Metab-Immune analysis of the Non-obese diabetic mouse

Viqar Banday

To my family

Don't be satisfied with stories, how things have gone with others. Unfold your own myth...

~ Rumi

Table of Contents

Abstract

Type 1 diabetes mellitus (T1D) is a chronic disease characterized by T cell mediated destruction of the insulin producing β cells in the islets of Langerhans. The classical symptoms include high glucose levels in urine and blood, polyuria and polydipsia. It is the leading cause of blindness, amputations, end-stage renal disease and contributes to premature death. The disease has a strong genetic influence which is heritable and shows a high concordance rate of 50% between monozygotic twins; however environmental factors have also been shown to be involved in diabetes.

The non-obese diabetic (NOD) mouse which was first described by Makino et al in 1980 is the most widely used model organism for T1D. NOD mice develop T1D spontaneously with incidence rates of up to 80% in females and 30% in males. These mice also show the classical symptoms of high glucose levels in urine and blood, polyuria and polydipsia. These symptoms are preceded by insulitis - the infiltration and destruction of the insulin producing β cells in the islets of Langerhans by mononuclear cells. T1D disease in the NOD mouse shares a number of similarities to human T1D, like dependence on genetic and environmental factors. More than 20 disease associated gene regions or loci [termed insulin dependent diabetes (*Idd*) loci] have been associated with T1D development in NOD and for some of these loci, the human chromosomal counterpart has been linked to the development of T1D in human.

T1D, both in humans and mice, is recognized as a T cell mediated disease however many studies have shown the importance of B cells in the pathogenesis of the disease. Appearance of autoantibodies in the serum of patients is the first sign of pathogenesis. However, molecular and cellular events precede this beta-cell immunity. It has been shown that patients who developed T1D had an altered metabolome prior to the appearance of autoantibodies. Although much is known about the pathogenesis of T1D, less is known about the environment/immune factors triggering the disease.

In the present thesis, both the metabolic and immune deviations observed in the NOD mouse were characterized. The serum metabolome analysis of the NOD mouse revealed striking resemblance to the findings reported in the human study. In the NOD mouse, the TCA cycle was significantly different from the non-diabetic control C57BL/6 mice with glutamic acid being one of the most distinguishing metabolite. In addition, other metabolites linked to glutamic acid metabolism were also affected. Furthermore, a detailed bioinformatics analysis revealed various genes/enzymes to be present in the *Idd* regions. When compared to the non-diabetic control C57BL/6 mice, many of genes correlated to the metabolic pathways showed single nucleotide polymorphism (SNP) which can eventually affect the functionality of the protein.

Continuing to analyze the metabolic perturbation in the NOD mouse, genetic analysis of the increased glutamic acid phenotype was done, which revealed several *Idd* regions to be involved. The regions mapped in the genetic analysis harbored important enzymes and transporters related to glutamic acid. These enzymes and transporters play an important role in maintaining glutamic acid levels in the serum and prevent glutamic acid induced toxicity. Interestingly, enzymes catabolizing glutamic acid were found to be decreased in the NOD mouse. *In vitro* islet culture with glutamic acid led to increased beta cell death indicating the toxic role of glutamic acid specifically towards insulin producing beta cells.

B cells from NOD mice are known to express high levels of TACI, a TNF super family receptor. TACI is known to regulate both B cell homeostasis as well as activation. *In vitro* stimulation of B cells by TACI ligand APRIL resulted in enhanced plasma cell differentiation accompanied with increased class switching and IgG production. In addition, TACI+ cells were observed in NOD germinal centres leading to increased BAFF uptake and production of low affinity antibodies.

It has been shown that NOD mice react vigorously to T-dependent antigens upon immunization. An enhanced and prolonged immune response to hen egg

lysozyme immunizations was elicited in NOD mice. Serum IgG levels were significantly increased in the NOD mice and were predominantly of the IgG1 isotype. Immunofluorescence analysis revealed increased germinal centers in the NOD mice. Adoptive transfer experiments of purified B and T cells from NOD into NOD.*Rag2*$^{-/-}$ (NOD.Rag) mice revealed the importance of B cell intrinsic defects in the reproduction of the original phenotype as seen in NOD.

Collectively, the aim of this thesis was to get an insight into the metabolomic and immune deviations observed in the NOD mice.

List of papers included in the thesis

Paper I

Altered Metabolic Signature in Pre-Diabetic NOD Mice.
Rasmus Madsen, <u>Viqar Showkat Banday</u>, Thomas Moritz, Johan Trygg, Kristina Lejon. PlosOne 2011

Paper II

Elevated Systemic Glutamic Acid Level in the Non-Obese Diabetic Mouse is Idd Linked and Induces Beta Cell Apoptosis
<u>Viqar Showkat Banday</u>, Kristina Lejon. (Submitted Manuscript)

Paper III

Increased expression of TACI in the NOD mouse results in enhanced plasma cell differentiation and immunoglobulin production.
<u>Viqar Showkat Banday</u>, Radha Thyagarajan, Mia Sundström and Kristina Lejon. (Manuscript)

Paper IV

B cell intrinsic defects lead to enhanced immune response in the NOD mice
<u>Viqar Showkat Banday</u>, Radha Thyagarajan and Kristina Lejon. (Manuscript)

List of papers not included in the thesis

Paper V

Contribution of autoallergy to the pathogenesis in the NOD mice.
Radha Thyagarajan, <u>Viqar Showkat Banday</u>, Zhouji Ding and Kristina Lejon. (Autoimmunity 2015)

Abbreviations

AID	Activation induced cytidine deaminase
Alt	Alanine transaminase
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
Ast	Aspartate transaminase
B cell	B lymphocyte
BAFF	B cell activating factor
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FDC	Follicular dendritic cell
FO	Follicular
GAD	Glutamic acid decarboxylase
GC	Germinal center
HEV	High endothelial venules
HLA	Human leukocyte antigen
HSPG	Heparan sulfate proteoglycan
IA-2	Insulinoma-associated antigen-2
IAA	Insulin autoantibody
IC	Immune complex
Idd	Insulin dependent diabetes
Ig	Immunoglobulin
IL	Interleukin
MHC	Major histocompatibility complex
MZ	Marginal zone
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOD	Non-obese diabetic
PTPN22	Protein tyrosine phosphatase non-receptor type 22
QTL	Quantitative trait locus
RAG	Recombination-activating genes

SCS	Subcapsular sinus
T cell	T lymphocyte
T1D	Type 1 diabetes
T1	Transitional 1
T2	Transitional 2
T3	Transitional 3
TACI	Transmembrane activator calcium modulator and cyclophilin ligand interactor
TCR	T cell receptor
TFH	T follicular helper
TNF	Tumor necrosis factor
VNTR	Variable number of tandem repeat
ZnT8	Zink transporter 8

Introduction

Immunity

Humans and many other multicellular organisms have over time developed various mechanisms to protect themselves against a variety of infections. Early Greeks noted that persons surviving a bout of plague did not contract the disease again (1). It was not until the works of Elie Metchnikoff (1883) and Emil von Behring (1890) that both cellular and serum contents were identified as contributing to the immune state of an animal.

The immune system, comprising of both cells and molecules, is a multifaceted system that has evolved to identify and kill pathogens and diseased cells. In this process, the immune system can distinguish between pathogens, tumor cells and healthy cells. The immune system can thus be divided into two fundamental parts:

- Innate immune system
- Adaptive immune system

The innate immune system represents the first line of defense against a pathogen. It comprises of physical barriers, molecules and specific immune cells. The cells of the innate immune system have evolved to recognize specific pathogen patterns leading to their activation and clearance of the pathogen. The secreted molecules include a variety of host defense peptides and enzymes.

The adaptive immune system, on the other hand, represents the more specific arm of the immune system. If the innate immune system fails to completely clear the pathogen, the adaptive arm of the immune system gets activated. The main hallmark of this system is the ability to adapt and respond in a specific manner. The ability of various cells to rearrange their receptors results in the capability to recognize essentially all molecules irrespective of their nature. The other important aspect of the adaptive immune response is

the creation of immunological memory. This leads to an enhanced and rapid response during a secondary exposure to the same antigen.

Cells of the adaptive immune system can be broadly grouped as B lymphocytes and T lymphocytes. Both of these cell types are equipped with specific cell receptors which help them to recognize and bind protein peptides but also other foreign molecules. B cells are equipped with the B cell receptor (BCR). The BCR is a membrane bound immunoglobulin which can signal downstream post recognition of the antigen. The T cells on the other hand possess the T cell receptor (TCR). Although B cells can directly bind and recognize an antigen independently, T cells require the presence of the antigen presented on major histocompatibility complex (MHC) for efficient activation (2). Signals from both the BCR and TCR lead to signals that induce proliferation and differentiation of cells leading to creation of immunological memory and clearance of the antigen.

B cells: Development and Subsets

B cells are one of the most important cells of the adaptive arm of the immune system. In adult mouse they arise in the bone marrow (BM) from the hematopoietic precursor cells. Embryonic B cell pool is seeded by hematopoietic stem cells from the fetal liver (3). Although all B cells, with the exception of B1-b cells, originate from the bone marrow as immature cells, they further develop into specialized B cell subsets in the spleen.

B cells like the T cells arise from a common lymphoid progenitor (CLP) in the bone marrow. Commitment to B cells is however mediated with the expression of lineage specific molecules such as B220, an isoform of CD45 (4). These B220+ nucleated BM cells, called the pre-pro B cells, account for about 1% of total nucleated cells in the BM (5). The development to pro-B cells is achieved by the activation of recombination activation genes 1 and 2 (Rag-1 and Rag-2) and development of the pre-BCR through V(D)J recombination (6). The expression of the pre-BCR serves as an important checkpoint in the

life of a pre-B cell as the V(D)J recombination often results in truncated or non-functional μ heavy chains (μHCs) (7). Once the heavy chain is correctly expressed, the Rag-1 and Rag-2 proteins commence the light chain rearrangement at Ig kappa or Ig lambda locus to yield the complete BCR (8). This stage of the B cell development demarcates the immature BM B cells stage.

The newly formed immature B cells exit the BM as transitional B cells and migrate to the spleen for further differentiation. Based on the expression of surface molecules, transitional (T) B cells are divided into different stages as summarized in Figure 1.

The transitional B cells serve as the precursors to the mature naïve B cells (9). Loder et al have neatly shown the series in which the diiferent transitional B cells differentiate (10). T1 cells differentiate to T2 which, based on various signals received, can develop into further subsets, either a follicular (FO) or marginal zone (MZ) B cell (11).

FO B cells
B220$^+$
AA4$^-$
IgM$^{low/int}$
CD21/CD35^{int}
CD23$^+$

T1 B cells
B220$^+$
AA4$^+$
IgM^{high}
CD21/CD35^{low}
CD23$^-$

T2 B cells
B220$^+$
AA4$^+$
IgM^{high}
CD21/CD35^{int}
CD23$^+$

MZ precursor B cells
B220$^+$
AA4^{low}
IgM^{high}
CD21/CD35^{high}
CD23$^+$

MZ B cells
B220$^+$
AA4$^-$
IgM^{high}
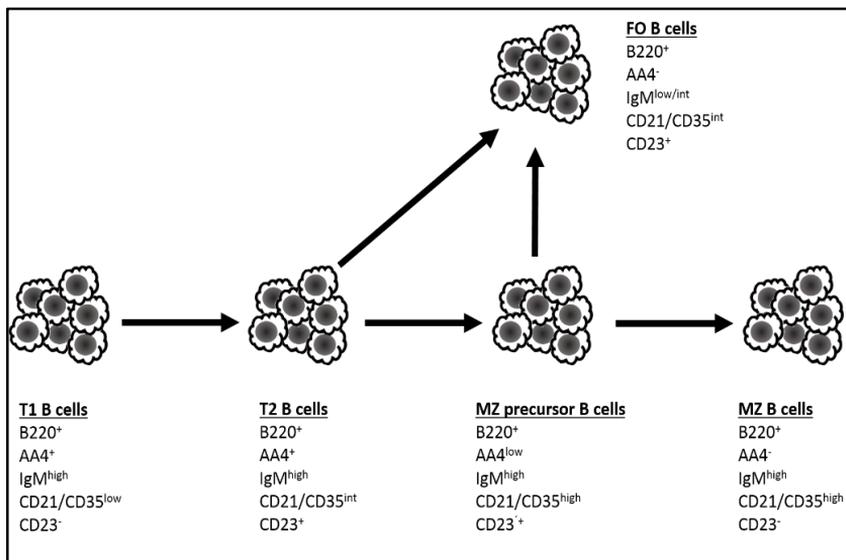CD21/CD35^{high}
CD23$^-$

Figure 1. Schematic diagram of B cells development in the spleen (Srivastava et al 2009).

The mature B cell subsets are differentiated based on the surface marker expression and function. Based on these attributes, B cells are broadly classified as to be composed of three distinct populations:

- B-1 B cells
- Marginal Zone B cells (MZ B cells)
- Follicular B cells (FO B cells)

B-1B cells form the majority of the B cells found in the peritoneal and pleural cavities. These constitute about 5% of the peripheral B cell pool and have an IgM^{hi} IgD^{low} CD23$^-$ B220^{low} phenotype. These are the first cells to be formed in the developing fetal liver and are believed to have a restricted BCR repertoire (12). Unlike other peripheral B cell subsets, B-1 B cells are self-renewing (13). These cells act as innate effector cells and produce mainly IgM and IgA in response to T cell independent antigens (14).

The Marginal zone B cells form roughly 5% of the peripheral splenic B cell population and have an IgM^{hi} IgD^{low} CD23^{low} B220$^+$ CD21^{hi} CD1d^{hi} CD9$^+$ phenotype (15). These cells develop from the MZ precursor cells which have dual potential of developing into either MZ or FO B cells (11). Notch2 signaling, signaling through BCR, BAFF-R have been shown to affect the development of T2 B cells into MZ B cells. While a lower BCR strength favors MZ B cells development (15), Notch2 signalling has also been shown to be essential for MZ B cell development (16). Marginal zone B cells do not re-circulate and reside in the marginal sinus of the spleen (17). MZ B cells, unlike other B cells having a monoreactive BCR, express a polyreactive BCR which can recognize multiple microbial molecular patterns (18, 19). Alongwith B-1 B cells, MZ B cells contribute significantly in responses to blood borne pathogens by mounting a strong IgM antibody response (17). MZ B cells, however, also have the capacity of producing high amounts of IgG2 and IgG3 antibodies (20). By expressing high levels of CD1d, MZ B cells are capable of presenting lipid antigens to invariant natural killer T cells (iNKT cells) (21). Not only do MZ B cells act as quick responders to pathogens, they also have

the capacity to transport antigen by shuttling from the marginal sinus to B cell follicles for an effective antibody response to antigen (22).

