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CHARACTERIZATION OF B CELL RESPONSE ALTERATIONS RESULTING FROM ABLATION OF IKBNS EXPRESSION

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Characterization of B cell response alterations resulting from ablation of IκBNS expression

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Today me will live in the moment
unless it’s unpleasant
in which case
me will eat a cookie

- Cookie Monster

ABSTRACT

The immune system is a sophisticated organization of cells and tissues that cooperate in safeguarding the integrity of our body by eliminating internal and external threats. Vaccines are the most impactful medical intervention and have contributed tremendously to lowering mortality rates of infectious diseases. The protective immunity induced by vaccination is mediated by eliciting neutralizing antibodies that are sustained for years or even for a lifetime. Antibodies are produced by plasma cells, which are generated from activated and terminally differentiated B cells. Many processes regulating B cell development and function are under the regulation of the NF-κB signaling pathway. Defects in NF-κB signaling have been shown, using mouse models, to be detrimental to the development of distinct B cell subsets as well as their ability to participate in humoral responses. In humans, mutations of components in the NF-κB pathway are increasingly associated with common variable immunodeficiencies. In this thesis, the role of IκBNS, a nuclear regulator of the NF-κB pathway, was investigated to gain a more comprehensive understanding of B cell development and function.

In **paper I**, we addressed the absence of B-1a cells in IκBNS-deficient *bumble* mice. We identified a precursor population in wildtype mice of $IgM^{+}CD93^{+}CD19^{+}CD5^{+}$ transitional B-1a cells (TrB-1a), which exhibited other indicators of B-1 lineage commitment, such as natural IgM secretion and increased Igλ expression. We did not detect TrB-1a cells in *bumble* mice whereas the neonatal B-1 progenitor cell (B-1p) population was intact. These results indicate that IκBNS is required for the transition from B-1p to the TrB-1a stage. In **paper II**, we investigated potential reasons for the impaired T cell-independent (TI) responses in *bumble* mice. We found impaired expression of the surface receptor TACI, which is essential in responses to TI antigens, and reduced responsiveness to its ligands in *bumble* mice. In addition, *bumble* B cells did not fully silence Pax5 expression and exhibited rapid upregulation of Blimp-1 during early division cycles. Thus, these results suggest that IκBNS is involved in TACI expression and function as well as in transcriptional regulation of PC differentiation. In **paper III**, we used $nfkbid^{B-}$ mice in which IκBNS was selectively depleted from B cells to distinguish between B cell intrinsic and extrinsic defects in T cell-independent (TD) responses. $Nfkbid^{B-}$ mice exhibited normal GC formation, but antigen-specific antibodies of the IgG2c and IgG3 isotype were reduced. Hence, IκBNS appears to be important for class switching during TD responses. In **paper IV**, we developed a protocol for evaluation of plasma cell differentiation from human primary B cells. Cells that exhibited a phenotype resembling plasma cells were identified as $CD38^{+}IRF4^{hi}Pax5^{lo}$ and $CD38^{+}IRF4^{int}Pax5^{lo}$. Both populations were generated when starting from fresh or cryopreserved samples, or isolated memory and naïve B cells. Application of the methods developed in this paper to patient samples could provide more insight into mechanisms underlying immune disorders.

Collectively, the results of this thesis contribute to our understanding of processes that are fundamental to establishing adequate humoral responses and are of direct relevance to immunodeficiency disorders.

LIST OF SCIENTIFIC PAPERS

I. Gabriel K. Pedersen, Monika Ádori, **Sharesta Khoenkhoen**, Pia Dosenovic, Bruce Beutler, Gunilla B. Karlsson Hedestam (2014); B-1a transitional cells are phenotypically distinct and are lacking in mice deficient in IκBNS. *Proc. Natl. Acad. Sci. USA* 111(39): E41119-26

II. **Sharesta Khoenkhoen**, Elina Erikson, Monika Ádori, Jean M. Scholz, Michael P. Cancro, Gabriel K. Pedersen, Gunilla B. Karlsson Hedestam (2019); TACI expression and plasma cell differentiation are impaired in the absence of functional IκBNS. *Immunol. Cell. Biol.* 97(5): 485-497

III. **Sharesta Khoenkhoen**, Monika Ádori, Juan Darío Solis Sayago, Juliette Soulier, Jamie Russel, Bruce Beutler, Gabriel K. Pedersen, Gunilla B. Karlsson Hedestam; IκBNS expression in B cells is dispensable for germinal center formation but required for optimal antibody responses to T-dependent antigens. *Manuscript*.

IV. **Sharesta Khoenkhoen**, Monika Ádori, Gabriel K. Pedersen, Gunilla B. Karlsson Hedestam; Flow cytometry-based protocols for the analysis of human plasma cell differentiation. *Manuscript in revision for Front. Immunol.*

LIST OF RELATED PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Gabriel K. Pedersen, Monika Ádori, Julian M. Stark, **Sharesta Khoenkhoen**, Carrie Arnold, Bruce Beutler, Gunilla B. Karlsson Hedestam (2016); Heterozygous mutation in IκBNS leads to reduced levels of natural IgM antibodies and impaired responses to T-independent type 2 antigens. *Front. Immunol.* 7:65

II. Monika Ádori, Gabriel K. Pedersen, Csaba Ádori, Elina Erikson, **Sharesta Khoenkhoen**, Julian M. Stark, J. H. Choi, Pia Dosenovic, Mikael C. I. Karlsson, Gunilla B. Karlsson Hedestam (2018); Altered marginal zone B cell selection in the absence of IκBNS. *J. Immunol.* 200(2): 775-787

III. Gabriel K. Pedersen, Xiaohong Li, **Sharesta Khoenkhoen**, Monika Ádori, Bruce Beutler, Gunilla B. Karlsson Hedestam (2018); B-1a cell development in splenectomized neonatal mice. *Front. Immunol.* 9:1738

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Ag
AID	Activation-induced deaminase
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
ASC	Antibody-secreting cell
B-1p	B-1 progenitor cell
BAFF	B cell-activating factor
BAFFR	B cell-activating factor receptor
Bcl-6	B cell lymphoma 6 protein
Bcl-10	B cell lymphoma 10 protein
BCMA	B cell maturation antigen
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
B_{reg}	Regulatory B cell
CARD11	Caspase recruitment domain-containing protein 11
CD	Cluster of differentiation
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CGG	Chicken gamma globulin
CLP	Common Lymphoid Progenitor
CSR	Class switch recombination
CXCL	C-X-C-motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
D	Diversity
DAMP	Danger associated molecular pattern
DNA	Deoxyribonucleic acid
DZ	Dark Zone
FDC	Follicular dendritic cell

FOB	Follicular B cell
GC	Germinal centre
HSC	Hematopoietic stem cell
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
Igκ	Immunoglobulin kappa
IgL	Immunoglobulin light chain
Igλ	Immunoglobulin lambda
IκB	Inhibitor of NF-κB
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
i.p.	intraperitoneal
IRF4	Interferon Regulatory Factor 4
i.v.	Intravenous
J	Joining
LN	Lymph node
LPS	Lipopolysaccharide
LZ	Light Zone
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MHC	Major histocompatibility complex
mROS	Mitochondrial reactive oxygen species
MZB	Marginal zone B cell
NFKBID	Nuclear Factor of Kappa light polypeptide gene enhancer in B cell Inhibitor Delta
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP	Non-human primate
NK	Natural killer cell
NKT	Natural killer T cell
NLS	Nuclear localisation signal

NP	4-hydroxy-3-nitrophenyl
PAMP	Pathogen associated molecular pattern
Pax5	Paired box protein 5
PB	Plasmablast
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PRR	Pattern recognition receptor
RAG	Recombination activating gene
RNA	Ribonucleic acid
rSFV-ßGal	Recombinant Semliki Forest virus ß-Galactosidase
ROS	Reactive oxygen species
RSS	Recombination signaling sequence
S1P	Spingosine-1-phosphate
s.c.	subcutaneous
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
SLC	Surrogate light chain
TACI	Transmembrane Activator and CAML interactor
T-bet	T-box expressed in T cells
TD	T cell-dependent
TdT	Terminal deoxynucleotidyl transferase
T_{FH}	T follicular helper cell
TI	T cell-independent
TLR	Toll-like receptor
TrB	Transitional B cell
TrB-1a	Transitional B-1a cell
T_{reg}	Regulatory T cell
V	Variable
VLP	Virus-like particle

1 INTRODUCTION

This thesis addresses the role of IκBNS, a nuclear regulator of the NF-κB pathway, in B cell development and function. This chapter provides background information relevant to the field of B cell immunology and the work presented in this thesis.

1.1 Overview of the immune system

The immune system consists of a wide variety of molecules, cells, and tissues that collaborate towards the common goal of protecting the body from infectious pathogens and maintaining homeostasis. The epithelial and mucosal surfaces outside and inside our body, antimicrobial peptides, acidity of the stomach, and commensal microbiota in the gut serve as a first line of defence. Once these barriers are breached by an invading pathogen or a wound, the immune system comes into play. Generally, the immune system is distinguished into two branches: the innate and adaptive immune system.

The innate immune system initiates immune responses based on the recognition of foreign structures or altered host structures, and thereby identifies potential threats. Soluble factors belonging to the innate immune system include anti-microbial peptides, complement proteins, and natural serum immunoglobulin, which control the early phase of infection or tissue damage and initiates immune responses. Many immune cells express pattern recognition receptors (PRRs) on their cell surface that enable them to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs and DAMPs are specific to infectious pathogens and damaged host cells, respectively. Neutrophils, monocytes, and macrophages can eliminate pathogens and debris through phagocytosis, whereas natural killer (NK) cells recognize and kill infected cells and (pre)cancerous cells through the release of cytotoxic factors or via death ligands.

Macrophages, dendritic cells (DCs), and B cells are referred to as professional antigen presenting cells (APCs) as they present antigen (Ag) on MHCII molecules to cells of the adaptive immune system to induce their activation, differentiation, and acquisition of effector functions. Key characteristics of the adaptive immune system are diversity, specificity, and memory. The antigen receptors of B cells and T cells are generated through recombination of gene segments, that allow for a high diversity of specificities in the adaptive immune repertoire. Moreover, B cells can undergo affinity maturation and class-switching to fine-tune the humoral response to the invading pathogen. $CD4^+$ T cells promote B cell activation, whereas $CD8^+$ T cells mediate cellular immunity against intracellular pathogens or eliminate altered cells. Both B cells and T cells can be re-programmed to become memory cells that circulate and survey the periphery. Upon secondary encounter of a pathogen, these memory cells rapidly differentiate to acquire effector functions. Due to their ability to produce highly effective binding and neutralising antibodies, B cells are key mediators of long-lasting protective immune responses induced by vaccines.

1.2 B cell subsets

Mature B lymphocytes can be distinguished into three main subsets: follicular B cells (FOBs), marginal zone B cells (MZBs), and B-1 cells. These B cell subsets are different in phenotypic and functional characteristics, anatomic localisation, migratory capacity, and ontogeny. The B-1 cell population, also referred to as the B-1 lineage, is further subdivided into B-1a and B-1b cells. The generation of B-1 cells occurs predominantly in the foetal liver and wanes after birth. Maintenance of a stable B-1 population is dependent on *in situ* self-renewal (Kantor et al., 1995). FOBs and MZBs, commonly referred to as the B-2 lineage, are generated and maintained via continuous replenishment from precursor cells residing in the bone marrow (Osmond, 1986). B-1 and MZB cells are generally activated in a T cell-independent manner while follicular B cells are activated in a T cell-dependent manner. Due to the ability of marginal zone B cells and B-1 cells to provide early responses, they are considered to be innate-like lymphocytes that functionally connect innate to adaptive immunity. Apart from antibody production, B cells also contribute to shaping the immune response by acting as APCs through Ag presentation on MHCII molecules and secretion of cytokines, such as IL-10.

1.2.1 Follicular B cells

Naïve follicular B cells circulate throughout the periphery and reside in follicular niches that are in close proximity to T cell zones in secondary lymphoid tissues, such as the spleen, lymph nodes, and Peyer's patches (Allman and Pillai, 2008). Homeostasis and survival of follicular B cells in the follicular area is dependent on BAFFR signaling (Rauch et al., 2009). FOBs are important for mediating TD antibody responses to protein antigens, which requires GC formation (see section 1.4.3). Activation and differentiation of FOBs into antibody secreting cells is achieved through synergistic signals from the BCR, CD40, and TLRs (Genestier et al., 2007). FOBs can differentiate into short-lived plasma cells during the early phase of the TD response, as well as become long-lived plasma cells after migration and homing to the bone marrow (Genestier et al., 2007).

1.2.2 Marginal zone B cells

Marginal zone B cells are important for humoral responses against blood-borne pathogens and can rapidly differentiate into plasmablasts and short-lived plasma cells upon antigen activation. MZB cells populate the splenic marginal zone near the marginal sinus, which is dependent on their expression of the spingosine-1-phospate receptors S1P1 and S1P3 (Kraal, 1992; Cinamon et al., 2004). Upon capture of antigen bound by immune complexes, MZBs can shuttle between the marginal zone and the follicular area for antigen presentation to naïve T cells and thereby promote TD responses (Attanavanich and Kearney, 2004; Cinamon et al., 2004). In addition, MZB cells express CD1d on their surface, which enables them to present lipid antigens to NK T cells and produce anti-lipid antibodies (Leadbetter et al., 2008).

1.2.3 B-1 cells

B-1 cells predominantly reside in peritoneal and pleural cavities and spontaneously secrete polyreactive and broadly neutralizing IgM, and are therefore the main source of natural antibodies found in serum (Hayakawa et al., 1986). These natural antibodies have specificities against self-antigens, such as oxidized lipids, annexin IV and phosphatidylcholine, and thereby contribute to clearance of atherosclerotic lesions, apoptotic cells and cellular debris (Chou et al., 2009; Kulik et al., 2009). Natural IgM can also assist in the clearance of (pre)cancerous cells through recognition of tumour-associated proteins (Brändlein et al., 2003). The presence of tumour-associated IgM antibodies in patients can be used as a diagnostic tool for early detection of cancer (Díaz-Zaragoza et al., 2015). In addition, common viral and bacterial antigens containing multivalent epitopes, such as phosphorylcholine and lipopolysaccharides, are also detected by natural IgM (Briles et al., 1981). The important role of B-1 cells in maintaining homeostasis and providing the first line of defence against infections is thus attributed to their production of natural IgM.

The majority of the B-1 cell lineage expresses intermediate levels of CD5 on their surface and are termed B-1a cells, while CD5-negative B-1 cells are termed B-1b cells (Hayakawa et al., 1986). In addition, B-1b cells are capable of generating protective long-term TI responses to infectious pathogens, and thereby contribute to adaptive immunity as well (Alugupalli et al., 2004; Haas et al., 2005). The homing of B-1 cells to the peritoneal and pleural cavity has been shown to depend on the chemokine CXCL13, which is produced by peritoneal macrophages and cells in the omentum (Ansel et al., 2002). During infections, migration of activated B-1a cells towards secondary lymph nodes and subsequent differentiation into IgM secreting cells is regulated via TLR signaling and type I IFNs, and followed by a loss of CD5 expression (Ha et al., 2006; Waffarn et al., 2015; Savage et al., 2019).

1.3 B cell development

The production of B cells is a process that commences *in utero* and continues throughout life. B cells, like all cells in the body, are derived from hematopoietic stem cells (HSCs) and transition through several developmental stages before becoming functional mature B cells. B cell development is characterized by expression of surface markers, the assembly of the BCR, and selection mechanisms. It is currently thought that B cell development occurs in three waves; the first wave originates from the yolk sac and the para-aortic splanchnopleura and generates B-1 cells, the second wave stems from foetal liver HSCs and predominantly produces B-1 and MZBs , whereas the third wave is comprised of bone marrow HSCs which gives rise to mainly the B-2 lineage (Fig. 1).

1.3.1 Foetal B cell development

As early as embryonic day (E) 9.5, cells are present in the yolk sac that have the potential to give rise to lymphoid lineages. These yolk sac cells have been defined as $CD45^{+}c\text{-}Kit^{hi}CD93^{+}$ but do not express Sca-1, a definitive marker of HSCs, and are therefore considered pre-haematopoietic stem cells (pre-HSCs) (Yamane et al., 2009; Ito et al., 2013). Cells derived from the aorta-gonad-mesonephros (AGM) region, which develops from para-aortic splanchnopleura (P-Sp), are able to reconstitute multiple myeloid and lymphoid lineages, including B cells, before cells from the yolk sac acquire the potential to generate lymphomyeloid cells (Cumano et al., 1996). For this reason, the P-Sp is considered the first tissue to harbour B cell potential, and by E10.5, it becomes an important source of definitive HSCs (Taoudi et al., 2005). Around E11, HSCs migrate from the yolk sac and AGM region, populate the foetal liver, and undergo expansion.

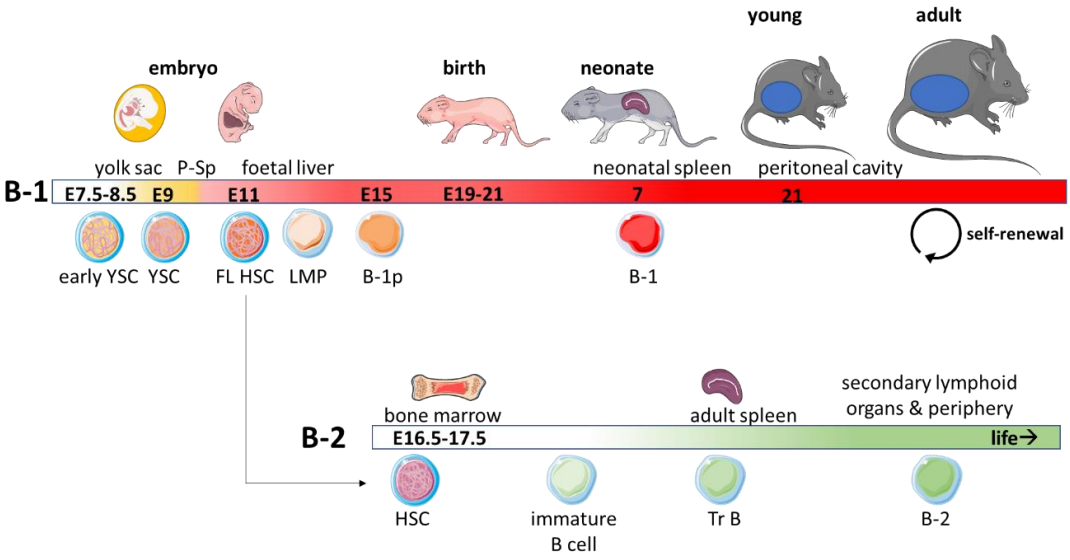

Figure 1. The emergence of cells in the haematopoietic system with B cell potential.
P-Sp = para-aortic splanchnopleura, YSC = yolk sac cell, FL HSC = foetal liver haematopoietic stem cell, LMP = lymphomyeloid progenitor cell, B-1p = B-1 progenitor cell, Tr B = transitional B cell.

