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# iNKT CELL REGULATION OF B CELL ACTIVATION IN INFLAMMATION

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by Eprint AB 2016

ISBN 978-91-7676-210-3

**Institutionen för Mikrobiologi, Tumör- och Cellbiologi**

# iNKT cell regulation of B cell activation in inflammation

AKADEMISK AVHANDLING som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Atrium, Nobels väg 12B, Karolinska Institutet, Solna Campus

## Fredagen den 5 februari 2016, kl. 09.00

av


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Till mamma och pappa

# ABSTRACT

The ability to combat infections through the generation of specific immune responses is critical to our survival. The immune system can react to and combat virtually any molecule presented to it, including those derived from our own body. The immune system can cause considerable damage to the structures it was designed to protect, thus causing autoimmune disorders. Examples include systemic lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis, among many others. Many autoimmune disorders are caused in part by autoreactive antibodies produced by B lymphocytes, and targeting the activation of B lymphocytes thus forms a target for ameliorating disease. Better understanding of how B lymphocytes are activated is essential not only in order to limit autoimmune disease, but also in order to harness their capabilities in the context of generating successful vaccines.

The work presented in this thesis deals with B lymphocyte activation in inflammation and in response to glycolipid antigens. The aim was to investigate how this activation is regulated and investigate the possibility of manipulating B cell activation in a desirable way. In papers I and III, mouse models were used to study the regulation of autoreactive and IgE-producing B lymphocytes using IL-18 to induce inflammation. In paper II, model glycolipid antigens were employed to investigate how different types of iNKT cell help affect the outcome for B cell activation, with the prospect of harnessing iNKT cell help as an adjuvants in vaccine therapy. In paper IV, the potential of using glycolipids in order to skew faulty B cell activation in inflammation was investigated.

In **paper I**, iNKT cells were identified as regulators of autoreactive and IgE-producing B cells. **Paper II** defines quantitative and qualitative differences between cognate and noncogante B cell help provided by iNKT cells. Cognate iNKT cell help, compared to noncognate, expands a larger number of B cells producing the cytokine Interleukin 10. In **paper III**, mechanisms of iNKT cell regulation of B cell activation were investigated. Neutrophils were found to license iNKT cells to adopt a killing phenotype required to restrict B cell activation in sterile inflammation. In **paper IV**, B cell activation in inflammation was studied using exogenous glycolipid iNKT cell antigens. Serum antibody levels and germinal center formation were found to be increased in inflammation when iNKT cells were stimulated with glycolipids.

In summary, the work presented in this thesis describes mechanisms controlling faulty B cell activation in inflammation. iNKT cells are critical in limiting B cell activation, and this was dependent on interaction with neutrophils. The interaction between neutrophils and iNKT cells plays a previously unappreciated role in the restriction of B cell activation, and thus serves as a potential target for new therapeutic strategies in autoimmune diseases. In addition, the use of glycolipids as vaccine adjuvants, or to target B cell activation is investigated.

# LIST OF SCIENTIFIC PAPERS

I. Lind Enoksson S, Grasset EK, Hägglöf T, Mattson N, Kaiser Y, Gabrielsson S, McGaha TL, Scheynius A, Karlsson MCI. **The inflammatory cytokine IL-18 induces self-reactive innate antibody responses regulated by natural killer T cells** *Proc Natl Acad Sci USA, 2011, 108, 1399-1407*

II. Vomhof-Dekrey EE, Yates JL, Hägglöf T, Lanthier PA, Amiel E, Veerapen N, Besra GS, Karlsson MCI, Leadbetter EA. **Cognate interaction with iNKT cells expands IL-10 producing B regulatory cells** *Proc Natl Acad Sci USA, 2015, 108, 1399-1407*

III. Hägglöf T, Sedimbi SK, Yates JL, Lanthier PA, Leadbetter EA, Karlsson MCI. **Neutrophils license *i*NKT cells to regulate self-reactive B cell responses** *Submitted*

IV. Sedimbi SK*, Hägglöf T*, Brenner MB, Leadbetter EA, Karlsson MCI. **Exogenous glycolipid agonists alter the inherent B cell regulatory function of iNKT cells in inflammation** *In manuscript*

**Equal contribution*

## PUBLICATIONS NOT INCLUDED IN THE THESIS

Sedimbi SK, Hägglöf T, Karlsson MCI. **IL-18 in inflammatory and autoimmune disease** *Cell Mol Life Sci, 2013, 70, 4795-4808*

Grasset EK, Duhlin A, Agardh HE, Ovchinnikova O, Hägglöf T, Forsell MN, Paulsson-Berne G, Hansson GK, Ketelhuth DF, Karlsson MCI. **Sterile inflammation in the spleen during atherosclerosis provides oxidation-specific epitopes that induce a protective B-cell response** *Proc Natl Acad Sci USA, 2015, 112, 2030-2038*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| αGalCer | Alpha-galactosylceramide |
| AFC | Antibody-forming cell |
| ASC | Apoptosis-associated speck-like protein containing a caspase recruitment domain |
| ADCC | Antibody-dependent cellular cytotoxicity |
| AFC | Antibody-forming cell |
| AID | Activation-induced cytidine deaminase |
| BAFF | B cell-activating factor of the TNF-family |
| Bcl6 | B-cell lymphoma protein 6 |
| BCR | B cell receptor |
| $B_{reg}$ | Regulatory B cell |
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CSR | Class-switch recombination |
| CXCR5 | CXC chemokine receptor 5 |
| DAMP | Damage-associated molecular pattern |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| ELA2 | Elastase 2, gene coding for neutrophil elastase |
| ELISA | Enzyme-linked immunosorbent assay |
| FDC | Follicular dendritic cell |
| FOB | Follicular B cell |
| Foxp3 | Forkhead box protein 3 |
| FRP1 | Formyl peptide receptor 1 |
| GC | Germinal center |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GSL-1 | Glycosphingolipid-1 |
| IFN | Interferon |
| IKK | Inhibitor of κB kinase |
| IL | Interleukin |
| IL-18BP | Interleukin-18 binding protein |
| iNKT cell | Invariant natural killer T cell |
| $iNKT_{FH}$ cell | Invariant natural killer T follicular helper cell |
| IRAK | Interleukin-1 receptor-associated kinase 1 |
| KLH | Keyhole limpet hemocyanine |
| KLRG1 | Killer cell lectin-like receptor subfamily G, member 1 |
| LPS | Lipopolysaccharide |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatibility complex |
| MMP9 | Matrix metalloproteinase 9 |
| MS | Multiple sclerosis |
| MyD88 | Myeloid differentiation factor 88 |
| MZB | Marginal zone B cell |
| nAb | Natural antibody |
| NET | Neutrophil extracellular trap |
| NFκB | Nuclear factor κB |
| NIK | NFκB-inducing kinase |
| NK cell | Natural killer cell |
| NLR | NOD-like receptor |

| | |
|---|---|
| NP | 4-hydroxy-3nitrophenyl acetyl |
| PALS | Periarteriolar lymphoid sheath |
| PAMP | Pathogen-associated molecular pattern |
| PC | Plasma cell |
| PD-1 | Programmed death 1 |
| PD-L1 | Programmed death ligand 1 |
| PD-L2 | Programmed death ligand 2 |
| PRR | Pattern recognition receptor |
| QCM | Quartz crystal microbalance |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| RA | Rheumatoid arthritis |
| RP | Red pulp |
| SHM | Somatic hypermutation |
| SLE | Systemic lupus erythematosus |
| T1 | Transitional type 1 |
| T2 | Transitional type 2 |
| TCR | T cell receptor |
| TD antigen | T cell-dependent antigen |
| $T_{FH}$ cell | T follicular helper cell |
| $T_{FR}$ | T follicular regulatory cell |
| Tg | Transgenic |
| TGFβ | Transforming growth factor β |
| $T_H$ | T helper cell |
| TLR | Toll-like receptor |
| TRAF6 | TNF receptor-associated factor 6 |
| $T_{REG}$ | T regulatory cell |

# 1 INTRODUCTION

Cellular organisms face constant challenges from other forms of life invading, in order to support their existence. Sometimes this is beneficial to the host, while other times it causes disease. Our immune system has evolved for millions of years in order to cope with both types of interactions. Through evolution, a highly intricate network of cells and organs has developed, appearing about 500 million years ago, to combat a diverse set of threats, extrinsic as well as intrinsic.

Our immune system can be roughly divided in two main branches: The innate and the adaptive immune system. The former has evolved over time to make up a pre-formed, rapid defense system directed against a range of different pathogen- or danger-associated molecular patterns, including viral and bacterial particles, as well as deleterious components originating from our own bodies, such as dying cells. The second, adaptive, branch of the immune system evolves during the course of days to weeks. It resembles an incredibly accelerated form of evolution taking place within our own bodies, entailing molecular rearrangement of DNA to generate cellular receptors able to recognize foreign matter which have never before been encountered. The receptors are able to combat threats from the ever-changing challenges of pathogens, and long-lived cells are generated, enabling the immune system to remember the pathogen and combat it rapidly upon the next encounter, thus generating immunity. However, this impressive capacity of the adaptive immune system, to bind virtually any molecular structure, comes at a cost - autoimmunity. As powerful as it is, and with the dangers associated with it, the adaptive immune system requires an abundance of regulatory mechanisms in order to keep it in check, so as to limit the destruction to pathogenic, rather than physiologic constituents of our bodies.

The innate and adaptive immune systems are heavily interconnected, and they have co-evolved to be able to combat infections together at different stages. The early stages are covered by the innate branch, while the adaptive immune system needs more time to evolve to become more efficient in fighting infection. However, it is clear that the innate system isn't just a sentinel standing guard, but has a massive impact on the ensuing adaptive response.

The work presented in this thesis is focused on cells of the immune system between the innate and adaptive branches. It investigates mechanisms of how these cells function to shape the outcome of inflammation in connection to diseases such as allergy and autoimmunity, as well as novel vaccine strategies.

## 1.1 THE IMMUNE SYSTEM

The cells and organs comprising the immune system have evolved in order to protect multicellular organisms from invading pathogens, e.g. viruses, bacteria and parasites. It is made up by a wide range of cells, which all play very different roles in the refined set of reactions that make up an immune response. Given that a pathogen manages to trespass the barriers to infection made up by the skin and mucosa, sentinel cells present at these sites recognize the intrusion and act accordingly. These cells include dendritic cells (DCs), neutrophils and macrophages, which are able to identify foreign molecular entities, or antigens, and initiate a cascade of events to counteract the infection. They produce and excrete signaling proteins that activate and recruit (cytokines and chemokines, respectively) cells downstream in the immune response, including neutrophils and monocytes. The cells recruited to sites of infection begin to combat the infection locally, while DCs migrate from the site of infection to draining lymph nodes, where they initiate the adaptive immune response. The goal is for the DC to select and activate the right lymphocytes, which are the main characters of the adaptive immune system. T and B lymphocytes reside in lymph nodes and spleen, and express receptors capable of targeting virtually any antigen. However, it takes time to select the right lymphocyte for the job, and once the right lymphocyte is found some additional time is required for cellular expansion and improvement of its ability to target foreign antigen. Following a successful reaction to an invading pathogen, lymphocytes able to recognize and neutralize the pathogen are generated which survive as long-lived cells conferring immunity to the host, which is the goal for vaccination programs.

## 1.2 INFLAMMATION

The term inflammation is essential to the concept of immunology. In response to tissue trauma, e.g. if the skin breaks and there is a local infection, the tissue will respond by initiating an inflammatory response. We have all experienced this at some point in our lives, and the signs are probably recognized by all of us: Rubor, Dolor, Calor, and Tumor. These are the terms by which the inflammatory process was described about two thousand years ago by Celsus, and correspond to redness of the skin, pain, fever, and swelling, respectively.

These hallmarks of inflammation can all be put into a functional context in order to explain and understand the process of inflammation. For example, when the skin breaks, cells proximal to the afflicted area will respond as to increase the blood flow to the area, resulting in heat and redness of the skin. It might seem counterproductive, but actually results in more cells of the immune system accessing the afflicted area. Cells of the vessel walls (endothelium) will also change so as to increase the permeability of vessels, enabling cells of the immune system to transmigrate across the vessel wall into the site of tissue damage, resulting in swelling of the tissue and again, more immune cells reaching the afflicted area[1]. Thus, inflammation serves as the kick-starter of the immune system in response to virtually any noxious stimulus, even non-infectious ones. However, the different constituents of the inflammatory tool box are very powerful, and can cause severely detrimental effects if not regulated tightly. There are numerous examples of diseases caused by inflammation, not only classical autoimmune diseases, where the body's own organs are damaged, but also vascular disease[2] and cancer[3] are influenced by inflammatory processes. Inflammation is directed by a wide variety of molecules, soluble and present in cell membranes. These molecules include chemokines and cytokines. Interleukin-18 is a cytokine essential in immune cell activation and is one of the main players in inflammation and in this thesis.

## 1.3 INNATE IMMUNITY

The division of our immune system into two main branches (innate and adaptive) is based on the mode of generation of the different receptors expressed by immune cells. Cells belonging to the adaptive immune system require extensive re-arrangement of their surface receptors in order to be able to perform their tasks. The receptors utilized by cells of the innate immune system are germline-encoded, meaning that we are born with this set of receptors which mediate protection, and hence, they are called "innate". The cells and receptors that make up the innate immune system constitute our first line of defense against invading pathogens in the case a breach of the physical barriers formed by our mucosa and skin, and include neutrophils, DCs, macrophages, monocytes, neutrophils and Natural Killer (NK) cells.

In case there is a breach of skin or mucosal barriers, innate immune cells have been equipped with tools able to identify the presence of foreign entities, enabling immediate recognition of infectious antigens. Throughout evolution, the innate immune system has selected recurring molecular patterns present in an abundance of microorganisms, which are the targets of receptors expressed by innate leukocytes. These recurring structures are referred to as pathogen-associated molecular patterns, or PAMPs.

The receptors used by cells of the innate immune system to recognize PAMPs are pattern-recognition receptors (PRRs), which are present both at the cell membrane and at intracellular compartments[4]. PRRs include toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors, among others. TLRs recognize a wide variety of PAMPs in the extracellular space as well as in the endosomal compartment. Examples of TLRs include TLR4, which binds lipopolysaccharide (LPS) present in in gram-negative bacteria [5], and TLR9, which binds unmethylated DNA[4]. While TLRs mainly recognize extracellular PAMPs, the NLRs are cytoplasmic receptors linked to assembly of a multi-protein complex known as the inflammasome, followed by downstream cytokine production[6].

