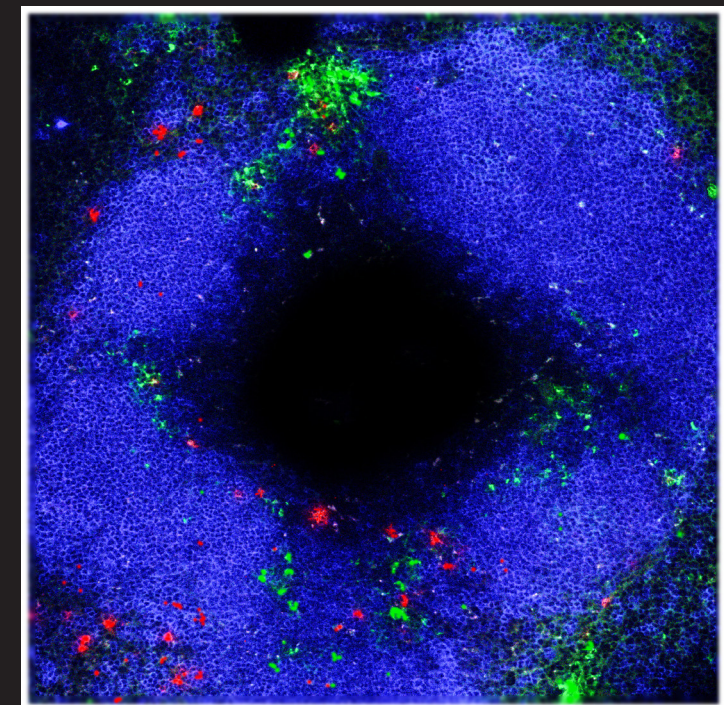


Thesis for doctoral degree (Ph.D.)  
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# INNATE MECHANISMS REGULATING B CELL ACTIVATION IN INFLAMMATORY DISEASES



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From the Department of Medicine Solna  
Clinical Allergy Research Unit,  
Karolinska Institutet, Stockholm, Sweden

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COVER ILLUSTRATION: IgG<sub>1</sub> (green) and IgE (red) extrafollicular foci in a spleen section of an IL-18-injected CD1d<sup>-/-</sup> mouse. The B cell follicle (B220<sup>+</sup> cells) is shown in blue.

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You miss 100 % of the shots you don't take  
- Wayne Gretzky



## ABSTRACT

Generation of powerful and highly specific immune responses against invading pathogens is essential to our survival. However, the immune system can cause disease if activated in an inappropriate manner. Examples include production of IgE antibodies against harmless environmental antigens in allergy and production of IgG antibodies against self-antigens in autoimmunity. Thus, as the antibody-producing cells of the immune system, B cells play a key role in the pathology of both allergic and autoimmune disease.

The aim of the work presented in this thesis was to investigate how B cell activation is regulated by components of the innate immune system in allergy and autoimmunity. In **papers I and II**, regulation of autoreactive and IgE-producing B cells by the innate-lymphocyte subset natural killer T (NKT) cells and the inflammatory cytokine IL-18 was studied in mouse models. In **papers III and IV**, the interplay between NKT cells and IL-18, as well as the B cell-activating cytokines BAFF and APRIL were studied *in vitro* and in patients with atopic eczema (AE).

NKT cells were found to regulate activation of autoreactive and IgE-producing B cells by limiting the formation of germinal centers (GCs; **papers I and II**). In **paper I**, the regulatory effect of NKT cells was shown to be mediated by interactions with CD1d<sup>+</sup> B cells before GC entry. Interestingly, the increased production of self-reactive antibodies in NKT cell-deficient mice could be reduced by injection of NKT cells (**paper I**), and IgE production could be reduced by injection of the NKT cell-activating ligand  $\alpha$ -GalCer (**paper II**). This indicates a potential for NKT cell-based therapies in autoimmune and IgE-mediated diseases. Several inflammatory conditions have been associated with elevated levels of IL-18, and the effects of this inflammatory cytokine on activation of B cells and NKT cells were studied in **papers II and III**. In **paper II**, injections of IL-18 were found to expand the innate marginal zone B cell subset and to induce production of self-reactive natural IgM and IgG antibodies as well as IgE in extrafollicular foci in the spleen. In **paper III**, IL-18 was found to skew the human invariant (i)NKT cell population towards the pro-inflammatory CD4<sup>+</sup> subset *in vitro*. In addition, patients with AE were found to have both elevated levels of IL-18 and a decreased CD4<sup>+</sup> iNKT cell population compared to healthy controls. A reduced CD4<sup>+</sup> iNKT cell population also coincided with elevated total-IgE levels, suggesting a role for IL-18 and NKT cells in regulation of the IgE response in AE. The regulation of B cell activation in AE was further investigated in **paper IV** by characterizing the expression of the cytokines BAFF and APRIL in eczema skin and peripheral blood. The levels of neither BAFF nor APRIL were elevated in the circulation compared to healthy controls. In the skin, both BAFF and APRIL were found to be expressed by keratinocytes, macrophages and T cells, and acute lesions had increased levels of BAFF while both acute and chronic lesions had reduced levels of APRIL. This indicates that the expression of these B cell-activating cytokines is altered in the local skin micro-environment in AE.

In conclusion, the work presented here identifies NKT cells and IL-18 as important regulators of B cells that produce IgE and autoreactive antibodies. While NKT cells limit inappropriate B cell activation, IL-18 drives such responses and skews the iNKT cell population towards pro-inflammatory effector functions. Finally, B cell-activating cytokines can be potential targets for new therapeutic strategies in AE.

## LIST OF PUBLICATIONS

- I. Wermeling F, Lind SM, Domange Jordö E, Cardell S, Karlsson MCI  
**Invariant NKT cells limit activation of autoreactive CD1d-positive B cells**  
*J. Exp. Med* 2010 May 10;207(5):943-52  
(Highlighted in Nat Rev Immunol 2010 Jun;10(6):384)
- II. Lind SM, Domange Jordö E, Hägglöf T, Mattsson N, Gabrielsson S, McGaha TL, Scheynius A, Karlsson MCI  
**IL-18 induces natural antibody responses regulated by NKT cells**  
*In manuscript*
- III. Lind SM, Kuylenstierna C, Moll M, Domange Jordö E, Winqvist O, Lundeberg L, Karlsson MA, Tengvall Linder M, Johansson C, Scheynius A, Sandberg JK, Karlsson MCI  
**IL-18 skews the invariant NKT cell population via autoreactive activation in atopic eczema**  
*Eur J Immunol* 2009 Aug;39(8):2293-301  
(Highlighted in News and Views, Eur J Immunol 2009 Aug;39(8):1988)
- IV. Chen Y, Lind SM, Johansson C, Karlsson MA, Lundeberg L, Scheynius A, Karlsson MCI  
**The expression of BAFF, APRIL and TWEAK is altered in eczema skin but not in the circulation of atopic and seborrheic eczema patients**  
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### **Publications not included in the thesis**

Lindh E, Lind SM, Lindmark E, Hässler S, Perheentupa J, Peltonen L, Winqvist O, Karlsson MCI

**AIRE regulates T-cell-independent B-cell responses through BAFF**

*Proc Natl Acad Sci U S A* 2008 Nov;105(47):18466-71

(Highlighted in Nature Reviews Immunology 2009 Jan(9):3)

Wilsson A, Lind S, Öhman L, Nilsson-Augustinsson A, Lundqvist-Setterud H

**Apoptotic neutrophils containing *Staphylococcus epidermidis* stimulate macrophages to release the proinflammatory cytokines tumor necrosis factor- $\alpha$  and interleukin-6**

*FEMS Immunol Med Microbiol.* 2008 Jun;53(1):126-35





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## LIST OF ABBREVIATIONS

AE	Atopic eczema
$\alpha$ -GalCer	Alpha-galactosylceramide
AHR	Airway hyperreactivity
AID	Activation-induced deaminase
APRIL	A proliferation-inducing ligand
APT	Atopy patch test
BAFF	B cell-activating factor of the TNF-family
BCMA	B cell maturation antigen
BCR	B cell receptor
Be cell	B effector cell
CSR	Class-switch recombination
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme linked immunosorbent assay
FDC	Follicular dendritic cell
FoB	Follicular B cell
GC	Germinal center
hMnSOD	Human manganese superoxide dismutase
IBD	Inflammatory bowel disease
iGb3	Isoglobotrihexosylceramide
IL-18BP	IL-18 binding protein
LPS	Lipopolysaccharide
MDDC	Monocyte-derived dendritic cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MZB	Marginal zone B cell
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	NOD-like receptor
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PRR	Pattern-recognition receptor
RA	Rheumatoid arthritis
RLR	Retonic acid-inducible gene (RIG)-I-like receptor
S1P(1)	Sphingosine 1-phosphatase receptor 1
SCORAD	Scoring atopic dermatitis
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
T1	Transitional type 1
T2	Transitional type 2

TACI	Transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TCR	T cell receptor
TD antigen	T cell-dependent antigen
Tfh cell	T follicular helper cell
Tg	Transgenic
Th cell	T helper cell
TI-I antigen	T cell-independent antigen type I
TI-II antigen	T cell-independent antigen type II
TLR	Toll-like receptor
TWEAK	TNF-like weak inducer of apoptosis
Yaa	Y-linked autoimmune acceleration



# 1 INTRODUCTION

Our immune system represents a highly evolved network composed of organs, cells and molecules that efficiently protects us from potential threats. Such threats include bacteria, parasites and virus (exogenous agents), and harmful compounds released from damaged cells within our body (endogenous agents).

The immune system consists of two branches; the innate immune system and the adaptive immune system. The cells of the innate branch have a fixed set of sensors (receptors) whereby they rapidly respond to microbial structures, such as bacterial or viral components, or to endogenous molecular compounds released from damaged cells. The kinetics of the adaptive part of the immune response are slower in onset, but once activated, it recognizes a huge variety of molecules with great specificity, and is thereby capable of generating a highly powerful response against the potential threats. It is evident that an efficient immune system is fundamental to our survival, but it can also cause severe diseases if activated in an inappropriate manner. An immune response against harmless exogenous or endogenous agents causes allergic and autoimmune disease, respectively. An example of the former is an immune response against the exogenous agent birch pollen while the latter could be exemplified by autoimmune-mediated destruction of the joints in rheumatoid arthritis. These unwanted actions of our immune system have been subject to investigations for several years, generating many important answers but also unresolved and additional questions.

It has become clear over recent years that the innate branch of the immune system can shape the adaptive branch, and that this plays an important role during both beneficial and harmful immune responses. This thesis deals with regulation of B cells by innate mechanisms in connection to inflammatory diseases such as allergy and autoimmunity.

## **1.1 THE IMMUNE SYSTEM**

The immune system is an important part of our body's defense. When potentially harmful agents, such as bacteria, enter our body, the leukocytes of the immune system are activated and eradicate the bacteria by a sophisticated chain of events. First, tissue resident cells, such as dendritic cells (DCs), macrophages and mast cells, recognize the bacteria and react by initiating an inflammatory response. This includes production of soluble factors (cytokines and chemokines) which activate and attract neutrophils and monocytes towards the site of infection. The innate immune cells fight the bacterial infection by various effector mechanisms as well as alert the adaptive immune system. Bacterial components are transported from the tissue to the local lymph nodes via the lymphatic system by DCs and as soluble antigen particles. The T cells and B cells of the adaptive immune system that reside in the lymph node and have antigen receptors specific for the bacterial components are activated by the antigen particles and start to expand. The expanded T and B cells contribute to the eradication of the bacteria by production of soluble factors (cytokines and antibodies) which increase the efficacy of the innate immune cells and target the bacteria for destruction. When the bacteria are eradicated, the immune system's job is (temporarily) done. A few of the activated T and B cells survive as long-lived memory cells which can be activated quickly if the same bacteria enter our body again.

### **1.1.1 Innate immunity**

The innate immune system, which constitutes our first line of defense against invading pathogens, consists of physical barriers, such as the skin and mucosal surfaces, and cells that phagocytose and kill the invading pathogen. The cellular part of the innate immune system includes neutrophils, monocytes, macrophages, DCs, eosinophils, basophils, mast cells and natural killer (NK) cells [1]. The innate immune system has evolved different strategies to recognize potentially dangerous agents based on germ-line encoded proteins. These proteins encode receptors which recognize pathogen-associated molecular patterns (PAMPs) which are highly conserved microbial components shared by entire classes of pathogens, danger-associated molecular patterns (DAMPs) which are endogenous components released from damaged cells, and absence of self-associated molecules such as major histocompatibility complex class I (MHC I) [2]. An accurate discrimination between dangerous and harmless agents by the innate immune system is essential to the survival of the host. For example, if the innate immune system is activated by self-antigens, activation of the adaptive immune system will follow, which can result in severe autoimmune disease [2]. The receptors that recognize PAMPs and DAMPs are called pattern-recognition receptors (PRRs) and have been most widely studied in antigen-presenting cells such as macrophages and DCs. It has been suggested that PAMPs mark the difference between self and microbial nonself, while DAMPs denote the difference between pathogenic (i.e. tissue destructing) and non-pathogenic microbes such as commensal bacteria or fungi [3].

PRRs include toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors [4]. Different PRRs recognize different PAMPs and/or DAMPs and this shapes both the innate and adaptive immune response by regulating cytokine/chemokine production and upregulation of MHC-, costimulatory- and adhesion molecules [2, 5]. The main function of TLRs is to recognize PAMPs in the extracellular space and in endosomes. TLRs that bind components of bacterial cell walls are located on the plasma membrane; TLR2 binds peptidoglycan from gram positive bacteria and TLR4 binds lipopolysaccharide (LPS) from gram negative bacteria. Endosomes hold TLRs that bind nucleic acids from bacteria or viruses; TLR3 binds double-stranded RNA, TLR7 binds single stranded RNA and TLR9 binds unmethylated DNA [1, 4]. How these TLRs discriminate between self and nonself nucleotides is still incompletely understood, but the endosomal localization of TLR7 and TLR9 has been suggested to prevent activation by self-nucleotides [6, 7]. The RLR RIG-I is another intracellular PRR that binds nucleic acids [4]. The NLRs are cytoplasmic receptors of the subfamilies NALP, IPAF and NOD [8]. The NALP- and IPAF-families of receptors are connected to assembly of the inflammasome and production of cytokines of the IL-1 family, which will be described in section 1.4.1 “The inflammasome”. The most well known NOD receptors are NOD1 and NOD2 which are activated by peptidoglycans from both gram positive and gram negative bacteria [8]. Furthermore, the C-type lectin receptor Mincle recognizes both damaged cells and the yeast *Malassezia*, and is thus important in both tissue homeostasis and anti-fungal immunity [9, 10].