Here, they develop into $CD45^+$c-$KIT^+FLT3^+IL7Ra^+$ lymphomyeloid progenitor cells (LMP) (Böiers et al., 2013; Kobayashi et al., 2014). Distinct progenitor cells of the B-1 lineage (B-1p) were identified as $Lin^-CD45^{lo/-}CD93^+CD19^+$ in foetal liver at E11 already (Montecino-Rodriguez et al., 2006). Shortly after osteogenesis is initiated in the embryo, FL HSCs migrate from the foetal liver to the bone marrow, where they expand and reside throughout life as adult HSCs. Transfer experiments of B-1p from the foetal bone marrow have shown that they preferentially reconstitute $CD5^+$ B-1a cells, whereas B-1p from adult bone marrow generates $CD5^-$ B-1b cells(Kikuchi and Kondo, 2006). Around the time of birth and up to two weeks after, the neonatal spleen becomes a prominent location of B-1 cell development. Between 7 and 21 days of age, B-1 cells from the spleen populate the peritoneal cavity, where they persist through self-renewal. Once the B-1 cell population is established in the peritoneal cavity, contribution of adult bone marrow HSCs to this population is limited (Lalor et al., 1989).

1.3.2 BCR rearrangement and selection in the bone marrow

During B cell development the progenitor and precursor cell stages can be characterized by expression of surface markers as well as the rearrangement status of the membrane-bound immunoglobulin molecule, which is known as the B cell receptor (BCR) (Fig.2). The generation of the BCR is achieved through step-wise recombination of gene segments encoded by the immunoglobulin loci (Tonegawa, 1983). The BCR consists of two immunoglobulin heavy chains (IgH) combined with two immunoglobulin light chains (IgL). Both chains contain a constant C-terminal region that is invariable between antibodies of the same immunoglobulin class, while the N-terminal regions are highly variable. The variable regions are encoded by variable (V_H), diversity (D_H), and joining (J_H) segments in the heavy chain. The light chain is only encoded by variable (V_L) and joining (J_L) segments. The variable regions of the heavy and the light chains form the antigen-binding site and determine the binding specificity of the BCR (Alt et al., 1987).

The sequential DNA rearrangement of the IgH and IgL loci, a mechanism referred to as V(D)J recombination, is pivotal for successful assembly and expression of the BCR. V(D)J recombination occurs primarily in foetal liver and bone marrow in progenitor (pro-) and precursor (pre-) B cells (Fig. 3). First, rearrangement of the D_H and J_H gene segments followed by rearrangement of the V_H gene segment to the D_HJ_H segment in the IgH loci occurs during the pro-B cell stage. After successful recombination, the IgH chain associates with the surrogate light chain (SLC), which will lead to translocation to the cellular membrane where they form the pre-BCR complex together with the Igα and Igβ chains. Signaling mediated by the pre-BCR complex is required for pre-B cell survival and serves as a checkpoint for successful recombination (Mårtensson and Ceredig, 2000).

Subsequently, rearrangement of the IgL gene segments occur at the pre-B cell stage during which preferentially the Igκ gene loci will be rearranged prior to the Igλ loci. The sequential order of V(D)J recombination ensures that only one particular BCR will be expressed by a B cell, a mechanism which is referred to as allelic exclusion (Nussenzweig et al., 1987). After expression of a functionally rearranged BCR, the pre-B cell will become an immature B cell and will be surface IgM positive (sIgM$^+$). These immature sIgM$^+$ B cells migrate to the spleen, from where they will develop further through transitional B cell stages and mature into follicular B cells, marginal zone B cells, or B-1 cells (Allman and Pillai, 2008; Montecino-Rodriguez and Dorshkind, 2011).

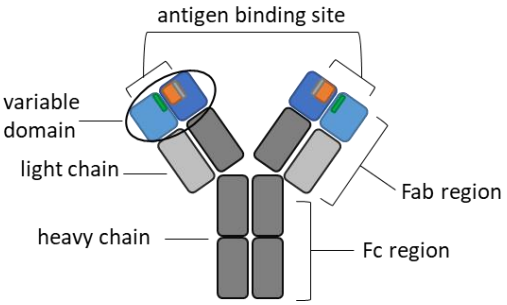

Figure 2. Antibody structure.

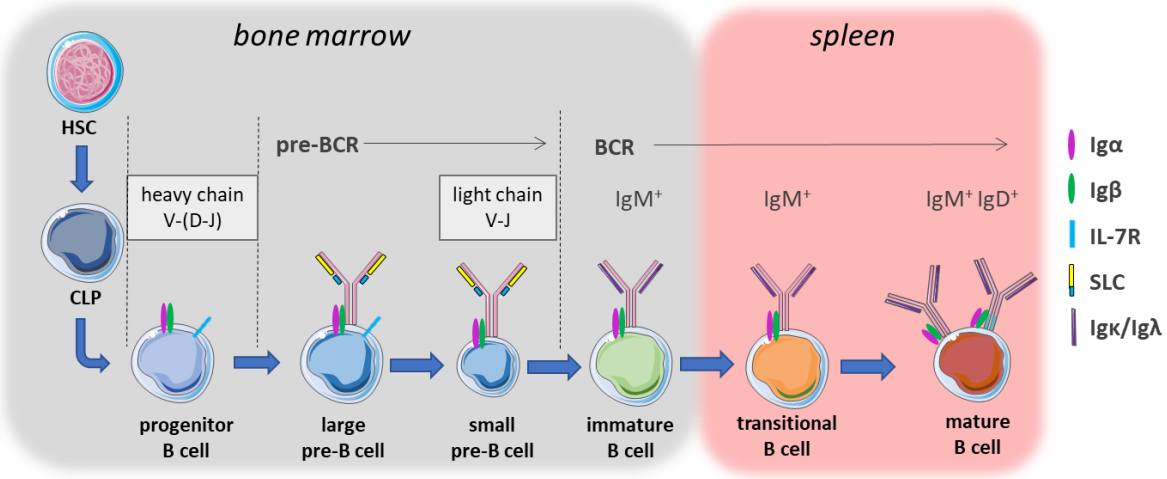

Figure 3. Stages of B cell development and maturation in bone marrow and spleen and the corresponding status of BCR rearrangement and expression.

1.3.3 B cell receptor rearrangement

The generation of a functional and unique BCR is dependent on the rearrangement of the germline V(D)J segments (Fig. 4). All V, D, and J gene segments are flanked by a recombination signal sequence (RSS), which are recognized by the enzymes recombination activating genes (RAG) 1 and 2 (Jung and Alt, 2004; Schroeder and Cavacini, 2010; Schatz and Ji, 2011). RAG1 and RAG2 locate to the RSS of the gene segments that recombine together and introduce double-strand DNA breaks, which form closed hairpin structures. Endonucleases are recruited to cleave the hairpin structures to allow for ligation of the gene segments through the DNA repair mechanism of non-homologous end joining (NHEJ).

During NHEJ, the two ends of the coding sequences need to be paired and thus palindromic sequences (P-nucleotides) could be added to enable pairing of otherwise non-matching ends. Additionally, the gene segment ends are exposed to exonucleases and the enzyme terminal deoxynucleotidyl transferase (TdT), which remove nucleotides and insert random N-nucleotides in the coding ends, respectively (Schroeder and Cavacini, 2010). These mechanisms contribute to further increase the diversity of the V(D)J junction. The diversity of the BCR repertoire forms the basis for our ability to mount protective immune responses to a broad range of infectious pathogens.

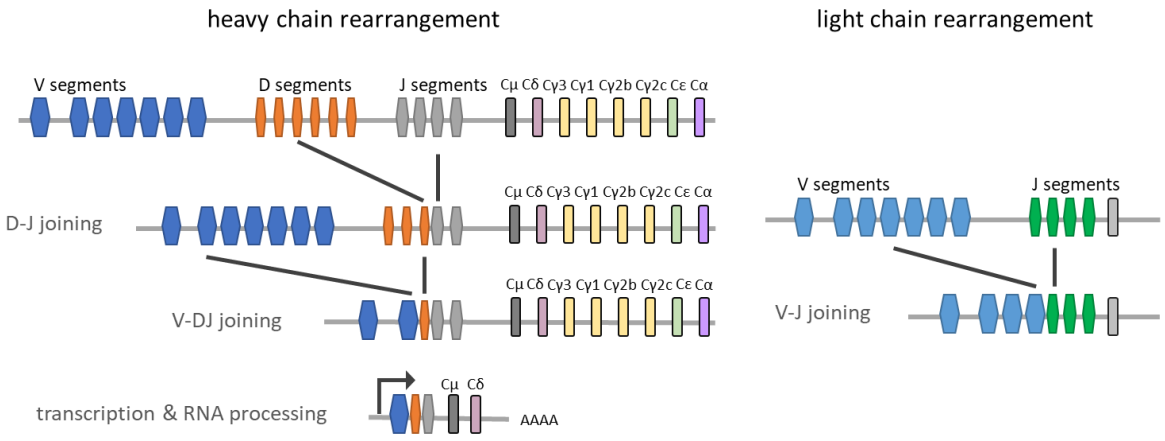

Figure 4. Rearrangement of heavy and light chain V(D)J genes.

1.3.4 Transcriptional regulation of B cell development

The transcriptional programme that controls foetal versus adult B cell development is not well characterized. Lin28b is an RNA-binding protein that has recently been described as a regulator of B-1a cell development during foetal haematopoiesis (Kristiansen et al., 2016). Lin28b prevents degradation of Arid3a mRNA transcripts by the Let-7b microRNA (Zhou et al., 2015). Expression of the transcription factor Arid3a promotes light chain recombination simultaneous to heavy chain recombination in pre-B cells, enabling them to bypass the pre-BCR checkpoint (Hayakawa et al., 2019; Wong et al., 2019). Arid3a has also been shown to induce the expression of the transcription factor Bhlhe41, which is involved in the regulation of B-1a cell development and persistence (Kreslavsky et al., 2017; Hayakawa et al., 2019). In addition, the transcription factor Oct2 has been shown to regulate IL-5Rα expression in B-1 cells, thereby contributing to their function and survival (Emslie et al., 2008).

1.4 B cell activation

B cells recognize antigen through the BCR, which is membrane-bound immunoglobulin. B cell activation occurs in secondary lymphoid organs where they encounter antigen and undergo proliferation, maturation, and differentiation. Considering the vast diversity of pathogens, humoral immune responses are adapted towards each individual pathogen for efficient clearance. The activation and differentiation of B cells largely depends on the type of antigen but is also influenced by interactions with other immune cells and cytokines. Ultimately, the quality of the antibody response is determined by antibody affinity, epitope specificity, titre, effector function mediated by the antibody isotype, and the kinetics of the response.

1.4.1 T cell independent and T cell dependent responses

Antibody responses are classified as either T cell-independent (TI) or T cell-dependent (TD) (Fig. 5). TI antigens are capable of inducing B cell responses in the absence of T cell help. TI antigens can be further designated as 'TI type 1' (TI-1) or 'TI type 2' (TI-2) antigens depending on whether they elicit antibody responses in CBA/N mice, which are unable to signal through the BCR as they are deficient in Bruton's tyrosine kinase (Btk) (Mosier et al., 1977). TI-1 antigens, such as lipopolysaccharide (LPS), CpG, and viral RNA, engage and signal through Toll-like receptors (TLRs) to activate B cells (Coutinho et al., 1974; Bekeredjian-Ding and Jego, 2009). TI-2 antigens are generally multivalent antigens that extensively ligate BCRs and thus require intact signaling through Btk in order to effectively activate B cells. Examples of TI-2 antigens are bacterial capsular polysaccharides from *Streptococcus pneumoniae*, *Haemophilus influenza* type b, and *Neisseria meningitides*, but also includes repetitive determinants on virus particles (Mond et al., 1995; García de Vinuesa et al., 1999b). TD responses are elicited by protein antigens that are recognized specifically by the BCR and require additional T cell help to activate B cells. Full activation and proliferation of B cells in response to these antigens depends on T cell help through CD40L-CD40 interaction and secretion of cytokines, such as IL-21, IL-4 or IFN-γ (Bryant et al., 2007; Cannons et al., 2010). A hallmark of TD responses is the formation of germinal centres (GCs) where B cells undergo affinity maturation and selection (see section 1.3.3).

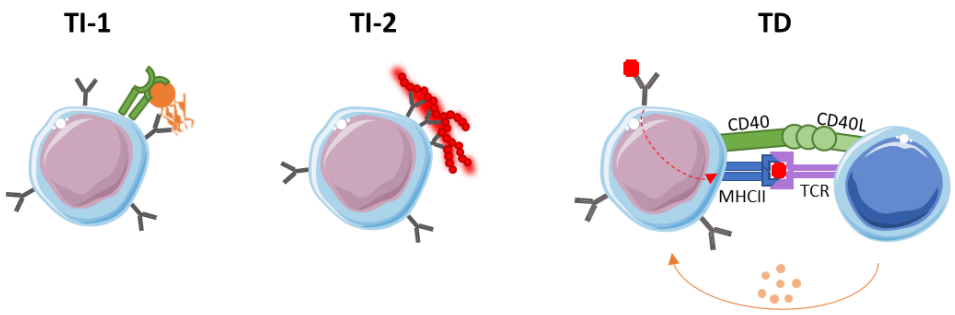

Figure 5. B cell activation during TI and TD responses.

1.4.2 Extrafollicular response

After antigen-induced activation of B cells in the spleen or lymph node, differentiation continues either in the follicular area as germinal centre B cells or in the extrafollicular area as plasmablasts (Fig. 6). B cells committing to differentiation via the extrafollicular route will localize at the boundary between the T cell zone and the red pulp within the spleen or the medullary cords within the lymph nodes (Toellner et al., 1996; Luther et al., 1997). Migration from the follicular zone to the extrafollicular area is mediated by CXCR4 expression, which increases the responsiveness to the chemokine CXCL12, and downregulation of CXCR5 and CCR7, which reduces their responsiveness to CXCL13, CCL19, and CCL21 respectively (Hargreaves et al., 2001). Migration into the extrafollicular area occurs within 24 hours of antigen encounter, and commitment to plasmablast differentiation is already observed at 48 hours as evidenced by high expression of B lymphocyte-induced maturation protein-1 (Blimp-1) which is sustained afterwards (Vinuesa et al., 2001). Extrafollicular plasmablast differentiation and survival is supported by $CD11c^{hi}$ DCs and completely independent of T cell help (García De Vinuesa et al., 1999a). The frequency of extrafollicular plasmablasts peaks at day 7-8 after immunization or infection and then declines as cells undergo apoptosis (Jacob et al., 1991; Smith et al., 1996). The rapid generation of plasmablasts and short-lived plasma cells from the extrafollicular response is essential for the early control of infections, whilst adaptive responses are initiated.

1.4.3 Germinal centre response

Affinity matured B cells are generated through germinal centres (GCs). GCs are structural microenvironments induced by antigen-activated B cells in the lymph nodes and spleens (MacLennan, 1994; Toellner et al., 2002). Geminal centre formation is observed four days after antigen encounter, peaks around day 14, and declines afterwards (Toellner et al., 1996). GCs facilitate interaction between B cells and T helper cells (T_{FH} cells) and follicular dendritic cells (FDCs) in order to support affinity maturation and selection (Garside et al., 1998; Cannons et al., 2010). Antigen-activated B cells exhibit induced expression of the chemokine receptors CCR7 and EBI2, allowing them to migrate to the border with the T cell zone where they encounter antigen-activated T cells (Reif et al., 2002; Pereira et al., 2009). High expression of the transcription factor B cell lymphoma protein 6 (Bcl-6) results in downregulation of EBI2, promoting B cell migration towards the centre of the follicle to initiate the GC reaction (Shaffer et al., 2000). In addition, migration of both B cells and T_{FH} cells requires upregulation of CXCR5 and downregulation of CCR7, which are also under the control of Bcl-6 (Vinuesa et al., 2005). After migration of antigen-activated B cells to the interface of the T and B cell zone, B cells present antigen-derived peptides on MHC class II molecules to T_{FH} cells (Victora et al., 2010). GC B cells with increased BCR affinity are more efficient at presenting antigen to T_{FH} cells, and those that are able to form stable interactions with T_{FH} cells, as demonstrated by increased levels of ICAM-1 and SLAM, exhibit higher levels of IRF4 (Victora et al., 2010; Ise et al., 2018).

The GC consists of two distinct areas referred to as the dark zone and the light zone. The dark zone is where B cells proliferate and undergo somatic hypermutation (SHM), while the light zone is where affinity-based selection takes place. GC B cells induce the expression of the enzyme activation induced cytidine deaminase (AID) which mediates SHM and class-switching. AID introduces random point mutations mainly into the immunoglobulin variable (V) region gene segments which could alter the affinity of the BCR. The processes of B cell selection and differentiation in the germinal centre is regulated by T_{FH} cells and follicular dendritic cells (FDCs). For example, T_{FH} cells express CD40L and thus induce CD40 signaling in B cells, and secrete cytokines essential for survival, differentiation, and proliferation, such as IL-4, IL-10, IL-21, and IL-2 (Bryant et al., 2007). In addition, GC B cells upregulate ligands for PD-1 and the interaction with PD-1 expressed on T_{FH} cells and FDCs seems important for selection and generation of plasma cells (Good-Jacobson et al., 2010). It was shown that the selection pressure arises from limited availability of T cell help. GC B cells with higher affinity are able to take up more antigens from FDCs, and consequently present more antigen-derived peptides on MHC class II to T cells, thereby outcompeting cells with lower affinity (Victora et al., 2010). The final outcome of GC responses are plasma cells, some of which become long-lived plasma cells upon migration and homing in the bone marrow niche where they can survive and confer protective immunity for years, and memory B cells that circulate through the periphery and induce rapid recall responses upon encountering the same or a similar antigen.

1.4.4 Plasma cell differentiation

Plasma cells (PCs) are terminally differentiated B cells that fulfil the highly specialized function of continuously producing and secreting antibodies. The morphology and gene expression pattern of plasma cells reflect their capacity for antibody production and is distinct from undifferentiated B cells. The process of plasma cell differentiation depends on both induction of a PC specific gene expression programme as well as silencing of the B cell gene expression programme. Therefore, the transition of an activated B cell into a plasma cell is coordinated and tightly regulated by several key transcription factors.

Expression of transcription factor paired box protein 5 (Pax5) is essential for development of the B cell lineage and the maintenance of B cell identity in mature B cells (Urbánek et al., 1994; Horcher et al., 2001). Induction of Pax5 in common lymphoid progenitor cells signifies commitment to the B cell lineage, in which it regulates expression of surface receptors that are essential to B cell function, such as CD19, CD21, CD23, and Igα. Simultaneously, Pax5 suppresses genes that are specific to progenitor cells, myeloid cells, T cells, and plasma cells (Carotta et al., 2006; Delogu et al., 2006). Even though increased Pax5 levels are associated with initial activation of B cells and early events of PC differentiation, efficient terminal PC differentiation is linked to Pax5 silencing (Lin et al., 2002; Nera et al., 2006). B cell differentiation is regulated in a dose-dependent manner by the transcription factor interferon-

regulatory factor 4 (IRF4). Low expression of IRF4 in activated B cells induces the germinal centre programme via Bcl-6, whereas high expression of IRF4 represses the GC programme and instead initiates plasma cell differentiation (Sciammas et al., 2006; Ochiai et al., 2013). High expression of IRF4 induces Blimp-1, which directly suppresses Pax5 expression. However, initiation of plasma cell differentiation has been shown to be induced upon reduced Pax5 levels in a Blimp-1 independent manner (Kallies et al., 2007). Blimp-1 function is crucial for terminal differentiation and maintenance of the PC phenotype (Minnich et al., 2016; Tellier et al., 2016). The migration of PCs generated in secondary lymphoid organs towards bone marrow depends on the expression of CXCR4 and its ligand CXCL12, which is expressed on BM stromal cells (Hargreaves et al., 2001). Signaling downstream of CXCR4 needs to be intact for terminal differentiation and longevity in the bone marrow niche (Good-Jacobson et al., 2015; Biajoux et al., 2016).