As innate immune cells express germline-encoded receptors constitutively, they are directly activated following the encounter of PAMPs. They immediately act to combat the source of PAMPs – bacteria or viral particles, for instance. One way in which innate immune cells combat infection is to engulf the pathogen by means of phagocytosis. Other mechanisms employed by innate immune cells to fight pathogens will be discussed in later sections. Even though innate immune cells express PRRs able to recognize a wide variety of PAMPs, pathogens evolve rapidly and have developed ways to evade the innate immune system.

## 1.4 ADAPTIVE IMMUNITY

The innate immune system combats invading pathogens, but also activates the adaptive immune system. In essence, adaptive immunity is made up of the cells that are generated through a selection process effectively involving evolution in a micro-format – a quite extraordinary process in biology. The key cells in adaptive immunity are T and B lymphocytes.

In the adaptive phase of an immune response, B and T lymphocytes are activated to undergo molecular programs enhancing their ability to fight infection. Unlike cells of the innate immune system, B and T lymphocytes express unique antigen-binding receptors (BCR and TCR, respectively), recognizing a distinct antigen (Figure 1). The receptor repertoire of lymphocytes is extensive, and it has been estimated that the T lymphocyte population can theoretically form up to $10^{15}$ different TCRs[7]. However, this is not actually achieved in a practical setting[8]. B and T cell receptors are generated by somatic rearrangement of the DNA of each lymphocyte through a process known as V(D)J recombination. During V(D)J

recombination, DNA strands open up and gene segments (labeled V, D, and J) are assembled to generate cell surface receptors capable of binding foreign and self-derived molecules. These functions are performed by RAG enzymes, and their expression is restricted to developing lymphocytes[9]. There is a limited number of V-, D-, and J-segments available for assembly, but the enzyme Terminal eoxynucleotidyl transferase adds random nucleotides in between segments assembled, in order to generate an incredible number of different receptor combinations. As the B and T cell receptor assembly process involves a certain amount of randomness, receptors recognizing molecular components derived from the host itself are generated, and for this reason, lymphocyte receptor selection is tightly regulated[10]. These include self-reactive proteins in membrane- and soluble form.

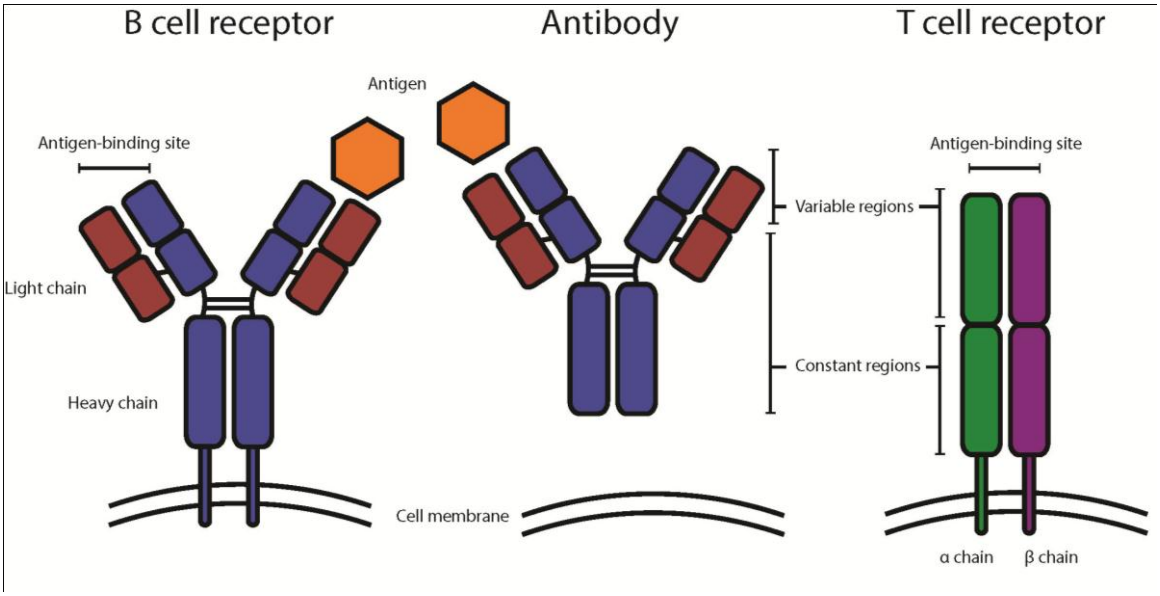



***Figure 1.*** *The structure of T and B lymphocyte receptors. BCRs and TCRs are similar in that they both are formed by two protein chains. However, only the BCR is found in a secreted form and is then referred to as an antibody. If an antibody was the size of an adult human, the cell membrane of a B cell would take up an area corresponding to three times the size of Central Park, NYC.*

The structures of completed B cell and T cell receptors are similar in that they both consist of two protein chains; heavy and light chain for B cells, and α/β chains for T cells. The receptors are generated while the cells are developing in the thymus (T lymphocytes) and in the bone marrow (B lymphocytes). Receptors are selected which can bind antigens presented at the site of development with a binding capacity, or affinity, not too high and not too low. This is in order to select BCRs and TCRs with the potential to bind antigens encountered in the future, while not self-reactive[11]. While the TCR serves as a solely a receptor conferring specificity to the T cell, the BCR serves the same function while also being able to be secreted once a B cell is activated. This soluble secreted form of the BCR is known as an antibody (or immunoglobulin, Ig) and is an essential product of the adaptive immune response which can confer immunity. The constant region of secreted antibodies can be of different subcategories, known as isotypes. Different isotypes serve different purposes, e.g. IgA is present at high proportions in tear ducts and intestine, while IgG is present in the blood. Thus, the same specificity derived from the variable region can perform different functions through changing the immunoglobulin isotype. Antibodies serve several functions, including binding to molecular structures and recruiting immune cells, conferring immunity to infection, or causing pathology in autoimmunity.

## 1.5 ANATOMY OF THE IMMUNE SYSTEM

As mentioned above, the immune system is comprised by a large variety of cells located all over the body, since we require protection from pathogens everywhere as all of our tissues represent potential sites of infection. The immunological organs are generally divided into primary and secondary organs, and the initial hematopoiesis takes place in primary lymphoid organs. Included in the category of primary lymphoid organs are the thymus and the bone marrow (Figure 2). Some developmental steps required for proper leukocyte generation do not take place in primary lymphoid organs, but require leukocytes to migrate to secondary lymphoid organs. These organs include the spleen, lymph nodes, mucosa-associated lymphoid tissue, tonsils, and several other sites present in many tissues.

### 1.5.1 The bone marrow

The bone marrow is a non-solid organ mainly found in the epiphysis part of the long bones (e.g. the femur). It is the production site of an impressive number of immune cells, including $5x10^{12}$ red blood cells per day. Importantly, the bone marrow is the site of granulocyte and lymphocyte formation.

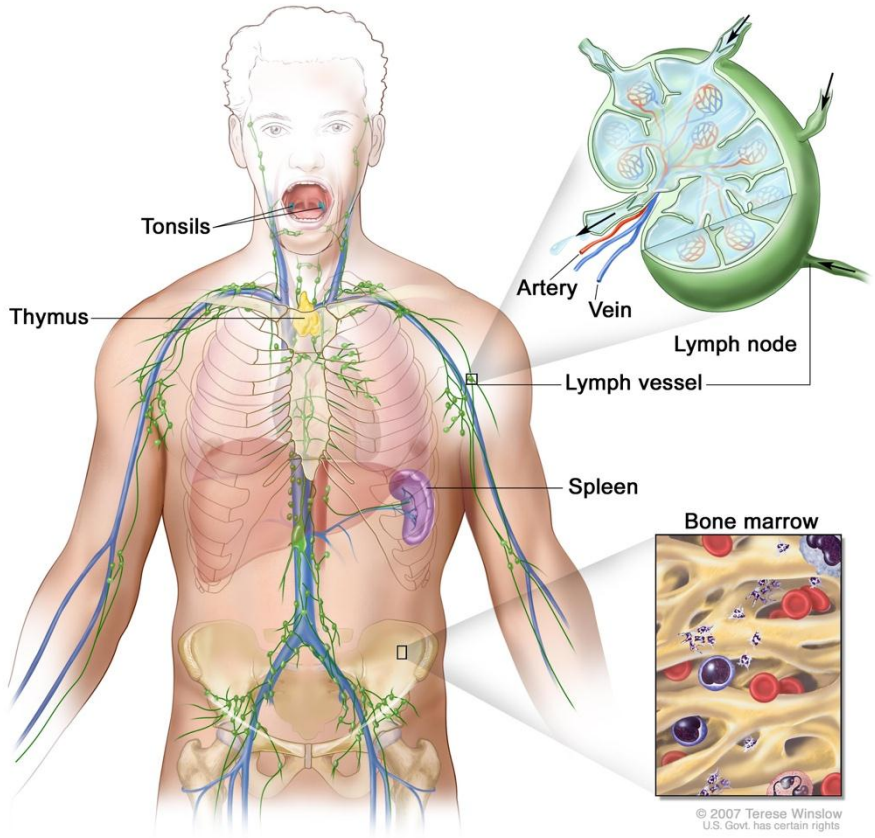



***Figure 2.*** *Anatomy of the immune system.*
*For the National Cancer Institute © 2007 Terese Winslow, U.S. Govt. has certain rights*

The bone marrow is the site for several early steps of B lymphocyte development including generation of the antigen-binding B cell receptor (BCR) [12]. For most developing B cells, the final maturation steps and adopting a mature phenotype take place in the spleen [13, 14]. The maturation stages of B cell development can be delineated according to differential expression of a set of cell surface proteins[15]. The B cell maturation sequence in the bone marrow is crucial to form a normal B cell repertoire as well as to limit the formation of self-reactive B cells[10]. The program includes heavy-chain gene rearrangement in pro-B cells

followed by selection and proliferation of pre-B cells which express appropriate BCRs. This is followed by light-chain rearrangement and further differentiation into immature T1 and T2 B cells [15, 16].

### 1.5.2 The thymus

The thymus is the site of maturation for T lymphocytes. It is located in the mediastinum, under the sternum and in front of the heart. The thymus is an organ which in time atrophies, and after the neonatal pre-adolescent phase of life, it is replaced by fat, with some residual T cell development lingering [17]. Lymphocytes precursors destined to develop into T lymphocytes are initially generated in the bone marrow, followed by migration to the thymus where they undergo a multistep selection program, in order to generate a desirable repertoire of T lymphocytes[11]. The generation of T cells in the thymus is critical in order to combat infection[18] as well as to reject foreign tissue and grafts, as famously shown with the athymic "Vacanti" mouse, growing a prosthetic ear on its back [19].

T cell development taking place in the thymus is essential for the formation of T cells, and without a thymus, T cells generally will not form. In the thymus, there is a unique expression pattern of a range of host-derived molecules, enabling negative selection of T cells which are too self-reactive[11]. This function is exerted by stromal cells known as thymic epithelial cells. T cells go through a double-negative stage, followed by a CD8+ single-positive and CD4+ CD8+ double-positive stage. T lymphocytes then differentiate into either CD8+ T cells, crucial in combating intracellular infections[20], or CD4+ T cells, which are the orchestrators of the immune system[21]. The thymus is also the site for iNKT development, which constitute an innate-like subset of T cells. iNKT cells, unlike other T cell subsets, do not recognize peptide antigens, but rather glycolipid molecules. In addition, they are not selected by cortical epithelial cells in the thymus, but rather by other double-positive thymocytes [22, 23].

### 1.5.3 The spleen

The spleen is the largest secondary lymphoid organ in humans, weighing about 150-200 grams and is located in the upper left quadrant of the abdomen, directly beneath the diaphragm. It has a wide range of functions, including storage of red blood cells, neutrophils and platelets, hemoglobin metabolism and antibody production[24]. Splenectomized individuals have an increased predisposition to certain bacterial infections, such as *Streptocuccus pneumoniae*[25], indicating the importance of this organ in combating blood borne pathogens[26].

Histologically, the spleen is divided into two distinct compartments – the red pulp and the white pulp, which are functionally and morphologically separate (Figure 3). The spleen is a highly vascularized organ and blood enters through the splenic artery and divides into smaller trabecular arteries which feed white pulp capillary beds. Some capillary beds end up in the marginal sinus, between the white pulp and the marginal zone (MZ), while others drain in the marginal zone itself. A few capillary beds extend beyond the white pulp and terminate in the red pulp of the spleen[27]. Unlike lymph nodes, there are no afferent lymphatic vessels leading to the spleen. Thus, this organ monitors the blood and performs janitorial tasks required in this tissue. The spleen contains about one-fourth of the body's lymphocytes, including several specialized cell types, which monitor and respond to sepsis, for instance.

The red pulp is the main site for removal of senescing erythrocytes and iron recycling. To uphold this function, there are numerous macrophages present in the red pulp, which take up old erythrocytes through phagocytosis. The red pulp also serves as a lodging site for

plasmablasts and plasma cells[24]. Following B cell activation by antigen, B cells transit via the red pulp and remain there temporarily while they produce and secrete antibodies allowing rapid entry into the bloodstream.

The white pulp is subdivided into follicles, periarteriolar lymphoid sheaths (PALS), and the marginal zone. These subcompartments contain B cells, T cells, macrophages, DCs, as well as other cell types. In the PALS, also known as T-cell zones, T cells constantly monitor passing cells, including DCs and B cells, in order to coordinate immune responses[28]. In the follicles, or B-cell zones, clonal expansion of B cells takes place following activation by antigen, leading to somatic hypermutation (SHM) and isotype switching[29].

