabetes development since increased gut permeability has been associated with T1D both in humans and animal models (Vaarala 2008). Additionally, the low numbers of IgA positive B cells in infancy, compared with the levels later in life, may contribute to the increased risk for inflammatory response at the mucosal surface early in life. IgA is the principal antibody in the immunologically mature intestine. This antibody has the capacity to bind to antigens and exclude them from the intestinal mucosa without

causing inflammation (Mestecky et al. 1999). Levels of IgA are very low at birth and increase relatively slowly, reaching adult levels around the age of 1 year in humans (Ouwehand et al. 2002).

It is thought that the above described factors render the intestinal immune system of newly weaned individuals particularly sensitive to immune disruption by environmental factors. Additionally, exposure to microbes when a baby is born through vaginal delivery may offer protection from T1D, since the diabetes incidence in children born through Caesarean section is significantly higher, and it is known that the composition of the microbiota is fundamentally different in children born through Caesarean section and vaginally born children (Grönlund et al. 1999). Ultimately, whatever the environmental triggers, they are likely to be something we are exposed to early on in childhood. This is because the greatest increase in T1D incidence has been observed in children diagnosed under 5 years of age, and the earliest signs of autoimmunity become evident under one year of age (Kimpimäki et al. 2001).

2.3 Implications of gut immune regulation for T1D development

Current knowledge about the physiology and immunoregulation of the intestine has provided important clues as to how dietary and microbial factors may affect the risk for developing diabetes. Research in both humans and animals has shown that TID is frequently associated with enteropathies in the small intestine (Graham et al. 2004; Maurano et al. 2005; Bosi et al. 2006). Increased permeability of the small intestine has been observed in both diabetic patients (Bosi et al. 2006) and in newly weaned BioBreeding diabetes sensitive rats (Graham et al. 2004). Increased gut permeability was also observed pre-diabetic patients. This indicates that rather than being a consequence of T1D, gut enteropathies precede diabetes onset and that the intestine may participate in the pathogenesis of the disease. Moreover, the expression of inflammatory cytokines is increased in the small intestinal lamina propria in diabetic patients (Westerholm-Ormio et al. 2003) as well as in NOD mice (Maurano et al. 2005). Inflammatory propensities in the lamina propria and impaired intestinal barrier functions may cause alterations in antigen responses and disrupt the immunological homeostasis of the intestine. Increased permeability in the gut may furthermore increase the antigenic load from the intestinal lumen and trigger T cell activation both inside and outside of the intestine. Since the pancreatic lymph nodes (PaLN) sample antigens from the gastrointestinal tract (Turley et al. 2005), it is possible that enteropathies in the intestine have a direct association with the presence of activated T cells in the PaLN, which are the cause of β cell destruction in the pancreatic islets (Turley et al. 2005; Vaarala 2008).

The studies related both to antigen-induced effects on the gut immune system as well as other defects in the intestine that may influence T1D, have focused primarily on the small intestine. The potential role of the large intestinal immune system in T1D

development has attracted less attention. The large intestine differs immunologically in several aspects from the small intestine. The disruptions in the immunity of the small intestine are generally linked foremost to ingested antigens or allergens, often with focus on insulin from cow milk and cereal based allergens. In the large intestine, the most immediate sources of immune disruption are indubitably the vast quantities of bacteria residing therein. The large intestine lamina propria lymphocytes (LPL) also differ markedly in terms of population dynamics and cytokine expression from the LPL of the small intestine (Resendiz-Albor et al. 2005). The effect of specific bacteria on the induction of inflammatory immune responses may furthermore differ between the small and large intestine (Ivanov et al. 2009).

2.3.1 Role of gut microbiota in the maintenance of homeostasis

The 'hygiene hypothesis', which states that exposure to bacteria and infections are the keys to a healthier immune system, is a widely recognised concept. Originally quite a general theory advocating the dangers of an environment deficient in bacterial stimuli, this theory is slowly being replaced by a more detailed understanding of the importance of the delicate balance between tolerance and immune response.

The intestinal immune system is continuously faced with the daunting task of protecting the body from harmful microbes whilst maintaining tolerance towards the commensal microbiota, food- and self antigens. With approximately 10^{14} bacteria residing in the human gut, discriminating between harmful and harmless bacteria and antigens is a complicated task. It is hence understandable that the intestine harbours the largest numbers of immune cells in the body.

The interactions between different microbes and host and the importance of specific bacterial strains for intestinal homeostasis are gradually being unravelled. In the field of T1D research some common themes are emerging that highlight the links between T1D development and intestinal immune response abnormalities. Yet, many questions remain concerning the precise role of gut microflora in protecting from or predisposing an individual to the disease.

Antibiotics can be used to alleviate intestinal inflammation (Videla et al. 1994). However, depletion of commensal bacteria by antibiotic treatment to ease inflammation may not be entirely unproblematic, since studies in mice have shown that this renders the colon more susceptible to chemically induced epithelial injury (Zaph et al. 2008). Moreover, proper immune recognition of bacteria, rather than just commensal bacteria *per se* is considered a critical element in the immune regulation of the colon (Wen et al. 2008; Zaph et al. 2008). The immune cells in healthy individuals are hyporesponsive to resident bacterial flora, but this immune tolerance is broken in patients suffering from inflammatory bowel disease (Duchmann et al. 1995; Duchmann et al. 1996). It is also thought, that disruption of the balance between potentially pathogenic and potentially

beneficial commensal bacteria may be an underlying factor behind inflammatory bowel disorders (Frank et al. 2007; Mazmanian et al. 2008).

2.3.2 Freedom from microbes – germ-free animal models

A number of different germ-free rat and mouse animal colonies have been established and studied to shed light on the impact of microbiota on intestinal immune regulation and physiology in the absence of microbes. Various significant morphological and physiological irregularities are common in all these models (Smith et al. 2007). The immune system in germ-free animals is immature, since its development requires microbial stimulation. Germ-free mice are deficient in CD4+ T cells and IgA producing plasma cells and the Peyer's patches and lymph nodes in these mice are reduced in size (Wagner 2008). The intestinal functions, including motility, absorption, electrolyte handling and bile metabolism are affected in germ-free conditions (Smith et al. 2007). Moreover, several morphological differences in the intestine can be observed. The most dramatic is the extremely enlarged caecum in germ-free animals, which is caused by undegraded mucus glycoproteins produced by the epithelia (Donowitz and Binder 1979). These profound differences caused by microbial deprivation illustrate the importance of microbiota on mammalian development. They also emphasize that caution must be exercised when extrapolating results from a germ-free animal model for a particular disease to the conventional animal population.

Based on evidence from animal models – in particular the NOD mouse model - it can be concluded that bacteria are not a prerequisite for developing diabetes. Germ-free NOD mice develop diabetes at least to the same extent as specific pathogen free (SPF) NOD mice. It has in fact long been assumed that germ-free NOD mice develop diabetes more quickly and consistently than SPF NOD mice. This view is based mainly on a single, rather small study dating back to the 1980s (Suzuki T. 1987). A few other reports on germ-free NOD diabetes incidence have since become available. Though they fail to show the same tendency of aggravated diabetes under germ-free conditions (Taniguchi et al. 2007; Wen et al. 2008; King and Sarvetnick 2011), this view still dominates the scientific discussions on the matter.

The reasons underlying the tendency to aggravated diabetes development in germ-free mice, if indeed such a tendency exists, have not been studied systematically. TLR signalling has been demonstrated to play an important role in T1D development, since *Myd88* knockout NOD mice, in which TLR signalling pathway is significantly impeded, are protected against T1D. The protective effect however, is dependent on microbiota, since germ-free *Myd88* knockout NOD mice develop diabetes almost to the same extent as wild-type germ-free NOD mice (Wen et al. 2008). For the most part, the existing knowledge on how germ-free conditions alter immune regulation in animals is based mostly on studies that are not directly related to T1D. These studies can however provide important insights into the effect of microbes on immune disorders in general.

2.3.3 Shifted balance between tolerance and inflammation in the absence of microbiota

It has been suggested, that germ-free conditions impede the development of oral tolerance, as well as the differentiation of T regulatory cells in the intestine and in mesenteric lymph nodes (Östman et al. 2006; Hrncir et al. 2008). A simultaneous increase in IL-17 levels, at least in the colon, has also been observed (Zaph et al. 2008). The elevated IL-17 levels in germ-free animals in the study by Zaph et al. was contributed to a lack of microbial-induced IL-25, which under normal circumstances would limit Th17 cell differentiation.

The above mentioned results would indicate that gut microbiota is required for the differentiation of T regulatory cells and for keeping the balance between tolerance and Th17-induced responses to pathogens. Since the differentiation of T regulatory and IL-17 cells is reciprocally regulated, it is understandable that low levels of T regulatory cells can be associated with increased IL-17 levels (Bettelli et al. 2006). The impact of microbiota on this balance however, is multifaceted, since interactions between immune cells and microbiota can have both pro- and anti-inflammatory effects. Monocolonisation of germ-free mice with segmented filamentous bacteria, for example can potently induce IL-17 responses in the small intestine (Ivanov et al. 2009). Moreover, IL-17 is more abundant in conventionally reared B6 mice that harbour high levels of segmented filamentous bacteria in the ileum, compared to those B6 mice in which these bacteria are absent (Ivanov et al. 2009). In contrast, *Bacteroides Fragilis* increases the suppressive activity of Treg cells and induces the production of the anti-inflammatory cytokine IL-10 in germ-free mice monocolonised with this bacterium. This immunomodulatory effect of *Bacteroides* is dependent on the bacterial polysaccharide, PSA (Round and Mazmanian 2010).

The complex role of different bacteria on the regulation of the gut immune system makes it difficult to unambiguously evaluate the impact of germ-free conditions on gut immune regulation. There are contradicting reports on the requirement of microbiota for the induction of IL-17 in the gut (Zaph et al. 2008; Ivanov et al. 2009). The studies by Östman an co-workers (2006) and Hrncir et al. (2008) showing a requirement for microbiota in the development of Tregs and oral tolerance, have likewise been challenged by another investigation which shows contradicting results (Min et al. 2007). The requirement of microbiota for IL-17 cell differentiation and the development of tolerance in the gut is hence still debated. Taking into consideration the immunological and functional differences of the small and large intestines, it is plausible that inflammatory responses are differentially regulated in the small and large intestines. Hence, it is possible that specific bacterial stimulation is required for IL-17 induction in the small intestine, but not in the colon.

2.3.4 *Bacteroides versus Firmicutes*

Recent discussion has focused on the importance of an appropriate balance between two bacterial phyla that are prevalent in the gut; namely, Bacteroides and Firmicutes. Bacteria belonging to these two phyla make up an estimated 60% of the colonic microbiota.

Segmented filamentous bacteria, which are a subset of the Firmicute-Clostridiae group of bacteria is, as discussed in the previous section (2.3.3), associated with a promotion of proinflammatory cytokines while Bacteroides fragilis is associated with anti-inflammatory responses (Ivanov et al. 2009; Round and Mazmanian 2010). Bacteroides fragilis is moreover capable of both preventing and curing colitis in an H. hepaticus induced colitis model (Mazmanian et al. 2008). A beneficial effect of Bacteroides is also suggested by Wen and colleagues (2008). The protective effect of Myd88 deficiency in NOD mice correlates with a higher ratio of Bacteroides to Fragilis induced by the TLR signalling aberration in this knockout NOD mouse strain (Wen et al. 2008).