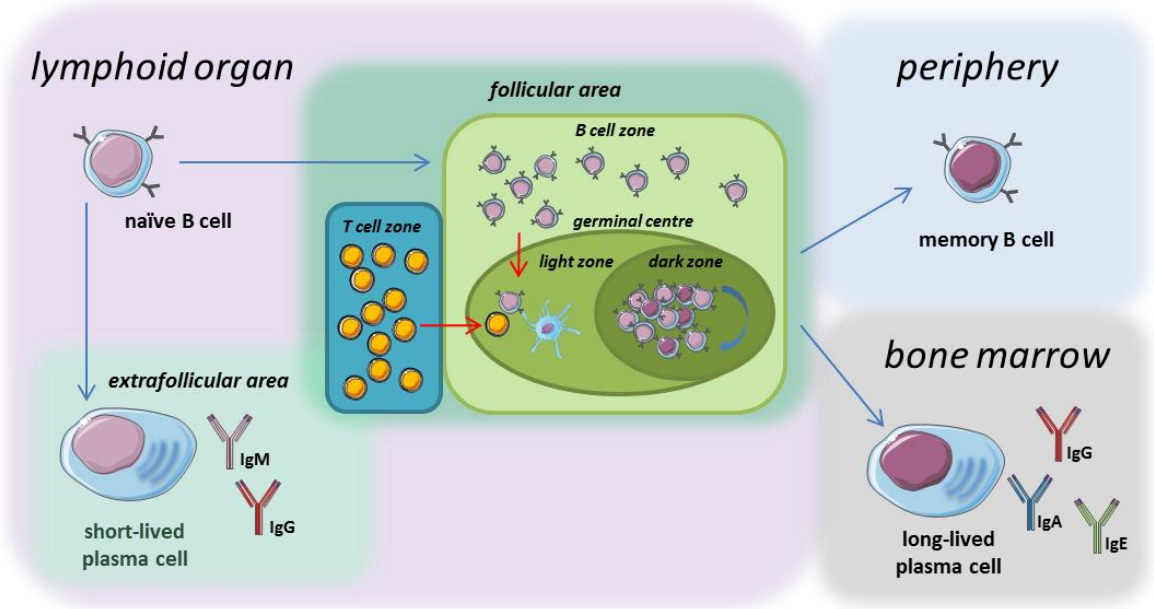

Figure 6. Schematic overview of B cell activation and differentiation.

1.5 NF-κB signaling in B cells

Many of the processes that occur during B cell development require intact signaling of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) proteins. Activation of the NF-κB pathway can be induced via signaling through the BCR (classical pathway) or via signaling through TLRs and cytokine receptors (classical and alternative pathway).

1.5.1 The role of NF-κB in B cells

The NF-κB signaling pathway has been implicated in several stages of B cell development. For instance, NF-κB is required for signaling induced by the pre-BCR, which will prevent elimination of pre-B cells expressing functionally rearranged pre-BCRs and thus allows for progression to the next developmental stage. Furthermore, NF-κB activity is indispensable for survival of immature B cells in the spleen (Franzoso et al., 1997). Studies using mice in which NF-κB signaling is compromised have led to increased understanding of the function of NF-κB in B cell development. Defects in the classical NF-κB signaling pathway primarily affect the development of B-1 and MZB cells, whilst defects in the alternative NF-κB signaling pathway disturb the B-2 compartment (Miosge et al., 2002; Pohl et al., 2002; Rauch et al., 2009; Montecino-Rodriguez and Dorshkind, 2011). Previous studies have identified several components required for functional NF-κB activity to be essential for B-1 and MZB cell development, such as the IKK, CARD11-Bcl-10-MALT1 (CBM), and LUBAC complexes (Egawa et al., 2003; Li et al., 2003; Ruefli-Brasse et al., 2003; Xue et al., 2003; Sasaki et al., 2013). With the identification of precursor cells that specifically give rise to cells of the B-1 lineage, it was suggested that the role of NF-κB signaling in the B-1 cell population is restricted to their self-renewal capacity (Montecino-Rodriguez et al., 2006; Montecino-Rodriguez and Dorshkind, 2011). Nevertheless, as the developmental process of the B-1 lineage and regulation thereof through NF-κB activity has not been fully elucidated, future studies are needed to define the differential requirements of distinct NF-κB components for development, maintenance and survival of B-1 cells.

1.5.2 The NF-κB protein family and regulation of their activity

The NF-κB protein family comprises five transcription factors: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB. These NF-κB proteins are capable of binding to DNA as various combinations of homo- and heterodimers, which determine their binding specificity for promotors and enhancers of target genes. RelA, RelB, and c-Rel contain transactivation domains (TADs) and are thought to be able to directly induce transcription of their target genes, whereas p50 and p52 homodimers are thought to function as repressors due to their lack of TADs.

NF-κB activity is regulated by inhibitor of NF-κB (IκB) proteins. The classical IκB family consists of IκBα, IκBβ, IκBε, and the NF-κB precursor proteins p105 (C-terminus IκBγ), p100 (C- terminus IκBδ). Classical IκB proteins bind and sequester NF-κB to retain it in an inactive state in the cytosol by masking their nuclear localization signals (NLS). These IκB proteins can be phosphorylated by the IκB kinase (IKK) complex, resulting in ubiquitination and degradation. Consequently, the NF-κB complexes will be released and translocated to the nucleus where they can induce or repress transcription of target genes. After translocation into the nucleus, the activity of NF-κB dimers is regulated by the atypical nuclear IκB family proteins Bcl-3, IκBζ, IκBNS, IκBη and IκBL. Each IκB protein binds with different affinities to particular hetero-/ homodimers of NF-κB proteins, thus further increasing the range and specificity by which NF-κB regulates its target genes.

1.5.3 IκBNS

IκBNS was first identified as an inhibitor of NF-κB signaling during negative selection in T cells (Fiorini et al., 2002). IκBNS is a protein of 327 amino acids with a molecular weight of 35 kDa. Similar to other IκB proteins, IκBNS contains seven ankyrin repeat domains, which are important for mediating the interaction with NF-κB proteins. IκBNS has been shown to interact with p50 and p52 homodimers, which act as inhibitors of NF-κB target genes by blocking access to the binding sites, as well as with RelA, RelB, and cRel (Fiorini et al., 2002; Touma et al., 2007; Schuster et al., 2012).

Mice deficient in IκBNS lack the B-1 cell population, have reduced frequencies of MZBs, and display impaired responses to TI antigens (Touma et al., 2011; Arnold et al., 2012; Adori et al., 2018). Apart from a role in B cells, IκBNS is also essential to the development of regulatory T cells (T_{regs}) and the function of $CD4^+$ effector cells. The transition of precursor T_{regs} to mature T_{regs} is regulated by IκBNS-induced expression of FoxP3 (Schuster et al., 2012; Schuster et al., 2017; Schuster et al., 2019). In $CD4^+$ effector T cells, IκBNS is involved in the proliferation and function of T_H1, T_H17, and T_{FH} cells (Annemann et al., 2015; Hosokawa et al., 2017). IL-2, IL-10, IL-17A, IL-17F, and GM-CSF production in T_H17 cells depend on IκBNS (Annemann et al., 2015). In T_{FH} cells, expression of CXCR5 as well as transcriptional regulation of Bcl-6 and IL-21 appear to be mediated by IκBNS (Hosokawa et al., 2017).

Finally, IκBNS is also required for dampening the pro-inflammatory immune response in the myeloid lineage. Prolonged p65 activity at the IL-6 promotor has been observed in LPS-activated $IκBNS^{-/-}$ macrophages, suggesting that IκBNS is required for the removal and/ or degradation of p65 (Kuwata et al., 2006). IκBNS associates predominantly with p50 in LPS-activated macrophages, which raises the possibility that alterations in NF-κB activity are established through recruitment of IκBNS to NF-κB target genes by p50 (Hirotani et al., 2005). Enhanced IL-6 production, as well as IL-12p40 and IL-12p70, was also reported for $IκBNS^{-/-}$ DCs (Kuwata et al., 2006). Additionally, TLR-induced IL-10 production in DCs and B cells is dependent on IκBNS (Hirotani et al., 2005; Fujita et al., 2006; Miura et al., 2016).

1.6 Disorders of the immune system

Defects in the immune system can result in failure to clear pathogens and (pre)cancerous cells and/ or the inability to maintain tolerance towards self-antigens. Depending on whether defects are caused by inborn errors or acquired, they are categorised as primary or secondary immune disorders. Secondary immunodeficiencies can develop from infections that interfere with normal functioning of the immune system, such as acquired immune disorder syndrome (AIDS) caused by human immunodeficiency virus (HIV), or from immunosuppressive drugs or cancers. Primary immunodeficiencies (PIDs) are caused by mutations in proteins critical to the development or function of immune cells, resulting in enhanced susceptibility to infections, increased risk of autoimmunity, dysregulated inflammation, and malignancies.

Common variable immunodeficiencies (CVID) are a heterogenous classification within PIDs (Cunningham-Rundles, 2012; Jolles, 2013). CVID patients are typically diagnosed with hypogammaglobulinemia, reduced numbers of isotype-switched memory B cells, suffer from increased susceptibility towards infections of the respiratory and gastrointestinal tract, and have poor vaccine responses (Bonilla et al., 2016). Causative mutations are identified in less than 20% of CVID patients, with the most common mutations found in genes encoding TACI, ICOS and BAFFR, and other proteins required for B cell activation and signaling or interaction with T cells (Castigli et al., 2005; Salzer et al., 2005; Bogaert et al., 2016). Homo- and heterozygous TACI mutations are associated with 8-10% of CVID cases, but these mutations are often also found in healthy individuals, suggesting that additional factors contribute to the development of CVID in a subset of individuals harbouring such mutations (Martinez-Gallo et al., 2013).

The application of next-generation sequencing (NGS) methods enabled the identification of novel mutations in many other genes now associated with PID and CVID (Maffucci et al., 2016; Meyts et al., 2016). Mutations in *NFKB1* and *NFKB2*, encoding the NF-κB precursor proteins p105 and p100, respectively, have been implicated in the pathogenesis of CVID (Chen et al., 2013; Fliegauf et al., 2015; Keller et al., 2017). Additionally, mutations in other components of NF-κB signaling, such as *CARD11*, *BCL10* , and *MALT1* have also been associated with immune dysfunction (Jabara et al., 2013; Stepensky et al., 2013; Torres et al., 2014). This highlights the importance of intact NF-κB signaling for normal immune function as well as the possibility that mutations in other components of the NF-κB pathway potentially cause immune disorders. Genetic and phenotypic characterization of novel mutations can advance our understanding of the human immune system and CVID pathogenesis.

1.7 Animal research models in immunology

Most, if not all, medical breakthroughs are rooted in scientific discoveries using animal research models. The use of animal models is crucial for basic research to increase our understanding of fundamental biological processes, as well as for translational and pre-clinical research to test the safety and efficacy of pharmaceutical compounds and vaccines before approving them for human use. In this thesis, the use of mouse models was essential to the research presented in **papers I-III** with key results forming the basis for the translational work presented in **paper IV**. This chapter provides an overview of the achievements, value, and ethics of experimental animal models in biomedical research with a focus on immunology.

1.7.1 The history, rationale and ethics in the use of animal models

Animals have been used as tools to understand anatomy as early as in the 5th century BC, once it was recognised that certain anatomical and physiological functions were preserved between humans and animals (Crivellato et al., 2007; Natale et al., 2017; Irschick et al., 2019). The fall of the Roman Empire and the subsequent spread of Christian and Islamic ideology halted the progress of medical knowledge. The Renaissance caused a renewed interest in the medical field which led to an increase in vivisections, autopsies, and experiments on animals (Natale et al., 2017). Ever since, the scientific use of animal models has greatly contributed to medical advancements concerning human health and continues to do so (Fig 7).

The necessity of using appropriate animal models has been exemplified by the medical tragedy caused by thalidomide, a popularly prescribed drug between 1957 and 1962 to treat morning sickness in pregnant women, which caused severe birth defects. Before use in humans, thalidomide was tested in rabbits and mice, as it was not known at the time that rodents are less sensitive to the drug than non-human primates (Merker et al., 1988; Vargesson, 2009). The catastrophic outcome of thalidomide usage due to insufficient testing in appropriate animal models, resulted in significant improvements in the procedures and legislation of safety testing of pharmaceutical drugs and advanced the field of toxicology. Nowadays, the proportion of animals used for safety, potency, toxicology, and reproductive studies for pharmaceutical substances as required by law comprises 23% of all animals used in research in Europe. A comparable number of animals are used in translational and applied research (23%), whereas basic research accounts for 48%, and the remainder (9%) is attributed to other purposes such as education, monoclonal antibody production, preservation of species, etc. (EC, 2020).

Naturally, the wellbeing of animals used in research is of great concern. In addition to humane treatment, the quality and reproducibility of experimental results obtained from animals are influenced by their psychological and physical health (Weed and Raber, 2005). The principles known as the 3Rs (replacement, refinement, and reduction) were established in support of animal welfare in research settings (Russell and Burch, 1959). In Europe, regulations concerning the use, wellbeing, (commercial) breeding, handling, and euthanasia methods of research animals are stated in the EU Directive 2010/63/EU, which have been implemented

into the Swedish legislation in 2013. Increased ethical awareness and stricter regulation of animal research over time, combined with technological advances to create transgenic animal models, has shifted the focus of animal research from exploratory to translational and clinical.

1.7.2 The use of animals in immunological studies

The immune system relies on interaction between many different cell types and their environment, and therefore, comprehensive analyses of immune responses require *in vivo* approaches. *In vitro* cell culture methods do not recapitulate the complexity of the immune system, but instead are useful for studying functional properties of immune cells such as their growth, activation, and differentiation (in response to cytokines). Many discoveries in immunology originate from studies using experimental animal models, such as the identification and development of lymphoid organs, haematopoiesis, and the existence and function of distinct lymphocyte subsets (Natale et al., 2017).

The discovery of lymphocytes responsible for antibody production was made in chickens, after the observation that removal of the bursa of Fabricius resulted in the inability to generate antibodies against *Salmonella typhimurium*. The bursa of Fabricius, a lymphoid organ in birds that is not present in mammals, was later found to be the site where B lymphocytes develop and mature (Glick, 1956). The role of the thymus in the production of T lymphocytes was recognized a few years later, in studies using mice that were neonatally thymectomized and lacked a population of smaller lymphocytes involved in the tolerance regulation of tissue grafts (Miller, 1961; Miller, 1962). Considering that mammals lack the bursa of Fabricius, it was initially thought that the thymus was required for the development of all mature lymphocytes. It was not until the late 60s when bone marrow was identified as the mammalian source of antibody producing cells, using cell transfer experiments in irradiated mice (Claman et al., 1966). In subsequent studies comparing chickens that were either bursectomized or thymectomized to chickens were subjected to combined bursectomy and thymectomy, it was shown that humoral immunity was facilitated by large lymphocytes originating from the bursa of Fabricius, and cellular immunity by smaller lymphocytes originating from the thymus (Cooper et al., 1966). Soon thereafter the large and small lymphocytes were renamed as B cells and T cells, referring to their organ of origin.

1.7.3 Mouse models

Mice are the commonly used model for addressing functionally relevant questions in biomedical research, especially within the field of immunology. Most mouse strains are inbred and well characterized, and compared to other species, mice are easy to handle and breed rapidly with a gestation period ranging between 19 and 21 days. Nevertheless, results from immunological research done in the mouse system are not always translatable to the human system due to differences of receptor expression on lymphocytes, differences in function or localisation of lymphocytes, susceptibility to pathogens, etc. In order to bridge the gap between the mouse models and human immune system, mice can be manipulated in such a way that they become more suitable to study human physiology. The transfer of human hematopoietic cells into immunodeficient mice to repopulate them with a functional human immune system for *in vivo* studies is an approach that has been in use for more than 30 years (McCune et al., 1988; Mosier et al., 1988). Depending on the nature and potential of the cells used for transfer, the human immune system can be reconstituted partially or almost completely (Lan et al., 2006; Chung et al., 2015). SCID/NOD IL2Rγ^{null} mice receiving human bone, thymus, and spleen transplants followed by transfer of foetal liver cells, so far seem to harbour the most accurate representation of a human immune system in mice (Chung et al., 2015).

More recent technological advances are focused on generating knock-in mouse strains in which human genes of interest are inserted into the mouse genome. For example, genes encoding the human immunoglobulin variable regions were inserted in the corresponding murine loci, generating mice that express antibodies of which the variable region is human and the constant region murine (Lee et al., 2014; Murphy et al., 2014). These transgenic humanised mice are used for the generation of antibodies for therapeutic purposes and studies of antibody responses against human pathogens. A major caveat of this system is that the expressed BCR repertoire in these mice are restricted to the immunoglobulin alleles of one human individual and is not representative of immunoglobulin diversity observed in the human population.

Another approach is to introduce targeted mutations, inserts, deletions, or recombinations that were identified in patients into the mouse genome to create transgenic mice. This way, genes of interest can be manipulated to recapitulate human disease and allow for mechanistic studies and development of therapeutics *in vivo* (Walsh et al., 2017; Lampreht Tratar et al., 2018). Nevertheless, the general concern for humanised and transgenic animals remains that cytokine and cytokine receptor expression, distribution and function differ between human and murine immune cells. Discrepancies between different model systems can result in an artificial environment with many confounding factors and should be taken into consideration when interpreting results obtained from studies using modified animals.

1.7.4 Other animal models in immunology

Despite their versatility and ease of use, mice are not always a suitable model for the human immune system. Apart from differences in immune cells, other limitations of mice models are

decreased or no susceptibility to many human diseases and the volume of tissues that can be sampled. For studies focusing on pathogenicity, the susceptibility of the animal model to the pathogen has to be taken into consideration. Other rodents commonly used in immunology are rats, guinea pigs, and rabbits1. In the case of influenza and other droplet/aerosol-transmitted infections, ferrets are the model of preference, because the physiology of their respiratory tract is similar to humans, they are naturally susceptible to most human respiratory viruses, and they resolve the infection in a manner similar to humans (Belser et al., 2011).

