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# B cells in Type 1 diabetes: Studies on cell surface antibody binding

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# Table of Contents

# Abstract

Type 1 diabetes (T1D) is a highly serious chronic illness where the pathophysiology involves an autoimmune destruction of the insulin producing β cells residing in the pancreatic islets of Langerhans. The non-obese diabetic (NOD) mouse has been extensively used as a model system for studying the pathogenesis of T1D, since it spontaneously develop diabetes with similar symptoms as human patients.

B cells are key players in T1D pathogenesis as they are important in disease onset and progression. NOD mice are protected from diabetes when rendered B cell free and at early disease progression, B cells are observed in the islets of Langerhans, forming tertiary lymphoid structures seen in other autoimmune diseases. Moreover, the production of anti-islet auto-antibodies is a strong prognostic marker in human T1D diagnosis. Ongoing clinical trials focused on selective B cell depletion by monoclonal antibodies report evidence for preservation of β cell function in humans. Clearly, B cells are important interacting partners in the autoimmune process that destroys the pancreatic β cells.

**Paper I** describes the finding of an aberrant capture of IgG and IgM to B cells of NOD compared to C57BL/6 (B6) mice. As a consequence, the level of extramembranous IgG monomers and IgM pentamers on peripheral blood B cells from NOD mice was significantly higher compared with B6 mice. In addition, the effect of this aberration was that all B cells in peripheral blood of (NOD.IgH$^{a}$ *x* B6(IgH$^{b}$))F$_1$ mice carried both IgM allotypes on their surface. Moreover, analysis of Immune complex binding using IgG- or IgM-opsonized bacterial particles revealed a higher degree of binding in NOD mice compared with B6.

**In Paper II**, we focused on identifying the responsible receptor for the aberrant capture of Igs to the B cell surface. We developed a so called re-tagging method for the application on isolated mouse cells and identified the novel Ig binding receptor as the “heat shock cognate protein 70” (HSC70). HSC70 has previously been shown to be involved in antigen presentation and highlighted in other autoimmune diseases such as rheumatoid arthritis. We believe that HSC70 is altered in NOD B cells,

which could lead to increased capture of autoantigen-immune complexes and thus enhance antigen presentation as part of the disease pathogenesis. We also demonstrate the presence of IgM on the cell surface of B cells in T1D patients in similarity with that seen in NOD as described in Paper I.

**In conclusion**, our findings indicate a B cell mechanism that could potentiate the autoimmune process in Type 1 diabetes and for that case is an important field to investigate in future studies.

# Abbreviations

| | |
|---|---|
| APC | Antigen presenting cell |
| B6 | C57BL/6 mouse strain |
| BCR | B cell receptor |
| FACS | Fluorescence activated cell sorting |
| HSC70 | Heat shock cognate protein |
| ICs | Immune complexes |
| NOD | Non-obese diabetic |
| PAGE | Poly acrylamide gel electrophoresis |
| SDS | Sodium dodecyl sulphate |
| SSBED | Sulfosuccinimidyl[2-6-(biotinamido)-2-(p-azido-benzamido)-hexanoamido]-ethyl-1,3´-dithiopropionate |
| T1D | Type 1 diabetes |
| MALDI-TOF | Matrix assisted laser desorption ionization Time of flight |

# Publication and Manuscript

The reprint of Paper I, published in *International Immunology*, is in accordance with the directions for authors stated by the *Oxford Journals* at their homepage, www.oxfordjournals.org, as per 2010-09-13. Following is a full acknowledgement to the original source and *Oxford University Press* and the learned society:

**Paper I: Ekici R,** Sundström M, Thay B, Lejon K, 2009, *International Immunology*, 21(5): 533-541: Enhanced capture of extrramembranous IgM and IgG on B cells in the NOD mouse – implications for immune complex trapping.

**Paper II: Ekici R**, Sundstrom M, Lejon K, Manuscript, HSC70 is a novel B cell surface receptor involved in the capture of immunoglobulins in the NOD mouse.

# Introduction

## The immune system

We are constantly surrounded by and exposed to microorganisms that constitute potential threats to our health. However, these threats are eliminated by our immune system in a controlled and regulated manner often without any notice to the individual. The immune system could be considered as an army consisting of different specialized units, the immune cells and areas outlining the first line defense such as the mucous. The destruction of foreign agents requires a fine tuned interplay among the different immune cells in order to ensure a controlled elimination and prevent them from attacking the own body (Janeway et al, 2002). The evolution of the immune system has equipped it with the capacity to tolerate our own self antigens but to immediately recognize specific pathogens and eliminate them. The immune cells have their designated mission to fulfill and some of the cells can gain a long-lasting memory after an infection, which is rapidly activated upon a second encounter (Cooper et al, 2006).

The main components of the blood are erythrocytes, platelets and leukocytes. Leukocytes are cells derived from a multipotent cell in the bone marrow known as the hematopoietic stem cell, a cell that can renew itself and differentiate into progenitors common to lymphoid and myeloid cell lineages and then further into a variety of specialized cells such as lymphocytes, myeloid cells (such as macrophages) and erythrocytes. Leukocytes are found within all organs of the body including the blood and lymphoid tissues (Abramson et al, 1977).