In other studies related to T1D, an increase in bacteria of the Bacteroides genus has been associated with the development of diabetes. Comparisons between BB diabetes prone rats that later went on to develop diabetes and those that did not, were shown to differ in their faecal flora composition in that the rats that did develop diabetes harboured significantly more bacteria of the Bacteroides genus (Brugman et al. 2006). Higher levels of Bacteroides were also found in Diabetes prone BB rats compared with diabetes resistant BB rats (Roesch et al. 2009). Likewise, in a comparison between children of the same HLA genotype, a higher ratio of Bacteroides to Firmicutes was seen in children who went on to developing T1D. Children who stayed healthy had more bacteria of the Firmicutes genus and less of Bacteroides (Giongo et al. 2011). However, despite the overall lower levels of bacterial of the Bacteroides genus in the study by Giongo et al. (2011) the Bacteroides species B.Vulgatus and B. Fragilis were much more common in the children who did not develop diabetes. The comparisons between diabetes resistant and diabetes prone rats by Roesch et al. (2009) showed similar differences within the Bacteroides genus. This shows the importance of looking beyond the genus level to accurately determine a diabetes promoting or preventive microflora.

2.4 B cells

B lymphocytes are derived from hematopoietic stem cells and are produced in foetal liver and postnatally throughout life in the bone marrow in most mammals. Newly formed B cells home to the spleen and undergo several stages of maturation. B cells are also found in significant numbers in the peritoneal and pleural cavities, as well as recirculating through the secondary lymphoid organs, including lymph nodes and the gut associated lymphoid tissues (GALT). B cells are the mediators of humoral immunity through their production of antibodies. In contrast to T lymphocyte receptors, which mostly recognise only protein antigens, B cell antibodies recognise a wide variety of antigens comprising different chemical structures including proteins, polysaccharides, lipids and small chemicals. Some B cell antibodies are autoreactive. It is thought that most individuals harbour some autoantibodies in their blood, but that they are kept inactivated through a mechanism of ‘peripheral tolerance’. If this immune tolerance is broken, autoimmunity may ensue (Ballotti et al. 2006).

2.4.1 B cell types

B cells can be divided into different subclasses; B2 cells (often referred to as 'follicular (FO) B cells'), marginal zone (MZ) B cells and B1 cells. These B cell classes differ in several ways. B1 cells develop early in ontogeny, during the foetal/ neonatal period and possess the capacity for self-renewal, while B2 cells and MZ B cells are produced at a later stage and only divide in response to antigen stimulation. Moreover, the receptor signalling pathways and activation requirements differ between B cell subtypes.

B2 cells constitute the majority of B cells and circulate through the secondary lymph organs. These B cells mainly recognise protein antigens and elicit T-cell dependent immune mechanisms. B1 cells on the contrary, are mainly found in the plural and peritoneal cavities and recognise a wider variety of antigens and account for most of the T-independent antibody responses (Fagarasan and Honjo 2000). Broadly speaking, B2 cells generate high-affinity antibodies and memory cells that offer long-term protection against pathogen invasion, whereas B1 cells produce natural antibodies and short-term low-affinity antibody responses that offer immediate and broad-ranged protection against pathogens. Hence, B1 cells provide the first line of defence against pathogens, while B2 cells provide a slower but much more efficient antibody response (Baumgarth et al. 2005). MZ B cells akin to B1 cells are capable of recognising non-protein antigens and like B1 cells, elicit rapid immune responses to invading pathogens. MZ cells furthermore display low levels of self-reactivity and maintain a 'pre-activated' status which allows them to respond quickly to antigens (Lopes-Carvalho et al. 2005).

In addition to producing natural antibodies, B1 cells also produce a wide range of autoreactive antibodies. These include antibodies to cell components such as membrane molecules (phosphorylcholine), cell surface molecules (CD90) and intracellular molecules (such as single-stranded DNA). Antibodies are also produced against phosphatidylcholine, carbohydrate epitopes, and immunoglobulins (Baumgarth et al. 2005). These polyreactive low-affinity antibodies are important in the first line of defence against pathogens and in the clearance of apoptotic cells. The polyreactivity, however, also renders these cells potentially capable of instigating autoimmunity (Duan and Morel 2006). B1 cells have, in fact, been associated with a number of autoimmune illnesses, such as Sjögren's syndrome, rheumatoid arthritis, lupus (Duan and Morel 2006) as well as T1D (Wong et al. 2004).

2.4.2 Peritoneal B cell subclasses and characteristics

Peritoneal B cells comprise B2-like and B1 B cells, the latter of which can be divided into three subclasses; B1a, B1b and a relatively recently discovered, B1c subclass (Kantor and Herzenberg 1993; Ghosn et al. 2008). B1a peritoneal cells are characterised by their expression of both CD5 and CD11b surface markers, while B1b cells do not express CD5, but only CD11b. Hastings and co-workers discovered a population of CD5 positive peritoneal B cells that lacked CD11b expression, and this population was named 'B1c

type B cells' (Hastings et al. 2006). Later work has however revealed that the expression of CD11b does not discern an altogether separate B cell subclass, but the expression of CD11b on B1 cells distinguishes sequential steps of B cell development in the peritoneum. The expression of CD11b is furthermore associated with the location of B cells in the peritoneum; increased time in the peritoneum increases CD11b expression, and migration out of that location is accompanied with loss of CD11b expression (Stoermann et al. 2007).

It is still debated whether peritoneal B1 cells exist as a separate lineage independently of MZ B cells, or whether the two B-cell subsets arise from the same progenitors, since evidence for both hypotheses exists (Figure 1) (Rothstein 2002; Stoermann et al. 2007). Though only little exchange between splenic and peritoneal B cells appears to take place, splenectomy affects the peritoneal B1 cell pool dramatically (Stoermann et al. 2007). Furthermore, peritoneal and splenic B1 cells express different surface markers and also demonstrate differences in gene expression patterns. These differences, however, are not necessarily indicative of that these two cell populations originate from alternative lineages, but may instead be caused by the influence of the microenvironment. Indeed, (Stoermann et al. 2007) have demonstrated the impact of the microenvironment on the imprinting of peritoneal B cells using the L2 mouse, in which B1 cells are the sole B cell type. Adoptive transfer of B1 cells from L2 mice to lymphopenic *Rag1*$^{-/-}$ mice showed that both splenic and peritoneal B1 cells have the capacity to acquire the phenotype of one or the other subpopulation, if forced to reside in the respective anatomical location (Stoermann et al. 2007).

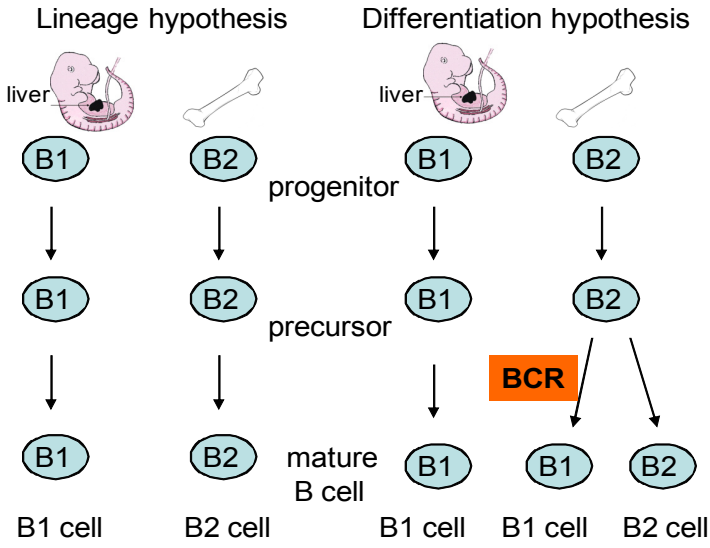

Figure 1. B1 cell origin. Schematic drawing representing two different views on B1 and B2 cell origin. According to the lineage hypothesis, B1 cells arise from progenitors in the foetal liver whereas B2 cells develop in the bone marrow postnatally. On the contrary, the differentiation hypothesis states that there is only one B cell lineage and that differentiation of B cell progenitors into B1 or B2 cells depends on the strength of the B cell receptor (BCR) signalling, such that stronger signalling promotes differentiation into B1 cells (Casola et al. 2004).

Peritoneal B2 cells differ in several respects from splenic B2 cells and appear to constitute a unique B cell subset that exhibits characteristics that are intermediate between peritoneal B1 cells and splenic B2 cells (Hastings et al. 2006). The study by Hastings and co-workers suggests that peritoneal B2 cells acquire characteristics of B1 B cells upon residing in the peritoneal cavity. They report that that the expression of the CD11b marker on the transferred cells is dependent upon their location in the peritoneal cavity. CD11b moreover seems to distinguish different developmental stages of peritoneal B cells, rather than completely distinct B cell subsets (Ghosn et al. 2008).

2.4.3 Peritoneal B cell function

Upon stimulation with chemokines, LPS or even whole bacteria, B cells rapidly migrate from the peritoneal cavity into lymph nodes and intestinal lamina propria and produce antibodies. The migration from the peritoneal cavity is controlled by biological signals and is coupled to downregulation of surface markers characteristic of peritoneal B cells, such as integrin α4β1 and CD11b (Kawahara et al. 2003; Ha et al. 2006).

Peritoneal B1 cells produce large amounts of natural antibodies of the IgM class. These are produced spontaneously, without prior antigen encounter, and are thus part of the innate immune function of B1 cells. As mentioned in section 2.4.1, their contribution to the initial response to pathogens is important (Boes 2000). These B1 cells have also been shown to migrate to the spleen and become IgM presenting cells (Kawahara et al. 2003). Peritoneal B cells are also important producers of intestinal IgA. It is estimated that around 50% of the IgA producing plasma cells in the lamina propria derive from peritoneal B cells (Kroese et al. 1989). Intestinal IgA is needed in the defence against pathogens at the mucosa as well as for the establishment of homeostasis and symbiosis with commensal bacteria. IgA producing B cells thus contribute to the mechanisms of tolerance to commensal microflora in the gut (Kunisawa et al. 2007). The migration of peritoneal B cells to intestinal lamina propria and the production of IgA is biologically regulated and is dependent on the chemokine sphingosine 1-phosphate (S1P) signalling. IgA levels in the intestine are drastically reduced upon stimulation with a high-affinity S1P receptor agonist, FTY720, which halts S1P-mediated signalling through evoking aberrant S1P receptor internalisation (Kunisawa et al. 2007).

B1 cells produce self-reactive antibodies capable of recognising DNA and other autoantigens which aid in the clearance of apopototic products (Duan and Morel 2006). However, because of their capacity to produce polyreactive, self recognising antibodies, peritoneal B1 cells are suspected to contribute to several autoimmune disorders, including lupus, rheumatoid arthritis and T1D. In line with this notion, elevated numbers of B1 cells have been associated with autoimmunity in humans as well as animal models (Duan and Morel 2006). Furthermore, NOD mouse sera contain high levels of polyreactive natural antibodies (Thomas et al. 2002).