For preclinical studies of pharmaceutical substances and vaccine development, often non-human primates (NHPs) are used due to the high degree of similarity with humans with regards to physiology, immunocompetence, and susceptibility to pathogens (Bluemel et al., 2015; Estes et al., 2018). The use of NHPs in vaccine immunology has tremendously increased since the 1950s, due to the realisation that certain therapeutics can act in a species-specific manner as well as major efforts to develop effective vaccines, for example the successful polio vaccines (Salk, 1956; Sabin, 1957; Merker et al., 1988). The identification of HIV-1 in 1983 as a novel human pathogen with the potential to cause serious disease further standardized the use of NHPs as a valuable model for human infections and vaccine design, even though only chimpanzees were found to be naturally susceptible to HIV-1 (Estes et al., 2018). The subsequent discovery of simian immunodeficiency virus (SIV), which caused disease in rhesus macaques that were housed with other species of NHPs, led to the creation of chimeric HIV/SIV viruses. These became the led preferred models for studies on AIDS, development of retroviral therapeutics, and vaccine design (Letvin et al., 1983; Chahroudi et al., 2012; Estes et al., 2018).

1.7.5 Studies on human research subjects

Ultimately, the most suitable model in which to study human pathogens and disease mechanisms, is the human system itself. Collection of cells or tissues from healthy volunteers and patients is useful for immediate analysis and *in vitro* studies. Some human tissues can be modelled *in vitro* by 3D culturing to achieve a cellular organisation similar to *in vivo* tissue structures (Fang and Eglen, 2017). Even though the application of 3D tissue culturing is limited to the cell types that can be generated through *in vitro* cultures of human induced pluripotent stem cells (HiPSCs), it has been a promising development for the fields of oncology, neurology, cardiology, and ophthalmology (Eglen and Reisine, 2019). For immunology and vaccine studies, *in vivo* studies remain the most suitable approach for understanding protective immune responses. Some *in vivo* studies require exposure of study subjects to pathogens, such as evaluation of malaria vaccine efficiency under controlled human malaria infection (CHMI) and influenza challenge studies in the interest of universal influenza vaccine design and development of novel antiviral therapeutics (Roestenberg et al., 2011; Sherman et al., 2019).

[1] Whether guinea pigs (Caviomorpha) and rabbits (Lagomorpha) should be defined as rodents is disputed.

The current COVID-19 pandemic has led to the proposal of human challenge studies with SARS-CoV-2 to improve and accelerate vaccine design (Eyal et al., 2020; Plotkin and Caplan, 2020). This has been perceived as controversial due to the potential risks to study participants, spread to third parties, and incomplete understanding of COVID-19 pathogenesis.

Most analyses from human studies rely on non-invasive sampling of peripheral blood, from which antibody responses, cytokines, and lymphocytes can be analysed. However, some fundamental processes, such as cellular interactions, such as germinal centre formation, are not detectible from blood samples. As these processes take place in secondary lymphoid organs, fine needle aspirations to collect cells from draining lymph nodes could be considered as a low-invasive method for tissue sampling (de Hair et al., 2012; Tatovic et al., 2015). Considering the ethics involved in human experimentation, clinical trials and *in vivo* studies using human subjects require ethical approval from an independent review board and informed written consent from subjects.

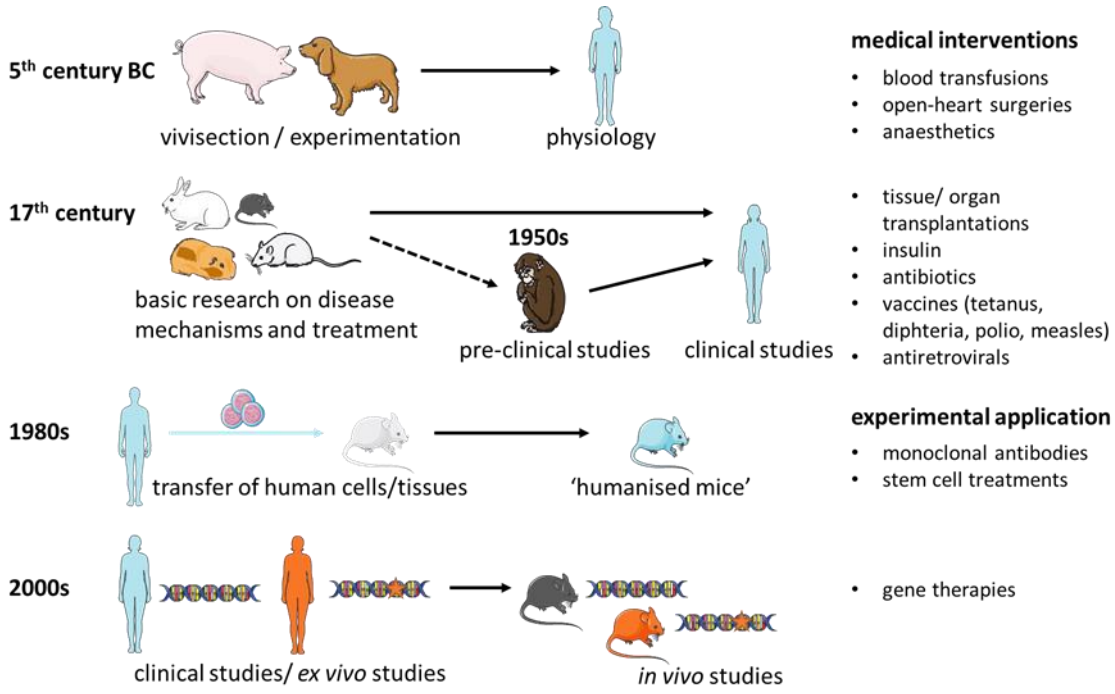

Figure 7. Medical interventions developed using experimental animal models and the advances made in translation to human relevance. *Early experimental work often used non-anaesthetised animals to gain knowledge on organ and tissue function, or to practice and refine operational approaches before applying them to human subjects. Rodents became popular since the 1650s when the renaissance caused a surge in scientific discoveries. Attempts to formulate vaccines that are safe and efficient for human use, introduced non-human primates, which are still valuable in pre-clinical models. The process of humanising mice dates back to 1988, when the first successful cell grafts were done in immunodeficient mice. Currently, generation of gene-targeted engineered mice are a popular method for understanding human pathogenesis and immune responses.*

1.7.6 Brief history of human research ethics

The earliest collection of medical literature dates back to the 5th century BC and includes an oath of ethics which is still symbolically taken by physicians as of today: the Hippocratic Oath. This includes several ethical principles that have remained relevant in modern-day society, such as medical confidentiality and using medical knowledge to help and not to harm.

Unfortunately, human history knows many examples of unethical research on human subjects. For example, the Tuskegee syphilis experiment was meant to study the natural course of syphilis infection in African American males between 1932 and 1971. Subjects were coerced into participation, withheld information, and deprived of treatment, despite the proven effectiveness of penicillin by 1942 (Brandt, 1978). Furthermore, studies on the pathogenesis and treatment of infectious diseases (incl. malaria, typhus, tuberculosis, yellow fever, hepatitis) and organ transplantations were part of the human experiments of horrifying nature that were conducted during the Nazi regime in concentration camps during 1939-1945 (Weindling et al., 2016). Twins, often children, were used for the sole purpose of using one as an experimental subject and the other as a genetic control (Weindling et al., 2016).

The inhumane experimentation came to light during the Nuremberg trials and resulted in prosecution of the responsible physicians and simultaneously in the establishment of the Nuremberg code in 1947. The Nuremberg code explicitly states informed consent as a prerequisite for inclusion of individuals in human experiments, and strongly emphasizes that studies should be designed to protect the wellbeing of participants.

Nowadays, the ethical principles that human experimentation should adhere to are similar in nature to the Hippocratic Oath and the Nuremberg Code, and are stated in the Declaration of Helsinki 1964 with the most recent amendment made in 2013. The Declaration emphasizes principles that are at the core of ethical medical research, e.g. the duty of physicians to act in their patient's best interest, confidentiality of patient data, independent reviews of the ethical standards of human experiments, and replacement by animal models or *in vitro* models whenever possible (WMA, 2018).

2 AIMS OF THIS THESIS

The overall aim of this thesis was to characterize the role of IκBNS in B cell development and antibody responses. The individual aims were as follows:

Paper I: To investigate the requirement of IκBNS in the development of B-1a cells.

Paper II: To evaluate TACI functionality and plasma cell differentiation as underlying mechanisms of defective T cell-independent responses caused by the absence of IκBNS.

Paper III: To determine the requirement for IκBNS in T cell dependent humoral responses using a B cell conditional knock-out mouse model.

Paper IV: To establish robust *in vitro* assays for the analysis of human plasma cell differentiation.

3 METHODOLOGICAL CONSIDERATIONS

Choosing the most suitable animal model to address a certain research question is crucial, as is designing experimental set-ups using the appropriate methods and conditions. This chapter describes general considerations regarding experimental approaches and selected methods. More detailed information regarding methods and experimental protocols can be found in the material and methods sections of respective papers.

3.1 Ethical considerations

All mice experiments were planned and performed according to institutional guidelines and with approval of the Committee for Animal Ethics (Stockholms Norra Djursförsöksetiska nämnd, ethical permit no. N521/12 for **paper I** and N4/16 for **paper II** and **III**). To ensure animal welfare, we followed the 3R guidelines of replacement, refinement, and reduction (Russell and Burch, 1959). We evaluated the level of discomfort caused by immunization or experimental approaches in the mice beforehand and monitored the wellbeing of the mice during experiments when necessary. For example, when analyzing plasma cell generation *in vivo* in **paper II**, we observed a significant reduction of discomfort in our mice when reducing the injected dose of LPS from 10 µg to 5 µg. In addition, considering the distress caused by alum adjuvants in mice, we decided to use AddaVax^{TM} as an adjuvant for the NP-CGG immunizations in **paper III** (Conrad et al., 2009). For **paper IV**, no ethical permits were required as all experiments were carried out using anonymized blood donor samples.

3.2 Mouse models

In this thesis, we studied the role of the protein IκBNS in B cells using mouse strains with genetic alterations in the *nfkbid* gene. IκBNS-deficient *bumble* mice were used in **paper I-III**. The *bumble* strain was generated and identified in the Beutler lab through ENU-mutagenesis and a screen for mice that displayed defects in antibody production following immunization (Arnold et al., 2012) (Fig. 8).

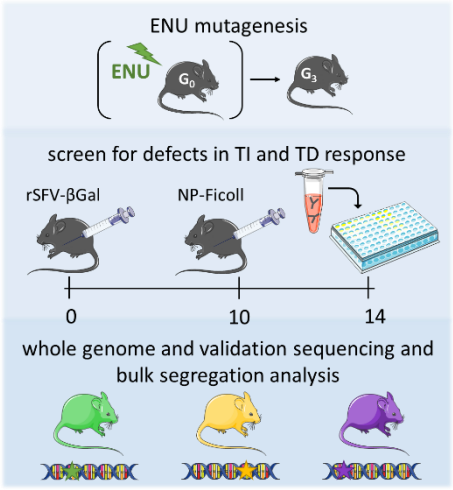

Figure 8. An overview of the forward genetic screen using ENU-mutagenesis in the Beutler lab. *Male C57BL/6 mice were injected with ENU and bred until the 3rd generation to ascertain mutations were genetically transferrable. G3 mice were immunised with rSFV-βGal at day 0, NP-Ficoll at day 10, and at day 14 antigen-specific antibody responses were evaluated. Sequencing was performed on mice with defective responses to identify causative ENU-induced mutations.*

Considering that IκBNS also affects the development and function of other immune cells, such as macrophages, dendritic cells, regulatory T cells and Th17 cells (Kuwata et al., 2006; Schuster et al., 2012; Annemann et al., 2015), the *bumble* strain is less suitable for studying alterations in TD responses as defects in the T cell compartment cannot be ruled out. Therefore, we generated a mouse strain with a B cell specific deletion of IκBNS using the Cre-*loxP* system in **paper III**. Our first effort of generating B cell conditional IκBNS knock-out mice utilized the *Cd19*^{Cre} strain. Early phenotypic characterisation of *Cd19*$^{Cre/+}$*nfkbid*$^{fl/fl}$ mice revealed variable frequencies of B-1a cells (unpublished data), suggesting inefficient or excision of *nfkbid* alleles too late during development. We then generated B cell conditional IκBNS knock-out mice using the *Cd79a*^{Cre} strain, since *Cd79a* is expressed prior to *Cd19* during B cell development and has been shown to be more efficient in Cre-mediated excision of target genes (Hobeika et al., 2006) (Fig. 9).

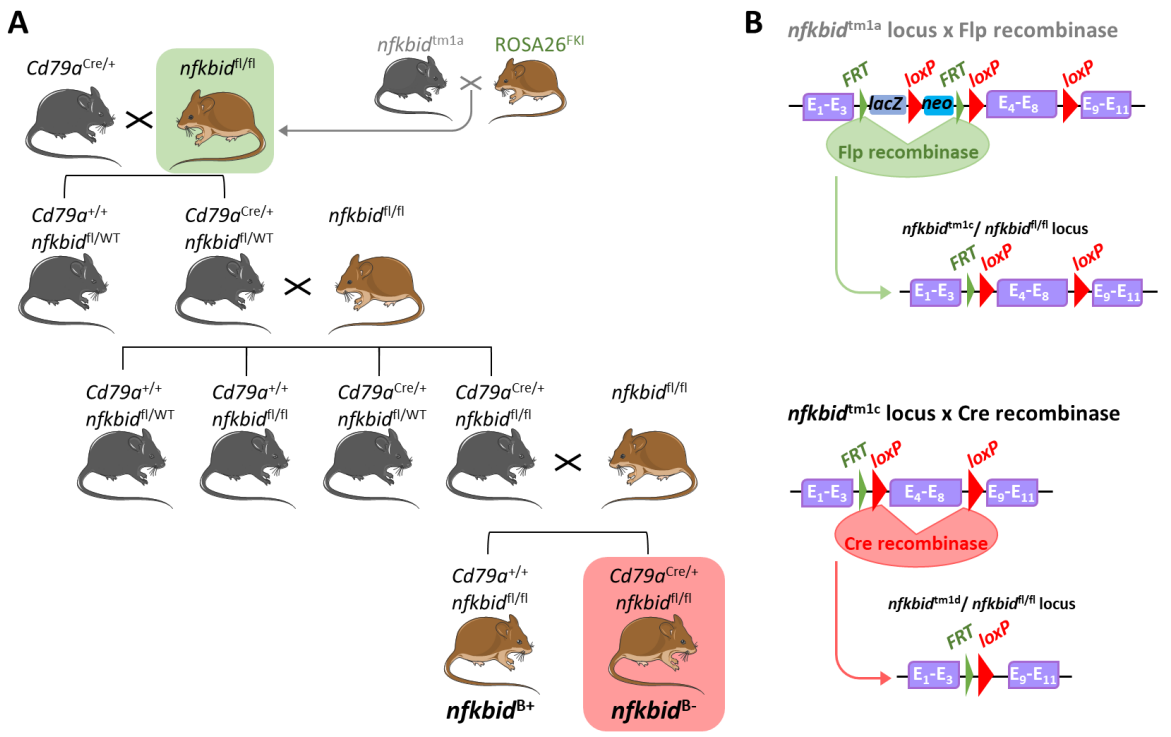

Figure 9. Generation of the Cd79a$^{Cre/+}$nfkbid$^{fl/fl}$ strain. *(A) To remove the lacZ and neo cassette from the nfkbid locus, nfkbid^{tm1a} mice were crossed to FLPe knock-in ROSA26^{Fki} mice in the Beutler lab, creating nfkbid^{tm1c} (nfkbid$^{fl/fl}$) mice which were shipped to the GHK group. Nfkbid$^{fl/fl}$ mice were crossed to Cd79a$^{Cre/+}$ mice (1st cross), Cd79a$^{Cre/+}$nfkbid$^{fl/WT}$ mice (2nd cross), and Cd79a$^{Cre/+}$ nfkbid$^{fl/fl}$ (3rd cross). As both the Cd79a gene and the nfkbid gene are located on chromosome 7, the 3rd cross was dependent on a chromosomal crossover event. Highlights indicate breeding stage where Flp-mediated FRT recombination (green) and Cre-mediated loxP recombination (red) occur. (B) Flp recombinase-mediated excision of the lacZ and neo cassettes to generate nfkbid^{tm1c} alleles (upper panel) and Cre recombinase-mediated excision of coding exons 4 to 8 of the nfkbid allele to generate nfkbid^{tm1d} alleles in nfkbid^{B-} mice (lower panel).*

During the breeding and crossing process, genotyping was performed on ear biopsies of weaned mice to detect *Cd79a* wildtype alleles, the *Cre* sequence, and *loxP* regions in the *nfkbid* alleles. B cell conditional knock-out mice with the *Cd79a*$^{Cre/+}$ *nfkbid*$^{fl/fl}$ genotype were named IκBNS^{B-} and their littermate controls with the *Cd79a*$^{+/+}$ *nfkbid*$^{fl/fl}$ genotype were named IκBNS^{B+}. B-1a cell frequencies from the peritoneal cavities of IκBNS^{B-} mice in **paper III** were evaluated as an indirect assessment of the loss of functional IκBNS, as in **paper I** it was shown that the loss of B-1a cells is an intrinsic defect. To verify the deletion of the *nfkbid* alleles in IκBNS^{B-} mice, we assessed *nfkbid* RNA levels by real-time PCR on isolated B cells that had been stimulated for 2 hours with anti-IgM. The strain was maintained by breeding *nfkbid*$^{B+}$ mice to *nfkbid*$^{B-}$ mice to preserve the *Cd79a*^{Cre} allele in a hemizygous state.

3.3 Immunizations

For studying humoral responses towards TI antigens, we used the model antigens TNP-LPS in **paper I**, LPS in **paper II**, and NP-Ficoll in **papers I, II and III**. LPS (a TI-1 antigen) is a potent activator of murine B cells with the advantage that it can be used to immunise mice *in vivo* and to stimulate isolated B cells *in vitro*, thus allowing us to evaluate plasma cell differentiation under both conditions in response to the same antigen. The use of the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) makes it possible to evaluate antibody affinity, since, for antibody detection, NP molecules can be coupled to BSA at different densities.

For studying humoral responses towards TD antigens in **paper III**, we used the model antigens NP-CGG in AddaVax^{TM} and rSFV-βGal. NP conjugated to the T cell antigen chicken γ-globulin has been in use for several decades to study immune responses in mice. AddaVax^{TM} is an oil-in-water based adjuvant similar to MF59®, which is used in various human vaccines and induces both Th1 and Th2 immune responses (Ott et al., 1995).

We had previously observed delayed GC responses in *bumble* (unpublished data) and therefore, to accurately follow the dynamics of the GC response over time, we evaluated the immune response to NP-CGG at 7, 14 and 21 days. rSFV-βGal is a Semliki Forest Virus-based vector encoding the model antigen β-Galactosidase (Smerdou and Liljeström, 1999). Recombinant Semliki Forest Virus (rSFV) particles were produced using a trans-replication system that prevents genes encoding viral structural proteins from being packaged into newly synthesized particles. As a result, rSFV particles are replication incompetent and only able to cause a single round of infection in cells (Smerdou and Liljeström, 1999).