In addition to molecules expressed on pathogens or released from damaged cells, the immune system can also be activated by products released by pathogens. Parasites, for example, release proteases to degrade and invade the tissue, and the innate immune system is activated by these proteases to induce a Th2-type immune response. This pathway is shared by protease allergens which are recognized by basophils which, in turn, induce the Th2-type response [11].

### **1.1.2 Adaptive immunity**

The adaptive immune system constitutes our specific defense and consists of T cells and B cells. Each T and B cell has a unique antigen-binding receptor which is generated through random joining of DNA gene fragments of the *tcr* and *Ig* loci, respectively. In this way, a large and diverse repertoire of antigen-binding receptors can be formed by a relatively small set of genes [1]. However, receptors that bind self structures and harmless environmental antigens can also be generated in this random process, which is why activation of T and B cells is tightly regulated. Hence, activation of the innate immune system is a prerequisite for successful activation of adaptive immunity. The ability of the innate immune system to sense molecules associated with nonself and danger thereby translates to activation of T and B cells [2].



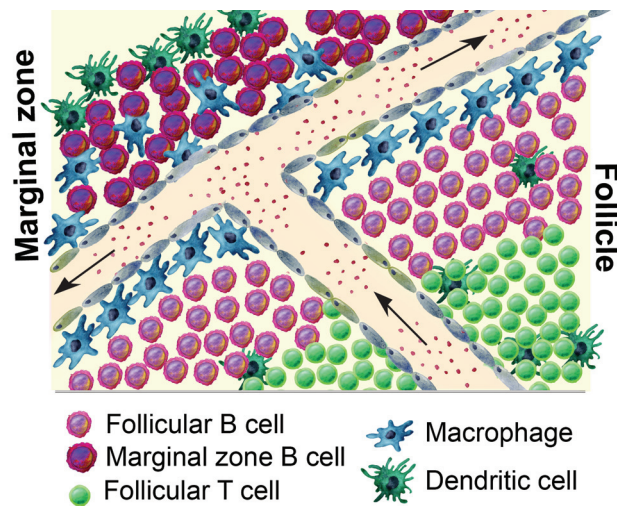
The T cell receptor (TCR) and B cell receptor (BCR) both consist of 2 chains;  $\alpha/\beta$  and heavy/light chains, respectively. The TCR is generated in the thymus and recognizes antigen peptides presented in the MHC molecule on antigen-presenting cells. MHC I is recognized by cytotoxic  $CD8^+$  T cells while MHC II is recognized by  $CD4^+$  T helper (Th) cells. Assembly of the BCR, on the other hand, starts in the bone marrow at the pre-B cell stage with generation of a heavy chain followed by rearrangement of the light chain genes. T and B cells with a TCR/BCR that bind self-antigens with neither too low nor too high affinity survive the positive and negative selection steps in the thymus/bone marrow, and populate peripheral lymphoid organs as naïve lymphocytes [1].

Continuous transport of antigens to the lymph nodes by DCs and presentation of the antigen to T cells as MHC-peptide complexes are essential for activation of the adaptive arm of the immune system. When the DCs have been activated by the antigen they are carrying, for example via PAMPs, the DCs upregulate costimulatory molecules (CD80 and CD86) which, in combination with specific TCR-antigen interaction, result in T cell activation [2]. B cells, on the other hand, recognize three-dimensional surfaces of soluble or surface bound antigen particles [12]. After initial binding to the BCR, the antigen is internalized and presented on MHC II. A second activation signal is usually needed for the B cell to become an antibody-producing effector cell, and this is provided by activated antigen-specific Th cells that have upregulated the costimulatory molecule CD40L. Activated T and B cells proliferate and expand to a large population with the same antigen specificity, i.e. clones. It takes about 5 days from initial antigen encounter with the innate immune system until adaptive effector cells are ready to start fighting the infection [1].

The  $CD4^+$  Th cells are divided into the Th1, Th2 and Th17 subsets based on the cytokine they produce. The classical view is that Th1 cells produce IFN- $\gamma$ , Th2 cells produce IL-4 and Th17 cells produce IL-17. These subsets also mediate immune responses to different sets of pathogens; the Th1 subset is important for cellular immunity against intracellular pathogens, the Th2 subset mediate antibody responses to extracellular pathogens and the Th17 subset is an important player in the immune response against fungi and gram negative bacteria [13]. Several other T cell subsets have been described and two that will be discussed in this thesis include the T follicular helper (Tfh) cells and the innate-like natural killer T (NKT) cells. Tfh cells are important in maturation of B cell responses and will be discussed in section 1.2.3 “B cell activation and antibody production” and the potent immune regulatory functions of NKT cells and the impact on B cell responses will be discussed in section 1.3 “NKT cells”.

### **1.1.3 The spleen**

The spleen is the largest peripheral lymphoid organ in our body, but in contrast to other peripheral lymphoid organs, it lacks afferent lymphatics and is instead supplied by the splenic arteries. The spleen is divided in the red pulp, which filters old/damaged erythrocytes from the blood, and the white pulp, which holds innate and adaptive immune cells. The cells in the white pulp are organized in a highly specialized structure (Fig. 1), optimized for efficient antigen-specific activation of T and B cells [14].



**Figure 1.** Schematic illustration of the innate and adaptive immune cells in the white pulp of the spleen. The splenic artery enters in the center of the white pulp and the blood flows up towards the red pulp and marginal sinus. The follicle is made up of the T cell zone in the center, with mainly Th cells and specialized follicular DCs, and the follicular B cells surrounding the T cell area. The outer boarder of the follicle is lined by the metallophillic macrophages and the endothelial cells of the marginal sinus. The blood flows from the central arteriole through the marginal sinus, which separates the follicle from the marginal zone. The marginal zone forms the interface between the white and red pulp and holds the marginal zone macrophages and the innate-like marginal zone B cells.

The marginal zone is in constant contact with the blood, and its inhabitants screen the systemic circulation for antigens and pathogens. The marginal zone B cells (MZBs) and especially the marginal zone macrophages express a number of TLRs and scavenger receptors for this purpose [15]. The MZBs shuttle between the marginal zone and the follicles and can thereby deliver antigen to the T and B cell areas of the white pulp [16]. The MZB-shuttling is regulated by expression of the sphingosine 1-phosphate receptor 1 (S1P(1)) and the production of CXCL13. S1P(1) is a G-protein coupled receptor expressed by MZBs and interactions with S1P in the blood retains MZBs in the marginal zone. However, activation of MZBs by antigen or LPS leads to down-regulation of the S1P(1) [17]. The MZBs can then migrate towards the follicle guided by the CXCL13-gradient produced by the follicular DCs (FDCs) and deliver antigen to this DC subset, which subsequently leads to activation of T and B cells. Antigen-specific differentiation of B cells in the follicles of the white pulp is followed by migration of the generated plasmablasts to the red pulp. Here, the plasmablasts form extrafollicular plasma cell foci together with CD11c<sup>hi</sup> DCs and produce antibodies that rapidly enter the circulation [14].

Defense against blood borne pathogens, and thus protection from development of sepsis, is one of the most important functions of the spleen. This is exemplified by the fact that asplenic patients have a higher risk of developing sepsis when infected with encapsulated bacteria [14]. The spleen is indispensable for the production of poly-reactive anti-polysaccharide IgM antibodies which are vital for the clearance of encapsulated bacteria by tissue macrophages. In humans, this is ascribed to the IgM memory B cells located in the marginal zone of the spleen [18]. In mice, on the other hand, absence of the spleen leads to absence of both the splenic MZBs and the peritoneal B1 cells, and the latter has been ascribed an important role in the production of anti-polysaccharide IgM antibodies [19].

## 1.2 B CELLS

B cells are mostly known as antibody-producing cells activated by Th cells during adaptive immune responses. In addition, B cells have several other important effector functions such as T cell-independent antibody production, cytokine production and antigen presentation [20]. However, a feature unique to B cells is the production of immunoglobulins and the use of these antigen-specific molecules both as membrane bound receptors (BCRs) and secreted effector molecules (i.e. antibodies). Production of antibodies is also the effector function most commonly connected to disease pathology, exemplified by production of self-reactive IgG in autoimmunity and allergen-specific IgE in allergy.

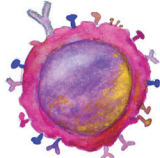
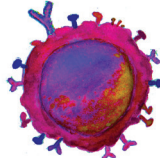
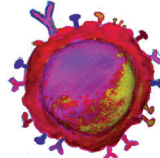
### 1.2.1 B cell development and tolerance

It has been estimated that the antibodies/BCRs in one individual can recognize as many as  $10^7$  different antigen epitopes. The development of B cells in the bone marrow is focused around assembly of the BCR, with emphasis on the generation of the variable antigen-binding domains of the heavy and light chains. The variable part of the Ig-molecule is created by rearrangement of the variable (V), diversity (D) and junctional (J) gene segments, a process known as V(D)J recombination. This process starts at the heavy chain locus and the functionality of the rearranged heavy chain is tested by formation of the pre-BCR by pairing with the surrogate light chain [1, 20]. Signals from the pre-BCR have been suggested to mediate negative selection of self-reactive B cells [21]. B cells with a functional pre-BCR that passes the negative selection step start rearranging the V and J segments at the light chain locus. Generation of a BCR with low affinity for self-antigen mediates survival of the developing B cell at this stage. However, cells that bind to self-antigens with too high affinity get a second chance at generating a less self-reactive BCR. This is known as receptor editing and involves expression of new, further rearranged, light chain genes. In addition to expressing a functional and not too self-reactive BCR, it is also important that all BCRs on a single B cell share a common antigen-binding site. This is assured by the process of allelic exclusion which shuts down further rearrangement of the VDJ genes when a functional rearrangement has been expressed [1, 20].

B cells that have succeeded in generating a BCR that passes both the positive and negative selection steps in the bone marrow eventually enter the circulation as immature B cells. The immature B cells are first referred to as transitional type 1 (T1) cells which circulate to the spleen where they differentiate into T2 cells. Here, the T2 cells then differentiate into different mature naïve B cell subsets [22]. Up to 40 % of the B cells that leave the bone marrow express BCRs that bind self-antigens in humans. This population of self-reactive B cells is decreased to 20 % by peripheral tolerance mechanisms during the differentiation from early emigrant to mature naïve B cell [23]. In patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), the frequency of autoreactive clones does not decrease from the early emigrant to mature naïve B cell compartment, indicating that B cell tolerance has been broken already before antigen-mediated activation [24].

### 1.2.2 B cell subsets

The mature B cell pool consists of several different types of B cells that belong either to the naïve B cell pool or to the antigen-experienced B cell pool which produces antibodies and cytokines. The majority of the mature naïve B cell pool consists of the follicular B cells (FoBs) which recirculate between the blood and the peripheral lymphoid organs and take part in classical adaptive immune responses together with Th cells. The MZB and B1 cell subsets, on the other hand, consist of innate-like resident B cells with specialized functions and characteristics (Fig. 2) [20].

Follicular B cell	Marginal zone B cell	B1 cell
		
IgD/IgM, CD23	IgM/IgD, CD21, CD1d	IgM, CD5, Mac1
Adaptive	Innate-like	Innate-like
Recirculating	Splenic	Peritoneal

**Figure 2.** Phenotypes and functions that are characteristic of the different B cell subsets in the mature naïve B cell pool. Follicular B cells recirculate between the blood and secondary lymphoid organs, express high levels of IgD, and participate in classical adaptive T cell-dependent immune responses. The marginal zone B cells and B1 cells are referred to as innate-like and preferentially participate in T cell-independent immune responses. They are resident cells located in the spleen and the peritoneum, respectively. Marginal zone B cells express high levels of CD21 (the high affinity complement receptor) and the antigen-presenting molecule CD1d, while expression of CD5 is a typical feature of B1 cells.

Molecules that activate B cells can be divided in T cell-dependent (TD) and T cell-independent (TI) antigens. TD antigens are proteins that contain both T and B cell epitopes and induce activation of antigen-experienced B cells first after costimulation by activated T cells. MZBs and B1 cells can be activated by and participate in TD responses but are specialized in activation by TI antigens. The TI antigens can be further divided into polyclonal activators (TI type I; TI-I) and multivalent antigens (TI type II; TI-II). TI-I antigens activate B cells independently of the BCR. One example is LPS which activates B cells by binding to TLR4. TI-II antigens are repetitive molecules, such as polysaccharides, that can bind several BCRs on a B cell simultaneously, inducing a signal strong enough to activate the B cell without the need for T cell help [1]. Interestingly, the TD antigen ovalbumin (OVA) can induce a TI-II B cell response when presented on FDCs [25]. TI-antibody responses are quicker compared to the TD counterpart but the lack of T cell help limits the maturation of the B cell response, as discussed in section 1.2.3 “B cell activation and antibody production”.

The early TI-antibody response by innate-like B cells bridges the gap between the rapid innate immune response and the slower TD adaptive antibody response. The BCR repertoire of innate-like B cells bears similarities with PRRs; it is rich in germ-line encoded specificities and reacts with conserved microbial carbohydrates and glycolipids. These antibodies are often polyreactive (i.e. bind several unrelated epitopes) and

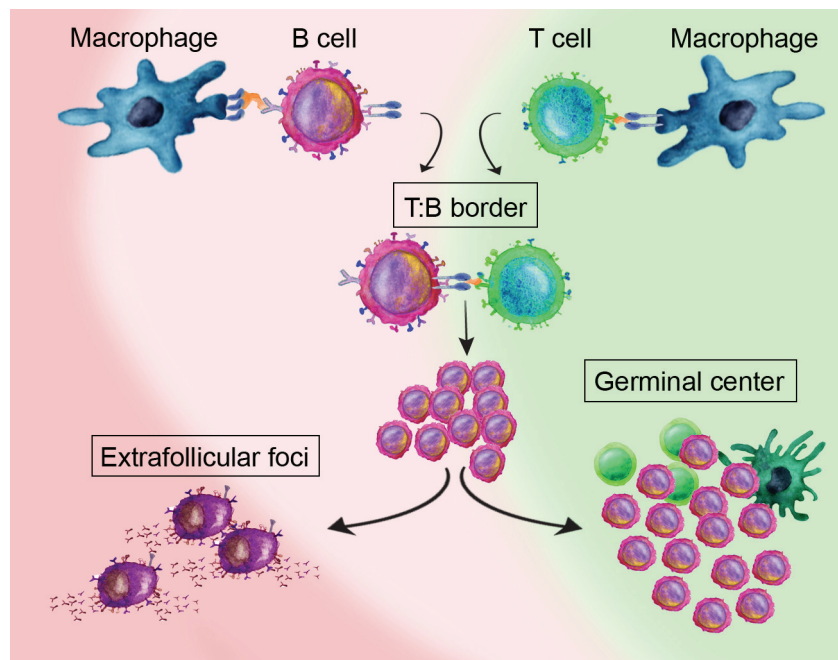
MZBs and B1 cells continuously produce IgM, without apparent antigenic stimulation. This is referred to as natural antibody production [26]. Natural antibodies react with several different pathogens and enhance antigen-trapping in secondary lymphoid organs and prevent dissemination of the pathogen to vital organs [27, 28]. However, natural antibodies also bind self-antigens such as DNA, phosphorylcholine and insulin, and have therefore been suggested to be a potential source of pathogenic autoantibodies in case they undergo affinity maturation and isotype switching [29].