Follicular B cells are the conventional B cells which constitute the major part of the B cell population. These cells are long lived and recirculate continuously through lymph and blood (23). FO B cells develop from the T2 B cell pool in the spleen and have a B220$^+$ CD19$^+$ CD21^{mid} CD23$^+$ IgM^{lo} IgD^{hi} CD1d^{low} CD9$^-$ phenotype (15). A strong BCR signaling has been found to favor FO B cell development (15). FO B cells are present in major lymphoid organs and places where they can encounter antigen including spleen, lymph nodes and payer's patches. They often reside near the T cell rich areas which results in efficient interaction between the two cell types. Due to this specific location, FO B cells are able to mount a robust antibody response to T cell-dependent antigens (24).

TACI, BAFF-R, BCMA and their ligands

During B cell development, the cells rely heavily on external signals for further differentiation. In addition to the signal from BCR, soluble factors play a major role in B cell survival, maturation and tolerance. B cell activating factor (BAFF) from the TNF family has long been identified as an important cytokine governing B cell survival (25, 26). Multimeric forms of BAFF and another TNF-like ligand: A proliferation-inducing ligand (APRIL, TNFSF13), mainly produced by the innate immune cells, are found as membrane bound or soluble proteins. Apart from BAFF receptor (BAFF-R), the primary receptor, BAFF binds to two other receptors: transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI, TNFRSF13B) and B cell maturation antigen (BCMA, TNFRSF17). Both TACI and BCMA also recognize APRIL (26, 27) (Figure 2).

BAFF-R is the main receptor for BAFF that promotes B cell survival as the mature B cell compartment is severely affected in BAFF-R knockout mice

(28). Signaling through BAFF-R induces primarily the non-canonical NFκB pathways but also the canonical pathway although with a slower kinetics (29, 30). The activation of either pathways leads to NFκB mediated B cell survival (31, 32). BAFF-R signaling also leads to the decrease in the levels of pro-apoptotic protein Bim (33). During the development of mature B cells, transitional B cells compete for the available BAFF. Availability of BAFF thus serves as an important selection mechanism for B cell clones as in presence of excessive BAFF, low-avidity self-reactive clonotypes survive (34). BAFF, primarily through BAFF-R, overcomes anti-IgM activation induced death signals in primary B cells (33) and can possibly play a role in the survival of self-reactive clones which thus become resistant to death signals.
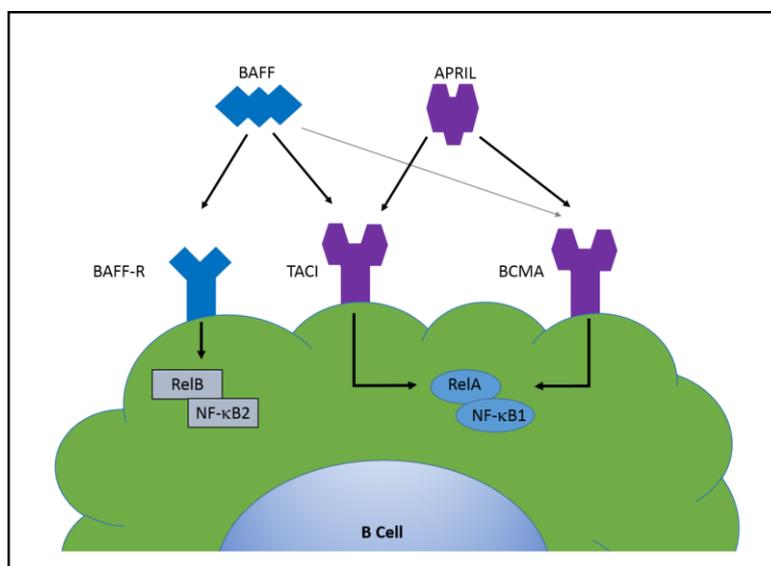
Figure 2. Schematic diagram of TACI, BAFF-R and BCMA alongwith their ligands.

TACI is another member of the TNF receptor family which is expressed on B cells. There is a subset variation in the expression of TACI with MZ and T2 B cells expressing the highest levels (35). TACI is primarily activated by oligomeric forms of BAFF and APRIL (36). TACI mainly activates the canonical NFκB pathway but can also trigger the non-canonical pathway through the activation of p100 (37). The activation of NFκB pathway leads to

similar effects on B cells as BAFF i.e., survival (37). Besides its ability to promote survival, TACI has been shown to have dual effects on mouse B cell development. TACI-/- mice display a major alteration in B cell compartment with lympho-proliferation, autoimmunity with increased numbers of hyper-reactive B cells, massive infiltration of lymphocytes in liver and kidney, suggesting a negative role of TACI in downregulating B cell activation and expansion (33-35). TACI, on the other hand, has been to shown to play an important role in mounting of both T cell-dependent and independent immune responses (38, 39). TACI also plays a positive role in B cell development, as it drives plasma cell differentiation, increases Ig production and induces isotype switch in response to the TACI-ligand APRIL (40, 41).

BCMA is another receptor which binds APRIL but has been shown to bind BAFF (42, 43). The expression of BCMA is however weak in most cell types. BCMA is upregulated in mouse plasma cells and plays an important role in the maintenance of long lived plasmablasts in the bone marrow (44, 45).

Immune response

The moment a pathogen or an external antigen is recognized, the immune system is activated leading to a series of processes, which finally clear out the pathogen. In this course of action, both the innate and the adaptive arms of the immune system are involved. The initial events that occur consist of complement activation, innate cell activation, antibody mediated clearance and secretion of soluble factors such as defensins.

Both B cells and T cells are required for an effective adaptive response to an antigen. As T cells only recognize processed antigen presented by an antigen presenting cell (APC), naïve T cells migrate through secondary lymphoid structures in search of cognate antigens presented by APCs. The movement of the T cells in the lymphoid tissues is governed by the T cell chemoattractant CXCL12 (46). These structures are specifically rich in APCs and increase the chance for a T cell to be activated. In addition to the signals through the TCR, T cells require additional signals including signals through the costimulatory

molecule CD28 as signaling solely through the TCR results in anergy (47). The costimulatory molecule CD28 cooperates with the CD80/CD86 molecules on the APC leading to a robust activation of the T cell (48).

B cells on the other hand recognize antigen through the BCR. This recognition of the antigen by a B cell induces the humoral immune response. Depending on the antigenic property, antigens for which the B cells require T cells are known a T-dependent antigens and antigens for which no T cell help is required are known as T-independent antigens.

Antigen capture and B cell activation

As mentioned above, induction of the humoral immune response requires the recognition of antigen by the B cell. Recirculating mature B cells populate secondary lymphoid organs which provide necessary structural and chemical microenvironment for antigenic encounter and activation (49). B cells migrating to the lymphoid organs such as spleen form organized structures called follicles. This directed migration of the B cells is mediated by CXCL13 chemokine produced by the stromal cells and its receptor CXCR5 (50).

B cell follicles in the secondary lymphoid organs provide a suitable environment for antigenic encounter. Here B cells can encounter soluble or large antigens on the surface of neighboring cells including macrophages, follicular dendritic cells (FDCs) and dendritic cells (51-53). How antigens of differing sizes reach the B cell follicle remains controversial. Entry through subcapsular sinus pores in lymph nodes (54) or simple diffusion (55) have been speculated to be the mechanisms for antigens of small sizes. Majority of larger antigens (>70 kDa) have limited access to the follicle. However *in vivo* evidence points to an efficient mechanism for transport of such antigens. These can be tethered to the surface of APCs and can induce efficient B cell responses (56).

After antigenic recognition through the BCR, the antigen is internalized, processed and presented on the MHC II complex. In the process the B cells migrate to the T-B cell boundary to receive signals required for full activation

of B cells (57). This migration of B cells is governed by the expression of the chemokines CCL19 and CCL21 produced by the stromal cells along with their receptor CCR7 (58). Activation of antigen specific B cells leads to the development of low affinity antibody producing short-lived plasmablasts (59). Some of the activated B cells continue to express high levels of CXCR5 and reenter the B cells follicle where they continue to proliferate and give rise to transient structures known as germinal centers.

Germinal Center

Germinal centers (GC) are transient but highly dynamic structures formed by the proliferating B cells during an immune response in the secondary lymphoid organs. These structures consist of a network of specialized cells such as T follicular helper cells (T_{FH} cells) and FDCs providing specific survival signals to the proliferating B cells (Figure 3). The complex network of helper cells provide CD40L, IL-4 (FDCs) and IL21 (T_{FH} cells) (60). Germinal centers thus provide a specific niche for the rapidly dividing B cells to undergo somatic hypermutation (SHM), differentiation to antibody-producing plasma cells and long lived memory B cells (61, 62). GC B cells express high levels of activation-induced deaminase (AID) which is responsible for the deamination of cytidine residues and leads to SHM and class switching (63, 64).

Another important activity taking place in the GCs is the deletion of self-reactive and low affinity B cell clones. This negative selection is achieved by limiting availability of cognate T_{FH} cell interaction (65) and pro survival factors such as BAFF (66). This results in the generation of high affinity B cell clones secreting class switched antibodies.
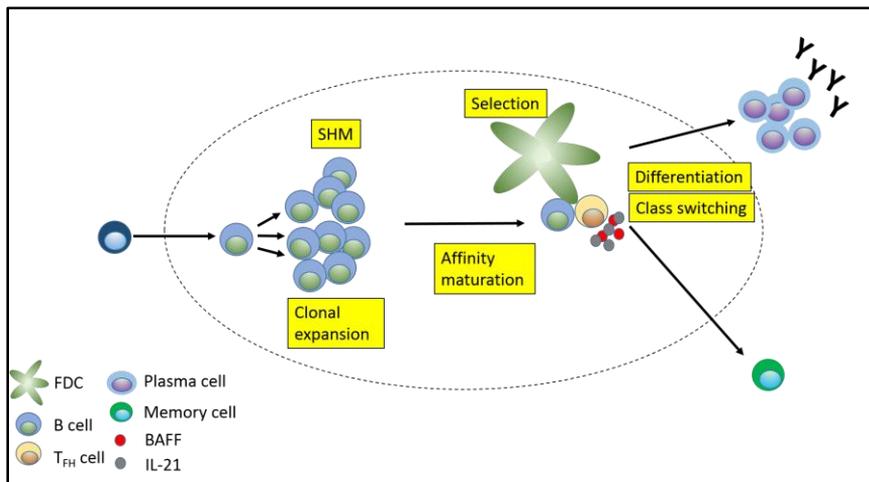
Figure 3. Schematic representation of a germinal center.

Metabolomics

Metabolism in cells and organisms is the term which describes all the biochemical processes going on to maintain life. The sum of these bio-reactions provides energy required for the functioning of vital processes and synthesizing new organic material. Metabolic processes including pathways, enzymes and structural motifs are conserved across a wide range of species signifying the importance of metabolism (67). It is a dynamic process with molecules being continuously broken down to obtain energy but at the same time new complex organic compounds are synthesized. Metabolism can thus be divided into catabolism, breakdown of molecules and anabolism, creation of complex molecules. During this breakdown and synthesis of compounds, in addition to complex byproducts, several small (<1 kDa) molecules called metabolites are produced. Collectively the metabolites present in a living system form the metabolome which is specific for that system.

Metabolomics is the comprehensive study and analysis of metabolites. These include carbohydrates, amino acids, nucleotides, organic acids and other compounds. The number of metabolites in the body is small as compared to

the total number of genes and proteins (68). Owing to the dynamic nature of metabolism, metabolite levels are affected by environmental factors such as nutrition, daily rhythms and pharmacological treatment (69, 70). Since similar metabolites are present in all organisms and tissues, it is highly unlikely to find single metabolite differences between individuals. It is this property of metabolites that makes their study multivariate as only patterns in metabolites can be studied.

It had long been understood that disease can affect body fluid composition although it has been technically challenging to identify all the metabolites. Advancement in analysis techniques has led to the rapid growth in the field. It has been recognized that metabolomics provides a possibility of identifying changes in the metabolome in course of disease and other pathological conditions (71, 72).

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the two most widely used analytical techniques in metabolomics. Although there has been development of new techniques, NMR and MS continue to dominate the metabolomics field as the two techniques allow simultaneous analysis of a multitude of small molecules in a sample (73). Due to limited sensitivity, use of NMR is limited in metabolomics. In contrast MS is more sensitive and allows for the identification of a variety of compounds in complex samples. To allow efficient analysis and identification of metabolites, MS is frequently coupled to high resolution techniques such as liquid chromatography (LC) or gas chromatography (GC) (74, 75).

Traditionally three different approaches have been adopted in metabolomics. These include: metabolic fingerprinting, metabolic profiling and targeted metabolomics (76). Among these, metabolic profiling is the most widely used as it allows for analysis of metabolites in a non-targeted manner. In this approach pre-defined set of metabolites are identified and measured. The metabolite sets are often members of specific class of compounds such as amino acids but can also include metabolites from a specific biochemical pathway. Metabolic profiling enables detection of changes in the non-targeted

parts of the metabolome which can eventually be traced to a specific biochemical pathway. The non-targeted approach of metabolic profiling often increases the possibility of identifying novel metabolic biomarkers (77, 78).

Autoimmunity

The hallmark of the immune system is its specificity in identifying and clearing foreign antigens. Several redundant checkpoints exist to prevent the immune system from self reactivity. During the development of B cells, overlapping mechanisms at several checkpoints tolerize cells against self-reaction. The checkpoints set a high threshold for B cells to be recruited into the peripheral B cell pool. As a result only 5% of newly formed B cells in the bone marrow are recruited into the peripheral pool (79).

The process of tolerance is carried out both in the bone marrow and in the secondary lymphoid organs such as spleen. Central tolerance which takes place in the bone marrow tests the specificity of the newly formed B cells against self-antigens. B cells reacting strongly to multivariate self-antigens are deleted from the repertoire or rescued through receptor editing (80, 81). In spite of the checks in the bone marrow, B cells with low affinity manage to escape and enter the naïve B cell pool (80). These low affinity B cells are regulated both intrinsically by checking BCR signal strength towards self-antigens and extrinsically by an increased competition to BAFF (34, 82).

Although strict mechanisms limit the escape of self-reactive cells into the peripheral pool, occasionally, failure of these checkpoints leads to autoimmunity – a condition where the immune system reacts to self-antigens. Most of the autoimmune diseases are characterized by the production of autoantibodies and/or autoreactive lymphocytes. Several autoimmune diseases are caused by a complex interaction of multiple factors. The major interacting components in any autoimmune disease are: genetic, environmental and regulatory (83).

The mechanistic role of the autoimmune processes is, however, not clear in most of the cases. Appearance of autoimmune disorders in families implies the underlying genetic susceptibility in such cases. The genetics in both animal models and humans is complex as many different genes or gene products interact in a complex manner. Only a few of the genes involved in autoimmune diseases are known (84). Among the genes thus far identified, few stand out due to their involvement in multiple diseases. Among these, MHC has been associated with autoimmune diseases where T cells play a major role in the pathogenesis. The association of MHC in insulin dependent diabetes mellitus (IDDM) has been proven sufficiently strong to allow for MHC typing being used as disease susceptibility marker (85).