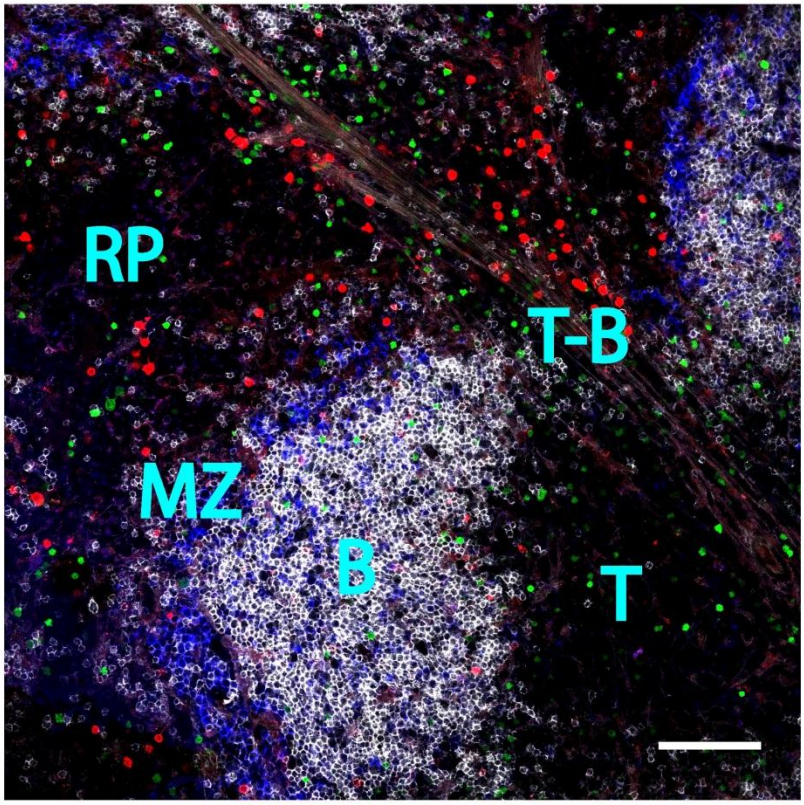



***Figure 3**. Histology of the spleen.*

*Spleen section from a CXCR6-GFP reporter mouse, stained for CD1d (blue - MZB), Ly6G (red - neutrophils), and B220 (white – FOB). CXCR6 is reported in green (iNKT).*

*RP – Red pulp*
*B – Follicle (B cell area)*
*T – PALS (T cell are)*
*MZ – Marginal zone*
*T-B – T zone-B-zone border*

The marginal zone is situated at the interface of the red pulp with the PALS and follicles. As the marginal zone contains specialized cells restricted to this area, the marginal zone is generally regarded as a separate compartment, rather than part of the white pulp. In the marginal zone there are at least two subsets of specific macrophages - marginal-zone macrophages and marginal-zone metallophilic macrophages[28], performing functions such as scavenging and iron recycling. Marginal zone B cells are also uniquely found in the marginal zone of the spleen. They shuttle between the marginal zone and follicles in order to transport and deposit antigen to the T and B cell areas of the white pulp[30, 31]. MZB cells are sentinels capable of rapidly responding to blood-borne antigens[32]. They express very high levels of the antigen-presenting molecule CD1d, compared to other leukocytes [33].

## 1.6 B CELLS

The concept of separate B cell and T cell lineages was first described by Max B. Cooper in the 1960's, although essential products of their activation - antibodies - have conceptually been around since the late 1800s[34]. B cells are mostly known for making antibodies, and this is indeed one of the major functions of this cells type. However, B cells serve other capabilities as well, including production of cytokines[35] and antigen presentation[36]. Every B cell expresses a B cell receptor unique to that particular B cell, which is generated during the development of the B cell. Cytokines produced by B cells help direct immune

responses and are important in the development of lymphoid organs[37]. Thus, B cells perform a wide variety of functions in inflammation and immune responses.

### 1.6.1 B cell development and subsets

During B cell development, the majority of precursors will not survive to maturity. It has been estimated that as much as 95% B cell precursors do not make it to the mature naïve stage of B cell development[10]. In the bone marrow, cells that are destined to become B cells initiate immunoglobulin heavy-chain rearrangements, ultimately generating a BCR which serves as the blueprint for secreted antibodies later on, following activation of the B cell[12]. In B cell development, there are two major checkpoints for negative selection of B cells. One central checkpoint in the bone marrow between early immature and immature B cells, and one peripheral checkpoint between the transition from new emigrant and mature naïve B cells[10]. B cells which generated strongly self-reactive BCRs are negatively selected and undergo clonal deletion and cell death[38].

Successful BCR rearrangement in the bone marrow generates a B cell which matures further, through transitional 1 and 2 B cell stages (T1 and T2, respectively). The T1 developmental stage for a B cell is characterized by the inability to recirculate and these B cells are found in the spleen and bone marrow[15]. In the following T2 stage, B cells are able to recirculate and are localized in follicles, while still expressing markers for immaturity. Most T2 cells mature into follicular B cells in the spleen, while about one-quarter of T2 cells acquire this phenotype in the bone marrow[39]. T2 cells migrating from bone marrow to spleen in order to mature further can also mature into Marginal Zone B cells (MZBs), which make up a distinct subset of B cells.

The mature pool of B cells is generally divided into three different subsets: Follicular B cells (FOB), Marginal zone B cells (MZB), and B1 cells[40]. These cover different functional aspects in antibody-mediated protection against noxious stimuli [12, 32, 41]. The FOBs make up the majority of mature naïve B cells, and are the ones that generally come to mind when discussing B cells. They are recirculating and considered the main B cells executing the classical thymus-dependent antibody-mediated immune response. In contrast to FOBs, MZBs are rather sessile in rodents, and found in the marginal zone of the spleen. MZBs are more innate-like, and are rapidly activated to produce antibodies, compared to FOBs[32]. Finally, B1 cells are located in the pleural and peritoneal cavities, and are also considered innate-like B cells, as they generally are easily activated and produce antibodies which tend not to be a product of SHM but rather, germline-encoded[41, 42].

Even in the absence of immunization, there are antibodies formed, called natural antibodies (nAb). nAbs are capable of binding antigens present in pathogens, for instance, bacterial polysaccharides. nAbs often cross-react with host-derived antigens, including DNA and phosphorylcholine, as well antigens not present in the host, e.g. 4-hydroxy-3nitrophenyl (NP). B1 and MZBs are believed to be responsible for the production of nAbs[43].

### 1.6.2 B cell activation

In T cell-dependent antibody responses, a mature B cell encounters its cognate antigen and is activated through BCR engagement. There are different ideas of exactly how this occurs, but current models propose that BCR engagement leads to receptor clustering and downstream signaling of intracellular molecules [44-46]. Depending on the context, and what stage the B cell is at during its lifespan, B cell activation will have different outcomes[47]. Germinal center (GC) and extrafollicular foci formation are the two major pathways taken by antibody producing B cells. One of the primary determinants for choosing between the GC and

extrafollicular foci is the strength of the interaction between the B cell receptor and its antigen[48]. B cells can also be activated to produce another class of proteins – cytokines[49].

#### *1.6.2.1 Germinal centers*

Germinal centers (GC) are structures found in the spleen and lymph nodes formed by activated B cells which undergo selection events and are stimulated to produce high-affinity antibodies[29]. The GC is a hallmark structure of immune system activation, as they participate in the generation of immunity against pathogens. However, the very process of generating high-affinity antibodies involves dangerous downsides: malignant transformation of B cells[50], and important to this thesis – the generation of self-reactive antibodies[51].

The germinal center can be clearly identified in histological preparations of lymphoid tissue. Structurally, the GC is comprised of a dark zone and a light zone. These zones demarcate areas with different functions in B cell activation, necessary to generate high-affinity antibodies[29]. Following antigen engagement by B cells and stimulatory signals delivered by CD4+ T cells at the T-B border in secondary lymphoid tissues (Figure 3), B cells accumulate in primary follicles to form germinal centers[29]. In the germinal center, B cells are selected and undergo immunoglobulin gene rearrangement programs, known as somatic hypermutation (SHM) and class-switch recombination (CSR). These gene-modulatory processes are required in order to generate high-affinity antibodies[52, 53] of different isotype classes[54], respectively. When a B cell is activated to form a GC, it induces the enzyme activation-induced cytidine deaminase (AID), which introduces random mutations in the antigen-binding site of the BCR. Sometimes this generates a BCR with higher binding-capacity, or affinity, for the antigen that induced B cell activation, while other times the affinity is lowered by a mutation. In the former case, the B cell will be selected and receive survival signals from follicular dendritic cells and T follicular helper cells ($T_{FH}$), while the latter case will result in induction of apoptosis in the B cell. The insertion of random mutations into the antigen-binding site of the BCR comes with the potential of generating self-reactive immunoglobulin[55]. GC B cells upregulate Fas, a protein inducing apoptosis when it is engaged by FasL[56]. GC B cells with high affinity for the antigen inducing the GC will receive supporting signals from $T_{FH}$ cells and upregulate anti-apoptotic proteins. Fas-FasL regulation is critical to the restriction of GC B cell formation[57], and a lack of FasL results in increased GC formation and production of autoreactive antibodies[58-60].

#### *1.6.2.2 Extrafollicular foci*

The majority of the early protective antibodies in an immune response derive from the extrafollicular foci response[61]. In the spleen, extrafollicular foci formation takes place in a different microanatomical compartment compared to the germinal center response. Extracellular foci are formed by antibody-forming cells (AFC) in the red pulp area, at the bridging channels in proximity of PALS and B cell areas.

Following B cell activation by antigen, the B cell migrates towards the T cell zone of the spleen where they present antigen to CD4+ T cells from which they receive signals to produce Ig heavy chain transcripts [62]. Next, they enter the cell cycle, upregulate the essential transcription factor Blimp-1[63], proliferate, followed by exit to the red pulp area[61]. Here, the activated B cell, now called plasmablast, undergoes class switch recombination and starts secreting antibody. B cells undergo somatic hypermutation outside germinal centers [64], and therefore, these structures also present potential sources of self-reactive antibodies.

### *1.6.2.3 Cytokine production*

A more recently described feature of B cell activation is the production of cytokines capable of directing immune system activation[65]. B cells have been identified as producers of a wide range of cytokines. These affect several crucial aspects of the immune system, including lymphoid tissue development and organization[66], dendritic cell cytokine production[67, 68], polarization of CD4+ T cells, and negative regulation of immune responses. The concept of B cells negatively regulating immune activation is not new, and the idea of B cells acting in a suppressive manner was suggested already in the 1970s[69, 70]. However, the mechanisms of B cell suppression were described fairly recently, with the first papers identifying B cell provision of immune regulatory IL-10 early this millennium[71-73].

Unlike the T cell branch of lymphocytes, no clear consensus regarding the phenotype of regulatory B cells has been established. For B cells, all of the following subsets have been identified as capable of producing regulatory cytokines; T2-MZP cells[74], $CD5^{+}$ $CD1d^{hi}$ B (B10) cells[75], MZB cells[76], CD138+ plasma cells[77], and others are being investigated. To this day, despite considerable efforts, no study has been able to identify a lineage-specific marker for regulatory B cells. As it seems, any B cell can potentially adopt a B regulatory ($B_{reg}$) phenotype, depending on local stimuli present in the microenvironment[65]. Thus, it seems like the primary factor affecting whether B cell will adopt a regulatory phenotype or not, is the environment in which it finds itself, prompting further investigation of the different types of stimuli capable of inducing this regulatory phenotype. TLR stimuli and CD40-mediated activation are the signals that have been characterized most extensively[35], while recent studies have shown that cytokines are also capable of inducing $B_{reg}$ cells producing IL-10[78].

## 1.7 T CELLS

T lymphocytes act as conductors of essentially all immune reactions, but also function as effector cells, directly combating pathogens as well as dangers arising from our own bodies – e.g. cancer[20]. The importance of T cells can be grasped when considering that the global health issue of AIDS arises due to a virus capable of eliminating a subpopulation of these cells[18]. T lymphocytes undergo a strict selection process through which they acquire a unique antigen-binding T cell receptor (TCR). This receptor is able to recognize antigen, self- and foreign, in the context of a family of proteins known as the major histocompatibility complex (MHC)[79]. Molecules presented to T cells in the context of MHC derive from all cellular compartments, so as to give the T lymphocytes an idea of what is going on inside other cells of the body[80]. There are two groups of MHC molecules – MHC class I (MHC I) and MHC class II (MHC II). MHC I molecules can be expressed by virtually any cell, and it is through the interaction between MHC and TCR that T cells are able to recognize infected or malignantly transformed cells and discriminate them from healthy, non-infected cells. Several different categories of T cells have been identified, including conventional $T_H1$, $T_H2$ and $T_H17$ cells, regulatory T cells (including Foxp3+ $T_{REG}$), PD-1+ CXCR5+ $T_{FH}$ cells, the innate-like iNKT cells, and others.

### 1.7.1 Regulatory T cells ($T_{REG}$)

T cells not only act as to amplify immune activation. A subset of regulatory T cells ($T_{REG}$) inhibit the actions of other immune cells. Their importance of $T_{REGS}$ is illustrated by the fact that mice lacking them succumb to autoimmune disease within months [81], and people deficient in $T_{REG}$ cells are afflicted with severely debilitating diseases [82]. These $T_{REG}$ cells can be induced in peripheral organs as well as lineage-committed at an early stage of T cell development[83]. The transcription factor Foxp3 serves as a marker for $T_{REG}$ cells, and is required for regulatory functions[84]. A subset of $T_{REG}$ cells localize to B cell follicles in order to exert their regulatory functions and express CXCR5 and PD-1[85]. $T_{REG}$ cells function by producing cytokines such as IL-10 and TGFβ which dampen immune responses[86].

### 1.7.2 T follicular helper cells ($T_{FH}$)

$T_{FH}$ cells are required for the development and function of germinal centers[87]. In the germinal center, B cells bind antigen which they present to $T_{FH}$ cells which, in turn, provide help to the B cell. These helping signals provided by $T_{FH}$ cells are critical to the survival and proliferation of GC B cells. The GC B cells with high antigen affinity receives supporting signals from $T_{FH}$ cells, followed proliferation in the germinal center and somatic hypermutation in order to mutate the BCR, to further increase antigen affinity[88, 89]. $T_{FH}$ cells can be defined by means of several different markers, including CXCR5, BCL6, PD-1, CD4, all of which are important to the proper function and migration of $T_{FH}$ cells[90]. The help signals provided by $T_{FH}$ cells include soluble molecules and receptors expressed on the cell surface. Major help molecules identified so far include IL-21, IL-4 and CD40L. These molecules provide survival and proliferation signals to B cells, and also stimulate class switch recombination[90].

### 1.7.3 iNKT cells

Invariant Natural Killer T (iNKT) cells form a subgroup of T cells which operate on slightly different premises than conventional T lymphocytes. They express a TCR formed by a very limited set of V(D)J regions, which in mice are comprised of the $V_{\alpha}14J_{\alpha}18$ chain, combined with a $V_{\beta}7$, $V_{\beta}8.2$ or $V_{\beta}2$ chain[91, 92]. This restricts the set of antigens which can be recognized by the iNKT cells. While conventional T lymphocytes recognize peptide antigens in the context of highly polymorphic MHC molecules, iNKT cells are selected to recognize glycolipids in the context of the non-polymorphic MHC I-like molecule CD1d[92]. In humans, there are several different CD1 molecules presenting antigen to CD1-restricted T cells[93]. In the developmental stages of iNKT cells, they are not selected by thymic epithelial cells expressing MHC molecules, but rather by other thymocytes which express CD1d[22]. They are also dependent on other cytokines than conventional T cells for their maturation and maintenance[91]. iNKT cells can be identified with tetrameric CD1d protein loaded with glycolipid ligands like α-Galactosylceramide (αGalCer)[94] In the spleen, the expression of CXCR6 identifies mostly iNKT cells[95].

