Anomalies in Humoral Immunity in the NOD mouse

Contribution to the progression of Type 1 Diabetes

Radha Thyagarajan

Department of Clinical Microbiology, Immunology
Umeå 2016

Responsible publisher under swedish law: the Dean of the Medical Faculty

ISBN: 978-91-7601-542-1
ISSN: 0346-6612
Front Cover: ”Primary Tones” by Radha Thyagarajan
Elektronisk version tillgänglig på http://umu.diva-portal.org/
Printed by: Print & Media, Umeå
Umeå, Sweden 2016

To my family

Table of Contents

Abstract

Type 1 diabetes (T1D) is a chronic inflammatory disease characterized by destruction of the insulin producing β cells in the islets of Langerhans by patients' immune cells. The classical symptoms include increased glucose levels in urine and blood, frequent urination and enhanced thirst. Diabetes is the leading cause of blindness, amputations, and kidney failures, contributing to premature death. The disease has a strong genetic component and is also influenced by the environment.

The non-obese diabetic (NOD) mouse 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. The disease occurs in two phases; insulitis - the infiltration of immune cells in the islets of Langerhans and overt diabetes caused by the destruction of insulin producing β cells. The disease in the NOD mouse shares several features with the human disease for example, strong genetic component, presence of autoantibodies towards islet antigens, insulitis and increased blood glucose. Several 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 in the NOD, the human chromosomal counterpart has been linked to the development of T1D in humans. Although, T1D is recognized as a T cell mediated disease in both mouse and man, many studies have shown the importance of B cells in the pathogenesis of the disease. Autoantibodies appear prior to islet infiltration and several molecular and cellular events precede this beta-cell autoimmunity. Although the pathogenesis of T1D is well

characterized, less is known about the environmental and immunological factors that trigger the disease.

In this thesis, we studied the contribution of B cell anomalies to the skewed immune response observed in the NOD mouse. Previously our group established that B cells in the NOD mice captured copious antibodies of IgG and IgM type on their surface. In this thesis, we have observed that NOD B cells also captured IgE on their surface. IgE is associated with allergy and seldom associated with autoimmunity. However, recent reports indicate the pathogenic role of IgE also in autoimmune diseases. In our study we observed that NOD mice display enhanced IgE in the serum already at one week of age. In addition, upon treatment of pre-diabetic NOD mice with anti-IgE antibodies, diabetes incidence was delayed. We hypothesize that the presence of IgE in the system may be explained due to enhanced class switching. Antibody feedback however, is an essential component of the immune response and can lead to either enhanced or dampened responses. Thus, increased IgE may provide positive feedback that might sustain an immune response.

Another feature of NOD B cells described by the Lejon lab is the enhanced expression of TACI, a TNF super family receptor. TACI regulates B cell homeostasis, activation and class switching. Here we have aimed to analyze the biological consequence of this feature. *In vitro* stimulation of B cells by the 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 centers facilitating increased BAFF uptake and subsequent escape of low affinity antibody producing clones.

NOD mice respond vigorously towards several T-dependent antigens upon immunization. In this thesis, we tested the response towards the conventional antigen hen egg lysozyme (HEL). Indeed, NOD mice elicited an enhanced and prolonged immune response to HEL immunizations. Serum HEL-specific IgG level was significantly increased and was predominantly of the IgG1 isotype. Immunofluorescence analysis of NOD spleen revealed the presence of spontaneous germinal centers which others have perceived to provide a ready niche for the entry of naïve B cells that encountered novel antigen. Adoptive transfer experiments of purified B and T cells from NOD into NOD.*Rag2*-/- (NOD-RAG) mice illustrated the importance of B cell intrinsic defects in the reproduction of the original phenotype as observed in NOD.

Through this thesis we aimed to understand how these B cell anomalies contributed to skewed immune response towards self and non-self-antigens in the NOD mice.

List of papers in thesis

Paper I

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

Paper II

Increased expression of TACI in the NOD mouse results in enhanced plasma cell differentiation and immunoglobulin production.
Viqar Showkat Banday, Radha Thyagarajan, Mia Sundström and Kristina Lejon (Immunology, 2016)

Paper III

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

*Equal Contribution

Abbreviations

AID	Activation induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
BAFF	B cell activating factor
BB rat	Biobreeding rat
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
CD	Cluster of Differentiation
CDR	Complementarity Determining Regions
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CXC	Chemokine
CXCR	Chemokine receptor
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FDC	Follicular dendritic cell
FO B	Follicular B cell
GAD	Glutamic acid decarboxylase
GC	Germinal center
HEL	Hen Egg Lysozyme
HLA	Human leukocyte antigen
IA-2	Insulinoma-associated antigen-2
IAA	Insulin autoantibody
Idd	Insulin dependent diabetes
Ig	Immunoglobulin
IL	Interleukin
MHC	Major histocompatibility complex
MZ	Marginal zone
NOD	Non-obese diabetic
NP	4-Hydroxy-3-nitrophenylacetyl hapten
PRR	Pattern recognition receptors
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RAG	Recombination-activating genes
SHM	Somatic Hypermutation
T1D	Type 1 Diabetes
TACI	Transmembrane Activator CAML (calcium modulator and cyclophilin ligand) Interactor
T_{FH}	T follicular helper
TNF	Tumour Necrosis Family

Introduction

Immunity

The molecules, cells, tissues and organs in our body that protect us from the attack by foreign elements and organisms are a part of the body's *Immune System*. The immune system is multifaceted and has evolved to identify and kill pathogens and altered self-cells (e.g. Tumour cells). It also plays an important role in overall tissue and organ homeostasis by clearing out apoptotic cells. The immune system is specialized to distinguish between self and foreign [1].

The process that the immune system uses to clear pathogens and/or repair a wound involves a series of physical events that are collectively called *Inflammation*. Inflammation occurs when the barriers are breached by pathogens or when there is a wound that makes the body vulnerable to infections. It is characterized by the following cardinal symptoms: heat, pain, redness, swelling and loss of function.

The immune system is broadly classified into:

- *Innate immunity*
- *Adaptive immunity*

The innate immunity corresponds to the physical barriers like skin, pH of body secretions, mucus layers, small hair for e.g. in the ear etc. – all of which prevent the entry and binding of pathogens in the body. It also comprises of the innate immune cells neutrophils, mast cells basophils, macrophages, eosinophils, natural killer (NK) cells, innate lymphoid cells (ILCs) and specialized epithelial cells in the gut which promote inflammation upon breach and are able to detect a wide

variety of pathogens with their pattern recognition receptors (PRRs) [1, 2]. These cells especially the epithelia are able to secrete anti-microbial peptides and molecules into the mucus layers, which function as irritants to pathogens. The complement system in sera is also part of innate immunity. Therefore, the innate immunity is also referred to as the first line of defence. Some form of innate immunity is present in all organisms including plants [1].

The adaptive immunity comprises of cells and organs of a more sophisticated and specific nature. Here the immune system selectively targets a specific pathogen and deals with the elimination of that pathogen. The cells involved are the lymphocytes which have specific receptors namely the antibodies and T-cell receptor (TCR) that recognize specific epitopes on the antigen. The antibodies can recognise soluble antigens and antigens in their native form; TCR recognises peptide antigens bound to specific display molecules known as major histocompatibility complex (MHC) in mice and human leukocyte antigen (HLA) in humans [3]. T cells are educated in the thymus to recognise both peptide antigen and the MHC by a process called MHC restriction. This unique process depends upon the accessory molecules CD4 or CD8, depending on the T cell type [4]. CD4+ T cells called T-helper (Th) are restricted to MHC class II and recognise peptide antigens bound to it and CD8+ T cells (Cytotoxic T cells/CTL) are restricted to MHC class I [5]. There are other cells like the NKT cells that are restricted to non-classical MHC class I molecules that display lipid antigens [6].

The adaptive immunity is triggered only when the innate immunity fails to clear the pathogen. The cells of the adaptive immunity include the variety of T cells (Th1, Th2, Th17, Treg etc.) and B cells (B1, MZ,

FoB, Breg) and natural killer like T (NKT) cells (that belong to both innate and adaptive immunity). The dendritic cells (DC) form the bridge between the innate and adaptive as they use their PRRs, pinocytosis and phagocytosis to detect pathogens. They however, process and present the antigen to the T cells and bring in the antigens from periphery for B cells in order to activate them. DCs also secrete cytokines and chemokines and provide the T cells with survival signals through co-stimulation that in turn provide survival signal to B cells. The most important feature of the adaptive immunity is the immunological *memory* that it forms, so that upon secondary encounter of the same pathogen the response is faster and more robust thereby providing lifelong immunity. Both and B and T cells form memory cells. Only vertebrates are known to have an adaptive immune system [1, 3]. A summary of immune cells and their functions is presented in Table 1.

Deviations or anomalies in the immune system components or cells can impair the immunity of the organism. A common deviation seen is the lack of tolerance to self-antigens. This leads to a condition called *Autoimmunity*. Even though autoimmunity is seen in varying degrees in healthy individuals, it is taken care of by the checkpoints in central and peripheral tolerance. However if this tolerance is broken, the individual will suffer from autoimmune diseases where the immune system reacts to self –antigens causing chronic inflammation [7]. Autoimmune diseases mostly occur when adaptive immunity fails to maintain self-tolerance.

Cell type		Identifying markers	Basic functions
Innate Immunity			
Granulocytes	Monocytes/ Macrophages	CD40, CD11b, CD64, F4/80 and CD68	Phagocytosis, tissue homeostasis, antigen presentation
	Neutrophils	CD11a, CD11b, Ly6C, Ly6G	Phagocytosis, netosis, release of inflammatory mediators to bacterial infections
	Eosinophils	CD11b, CD193, F4/80, Siglec-F	Associated with allergy and asthma, release of inflammatory mediators to parasitic infections
	Basophils	CD200R3, FcεRIα	Phagocytosis, associated with allergy and asthma, release serotonin and heparin
	Mast cells	FcεRIα, Ly6G, histamine	Associated with allergy and asthma, release histamine
	Natural Killer Cells	CD49b, CD335, NKG2A/C/E/D, CD94	Cytotoxic cells of the innate immunity, anti-tumour immunity, anti-viral immunity
Between Innate and Adaptive Immunity			
Lymphocytes	γδT cells	RORγT, CD3, CD16, CD56	Intraepithelial leucocytes in the mucosa, amongst others provide immunity against listeria
	Natural Killer T cells	αGalSer. CD1dcomplex, PLZF, CD160, CD94	Recognize lipid antigens and provide immunity against several bacteria
	Dendritic cells	CD11c, CD123	Antigen presenting cells, phagocytosis, activate adaptive immunity
Adaptive Immunity			
Lymphocytes	B cells	CD19, B220, IgM, IgD	Responsible for humoral immunity – produce antibodies and have memory
	T helper cells	CD4, CD3	Restricted to MHC class II, induce CD8 T cells to CTLS and B cells to produce antibody
	Cytotoxic T cells	CD8, CD3	Cell mediated immunity, anti-tumour immunity, anti-viral immunity

Table 1: A summary of the cells of the immune system and their function

Autoimmunity

Autoimmunity is characterized by the failure of effective self-tolerance, usually under environmental stress. Autoimmune diseases affect approximately 5-7% of the western world's population making them one of the major causes for chronic illness [7, 8]. These diseases are broadly classified based on their final effector mechanism as:

- *Systemic diseases* – ex. Systemic Lupus Erythematous (SLE) that affects all organs.

- *Organ-specific diseases* – ex. Type 1 Diabetes (T1D) that affects usually the pancreas of the individual.