B cell regulation of T cell responses have not been without controversy but the use of the B cell-depleting anti-CD20 antibody in autoimmune disease have highlighted antibody-independent effector mechanisms of B cells in disease pathogenesis [30]. For example, a study in mice showed that anti-CD20 mediated B cell depletion impaired both the adaptive and autoreactive activation of CD4<sup>+</sup> T cells [31]. A subset of B cells that has emerged as important regulators of immune responses is the cytokine-producing B cells which include both B effector (Be) cells and regulatory B cells [32]. The Be cells have been further divided into Be-1 and Be-2 depending on if they produce IFN- $\gamma$  or IL-4. For example, Be-1 cells have been primed by antigen in combination with Th1 cells and amplify the Th1 response by priming naïve Th cells to differentiate into Th1 cells [33]. The Be cells have been suggested to develop from the FoBs, which are commonly involved in TD B cell responses [32]. The IL-10-producing regulatory B cells, on the other hand, have attributes of MZBs and B1 cells and have also been suggested to develop from these innate-like B cell subsets. The IL-10-producing regulatory B cells control TD responses by inhibiting the activation of FoBs by Th cells and attenuating the Th1 priming by DCs [32, 34].

### **1.2.3 B cell activation and antibody production**

Antigen-mediated activation of B cells is initiated following engagement of the BCR with unprocessed, intact antigen. The activation is most efficient when the antigen is membrane bound, but soluble antigen in high enough concentration has also been reported to activate B cells [12]. Membrane bound antigens are often in the form of antigen-antibody immune complexes bound to Fc receptors or complement receptors on cell membranes [12]. During the last three years, several studies have provided important insights regarding how antigens reach FDCs and B cells in the lymph node follicles. Small antigens that reach the subcapsular sinus of the lymph node can either diffuse through pores between the subcapsular sinus macrophages [35], or travel through conduits (max 70kD) that extend from the subcapsular sinus into the follicle [36]. Larger antigens, such as immune complexes, are made accessible to B cells by transport on the subcapsular sinus macrophages, which constitute the border between the subcapsular sinus and B cell follicle [37]. Antigen presented on subcapsular sinus macrophages can either be engaged by antigen-specific B cells directly or be transported by non-cognate B cells to FDCs [37, 38]. In the spleen, the transportation of blood-born antigen to the FDCs is carried out by MZBs in an IgM and complement-dependent fashion [39]. The FDCs are very efficient at presenting antigen to antigen-specific B cells and BCR-mediated endocytosis and presentation of the antigen on MHC II is followed by proliferation at the border between the B and T cell areas [12]. After this initial burst of proliferation, the activated B cells continue to differentiate into effector cells in extrafollicular foci or germinal centers (GCs) (Fig. 3) [40].





**Figure 3.** B cell activation in the spleen leads to formation of extrafollicular foci and germinal centers. After antigen encounter (top left), B cells migrate to the T:B border where they proliferate together with antigen-activated Th cells. The B cells then migrate either to the follicle and form germinal centers or to the bridging channels in the boarder between the white and red pulp and form extrafollicular foci.

Differentiation to antibody-producing plasmablasts and plasma cells (PCs) in extrafollicular foci is supported by CD11c<sup>high</sup> DCs and thus occur independent of T cell help [41]. B cells with high antigen affinity have been suggested to be selected into the extrafollicular foci pathway, which is a fast route to antibody production [42]. B cells in extrafollicular foci produce both IgM and isotype-switched antibodies, starting at 3-4 days after antigen encounter. The antibodies produced there are germ-line encoded, since affinity maturation is normally confined to the GC reaction [40, 43]. Plasmablasts in extrafollicular foci make an initial wave of antibodies in TD responses and production of antibodies against TI-antigens are largely confined to this site [41, 44]. For example, MZBs activated by TI-antigen differentiate into plasmablasts in association with DCs at extrafollicular sites [45]. Self-reactive antibody responses have also been shown to take place in extrafollicular foci, and both somatic hypermutation (SHM) and T cell help have been demonstrated during B cell activation at extrafollicular sites in mouse models of autoimmunity [46-48]. Nevertheless, high affinity isotype-switched autoantibodies have been associated with the GC pathway of B cell activation in several mouse models and in patients with autoimmunity [46].

GCs are specialized structures where B cells undergo affinity maturation and isotype switching [43, 49]. Clones with BCRs that bind antigen with high affinity are selected to become antibody-producing PCs and memory B cells. Affinity maturation of the BCR is achieved through a combination of limited access to antigen and SHM of genes in the V gene segments of the BCR. SHM changes the antigen-binding site of the BCR and sufficient survival signals by BCR-antigen interactions are restricted to those B cells that acquire the highest affinity for the antigen. Increased antigen-binding by the BCR results in increased antigen presentation on MHC II and thereby increased access to T cell help. In addition to changes in the antigen-binding site, the constant part of the

BCR (and thus the effector function of the antibodies) is also altered in the GC. Isotype switching from IgM to other subclasses such as IgG<sub>1</sub> or IgE occurs through the process of class-switch recombination (CSR) [1, 20]. Both SHM and CSR involve genetic alterations in the BCR heavy chain and these are mediated by the enzyme activation-induced deaminase (AID) [50]. T cell help to B cells in GCs is provided by Tfh cells that are initially primed when interacting with B cells at the T-B boarder and migrate together with the B cells to the follicle and the GC reaction. The hallmarks of Tfh cells are high level expression of CXCR5, PD-1 and IL-21. IL-21 is important to sustain the Tfh phenotype as well as for the differentiation of B cells in the GC. It has been suggested that Tfh cells may consist of functionally distinct subsets, as this cell type has been shown to produce IFN- $\gamma$  during *Leshmania* infection, IL-4 during helminth infection and IL-17 in a mouse model of experimental autoimmune encephalomyelitis (EAE) [51]. The cytokine profile of the Tfh cells has been suggested to shape B cell responses and conjugates of IL-4<sup>+</sup> Tfh cells and IgG<sub>1</sub><sup>+</sup> B cells as well as IFN- $\gamma$ <sup>+</sup> Tfh cells and IgG<sub>2a</sub><sup>+</sup> B cells have been identified. This indicates that Tfh cells could direct isotype switching [52].

The output of a GC is affinity matured B cells with either a memory B cell or PC phenotype. A high affinity BCR is associated with increased antigen-mediated BCR signaling, which in turn activates transcription of the *blimp-1* gene, which promotes the PC phenotype. No such “master switch” has yet been identified for memory B cells and the factors that control memory B cell vs. PC fate are still poorly understood. GC B cells that differentiate into memory B cells leave the GC earlier compared to those that take on a PC phenotype, and the unswitched IgM memory B cells exit prior to the more affinity mature switched memory B cells [53]. Upon re-challenge with antigen, the IgM<sup>+</sup> memory B cells reenter the GC, while the switched memory B cells differentiate into antibody-producing PCs [54]. A GC-reaction with T cell help was for long considered to be a prerequisite for B cell memory. However, polysaccharide TI-II antigens also give rise to memory B cells [55]. If such memory B cells are a product of short lived abortive GCs or extrafollicular PC responses is, however, not known. Memory B cells can quickly differentiate into antibody-producing PCs upon re-encounter with antigen or if the PCs in the bone marrow are depleted [20]. In addition, a combination of the cytokines BAFF (B cell-activating factor of the TNF family) and IL-21 has been shown to stimulate the differentiation of IgG<sup>+</sup> memory B cells into antibody-producing PCs [56].

#### **1.2.4 Regulation of B cell activation**

In addition to antigen recognition by the BCR and costimulatory signals from cognate Th cells, B cell activation is regulated by several other factors including cytokines, microbial products and the balance between activating and inhibitory receptors. Two cytokines that regulate peripheral B cell survival, and thus impact B cell development and activation, are the TNF-family members BAFF and APRIL (a proliferation-inducing ligand). The receptors for BAFF and APRIL are expressed by B cells and include TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen) which are shared by both BAFF and APRIL. In addition, BAFF binds BAFF receptor and APRIL interacts with proteoglycans. During peripheral B cell development, BAFF is necessary for the transition

from the T1 to T2 stage in the spleen, and thus also for the development of the mature FoB and MZB populations. Both BAFF and APRIL contribute to the survival of plasmablasts in TI-II antibody responses, while long-lived PCs are more sensitive to APRIL-mediated survival signals [57]. BAFF- and APRIL-mediated signals can also induce CD40L-independent isotype class-switch to IgG<sub>1</sub> and IgA, and together with IL-4 also to IgE [58]. Studies in mice transgenic for BAFF (BAFF Tg) revealed that elevated levels of BAFF are associated with development of autoimmune disease and BAFF has been shown to support survival of autoreactive B cells with low/intermediate affinity for self-antigens into the MZB pool [59, 60]. Likewise, elevated levels of BAFF have also been reported to correlate with disease progression in patients with autoimmune disorders. In addition, strategies to neutralize BAFF are showing promising results in patients with SLE and RA [61]. Interestingly, autoimmunity and altered B cell tolerance in BAFF Tg mice is independent of T cell help but requires TLR signaling, indicating that activation of self-reactive B cells can be innate-driven [62].

TLR ligands can act as TI-I antigens and activate B cells to proliferate, upregulate costimulatory molecules, and to produce cytokines and antibodies. In mice, the naïve mature B cell subsets have been shown to express TLR 1, 2, 4, 7 and 9 [63, 64]. Although TLR ligands can induce antibody production on their own, co-engagement of the BCR and CD40L-mediated signals are needed for optimal antibody responses. In addition, B cell stimulation by TLRs has been shown to enhance TD antibody responses, presumably by sustaining the expansion of B cells during the response [65, 66]. TLR-signals have therefore been suggested to be “signal 3” in B cell activation, in addition to BCR engagement (signal 1) and T cell help (signal 2) [66]. The connection between increased activation of TLRs and activation of autoreactive B cells is well established, and TLR ligands have even been shown to generate T cell-independent activation of autoreactive B cells [67]. The autoimmune phenotype in mice carrying the *Yaa* (Y-linked autoimmune acceleration) allele has also been attributed to increased TLR-signaling, more specifically to duplication of TLR7 [68]. Although this TLR7 duplication is not unique to B cells and will affect multiple components of the immune system, it specifically biases B cells to produce anti-RNA antibodies in a B cell intrinsic manner [68]. Likewise, TLR9 was shown to be important for production of anti-DNA antibodies after BCR-mediated uptake of DNA-containing immune complexes [69].

In addition to increased activation signals, insufficient inhibitory signals can also contribute to activation of self-reactive B cells. One example is the inhibitory Fc receptor FcγRIIB which has been shown to prevent autoimmune disease by regulating activation of autoreactive B cells in the periphery [70, 71]. This has been suggested to be mediated by dampening of B cell activation induced by both BCR- and TLR-mediated signals. For example, B cells are normally only activated by DNA-containing immune complexes where the DNA is rich in unmethylated CpG sites. However, immune complexes containing CpG-poor DNA (i.e. similar to self-DNA) have been shown to be able to activate FcγRIIB-deficient B cells [72].



### 1.3 NKT CELLS

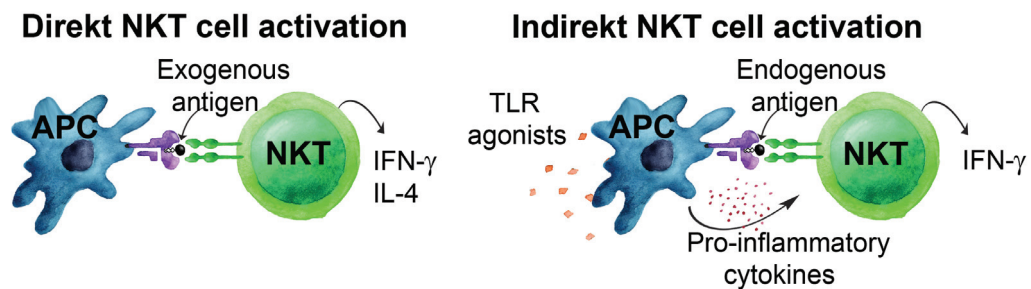
NKT cells are innate-type lymphocytes that express both NK and T cell markers such as CD161 (NK1.1), CD3 and a TCR. NKT cells are also different from conventional T cells in that they recognize lipid antigens presented by CD1d (an MHC I-like molecule expressed by antigen-presenting cells) rather than MHC-restricted peptides. Antigen-mediated activation of NKT cells induces rapid production of cytokines (within hours), which has attributed potent immunomodulatory functions to this innate T cell subset. The size of the NKT cell population is highly variable between individuals, from undetectable (approximately 0.001 %) to 3 % of the lymphocytes in peripheral blood [73].

NKT cells develop in the thymus and segregate from the conventional T cells at the double positive ( $CD4^+CD8^+$ ) stage if a TCR that recognizes CD1d is generated. The positive selection of NKT cells is mediated by CD1d-expressing double positive cortical thymocytes presenting a self-lipid of yet unknown identity [73]. It has been proposed that the self-lipid and CD1d-ligand isoglobotrihexosylceramide (iGb3) mediates positive selection of NKT cells [74]. However, this idea has been challenged by a study demonstrating normal development of NKT cells in iGb3-deficient mice [75]. A TCR with an  $\alpha$ -chain that consists of  $V\alpha14J\alpha18$  paired with a  $\beta$ -chain that is either  $V\beta8.2$ ,  $V\beta7$  or  $V\beta2$  will recognize glycolipids presented by CD1d in mice. Cells that express such a TCR are referred to as type 1 or invariant (i)NKT cells. In humans, iNKT cells are defined by a TCR consisting of  $V\alpha24J\alpha18$  paired with  $V\beta11$  [73]. The prototypic ligand for iNKT cells in both mice and humans is the glycosphingolipid  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), originally identified as an anti-cancer compound isolated from the marine sponge *Agelas mauritianus* [76, 77]. NKT cells that do not recognize  $\alpha$ -GalCer presented by CD1d are referred to as type 2 NKT cells. These have a more diverse TCR repertoire, and no prototypical ligand has yet been identified. Antigens presented by CD1d and recognized by type 2 NKT cells include sulfatide and lysophosphatidylcholine. Both iNKT cells and type 2 NKT cells can be either  $CD4^+$  or double negative ( $CD4^-CD8^-$ ), while iNKT cells also can be  $CD8^+$  in humans. These subsets produce different cytokines and have thus been attributed different functions. Studies of human iNKT cells have revealed that  $CD4^+$  iNKT cells are more tolerogenic while double negative iNKT cells are more inflammatory [73].