### 1.7.4 iNKT cell activation

iNKT cells are able to act rapidly and potently when stimulated[96]. iNKT cells resemble memory T cells in that they are rapidly activated and express receptors which normally distinguish antigen-experienced T cells[91]. Their threshold of activation is also lower than for conventional T cells, making iNKT cell activation slightly more versatile [97]. Conventional T cells undergo phenotypic and functional changes when they become memory T cells, which partly form the basis of immunity. iNKT cells have been assumed not to form memory cells. However, iNKT cells expressing killer lectin-like receptor subfamily G, 1-positive (KLRG1) were shown to possess memory qualities, conferring long-lasting antitumor capability [98].

While clonal expansion and cytokine expression by conventional T cells requires several signals, including TCR engagement and co-receptor stimulation, iNKT cells can be activated solely through their TCR, cytokine receptors, or a combination of the two[97]. The way in which the iNKT cell responds will depend on the combination of signals present, and site of these stimuli [96, 99]. In the context of an infection, the lipid presented to iNKT cells by CD1d can be derived from the invading pathogen[100], but iNKT cells are also inherently autoreactive[42]. There are several glycolipids identified able to activate iNKT cells, and depending on their structure they generate slightly different immune responses. Examples include αGalCer[101], GSL-1[102] and OCH[103], which all induce different cytokine outputs when activating iNKT cells.

The most dramatic effect seen following iNKT cell activation with glycolipids is a massive production of cytokines. The type and amount of cytokine produced depends on the activating ligand, but the main ones are IL-4[104] and IFNγ[102]. However, iNKT cells are able to produce a much wider range of cytokines (e.g. IL-2, IL-3, IL-6, IL-10, IL-21, GM-CSF [105]), indicating that this feature of iNKT cell activation is highly context-dependent. The context-dependent activation of iNKT cells has been further corroborated in vivo[99], and it has been suggested that different lineages of iNKT cells are responsible for the production of different cytokines at steady state[106, 107].

The role played by iNKT cells in B cell responses to foreign antigens recently took dramatic turn. Two studies using different approaches identified a B cell-helping role of iNKT cells [108, 109]. iNKT cells adopted a follicular helper phenotype, including expression of CXCR5 and PD-1[109]. Importantly, iNKT cells were essential in providing IL-21[108], a cytokine

critical to support antibody production and isotype switching by B cells[110]. In mice injected with αGalCer, a proportion of iNKT cells adopt a phenotype resembling $T_{FH}$ cells. There are two ways in which an iNKT cell proved help to B cells; cognate and noncognate. Cognate iNKT cells help takes place when the B cell directly provides antigen in the context of CD1d, and receives activating signals from the iNKT cell. For noncognate help, another cell presents antigen to the iNKT cell, which gets activated and activates the B cell without necessarily interacting directly[97].

## 1.8 NEUTROPHILS

We have all seen neutrophils at some point in our lives - dead or dying neutrophils form the major cell constituent of pus. Neutrophils are typically the first leukocyte responders in response to infections, and their role in the initial phase of an immune response has been recognized for a very long time. Neutrophils have a very high turnover rate, with a bone marrow output of about $10^{11}$ cells per day[111]. In mice, neutrophils constitute 10-25% of circulating leukocytes while in humans, the proportion is considerably higher at 50-70%[112]. Neutrophils have a distinct phenotype, with a multi-lobular nucleus[113] and high content in granules containing pro-inflammatory proteins[114] serving a wide range of functions[115]. Several of the proteins have been identified as proteases, capable of degrading tissue components in order to allow for neutrophil movement to inflammatory sites, while others are capable of directly killing bacteria[114, 116].

It is well-established that neutrophil localization to inflammatory sites is required following infection in order to efficiently clear infection. The classical view of neutrophils regard their sole mission as engulfing pathogens followed by neutrophil death, and this is indeed a part of their function[117]. However, recent advances within the field have shown that neutrophils have in their arsenal a much more sophisticated set of tools than previously expected[118].

### 1.8.1 Neutrophil development

Under homeostatic conditions, neutrophils develop exclusively in the bone marrow and, unlike T and B lymphocytes, they are normally not released from the bone marrow before maturity [119]. Being part of the myeloid lineage of cells, neutrophils develop from a common myeloid progenitor stem cell in the bone marrow followed by a series of developmental steps on the way to becoming a mature neutrophil[120]. CXCR4 is the main chemokine receptor for the chemokine CXCL12, and these molecules are expressed by neutrophils and bone marrow stromal cells (e.g. osteoblasts, endothelial cells), respectively[121]. This axis is important in the life of a neutrophil, as the expression level of CXCR4 by neutrophils increase during their lifespan, thus homing ageing neutrophils back to the bone marrow for clearance[121, 122].

Compared to T lymphocytes, neutrophils have classically been regarded as a far more homogenous population of cells, where distinctions analogous to $T_H1$ and $T_H2$ are not applicable. Recently however, there has been a renaissance within the field of neutrophil biology, describing several different subtypes of neutrophils [123]. Evidence has accumulated supporting the notion that different neutrophil subsets or even lineages have distinct functions in infection[124], inflammation and cancer immunology[125]. However, it remains to be demonstrated if the different neutrophil populations demonstrated so far represent different lineages, or whether they are rather a product shaped by the course of inflammation.

### 1.8.2 Neutrophil activation

When inflammation is triggered, molecules are released from the inflicted tissue in order to recruit leukocytes, including neutrophils, to help fight the triggering event. Neutrophils are activated by a wide range of stimuli since they express all TLRs described, except for TLR3 [126]. They also express a G protein-coupled receptor called formyl peptide receptor 1, important in sensing sterile inflammatory components[115, 127]. Once activated, dramatic changes take place, concerning many different aspects of neutrophil biology. One crucial parameter is their lifespan. The average lifespan for neutrophils in mice has been estimated to hours[128], but increase several-fold i inflammation, ensuring that activated neutrophils are present at the inflammatory site. Activation by bacterial products, as well as various cytokines and growth factors can all have this effect[129]. Another important function of neutrophils is cytokine production and release, and they can produce a roster of cytokines bested by few other leukocytes[130]. The cytokines they produce include IL-1β, IL-18, BAFF, IL-4, IFNγ, and many more[115]. Cytokine contribution by neutrophils can be controlled at the transcriptional level (mRNA transcription), translational level (e.g. through targeting of microRNA), and at the level of secretion of protein [131].

Neutrophils can also release intracellular components to form structures called neutrophil extraceullular traps (NETs)[132]. These structures have since their discovery in the early 2000s been the focus of intense research to further our understanding regarding their function[133]. NETs are formed following neutrophil expulsion of nuclear components, such as DNA and histones, which are covered by proteins derived from neutrophil granules, such as neutrophil elastase (ELA2)[132, 134] and matrix metalloproteinase 9 (MMP9)[132]. Several different pathogens have been shown to be trapped by NETs, including *S. aureus*, *S. enterica*, *C. albicans*, and others [132, 134, 135]. Trapping of microbes in NETs promote the action of proteins derived from neutrophil granules which act to lyse the trapped bacteria, which are then disposed of [115]. Although NETs seem beneficial in settings of infection, they contain several self-derived molecules, including antigens forming the main targets of autoreactive antibodies which underlie pathology in diseases such as SLE and Sjögren's syndrome, including DNA and histones[136]. Thus, NETs can potentially contribute to the development and propagation of autoimmune disease [137, 138].

Another aspect of neutrophil functionality is their capacity to present antigen to other cells of the immune system. The role of neutrophils as antigen presenting cells has been largely overlooked in the past. However, neutrophils express MHC II, like professional antigen presenting cells[139]. In bone marrow chimeric mice it was demonstrated that neutrophils can be activated in a CD1d-dependent way, to produce IL-10[140]. However, little is known about the expression of this group of antigen-presenting molecules on neutrophils.

Early neutrophil activation can be evaluated by monitoring the expression of cell surface proteins. These proteins include molecules critical to the function of neutrophils like CD11b, CD18 and CD62L. CD11b and CD18 are integrins which mediate firm adhesion to endothelium and are required for neutrophils to enter tissues[141]. CD62L, or L-selectin, plays a role in tethering and rolling of neutrophils before crossing vascular endothelium to sites of inflammation[142]. Following activation by chemokines, neutrophils rapidly shed CD62L [143].

## 1.9 IL-18 AND THE INFLAMMASOME

Cytokines are critical in the orchestration of immune responses, both with respect to initiating an inflammatory response and triggering leukocyte activation, as well as inducing tolerogenic, immune-dampening responses[144]. Within the larger family of cytokines, subgroups of related proteins have been established. In this thesis, one of the main characters – Interleukin-18 – belongs to the IL-1-family of cytokines[145]. IL-18 is constitutively expressed in several different cell types (e.g. DCs, macrophages) and requires post-translational processing before becoming biologically active [146]. This is accomplished by a multiprotein complex known as the inflammasome[6]. Known inducers of inflammation such as asbestos, aluminum complexes, toxins, bacteria, viruses can all induce inflammasome-assembly [147-150], and thus, the cleaving of IL-18 into its biologically active form. Inflammasomes can be assembled and transferred through the extracellular space to another cell and exert its function in the recipient cell [151, 152].

There are a number of different inflammasomes, differing in the type of stimuli inducing them, and their downstream products[6]. Nod-like receptor 3 (NLRP3) is an extensively studied inflammasome activated by viruses[148], bacteria[147], fungi[153], toxins[150], and dying cells[154]. NLRP3 oligomerizes when it senses intracellular danger signals (Figure 4). Subsequently, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is recruited and incorporated as another domain of the assembling inflammasome[155]. The ASC domains recruit pro-caspase-1, which is cleaved and activated to caspase-1, generating a mature inflammasome[156]. Caspase-1 is a cysteine protease that cleaves pro-IL-18 into biologically active IL-18[146].

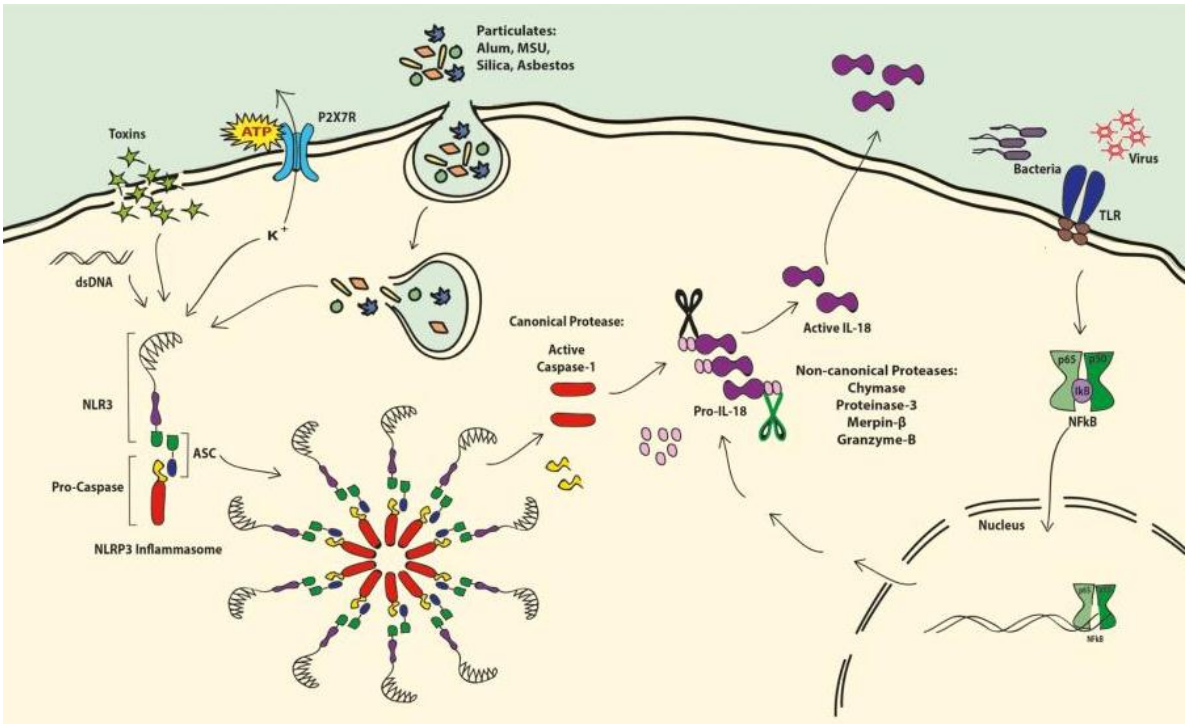



***Figure 4.*** *Inflammasome activation and assembly. Formation of the multiprotein complex NLRP3 is induced by several different stimuli, including toxins, asbestos and pathogen-associated molecular patterns. Downstream of Caspase-1 activation, pro-IL-18 is cleaved to produce biologically active IL-18.*

### 1.9.1 IL-18 in immune activation

In general, IL-18 functions as to enhance innate and adaptive immune responses. This enhancement includes the upregulation of MHC molecules and migration of leukocytes[157]. The biological effects of IL-18 are mediated through binding of IL-18 to the IL-18 receptor (IL-18R), which is formed by a heterodimer consisting of the IL-18Rα and ß chains [158, 159]. This is achieved by inflammasome-cleaved IL-18 binding to non-occupied IL-18Rα on cells expressing this receptor, followed by recruitment of the IL-18Rß chain, which does not bind the cytokine but is required for intracellular signaling[160, 161].

IL-1 and IL-18 are similar in that they both make use of the myeloid differentiation factor 88 (MyD88)-signaling pathway [162]. MyD88 functions as an adaptor molecule in TLR- and IL-1R signaling pathways[163], and for the IL-18 pathway, it anchors IL-1R-associated kinases (IRAKs) to the protein complex established at the cell membrane[164]. Once phosphorylated, IRAKs bind tumor necrosis factor receptor-associated factor 6 (TRAF6) [165], which is in turn thought to phosphorylate NFkB-inducing kinase (NIK)[166]. NIK phosphorylates Inhibitor of κB kinases (IKK) which are thus targeted for destruction via ubiquitination and proteasomal degradation, resulting in a lack of inhibition of the NFκB protein which is set free to enter the nucleus and alter gene expression of downstream targets such as e.g. IFNγ[167].

IL-18 is a very potent cytokine requiring multiple regulatory mechanisms avoiding improper and detrimental activation. One of these is found in the intrinsic lack of biological activity of IL-18 not yet processed by the inflammasome. Another mode of regulation is the production of regulatory proteins. IL-18 binding protein (IL-18BP) is constantly being produced and is present in serum of healthy humans at a 20-fold molar excess compared to IL-18[168]. IL-18BP binding to IL-18 effectively inhibits the actions of IL-18, thus protecting the body against detrimental effects of untimely secretion of biologically active IL-18[169].

