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Regulation of Autoimmunity and Inflammation by microRNAs and Environmental Factors

Tormo-Badia, Neivis

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Neivis Tormo Badia

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Abstract

Tolerance is crucial for maintaining immunological balance and avoid autoimmune diseases like type 1 diabetes and the inflammatory bowel disease, ulcerative colitis (UC). **In Study I**, we have investigated the regulatory role of a class of non-coding RNAs, the miRNAs, during thymocyte development of the where central tolerance is established. We used the non-obese diabetic mouse (NOD) which is spontaneously developing T1D and have been described to have defects in the T cell maturation. By studying the apoptosis response of NOD lymphocytes (which activates similar cell-cycle checkpoints and apoptosis pathways as during thymocyte maturation) we showed differential expression of the miRNA-34a/b/c gene family, miR-125 and miR-155 in the DNA damage response between NOD and wild-type mice. We believe that these differentially expressed miRNAs may contribute to defect p53 expression in NOD thymocytes after DNA damage, which we also demonstrated in this study. **In Study II**, we studied the importance of global canonical miRNA regulation in the NOD mice for the development of T1D development by deleting *Dicer1* (an enzyme needed for miRNA maturation) early in thymocyte development. We showed that these NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice had phenotype alterations including markedly decreased amount of αβ $CD4^+$ and $CD8^+$ T cells in the secondary lymph nodes but not a similarly large decrease in nTregs. No difference in diabetes incidence between female NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice and control littermates could be found as a result of these phenotypic changes but surprisingly a significant increase in the male mice diabetes incidence. **In Study III**, we investigated whether the maternal intestinal microbiota is an environmental factor influencing T1D development in the offspring. By modulating the intestinal gut microbiota with antibiotics during pregnancy of NOD mice we showed decreased diversity and a persistent modulation of the intestinal microbial pattern in the offspring. Possibly resulting in the immunological alterations of $CD8^+$ and $CD4^+CD25^+$ T cell frequencies in the mesenteric lymph nodes respectively Peyer's patches, which we demonstrated. The diabetes incidence seems to have increased in the offspring to treated mothers at 20 weeks of age but the effect was not persistant. **In study IV**, the relationship between the global intestinal microbiota and the immune system was investigated in the dextran sulfate sodium induced UC mouse model. We demonstrated changes in the colonic intestinal microbiota pattern and immunological alterations of different populations of T cells, dendritic cells and natural killer cells after UC induction.

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Neivis Tormo Badia

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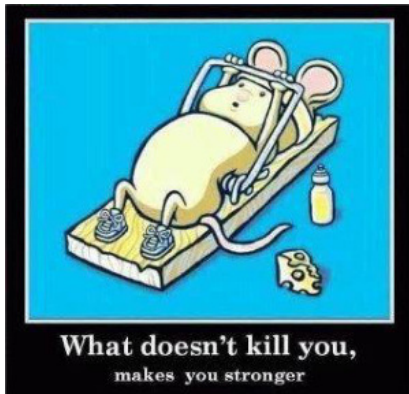
What doesn't kill you, makes you stronger

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LIST OF ORIGINAL PUBLICATIONS

I.   Apoptosis resistance of non-obese diabetic thymocytes is mediated by a defective p53 expression and altered miRNAs network controlling cell death and proliferation, Tormo-Badia N.*, Bolmeson C.*, Carpanetto A., Makowska A., Carlsén M., Ahmad S., Cilio C.M. (Submitted for publication)

II.   Deletion of Dicer1 in T cells of non-obese diabetic mice leads to T cell alterations and dramatic increase of autoimmune diabetes in male., Tormo-Badia N., Uvebrant T., Arvastsson J., Brahimi Q., Bertilsson P-A., Eliasson L., Cilio C.M. (Manuscript)

III.   Antibiotic treatment of pregnant non-obese diabetic mice leads to altered gut microbiota and intestinal immunological changes in the offspring., Tormo-Badia N., Håkansson Å., Vasudevan K., Molin G., Ahrné S and Cilio C.M., Scandinavian Journal of Immunology. 2014 Jun 26; 10.1111/sji.12205.

IV.   Immunological alteration and changes of gut microbiota after dextran sulfate sodium (DSS) administration in mice., Håkansson Å.*, Tormo-Badia N.*, Baridi A., Xu J., Molin G., Hagslätt M-L., Karlsson C., Jeppsson B., Cilio C.M., Ahrné S., Experimental Clinical Medicine. 2014;10.1007/s10238-013-0270-5.

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I. Beta-cell specific deletion of Dicer 1 leads to defective insulin secretion and diabetes mellitus., Kalis M., Bolmeson C., Esguerra J.L., Gupta S., Edlund A., Tormo-Badia N., Speidel D., Holmberg D., Mayans S., Khoo N.K., Wendt A., Eliasson L., Cilio C.M., PLoS One. 2011;6(12):e29166.

ABBREVIATIONS

APC	Antigen-presenting cell
Bcl-2	B-cell lymphoma 2
CDK4, 6	Cyclin-dependent kinase 4, 6
CLP	Common lymphoid progenitor
cSMAC	Central supramolecular activation cluster
cTEC	Cortical epithelial cells
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DC	Dendritic cell
DGCR8	DiGeorge Syndrome Crital Region 8
DN	Double negative T cell stage
FACS	Flow automated cell sorting
FasL	Fas ligand
Foxp3	Forkhead box p3
GADA	Glutamic carboxylase acid
GALT	Gut associated immune system
GF	Germ-free
HLA	Human leukocyte antigen
IA-2A	Insulinoma-associated protein 2
IBD	Inflammatory bowel disease
IDO	Tryptophan-degrading enzyme indoleamine 2,3-dioxygenase
IEL	Intra-epithelial lymphocytes
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
IP3	Inositol-tri-phospate

IS	Immunological synapse
ITAMs	Immunoreceptor tyrosine-based activation motifs
iTreg	Inducible Treg
LAT	Linker for activation of cells
Mφ	Macrophage
miRNA, miR-	microRNA, miRNA
MHC	Major histocompatibility complex
MLN	Mesenteric lymph nodes
MCFA	Medium-chain-length fatty acid
MPO	Myeloperoxidase
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
ncRNA	Non-coding RNA
NHEJ	Non-homologous end joining
NK cells	Natural killer cells
NOD	Non-obese diabetic mice
nTreg	Natural Treg
PAMP	Pathogen-associated molecular patterns
PCA	Principal component analysis
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
pSMAC	Peripheral supramolecular activation cluster
pre-TCR	Pre-T cell receptor
RAG1, RAG 2	Recombination activation gene 1, 2
SFB	Segmented filamentous bacteria
SHIP	SH2-containing Inositol 5′-Phosphatase
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism

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STAT	Signal transducer and activators of transcription
TCR	T cell receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-β	Transforming growth factor- β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells
TRFL-P	Terminal Restriction Fragment Length Polymorphism
TRF	Terminal Restriction Fragment
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick labeling
UC	Ulcerative colitis
3'UTR	3' untranslated region
VNTR	Variable number tandem repeats
ZNT8	Zinc transporter 8

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en sjukdom som drabbar nästan 177 miljoner människor världen över och siffran förväntas stiga till 366 miljoner år 2030. Vid typ 1 diabetes (T1D) har kroppen helt eller nästan helt slutat producera insulin och alla som har T1D måste därför dagligen injicera insulin. Insulin behövs i kroppens celler för att kunna ta upp näring (socker) från blodet. T1D uppstår till följd av en obalans i kroppens immunsystem som kallas autoimmunitet. Kroppens immunceller uppfattar då insulin producerande betaceller i bukspottskörteln som ett hot och angriper dem vilket leder till att de förstörs. Anledningen till varför betacellerna förstörs är inte fullständigt känd men man tror att det finns defekter i mekanismerna som i normala fall hjälper till att hålla immunsystemet under kontroll i ett tillstånd som kallas tolerans. Tolerans skapas bland annat under utvecklingen och mognaden av en viss typ immunceller, så kallade T-celler. T-celler som eventuellt kan komma att utvecklas till att angripa kroppens egna organ dödas i en kroppsegen process som kallas programmerad celldöd.

microRNA (miRNA) är en ny typ av små RNA molekyler (en kusin till DNA molekylen som kodar för vår arvsmassa) som först upptäcktes 1993 och som sedan dess har påvisats reglera genuttryck i både växter, djur och människor. Små RNA som inte kodar för några gener troddes förut vara skräp i vår arvsmassa eftersom de oftast låg mellan våra genetiska DNA sekvenser. Detta är något som på senare år har omprövats då dessa "icke-kodande" RNA har uppskattats styra ~60% av våra gener. I den första studien har vi tittat på vilka miRNA som är involverade i reglering av programmerad celldöd (såkallad apoptos) av T-celler under utvecklingen i thymus. Vi jämförde thymocyt-apoptosen i en T1D musmodell, NOD musen, med viltypsmöss och upptäckte att NOD thymocyter har mycket svårare att begå programmerad celldöd än

vildtypsmusens thymocyter. Detta kan medföra att de utvecklade T-cellerna i NOD musen möjligtvis kan vara självreaktiva (eftersom de har svårare att elimineras av kroppen under utvecklingen i thymus) och detta kan bidra till det autoimmuna sjukdomsförloppet i NOD musen. Vi upptäckte också att miR-34 a, b, c familjen, miR-125b samt miR-155 hade ett annorlunda uttryck i NOD thymocyter före och efter DNA skada och att proteinet p53 som kan regleras av dessa miRNAs och är involverad i att initiera programmerad celldöd, inte ökade i uttryck efter DNA skada i NOD thymocyter, i jämförelse med vildtypsmöss.

I den andra studien har vi skapat en genmodifierad NOD musmodell som saknar funktionella miRNA i thymocyter och karaktäriserat alla T-cellerna från thymus och olika lymfnoder genom att titta på deras antal och uttrycket av deras olika markörer (som representerar olika T-cell populationer). Vi har studerat betydelsen av miRNA reglering i de olika T-cell populationerna för utvecklingen av T1D i dessa möss och fann överraskande att avsaknaden av miRNA-reglering bidrog till minskad utveckling av diabetes. Eftersom cytotoxiska T-celler anses vara de celler som initierar immunattacken på β-cellerna så trodde vi att miRNA reglering hade större betydelse för funktionen av dessa T-celler och gick därför vidare genom att undersöka miRNA innehållet i dessa celler och ska i framtiden även undersöka hur avsaknaden av miRNA påverkar funktionen av både de cytotoxiska T-cellerna och regulatoriska T celler.

I den tredje studien så studerade vi effekten av miljöpåverkan på en hälsosam bakterieflora i tarmen under graviditeten i NOD möss, på ungarnas immunsystem och utvecklingen av T1D. Detta är ytterst intressant att studera då många gravida kvinnor idag behandlas med antibiotika för diverse infektioner och ingen har studerat vilken effekt detta har för utvecklingen av barnens immunsystem och om detta kan influera utvecklingen av T1D. De gravida mössen behandlades med antibiotika, som förändrade bakteriefloran i mammorna genom att döda vissa bakteriesorter. Ungarna
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från dessa mammor som behandlats med antibiotika under sin graviditet hade förändringar i vissa T-cell populationer i lymfnoderna i tarm-regionerna och deras bakterieflora var annorlunda samt att den hade lägre diversitet jämfört med ungar till mammor som inte behandlats med antibiotika. Ungarna till mammor som behandlats med antibiotika under graviditeten hade också ett annorlunda diabetesutvecklingsmönster.

I den fjärde studien användes en kemikalie, dextran sulfate sodium (DSS) för att inducera inflammation i tarmen på möss som en modell för ulcerös kolit som är en inflammatorisk sjukdom i tarmen på människor. Symptomen av ulcerös kolit är bland annat smärtor i magen, diarré, blod i avföringen och viktnedgång. Vi studerade hur immunsystemet samt bakteriefloran i tarmen medverkade till den inflammatoriska processen i tarmen vid sjukdomsförloppet. Inflammationen bidrog till förstörelse av tjocktarmens ytceller och förändrade vilka typer av bakterier som fanns närvarande i tarmen. Inflammationen i tarmen ledde till att vissa immunceller ökade i antal i lymfnoderna i tarmregionen, vilket ökade mängden inflammatoriska signalsubstanser (cytokiner) i mössen, medan andra typer av immunceller minskade i antal. Genom att studera samspelet mellan tarmens bakterieflora och immunsystemets bidrag till inflammationen har vi ökat förståelsen av sjukdomen.

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INTRODUCTION

The immune system

Our immune system is crucial for fighting infections and to do that in an efficient way it is divided into an innate and an adaptive immune system. The innate immune system includes cell types like monocytes/macrophages (Mϕ) which are the first to arrive to an inflammatory site and initiate an unspecific immune response. For instance, Mϕ recognize pathogen-associated molecular patterns (PAMPs) via Toll-like receptors (TLRs) and initiates an inflammatory response with secretion of pro-inflammatory cytokines and chemokines (Takeda et al. 2003). The effect of inflammatory cytokines results in physiological changes, such as increased blood flow to facilitate the transportation of more inflammatory cells to the inflammatory site. The subsequent rise in body temperature as the result of cytokine production is one of the preventative measures to inhibit the replication of invading microorganisms. In addition, released chemokines attract more effector cells to the site of inflammation. The innate immune response is the first line of defense of the immune system. Owing to the innate immune response the body is given more time to raise a secondary and more specific line of defense called the adaptive immune response(Janeway et al. 2001). Here, the main effector cells are the T cells and the B cells which are the producers of specific antibodies against pathogenic antigens. These adaptive immune cells act together with the antigen-presenting cells (APCs) the dendritic cells (DCs), which have migrated to the site of inflammation. The migration allows the DCs to encounter pathogens and process into peptides. Once achieved, the DCs further migrate to the secondary lymphoid organs from the site of inflammation to present the antigens to naïve T cells (Guermonprez et al. 2002). The naïve T cell recognition of the antigen and activation leads to

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proliferation of the T cells and differentiation into different effector T cell populations with different functions that are able to eliminate the pathogens during the adaptive immune response.

The immune system is very effective in eliminating pathogens but mechanisms to clear the immune responses in order to maintain immunological balance (tolerance) is however one the most important features of the immune system. Similarly, tolerance is an essential feature of the gut immune system. With the high presence of 10^{11} intestinal bacteria per gram intestinal content of the colon, bacterial antigens that would normally induce an immune response are tolerated and do not induce immune reaction. This is an evolutionary symbiosis between intestinal bacteria and the host. Moreover, the commensal intestinal bacteria are not only passive bystanders, but can actively interact with the immune system to protect the body from pathogenic bacteria (Belkaid and Tarbell 2009). However, in genetically susceptible individuals, the breakdown of tolerance in the gut leads to inflammatory bowel diseases like ulcerative colitis (UC). On the other hand, breakdown of immunological tolerance in the pancreatic islets leads to the autoimmune disease type 1 diabetes (T1D).

T cells

The T cells are divided into the two different types, the αβ T cells and γδ T cells. The γδ T cells have a more restricted antigen recognition than αβ T cells and respond more rapidly than the αβ T cells. For this reason, the γδ T cells are often referred to as "innate-like" T cells. The γδ T cells, in contrast to the αβ T cells, are able to recognize antigens without the need of pre-processing and presentation of pathogenic antigens by the APCs (Schild et al. 1994). Both the γδ T and αβ T cells play a role T1D development in the main mouse model for human T1D called the non-obese diabetic

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(NOD) mice the (Markle et al. 2013). This is closer described in the future section "Non-obese diabetic mouse (NOD)". However, the role of γδ T cells in NOD mice is complicated because they have also been shown to also have a protective function against T1D when isolated from the intestinal intraepithelial lymphocyte (IEL) populations (Locke et al. 2006). The involvement of γδ T cell lineage in the gut immune system is closer described in the section "Gut immune system".