Traditionally, the immune system is divided into two components consisting of an innate and adaptive part. These two lines of defense are tightly intertwined and complement each other to protect the body against pathogens and foreign materials.

## Innate and adaptive immune system

The innate immune system is present at birth and provides an immediate response to foreign molecules and organisms. Its components treat all foreign substances in a generic but specific manner and respond to a defined number of antigens and structures, both from pathogens but also from ourselves. Innate immunity has no memory of the encounters and does not provide any lasting protection against future infection. (Medzhitov *et al*, 2000).

The replication of an infecting pathogen in the body is rapidly controlled by the effector mechanisms of innate immunity. These include antimicrobial peptides, phagocytes, and the alternative complement pathway. One example of where these mechanisms function is the mucous layers of the body, which with their barrier function are also part of innate immunity. The mucosa of e.g. pharynx is severely loaded by microorganisms each day without them causing any infection. However, whenever microorganisms have invaded the outer tissue layer and caused infection, the adaptive immune system is induced to fight against the pathogens. This system takes several days to be fully active and consists of many cells with specific responses. Because of this, containing the infection until the adaptive immune system can begin to deal with it is one of many important roles of the innate immune system (Loker *et al*, 2004. Flajnik *et al*, 2004, Medzhitov *et al*, 2000).

The overwhelming variability of antigenic structures, as well as the ability of pathogens to mutate to avoid host detection, has driven the evolution of the adaptive immune system to rely on lymphocytes with diverse receptors capable of recognizing and removing potential pathogens. It involves a tightly regulated interplay between antigen-presenting cells and T and B cells, which facilitate pathogen-specific immunologic effector pathways, generation of immunologic memory, and regulation of host immune homeostasis (Bonilla et al, 2009). Invading pathogens are taken up by APCs, degraded and presented to T helper cells. In order for a B cell to produce antibodies, aid from a T helper cell is demanded. The antigen epitope, of which the T helper cell receptor is specific for, must also be recognized by the BCR of its interacting B cell, i.e. they must have the specificity for the same antigen. Upon activation, the production of

cytokines from the T helper cell allows the B cell to proliferate and produce antibodies that will disable the bacteria from harming the cells of the body. (Pancer *et al*, 2006).

## Lymphocytes and B cells

Cells of the adaptive immune system include the effectors of cellular immune responses, the T cells, and antibody-producing cells, the B cells (plasma cells) (Hedrick, 2008). The high mobility of lymphocytes ensures their transport between different tissues and organs of the body. After developing in the primary lymphoid organs (thymus and bone marrow, respectively), they traffic to secondary lymphoid organs, including lymph nodes and the spleen, which serve to capture circulating antigens from lymph and blood, respectively (LeBien et al, 2008).

B cells are the main players in the humoral response of the adaptive immune system. Upon infection, the differentiation of activated B cells into plasma cells, under the direction of signals received from T cells, enables the production of antigen specific antibodies. These antibodies are produced during the whole infectious period and often a long time after the defeat of the pathogens (Strugnell et al, 2010).

In the bone marrow, B cells pass through several developmental stages of which they gain their antigen specificity. When reached the immature stage, B cells enter the egress phase where they leave the marrow and complete the development to mature or naïve cells. This is demonstrated by the appearance of IgD in addition to IgM on the B cell surface. Importantly, all these developmental stages occur in the absence of any exogenous antigen encounter and thus referred to as antigen-independent B cell development (Janeway et al, 2005). The second phase of development takes part upon antigen encounter and activation and is called the antigen-dependent phase. Some antigens that B cells encounter can induce antibody production without the presence of T cells and are called T cell-independent antigens (Richards et al, 2008). Bacterial polysaccharides are examples of such antigens that can cross-link several BCRs on the surface and initiate the formation of memory cells or plasma cells only under the additional stimuli provided by dendritic cell cytokines (Maddaly et al, 2010).

## B cell activation

When matured, B cells recirculate through secondary lymphoid organs such as lymph nodes and the spleen. In the lymph nodes B cells are mainly gathered in the cortex in areas called primary follicles. Here they are in close contact with follicular dendritic cells which can carry antigens on their surface, bound to IgM, IgG or complement. These antigens can stimulate B cells through BCR crosslinking, expression of other interacting surface molecules or cytokine expression. This transportation to the B cell areas can also be performed by B cells themselves who like the specialized dendritic cells express receptors for IgG Fc on their surface (Janeway et al, 2005).

There are two principal signals that B cells require in order to become activated. The first signal is derived from the cross-linking of the BCR as mentioned above and leads to a downstream activation of intracellular signaling pathways that enable the B cell to interact with T cells and thereby receive the second signal (Fearon et al, 1996). B cells express peptides on their surface along with MHC class II molecules. These peptides can arise from antigens internalized and processed after bound to the MHC class II molecule. When the B cell is in close contact with a CD4+ T cells that is specific for such a peptide on the MHC class II molecule and which have been previously activated by an APC (such as a macrophage), the T cell is able to provide help and the B cell through direct cellular contact to activate it for further differentiation into memory or plasma cells. This cognate interaction between T cells and B cells is similar to the interaction that occurs initially between T cells and dendritic cells (APCs) (Bonilla et al, 2009). This initial interaction takes place at the margin between primary follicles and T cell areas in secondary lymphoid organs. The activated B cell stands in front of two pathways; either immediately become a short lived plasma cell that secrets low-affinity antibodies without undergoing somatic mutation or the B cell enter a follicle to establish a germinal center (Allen et al, 2007). In the germinal center, B cells can change from the production of IgM and IgD, which are the Ig isotypes they carry on the surface when they egress from the bone marrow, to other isotypes such as IgG, IgA and IgE. This is called Ig class-switching and occurs through a mechanism of gene rearrangement (Stavnezer et al, 2008).