Traditionally, cytokines have been divided into $T_H1$- or $T_H2$-type cytokines, based on their functions enhancing T helper responses[170]. The discovery of alternative cytokines, which do not allow such classification[144] has made it increasingly complicated to describe and model the functions of individual cytokines according to $T_H1$- or $T_H2$-properties. IL-18 is one of those cytokines not allowing simple classification, but exerts functions dependent on the inflammatory milieu [171, 172]. For instance, if co-administered with IL-12, IL-18 enhances a $T_H1$-skewed inflammatory response with production of IL-13, while IL-18 alone stimulates IL-4 release and a $T_H1$-response [172]. The functions of IL-18 have been tied to pathological processes in a multitude of autoimmune and inflammatory disease, including Sjögren's syndrome, systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA) and others[157, 173]. It has been shown that IL-18 levels in serum or in the target organ correlate with disease severity in patients suffering from SLE[174], MS[175], and RA[176, 177].

## 1.10 SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is a potentially fatal autoimmune disease afflicting about 1 in 2,500 people, and the prevalence is highly variable depending on geographical location and ethnicity[178]. An even stronger factor than ethnicity is sex, as about 90% of SLE patients are women. A hormonal component is suggested to be causing this difference while underlying mechanisms remain unclear[179]. SLE is highly variable in its manifestations, and several diagnostic criteria need to be fulfilled for accurate diagnosis. Among these are clinical symptoms, including arthritis, rashes, photosensitivity, as well as laboratory tests of kidney and liver function, and the presence of autoantibodies in serum[180]. Clinical symptoms of SLE are caused by the presence of antibodies binding self-antigens, which form immune complexes deposited in fine vessels where they initiate inflammation and cause disease[181]. This feature is a hallmark of SLE, where immune complexes usually end up in the kidneys causing nephritis[182]. There is a range of antigens to which autoantibodies bind, but DNA is remains the essential biomarker for SLE[183]. Patients usually suffer from disease flares, where sudden outbursts of severe disease can be triggered by environmental agents, e.g. UV-light[184].

There is no cure for SLE, but there are several therapeutic strategies employed in order to ameliorate disease progression as well as symptomatic treatment[185]. These include non-steroidal anti-inflammatory drugs for mild disease, and corticosteroids and cyclophosphamide for treating flares and serious disease[186]. New strategies for managing disease include antibody-based treatments targeting inflammatory cytokines, like BAFF, IL-10, and TNFα, which drive disease progression[186]. These therapies have shown beneficial effects and are still under development[185].

The etiology of SLE is still unclear, but it is well-established that patients afflicted are genetically predisposed[187] and environmental factors also contribute [188]. Mouse models used to study SLE include the $MRL^{lpr/lpr}$ mouse strain[189] which is deficient in Fas[190], and thus has a defective apoptotic program in activated B lymphocytes. These mice develop high titers of autoantibodies[189], indicating the importance of this pathway in regulating autoreactive B cells. In a model of lupus nephritis in mice, it was described that IL-18 had a negative role for the induction, prompting further investigation of this cytokine in relation to autoimmunity[191].

# 2 THE PRESENT STUDY

## 2.1 AIM

The general aim of the present study was to investigate how innate immune cells regulate B cell activation in inflammation and when using novel vaccine adjuvants.

Specific aims:

Paper I**.** To investigate how the B cell response in IL-18-induced antibody production is initiated and regulated by iNKT cells.

Paper II. To investigate how cognate and noncognate iNKT-cell help provided to B cells shape ensuing antibody responses.

Paper III**.** To identify how iNKT cells and neutrophils collaborate to restrict B cell activation induced by sterile inflammation

Paper IV**.** To investigate the possibility of harnessing glycolipid stimulation of iNKT cells in order to regulate B cell activation in sterile inflammation

## 2.2 RESULTS AND DISCUSSION

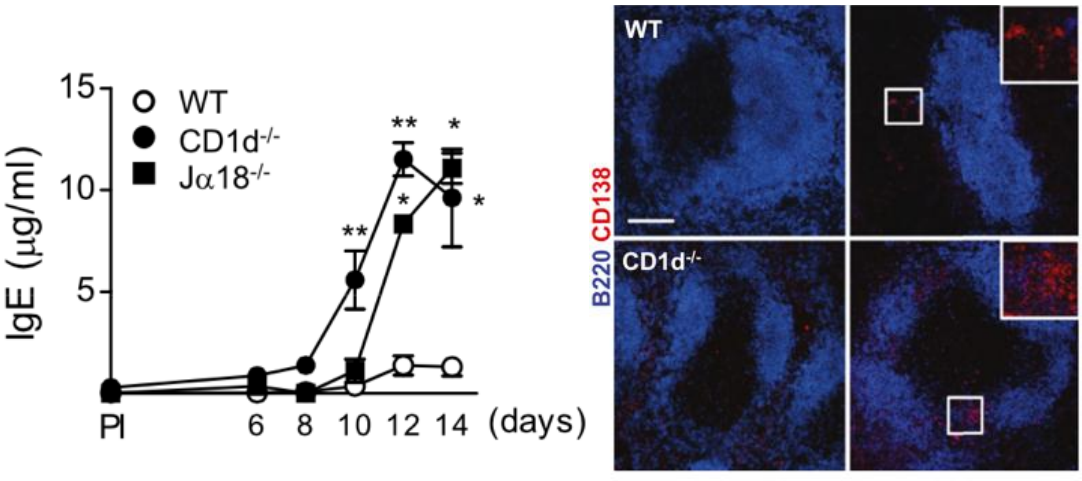
### 2.2.1 The inflammatory cytokine IL-18 induces self-reactive innate antibody responses regulated by natural killer T cells (Paper I)

Elevated levels of IL-18 have been reported in several autoimmune disorders [174-177]. Although originally identified as IFNγ-inducing factor, IL-18 is a cytokine that enhances different types of immune responses depending on the context [171]. If combined with IL-12, IL-18 gives rise to a significant IFNγ production – the archetypical $T_H1$ cytokine, while injecting IL-18 alone results in a pronounced $T_H2$ response which depends on IL-4 and IL-13[172]. While the T cell response in IL-18-mediated inflammation has been extensively studied, little was known about how B cells are activated in response to increased levels of this cytokine. Thus, we set out to investigate the B cell response in settings of elevated systemic levels of IL-18.

Intraperitoneal injections of IL-18 alone for 10 consecutive days resulted in IgE and IgG1 production, as well as increased serum levels of IgM. In addition, serum levels of autoreactive antibodies were significantly elevated in mice injected with IL-18. Antibody levels against NP coupled to bovine serum albumin (BSA) were significantly increased in mice injected with IL-18 alone. In summary, these findings indicate an induction of a polyclonal natural antibody response.

To determine the type of B cell activation induced by elevated levels of IL-18, we investigated how different B cell populations expanded in response to repeated injections of IL-18. We found that mice injected with IL-18 had a transient increase in the innate type marginal zone B cell population, peaking at day 6 after induction of inflammation. Later in response to elevated levels of IL-18, plasma cells and germinal center B cells populations also expanded. These B cell-activating features following IL-18 injections could be mediated by the cytokine BAFF [39, 192], of which we found increased levels in serum of mice injected with IL-18.

***Figure 5.*** *Increased serum levels of IgE in iNKT cell-deficient mice (Jα18-/- and CD1d-/-) injected with IL-18, compared to wild type mice (left). Increased formation of CD138+ extrafollicular foci in the absence of iNKT cells. Immunohistochemistry of spleen sections from wild type and CD1d-/- mice pre-immunization (PI) and following injection with IL-18. (Scale bar, 150μm) (right)*



iNKT cells restrict GC formation in autoreactive B cell responses[33]. Moreover, in several autoimmune diseases where increased levels of IL-18 have been reported, there are alterations in the iNKT cell compartment[193]. This prompted us to further investigate the role of iNKT cells in regulation of B cell activation induced by IL-18. Injecting iNKT cell-deficient mouse strains with IL-18 resulted in a significant increase in serum antibody levels as compared to wild type. Histological investigation of the B cell activation revealed that extrafollicular foci and germinal center formation was increased in iNKT cell-deficient mice.

In iNKT cell-deficient CD1d-/- mice we found an even more pronounced activation of B cells, with increased GC and extrafolicular foci formation, shown by immunohistochemistry. By means of quantitative RT-PCR we found that CD1d-/- mice injected with IL-18 had higher expression levels of aid, unlike wild type mice

However, the qualitative changes of the antibody response and whether iNKT cells regulated these was still unknown. Therefore, we next used quartz crystal microbalancing (QCM) to measure affinity. QCM is a technique similar to surface plasmon resonance, with a difference in that it employs the piezoelectric effect, instead of resonant oscillation of conduction electrons[194]. We investigated the ability of sera pooled from wild type and CD1d-/- mice to bind to carboxylchips coated with the NP-hapten coupled at a low ratio to BSA ($NP_4$-BSA, i.e. 4 NP haptens per BSA molecule). Using this approach, we found no difference in the capability of pooled sera from wild type mice injected with IL-18 to bind to the high affinity $NP_4$-BSA antigen, as compared to pre-immunized controls. However, in pooled sera from CD1d-deficient mice, there was increased binding to the high affinity antigen, suggesting a qualitative change in the antibody response in the absence of iNKT cells.

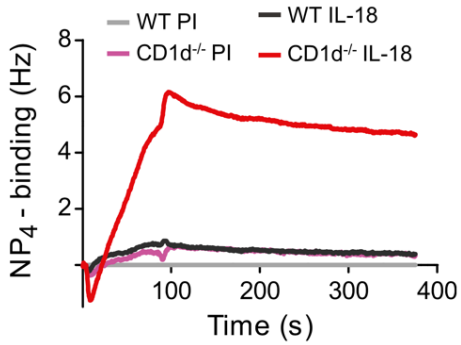



***Figure 6.*** *Presence of high-affinity anti-NP antibodies determined by quartz crystal microbalance. Pooled sera from indicated groups were tested for binding to $NP_4$-BSA immobilized on a carboxyl-chip, detected as changes in frequency measured with an Attana100*

How iNKT cells regulated B cell activation was still unknown. iNKT cells produce and respond to IL-21[108], and express higher levels of cytotoxic molecules in response to this cytokine[105]. As IL-21 inhibits IgE production[110], we measured expression levels of this cytokine and found a significant increase in splenic levels of *Il-21* mRNA. Next, we examined mice deficient in cytotoxic pathways important in mediating cell death. Perforin and FasL (CD178) are two cytotoxic molecules known to be utilized by iNKT cells in a CD1d-dependent manner[195]. Injecting IL-18 into strains of mice deficient in perforin or FasL resulted in significantly elevated levels of serum IgE compared to wild type controls, indicating that these pathways are mechanistically linked to the regulation of B cell antibody production in response to elevated levels of IL-18.

In summary, we found that elevated levels of IL-18 induce B cell activation that results in production of self-reactive antibodies. B cell activation is strictly regulated by iNKT cells that prevent GC entry and formation of extracellular foci. iNKT cells limit expression of *AID* and the formation of antibodies capable of binding self- and natural antigens.

### 2.2.2 Cognate interaction with iNKT cells expands IL-10-producing B regulatory cells (Paper II)

The ultimate goal with a vaccine is to generate immunological memory against harmful pathogens, thus generating immunity against future encounters with the same pathogens. There are highly efficient vaccines which enabled eradication of previously deadly diseases, such as the smallpox vaccine. However, there are still many infectious diseases for which there are currently no efficient vaccines, and conventional methods for eliciting protection against these harmful infections have proved inefficient. Therefore, much effort has been aimed at exploring novel strategies to increase the efficiency and immunogenicity of vaccination protocols [196-198].

iNKT cells stimulated with cognate lipid antigen provide help to B cells, and thus stimulate antibody production[108, 109]. Glycolipids that stimulate iNKT cells could function as vaccine adjuvants and are therefore attractive compounds in novel vaccine design. iNKT-cell help to B cells generates a robust primary antibody response with both cognate and non-cognate iNKT-cell help. However, cognate iNKT-cell help cannot induce memory B cell responses[108, 109]. The fundamental difference in the B cell help delivered by iNKT cells activated either in a cognate or noncognate fashion remains elusive. Understanding the difference between these types of help provided by iNKT cells to B cells may allow us to harness this type of help for future vaccine development.

To address how cognate iNKT-cell help differs from noncognate, we used two different model antigens. First, we immunized mice with a haptenated protein (NP-Keyhole Limpet Hemocyanin (KLH)) plus αGalCer, in order to deliver noncognate signals to iNKT cells. Second, we used directly haptenated lipid (NPαGalCer) alone, to deliver cognate iNKT cell stimulus. Immunizing mice with NPαGalCer alone resulted in a quick, but transient GC response. Immunizing mice with NP-KLH plus αGalCer induced more $T_{FH}$ cells and later development of germinal center B cells, compared to NPαGalCer alone. Additionally, cognate iNKT-cell help induced more $T_{FR}$ cells than noncognate iNKT cell-help. TFR and TFH cells are important in negative and positive regulation of GC responses, respectively[86]. Thus, higher numbers of $T_{FR}$ cells and lower number of $T_{FH}$ cells following cognate iNKT-cell, compared to noncognate, help could potentially contribute to an impaired induction of B cell memory.