In addition to producing antibodies, B cells, similarly to dendritic cells and macrophages, function as antigen presenting cells (APC) for major histocompatibility class II (MHCII) restricted T cells. The APC function seems to be of importance for the development of diabetes, at least in animal models. This is demonstrated by the poor response of CD4 T cells in B cell deficient NOD.Igμ^{null} mice to glutamic acid decarboxylase 65 (GAD65) and heat shock protein 60 (HSP60), which are major autoantigens involved in the pathogenesis of T1D (Falcone et al. 1998). Further evidence emphasising the role of B cell APC function in diabetes development has come from the observation that rendering NOD mouse B cells MHCII deficient protects these mice from diabetes despite the presence of MHCII sufficient dendritic cells and macrophages (Serreze et al. 1998).

Finally, B cells in the peritoneum may influence the differentiation of peritoneal macrophages (Wong et al. 2010). Macrophages can be divided into two main categories, termed M1 and M2. M1 type macrophages, which have inflammatory properties, are induced by proinflammatory cytokines such as IFNγ and IL1β, whereas the M2 type is induced by anti-inflammatory molecules such as IL-10. B1 cells have been shown to induce the differentiation of macrophages into the M2 type and thus drive the expansion of macrophages with anti-inflammatory propensities (Wong et al. 2010).

2.4.4 B cell migration

Peritoneal B cells respond to activation by migrating to lymphoid organs, intestinal lamina propria and spleen (Kawahara et al. 2003; Itakura et al. 2005; Berberich et al. 2007). The migration is induced very rapidly in response to various stimuli, including skin immunisation with contact sensitising antigen, oral LPS or indomethacine administration or S1P agonist FTY720 (Itakura et al. 2005; Ha et al. 2006; Kunisawa et al. 2007).

Integrins are known to play important roles in lymphocyte homing to lymphoid tissues. Inactivated peritoneal B cells, in particular B1 type cells, express high levels of integrin α4β1. Adaptive transfer experiments in which B cells were treated with α4-neutralising antibody have indicated that α4β1 integrin is vital for the entry of B cells into the peritoneum. Moreover, neutralisation of α4 increased the recovery of i.p transferred B cells in the lymph nodes, indicating that the expression of this integrin is necessary for retaining B cells in the peritoneal cavity (Berberich et al. 2008). An association between α4β1 expression, B cell activation and exit from the peritoneum has also been demonstrated by the rapid α4β1 downregulation and clearance of peritoneal B cells in response to LPS (Ha et al. 2006).

Sphingosine 1-phosphate (S1P) has received considerable attention in recent years as being a regulator of lymphocyte trafficking. In the peritoneum, signalling through S1P helps retain B lymphocytes within the peritoneal cavity (Kunisawa et al. 2007). B cell unresponsiveness to chemotactic signals from S1P thus facilitates the emigration of peritoneal lymphocytes. Downmodulation of S1P signalling, moreover, is associated

with B cell activation, since activating signals such as LPS impede S1P signalling (Cinamon et al. 2004) and consequently permit B cell exit.

2.4.5 B cells and type 1 diabetes

Autoantibodies have long been used as a diagnostic marker for T1D prediction. These autoantibodies, however are considered not be pathogenic in themselves, since T1D can not be transmitted through mere transfer of these autoantibodies. It is perhaps for this reason that B cells have in the past been viewed mainly as insignificant bystanders in the process of T1D development (Silveira and Grey 2006).

T1D has traditionally been considered primarily a T-cell mediated disease. T-cells are important mediators of the β-cell damage in pancreatic islets, which is ultimately what defines this autoimmune disease. However, several other cell types, such as dendritic cells, macrophages and B cells, unfalteringly contribute to the disease progress.

The importance of B cells in T1D development has been demonstrated in several animal studies using NOD mice, BB rats as well as transgenic DO11.10 RIP-mOVA mice. B cells are abundant in pancreatic islet infiltrates of diabetes-prone animals (Kendall et al. 2004; Ryan et al. 2010). B-cell deficient NOD.Igμ^{null} mice are protected from T1D and develop little insulitis (Serreze et al. 1996). Protection from T1D and even reversal of the disease can also be achieved in animal models through depletion of B cells by antibody therapy using anti-CD20 treatment or anti-CD22/cal monoclonal antibody (Hu et al. 2007; Fiorina et al. 2008). A similar effect can be accomplished through hypotonic lysis of peritoneal B cells. This treatment dramatically decreases the number of B1 type B cells in infiltrates and also protects animals from T1D (Kendall et al. 2004).

The specific mechanisms through which B cells exert their effect in T1D progression are not fully understood, but several lines of evidence suggest that antigen presentation by B cells is central for this process (Falcone et al. 1998; Serreze et al. 1998). Other studies indicate that B cells may promote T1D through aiding T cell survival in the pancreatic islets (Brodie et al. 2008). B cells may also be important for allowing entry of autoreactive T cells into the pancreas, since islet specific T cells in transgenic DO11 RIP-mOVA mice fail to migrate from the pancreatic lymph nodes into the pancreas when B cells are depleted (Ryan et al. 2010). In their study Ryan et al. further show that the ability to trigger T cell infiltration is specific to the B1 type B cell subset. Because of the close resemblance of islet-infiltrating B cells and peritoneal B1 cells in DO11 RIP-mOVA and NOD mice, it is suggested that many of the infiltrating B cells in the pancreas may originate from the peritoneal B cell population. These islet-infiltrating CD19^{+}B220^{low} B cells express the CD5 antigen as well as IgM and CD11b, making them identical to the peritoneal B1 type cells (Kendall et al. 2004; Ryan et al. 2010). These B1-like B cells in the pancreatic infiltrates dramatically decrease in number following hypotonic lysis of peritoneal cells. As discussed above, depletion of peritoneal B cells through hypotonic lysis also efficiently protects NOD mice from T1D (Kendall et al. 2004).

In addition to B1 type peritoneal B cells, marginal zone splenic B cells have also been implicated in T1D pathogenesis in NOD mice (Puertas et al. 2007; Marino et al. 2008). These B cells show several signs of hyper-responsiveness, expand rapidly with the onset of diabetes (at 12-16 weeks of age in NOD mice) and are able to present insulin peptides to diabetogenic T cells (Marino et al. 2008). Ryan and co-workers report that B1 type cells are present in the pancreas at very early stages of insulitis, whereas marginal zone B cells predominate at later stages, at 14-16 weeks, consistent with the timing of the expansion of this cell population in the spleen (Ryan et al. 2010).

While the importance of B cells in animal models of T1D is widely accepted, the significance of B cells in human T1D is still a subject of considerable debate. Recent evidence indicates that B cells are also present in pancreatic infiltrates of humans with T1D (Willcox et al. 2009). Moreover, B cell depleting anti CD20 antibody (rituximab) treatment may have a beneficial effect on β-cell function, particularly in newly diagnosed T1D patients, according to ongoing clinical phase 2 trials (Pescovitz et al. 2009). These data emphasise that B cells contribute in an important way to the development of T1D in humans as well as in animal models. Nevertheless, B cells may not be an absolute requirement for T1D development in animals or in humans, and the exact mechanisms through which B cells function in this process are not entirely clear.

2.5 Gut-peritoneum-pancreas link

Some evidence exists that directly associates the colon with the pancreatic lymph nodes (PaLN). It has been proposed that the pancreatic lymph nodes are the primary draining sites for the transverse colon (Carter and Collins 1974). Moreover, studies in BDC2.5/NOD mice have indicated that dextran sodium sulphate, which disrupts the barrier functions of colonic epithelium, enhances activation of islet reactive T-cells in PaLN of NOD mice (Turley et al. 2005). Furthermore, lymphocytes accumulating in the islets share homing characteristics with gut-associated lymphocytes. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which mediates lymphocyte homing specifically to the mucosal lymphoid tissues is particularly important in the early stages of diabetes development in NOD mice. It is hence suggested that priming of lymphocytes in the mucosal lymphoid tissue plays a role in TID pathology (Hänninen et al. 1996; Yang et al. 1997; Hänninen et al. 1998). Taken together, these pieces of evidence indicate that there is a direct connection between NOD colonic immune interruptions and the onset of autoimmune events in the pancreatic lymph nodes which ultimately lead to T1D.

Evidence exists also for the involvement of the peritoneal B cells in the trafficking routes of immune cells from the gut to the pancreas. Indomethacine, which disrupts the epithelial barrier mainly in the small intestine, causes rapid changes in the character and the behaviour of the peritoneal cells (Ha et al. 2006). In fact, subcutaneous administration of indomethacine, or lipopolysaccharide (LPS) results in similar effect on peritoneal

B cells. Both of these treatments increase the surface expression of various B cell activation markers, decrease α4β1 integrin expression and increase B cell migration out of the peritoneal cavity. Disruptions in gut barrier properties have been linked to T1D in humans as well as in animal models (Graham et al. 2004; Bosi et al. 2006; Vaarala 2008; Lee et al. 2010) and it has been suggested, that increased permeability of the gut epithelial layer may affect T1D development through the increased antigenic load on the immune system through bacteria or bacterial components leaking out through the gut epithelium (Vaarala 2008). Peritoneal B cell involvement in this process has not been scrutinised, but given that these B cells are activated in response to a chemically induced gut barrier disruption, it is plausible that the deficiencies in gut barrier properties observed in T1D patients and animal models would likewise trigger a response in the peritoneal cavity B cells.

Activation of peritoneal B cells triggers their migration from the peritoneal cavity, as discussed in section 2.4.4. The migration of B cells to different lymph nodes is, however, strongly skewed towards trafficking to the pancreatic lymph nodes. This preferential migration of peritoneal B cells is developmentally regulated. The favoured draining to the pancreatic lymph nodes cannot yet be seen in one week old mice, but by three weeks of age the ratio of migration to PaLN over other lymph nodes is 30-fold (Turley et al. 2005). The peritoneal cavity B cells are thus closely associated with the gastrointestinal tract, but also intimately connected with the pancreatic lymph nodes. They may hence provide a route of transmission between environmental triggers of T1D and the induction of autoimmune responses in the pancreatic lymph nodes.

3 AIMS OF THE STUDY

It has been hypothesised that the increase in T1D incidence comes down to dietary or microbial factors. This hypothesis is scrutinised using the NOD mouse model.

Experimentally induced colitis has a profound effect on peritoneal cell activation. However, there is a possibility that even a moderate irritation or inflammation of the intestinal wall may disrupt its barrier properties and cause the activation of peritoneal cells in a way that enhances T1D progression. An irritation/ inflammation of this sort could be caused by dietary and/ or microbial factors. It is thought that the immature intestine of newly-weaned infants might be particularly sensitive to inflammation caused by the newly introduced food substances and/ or the diet-induced changes to the microflora.

This thesis work aims to answer the following questions:

1) Do NOD mice exhibit characteristics that may be indicative of colonic inflammation? If so, are these characteristics associated with dietary or microbial factors?
2) B1 cells, which are ubiquitous in the peritoneum, have been linked to type 1 diabetes in NOD mice. Do these cells show abnormal characteristics in terms of cell phenotype, activation status or migration patterns that may influence diabetes development?
3) What kind of influence, if any, does a germ-free environment have on the above mentioned features of the gut immune system or peritoneal cell characteristics? How do germ-free conditions influence immune cell islet infiltration into pancreatic islets and diabetes incidence in NOD mice?