Susceptibility to nearly all autoimmune diseases is strongly influenced by genes in the MHC/HLA loci [7]. Despite the strong genetic predisposition, autoimmune disease incidence between monozygotic twins is less than 50%, suggesting susceptibility to be multifactorial involving environmental stimuli [9, 10]. Infections can also induce autoimmunity through molecular mimicry of foreign antigens. In T1D, for example, cross reactivity is common between the Coxsackie virus non-structural protein 2C viral protease and the human antigen GAD65, a common target for auto-antibodies and T cells in T1D [11-13].

Type 1 Diabetes

Type 1 Diabetes (T1D) or insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disease, where the insulin producing pancreatic β-cells are selectively destroyed. Consequently, this leads to low insulin levels and increased glucose in blood and urine which

cause several complications such as blindness, renal failure etc. in the patient, leading to premature death. The actual disease trigger is not fully understood however; susceptibility is influenced by both genetic and environmental factors. The highest incidence of T1D is seen in Northern Europe compared to other parts of the world [14-16]. Insulin is required to be administered throughout a patient's life as there is no known cure for T1D.

Clinically, a patient with T1D is often diagnosed only after the diabetes onset. However, the immunological events leading to diabetes occur several years or months ahead of the clinical symptoms. Pancreatic islets in patients are inflamed and infiltrated with immune cells [16]. T1D has a type 1 interferon signature and $CD4^+$ T cells are considered the effector cells [17]. An exacerbated IFN response is observed in the earlier stages of the autoimmune response in T1D and this disease-associated transcriptional signature can be detected in children with genetic predisposition to T1D before the development of autoantibodies [17]. Indeed, the key feature for T1D is the presence of autoantibodies to several islet antigens, highlighting the importance of B cells. Autoantibodies are formed mainly towards insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated antigen-2 (IA-2) and zinc transporter 8 (ZnT8) [18, 19]. These auto-antibodies till date are used to predict onset of T1D in familial cases [17, 19].

T1D, like other autoimmune diseases, is multifactorial involving several genes and environmental triggers. First-degree relatives of patients with T1D have a 6% higher risk of developing the disease. Concordance rate for monozygotic twins is reported to be around 30-

50%, indicating the need to define the environmental factors contributing to the disease [20].

Genetics of T1D

In the human disease, about 50 genetic loci have been associated with T1D [21, 22]. The *HLA* region is the strongest risk factor, while regions outside this have smaller contributions. The *HLA*-class II alone is estimated to explain 40% of the inherited disease risk especially genes encoding for HLA-DR and HLA-DQ molecules [23]. Most T1D patients express the DR3/4-DQ2/8 genotype [24, 25].

The mechanisms with which DR/DQ contribute to autoimmune disease may be explained in the following ways:

1. Specific or limited set of self-peptides may preferentially be presented, which may allow many autoreactive T cells to escape central tolerance in the thymus leading to a skewed population of T cells that do not recognize all self-molecules [26].
2. Failure of polymorphic DR/DQ molecules to select a good T regulatory cell (Treg) population could also make the body vulnerable to autoimmunity.
3. Presentation of endogenous antigens by HLA class II. Although class II molecules traditionally present exogenous antigens and class I present endogenous antigens, this system is not absolute and cross presentation can occur. This could alter how these antigens are presented to the immune system and the response triggered [26].

These hypotheses suggest the potential pathways by which the HLA class II molecules can be involved in disease onset by modifying the

Th (T helper) and Treg cell repertoire and/or through alterations in how the antigen is recognized in the periphery [23, 25, 26].

Non-HLA loci that prominently contribute to disease development are the genes encoding for insulin, protein tyrosine phosphate non-receptor type 22 (PTPN22), IL-2 receptor α (IL2Rα) that is essential for T cell proliferation and differentiation, interferon induced helicase 1 (IFIH1) and cytotoxic T lymphocyte associated protein 4 (CTLA4) that is an important regulator for T cell responses[21, 23, 27].

Being a complex disease, T1D susceptibility is based on multiple genetic components and their interaction with each other and the environment. Although genome-wide association studies (GWAS) have successfully identified common variants involved in complex disease etiology, for the majority of complex diseases including T1D, <10% of genetic variance is explained by common variants. These variants however, contribute to disease phenotype through small additive effects. The complexity in inheritance may be attributed to epigenetic inheritance, single nucleotide polymorphisms (SNP), phase-dependent interactions between genetic variants, non-coding microRNAs and post translational modifications [27-29].

Environmental factors contributing to T1D

Several environmental factors have been linked to trigger and precipitate T1D in humans. Persistent viral infections have been considered risk factors either as a trigger or as an accelerator of the disease process, in this case destruction of pancreatic β cells. Molecular mimicry of viral antigens by self-antigens either due to alternate splicing or other post translational modification lead to cross reactivity and immune recognition of self [9, 12, 13].

Childhood obesity is also a contributor to type 1 diabetes and is associated with younger age of onset [30]. Changes in metabolic profile have been observed in familial T1D in Finland. Altered metabolism with high levels of glutamic acid and other amino acids and lipids is observed in children with genetic pre-disposition for T1D progression [31]. Dietary gluten has also been associated with T1D commonly due to the leaky gut phenomenon, also increasing the risk for Coeliac Disease amongst these individuals [32]. Gut microbiota is currently thought to be an important factor influencing many autoimmune diseases including T1D [33].

Animal models for T1D

T1D pathophysiology is extremely difficult to study in human patients, as the target organ is the pancreas. This means that the pancreas is available for study only post mortem. For the ease of studying this complex disease the two most commonly used spontaneous models are the BioBreeding rat (BB rat) and the non-obese diabetic mouse (NOD; Figure 1). Table 2 summarizes the similarities of the pathogenesis in these models with human T1D [34, 35].

Figure 1: Image of a NOD mouse

Type 1 diabetes characteristics	Humans	Biobreeding (BB) RAT	Non-obese diabetic (NOD) Mouse
Genetic predisposition and polygenic trait	Yes	Yes	Yes
MHC-Loci contribution	HLA-DR and DQ	$RT1^{u}$	$I\text{-}Ag^{7}$, absent I-E
Environmental influence	Diet and viral infection implicated in pathogenesis (lacking definitive proof)	LCMV (lymphocytic choriomeningitis virus) prevents disease, diet and bacterial vaccines reduce diabetes frequency	Coxackie virus implicated in pathogenesis. Over 120 interventions known to prevent diabetes
Autoantibodies towards islet antigens	GAD65, IA-2, Insulin, ICA	ICA	Insulin, GAD65, IA-2, ICA
Islet autoimmunity	Yes, peri-insulitis	Yes, peri-insulitis	Yes, full blown insulitis
Age of onset	Adolescence	7-14 weeks	12-15 weeks
Ketosis	Severe	Severe	Mild
Table adapted from Roep et al (2004) and Mordes et al (2004)			

Table 2: Similarities of the BB rat and NOD models with human T1D Adapted from Roep et al (2004) and Mordes et al (2004)

The Non-obese Diabetic (NOD) mouse

The most commonly used model for T1D, the NOD mice develop spontaneous diabetes caused by the combination of genetic and environmental factors. These mice have auto-antibodies towards islet antigens and have autoreactive T cells. The diabetes incidence is about 80% for females and 30% for males [36-38].

Bacterial and viral infections and the diet constitute the environmental influence for T1D of the NOD mouse [39]. These mice are known to be more prone to T1D depending on their gut flora and animal house environment. It is known that presence of segmented filamentous bacteria protects them from autoimmunity [40]. Sex hormones also seem to influence diabetes development in NOD mice, a feature not observed in humans. Upon castration of NOD males,

there was increased susceptibility to T1D and androgen treatment of NOD females was found to be protective [41, 42]. Metabolic deviations in pre-diabetic NOD mice also contribute to T1D development, a feature similar to humans. One example is glutamic acid levels, which are higher in pre-diabetic NOD mice compared to C57BL/6 mice, and an altered metabolic signature also observed in humans with genetic pre-disposition to T1D [43].

The genetics of T1D in NOD mice is polygenic, similar to that in humans. More than 50 loci on 15 chromosomes have been linked to disease susceptibility in NOD mouse, designated insulin dependent diabetes (*Idd)* loci [22, 44]. Similar to that in humans, the major risk factor in the NOD mouse resides in the MHC region on chromosome 17 namely *Idd1* [45]. Other regions that are strongly linked to disease development are *Idd3* on chromosome 3 that includes genes that code for the immunoregulatory cytokines IL-2 and IL-21 [44]. Another important region is *Idd5* on chromosome 1, that includes genes coding for CTLA-4 and Inducible T cell co-stimulator (ICOS), which are important for T cell response regulation [46, 47].

Immune response in the NOD mouse

In the NOD mouse, the immune response towards the pancreas is similar to that in humans (Table 2). The disease progression has two phases – the infiltration of pancreas with immune cells called insulitis and overt diabetes, marked by the death of approximately 80% of β cell mass. The cells participating in the immune response include T cells, B cells, dendritic cells, macrophages and NKT cells [48, 49]. Although $CD4^+$ T cells are the main effector cells that drive the destruction of β cells in pancreatic islets of diabetic NOD mice, the B cells are also important to the initiation of disease. The B cells

produce autoantibodies towards several islet antigens in NOD [50-53].

In the Lejon lab, the focus has been to study the B cell related defects in the NOD mouse and B cell contribution to T1D. In this thesis, the B cell anomalies and mechanisms by which they possibly contribute to T1D in NOD mice are discussed further.

B cells in mice

B cells arise in the bone marrow (BM) of the adult mouse from the common lymphoid progenitor (Figure 2). Expression of B220 (CD45 isoform) ensures commitment to B cell lineage [54]. These are termed *pre-pro*-B cells and form about 1% of total nucleated cells in BM [55]. These then mature to large pre-B cells that express the pre-BCR. This serves as an important checkpoint in B cell development as non-functional pre-BCR results in rearrangement of the other heavy chain allele or apoptosis. The μ-heavy chain is the first one to be recombined through the VDJ recombination process [56, 57]. Once a functional heavy chain is expressed, the rearrangement of the light chains, either at κ (kappa) or λ (lambda) loci, by Rag-1 and Rag-2 is initiated [58]. Upon expression of a functional BCR, the immature B cells exit the BM and home to spleen as transitional (T1) B cells. These then differentiate to T2 B cells that serve as a precursor to mature B cells i.e follicular B (FoB) cells or marginal zone (MZ) B cells [59-62].

Notch2 signalling in T2 B cells results in MZ B cell development along with low BCR signalling strength and BAFF-R (B cell activating factor-receptor) signalling for survival. MZ B cells are localised to the marginal sinus of the spleen (Figure 3) [63, 64]. They are

characterized as IgM^{hi} IgD^{low} $CD23^{low}$ $B220^{+}$ $CD19^{+}$ $CD21^{hi}$ $CD1d^{hi}$ $CD9^{+}$ and constitute about 5% of splenic B cells [63]. Recent reports have also indicated the presence of B cells with MZ like phenotype in mouse lymph nodes which express low-affinity polyreactive BCRs on their surface [65]. MZ B cells mostly recognise and respond to T independent antigens and blood borne pathogens. Since they express high CD1d on their surface they can present lipid antigens to invariant NK-T (iNK-T) cells [66-68]. They also transport antigens from the marginal sinus to the follicle for an effective antibody response. Usually they form short-lived antibody secreting plasma cells that secrete copious amounts of IgM and IgG [69].