3.4 Sample processing and cell isolations

To study functional properties of B cells, we performed *in vitro* culture experiments using primary cells isolated from the spleen (for mouse studies) or blood (for human studies). Murine tissues and organs were homogenised through a cell strainer to obtain single cell suspensions and, in case of spleen and blood samples, treated with red blood cell lysis buffer to deplete erythrocytes. Foetal liver cells were prepared using Lymphoprep in **paper I**. Density gradient separation with Ficoll-Paque was used for human blood to isolate PBMCs in **paper IV** (Fig. 10).

The composition of cells within the PBMC fraction varies on an individual basis, but usually consists of 70-90% leukocytes, 10-20% monocytes, and 1-2% dendritic cells. Within the lymphocyte population, 70-85% consists of T cells, 5-10% of B cells, and 5-20% of NK cells (Kleiveland, 2015). For isolation of murine and human B cells, we used EasySep isolation kits as these allow for enrichment of cells within 20 minutes with high yield and purity. Furthermore, we used negative selection kits to avoid unintentional activation of cells by antibody-binding.

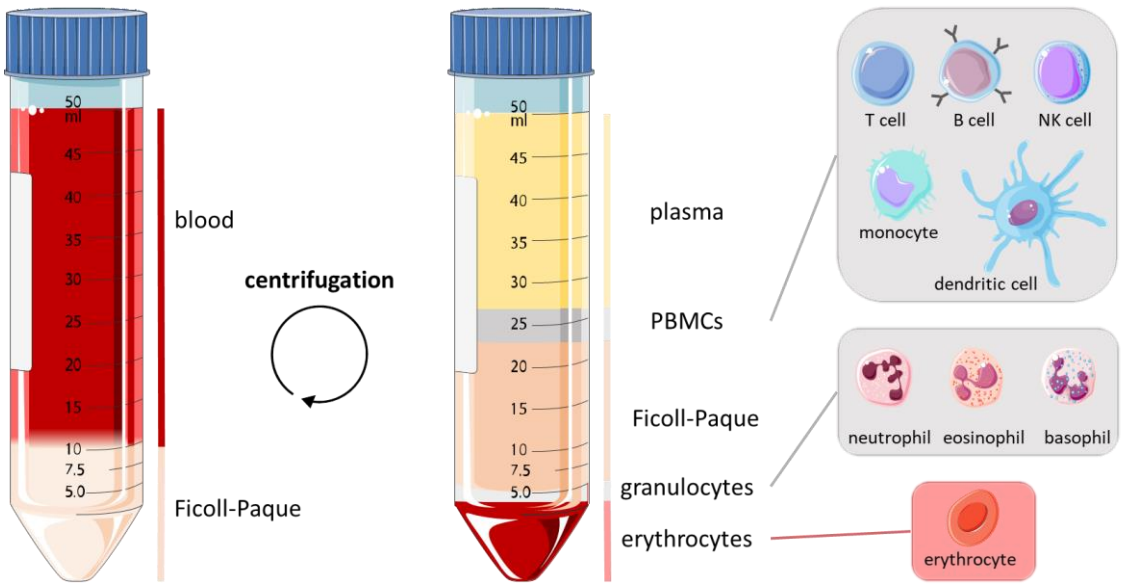

Figure 10. Separation of PBMCs using Ficoll-Paque density gradient centrifugation. *Blood is diluted in PBS and carefully layered on top of 10ml Ficoll-Paque. The density of Ficoll-Paque is 1.077 g/mL and during centrifugation cells with a higher density (erythrocytes and granulocytes) are able to migrate through Ficoll-Paque and sediment at the bottom of the tube. Mononuclear cells of the lymphoid and myeloid lineage have a lower density than Ficoll-Paque and thus remain in the upper fraction.*

3.5 *In vitro* B cell stimulations

Cells were stimulated *in vitro* with LPS to model TI-1 antigen and anti-IgM to model TI-2 antigen activation in **paper II**. For read-outs on plasma cell differentiation, cells were cultured with LPS, as anti-IgM alone is not sufficient for survival and differentiation of cells.

To induce *nfkbid* mRNA expression in **paper III**, we cultured cells for 2 hours with anti-IgM as we previously observed the highest expression levels at this timepoint (unpublished data).

For **paper IV**, the stimulations chosen were based on B cell activating antigens and cytokines that are known to induce plasma cell differentiation. Stimulation I and II had been optimised previously within our group for differentiation of macaque memory B cells into antibody secreting cells (Sundling et al., 2010), stimulation III was selected based on its ability to induce Blimp-1 expression (Banko et al., 2017), and stimulation IV was selected as it potently induced plasma cell differentiation *in vitro* (Le Gallou et al., 2012). We did not consider differentiation protocols requiring long-term cultures and multiple media replenishments, as the objective for this paper was to establish a robust and efficient yet simple method suitable for application in the clinic.

3.6 Evaluation of cell populations by flow cytometry and FACS

Flow cytometry is a well-established method for the detection, measurement and isolation of cell populations. Cells are stained with fluorescently labelled antibodies and then acquired in suspension on a flow cytometer, which uses lasers to excite the fluorophores and detectors to record the intensity of fluorescence emitted. This technique allows for the identification, quantification and phenotyping of cells. However, because single cells are analysed in suspension, information regarding tissue structure, cellular distribution, and intercellular interaction is lost. We have used flow cytometry in this thesis for the identification and quantification of distinct subsets of B cell populations, and the activation and differentiation status of cells after *in vitro* stimulation or *in vivo* immunization.

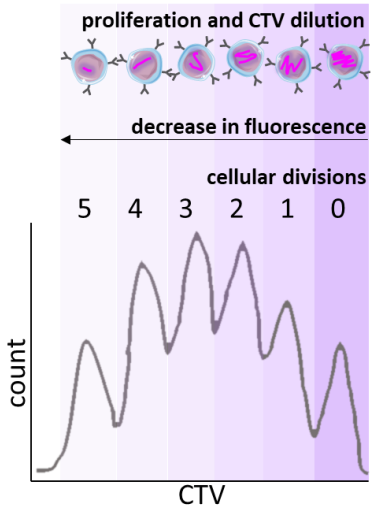

Figure 11. Evaluating cellular proliferation by flow cytometry. *Cells are labelled with CTV, stimulated, and allowed to proliferate in vitro. During each division, the dye is distributed equally over the two daughter cells. This results in a sequential decrease in fluorescence that is proportional to the number of divisions that a cell has undergone.*

In **papers II** and **IV** we used flow cytometry to assess proliferation of cells using the CellTrace^{TM} Violet dye (CTV) (Fig. 11). In contrast to murine cells, primary human B cells were more prone to cell death during CTV labelling, most likely due to DMSO exposure, and we optimised the concentration to 0.25 µM instead of the 1 µM dose used for murine cells. For intracellular Blimp-1 stains in **paper IV**, incubation of cells with the Blimp-1 antibody required 6 hours for the signal to be detectable.

A major advantage of flow cytometry is the possibility of sorting out cell populations for downstream applications. We have used flow cytometry sorts in **paper I** to purify transitional B cell populations ($CD93^+IgM^+$, $CD93^+IgM^+CD5^-$, and $CD93^+IgM^+CD5^+$ cells) for cell transfer experiments, and in **paper II** to purify follicular B cells ($L/D^-B220^+CD21^{lo}CD23^{hi}$) for *in vitro* cultures.

3.7 Detection of antibodies by ELISA

Immunoassays are based on the principle of detecting antigens through binding by antigen-specific antibodies that are labelled with a detectable component. The earliest immunoassays were developed during the 1950s and used radioactive isotopes (Yalow and Berson, 1960). Conjugation of antibodies to enzymes, such as alkaline phosphatase (ALP) and horseradish peroxidase (HRP), popularised and standardized the use of immunoassays (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971).

Enzyme-linked immunosorbent assay (ELISA) is a method that allows for both quantitative and qualitative measurement of proteins of interest, such as antigens, antibodies, or cytokines. ELISA measurements are sensitive and commonly used to determine the concentration of antibodies in samples. However, it is not possible to identify the cells that produced and secreted the antibodies. The general principle of ELISA is to capture the protein of interest on wells pre-coated with primary antibody, detect the captured protein by a secondary antibody conjugated to an enzyme, and then add substrate which is converted into coloured product that can be measured.

The ELISA methods used in this thesis were developed to detect the presence of natural antibodies in **papers I** and **III**, antibodies produced upon *in vitro* stimulation in **paper II** and **IV**, and antigen-specific antibodies induced by immunization in **paper I**, **II** and **III.**

3.8 Detection of antibody-producing cells by ELISpot

Enzyme-linked immunosorbent spot (ELISpot) assays enable the quantification of cells that are producing proteins of interests, such as antibodies or cytokines. This method was developed in the 1980s and replaced the plaque-forming assay that was based on haemolysis of sheep red blood cells (SRBCs) for the detection of IgM-producing cells (Czerkinsky et al., 1983).

Cells are seeded in wells that have been pre-coated with antibodies specific for the protein of interest, so that the proteins are bound on the spot where they are produced by a cell. A secondary antibody conjugated to an enzyme is allowed to bind to the proteins, and development of the assay is done by adding substrate that, once converted by the enzyme, visually marks the spot where the protein has been produced by the cell. The number of spots in a well is used to determine the numbers of cells capable of producing the protein of interest, while the size of the spot is indicative of the amount of protein produced by an individual cell.

We have used ELISpot in **paper I** to detect and enumerate IgM producing cells.

3.9 Statistics

We applied two-tailed unpaired *t*-test in **paper I** and **II** to detect differences in the mean values measured between samples. In **paper II** we also used Mann-Whitney *U*-test for data sets that showed unequal distributions between samples. To account for differences in variations caused by mouse strain backgrounds in **paper III**, we applied the Student's *t*-test with Welch's correction, which is more reliable in cases of unequal variation and unequal sample sizes between tested groups. Biological variation in humans is far greater than in laboratory mouse strains that have been inbred to the extent of being genetically identical, and therefore, in **paper IV**, we used the Mann-Whitney *U*-test that tests the hypothesis of equal/ unequal distribution and is less sensitive to skewed data sets with outliers compared to the unpaired Student's *t*-test.

4 RESULTS AND DISCUSSION

Our studies have focussed on a nuclear regulator of the NF-κB pathway, IκBNS, which was originally identified 18 years ago (Fiorini et al., 2002). IκBNS is presumed to have an inhibitory function in the myeloid lineage in response to LPS as IκBNS KO macrophages and dendritic cells exhibit increased production of pro-inflammatory cytokines (Kuwata et al., 2006). In contrast, in the lymphoid lineage IκBNS has been shown to play a role in the development, proliferation and function of B cells and T cells (Touma et al., 2011; Arnold et al., 2012; Schuster et al., 2012; Annemann et al., 2015). The work included in this thesis focuses on the role of IκBNS in B cell development and function, as well as the development of methods to evaluate PC differentiation of human primary B cells. In this chapter, the main findings of **papers I-IV** and relevant unpublished data are presented and discussed.

4.1 The absence of B-1a cells in *bumble* is an intrinsic developmental defect

Bumble mice were identified in an ENU-mutagenesis screen based on their impaired antibody responses towards NP-Ficoll and rSFV-βGal. The causative mutation was mapped to the fourth intron of the nfkbid gene, causing a T→G transversion in the splice region preventing the removal of this intron in mRNA transcripts. The stop codon present in this intron presumably terminates transcription after 65 of the 327 aa full-length IκBNS protein. Examination of the lymphocyte compartment in the *bumble* mice revealed a severely reduced B-1 cell population in addition to the impaired humoral responses (Arnold et al., 2012).

To understand the role(s) of IκBNS in B-1a cells, we examined B-1 cell development and TI responses in *bumble* in **paper I**. Development of the B-1 lineage begins in the foetus, with the foetal liver playing a prominent role in the expansion of B-1 progenitor (B-1p) cells, whereas after birth, the neonatal spleen is the main location for transitional B cells to mature, after which they migrate and take residency in the peritoneal and pleural cavities.

First, we showed that B-1a cells were completely lacking and B-1b cells were severely reduced in the peritoneal cavity of *bumble* mice (Fig. 12A). In addition, we found no B-1a cells and reduced B-1b cells in the spleen and bone marrow, rendering it unlikely that the B-1 cell population was lacking in these tissues due to defects in migration. Next, we transferred wildtype B-1 cells into *bumble* mice and observed an expansion of donor B-1 cells in the peritoneum and in the spleen. Thus, possible defects in microenvironment were also excluded as a possible cause for the lack of B-1 cells in *bumble*.

In contrast to cells of the B-2 lineage, B-1 cells are not continuously generated from bone marrow, but instead rely on self-renewal to preserve the B-1 pool throughout life. We therefore evaluated B-1a cell frequencies at different intervals between 1 and 16 weeks of age, as a loss of these populations over time would indicate a limited potential of self-renewal. Whilst we were able to detect an increase of peritoneal B-1a cells from week 1 to 5 followed by sustained

frequencies in wildtype, no B-1a cells were detected in *bumble* at any time point. Similarly, in wildtype mice we observed a pronounced population of B-1a cells in the spleen already at week 1 but not in *bumble*. Given the absence of B-1a cells at all time points, we concluded that rather than a defect in self-renewal, it was more likely a defect in B-1a cell development that caused the *bumble* phenotype. Moreover, chimera studies revealed that the inability of *bumble* foetal liver and bone marrow cells to generate B-1a cells was cell-intrinsic, as the presence of wildtype cells did not restore their ability to develop into B-1a cells.

4.2 B-1a cells develop via a transitional B-1a cell stage which is missing in *bumble*

In order to identify at which developmental stage IκBNS is required for the generation of B-1 cells, we examined the frequencies of progenitor cells known for their capacity to give rise to the B-1 lineage. We found normal frequencies of $Lin^-CD93^+CD19^+B220l^{o/-}$ B-1p in foetal liver at E15 as well as of IgM^+CD93^+ splenic TrB cells at week 1 in *bumble*, suggesting that IκBNS is not required for B-1 cell development until after the transitional B cell stage. Upon further examination of the TrB cells, we could distinguish two phenotypically distinct populations of $B220^{lo}CD5^+$ and $B220^+CD5^-$ cells. However, of these two populations, the transitional $B220^{lo}CD5^+$ cells were not present in *bumble*.

Transfer experiments of wildtype cells of each population into *bumble* mice demonstrated the capacity of $CD5^+$ TrB cells to give rise to mature B-1a cells, whereas $CD5^-$ TrB gave rise to both the B-1 and B-2 lineage (Fig. 12B). Hence, we named the $B220^{lo}CD5^+$ subpopulation transitional B-1a (TrB-1a). Considering their potential for B-1a cell generation, we sought to identify more evidence of their commitment to the B-1 lineage. We found the TrB-1a population to express CD43, exhibit increased λ light chain usage, and naturally secrete IgM, all of which are considered features of mature B-1a cells.

A reduction in B-1 cells has also been observed in mice deficient in components of the classical NF-κB pathway, such as p50, the CARD11-Bcl-10-MALT1 complex, the IKK complex, and the LUBAC complex (Pohl et al., 2002; Egawa et al., 2003; Hara et al., 2003; Li et al., 2003; Xue et al., 2003). Intact frequencies of neonatal TrB cells in these mice can incorrectly lead to the assumption that signaling through the classical NF-κB pathway is essential to the survival and maintenance of B-1a cells instead of their generation. Therefore, identification of the intermediate TrB-1a stage can help determine developmental defects caused by other NF-κB components in the classical and alternative pathway.

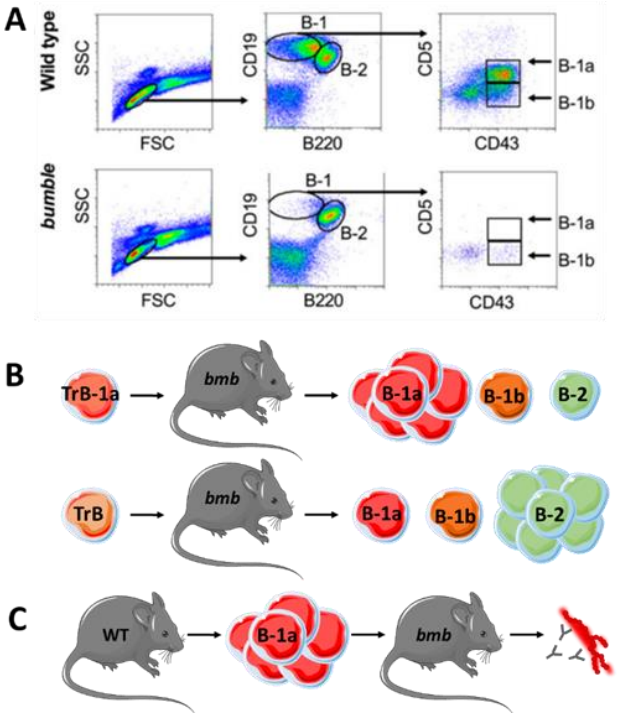

Figure 12. Overview of key findings in paper I. *(A) $CD19^+B220^{lo}CD43^+CD5^+$ B-1a cells and $CD19^+B220^{lo}CD43^+CD5^-$ B-1b cells are absent in the peritoneal cavity of bumble mice. (B) Wildtype neonatal $IgM^+CD93^+CD5^+$ and $IgM^+CD93^+CD5^-$ transitional B cells were sorted and transferred into bumble mice to investigate their potential to give rise to the mature B-1a, B-1b and B-2 lineages. Donor cells were analysed 4-6 days post-transfer. $CD5^+$ TrB predominantly generated B-1a cells whilst $CD5^-$ TrB were more efficient in generating B-2 cells. (C) Transfer of wildtype B-1a cells into bumble mice partially restored the antibody response upon immunization with the TI-2 antigen NP-Ficoll.*

Unlike B-1a cells, which were completely absent, B-1b cells were detectable in *bumble*, albeit at reduced frequencies compared to wildtype. Differential requirements for the development of B-1b cells are not well studied. Our data indicated that B-1b cells were generated from both $CD5^+$ and $CD5^-$ TrB cells. Therefore, it might be possible that in *bumble* B-1b cell generation from $CD5^-$ TrB cell population is intact and that their reduced frequencies reflect the loss of B-1b cells derived from the $CD5^+$ TrB population.

4.3 Impaired TI responses in the absence of IκBNS

Clearance of TI antigens is predominantly facilitated by B-1 cells and MZBs. In **paper I**, we showed that *bumble* mice do not produce NP-specific IgM or IgG responses to NP-Ficoll, and no IgM to TNP-LPS. Transfer of wildtype peritoneal cells to *bumble* partially restored NP-Ficoll responses, suggesting that the lack of B-1 cell contributes to the observed impairment in TI responses (Fig. 12C). In a follow-up study not included in this thesis, we demonstrated that in mice heterozygous for the *bumble* mutation, the B-1 cell compartment was intact yet antibody responses towards NP-Ficoll and Pneumovax were attenuated (Pedersen et al., 2016). Taken together, these results indicated that the impaired TI responses observed in *bumble* are due to the lack of B-1a cells as well as defects in B cell function. NP-Ficoll induced antibody titres were also undetectable in *nfkbid*B^- mice, in which IκBNS is selectively deleted from the B cell compartment, further strengthening the notion that the impaired TI response is intrinsic.