#### 1.3.1 Cytokine production by activated NKT cells

NKT cells migrating from the thymus continue to develop in the periphery, and eventually populate the blood, liver, lymph nodes and spleen. Cells that express CD1d and thus are able to present antigen to NKT cells include macrophages, DCs and B cells (especially the CD1d-high MZB subset) [78]. A recent study showed that the  $CD169^+$  subcapsular sinus macrophages constitute the celltype that present lipid antigens to NKT cells in lymph nodes [79]. CD1d presents both endogenous and exogenous lipids; examples of the latter include glycosphingolipids from the  $\alpha$ -proteobacteria *Sphingomonas*. The most used method to study activation of NKT cells is to use  $\alpha$ -GalCer or anti-CD3 antibodies. TCR-mediated activation of NKT cells results in rapid production of large amounts of cytokines, induction of cytotoxic activity and upregulation of costimulatory molecules [78]. Activation of NKT cells with anti-CD3/CD28 has been shown to stimulate production of the following cytokines; IL-2,

IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17, IL-21, IFN- $\gamma$ , TNF- $\alpha$  and GM-CSF [80]. Individual NKT cells can produce both Th1 and Th2 cytokines simultaneously. Whether an immune response will be polarized towards Th1 or Th2 depends on which subset of NKT cells that has been activated, as well as on the nature of the ligand [78]. For example, human CD4<sup>-</sup> iNKT cells produce both IL-4 and IFN- $\gamma$  while CD4<sup>+</sup> iNKT cells mainly produce IFN- $\gamma$  and less IL-4, thus facilitating Th1 polarization [81, 82]. Furthermore, the  $\alpha$ -GalCer analog OCH preferentially induces production of Th2 cytokines. In addition to TCR-mediated activation by exogenous antigens, NKT cells can also be activated by a combination of pro-inflammatory cytokines and endogenous antigens presented by CD1d. This is referred to as indirect NKT cell activation and mainly results in production of IFN- $\gamma$  (Fig. 4) [78].



**Figure 4.** Schematic illustration of direct and indirect NKT cell activation. Direct NKT cell activation involves interaction between the NKT cell TCR and exogenous lipid or glycolipid antigen presented by CD1d. This leads to production of both IFN- $\gamma$  and IL-4. Indirect NKT cell activation involves stimulation of antigen-presenting cells with TLR-agonists, which induce production of pro-inflammatory cytokines. NKT cells are moderately autoreactive to endogenous ligands presented by CD1d, and the combination with pro-inflammatory cytokines and low-level autoreactivity induces production of IFN- $\gamma$ .

### 1.3.2 Regulation of B cell responses by NKT cells

NKT cells have been shown to both enhance and inhibit humoral immune responses, and conflicting results in different studies have been reported. Models and methods used to study the impact of NKT cells on B cell responses include:

- NKT cell-deficient mice; CD1d<sup>-/-</sup> mice which are deficient in all NKT cells and J $\alpha$ 18<sup>-/-</sup> mice which only lack iNKT cells.
- NKT cell Tg mice; V $\alpha$ 14 Tg mice are enriched in iNKT cells.
- Activation of iNKT cells by the prototypic ligand  $\alpha$ -GalCer.

Several studies have shown that iNKT cells activated by  $\alpha$ -GalCer provide B cell help and enhance antibody responses to protein antigens [83]. The mechanism(s) by which iNKT cells provide B cell help remains to be fully elucidated, but presentation of  $\alpha$ -GalCer by CD1d on B cells has been shown to be required [84]. The rapid production of IL-4 by activated NKT cells has been suggested to be important for the initiation of IgE responses, and the absence of IL-4-producing NKT cells has been associated with a reduced IgE response [85]. However, the absence of IL-4-producing NKT cells had no effect on the production of IgE in CD1d<sup>-/-</sup> mice [86]. It is highly likely that the rapid production of large amounts of cytokines by activated NKT cells is involved in the mechanism by which they regulate immune responses. An *in vivo* system where IFN- $\gamma$  and/or IL-4 are knocked-out only in NKT cells would be very useful to outline in what way these cytokines contribute to the stimulating and suppressive effects of NKT cells.

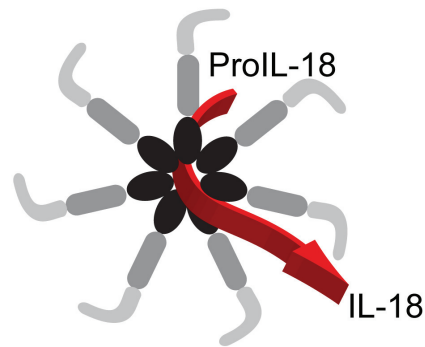
The role of NKT cells in IgE-associated immune responses has been further investigated in mouse models of airway hyperreactivity (AHR), as well as in asthmatic patients. Several studies have proposed that NKT cells contribute to the Th2 response and development of AHR during the sensitization phase but inhibit AHR when activated at the time of allergen challenge [87-89]. However, it has also been reported that the antigen-specific IgE response does not differ between NKT cell-deficient and WT mice in a mouse model of airway inflammation [90]. Furthermore, NKT cells have been identified in the lungs and have been reported to be increased in the airways of patients with asthma [91, 92]. However, other studies have failed to show a difference in the NKT cell population in the lungs between healthy controls and asthmatic patients [93, 94]. Production of IL-21 has been suggested as the mechanistic link between NKT cells and regulation of IgE responses since it has been shown to induce apoptosis in IgE<sup>+</sup> B cells. IL-21 has numerous effects on both innate and adaptive immune responses and both positive and negative effects have been attributed to this cytokine in B cell activation. It has been suggested that B cells activated via the BCR are stimulated by IL-21 while B cells activated by TLRs are inhibited by IL-21 [95].

Perturbed numbers and functions of NKT cells have been reported in a variety of autoimmune diseases. In addition, NKT cell deficiency leads to disease exacerbation in several mouse models for autoimmunity. However, studies in mouse models have also shown that NKT cells can contribute to autoimmune disease or have no effect on autoimmune responses [96]. One such example is SLE where patients have been reported to have a reduced NKT cell population in the blood [97], while conflicting results exist on the role of NKT cells in mouse strains that spontaneously develop lupus-like disease. Data from the MRL/*lpr* model indicates a protective role for NKT cells while studies of (NZB/NZW)F1 mice suggest that NKT cells play a pathogenic role [96]. In addition, aged NKT cell-deficient mice develop lupus-like disease, which supports a protective role for NKT cells in SLE [98]. Several studies have also found a protective role for NKT cells in mouse models of diabetes and multiple sclerosis (MS). Although conflicting results exist, the majority of the data indicate that increased numbers of V $\alpha$ 14<sup>+</sup> T cells or activation of iNKT cells by  $\alpha$ -GalCer reduce disease in non-obese diabetic (NOD) mice as well as in the EAE model of MS. [96]. Type 2 NKT cells have also been suggested to play a protective role in both diabetes and MS. Injections of sulfatide prevent development of EAE in a CD1d-dependent manner [99] and type 2 NKT cells have been shown to be reactive with sulfatide isoforms expressed in pancreatic islet  $\beta$ -cells as well as in myelin sheets of the nervous system [100].

## **1.4 IL-18**

IL-18 is a member of the IL-1 family of cytokines which also includes IL-1 $\beta$  and the more recently discovered IL-33. IL-18 is produced by cells of the innate immune system and is one of the most potent amplifiers of innate and adaptive immune responses. This is achieved by acting on innate and adaptive immune cells as well as non-immune cells [101, 102]. The ability of IL-18 to significantly enhance inflammation is beneficial when it comes to pathogen protection, but is also potentially harmful since it exacerbates autoimmune and inflammatory diseases, as discussed below in section 1.4.3, “IL-18 and inflammatory disease”.

IL-18-expressing cells include neutrophils, macrophages, dendritic cells, keratinocytes, Kupffer cells, chondrocytes, synovial fibroblasts and osteoblasts [103]. Production of IL-18 starts with synthesis of proIL-18 which requires cleavage by caspase 1 to generate a biologically active protein [104]. ProIL-18 is constitutively expressed and the levels can be increased by TLR ligands such as LPS [105]. This is in contrast to IL-1 $\beta$ , where transcription of proIL-1 is initiated first after TLR-mediated activation of NF- $\kappa$ B [101, 106]. The rate-limiting step in production and secretion of active IL-18 is thus the activity of caspase 1 which, in turn, is activated upon stimulation of the inflammasome (Fig. 5) [107].



**Figure 5.** Schematic picture showing generation of biologically active IL-18 from proIL-18 by the inflammasome. Several inflammasome components are assembled to an active complex.

#### 1.4.1 The inflammasome

The concept of the inflammasome was introduced when Martinon *et al.* identified a caspase-activating complex in the cytosol which is connected to sensors of the NLR family and essential for the generation of active IL-1 $\beta$  [107]. This was the so-called NALP1 inflammasome, and since then several different inflammasomes have been identified and defined by the NLR protein they contain [8]. The NALP3 inflammasome has been tightly associated with autoinflammatory disorders. For example, mutations in the NALP3 gene, which causes enhanced caspase 1 activity and overproduction of IL-1 $\beta$ , have been identified in Muckle-Wells autoinflammatory disorder [108].

The inflammasome is activated upon exposure to whole pathogens (fungi, bacteria and virus), PAMPs, DAMPs (ATP, uric acid) and environmental irritants (skin irritants, UV-irradiation, alum, asbestos and silica) [8]. These agonists have been suggested to activate the inflammasome by three different mechanisms; *i*) PAMPs and DAMPs interact directly with the NLRs and gain access to the cytosol through membrane pores opened by increase in extracellular ATP, *ii*) the NLRs are activated by factors released after lysosomal rupture, which occurs upon phagocytosis of inflammasome agonists, or *iii*) the inflammasome agonists trigger production of reactive oxygen species which, in turn, activate the NLRs [8]. Activation of the inflammasome by the environmental toxins asbestos and silica has been suggested to be a crucial step in chemically induced autoimmunity. This sheds light on the important effects on the immune system by the cytokines produced upon inflammasome activation. [109].

### 1.4.2 Effects of IL-18 on cells of the immune system

The plentiful effects of IL-18 all aim to potentiate innate and adaptive immune responses. The effects on the innate immune system include migration, upregulation of MHC and costimulatory molecules and enhanced effector functions [102]. These effects are mediated through signaling from the IL-18 receptor which shares many components and characteristics with TLRs, such as the cytoplasmic Toll/IL-1 receptor (TIR) domain and the MyD88 adaptor protein [110]. The IL-18 receptor has been reported to be expressed by cells of the innate immune system (neutrophils, macrophages, DCs, basophils, mast cells and NK cells), the adaptive immune system (T cells and NKT cells) and by non-immune cells (chondrocytes, endothelial cells, epithelial cells, keratinocytes, smooth muscle cells and synovial fibroblasts) [103].

In adaptive immune responses, IL-18 was first described to potentiate Th1 polarization by enhancing the IFN- $\gamma$  production from IL-12 stimulated CD4<sup>+</sup> T cells, NK cells and NKT cells [111-114]. However, it has become increasingly clear that the default action of IL-18 seems to be to induce production of Th2-associated cytokines and that IL-12 shifts the activity of IL-18 towards an IFN- $\gamma$  response [101]. The Th2-promoting effect is especially pronounced together with IL-2 and includes production of IgG<sub>1</sub> and IgE by B cells [115, 116] and IL-4, IL-5, IL-10 and IL-13 by CD4<sup>+</sup> T cells, NKT cells, basophils and mast cells [115-119]. The dual role of IL-18 on both Th1 and Th2 type responses is exemplified by that IL-18 Tg mice have increased serum levels of both IFN- $\gamma$  and IL-4 as well as IgE and IgG<sub>1</sub> [120].

### 1.4.3 IL-18 and inflammatory disease

IL-18 has been ascribed an important role in the pathology of several autoimmune and inflammatory diseases including allergic contact dermatitis, asthma, atopic eczema (AE), inflammatory bowel disease (IBD), MS, RA, Sjögren's syndrome and SLE [102]. This is based on studies showing that:

- Eliciting factors activate the inflammasome which leads to production of IL-18 at the site of inflammation and/or in the target organ. Examples of such eliciting factors include contact sensitizers in allergic contact hypersensitivity [121], *S. aureus* in AE [122], uric acid in IBD [123] and DAMPs released from apoptotic cells in SLE [124].
- Elevated levels of IL-18 in serum or in the target organ correlate with disease activity/severity in patients with asthma [125], AE [126, 127], IBD [128-130], MS [131], RA [132, 133], Sjögren's syndrome [134] and SLE [135].
- Elevated levels of IL-18 increase the pathology while deletion/blocking of IL-18 ameliorates disease in animal models of allergic contact dermatitis [136], asthma [137, 138], AE [122, 139], IBD [140], MS [141], RA [133, 142, 143] and SLE [144].
- Polymorphisms in the IL-18 gene have been linked to asthma [145] and AE [146].



While it seems increasingly clear that IL-18 exacerbates autoimmune/inflammatory disorders, this cytokine has a dual role in atherosclerosis and cancer. IL-18 is protective from initiation of the metabolic syndrome [147] but contributes to destabilization of atherosclerotic plaques [148]. In cancer, IL-18 contributes to the anti-tumor immune response through stimulation of IFN- $\gamma$ /Th1 responses but can also favour tumor spread [149].

The connection between IL-18 and several severe diseases makes it an attractive therapeutic target. IL-18 binding protein (IL-18BP) is a natural inhibitor present in the circulation which binds IL-18 with high affinity and thereby prevents its biological activity [150]. Recombinant IL-18BP has been proven safe in a Phase 1 study with healthy controls and RA patients [151]. A fusion protein of IL-18BP and the Fc part of IgG<sub>1</sub> is also in clinical trials for arthritis and other inflammatory diseases [103].