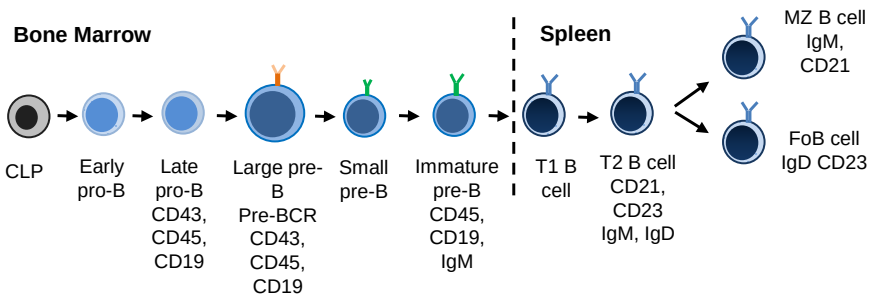

Figure 2: B cell development in mouse in bone marrow and spleen

Follicular B (FoB) cells constitute the majority of the B cells in the lymphoid organs. These cells recirculate through the lymph and blood and are long-lived. A strong BCR signal to T2 B cells allows them to differentiate to FoB cells [63]. They populate the B cell follicles of all major lymphoid organs i.e spleen, lymph nodes and Peyer's patches (Figure 4). These cells are characterized as IgM^{lo} IgD^{hi} $CD23^{+}$ $B220^{+}$ $CD19^{+}$ $CD21^{low}$ $CD1d^{low}$ $CD9^{-}$ [63]. FoB cells are responsible for providing immunity to T dependent antigens and reside in proximity to T cell zones in the lymphoid organs. They interact with $CD4^{+}$ T

cells, give and receive co-stimulation and are able to class switch into other antibody classes (IgG, IgA and IgE). Upon activation, they differentiate in to antibody secreting plasma cells and memory cells [70].

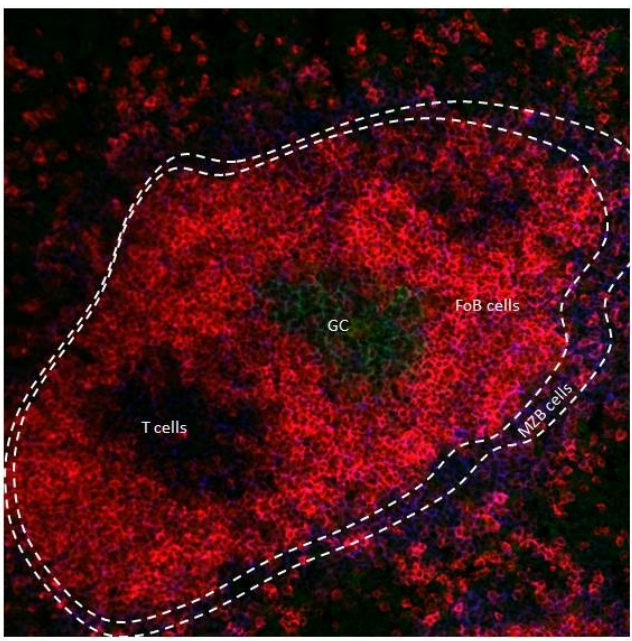

Figure 3: Representative image of a splenic follicle in NOD mouse. The FoB cells (red) are stained with anti-IgD, the germinal center (GC; green) is stained with GL7 and the BAFF (blue) bright MZ B cells are seen as a ring around the follicle.

All B cell responses require co-stimulation and/or survival signals from the microenvironment for antibody production, memory formation and antibody class switch. Some important molecules that aid in these responses are transmembrane activator and calcium

modulator and cyclophilin ligand interactor (TACI; TNFRSF13B), B cell maturation antigen (BCMA, TNFRSF17) and BAFF-R. TACI and BCMA bind a proliferation inducing ligand (APRIL). BAFF-R and TACI bind B cell activating factor (BAFF). Both APRIL and BAFF promote B cell survival, maturation and tolerance [71-73]. TACI deficient mice are unable to class switch in response to APRIL [74] and generate long-lived plasma cells [75, 76] emphasizing its role in germinal centre (GC) responses.

Several overlapping tolerance mechanisms at distinct checkpoints in B cell development and differentiation ensure the elimination of autoreactive B cells. In fact, only 5% of the newly formed bone marrow B cells are recruited into the peripheral B cell pool [77]. Both central and peripheral tolerance mechanisms are in place for this purpose. Central tolerance mechanisms are present in the bone marrow during the development B cells, and include receptor editing, anergy and deletion of developing B cells [78-80].

Despite the stringent central tolerance, some autoreactive B cells escape negative selection in the bone marrow. Especially B cells with low-affinity to self-antigens and autoreactive B cells that have not encountered their antigen in the bone marrow, can enter into the naïve B cell compartment in the periphery [78]. To ensure elimination of such autoreactive cells, tolerance mechanisms are also established in the periphery. As in the bone marrow, naïve B cells in the spleen that encounter a strongly cross-linking self-antigen will undergo clonal deletion, and those that meet soluble self-antigens (low or very low avidity interactions) become anergized [80]. Self-reactive B cells are also extrinsically regulated through competition for BAFF. With

large numbers of circulating B cells the autoreactive cells fail to receive enough BAFF and are competitively eliminated [81].

During an immune response, activated B cells form GCs and undergo somatic hypermutation (SHM). Variable region mutations during SHM can at times create or increase antibody affinity for self-antigens. Autoantibodies are not often seen in high affinity antibody responses, indicating that tolerance mechanisms operate at this level too [82]. Apoptosis is triggered in GC B cells that bind too avidly to self-antigens [83]. GC B cells are also negatively selected against self-reactivity due to local competition for antigen presented on FDCs and survival cytokines secreted by T_{FH} cells [80].

Antibody classes and their functions

There are five known classes of antibodies named after their heavy chain isotype - IgM, IgD, IgG, IgE and IgA. IgM is the first immunoglobulin to be expressed during B cell development through the VDJ rearrangement by RAG-1 and RAG-2 genes. IgM, IgG, IgE and IgA are present both as secreted and membrane bound (BCR) however, IgD is mostly membrane bound. IgM is pentameric in its secreted form. IgA is both monomeric and dimeric in its secreted form. IgG is the most common antibody present in large titres in the serum and has the longest half-life [62]. IgG and IgA provide passive immunity to the infant through transplacental transport and breast milk [62, 84]. IgM and IgG are the major antibodies in circulation usually secreted against a variety of pathogens including viruses. IgA is present in all mucosal secretions, saliva and breast milk. IgE, which has the shortest half-life, is usually secreted against parasitic infections and allergies. The half-life of IgE increases when it binds to the FcεRI on mast cells [62].

Each of the antibody class has the typical 'Y' shape of the antibody comprising the antigen-binding fragment (Fab) at the 'v' part and effector part (Fc) formed by the constant stem of the 'Y'. The secreted antibodies can bind to immune cells that have Fc receptors on their surface. The Fc part specifies the effector function of the antibody i.e. neutralization, agglutination, opsonisation, precipitation, complement activation and antibody induced cell death. Antibodies when bound to their Fc receptors provide feedback mechanisms either by enhancing the immune response or by regulating it [62]. Immunoglobulin feedback mechanism is essential for maintenance and regulation of immune response [85]. For example, IgG subclasses are known to dampen an ongoing immune response when bound to their inhibitory FcγRII, on the other hand IgE is known to enhance the immune response when bound to FcεRII commonly known as CD23, the low affinity receptor for IgE on B cells and other cell types [85, 86]. IgG3 and IgM can activate the complement [85].

Antibodies have a role in homeostasis. Natural IgM and IgG coat apoptotic cells to enhance phagocytosis by macrophages [87]. Natural antibodies (NAbs) are directed towards self-antigens and other antibodies (anti-idiotypes) to ensure maintenance of tissue homeostasis and regulation of immune response. Usually when an anti-idiotype antibody binds to both the Fcγ receptor and the BCR, it inhibits BCR mediated signaling in the B cell [87-89]. Idiotype- anti-idiotype interactions aid in regulating B cell self-reactivity. This is a correction mechanism, where the anti-idiotype antibody aids in clearance of the autoantibody. Impaired idiotype- anti-idiotype interactions form the basic feature of many autoimmune diseases including T1D, thereby promoting continued existence of autoantibodies which can contribute to disease [89].

Humoral response and germinal centres

B cells can recognize both membrane bound antigens and soluble antigens. T cells, however, can only recognize antigens bound on MHC class I/II molecules. B cell follicles in the secondary lymphoid organs provide a favorable environment for antigenic encounter. Here B cells can encounter soluble or large antigens on the surface of macrophages, follicular dendritic cells (FDCs) and dendritic cells [90-92]. Mechanism by which antigens of differing sizes reach the B cell follicle remains unclear. Entry through subcapsular sinus pores in lymph nodes or simple diffusion have been speculated to be the mechanisms for small antigens (<70 kDa) [93, 94]. The larger antigens (>70 kDa) have limited access to the follicle. However *in vivo* evidence points to an efficient mechanism for transport of such antigens usually tethered to the surface of antigen presenting cells (APC) [95]. Antigen recognition by the BCR results in activation of the B cells. In the process the B cells migrate to the T-B cell boundary for full activation [96]. This migration of B cells is directed by the expression of the chemokines CCL19 and CCL21 by the stromal cells along with their receptor CCR7 [97].

Some of the activated B cells continue to express high levels of CXCR5 and reenter the B cells follicle where they continue to proliferate. B cells can either differentiate to form extrafollicular plasma blasts that are essential for rapid antibody production and early immune responses [98]. Or activated B cells can enter germinal centres, where they either differentiate into plasma cells, which can secrete high-affinity antibody following affinity maturation, or memory B cells, which confer long-lasting protection from secondary challenge with

antigen [98-100]. A schematic of germinal centre events is presented in Figure 4.

Germinal centers (GC) are highly dynamic and transient structures in which B cells proliferate to undergo clonal expansion, class switch recombination, SHM, and affinity maturation during an immune response. These structures consist of a network of specialized cells such as $CD4^+$ $CXCR5^+$ T follicular helper cells (T_{FH} cells) and FDCs (Figure 6). The complex network of helper cells provide CD40L, IL-4 (FDCs) and IL21 (T_{FH} cells) which are essential for B cell survival [82]. Germinal centers provide a specific niche for the highly proliferative B cells to undergo SHM, differentiation to plasma cells and long lived memory B cells [99, 101, 102]. During an immune response, newly activated B cells can reuse the existing germinal centers in presence of T cell help [103]. GC B cells express high levels of activation-induced deaminase (AID) which is responsible for the deamination of cytidine residues which enables SHM and class switching [104-106].

Deletion of self-reactive and low affinity B cell clones also occurs simultaneously in the GC. This negative selection is achieved by limiting availability of cognate T_{FH} cell interaction [107] and pro survival factors such as BAFF [108]. This results in the generation of high affinity B cell clones secreting class switched antibodies. This mechanism is affected in autoimmunity and the self-reactive clones escape the negative selection.

Majority of the plasma cells migrate to the bone marrow or local mucosa-associated lymphoid tissues and a smaller proportion of GC-derived plasma cells is retained in the spleen. Cytokines important for plasma cell survival include IL-5, IL-6, BAFF, APRIL and TNF [82].

Memory B cells persist in the body for a long time after antigen exposure and recirculate through secondary lymphoid organs to increase the probability of meeting their cognate antigen. They respond to antigen re-encounter with secretion of high affinity antibodies [109].