4 MATERIALS AND METHODS

4.1 Mice

Non-obese diabetic mice were used as a model for type 1 diabetes. Age-matched BALB/c and C57BL/6 mice were used as control mouse strains. All mice were raised and bred in the Central Animal Laboratory of Turku University.

NOD mice were raised under SPF conditions and were maintained either on a regular diet (CRM-E, SDS, Tapvei) or ProSobee infant formula (Mead Johnson Nutritionals) (referred to as "PNOD" mice). The ProSobee formula was given to the mothers from 2 weeks after giving birth and to the offspring immediately after weaning and continued throughout the study period. The average weight of 4.5 week old NOD and PNOD mice was not significantly different; NOD, 21.0 g (±1.1 g), and PNOD, 19.6 g (±1.2 g). The diet modifications therefore did not impede growth in mice.

For the establishment of a germ-free NOD mouse colony, germ-free inbred Swiss mice were kindly provided by The Central Animal Laboratory of Radboud University, Nijmegen, Netherlands. Sterile NOD pups, derived through caesarean section, were then cross fostered with the germ-free mice. Germ-free NOD mice used for experiments were of second or subsequent generations. Faecal samples from germ-free animals were collected weekly. These samples were analysed by PCR using 16sRNA primers for conserved bacterial sequences, as well as cultured under both aerobic and anaerobic conditions, to monitor the sterility of the colony.

All animal experiments were approved by the National Laboratory Animal Care and Use Committee in Finland and conformed to the legal acts, regulations and requirements of the European Union concerning the protection of animals used for research.

4.2 Cell isolation

Lamina propria lymphocytes and myeloid cells: Colons were excised, washed, and cut into pieces. The pieces were incubated for 3 × 20 min at 37°C in Hanks' balanced salt solution supplemented with 2% foetal calf serum (FCS) (Life Technologies) and 2 mmol/l EDTA to remove the epithelial layer and intraepithelial lymphocytes. The colon pieces were then washed with RPMI-1640 (Life Technologies) and digested with Collagenase A (Roche) (0.5 mg/ml) for 1 h at 37°C in RPMI-1640 supplemented with 10% FCS. Undigested pieces were minced and filtered through a nylon mesh. Leukocytes were purified from the resulting cell suspension using Lympholyte-M (Cedarlane) gradient centrifugation (1250 g, room temperature) and thereafter washed twice in culture medium before further use.

Peritoneal lavage cells: Peritoneal cells were obtained by washing out the peritoneum with cold RPMI-1640 medium (Gibco). B cells were isolated from the peritoneal cells using B220 MicroBeads for experiments that required purified peritoneal B cells (Miltenyi Biotec).

Splenic T and B cells: Single-cell suspensions of spleen cells were acquired by gently pressing the tissues through a metal mesh. Erythrocytes were extracted by hypotonic lysis using 0.2% wt/vol NaCl. For some experiments, where purified splenic T- or B cell were required, $CD4^+$ T cells and B cells were isolated from the spleen cell suspension using CD4 and B220 MicroBeads, respectively. (Miltenyi Biotec).

4.3 Flow cytometry (I, II, III)

Flow cytometry is a versatile method and it was used extensively throughout the research project. Its applications included; identification of cell populations, determination of surface markers for cell activation, intracellular staining, cell proliferation and detection of cells for *in vivo* and *in vitro* migration assays.

4.3.1 Cell population dynamics and cell activation (I, II, III):

Colon lamina propria T cell analysis: Anti-CD4 and anti-CD8 conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used to detect T cell populations. Cell populations were further stained using PE-conjugated anti-α4 or PE-conjugated anti-CD86 (BD Pharmingen), or FITC-conjugated anti-CD44, PE-conjugated anti-CD69 or FITC-conjugated anti-CD62L (Immunotools).

Colon lamina propria myeloid cell populations: Subsets of myeloid antigen presenting cells were characterised as follows: CD11b+ F4/80+ macrophages, F4/80-CD11b+CD11c+ myeloid dendritic cells and F4/80-CD11b- CD11c+ lymphoid/plasmacytoid dendritic cells (all antibodies for this characterisation were either FITC or PE conjugated and purchased from Immunotools).

Peritoneal B cell characterisation: Peritoneal wash-out cells were stained with FITC-conjugated anti-CD11b (Immunotools) and allophycocyanin conjugated anti-CD45R (Caltag Laboratories) to identify B1 cells. PE-conjugated anti-CD40 or anti-CD86 (Immunotools), integrin α4 (BD Biosciences and AbD Serotec, Oxford, UK), Integrin β1 (AbD Serotec), integrin α4 β7 heterodimer (DATK32; BD PharmingenTM) and CD69 (Immunotools) were used for peritoneal B cell activation marker detection. The L-selectin antibody that was used was FITC-conjugated (Immunotools)

In vitro S1P-induced CD69 downregulation: Peritoneal cells were incubated overnight in serum-free medium supplemented with 0.1 % fatty acid free BSA, 2mM L-Glutamine and 100 units/ml penicillin and streptomycin either in the presence or in the absence of S1P (200 nM). The cells were collected and incubated with antibodies against B220/CD45R, CD11b and CD69.

All samples were analysed with a FACS Calibur apparatus and CellQuest software (Becton Dickinson).

4.3.2 Intracellular staining (II, III)

Intracellular staining for IFN-γ, IL-17A and Foxp3 was performed for MLN, PaLN and purified colonic lamina propria lymphocytes (LPLs). Single-cell suspensions of lymph node cells and purified colonic LPLs were incubated in complete RPMI 1,640 (supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and streptomycin) containing 0.1 μmol/l PMA, 1 μmol/l ionomycin, and 10 μg/ml Brefeldin A (Sigma-Aldrich) for 4 h at 37°C. Stimulated cells were surface-stained using FITC-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25. The cells were then fixed with 2% paraformaldehyde and permeabilised with 0.5% saponin. Fc block was used to block nonspecific binding. PE-conjugated anti-IFN-γ, PE-conjugated anti-FOXP3, or PE-conjugated anti-IL-17A and appropriate isotype controls (all reagents from eBiosciences) were used for the intracellular staining.

4.3.3 Proliferation assay using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling (I)

Peritoneal and spleen cells from BALB/c and NOD mice were labelled with CFSE (5 μmol/l; Molecular Probes), diluted to a concentration of 1×10^6 cells/ml and cultured for 72 h on 96-well culture plates in culture medium alone or with the addition of anti-mouse CD40 antibody (3 μg/ml; BD Biosciences), CpG oligodeoxynucleotide (CpG) (1 μg/ml; CpG 1826; Coley Pharmaceutical) or lipopolysaccharide (10 μg/ml; Sigma-Aldrich). Finally, cells were collected from the wells, stained with anti CD45RB/B220-APC and CD5-PE conjugated antibodies, and subjected to flow cytometric analysis.

4.3.4 Adoptive transfer (I)

Peritoneal cells from BALB/c and NOD mice were labelled with Vybrant DiI dye (Molecular Probes) according to the manufacturer's recommendations. 2×10^6 cells were injected intraperitoneally into each recipient mouse. The mice were killed 20 h later and peritoneal cells, mesenteric lymph nodes, spleens and PaLN were isolated. The cells obtained were stained with fluorochrome-conjugated CD45R and CD11b antibodies. Frequencies of adoptively transferred B cells in collected samples were analysed by flow cytometry and the results expressed as ‰ of total (DiI-negative and DiI-positive) B cells collected for each sample of mesenteric lymph nodes, spleen and PaLN. The decrease in DiI-labelled B cells in the peritoneum at 20 h after transfer was determined by measuring DiI-positive B cells as percentage of all peritoneal B cells at the time of transfer vs 20 h post-transfer. Results are expressed as per cent DiI-labelled B cells left in peritoneum relative to input.

4.3.5 *In vitro* transwell migration assay (I)

Migration experiments were performed using 96-well Transwell Permeable Supports (Corning) with 5 μm pore sizes. Peritoneal cells from NOD and BALB/c mice were

isolated. Macrophages were removed by adherence to plastic for 1 to 2 h in RPMI medium containing 10% FCS. This step typically yielded a cell suspension with approximately 90% purity of B cells. Serum-free medium supplemented with 0.1% fatty acid free BSA was added to the lower wells with different concentrations (1, 5, 10, 30, 100, 500 or 1,000 nmol/l) of S1P (Biomol International) or without S1P (control). The enriched B cells were washed in RPMI, resuspended in serum-free medium supplemented as described above and added to the upper wells (75,000 cells/well). In some experiments, B cells were pre-incubated for 1 h with 1 μmol/l VPC23019 (Avanti Polar Lipids), an antagonist of S1P receptors 1 and 3. VPC23019 was additionally added at 1 μmol/l to the upper and lower wells of the migration assay in these experiments. Cell migration was allowed to proceed for 1 h at 37ºC. Cells that had migrated to the lower wells were then collected and quantified by flow cytometry. Quantification was based on cell counts obtained by flow cytometry during a set time constant (100 s) with identical cell-suspension volumes. The margin of error using this method of analysis was approximately 10%, which was roughly the same as when using TruCount fluorescent beads for quantification with flow cytometry.

4.4 Western blotting (I)

Peritoneal cells from NOD and BALB/c mice were isolated and macrophages were removed by adherence to plastic. Whole-cell lysates from the B cell-enriched peritoneal cells were obtained by incubating the cells on ice for 1 h, under agitation in a lysis buffer containing 20 mmol/l tris base, 150 mmol/l NaCl, 0.5% (vol/vol) nonidet P-40, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulphonyl fluoride and aprotinin 0.01 trypsin inhibitor units per ml (pH 8.0). The cell suspension was then centrifuged at 15,000×*g* for 15 min at 4ºC, after which the supernatant fraction was collected. The protein concentration in the sample was determined with a BSA protein assay reagent kit (Pierce) according to the manufacturer's recommendations.

To determine peritoneal cell S1P receptor 1 and 3 levels, protein lysates were mixed 3:1 in 3× SDS buffer (30% [vol/vol] glycerol, 3% [wt/vol] SDS, 187.5 mmol/l Tris/HCl, pH 6.8, 0.015% (wt/vol) Bromophenol Blue and 3% [wt/vol] 2-mercaptoethanol). The mixture was then heated for 4 min at 95°C. Western blotting was performed in accordance with Balthasar and colleagues (Balthasar et al. 2006) Rabbit anti-EDG1 and rabbit anti-EDG3 (Santa Cruz Biotechnology) were used as primary antibodies and HRP-conjugated goat anti-rabbit IgG (BioRad Laboratories) as the secondary antibody. Finally, membranes were stripped as described (Balthasar et al. 2006) and secondary probing with the loading control β-actin (Cell Signalling Technology) was performed. Densitometric analysis of the blots was performed using ImageJ 1.41 software and S1P receptor blot intensities were correlated to the β-actin loading control blot intensities.