The αβ T cells are the most common cells in the immune system. These cells are divided into CD4+ and CD8+ T cells based on their co-receptors. CD4+ T cells interact with the major histocompatibility complex (MHC) class II and differentiate into T helper cells (Th1 or Th2) upon activation. The differentiation into Th1 or Th2 cells depends on the types of cytokine present in milieu during activation (Constant and Bottomly 1997). Th1 cells are induced by interferon (IFN)-γ and interleukin (IL)-12 and their differentiation is regulated by the key transcription factor Signal transducer and activators of transcription (STAT)-4, which in its turn is regulating T-bet (Murphy et al. 2000). Differentiated Th1 cells secrete IL-2 and the pro-inflammatory cytokine IFN-γ, which is involved in activation of monocytes during inflammatory response and is involved in the pathology of T1D in pancreatic islets (El-Sheikh et al. 1999). This is further described in the "Pathogenesis of T1D" section.

Cytokine milieus with IL-4 differentiates naïve T cells into Th2 cells. This transformation process is mediated by the key transcription factors STAT-6 and GATA-3 which results in secretion of cytokines such as IL-4, IL-5, IL-13 and IL-10 (Murphy et al. 2000). Th2 cytokines promotes B cell differentiation and antibody production against specific pathogenic antigens. However, both Th1 and Th2 cells can participate in antibody responses. For example IL-4 promotes class-switch to IgE in response to parasites and IFN-γ can mediate class-switch to IgG2a against viral infections (Vinuesa et al. 2005).

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In a state of tolerance breakdown the Th1 cells have been shown to be involved in pathogenesis of autoimmune diseases like type 1 diabetes (T1D), Crohn's disease, multiple sclerosis (MS), rheumatoid arthritis (RA) etc. (Peluso et al. 2006; Larsen et al. 2012; Oreja-Guevara et al. 2012). In contrast, Th2 cells are involved in pathogenesis of for instance UC, allergic disease, systemic lupus erythematosus (SLE) (Funauchi et al. 1998; Georas et al. 2005; Heller et al. 2005).

There is another class of T cells, the Th17 cells. Naïve T cells are differentiated into Th17 cells in the presence of IL-23 and IL-6. However, the participation of transforming growth factor (TGF)-β and IL-21 have also been demonstrated in the induction of Th17 cells (Korn et al. 2009). The process of differentiation in this case is mainly under transcriptional regulation by STAT-3 and matured Th17 cells are able to produce the cytokine IL-17. The dual role of Th17 cells in the intestine is described in the section "Gut immune system". Notably, the involvement of Th17 cells in the pathogenesis of T1D has been confirmed in animal models and has also been found to be increased as circulating cells in patients (Shao et al. 2012). The mechanisms by which Th17 cells are believed to mediate T1D development involves the suppression of regulatory T cells (Tregs) expansion, mediates T cell shift to Th1 phenotype and stimulates $CD8^+$ cytotoxic T lymphocyte (CTL) responses (Shao et al. 2012).

Tregs are necessary to keep the immunological balance to "self" and they are the main tolerance inducers. Natural $CD3^+CD4^+CD25^+$ Tregs (nTregs) are derived from thymic differentiation and express the master regulator transcription factor Forkhead box p3 (Foxp3). However, Tregs can also be induced in the periphery and are then referred as iTregs. Moreover, as not all iTregs express Foxp3, cells lacking Foxp3 expression are further sub-divided into Tr1 and Th3 cells which are characterized by their ability to secrete IL-10 and/or TGF-β (Curotto de Lafaille and Lafaille 2009). The mechanisms through which Tregs can suppress and regulate T cell responses as well as innate

immune responses are described in more detail in the "Tolerance versus autoimmunity" section.

One of the main functions of the CD8^{+} T cell is the recognition of virus infected cells and abnormal cells such as cancer cells (Klenerman and Hill 2005; Gattinoni et al. 2012). The recognition is mediated through the interaction with MHC class I, which is expressed on nearly all cells in the body. Naïve CD8^{+} T cells mature into cytotoxic T cells that have the ability to induce cellular apoptosis by releasing lytic granules containing perforins. These enzymes create pores in the lipid bilayer of the target cell and subsequently release proteases referred as granzymes. Also, CD8^{+} T cells could employ perforin-independent mechanism of cytotoxicity that involves binding of membrane incorporated Fas ligand (FasL) to Fas on the target cell. Both the release of perforin/granzymes and the Fas-FasL interaction induce the intrinsic apoptotic pathway by cleaving and activating pro-caspases (Russell and Ley 2002).

T cell development

All immune cells develop from hematopoietic stem cells and a common lymphoid progenitor (CLP) has been suggested for T and B cells (Kondo et al. 1997; Wada et al. 2008). The differentiation of T cells occurs while the thymocytes are migrating through the cortical and medullary regions of the thymus (Figure 1). During migration through the cortex the thymocytes are characterized into different developmental stages based on their expression of extracellular and intracellular markers. The early maturation stages are defined mainly by the expression of KIT (CD117) and CD24 and later CD44 and CD25: CD44^{+}CD25^{-} (DN1), CD44^{+}CD25^{+} (DN2), CD44^{-}CD25^{+} (DN3) and CD44^{-}CD25^{-} (DN4). At the DN3 stage, T cells differentiate into either αβ T cells or γδ T cells (Taghon et al. 2006).

During the DN3 stage the pre-T cell receptor (pre-TCR) β-chain is rearranged with V(D)J recombination by joining the invariant D and J chain segments with the variable V chain. The recombination maximizes the T cell receptor (TCR) repertoire and increases the probability of antigen recognition. This process is called "β-selection", in which the rearranged β-chain is combined with the invariant α-chain in the pre-TCR. The early pre-TCR is then tested for the ability to interact with the MHC molecule presented by the cortical thymic epithelial cells (cTECs) (Hoffman et al. 1996; Dudley et al. 2005). DN3 cells express T cell specific genes like the Recombination activation genes 1 and 2 (Rag1 and Rag2) which encode for endonucleases that are active during the V(D)J recombination of the TCRβ chain as well as genes for CD3ε and Lck molecules (David-Fung et al. 2006). Collectively, these molecules are part of the T cell receptor which plays a critical role in the process of positive thymocyte selection. If the V(D)J recombination of the α and β chains is unsuccessful and TCR signaling fails, the thymocyte is eliminated by apoptosis that might involve p53. The involvement of p53 in the apoptosis of thymocytes during development is described further in the next section. Successful recombination, on the other hand, results in proper TCR signaling that mediates transition of the DN cells into the next maturation step (Jiang et al. 1996; Haks et al. 1999). Before the thymocytes fully gain CD4$^+$CD8$^+$ double positive (DP) expression, they undergo an intermediate stage of differentiation called immature-single positive (ISP) and transiently express CD4$^-$CD8^{lo} (Paterson and Williams 1987; Rothenberg and Taghon 2005). The commitment to the CD4$^+$ or CD8$^+$ lineage has been shown to be influenced by the Lck activity strength in the interaction with the MHC complex. High Lck activity gives stronger interaction with MHC, thus stronger TCR signal which mediates the differentiation into CD4$^+$

T cells whereas lower Lck activity will give rise to CD8^{+} T cells (Hernandez-Hoyos et al. 2000).

After passing positive selection, DP thymocytes migrate towards the medullary parts of the thymus. The migration of the DP thymocytes is directed by chemokines as for instance CCR7, binding to receptors expressed on medullary thymic epithelial cells (mTECs) (Takahama 2006; Nitta et al. 2008). In the medulla, thymocytes that interact with high-affinity to self-derived peptides are usually deleted by apoptosis in a process defined as the negative selection (Sebzda et al. 1999). Self-antigens are presented by the mTECs and dendritic cells in the medulla where they can provide the right co-stimulation by expression of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) which are necessary for optimal negative selection (Page et al. 1993; Nitta et al. 2008). Finally, the naïve CD4^{+} or CD8^{+} single-positive T cells migrate into the periphery. Ultimately, only ~1% of thymocytes survive the maturation and migrate into the periphery (Berzins et al. 1999).
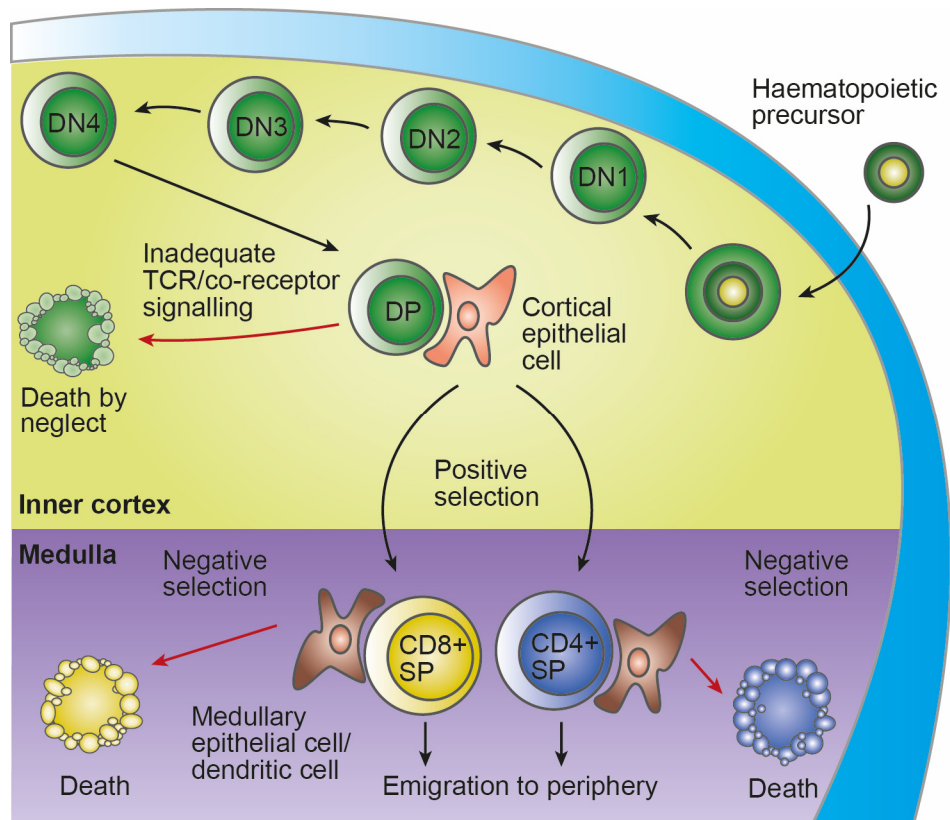
Figure 1. T cell maturation in the thymus. As the thymocytes migrate through the thymic cortex they go through early DN1-DN4 maturation stages characterized mainly by the sequential expression of CD25 and CD44. The re-arrangement of the β-chain of the pre-TCR initiates between the DN3 to DN4 stage. Upon successful re-arrangement of the β– and α-chains, the TCR is tested for the ability to recognize the MHC-peptide complex presented by the cTECs in a process defined as positive selection. At this developmental stage the thymocyte is double positive and express both CD4^{+} and CD8^{+} co-receptor molecules. As the DP thymocytes pass positive selection and migrate towards the medullary regions of the thymus they commit to either CD4^{+} or CD8^{+} T cell lineage. In the medullary regions the thymocytes go through the process of negative selection where they are eliminated by apoptosis if the affinity to self-antigens presented by the mTECs/DCs is too high. Only ~1% of the thymocytes pass both positive and negative selection and can migrate into the periphery as mature naïve T cells.

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Cell cycle checkpoint and apoptosis during thymocyte selection

The TCR chain re-arrangement involves V(D)J recombination and is tightly linked with the non-homologous end joining (NHEJ) machinery. The NHEJ machinery is ligating the double strand breaks (DSB) during the recombination to avoid abnormal joining and chromosomal translocations (Lieber 2010). Following the V(D)J recombination in the β-selection, the cell cycle is arrested at a checkpoint to minimize the chance of proliferation in case of abnormal V(D)J recombination (Lin and Desiderio 1995). A role for the tumor suppressor protein p53 is suggested in the regulation of this cell cycle progression and apoptosis (Figure 2) (Guidos et al. 1996; Jiang et al. 1996; Haks et al. 1999; Costello et al. 2000). In contrast, it has also been shown that V(D)J recombination *in vivo* does not induce p53 expression in wild-type mice and that $p53^{-/-}$ mice have normal T cell development (Donehower et al. 1992; Guidos et al. 1996). In addition, Haks *et al.* showed that cell cycle arrest can be overcome. In $CD3\gamma^{-/-}$ mice the thymocytes can proceed with maturation even in the absence of functional pre-TCR receptor signaling (Haks et al. 1999). On the other hand, the cell cycle checkpoint seem to involve p53 activation in the immunodeficient SCID mice (Guidos et al. 1996). SCID mice have previously been shown to lack functional TCR and B cell receptors (Bosma and Carroll 1991). More recently, Haines *et al.* demonstrated that blocking of lymphocyte development at the cell cycle checkpoints where RAG activity is present could provoke lymphogenesis in a genetic background of mice with deficient DNA damage response (Haines et al. 2006).

Additionally, p53 was shown to be involved in apoptosis induction during negative selection (Figure 2) (Quaglino and Ronchetti 2001). Also, Zhu *et al.* demonstrated the involvement of E2F1 regulation of ARF and p53 in the

negative selection of thymocytes (Zhu et al. 1999). However, thymocyte apoptosis during negative selection have also been shown to involve the mechanism of Fas-FasL interaction (Kishimoto et al. 1998). Also, Fas independent mechanism of apoptosis of semi-mature thymocytes has been shown involving Bim (Bouillet et al. 2002; Villunger et al. 2004).
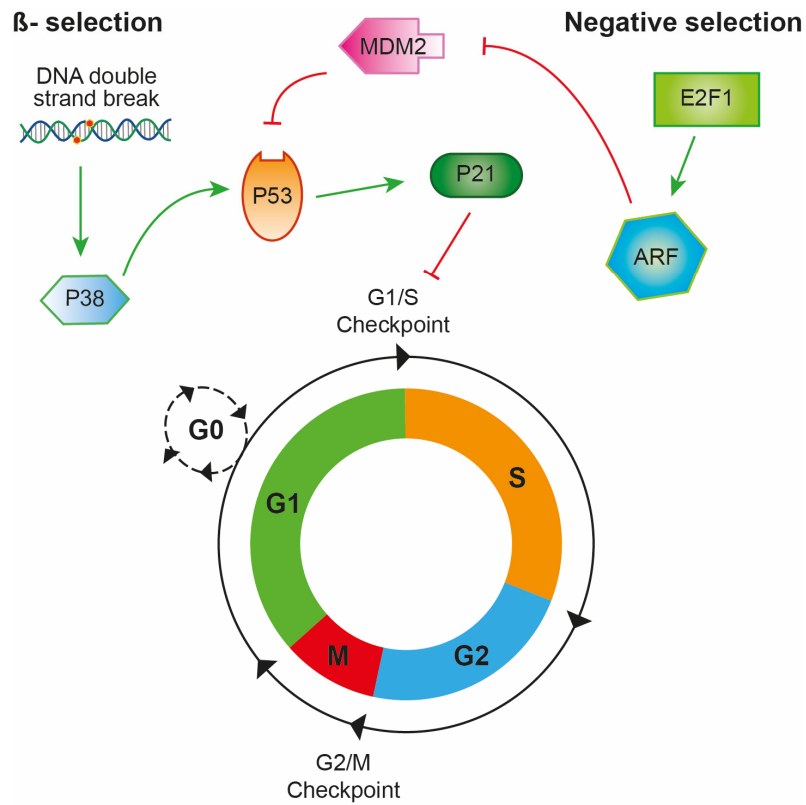
Figure 2. Illustration of the proposed cell cycle progression and G1 checkpoint during β-selection respectively negative selection in thymocyte development involving p53. Depending on different signaling involving p53 this can lead to cell cycle arrest and apoptosis of the thymocyte.