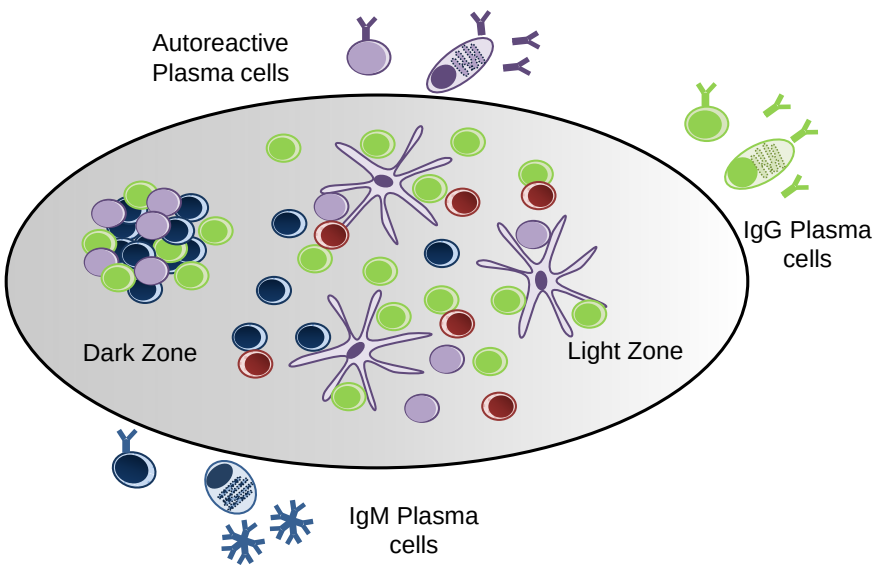

Figure 4: Schematic of germinal centre reaction. About five clones enter the GC during an immune response. They undergo proliferation, class switch and SHM in the dark zone and are selected for affinity by FDC and T_{FH} in the light zone. High affinity IgM and IgG clones are allowed to exit whereas autoreactive clones are usually deleted. However, some autoreactive clones may escape the selection in the GC and form memory and plasma cells, a process that may be increased in an autoimmune disease. B cells – circular blue, green and purple cells; B cells with BCR – memory cells; T_{FH} cells – circular red cells; FDC – purple cells with long dendrites.

B cell deviations in the NOD mouse

The B cells in NOD mouse have defects that aid in autoimmunity towards islet antigens [53, 110, 111]. Moreover, depletion of B cells with monoclonal antibodies like anti-CD20, supresses T1D development in NOD mice [52, 112]. This established the role of B cells in T1D autoimmunity although how B cells participate in T1D pathogenesis is not entirely deciphered. They may serve as APC to autoreactive T cells and/or produce autoantibodies that skew the immune response [113].

Compared to other mouse strains, the NOD mouse has an altered Ig-repertoire with an altered VH gene utilization pattern, which is also reported in humans [114, 115]. The natural antibody repertoire in NOD is multireactive corresponding to an altered B cell selection and activation [116]. Transgenic NOD mice that produce B cells expressing a mutant membrane bound IgM (unable to secrete), have increased insulitis and T1D incidence, suggesting the role of B cells in promoting the disease which is independent of Ig-secretion [49]. The APC function of B cells is probably more critical for diabetes development than autoantibody secretion. NOD mice with MHC class II deficient B cells have reduced T1D incidence, despite the ability of DCs and macrophages to express MHC class II [117]. A $CD4^+$ T cell response to several autoantigens is impaired in B cell deficient NOD mice, emphasizing the role of B cells as APC [111, 118, 119].

Engagement of CD28 on T cells with its ligand CD80/ CD86 on APCs is a critical costimulatory signal for T cell proliferation [96]. Resting splenic B cells express low levels of CD80 and CD86. However, it has been shown that splenic and islet-infiltrating B cells in the NOD mouse have enhanced expression of CD80 before insulitis, ensuring

potent T cell activation and islet infiltration [120]. Self-reactive B cells constitute the major APC subpopulation in NOD islets before insulitis is established [121]. From these studies the ability of NOD B cells to capture and present specific islet antigens to islet-reactive NOD T cells is evident [117, 118, 122].

Defects in B cell tolerance could also contribute T1D in NOD mice. NOD B cells are resistant to BCR-dependent activation-induced cell death and hyperproliferate upon BCR stimulation. These hyper activated B cells are mostly found in the spleen, pancreatic lymph nodes and islets [121]. For e.g. NOD mice fail to eliminate transgenic autoreactive B cells, as HEL-specific NOD B cells that encounter HEL as a neo self-antigen in soluble form are not as efficiently deleted or anergized, compared to transgenic B cells from C57BL/6 mice [50]. However, through T cell help, anergy is reversed in NOD *IgHEL*-transgenic B cells, a feature not observed for self-reactive B cells from C57BL/6 [119].

The spleen and the pancreatic lymph node are most likely the sites of primary interaction between autoreactive B cells and $CD4^+$ T cells in the NOD mouse since, the pancreatic lymph nodes are important sites for T cell activation and expansion and the spleen is the largest reservoir of diabetogenic T cells [51]. B cells and T cells that infiltrate the islets can also organize into tertiary lymphoid structures with GCs that contain B cells enriched for the GC marker GL7 and for sequences containing multiple mutations within CDRs [123]. NOD B cells display enhanced expression of TACI on their cell surface [124], a feature that may rescue the autoreactive B cells from the GCs.

The B cells in NOD mouse are also known to have a prolonged response towards both self and non-self-antigens, a feature mapped

to multiple *Idd* regions [125]. They also have the capacity to capture immunoglobulins on their surface [126] which might enhance their capacity to carry immune complexes to the secondary lymphoid organs and enhance immune responses. The NOD mice also display an expanded MZ B cell compartment which can present lipid antigens to NKT cells through CD1d [127]. The inhibitory FcγRII receptor is defective in NOD, impairing the feedback mechanisms and is associated with increased serum IgG1 and IgG2a [128]. Further investigations are still needed to understand the precise role B cells play in contributing to Type 1 Diabetes in NOD mouse and in humans.

Aim of the thesis

The aim of the thesis is to understand anomalies of the NOD B cell compartment, which in turn could contribute to immune perturbations in general and potentially to the development of T1D.

The specific aims are:

- To understand the role of B cells in the initiation phase of the T1D in NOD mouse and the influence on the immune response.

- To identify the molecules that may contribute to B cell hyper responsiveness, more specifically TACI.

Methodological considerations

Mice

All the mice used in the study were bred and housed in Umeå Transgenic Core Facility (UTCF), Umeå University. NODShilt/J (NOD), NOD.*Rag2*-/- (NOD.Rag), C57BL/6J (B6), C57BL/6.H2g7 (B6g7) mice have been bred at UTCF for over ten generations. The CD23 knockout mice on BALB/c background and DO11.10 TCR transgenic mice were obtained as breeding pair from Professor Birgitta Heyman, Uppsala University and bred in the our animal facility. Only 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).

As discussed previously, the NOD mouse is a model for T1D that mimics the human disease and is a preferred tool due to the ease of study. This mouse model, like the others is inbred and has been essential in deciphering several immune mechanisms in T1D.

B and T cell adoptive transfers

To study the role of B cells in IgE- immune complex transport, adoptive transfers in CD23-/- KO mice were performed as described in Henningsson et al. Briefly, CD4+ T cells were isolated from DO11.10 spleens and transferred to 8 week old CD23-/- mice. 24 hours later, B cells isolated from NOD and B6 spleens transferred in the mice that already received T cells. Cells were transferred into the recipient mice through the intra venous (*i.v*) route. Three hours post

B cell transfer; the mice were injected *i.v* with 20µg of OVA-TNP and 50 µg of IgE anti-TNP immune complex. Spleens were harvested 72 hours post immunization and flash frozen in liquid Nitrogen. This allows the understanding of the role of CD23, the low affinity receptor for IgE on NOD B cells.

To study the cells playing a major role in the immune response, B and T cells from NOD and B6g7 were transferred in to immune deficient NOD.Rag mice. Untouched B and T cells were isolated from HEL primed NOD or B6g7 mice using commercially available kits and transferred to the NOD.Rag mice. In some experiments, FACS sorting was employed to isolate untouched B and T cells prior to transfer. Cells were transferred into the recipient mice through the *i.v* route. This allows for the study of only the transferred cells as the recipient mice lack both B and T cells. Recipient mice were immunized with HEL three days post cell transfer and the antibody response was measured four weeks later by ELISA.

A major limitation here has been to get enough cell numbers for transfer. Cell mortality was higher when isolation kits were used for cell purification; on the other hand, the time taken to purify using FACS sorting was very long.

23G3 Hybridoma culture and anti-IgE purification

B cell hybridoma producing murine anti-IgE (clone 23G3) was obtained from Prof. D.H Conrad, Virginia University and were cultured in DMEM with 5% FCS. Supernatant of the cell culture was collected and ran through a HiTrap™ protein-L column that captures κ light chain antibodies selectively. This ensured the purity of the antibody being isolated, eliminating possible contamination with

bovine IgG present in the growth media, which could potentially hinder the binding of anti-IgE to the cells.

ELISA

ELISA was used to determine serum levels of IgE (Paper I), antibody response to HEL and NP-HEL (paper II & III), determine antibody clearance of anti-KLH IgG1 (paper III). This method is preferred for its sensitivity and robustness. Sandwich and Indirect ELISA were mostly used.

Immunizations

Most immunizations were performed intra peritoneally (*i.p*) - Anti-IgE (paper I), NP-HEL (Paper II), HEL (Paper II and III) and anti-KLH IgG1 (Paper III), as larger volumes and more viscous solutions could be easily administered.

Flow cytometry

Flow cytometry was used for analysis of cell surface expression of IgE (Paper I), TACI and BAFF-R (Paper II), calcium release upon anti-IgM stimulation in B cells (Paper III) and all cell surface markers. This method allows for separation and distinction based on cellular and surface molecular staining properties and is very sensitive as well as specific.

Immunofluorescence and Immunohistochemistry

To determine the histological location of transferred CD4+ T cells in spleens of CD23-/- mice and IgE in NOD and B6 spleens (paper I), germinal centers in NOD and B6 spleen (paper II & III), BAFF in

spleen (paper II) 8µm thick cryosections obtained from frozen spleens were stained. NOD and B6 pancreas sections (8µm thick) were also stained for infiltrating B cells, T cells and IgE.

This method allows for visualization of location of cells and structures formed during an active immune response. This is important as location directs the microenvironment and signals that are essential for SHM and class switching. However, only one layer can be observed at a given time point and hence there is loss of information from subsequent time points. Sectioning of organs like the pancreas may also lead to loss of information in other lobules. A technique such as optical projection tomography as demonstrated by Hörnblad et al [129] may be better alternative to view the infiltration and tertiary lymphoid structures in whole mouse pancreas.

Statistics

Mann–Whitney test for unpaired samples or Student's t-test or two-way ANOVA or Chi-square test were performed where applicable and a two-tailed p value was calculated. A p value < 0.05 was considered significant.

Results and discussion

IgE in NOD mouse – implications in T1D progression

IgE has been widely associated with allergy. Autoimmunity and allergy have been previously considered mutually exclusive, a notion now challenged by the increased observation of IgE antibodies in patients with autoimmune diseases [130]. IgE binds to the FcεRI on mast cells and upon being crosslinked by an antigen causes the mast cell to release the mediators of allergy for e.g. histamine. Valenta et al coined the term '*autoallergy*' when IgE was found directed towards self-antigens in patients with autoimmune diseases [130]. Presence of IgE has been associated with poor disease prognosis in patients with bullous pemphigoid and SLE [131, 132]. IgE directed towards GAD65 has also been observed in patients with T1D [133].