4.5 [3H] Thymidine incorporation assays (I, II)

Proliferation tests using radioactive thymidine incorporation was used in two different types of experimental settings:

4.5.1 Cell proliferation in response to commensal bacteria

Fresh faecal pellets were collected from individual BALB/c and NOD mice, incubated in PBS (1 pellet/ 0.1ml) for 30 minutes at 37ºC, then vortexed and centrifuged to remove undissolved fibrous pieces. The suspension was further incubated for 2 hours at 60ºC to inactivate the bacteria and sonicated to produce a suspension of dead bacterial components. The bacterial density was adjusted using absorbance measurements relying on a standard curve created on the basis of titration of colony-forming unit values for different absorbance values. Bacterial sonicate was added to cell culture plates containing MLN cells (200000 cells/well) from the same mouse from which the pellets were collected (bacterial sonicate from autologous intestine; BsA) or from a littermate (bacterial sonicate from heterologous intestine; BsH). The cells were incubated in 37ºC for 72 hours with the addition of [H^3] thymidine (0.4 μCi/ml) during the last 6 hours of incubation. Finally, cells were collected using an automatic cell harvester (Tomtec Harvest 96) and the radioactivity was counted using a beta counter (Wallac). Each experimental condition was performed in triplicate.

4.5.2 Antigen presentation assay

NOD, PNOD and BALB/c mice were immunised with 50 μg insulin peptide (Insulin B (9-23), Anaspec) subcutaneously on the hind flank. Ten days later, spleens were collected from these animals and the splenic $CD4^+$ T cells were purified using CD4 MicroBeads (Miltenyi Biotec). These purified T cells were co-cultured (200000 cells/ well) with either peritoneal- or splenic B cells (150000 cells/well) purified with B220 MicroBeads (Miltenyi Biotech). Additionally, either insulin peptide (4μmol/l or 40μmol/l; Insulin B 9-23; Anaspec) or intact insulin (20 μg/ml or 200 μg/ml Sigma-Aldrich) was added to some of the wells. The cells were incubated in 37 ºC for 72 hours with the addition of [H^3] thymidine (0.4 μCi/ml) during the last 16 hours of incubation. Finally, cells were collected using an automatic cell harvester (Harvest 96, Tomtec) and the radioactivity was counted using a beta counter (Wallac). Each experiment was performed in triplicate.

4.6 Gas chromatographic analysis (II)

The intestinal flora of NOD and PNOD mice were assessed for overall differences using gas–liquid chromatographic (GLC) techniques. This method allowed computerised profiling of cellular fatty acids of bacteria in NOD and PNOD stool samples. Differing

fatty acid profiles correlate to differences in bacterial species because the fatty acid composition is species specific (Eerola and Lehtonen 1988). To assess the differences in gut flora, stool samples were collected from NOD and PNOD mice and stored at -70°C until processing. Before proceeding to GLC analysis, bacterial mass was separated from other fatty acids present in the faeces as described (Toivanen et al. 2001) using sedimentation and centrifugation steps. The bacterial mass was further saponified and methylated, and the GLC was run as previously described (Eerola and Lehtonen 1988).

4.7 rtPCR (II, III)

Mouse colon samples were cut into pieces and stored in RNA Later (Qiagen). Total RNA was purified with RNeasy Mini Kit (Qiagen). RNA purity and quantity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). cDNA was synthesised with DyNAmo cDNA Synthesis Kit (Finnzymes), using oligo-dT primers provided with the kit. Levels of cytokine expression in colons of individual mice were evaluated with real-time quantitative PCR using Maxima SYBR Green qPCR Master Mix (Fermentas) and RotoGene cycler (Corbett Research). Ct-values were normalised to the endogenous housekeeping gene GAPDH and are expressed as copy numbers relative to the GAPDH copy numbers. Primer sequences are given in Table 2.

Table 2. Sense and anti-sense primer sequences used for rtPCR analysis.

Gene	Primer name		Sequence (5'→ 3')
Gapdh	GAPDH-s	sense	AACGACCCCTTCATTGAC
	GAPDH-a	antisense	TCCACGACATACTCAGCAC
Il12p35	IL12p35-s	sense	CCACCCTTGCCCTCCTAAAC
	IL12p35-a	antisense	GGCAGCTCCCTCTTGTTGTG
Il17a	IL17-s	sense	TCCCTCTGTGATCTGGGAAG
	IL17-a	antisense	CTCGACCCTGAAAGTGAAGG
Ifnγ	INF-s	sense	AACGCTACACACTGCATCT
	INF-a	antisense	GAGCTCATTGAATGCTTGG
Il10	IL10-s	sense	AGGGCCCTTTGCTATGGTGT
	IL10-a	antisense	TGGCCACAGTTTTCAGGGAT
Il6	IL6-s	sense	CTGCAAGAGACTTCCATCC
	IL6-a	antisense	TATATCCAGTTTGGTAGCATCC
Tgfβ	TGF-s	sense	GACCGCAACAACGCCATCTA
	TGF-a	antisense	AGCCCTGTATTCCGTCTCCTT
Foxp3	FoxP3-s	sense	GGCCCTTCTCCAGGACAGA
	FoxP3-a	antisense	GCTGATCATGGCTGGGTTGT
Il1-β	IL1-s	sense	CAACCAACAAGTGATATTCTCCATG
	IL1-a	antisense	GATCCACACTCTCCAGCTGCA

4.8 Histology (II, III)

Colon and pancreas histology: Colon and pancreas tissue from NOD, PNOD, germ-free NOD and BALB/c mice of different ages were excised, washed with PBS and fixed in 10% buffered formalin. After rehydration, 4–5 μm thick paraffin-embedded sections were stained with hematoxylin and eosin. Colon samples were analysed for hyperplasia or other signs of inflammation and pancreas tissues were assessed for insulitis. Insulitis was assessed by giving a score from 0-3 for each islet, whereby 0 = no infiltration, 1 = peri insulitis, 2 = infiltration covering approximately half of the islet, 3 = full insulitis. 50 islets per pancreas were scored from each NOD mouse group.

4.9 Immunofluorescence (III)

Insulitis characterisation: Pancreas tissue from 6 and 13 week old NOD and germ-free NOD mice were dissected, immersed in embedding medium (Sakura Finetek) and frozen in liquid nitrogen. Cryosections of 6-7 μm thickness were acetone-fixed and subsequently stained either with CD4-FITC and CD19-PE (Immunotools) or with CD4-FITC, FOXP3-PE (eBioscience) and anti-rabbit Insulin H-86 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by donkey anti-rabbit fluor 647 (Invitrogen). Analysis was performed using a UV microscope (Olympus) or a confocal microscope (Zeiss LSM510 META).

5 RESULTS

5.1 Diabetes incidence, insulitis and gut microbiota in NOD mice

5.1.1 NOD mice

The diabetes incidence for the NOD mice used in this study was 60-65% by the age of 30 weeks and 70-75% if the NOD mice were followed up until 50 weeks of age. Insulitis was observed in 5% of pancreatic islets of Langerhans in 6 week old mice and in roughly 40% of islets at 13 weeks of age (III, Figure 1, 5A). CD19+ B cells were present in 40% of infiltrated islets at 6 weeks and in 70% of affected islets at 13 weeks.

5.1.2 ProSobee NOD mice

The antidiabetogenic effect of ProSobee has previously been demonstrated in NOD mice (Reddy et al. 1995). In our NOD colony, ProSobee diet decreased diabetes incidence at 50 weeks from 75% in mice that were fed conventional murine pellet food to approximately 20% in mice that were fed only ProSobee from the time of weaning (II, Figure 3A).

The diet change also significantly affected the gut microflora in NOD mice. Gas chromatographic analysis of gut bacteria revealed substantial differences in the composition of the microflora in NOD mice maintained on conventional food and the mice that were given the ProSobee diet (II, Figure 3B-C). This method of analysis permits computerised profiling of cellular fatty acids from gut bacteria. These fatty acids are components of the bacterial cell walls and their composition is species-specific. A different bacterial fatty acid profile hence indicates a difference in the prevalence of bacterial species in the gut.

5.1.3 Germ-free NOD mice

The diabetes incidence did not differ significantly between NOD mice and germ-free NOD mice. Frank diabetes, determined by blood glucose measurement in NOD mice and by analysis of glucosuria in germ-free NOD mice started occurring around 15 weeks of age and increased gradually to 60-65% in both colonies by 30 weeks of age (III, Figure 1A).

In contrast to the coincident diabetes development in the NOD and germ-free NOD mice, there was a significant difference in the timing and extensiveness of the insulitis progression in these two mouse colonies. Insulitis was more widespread in germ-free mice both in the early stages (6 weeks of age) and in later stages of T1D development (13 weeks). At 6 weeks, only 5% of observed islets were affected in NOD mice, compared to 15% in germ-free NOD mice. At 13 weeks, the proportion was 35% in NOD versus 75% in germ-free NOD mice (III, Figure 1B-E).

The pancreas-infiltrating cells were dominated by $CD4^+$ T cells, with $CD8^+$ T cells being in minority both in NOD and germ-free NOD mice (our unpublished observation). Co-infiltrating B cells were found frequently in NOD mice at 6 weeks of age, with 40% of CD4-positive islets also containing CD19-positive B cells. In germ-free NOD mice, on the contrary, only 15% of islets were co-infiltrated with B cells at this age. This trend was consistently observed in older mice as well, albeit the difference was less accentuated, with 70% of affected islets containing CD19-positive cells in NOD mice, compared to 60% in germ-free NOD mice (III, Figure 5A-B).

Immunohistochemical staining of pancreas sections showed that insulin staining was preserved to the same level in 13 week old NOD and germ-free NOD mice despite the more extensive insulitis in the germ-free mice (III, Figure 5E) Staining of the pancreas sections for CD4 and FOXP3 moreover revealed that FOXP3+ cells were present in a significantly higher percentage of insulitic islets in germ-free NOD compared to NOD mice (Figure 2 and III, Figure 5C,D). The presence of Tregs in the pancreas is known to be a critical factor for maintaining insulitis in an innocuous state (Ryan et al. 2010). The increased number of Tregs in the pancreas could thus provide an explanation as to why germ-free NOD mice did not develop diabetes earlier than their SPF counterparts despite the increased insulitis in the germ-free NODs.

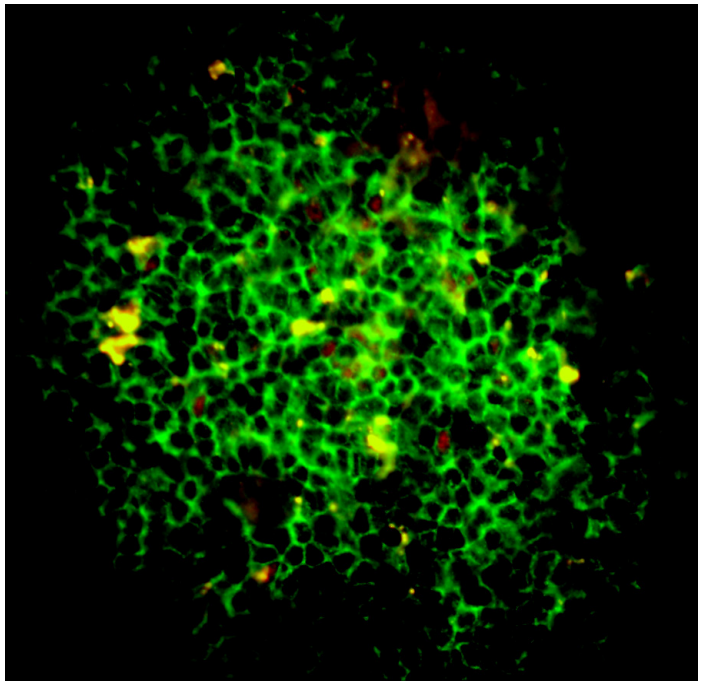

Figure 2. Representative picture of a pancreas section stained with CD4-FITC (green) and FOXP3-PE (red), from a 13 week old germ-free NOD mouse.