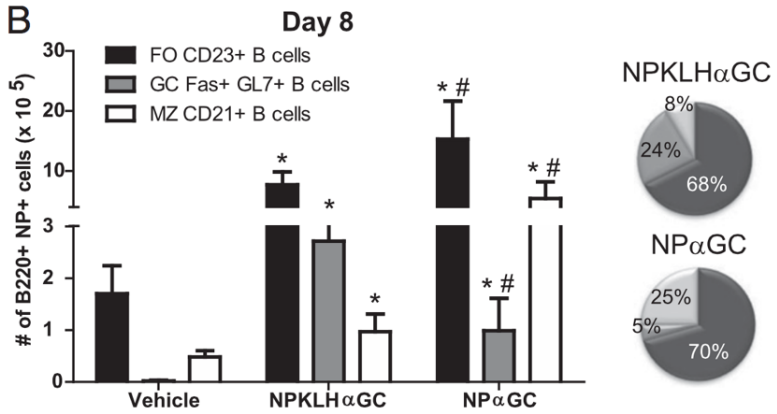



***Figure 7.*** *Cognate iNKT-cell help preferentially expands innate B-cell populations. Splenocyte FACS data from mice immunized with vehicle, 100μg NP-KLH+ 0.5μg αGalCer, or 0.5μg NP-αGalCer. Pie charts show B-cell subsets as percentages of total B cells in the spleen.*

The reason for the failure of cognate iNKT-cell help to induce memory B cell responses could be that there were too few $iNKT_{FH}$ cells. PD-1 and CD25 are negative regulators of $T_{FH}$ development[88, 199]. In order to increase the number of $iNKT_{FH}$ cells, we generated mixed

bone marrow chimeras deficient in PD-1 or CD25, specifically in iNKT cells. We found that these chimeras had increased numbers of $iNKT_{FH}$ cells. However, these mice could still not generate B cell memory following immunization with NPαGalCer, indicating that there are qualitative differences between cognate and noncognate help from iNKT cells.

iNKT cells localize in the marginal zone of the spleen following immunization with cognate antigen[96]. We therefore investigated how noncognate and cognate iNKT cell help affected the expansion of different B cell populations following immunization with NP-KLH plus αGalCer or with NPαGalCer alone. We found that cognate iNKT cell help preferentially expands innate B cell populations. Both noncognate and cognate iNKT cell help induced an expansion of MZB cells, but nocognate iNKT cell help only transiently, and would instead support a later germinal center B cell response. Conversely, cognate iNKT cell help induced a transient germinal center B cell expansion. Instead cognate iNKT-cell help was able to support a MZB cell expansion for a more extended period of time, compared to noncognate iNKT-cell help.

Since cognate iNKT cell help preferentially expanded $CD1d^{hi}$ MZB cells in the spleen, we next investigated the difference between cognate and noncognate iNKT cell help regarding a related splenic subset of B cells – the regulatory B cells[65]. To this end, we used VertX IL-10 reporter mice, reports IL-10 production via green fluorescent protein[200]. Following immunization of these mice, we found that NPαGalCer induced more antigen-specific B10 cells compared to NP-KLH plus αGalCer, and that higher numbers of these cells were IL-10 positive. However, cognate iNKT cell help is not specifically focused on the induction of IL-

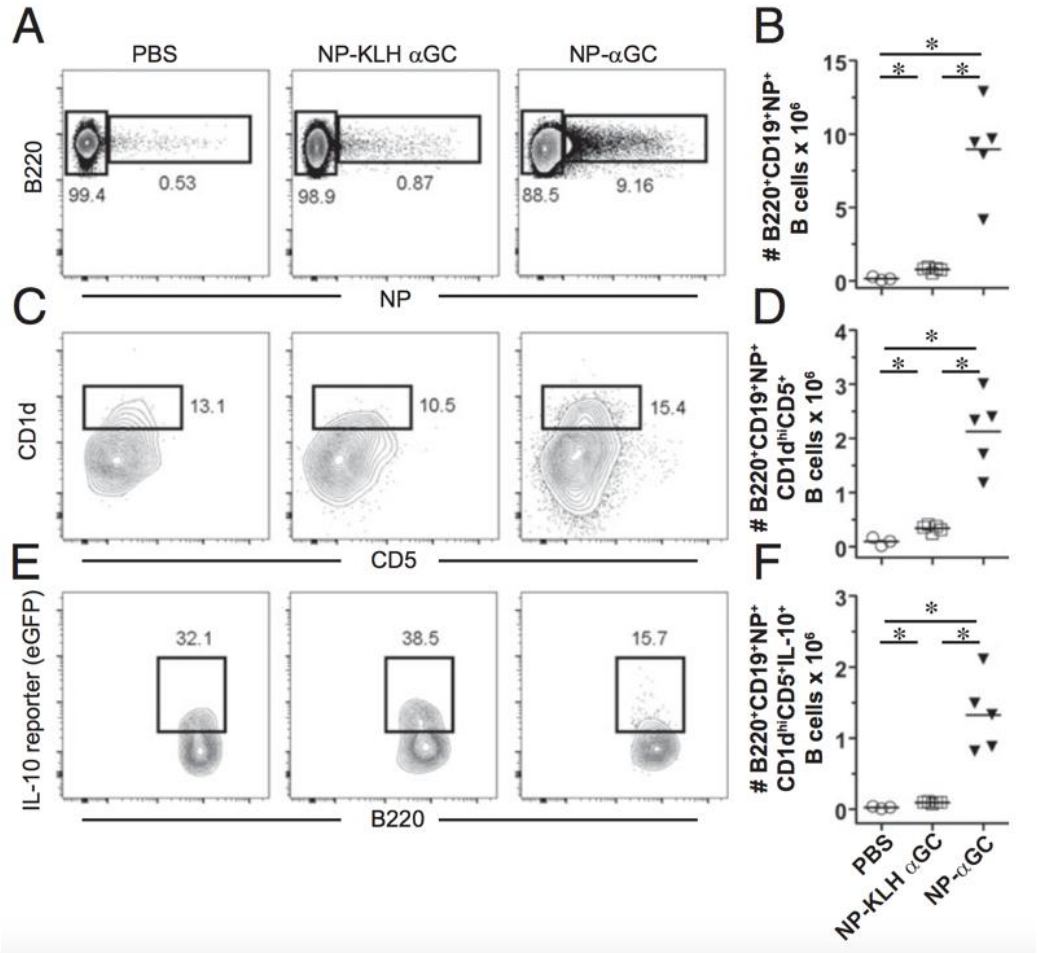



***Figure 8.*** *Cognate iNKT-cell help expands antigen-specific IL-10+ B10 B cells. FACS data of splenocytes 4d after immunization with vehicle, NP-KLH+αGalCer, or NPαGalCer. FACS analysis showing B220+NP+(A and B), B220+NP+CD1d+CD5+(C and D), and B220+NP+CD1d+CD5+IL-10+(E and F) subsets of cells.*

10-secreting B cells, since the proportion of IL-10+ B cells expanded in the antigen-specific CD5+CD1d+ B regulatory cell population is lower, rather than higher in mice immunized with NPαGalCer.

This feature of cognate iNKT cell help provided to B cells - generating a higher number of regulatory B cells - might explain the different outcomes regarding memory B cell formation following cognate and noncognate iNKT cell help.

In conclusion, there are fundamental qualitative and quantitative differences between cognate and noncognate iNKT cell help provided to B cells. Cognate iNKT cell help provided to B cells fails to generate B cell memory, possibly due to differences in B and T cell activation induced by cognate iNKT cell help as compared to noncognate. These findings have implications for the design of vaccines employing glycolipids as adjuvants, indicating that the way signals are delivered will affect the outcome in terms of B cell memory.

### 2.2.3 Neutrophils license iNKT cells to regulate self-reactive B cell responses (Paper III)

In paper I, we found increased levels of BAFF, a cytokine crucial to activation of B cells[201]. Therefore, we investigated potential sources of BAFF. Many cells are capable of producing BAFF, including DCs, macrophages and neutrophils[131]. Employing our inflammatory model with daily i.p. injections of IL-18, we investigated the kinetics of the systemic increase in BAFF and found that serum BAFF levels were significantly increased four days following injections of IL-18. Neutrophils stimulate B cells by producing BAFF and provide help to innate type B cells [123]. Neutrophils are thus potential candidate cells involved in controlling B cell activation in response to increased systemic levels of IL-18. Neutrophils accumulate in the spleens of mice injected with IL-18 compared to vehicle-injected controls. We investigated the presence of IL-18R on splenic neutrophils and found that they express this receptor, indicating that elevated levels of IL-18 could influence splenic accumulation of neutrophils directly. We cultured sorted neutrophils *in vitro* in the presence of IL-18 and identified an increase in the surface expression of BAFF. Array data of sorted splenic neutrophils from mice injected with IL-18 showed that neutrophils upregulate *Tnfsf13b* mRNA following elevated systemic levels of IL-18. Additionally, splenic neutrophils expressed BAFF on their surface.

In paper I, we found that iNKT cells negatively regulate B cell activation. The splenic accumulation of neutrophils identified here prompted us to investigate the expression of CD1d on these cells. Expression of CD1d is a requirement for cognate engagement of iNKT cells[92]. At steady state, splenic neutrophils did not express detectable CD1d on the cell surface. However, sorted neutrophils exposed to IL-18 *in vitro* upregulated cell surface CD1d.

CXCR6+ is a chemokine receptor expressed almost exclusively by iNKT cells in the spleen [95, 96]. Therefore, we used CXCR6-GFP knock-in mice to address if iNKT cells and neutrophils were located close enough to permit interaction. In the spleens of these mice we found numerous examples of CXCR6+ iNKT cells in close proximity to neutrophils in the splenic red pulp.

Neutrophils and iNKT cells can potentially communicate in several different ways, as both cells express many different cytokines[105, 115], and cell surface receptors known to interact, for instance CD40-CD40L[109, 202]. However, since we found that IL-18 induced CD1d on neutrophils *in vitro*, we first address the prospect of cognate interaction between neutrophils and iNKT cells. Using mixed bone marrow chimeras we detected an upregulation of CD1d in response to injections with IL-18, enabling cognate interaction with iNKT cells *in vivo* (Figure 9). Strikingly, CD1d-deficient neutrophils had a compromised ability to induce an activated phenotype in response to elevated levels of IL-18. This suggests that cognate interaction between iNKT cells and neutrophils is required in order for neutrophils to be fully activated in IL-18-mediated inflammation.

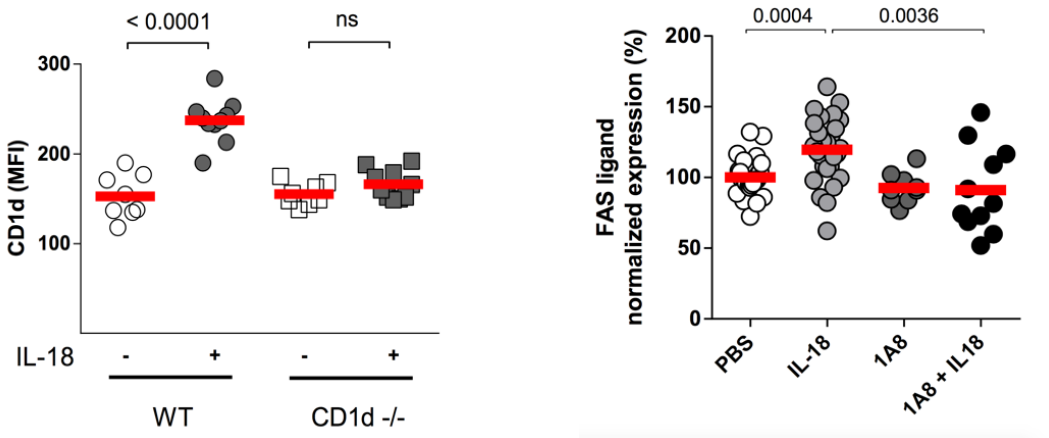



***Figure 9.*** *CD1d expression by neutrophils in mixed bonew marrow chimeras and iNKT cell expression of FasL following depletion of neutrophil using the antibody 1A8. Expression of CD1d measured by FACS, following exposure to IL-18* in vivo *in neutrophils derived from the indicated genetic background (left).Normalized FasL expression by iNKT cells harvested from spleens of mice injected with vehicle, IL-18, neutrophil-depleting 1A8 antibody, or 1A8 + IL-18 (right).*

Next, we investigated phenotypic changes of iNKT cells and found that iNKT cells, specifically, upregulated FasL following injections with IL-18 (Figure 9). Since neutrophils upregulated CD1d in response to IL-18, we tested whether the presence of neutrophils had any impact on the expression of FasL by iNKT cells. By means of antibody-dependent cellular cytotoxicity (ADCC) - using antibodies to selectively deplete neutrophils – we found that FasL upregulation was abrogated in mice with elevated IL-18-levels depleted of neutrophils. Thus, neutrophils license iNKT cells to adopt a killing phenotype.

We next sought to establish whether FasL expressed specifically by iNKT cells was required to control faulty B cell activation leading to autoantibody production. Using mixed bone marrow chimeric mice, we found that mice reconstituted with FasL-deficient iNKT cells had significant increases in the proportion of plasma cells as well as germinal center B cells. However, mice receiving wild type iNKT cells were able to control expansion of these B cell subsets. These differences were reflected in the serum levels of anti-DNA IgG1 and IgG3 (Figure 10). Mice receiving wild type iNKT cells showed no differences in serum levels of autoantibodies in response to increased levels of IL-18. However, mice receiving FasL-/- iNKT cells had an increase in both serum anti-DNA IgG1 and IgG3. Thus, iNKT cells transferred in these experiments blocked B cell activation and self-reactive antibody formation using FasL.

B cells could potentially be regulated by more than just FasL, considering that some B cell subsets express very high levels of CD1d[33]. In paper I, we established that B cells enter germinal centers in response to elevated levels of IL-18. Therefore, we used mixed bone marrow chimeras to investigate how CD1d expression by B cells affected the propensity to form germinal centers. We found that germinal center B cell formation was significantly increased in the population of B cells lacking CD1d. This indicates that iNKT cells directly interact with B cells in a cognate fashion in order to restrict this type of B cell activation. In order to test if iNKT cells moved to germinal centers to exert this regulation, we injected the CXCR6-GFP knock-in mice with IL-18. Surprisingly, we could detect no increase in the density of iNKT cells in germinal center areas of the spleen on day 12 in mice injected with IL-18 compared to controls. However, in CD138+ extrafollicular foci, there was a significant increase in the density of iNKT cells. This suggests that iNKT cells redistribute to extrafollicular foci following increased levels of IL-18. However, iNKT cells could also be present at higher densities in the GC, earlier in the response.