We, in the Lejon lab, have looked at several B cell anomalies in the NOD mouse. These include excess capture of IgM and IgG on the B cell surface and increased expression of TACI on B cells [124, 126]. In line with previous studies from our lab, we observed increased capture of IgE on NOD B cells (Paper I) [126]. Moreover, an increased level of IgE was also detected in the red pulp of the spleen of NOD mice, implying that additional cell types also displayed this feature (Figure 1 A-D, Paper I). However, IgE capture was partly but not exclusively mediated by CD23. It is known that CD23 (FcεRII) is found on several cell types residing in the red pulp including macrophages and eosinophils [86]. IgE levels on B cells in NOD mice were increased through all ages starting from one week and did not correlate with serum levels of IgE (Figure 2A-B, Paper I). This increased availability of IgE may be explained due to the preferential

production of IgG1 in NOD mice, resulting in some B cells to develop to IgE producing plasma cells which is usually the next isotype these B cells switch to [128, 134]. We and others have observed an increased IgG1 response following immunization with antigens such as HEL, associated with the escape of these cells from the GC (Paper III and [128, 134]).

In NOD mice, it was observed that early depletion of B cells using anti-IgM antibodies prevents diabetes development [135]. In paper I, we administered anti-IgE antibodies for duration of 5 weeks, starting when the mice were 3 week old. Depletion of IgE using anti-IgE delayed diabetes development (Figure 4, Paper I). It has been suggested that IgE mediates feedback on the immune system by increasing antigen presentation and thereby, enhancing the immune response [85, 136-139].

We stained pancreas section from NOD and B6 mice to clarify the occurrence of IgE at the site of inflammation. We observed a distinct IgE staining in one NOD mouse (13 weeks) (Figure 5, Paper I). This implies that localized IgE production may occur. The presence of tertiary lymphoid structures in and around the infiltrated pancreatic islets as observed by us and others [140] suggests that class switching could occur at the site of inflammation and that the IgE might be specific to islet antigens.

In conclusion, we suggest that IgE antibodies could contribute to the pathogenesis of T1D in the NOD mouse either through sustained inflammation due to the IgE feedback mechanism or aid in destruction of the β cells by opsonization, if they are β cell antigen specific. In addition, it is plausible that excessive class-switching in germinal centers due to sustained inflammation in tertiary follicles

could initiate production of non-specific IgE. However, more work needs to be done to identify the antigen targeted by IgE.

Enhanced expression of TACI on NOD B cells – implications for better survival of autoreactive B cells

Autoreactive B cells escape the checkpoints during a GC reaction in autoimmune diseases. One reason for better survival of these clones could be increased survival signals in the environment. TACI is a molecule that regulates B cell survival and class switching. TACI deficient mice have been reported to be unable to produce IgG1, IgA and IgE in response to APRIL [74] and generate long-lived plasma cells [75, 76]. TACI has been revealed to function as a negative regulator of B cell homeostasis [141-143].

Previously we reported that NOD B cells contain an increased percentage of $TACI^{high}$ cells [124]. This upregulation of TACI in the NOD mouse may reflect an attempt to downregulate the generally activated B cell. However, as a consequence of the $TACI^{high}$ phenotype in the NOD mouse, other effects such as increased plasma cell differentiation, isotype switch and immunoglobulin production could also be expected. This was supported by our observation (Paper II) of an increased plasma cell differentiation and Ig production in APRIL-stimulated B cells of NOD mice compared to B6 mice. In addition $TACI^{+}$ NOD B cells populated GCs, bound more BAFF and produced low affinity antibodies against T-dependent antigen (Figure 4-6, Paper II).

As described in Paper II, we stimulated the cells with APRIL to avoid the potential contribution of other factors that may differ between NOD and B6 mice (i.e. signaling pathways) as it has been previously demonstrated that there is a synergy between TACI, TLR, CD40-

ligation and/or cytokine signaling (i.e. IL-4) [144, 145]. Although the contribution of the APRIL binding receptor BCMA cannot be excluded, the difference we observed was most likely was due to enhanced TACI expression on NOD B cells (Figure 2 and 3, Paper II).

As previously mentioned, increased TACI expression could contribute to highly activated B cell repertoire prone to plasma cell differentiation and isotype switch. Our lab and others have reported that NOD mice exhibit an enhanced and prolonged immune response [125, 146, 147]. However, based on the fact that the immune response trait was assayed 21 days after immunization, versus the recent report that TACI was involved in generating long lived plasma cells (i.e. assayed 35 days after immunization) [75], we speculate that TACI contributes to a prolonged and enhanced immune response at a later stage.

In order to ensure survival of high affinity clones in GC, survival factors such as BAFF is limited. However, excess BAFF allows for survival of low affinity and autoreactive clones [81, 108]. Sequestering of BAFF in GCs has been attributed to TACI expression in GC B cells [108]. Correspondingly, we observed that B6 mice immunized with HEL display little or no $TACI^+$ B cells in the GC. However, both unimmunized and immunized NOD mice failed to downregulate TACI expression on GC B cells (Figure 4, Paper II). This led us to analyze the expression of BAFF in GCs. Indeed, both unimmunized and HEL immunized NOD mice stained positively for BAFF in GC (Figure 5, Paper II). In addition, BAFF staining was observed in the marginal zone reflecting the TACI bright nature of MZ B cells [148].

Low affinity IgG form the bulk of the natural antibodies (for e.g. against insulin) in NOD mice [116, 149]. We hypothesized that the

increased presence of BAFF in the NOD GCs could affect affinity maturation against a conventional antigen and therefore, we immunized NOD and B6 mice with NP-HEL. We observed that NOD mice produced proportionally more low affinity antibodies as deduced from the ratio between NP_4-BSA and NP_{20}-BSA signals, supporting our hypothesis (Figure 6, Paper II). Targeting of BAFF in the NOD mouse has been shown to protect against T1D [150]. We speculate that the property of NOD B cells to respond with higher titers during an (auto)immune response and to generate low affinity antibodies may contribute to epitope spreading towards autoantigens as observed in these mice [116].

Immune response in the NOD mouse is more robust – B and T cell interplay

Previously, we and others have observed that NOD mice respond intensely to both foreign and self-antigens by producing large amounts of antigen specific IgG [151, 152]. Autoantibodies in NOD mice and humans are essentially of IgG isotype and have been shown to arise from germinal center reaction [149]. This response was also found to be T-cell dependent, as NOD.nu/nu mice (that lack thymus) did not respond [125].

In Paper III, we aimed at analyzing the mechanism contributing to the enhanced and prolonged response in the NOD mouse towards the conventional antigen, HEL. Our control B6 mice have previously been reported to be low responders to HEL+CFA as compared to several other strains, and the MHC has been suggested to be an important factor in this response [153]. The use of CFA alone in NOD mouse skewed the immune response and prevented the mice from developing T1D [154]. In our protocol we have used HEL+IFA, a

combination that is not sufficient to promote a robust response in almost any strain [155]. However, as previously described, NOD mice responded well to antigen given with this adjuvant [155]. In line with our previous finding, MHC was observed to have a potent influence on this trait [125] as B6g7 mice (which shared the I-Ag7 with NOD mice) responded to HEL with titers comparable to that in NOD mice.

A potential cause for the high serum levels of IgG1 against HEL observed over time (Figure 1A, Paper III) could be the inefficient clearance of antibodies in the NOD mouse. To test this, we injected monoclonal anti-KLH IgG1 antibodies as described by Viera et al [156], and determined the remaining level of anti-KLH antibodies in serum at several time points. We observed a slower clearance of the IgG1 antibodies in both strains. In our study we have not included the B6g7 mice as we expect the strain to behave similarly. We, therefore, excluded inefficient clearance as a contributor to the phenotype.

Since, NOD mice have a highly active B cell repertoire and pre-existing GCs [116, 157], this preset may provide a favorable milieu for the maintenance of an immune response. It has also been shown that pre-existing GCs are reused upon activation of B cells by a new antigen and relevant T cell help [103]. As described previously by Luzina et al [157], we also observed that non-immunized NOD mice had a large number of pre-existing GCs compared to B6 mice (Figure 3, Paper III). This difference was not evident post-immunization, supporting the theory of GC reuse. However, we did not look for spontaneous GC formation in B6g7 mice, which might give an insight to if the MHC alone is sufficient, to get T cell help or if the environment contributes in the availability of this privilege.

Increased BAFF availability in GC (Paper II) and enhanced TACI expression on GC B cells could contribute to B cell survival, plasma cell differentiation, as well as class switch to IgG (Paper II). In line with this it has also been described that the presence of excess BAFF can activate transitional B cells and promote autoantibody production in a TACI dependent manner [158]. Accumulation of long-lived plasma cells in the spleen promoted the differentiation of $CD4^+$ T cells to T_{FH} cells that in turn contributed to the persistence of humoral autoimmunity [159].

We also tested BCR induced activation of B cells using anti-IgM antibodies. Strangely, we noticed that NOD B cells were hypo-responsive, in particular to low concentrations of anti-IgM. BCR stimulation with low concentrations of anti-IgM may mimic physiological stimulation by foreign or self-antigens. So it is plausible that this hypo-responsiveness had consequences during B cell development in the bone marrow. As previously described, NOD B cells escaped negative selection during development [50] an indication of autoreactive clones and low-affinity clones (Paper II) released in the periphery. This feature may lead to the avoidance of antigen induced anergy in the periphery and activation induced cell death thereby contributing to the prolonged and enhanced immune response.

In order to confirm that B cell anomalies contributed to the skewed immune response in HEL, we adoptively transferred NOD and B6g7 cells into NOD Rag mice in various combinations as described in Paper III. We observed that B cells of NOD origin seemed sufficient to respond to HEL irrespective of the origin of the T cells. On the contrary, B6g7 B cells in combination with NOD T cells did not

respond. It is plausible that the TACI^{+} B cells along with the hypo-responsive BCR may be better at responding to low amounts of survival signals as well as producing antibodies towards HEL, although other explanations cannot be excluded at this point. We also speculate that B6g7 B cells may have a lower expression of TACI, and hence would not have access to sufficient amounts of BAFF to survive or class switch. The fact that the B6g7 spleen cells had the ability to respond if they were located in a NOD-RAG environment suggests that the myeloid compartment and/or stromal cells could provide a milieu promoting an immune response.

The proposed explanation for the cause of the prolonged and enhanced immune response towards HEL in NOD mice could be extended to an immune response towards self-antigens, thereby providing additional understanding of the mechanisms promoting autoimmunity.

Conclusion

The thesis covers some important B cell anomalies that might contribute to the skewed immune response towards both self and non-self-antigens in NOD mice. The immune response is highly dependent on cell-cell interactions, reception of survival signals and regulation of inflammatory mediators such as cytokines. There may be several deviant aspects that could contribute to this complex interplay and lead to autoimmunity. Some of them are listed in this thesis and are as follows:

- The TACI^{high} phenotype of NOD B cells could enhance survival of low-affinity clones and result in an increased class switching due to an increased availability of BAFF. Pre-existing GCs in NOD mice may contribute to the robust response to novel antigens.

- The immune response to HEL usually promoted antibodies of the IgG1 isotype and might, along with decreased BCR signalling strength, serve to maintain the low-affinity pool.

- Excessive class switching in the NOD mouse may yield IgE antibodies. IgE is increased in the NOD mouse suggesting potential autoallergy, as it was also found in tertiary lymphoid structures in the pancreas. Depletion of IgE antibodies with anti-IgE treatment in pre-diabetic NOD mice delayed T1D disease onset.

The mechanisms together may contribute to a skewed immune response in the NOD mice leading to events that facilitate precipitation of T1D in these mice. A model based on these anomalies is hereby proposed (Figure 5).