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Nevertheless, changes in p53 levels can be involved in determining the outcome of apoptotic signals during specific stages of thymic development which can influence the susceptibility of thymocytes to apoptosis. In Study I, we tried to elucidate the control mechanism of p53-dependent DNA damage response in the presence of DNA strand breaks after V(D)J recombination as well as during negative selection in non-obese diabetic (NOD) mice.

T cell activation

The T cells scan for antigens that are presented by the DCs in the lymph nodes, matching to their TCR specificity. When a fitting antigen is presented to the T cell an immunological synapse (IS) or contact is formed between the T cell and the APC. This mechanism is responsible for activation of T cells. The IS is divided into the central supramolecular activation cluster (cSMAC) and peripheral supramolecular activation cluster (pSMAC). The cSMAC includes the TCR, co-receptor CD4/CD8, co-stimulatory molecule CD28 and cytotoxic T lymphocyte activated antigen 4 (CTLA-4) (Figure 3) (Chen and Flies 2013). In this cluster, the CTLA-4 molecule is a negative regulator of T cell activation and is up-regulated upon T cell activation and leads to reduced T cell proliferation by inhibition of IL-2 transcription and subsequent cell cycle arrest through inhibition of cyclin D3 and cyclin dependent kinases (Brunner et al. 1999). Moreover, the pSMAC includes other co-stimulatory molecules such as CD2 that interacts with CD48/59 expressed on APCs and adhesion molecules like LFA-1 that interacts with ICAM-1 on the APC (Chen and Flies 2013). T cell activation engages the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR-associated CD3 chains by Fyn and CD4/CD8 co-receptor by Lck. As a consequence, the tyrosine kinase ZAP-70 is

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activated and in turn is phosphorylating several adaptor proteins including linker for activation of cells (LAT). The adaptor proteins mediate activation of phospholipase C (PLC)-γ that generates diacylglycerol (DAG) and inositol-tri-phospate (IP3) activate the downstream protein kinase C (PKC). Engaged PKC through various signaling pathways ultimately generates elevated cytosolic Ca^{2+} influx and activation of transcription of transcription factors including NF-κB, NF-AT and AP-1 (Kane et al. 2001; Srikanth and Gwack 2013; Gerondakis et al. 2014). These transcription factors in turn induce different effector molecules that cause T cell differentiation with effector functions as well as IL-2 production necessary for T cell proliferation and expansion.
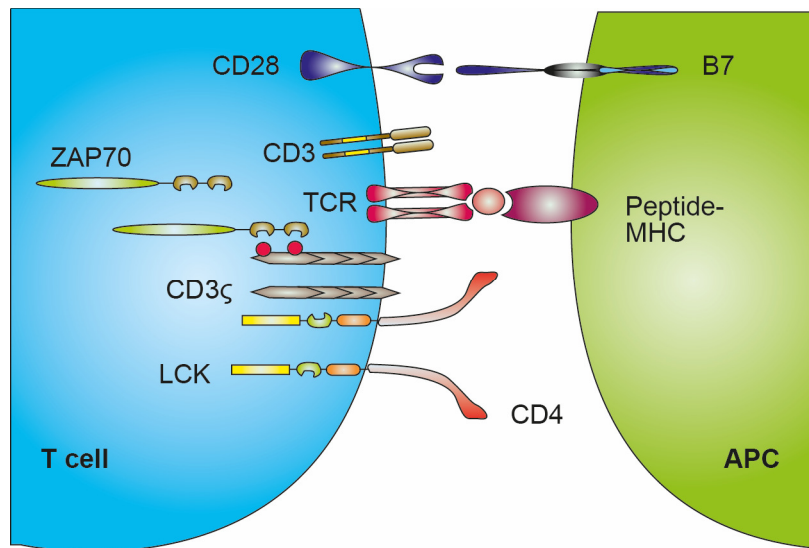
Figure 3. The TCR interaction with an appropriate MHC-peptide complex leads to T cell activation initiated by formation of the immunological synapse (IS) which is a contact area between the T cell and the APC. At the stage of T cell activation the TCR-associated CD3 chains is phosphorylated by the tyrosine kinase Lck. CD28 interaction with B7.1 or 2 provides a necessary co-stimulatory signal. ZAP-70 becomes activated by phosphorylation that leads to recruitment of the adaptor protein LAT. LAT then induces a downstream signaling cascade that ultimately activates the nuclear transcription factors NF-κB, NF-AT and AP-1.

Tolerance versus autoimmunity

Central tolerance is established by eliminating potentially self-reactive T cells that TCR binds to with high affinity to self-antigens during thymocyte development. However, all potentially auto-reactive T cells are not deleted because all self-antigens are not present in the thymus. Therefore, mechanisms exists in the periphery to maintain peripheral tolerance. This include induction of T cell anergy, T cell deletion and T cell suppression. Tregs are responsible for the suppression of self-reactive T cells by several mechanisms. For example, anergy is characterized by the total lack of T cell response and can be induced by the absence of co-stimulation from B7 family molecules on the

APC cell at T cell activation and by CTLA-4 up-regulation (Rudd et al. 2009). Tregs have been shown to suppress T cell responses by inducing down-regulation of co-stimulatory molecules and IL-2 production by APC's through direct cell-cell mediated interaction (Cederbom et al. 2000; Vendetti et al. 2000). CTLA-4 which is constituently expressed on Tregs may be also involved in suppression of T cell function (Read et al. 2000; Takahashi et al. 2000). For instance, Tregs have been described to suppress tryptophan catabolism in DCs in CTLA-4 mediated fashion and direct cell-cell interaction with B7 family molecules (Fallarino et al. 2003). The enzyme tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) that catabolizes tryptophan in DCs, has been shown to be involved in suppression of antigen-driven proliferation of T cells (Munn et al. 2004). Alternatively, CTLA-4 could also directly give a negative proliferation signal of T cell responses through interaction with the B7 family molecules (Paust et al. 2004).

Direct T cell deletion is achieved through Fas-FasL interaction which initiates intrinsic apoptosis program of the cell including downstream caspase cascade activation (Russell and Ley 2002). Similarly, Tregs could potentially be used to induce apoptosis of effector T cells by using FasL. For instance, Tregs primed with FasL administrated to mice with chronic colitis, were shown to severely reduce the pathogenic burden of the animals (Kaminitz et al. 2013).

Tregs have also been shown to suppress both T cell responses and innate immune cells through secretion of IL-10 and TGF-β. The anti-inflammatory functions of IL-10 include inhibition of Th1 activation and reduction of cytokine secretion from effector T cells and additionally inhibiting antigen-induced proliferation by down-regulation of co-stimulatory molecules on APCs (Letterio and Roberts 1998; Moore et al. 2001; Maloy et al. 2003). The IL-10 and TGF-β immunosuppressive mechanisms are

particularly crucial for maintaining intestinal tolerance and inhibiting development of intestinal inflammation (Asseman et al. 1999). Further description of this subject is outlined in the "Gut immune system" section.

Defects in the mechanisms responsible for maintenance of central and peripheral tolerance, as described above, leads to breakdown of tolerance which results in autoimmune diseases like T1D and inflammatory bowel disease (IBD).

Autoimmune diabetes (type 1 diabetes)

Autoimmune diabetes, also known as T1D is characterized by insulin deficiency due to β-cell destruction caused by immune cells infiltrating the pancreatic islets. The loss of β-cell mass results in elimination of insulin production and consequently impaired uptake of glucose to the cells which give rise to systemic hyperglycemia and ketoacidosis. As the result of failure of insulin production, T1D leads to a lifelong insulin dependency. Until 1920's T1D was untreatable and a therefore considered as a fatal disease. The scientists Frederick Banting, Charles Best and John Macleod changed the paradigm by successfully treating a diabetic dog with pancreatic extract from a healthy dog. They also managed to treat the first human, a boy dying from diabetes, by injecting a more refined extract of insulin (Banting et al. 1922; King and Rubin 2003). A better understanding of the disease causing mechanism of came with the discovery of autoantibodies in 1974 which suggested that T1D was an autoimmune disease (Bottazzo et al. 1974). To date, the major autoantibodies against β-cell antigens target insulin (IAA), glutamic carboxylase acid 65 (GADA), insulinoma-associated protein 2 (IA-2A) and Zinc transporter 8 (ZnT8A) (Atkinson et al. 2014).

The triggers that initiate the autoimmune attack on the β-cells by the T cells and other mononuclear cells to date remains elusive. However, it is believed that potentially

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autoreactive T cells that escaped clonal deletion are activated by DCs presenting β-cell antigens which they encounter in pancreatic islets during very early insulitis. Alternatively, T cells may be activated by β-cell antigens that are released after a virus infection from damaged β-cells as a result of the inflammatory response.

Epidemiologically, the highest prevalence of T1D is observed particularly in Finland, but also in the other Scandinavian countries and Sardinia, whereas the disease prevalence is less frequent in Asia, Southern Europe and Middle East (Tuomilehto 2013). Although, there is a strong genetic basis of the disease, the incidence of T1D is increasing globally with approximately 2.8 % each year (Group 2006). Thus, triggering environmental factors in addition to genetic factors are important for the initiation of T1D.

Pathogenesis of T1D

Histological similarities but also differences of T1D have been observed between humans and mice. For example, diabetogenic $CD8^+$ T cells, $CD4^+$ T cells, monocytes and B cells have been observed in pancreatic islets of humans and mice during insulitis (Wong et al. 1999; Willcox et al. 2009). However, peri-islet insulitis and the massive infiltration of pancreatic islets in NOD mice have in contrast not been found in humans in which insulitis has only been observed in <10 % of islets (In't Veld 2014).

Animal studies show that the inflammatory process in the pancreatic islets during early insulitis is driven by diabetogenic T cells and mononuclear cells. Diabetogenic T cells primarily induce β-cell death through the Fas-dependent mechanism, as described earlier. In addition, T effector cells secrete IFN-γ and tumor necrosis factor (TNF-α) (Varanasi et al. 2012) and monocytes secrete pro-inflammatory cytokines including IL-1β and TNF-α (El-Sheikh et al. 1999; Calderon et al. 2008). These cytokines are

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responsible for both maturation and activation of immune cells and attraction of more immune cells to the pancreatic islets (Bergholdt et al. 2004; Cantor and Haskins 2007; Schoenborn and Wilson 2007; Ben-Sasson et al. 2009). The released pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ activate several intrinsic apoptosis pathways in the β-cells that induce cell death (Eizirik and Mandrup-Poulsen 2001; Gurzov et al. 2009). Further, in response to secreted IL-1β and TNF-α, activation of the NF-κB pathway results in the expression of genes of several chemokines and inducible nitric oxide synthase (iNOS) (Kurrer et al. 1997; Mandrup-Poulsen 2003; Ortis et al. 2008; Quintana-Lopez et al. 2013). Moreover, β-cell exposure to TNF-α and IFN-γ induce apoptosis through the activation of the transcription factor STAT-1 and pro-apoptotic protein Bim (Barthson et al. 2011).

In short, the β-cell inflammatory response is actively involved in the pathogenesis of T1D and ultimately resulting in the loss of β-cell mass. The decrease in β-cell mass due to β-cell death in turn causes insulin deficiency and chronic hyperglycemia.

Genetics of T1D

The strongest genetic association with T1D susceptibility is found in the human leukocyte gene (HLA) class II and in NOD the mouse analogue I-A^{g7}, which encodes the MHC II molecule. In humans the HLA genes account for approximately 40-50 % of the T1D risk (Noble and Valdes 2011). The HLA class II gene is situated on chromosome 6p21 locus and has several susceptibility regions. The DR3-DQ2 and DR4-DQ8 regions confer the highest T1D risk and are present in 90 % of Caucasian T1D patients (Kockum et al. 1999; Devendra et al. 2004).

Also, genetic variation in non-HLA genetic susceptibility genes is associated with human T1D. For instance, the variable number tandem repeats (VNTRs) upstream of

the insulin gene (INS) which are located on chromosome 11p15.5 is a known genetic susceptibility region (Undlien et al. 1995). The PTPN22 gene is a second non-HLA susceptibility gene for T1D (Smyth et al. 2004). PTPN22 gene is located on chromosome 1p13 and is encoding the lymphoid tyrosine phosphatase LYP that is involved in down-regulation of T cell activation. Also, the CTLA-4 gene (located on chromosome 2q33) has also been associated to T1D (Nistico et al. 1996). In addition, in the NOD mouse CTLA-4 is suggested to be one of the candidate susceptibility genes (located in Idd5 on chromosome 1). Moreover, CTLA-4$^{-/-}$ mouse lymphocytes are similarly to NOD lymphocytes resistant to γ-irradiation induced apoptosis (Bergman et al. 2001). Apoptosis resistance of NOD lymphocytes is further described in the section "Non-obese diabetic mouse (NOD)".

Environmental factors in T1D and inflammation

Although the genetic factors are established to be the strongest contributor to increased T1D risk, it is not clear how the disease is initiated. Environmental factors that have been considered in triggering T1D include for instance virus infections by enteroviruses, cytomegaloviruses, rotaviruses and herpesviruses. Viruses with the strongest association to T1D risk are enteroviruses. The probability of finding enteroviruses is 10 times higher in T1D patients than in healthy individuals (Yeung et al. 2011). The presence of Coxackie virus B1 has been associated with two or more autoantibiodies found in the serum of children that were progressing towards T1D (Lonnrot et al. 2000; Laitinen et al. 2014). Recently, Ferreira *et al.* and Kallionpää *et al.* reported an increased innate IFN-γ expression pattern that are associated with virus infections in children with genetic predisposition prior to progression to T1D (Ferreira et al. 2014; Kallionpaa et al. 2014). Although, virus infections notably seem to trigger

β-cell autoimmunity in some T1D susceptible individuals, only a low percentage develop the disease considering the high frequency of virus infections.

Other environmental factors which could influence the development of T1D are infant and maternal diet as well as intestinal microbiota (Vaarala 2004; Norris 2010; Vaarala 2013). For instance, hydrolyzed casein in the diet increased plasma insulin concentration and insulin content in islets as well as reduce L-glutamine oxidation in both islets and mesenteric lymph nodes (MLNs) resulting in decreased diabetes incidence in diabetes prone BB rats (BBdp) (Malaisse et al. 2000; Scott et al. 2000). A certain type of wheat protein diet has on the other hand been shown to accelerate diabetes incidence in BBdp rats (Chakir et al. 2005). In children with genetic risk for T1D, a recent association study could not show any difference in risk to develop β-cell autoimmunity after use of hydrolyzed casein formula and autoantibodies (Knip et al. 2014). Another dietary factor that is associated with increased risk of T1D in humans is low intake of vitamin D (Littorin et al. 2006). In NOD mice the administration of oral vitamin D has been shown to protect NOD mice from T1D. More specifically, the intake of 50 ng of vitamin D per day prevented the onset of T1D through 200 days of age (Zella et al. 2003). Supplementation of vitamin D in humans was observed to benefit T1D patients with vitamin D deficiency and intake of vitamin D combined with calcium resulted in improved glycemic control (Aljabri et al. 2010). In short, the dietary effects of vitamin D seem to benefit both NOD mouse and human with T1D.

Dietary components have the ability to modulate the gut microbiota. The connection of the intestinal microbiota to development of autoimmune disease as well as inflammatory bowel disease is addressed in the next couple of sections.