## Ig rearrangement

Ig molecules comprise heavy (H) and light (L) chains with both constant (C) and variable (v) regions that are encoded by genes residing in three loci. The possible rearrangement combinations of the heavy chain with the addition of the variability of the light chains provide an enormous capacity of Ig repertoire (Dudley et al, 2005). Assembly of the Ig μ heavy chain and one of the light chains results in the formation of IgM that is expressed on the immature B cell surface. It is noteworthy to bear in mind that one certain B cell can only gain one certain epitope specificity through its lifetime and therefore the Ig heavy chain can only be of one allotype in a B cell (Shapiro-Shelef et al, 2005).

A naïve B cell expresses IgM on its surface, constituting the BCR, and IgD that is frequently associated with the B cells immature developmental stage. Upon antigen encounter, from foreign invaders or from endogenous cells such as tumours, the B cells undergo a transition as described above and gene rearrangement within the Ig locus occurs, which enables them to generate a diverse repertoire of Igs in order to eliminate and neutralize the antigens (Tonegawa et al, 1983).

## Fc receptors and HSC70

Cellular receptors for the different immunoglobulin isotypes (IgA, IgE, IgM and IgG), so called Fc-receptors are involved in regulating and executing antibody mediated responses, such as antibody dependent cytotoxicity, mast cell degranulation and phagocytosis. All these interactions are initiated through the binding of the Fc domain of antibodies or ICs to the specialized surface receptors, e.g. to Fc receptors on dendritic cells, which results in phagocytosis and presentation of antigen peptides on MHC class I and II molecules. Fc-receptors are widely expressed on cells of the immune system and select other cell types, such as endothelial cells, mesangial cells and osteoclasts; one of the few hematopoietic cell types that do not show expression of Fc-receptors are T cells (Nimmerjahn et al, 2007).

There may be multiple undiscovered receptors on the B cell surface that bind immunoglobulins. In this thesis we descibe HSC70 as a B cell surface protein that binds immunoglobulins and ICs. HSC70 is a constitutively expressed protein belonging to the heat shock 70 protein family (Chappell et al, 1986). HSC70 was originally identified as a water soluble cytosolic protein and due to its molecular mass and sequence homology, placed in the family of heat shock proteins (Hsps). Because of that, HSC70 was long considered solely as an intracellular protein (Weigl et al 1999 and Haberstroh, 1995). Within the cell, HSC70 has a key role in clathrin-mediated endocytosis and chaperone mediated autophagy (Eisenberg et al, 2007). However, HSC70 is also associated to the cell surface and involved in immunologically significant events such as viral cell entry (Jang et al, 2003).

HSC70 has been described in the context of autoimmune conditions such as rheumatoid arthritis, SLE and T1D (Auger et al, 2005, Page et al, 2009, Alam et al, 2009). Because of the ubiquitous expression and the high degree of homology between prokaryotic HSC70 and eukaryotic, HSC70 has long been suspected of inducing autoimmune disease (Fugger et al, 1996). The potency of HSC70 to promote autoimmune disease could be supported by findings indicating a powerful property of HSC70 in the immune response against tumor cells. Vaccination with HSC70, even without adjuvants, protects mice from challenge by the tumors from which the Hsc70 was originally isolated (Mizukami et al, 2008).

## Autoimmunity and T1D

Whenever immune regulation is disturbed in one of several check-points, the immune cells gain an inclination for self structures and are able to direct immune responses to cause damage to tissues, leading to disease. The consequence is autoimmunity. Several core concepts in human autoimmunity are illustrated by these regulatory check-points, which control the likelihood of disease-initiating events, the transition from immune autoimmune susceptibility to autoimmune progression and finally a failure in peripheral immune regulation (Ermann et al, 2001). One of the checkpoints is called central tolerance induction, where T cells that are reactive to self-antigens are largely deleted in the thymus in an

active process (Kisielow et al, 1988). This process is not absolute and self-reactive T cells can be found in the periphery of even healthy individuals. However, the development of autoimmune disease is commonly prevented in most of us due to distinct safety mechanisms in the periphery that result in a tolerance against these potentially dangerous T cells (Hammerling et al, 1991). Infections or other form of overt stimulation of APCs can break peripheral tolerance and induce the formation of self-reactive T cells in draining lymph nodes, which in predisposed individuals may lead to autoimmunity (Ohashi et al, 1991).