5.2 Immune regulation in the colon and MLN

5.2.1 Proinflammatory bias and increased cell activation in young NOD mice (II)

Intestinal enteropathy and permeability impairment in the small intestine have been linked to T1D in several previous studies (Maurano et al. 2005; Bosi et al. 2006; Vaarala 2008). Little is known about the association between the immune regulation of the colon and T1D. The increasing evidence emphasizing the importance of the microbiota in T1D development, however, argues for a fundamental role for the immune regulation of the colonic mucosa.

The results presented in this thesis indicate that proinflammatory tendencies exist in the colon of newly weaned NOD mice. When young NOD mice were compared with age-matched BALB/c mice, a number of inflammatory changes were observed exclusively in the NOD mice, such as a loss of tolerance to commensal microbiota, hyperplastic changes in the colonic epithelium, increased levels of *Il-17a* and *Il-23*, higher ratio of inflammatory dendritic cells and increased expression of costimulatory molecules on lamina propria lymphocytes (II, Figures 1-2,4). Treg cells, which contribute to immune tolerance, nevertheless, were not reduced in the intestine of NOD mice. In fact, slightly higher levels of *Foxp3* as well as anti-inflammatory cytokine *Il-10* mRNA were found in NOD mice compared to BALB/c control mice (II, Figure 5). This increase may be induced by the inflammatory changes. The accumulation of naturally-occurring T reg cells has been detected in inflamed pancreatic lymph nodes and in the pancreas (Tritt et al. 2008) and increased numbers of Foxp3 T cells have also been detected in the small intestine of children with both celiac disease and T1D (Vorobjova et al. 2009). An increase in IL-10 in inflamed mucosa of patients with ulcerative colitis has additionally been reported (Matsuda et al. 2009). The increased *Foxp3* and *Il-10* in NOD mice may thus present a counter effect to the ongoing inflammation in the colon.

The above described proinflammatory tendencies in the colon of NOD mice seem to culminate at the age of 4-5 weeks. The colon hyperplasia and increase in pro-inflammatory cytokine mRNA levels were detected in 4-5 week old but not in 6 or 10 week old NOD mice (II, Figure 2). The time period at 4-5 weeks of age coincides with the time straight after weaning in NOD mice.

5.2.2 The proinflammatory bias in NOD colons can be reversed through diet manipulation (II)

The inflammatory changes observed in NOD colons were profoundly alleviated when the standard mouse diet was changed to the antidiabetogenic ProSobee diet from time of weaning. This was evidenced by a reversal of the hyperplastic changes, decreased levels of *Il-17a* and *Il-23* mRNA, a decreased percentage of activated CD4 T cells and inflammatory dendritic cells in the lamina propria of ProSobee fed NOD mice (II,

Figures 2,4-5). Hence, the proinflammatory bias in NOD colons seems to be highly diet-dependent.

5.2.3 Effects of germ-free conditions on immune regulation in the gut and gut-draining lymph nodes (MLN/PaLN)

The mRNA levels of both *Il-17a* and *Foxp3* were lower in the ileum when NOD mice were kept germ-free. In the colon, *Foxp3* levels were likewise lower in germ-free NOD mice, but *Il-17a* was significantly higher in germ-free NOD than in NOD mice (Figure 3 and III, Figure 2A-D). It would thus seem that germ-free conditions restrain Treg cell differentiation in the small and large intestine as well as promote a proinflammatory *Il-17a* response in the large intestine. Despite this, the crypt hyperplasia that was observed in the colons of young NOD mice could not be detected in germ-free NOD mice. Germ-free NOD colons from 5 week old animals were lean and uniform with roughly 50% thinner crypts compared with age matched NOD mice (III, 2E-F).

In accordance with the rtPCR results from ileum and colon samples, intracellular (IC) staining from pooled MLN/PaLN cells also showed a reduction in FOXP3 in germ-free NOD mice, an average of 2% of $CD4^+$ T cells were $FOXP3^+$, compared with 4% in NOD mice. IC staining for IFNγ and IL-17A moreover demonstrated a significantly higher percentage of both IFNγ and IL-17A $CD4^+$ T cells in the MLN/PaLN in germ-free NOD than in NOD mice (III, Figure 3). Germ-free conditions thus increase the percentage of Th1 and Il-17a lymphocytes in the MLN/PaLN as well as limit the number of T regulatory cells.

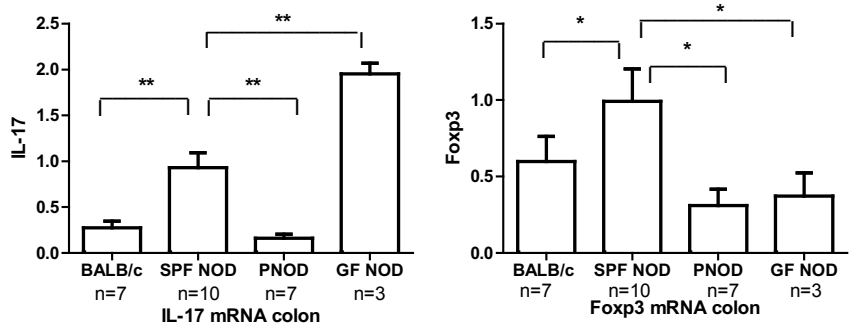

Figure 3. Summary of rtPCR results of IL-17A and Foxp3 mRNA levels in the colon of BALB/c, (SPF) NOD, PNOD and germ-free (GF) NOD mice. *Il-17a* (left) and *Foxp*3 (right) mRNA expression in colon of BALB/c, NOD, PNOD and germ-free NOD mice measured with real time PCR. Bars represent normalised mean values ± SEM. *=p<0.05 and **=p<0.01 as calculated using One way ANOVA and Bonferroni's post hoc test.

5.3 Peritoneal cavity B cells

5.3.1 NOD peritoneal B cells have increased expression of costimulatory molecules and augmented response to CD40 ligation (I)

Signs of an increased activation status were observed in the peritoneal B cell pool. Peritoneal B cells from NOD mice expressed higher levels of several activation markers compared with BALB/c and C57BL/6 mice. The expression level of the costimulatory receptor CD40 was significantly higher on B1 as well as B2 cells from NOD than from BALB/c and C57BL/6 mice. CD86, which could only be detected on B1 cells, was also over-expressed in the NOD mouse (I, Figure 5A-B, E-F).

The majority (56%) of NOD B2 cells expressed CD69, as compared to 24% of C57BL/6 B2 cells and only 8% of B2 cells from BALB/c mice. A smaller, yet significant increase in CD69 expression was also observed on NOD B1 cells. Less than 30% of NOD B1 cells expressed L-selectin, as compared to over 75% of BALB/c and C57BL/6 B1 cells. There was no significant difference in L-selectin expression between B2 cells from NOD, BALB/c and C57BL/6 mice (I, Figure 5 C-D, G-H).

The increased expression of CD69 and L-selectin downregulation was seen at every time point in NOD mice, from 3 weeks up to >17 weeks of age. Increased expression of CD40 and CD86 however was observed from 4.5-5 weeks of age and onwards, but not yet at 3 weeks (unpublished results).

To evaluate the responsiveness of NOD peritoneal B cells to stimulation through CD40, TLR 4 or TLR 9, the proliferative responses of peritoneal B cells and splenic B cells from NOD mice to anti-CD40 antibody, LPS and CpG were compared. There was no significant difference in the response of peritoneal or splenic B cells to CpG, LPS stimulation or CD40 ligation. When NOD and BALB/c peritoneal B cells were compared, the response of NOD peritoneal B cells to CD40 ligation was nevertheless stronger (I, Figure 6).

5.3.2 The expression of costimulatory molecules on peritoneal B cells in PNOD and germ-free NOD mice (II, III)

The expression of costimulatory molecules CD40 and CD86 was significantly decreased on PNOD peritoneal B1 cells compared to NOD B1 cells (II, Figure 6A-B). CD40 and CD86 were similarly decreased on B1 cells from germ-free NOD mice, compared to NOD mice (Figure 4). The expression of CD69 was equally high on NOD and PNOD B cells, but significantly lower in germ-free NOD mice (III, Figure 4).

These results suggest that gut immunity or microbial factors may be the cause for the upregulated expression of activation markers in conventionally reared NOD mice. It is possible that the inflammatory milieu in the colon works as a trigger for the activation of the peritoneal B cell pool.

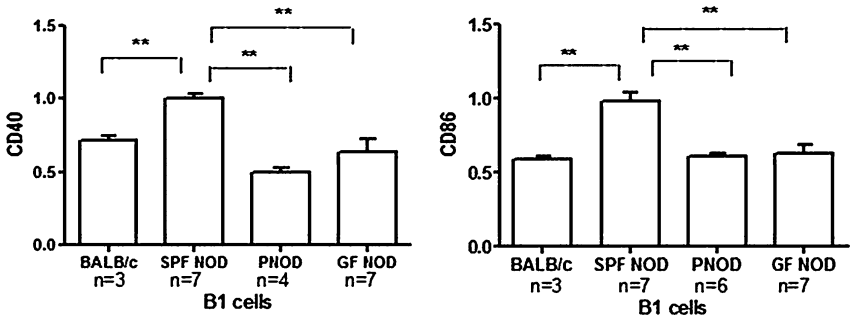

Figure 4. Relative CD40 (left) and CD86 (right) surface expression on peritoneal CD11b$^+$B220$^+$ B1 cells from BALB/c, (SPF) NOD, (SPF) PNOD and germ-free (GF) NOD mice. Bars represent normalised mean values ±SEM, **=p<0.01 as calculated using One Way ANOVA and Bonferroni's post hoc test.

5.3.3 Increased trafficking of peritoneal B cells to the pancreatic lymph nodes and enhanced antigen presentation capacity of NOD mice (I,II)

The peritoneal B cell pool in NOD mice does not undergo a normal age-dependent expansion, but the number of B1 cells (B220+CD11b+) in particular, remains reduced throughout development. B2 type (B220+CD11b-) B cells are also reduced in numbers up to 15 weeks of age in NOD mice, compared to BALB/c mice (I, Figure 1). The number of peritoneal macrophages, in contrast, increased rapidly after weaning and the number of macrophages was 3-fold in NOD mice at the age of 4-5 weeks of age, compared to BALB/c mouse macrophages (unpublished data).

The decrease in B cell numbers was accompanied by a highly increased migration rate of these cells to the pancreatic lymph nodes. Labelled peritoneal cells from NOD and BALB/c mice were transferred intraperitoneally to intact recipients of the same strain. The transferred cells migrated preferentially to the pancreatic lymph nodes (PaLN) over spleen and MLN in NOD mice. The migration rate of peritoneal B cells to PaLN was enhanced 10-fold in NOD mice compared to BALB/c mice. In line with this, the percentage of DiI labelled B cells left in the peritoneum 20 hours after transfer was significantly lower in NOD mice, compared with BALB/c mice (I, Figure 2). The accumulation of transferred cells in the pancreatic lymph nodes was observed only when peritoneal B cells from a NOD donor was transferred to a NOD recipient, but not if NOD B cells were transferred to a BALB/c recipient or *vice versa* (Figure 5). This suggests that the increased trafficking of NOD peritoneal B cells depends on both the characteristics of peritoneal B cells as well as on the peritoneal micro milieu in NOD mice.