## 1.5 ATOPIC ECZEMA

AE, also known as atopic dermatitis, is a chronic relapsing inflammatory skin disease affecting 15 – 30 % of children and 2 – 10 % of adults in industrialized countries [152]. Atopy is defined by a hereditary tendency to produce IgE antibodies against allergens, and AE is often associated with allergic rhinitis and asthma [153]. Production of IgE antibodies by allergen-specific B cells involves interaction with Th2-polarized cognate Th cells. The Th2 phenotype includes upregulation of CD40L and production of IL-4 and IL-13, which together promotes germ-line transcription of the  $\epsilon$ -heavy chain and expression of AID, leading to B cell class-switch to IgE. Most of the produced IgE is bound by its high-affinity Fc receptor, Fc $\epsilon$ RI, expressed by mast cells and basophils [154]. This is referred to as the sensitization phase, while clinical manifestations of atopy occur upon re-exposure to the allergen. Crosslinking of Fc $\epsilon$ RI-bound IgE by antigen (allergen) on mast cells results in degranulation (release of preformed mediators, including histamine and proteases) and production of eicosanoids and cytokines. This results in a strong inflammatory response manifested by bronchoconstriction, vasodilation, increased vascular permeability and increased mucus production, as well as influx of inflammatory leukocytes [155]. Together, this leads to clinical manifestations such as itching, sneezing and wheezing [155, 156].

Eczema lesions in AE are characterized by very itchy, red, dry and crusted skin, and a disturbed epidermal barrier function is a typical hallmark of AE. If the disturbed epidermal barrier is a consequence of IgE-sensitization and local inflammation or vice versa remains to be fully elucidated [152]. Nevertheless, both aeroallergens and microbes can easier penetrate an impaired epidermal barrier and thereby add to the pathology of AE [157]. The etiology of AE is not completely known but has been shown to involve both genetic predisposition and environmental factors. The initiation of AE lesions is associated with production of Th2-type cytokines such as IL-4, IL-5 and IL-13. This cytokine profile shifts to a more Th1-like pattern, characterized by IFN- $\gamma$  and IL-12, in chronic lesions [152, 157]. Examples of environmental factors that have been associated with the etiology of AE include microorganisms such as the bacteria *S. aureus* and the yeast *Malassezia* [152, 157]. Approximately 50 % of adult AE patients have positive skin prick test, atopy patch test and/or specific serum IgE against *Malassezia*, a reactivity that is rare in other allergic diseases [158]. To date,

thirteen allergens have been cloned from *Malassezia*, designated Mala f/s 1-13 [158]. Interestingly, some of these allergens show high homology to human proteins. For example, Mala s 11 shares 50 % identity with human manganese superoxide dismutase (hMnSOD) [159]. Sequence homology to self-proteins has also been shown for allergens from other species such as the birch pollen allergen Bet v 2 and the aspergillus allergens Asp f 6, Asp f 8 and Asp f 11 [160]. In AE, about 25 % of the patients have IgE antibodies against self-proteins, but it is unclear how/if such auto-reactive IgE antibodies contribute to the pathogenesis. A possible role for self-antigens in AE is suggested by the fact that IgE reactivity against hMnSOD correlate strongly with activity of AE [161]. Molecular mimicry between self-proteins and allergens, as in the case of Mala s 11 and hMnSOD, is a likely cause for the occurrence of self-reactive IgE antibodies in AE [160]. Furthermore, it has been suggested that self-reactive IgE antibodies can be induced by immune responses raised against self-antigens that are released from damaged skin cells in atopic individuals upon scratching [152].

Studies in mouse models of AE have provided many important insights into the pathogenesis of this inflammatory skin disease. AE-like skin inflammation and elevated serum levels of IgE have been shown to be induced in WT mice by epicutaneous application of sensitizers such as the model antigen OVA (in combination with mechanical injury) and recombinant mite allergens [162]. Furthermore, there are also several mouse strains that spontaneously develop AE-like skin lesions along with elevated serum IgE levels. For example, Tg mice which overexpress IL-4, IL-31, caspase-1 or IL-18, and the inbred strain Nc/Nga [162, 163]. Nc/Nga is a Th2-prone strain that has several similarities with human AE. These mice must be kept under conventional conditions for the disease to develop, indicating an important role for microbes in the disease development [164]. Increased production of IL-18 has been shown to be able to cause AE-like skin lesions and elevated serum IgE levels in mice where keratinocytes overexpress caspase-1 or active IL-18 protein [163].

## 1.6 SLE

SLE is a systemic autoimmune, relapsing disease that affects 0.04 % of individuals in northern Europe. A large majority (90 %) of these are females [165]. Autoantibodies reactive with DNA represent a hallmark of SLE, and can be found in 70 % of SLE patients [165]. The autoreactive immune response in SLE affects most organs in the body and causes tissue damage in the skin, kidneys, joints, nervous system, etc. The pathogenic role for B cells in SLE has been attributed to production of autoantibodies which form immune complexes with cellular debris; these immune complexes get stuck in the kidneys and joints and subsequently initiate inflammatory responses [165, 166]. However, B cells have also been shown to contribute to SLE independently of antibody production [167] and autoreactive T cells have been suggested to have an important role in the tissue damage of the kidney [168].

Although the etiology of SLE remains to be fully elucidated, it is known that both genetic predisposition and environmental factors contribute to an inappropriate activation of the immune system [166]. The susceptibility loci include genes involved in development and activation of lymphocytes as well as clearance of apoptotic cells [165, 166]. One example of a strong risk factor for SLE is deficiency in the early

complement component C1q, which is important for proper clearance of apoptotic cells [169]. Activation of autoreactive B cells by an increased load of apoptotic cells can be studied in mice as repeated injections of apoptotic cells has been shown to induce production of anti-DNA antibodies and transient SLE-like disease [170]. Other mouse models used to study SLE include strains that spontaneously develop SLE-like disease, such as MRL<sup>lpr</sup> and (NZB/NZW)F1 mice. The MRL<sup>lpr</sup> mice are deficient in Fas (also known as CD95 and encoded by the *lpr* locus), leading to defective apoptosis in autoreactive lymphocytes during negative selection [171]. The (NZB/NZW)F1 mice are referred to as a multigenic model where a combination of different genes contribute to the autoimmune disease, resembling the situation in SLE patients [172].

That an increased load of apoptotic cells can induce an autoimmune response might seem paradoxal given the anti-inflammatory response normally associated with apoptotic cell death. However, if apoptotic cells are not removed by phagocytosis, for example due to defective clearance mechanisms, they undergo secondary necrosis which is associated with a pro-inflammatory response [173]. However, injections of necrotic cells do not mimic the autoreactive immune response induced by an increased load of apoptotic cells [170]. Thus, the induction of an autoimmune response by defect clearance of apoptotic cells can not solely be explained by a switch from an anti-inflammatory to a pro-inflammatory response. The process of apoptosis is associated with modification of self-antigens which results in new self structures referred to as neo-epitopes [174]. Lymphocytes reactive with such neo-epitopes would not have been subjected to negative selection during central tolerance and could thus be activated and induce an autoimmune response. Antigens that are normally hidden inside the cell can be exposed on blebs formed on the surface of apoptotic cells, which could lead to that the apoptotic cells are coated with autoantibodies [175]. Complexes of apoptotic cells and autoantibodies can induce an inflammatory response by activating complement and crosslinking Fc $\gamma$  receptors on leukocytes [169].





## **2 THE PRESENT STUDY**

### **2.1 AIM**

The overall aim of the work presented in this thesis was to investigate how innate mechanisms can regulate B cell activation in allergy and autoimmunity.

The more specific aims were as follows:

Paper I – To investigate how autoreactive B cell activation induced by apoptotic cells is regulated by NKT cells.

Paper II – To investigate how the B cell response in IL-18-induced antibody production is initiated and regulated by NKT cells.

Paper III – To investigate the effect of IL-18 on human iNKT cells and how this is connected to atopic eczema.

Paper IV – To investigate the expression of BAFF, APRIL and TWEAK in the skin and in the circulation of patients with atopic eczema and seborrheic eczema, as well as in healthy controls.

## 2.2 METHODOLOGY

The methods used for this work are described in detail in the “materials and methods” sections of paper I-IV:

### *Mice (Papers I-III)*

The following mouse strains were used; C57Bl/6 and 129/SvEv wild type mice. CD45.1 congenic mice, Tg V $\alpha$ 14 mice, and CD1d<sup>-/-</sup>, J $\alpha$ 18<sup>-/-</sup>, CD19<sup>-/-</sup> and IFN- $\gamma$ R<sup>-/-</sup> knock-out mice on C57Bl/6 background, as well as CD19<sup>-/-</sup> knock-out mice on 129/SvEv background.

### *Patients (Papers III- IV)*

Blood samples (serum/plasma and peripheral blood mononuclear cells; PBMCs) and skin biopsies were collected from patients with AE and SE as well as from healthy controls. All patients and controls gave their informed consent.

### *Adoptive cell transfer (Paper I)*

NKT cells and B cells were purified with antibody coated MACS-beads and injected i.v. in recipient mice.

### *$\beta$ -hexosaminidase assay (Paper II)*

Degranulation of mast cells following crosslinking with anti-IgE was analyzed by measuring the granular enzyme N-acetyl- $\beta$ -D-hexosaminidase.

### *Bone marrow transfer (Paper II)*

Recipient mice irradiated with 900 rad were injected i.v. with bone marrow cells originating from femurs of donor mice.

### *Enzyme linked immunosorbent assay (ELISA) (Papers I-IV)*

Antibodies and cytokines in cell culture supernatants as well as serum/plasma from mice and humans were measured with ELISA.

### *Ex vivo splenocyte cultures (Papers I-II)*

In paper I, splenocytes were isolated from mice injected with apoptotic cells and stimulated with *i)*  $\alpha$ -GalCer and analyzed for survival and cytokine release or *ii)* PMA and ionomycin and analyzed for intracellular cytokines by flow cytometry. In paper II, splenocytes from mice injected with IL-18 were cultured without additional stimuli and the supernatants were analyzed for antibodies.

### *Flow cytometry (Paper I-III)*

Cells from mouse spleens, inguinal lymph nodes and Peyer's patches were strained through a nylon mesh and human PBMCs were purified from blood by centrifugation on a Ficoll gradient. Cells in single cell suspension were stained with fluorochrome-conjugated antibodies and analyzed on a FACSaria or FACSCalibur using FACSDiva, CellQuestPro or FlowJo software.

#### *Histology (Papers I-II, IV)*

Sections of tissue samples from spleen and kidney (mouse) or skin (human) were stained with either hematoxylin and eosin (H&E) followed by light microscopy or with fluorochrome-conjugated antibodies and analyzed by confocal microscopy.

#### *In vitro cell culture (Papers II-III)*

In Paper II, bone marrow-derived mast cells were obtained by culturing bone marrow from mouse femurs in media conditioned with IL-3. In Paper III, human iNKT cells were expanded from PBMCs using  $\alpha$ -GalCer and IL-2 followed by MACS purification. Monocyte-derived dendritic cells (MDDCs) were obtained by culturing CD14<sup>+</sup> monocytes in media conditioned with IL-4 and GM-CSF. The iNKT cells were then co-cultured with MDDCs or CD1d-transfected 293T cells.

#### *In vivo activation of B cells in mice (Papers I-II)*

- Injections of apoptotic cells were used in Paper I to induce an autoreactive anti-DNA response. Syngenic apoptotic cells were injected weekly i.v. for 4 weeks and the autoantibodies were measured in serum with ELISA.
- Injection of TNP-Ficoll was used in Paper I to study a TI-II response. Mice were injected with TNP-ficoll i.v. and TNP-specific antibodies in serum were analyzed with ELISA.
- Injections of NP-OVA in Alum (Al) adjuvant were used in Paper I to study a TD response. Mice were injected with NP-OVA/Al i.p. and the NP-specific antibody response in serum was analyzed with ELISA.
- Injections of IL-18 were used in Paper II to induce an early antibody response. Mice received daily i.p. injections of IL-18 for 10 days and the IgM, IgG and IgE antibody responses in serum were analyzed with ELISA.

#### *Mixed bone marrow chimeras (Paper I)*

Recipient mice irradiated with 900 rad were injected i.v. with a mixture of femoral bone marrow cells from WT (CD45.1 congenic) and CD1d<sup>-/-</sup> mice (CD45.2 congenic).

#### *Statistical analysis (Papers I-IV)*

The data was analyzed using non-parametric tests; Mann Whitney U-test (two unpaired samples), Wilcoxon's matched pairs test (two paired samples), Kruskal-Wallis test with Dunn's post-hoc test (multiple samples) and Spearman's rank correlation (correlation). P<0.05 was considered significant.

#### *Western blot (Paper III)*

Proteins in cell lysates were separated by gel electrophoresis, transferred to protein binding membranes and detected using HRP-conjugated antibodies and ECL-reagents.

#### *Quantitative real time PCR (Papers II, IV)*

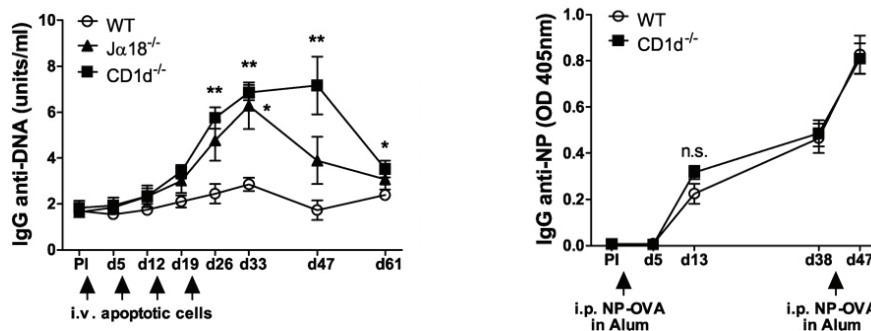
Total RNA was extracted by the TRIzol method and synthesized to cDNA by reverse transcription. RNA transcripts were amplified using specific primer pairs.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Invariant NKT cells limit activation of autoreactive CD1d-positive B cells (Paper I)

Efficient removal of apoptotic material and an adequate NKT cell population are two features that have been suggested to restrict activation of autoreactive B cells. This can be exemplified in SLE patients, in which both defective clearance of apoptotic cells and decreased numbers of iNKT cells have been reported [97, 176]. The process of apoptosis is associated with modifications of cell membrane lipids, and presentation of lipid antigens by CD1d is known to activate NKT cells to produce immunoregulatory cytokines [73, 174]. We therefore set out to investigate whether NKT cells regulate autoreactive B cell activation in response to increased levels of apoptotic cells.