Studies have shown that more than half of all newly generated BCRs are capable of binding auto-antigens (Nemazee 1995). However, it is complicated to define the reactivity or responsiveness of B cells to autoantigens because the correlation between the binding affinity of an antigen to a BCR and the functional response of that B cell's BCR cannot be defined as a quantitative term. In human B cell development, many polyreactive cells (e.g B cells binding intracellular proteins, DNA, lipopolysaccharides) and some autoreactive cells are lost during the transition from pre-B2 to immature B cells. B2 B cells are generated in the bone marrow throughout life, can respond to T cell-dependent antigens in germinal centers and are required for adaptive immunity (Boehmer et al, 2010). However, in patients with the autoimmune disease systemic lupus erythematosus, these autoreactive or polyreactive BCRs are not lost at this checkpoint, suggesting a strong role for this checkpoint in preventing autoimmune disease (Witsch et al, 2006).

T1D is a progressive autoimmune reaction towards the insulin-secreting pancreatic β cells in the islets of Langerhans. The disease is the end result of a sequential series of failed homeostatic check-points for selection and activation of immunity. It is primarily thought to be caused by pro-inflammatory autoreactive T cells, which mediate the destruction of the β cells. The development of T1D is genetically controlled and is thought to be initiated in susceptible individuals by environmental factors such as virus infections, although a viral cause has not been clearly identified (von Herrath, 2009). Both humoral and cell-mediated immune mechanisms are involved in diabetes development. Prior to overt diabetes diagnosis, the pancreatic islets are infiltrated by inflammatory

cells including CD4+ T cells (Kent et al, 2005) and antibodies to various β cell antigens are demonstrable in the sera of patients at risk (Achenbach et al, 2005). In NOD mice, the destruction of pancreatic β cells has been identified to be mediated by several immune cells. $CD4^+$ and $CD8^+$ T cells, as well as macrophages and B cells have been shown to play a role in β cell death. B cells are present in the pancreatic infiltrate and in the pancreatic draining lymph node, where the initial presentation of islet antigen by dendritic cells to islet antigen-specific T cells occurs (Turley et al, 2003). Although T cells, especially the effector $CD8^+$, are considered crucial for T1D development, there is substantial evidence suggesting B cells as important players in disease development. B cell depletion in NOD mice, either through gene modification or antibody treatment, impairs the development of T1D (Hu et al, 2007, Serreze et al, 1996, Forsgren et al, 1991).

## Clinical aspects of T1D

Over the past decades, the incidence of T1D has increased, afflicting millions of people worldwide. Typically, the disease is manifest in children and young adults when clinical signs such as polydipsia, polyuria and weight loss are seen. The symptoms are due to the lack of insulin resulted by autoimmune pancreatic β cell destruction which is initiated by a series of complex mechanisms consisting of genetics, environmental factors and loss of immune regulation. Untreated, the condition is acute with deadly outcome. However, with modern insulin analogues, the short-term mortality in Type 1 diabetes is low. In the long-term, Type 1 diabetes is associated with complications such as general vasculopathy, nefropathy and neuropathy, which the majority of patients develop despite of satisfactory treatment (Shogbon et al, 2010). This is because of the fact that exogenous insulin cannot match the precision of endogenous insulin secretion. The co-morbidity that lies ahead with the disease impacts on life quality and expected life time, which underscores the severity of T1D and the need for a cure.

To this date, it is still evident that the etiology of T1D is not fully established. It is considered to have a strong genetic association but also to be affected by environmental factors. T1D is overrepresented in the

western world, a notion that has led to the “hygiene hypothesis”, where children are under-exposed to infectious agents depriving the immune system its useful stimulation to later strictly regulate immune responses (Ludvigsson, 2006). In animal models, it has been shown that NOD mice are more protected from diabetes when bred in a non-clean environment (Todd, 1991). It is also proposed that T1D genetically identical (or monozygotic) twins would be much more concordant for T1D than average 50 % recorded in standard environmental conditions if, in the quite unlikely scenario, they could be reared microbe-free (Wen et al, 2008).

The most important clinical sign of a change in T1D risk status of a child (much of what we know about T1D in humans is derived from studies on child material) occurs when islet autoantibodies develop. Autoantibodies to four islet antigens have been identified so far: GAD65 or GAD67, Insulin or proinsulin, IA-2 and ZnT8 (Ziegler et al, 2010). These are the autoantibodies for disease prediction that are currently adequate for T1D that develop in children and young adults (LaGasse et al 2002). The presence of autoantibodies to just one of the four antigen groups alone is associated with only a marginal risk increase and autoantibodies to single antigens are not rare among non-clinical individuals. An important checkpoint in disease is the progression to the multiple islet autoantibodies stage. The risk of T1D is therefore markedly increased in a child that presents autoantibodies to two or more islet antigens. The presence of autoantibodies to IA-2 is associated with highest risk (Achenbach et al, 2004). In overall, T1D risk is a combination of the probability of disease development and the rate at which it will develop. It is an average likelihood, meaning that some will develop T1D within days of being identified as positive for islet autoantibodies, whereas others will take decades to develop the disease and some not at all.

# Aims

The aim of this work was to elucidate the mechanism behind the aberrant binding of IgM on NOD B cell and the contribution of this to progression of autoimmunity. Thus, the specific aims of the work are presented in Paper I and Paper II, respectively, and were:

- To investigate in detail the observed dual BCR appearance on NOD B cells.
- To determine the receptor responsible for the above mentioned trait.