NOD peritoneal B cells were furthermore characterised with lower expression of integrin α4β1 compared with BALB/c mice (I, Figure 3A-B). It is thought that the low surface expression of α4β1 is one factor that permits the efflux of these cells. In addition,

insensitivity to the important chemotactic cue, S1P, was observed in NOD peritoneal B cells, which also helps explain their augmented efflux from the peritoneum (I, Figure 3D-E). S1P signalling is needed for keeping B cells in the peritoneal cavity. Loss of sensitivity to S1P, hence, allows their emigration. Surprisingly, however, NOD peritoneal B cells were not deficient in S1P receptor 1 or 3 proteins (I, Figure 4A-C). The surface expression of the S1P1 receptor was lower on NOD than on BALB/c B cells, but the difference was not statistically significant (I, Figure 4D). A cross-communication between CD69 and S1P receptors on NOD B cells may be the cause of the loss of sensitivity to S1P chemotactic cues on B cells, since NOD B cells express high levels of CD69 and this early activation marker is known to cross-react with S1P receptors. Indeed, incubation with S1P did significantly reduce CD69 expression on NOD peritoneal B cells, indicating that cross-communication between S1P and CD69 receptors does occur on these cells (I, Figure 4E).

It is known that activation of peritoneal B cells by direct exposure to LPS, or by chemically inducing an inflammation in the gut with indomethacine, causes internalisation of B cell integrin α4β1 surface expression and a rapid efflux of these cells out of the peritoneum (Ha et al. 2006). LPS stimulation furthermore causes loss of sensitivity to S1P chemotactic signals in B cells (Cinamon et al. 2004). The results presented in this thesis show similar tendencies of the peritoneal B cell population in NOD mice under unmanipulated conditions.

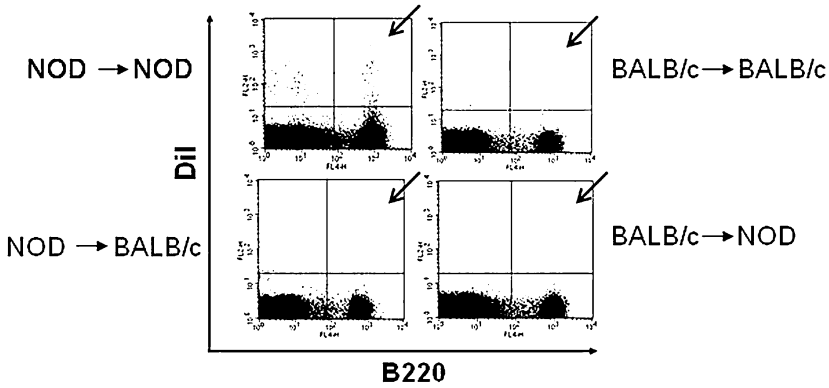

Fig 5. Adoptive transfer of DiI labelled peritoneal B cells. Dot plots depict the cell populations in pancreatic lymph nodes 20 hours after i.p. transfer of NOD B cells into a NOD recipient, BALB/c B cells into a BALB/c recipient, NOD B cells into a BALB/c recipient or BALB/c B cells into a NOD recipient mouse. Arrows indicate the DiI-labelled $B220^{+}$ B cells.

6 DISCUSSION

While it is broadly accepted that environmental factors, including diet and microbes, likely contribute to the increasing incidence of T1D witnessed over the last decades, relatively little is known about the specific mechanisms relating these factors to T1D development. The work presented in this thesis describes several anomalies in the immune regulation of the intestine and in the adjacent peritoneal cavity B cell population in diabetes-prone NOD mice. The significance of dietary and microbial factors on this immune imbalance was demonstrated through dietary manipulation and microbial deprivation. It is evident that the diet profoundly affected the microflora and had considerable effects on inflammatory responses in the colonic mucosa in NOD mice. Changing the diet from a conventional murine pellet feed to a soy-based formula significantly downregulated the expression of peritoneal B cell activation markers, reduced the antigen presenting efficiency of these B cells and decreased diabetes incidence in NOD mice. Complete microbial deprivation, achieved by rearing NOD mice in germ-free conditions, also significantly affected the immune regulation in the intestine as well as in the peritoneum. Germ-free conditions, however, did not significantly alter the diabetes incidence in NOD mice.

6.1 Inflammatory changes in NOD mouse intestine

The evidence presented herein indicates that young NOD mice suffer from a mild colitis, which disrupts the immune homeostasis of the large intestine. The signs of inflammation included tolerance to autologous microbiota, colonic hyperplasia, increased numbers of dendritic cells and increased levels of *Il-17a* and *Il-23* in the NOD colon. The hyperplastic changes and the increase in proinflammatory cytokine levels were most distinct at the age of 4-5 weeks, which coincides with the time immediately after weaning, as well as with the time when the signs of insulitis manifest in the pancreas. It is plausible that the inflammatory changes are triggered by the introduction of solid food to mice whose intestinal immunity is, at this age, still partially immature. Remarkably, this pro-inflammatory condition was alleviated when the standard mouse diet was changed to an antidiabetogenic soy-based diet (ProSobee) from the time of weaning. It is suggested that the effects of ProsoBee on colon histology and immunology in NOD mice are a consequence of the significantly different microflora in the mice maintained on this diet. Several recent studies have emphasized the importance of specific bacterial species in the maintenance of immune homeostasis as well as in the induction of an inflammatory response in the intestine (Mazmanian et al. 2008; Wen et al. 2008; Ivanov et al. 2009). The idea that a particular gut microbiome may protect from or promote the development of T1D has been hypothesised, but further investigations are needed to accurately define the composition of an ‘autoimmune microbiome’ or a microbiome that may protect from

T1D (Arumugam et al. 2011; Giongo et al. 2011). The gut microbiome in the human population nevertheless, appears to be clustered into a limited number of enterotypes, rather than constituting a continuum of arbitrary bacterial species resident in the gut. These enterotypes may influence the susceptibility to various diseases, including T1D, and may furthermore respond differently to diet or medication (Arumugam et al. 2011).

Mucosal inflammation in the small intestine has been associated with T1D in humans in a number of studies (Westerholm-Ormio et al. 2003; Auricchio et al. 2004). Frequently, when associations between diet or dietary antigens and T1D have been suggested, the immediate focus has been on the immune regulation of the small intestine in response to these triggers. The involvement of the colonic immune cells in T1D development has been less studied. It has nevertheless been proposed, that children whose mothers have been diagnosed with ulcerative colitis have a slightly increased risk of T1D (Hemminki et al. 2009). Moreover, it is important to note, that the colonic inflammation observed in NOD mice in this study was relatively mild and possibly only transient. A subclinical inflammation of this kind would easily escape diagnosis in human T1D patients.

6.2 Peritoneal cavity B cells in NOD mice

Signs of a spontaneously increased activation status were also observed in the peritoneal cavity immune cells in NOD mice. An abrupt increase in the number of macrophages occurred at 4-5 weeks of age and slowly levelled out over the subsequent weeks (unpublished data). Peritoneal B cells in NOD mice showed persistent activation, exhibiting high surface expression of the costimulatory molecules CD40, CD86 and CD69, low expression of integrin α4β1 and L-selectin and a strong response to CD40 ligation from the age of 3-5 weeks up until at least 15 weeks of age. Besides indicating overall cell activation, costimulatory molecules are important factors in diabetes pathology. NOD T cells that express high levels of CD40 are capable of inducing clinical diabetes (Wagner et al. 2002) while CD86 upregulation on islet infiltrating NOD B cells is associated with development of insulitis (Hussain and Delovitch 2005). CD69 is an early activation marker but its sustained up-regulation has been associated with a number of inflammatory and autoimmune diseases (Marzio et al. 1999). NOD mice reared from the time of weaning on soy-based ProSobee diet and NOD mice reared under germ-free conditions exhibited lower activation marker expression on the peritoneal B cell surface. Furthermore, the abrupt influx of macrophages into the peritoneum that was witnessed in young NOD mice was entirely lacking in germ-free NOD mice. These observations strongly suggest that the consistently elevated activation status in NOD peritoneal B cells is dependent on the gut microflora.

Peritoneal B cells were significantly reduced in number in NOD mice and the migration rate of these cells from the peritoneum to the pancreatic lymph nodes was significantly increased. The low expression of integrin α4β1 on the peritoneal B cells is probably partially responsible for this, since integrin α4β1 is important for the retention

of B cells in this location. This relationship has been demonstrated by the rapid clearance of peritoneal B1 cells in response to LPS-stimulated down-regulation of α4β1 (Lenschow et al. 1995). Another important chemotactic regulator of peritoneal B cells is S1P. S1P is a bioactive lipid that plays a central role in many cell physiological processes (Strub et al. 2010). The importance of S1P for cell migration in different cancers is well documented (Pyne and Pyne 2010), but the significance of S1P chemotactic cues for B lymphocyte migration has only recently been emphasized (Cinamon et al. 2004; Kunisawa et al. 2007; Allende et al. 2010; Donovan et al. 2010). The function of S1P in the peritoneal cavity is to retain the B cells in the peritoneum (Kunisawa et al. 2007). Insensitivity to S1P thus allows an increased migration of these cells out of the cavity. In contrast to BALB/c peritoneal B cells, NOD peritoneal B cells showed no *in vitro* migratory response to S1P. Of the five known S1P receptors, it is S1P receptors 1 and/ or 3 which communicate the migratory response (Cinamon et al. 2004; Matloubian et al. 2004). The lacking response to S1P in NOD peritoneal B cells could be caused by a diminution of these receptors or by a functional defect in the signalling through these receptors. No deficiency in S1P1 or S1P3 receptor proteins was observed in NOD peritoneal B cell lysate, but the surface expression of S1P1 was lower on NOD than on BALB/c peritoneal B cells. The high expression of CD69 on the these B cells could be the underlying reason for the lower surface expression of S1P1, since the S1P1 receptor can cross-communicate with the CD69 receptor (Shiow et al. 2006). Transmembrane and membrane proximal regions of CD69 interact with the transmembrane helix 4 of the S1P receptor. This cross-communication mimics some of the aspects of the ligand-bound state of the S1P receptor and thereby causes internalisation and eventually degradation of the S1P receptor (Bankovich et al. 2010) However, high levels of CD69 down-regulates the surface expression without significantly affecting the mRNA expression of S1P1 (Shiow et al. 2006). These results are in accordance with the differing results presented in this thesis for S1P1 protein levels using Western blotting techniques, and S1P1 surface expression. Furthermore, it has been shown that high levels of CD69 functionally inhibit S1P1, rendering the cells unresponsive to chemotactic signals from S1P (Shiow et al. 2006). This relationship is coherent with the functional characteristics of peritoneal B cells which involve rapid upregulation of CD69 and migration out of the peritoneal cavity and subsequent differentiation into antibody-producing cells (Watanabe et al. 2000). This activation response in peritoneal B cells may be facilitated by a reciprocal cross-communication between activation marker molecules (CD69) and S1P receptors on the cell surface.