4.4 *Bumble* displays lower Igλ usage in all B cell subsets

B-1a and MZB cells in B6 mice have been reported to exhibit an increased proportion of Igλ usage compared to the FOB cell population, which consists of $Ig\kappa^+$ cells and $Ig\lambda^+$ cells in a 95 to 5 ratio (Hayakawa et al., 1986; Arakawa et al., 1996; Carey et al., 2008). Igκ rearrangement occurs prior to Igλ rearrangement, and prolonged survival mediated through NF-κB signaling seems to be required for Igλ rearrangement but not Igκ rearrangement (Derudder et al., 2016).

To support our finding that increased immunoglobulin light chain usage is an indication of early B cell lineage predisposition in the TrB cell population, we determined the frequencies of $Ig\kappa^+$ and $Ig\lambda^+$ cells in the mature B cell subsets and expanded our analysis to *bumble* B cells. Examination of the light chain usage in wildtype subsets confirmed previous findings that the B-1 cell population contains a higher proportion of $Ig\lambda^+$ cells than the B-2 cell population and revealed a modest increase of Igλ usage in the MZB cell subset compared to the FOB cell subset. As may be expected from the lack of B-1a cells in *bumble*, the peritoneal B-1 population exhibited decreased Igλ usage (Fig. 13). The $CD19^+B220^{lo}$ *bumble* cells that were detectable might be a heterogeneous population consisting of B-1b and immature B cells. The possibility remains that *bumble* B-1b cells are affected in their capacity to generate $Ig\lambda^+$ cells. When comparing the B-2 cell population, *bumble* B-2 cells displayed reduced levels of Igλ usage compared to wildtype B-2 cells in the peritoneal cavity but not in the spleen (Fig. 13).

To examine Igλ usage in the FOB and MZB population, we used 5 months old mice as MZBs are initially reduced but increase in frequency upon aging. Again, we found decreased Igλ usage in *bumble* in both subsets (Fig. 13). As alterations in the $Ig\kappa^+$ to $Ig\lambda^+$ cell ratio extended beyond the B-1 lineage in *bumble*, these results suggest that disturbed Igλ usage results from a general impairment in the absence of IκBNS. Considering that overexpression of pro-survival signals such as Bcl-2 restored Igλ usage in mice deficient for NEMO and IKK1/IKK2, it would be worthwhile investigating whether IκBNS-dependent NF-κB activity is necessary in mediating survival signals. Alternatively, IκBNS could be involved in Igλ locus accessibility, as Vλ and Jλ germline gene segments were more efficiently transcribed upon IκBα-mediated NF-κB activity (Bendall et al., 2001).

Interestingly, activation causes *bumble* B cells to rapidly upregulate Blimp-1 (see section 4.6) and recent data indicate that Blimp-1 limits the repertoire of autoreactive B-1a cells presumably via repression of the anti-apoptotic protein Bcl-2 (Hug et al., 2014; Setz et al., 2018). Pre-BCR signaling induces IRF4 expression, which facilitates RAG-mediated light chain recombination in pre-B cells in favour of Igλ recombination and independently of IL-7 (Johnson et al., 2008). It has been suggested that high IRF4 levels potentially induce Blimp-1 in pre-B cells, which then interfere with proliferation and survival of autoreactive cells. As B-1 cells are known to be selected on self-antigen, this could be a mechanism to deplete autoreactive cells that are harmful.

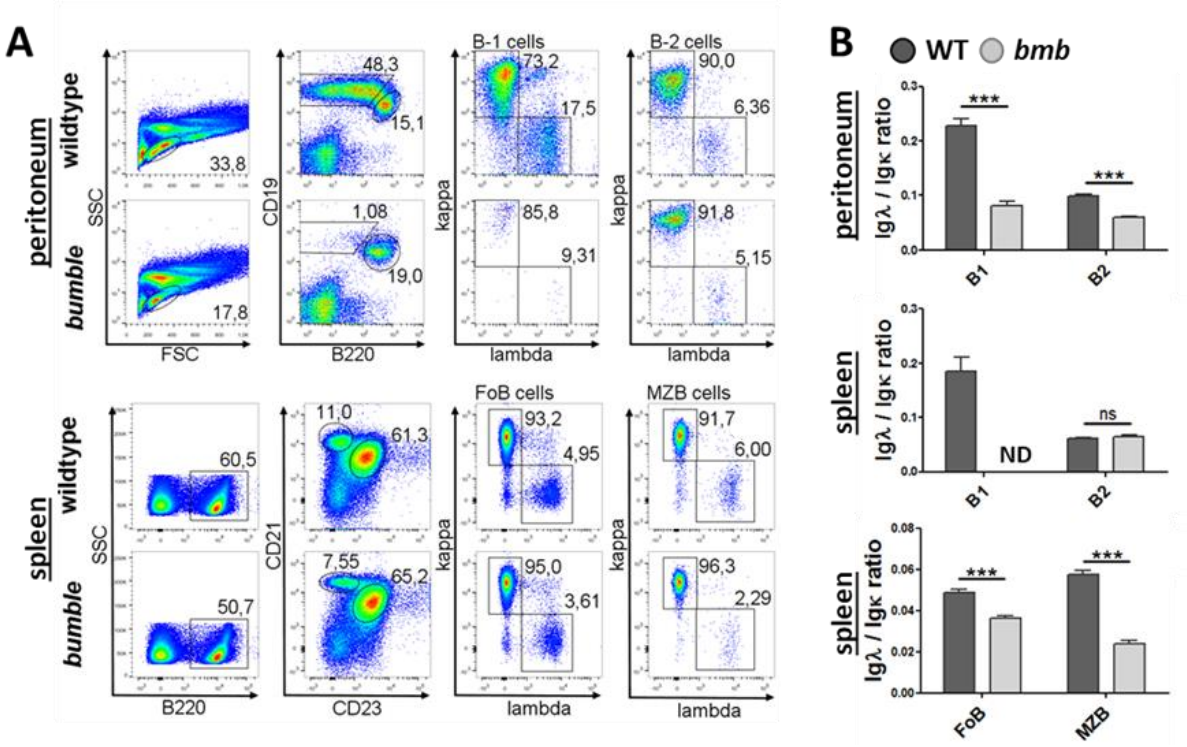

Figure 13. Igλ to Igκ ratio is reduced in the absence of IκBNS (unpublished data). *(A) Gating strategy for examining Igκ and Igλ usage in peritoneal CD19$^+$B220^{lo} B-1 and CD19$^+$B220$^+$ B-2 cells (upper panel) and splenic B220$^+$CD21^{lo}CD23$^+$ FOB and B220$^+$CD21$^+$CD23^{lo} MZB cells (lower panel). Numbers denote frequencies of gated populations. (B) Bar graphs show the ratio of Igλ to Igκ expressing cells within the peritoneal B-1 and B-2 population (upper panel) and splenic B-1 and B-2 population (middle panel) in 8 week old mice, and splenic FOB and MZB population (lower panel) in 5 month old mice. Statistical differences were determined using the Student's t-test and indicated by ns (not significant) and *** ($p<0.001$). Splenic B-1 cells were not detected (ND) in bumble.*

In light of the rapid Blimp-1 expression observed during PC differentiation in *bumble* B cells, it is plausible that during B cell development Blimp-1 is disproportionally expressed in progenitor cells of the B-1 lineage and triggers cell death through Bcl-2 repression. The transitional window in which IκBNS is required, e.g. between the B-1p and TrB-1a stage, coincides with surface expression of IgM. Thus, BCR-induced negative selection mediated via elevated IRF4/ Blimp-1 expression would correspond to the developmental time point at which cells with B-1a potential are lost in *bumble*. Future studies using Blimp-1 repression and/ or Bcl-2 overexpression in *bumble* to verify whether B-1a and Igλ$^+$ cells generation can be rescued, could help elucidate the underlying mechanism that causes the loss of B-1a cells.

4.5 TACI expression and function is dependent on IκBNS

The absence of antibody production towards TI antigens in *bumble* mice made us question whether *bumble* B cells were able to generate ASCs at all. In **paper II**, we demonstrate that, despite relatively normal early activation, LPS-induced PB and PC populations were strongly reduced in *bumble* mice (Fig. 15A). Similar to *bumble*, XID mice that are deficient in Bruton's tyrosine kinase (Btk) are also unresponsive to TI-2 antigens. This has been linked to reduced

expression of the surface protein TACI, which could be partially rescued through CpG stimulation (Uslu et al., 2014). Studies using TACI$^{-/-}$ mice have established TACI as an important surface receptor involved in the generation antibody responses towards both TI-1 and TI-2 antigens (von Bülow et al., 2001; Yan et al., 2001; Mantchev et al., 2007). In TACI$^{-/-}$ mice, both the loss of Blimp-1 expression and the inability to downregulate BIM have been suggested to contribute to increased apoptosis of plasma cells and thus decreased antibody titres (Ou et al., 2012).

In agreement with previous studies, we found that TACI was expressed on all mature B cell subsets but at higher levels on B-1 and MZB cells (Katsenelson et al., 2007). However, on *bumble* MZB and FOB cells, TACI was not detectable at steady state. Inducible TACI expression is achieved through BCR and TLR ligation, but we found that on *bumble* B cells TACI was upregulated poorly in response to anti-IgM and CpG and only partially in response to LPS (Fig. 12A). Furthermore, despite their partial upregulation of TACI, *bumble* B cells remained unresponsive towards TACI ligands (Fig. 14B).

APRIL and BAFF, through TACI, mediate downregulation of the pro-apoptotic protein BIM to promote plasma cell survival. In TACI$^{-/-}$ mice, both the loss of Blimp-1 expression and the inability to downregulate BIM have been suggested to contribute to increased apoptosis of plasma cells and thus decreased antibody titres (Ou et al., 2012). BAFF and BCMA expression were normal in *bumble*, further suggesting that the defect can be attributed to TACI expression and function. Altogether, these observations hint at a role for IκBNS in TACI-mediated survival, PC generation, and antibody production in TI responses.

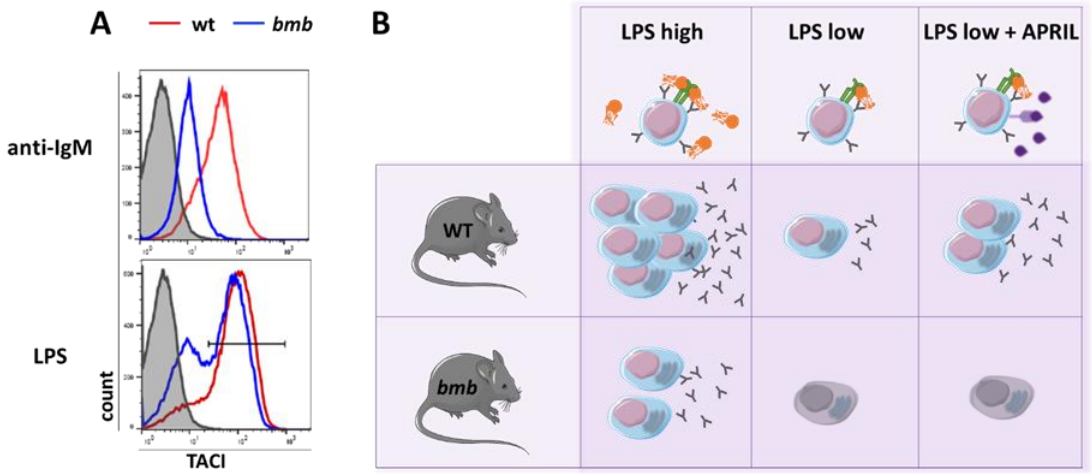

Figure 14. TACI expression and function in bumble is attenuated. *(A) TACI expression on wildtype and bumble B cells after 48 hours of anti-IgM and LPS stimulation. (B) High concentrations of LPS (10 µg/ mL) induced the generation of antibodies and CD138^{+} cells, whereas low concentrations of LPS (100 ng/ mL) were less efficient in both read-outs. TACI ligation is known to enhance antibody responses towards low concentrations of LPS. Wildtype B cells exhibited increased antibody production, PC generation, and survival when APRIL was added to cultures with low LPS concentrations. However, bumble B cells did not respond to APRIL co-stimulation as they were unable to produce antibodies and CD138^{+} cells and exhibited increased cell death compared to wildtype.*

4.6 PC differentiation is dysregulated in IκBNS-deficient B cells

To gain further insight into the absence of PCs in *bumble*, we evaluated expression of transcription factors that are necessary for the regulation of PC differentiation. Pax5, which controls the expression of the B cell specific gene programme, is downregulated in cells committing to PC fate to allow for efficient PC differentiation (Nera et al., 2006). On the other hand, IRF4 and Blimp-1 are upregulated and act in concert to establish expression of PC-specific genes (Ochiai et al., 2013; Minnich et al., 2016).

Despite being able to upregulate IRF4, *bumble* B cells were unable to fully downregulate Pax5 in IRF4^{+} cells. Since differentiation into PCs is a process that has been linked to cellular divisions, we investigated changes in Pax5, IRF4, and Blimp-1 expression per division cycle. *Bumble* B cells exhibited higher Pax5 expression consistently throughout all divisions and upregulated Blimp-1 more rapidly than wildtype B cells (Fig. 15B). Nevertheless, after the 6th division cycle, cells committing to PC fate were drastically reduced.

Blimp-1 has been shown to gradually increase during differentiation, with splenic ASCs being defined as both Blimp-1^{int} and Blimp-1^{hi} and bone marrow plasma cells as Blimp-1^{hi} (Kallies et al., 2004). Considering that c-myc is a direct target of Blimp-1 repression (Lin et al., 1997), high Blimp-1 expression is thought to contribute to cell-cycle exit during plasma cell generation whereas intermediate Blimp-1 expression potentially permits proliferation of cells in transient stages of PC differentiation. Thus, the accelerated induction of Blimp-1 expression in *bumble* mice might compromise the proliferative capacity of differentiating cells. In addition, premature expression of Blimp-1 has been associated with increased apoptosis (Setz et al., 2018). Investigations in the expression of proteins associated with apoptosis and survival, such as and Bcl-2, might offer clues as to whether rapid increase of Blimp-1 expression in the absence of IκBNS results in enhanced cell death and thereby limits terminal differentiation.

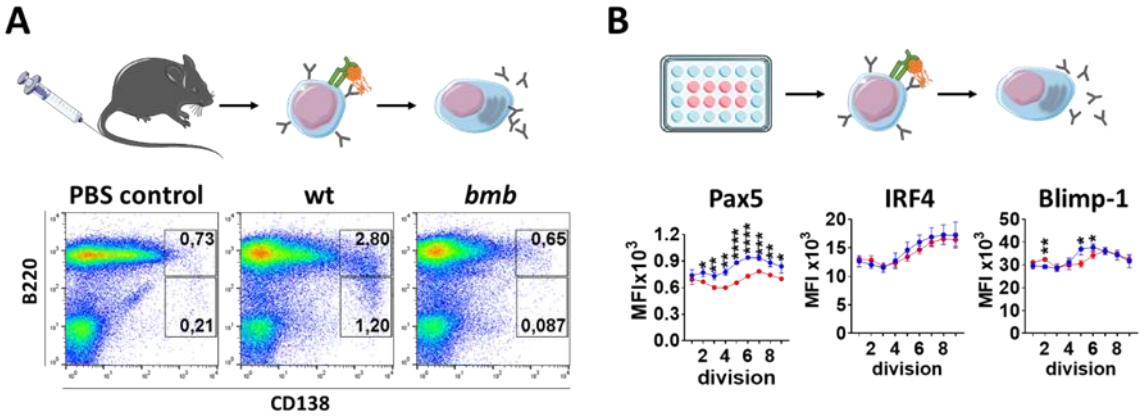

Figure 15. Reduced PC generation and alterations in regulation of PC differentiation in bumble. *(A) LPS was injected intravenously in mice and the presence of PBs and PCs was evaluated after 3 days. LPS induced B220^{+}CD138^{+} PBs and B220^{-}CD138^{+} PCs in wildtype but not in bumble mice. (B) B cells were labelled with CTV, cultured for 3.5 days with LPS, and then stained for the expression of Pax5, IRF4, and Blimp-1. Expression is shown for the Pax5^{lo}, IRF4^{+}, and Blimp-1^{+} subpopulations per division cycle.*

4.7 Validation and phenotypic characterisation of *nfkbid*$^{B-}$ mice

The failure to mount humoral responses in *bumble* mice also affected TD antigen responses (Arnold et al., 2012). To distinguish between defects in the B cell compartment from those in the T cell compartment, we generated the B cell conditional *Cd79a*$^{Cre/+}$*nfkbid*$^{fl/fl}$ strain in **paper III** to address the role of IκBNS in TD responses. Cre-mediated excision of the *LoxP* flanked *nfkbid* alleles was validated by *nfkbid* mRNA measurements, as we did not detect *nfkbid* mRNA in B cells isolated from *nfkbid*$^{B-}$ mice. To our surprise, as we presumed that the non-functional *nfkbid* mRNA transcripts in *bumble* were degraded, we detected *nfkbid* transcripts in *bumble* at much higher levels compared to wildtype.

As shown in **paper I**, the loss of B-1a cells in the absence of IκBNS is an intrinsic effect. The frequencies of the B-1a cell population in *nfkbid*$^{B-}$ mice could thus be considered an indirect validation of *nfkbid* deletion efficiency. Although severely reduced, a small proportion of B-1a cells were detectable in the *nfkbid*$^{B-}$ mice, suggesting that not in all B cells excision was successful. Frequencies of B-1a cells correlated with serum IgM titres, which were also severely reduced in *nfkbid*$^{B-}$ mice. We found normal frequencies of the transitional B cell subsets and MZB cells in *nfkbid*$^{B-}$ mice, unlike in *bumble*, where these compartments are affected (Ádori et al., 2018).

4.8 TD responses in *nfkbid*$^{B-}$ mice reveal normal GC formation but attenuated production of antigen-specific antibodies of the IgG2c and IgG3 isotype

We examined GC formation and antibody responses to the TD antigens rSFV-βGal and NP-CGG. Both βGal-specific and NP-specific IgG3 titres were reduced, and in line with reduced natural serum IgG3 levels, suggests a general class switching defect to the IgG3 isotype (Fig. 16A). Switching to IgG3 is rapidly triggered during infections and is promoted by IL-4, IL-10, and IL-21 (Brière et al., 1994; Fujieda et al., 1995; Malisan et al., 1996; Pène et al., 2004). As IL-10 production in IκBNS-deficient mice is compromised (Miura et al., 2016), this could explain their inability to produce antibodies of the IgG3 isotype. However, it has been shown that in LPS cultures supplemented with IL-4, switched transcripts of IgG3 were undetectable in IκBNS KO B cells, suggesting that even in the presence of IgG3-inducing cytokines, switching is defective (Touma et al., 2011).