Although most of the autoimmune diseases present a strong genetic association, genetics alone does not explain the lack of concordance in identical twins. The diseases often manifest in less than 50% of the twin siblings indicating that some non-genetic elements are associated with disease development. Environmental factors such as infections have been considered to play an important role in predisposing or triggering autoimmune diseases (86). Infections can induce autoimmunity by either mimicking a self-antigen or by an antigen-nonspecific process collectively termed as ¨bystander activation¨. Bystander activation can occur in a number of ways. For example, an increased microbial load can lead to release of sequestered proteins identified as foreign by immune cells or by altering APC phenotype leading to production of TNF-α and IL-1, two important cytokines involved in lymphocyte activation (86).

Type 1 Diabetes

Type 1 diabetes mellitus or T1D is a chronic disease characterized by T cell mediated destruction of the insulin producing β cells in the islets of Langerhans. Loss of insulin producing β cells leads to elevated blood glucose levels. The classical symptoms include high glucose levels in urine and blood, polyuria, polydipsia (87). It is the leading cause of blindness, amputations, end-stage renal disease and contributes to premature death (88). There is no

treatment available for T1D making daily injections of insulin an absolute requirement throughout the life of the patient.

The pathogenesis in T1D involves the gradual loss of insulin producing β cells. Clinical diabetes is manifested only after the loss of 80-90% of the β cell mass (89). However, before the appearance of clinical symptoms of T1D, many immune events take place. Inflammation of islets associated with the activation of self-reactive lymphocytes starts the destruction of the β cells. The insulitic infiltrates have been shown to contain CD8 T cells, CD4 T cells, B cells and macrophages (90). Another hallmark of pathogenesis in T1D is the appearance of autoantibodies before any clinical symptoms. Their appearance has been used clinically for the prediction of T1D as they represent the self-activation of B cells in T1D (89). Autoantibodies in T1D are mainly directed to four islet autoantigens: glutamic acid decarboxylase (GAD) 65, insulin, insulinoma-associated antigen-2 (IA-2) and zinc transporter 8 (ZnT8) (91). Although appearance of autoantibodies has not been linked to T1D pathogenesis directly, B cells however are important, as B cell depleting therapies have shown efficacy in T1D (92).

Metabolomics in T1D

As mentioned above, metabolome of an organism is highly dynamic and sensitive to changes induced by disease or environment. Given its dynamic nature and sensitivity to both internal and environmental factors, metabolomics has been used to detect novel biomarkers for various diseases like prostate cancer (93), type 2 diabetes (94), Alzheimer's disease (95), rheumatoid arthritis, (96) and type 1 diabetes (97).

In T1D research, metabolomics has been used to study disease progression and development of diabetic complications. However, many studies have focused on events preceding seroconversion (97, 98). Identification of biomarkers specific to islet immunity before the appearance of autoantibodies can lead to the development of interventions which can slow or stop autoimmunity.

To decipher the changes in metabolome in T1D patients, Oresic et al employed metabolomics approach to a longitudinal series of samples collected from birth to the diagnosis of overt T1D. Children who progressed to T1D showed major differences in lipid and amino acid metabolism prior to the appearance of autoantibodies (97). This study showed that an altered metabolome was visible in patients and pointed to the potential application of metabolomics in clinical diagnosis.

A similar approach was used to study serum metabolic differences in congenic bio-breeding rats (99). This study focused on metabolic events before appearance of insulitis. The authors found a diabetes prediction pattern in rats >40 days of age with changes in fatty acids, phospholipids and amino acids.

NOD as a model

The non-obese diabetic mouse is the most commonly used animal model for T1D (Figure 4). It was developed by Makino et al during the selection of a cataract prone strain (100, 101). NOD mice develop spontaneous diabetes with females being most affected indicating the protective effects of androgens in this model. The incidence of diabetes in the NOD mice ranges from 80% in females to around 30% in males (102).

Figure 6. The Non-obese diabetic mouse.

Owing to the similarity of disease pathogenesis to human T1D, the NOD mouse has been the source of insight into the various complex processes involved in T1D. Insulitis in NOD mice starts around 3-4 weeks of age and by 10 weeks most mice demonstrate severe insulitis with majority of the islets infiltrated by mononuclear cells (103). Similar to human T1D, the islet infiltrates in the NOD mice consist primarily of CD4 T cells although other cell types like CD8 T cells, NK cells, B cells, dendritic cells and macrophages have been observed as well (104).

Diabetes incidence in the NOD mouse is influenced by a number of factors. Infections, bacterial and viral, prevalent in the animal housing facilities have been shown to lower the incidence of diabetes in NOD mice. Although germ free mice show increased diabetes incidence, the presence of specific gut commensal bacterium have been shown to be protective in NOD mice (105, 106). In addition to the infection status of the NOD mouse colony, diet has also been shown to influence diabetes incidence. NOD mice exclusively fed on gluten free diet have lower diabetes incidence signifying the importance of diet in T1D (107). Introduction of gluten free diet during pregnancy and lactation in NOD mice also influences diabetes in the offspring (108). This maternal imprinting in the offspring can potentially lead to the development of interventions aimed at the preventing diabetes before birth. Although diet seems to directly influence the immune system in NOD mice, studies have shown that the effect of diet is indirect as it influences the gut microbiota which in turn can affect diabetes incidence (109).

Genetics of T1D in NOD

NOD mice have been extensively used to dissect the genetic components involved in the development of T1D. Genetic engineering has enabled the limitless manipulation of this model in providing mechanistic insights into the development of autoimmunity in general and T1D in particular. To score for potential susceptibility genetic regions involved in T1D, NOD mice have been outcrossed to disease-resistant inbred strains. This approach has identified more than 30 genetic loci on different chromosomes which have been

designated as *Idd* or insulin-dependent diabetes as summarized in Table 1 (110). The generation of subcongenic strains has revealed the presence of multiple genes in the *Idd* regions.

Among the *Idd* regions, the strongest linkage is found to the *Idd1* as its presence is essential for the development of T1D (111). *Idd1* region in NOD mice harbours the unique MHC, H2^{g7}, which in interaction with other genes contributes to T1D development. This MHC haplotype is considered unique as it lacks the I-E class II due to exon deletion in the I-E α-chain (112). In addition to the absence of I-E molecule, there is a substitution of proline and aspartic acid at positions 56 and 57 respectively of the beta chain in the I-A molecule (113). The substitution of aspartic acid in particular results in a wider peptide binding groove (114) which substantially alters the repertoire of peptides presented by this allele (115). Interestingly, a similar substitution is also seen in the human T1D susceptibility loci in DQ beta chain (116).

It is not only the class II region that is important in T1D, NOD class I alleles (H2k^d and H2d^b) have been shown to contribute to T1D (117, 118). These molecules when expressed in the NOD mice select and target autoreactive CD8$^+$ T cells (118). In addition to the class I and class II molecules, MHC class II like molecule H2-DO has been shown to be important in development of T1D in NOD mice as overexpression of this molecule in NOD prevents the development of diabetes (119). As this molecule is involved in the selection of peptides presented on MHC class II (120), modulating the H2-DO molecule can alter the repertoire of peptides presented on the MHC.

Idd3 on chromosome 3 is the non MHC *Idd* region linked to T1D in NOD. Diabetes incidence drops to about 25% in NOD mice congenic for *Idd3* indicating the importance of genes present in this region to modulate T1D (121). *Idd3* region has been narrowed to an interval of ~650 kb containing five known genes (121). Among these genes only IL2 and IL21 have been linked to T1D in both NOD and humans (122-125). Although it has been shown that islet specific expression of IL2 exacerbates diabetes, loss of IL21 receptor prevents diabetes development (126, 127).

Idd5.1 located on chromosome 1 confers the most susceptibility among other *Idd* regions in the vicinity i.e., *Idd5.2, Idd5.3* and *Idd5.4* as NOD mice congenic for this region have lower incidence of diabetes (128). *Ctla-4* gene which plays an important role in downregulating effector T-cell responses has been identified as the potential candidate gene in this region (129-131). Interestingly, this gene has been strongly linked to T1D susceptibility in humans (132).

In the case of other *Idd* regions, potential candidate genes have been identified. These include CD101 in *Idd10* (133), *Vav3* in *Idd18.3* (134), *Ptpn22* and *Cd2* in *Idd18.2* and *Idd18.4* respectively (135) and *Nramp1* in *Idd5.2* (136). In spite of identification of susceptibility loci, none of the genes have been shown to be dysregulated in NOD mice. This highlights the complexity of multiple gene interactions involved in development of T1D in NOD mice.

Table 1. *Idd* regions

Idd-region	Chr.	Region Mb	Interval Size Mb	Congenic strain effect on T1D	Reference
Idd1	17	34.133 - 35.405	1.27	B10-resistance	Todd et al., 1991,*
Idd2	9	32.308 - 98.698	66.39	B10-resistance	Pearce, 1998
Idd3	3	36.627 - 37.277	0.65	B6-resistance	Yamanouchi et al.,2007
Idd4.1	11	69.76 - 71.152	1.39	NOR-resistance	Ivakine et al., 2006
Idd4.3	11	44.553 - 55.855	11.30	C57L-resistance	Litherland et al., 2005
Idd5.1	1	60.833 - 62.840	1.96	B10-resistance	Wicker et al., 2004
Idd5.2	1	73.984 - 75.465	1.48	B10-resistance	Wicker et al., 2004
Idd5.3	1	66.530 - 70.084	3.55	B10-resistance	Hunter et al., 2007
Idd5.4	1	77.143 - 147.307	70.16	B10-susceptiblity	Hunter et al., 2007
Idd5.4	1	152.632 - 157.938	5.31	B10-susceptiblity	Hunter et al., 2007
1dd6.1	6	146.378 - 149.517	3.14	C3H-resistance	Hung et al., 2006
Idd6.2	6	137.404 - 146.386	8.99	B6-resistance	Bergman et al., 2003
Idd6.3	6	146.262 - 147.388	1.13	C3H-resistance	Hung et al., 2006
Idd7	7	21.0 - 43.0	22.0	B6-susceptiblity	Serreze et al., 2008
Idd8	14	Peak at 21.66		B10-susceptiblity	Ghosh et al., 1993; Liston et al., 2004
Idd9.1	4	128.365 - 131.179	2.81	B10-resistance	Lyons et al., 2000
Idd9.2	4	144.968 - 149.098	4.13	B10-resistance	Siegmund et al., 2000
Idd9.3	4	149.300 - 150.522	1.22	B10-resistance	Cannons et al., 2005
Idd10	3	99.699 - 100.577	0.88	B6-resistance	Penha-Goncalves et al., 2003
Idd11	4	125.017 - 132.983	7.97	B6-resistance	Brodnicki et al., 2005
Idd12	14	Peak at 35.170		B6-resistance	Liston et al., 2004; Morahan et al., 1994
Idd13	2	114.118 - 158.330	44.21	NOR-resistance	Chen et al., 2007; Serreze et al., 1998
Idd14	13	25.424 - 120.284	94.86	B6-susceptiblity	Brodnicki et al., 2003
Idd15	5	Peak at 8.798		NON-resistance	McAleer et al., 1995
Idd16	17	26.318-29.405	3.09	B6-resistance	Deruytter et al., 2004
Idd17	3	79.484 - 87.106	7.62	B6-resistance	Podolin et al., 1997
Idd18.1	3	108.993 - 109.597	0.60	B6-resistance	Lyons et al., 2001, *

Idd18.2	3	100.95 - 108.222	7.27	B6-susceptibility	Lyons et al., 2001, *
Idd18.3	3	108.24 - 109.24	1.0	B6-resistance	Fraser et al., 2010
Idd18.4	3	101.11 - 102.06	0.95	B6-resistance	
Idd19	6	117.439 - 128.468	11.03	C3H-susceptibility	Morin et al., 2006; Rogner et al., 2001
Idd20	6	83.595 - 91.990	8.39	C3H-resistance	Morin et al., 2006
Idd21.1	18	69.192 - 90.722	21.58	ABH-resistance	Hollis-Moffatt et al., 2005
Idd21.2	18	64.618 - 74.588	9.97	ABH-susceptibility	Hollis-Moffatt et al., 2005
Idd21.3	18	0 - 21.671	21.67	ABH-resistance	Hollis-Moffatt et al., 2005
Idd22	8	Peak at 90.626		ALR-resistance	Mathews et al., 2003
Idd23	17	3.924 - 26.318	22.39	B6-resistance	Deruytter et al., 2004
Idd24	17	35.340 - 44.938	6.10	B6-resistance	Deruytter et al., 2004
Idd25	4	Peak at 133.341		NOR-resistance	Reifsnyder et al., 2005
Idd26	1	19.802 - 40.319	20.52	NOR-resistance	Reifsnyder et al., 2005
Idd27	7	86.521 - 127.029	40.51	CBA-resistance	Chen et al., 2005
Not assigned	7	117.936 - 152.524	34.59	C57L-resistance	Chen et al., 2005

Table 1. Summary of size and location of *Idd* regions according to T1dbase.org. Resistance and susceptibility refers to the diabetes phenotype associated with the *Idd*-allele in the non-NOD strain.

Immune regulation in NOD

The inherent complexity of autoimmune process and presence of multiple susceptibility loci in NOD indicate the failure of multiple tolerance networks in this strain. Although the pancreatic infiltrates from NOD mice contain a variety of immune cells, T cells have been shown to play a central role as transferring T cells alone to non-diabetic mice can transfer diabetes (137). However, experiments using genetically modified NOD mice and cell transfers have highlighted the importance of other cells in the initiation and development of T1D (138-141).

It is now widely accepted that T cell intrinsic defects in the NOD mouse allow the survival of autoreactive T cells in the thymus. This break in tolerance has been attributed to the pro-apoptotic protein Bim which fails to be upregulated post TCR stimulation to mediate deletion (142). The survival among the pathogenic T cells has also been shown to be correlated with reduced expression of IL2, IL4, Fas, FasL (143, 144). Although the Treg numbers are normal in NOD mouse (145), the regulatory compartment in NOD mouse has a reduced capacity to suppress the pathogenic conventional T cells (146). NKT-cells, recognizing glycolipids presented on MHC class-I like CD1d molecule (147), also form a part of regulatory subset that is both quantitatively and qualitatively deficient in NOD mice compared to other inbred strains (148, 149).

Dendritic cells play a major role in selection of T cells leading to deletion or anergy among self-reactive clones (150). NOD dendritic cells stimulated with various signals fail to mature properly which can inhibit their ability of inducing cell death in pathogenic T cell clones (151, 152). Macrophages in the NOD mouse, similar to dendritic cells, suffer from both developmental and functional defects. NOD macrophages fail to upregulate CSF-1 and IL1 production in response to LPS stimulation (153). In addition, macrophages in NOD mice fail to clear apoptotic cells which for long have been considered to trigger the immune cascade that leads to T1D (154, 155).