Commensal gut microbiota

The gut microbiota is largely heterogeneous and consists of anaerobic, aerobic and facultative aerobic bacteria with increasing proportion of anaerobic bacteria in the distal areas of the intestine. The dominating phyla in the colon are the Firmicutes (including genera of *Clostridium, Enterococcus, Lactobacillus* and *Lactococcus*) and Bacteroidetes with mainly *Bacteroides* species (Hold et al. 2002; Eckburg et al. 2005). The minorities (approximately 10 %) of the colonic microbiota include *Bifidobacterium, Enterobacter* and *Fusobacterium* genera (Backhed et al. 2005; Eckburg et al. 2005). Although there are variations of bacterial strains in different individuals and also between family members, the overall phylotypes are conserved (Turnbaugh et al. 2009), which suggests similar metabolic function. The symbiosis between the gut microbiota and the host gives the benefit of maximizing extraction of dietary energy by fermentation of dietary carbohydrates, vitamin synthesis (K, B12, biotin, folic acid) and amino acid synthesis (Hooper et al. 2002). Another advantage of the gut microbiota is their ability to act as a protective barrier against pathogenic bacteria which is further described in the "Gut immune system" section. In addition, the gut microbiota is necessary for proper development of the immune system, demonstrated by the fact that mice kept in germ-free (GF) conditions have underdeveloped gut-associated immune tissues, lower amount of $CD8^+$ intraepithelial T cells with impaired function and fewer plasma cells (Round and Mazmanian 2009). This indicates that the maternal gut microbiota is important for development of the fetal immune system as well as early priming against commensal microbes.

Previously it has been shown that already after 48h after birth the gastro-intestinal tract of a child is colonized with strains of *Lactobacillus, Bifidobacterium, Bacteroides fragilis, Enterococcus and Enterobacteriaceae* (Karlsson et al. 2011). The maternal, gut microbiota, vaginal flora and skin are important sources but also hospital environment

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can be a bacterial source (Mackie et al. 1999; Favier et al. 2002; Fanaro et al. 2003). Recently, evidence points out that transplacental transmission of the microbiota of mothers starts already during pregnancy (Satokari et al. 2009) and also postnatally with the mothers' milk in both humans (Martin et al. 2003) and mice (Zhou et al. 2000). There are even new evidence that the placenta itself harbors microbiota similar to the mother's oral microbial pattern (Aagaard et al. 2014). Therefore in Study III, we investigated whether derangement of the maternal gut microbiota during pregnancy could impact on the development of the offspring's gut microbial pattern, gut immune system and impact the incidence of autoimmune diabetes.

Gut microbiota and T1D

In the state of disease the gut microbial pattern is often altered compared to healthy individuals. For example, it has been observed that children progressing towards T1D had decreased proportion of Firmicutes which was accompanied by an increase in Bacteroidetes phyla (Giongo et al. 2011). Other observations show that *Bacteroides*, *Clostridium* and *Veillonella* genera were more abundant in children with β-cell autoimmunity with lower abundance of the *Bifidobacterium*, *Lactobacillus* and *Prevotella* genera's compared to healthy children (de Goffau et al. 2013; Murri et al. 2013). In the NOD mouse, it has been shown that the gut microbiota has the potential to modulate diabetes development (King and Sarvetnick 2011; Kriegel et al. 2011). In the same line, NOD mice bred in germ-free conditions displayed higher incidence of T1D or increased insulitis compared to specific pathogen-free (SPF) conditions (Pozzilli et al. 1993; Alam et al. 2011; Tlaskalova-Hogenova et al. 2011). In addition, modulation of the gut microbiota by the use of antibiotics has been shown to alter diabetes incidence in both NOD mice (Wen et al. 2008; King and Sarvetnick 2011;

Kriegel et al. 2011) and BB rat models of type 1 diabetes (Brugman et al. 2006; Roesch et al. 2009).

The gut immune system

The intestines are among the largest areas of contact of the body to external antigens (both dietary and potential pathogens) and not surprisingly the largest amount of immune cells can be found in the gut-associated lymphoid tissues (GALT), (Figure 4.) The GALT mainly consists of the Peyer's patches, MLN (that drain the intestines more distally), isolated lymphoid follicles and the lymphocyte populations in the connective tissue of the intestine, the lamina propria (LP) (Izcue et al. 2009). The Peyer's patches have the anatomical structure of a lymph node with B cell and T cell areas and the development of the Peyer's patches in intestine is not dependent on the gut microbiota (Mowat 2003). In contrast, the number of isolated lymphoid follicles could be triggered by intestinal microbiota and the number of follicles increases during chronic inflammation (Lorenz et al. 2003).

The gut microbiota is actively involved in the gut-immune responses to maintain the immunological balance in the GI tract. The commensal gut microbiota can actively defend the intestinal epithelial cells (IEC) against pathogenic bacteria by inducing the secretion of antimicrobial peptides like α-defensins and lysozyme from a type of epithelial cells, the Paneth cells (Ayabe et al. 2000). Another type of epithelial cells, the Goblet cells, secrete mucin with high quantities of glycosylated proteins that forms a gel-like mucus layer on the epithelial surface that limits the contact with the intestinal bacteria (Hansson and Johansson 2010).
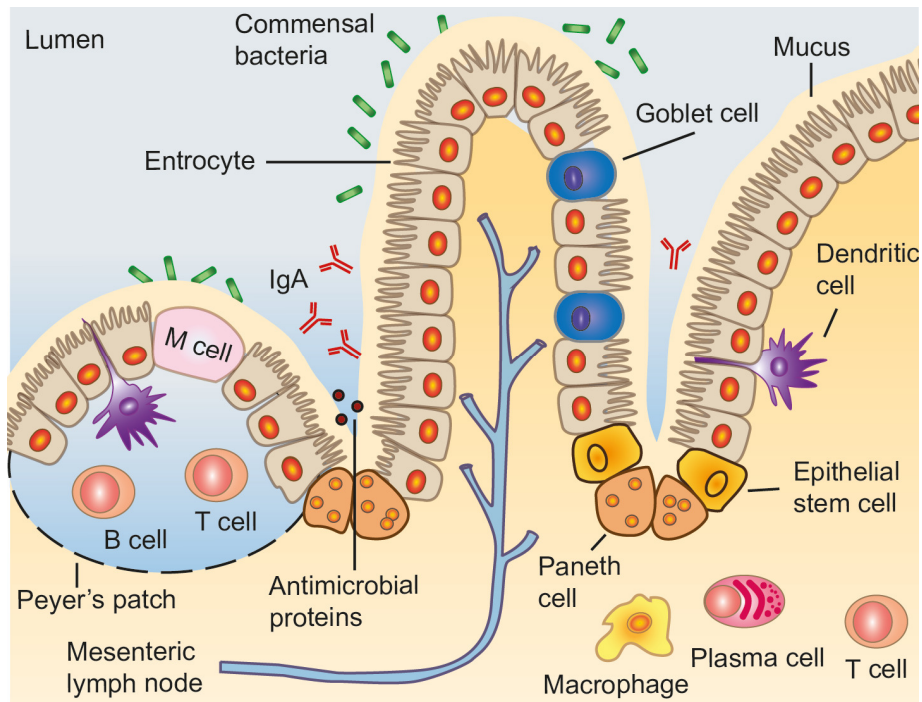
Figure 4. The figure shows the intestinal architecture and the gut-associated lymphoid tissues (GALT), the Peyer's patches and mesenteric lymph nodes (MLN). The monolayer of several intestinal epithelial cells connected with tight junctions form the architecture of the villi and crypts. The Goblet cells secrete mucus that covers the epithelial cell layer to limit the contact with the bacteria in the lumen. The Paneth cells secrete antimicrobial peptides which adds to another line of defense to the intestinal barrier together with the IgA antibodies secreted from differentiated B cells, the plasma cells. Peyer's patches (located to the left in the illustration) have distinct B cell and T cell areas and specialized type of cells towards the lumen called microfold (M) cells. The M cells have the ability to catch and transmit soluble antigens and bacteria to the Peyer's patch where they can either be processed by dendritic cells or ingested by macrophages (Mϕ). DC, Mϕ and intraepithelial T cells (IEL) populations are also present within the epithelial cell layer and in the lamina propria (LP), situated between the epithelium and the deeper submucosa. The DC have the ability to stretch between the tight junctions of the epithelial cells and collect soluble antigens directly from the lumen.

Innate immune responses to limit intestinal barrier penetration by bacteria can be a MyD88-dependent TLR activation that leads to the production of antimicrobial peptides (Vaishnava et al. 2008). Moreover, TLR2 signaling has also shown to enhance tight junctions between the IEC and contribute to an even more restricted environment

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against bacterial penetration (Gibson et al. 2008). Another mechanism by which commensal bacteria, particularly SFB, limit bacterial expansion is through the induction of IgA via the adaptive immune response. (Suzuki et al. 2004). Secreted IgA could be involved in trapping the bacteria in the mucus layer (Fagarasan and Honjo 2003). SFB in LP and small intestine have also been demonstrated to induce the expansion of Th17 cells leading to protection against *Citerobacter rodentium* infection (Ivanov et al. 2009). In addition, the recruited Th17 cells from secondary lymphoid organs to Peyer's patches have recently shown to also be involved in induction of IgA response (Hirota et al. 2013). However, Th17 cells are also involved in the chronic inflammation and development of IBD in the colon by mediating recruitment of neutrophils (Honda and Takeda 2009). Also, Th17 differentiation could further the disease progression by inhibiting Treg differentiation (Izcue et al. 2008). Thus, the Th17 cells in the gut environment is complex and demonstrated to have a dual role in disease protection and amplification.

With respect to the symbiosis of commensal bacteria, there is a mechanism to mediate T cell tolerance towards commensal gut microbiota and in the intestine. This mechanism is controlled by Foxp3^{+} Tregs or IL-10/TGF-β producing iTreg cells. The production of IL-10 prevents the activation of Mφ that inhibits the recruitment of T effector cells to the intestine (Asseman et al. 1999; Powrie 2004). In addition, intraepithelial γδ T cells have been demonstrated to induce and maintain oral tolerance by secretion of IL-10 (Kapp et al. 2004; Yue et al. 2013). Breakdown of tolerance in the intestines causes intestinal inflammation because the immune system overreacts to commensal bacterial antigens in genetically susceptible individuals or fail to down-regulate immune response. Inflammation reduces intestinal mucus layer which allows for more contact of the bacteria to the intestinal epithelial cells and increases interaction

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with the immune system. Chronic intestinal inflammation ultimately manifests in ulcerative colitis (UC), an inflammatory bowel disease.

Ulcerative Colitis (UC)

Ulcerative colitis and Crohn's disease are two major forms of IBD. The clinical symptoms include diarrhea, rectal bleeding, weight loss and abdominal pain (Podolsky 2002). The development of UC is influenced by multiple factors including genetic susceptibility and environmental factors like intestinal microbiota and the relationship of the gut microbiota and the immune system. Genetic susceptibility regions associated with UC are *IL-10* and *ARPC2* regulatory pathways as well as the known T1D susceptibility regions *HLA/MHC* and PTPN2 and PTPN22 (Kaser et al. 2010). Although it is not known whether altered gut microbiota is a causative factor of UC, it has been shown that the intestinal microbiota in mucosa of UC patients contains a high proportion of *Pseudomonas aeruginosa, Bacteroides fragilis and Clostridium difficile* while strains of *Lactobacillus* decrease during active colitis (Fabia et al. 1993; Wang et al. 2007).

Colitis in animal models can be pathogen induced (*Citrobacter rodentium*), genetically induced through *IL-10* deletion or alternatively chemically induced with dextran sodium sulfate (DSS) (Kaser et al. 2010). The DSS *in vivo* model has widely been used since the 1990's because when administered orally it can both induce acute and chronic colitis in rodents and because that the histopathological characteristics are similar to human UC (Okayasu et al. 1990). The mechanism by which DSS induces colitis is to the present day elusive. However, recent studies show that DSS can form nanovesicles with medium-chain-length-fatty acids (MCFA) which can fuse with colonocyte membrane. Delivery of the DSS into the epithelial cells can negatively affect the function and activate inflammatory signaling pathways which ultimately leads to

increased intestinal permeability (Laroui et al. 2012). Interestingly, MFCAs are present at high concentrations in the colonic lumen (Schmidt et al. 2011) and salts of the MFCAs capric and lauric acids have been shown to increase rectal drug absorption, probably due the effects on the intestinal tight junction barrier function (Laroui et al. 2012). Increased colonic mucosal permeability also allows increased translocation of bacteria and endotoxin from the lumen which can both induce inflammatory reaction and impinge on the function of the epithelial cells (Kitajima et al. 1999). Increased TLR recognition as the result of translocation of bacteria and endotoxins mediates increased cytokine production by monocytes that further amplifies the inflammatory process (Cario 2005). Thus, chemical initiation of inflammation by DSS could trigger and exaggerate the biological events of inflammation.

The cells present in the ulcerative lesions in the intestinal mucosa during acute inflammation include lymphocytes, mast cells, Mϕ and neutrophils (Fiocchi 1997). In children with UC, an increase of Th-17 cells is observed in the colonic mucosa together with increased concentrations of the cytokines IL-6, IL-22 and IL-17 (Holtta et al. 2013). Interestingly, Hölttää *et al.* reported an increase in the number Foxp3^{+} Tregs and this finding is further discussed in Study IV. Previously, it was believed that the Th-2 cells were the main mediators of UC. However, at present it is well-known that Th-17 cells and the cytokines they secrete are also crucially involved in UC to initiate and enhance the inflammatory process (lvez 2014). The inflammatory Mϕ are well-known contributors to inflammation in IBDs. Another type of mediator in IBD are mast cells. In DSS-induced mouse model these cells release Mast cell protease 6, a tryptase that acts upstream of many factors implicated in IBD. The production of the enzyme after DSS treatment enhances pro-inflammatory cytokines and chemokines that specifically attract neutrophils and other extracellular matrix remodeling enzymes that partake in the DSS-mediated loss of epithelial cells. Thus, mast cells and their

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production of tryptase are also important inflammatory instigator of IBD (Hamilton et al. 2011).

microRNAs

MicroRNAs (miRNAs) belong to a class of small endogenously expressed non-coding RNAs (ncRNAs) and were first identified in 1993 by Lee *et al.* and Wightman *et al.*. They identified previously unknown RNA element regulating the developmental timing of *C. elegans*, the lin-4 and lin-14 (Lee et al. 1993; Wightman et al. 1993). In 1998, RNA interference (RNAi) was described by Fire *et al.* as the mechanism by which miRNAs regulate target mRNAs (Fire et al. 1998). After the discovery of the first miRNAs, in years 2000-2003, a set of papers was the big kick-off for the miRNA field showing that many miRNAs were not only expressed in *C. elegans* but in fact in all mammals and were very evolutionary conserved between species (Pasquinelli et al. 2000; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Lagos-Quintana et al. 2003). Today, there are around ~2000 identified miRNAs in the database miRbase (http://www.mirbase.org) and notably, it is estimated that miRNAs can regulate approximately 60 % of the human genome (Friedman et al. 2009).