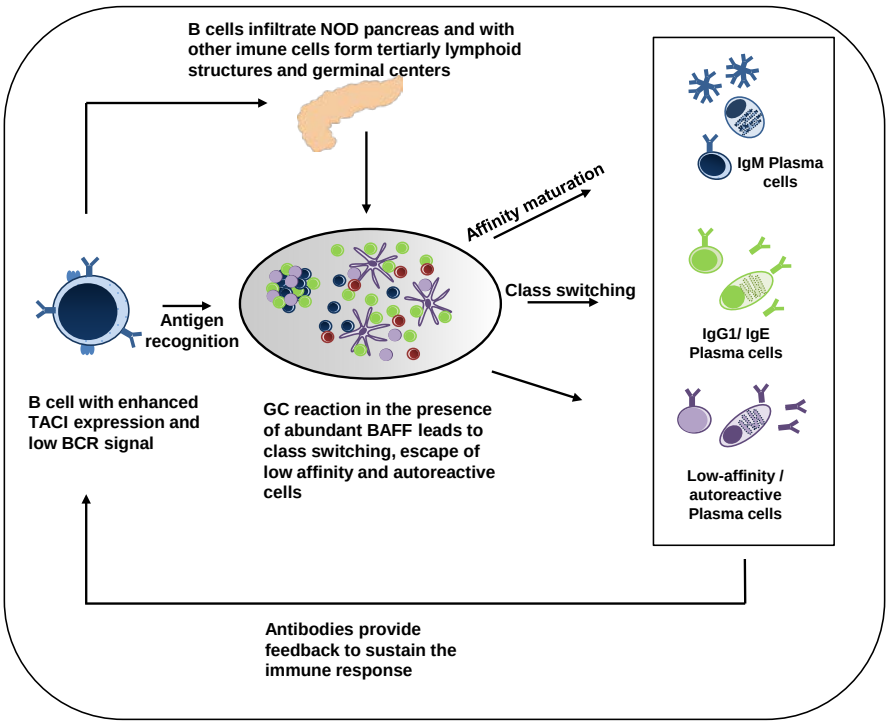

Figure 5: Schematic model of the skewed immune response in the NOD mouse towards self and non-self-antigens

Acknowledgements

This journey would have not been possible without the involvement of the many people who have contributed both to my professional and personal growth. I would especially like to thank the people who have been an important part of my Ph.D time here in Umeå.

Heartiest thanks to my supervisor ***Kristina*** for the opportunity to do a Ph.D and for the support during these years. Your optimism, cheerfulness and enthusiasm have been very much a part of your supervision and discussions. It has been a fantastic learning experience to have worked with you. Sincere thanks to my co-supervisor ***Per-Arne*** for the constant support and for sharing your knowledge and ideas with me during group meetings and journal clubs. ***Marie-Louise, Sten and Torgny*** – Thank you so much for the invaluable knowledge you bring into every discussion.

Viqar – Your knowledge, ideas and love for details stands out and is most admirable. Thank you for all these years of friendship, arguments, long discussions on science and every other topic imaginable, help and encouragement. It has been oodles of fun [annoying too at times ;)] working [and fooling around] with you. You have always been there for me no matter what. ***Mia*** – Your bubbly personality always cheered me up. You are missed! I wish we had worked longer together. Sincere thanks to all the students that I have had the opportunity to work with. You guys made life fun and science a lot more interesting. I have also learnt a lot myself during the time spent with you people. ***Shrikant*** – Thanks a lot for all the encouragement, discussion and ideas. I am so glad that you moved into this corridor to work amongst us! You have always been very kind and helpful. ***Barbro*** – Thank you for teaching me all that I know about immunohisto.

At UTCF ***Ulla-Brit, Carola and Maria***– Thank you so much for all the help over these years.

Anne – You are wonderful! As I have said a thousand times before, I cannot imagine Immunology without you. You have been my mentor and support system during these years. You have always been there for me to share joys, sorrows and all in-between. Saying a mere thank you will not justify the immense gratitude and respect I feel for you. ***Lena*** – Another person I cannot imagine Immunology without! You are lovely! You have always been there to answer any and all administrative questions. It has also been fun discussing culture and films with you and of course our mutual interest in Indian designs.

Greg – It has been a lot of fun sitting with you at work and talking about science and everything else! You have always been kind, funny and helpful. Thank you for these years. I shall remember you and “lille Hampus” very fondly! Wish you the very best in life. ***Aziz, David, Fredrik*** – The times

we have spoken has always been filled with laughter. I have had interesting discussions and very positive insights to both research and life from you all. Thank you so much for these memorable years.

Lina, Theres and Veronika – Thanks for the enjoyable conversations, fikas, dinners and lively times in Immunology. You are really missed!

Pedro - We make the best team ever! I have had a lot of fun with you during course labs. Remember- Do NOT joke with the students, they take it too seriously! It has been fun knowing and working with you and of course becoming "friends" from "almost friends".

Madhu, Sujan, Sandra, Ava and Marios – It has been a short time with you guys here but great fun. Glad you guys moved to this corridor! ***Anne, Priscilla, Tora and Andy*** – It has been really nice to know you all, though for a short while. I wish you the best for your futures.

Eva – I have enjoyed our conversations on work life, post docs, life experiences, scientific methods, travel and culture! Thank you for being friendly, kind and helpful at all times! ***Sofia*** – The interesting discussions with you always leave me energized and inspired. ***Mattias*** – Thank you for sharing your expertise on B cells and Flow Jo. Thanks to present and past members of the Immunology and Clinical Immunology corridor for the discussions and support – ***Mari, Maria, Åsa, Lucia, Evan, Olga, Ali, Dan, Nina, Lisbeth, Constantin,Veronica, Marianne, Basel and Ritu.***

Jonathan – Thanks for your help and discussions on microscopy. ***Rutan*** – Thanks a lot for sharing your expertise and knowledge with me. You have always been very forthcoming and kind whenever I have had any question.

Emma and Katarina – Thanks for the dinners, the tie and dye socks and for introducing me to some much needed silly TV shows. ***Anandi, Mridula, Steffi, Mahesh and Ala –*** Thanks for adding sunshine and smiles to the otherwise cold Umeå! You have been really fun to hang out with. I will cherish the dinners, dances, card games and more. ***Tanu, Christophe, Gautam, Lars Geetanjali, Chinmay, Arun, Agi, Tamas, Chaitanya, Zahra, Asim, Sandrine, Richard, Naresh, Nabil and Manu –*** Thanks for the good times, the Diwali parties, birthday celebrations, BBQs, bowling sessions and the get-togethers. Friends at MolBio – ***Anne-Laure, Roshani, Akbar, Nicola, Ievgen, Svitlana, Sabine and Victoria*** I'm so glad to know all you cheerful people on the other side and for being able to discuss science and just life in general as Ph.Ds and Post Docs. Wish you all the best for the future. A lot of you have moved out of Umeå. Hope we cross paths many times in life.

To the people of ***NCRSCID, KI***! What a wonderful 2 years have I had at the research school! You people are inspirational. ***Helena*** and ***Jan-Alvar***,

thank you for coming up with this idea! I have learnt a lot during this time. The people we have had the opportunity to meet through the workshops and courses have been really awe-inspiring. I sincerely hope I have the opportunity to work with some of them. I would like to mention some people I have spent most time in the school with – ***Sandra***, my roomie most times☺, ***Daniel, Simon, Erik, Harald, Sabrina, Christina*** for the really good discussions and Erik also for introducing me to a lot of card/board games. ***Vivek and Behdad*** – your good humour always cheered me up. Some close friends I made during this time – ***Mikael*** – I am so happy to know you! It's been wonderful talking to you about everything. You are always so positive, practical and encouraging. ***Johanna*** – I will cherish the good times we have had together. It's been so much fun doing things with you, travelling, wall papering. I hope we will go through all other plans we have – specially exploring Sweden. ***Xia*** – my quite yet funny friend! The last time spent with you before you moved to Boston was really emotional! I hope to see you again soon! ***Nastya –*** you are so insightful! It's calming to be around you. I hope to meet you soon! ***Mahsa*** – from the second batch, you are so friendly and sweet! It has been really nice to know you. I wish you the best with your thesis and life.

Amez and Nils – you both are wonderful people, fun, helpful, knowledgeable and kind! Thanks for all the lovely times we have had. I hope there will be more! ***Personnel at Kahls*** – your coffee has fueled me through my PhD. Your lovely smiles and conversations always made my day. My friends from far away – ***Vasu –*** my support! You have always been at my side not matter what I chose to do in life. ***Nora*** – my sweet friend! You are so caring and lovely. You have always been positive and encouraging. ***Sohini*** – a person I can thoroughly count on for advice, for discussing books (and a lot more) and great times! I am blessed to have you women in my life. Thanks a ton. ***Sunu*** – my childhood friend! We've always managed to keep contact through all these years in spite of our different life paths; I hope we keep it up! Time and distance seem irrelevant with friendships like these.

To my loveliest, kindest, best and most caring friend ***Damini*** – You have been an important part of my PhD and life here in Sweden. I cannot thank you enough for your unwavering support through these years. I love you! It is amazing to have a friend/sister in life that you can count on at all times. Anything I write here falls short to describe the friendship we share. Just know I will always be there too.

To ***Amma, Appa*** – I love you! Your unconditional love and support is what gives me strength. ***Anand*** – you are awesome!

"*No matter how much time passes, no matter what takes place in the interim, there are some things we can never assign to oblivion, memories we can never rub away.*"– Haruki Murakami

And that is what I take with me from my time here – a lot of memories.

References

1. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts.* N Engl J Med, 2000. **343**(1): p. 37-49.

2. Walker, J.A., J.L. Barlow, and A.N. McKenzie, *Innate lymphoid cells--how did we miss them?* Nat Rev Immunol, 2013. **13**(2): p. 75-87.

3. Delves, P.J. and I.M. Roitt, *The immune system. Second of two parts.* N Engl J Med, 2000. **343**(2): p. 108-17.

4. Kisielow, P., et al., *Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes.* Nature, 1988. **333**(6175): p. 742-6.

5. Pircher, H., et al., *Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen.* Nature, 1989. **342**(6249): p. 559-61.

6. Kronenberg, M., *Toward an understanding of NKT cell biology: progress and paradoxes.* Annu Rev Immunol, 2005. **23**: p. 877-900.

7. Davidson, A. and B. Diamond, *Autoimmune diseases.* N Engl J Med, 2001. **345**(5): p. 340-50.

8. Cooper, G.S., M.L. Bynum, and E.C. Somers, *Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases.* J Autoimmun, 2009. **33**(3-4): p. 197-207.

9. Benoist, C. and D. Mathis, *Autoimmunity provoked by infection: how good is the case for T cell epitope mimicry?* Nat Immunol, 2001. **2**(9): p. 797-801.

10. Ermann, J. and C.G. Fathman, *Autoimmune diseases: genes, bugs and failed regulation.* Nat Immunol, 2001. **2**(9): p. 759-61.

11. Atkinson, M.A., et al., *Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes.* J Clin Invest, 1994. **94**(5): p. 2125-9.

12. Coppieters, K.T., A. Wiberg, and M.G. von Herrath, *Viral infections and molecular mimicry in type 1 diabetes.* APMIS, 2012. **120**(12): p. 941-9.

13. Fujinami, R.S., et al., *Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease.* Clin Microbiol Rev, 2006. **19**(1): p. 80-94.

14. Kelemen, K., *The role of T cells in beta cell damage in NOD mice and humans.* Adv Exp Med Biol, 2004. **552**: p. 117-28.

15. Kraine, M.R. and R.M. Tisch, *The role of environmental factors in insulin-dependent diabetes mellitus: an unresolved issue.* Environ Health Perspect, 1999. **107 Suppl 5**: p. 777-81.