The fact that B cell deficient NOD.*Igμ^null* mice fail to develop diabetes highlights the importance of B cells in the pathogenesis of T1D (138, 139). In addition, therapies targeting B cells have shown positive effects in both NOD mice and humans (92, 156). B cells in NOD act as efficient APC as a B cell specific deficiency in MHC II protects mice from developing T1D (157). This ability of NOD B cells to act as efficient APCs in T1D is dependent on their ability to bind auto-antigens through their autoreactive Ig-molecules. B cells in NOD.*IgHEL* mice which contain BCR specific for hen-egg lysozyme (HEL) fail to bind autoantigens and protect NOD mice from T1D (158). In addition, NOD B cells display increased expression of co-stimulatory molecule CD80 which in turn is associated with increased T cell stimulation (159).

The production of autoantibodies in the NOD mice is an active process as most of the autoantibodies are of the IgG isotype (160). This indicates the presence of active centers for class switching. Interestingly, spontaneously formed germinal centers have been reported from NOD and other autoimmune mice (161). Although autoantibodies have not directly been shown to be involved or transfer diabetes in NOD mice, elimination of maternally transmitted autoantibodies has been shown to protect the offspring from developing T1D (162).

Aims of the study

The aim of this study was to study the metabolic and immune perturbations seen in the NOD mouse. The specific aims of this study were to:

- Investigate the metabolic differences observed in the NOD mouse and determine the genetic basis of the increased glutamic acid in the NOD mouse.

- Dissect the functional concequence of TACI^{high} B cells in the NOD mouse focusing on *in vitro* stimulations.

- Explore the cellular and molecular mechanisms that contribute to the enhanced immune response in the NOD mouse.

Methodological considerations

Mice

To study the metabolic and immune deviations in type 1 diabetes, we used the NOD mouse. As discussed earlier, the NOD mouse mimics the disease pathogenesis as seen in humans. All the mice used in the study were bred and housed in our animal house.

NODShilt/J (NOD), NOD.*Rag2*$^{-/-}$ (NOD.Rag), C57BL/6J (B6), C57BL/6.H2g7 (B6g7) mice have been bred for more than ten generations in our own animal facility. B6 mice were chosen as controls since most of the genetics is known and the mice remain insulitis free. In addition, most of the congenic studies including NOD have utilized B6 mice as source of congenes. (NODxB6)F2 mice were established by first crossing NOD female mice with B6 male mice to obtain (NODxB6)F1 mice and then intercrossing the (NODxB6)F1 mice. Female mice of different ages were used in all the experiments. Experimental procedures were performed in strict compliance with the relevant Swedish and Institutional laws and guidelines and approved by the Umeå research animal ethic committee (A44-12;03/07/2012, A2-15;15/1/2015).

Extraction and LC-MS

Metabolite (Paper I) and amino acid (Paper II) measurements were performed at the Swedish Metabolomics centre at Swedish University of Agricultural Sciences, Umeå, Sweden. A methanol:water mixture was used to extract the metabolites. This extraction mixture essentially extracts polar metabolites. However, the disadvantage of using this mixture is the failure to extract non-polar metabolites such as lipids. Post extraction, all the amino acids were quantified using commercially available kit (Waters, Milford, US). Quantification was achieved using an internal standard (Cambridge Isotope Laboratories, US). Measurements were done using UPLC (ultra-performance liquid chromatography) coupled to a mass spectrophotometer. This method allows for efficient separation and identification of metabolites. The main

advantage of this method is that high resolution of separation is achieved requiring less or no mathematical resolution.

Genotyping

To study the genetic control of glutamic acid levels in the NOD mouse (Paper II), a sub phenotype approach was used. This approach utilizes the classical Mendelian law of segregation. By breeding NOD and the non-diabetic control B6 mice, we obtained the F1 generation. This generation, depending on the genetic control, can show an intermediate phenotype indicating a co-dominant effect from both parents. To dissect the genetics further, two F1 generation mice were bred and the corresponding F2 generation was achieved. The phenotype and the corresponding genotype segregate in this generation. The F2 generation was analyzed for the phenotype by measuring the glutamic acid levels. For the genotypic analysis, microsatellite markers were used. These oligos recognize a specific region in the genome and are known to have polymorphism among different strains. The markers were spaced at ~10cM, a distance considered to account for possible crossovers.

Protein expression

To analyze multiple parameters on a single cell basis, flow cytometry was used. This method provides speed but also a high degree of sensitivity. The method uses the principles of light scatter, electron excitation and fluorescence. This method was used to detect specific cell subsets and also to provide information about cell proliferation and survival. This was achieved by staining the cell with specific fluorescently labeled antibodies and dyes. In some instances, cells were fixed with paraformaldehyde and permeabilized with detergent prior to staining. This allows for the staining of intracellular proteins.

Western blotting is another technique to analyze protein expression. The main advantage of this method is the possibility to determine size of the protein of interest. The proteins are separated on a gel and then transferred to a membrane. The detection is achieved using specific antibodies. A loading

control is often used to verify equal protein loading in all samples. A size ladder may also be included to verify the right size of target protein. We analyzed the membrane glutamate transporters after making whole lysates from liver and kidney tissues.

In vitro B cell culture and stimulation

An *in vitro* system allows us to study specific function of a cell under strict conditions. We isolated untouched B cells from mice spleen and cultured them with specific condition and stimulations. It was important to isolate untouched B cells as staining with antibodies for isolation can lead to undesirable signaling events downstream. As a positive control, B cells were stimulated by LPS, a protein of bacterial origin, to which the cells react in an unspecific manner.

Imunization and ELISA

To study the cells and pathways involved in an immune response, a model antigen is needed which elicits a strong but stable immune response. NOD and B6 mice were immunized with HEL, one of the most commonly used antigen, emulsified in Freund's incomplete adjuvant intraperitoneally. This antigen elicited a strong immune response in the NOD mouse which was stable over few weeks.

To measure the antibody response to the immunized antigen, enzyme linked immunosorbent assay (ELISA) was used. ELISA is a frequently used technique both in the lab and clinics. Not only is ELISA very sensitive, it can also be used for quantification of proteins or antibodies present in a sample (serum in this case). The method utilizes the specificity of antibodies and enzyme assays. Both IgG and IgM antibodies were analyzed in the serum post immunization. Pre-immunization serum was used as negative control.

To determine the affinity of antigen specific antibodies, a hapten (nitrophenyl) bound HEL was used. For detection, NP_4-BSA (where four NP molecules are bound to BSA) and NP_{20}-BSA were used. Antibodies binding to

NP_4-BSA are considered high affinity while antibodies binding to NP_{20}-BSA consist of both high and low affinity antibodies. A ratio of NP_4-BSA/NP_{20}-BSA gives a qualitative measurement of the affinity of the antibodies produced.

B and T cell isolation and adoptive transfers

Adoptive cell transfers to immune deficient mice were made to study the cells playing a major role in the immune response. Untouched B and T cells were isolated from HEL primed NOD or B6g7 mice using commercially available kits and transferred to the immune deficient NOD.Rag mice. In some experiments, FACS sorting was employed to isolate untouched B and T cells prior to transfer. This allows for the study of only the transferred cells as the recipient mice lack both B and T cells. Recipient mice were later immunized with HEL and the antibody response measured by ELISA.

Immunofluorescence

To determine the histological location of germinal centres in the spleen, we stained 8μm thick cryosections obtained from frozen NOD and B6 spleens. This method allows for visualization of cells and structures that are formed during an active immune response. The location of these cells and structures are important as they dictate the microenvironment and signals that are essential for SHM and class switching. However, we can only observe one layer at a given time point and hence lose out informations from subsequent timepoints.

Statistics

In Paper II, for association analysis χ2 test was used. This test compares the observed data with the expected data. The χ2 test gives the ¨goodness to fit¨ between the observed and expected. It always tests the null hypothesis that states that there is no difference between the observed and expected frequencies. In the χ2 test, a *p*-value of <0.05 was considered significant. For most grouped analysis, we have used Two-way ANOVA, a *p*-value of <0.05 was considered significant.

Results and Discussion

Paper I & II: Altered metabolome in the NOD mouse: Genetic regulation and consequence of increased glutamic acid

In metabolomics, the chemical fingerprint of biological fluids including intracellular compartments is studied. The sensitive nature of the metabolic phenotype towards various factors such as age, lifestyle, nutrition and gut microbiome makes it possible to determine variations associated with pathological factors associated with disease (163-165). Metabolic perturbations early in the childhood may thus reflect influences of both genetic and environmental factors that may affect later susceptibility to chronic disease (166).

As mentioned earlier, longitudinal studies involving genetically susceptible children have highlighted the metabolic changes prior to the appearance of autoantibodies and diabetes development (97). This study highlighted the importance of early diagnosis as the appearance of autoantibodies indicates the activation of the immune system.

In paper I, we hypothesized that NOD mice would display similar metabolic profile as T1D progressors. In accordance with the human metabolic profile, NOD mice grouped separately over different ages compared to the non-diabetic control strain C57BL/6 (B6) (Paper I, Figure 1 A-C). Samples were taken at specific time points to study metabolic changes associated with pancreatic inflammation. The 0-3 week old mice were considered autoimmunity free (insultis free) and the only difference between these two ages was weaning. Four to five old mice were assumed to have mild or early insulitis (103). By 6 weeks the mice develop heavy insulitis. None of the mice developed overt diabetes during the study.

Using a non-targeted strategy, levels of 89 metabolites were identified. The separation between NOD and B6 was already evident in newborn mice, indicating a genetic control of metabolites (Paper I, Figure 1 C, 0w). In general, NOD mice had increased levels of amino acids post weaning, a trend that was

stable till week 15 (Paper I, Figure 2). Among the increased amino acids, the branched chain (BCAA) group of isoleucine and leucine were already increased at birth and continued to be increased post weaning. BCAAs have been associated with increased insulin secretion (167) which can lead to ER stress among β-cells (168). Furthermore, using backward variable selection, metabolites belonging to TCA cycle were found to be among the most discriminative between the two groups (Paper I, Figure 3). Interestingly, among the most discriminative metabolites, glutamic acid was increased in the NOD mice. Glutamic acid is an important amino acid as it links the amino acid metabolism with the TCA energy cycle via alpha-ketoglutaric acid, a metabolite lower in NOD mice. Increased serum glutamic acid has been previously associated to T1D progression among children and NOD mice (97, 169). To explore the genes/enzymes that are possibly involved in the observed metabolic variations, we performed a detailed bioinformatics analysis where enzymes in the metabolic pathway were identified to be present in previously described *Idd* regions (Paper I, Figure 4).

In addition, the enzyme sequences were analyzed for any genetic differences using the dbSNP database (170). In total 1 274 SNPs were found, out of which 9 SNPs were present in exons, 71 in the 59, 39 UTRs and the remaining 1 194 in the intron regions (Paper I, Table 1). Although not tested *in vivo*, we hypothesize that SNPs in some of these enzymes may be involved in regulating enzyme function. Interestingly, SNPs present in introns can affect splicing and enzyme activity (171) as well as expression levels of proteins (172).

In paper II, we analyzed the genetic basis for the elevated levels of glutamic acid, one of the most discriminative metabolite in our previous study (Paper I), in NOD mice. Glutamic acid is the most common neurotransmitter in mammalian brain. It has previously been shown that glutamic acid can exert toxicity through over-activation of its receptors, the phenomenon termed as excitotoxicity (173). In addition glutamic acid is also toxic to insulin producing islets at levels which are physiologically relevant (174). It is plausible that increased level of glutamic acid potentiates beta-cell apoptosis in NOD mice. Increased apoptosis and defective clearance by macrophages can thus form a

part of the triggering mechanism leading to activation of immune system (154, 175).

To perform a genetic analysis of the elevated glutamic acid trait, we first measured glutamic acid levels in four week old females from (C57BL/6xNOD)F1 and (NODxC57BL/6)F1 cohorts. The use of two cohorts was done to account for the effect of mother on the metabolome of the pups. This imprint on the offspring can in part be modulated by the maternal microbiota as newborns are colonized early in life by the microbiota encountered during birth (176). However, no significant difference in glutamic acid level was observed between the two cohorts (Figure 8, unpublished data).
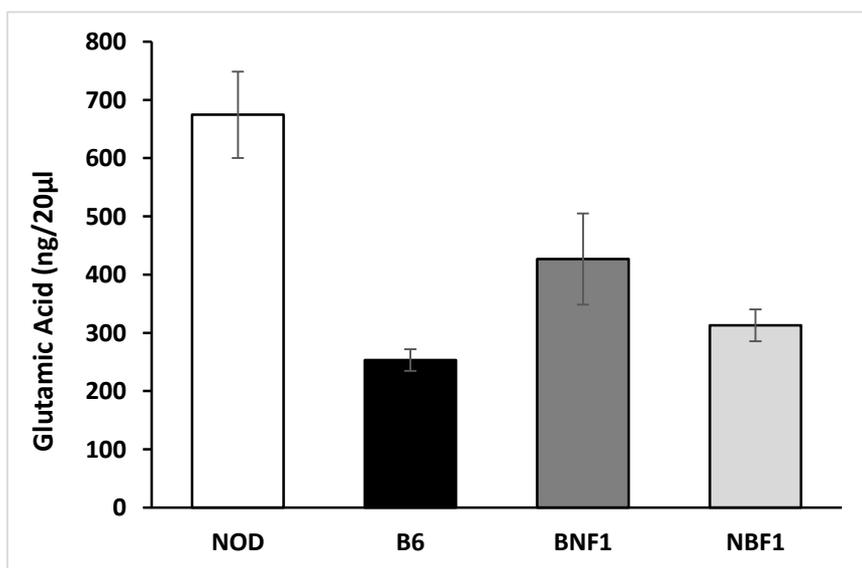

Figure 8. Glutamic acid levels in parental and F1 cohorts.

Intermediate levels of glutamic acid were found in both the F1 cohorts, indicating a codominant effect of the parental strains on the phenotype. To further analyze the genetic components involved, we bred a (C57BL/6xNOD)F2 cohort and measured the serum glutamic acid levels. The glutamic acid levels in the (C57BL/6xNOD)F2 cohort were normally

distributed with a variance larger than the parental strains (Paper II, Figure 2).

We performed a genetic screen covering the whole genome of the F2 cohort. The mice in this cohort were phenotypically classified as NOD-like or B6-like based on the glutamic acid levels. The classification was done as selective genotyping of individuals with extreme phenotype imparts similar power as genotyping the whole cohort (177, 178). The genetic association of the glutamic acid levels was analyzed by χ^2 test. Significant association ($p<0.05$) was observed to six chromosomes of which three regions were within *Idd22, Idd2* and *Idd4* (Paper II, Table 1). Correlation to multiple regions/genes was in line with the normal distribution of the glutamic acid levels among the F2 cohort.