# Materials and Methods

## NOD mouse

A true understanding of Type 1 diabetes in humans has been difficult to study mainly because the disease develops as a result of an interaction between several genes and environmental factors. To study such a complex disease, it is both an advantage and a necessity to use a model organism with a disease pathogenesis that is highly resemblant to that seen in human patients. This became available in the 1980s when Makino and colleagues (reviewed in Tanaguchi *et al*, 2007) developed the spontaneous inbred mouse model of the disease, the non-obese diabetic mouse (NOD), which advanced the research field of autoimmune diabetes. The NOD mouse has enabled us to study the cellular and molecular processes of B cell directed autoimmunity in diabetes and allowed us to easily access organs of interest which is complicated in humans due to the remoteness of e.g. the pancreatic lymphoid organs.

To this date, there is no other model organism or a substitute way of studying the etiology and pathogenesis of Type 1 diabetes than the NOD mouse. The spleen and blood of NOD were organs from which B cells were frequently extracted for this work. This allowed us to study the cells ex-vivo and in a convenient and reproducible way perform our experiments. Thus, the NOD mouse was truly indispensible for the study of B cells in Type 1 diabetes pathogenesis.

## SDS-PAGE and western blot

The development of the SDS-PAGE technique has certainly improved our way to visualize proteins in single or complex forms. The assay is designated to run solutions of proteins and separate them on the basis of their molecular weight (Laemmli, 1970). A subsequent step in the visualization process is to transfer the proteins to another membrane and by the aid of antibodies visualize a specific protein of interest. The latter procedure, called western blot, was frequently used in this work because of the ability it provided to detect captured IgM pentamers on the B cell surface, and more important to distinguish the IgM pentamers from the endogenously produced B cell receptors (IgM monomers). IgM is

secreted as a large molecule consisting of five IgM monomers linked to each other through disulfide bonds (Dudley et al, 2005), which are destroyed in reducing conditions leaving monomeric remnants. Thus, when studying the presence of IgM pentamers on isolated $CD19^+$ B cells, we run the samples on native gels and in non-reducing conditions as to keep the pentamers intact.

Moreover, samples that contained the presumptive receptor for the Igs were assayed with SDS-PAGE in reducing conditions as to get a good separation of proteins as possible. These gels were further subjected to silver staining, a procedure that would visualize small amounts of protein, which indeed was necessary for our cause since the protein outcome in the re-tagging procedure were under the detection limit of conventional coomassie blue staining.

## FACS

A powerful method to separate cells that are phenotypically different from each other and at a high speed is to use FACS. In addition to this, the FACS machine provides information on how many cells that express a certain protein and how much of the protein that is expressed. Protein expression can be analyzed both intracellularily and at the cell surface depending on how you design your assay.

In this work, we have used the FACS technique to analyze predominantly B cell populations. By staining an ex vivo cell solution with appropriate antibodies for a certain B cell population, they are easily detected later in the FACS machine and analyzes can be performed. In early experiments, we came across a dominating phenotype of a B cell population in (NOD.$IgH^a$ x B6($IgH^b$))$F_1$ mice that displayed both the $IgH^a$ and the $IgH^b$ allotypes on their surface, which made us suspect a disturbance in the Ig gene rearrangement in B cells of these mice. Hence, we permeabilized the cell membrane of peripheral blood lymphocytes from these mice and stained for the both IgH allotypes. The FACS analysis revealed two distinct populations of B cells, one that produced $IgH^a$ and another $IgH^b$. A situation that would have been expected for the surface of these B cells. From these experiments, we gained the information that the B cells of (NOD.$IgH^a$ x B6($IgH^b$))$F_1$ had only one functioning Ig gene

rearrangement and that the phenotype seen on the surface of these cells must have been due to conditions restricted to the membranous or extrramembranous parts of the B cells. The FACS technique provided us the ability to precisely calculate and distinguish the B cells from other cells on the basis of which proteins they expressed.

We also wanted to rapidly analyze the binding of ICs to NOD B cells when opsonized with IgG or IgM and compare the level of binding with that of B6 B cells. The result of this assay is presented in Paper I. It illustrates how $CD19^+$ B cells are analyzed on the basis of bound opsonized S. aureus particles in the two mouse strains. To further visualize this binding and its presumptive effect, we analyzed the B cells in a confocal microscope as described below.

## Receptor re-tagging

The re-tagging technique is an interesting way to isolate an unknown protein in a known molecular interaction. Briefly, the method is based on a commercially available photoactive reagent, SSBED, which is cross-linked to a known protein that acts as a bait for its unknown interacting partner. By the impact of short wave UV-light, the biotin group of the SSBED is transferred to the unknown protein which can later be affinity purified and further identified by MALDI-TOF.