The size of miRNAs is around ~22 nucleotides and they regulate gene expression by direct binding of the miRNA to the 3'UTR of a target messenger RNA (mRNA). MiRNA binding to target mRNA typically leads to degradation of the mRNA or hindering the translation of the mRNA to protein (He and Hannon 2004). One miRNA can have several mRNA targets and miRNAs are often transcribed in clusters that work in networks in fine-tuning the regulation of for example cellular processes such as proliferation, developmental timing, cell differentiation, apoptosis and immune function (Bartel 2009).
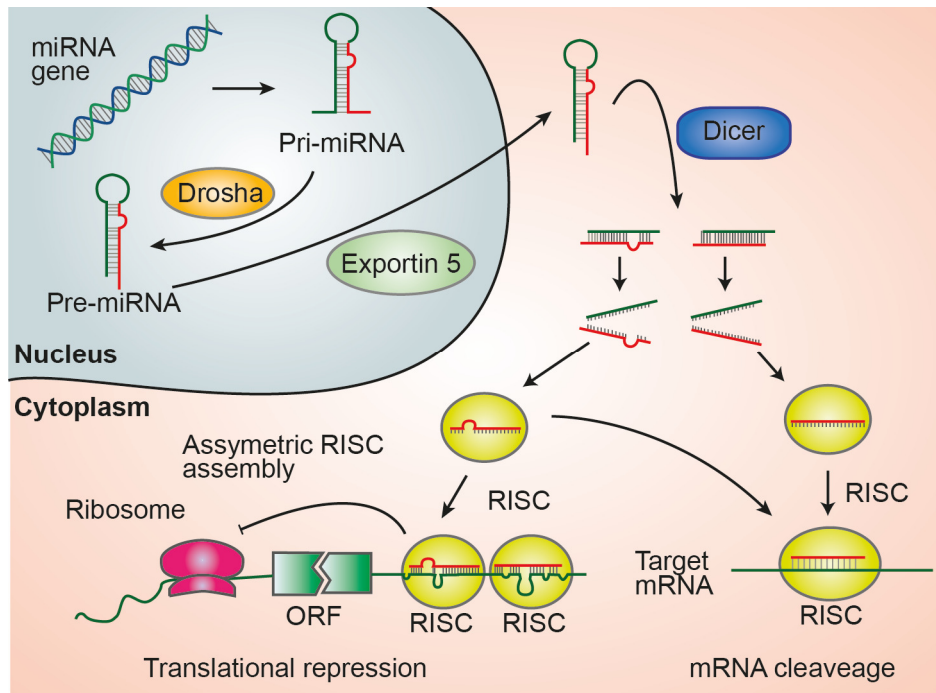
miRNA biogenesis and function

During the canonical miRNA biogenesis pathway the miRNA gene is transcribed in the nucleus of the cell and the pri-miRNA is cleaved by the Drosha/DGCR8 microprocessor complex into a ~70 nucleotide long pre-miRNA (Lee et al. 2003; Cullen 2004; Han et al. 2004). The hairpin-looped pre-miRNA is then transported to the cytoplasm by Exportin 5 (Lund et al. 2004) and further processed by RNAse III endonuclease Dicer to a single stranded mature miRNA (Tomari and Zamore 2005). At this stage, the mature miRNA is incorporated into the RISC protein complex which directs the binding of the "seed region" of the miRNA to the 3'UTR of the target mRNA (Figure 5). This process results in the destabilization and degradation of mRNA or directly hinder translation (Pasquinelli et al. 2005; Bartel 2009).

Moreover, evidence suggests that miRNAs could exert their inhibitory function by binding to the 5'UTR of target mRNAs (Lytle et al. 2007; Orom et al. 2008). In contrast, miRNAs are also able to increase translation of target mRNA. Ørom *et al.* showed that miR-10a increased translation of mRNA encoding ribosomal proteins and by increasing ribosomal biogenesis, the level of global protein synthesis was also raised (Orom et al. 2008). Furthermore a "decoy function" has been suggested for miRNA-328 which independently of the 3' UTR seed sequence competitively interacts with a RNA binding protein, that normally inhibits translation and thereby indirectly lead to up-regulation of translation (Eiring et al. 2010). Also, epigenetic regulation by miRNAs

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is indicated by direct binding to genomic DNA thus directing DNA methylation (Kim et al. 2008; Khraiwesh et al. 2010).

Figure 5. The miRNA gene is transcribed and the pri-miRNAs transcript is cleaved by the Drosha/DGCR8 microprocessor complex into a pre-miRNA that is exported to the cytoplasm by Exportin 5 and processed by Dicer into shorter mature miRNAs. The matured miRNA is then incorporated into the RISC protein complex that silence either translation of genes or induces the degradation of mRNAs.

In addition to the canonical miRNA biogenesis pathway, Dicer-independent miRNA biogenesis has been shown for miRNA-451 (Cheloufi et al. 2010; Cifuentes et al. 2010). The Argonaute2 (Ago 2) protein which is a member of the RISC complex contains a Piwi domain that acts as catalytic center for "slicer" activity that can cleave pre-miRNA transcripts (Cheloufi et al. 2010; Cifuentes et al. 2010). There are also Drosha-independent miRNAs transcribed from introns called the mirtrons. The mirtrons are Dicer dependent but bypasses Drosha processing by using RNA

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sliceosome activity for pre-miRNA cleavage (Okamura et al. 2007; Flynt et al. 2010). Lately, many other types of small non-coding RNAs have been discovered including tRNAs, piRNAs, qiRNAs, vault RNAs, lincRNAs etc. and their biogenesis include both Dicer and/or Drosha dependent and independent biogenesis pathways (Carthew and Sontheimer 2009; Lee et al. 2009; Cesana et al. 2011; Yang and Lai 2011).

miRNAs in β-cells and T1D

The absence of mature miRNAs after deletion of Dicer 1 demonstrates that canonical miRNA expression overall is necessary for normal development of the endocrine pancreas (Lynn et al. 2007) and for maintenance of β-cell mass as well as glucose homeostasis including insulin expression and insulin secretion (Morita et al. 2009; Kalis et al. 2011; Mandelbaum et al. 2012).

The miR-375 was among the first key miRNAs found to be highly abundant and important for the pancreatic β-cell function. It was shown that miR-375 regulates insulin secretion and maintenance of both β-and α-cell mass (Poy et al. 2004; El Ouaamari et al. 2008; Poy et al. 2009). The crucial role of miR-375 was also demonstrated during pancreatic islet development in which the absence of miR-375 resulted in malformation of the pancreatic endocrine (Kloosterman et al. 2007). The miRNAs miR-24, miR-26, miR-182 and miR-148 are involved in insulin synthesis by inhibiting transcriptional repressors consequently resulting in increased insulin promoter activity (Melkman-Zehavi et al. 2011). The regulation of insulin secretion also involves miRNAs. Namely miRNAs miR-124, miR-96, miR-130a, miR-132, miR-212 and miR-335 have been linked to the regulation of insulin exocytosis (Lovis et al. 2008; Esguerra et al. 2011).

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MiRNAs miR-21, miR-34, miR146a/b and miR-29 are pinpointed in the regulation of β-cells inflammatory response to pro-inflammatory cytokines in mice and humans, thus contributing to loss of β-cell mass during the progression of diabetes. For instance, miR-29 is involved in the down-regulation the anti-apoptotic protein Monocarboxylase transporter 1 (Mcl-1) that belongs to the Bcl-2 family (Roggli et al. 2010; Roggli et al. 2012). Recently, it was shown that a single nucleotide polymorphism (SNP) in the pre-miR-34a gene in human and mouse insulinoma cell lines, led to increased amount of miR-34a in islets causing increased β-cells apoptosis (Locke et al. 2013). Additionally, miR-21 has been shown to be up-regulated by NF-κB activation which by mediating suppression of programmed cell death 4 (PDCD-4) increases β-cell apoptosis through Bax (Ruan et al. 2011).

miRNAs in T cells and the immune system

The role of miRNA regulation in T cells has been investigated in various Dicer deletion models. Cobb *et al.* demonstrated by deleting Dicer during early thymocyte development (mediated by Lck or CD4 promoters) that canonical miRNAs are essential for the number of surviving αβ T cells but redundant for CD4^{+} and CD8^{+} lineage commitment (Cobb et al. 2005). While Dicer deletion from DP cortical thymocytes showed a moderate effect on CD4^{+} and CD8^{+} T cell numbers, the invariant NK T cell development was affected with largely diminished numbers (Fedeli et al. 2009).

Deletion of Dicer and DGCR8 in CD4^{+} T cells illustrated that miRNAs are also important for nTreg development and function (Cobb et al. 2006) as well as Foxp3 expression stability (Jeker et al. 2013). Cobb *et al.* showed that the miRNA expression profile in activated CD4^{+} T cells differed from the miRNA expression in regulatory T cells (Cobb et al. 2006). More recent evidence suggests that miRNAs expressed in

activated T cells mediate regulation of pathways that would be involved in inactivation of T cell activity (Grigoryev et al. 2011).

Moreover, Dicer has been shown to be crucial for CD8⁺ T cell proliferation, function and migration (Zhang and Bevan 2010). Zhang *et al.* found this to be a consequence of the inability of Dicer deficient cells to down-regulate CD69 after activation. CD69 down-regulation was then demonstrated to be regulated by miR-130/miR-301 cluster (Zhang and Bevan 2010). In CD8⁺ T cells, miR-17-92 gene cluster was up-regulated after T cell activation, but instead down-regulated in CD8⁺ memory T cells (Wu et al. 2012). Wu *et al.* demonstrated that on overexpression of miR-17-92 the CD8⁺ T cells are driven towards effector T cell function and the development of CD8⁺ memory T cells is impaired (Wu et al. 2012). In contrast, the miR-150, miR-155 and let-7 family need to be up-regulated in a differentiation window during development of CD8⁺ memory T cells (Almanza et al. 2010).

The miR-155 is essential miRNA in the regulation of overall immune system and is necessary for the immune function of B-cells, T cells, DCs (Tlaskalova-Hogenova et al.) and Tregs (Rodriguez et al. 2007; Thai et al. 2007; Vigorito et al. 2007; Lu et al. 2009) as well as macrophage inflammatory response (O'Connell et al. 2007; Tili et al. 2007). Another key miRNA, miR-181a, is expressed in high levels during T cell development and is involved in altering TCR signaling threshold (Li et al. 2007). Inhibition of miR-181a expression impaired both positive and negative selection. By changing the sensitivity of the TCR signaling during thymocyte maturation, inhibition of miR-181 was shown to lead to maturation of autoreactive CD4⁺ T cells (Ebert et al. 2009). Li *et al.* also demonstrated that miR-181a reduced expression of several phosphatases which negatively regulates TCR signaling e.g. DUSP5/6, SHP2 and PTPN22. In other words, miR-181a increases TCR signaling strength (Li et al. 2007). However, the deletion of miR-181a/b in early thymocyte development only reduced

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the overall numbers but did not change the percentages of TCRαβ cell compartment (Henao-Mejia et al. 2013). This indicates the complexity of miRNA networks and the role of miRNAs as fine-tuning regulators of gene expression.

Recently, miR-146a was shown to be up-regulated after T cell activation of both CD4^{+} and CD8^{+} cells in a NF-κB dependent manner and proposed to be involved in a negative feedback loop where miR-146 up-regulation, down-regulated NF-κB partially by TRAF6 and IRAK1 signaling (Yang et al. 2012). In addition Yang *et al.* found that T cells lacking miR-146 are hyperactive and caused systemic T cell mediated autoimmunity and chronic inflammation *in vivo*.

MiR-10a has been shown to be preferentially expressed in Tregs, although miR-10 (as well as previously mentioned miR-155) alone seems to be dispensable for Foxp3 induction in naïve T cells and overall Treg function (Jeker et al. 2012). This supports the notion of miRNA regulation by networks. In Study II, we investigated the role of the canonical miRNA network for early T cell development in NOD mice and the consequences for development of T1D.

miRNAs in cell cycle checkpoint control and apoptosis

Several lines of evidence suggesting that miRNAs are involved in the regulation of targets in the cell cycle check point and in apoptosis. The first miRNA gene families shown to regulate the p53 apoptosis pathway (implicated in G1 cell cycle checkpoint during thymocyte development) includes the miR-29 (miR-29a/b/c) and miR-34 (miR-34a/b/c) gene families (Tarasov et al. 2007). In addition, up- and down-regulation of genes by these two miRNA gene families are significantly overlapping with genes regulated in DNA damage response.

MiRNA-29 family genes were demonstrated to increase p53 activation in human cell lines through the direct suppression of two negative regulators of p53, the p85a subunit of phosphatidylinositol-3-kinase (PI3K) and CDC42 (a Rho family GTPase), involved in controlling cellular survival and apoptosis (Park et al. 2009). In addition, up-regulation of miR-29 was demonstrated in response to DNA damage by suppression of the protein phosphatase Ppm1 leading to stabilization of p53 (Ugalde et al. 2011).

Upon DNA damage the miR-34 gene family (miR-34a/b/c) up-regulates p53 in both human and mice. MiR-34-dependent up-regulation of p53 induces cell-cycle arrest and apoptosis by suppression of amongst others cyclin-E2, cyclin-dependent kinase 4 (CDK4), E2F3 and p53 downstream target b-cell lymphoma 2 (Bcl-2) genes (Bommer et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007).

MiRNAs being negatively regulated after DNA damage have also been reported. For instance, miR-155 and miR-125 that have been demonstrated to be down-regulated after DNA damage in a p53-dependent manner in limbs of *Drosophila melanogaster* (Gueta et al. 2010). Additionally, Le *et al.* reported that miR-125b is a negative regulator of p53 by directly suppressing p53 protein translation in human cell lines and zebra fish (Le et al. 2009).

In a human cancer cell line, the over-expression of miR-16 family genes resulted in G0/G1 cell cycle arrest by targeting CDK6 (Linsley et al. 2007). In addition, miR-15a and miR-16-1 have shown to directly regulate Bcl-2 mRNA in chronic lymphocytic leukemia (CLL) patients (Cimmino et al. 2005).

Additionally, inhibition of miR-17-92 in a lung cancer cell line decreased the suppression of E2F1 leading to induction of apoptosis (O'Donnell et al. 2005;

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Matsubara et al. 2007). E2F1 is an upstream target of p53 with a demonstrated role during negative selection in thymocyte development (Garcia et al. 2000).

It is highly likely that the expression pattern of a miRNAs network is regulating several targets in the G1 cell cycle checkpoint /p53 apoptosis pathways initiating cell cycle progression or apoptosis in thymocytes. In Study I, we are investigating the contribution of miR-29 (a/b/c), miR-34 (a/b/c), miR-125b, miR-155 and miR-203 to the apoptosis resistant phenotype of the NOD lymphocytes in response to DNA damage induced by γ-irradiation as a model for thymocyte apoptosis induction during development.

AIMS OF THIS THESIS

The general overall objective of this thesis was to investigate the regulatory role of miRNAs in autoimmunity, particularly in the development of T1D, as well as the influence of environmental factors like gut microbiota on intestinal inflammation and T1D.

Specific aims:

- To investigate whether miRNAs could be involved in the molecular mechanism of thymocyte apoptosis in NOD mice

- To study the overall regulatory role of miRNAs in T cells and their effects on the development of T1D in NOD mice

- To investigate the effect of antibiotics treated pregnant NOD mice on the immune system of the offspring, intestinal microbiota and development of T1D

- To study the interplay between the gut microbiota and the immune system in the development of colitis, an experimental model of IBD

METHODOLOGY

Animal models

Non-obese diabetic mouse (NOD)

Since the discovery in the 1980's by the Japanese group Makino *et al.* that the inbred NOD mouse strain spontaneously develops diabetes, it has been the most commonly used model of human T1D. Main reasons for this are the similarities of strong genetic MHC basis and pathogenesis including islet infiltration by mononuclear cells. The infiltration in the NOD mouse occurs at approximately 6-8 weeks of age leading to a progressive decrease of β-cell mass and development of diabetes and ketoacidosis from 12-17 weeks of age (Makino et al. 1980). The involvement of T cells in the initiation of T1D in the NOD mouse is very well studied. For example, it is known that both CD4^{+} and CD8^{+} T cells have been shown to be involved in the pathogenesis of T1D in NOD mice (Tisch and McDevitt 1996). Recently, γδ T cells (expressing CD27^{-}CD44^{hi} or CD27^{+}CD44^{lo}) have also been shown to infiltrate islets in pre-diabetic NOD mice and mediate diabetes development together with the αβ T cells. Interestingly, the combined effect of both cell types on disease development is more significant than the effect caused the αβ T cells alone. In addition, the γδ T cells were shown to be pre-programmed to secrete IL-17 or IFN-γ upon activation, which may be involved in contributing to the pathogenesis of T1D by increasing inflammation in the pancreatic islets. Moreover, γδ T cells isolated from spleen and pancreatic lymph nodes are reactivity against the insulin peptide (Zhang et al. 2010). In contrast, the γδ T cells have also been demonstrated to be involved in the protection against T1D by up-regulation of TGF-β production (Han et al. 2010).