In the *in silico* analysis, enzymes, receptors and transporters associated with glutamic acid metabolism were analyzed. These were present in and/or around the mapped regions (Paper II, Table 2). Search for genetic differences in potential genes yielded 143 SNPs. Among the potential enzymes involved, Alanine aminotransferase (ALT) and glutamic-oxaloacetic transaminase (AST) were considered important as they link glutamic acid to the energy cycle via formation of alpha-ketoglutarate, a metabolite present in low levels in NOD mice. Interestingly, ALT levels are low in NOD serum which might affect glutamic acid catabolism (179, 180). Supporting our linkage data and *in silico* analysis, we found the expression of high affinity transporter EAAC1 to be increased in NOD liver. The increased expression of EAAC1 can possibly enhance the absorption of glutamic acid from the hepatic portal vein. Finally, we tested the effect of the glutamic acid levels on beta cells. Culturing insulin producing islets from NOD mice in physiologically relevant glutamic acid levels (1mM), we observed increased apoptosis. In addition, beta cells were specifically sensitive to glutamic acid mediated toxicity, compared to the other islet derived cells, as was evident from the flow cytometric analysis (Paper II, Figure 4). Apoptosis, as mentioned earlier, can initiate autoimmune events as novel self antigens become available which have the ability of activating the immune system. In addition apoptotic cells have the ability to activate interferon alpha production from plasmacytoid dendritic cells (181). An early

interferon signature has been detected in both humans and NOD mice strengthening the case for apoptotic cell mediated activation of immune system (182, 183).

Paper III: Consequence of TACI^{high} B cells in the NOD mouse

Our lab has previously demonstrated that splenocytes from NOD mice contain a high proportion of TACI^{high} B cells as compared to B6 mice and this phenotype has been shown to map to regions on chromosome 1 and 8 (184). The role of TACI in B cell homeostasis and function has however been contradictory. On one hand, TACI^{-/-} mice display major lymphoproliferation and autoimmunity, on the other hand TACI plays an important role in driving plasma cell differentiation, Ig production and isotype switching (32, 34, 40, 41). The dual role that TACI seems to play in B cell homeostasis might be attributed to the availability of BAFF for BAFF-R in absence of TACI. Presence of two BAFF binding sites as compared to one for BAFF-R, allows TACI to bind higher orders of BAFF making it unavailable for signaling through the BAFF-R (36). Interestingly serum BAFF levels are increased in TACI knockout mice highlighting the importance of BAFF binding capability of TACI (66). Thus in absence of TACI, more BAFF can bind to BAFF-R leading to cell proliferation. On the other hand by sustaining Blimp-1 expression in plasmablasts, TACI promotes Ig production and class switching (39).

To confirm the previous mapping of the TACI[high] phenotype, we bred double congenic NOD.*C1/Idd22* mice containing B6 derived genetic regions on chromosome 1 and 8 (Paper III, Figure 1A). The splenic B cells compartment in these mice show a significant reduction in percentage of TACI^{high} B cells compared to regular NOD mice (Paper III, Figure 1D), thus supporting our genetic mapping. To further evaluate any potential genes present in these two regions, breeding of NOD.*C1/Idd22* mice with smaller B6 derived regions was initiated. However, after multiple attempts over several years, the establishment of double congenic NOD.*C1/Idd22* strain was not possible, as the mice appeared to be infertile.

To analyze the functional effects of the increased proportion of TACI^{high} B cells in NOD mice, we used an *in vitro* system. We stimulated B cells from NOD and B6 with APRIL, with media serving as control. NOD B cells produced higher amounts of IgG and IgA in response to APRIL (Paper III, Figure 2A-B). The relative production of IgM in both the strains was similar (Paper III, Figure 2C). Analyzing the number of CD138^{+} plasma cells/plasmablasts, both strains showed significant increase in the number of CD138^{+} cells. However, NOD mice showed more than 10-fold increase in plasma cells upon APRIL stimulation (Paper III, Figure 3).

APRIL and BAFF both bind TACI and downregulation of TACI in germinal center B cells has been shown to create a BAFF poor region (66). This essentially creates competition for the pro survival BAFF cytokine and as a result only high affinity clones survive (34). As NOD mice have high proportion of TACI^{high} B cells, we analyzed expression of TACI on germinal center B cells from NOD and B6. Increased numbers of B220^{+} CD95^{+} GL7^{+} TACI^{+} B cells were found in unimmunized NOD and B6 mice. To determine if the loss of TACI from germinal center B cells is in response to antigen, we analyzed germinal center B cells from HEL immunized NOD and B6 mice. Percentage of B220^{+} CD95^{+} GL7^{+} TACI^{+} B cells post immunization decreased in B6 as has been reported. However, NOD mice continued to display increased numbers of B220^{+} CD95^{+} GL7^{+} TACI^{+} B cells indicating a general failure in downregulation of TACI on germinal center B cells (Paper III, Figure 4A-B).

As TACI failed to be downregulated in NOD mice and this molecule potentially can capture and thus be a source of BAFF signaling, we were interested to analyze the presence of BAFF in the germinal center. Indeed, immunofluorescence staining of spleen sections from hen egg lysozyme immunized mice revealed increased presence of BAFF in germinal centers from NOD mice. Germinal centers from unimmunized NOD mice also stained positively for BAFF (Paper III, Figure 5A-B). It is yet to be proven if the increased staining for BAFF in the germinal center is due to TACI^{+} B cells or if some other cells residing in the germial center could mediate this function.

However, since a germinal center mainly consists of B cells, T cells and FDCs, it is plausible that increased BAFF binding in the germinal center maybe a consequence of TACI^{+} B cells present at this location. To test whether increased presence of BAFF in germinal centers would affect the antibody affinity maturation, we utilized the response of B cells towards haptenated antigens. Challenging NOD and B6 mice with NP-HEL resulted in affinity maturation in both strains (Paper III, Figure 6A-B), NOD however produced higher titres of low affinity antibodies as compared to B6 mice (Paper III, Figure 6C). In line with our findings, an increased presence of BAFF in the germinal centers can enhance survival of low affinity and autoreactive clones (34, 66) supporting our hypothesis that increased presence of TACI^{+} B cells in the GCs and consequent BAFF binding leads to altered affinity maturation in NOD mice.

Paper IV: Cellular players in the enhanced immune response in the NOD mouse

We and others have shown that NOD mice mount an enhanced and prolonged response to both self and non-self protein antigens (185-187). This response involves antigen specific IgG1 production as well as a total increase in IgE levels in serum (186). In addition, upon re-challenge with the immunizing peptide, NOD mice develop severe anaphylaxis (186, 188), a manifestation we have observed ourselves (data not shown). To study the cellular factors that play a major role in this response, we immunized NOD and B6 mice with HEL emulsified in Freund's incomplete adjuvant intraperitoneally. This specific composition and route of administration was chosen to avoid the anaphylaxis reaction from NOD mice. High levels of HEL specific IgG antibodies were present in sera from NOD mice one week post immunization and increased steadily at least up to eight weeks (Paper IV, Figure 1A) post immunization. The antibody response to HEL was dominated by the IgG1 isotype (Paper IV, Figure 1B) although a delayed appearance of IgG2b was also observed (data not shown).

The steady increase in antigen specific antibodies observed in NOD can result from either increased turnover of antibody forming cells or a defect in the antibody clearance mechanism. Macrophages in NOD mice carry a defective Fc gamma RII gene resulting in increased serum IgG levels (189). To determine if the increase in antigen specific antibody results from defective clearance, we injected NOD and B6 mice with anti-KLH IgG1 antibodies. The use of anti-KLH antibodies allowed for their detection from whole serum using ELISA. We, however, did not find any difference in clearance of this antibody between NOD and B6 (Paper IV, Figure 2).

As the response towards HEL was apparent at one week post immunization, this led us to analyze the germinal center formation in NOD. B cells have been shown to re-use the pre-existing germinal centers and obtain pre-existing T cell help (190). We analyzed germinal centers both in unimmunized and HEL immunized NOD and B6 mice. Spleens from unimmunized NOD mice contained more number of germinal centers (Paper IV, Table 1) and PNA+ B cells as observed with flowcytometry (Paper IV, Figure 3B) as compared to B6 mice. However, this difference was not seen after immunization (data not shown). It is plausible that the B cells in NOD mice reutilize pre-existing germinal centers resulting in no apparent increase in their numbers. In addition, readily available T cell help and other survival factors can result in the rapid response seen within one week in NOD mice.

To dissect the cellular players involved in this phenotype, we adoptively transferred purified B and T cells from HEL primed NOD and B6.g7 mice to NOD.Rag mice. Upon re-challenge with HEL, mice which received purified B cells from NOD irrespective of T cell source and splenocytes from B6.g7 and NOD mice responded (Paper IV, Figure 5). This indicates that intrinsic B cell defects contribute to the phenotype. Although B6.g7 mice respond moderately to vaccination (Paper IV, Figure 1C) (187), the NOD milieu available in NOD.Rag mice seems to promote cells of B6.g7 origin to respond to HEL antigen (Paper IV, Figure 5). These findings indicate the need for both cells and milieu of NOD origin to contribute to the phenotype. This robust response

to a single immunization of non-self antigen indicates intrinsic defects in the B cell compartment.

Conclusions

In this thesis the various deviations seen in the metabolic and the immune system of the NOD mice were studied. The major findings from the thesis can be summarized as follows:

- Serum metabolome in NOD mice varied significantly from B6 mice and this variation seemed to be stable throughout the ongoing autoimmunity. The major pathway affected included the TCA cycle.
- Serum glutamic acid was increased in NOD mouse compared to B6 mouse. This trait mapped to six chromosomes of which three regions were within *Idd22, Idd2* and *Idd4*. In addition, glutamic acid was shown to be toxic to beta-cells *in vitro*.
- APRIL stimulated NOD splenic B cells displayed increased numbers of plasma cells and produced higher amounts of IgG and IgA compared to B cells from C57BL/6 mice. NOD germinal center B cells failed to downregulate TACI and bound more BAFF.
- NOD mice showed an enhanced and prolonged response to hen egg lysozyme challange. This response was of the IgG1 isotype and required B cells of NOD origin.

Based on the finding in this thesis, a proposed model how the metabolic and immune systems interact, the following model can be proposed.
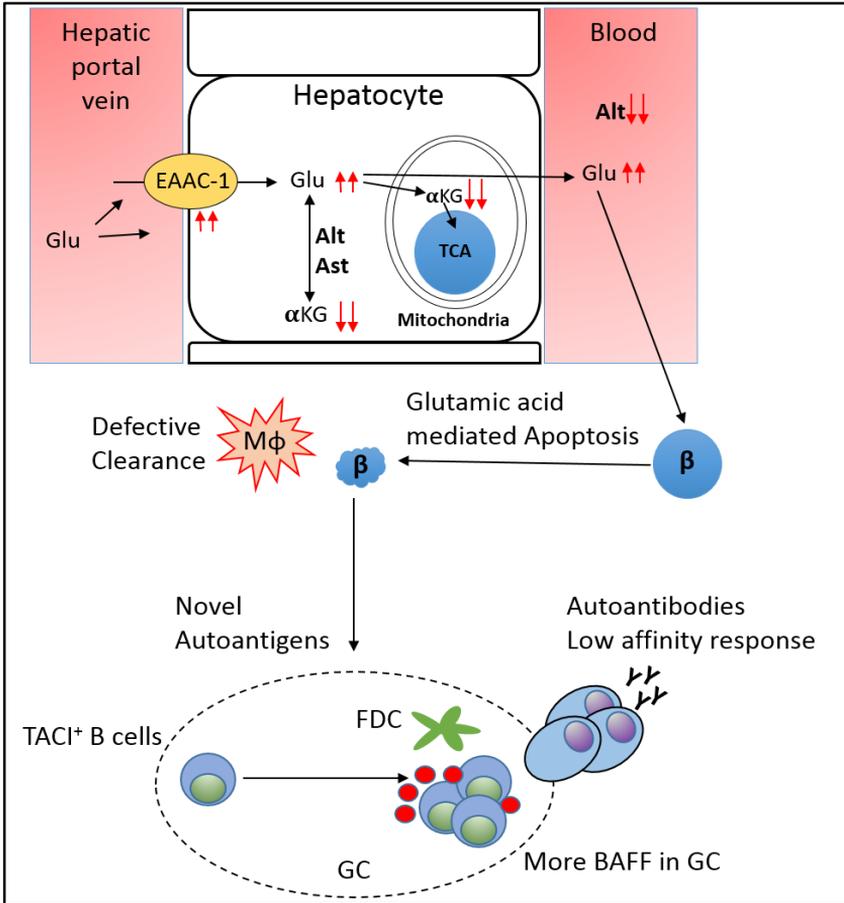
Figure 9. Metabolic and Immune interaction. The increased expression of EAACI leads to enhanced glutamate absorption from portal vein from the intestine. A defect in glutamate metabolizing enzymes results in increased levels of glutamate in blood. This increased glutamate is toxic to beta cells in the pancreas. NOD macrophages are defective in clearing out apoptotic cells and as a result self-antigens become available and activates B and T cells in the germinal center. In addition, the increased percentage of TACI^{high} B cells and BAFF in germinal center of NOD mice favors survival of autoreactive B cells and production of autoantibodies.

Acknowledgements

The road to PhD has been a long one but with all the people I have had around me made it much easier and FUN.

Kristina, thank you for giving me this chance to work with you. It has been a fantastic time being your PhD. Your energy at work and way of teaching is truly inspiring. Your guidance, training in the lab and encouragement has helped me to work independently and think as a researcher. Thank you for always answering my emails and questions even at odd hours.

Per-Arne, thank you for being my co-supervisor. Your ideas and thoughtful insights during group meetings and journal clubs have been truly inspiring.

Radha, it would have been a tough road if it wasn't for your discussions and encouragement. Your ideas and knowledge for our projects has been amazing. **Mia** thank you for all the help and support. Your energy at work was truly inspiring. It was a lot of fun working with you.

Damini, thank you for the lovely friendship over these years. Your cheerful and caring nature has always lifted my spirits.

Shrikant, thank you for your support and suggestions. Thank you for always being ready to help whenever I needed it. Thank you for keeping me up to date with all the cricket matches.

Anne, thank you for all your help during the whole time. Immunology would be in chaos if it wasn't for you! Thank you for always listening with interest and helping me out in my Swedish. **Mariane**, thank you for introducing me to FACS.

Greg, our DOCTOR in the PhD room. Thank you for all the help and support during these years. **David**, thank you for the support and help during my PhD. If it wasn't for you, I would have never caught my first GÄDDA. We sure need to go for more fishing trips.