6.3 Effects of germ-free conditions on colonic immune regulation, peritoneal B cell activation and diabetes development in NOD mice

It is a widely accepted phenomenon, that NOD mice maintained under SPF conditions develop diabetes more often than NOD mice under conventional maintenance. It is also

often thought, that completely sterile (germ-free) conditions further exacerbate diabetes in NOD mice. However, significant aggravation of diabetes in NOD mice reared under germ-free conditions has been reported only in one germ-free NOD mouse colony (Suzuki et al. 1987). This early study by Suzuki (1987) was for more than a decade the sole reference for germ-free NOD mouse diabetes incidence, and even to date only a small number of studies worldwide have presented further data on the diabetes incidence in de novo germ-free NOD colonies (Taniguchi et al. 2007; Wen et al. 2008). However, the few other reports that do exist fail to show any clear indications that germ-free conditions exacerbates diabetes in NOD mice. In fact, according to a study based on a large germ-free NOD colony at Shionogi Aburahi Laboratories in Japan, no difference in diabetes incidence was observed in male or female SPF and germ-free NOD mice (Taniguchi et al. 2007). Moreover, despite the fact that the diabetes incidence by 30 weeks in germ-free NOD female mice in the study by Wen and colleagues was 100%, this was not notably different to SPF NOD females, nor was the time of diabetes onset earlier in the germ-free compared with SPF NOD mice (Wen et al. 2008). In our study, no significant difference in diabetes incidence could be observed in germ-free and SPF NOD females, in accordance with previous reports by Taniguchi (2007) and Wen et al. (2008). Whether the unchanged diabetes incidence observed in the more recent studies, or the considerably aggravated disease outcome observed by Suzuki and co-workers (Suzuki et al. 1987) is a more common effect of microbial deprivation in NOD mice, will probably become more clear as a higher number of germ-free NOD colonies are established. The pedigree of the mice and/ or the animal maintenance practices at different breeding facilities may play a significant role, since the diabetes incidence of conventional NOD mice varies depending on the breeding facility, from 20% to 100% by 30 weeks of age (Pozzilli et al. 1993). It is also possible that the differences observed come down to factors such as genetic drift in colonies that have been reared for several years in a particular facility, like the NOD mice used in the present work.

The results presented herein suggest that even though the diabetes incidence remains the same in NOD mice, regardless of the presence or absence of microbes, insulitis develops at a faster rate in germ-free NOD mice. Despite this, no difference was found in the preservation of insulin in the infiltrated islets. A closer analysis of the islets revealed further differences in the infiltrating cells in the islets of Langerhans. B cells, which are found very early and abundantly in the pancreatic islets of NOD mice, were significantly reduced in the islets of germ-free NOD mice especially at the early stages of insulitis (6 weeks of age). Treg cells, defined as $CD4^{+}FOXP3^{+}$ cells were, on the contrary, present more frequently in the islets of germ-free NOD mice. The increased occurrence of Treg cells in the islet infiltrates may explain why transition to overt diabetes remained the same in germ-free NOD and NOD mice despite the extensive insulitis in the germ-free mice. Treg cells play an important role in keeping islet-infiltrating lymphocytes in an innocuous state, as well as in preserving insulin in the islets (Chen et al. 2005; Ryan et

al. 2010). This is demonstrated by the development of aggressive insulitic lesions, loss of insulin staining in islets and conversion to overt diabetes in DO11 × RIP-mOVA/rag^{-} mice upon depletion of FOXP3^{+} cells (Ryan et al. 2010). B cell depletion is, moreover, accompanied by an expansion of Treg cells (Hu et al. 2007; Fiorina et al. 2008). Hence the reduction in infiltrating B cells in the germ-free NOD pancreatic islets could contribute to the observed increase in T reg cells, and subsequently to the longer preservation of pancreatic islet lesions in an innocuous state in the germ-free NOD mice.

The IL-17A level was increased in the colon of NOD mice compared with BALB/c mice, as discussed above. In the colon of germ-free NOD mice, *Il-17a* was even more abundant than in NOD mice. The level of *Foxp3*, contrarily, was highest in NOD colons and lowest in germ-free NOD mice, and intermediate in BALB/c. A reciprocal relationship exists between IL-17 and production and FOXP3 induction whereby the balance of cytokines present in the environment cause the differentiation of the same precursor T cells into either IL-17 effector cells or FOXP3^{+} regulatory cells (Bettelli et al. 2006). However, the diminished *Foxp3* and increased *Il-17a* levels observed in germ-free NOD mice may depend on other factors as well. T cells in the intestine may require antigenic stimuli from commensals to differentiate into Treg cells (Ishikawa et al. 2008). Moreover, a previous study with germ-free mice indicates that the increased Il-17 production in the colon of germ-free mice is due to a lack of commensal-dependent Il-23, which under normal circumstances limits Il-17 production (Zaph et al. 2008).

In accordance with previous reports for other germ-free mouse strains (Zaph et al. 2008; Gaboriau-Routhiau et al. 2009), mRNA levels of *Il-17a* were not increased in the ileum of germ-free NOD mice. Il-17 production can potently be induced in the small intestine by monocolonisation with a segmented filamentous bacteria with the candidate name Artromitus (Ivanov et al. 2009). However, the same study by Ivanov et al. also showed that colonisation with Artromitus has a much smaller effect on Il-17 levels in the colon. Given the different effects of germ-free conditions on Il-17 regulation as well as the different impact of monocolonisation with Artromitus on the small and the large intestine, it seems likely that Il-17 induction is differentially regulated at these two sites.

The finding that Treg cells were significantly reduced in the intestine and draining lymph nodes but enriched in the pancreas may be explained by a different origin of these two sets of Treg cells. Tregs can be produced in the thymus through selection that is independent of antigenic stimuli, or in the periphery in response to antigenic challenge (Walker et al. 2003). Previous studies with other germ-free mouse strains have shown that Treg cell numbers in the thymus or spleen are not affected by germ-free conditions, but rather, the reduction is limited to MLN and Peyer's patches (Hrncir et al. 2008). It is thus conceivable that the FOXP3+ CD4+ T cells in islets represent natural Treg cells. Nevertheless, the faster rate of insulitis development in young germ-free NOD mice may be linked to the lack of antigen-induced FOXP3 T reg cells and/ or the increased *Il-17a* levels in the intestine and MLN/ PaLN during the initiation of islet-specific immune responses.

Peritoneal B cells in NOD mice were hyper-activated, and decreased in numbers in the peritoneal cavity. In peritoneal B cells from germ-free NOD mice however, no such signs of activation were observed. The expression levels of CD69 and of costimulatory molecules CD40 and CD86, which were abnormally increased in NOD mice, were significantly lower on both B1 and B2 peritoneal cells in germ-free NOD mice. Peritoneal B cells characteristically migrate out of the peritoneum upon activation (Ha et al. 2006) and the reduced number of these cells in NOD mice is hence indicative of cell activation. In germ-free NOD mice however, B cell numbers were markedly higher than in NOD mice. The increased number and the reduced surface expression of costimulatory molecules would thus indicate that the peritoneal B cells in germ-free NOD mice may not exhibit the same heightened activation status as their SPF counterparts. This is intriguing, since it suggests that the activation of peritoneal B cells in NOD mice may be dependent on the presence of microbes. Peritoneal B cells and/ or other innate B1 cells are thought to play an important role in the development of diabetes in the NOD and RIP-OVA transgenic mouse model, since B cells in the pancreatic infiltrates closely resemble peritoneal B1 cells (Kendall et al. 2004; Ryan et al. 2010) and hypotonic lysis of peritoneal B cells has been reported to delay diabetes (Kendall et al. 2004). The results presented here raise the question as to whether microbial stimulation may be needed for peritoneal B cells to become activated, efficiently infiltrate pancreatic islets and promote islet damage in NOD mice (Figure 6).

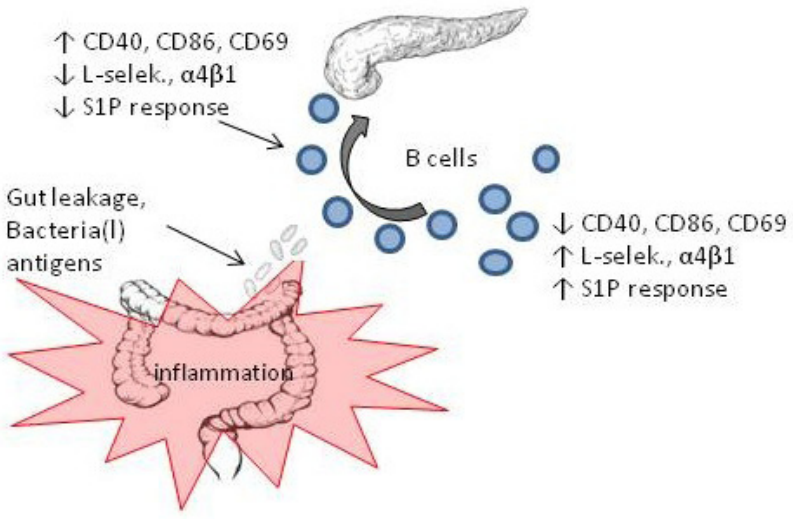

Figure 6. Schematic illustration of the proposed hypothesis for peritoneal B cell activation and migration to the pancreatic lymph nodes. Colitis- induced impaired barrier properties of the colon may cause leakage of bacteria or bacterial antigens into the peritoneal cavity. These stimuli activate the peritoneal B cells and promote their migration to the pancreatic lymph nodes.

7 CONCLUSIONS

The research presented in this thesis suggests that newly weaned (4.5 week old) NOD mice suffer from a mild colitis, which disrupts the immune cell homeostasis, causing a shift towards a proinflammatory status. Concomitantly, several changes in the peritoneal immune cells of NOD mice are observed that do not occur in healthy mice (BALB/c); a substantial and rapid influx of macrophages to the peritoneal cavity and an increase in activation marker expression on peritoneal B cells. NOD peritoneal B cells also exhibit enhanced spontaneous trafficking to the pancreatic lymph nodes and a significantly higher APC efficiency towards insulin-specific T cells than spleen-derived conventional B cells. Remarkably, most of the abnormalities in the colon, peritoneal macrophages and the peritoneal B cell APC activity of NOD mice are abrogated when NOD mice are fed an antidiabetogenic soy-based diet (ProSobee) from the time of weaning.

Several differences were observed in the immune cells of the gut, gut-draining lymph nodes, peritoneal cavity and in the pancreatic islets in germ-free NOD and NOD mice. Evidence of aggravated inflammation was seen in the gut and gut-draining lymph nodes (MLN/PaLN) but not within the peritoneal B cells or macrophages.

The insulitic lesions were more extensive in germ-free NOD mice, but diabetes incidence in germ-free NOD mice was not markedly different from that in NOD mice. The similar rate of progression to overt diabetes despite more extensive insulitis is hypothesised to depend on the larger number of Treg cells in the islets and perhaps also a weaker involvement of peritoneal B cells in the absence of microbial stimuli.

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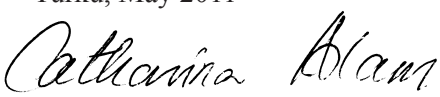

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