We chose to utilize the re-tagging method to identify the unknown B cell surface receptor responsible for the aberrant binding of immunoglobulins in NOD. The advantage here was that marking the presumptive receptor with biotin using a well defined ligand as bate, the receptor could be purified and enriched in monomeric avidin columns for further analysis. Mouse Isotype control $IgG_1\kappa$ antibody was cross linked to the SSBED and incubated with purified B cells to bind to its receptor. The antibody lacks any defined specificity and was therefore an appropriate ligand to choose. We developed and optimized an assay to detect HSC70 as a receptor for immunoglobulins on $CD19^+$ B cells in NOD mice. To our knowledge, this has not yet been done in this model species and gives us the possibility to apply the technique on other mammal cells such as human diabetic B cells.

## Confocal microscopy

Through an affinity competition assay, we determined that IgM and IgG antibodies bound to the same receptor. To further see if the antibodies bound to antigens resulted in endocytosis, we opsonized fluorescent *S. aureus* particles with IgG. With a confocal scanning laser microscopy, it is convenient to visualize the opsonized bacterial particles and the plasma membrane. The experiment revealed that the ICs were localized to the exterior part of the plasma membrane of NOD splenic B cells. Additionally, the B cells were visualized to capture more ICs compared when opsonized with IgG than without. This method provides a macroscopic view of the situation on and around the plasma membrane.

# Results and Discussion

The intricate nature of the immune system is an evolutionary masterpiece of where the immune cells are tightly regulated through mechanisms that provide surveillance of the body but also direct effector functions against harmful agents. Unfortunately, there are conditions that disturb the immune homeostasis in the body and therefore make the individual susceptible to autoimmunity. T1D is a well studied disease but yet there remain important discoveries to fully understand the mechanisms involved in disease onset and progression.

T cells have long been considered as the main cells responsible for the initiation and development of the disease. However, studies over the past decade indicate B cells as critical players in disease progression both in NOD and in humans. Auto-antibody production is a strong marker for disease progression and the entrance of B cells to the pancreatic islets is indeed a critical event for diabetes onset. Moreover, a recent study demonstrated the necessity for B cells to remain within the islets of Langerhans for the survival of intra-islet $CD8^+$ cytotoxic T cells to enhance T1D (Brodie et al, 2008). Thus, the role of B cells in the diabetic autoimmune process is truly diverse.

In Paper I, we describe a trait observed on the surface of peripheral blood B cells in NOD. During the establishment of the NOD.$IgH^a$ strain, we observed that NOD.$IgH^{a/b}$ heterozygote mice displayed both the a- and the b-IgM allotype on the surface of peripheral blood B cells. To examine this feature in detail, we bred (NOD.$IgH^a$ x B6($IgH^b$))$F_1$ mice and compared these with (BALB/c($IgH^a$) x B6($IgH^b$))$F_1$ mice (Paper I, figure 1A). Analysis of peripheral blood of the $F_1$ progeny revealed that the majority of B cells in (NOD.$IgH^a$ x B6($IgH^b$))$F_1$ mice had both $IgM^a$ and $IgM^b$ at the cell surface, in contrast to the expected pattern observed for (BALB/c($IgH^a$) x B6($IgH^b$))$F_1$ mice (figure 1A). This latter progeny had two separate populations of B cells, one expressing the a allotype and the other the b allotype, which lead us to suspect a defect in allelic exclusion in B cells of (NOD.$IgH^a$ x B6($IgH^b$))$F_1$. Seemingly, they did not fulfill the criteria of one B cell = one antigen specificity. However, following

experiments of intracellular staining (Paper I, figure 1B) revealed an IgM allotype distribution as seen in (BALB/c($IgH^a$) x B6($IgH^b$))$F_1$ mice. This left us with the question if the surrounding serum of NOD B cells could be the reason for the dual surface-associated IgMs. When assaying for this, by incubating NOD or B6 B cells with serum from BALB/c or NOD.$IgH^a$, we observed a higher $IgM^a$ binding to NOD B cells compared to B6 (Paper I, figure 2). Interestingly, NOD B cells bound even more $IgM^a$ when incubated with Balb/c serum. These findings indicated a B cell intrinsic property responsible for the observed trait in NOD and did not rely on the potential multi-reactivity of NOD antibodies or BCRs (Leijon et al, 1993, Thomas et al, 2002).

Further, we analyzed the binding of isotype control IgG and IgM antibodies respectively to B cells of NOD and B6 mice (Paper I, figure 3). The results from this experiment were in line with above described notions where NOD B cells capture antibodies to a greater extent than B6. Taking this to another level, we wanted to investigate the IgM binding situation *in vivo* ( Paper I, figure 4). Thus, we purified and lysed NOD and B6 $CD19^+$ B cells and run the samples on SDS-PAGE whereafter visualization of surface bound IgM pentamers (IgM is secreted in pentameric form, figure 1B) was done with the Western blot technique. The results demonstrated an increased occurrence of IgM pentamers on NOD B cells compared to B6. The mechanism behind the increased association of Igs to the B cell surface in NOD could presumably lead to an increased ability of binding ICs (figure 1C). The confocal microscopy experiments indicated that this was the case in NOD compared to B6 (Paper I, Figure 6).