It would have been possible to do all this with the love, support and prayers of my beloved **MOM** and **DAD.** Thank you for believing in me and letting me go after my dream. My saying thank you is not enough for all the sacrifices you have made for me. Thank you my lovely brothers **VIHAJ** and **WAJAHAT.** Your love and support has always kept me going. Thank you to all my family members and friends back in Kashmir, **Adeel, Zarnain, Zartab, Uncle, Aunty, Ammi, Daddy, Bada Mamu, Chota Mamu, Maami, Pyaari, Saqib, Umer and Nazaan.**

Last but not the least, thank you my four legged babies **BELLA** and **ZELDA**. You have given me immense joys. Thank you for listening and giving company at odd hours.

References

1.   Retief, F. P., and L. Cilliers. 1998. The epidemic of Athens, 430-426 BC. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* 88: 50-53.

2.   Haskins, K., J. Kappler, and P. Marrack. 1984. The major histocompatibility complex-restricted antigen receptor on T cells. *Annu Rev Immunol* 2: 51-66.

3.   Kincade, P. W. 1981. Formation of B lymphocytes in fetal and adult life. *Advances in immunology* 31: 177-245.

4.   Li, Y. S., R. Wasserman, K. Hayakawa, and R. R. Hardy. 1996. Identification of the earliest B lineage stage in mouse bone marrow. *Immunity* 5: 527-535.

5.   Hardy, R. R., C. E. Carmack, S. A. Shinton, J. D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med* 173: 1213-1225.

6.   Oettinger, M. A., D. G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* 248: 1517-1523.

7.   Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature* 302: 575-581.

8.   Ehlich, A., V. Martin, W. Muller, and K. Rajewsky. 1994. Analysis of the B-cell progenitor compartment at the level of single cells. *Curr Biol* 4: 573-583.

9.   Tussiwand, R., N. Bosco, R. Ceredig, and A. G. Rolink. 2009. Tolerance checkpoints in B-cell development: Johnny B good. *European journal of immunology* 39: 2317-2324.

10.   Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J Exp Med* 190: 75-89.

11.   Srivastava, B., W. J. Quinn, 3rd, K. Hazard, J. Erikson, and D. Allman. 2005. Characterization of marginal zone B cell precursors. *J Exp Med* 202: 1225-1234.

12.   Rowley, B., L. Tang, S. Shinton, K. Hayakawa, and R. R. Hardy. 2007. Autoreactive B-1 B cells: constraints on natural autoantibody B cell antigen receptors. *Journal of autoimmunity* 29: 236-245.

13.   Stall, A. M., S. M. Wells, and K. P. Lam. 1996. B-1 cells: unique origins and functions. *Seminars in immunology* 8: 45-59.

14.   Martin, F., and J. F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunological reviews* 175: 70-79.

15.   Pillai, S., and A. Cariappa. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nature reviews. Immunology* 9: 767-777.

16.   Saito, T., S. Chiba, M. Ichikawa, A. Kunisato, T. Asai, K. Shimizu, T. Yamaguchi, G. Yamamoto, S. Seo, K. Kumano, E. Nakagami-Yamaguchi, Y. Hamada, S. Aizawa, and H. Hirai. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* 18: 675-685.

17.   Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 14: 617-629.

18.   Martin, F., and J. F. Kearney. 2002. Marginal-zone B cells. *Nature reviews. Immunology* 2: 323-335.

19.   Cooper, M. D., and M. N. Alder. 2006. The evolution of adaptive immune systems. *Cell* 124: 815-822.

20.   Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol* 1: 31-36.

21.   Leadbetter, E. A., M. Brigl, P. Illarionov, N. Cohen, M. C. Luteran, S. Pillai, G. S. Besra, and M. B. Brenner. 2008. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* 105: 8339-8344.

22.   Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, Jr., and J. G. Cyster. 2008. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat Immunol* 9: 54-62.

23.   Hao, Z., and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J Exp Med* 194: 1151-1164.

24. Allman, D., and S. Pillai. 2008. Peripheral B cell subsets. *Current opinion in immunology* 20: 149-157.

25. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, D. Valmori, P. Romero, C. Werner-Favre, R. H. Zubler, J. L. Browning, and J. Tschopp. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* 189: 1747-1756.

26. Mackay, F., P. Schneider, P. Rennert, and J. Browning. 2003. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 21: 231-264.

27. Mackay, F., and J. L. Browning. 2002. BAFF: a fundamental survival factor for B cells. *Nature reviews. Immunology* 2: 465-475.

28. Schiemann, B., J. L. Gommerman, K. Vora, T. G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M. L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111-2114.

29. Morrison, M. D., W. Reiley, M. Zhang, and S. C. Sun. 2005. An atypical tumor necrosis factor (TNF) receptor-associated factor-binding motif of B cell-activating factor belonging to the TNF family (BAFF) receptor mediates induction of the noncanonical NF-kappaB signaling pathway. *J Biol Chem* 280: 10018-10024.

30. Sasisekharan, R., Z. Shriver, G. Venkataraman, and U. Narayanasami. 2002. Roles of heparan-sulphate glycosaminoglycans in cancer. *Nat Rev Cancer* 2: 521-528.

31. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat Immunol* 3: 958-965.

32. Mackay, F., and P. Schneider. 2008. TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties. *Cytokine Growth Factor Rev* 19: 263-276.

33. Craxton, A., K. E. Draves, A. Gruppi, and E. A. Clark. 2005. BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway. *J Exp Med* 202: 1363-1374.

34. Lesley, R., Y. Xu, S. L. Kalled, D. M. Hess, S. R. Schwab, H. B. Shu, and J. G. Cyster. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* 20: 441-453.

35. Ng, L. G., A. P. Sutherland, R. Newton, F. Qian, T. G. Cachero, M. L. Scott, J. S. Thompson, J. Wheway, T. Chtanova, J. Groom, I. J. Sutton, C. Xin, S. G. Tangye, S. L. Kalled, F. Mackay, and C. R. Mackay. 2004. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *Journal of immunology* 173: 807-817.

36. Bossen, C., T. G. Cachero, A. Tardivel, K. Ingold, L. Willen, M. Dobles, M. L. Scott, A. Maquelin, E. Belnoue, C. A. Siegrist, S. Chevrier, H. Acha-Orbea, H. Leung, F. Mackay, J. Tschopp, and P. Schneider. 2008. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood* 111: 1004-1012.

37. Chang, S. K., B. K. Arendt, J. R. Darce, X. Wu, and D. F. Jelinek. 2006. A role for BLyS in the activation of innate immune cells. *Blood* 108: 2687-2694.

38. von Bulow, G. U., J. M. van Deursen, and R. J. Bram. 2001. Regulation of the T-independent humoral response by TACI. *Immunity* 14: 573-582.

39. Tsuji, S., C. Cortesao, R. J. Bram, J. L. Platt, and M. Cascalho. 2011. TACI deficiency impairs sustained Blimp-1 expression in B cells decreasing long-lived plasma cells in the bone marrow. *Blood* 118: 5832-5839.

40. Castigli, E., S. A. Wilson, S. Scott, F. Dedeoglu, S. Xu, K. P. Lam, R. J. Bram, H. Jabara, and R. S. Geha. 2005. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med* 201: 35-39.

41. Castigli, E., S. A. Wilson, A. Elkhal, E. Ozcan, L. Garibyan, and R. S. Geha. 2007. Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation. *J Allergy Clin Immunol* 120: 885-891.

42. Shu, H. B., and H. Johnson. 2000. B cell maturation protein is a receptor for the tumor necrosis factor family member TALL-1. *Proc Natl Acad Sci U S A* 97: 9156-9161.

43. Marsters, S. A., M. Yan, R. M. Pitti, P. E. Haas, V. M. Dixit, and A. Ashkenazi. 2000. Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI. *Curr Biol* 10: 785-788.

44. Deng, S., T. Yuan, X. Cheng, R. Jian, and J. Jiang. 2010. B-lymphocyte-induced maturation protein1 up-regulates the expression

of B-cell maturation antigen in mouse plasma cells. *Mol Biol Rep* 37: 3747-3755.

45.   O'Connor, B. P., V. S. Raman, L. D. Erickson, W. J. Cook, L. K. Weaver, C. Ahonen, L. L. Lin, G. T. Mantchev, R. J. Bram, and R. J. Noelle. 2004. BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med* 199: 91-98.

46.   Bleul, C. C., R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti, and T. A. Springer. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 184: 1101-1109.

47.   Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T cell activation. *Annu Rev Immunol* 27: 591-619.

48.   Bour-Jordan, H., J. H. Esensten, M. Martinez-Llordella, C. Penaranda, M. Stumpf, and J. A. Bluestone. 2011. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunological reviews* 241: 180-205.

49.   von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nature reviews. Immunology* 3: 867-878.

50.   Cyster, J. G. 2010. B cell follicles and antigen encounters of the third kind. *Nat Immunol* 11: 989-996.

51.   Carrasco, Y. R., and F. D. Batista. 2007. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* 27: 160-171.

52.   Suzuki, K., I. Grigorova, T. G. Phan, L. M. Kelly, and J. G. Cyster. 2009. Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med* 206: 1485-1493.

53.   Qi, H., J. G. Egen, A. Y. Huang, and R. N. Germain. 2006. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* 312: 1672-1676.

54.   Clark, S. L., Jr. 1962. The reticulum of lymph nodes in mice studied with the electron microscope. *The American journal of anatomy* 110: 217-257.

55.   Gretz, J. E., C. C. Norbury, A. O. Anderson, A. E. Proudfoot, and S. Shaw. 2000. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized

conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J Exp Med* 192: 1425-1440.

56.   Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature* 411: 489-494.

57.   Mitchison, N. A. 2004. T-cell-B-cell cooperation. *Nature reviews. Immunology* 4: 308-312.

58.   Reif, K., E. H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J. G. Cyster. 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* 416: 94-99.

59.   Cunningham, A. F., F. Gaspal, K. Serre, E. Mohr, I. R. Henderson, A. Scott-Tucker, S. M. Kenny, M. Khan, K. M. Toellner, P. J. Lane, and I. C. MacLennan. 2007. Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection. *Journal of immunology* 178: 6200-6207.

60.   Goodnow, C. C., C. G. Vinuesa, K. L. Randall, F. Mackay, and R. Brink. 2010. Control systems and decision making for antibody production. *Nat Immunol* 11: 681-688.

61.   Allen, C. D., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* 27: 190-202.

62.   MacLennan, I. C. 1994. Germinal centers. *Annu Rev Immunol* 12: 117-139.

63.   Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 102: 553-563.

64.   Pavri, R., and M. C. Nussenzweig. 2011. AID targeting in antibody diversity. *Advances in immunology* 110: 1-26.

65.   Schwickert, T. A., G. D. Victora, D. R. Fooksman, A. O. Kamphorst, M. R. Mugnier, A. D. Gitlin, M. L. Dustin, and M. C. Nussenzweig. 2011. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J Exp Med* 208: 1243-1252.

66.   Goenka, R., A. H. Matthews, B. Zhang, P. J. O'Neill, J. L. Scholz, T. S. Migone, W. J. Leonard, W. Stohl, U. Hershberg, and M. P. Cancro. 2014. Local BLyS production by T follicular cells mediates retention

of high affinity B cells during affinity maturation. *J Exp Med* 211: 45-56.

67. Peregrin-Alvarez, J. M., C. Sanford, and J. Parkinson. 2009. The conservation and evolutionary modularity of metabolism. *Genome Biol* 10: R63.

68. Aboud, O. A., and R. H. Weiss. 2013. New opportunities from the cancer metabolome. *Clin Chem* 59: 138-146.

69. Krug, S., G. Kastenmuller, F. Stuckler, M. J. Rist, T. Skurk, M. Sailer, J. Raffler, W. Romisch-Margl, J. Adamski, C. Prehn, T. Frank, K. H. Engel, T. Hofmann, B. Luy, R. Zimmermann, F. Moritz, P. Schmitt-Kopplin, J. Krumsiek, W. Kremer, F. Huber, U. Oeh, F. J. Theis, W. Szymczak, H. Hauner, K. Suhre, and H. Daniel. 2012. The dynamic range of the human metabolome revealed by challenges. *FASEB J* 26: 2607-2619.

70. Heinzmann, S. S., C. A. Merrifield, S. Rezzi, S. Kochhar, J. C. Lindon, E. Holmes, and J. K. Nicholson. 2012. Stability and robustness of human metabolic phenotypes in response to sequential food challenges. *J Proteome Res* 11: 643-655.

71. Williamson, M. P., G. Humm, and A. J. Crisp. 1989. 1H nuclear magnetic resonance investigation of synovial fluid components in osteoarthritis, rheumatoid arthritis and traumatic effusions. *Br J Rheumatol* 28: 23-27.

72. Nicholson, J. K., and J. C. Lindon. 2008. Systems biology: Metabonomics. *Nature* 455: 1054-1056.

73. Dunn, W. B., N. J. Bailey, and H. E. Johnson. 2005. Measuring the metabolome: current analytical technologies. *Analyst* 130: 606-625.

74. Gika, H. G., G. A. Theodoridis, R. S. Plumb, and I. D. Wilson. 2014. Current practice of liquid chromatography-mass spectrometry in metabolomics and metabonomics. *J Pharm Biomed Anal* 87: 12-25.

75. Putri, S. P., S. Yamamoto, H. Tsugawa, and E. Fukusaki. 2013. Current metabolomics: technological advances. *J Biosci Bioeng* 116: 9-16.

76. Fiehn, O. 2002. Metabolomics--the link between genotypes and phenotypes. *Plant Mol Biol* 48: 155-171.

77.   Blekherman, G., R. Laubenbacher, D. F. Cortes, P. Mendes, F. M. Torti, S. Akman, S. V. Torti, and V. Shulaev. 2011. Bioinformatics tools for cancer metabolomics. *Metabolomics* 7: 329-343.

78.   Monteiro, M. S., M. Carvalho, M. L. Bastos, and P. Guedes de Pinho. 2013. Metabolomics analysis for biomarker discovery: advances and challenges. *Curr Med Chem* 20: 257-271.

79.   Srivastava, B., R. C. Lindsley, N. Nikbakht, and D. Allman. 2005. Models for peripheral B cell development and homeostasis. *Seminars in immunology* 17: 175-182.

80.   Goodnow, C. C., J. Sprent, B. Fazekas de St Groth, and C. G. Vinuesa. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* 435: 590-597.

81.   Tiegs, S. L., D. M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J Exp Med* 177: 1009-1020.

82.   Basten, A., and P. A. Silveira. 2010. B-cell tolerance: mechanisms and implications. *Current opinion in immunology* 22: 566-574.

83.   Ermann, J., and C. G. Fathman. 2001. Autoimmune diseases: genes, bugs and failed regulation. *Nat Immunol* 2: 759-761.