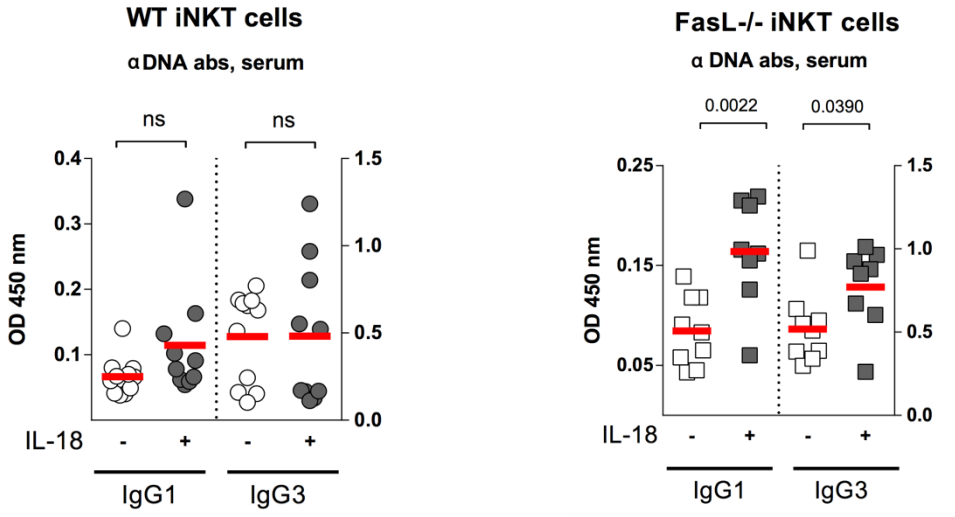



***Figure 10.*** *Increased serum levels of autoantibodies in mice with an iNKT-cell specific FasL-deficiency. Serum levels of anti-DNA IgG1 and IgG3 in mice reconstituted with wild type iNKT cells (left). Serum levels of anti-DNA IgG1 and IgG3 in mice reconstituted with FasL-/- iNKT cells (right).*

We next investigated how a lack of neutrophils available to license iNKT cells affected B cell activation in response to increased levels of IL-18. We found that AFC and GC responses increased in the spleens of neutrophil-depleted mice injected with IL-18, compared to IL-18 alone. This suggested that the presence of neutrophils was required to restrict B cell activation. The lost restriction of B cell activation seen in neutrophil-depleted mice injected with IL-18 also had significant effects on the serum levels of self-reactive antibodies. Serum levels of anti-DNA IgG1 and IgG3 increased compared to mice injected with IL-18 alone. This indicated that the presence of neutrophils which license iNKT cells to restrict faulty B cell activation is essential to keep the levels of autoreactive antibodies in check.

In conclusion, in paper III we have identified a previously unappreciated crosstalk between iNKT cells and neutrophils in sterile inflammation. Neutrophils were required in order to license iNKT cells to adopt a killing phenotype in response to elevated systemic levels of IL-18, and this was critical to restrict faulty B cell activation and the production of autoreactive antibodies. Thus, the findings in paper III suggest mechanisms potentially underlying autoimmune disease.

### 2.2.4 Exogenous glycolipid agonists alter the inherent B cell regulatory function of iNKT cells (Paper IV)

Given the different roles played by iNKT cells, we tested the potential of harnessing the activity of iNKT cells to redirect faulty immune activation in sterile inflammation in paper IV. To this end, a combinatory model was employed where inflammation was induced by daily intraperitoneal injections with IL-18 combined with one injection of glycolipid iNKT cell ligand at onset of inflammation, at the time of the first injection of IL-18.

Total serum levels of IgE and IgG1 increase in response to intraperitoneal injections of IL-18. Compared to IL-18 alone, serum antibody levels were significantly increased when iNKT cells were simultaneously stimulated with the iNKT cell ligands OCH[103], GSL-1[102] or αGalCer[101]. The formation of autoreactive antibodies of the IgE and IgG1 isotypes also increased significantly when activating glycolipids were coadministered with IL-18.

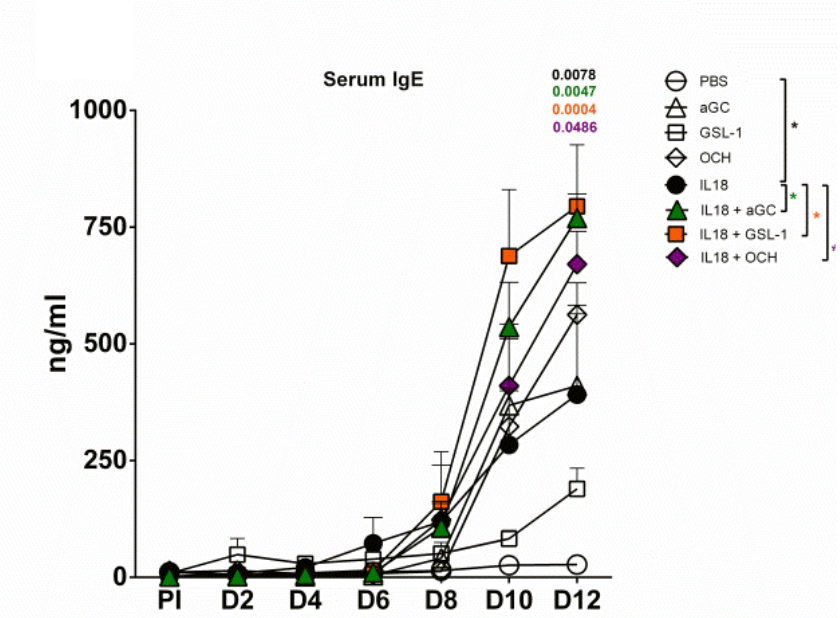



***Figure 11.*** *Increased serum IgE levels in wild type mice receiving glycolipids in inflammation. Injection of OCH, αGalCer (αGC), and GSL-1 into wild type mice receiving IL-18, all result in increased production of IgE, compared to IL-18 alone.*

In paper I, we found that the MZB cell population expanded in response to elevated levels of IL-18. Therefore we investigated changes in B cell proportions and phenotypes in response to IL-18 plus glycolipid. We found no differences in the proportion of MZBs with glycolipid and IL-18 combined, while MZB expression of CD1d was significantly increased in response to IL-18 alone. However, when αGalCer was administered concomitantly with IL-18, the increase in CD1d expression following IL-18 alone was abrogated. The proportion of plasma cells in the spleen significantly increased in response to IL-18 alone and was even higher when OCH was co-administered. The frequency of germinal center B cells was increased in response to IL-18 alone, and all the activating glycolipids tested, further enhanced the proportion of germinal center B cells. We next investigated the switching patterns exerted by plasma cells and found that αGalCer combined with IL-18 resulted in significantly increased proportions of plasma cells switching to IgE and IgG1.

Next, we investigated the proportion of iNKT cells. We found that, as has been reported before, the iNKT cell proportion was significantly diminished in response to injection with αGalCer alone[203]. However, when αGalCer was co-administered with IL-18 there was no change in the proportion of iNKT cells in the spleen. Several phenotypic changes following activation of iNKT cells have been reported [98, 107, 109]. Therefore, we next investigated phenotypic changes in the iNKT cell population and found that αGalCer was a highly potent inducer of the CXCR5+ PD-1+ $iNKT_{FH}$ phenotype. Additionally, a pronounced population of KLRG1+ iNKT cells formed when injecting αGalCer, with or without IL-18.

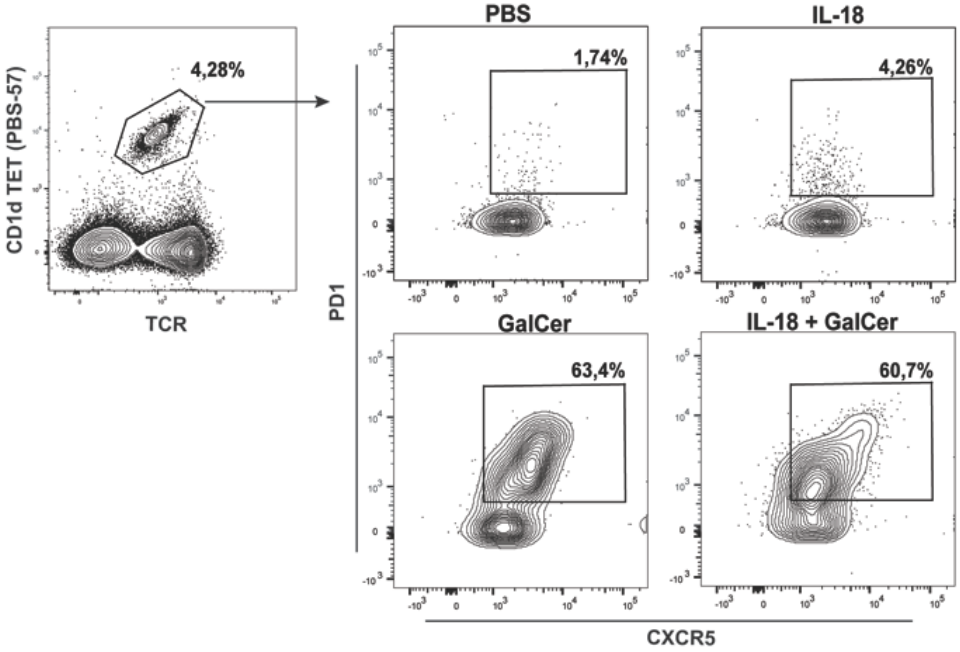



***Figure 12.*** *iNKT cell phenotype following glycolipid stimulation of iNKT cells in inflammation. Representative FACS plots indicating expression of $iNKT_{FH}$-markers PD1 and CXCR5 in population gated according to left panel. Percentages indicate frequency of parent.*

$iNKT_{FH}$ cells provide help to B cells in immune responses so as to boost antibody production[108, 109]. The dramatic change seen in the iNKT cell population of mice immunized with αGalCer with or without IL-18 might in part explain the differences seen in serum antibody levels seen compared to controls in these different treatment groups. The induction of $iNKT_{FH}$ cells in the iNKT cell population could potentially lead to B cell-helping functions being activated in iNKT cells. This could in turn lead to increased stimulatory cytokine production by iNKT cells enabling B cell activation and antibody production[204]. The induction of KLRG1+ iNKT cells is interesting with respect to the antitumor activity reported in this subset of iNKT cells[98]. It seems like IL-18 could be able to induce an even higher proportion of KLRG1+ iNKT cells than αGalCer alone. While more experiments are required to address this, it certainly would be interesting to use IL-18 as an adjuvant to αGalCer in the generation of antitumor memory iNKT cells.

Our discovery in paper III that neutrophils upregulated prompted us to investigate how the administration of glycolipids in addition to IL-18 affected neutrophils and BAFF. We found that the only lipid with significant effects by itself was αGalCer, which induced a significant accumulation of splenic neutrophils. In combination with IL-18, there was a combinatory effect of IL-18 and αGalCer, so that the proportion of neutrophils increased compared to IL-18 or αGalCer alone, respectively. Although GSL-1 and OCH did not affect the proportion of neutrophils when administered alone, OCH in combination with IL-18 significantly increased

the number of neutrophils present in the spleen, compared to OCH or IL-18 alone, respectively. Following IL-18 injections, adding activating iNKT cell ligands induced elevated levels of the cytokine BAFF. Since we established in paper III that IL-18 is capable of directly inducing BAFF expression by neutrophils, the increase in serum BAFF in mice injected with IL-18 plus αGalCer could possibly be explained by the increased accumulation of neutrophils in the combined treatment group, compared to IL-18 alone.

In conclusion, the activation of iNKT cells in inflammation using glycolipids results in an increase in B cell activation and production of autoreactive antibodies. Thus, in a setting of autoinflammatory disorders involving IL-18, different glycolipid administration protocols should need to be investigated further for their capacity to skew antibody production.

### 2.2.5 Concluding remarks and future perspectives

The major findings of the work presented in this thesis are:

- Increased systemic levels of IL-18 lead to the activation of autoreactive B cells. This activation is restricted by iNKT cells
- Differences between cognate and noncognate iNKT cell help to B cells depend on qualitatively and quantitatively diverse responses
- In inflammation, iNKT cells are licensed by neutrophils to adopt a regulatory phenotype required to restrict autoreactive B cells
- Glycolipid agonists of iNKT cells affects production of antibodies in inflammation

Many conclusions in this thesis are based on experiments involving injections of large amounts of IL-18. The amounts of IL-18 used in experiments will rarely be achieved in a physiological or even pathological setting. In an adult person afflicted with sepsis, amounts of up to 10ng of IL-18 per milliliter serum have been reported[168]. Therefore, the model of elevated systemic levels of IL-18 serves a highly relevant purpose in demonstrating essential effects of IL-18 *in vivo*.

The work in paper I identified iNKT cells as negative regulators of self-reactive B cell responses and autoantibody production in settings of increased levels of IL-18. As the levels of IL-18 are increased in several autoimmune diseases, IL-18 neutralization could potentially be a target for therapeutic intervention. Clinical trials are underway, testing the potential for the investigational drug Tadekinig alfa (recombinant IL-18BP) in order to treat the inflammatory adult-onset Still's disease. In adult-onset Still's disease, high levels of IL-18 are thought to play a critical role in pathogenesis. Thus, supplementing the body with an endogenously available regulator of IL-18 might prove beneficial in disease where this cytokine causes pathology. Phase 1 clinical trials have proven safety, although side effects remain to be investigated. Potentially, side effects of blocking IL-18 could include obesity and insulin resistance, as IL-18-/- mice present with these phenotypes[205]. The function of IL-18 in priming immune responses against tumors is also a factor to consider for IL-18-blocking therapies. Il-18 depletion could possibly lead to increased tumor progression, while the opposite is also true, due to the context-dependent functions of this cytokine[206].

In paper III, we found that part of the mechanism by which iNKT cells negatively regulate autoreactive B cell activation is through a change in phenotype. In response to IL-18, iNKT cells increase the expression of molecules mediating cellular cytotoxicity, in this case FasL. The conditional knock-out of FasL specifically on iNKT cells, resulted in increased production of autoreactive antibodies, but other molecules involved in cytotoxicity could also be involved. When Perforin-deficient mice were injected with IL-18 (paper I), serum levels of IgE were significantly increased compared to controls. Thus, it would be highly relevant to also investigate this molecule in the regulation of faulty B cell activation in response to IL-18, and specifically, whether iNKT cells make use of this pathway. However, the potential receptors employed by iNKT cells for regulating cell populations through killing seems to not be redundant, as knocking out FasL alone in the iNKT cell population still results in a significant B cell activation not permitted in mice with FasL-proficient iNKT cells. Possibly, the different killing pathways are used in different contexts. It has been suggested that antigenic potency correlates with in vivo cytotoxicity mediated by iNKT cells[195]. However, signals other than TCR strength are likely to be involved, as iNKT cells are activated by cytokines as well as other receptors [97]. Were it possible to pharmacologically induce a killing phenotype in iNKT cells, it might be possible to restore cytotoxic functions and thus restrict faulty B cell activation in autoimmune disorders. However, IL-18 might have too pleiotropic effects in order to be a candidate.

The reason for failure to restrict B cell activation might in part depend on neutropenia, which is found in about one-half of SLE patients[207]. As we found that the presence of neutrophils is required in order to license iNKT cells to adopt a killing phenotype, this could explain mechanisms underlying SLE. This finding also holds promise as a target for future therapeutic approaches. Inducing normal neutrophil numbers in combination with stimulating a killing phenotype by iNKT cells at the same time might be effective in re-establishing restriction of B cell activation in antibody-mediated diseases. This could be achieved by means of neutropoiesis-stimulating cytokines like GM-CSF or G-CSF. It has been reported that GM-CSF protects neutrophils against apoptosis[208], and this could be a highly desirable effect. Dead or dying neutrophils potentially provide self-antigens, thus propagating disease [138]. However, as many patients do not present with neutropenia, even complete success of these hypothetical treatments will probably not be able to cure all patients. There is also the caveat that comes with BAFF provision by neutrophils. Possibly, G-CSF would generate more neutrophils able to deliver BAFF, thus exacerbating disease.