Four weekly i.v. injections of apoptotic cells result in production of anti-DNA antibodies and transient SLE-like disease in WT mice [170]. We investigated this autoreactive B cell response in two different NKT cell-deficient mouse strains; CD1d<sup>-/-</sup> mice which lack all NKT cells as well as J $\alpha$ 18<sup>-/-</sup> mice which lack only iNKT cells. Absence of NKT cells resulted in increased levels of IgG anti-DNA antibodies (Fig. 6) as well as in elevated levels of antibodies against the lipid autoantigens cardiolipin and phosphorylcholine. However, there was no difference in the antibody response to exogenous TD (Fig. 6) or TI-II antigens. This indicates that absence of iNKT cells increases the autoreactive B cell response to apoptotic cells, but has no effect on general B cell activation.

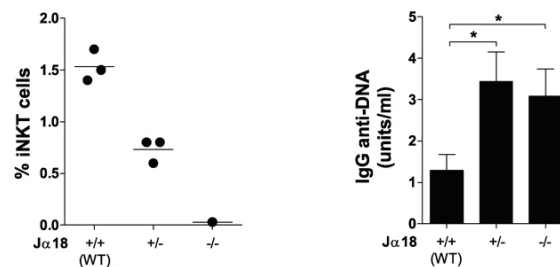


**Figure 6.** Antibody response in NKT cell-deficient mice injected with self-antigen or exogenous antigen. Left panel: IgG anti-DNA response following four injections with apoptotic cells in NKT cell-deficient (CD1d<sup>-/-</sup> and J $\alpha$ 18<sup>-/-</sup>) mice and WT mice. Right panel: IgG anti-NP response following two injections of the TD-antigen NP-OVA in Alum in NKT cell-deficient (CD1d<sup>-/-</sup>) and WT mice. Results shown as mean  $\pm$  s.e.m. n = 6-8. \*P<0.05, \*\*P<0.01, n.s. = not significant.

It has been reported that iNKT cells can provide help for B cell activation and that this requires the expression of CD1d on B cells [84]. This led us to investigate if this also applies to the regulatory effect of iNKT cells on autoreactive B cell activation to apoptotic cells. The activation of WT and CD1d<sup>-/-</sup> B cells in iNKT cell-sufficient mice was compared using adoptive B cell transfer models as well as mixed bone marrow chimeras. These experiments revealed that absence of CD1d-expression on B cells resulted in an increased anti-DNA response and GC entry in mice injected with apoptotic cells. Furthermore, we found that absence of iNKT cells resulted in an

increased amount of B cells with a GC phenotype in mice injected with apoptotic cells. In addition, we found that activated B cells downregulate CD1d as they enter GCs in WT mice. Taken together, this suggests that iNKT cells limit autoreactive B cell activation via a CD1d-dependent mechanism that takes place before GC entry.

Examination of iNKT cell-deficient mice has been widely used to investigate the role of this innate-like T cell subset in various models of autoimmune disease. However, patients with SLE do not have total absence of iNKT cells, but rather a reduced iNKT cell population. We therefore studied the autoreactive B cell activation to apoptotic cells in mice heterozygous for the  $J\alpha 18$  allele ( $J\alpha 18^{+/-}$ ), which have a 50 % reduced iNKT cell population (Fig. 7). The IgG anti-DNA response and GC B cell population were similarly increased in mice heterozygous ( $J\alpha 18^{+/-}$ ) and homozygous ( $J\alpha 18^{-/-}$ ) for the  $J\alpha 18$  deletion (Fig. 7). This supports that a reduction in iNKT cells is sufficient to affect B cell activation, and potentially disease, in lupus patients.



**Figure 7.** Reduced levels of iNKT cells are sufficient to see increased autoreactive B cell activation to injected apoptotic cells. Left panel: Splenic iNKT cell populations in  $J\alpha 18^{+/+}$  (WT),  $J\alpha 18^{+/-}$  and  $J\alpha 18^{-/-}$  mice. Right panel: IgG anti-DNA response in  $J\alpha 18^{+/+}$  (WT),  $J\alpha 18^{+/-}$  and  $J\alpha 18^{-/-}$  mice following four injections with apoptotic cells. Results shown as mean and individual mice  $n = 3$  (left panel) or mean  $\pm$  s.e.m.  $n = 7$  (right panel). \* $P < 0.05$ .

To establish whether iNKT cells are a potential therapeutic target in SLE, we transferred iNKT cells to  $J\alpha 18^{-/-}$  mice and analyzed the autoimmune response to repeated injections of apoptotic cells. Repopulation of the iNKT cell population significantly decreased all studied autoreactive B cell activation parameters, including the anti-DNA response, the size of the GC population and the amount of GCs.

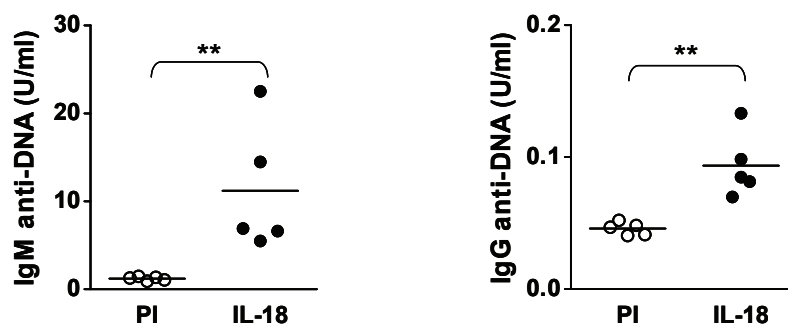
iNKT cells have been shown to both enhance and reduce B cell activation depending on the model system studied. Our data support the view that iNKT cells limit activation of autoreactive B cells [96]. Interactions between iNKT cells and B cells that present lipid antigens in CD1d is well established in models for iNKT cell-mediated B cell help [83]. We now extend this concept to iNKT cell-mediated restriction of autoreactive B cell activation. However, whether the tolerogenic effect of iNKT cells is induced by presentation of lipids derived from apoptotic cells, or by a combination of endogenous antigens and factors (e.g. cytokines) induced by apoptotic cell death remains to be elucidated.

*In conclusion*, we have identified iNKT cells as mediators of a novel peripheral tolerance checkpoint of B cell activation in autoimmune disease, and as a potential therapeutic target in patients with B cell-driven autoimmunity.

### 2.3.2 IL-18 induces natural antibody responses regulated by NKT cells (Paper II)

An important event in inflammatory responses is the activation of the inflammasome, which leads to production of biologically active IL-18 [8]. Repeated injections of IL-18 in mice induce an early Th2-type antibody response, and elevated levels of this cytokine have been reported in several diseases where antibodies play a detrimental role [115, 116, 126, 177]. Activation of CD4<sup>+</sup> Th cells that upregulate CD40L and produce IL-4 and IL-13 have been shown to be important for IL-18-induced antibody production [115, 116], but the nature and function of the B cell response have not been studied. We set out to investigate the antibody response induced by IL-18 with focus on antibody reactivity, which B cell subset(s) that are activated, where the activation takes place and how these events are regulated.

IL-18 was injected i.p. daily for 10 days to induce an early antibody response. We found increased levels of total-IgM and IgG after 10-12 days in IL-18-injected mice, which is in line with the early IgE response reported previously [115, 116]. Since no exogenous antigen is introduced in this model system, we hypothesized that the increased levels of IgM and IgG would be derived from the natural repertoire and/or be self-reactive. In line with this, antibodies reactive with phosphorylcholine, 4-hydroxy-3-nitrophenyl (NP) and DNA were elevated in IL-18-injected mice (Fig. 8), indicating a polyclonal natural antibody response.



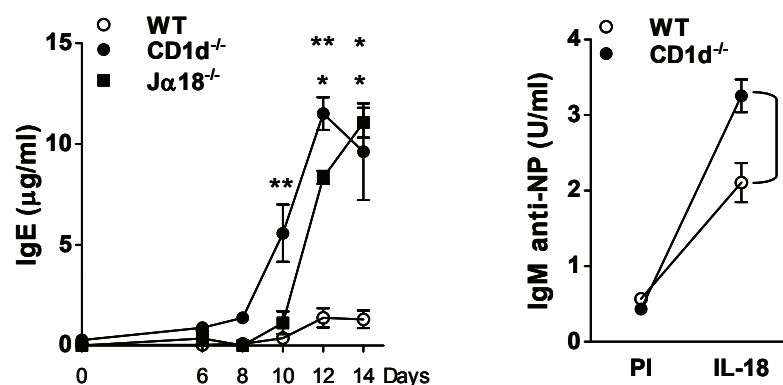
**Figure 8.** IL-18 induces production of self-reactive antibodies. Serum levels of IgM and IgG antibodies reactive with DNA in mice injected with IL-18 i.p. daily for 10 days. Results shown as mean and individual mice  $n = 5$ . \*\* $P < 0.01$ . PI; pre immune

The spleen is a common site for B cell activation, and we found production of IgM, IgG and IgE antibodies in *ex vivo* splenocyte cultures from IL-18-injected mice. This suggests that IL-18 has a direct impact on activation of splenic B cells, and analysis of the splenic B cell subsets revealed that expansion of the MZB subset preceded the increase in serum antibody levels. In addition, we found that the IL-18-induced antibody response was delayed in MZB-deficient (CD19<sup>-/-</sup>) mice, indicating a role for MZB in the early stage of IL-18-induced antibody production. Unexpectedly, we observed that a B cell population with MZB phenotype developed in the spleen of IL-18-injected CD19<sup>-/-</sup> mice. This could be mediated by the cytokine BAFF which we found to be increased in serum of IL-18-injected mice and is known to drive expansion of MZBs [59].



Activated B cells can either differentiate into antibody-producing cells in follicles where they form GCs or in foci in extrafollicular sites [41]. Analysis of the spleen of IL-18-injected mice by histology, flow cytometry and real time PCR revealed that B cell activation takes place in extrafollicular foci and immature GCs. Taken together, these findings indicate that injections of IL-18 expand the MZB compartment and lead to isotype-switch and production of self-reactive natural antibodies in the spleen. In paper I, we found that NKT cells regulate GC entry in autoreactive B cell responses, and alterations in the NKT cell compartment as well as increased levels of IL-18 have been reported in several autoimmune diseases [96, 177]. This prompted us to test whether NKT cells regulate the autoreactive B cell activation induced by IL-18.

The IL-18-induced antibody response was investigated in the two NKT cell-deficient mouse strains  $CD1d^{-/-}$  (deficient in all NKT cells) and  $J\alpha18^{-/-}$  (deficient in iNKT cells). In the absence of NKT cells, the isotype-switched antibody response and production of natural antibodies were significantly increased (Fig. 9). In addition, the IL-18-induced IgE response was decreased by injection of the iNKT cell-activating ligand  $\alpha$ -GalCer. This demonstrates that NKT cells negatively regulate activation of B cells in IL-18-induced antibody responses. We found that the increased antibody response in NKT cell-deficient mice was accompanied by a shift to a more mature GC reaction, indicating that NKT cells balance self-reactive natural antibody responses by regulating GC formation.



**Figure 9.** Absence of NKT cells results in increased levels of total-IgE and IgM anti-NP in IL-18-injected mice. Serum levels of total-IgE (left panel) and IgM anti-NP (right panel) in NKT cell-deficient ( $CD1d^{-/-}$  and  $J\alpha18^{-/-}$ ) mice and WT (C57Bl/6) mice injected with IL-18 i.p. daily for 10 days. Results shown as mean  $\pm$  s.e.m.  $n = 4-5$ . \* $P < 0.05$ , \*\* $P < 0.01$ . PI; pre immune.

In this paper, we describe that elevated levels of a cytokine produced upon activation of the inflammasome induce production of self-reactive natural antibodies. In this way, inflammasome activation could stimulate rapid production of polyreactive antibodies which are important in the defense against invading pathogens [29]. However, this is also a plausible mechanism by which inflammasome activation could contribute to the production of pathogenic self-reactive antibodies in autoimmune disease. This could be the case in autoimmunity induced by environmental toxins, where the pathology has been suggested to be mediated by inflammasome activation [109].

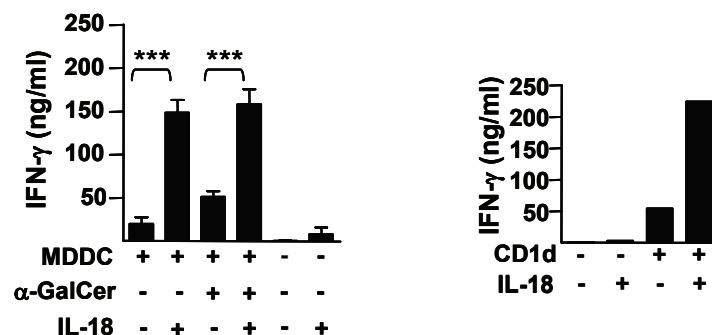
*In conclusion*, we have identified that elevated levels of IL-18 induce production of natural antibodies by autoreactive B cells, and that NKT cells regulate this response by preventing the formation of mature GCs.

### 2.3.3 IL-18 skews the invariant NKT cell population via autoreactive activation in atopic eczema (Paper III)

AE patients have been reported to have both elevated levels of IL-18 and a reduced proportion of iNKT cells in peripheral blood [126, 178, 179]. Furthermore, IL-18 has been shown to stimulate ligand-mediated activation of mouse iNKT cells *in vitro* [119]. This led us to look into if the levels of IL-18 and iNKT cells were connected in AE patients. We investigated the interplay between IL-18 and human iNKT cells *in vitro*, as well as how these factors correlated with AE disease measures.

IL-18 can be present in serum as a free cytokine or bound to the natural inhibitor IL-18BP [101]. We found significantly elevated levels of IL-18, but unchanged levels of IL-18BP, in peripheral blood of AE patients compared to healthy controls. This suggests a potential for free IL-18 in plasma of AE patients to contribute to AE pathogenesis, and we found that the plasma levels of IL-18 in AE patients were sufficient to activate human iNKT cells *in vitro*.

To further investigate the effect of IL-18 on human iNKT cells, iNKT cell lines were generated from peripheral blood from healthy individuals by expansion of the iNKT cell population with IL-2 and  $\alpha$ -GalCer. An *in vitro* co-culture system with iNKT cells and CD1d<sup>+</sup> antigen-presenting cells was used as a model for iNKT cell activation. IL-18 was found to greatly enhance the cytokine responses by iNKT cells in these co-cultures in a CD1d-dependent manner (Fig. 10). This was particularly pronounced for IFN- $\gamma$  where IL-18 enhanced the response to similar levels in co-cultures both with and without addition of the exogenous ligand  $\alpha$ -GalCer (Fig. 10).