84.   Wandstrat, A., and E. Wakeland. 2001. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nat Immunol* 2: 802-809.

85.   Wucherpfennig, K. W., and G. S. Eisenbarth. 2001. Type 1 diabetes. *Nat Immunol* 2: 767-768.

86.   Benoist, C., and D. Mathis. 2001. Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry? *Nat Immunol* 2: 797-801.

87.   van Belle, T. L., K. T. Coppieters, and M. G. von Herrath. 2011. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev* 91: 79-118.

88.   Nathan, D. M. 1993. Long-term complications of diabetes mellitus. *N Engl J Med* 328: 1676-1685.

89.   Gianani, R., and G. S. Eisenbarth. 2005. The stages of type 1A diabetes: 2005. *Immunological reviews* 204: 232-249.

90. Willcox, A., S. J. Richardson, A. J. Bone, A. K. Foulis, and N. G. Morgan. 2009. Analysis of islet inflammation in human type 1 diabetes. *Clinical and experimental immunology* 155: 173-181.

91. Wenzlau, J. M., K. Juhl, L. Yu, O. Moua, S. A. Sarkar, P. Gottlieb, M. Rewers, G. S. Eisenbarth, J. Jensen, H. W. Davidson, and J. C. Hutton. 2007. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci U S A* 104: 17040-17045.

92. Pescovitz, M. D., C. J. Greenbaum, H. Krause-Steinrauf, D. J. Becker, S. E. Gitelman, R. Goland, P. A. Gottlieb, J. B. Marks, P. F. McGee, A. M. Moran, P. Raskin, H. Rodriguez, D. A. Schatz, D. Wherrett, D. M. Wilson, J. M. Lachin, J. S. Skyler, and C. D. S. G. Type 1 Diabetes TrialNet Anti. 2009. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med* 361: 2143-2152.

93. Sreekumar, A., L. M. Poisson, T. M. Rajendiran, A. P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R. J. Lonigro, Y. Li, M. K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G. S. Omenn, D. Ghosh, S. Pennathur, D. C. Alexander, A. Berger, J. R. Shuster, J. T. Wei, S. Varambally, C. Beecher, and A. M. Chinnaiyan. 2009. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 457: 910-914.

94. Wang, T. J., M. G. Larson, R. S. Vasan, S. Cheng, E. P. Rhee, E. McCabe, G. D. Lewis, C. S. Fox, P. F. Jacques, C. Fernandez, C. J. O'Donnell, S. A. Carr, V. K. Mootha, J. C. Florez, A. Souza, O. Melander, C. B. Clish, and R. E. Gerszten. 2011. Metabolite profiles and the risk of developing diabetes. *Nat Med* 17: 448-453.

95. Oresic, M., T. Hyotylainen, S. K. Herukka, M. Sysi-Aho, I. Mattila, T. Seppanan-Laakso, V. Julkunen, P. V. Gopalacharyulu, M. Hallikainen, J. Koikkalainen, M. Kivipelto, S. Helisalmi, J. Lotjonen, and H. Soininen. 2011. Metabolome in progression to Alzheimer's disease. *Transl Psychiatry* 1: e57.

96. Madsen, R. K., T. Lundstedt, J. Gabrielsson, C. J. Sennbro, G. M. Alenius, T. Moritz, S. Rantapaa-Dahlqvist, and J. Trygg. 2011. Diagnostic properties of metabolic perturbations in rheumatoid arthritis. *Arthritis Res Ther* 13: R19.

97. Oresic, M., S. Simell, M. Sysi-Aho, K. Nanto-Salonen, T. Seppanen-Laakso, V. Parikka, M. Katajamaa, A. Hekkala, I. Mattila, P. Keskinen, L. Yetukuri, A. Reinikainen, J. Lahde, T. Suortti, J. Hakalax, T. Simell, H. Hyoty, R. Veijola, J. Ilonen, R. Lahesmaa, M. Knip, and O. Simell. 2008. Dysregulation of lipid and amino acid metabolism precedes

islet autoimmunity in children who later progress to type 1 diabetes. *J Exp Med* 205: 2975-2984.

98.   Pflueger, M., T. Seppanen-Laakso, T. Suortti, T. Hyotylainen, P. Achenbach, E. Bonifacio, M. Oresic, and A. G. Ziegler. 2011. Age- and islet autoimmunity-associated differences in amino acid and lipid metabolites in children at risk for type 1 diabetes. *Diabetes* 60: 2740-2747.

99.   Åkesson, L., J. Trygg, J. Fuller, R. Madsen, J. Gabrielsson, S. Bruce, H. Stenlund, T. Tupling, R. Pefley, T. Lundstedt, Å. Lernmark, and T. Moritz. 2011. Serum metabolite signature predicts the acute onset of diabetes in spontaneously diabetic congenic BB rats. *Metabolomics* 7: 593-603.

100.   Makino, S., K. Kunimoto, Y. Muraoka, Y. Mizushima, K. Katagiri, and Y. Tochino. 1980. Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* 29: 1-13.

101.   Kikutani, H., and S. Makino. 1992. The murine autoimmune diabetes model: NOD and related strains. *Advances in immunology* 51: 285-322.

102.   Bach, J. F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15: 516-542.

103.   Leiter, E. H. 1993. The NOD Mouse: A Model for Analyzing the Interplay Between Heredity and Environment in Development of Autoimmune Disease. *ILAR Journal* 35: 4-14.

104.   Marino, E., P. A. Silveira, J. Stolp, and S. T. Grey. 2011. B cell-directed therapies in type 1 diabetes. *Trends Immunol* 32: 287-294.

105.   King, C., and N. Sarvetnick. 2011. The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions. *PloS one* 6: e17049.

106.   Kriegel, M. A., E. Sefik, J. A. Hill, H. J. Wu, C. Benoist, and D. Mathis. 2011. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 108: 11548-11553.

107.   Funda, D. P., A. Kaas, T. Bock, H. Tlaskalova-Hogenova, and K. Buschard. 1999. Gluten-free diet prevents diabetes in NOD mice. *Diabetes Metab Res Rev* 15: 323-327.

108.   Hansen, C. H., L. Krych, K. Buschard, S. B. Metzdorff, C. Nellemann, L. H. Hansen, D. S. Nielsen, H. Frokiaer, S. Skov, and A. K. Hansen. 2014. A maternal gluten-free diet reduces inflammation and diabetes incidence in the offspring of NOD mice. *Diabetes* 63: 2821-2832.

109.   Marietta, E. V., A. M. Gomez, C. Yeoman, A. Y. Tilahun, C. R. Clark, D. H. Luckey, J. A. Murray, B. A. White, Y. C. Kudva, and G. Rajagopalan. 2013. Low incidence of spontaneous type 1 diabetes in non-obese diabetic mice raised on gluten-free diets is associated with changes in the intestinal microbiome. *PloS one* 8: e78687.

110.   T1dbase.   2016.
http://www.t1dbase.org/page/Regions/display/species/mouse/dise ase/1/type/linkage.

111.   Wicker, L. S., J. A. Todd, and L. B. Peterson. 1995. Genetic control of autoimmune diabetes in the NOD mouse. *Annu Rev Immunol* 13: 179-200.

112.   Hattori, M., J. B. Buse, R. A. Jackson, L. Glimcher, M. E. Dorf, M. Minami, S. Makino, K. Moriwaki, H. Kuzuya, H. Imura, and et al. 1986. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* 231: 733-735.

113.   Acha-Orbea, H., and H. O. McDevitt. 1987. The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique. *Proc Natl Acad Sci U S A* 84: 2435-2439.

114.   Corper, A. L., T. Stratmann, V. Apostolopoulos, C. A. Scott, K. C. Garcia, A. S. Kang, I. A. Wilson, and L. Teyton. 2000. A structural framework for deciphering the link between I-Ag7 and autoimmune diabetes. *Science* 288: 505-511.

115.   Kanagawa, O., S. M. Martin, B. A. Vaupel, E. Carrasco-Marin, and E. R. Unanue. 1998. Autoreactivity of T cells from nonobese diabetic mice: an I-Ag7-dependent reaction. *Proc Natl Acad Sci U S A* 95: 1721-1724.

116.   Todd, J. A., J. I. Bell, and H. O. McDevitt. 1987. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329: 599-604.

117.   Ikegami, H., S. Makino, E. Yamato, Y. Kawaguchi, H. Ueda, T. Sakamoto, K. Takekawa, and T. Ogihara. 1995. Identification of a new susceptibility locus for insulin-dependent diabetes mellitus by ancestral haplotype congenic mapping. *J Clin Invest* 96: 1936-1942.

118. Serreze, D. V., H. D. Chapman, D. S. Varnum, I. Gerling, E. H. Leiter, and L. D. Shultz. 1997. Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. *Journal of immunology* 158: 3978-3986.

119. Yi, W., N. P. Seth, T. Martillotti, K. W. Wucherpfennig, D. B. Sant'Angelo, and L. K. Denzin. 2010. Targeted regulation of self-peptide presentation prevents type I diabetes in mice without disrupting general immunocompetence. *J Clin Invest* 120: 1324-1336.

120. Perraudeau, M., P. R. Taylor, H. J. Stauss, R. Lindstedt, A. E. Bygrave, D. J. Pappin, S. Ellmerich, A. Whitten, D. Rahman, B. Canas, M. J. Walport, M. Botto, and D. M. Altmann. 2000. Altered major histocompatibility complex class II peptide loading in H2-O-deficient mice. *European journal of immunology* 30: 2871-2880.

121. Lyons, P. A., N. Armitage, F. Argentina, P. Denny, N. J. Hill, C. J. Lord, M. B. Wilusz, L. B. Peterson, L. S. Wicker, and J. A. Todd. 2000. Congenic mapping of the type 1 diabetes locus, Idd3, to a 780-kb region of mouse chromosome 3: identification of a candidate segment of ancestral DNA by haplotype mapping. *Genome Res* 10: 446-453.

122. McGuire, H. M., A. Vogelzang, N. Hill, M. Flodstrom-Tullberg, J. Sprent, and C. King. 2009. Loss of parity between IL-2 and IL-21 in the NOD Idd3 locus. *Proc Natl Acad Sci U S A* 106: 19438-19443.

123. Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V. E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S. L. Chen, R. Rosa, A. M. Cumiskey, D. V. Serreze, S. Gregory, J. Rogers, P. A. Lyons, B. Healy, L. J. Smink, J. A. Todd, L. B. Peterson, L. S. Wicker, and P. Santamaria. 2007. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* 39: 329-337.

124. Todd, J. A., N. M. Walker, J. D. Cooper, D. J. Smyth, K. Downes, V. Plagnol, R. Bailey, S. Nejentsev, S. F. Field, F. Payne, C. E. Lowe, J. S. Szeszko, J. P. Hafler, L. Zeitels, J. H. Yang, A. Vella, S. Nutland, H. E. Stevens, H. Schuilenburg, G. Coleman, M. Maisuria, W. Meadows, L. J. Smink, B. Healy, O. S. Burren, A. A. Lam, N. R. Ovington, J. Allen, E. Adlem, H. T. Leung, C. Wallace, J. M. Howson, C. Guja, C. Ionescu-Tirgoviste, F. Genetics of Type 1 Diabetes in, M. J. Simmonds, J. M. Heward, S. C. Gough, C. Wellcome Trust Case Control, D. B. Dunger, L. S. Wicker, and D. G. Clayton. 2007. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat Genet* 39: 857-864.

125. Zhernakova, A., B. Z. Alizadeh, M. Bevova, M. A. van Leeuwen, M. J. Coenen, B. Franke, L. Franke, M. D. Posthumus, D. A. van Heel, G.

van der Steege, T. R. Radstake, P. Barrera, B. O. Roep, B. P. Koeleman, and C. Wijmenga. 2007. Novel association in chromosome 4q27 region with rheumatoid arthritis and confirmation of type 1 diabetes point to a general risk locus for autoimmune diseases. *Am J Hum Genet* 81: 1284-1288.

126. Elliott, E. A., and R. A. Flavell. 1994. Transgenic mice expressing constitutive levels of IL-2 in islet beta cells develop diabetes. *International immunology* 6: 1629-1637.

127. Sutherland, A. P., T. Van Belle, A. L. Wurster, A. Suto, M. Michaud, D. Zhang, M. J. Grusby, and M. von Herrath. 2009. Interleukin-21 is required for the development of type 1 diabetes in NOD mice. *Diabetes* 58: 1144-1155.

128. Wicker, L. S., G. Chamberlain, K. Hunter, D. Rainbow, S. Howlett, P. Tiffen, J. Clark, A. Gonzalez-Munoz, A. M. Cumiskey, R. L. Rosa, J. M. Howson, L. J. Smink, A. Kingsnorth, P. A. Lyons, S. Gregory, J. Rogers, J. A. Todd, and L. B. Peterson. 2004. Fine mapping, gene content, comparative sequencing, and expression analyses support Ctla4 and Nramp1 as candidates for Idd5.1 and Idd5.2 in the nonobese diabetic mouse. *Journal of immunology* 173: 164-173.

129. Vijayakrishnan, L., J. M. Slavik, Z. Illes, R. J. Greenwald, D. Rainbow, B. Greve, L. B. Peterson, D. A. Hafler, G. J. Freeman, A. H. Sharpe, L. S. Wicker, and V. K. Kuchroo. 2004. An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 20: 563-575.

130. Araki, M., D. Chung, S. Liu, D. B. Rainbow, G. Chamberlain, V. Garner, K. M. Hunter, L. Vijayakrishnan, L. B. Peterson, M. Oukka, A. H. Sharpe, R. Sobel, V. K. Kuchroo, and L. S. Wicker. 2009. Genetic evidence that the differential expression of the ligand-independent isoform of CTLA-4 is the molecular basis of the Idd5.1 type 1 diabetes region in nonobese diabetic mice. *Journal of immunology* 183: 5146-5157.

131. Sansom, D. M., and L. S. Walker. 2006. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunological reviews* 212: 131-148.

132. Ueda, H., J. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B. Rainbow, K. M. Hunter, A. N. Smith, G. Di Genova, M. H. Herr, I. Dahlman, F. Payne, D. Smyth, C. Lowe, R. C. Twells, S. Howlett, B. Healy, S. Nutland, H. E. Rance, V. Everett, L. J. Smink, A. C. Lam, H. J. Cordell, N. M. Walker, C. Bordin, J. Hulme, C. Motzo, F. Cucca, J. F. Hess, M. L. Metzker, J. Rogers, S. Gregory, A. Allahabadia, R. Nithiyananthan, E. Tuomilehto-Wolf, J.

Tuomilehto, P. Bingley, K. M. Gillespie, D. E. Undlien, K. S. Ronningen, C. Guja, C. Ionescu-Tirgoviste, D. A. Savage, A. P. Maxwell, D. J. Carson, C. C. Patterson, J. A. Franklyn, D. G. Clayton, L. B. Peterson, L. S. Wicker, J. A. Todd, and S. C. Gough. 2003. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423: 506-511.