Another advantage of the animal model is the high diabetes incidence of 60-80 % in NOD females (Makino et al. 1980). However, the sex-bias in the disease incidence in the NOD mouse differs compared to human T1D where the incidence is fairly similar in both sexes. The NOD mouse has similar genetic pre-disposition as humans with T1D susceptibility linked dominantly to the MHC class II, which is encoded by the I-A^{g7} gene in mouse and is equivalent to the HLA class II gene in human (Wicker et al. 1995). In addition, recessive genes have been mapped in more than 20 susceptibility regions- the Idd regions. However, new data suggests that NOD mouse has more association to T2D genes than T1D which are related to insulin resistance, vascular pathology and endoplasmic reticulum stress (Chaparro et al. 2006).

Some developmental defects affecting central tolerance of the NOD mouse have previously been highlighted. Yui *et al.* demonstrated that defective β-selection checkpoint control which allows further differentiation of NOD thymocytes in the absence of functional pre-TCR signaling (Yui and Rothenberg 2004). Further, Kishimoto *et al.* reported that defective thymic negative selection caused by decreased sensitivity of thymocytes to undergo apoptosis, involves both Fas-dependent and independent pathways (Kishimoto and Sprent 2001). Peripheral NOD lymphocytes have also been shown to be apoptosis resistant in response to treatment with apoptosis inducing agents (Colucci et al. 1996). These defects in apoptosis of NOD lymphocytes may allow escape of autoreactive T cells and contribute to the breakdown of central and peripheral tolerance in the NOD mouse and finally resulting in the pre-disposition to the development of autoimmune diabetes.

C57BL/6

The C57BL/6 (also referred to as B6) is one of the most commonly used inbred strains of wild-type mice. In Study I, where NOD lymphocyte apoptosis resistance in response

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to γ-irradiation was investigated, the C57BL/6 mice were used as control mice. In Study IV, DSS treatment was performed on C57BL/6 mice to induce intestinal inflammation. This genetic background is susceptible for induction of inflammatory pathology of ulcerative colitis with DSS (Melgar et al. 2005).

NOD.Lck-Cre *Dicer 1* $^{\Delta/\Delta}$ mice

In Study II, we used Dicer$^{flox/flox}$ mice and backcrossed for 11 generations with NOD mice to generate a NOD.DicerFlox mouse. Then Cre-Lox system was used to mediate deletion of Lck (involved in signaling of the T cell receptor) by the Cre recombinase. This generated a mouse with T cell specific Dicer 1 deletion and thus also the absence of mature canonical miRNAs in T cells from the DN4 stage of T cell development. The NOD.Lck-Cre *Dicer 1*$^{\Delta/\Delta}$ mouse was characterized in Study II.

Flow Cytometry

Fluorescence assisted cell sorting (FACS) is a method used in all our studies to *ex vivo* detect different cell T cell populations in thymus and peripheral lymph nodes based on the expression of cell surface markers, as well as intracellular markers like the transcription factor Foxp3, using fluorescently labeled antibodies. FACS was also used in Study I to assess the amount of apoptotic cells in thymus respectively pancreatic islets of different mouse strains, which is further described in the next section about apoptosis assays.

FACS is based on a system of lasers that hit a stream of single cells which flow in a suspension through the nozzle. The light is deflected by the cells into mirrors that directs specific wavelengths of light to detectors by filters (Figure 6). The forward scatter (FSC) is giving information about the size of the cell whereas side scatter gives

information about the granularity of the cell (Ortis et al.). The color detectors collect emitted light from the excited flurochromes on the antibodies used to detect specific cell markers. Different fluorochromes have different emission spectra and each marker can therefore be detected in separate channels. The computer software converts the information relayed by the detectors to digital information that can be visualized and analyzed in histograms and plots.
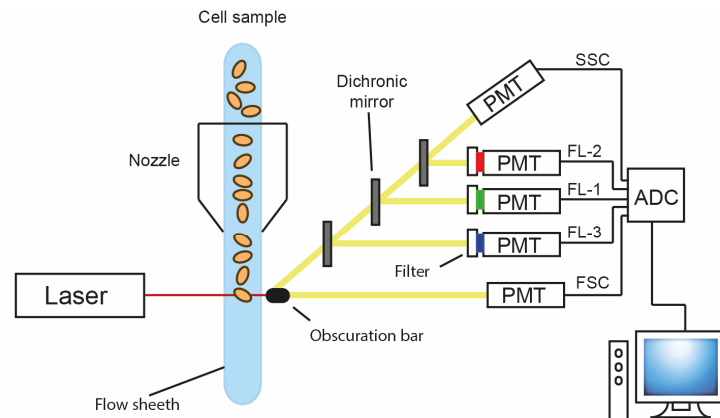
Figure 6. FACS is a laser-based technique used to study cell populations defined by expression of specific cellular markers that are fluorescently labeled by antibodies. As the single-cell suspension is passing through the laser beam fluorochrome on the antibodies is excited and emits a specific wavelength of light which after being deflected by mirrors is passing through filters into detectors. Forward scatter detects the size of the cell and side scatter detects the granularity. The color detectors can catch emission spectra from different fluorochromes in the different channels (FL-1 to FL-3 in the figure) used to identify different cellular markers. The signals are visualized in computer software as histograms and plots.

Apoptosis assays

Propidium Iodide staining

PI staining is used in Study I to assess the cellular apoptosis of thymocytes by measuring the percentage of cells in the subdiploid peak. The fluorescent stain binds to the major groove of DNA and thus shows the DNA content of the cell and is also used to visualize the cell cycle progression of the cells in the sample.
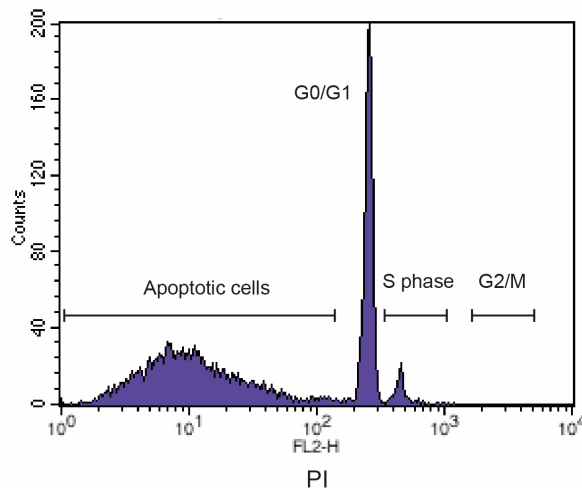
Figure 7. Dotplot of propidium iodide staining showing the subdiploid peak and the percentage of gated cells in the G0/G1 phase, M phase and G2 phase.

Terminal deoxynucleotidyl transferase dUTP nick labeling (TUNEL)

TUNEL staining is used in Study I to measure the amount of apoptotic thymocytes in NOD respectively wild-type mice after γ-irradiation. The cell sample was incubated with the deoxynucleotidyl transferase that incorporate nucleotides that cannot be further extended, the dUTPs, into single-strand breaks in the DNA of apoptotic cells therefore giving the reaction the name terminal nick labeling. The dUTPs are

fluorescently labeled and the fluorescence of the dUTPs incorporated in the DNA of the cells in the sample can be measured for example with flow cytometry.
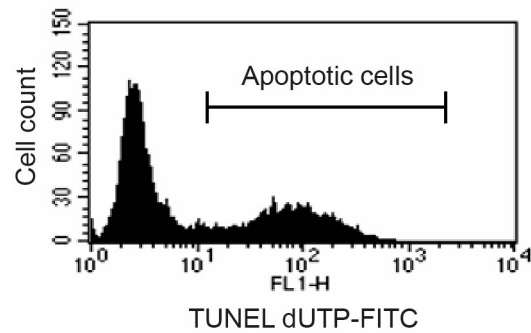
Figure 8. Dotplot of TUNEL staining showing the percentage of gated apoptotic cells. The number apoptotic cells were assessed as the percent of FITC-positive cells.

TaqMan quantitative PCR (qPCR)

Analysis of miRNA expression in Study I and II was performed using the TaqMan technology from Applied Biosystems which can differentiate between precursor and mature miRNAs and also between different miRNAs in a gene family that only differes by one nucleotide. Target specific stem-loop primers are used in the reverse transcription reaction to amplify miRNA sequence from total RNA including short <200 nt fragments (prepared with Qiagen miRNAeasy). The product is then used in a quantitative real-time PCR with fluorescently labeled probes using the reporter dye FAM (6-carboxy-fluorescein) and ROX as the quencher dye.
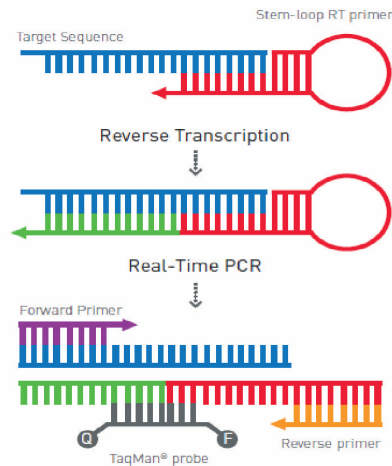
Figure 9. The principle of TaqMan technology. Stem-loop primers used in miRNA qPCR to analyse miRNA expression. *Adapted from Applied Biosystems online tutorial of miRNA research tools.*

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

TRFL-P is a PCR based method used to analyze intestinal microbiota and was used in Study III and IV. First, a PCR reaction is performed with fluorescently labeled, random hexamer primers that are used to amplify the bacterial genomic DNA and the PCR product is then cleaved by a restriction enzyme (Msp I was used in studies III-IV) and different bacterial strains will be restricted differently based on the genomic sequence of the bacteria. The fragments formed are sequenced and based on the length of the restricted fragments; the fluorescence from the sequences will be different which is represented as peaks in the analysis program. Since the peak area will be different for different bacterial strains different bacterial taxa will have lower or higher relative peak area (peak area of bacterial strain/total peak area). A principal component analysis (PCA), which is a statistical method, was used to visualize differences in samples with

same the properties. PCA clusters the bacterial taxa present in intestine based on the relative peak area in the sample.
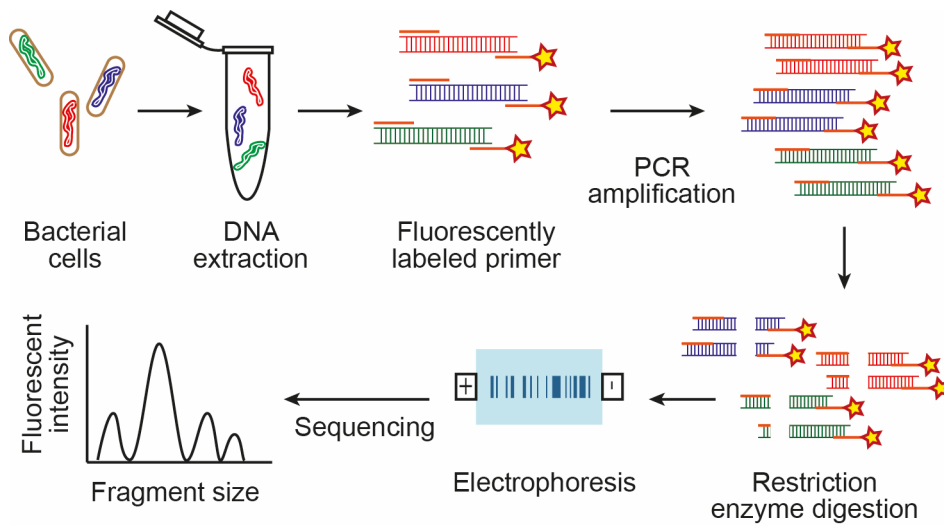
Figure 10. The principle of TRFL-P. Bacterial genomic DNA is extracted and PCR amplification with strain specific, fluorescently labeled primers is performed. The PCR product is digested with a restriction enzyme and restriction fragment sequenced after gel electrophoresis. The detected fluorescence is presented by a computer software as peaks in graph plotting fragment size against fluorescence intensity. Different bacterial strain DNA will be restricted differently and therefore one fluorescence peak detected will correspond to the relative amount of a specific bacterial taxa.

Cytokine detection assay (Luminex)

Cytokine analysis was performed on Serum from mice (study IV) using the bead-based Luminex platform. The beads which are bound to the primary catch antibody, were first incubated with the serum. After washing away excess substrate not bound to the beads, the secondary antibody coupled with phycoerythrin (PE), were added. After a final wash the plate was read similarly to an ELISA assay. The advantages the Luminex assay is its multiplexing capability which can analyze several protein analytes at the same time. This is made possible due to the different regions of the bead that emit

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fluorescence when excited by a laser. The measured fluorescence is re-calculated by the software based on a standard curve using regression-analysis into concentrations of cytokines in the serum.
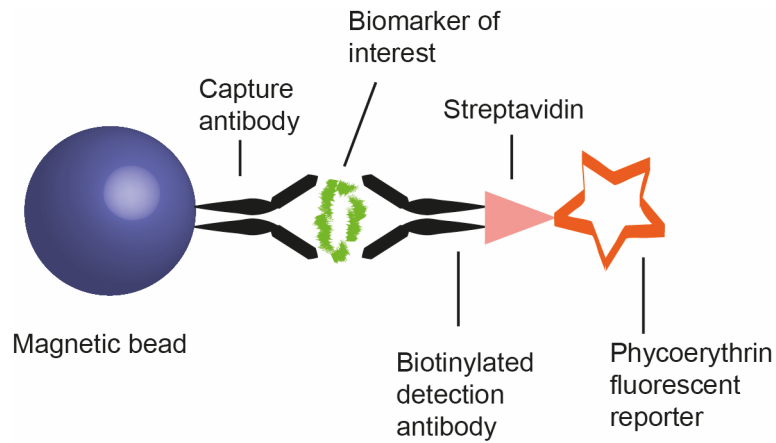
Figure 11. The principle of the Bio-Plex system. A magnetic bead bound to a catch antibody is incubated with the substrate (biomarker of interest). Excess unbound substrate is washed away and a secondary antibody coupled to a flourochrome (PE), is added. After incubation with the secondary antibody and a final washing step, a plate reader is used to detect the biomarker of interest. The emitted fluorescence is detected after being excitation by a laser. The computer software then employs a mathematical algorithm to calculate the concentration of the biomarker of interest in the sample.

RESULTS AND DISCUSSION

Study I

Clonal deletion during thymocyte maturation is essential to avoid defective and potentially self-reactive T cells. Defects in T cell maturation in the NOD mouse has previously been described, both at the cell-cycle checkpoint after β-selection and during negative selection. More specifically, Yui *et al.* have demonstrated defects in the β-selection checkpoint control allowing continued thymocyte development even in absence of proper pre-TRC re-arrangement (Yui and Rothenberg 2004). Also, Kishimoto *et al.* have shown that NOD thymocytes have increased apoptosis resistance during negative selection (Kishimoto and Sprent 2001). This might ultimately contribute to breakdown of the central tolerance and predispose the NOD mouse for the development of spontaneous T1D.

There are reports suggesting that p53 is involved in regulation of the cell-cycle checkpoint and apoptosis after β-selection (Guidos et al. 1996; Jiang et al. 1996; Haks et al. 1999; Costello et al. 2000). Additionally, during negative selection thymocytes with high affinity to self-antigens are deleted by apoptosis potentially via p53 regulation (Zhu et al. 1999; Quaglino and Ronchetti 2001).