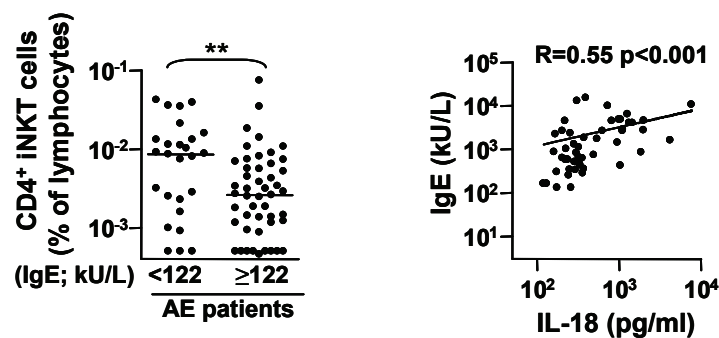


**Figure 10.** IL-18 enhances IFN- $\gamma$  production by iNKT cells in a CD1d-dependent manner, but independent of exogenous ligands. IFN- $\gamma$  production by iNKT cells co-cultured with MDDCs  $\pm$   $\alpha$ -GalCer (left panel) or with CD1d-transfected 293T cells without antigen addition (right panel). Results shown as mean  $\pm$  s.e.m.  $n = 5-7$  (left panel) or one representative experiment out of three (right panel). \*\*\* $P < 0.001$ .

Autoreactive activation to endogenous ligands presented by CD1d has been suggested to be important for the development and function of iNKT cells [78]. It is intriguing that this autoreactive activation can be enhanced by IL-18 to induce a three-fold higher response compared to the potent ligand  $\alpha$ -GalCer. These strong effects of IL-18 on iNKT cell activation led us to investigate the more long-term effect of IL-18 on iNKT cells. *In vivo* experiments in IFN $\gamma$ R<sup>-/-</sup> mice revealed that IL-18 decreases the iNKT cell population in an IFN- $\gamma$  dependent manner. In addition, long-term culture of human iNKT cells in the presence of IL-18 showed that the growth disadvantage was selective for CD4<sup>+</sup> iNKT cells. Prolonged stimulation by IL-18 thus skewed the iNKT cell

population towards a predominant CD4<sup>+</sup> profile *in vitro*. Human CD4<sup>+</sup> iNKT cells have been suggested to be more pro-inflammatory compared to the CD4<sup>+</sup> counterpart since CD4<sup>+</sup> iNKT cells mainly produce IFN- $\gamma$  while CD4<sup>+</sup> iNKT cells produce both IFN- $\gamma$  and IL-4 [81, 82]. This suggests that autoreactive activation of iNKT cells by IL-18 skews the iNKT cell population towards pro-inflammatory effector functions.

Taken together, these findings suggest a scenario where chronically elevated levels of IL-18 alone could suffice to induce chronic stimulation and subsequently change the iNKT cell population. To investigate this possibility, we analyzed the percentage of iNKT cells in peripheral blood of the AE patient cohort. We did not find a difference in the total iNKT cell population in AE patients with elevated total-IgE levels ( $\geq 122$  kU/L) compared to healthy controls. However, the CD4<sup>+</sup> iNKT cell subset was found to be significantly decreased. Comparison of the CD4<sup>+</sup> iNKT cell pool between AE patients with elevated IgE levels and AE patients with IgE within the reference range ( $< 122$  kU/L) showed that a small CD4<sup>+</sup> iNKT cell subset was indicative of elevated plasma IgE levels (Fig. 11). Furthermore, we also found that the plasma levels of IL-18 were connected to the plasma levels of IgE, as these two parameters correlated in AE patients with elevated total-IgE levels (Fig. 11).



**Figure 11.** A small CD4<sup>+</sup> iNKT cell population and elevated levels of IL-18 in peripheral blood are connected to elevated plasma levels of IgE in AE patients. Left panel: CD4<sup>+</sup> iNKT cells in AE patients with total plasma IgE levels within the reference range ( $< 122$  kU/L;  $n = 29$ ) and those with elevated IgE levels ( $\geq 122$  kU/L;  $n = 49$ ). Results shown as median and individual patients. \*\* $P < 0.01$ . Right panel: Correlation between the plasma levels of IL-18 and total-IgE in AE patients with elevated IgE levels ( $\geq 122$  kU/L).

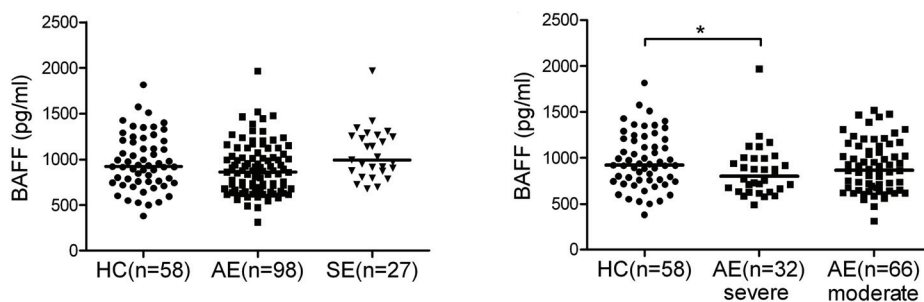
These findings suggest a novel model where IL-18 contributes to AE pathogenesis by dual effects on human iNKT cells: In the initiation of lesions, production of IL-18 is induced by microbes which activate the inflammasome, for example in keratinocytes. The elevated levels of IL-18 stimulate iNKT cells to produce pro-inflammatory cytokines to both self and foreign ligands presented by CD1d. Later, in the chronic stage, the continuous stimulation via IL-18 skews the iNKT cell repertoire with selective suppression of the tolerogenic CD4<sup>+</sup> iNKT cells. An iNKT cell repertoire skewed towards pro-inflammatory effector functions could contribute to the inflammatory response in the chronic stage of AE.

*In conclusion*, IL-18 skews the human iNKT cell population via autoreactive activation towards pro-inflammatory effector functions, and a skewed iNKT cell pool is associated with elevated plasma levels of IgE in AE patients.

### 2.3.4 The expression of BAFF, APRIL and TWEAK is altered in eczema skin but not in the circulation of atopic and seborrheic eczema patients (Paper IV)

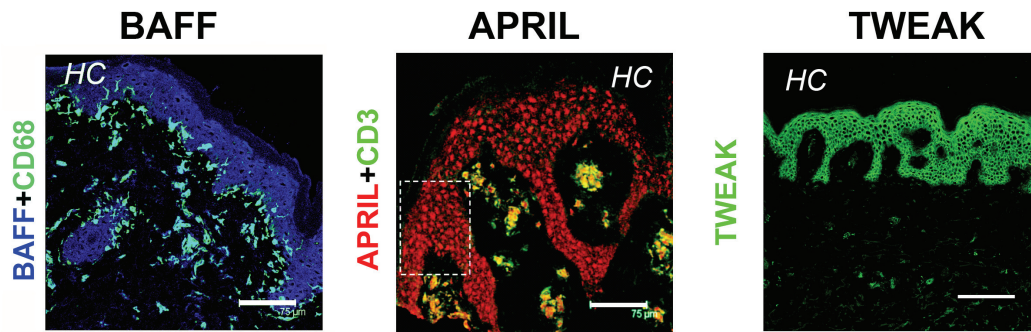
Treatment of AE patients with the B cell-depleting antibody anti-CD20 has been shown to improve eczema lesions [180], which suggests that B cells play a role in the pathology of skin inflammation. The TNF-family cytokines BAFF and APRIL are important regulators of B cell activation and survival [57]. We therefore investigated the expression of these cytokines in peripheral blood and skin of healthy controls and patients with AE and seborrheic eczema (SE). SE is a chronic inflammatory skin disease with eczema lesions that is not connected to atopy (i.e. production of IgE) [181].

We found that the levels of BAFF, APRIL and the closely related TNF-family member TWEAK (TNF-like weak inducer of apoptosis) were not elevated in plasma/serum of AE or SE patients compared to healthy controls (Fig. 12). This is different from most other inflammatory diseases where increased levels of BAFF and/or APRIL in serum and target tissues have been reported to correlate with disease progression [61]. Instead, the levels of BAFF were found to be significantly decreased in subgroups of AE patients with severe AE (Fig. 12) or elevated total-IgE levels. However, the serum/plasma levels of these cytokines were not found to correlate with AE disease measures such as the SCORAD (scoring atopic dermatitis) index, total-IgE levels or *Malassezia*-specific IgE levels. This suggests that elevated levels of BAFF, APRIL and TWEAK in peripheral blood is not a part of the pathogenesis of AE and SE in adult patients.



**Figure 12.** The plasma levels of BAFF are decreased in AE patients with severe disease. Left panel: Plasma levels of BAFF in healthy controls (HC) and patients with AE and SE. Right panel: Plasma levels of BAFF in healthy controls and AE patients with severe or moderate AE. Results shown as median and individual patients or healthy controls. \*P<0.05

We next turned our focus to the expression of BAFF, APRIL and TWEAK in the skin of healthy controls and patients with AE and SE. We found that these cytokines were expressed by keratinocytes and cells in the dermis in skin biopsies from healthy controls (Fig. 13). In the dermis, both BAFF and APRIL were expressed by CD68<sup>+</sup> macrophages, while APRIL also was expressed by CD3<sup>+</sup> T cells. In addition, BAFF was expressed by T cells in lesional skin from AE and SE patients.



**Figure 13.** Expression of BAFF, APRIL and TWEAK in skin of healthy controls. Left panel: BAFF expressed by keratinocytes in the epidermis and CD68<sup>+</sup> macrophages in the dermis. Center panel: APRIL expressed by keratinocytes in the epidermis and CD3<sup>+</sup> T cells in the dermis. Right panel: TWEAK expressed by keratinocytes in the epidermis. The scale bar represents 75  $\mu$ m (left and center panels) or 150  $\mu$ m (right panel).

To further characterize the expression of BAFF, APRIL and TWEAK in the skin, we analyzed the mRNA levels of these cytokines in biopsies of lesional skin from AE and SE patients, as well as in skin biopsies from healthy controls. To get a more complete picture of the dynamic regulation of these factors in AE, we also analyzed the mRNA expression of these cytokines in positive atopy patch test (APT) reactions, which mimic the acute (early) stage of AE lesions [182]. We found the mRNA levels of BAFF to be increased in SE lesions as well as in APT reactions, suggesting that this cytokine plays a role in SE and in the early stage of AE. This is in line with a study showing that the serum levels of BAFF are elevated in children with AE [183]. In contrast to BAFF, the mRNA levels of APRIL and TWEAK were found to be decreased in AE and SE lesions. Inflamed skin has been associated with increased levels of the inflammatory cytokine IL-18 [124], and we found that the levels of BAFF and APRIL correlated with IL-18 in AE lesions and APT reactions.

Taken together, these findings demonstrate that the expression of BAFF, APRIL and TWEAK is altered in eczema skin but not in the circulation of AE and SE patients. In line with this, the expression level of these cytokines in the skin did not correlate with the levels in the circulation. BAFF and APRIL are close homologues that share many functions and receptors, and strategies targeting both these factors are currently in clinical trials for inflammatory diseases [184]. One example is TACI-Ig, a receptor fusion protein that binds to both BAFF and APRIL. Our results demonstrate that inflamed skin lesions are associated with upregulation of BAFF and downregulation of APRIL. This suggests that a more selective approach should be considered to target BAFF and/or APRIL in AE and SE, such as the use of soluble BAFF receptor or monoclonal anti-BAFF antibodies.

*In conclusion*, the eczema skin lesions in AE and SE show altered levels of BAFF, APRIL and TWEAK. These cytokines could thus be considered as potential therapeutic targets in these inflammatory skin disorders. However, elevated levels of these cytokines in the circulation are not associated with the chronic phase of AE.



## 2.4 FINAL REFLECTIONS AND FUTURE PERSPECTIVES

The work presented in this thesis includes three major findings. First, IL-18 can act as a link between inflammatory responses and activation of both autoantibody- and IgE-producing B cells. Second, NKT cells keep autoreactive and IgE immune responses in check by limiting formation of germinal centers. Third, elevated levels of IL-18 lead to a pro-inflammatory NKT cell population which could contribute to the pathogenesis of autoimmune- and IgE-mediated diseases.

The autoimmune response induced by environmental toxins, such as mercury, has been suggested to involve activation of the inflammasome [109]. The mechanism by which this would lead to activation of autoreactive B and T cells is, however, unknown. In paper II, we show that elevated levels of IL-18, which is produced upon inflammasome activation, activate autoreactive innate B cells. IL-18 could thus be the link between activation of the inflammasome and production of autoreactive antibodies in inflammatory responses. This finding suggests that agents that neutralize the effects of IL-18 could be useful in the treatment of patients with inflammatory autoimmune disease. Our findings suggest that the innate splenic MZB subset gives rise to the antibody-producing cells in IL-18-induced antibody responses. However, the fact that a MZB population develops when MZB-deficient (CD19<sup>-/-</sup>) mice are injected with IL-18 makes it hard to evaluate the contribution of MZBs to IL-18-induced antibody responses using this mouse strain. It would therefore be of great interest to study IL-18-induced antibody production in other MZB-deficient strains, such as mice deficient in Pyk-2 [185] or Lsc [186]. Enrichment of BCRs with natural reactivates is not only characteristic for MZBs. It is also an attribute of the other innate B cell subset, the B1 cells [26]. We have not been able to detect an expansion of the B1 cells in the spleen of IL-18-injected mice (preliminary observations). However, a better way to investigate the role of B1 cells in IL-18-induced antibody responses would be to analyze the B cell populations and antibody production in the peritoneum.

The studies of NKT cell-deficient mice in papers I and II revealed that NKT cells negatively regulate B cell responses that involve production of autoreactive or IgE antibodies. However, we found that absence of NKT cells did not affect responses to nonself-antigens such as OVA or TNP-ficoll. This suggests that NKT cells might have different roles in different types of B cell responses, and that their inhibitory effects may be restricted to responses involving faulty B cell activation such as in autoimmunity and IgE-mediated diseases. An antibody response is commonly evaluated by measuring antibody quantity. However, quality is also an important parameter in humoral immune responses. Studies addressing the affinity of the antibodies produced in response to self and nonself-antigens in NKT cell-deficient mice would provide valuable insights into how this innate T cell subset regulates B cell responses.