16. Tisch, R. and H. McDevitt, *Insulin-dependent diabetes mellitus.* Cell, 1996. **85**(3): p. 291-7.

17. Ferreira, R.C., et al., *A type I interferon transcriptional signature precedes autoimmunity in children genetically at risk for type 1 diabetes.* Diabetes, 2014. **63**(7): p. 2538-50.

18. Wenzlau, J.M., et al., *The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17040-5.

19. Willcox, A., et al., *Analysis of islet inflammation in human type 1 diabetes.* Clin Exp Immunol, 2009. **155**(2): p. 173-81.

20. Todd, J.A., *Etiology of type 1 diabetes.* Immunity, 2010. **32**(4): p. 457-67.

21. Barrett, J.C., et al., *Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes.* Nat Genet, 2009. **41**(6): p. 703-7.

22. Burren, O.S., et al., *T1DBase: update 2011, organization and presentation of large-scale data sets for type 1 diabetes research.* Nucleic Acids Res, 2011. **39**(Database issue): p. D997-1001.

23. Pociot, F. and M.F. McDermott, *Genetics of type 1 diabetes mellitus.* Genes Immun, 2002. **3**(5): p. 235-49.

24. Aly, T.A., et al., *Extreme genetic risk for type 1A diabetes.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14074-9.

25. Risch, N., *Assessing the role of HLA-linked and unlinked determinants of disease.* Am J Hum Genet, 1987. **40**(1): p. 1-14.

26. Gough, S.C. and M.J. Simmonds, *The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action.* Curr Genomics, 2007. **8**(7): p. 453-65.

27. Pociot, F., et al., *Genetics of type 1 diabetes: what's next?* Diabetes, 2010. **59**(7): p. 1561-71.

28. Eichler, E.E., et al., *Missing heritability and strategies for finding the underlying causes of complex disease.* Nat Rev Genet, 2010. **11**(6): p. 446-50.

29. Trerotola, M., et al., *Epigenetic inheritance and the missing heritability.* Hum Genomics, 2015. **9**: p. 17.

30. Vehik, K. and D. Dabelea, *The changing epidemiology of type 1 diabetes: why is it going through the roof?* Diabetes Metab Res Rev, 2011. **27**(1): p. 3-13.

31. Oresic, M., et al., *Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes.* J Exp Med, 2008. **205**(13): p. 2975-84.

32. Serena, G., et al., *The Role of Gluten in Celiac Disease and Type 1 Diabetes.* Nutrients, 2015. **7**(9): p. 7143-62.

33. Paun, A., C. Yau, and J.S. Danska, *Immune recognition and response to the intestinal microbiome in type 1 diabetes.* J Autoimmun, 2016. **71**: p. 10-8.

34. Roep, B.O., M. Atkinson, and M. von Herrath, *Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes.* Nat Rev Immunol, 2004. **4**(12): p. 989-97.

35. Mordes, J.P., et al., *Rat models of type 1 diabetes: genetics, environment, and autoimmunity.* ILAR J, 2004. **45**(3): p. 278-91.

36. Bach, J.F., *Insulin-dependent diabetes mellitus as an autoimmune disease.* Endocr Rev, 1994. **15**(4): p. 516-42.

37. Kikutani, H. and S. Makino, *The murine autoimmune diabetes model: NOD and related strains.* Adv Immunol, 1992. **51**: p. 285-322.

38. Makino, S., et al., *Breeding of a non-obese, diabetic strain of mice.* Jikken Dobutsu, 1980. **29**(1): p. 1-13.

39. King, C. and N. Sarvetnick, *The incidence of type-1 diabetes in NOD mice is modulated by restricted flora not germ-free conditions.* PLoS One, 2011. **6**(2): p. e17049.

40. Kriegel, M.A., et al., *Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice.* Proc Natl Acad Sci U S A, 2011. **108**(28): p. 11548-53.

41. Fox, H.S., *Androgen treatment prevents diabetes in nonobese diabetic mice.* J Exp Med, 1992. **175**(5): p. 1409-12.

42. Makino, S., et al., *Effect of castration on the appearance of diabetes in NOD mouse.* Jikken Dobutsu, 1981. **30**(2): p. 137-40.

43. Madsen, R., et al., *Altered metabolic signature in pre-diabetic NOD mice.* PLoS One, 2012. **7**(4): p. e35445.

44. Driver, J.P., D.V. Serreze, and Y.G. Chen, *Mouse models for the study of autoimmune type 1 diabetes: a NOD to similarities and differences to human disease.* Semin Immunopathol, 2011. **33**(1): p. 67-87.

45. Hattori, M., et al., *The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex.* Science, 1986. **231**(4739): p. 733-5.

46. Vijayakrishnan, L., et al., *An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells.* Immunity, 2004. **20**(5): p. 563-75.

47. Wicker, L.S., et al., *Genetic control of diabetes and insulitis in the nonobese diabetic (NOD) mouse.* J Exp Med, 1987. **165**(6): p. 1639-54.

48. Delovitch, T.L. and B. Singh, *The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD.* Immunity, 1997. **7**(6): p. 727-38.

49. Wong, F.S., et al., *Investigation of the role of B-cells in type 1 diabetes in the NOD mouse.* Diabetes, 2004. **53**(10): p. 2581-7.

50. Silveira, P.A., et al., *B cell selection defects underlie the development of diabetogenic APCs in nonobese diabetic mice.* J Immunol, 2004. **172**(8): p. 5086-94.

51. Silveira, P.A. and S.T. Grey, *B cells in the spotlight: innocent bystanders or major players in the pathogenesis of type 1 diabetes.* Trends Endocrinol Metab, 2006. **17**(4): p. 128-35.

52. Xiu, Y., et al., *B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in Fc gamma R effector functions.* J Immunol, 2008. **180**(5): p. 2863-75.

53. Yang, M., B. Charlton, and A.M. Gautam, *Development of insulitis and diabetes in B cell-deficient NOD mice.* J Autoimmun, 1997. **10**(3): p. 257-60.

54. Li, Y.S., et al., *Identification of the earliest B lineage stage in mouse bone marrow*. Immunity, 1996. **5**(6): p. 527-35.

55. Hardy, R.R., et al., *Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow*. J Exp Med, 1991. **173**(5): p. 1213-25.

56. Oettinger, M.A., et al., *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.

57. Tonegawa, S., *Somatic generation of antibody diversity*. Nature, 1983. **302**(5909): p. 575-81.

58. Ehlich, A., et al., *Analysis of the B-cell progenitor compartment at the level of single cells.* Curr Biol, 1994. **4**(7): p. 573-83.

59. Tussiwand, R., et al., *Tolerance checkpoints in B-cell development: Johnny B good.* Eur J Immunol, 2009. **39**(9): p. 2317-24.

60. Srivastava, B., et al., *Characterization of marginal zone B cell precursors.* J Exp Med, 2005. **202**(9): p. 1225-34.

61. Loder, F., et al., *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, 1999. **190**(1): p. 75-89.

62. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.

63. Pillai, S. and A. Cariappa, *The follicular versus marginal zone B lymphocyte cell fate decision.* Nat Rev Immunol, 2009. **9**(11): p. 767-77.

64. Saito, T., et al., *Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development*. Immunity, 2003. **18**(5): p. 675-85.

65. Palm, A.K., H.C. Friedrich, and S. Kleinau, *Nodal marginal zone B cells in mice: a novel subset with dormant self-reactivity*. Sci Rep, 2016. **6**: p. 27687.

66. Leadbetter, E.A., et al., *NK T cells provide lipid antigen-specific cognate help for B cells*. Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8339-44.

67. Martin, F. and J.F. Kearney, *Marginal-zone B cells*. Nat Rev Immunol, 2002. **2**(5): p. 323-35.

68. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens*. Immunity, 2001. **14**(5): p. 617-29.

69. Cinamon, G., et al., *Follicular shuttling of marginal zone B cells facilitates antigen transport*. Nat Immunol, 2008. **9**(1): p. 54-62.

70. Allman, D. and S. Pillai, *Peripheral B cell subsets*. Curr Opin Immunol, 2008. **20**(2): p. 149-57.

71. Mackay, F. and J.L. Browning, *BAFF: a fundamental survival factor for B cells*. Nat Rev Immunol, 2002. **2**(7): p. 465-75.

72. Mackay, F., et al., *BAFF AND APRIL: a tutorial on B cell survival*. Annu Rev Immunol, 2003. **21**: p. 231-64.

73. Schneider, P., et al., *BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth*. J Exp Med, 1999. **189**(11): p. 1747-56.

74. Castigli, E., et al., *TACI and BAFF-R mediate isotype switching in B cells*. J Exp Med, 2005. **201**(1): p. 35-9.

75. Tsuji, S., et al., *TACI deficiency impairs sustained Blimp-1 expression in B cells decreasing long-lived plasma cells in the bone marrow*. Blood, 2011. **118**(22): p. 5832-9.

76. Ou, X., S. Xu, and K.P. Lam, *Deficiency in TNFRSF13B (TACI) expands T-follicular helper and germinal center B cells via increased ICOS-ligand expression but impairs plasma cell survival.* Proc Natl Acad Sci U S A, 2012. **109**(38): p. 15401-6.

77. Srivastava, B., et al., *Models for peripheral B cell development and homeostasis.* Semin Immunol, 2005. **17**(3): p. 175-82.

78. Goodnow, C.C., et al., *Cellular and genetic mechanisms of self tolerance and autoimmunity*. Nature, 2005. **435**(7042): p. 590-7.

79. Nemazee, D. and K. Buerki, *Clonal deletion of autoreactive B lymphocytes in bone marrow chimeras.* Proc Natl Acad Sci U S A, 1989. **86**(20): p. 8039-43.

80. Basten, A. and P.A. Silveira, *B-cell tolerance: mechanisms and implications.* Curr Opin Immunol, 2010. **22**(5): p. 566-74.

81. Lesley, R., et al., *Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF.* Immunity, 2004. **20**(4): p. 441-53.

82. Goodnow, C.C., et al., *Control systems and decision making for antibody production.* Nat Immunol, 2010. **11**(8): p. 681-8.

83. Shokat, K.M. and C.C. Goodnow, *Antigen-induced B-cell death and elimination during germinal-centre immune responses.* Nature, 1995. **375**(6529): p. 334-8.

84. Woof, J.M. and J. Mestecky, *Mucosal immunoglobulins.* Immunol Rev, 2005. **206**: p. 64-82.

85. Hjelm, F., et al., *Antibody-mediated regulation of the immune response.* Scand J Immunol, 2006. **64**(3): p. 177-84.

86. Conrad, D.H., *Fc epsilon RII/CD23: the low affinity receptor for IgE.* Annu Rev Immunol, 1990. **8**: p. 623-45.

87. Lutz, H.U., *Homeostatic roles of naturally occurring antibodies: an overview.* J Autoimmun, 2007. **29**(4): p. 287-94.

88. Notkins, A.L., *Polyreactivity of antibody molecules.* Trends Immunol, 2004. **25**(4): p. 174-9.

89. Hampe, C.S., *Protective role of anti-idiotypic antibodies in autoimmunity--lessons for type 1 diabetes.* Autoimmunity, 2012. **45**(4): p. 320-31.

90. Carrasco, Y.R. and F.D. Batista, *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, 2007. **27**(1): p. 160-71.

91. Suzuki, K., et al., *Visualizing B cell capture of cognate antigen from follicular dendritic cells.* J Exp Med, 2009. **206**(7): p. 1485-93.

92. Qi, H., et al., *Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells.* Science, 2006. **312**(5780): p. 1672-6.

93. Clark, S.L., Jr., *The reticulum of lymph nodes in mice studied with the electron microscope.* Am J Anat, 1962. **110**: p. 217-57.