133. Rainbow, D. B., C. Moule, H. I. Fraser, J. Clark, S. K. Howlett, O. Burren, M. Christensen, V. Moody, C. A. Steward, J. P. Mohammed, M. E. Fusakio, E. L. Masteller, E. B. Finger, J. P. Houchins, D. Naf, F. Koentgen, W. M. Ridgway, J. A. Todd, J. A. Bluestone, L. B. Peterson, J. Mattner, and L. S. Wicker. 2011. Evidence that Cd101 is an autoimmune diabetes gene in nonobese diabetic mice. *Journal of immunology* 187: 325-336.

134. Fraser, H. I., C. A. Dendrou, B. Healy, D. B. Rainbow, S. Howlett, L. J. Smink, S. Gregory, C. A. Steward, J. A. Todd, L. B. Peterson, and L. S. Wicker. 2010. Nonobese diabetic congenic strain analysis of autoimmune diabetes reveals genetic complexity of the Idd18 locus and identifies Vav3 as a candidate gene. *Journal of immunology* 184: 5075-5084.

135. Fraser, H. I., S. Howlett, J. Clark, D. B. Rainbow, S. M. Stanford, D. J. Wu, Y. W. Hsieh, C. J. Maine, M. Christensen, V. Kuchroo, L. A. Sherman, P. L. Podolin, J. A. Todd, C. A. Steward, L. B. Peterson, N. Bottini, and L. S. Wicker. 2015. Ptpn22 and Cd2 Variations Are Associated with Altered Protein Expression and Susceptibility to Type 1 Diabetes in Nonobese Diabetic Mice. *Journal of immunology* 195: 4841-4852.

136. Kissler, S., P. Stern, K. Takahashi, K. Hunter, L. B. Peterson, and L. S. Wicker. 2006. In vivo RNA interference demonstrates a role for Nramp1 in modifying susceptibility to type 1 diabetes. *Nat Genet* 38: 479-483.

137. Bendelac, A., C. Carnaud, C. Boitard, and J. F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *J Exp Med* 166: 823-832.

138. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. *J Exp Med* 184: 2049-2053.

139. Akashi, T., S. Nagafuchi, K. Anzai, S. Kondo, D. Kitamura, S. Wakana, J. Ono, M. Kikuchi, Y. Niho, and T. Watanabe. 1997. Direct evidence

for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice. *International immunology* 9: 1159-1164.

140. Clare-Salzler, M. J., J. Brooks, A. Chai, K. Van Herle, and C. Anderson. 1992. Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer. *J Clin Invest* 90: 741-748.

141. Hammond, K. J., L. D. Poulton, L. J. Palmisano, P. A. Silveira, D. I. Godfrey, and A. G. Baxter. 1998. alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* 187: 1047-1056.

142. Liston, A., S. Lesage, D. H. Gray, L. A. O'Reilly, A. Strasser, A. M. Fahrer, R. L. Boyd, J. Wilson, A. G. Baxter, E. M. Gallo, G. R. Crabtree, K. Peng, S. R. Wilson, and C. C. Goodnow. 2004. Generalized resistance to thymic deletion in the NOD mouse; a polygenic trait characterized by defective induction of Bim. *Immunity* 21: 817-830.

143. Decallonne, B., E. van Etten, A. Giulietti, K. Casteels, L. Overbergh, R. Bouillon, and C. Mathieu. 2003. Defect in activation-induced cell death in non-obese diabetic (NOD) T lymphocytes. *Journal of autoimmunity* 20: 219-226.

144. Arreaza, G., K. Salojin, W. Yang, J. Zhang, B. Gill, Q. S. Mi, J. X. Gao, C. Meagher, M. Cameron, and T. L. Delovitch. 2003. Deficient activation and resistance to activation-induced apoptosis of CD8+ T cells is associated with defective peripheral tolerance in nonobese diabetic mice. *Clin Immunol* 107: 103-115.

145. Mellanby, R. J., D. Thomas, J. M. Phillips, and A. Cooke. 2007. Diabetes in non-obese diabetic mice is not associated with quantitative changes in CD4+ CD25+ Foxp3+ regulatory T cells. *Immunology* 121: 15-28.

146. You, S., M. Belghith, S. Cobbold, M. A. Alyanakian, C. Gouarin, S. Barriot, C. Garcia, H. Waldmann, J. F. Bach, and L. Chatenoud. 2005. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* 54: 1415-1422.

147. Brigl, M., and M. B. Brenner. 2004. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 22: 817-890.

148. Godfrey, D. I., S. J. Kinder, P. Silvera, and A. G. Baxter. 1997. Flow cytometric study of T cell development in NOD mice reveals a

deficiency in alphabetaTCR+CDR-CD8- thymocytes. *Journal of autoimmunity* 10: 279-285.

149.   Ogasawara, K., J. A. Hamerman, H. Hsin, S. Chikuma, H. Bour-Jordan, T. Chen, T. Pertel, C. Carnaud, J. A. Bluestone, and L. L. Lanier. 2003. Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity* 18: 41-51.

150.   Reis e Sousa, C. 2006. Dendritic cells in a mature age. *Nature reviews. Immunology* 6: 476-483.

151.   Lee, M., A. Y. Kim, and Y. Kang. 2000. Defects in the differentiation and function of bone marrow-derived dendritic cells in non-obese diabetic mice. *J Korean Med Sci* 15: 217-223.

152.   Dahlen, E., G. Hedlund, and K. Dawe. 2000. Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation. *Journal of immunology* 164: 2444-2456.

153.   Serreze, D. V., J. W. Gaedeke, and E. H. Leiter. 1993. Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: defective regulation of cytokine receptors and protein kinase C. *Proc Natl Acad Sci U S A* 90: 9625-9629.

154.   O'Brien, B. A., Y. Huang, X. Geng, J. P. Dutz, and D. T. Finegood. 2002. Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. *Diabetes* 51: 2481-2488.

155.   Trudeau, J. D., J. P. Dutz, E. Arany, D. J. Hill, W. E. Fieldus, and D. T. Finegood. 2000. Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? *Diabetes* 49: 1-7.

156.   Hu, C. Y., D. Rodriguez-Pinto, W. Du, A. Ahuja, O. Henegariu, F. S. Wong, M. J. Shlomchik, and L. Wen. 2007. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J Clin Invest* 117: 3857-3867.

157.   Noorchashm, H., Y. K. Lieu, N. Noorchashm, S. Y. Rostami, S. A. Greeley, A. Schlachterman, H. K. Song, L. E. Noto, A. M. Jevnikar, C. F. Barker, and A. Naji. 1999. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. *Journal of immunology* 163: 743-750.

158. Silveira, P. A., E. Johnson, H. D. Chapman, T. Bui, R. M. Tisch, and D. V. Serreze. 2002. The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. *European journal of immunology* 32: 3657-3666.

159. Hussain, S., and T. L. Delovitch. 2005. Dysregulated B7-1 and B7-2 expression on nonobese diabetic mouse B cells is associated with increased T cell costimulation and the development of insulitis. *Journal of immunology* 174: 680-687.

160. Quintana, F. J., and I. R. Cohen. 2001. Autoantibody patterns in diabetes-prone NOD mice and in standard C57BL/6 mice. *Journal of autoimmunity* 17: 191-197.

161. Luzina, I. G., S. P. Atamas, C. E. Storrer, L. C. daSilva, G. Kelsoe, J. C. Papadimitriou, and B. S. Handwerger. 2001. Spontaneous formation of germinal centers in autoimmune mice. *Journal of leukocyte biology* 70: 578-584.

162. Greeley, S. A., M. Katsumata, L. Yu, G. S. Eisenbarth, D. J. Moore, H. Goodarzi, C. F. Barker, A. Naji, and H. Noorchashm. 2002. Elimination of maternally transmitted autoantibodies prevents diabetes in nonobese diabetic mice. *Nat Med* 8: 399-402.

163. Nikkila, J., M. Sysi-Aho, A. Ermolov, T. Seppanen-Laakso, O. Simell, S. Kaski, and M. Oresic. 2008. Gender-dependent progression of systemic metabolic states in early childhood. *Mol Syst Biol* 4: 197.

164. Lenz, E. M., J. Bright, I. D. Wilson, A. Hughes, J. Morrisson, H. Lindberg, and A. Lockton. 2004. Metabonomics, dietary influences and cultural differences: a 1H NMR-based study of urine samples obtained from healthy British and Swedish subjects. *J Pharm Biomed Anal* 36: 841-849.

165. Backhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A* 101: 15718-15723.

166. Gluckman, P. D., M. A. Hanson, C. Cooper, and K. L. Thornburg. 2008. Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* 359: 61-73.

167. Floyd, J. C., Jr., S. S. Fajans, R. F. Knopf, and J. W. Conn. 1963. Evidence That Insulin Release Is the Mechanism for Experimentally Induced Leucine Hypoglycemia in Man. *J Clin Invest* 42: 1714-1719.

168. Fonseca, S. G., F. Urano, M. Burcin, and J. Gromada. 2010. Stress hypERactivation in the beta-cell. *Islets* 2: 1-9.

169. Sysi-Aho, M., A. Ermolov, P. V. Gopalacharyulu, A. Tripathi, T. Seppanen-Laakso, J. Maukonen, I. Mattila, S. T. Ruohonen, L. Vahatalo, L. Yetukuri, T. Harkonen, E. Lindfors, J. Nikkila, J. Ilonen, O. Simell, M. Saarela, M. Knip, S. Kaski, E. Savontaus, and M. Oresic. 2011. Metabolic regulation in progression to autoimmune diabetes. *PLoS Comput Biol* 7: e1002257.

170. Sherry, S. T., M. H. Ward, M. Kholodov, J. Baker, L. Phan, E. M. Smigielski, and K. Sirotkin. 2001. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 29: 308-311.

171. Field, L. L., V. Bonnevie-Nielsen, F. Pociot, S. Lu, T. B. Nielsen, and H. Beck-Nielsen. 2005. OAS1 splice site polymorphism controlling antiviral enzyme activity influences susceptibility to type 1 diabetes. *Diabetes* 54: 1588-1591.

172. Duan, J., A. R. Sanders, J. E. Molen, L. Martinolich, B. J. Mowry, D. F. Levinson, R. R. Crowe, J. M. Silverman, and P. V. Gejman. 2003. Polymorphisms in the 5'-untranslated region of the human serotonin receptor 1B (HTR1B) gene affect gene expression. *Mol Psychiatry* 8: 901-910.

173. Choi, D. W. 1992. Excitotoxic cell death. *J Neurobiol* 23: 1261-1276.

174. Di Cairano, E. S., A. M. Davalli, L. Perego, S. Sala, V. F. Sacchi, S. La Rosa, G. Finzi, C. Placidi, C. Capella, P. Conti, V. E. Centonze, F. Casiraghi, F. Bertuzzi, F. Folli, and C. Perego. 2011. The glial glutamate transporter 1 (GLT1) is expressed by pancreatic beta-cells and prevents glutamate-induced beta-cell death. *J Biol Chem* 286: 14007-14018.

175. O'Brien, B. A., B. V. Harmon, D. P. Cameron, and D. J. Allan. 1997. Apoptosis is the mode of beta-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. *Diabetes* 46: 750-757.

176. Palmer, C., E. M. Bik, D. B. DiGiulio, D. A. Relman, and P. O. Brown. 2007. Development of the human infant intestinal microbiota. *PLoS Biol* 5: e177.

177. Darvasi, A., and M. Soller. 1992. Selective genotyping for determination of linkage between a marker locus and a quantitative trait locus. *Theor Appl Genet* 85: 353-359.

178. Huang, B. E., and D. Y. Lin. 2007. Efficient association mapping of quantitative trait loci with selective genotyping. *Am J Hum Genet* 80: 567-576.

179. Laboratory, T. J. 2006. Blood chemistry survey of 11 inbred strains of mice. . Mouse phenome database website.

180. Harrill, A. 2011. Drug study: Clinico-pathological survey of the liver and adverse reactions in response to isoniazid treatment in females of 34 inbred strains of mice., Mouse phenome database web site.

181. Lovgren, T., M. L. Eloranta, U. Bave, G. V. Alm, and L. Ronnblom. 2004. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum* 50: 1861-1872.

182. Ferreira, R. C., H. Guo, R. M. Coulson, D. J. Smyth, M. L. Pekalski, O. S. Burren, A. J. Cutler, J. D. Doecke, S. Flint, E. F. McKinney, P. A. Lyons, K. G. Smith, P. Achenbach, A. Beyerlein, D. B. Dunger, D. G. Clayton, L. S. Wicker, J. A. Todd, E. Bonifacio, C. Wallace, and A. G. Ziegler. 2014. A type I interferon transcriptional signature precedes autoimmunity in children genetically at risk for type 1 diabetes. *Diabetes* 63: 2538-2550.

183. Li, Q., B. Xu, S. A. Michie, K. H. Rubins, R. D. Schreriber, and H. O. McDevitt. 2008. Interferon-alpha initiates type 1 diabetes in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 105: 12439-12444.

184. Sundstrom, M., and K. Lejon. 2007. Idd-linked genetic regulation of TACIhigh expressing B cells in NOD mice. *Journal of autoimmunity* 29: 116-124.

185. Liu, E., H. Moriyama, N. Abiru, D. Miao, L. Yu, R. M. Taylor, F. D. Finkelman, and G. S. Eisenbarth. 2002. Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23. *J Clin Invest* 110: 1021-1027.

186. Overbergh, L., B. Decallonne, D. D. Branisteanu, D. Valckx, A. Kasran, R. Bouillon, and C. Mathieu. 2003. Acute shock induced by antigen vaccination in NOD mice. *Diabetes* 52: 335-341.

187. Sundstrom, M., and K. Lejon. 2010. The prolonged and enhanced immune response in the non-obese diabetic mouse is dependent on genes in the Idd1/24, Idd12 and Idd18 regions. *Journal of autoimmunity* 35: 375-382.

188. Pedotti, R., M. Sanna, M. Tsai, J. DeVoss, L. Steinman, H. McDevitt, and S. J. Galli. 2003. Severe anaphylactic reactions to glutamic acid decarboxylase (GAD) self peptides in NOD mice that spontaneously develop autoimmune type 1 diabetes mellitus. *BMC immunology* 4: 2.

189. Luan, J. J., R. C. Monteiro, C. Sautes, G. Fluteau, L. Eloy, W. H. Fridman, J. F. Bach, and H. J. Garchon. 1996. Defective Fc gamma RII gene expression in macrophages of NOD mice: genetic linkage with up-regulation of IgG1 and IgG2b in serum. *Journal of immunology* 157: 4707-4716.

190. Schwickert, T. A., B. Alabyev, T. Manser, and M. C. Nussenzweig. 2009. Germinal center reutilization by newly activated B cells. *J Exp Med* 206: 2907-2914.