Also, peripheral NOD lymphocytes have been observed to have a defect apoptosis induction in response to apoptosis inducing agents (Leijon et al. 1994; Colucci et al. 1996; Lamhamedi-Cherradi et al. 1998).

In Study I, we confirmed previous observations by showing that NOD thymocytes and lymphocytes are apoptosis resistant in response to treatment with low dose (6 Gy)

whole body γ-irradiation. Apoptosis of thymocytes and peripheral lymphocytes from NOD and wild-type (C57BL/6) mice was analyzed with TUNEL and PI staining and visualized with flow cytometry. We show that there are lower percentage of cells in both thymus and lymph nodes of NOD mice that underwent apoptosis ten hours after γ-irradiation compared with B6 wild-type mice.

Several miRNA gene families have been shown to be involved in p53-mediated apoptosis pathways. Examples are the miR-29 (miR-29a/b/c) and miR-34 (a/b/c) gene families. MiR-29a has been demonstrated to stabilize p53 by suppressing a regulatory subunit (p85α) of PI3K and the Rho family GTPase CDC42 (Park et al. 2009). Additionally, Bommer *et al.* showed that miR-34a expression is directly up-regulated by p53 (Bommer et al. 2007). MiR-34a is proposed to be involved in a positive feedback loop with p53 by suppressing the Silent information regulator 1 (SIRT1) (Yamakuchi et al. 2008; Yamakuchi and Lowenstein 2009). Conversely, miR-125b and miR-155 were demonstrated to be negative regulators of p53 and are down-regulated after DNA damage (Le et al. 2009; Gueta et al. 2010). In this study, we investigated the expression of miRNAs 34 a/b/c, miR-29a, miR-125b, miR-155 and miR-203 with qPCR. We show that the miR-34 gene family (miR-34a/b/c) expression profile is different in NOD thymocytes compared to wild-type B6 thymocytes (Figure 12a). In addition, miR-34b and miR-34c were not properly up-regulated in NOD thymocytes after γ-irradiation compared to B6 thymocytes (Figure 12b). There is also differential expression of miR-125b in NOD thymocytes compared to B6 mice both before and after γ-irradiation (Figure 12c) resulting in a net down-regulation of miR-125b in NOD thymocytes compared to B6 mice (Figure 12d). Moreover, the expression of miR-155 were higher in NOD thymocytes compared to B6 after γ-irradiation (Figure

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12e) resulting in a net up-regulation in miR-155 in NOD thymocytes after γ-irradiation (Figure 12f).
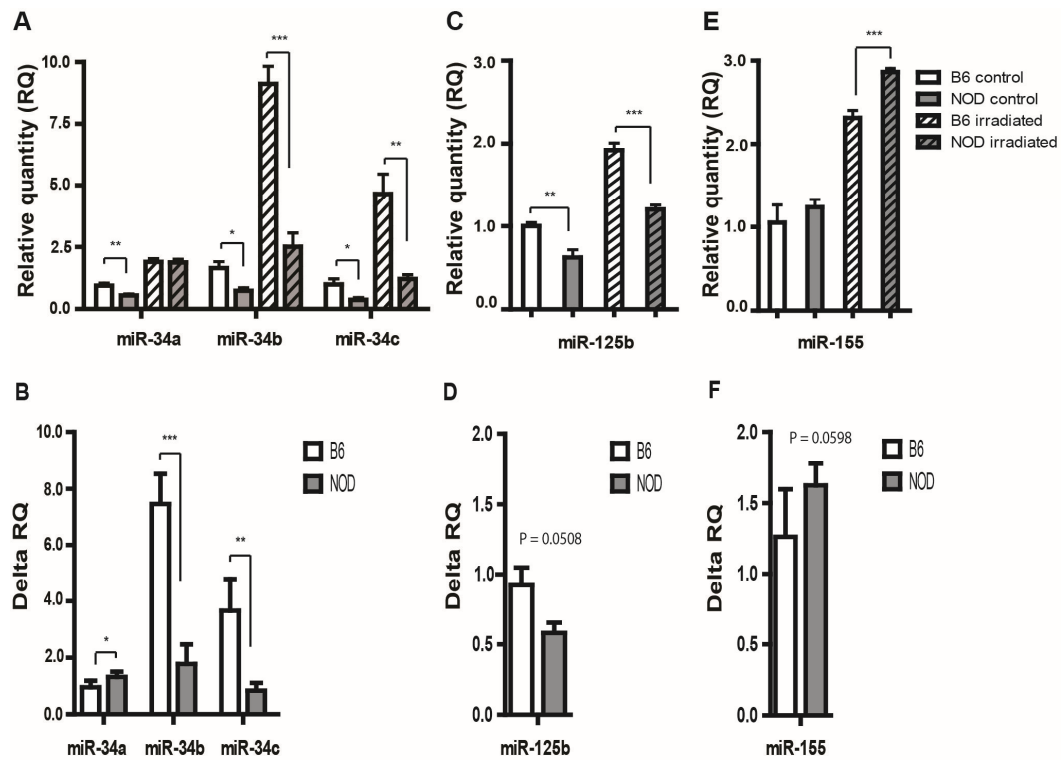
Figure 12. (a) miRNA profiling of NOD thymocytes with qPCR shows differential expression of the miR-34a/b/c family in the NOD thymocytes both before and after γ-irradiation. (b) The delta expression of miR-34 gene family, comparing before and after irradiation, shows that miR-34b and miR-34c are not properly up-regulated in NOD thymocytes compared to B6 mice. (c) The miR-125b is differentially expressed in NOD thymocytes before and after γ-irradiation compared to B6 mice. (d) The delta expression of miR-125b shows a down-regulation in NOD thymocytes compared to B6 thymocytes after irradiation. (e) The miR-155 is differentially expressed in NOD thymocytes after γ-irradiation compared to B6 mice. (f) The delta expression of miR-155 shows that the miRNA is up-regulated in NOD thymocytes efter γ-irradiation. Student's t-test (two-tailed), $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$.

We believe that altered miRNA regulation network in NOD lymphocytes could contribute to defective apoptosis regulation in NOD lymphocytes. Particularly, the defect up-regulation of the miR-34b and c after γ-irradiation in NOD thymocytes

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(Figure 11c, d). However, it has also been reported that miR-34 gene family is redundant in the regulation of p53 and down-streams targets p21, Bax, Mdm2 as well as PUMA *in vivo* in mouse thymus (Concepcion et al. 2012).

We therefore investigated the protein expression of several targets in the cell-cycle (c-abl, cyclin D1, p21, p27) and p53 in NOD lymphocytes by Western blot. We could show that p53 was the only target which was not properly up-regulated in NOD thymocytes and peripheral lymphocytes compared to wild-type mice after γ-irradiation (figure 13).
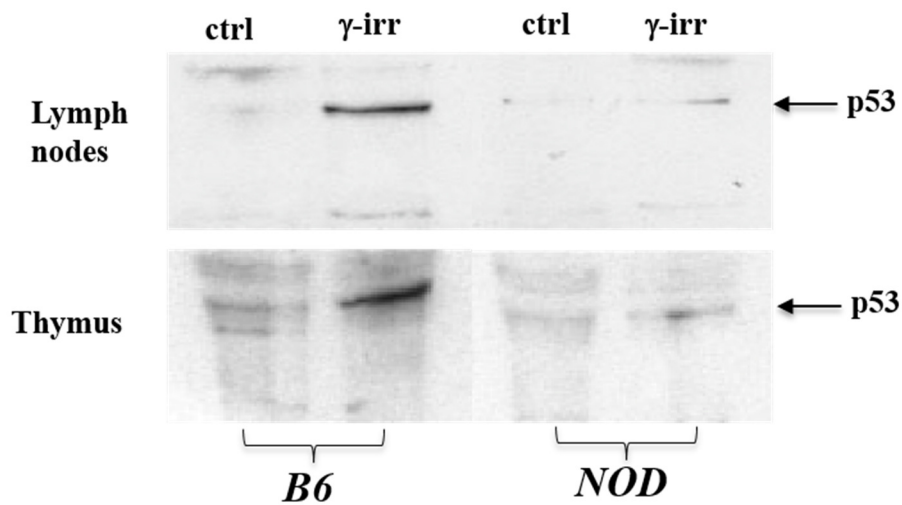
Figure 13. Defective up-regulation of p53 in NOD thymocytes and peripheral lymphocytes after γ-irradiation compared to wild-type B6 mice.

In conclusion, our data suggest that altered expression level of miR-34a/b/c and miR-125b in NOD thymocytes compared to B6 thymocytes could explain the defective up-regulation of p53 in NOD lymphocytes and thus contribute to the apoptosis resistance phenotype in NOD lymphocytes. This could ultimately contribute to impairing central and peripheral tolerance and predispose the NOD mouse to autoimmune diabetes.

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Study II

Canonical miRNAs have been shown to be important in the early development of T cells and the absence of miRNAs greatly affect αβ T cell numbers but does not impair the CD4/CD8 lineage commitment (Cobb et al. 2005). Deletion of canonical miRNAs from CD4$^+$ T cells results in defective differentiation of nTregs in the thymus and induction/stability of Foxp3 in peripheral Tregs (Cobb et al. 2006; Jeker et al. 2013).

Moreover, T cell response *in vivo* in CD8$^+$ T cells that lack canonical miRNAs is reduced (Zhang and Bevan 2010). Zhang *et al.* also demonstrated that despite increased activation and proliferation *in vitro* the CD8$^+$ T cells lacking canonical miRNAs failed to down-regulate CD69 which probably inhibited the cells migration ability *in vivo* (Zhang and Bevan 2010).

In this study, we investigated how deletion of canonical miRNAs during early thymocyte development in NOD mice would affect the overall immunological balance between the effector T cells and Tregs and how this would affect the outcome of diabetes development. Initially, we validated the deletion of *Dicer1* in sorted DP thymocytes and CD3$^+$CD4$^+$ splenocytes of the NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mouse. We then performed an immunophenotyping of the T cell populations of primary and secondary lymphoid organs. We found that the total cellularity of the thymus, spleen and MLN but not peri-pancreatic LN (pLN) of the NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mouse was decreased. The immunophenotyping by flow cytometry showed a significant decrease in the DP thymocytes but only a slight and not significant decrease in the amount of nTregs in the thymus of the NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mouse compared to NOD.*Dicer1*$^{\text{flox/flox}}$ control littermates. Similarly to published data by Cobb *et al.,* we show that the cell populations that decreased mainly belonged to the αβ T cells with no significant increase in the γδ T cells. Furthermore, we observed a significant

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reduction of CD8$^+$ T cells in both spleen and MLN in addition to a significant decrease of CD4$^+$ αβ T cells in the spleen of NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice compared to NOD.*Dicer1*$^{flox/flox}$ mice. However, there was only a slight non-significant decrease of nTregs in the secondary lymphoid organs (spleen, MLN and pLN). Interestingly, the percentage of CD4$^+$ and CD8$^+$ effector memory T cells was increased in spleen and MLN of NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice. However, when the results were normalized for the amount of cells there was a reduction in the total number of effector memory T cells. This indicates that the lack of canonical miRNA network is driving the CD4$^+$ and CD8$^+$ T cells into memory phenotype. Unfortunately, we have not yet elucidated the importance of specific miRNAs in the different immunophenotyped T cell populations. We will next perform a miRNA profiling to be able to study the regulatory role of particular miRNAs or groups of miRNAs for the function of different T cell populations. In the future we will also study the functionality of the NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ effector cells respectively Treg cells *in vivo* by adoptive transfer experiments.

Here, we show the overall effects of canonical miRNA deletion in different T cell populations for the development of autoimmune diabetes. Also, we showed a significantly decreased leukocyte infiltration in NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ pancreatic islets compared to NOD.*Dicer1*$^{flox/flox}$ control littermates. Finally, we investigated the diabetes incidence of NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice. We did not observe a difference in the diabetes incidence of NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ females compared with control littermates but a significant increase in the diabetes incidence of male NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice.
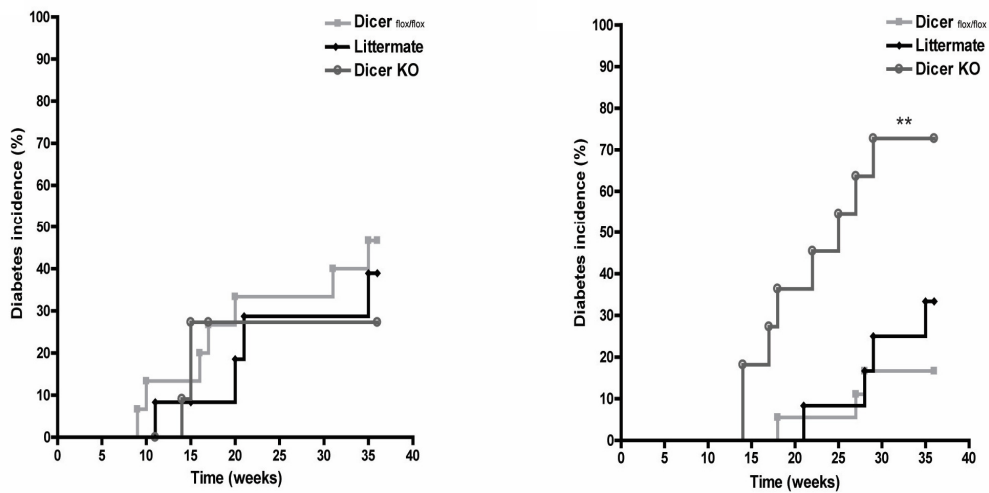
Figure 14. Diabetes incidence of the female and male NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice. There is no difference in diabetes incidence of the female NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice compared to mice NOD.*Dicer1*$^{flox/flox}$ control littermates and NOD.Lck-Cre *Dicer1*$^{\Delta/wt}$ heterozygote mice (left panel). Male NOD.Lck-Cre *Dicer1*$^{\Delta/\Delta}$ mice displayed significantly higher diabetes incidence (Student's *t*-test, two-tailed, p<0.01=**) compared to NOD.*Dicer1*$^{flox/flox}$ control littermates and NOD.Lck-Cre *Dicer1*$^{\Delta/wt}$ heterozygote littermates.

Due to the differential diabetes incidence between the genders we can speculate on the potential involvement of sex hormone-mediated regulation of miRNAs in our model. Sex-mediated differential miRNA expression patterns are present in both humans and mice (Ilnytskyy et al. 2008; Ciaudo et al. 2009; Langevin et al. 2010). More specifically, estrogen up-regulate transcription of a group of miRNAs and there are indications that Dicer might be regulated by estrogen (Bhat-Nakshatri et al. 2009; Shao et al. 2011). Further, certain miRNAs have also been shown to regulate the levels androgen expression in prostate cancer cells (Ostling et al. 2011; Hagman et al. 2013).

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Additionally, androgen has been demonstrated to regulate Dicer and is proposed to be involved in a positive feedback-loop in which androgen is increasing miRNA expression which in turn increases androgen levels (Narayanan et al. 2010). Indeed, there seems to be a link between sex-hormone regulation and miRNAs that could potentially generate differential outcome in T cell proliferation and ultimately result in different disease incidence of T1D.