In paper II we identified fundamental differences between cognate and noncognate iNKT cell help to B cells that have important implications for future vaccine design. Glycolipids which stimulate iNKT cells, boosting desirable immune responses hold great promise as compounds that can be used as adjuvants for new vaccines. We found an increase in IL-10 expressing B cells following cognate iNKT-cell help. Recently, iNKT cells were described to produce the inflammation-dampening cytokine IL-10 following a pre-activation protocol with αGalCer, showing that stimulation with iNKT cell ligands does not necessarily result in amplification of immune responses[107]. The reason why cognate iNKT-cell help to B cells fails to generate a robust memory response still remains partly unsolved. Potentially, the signals generated following cognate stimulation are too strong, therefore leading to an induction of IL-10 production by B cells. This would be analogous to the induction of IL-10 iNKT cells, where a strong induction leads to a regulatory phenotype, thus innate B cells mirroring the behavior of innate T cells. Since CD1d-expressing B cells and iNKT cells both are enriched for self-reactivites, cognate iNKT-cell help generating memory efficiently, could be a highway to autoimmunity. iNKT cell-stimulating glycolipids potentially cause unwanted activation of B cells in settings of autoimmune disease, as indicated by findings from paper IV.

In paper IV we tested the possibility of skewing the inflammatory response following elevated levels of IL-18 by means of glycolipid iNKT cell agonists. The iNKT cell regulatory role established in paper I, and the underlying mechanisms identified in paper III, prompted us to test different iNKT cell ligands in our inflammation model. However, when injecting a bolus dose of glycolipid at the beginning of inflammation onset, the levels of antibodies and B cell activation actually increased. Future studies should make use of several different glycolipid administration protocols, maybe similar to Sag et al.[107],as this has profound implications on how iNKT cells respond.

All studies in this thesis were performed using mouse models and mouse-derived cells, *in vivo* and *in vitro*. This forms a caveat, since different types of antigen-presenting molecules exist in mouse and man. While mice only express CD1d, humans express several different CD1 molecules able to present a wider array of antigens[93]. Thus, there is a potential loss in translation. However, a significant part of the iNKT cell repertoire in humans is able to bind antigens which typically are used to define murine iNKT cells. Thus, studies performed in mice have without question generated new insights concerning the biology of iNKT cells, which are currently applied even in clinical trials involving compounds first investigated in mice.

Taken together, the work presented in this thesis expands our knowledge about how B cell activation is regulated in inflammation, and how the activation of B cells can be fundamentally different depending on how helping signals are delivered.

# 3 ACKNOWLEDGEMENTS

I am truly grateful to many people who in one way or another contributed to this thesis:

My supervisor **Mikael Karlsson** for accepting me to LeGroupe. We've had some awesome times around the world including skiing, skeet shooting with a count, Korean food, squash lessons, to name a few. Your enthusiasm for science and simultaneously having a good time is great! And yes, scientists are a little bit like rock stars. RIP Lemmy.

My co-supervisor **Lisa Westerberg** for signing me up for marathons and having a sound relationship to your own data.

My co-supervisor **Annika Scheynius** for being an outstanding role model and a strong person standing up for what's right with a frank attitude.

My other supervisor, **Elizabeth Leadbetter** for letting me visit your lab, your curiosity and interest science, and for giving me media training. And for reminding me that the PhD is a marathon, not a sprint. The Leadbetter lab – **Emilie**, **Jen** and **Paula**, for welcoming me into the lab family and always helping me out, in the lab and outside.

My mentor, **Dan Grandér** for never getting tired of sharing stories and complaints, and for giving truehearted advice and being a great gym buddy.

The great people of LeGroupe: **Emilie**, for your patience, joy and wisdom when trying to teach me everything in immunology. **Sara**, for your tough love and teaching me that "Det man inte har i huvudet får man ha i benen". **Emma**, for not shying away from any discussion topic and being the sound mind in the office (sic). **Kajsa**, for your care for absolute things and directness. **Kiran**, my brother in arms, for your over-the-top thoughtfulness and kindness **Amanda**, for sharing crime docs and music, and being a great friend to have on a road trip. **Mattias F**, for always appreciating my Spotify-DJ skills and being a fun big brother in the lab. **Eva**, for teaching me to appreciate team work. **Yunying**, for stirring the LeG-pot. Knowingly or not. **Anna-Maria**, defining the term "all-in" in many aspects of life – I wish I could me more like that. **Vanessa**, for reinstating hope in machine-human relationships. **Silke**, for keeping vinyl 107 and Christmas strong. **Femke**, for adding to the great LeG athmosphere. **Fredrik**, for creating a nice shade for the rest of us to work in.

WASp members – **Magda-Liz**, for always being a genuine fan of my singing. **Mariana**, for creating drama and laughing about it. And for offering food. **Marisa**, for your laugh, enthusiasm for good Brazilian dance shows, and lack of enthusiasm for Christina Ronaldo. Captain **Marton** for making me feel calm. **Laura**, for joyfully participating in discussions and needless lingo competitions without hesitation. **Jamie**, for always being able to deliver great insults with tremendous grace. **Paul**, for dirty German proverbs. **Joanna** and honorary member **Milind**, for great light bulb moments, both on and off the dance floor. **Carin**, for banana guard, magic egg timer, and endless help. **Ming**, for your scientific appetite. **Anton2 (♥)**, for increasing the average IQ, amount of laughs, and amount of good music in the lab at the same time. **Nikolai**, for your immense consideration and bringing treats to us all. **Hanna**, for being kind and neat office mate.

The Trudeau Institute, especially **Mike Tighe** for fantastic technical assistance, and for being a great microscopy/drummer friend. **Cody**, for skiing, diner breakfasts, AWC, and almost fishing. **Ron LaCourse**, for letting me borrow your guitar when FACSing. NY State Governor **Andrew Cuomo**, for letting me participate in the Adirondack Winter Challenge and handing me a nice plaque. **Ron Goldfarb** for replacing hockey sticks broken in the name

of The Trudeau Institute. My housemates **Mariana** and **Mingfeng**, for building a temporary home together and reminding me that the solution to all problems is that "I should be a man".

From the L2:04 dynasty: **Sang**, for being adventurous and having a good sense of humor. **Stefanie**, for knowing what you want and having a nice zero-fucks-given attitude. **Ulf**, for great questions, being a role model in how to make your life an adventure, and your frank way about science and life. **Patricia**, for listening as a true friend when needed. **Tiiu**, for being incredibly brave. **Mattias E**, for deep knowledge of imaging software, human IgA, and always being up for a laugh. **Anna A**, for cheering me on, even when I'm crawling in mud. **Lill-Emma**, for knowing how to party, be a mom, and a PhD-student at the same time. **Ali,** (my old corridor mate) for teaching me that you never have to be stressed. **Catharina**, for sharing sparkling wine and immense administrative help. **Agneta**, for never seizing to tell stories. **Ulrika**, for helping out with my arbetsträning and great statements about heroin and honey. **Jeanette**, for your fun and skeptic way. **Anna Z**, for fun collaboration and always being a kind friend. **Ola Nilsson**, for your sense of humor, and for standing up for students. **Anne-Laure**, for laughs and lessons given about how everything will solve itself. **Reiner**, for sharing the passion for Galapagos and music. **Cindy**, for forgotten purses, appreciating details, and for being an example of how people in science should be passionate. **Katarina Lyberg**, for bringing style and fun (and) general knowledge to Versailles. **Tanja**, for making fun of Ulf. **Kurt**, for great shoulder massages and stories about religion schizophrenia and probes. **Ludvig**, for being the most relaxed person on the planet. **Evelina**, for a happy and tenacious spirit. **Malin**, for beautifully illustrating immunology. **Sven**, for inspiring everyone to be more of a gentleman.

Banff-buddies **Eric Meffre**, for making your way out of jail to inspire with great humor and intelligence. **Pierre Bruhns**, for teaching me what a jacket and shirt are really made for when on a dance floor.

Our new home at MTC, including **Torunn,** for countless great little talks. **Sabina,** for making the animal facility a nice place. **Helen,** for reminding me that I need to get groomed. **Kenth,** for not wasting time, but also for stories about The Matrix. **Anna-Karin**, for being a kind, always. **Per**, for being an awesome DJ and enabler/partaker of fun events. **Magnus**, for being able to fix everything, especially barbeques. **Åsa Belin**, for being an awesome and quirky administrator. **Kristina Gullsby**, for shrewdness in economics. **Lina Rejnö**, for being a great friend of LeG in spite of having to deal with scientists.

The commonwealth mafia: **Jonathan**, for being a fellow man of steel and for keeping check on the boys. **Gerry**, for fun stories about diving. **Benedict**, for handing out hugs when (not) needed and insults despite not even being close to the top in MTC table tennis.

**Susanne Gabrielsson**, for reimbursing me for sauna-beer. **Gunnar Nilsson**, for fun collaborations. **Guro Gafvelin**, for adding kindness to L2:04. **John Andersson**, for much needed harsh humor and for sincerely wondering how things are going. **Hans Grönlund** & Hersby backe, for letting me play some hockey-bockey before I got too young and for being an excellent President of the KiiM sauna club. **Eduardo Villablanca**, for being an astute and attentive PI, always asking questions. **Gunilla Karlsson Hedestam**, for being a strong science person, inspiring me and others.

The Karlsson Hedestam group – **Martina**, for giving out loans when much needed. **Néstor**, for attending my birthday ceremony even though not initially invited. **Lotta**, for being the attending, caring physician and going after what you want. **Martin**, for recognizing the true sound of a Les Paul, and having a hidden sense of humor. **Ganesh**, for your love of headphones and look on life. And for an awesome moustachio **Paola**, for your a playful attitude :-P. **Monika**, for sharing an interest in Hjulet and B cell transcription factors. **Elina**,

for finally joining the clan of mothers taking care of me. **Sharesta**, for liking my knullrufs, and your linguistic knockouts (in any language). **Gabriel**, for being a true tough Viking.

**Anton1 Gisterå**, for inspiration to become more disciplined and good times wrestling and lifting. **Marina**, for not giving a shit about what other people think. **Jacob**, for truly loving Swedish weather and karaoke, and being a kind friend. **Rosa**, for rocking the entrance of MTC and joining bar crawls. **Jonas**, for adding a pub touch to the pub. **Indira**, for having patience with Dr. K even when it's because of me, and still bring great spirits. The dudes at **Hagagrillen**, for all the burgers. **Domingos**, for handing out decent portions of food and a good beating. The other tough Vikings, **Ben**, **Malte**, and honorary viking **Lifeng**, for great days at MTC, and for running going through fire and getting electrocuted together.

The Coquet group – **Chris**, even though I sometimes don't understand Scottish, I can always understand a fellow Zubrowka-fan. Djinghis **Ting** Khan, for being a good sport at the pub and inspiring others with your ambition. **Julian**, for staying true to the beard and esoteric knowledge cravings.

My much missed second family, **Liz**, **Tam**, **Winslow**, **Sebastian**. Thank you for taking care of me and making me feel at home away from home and being the kindest people one could ever hope to meet. For great dinners, ice skating, and welcoming me into your family.

**Adil**, for being the bigger man, handing out second chances and Jäger, and giving me thesis writing aids. **Ninos**, the champion of scissors, for making me feel like a professor. **Tianqi**, for creating memories in Melbourne and making Oxford and China look good. See you there soon. **Mats Sundin**, for enabling science through your contributions, hopefully leading others of your kind to do the same. **Emma Svedin**, for fun collaboration and always reminding me of the great place where we learned about biology. **Markus Larsson**, **Gustaf Wigerblad**, **Jeanette Roos, Emil Wiklundh** for having my back, inspiring me to pursue a PhD, and molding me into something grittier.

My ghey friends from the Bahamas, **Curt** and **Clay**, for fantastic times lobstering, diving and sharing a passion for dive things.

Team Croatia/Anchor & Salsa crew – **AMG**, **CD86**, **AmandaPanda44**, **Anto\m/**, and honorary member **Bojan**, for the greatest adventures ever – including head banging, almost-broken-feet, a donkey, singing nordman on stage, diving, emotional roller coasters and great (after)parties! An extended hand also to Slovenian border control, for enabling the adventures of Team Croatia.

**KP** for tricking me into driving your Porsche and always being ready to celebrate. **Lalle**, for providing helping hands in private life. **Rasmus** for having the biggest heart of gold. **Marie** and **Chris** for epic nights including Averna sours and banana shots, and showing the world the reach of love. **Dawn** and **Andy**, I miss you. **Björn (Daniel)**, for discussing deep things and having a sincere opinion and interest in music. **Hellsing**, for making me feel cool and smart. **Lars**, for rocking the 80s together and looking like Christian Bale. **Niko**, for having the guts to change the course of life. **Siamak**, for inspiring to conquer life, in spite of obstacles. **Östberg**, for fun times in Berlin and elsewhere. **Yashar**, my twin soul in innebandy, for teaching me how to make mayo, and sharing my passion for beer. **Joel**, for liking science and being norrländsk in the best way. **Sebastian**, for multminding. **Ola (Alexander)**, for an awesome voice combined with a truly kind person.

**Alf**, and **Britt-Inger**, for the love, support and infinite kindness.

The Lundberg family – **Elisabet**, **Ronald**, **Johan**, **Henrik**, for good times at Saltarö, a beautiful wedding, concerts, and being a second family, and especially my bfam **Marcus** – for TV adventures and being there when I need ÅJ and everything between.

**Nipor**, for staying true to art, friends and family. **Majkel**, for being the best friend imaginable, always ready to help or listen or up for some boxing. **Olof**, for sharing doubts, an appetite for destruction and adventure, and co-creating a new force in music. **Linus**, for creating memories that will make a great book someday.

**Carro**, for the true love we've shared, and for being a source of inspiration.

**Hjertquists** and **Bäcks** – For the far too rare get-togethers and occasional bumping into each other, at Arlanda, Bromma, Sundsvall, Lemon or wherever. Let's not be strangers.

**Henrik**, **Cissi**, **Agnes** and **Eskil**, for dinners, play, brutal honesty, and building lego houses together. **Andreas**, the first incarnation of us, for always looking after me, and being enthusiastic about whatever I do in life. **Maria**, my sister (in-law), for your interest in my progress and for teaching me how to peel shrimps. **Liam**, for lighting up Christmas. **Erik** and **Hannalis**, for Cubans, and for hosting me in hellhole soon. I am so happy for you guys! **Kiara**, woof woof.

My **mother** and my **father** - you have given me everything. Thank you.

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