These data describe that B cells of NOD carry antibodies of IgG and IgM isotypes on their surface to a greater extent than normal mice. One function of this alteration is enhancing the B cell capacity to trap ICs, which in turn could potentiate the antigen presentation. The consequences of this could be an increased presentation of self-antigens

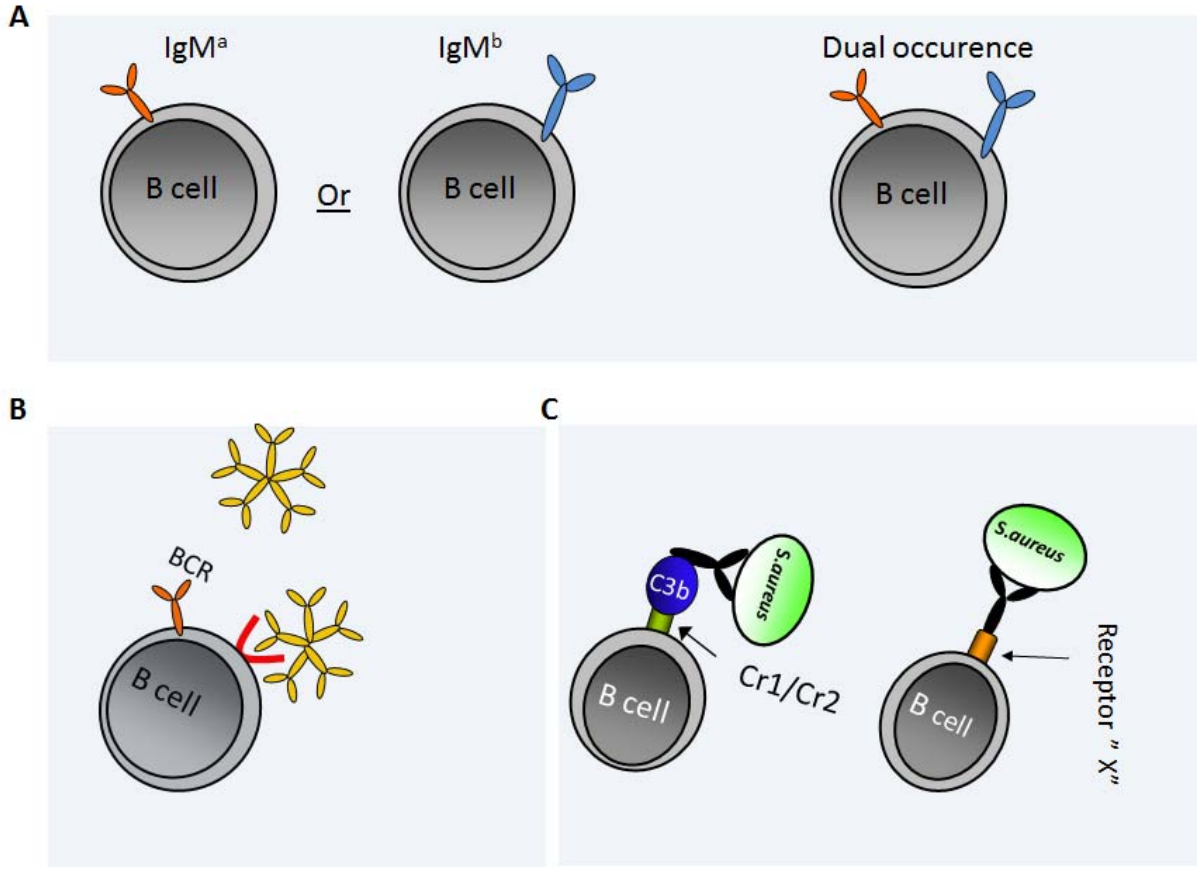


**Figure 1** Expected pattern of surface IgM in (NOD.IgH$^a$ x B6(IgH$^b$))F$_1$ mice and the observed (A). IgM molecules are secreted in pentameric form (B). Opsonized *S. aureus* particles bind to the unknown receptor (C), as well as to other receptors such as complement receptors (Cr1/Cr2).

to T cells and thereby initiate a strong autoimmune response. B cells are able to perform as professional antigen presenting cells and can transport and deposit antigens to follicular dendritic cells within the lymph nodes in the initiation of an immune response.

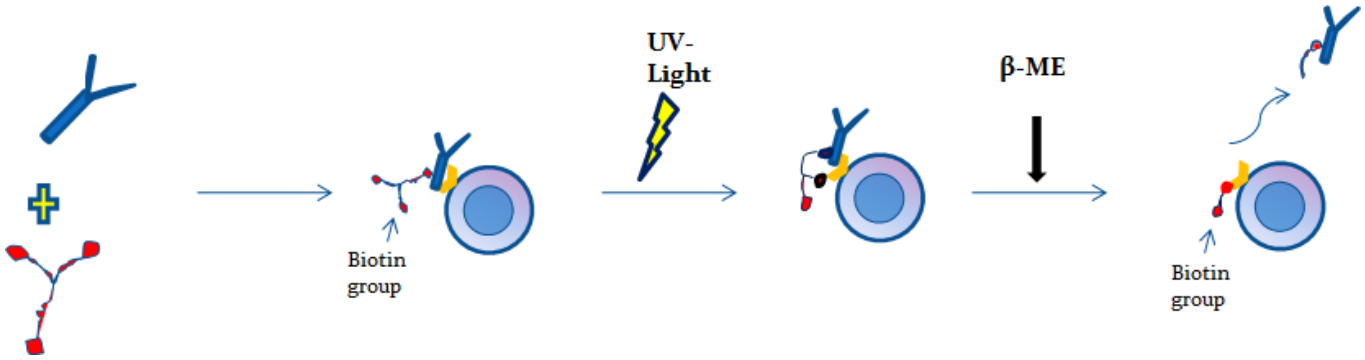


**Figure 2** Schematic procedure of the re-tagging method. The red Y-shaped molecule represents the SSBED cross-linker. The blue Y-shaped molecule represents an IgG antibody. The round shaped figures are equal to B cells.