Furthermore, serum titres of IgG2c in naïve as well as in rSFV-βGal immunized *nfkbid*$^{B-}$ mice were also reduced (Fig. 16A). Antibodies of the IgG2a/ IgG2c are preferentially induced by viral infection but were not elicited in IκBNS KO mice exposed to the murine-adapted influenza virus PR8 either (Coutelier et al., 1988; Touma et al., 2011). It has been shown that virus-like particles directly act on B cells to produce IgG2b and IgG2c antibodies through

TLR7/9-induced MyD88 signaling (Hou et al., 2011). Intact MyD88 signaling in B cells is necessary for their ability to prime T cells to differentiate and produce IFN-γ, which in turn acts on B cells to promote IgG2a/ IgG2c class switching via T-box transcription factor (T-bet) expression (Finkelman et al., 1988; Peng et al., 2002; Barr et al., 2009). It is possible that NF-κB activation upon MyD88 signaling is altered in the absence of IκBNS and interferes with the ability of B cells to support T cell differentiation and/ or function. Another possibility is that attenuated signaling downstream of IFN-γR, TLR7/9 or other PRRs restricts T-bet expression in IκBNS-deficient B cells. Measurements of IFN-γ in serum, IFN-γ^+ T cells, and T-bet expression in B cells from rSFV-βGal immunized mice could offer more insight into these mechanisms.

As for NP-specific and βGal-specific IgG1, titres were comparable between *nfkbid*$^{B-}$ and *nfkbid*$^{B+}$ mice, suggesting that IκBNS is not required for switching to IgG1. We noticed a tendency towards lower GC B cell frequencies in response to rSFV-βGal, but similar frequencies in response to NP-CGG (Fig. 16B). TACI$^{-/-}$ mice have been shown to produce larger numbers of GC B cells and T_{FH} cells in response to NP-CGG. TACI is able to sequester BAFF and prevent its binding to BAFFR, which would induce ICOSL surface expression on B cells (Ou et al., 2012). We have shown in **paper II** that TACI upregulation is more severely impaired upon BCR than TLR engagement. Possibly, NP-CGG and rSFVβGal could differentially induce TACI upregulation and/or signal through PRRs, explaining the observed differences in GC B cell expansion. Thus, as there were no further indications that GC formation or plasma cell differentiation was defective, the results from antigen-specific antibody titres only revealed a defect in class switching. Thus, the finding that *nfkbid*$^{B-}$ mice were able to form GCs and produce Ag-specific IgG1, suggests that the impaired TD responses observed in *bumble* were primarily due to defects in the T cell compartment.

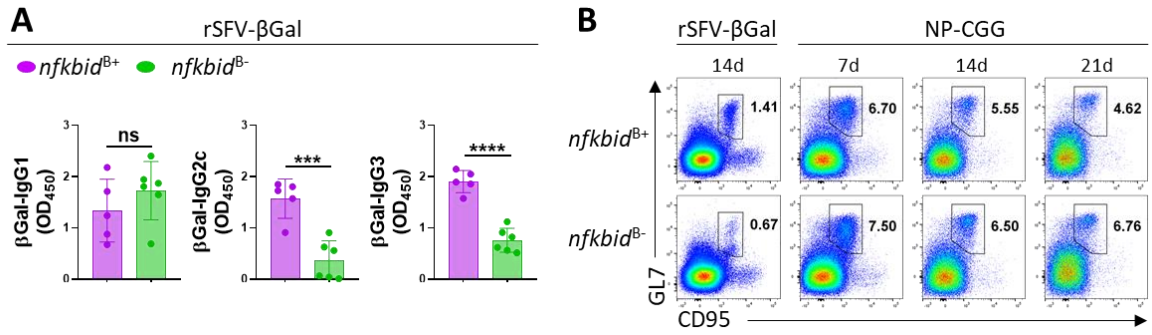

Figure 16. GC B cell frequencies*. Mice were immunised with rSFV-βGal i.p. or NP-CGG in Addavax s.c. (A) β-Gal-specific IgG1, IgG2c, and IgG3 titres. (B) Representative flow cytometry plots showing B220$^+$GL7$^+$CD95$^+$ GC B cells at indicated time points.*

4.9 Human plasma cell differentiation *in vitro*

Certain phenotypic characteristics of the *bumble* mice, most notably low serum IgM titres and poor antibody responses towards polysaccharide antigens, are similar to symptoms of patients suffering from immunodeficiencies. Nowadays, the application of next generation sequencing methods has allows for identification of an increasing number of mutations associated with CVID, including mutations in the NF-κB pathway (Chen et al., 2013; Fliegauf et al., 2015; Boztug et al., 2016; Kuehn et al., 2017; Klemann et al., 2019). As highlighted by our findings in **paper II**, defects in the NF-κB pathway can result in altered transcriptional regulation of PC differentiation which contributes to impaired humoral responses. Thus, our aim for **paper IV** was to translate our PC differentiation assays to the human context.

To develop a robust protocol, we compared several stimulation conditions and optimised a flow cytometry panel for the detection of *in vitro* generated plasma cells. Two populations with PC characteristics were detectable, namely $CD38^{+}IRF4^{hi}Pax5^{lo}$ cells and $CD38^{+}IRF4^{int}Pax5^{lo}$ cells. The latter population contained both Blimp-1^{+} and Blimp-1^{-} cells at day 3.5, and on day 6 all cells were Blimp-1^{-} (Fig. 17). This suggests that they developed from the $CD38^{+}IRF4^{hi}Pax5^{lo}$ population and gradually lost Blimp-1 expression as well as stopped cycling. Both the $CD38^{+}IRF4^{hi}Pax5^{lo}$ and $CD38^{+}IRF4^{int}Pax5^{lo}$ population contained cells that expressed IgG intracellularly.

To ensure that our protocol was applicable to patient samples, which are commonly stored at -80° C or in liquid nitrogen, we evaluated differentiation of cells from cryopreserved samples compared to fresh samples. Additionally, a genuine concern for working with CVID patient samples is that often circulating memory B cells are reduced, and as memory B cells have a faster differentiation kinetic than naïve B cells, samples with low memory B cell numbers could skew the outcome of the assay. Memory B cells did indeed differentiate more rapidly than naïve B cells, but both cell subsets generated $CD38^{+}IRF4^{hi}Pax5^{lo}$ cells and secreted IgG.

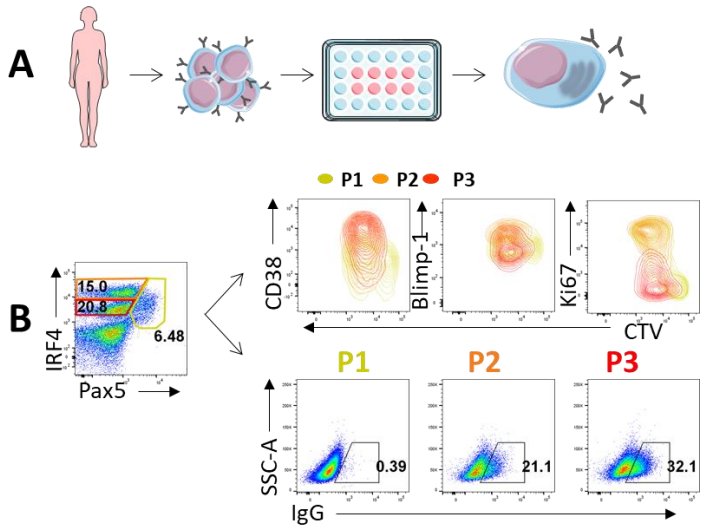

Figure 17. Human PC differentiation in vitro. *(A) Isolated B cells from healthy donor blood were stimulated in vitro to promote PC differentiation. (B) Expression of IRF4 and Pax revealed three distinct cell populations. Population P2 and P3 contained cells undergoing PC differentiation, as evidenced by their upregulation of surface CD38 expression and intracellular IgG levels.*

4.10 Evaluation of metabolic state in differentiated human B cells

Differentiation of activated cells is characterized by a profound switch in intracellular metabolic activity. Activated B cells, via the PI3K signaling pathway, increase their glycolysis and oxidative phosphorylation to support ER expansion and proliferation (Doughty et al., 2006; Caro-Maldonado et al., 2014). Cellular division of lymphocytes generates an uneven distribution of PI3K signaling strength in progenitor cells with diverging fates; cells exhibiting low PI3K activity continue to self-renew whereas cells exhibiting high PI3K activity favour differentiation (Lin et al., 2015; Adams et al., 2016). Subsequent commitment to the plasma cell fate has been shown to coincide with reduced levels of mitochondrial reactive oxygen species (mROS), as well as decreased mitochondrial mass and potential (Jang et al., 2015).

Mutations in both catalytic and regulatory PI3K subunits have been identified in patients with primary immunodeficiency and are associated with impaired polysaccharide responses, respiratory tract infections, and decreased frequencies of switched memory B cells (Lucas et al., 2014a; Lucas et al., 2014b; Deau et al., 2015; Elgizouli et al., 2016; Avery et al., 2018). The strong link between factors involved in regulation of metabolic activity and differentiation fate prompted us to evaluate the mitochondrial metabolic state and mitochondrial ROS (mROS) production. Human cells were subjected to fixation before flow cytometry acquisition and the mitochondrial dyes that were compatible with paraformaldehyde fixation were limited. We used MitoTracker Deep Red (MTDR) to measure mitochondrial mass and potential, and MitoSOX to measure mROS production. At day 3.5, B cells that had proliferated and upregulated CD38 had increased mitochondrial mass and potential. We observed a similar trend at day 6 albeit at lower expression compared to day 3.5. We found increased mROS levels in proliferated $CD38^-$ cells, whereas $CD38^+$ cell exhibited reduced mROS (Fig. 18).

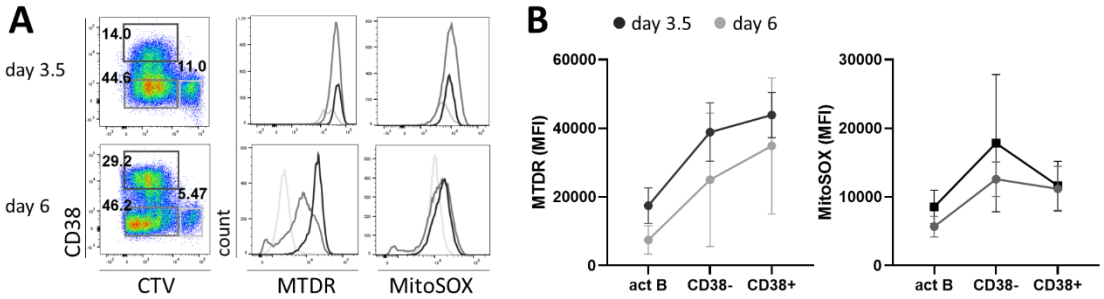

Figure 18. Changes in metabolic state in differentiating human B cells (unpublished data). *Cells were labelled with CTV, cultured with stimulation III and stained with the MTDR and MitoSOX dyes in addition to anti-CD38. (A) Flow cytometry plots showing gating strategy to identify unproliferated activated B cells (act B), proliferated $CD38^-$ cells and proliferated $CD38^+$ B cells. (B) Expression levels of MTDR and MitoSOX.*

The read-outs that were based on MTDR and MitoSOX resulted in considerable variation between donors but also between experiments. As a result, we omitted this assay from **paper IV** to ascertain that all included assays were reliable and applicable to patient samples. Nevertheless, further optimization of assays to evaluate metabolic alterations during plasma cell differentiation in human B cells are of interest for future investigations.

5 CONCLUDING AND PROSPECTIVE REMARKS

The work described in this thesis contributes to our understanding of the function of IκBNS in humoral responses and addresses relevant implications for human immune disorders. In this chapter, the key findings are briefly summarised.

First, we addressed the absence of B-1a cells in *bumble* mice and aimed to identify at which stage during B-1 a cell development IκBNS expression is essential. In **paper I**, we have demonstrated that the development of B-1a cells occurs via a $B220^{lo}CD5^{+}$ transitional B cell stage which was lacking in mice deficient in IκBNS. Considering that the $IgM^{+}CD93^{+}$ splenic TrB cell population was intact, IκBNS is most likely required for the transition from TrB cell to TrB-1a cell. Using chimeric cell transfer experiments, we showed that the inability of IκBNS-deficient bone marrow and foetal liver cells to generate B-1a cells is cell-intrinsic. In addition, the absence of B-1a cells in *bumble* partially explains why they are unable to respond to TI antigens, as the transfer of wildtype peritoneal cells restored the NP-Ficoll response. Even though the identification of a B-1a lineage committed transitional B cell population in this paper contributes to our understanding of B-1a cell development, how the development of B-1b cells is differentially regulated is as of yet still poorly understood. Furthermore, it is widely accepted that B-1a cells preferentially express $V_H12/V_κ4$ and $V_H11/V_κ14$ BCRs, are selected on self-antigen, and exhibit stronger tonic BCR signaling relative to B-2 cells. Nevertheless, to which extent the BCR contributes to the B-1 and B-2 lineage commitment continues to be a controversial topic.

Next, we investigated potential reasons for the impaired humoral responses to TI antigens in *bumble* mice. The surface receptor TACI is known to be important for optimal TI responses and TACI expression and function was shown to be compromised in mice with defective BCR signaling. This led us to examine TACI expression and function in *bumble*. In **paper II**, we show that TACI was not expressed on *bumble* FOB and MZB cells at steady state and upregulation upon stimulation was attenuated. Furthermore, antibody responses to low concentrations of LPS combined with TACI ligands APRIL or BAFF were enhanced in wildtype cells but not in *bumble* cells. As there have not been many studies aimed at identifying binding partners or target genes of IκBNS, it is unknown whether IκBNS is directly or indirectly involved in the expression of TACI. Additionally, since ligand binding to TACI induces activation of the classical NF-κB pathway, it is possible that IκBNS is needed downstream of TACI signaling.

We then proceeded to evaluate PC differentiation in response to LPS. Frequencies of PBs and PCs in *bumble* were severely reduced compared to wildtype *in vivo*. Similarly, B cells derived from *bumble* proliferated less and generated less $CD138^{+}$ cells compared to wildtype B cells *in vitro*. We therefore assessed expression of transcription factors that are central to regulation of the PC differentiation, namely Pax5, IRF4, and Blimp-1. We showed that LPS-stimulated cells that expressed high levels of IRF4 were unable to fully downregulate Pax5. In addition,

evaluation of Pax5, IRF4, and Blimp-1 expression levels in relation to cellular division revealed a pattern of rapid differentiation in the early division stages but a failure to commit to terminal differentiation in the late division stage. As Blimp-1 is imperative for terminal differentiation of cells committing to PC fate, and Blimp-1 is present in *bumble* cells yet terminal PC differentiation is dysregulated, ongoing studies in the group are focussing on the function of Blimp-1 in *bumble* cells.

The generation of *nfkbid*$^{B-}$ mice, in which IκBNS is selectively deleted in B cells, allowed us to interrogate the role of IκBNS in TD responses. In **paper III**, we show that *nfkbid*$^{B-}$ mice have severely reduced frequencies of B-1a cells, decreased natural IgM serum levels, and fail to respond to NP-Ficoll immunization. These results are in line with the intrinsic requirement of IκBNS for B-1a cell development shown in **paper I**. Upon immunization with the TD antigen rSFV-βGal, we observed normal βGal-specific IgG1 titres, but reduced βGal-specific IgG2c and IgG3 titres in *nfkbid*$^{B-}$ mice compared to *nfkbid*$^{B+}$ litter mate control mice. Additionally, *nfkbid*$^{B-}$ mice exhibited lower frequencies of GC B cells, although the distribution of light zone and dark zone GC B cells was normal. The differential requirement for IκBNS in class-switching to distinct isotypes remains a topic for future exploration. The modest alterations observed in GC responses combined with the reduction in antigen-specific antibody titres in *nkfbid*$^{B-}$ mice suggests that the role of IκBNS in B cells during TD responses is limited to class switching, and that the absence of GC formation and antibody production in *bumble* mice to TD antigens are due to extrinsic requirements.

Our finding that PC differentiation was dysregulated in *bumble* mice together with the strong association between mutations in NF-κB components and CVID, prompted us to design and validate robust methods suitable for evaluation of PC differentiation defects in immunodeficient patients. In **paper IV**, we compared four different stimulation conditions for their potential to induce PC differentiation from primary human B cells *in vitro*. We demonstrated that *in vitro* induced PC differentiation generated a CD38^{+}IRF4^{+}Blimp-1^{+} population expressing intracellular IgG. We have showed that the protocol is applicable to B cells from cryopreserved PBMCs and naïve B cells, indicating that it is suitable also for patient material with low memory B cell counts. Currently, how certain mutations contribute to the development of CVID is not well understood. The methods developed in **paper IV** could help identify defects in PC differentiation in B cells from immunocompromised patients and provide more insight into how different mutations result in distinct clinical phenotypes.

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7 POPULAR SCIENCE SUMMARY

7.1 English

From the moment we are born and each day for the rest of our lives, we are surrounded by microbes in our environment. The majority of microbes are harmless, but current estimates suggest that there are around 1400 microbes that cause human diseases (Microbiology by numbers, 2011). Harmful microbes are referred to as pathogens, and include viruses, bacteria, fungi, protozoa and helminths. It is thought that half of all humans that have lived have died from infectious diseases, and even now, 17 million people a year are lost to infectious diseases. New pathogens emerge repeatedly and drug resistance in pathogens is unfortunately becoming an increasing problem. It is not only pathogens that are a threat to our health though, cancer, heart conditions and metabolic diseases arise from within the body, sometime influenced by our lifestyle.

So how do we stay healthy and recover from infections? The body has defence mechanisms that recognise and eliminate threats, both internal and external. The immune system is composed of several cell types and tissues, each with their own unique function and characteristics, that collaborate to achieve their common goal of keeping us safe. The early stage of an infection is dealt with by the innate part of the immune system, which distinguishes self from non-self through the recognition of molecules that are foreign to healthy cells and tissues. Proteins, such as complement, anti-microbial peptides, and antibodies, bind and inhibit pathogens and abnormal cells. Binding also targets pathogens to cells that are specialised in absorbing and breaking down external particles and cells that induce cell death. When dealing with complex pathogens, cells of the innate immune system recruit and activate cells of the adaptive immune system. Key characteristics of the adaptive immune system are specificity and memory: the adaptive immune system targets elements that are unique to a particular pathogen and is capable of swiftly eliminating that same pathogen upon a second encounter. This specific and protective response is established by, on one hand, the elimination of infected and abnormal cells by T cells, and on the other hand, the production of antibodies by B cells. Due to their production of highly effective neutralising antibodies, B cells are key mediators of long-lasting protective immune responses induced by vaccines.

Immune responses can be extremely potent and precisely therefore it is essential that they are well-balanced and tightly regulated. Cells need to communicate and instruct each other, respond to pathogens in an appropriate manner, and dissolve the immune reaction as soon as the threat is eliminated, in order to prevent excessive collateral damage to host cells and tissues. Defects in the immune system can leave us vulnerable to infections and prone to developing immune deficiencies, autoimmune diseases or even cancer.

The focus of my thesis work has been understanding immune responses mediated by antibody-producing B cells. Different types of B cells exist based on how they develop, their function, and their localisation in the body. Those known as B-1 cells contribute to the innate immune system by producing antibodies at steady-state which easily recognize and respond to components of bacterial cell walls. Those known as B-2 cells contribute to the adaptive immune system by secreting antibodies that are more finetuned to specifically recognise particular pathogen components but also exert appropriate effector functions. In general, B-1 cells respond to antigens that can activate B cells without the help of T cells, which are referred to as T cell-independent (TI) antigens. B-2 cells respond to protein antigens, which generally require the help of T cells to induce effective antibody responses, and are therefore referred to as T cell-dependent (TD) antibody responses.