In paper I, we find that the ability of NKT cells to negatively regulate autoreactive B cell activation is depended on the interaction with CD1d on B cells. This led to the identification of a new pre-GC checkpoint that is important for the regulation of self-reactive B cells in the periphery. In this study, the autoimmune response was induced by repeated injections of apoptotic cells, which are known to contain a lot of modified lipids [174]. This led to the hypothesis that presentation of lipids from apoptotic cells

by CD1d on B cells could induce a tolerogenic NKT cell response. It would be very interesting to investigate whether apoptotic cells contain NKT cell ligands, and how NKT cells respond to such ligands. It is well known that different CD1d-ligands induce different cytokine responses by NKT cells. For example, the prototypic antigen  $\alpha$ -GalCer induces both IFN- $\gamma$  and IL-4, while the  $\alpha$ -GalCer derivatives  $\alpha$ -C-GalCer and OCH preferably induces IFN- $\gamma$  or IL-4, respectively [187]. NKT cells are able to produce a wide range of cytokines and the response induced by apoptotic cells is clearly beneficial to prevent activation of autoreactive B cells. The identification of NKT cell ligands from apoptotic cells would therefore provide a tool that could be used to reduce the pathologic activation of self-reactive B cells in autoimmune disease. Although the hypothesis that an apoptotic cell-derived ligand would mediate the tolerogenic NKT cell response to injections of apoptotic cells is quite feasible, the observed NKT cell response could be induced in other ways. An alternate mechanism could be that apoptotic cell death affects other cells to produce mediators, such as cytokines, which together with endogenous lipids presented by CD1d induce a tolerogenic NKT cell response. Studies of the NKT cell-regulated pre-GC checkpoint in other models of autoimmunity will be important to elucidate whether this is specific for increased load of apoptotic cells (and thus may involve presentation of apoptotic cell-derived ligands) or a common phenomenon in autoimmune responses.

In paper III, we identify that the combination of IL-18 and presentation of endogenous lipids by CD1d induces a potent pro-inflammatory NKT cell response. Furthermore, this paper illustrates a link between elevated levels of IL-18 and a NKT cell population that is skewed towards pro-inflammatory effector functions. The fact that elevated levels of IL-18 have been shown to correlate to disease severity in inflammatory diseases [177] indicates that this could be a general mechanism that contributes to disease pathology. It would be interesting to investigate whether elevated levels of IL-18 in serum affect the effector functions of individual NKT cells in addition to skewing the NKT cell repertoire towards the pro-inflammatory CD4<sup>+</sup> subset. This could for example be investigated by comparing the activation of NKT cells from AE patients with high and low plasma levels of IL-18. A complication factor, however, is that the AE patients with the highest plasma levels of IL-18 also had the smallest NKT cell populations. This makes it technically difficult to expand and study NKT cells from those patients *in vitro*. CD4-mediated signals have been shown to increase cytokine production by NKT cells *in vitro* [188]. Another way to continue the investigation of the consequences of elevated levels of IL-18 on NKT cell functions would thus be to compare the effects of this cytokine on indirect activation of purified CD4<sup>+</sup> vs. CD4<sup>-</sup> NKT cells from healthy donors.

Paper IV identifies that the B cell-activating cytokines BAFF and APRIL are not elevated in peripheral blood of AE patients but rather could play a role locally in eczema skin. The increased levels of BAFF in skin biopsies taken from positive APT-reactions, which mimic the early acute stage of AE lesions, suggest a role for this cytokine in initiation of AE lesions. In future studies of AE patients, it would be interesting to compare the blood levels of BAFF during exacerbations of the eczema to the levels during non-acute periods. In addition, it would be interesting to delineate if the altered expression of TNF-family cytokines that we observed in eczema skin of AE and SE patients are specific for the eczema lesions or a common feature in the skin of



AE and/or SE patients. This could be investigated by measuring the levels of BAFF, APRIL and TWEAK also in non-lesional skin from the AE and SE patients included in this study.

Studies of how a certain cell type affects B cell responses can yield different results depending on the approach. To get a more complete picture of the involvement of e.g. NKT cells in different types of B cell responses, it is important to study this from different perspectives. For example, in the absence of NKT cells, in the presence of elevated numbers of NKT cells, and in combination with NKT cell activation by different types of ligands. It would also be beneficial to combine studies in mouse models with *in vitro* experiments and patient samples. The data presented in this thesis has been generated using several of these approaches and the results shed light on the complex interactions involved in the regulation of B cells in allergic and autoimmune disease.

### 3 POPULÄRVETENSKAPLIG SAMMANFATTNING

Sjukdomar som orsakas av vårt eget immunförsvar är vanliga i dagens samhälle och drabbar 30 % av västvärldens befolkning. Var fjärde person har någon form av allergisk sjukdom där immunförsvaret reagerar på ofarliga ämnen i omgivningen från växter, djur eller födoämnen. Allergiska sjukdomar orsakar besvärande symptom som eksem, hösnuva och astma, vilket leder till kraftigt försämrad livskvalitet. En mindre grupp, ungefär 1 av 20 individer, har någon form av självreaktiv (autoimmun) sjukdom där kroppen angrips av sitt eget immunförsvar. Reumatism, typ 1-diabetes och systemisk lupus erythematosus (SLE) är exempel på sjukdomar där den självreaktiva immunreaktionen förstör funktionen hos viktiga organ i kroppen. Detta kan leda till livshotande skador, som till exempel hos patienter med SLE, där immunreaktionen bland annat förstör njurarna. Att immunförsvaret kan orsaka sjukdomar om det inte regleras på ett optimalt sätt är en besvärande bieffekt av de funktioner som gör det till ett effektivt försvar mot bakterier och andra sjukdomsalstrare (patogener). En avsaknad av immunförsvaret skulle å andra sidan medföra att även den mest harmlösa bakterie skulle kunna bli livsfarlig.

Immunförsvaret delas vanligtvis in i två delar, det medfödda och det adaptiva. Det medfödda kallas även för "innate" och som namnet antyder föds vi med de gener som bygger upp dessa celler och molekyler. Det medfödda immunförsvaret ärvt alltså från generation till generation och detta system kan snabbt och enkelt försvara oss mot en mängd olika patogener. Cellerna och molekyler i det medfödda immunförsvaret känner igen egenskaper som är vanliga hos flera olika patogener och en molekyl kan på detta sätt skydda oss mot många olika sjukdomar. Men det finns en begränsning för hur effektivt detta system är, och om man skulle ha gener för alla molekyler som behövs för ett komplett skydd mot alla patogener som finns skulle vår arvs massa mångdubblas. Som en lösning på detta problem har det adaptiva, eller anpassningsbara, immunförsvaret utvecklats. Där bildas många olika molekyler från en liten mängd gener, vilket på ett effektivt sätt ger stor variabilitet i vilka patogener kroppen kan känna igen och försvara sig mot. Priset vi får betala för detta system, som bildar många olika molekyler från få gener, är att det slumpvis även bildas molekyler som kan känna igen kroppsegna eller ofarliga substanser. Sådana molekyler gör tyvärr mer skada än nytta. Immunförsvaret har utvecklat sofistikerade system för att minska mängden skadliga molekyler, men dessa bildas alltid till en viss grad. För att minska risken för aktivering av självreaktiva delar av det adaptiva immunförsvaret kontrolleras det av det medfödda. Det kan beskrivas på följande vis: Först aktiveras det medfödda immunförsvaret av till exempel en bakterie, som det känner igen som farlig eller främmande. Det medfödda immunförsvaret skickar då signaler som aktiverar det adaptiva immunförsvaret att börja bilda molekyler som hjälper det medfödda immunförsvaret att bekämpa bakterieinfektionen.

Jag har studerat hur vissa celler och proteiner som tillhör det medfödda immunförsvaret aktiverar den adaptiva delen i autoimmuna och allergiska immunreaktioner. Närmare bestämt har jag studerat aktivering av B-lymfocyter, eller B-celler, i det adaptiva immunförsvaret. B-celler producerar proteiner som kallas för antikroppar. Dessa fäster mycket hårt och specifikt till olika patogener. Man brukar säga att de binder till

patogener, och detta är viktigt för ett effektivt immunförsvar. Emellertid kan B-celler även bilda antikroppar som binder kroppsegna strukturer och då bidra till autoimmuna sjukdomar. I allergiska sjukdomar orsakas symptomen av att det finns en speciell typ av antikropp (IgE) mot ofarliga ämnen i omgivningen. När B-celler har bildat IgE-antikroppar binder dessa till ytan av mastceller som är en del av det medfödda immunförsvaret. När mastcellerna kommer i kontakt med ämnen som IgE-antikropparna binder till, släpper de ut ämnen som orsakar det vi känner igen som symptom på allergi. Det vill säga att det kliar, luftvägarna drar ihop sig och det rinner ur ögon och näsa. För att hitta nya sätt att påverka den felaktiga aktiveringen av B-celler i autoimmuna och allergiska sjukdomar behöver vi veta mer om hur den går till. Studier av djurmodeller för och patienter med autoimmun och allergisk sjukdom har visat på förändringar i många delar av både det medfödda och det adaptiva immunförsvaret. Jag har studerat vissa av de kända förändringarna i det medfödda immunförsvaret för att försöka förstå hur de bidrar till den felaktiga aktiveringen av B-celler i dessa sjukdomar. De delar av det medfödda immunförsvaret som jag forskat om är de så kallade "natural killer T-cellerna" (NKT-cellerna) samt proteinerna "interleukin-18" (IL-18), "B-cells-aktiverande faktor" (BAFF) och "en proliferationsinducerande ligand" (APRIL). NKT-celler har egenskaper av både det medfödda och det adaptiva immunförsvaret och är mycket effektiva på att reglera immunreaktioner. IL-18, BAFF och APRIL är så kallade cytokiner, det vill säga små proteiner som fungerar som signalmolekyler mellan olika delar av immunförsvaret. Denna avhandling består av fyra studier och jag kommer nu att beskriva de upptäckter som gjordes i respektive studie samt hur de bidrar till kunskapen om felaktig reglering av B-celler i autoimmuna och allergiska sjukdomar.

I den första studien kom jag och mina forskarkollegor fram till att en interaktion mellan NKT-celler och självreaktiva B-celler är ett viktigt steg för att hindra att dessa B-celler aktiveras och bildar antikroppar som kan attackera kroppens organ. För att studera detta använde vi oss av en musmodell för autoimmunitet där aktiveringen av immunförsvaret har flera likheter med SLE-patienters. Vi studerade aktivering av självreaktiva B-celler genom att ta blodprover och mäta antikroppar samt genom att analysera aktivering av B-celler i mjälten. Genom att studera möss som saknar NKT-celler, eller saknar molekyler på B-celler som är viktiga för att interagera med NKT-celler, upptäckte vi att B-celler som interagerar med NKT-celler hindras från att bidra till autoimmuna sjukdomar.

I den andra studien upptäckte vi att cytokinen IL-18 aktiverar självreaktiva B-celler att bilda antikroppar som kan attackera kroppens organ. Detta studerade vi genom att simulera ökade nivåer av IL-18 i blodet genom att injicera det upprepade gånger i möss som vi sedan tog blodprov på. I blodproverna mätte vi sedan vilka antikroppar B-cellerna bildat. Det var känt sedan tidigare att IL-18 bildas vid inflammation och kan aktivera B-celler att producera IgE-antikroppar. Våra resultat visar att förhöjda nivåer av IL-18 kan bidra till den felaktiga aktiveringen av immunförsvaret i både autoimmuna och allergiska sjukdomar. En annan viktig upptäckt i denna studie var att NKT-celler är viktiga för att dämpa bildandet av såväl självreaktiva som IgE-antikroppar när nivåerna av IL-18 är förhöjda. Sammantaget visar denna studie hur cytokinen IL-18 kan bidra till att inflammation bidrar till felaktig B-cellsaktivering samt hur detta regleras.

I den tredje studien visade vi att patienter med hudsjukdomen atopiskt eksem har färre inflammationsdämpande NKT-celler och att cytokinen IL-18 bidrar till detta. Vi började med att studera hur IL-18 påverkar NKT-celler *in vitro* (i provrör). Det finns olika typer av NKT-celler och vi observerade att förhöjda nivåer av IL-18 bidrog till att det fanns färre NKT-celler som var av inflammationsdämpande typ. Sedan jämförde vi nivåerna av IL-18 och NKT-celler i patienter med atopiskt eksem med nivåerna hos friska kontrollpersoner. Patienter med atopiskt eksem har en ärftlig benägenhet att bilda IgE-antikroppar, så kallad atopi. Vi upptäckte ett samband mellan nivåerna av IgE-antikroppar och både färre inflammationsdämpande NKT-celler och förhöjda nivåer av IL-18 i dessa patienter. Sammantaget visar detta att aktivering av B-celler till att producera IgE-antikroppar i allergiska sjukdomar kan gynnas av att NKT-cellspopulationen är mer inflammatorisk samt att förhöjda nivåer av cytokinen IL-18 kan bidra till en sådan förändring.

I den fjärde studien kom vi fram till att eksemhud hos patienter med atopiskt eksem har förändrade nivåer av de B-cellsaktiverande cytokinerna BAFF och APRIL. Förhöjda nivåer av BAFF och APRIL har visats bidra till felaktig B-cellsaktivering i ett flertal allergiska och autoimmuna sjukdomar och flera läkemedel som motverkar dessa två cytokiner är under utveckling. Vi upptäckte att nivåerna av dessa cytokiner inte var förhöjda i blodet hos patienter med atopiskt eksem men att det däremot fanns skillnader mellan eksemhud och frisk hud. Framförallt var nivåerna av BAFF förhöjda i ett tidigt stadium av eksemutvecklingen, medan nivåerna av APRIL var lägre i eksemhud. Detta tyder på att om läkemedel som neutraliserar BAFF ska ges till patienter med atopiskt eksem bör detta ske i ett tidigt skede och behandlingen bör ges lokalt i eksemhuden.

Studierna i denna avhandling har bidragit till flera nya viktiga upptäckter om hur det medfödda immunförsvaret bidrar till den felaktiga B-cellsaktiveringen i autoimmuna och allergiska sjukdomar. Studierna beskriver mekanismer för hur inflammation kan orsaka aktivering av B-celler som producerar självreaktiva antikroppar och IgE-antikroppar, samt hur NKT-celler hämmar sådan felaktig aktivering av B-celler i autoimmuna och allergiska sjukdomar. De visar även att det bildas cytokiner i tidiga stadier av eksemutveckling som kan bidra till felaktig B-cellsaktivering. Dessa mekanismer kan vara intressanta för nya behandlingar av patienter med autoimmuna och allergiska sjukdomar. Till exempel skulle ett läkemedel som neutraliserar IL-18 kunna användas för att förhindra att inflammation leder till aktivering av det adaptiva immunförsvaret och produktion av skadliga antikroppar. Ett annat tänkbart scenario är att stimulera NKT-cellernas inbyggda förmåga att hämma aktivering av B-celler som producerar självreaktiva antikroppar eller IgE-antikroppar.

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## 5 REFERENCES

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