94. Gretz, J.E., et al., *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, 2000. **192**(10): p. 1425-40.

95. Batista, F.D., D. Iber, and M.S. Neuberger, *B cells acquire antigen from target cells after synapse formation.* Nature, 2001. **411**(6836): p. 489-94.

96. Mitchison, N.A., *T-cell-B-cell cooperation.* Nat Rev Immunol, 2004. **4**(4): p. 308-12.

97. Reif, K., et al., *Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position.* Nature, 2002. **416**(6876): p. 94-9.

98. Cunningham, A.F., et al., *Salmonella induces a switched antibody response without germinal centers that impedes the extracellular spread of infection.* J Immunol, 2007. **178**(10): p. 6200-7.

99. MacLennan, I.C., *Somatic mutation. From the dark zone to the light.* Curr Biol, 1994. **4**(1): p. 70-2.

100. Rajewsky, K., *Clonal selection and learning in the antibody system*. Nature, 1996. **381**(6585): p. 751-8.

101. Allen, C.D., T. Okada, and J.G. Cyster, *Germinal-center organization and cellular dynamics.* Immunity, 2007. **27**(2): p. 190-202.

102. MacLennan, I.C., *Germinal centers.* Annu Rev Immunol, 1994. **12**: p. 117-39.

103. Schwickert, T.A., et al., *Germinal center reutilization by newly activated B cells.* J Exp Med, 2009. **206**(13): p. 2907-14.

104. Muramatsu, M., et al., *Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme.* Cell, 2000. **102**(5): p. 553-63.

105. Pavri, R. and M.C. Nussenzweig, *AID targeting in antibody diversity*. Adv Immunol, 2011. **110**: p. 1-26.

106. Isakson, P.C., et al., *Pillars article: T cell-derived B cell differentiation factor(s). Effect on the isotype switch of*

murine B cells. J. Exp. Med. 1982. 155: 734-748. J Immunol, 2013. **190**(3): p. 849-63.

107. Schwickert, T.A., et al., *A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center.* J Exp Med, 2011. **208**(6): p. 1243-52.

108. Goenka, R., et al., *Local BLyS production by T follicular cells mediates retention of high affinity B cells during affinity maturation.* J Exp Med, 2014. **211**(1): p. 45-56.

109. Pape, K.A., et al., *Different B cell populations mediate early and late memory during an endogenous immune response.* Science, 2011. **331**(6021): p. 1203-7.

110. Akashi, T., et al., *Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice.* Int Immunol, 1997. **9**(8): p. 1159-64.

111. Serreze, D.V., et al., *B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice.* J Immunol, 1998. **161**(8): p. 3912-8.

112. Hu, C.Y., et al., *Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice.* J Clin Invest, 2007. **117**(12): p. 3857-67.

113. Gianani, R. and G.S. Eisenbarth, *The stages of type 1A diabetes: 2005.* Immunol Rev, 2005. **204**: p. 232-49.

114. Leijon, K., A. Freitas, and D. Holmberg, *Analysis of VH gene utilisation in the non-obese diabetic mouse.* Autoimmunity, 1993. **15**(1): p. 11-8.

115. Hillorn, V., et al., *Aberrant V(H) gene utilization in patients with established insulin dependent diabetes mellitus.* J Autoimmun, 1997. **10**(2): p. 157-63.

116. Thomas, J.W., P.L. Kendall, and H.G. Mitchell, *The natural autoantibody repertoire of nonobese diabetic mice is highly active.* J Immunol, 2002. **169**(11): p. 6617-24.

117. Noorchashm, H., et al., *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.* J Immunol, 1999. **163**(2): p. 743-50.

118. Falcone, M., et al., *B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice.* J Immunol, 1998. **161**(3): p. 1163-8.

119. Cox, S.L., et al., *Enhanced responsiveness to T-cell help causes loss of B-lymphocyte tolerance to a beta-cell neo-self-antigen in type 1 diabetes prone NOD mice.* Eur J Immunol, 2010. **40**(12): p. 3413-25.

120. Hussain, S. and T.L. Delovitch, *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.* J Immunol, 2005. **174**(2): p. 680-7.

121. Hussain, S., K.V. Salojin, and T.L. Delovitch, *Hyperresponsiveness, resistance to B-cell receptor-dependent activation-induced cell death, and accumulation of hyperactivated B-cells in islets is associated with the onset of insulitis but not type 1 diabetes.* Diabetes, 2004. **53**(8): p. 2003-11.

122. Greeley, S.A., et al., *Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice.* J Immunol, 2001. **167**(8): p. 4351-7.

123. Kendall, P.L., et al., *Tertiary lymphoid structures in the pancreas promote selection of B lymphocytes in autoimmune diabetes.* J Immunol, 2007. **178**(9): p. 5643-51.

124. Sundstrom, M. and K. Lejon, *Idd-linked genetic regulation of TACIhigh expressing B cells in NOD mice.* J Autoimmun, 2007. **29**(2-3): p. 116-24.

125. Sundstrom, M. and K. Lejon, *The prolonged and enhanced immune response in the non-obese diabetic mouse is dependent on genes in the Idd1/24, Idd12 and Idd18 regions.* J Autoimmun, 2010. **35**(4): p. 375-82.

126. Ekici, R., et al., *Enhanced capture of extramembranous IgM and IgG on B cells in the NOD mouse--implications for immune complex trapping.* Int Immunol, 2009. **21**(5): p. 533-41.

127. Rolf, J., et al., *The enlarged population of marginal zone/CD1d(high) B lymphocytes in nonobese diabetic mice maps to diabetes susceptibility region Idd11.* J Immunol, 2005. **174**(8): p. 4821-7.

128. Luan, J.J., et al., *Defective Fc gamma RII gene expression in macrophages of NOD mice: genetic linkage with up-regulation of IgG1 and IgG2b in serum.* J Immunol, 1996. **157**(10): p. 4707-16.

129. Hornblad, A., A. Cheddad, and U. Ahlgren, *An improved protocol for optical projection tomography imaging reveals lobular heterogeneities in pancreatic islet and beta-cell mass distribution.* Islets, 2011. **3**(4): p. 204-8.

130. Valenta, R., et al., *Linking allergy to autoimmune disease.* Trends Immunol, 2009. **30**(3): p. 109-16.

131. Messingham, K.A., H.M. Holahan, and J.A. Fairley, *Unraveling the significance of IgE autoantibodies in organ-specific autoimmunity: lessons learned from bullous pemphigoid.* Immunol Res, 2014. **59**(1-3): p. 273-8.

132. Atta, A.M., et al., *Autoimmune response of IgE antibodies to cellular self-antigens in systemic Lupus Erythematosus.* Int Arch Allergy Immunol, 2010. **152**(4): p. 401-6.

133. Petersen, J.S., et al., *Progression to type 1 diabetes is associated with a change in the immunoglobulin isotype profile of autoantibodies to glutamic acid decarboxylase (GAD65). Childhood Diabetes in Finland Study Group.* Clin Immunol, 1999. **90**(2): p. 276-81.

134. Butt, D., et al., *FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production.* Immunity, 2015. **42**(5): p. 890-902.

135. Forsgren, S., et al., *Immunoglobulin-mediated prevention of autoimmune diabetes in the non-obese diabetic (NOD) mouse.* Scand J Immunol, 1991. **34**(4): p. 445-51.

136. Kehry, M.R. and L.C. Yamashita, *Low-affinity IgE receptor (CD23) function on mouse B cells: role in IgE-dependent antigen focusing.* Proc Natl Acad Sci U S A, 1989. **86**(19): p. 7556-60.

137. Mudde, G.C., R. Bheekha, and C.A. Bruijnzeel-Koomen, *IgE-mediated antigen presentation.* Allergy, 1995. **50**(3): p. 193-9.

138. Mudde, G.C., et al., *IgE: an immunoglobulin specialized in antigen capture?* Immunol Today, 1990. **11**(12): p. 440-3.

139. Pirron, U., et al., *IgE-dependent antigen focusing by human B lymphocytes is mediated by the low-affinity receptor for IgE.* Eur J Immunol, 1990. **20**(7): p. 1547-51.

140. Astorri, E., et al., *Evolution of ectopic lymphoid neogenesis and in situ autoantibody production in autoimmune nonobese diabetic mice: cellular and molecular characterization of tertiary lymphoid structures in pancreatic islets.* J Immunol, 2010. **185**(6): p. 3359-68.

141. Seshasayee, D., et al., *Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor.* Immunity, 2003. **18**(2): p. 279-88.

142. von Bulow, G.U., J.M. van Deursen, and R.J. Bram, *Regulation of the T-independent humoral response by TACI.* Immunity, 2001. **14**(5): p. 573-82.

143. Yan, M., et al., *Activation and accumulation of B cells in TACI-deficient mice.* Nat Immunol, 2001. **2**(7): p. 638-43.

144. Castigli, E., et al., *Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation.* J Allergy Clin Immunol, 2007. **120**(4): p. 885-91.

145. Ozcan, E., et al., *Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in LPS-activated B cells.* J Allergy Clin Immunol, 2009. **123**(6): p. 1277-86 e5.

146. Andersson, Å., *B cell repertoire development in normal physiology and autoimmune disease*, 1993, Umeå University.

147. Leijon, K., B. Hammarstrom, and D. Holmberg, *Non-obese diabetic (NOD) mice display enhanced immune responses and prolonged survival of lymphoid cells.* Int Immunol, 1994. **6**(2): p. 339-45.

148. Groom, J.R., et al., *BAFF and MyD88 signals promote a lupuslike disease independent of T cells.* J Exp Med, 2007. **204**(8): p. 1959-71.

149. Quintana, F.J. and I.R. Cohen, *Autoantibody patterns in diabetes-prone NOD mice and in standard C57BL/6 mice.* J Autoimmun, 2001. **17**(3): p. 191-7.

150. Marino, E., et al., *BAFF regulates activation of self-reactive T cells through B-cell dependent mechanisms and mediates protection in NOD mice.* Eur J Immunol, 2014. **44**(4): p. 983-93.

151. Overbergh, L., et al., *Acute shock induced by antigen vaccination in NOD mice.* Diabetes, 2003. **52**(2): p. 335-41.

152. Branisteanu, D.D., et al., *Hen egg white lysozyme vaccination induces acute shock in NOD mice.* Ann N Y Acad Sci, 2003. **1005**: p. 215-7.

153. Moudgil, K.D. and E.E. Sercarz, *Dominant determinants in hen eggwhite lysozyme correspond to the cryptic determinants within its self-homologue, mouse lysozyme: implications in shaping of the T cell repertoire and autoimmunity.* J Exp Med, 1993. **178**(6): p. 2131-8.

154. Sadelain, M.W., et al., *Prevention of type I diabetes in NOD mice by adjuvant immunotherapy.* Diabetes, 1990. **39**(5): p. 583-9.

155. Billiau, A. and P. Matthys, *Modes of action of Freund's adjuvants in experimental models of autoimmune diseases.* J Leukoc Biol, 2001. **70**(6): p. 849-60.

156. Vieira, P. and K. Rajewsky, *The half-lives of serum immunoglobulins in adult mice.* Eur J Immunol, 1988. **18**(2): p. 313-6.

157. Luzina, I.G., et al., *Spontaneous formation of germinal centers in autoimmune mice.* J Leukoc Biol, 2001. **70**(4): p. 578-84.

158. Jacobs, H.M., et al., *Cutting Edge: BAFF Promotes Autoantibody Production via TACI-Dependent Activation of Transitional B Cells.* J Immunol, 2016. **196**(9): p. 3525-31.

159. Jang, E., et al., *Splenic Long-Lived Plasma Cells Promote the Development of Follicular Helper T Cells during Autoimmune Responses.* J Immunol, 2016. **196**(3): p. 1026-35.