Study III

Commensal gut microbiota is important for proper development of the immune system and maintenance of tolerance in the intestinal tract in several ways. Certain gut microbiota is linked to T1D and children with T1D (or progressing towards T1D) display altered gut microbiota with decreased amount of bacteria of the Firmicute phylum and increased amount of bacteria belonging to the Bacteroidetes phylum (Giongo et al. 2011). The maternal gut microbiota is the first source of bacteria which the child comes in contact with and could influence the development of the immune system of the infant. Therefore, in order to understand how the gut microbiota could potentially affect the immune system and diabetes development in the offspring, we investigated the effect of induced disturbance of commensal maternal gut microbiota by antibiotic treatment. This is an important subject because it is common for pregnant women to receive antibiotics for different infections during pregnancy (Lim et al. 2003; Andrade et al. 2004; Le et al. 2004; Chan et al. 2013). In addition, antibiotics treatment of pregnant mothers has recently been associated with increased risk of allergic disease but little is known about the effects for the development of T1D (McKeever et al. 2002; Metsala et al. 2013).

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We treated the pregnant NOD mice with a broad-spectrum antibiotics mix consisting of neomycin, polymyxin and metronidazol to alter the normal maternal gut microbiota. Next, we followed the development of spontaneous T1D development in the NOD offspring. By performing a TRFL-P analysis we found that the offspring to NOD mice treated with antibiotics displayed decreased diversity of intestinal microbiota and also differential clustering of the present microbiota, which was illustrated by clustering of the intestinal gut microbiota (TRFs) with a PCA analysis. This means that modulation of the maternal intestinal microbiota with antibiotics resulted in both decreased diversity and changed the composition of the intestinal microbiota in the offspring.
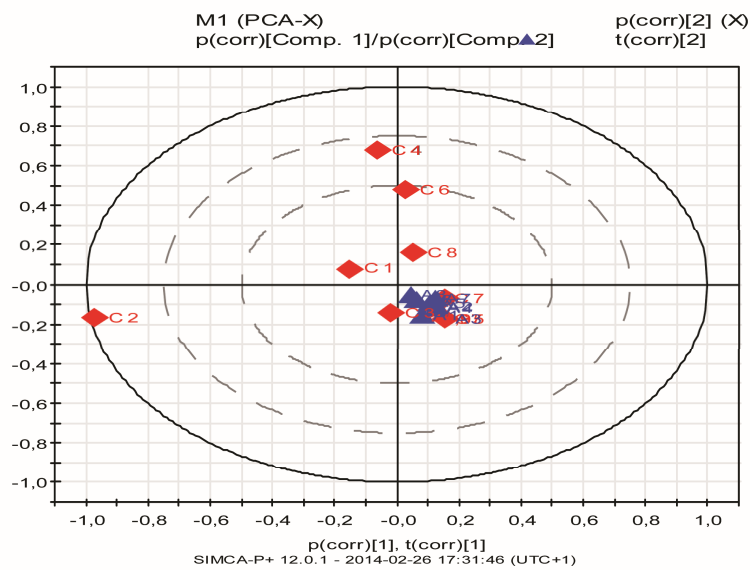
Figure 15. The clustering of the intestinal gut microbiota with PCA. There is different clustering of the TRFs in offspring to mothers treated with antibiotics mix during pregnancy compared to offspring to control mothers.

Next, we performed T cell analysis of different T cell populations from MLN and Peyer's patches in offspring to mothers treated with antibiotics and control mothers. We show that there was a significant decrease of percentage gated CD3$^+$CD8$^+$ T cells

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in the MLN of offspring to mothers treated with antibiotics. In the Peyer's patches we show a significant reduction in the percentage gated $CD3^+CD4^+CD25^+$ T cells in the offspring to mothers treated with antibiotics during pregnancy. Additionally, there was a significant decrease in the percentage gated naïve $CD4^+CD62L^+$ T cells and $CD4^+CD62L^-$ memory T cells in the Peyer's patches of offspring to treated mothers. Interestingly, the modulation of intestinal microbiota during pregnancy due to antibiotics treatment seems to have influenced alterations in T cell populations in MLN and Peyer's patches.

Additionally, we investigated if these changes in the intestinal gut microbiota and T cell alterations of the NOD offspring would contribute to altered development of T1D diabetes. We show a modulation of diabetes in the offspring to mothers treated with antibiotics at 20 weeks of age but at the end of the monitoring period at 30 weeks of age there is no significant difference in diabetes incidence between the offspring groups.
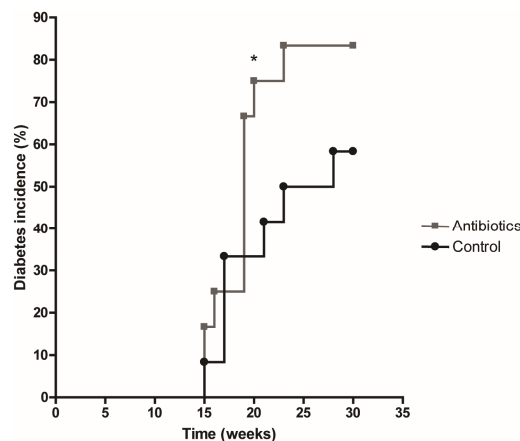
Figure 16. In the offspring from mothers treated with antibiotics during pregnancy there seems to be a modulation of diabetes incidence ($p<0.05$= *) at 20 weeks of age but at the end of the monitoring period there is no significant difference in diabetes incidence between the two offspring groups.

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In conclusion, we show that the modulation of the maternal gut microbiota during pregnancy with antibiotics treatment generated persistent changes in the intestinal gut microbiota diversity and composition of the offspring. These changes seem to have induced the demonstrated alterations of different T cell populations in the MLN and Peyer's patches in offspring to mothers treated with antibiotics during pregnancy. Although we believe that these effects modulate the development of T1D in the offspring from the antibiotics treated mothers at 20 weeks of age, we could not observe persistent effects. Further studies in a human population would be needed to fully understand the potential risk factors of antibiotics treatment during pregnancy for the development of autoimmune diabetes.

Study IV

It is known that the interaction between the immune system and the intestinal microbiota can be important for the outcome of intestinal inflammation and UC. However, how the global composition of intestinal microbiota changes and ultimately contribute to the disease is not completely understood. To study this we used the DSS model because it is the most commonly used animal model for UC and because of the many clinical and histopathological similarities with human UC (Okayasu et al. 1990; Melgar et al. 2005).

We induced UC in wild-type C57BL/6 mice through chemical induction by administration of 4 % DSS in the drinking water. The mice were then analyzed for the relationship between UC and gut microbiota as well as the immune response. The analysis of the colonic intestinal microbiota was performed with TRFL-P. Although we

did not find any decrease in overall diversity we show a differential clustering pattern after PCA of the intestinal gut microbiota in the DSS group comparing to control mice.
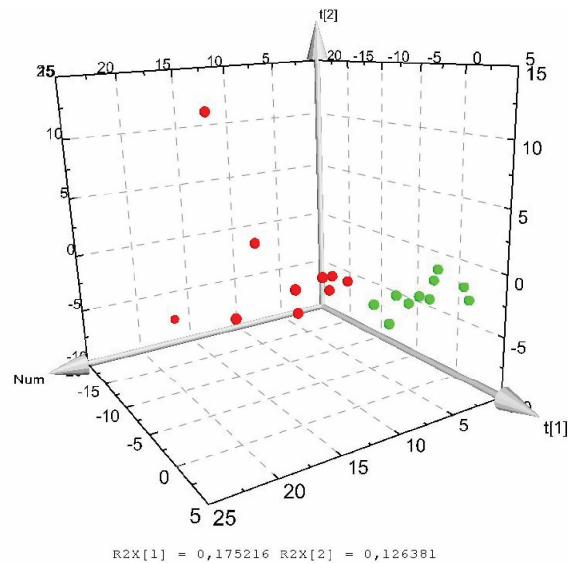
Figure 17. PCA 3D plot showing the bacterial clustering pattern. There is differential clustering of the DSS treated animals (green circles) and control animals (red circles).

Our qPCR analysis showed that the amount of *Lactobacillus* decreased whereas the number of bacteria belonging to the *Akkersmansia* and *Desulfovibrio* genera increased after DSS treatment. Additionally, *Enterobacteriaceae* (*Escherichia coli*) was present at a much higher incidence in the DSS group compared to control group. In concordance with our results an increase in *Bacteroides*, *Clostridium* and decrease in the *Lactobacillus* genera is reported to be associated with UC in humans and mice (Fabia et al. 1993; Garcia-Lafuente et al. 1997). Moreover, in support to our qPCR data, the increased presence of the *Desulfovibrio* and *Enterobacteriaceae* genera has been associated with UC in humans (Mylonaki et al. 2005; Rowan et al. 2010). Interestingly, *Akkersmansia*

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muciniphila is a mucin degrading bacteria (Eckburg et al. 2005) and could thus be involved in the degradation of the colonic mucous layer during UC.
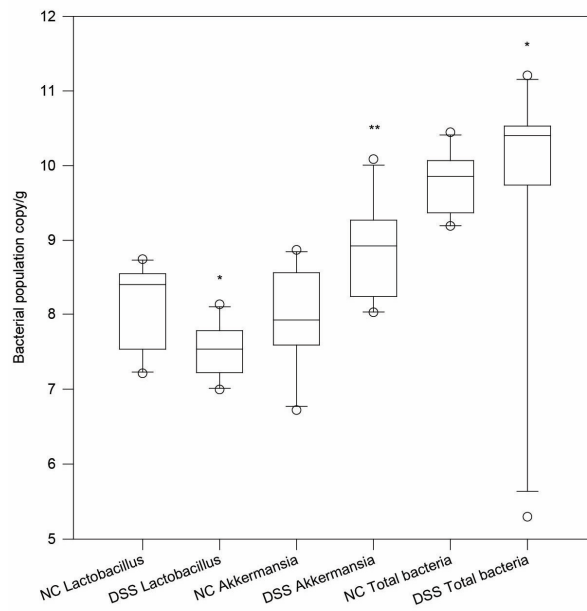
Figure 18. Mucosal bacterial populations identified with qPCR. Mann-Whitney rank sum test, $p < 0.05 = $ *; $p < 0.01 = $ **.

The intestinal neutrophil infiltration was assessed by myeloperoxidase (MPO) activity and was found to be increased in colon but not in small intestine. MPO is a neutrophil enzyme and has previously been associated with increased neutrophil infiltration during UC (Kristjansson et al. 2004). To assess the level of systemic inflammation, inflammatory cytokines in serum were investigated. We showed an increase in pro-inflammatory cytokines IL-6, IL-17 and the chemokine KC (a neutrophil attractant). In concordance to our results, these pro-inflammatory cytokines have previously been shown during the pathogenesis of chronic IBD in humans and mice and more specifically, IL-17 is connected to induction of Th17 cells (Yen et al. 2006; Alex et al. 2009; Holtta et al. 2013).

We additionally analyzed different lymphocyte populations, Mφ, and DCs in spleen, Peyer's patches and MLN using flow cytometry. We found significantly increased percentage of Tregs (co-expressing CTLA-4) in spleen, MLN, Peyer's patches. In spite of the presence of intestinal inflammation, we observed increased frequency of Tregs. In support, similar findings have been reported by Hölttä *et al.* in children with UC who demonstrated an increase in colonic Tregs (Holtta et al. 2013). It is possible that even in the presence of the increased frequency of Tregs these cells cannot reverse the disease progression (Yu et al. 2007). Alternatively, the functionality of the Tregs might be inhibited by TLR signaling.

We also showed increased percentage of CD11b^{+}CD11c^{+} expressing and CD11c^{+}TLR-4^{+} DCs cells in the MLN. Increased TLR signaling may indicate increased interaction between intestinal microbiota and the immune system. In other words, the enhanced interaction could be an effect of altered gut microbiota which results in greater immune responses leading to intestinal inflammation (Hart et al. 2005).

By investigating the changes in gut microbiota during DSS-induced colitis and the immunophenotype we can contribute to a more clear understanding of the active role that the intestinal microbiota play in the interaction with the gut immune system during intestinal inflammation in UC.

SUMMARY AND CONCLUSIONS

In the first two studies of this thesis the role of miRNA regulation was investigated in NOD mice. In Study I, we investigated the role of miRNA-34 gene family (miR-34a/b/c), miR-29 gene family (miR-29a/b/c), miR-125b, miR-155 and miR-203 in the regulation of p53-dependent DNA damage response in thymocytes in response to γ-irradiation. We show that NOD lymphocytes are apoptosis resistant in response to γ-irradiation compared to wild-type B6 mice. There is differential expression of miR-34a before γ-irradiation and miR-34b/c before and after γ-irradiation in NOD thymocytes compared to B6 thymocytes. Additionally, we show that miR-125b was differentially expressed before and after γ-irradiation as well as miR-155 after γ-irradiation in NOD thymocytes compared to B6 mice. Finally, we also show that p53 is not properly up-regulated in NOD thymocytes in response to γ-irradiation. We believe that this might be mediated by the miRNAs differentially expressed between the NOD and B6 thymocytes during the apoptosis response to γ-irradiation. In conclusion, altered miRNA network affecting the level of p53 expression in NOD thymocytes during cell-cycle checkpoint and apoptosis during development may influence the establishment of central tolerance and predispose the NOD mouse to T1D development.

In Study II, the overall role of canonical miRNAs in the NOD mouse was investigated by deletion of the mature miRNAs using the Cre-Lox system at the DN3 stage of early thymocyte development. The effect of lacking mature canonical miRNAs resulted in reduced overall cellularity of secondary lymphoid organs spleen, MLN and pLN. The immunophenotyping of the NOD.Lck-Cre $Dicer1^{\Delta/\Delta}$ mice show a significant decrease of αβ CD4$^+$ and CD8$^+$ T cell populations in the thymus, spleen and MLN. Additionally, we show a slight non-statistically decrease in nTregs in thymus, spleen,

MLN and pLN. There was also an increased ratio of memory T cells in the spleen and MLN of the NOD.Lck-Cre $Dicer1^{\Delta/\Delta}$ mice. Although there was lower leukocyte infiltration in the pancreatic islets, we could not show any difference in the diabetes incidence between the female NOD.Lck-Cre $Dicer1^{\Delta/\Delta}$ mice and control littermates. However, there was an increase in diabetes incidence of male. In short, canonical miRNAs are important for generation of αβ CD4$^+$ and CD8$^+$ T cells as well as nTregs in the primary and secondary organs of the NOD.Lck-Cre $Dicer1^{\Delta/\Delta}$ mice. It is probable that canonical miRNAs are also important for the function of the different T cell populations, which will be investigated in future studies.

In Study III, we studied the effect of derangement of maternal intestinal microbiota on the offspring, particularly for the development of T1D. The maternal intestinal microbiota was modulated by treating the pregnant NOD mice with antibiotics and we found decreased diversity and a persistent modulation of the intestinal microbial pattern in the offspring. We also demonstrated immunological alterations including CD8$^+$ T cell frequencies in the MLN and CD3$^+$CD4$^+$CD25$^+$ as well as CD4$^+$CD62L$^-$ T cell frequencies and in Peyer's patches. These changes may have influenced the increase in the diabetes incidence of offspring to treated mothers at 20 weeks of age although the effect was not persistent. In conclusion, our results indicate the importance of the maternal gut microbiota for the immunological tolerance in the offspring.

In Study IV, we wanted to clarify the relationship between the global intestinal microbiota and the immune system in the DSS induced UC mouse model. We showed changes in the overall colonic intestinal microbiota pattern after DSS treatment. In addition, we a showed a significant decrease in *Lactobacillus* and significant increase in *Akkersmansia* and *Desulfovibrio* as well as higher incidence of *Enterobacteriaceae*. We

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demonstrated immunological alterations of different populations of T cells, DCs and NK cells after UC induction. These include significant increase of T cells, particularly CD8$^+$ T cells in the MLN of DSS treated mice. Additionally, we showed significant increase in Tregs co-expressing CTLA-4 in the spleen, Peyer's patches and MLN in DSS group. Finally, we demonstrated significant increase in monocytes and DCs co-expressing TLR-4 in the MLN. In conclusion, our results indicate that the intestinal microbiota actively interacts with the immune system in UC.

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