The results presented in Paper II, describe the search for the receptor that could be responsible for the binding of Igs to the B cell surface. There are several known surface receptors for binding different Ig isotypes, such as FcamR and FcgammaR. We assayed for the possibility of these receptors to be responsible for the binding of IgG and IgM to the B cell surface but despite blocking them in vitro, the B cells were rendered with the same pattern of surface Igs after incubation with commercial Igs. In the final stage of the project, we indentified HSC70 to be the membrane bound molecule responsible for the observed B cell phenotype. HSC70 was discovered at the B cell surface with the so called re-tagging method (Paper II, figure 2). This method was developed by us to be used on mammalian *ex vivo* cells. Briefly, a commercially available isotype control $IgG_1\kappa$ antibody was cross-linked to SSBED (commercially provided by *Pierce*) (figure 2). Through photoactivation by UV-light, the biotin group of SSBED was transferred to the unknown surface receptor and could thus be affinity purified in avidin columns. This assay is fascinating as it provides the possibility to use a bait protein to catch its unknown interacting partner.

HSC70 has by many authors been demonstrated to be localized at the plasma membrane, acting as a receptor for e.g. Rota virus cell entry or passage through tissue by *Staphylococcus aureus* (Hirschhausen et al, 2010 & Baker et al, 2010). Ongoing studies in our lab will further reveal whether the expression of HSC70 is increased compared to normal mice or if HSC70 of NOD has a stronger affinity for Ig-antigen complexes,

leading to the increased immune complex trapping described in paper I. Indeed, it has been shown that young NOD mice at the age of six to seven weeks, upon entry into the adult stage, display an increase in HSC70 gene expression in B cells. The transition is co-incident with the initiation of islet inflammation, indicating an important role of HSC70 in diabetes onset (Eckenrode et al, 2004). Another report, published by Haberstroh et al (1995) described the drug 15-deoxysupergualin which reversed or ameliorated diabetes progression in NOD. However, at that time, the authors did not mention that 15-deoxyspergualin was a specific inhibitor of HSC70. Assumingly, the inhibition of HSC70 lead to decreased self-antigen-Ig complex binding to B cells, causing less antigen processing and presentation, and thus did abate the B cell contribution to diabetes progression. Moreover, 15-deoxyspergualin has been shown to have potent effects on B cell differentiation *in vitro* (Tepper et al, 1995). Being an immunosuppressant, the drug is probably modulating B cell differentiation through inhibition of HSC70. Indeed, it is shown that overexpression of HSC70 in macrophages enhances their antigen processing and presentation through the interaction with MHC II (Panjwani et al, 1999) and this could presumably be the case in NOD B cells. A recent publication by Alam et al, 2009, describes an enhancement of autoimmune diabetes in mice with transgenic expression of HSC70 in Pancreatic Islets, supporting its important role in autoimmune progression.

Although little has been studied about HSC70 in the context of T1D, the discovery of its presence on NOD B cell surface and the interaction with ICs brings intriguing insights into the potential of this immune pathway as a presumptive target for clinical therapy and intervention. HSC70 has also been implicated to play a role in other autoimmune diseases, such as rheumatoid arthritis. It has been described as a protein over-expressed by synovial cells in the joints. Moreover, HSC70 of rheumatoid B cells interacts with the disease susceptibility molecule HLA-DRB1*0401 and causes abnormal trafficking of the molecule (Auger et al, 2005).

HSC70 is also present on B cells of non-autoimmune mice, indicating its necessity in normal immune homeostasis. Our findings that HSC70 acts as a cell surface Ig-binding receptor may contribute to other research

fields studying the mechanisms of B cells. However, in NOD, HSC70 is seemingly enhancing the binding of ICs. This could be a step in the pathway of MHC class I and II processing of antigens. HSC70 could additionally function as an adjuvant for the antigenic peptide that they bind, triggering the immune response more powerfully. Moreover, pioneering experiments in our lab on human B cells, presented in Paper II, indicate a similar situation where surface IgM pentamers are present in T1D patients as seen in NOD mice. Although further human experiments have to be done to conclude the role of this observation, it is tempting to propose that an increase in HSC70 activity in an individual could switch tolerance into autoimmunity.

# Conclusions

1. NOD B cells display enhanced capture of IgM and IgG on their cell surface compared to normal mice, a feature that is not mediated by conventional Fc receptors such as Fc-α/μ receptor.

2. The binding of Igs to the cell surface is mediated by cell surface expressed HSC70.

3. The Ig binding phenotype of B cells in NOD is also seen in human T1D patients.

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