The process of a B cell to develop into a plasma cell whose sole purpose is to secrete large quantities of antibodies with a single specificity is a very meticulous one. This process requires B cells to integrate cues from their environment and communicate with other immune cells through receptors on their surface that induce signaling via intracellular proteins. Often, these signals transmit into the nucleus where they change expression of genes. This leads to alterations in the cellular state from inactive to active, promoting cell migration, enabling interactions between immune cells, proliferation of cells to expand the magnitude of the ongoing response, and supports their effector functions.

An important signaling pathway in B cells is the NF-κB pathway, named after the NF-κB proteins that are central to this pathway and alter gene expression upon their activation. The protein IκBNS regulates the activity of NF-κB proteins in the nucleus and modulates their effect on B cell development and function. Over the course of six years, I have studied the role of IκBNS by using mice that are entirely deficient in IκBNS, the *bumble* mice, and mice that are only deficient in IκBNS in their B cells, the *nfkbid*$^{B-}$ mice.

In **paper I**, we show that without IκBNS a specific subset of B-1 cells, the B-1a cells, do not develop. B cells develop from stem cells and pass through several precursor stages before they are fully matured and functional. We identified a precursor stage that mainly generated B-1a cells but which was missing in *bumble* mice. B-1 cells are the main responders to TI antigens and, consistent with this, we found that the *bumble* mice were unable to establish antibody responses against TI antigens.

In **paper II**, we found that activation of B cells from *bumble* mice was relatively normal up to 24 hours, but cellular division was noticeably affected. TACI is a receptor expressed on the surface of B cells and is essential for efficient TI antibody responses. We found that TACI was not expressed on the surface of *bumble* B cells and not upregulated efficiently upon activation. *Bumble* B cells were also not able to respond to the proteins that bind and activate TACI. B cells destined to become plasma cells need to modify their gene expression to support their future function as antibody secreting cells. The proteins involved in these modifications, IRF4 and Blimp-1, were induced rapidly in *bumble* B cells. Regardless, *bumble* B cells did not

develop into plasma cells. This suggests that 'over-activation' in *bumble* B cells is detrimental to the process of becoming plasma cells.

In **paper III**, we used the *nfkbid*$^{B-}$ mice to better understand to which extent IκBNS was necessary for B cell function during TD antibody responses. We immunized *nfkbid*$^{B-}$ mice with TD antigens and found that although these mice produced antibodies, they were not able to change their antibody isotype, which is the part that determines their effector function. T cells behaved normally in these mice, suggesting that the role of IκBNS during TD responses is limited to plasma cell differentiation and isotype switching in B cells.

In **paper IV**, with the knowledge we gained from papers **I-III** , we developed a method for evaluating the process of B cell activation and development into plasma cells for human B cells. Testing plasma cell development from patients suffering from immune disorders would provide more insights into how the disease develops. This would be beneficial to the diagnostic process or even for the identification of suitable targets for therapeutic interventions.

In summary, the work presented in this thesis contributes to our understanding of processes that are fundamental to effective antibody responses and of direct relevance to immune disorders.

7.2 Svenska

Under hela livet är man omgiven av mikrober från vår miljö. Samtidigt som mest av mikrober är ofarligt, nuvarande beräkningar föreslå att det finns cirka 1400 mikrober som kan förorsaka sjukdom i människor (Microbiology by numbers, 2011). Farliga mikrober, hänvisa som patogen, inkludera virus, bakterier, svampar, protozoer och helminter (parasitiska maskar). Halv av människor som levde är tänkt att ha dött från smittsam sjukdomar. Även nu finns det 17 miljoner av människor som döda från smittsam sjukdom varje år. Läkemedel resistans blir varje år en större problem med uppkomsten av ny patogen. Och patogener inte är ensam att hota hälsa av människor. Även om de utveckla från insidan av vår kroppen, cancer, kardiovaskulär och metabolisk sjukdomar kan vara också påverkat från miljö.

Så hur kan man slå tillbaka smittsam sjukdomar och blir frisk? Kroppen äga försvarsmekanismer som kan identifiera och ta bort internt och externt hot: immunsystemet. Det system involvera flera typ av celler och vävnader som ha unik funktion och kännetecken medan fungera tillsammans att hålla oss frisk.

Medfödd del av immunitet ta hand om tidigt stegar av infektion och särskilja intern mot externt komponenter genom erkännande av molekyler som frisk celler och vävnader bruka inte ha. Komplementproteiner, antimikrobiellt peptider och antikroppar binda och inhibera patogen och sjuk celler. Bindning också hjälpa att driva patogen mot celler som specialisera att internalisera och förgöra externt partiklar som kan inducera celldöd.

När medfödd immunsystemet hantera komplex patogen, det aktivera och rekrytera celler från adaptivt immunsystemet. Viktigt kännetecken av adaptiv immunsystemet är sina specificitet och minne: det kan identifiera och sikta partiklar som är unik för en specifik patogen att utlösa en snabb immunrespons när samma patogen smitta kroppen igen. Specifik och skyddande immunrespons är baserat på 1) ta bort smittat och onormal celler med T celler, 2) antikroppar produktion med B celler. B celler är viktigast för långvarigt effekt immunrespons efter vacciner emedan de producera väldigt effektiv neutraliserande antikroppar.

Immunrespons kan vara extremt effektiv så de måste vara väl balanserat och tätt kontrollerad. Celler behöver kommunicera och informera med varandra, avvanda lämplig immunrespons för specifik patogen. De måste också stoppa immunrespons när hot är bort att förhindra indirekta skador för celler och vävnader. Om det finns problem i immunsystemet, man kan ha höger risk för infektion och vara benägna at immunbrister, autoimmuna sjukdomar och även cancer.

Min PhD fokuserade om förståelse av immunrespons som är medierat med antikropp-producerande B celler. Olika typ av B celler skillna med hur de utveckla, funktion och lokalisering i kroppen. B-1 celler medverka medfödd immunsystemet emedan de ständigt producera antikroppar som kan identifiera och slåss mot partiklar från bakteriecellväggar. B-1 celler svara mot antigen som aktivera B celler utan hjälpa av T celler (T cell oberoende antigen; TI). B-2 celler medverka adaptivt del av immunsystemet med finstämd antikroppar som kan identifiera specifikt patogenpartiklar och leda till lämplig svar. B celler svara mot antigen som behöver T celler hjälp att inducera effektivt antikropprespons (T cell beroende antigen; TD).

Att utveckla till plasmacell som kan producera stora mängder av antikroppar med unik specificitet, B celler behöver följa process som skulle integrera signaler från miljön, kommunikation med andra immunceller. Kommunikationen fungera genom receptor som inducera signalvägar i immuncellen och kan leda att andra genexpression i cellkärnan. Det kan aktivera cellen, inducera cellmigrering, stödja immuncellinteraktioner, cellspridning som leda till ökade immunrespons och support till effektfunktioner.

NF-κB signalvägar är viktigt i B celler med centralt NF-κB proteiner som spela en stor roll och förändra genexpression efter de är aktiverat. IκBNS proteiner styra NF-κB aktivitet i cellkärnan och modulera NF-κB effekt över B celler utveckling och funktion. Över min 6 års-projekt studerade jag IκBNS roll i immunrespons och komparerade en musmodell helt utan IκBNS (*bumble* mus) mot en andra musmodell som inte ha IκBNS bara i B celler (*nfkbid*$^{B-}$ mus).

I **artikel I**, vi demonstrerade att en subset av B-1 celler, B-1a celler, kan inte utveckla utan IκBNS. B celler utveckla från stamceller i flera stegar innan att vara fullt mogna och funktionellt. Vi identifierade en föregångare steg som generera huvudsaklingen B-1a celler och som saknas i *bumble* möss. Eftersom B-1 celler är ansvarig för TI antigen immunrespons, vi konfirmerade att *bumble* möss kunde inte utveckla antikropprespons mot TI antigen.

I **artikel II**, vi hittades att B celler från *bumble* möss kan vara aktiverat för 24 timmar men att celldelning är påverkat. Vi studerade en receptor, TACI, som är uttryckat på cellytor av B celler

och är grundläggande för effektivt TI antikropprespons. I *bumble* möss, TACI är inte uttryckat på cellytor av B celler och inte uppreglerad effektiv efter aktivering. *Bumble* möss kunde inte utveckla immunrespons som binda och aktivera TACI. Att bli plasmaceller, B celler måste förändra deras genexpression att stödja ny funktion som antikropp utsöndrande celler. Proteiner involverande i dessa ändringar, IRF4 and Blimp-1, var snabb inducerat i *bumble* B celler. Oavsett kunde inte *bumble* B celler utveckla till plasmaceller. Det föreslår att ”överaktivering” av *bumble* B celler ha en skadlig effekt över process av blivande plasmaceller.

I **artikel III**, *nfkbid*$^{B-}$ var använda att förstå hur IκBNS deltar i TD antikropprespons medlat av B celler. Vi immuneserade *nfkbid*$^{B-}$ möss med TD antigen och hittades att möss kunde producera antikroppar men de kunde inte förändra antikropp isotyp, som är viktigt att bestämma effektfunktion. T celler bete sig normalt i *nfkbid*$^{B-}$ möss som föreslår att IκBNS roll i TD antigenrespons är limiterat till plasmaceller differentiering och isotyp ändring i B celler.

I **artikel IV**, vi samlade kunskap från artiklar I till III och utveckla en metod att evaluera process av human B celler aktivering och utveckling till plasmaceller. Att testa plasmaceller utveckling från patienter med immunsjukdomar skulle hjälpa att förstå hur sjukdomen utveckla. Det skulle också hjälpa med diagnosprocess och eventuellt identifiera mål för terapeutiskt interventioner.

För att sammanfatta, detta avhandling bidraga med vår uppfattning av fundamentalt processer för en effektivt antikropprespons och är relevant för immunsjukdomar.

7.3 Nederlands

Vanaf het moment dat we geboren worden, worden we elke dag van ons leven omgeven door micro-organismen in onze omgeving. De meerderheid van deze micro-organismen is ongevaarlijk, maar huidige schattingen suggereren dat er ongeveer 1400 micro-organismen ziektes kunnen veroorzaken bij mensen (Microbiology by numbers, 2011). Schadelijke micro-organismen worden pathogenen genoemd, en deze bestaan uit virussen, bacteriën, schimmels, protozoa, en parasitaire wormen. Er wordt gedacht dat de helft van alle mensen die ooit hebben geleefd overleden zijn aan de gevolgen van infectieziektes, en dat ook nu 17 miljoen mensen per jaar overlijden aan infectieziektes. Nieuwe pathogenen komen vaak op en resistentie tegen geneesmiddelen is een zich ontwikkelend probleem. Het zijn echter niet alleen pathogenen die een gevaar vormen voor onze gezondheid, kanker, hartkwalen, en metabole ziekten kunnen ontstaan in het lichaam, soms als gevolg van onze levensstijl.

Maar hoe blijven we dan gezond en herstellen we van infecties? Het lichaam heeft zowel interne als externe verdedigingsmechanismen die gevaren herkennen en onschadelijk maken. Het immuunsysteem bestaat uit verscheidene soorten cellen en weefsels, elk met eigen unieke functies en eigenschappen, die samenwerken om hun gezamenlijke doel om ons te beschermen te bereiken. In het eerste stadium van een infectie treedt het aspecifieke, ofwel aangeboren, immuunsysteem op. Deze onderscheidt eigen van vreemd door het herkennen van moleculen

die gezonde cellen en weefsels niet hebben. Eiwitten zoals het complement systeem, antimicrobiële eiwitten, en antilichamen kunnen pathogenen en abnormale cellen binden en tegenwerken. Binding van deze eiwitten zorgt er daarnaast ook voor dat pathogenen herkend worden door gespecialiseerde cellen die deeltjes kunnen opnemen en afbreken, en andere gespecialiseerde cellen die ongezonde cellen kunnen aansturen tot celdood. Wanneer het om complexe pathogenen gaat, werven en activeren cellen van het aspecifieke immuunsysteem cellen van het adaptieve, ofwel verworven, immuunsysteem. De belangrijkste eigenschappen van het adaptieve immuunsysteem zijn specificiteit en geheugen. Dat wil zeggen dat het adaptieve immuunsysteem zich richt op unieke elementen van een pathogeen en het daarmee gemakkelijk en snel het pathogeen onschadelijk kan maken kan opruimen wanneer deze zich een volgende keer voordoet. Deze specifieke en beschermende respons is opgebouwd uit aan de ene kant het uit de weg ruimen van geïnfecteerde en abnormale cellen door T cellen, en aan de andere kant het produceren van antilichamen door B cellen. Door hun productie van zeer effectieve en neutraliserende antilichamen vervullen B cellen ook een essentiële rol in langdurige beschermende reacties op vaccinaties.

Immuunreacties kunnen zeer krachtig zijn en daarom is het van belang dat deze goed gebalanceerd zijn en goed gecontroleerd worden. Cellen moeten met elkaar communiceren en elkaar instrueren, reageren op pathogenen op de juiste manier, en de immuunrespons beëindigen zodra het gevaar geweken is om zo schade aan eigen cellen en weefsels te minimaliseren. Defecten in het immuunsysteem kunnen ons vatbaar maken voor infecties en het ontwikkelen van immuundeficiënties, auto-immuniteit, en zelfs kanker.

De focus van mijn thesis ligt op het begrijpen van de immuunrespons door antilichaam-producerende B cellen. Er bestaan verschillende typen B cellen gebaseerd op hun ontwikkeling, functie, en plaats in het lichaam. Een bepaald type cellen, bekend als B-1 cellen, dragen bij aan het aangeboren immuunsysteem met een constante productie van antilichamen die algemene componenten van de bacteriële celwand herkennen. B-2 cellen dragen daarentegen meer bij aan het adaptieve immuunsysteem met een productie van antilichamen die preciezer en specifieke pathogenen herkennen, en ook gepaste effect mechanismen teweegbrengen. Over het algemeen reageren B-1 cellen op antigenen die B cellen kunnen activeren zonder de hulp van T cellen, ook wel T cel-onafhankelijke antigenen genoemd. B-2 cellen reageren daarentegen op eiwit-antigenen welke gewoonlijk de hulp van T cellen nodig hebben of effectieve antilichamen te produceren, dit wordt daarom ook wel T cel-afhankelijke antilichaam respons genoemd.

Het proces waarin een B cel zich ontwikkelt tot een plasma cel, welke als enig doel heeft om grote hoeveelheden antilichamen uit te scheiden met een enkele specificiteit, is een zeer nauwkeurig proces. Dit proces vereist de B cel om signalen uit de omgeving te integreren en te communiceren met andere immuuncellen via de receptoren op zijn oppervlakte en daaropvolgend eiwitten binnenin de cel. Meestal dragen deze signalen door tot in de celkern waar ze de expressie van genen veranderen. Dit leidt er vervolgens toe dat de cel wordt

geactiveerd, gaat migreren, interacties met andere cellen aangaat, zich vermenigvuldigt om de grootte van de reactie te vergroten, en effector functies ontwikkelt.

Een belangrijke signaaltransductie in B cellen is de zogenaamde 'NF-κB pathway', vernoemd naar de centrale NF-κB eiwitten die de genexpressie kunnen aanpassen als ze geactiveerd worden. Het eiwit IκBNS reguleert de activiteit van de NF-κB eiwitten in de celkern en regelt het effect op de ontwikkeling en activiteit van B cellen. De afgelopen zes jaar heb ik de rol van IκBNS bestudeerd door muizen te gebruiken die helemaal geen IκBNS hebben, genaamd *bumble* muizen, en muizen die alleen IκBNS missen in hun B cellen, genaamd *nfkbid*$^{B-}$ muizen.

In **artikel I**, laten we zien dat zonder IκBNS een bepaalde tak van B-1 cellen, de B-1a cellen, zich in zijn geheel niet ontwikkelen. B cellen ontwikkelen vanuit stamcellen en doorgaan verschillende voorloper stadia voordat ze gerijpt en functioneel zijn. We hebben een voorloper stadium geïdentificeerd welke vooral B-1a cellen produceert maar welke misten in de *bumble* muizen. B-1 cellen zijn de belangrijkste cellen voor een reactie op T cel-onafhankelijke antigenen en we vonden dan ook dat de *bumble* muizen in zijn geheel geen antilichamen konden produceren tegen deze soort antigenen.

In **artikel II**, laten we zien dat activatie van B cellen in *bumble* muizen de eerste 24 uur normaal verloopt, maar dat de celdeling daarna is aangetast. TACI is een receptor op de oppervlakte van B cellen en is essentieel voor een T cel-onafhankelijke antilichaam respons. We hebben aangetoond dat TACI niet op de oppervlakte van *bumble* B cellen voor komt, ook niet na activatie van de B cel. *Bumble* B cellen waren ook niet in staat te reageren op eiwitten die normaalgesproken binden aan TACI. B cellen die zich ontwikkelen tot plasmacellen moeten hun genexpressie aanpassen om hun toekomstige functie als antilichaam producerende cel te ondersteunen. De eiwitten die hierin betrokken zijn, IRF4 en Blimp-1, werden snel geactiveerd in *bumble* B cellen. Toch lukte het *bumble* B cellen niet om zich te ontwikkelen tot plasmacellen. Dit suggereert dat 'over-activatie' in *bumble* B cellen nadelig is voor het proces om een plasmacel te worden.

In **artikel III**, hebben we *nfkbid*$^{B-}$ muizen gebruikt om een beter inzicht te krijgen in hoeverre IκBNS nodig is voor B cellen om te functioneren in een T cel-afhankelijke respons. We hebben *nfkbid*$^{B-}$ muizen geïmmuniseerd met T cel-afhankelijke antigenen en zagen dat ondanks dat deze muizen antilichamen produceerden, ze niet in staat waren van type antilichaam te wisselen wat belangrijk is voor het effect van het antilichaam. T cellen gedroegen zich normaal in deze muizen wat suggereert dat de rol van IκBNS tijdens een T cel-afhankelijke respons zich beperkt tot plasmacel ontwikkeling en het type antilichaam wat geproduceerd wordt.

In **artikel IV**, met de kennis van artikelen **I-III**, ontwikkelden we een methode om het proces van B cel activatie en ontwikkeling tot plasmacel te evalueren voor humane B cellen. Het testen van de ontwikkeling van plasmacellen van patiënten met immuunstoornissen kan meer inzicht geven in hoe deze stoornissen zich ontwikkelen. Dit zou voordelig zijn voor het diagnostiek-proces of zou zelfs voor identificatie van potentiële doelwitten voor therapeutische ingrijpen kunnen zorgen.

In het kort, het werk in deze thesis draagt bij aan onze kennis over de fundamentele processen van effectieve antilichaam respons en is van direct belang voor immuunstoornissen.

7.4 Deutsch

Schon von Geburt an sind wir unser ganzes Leben von Mikroben in unserer Umwelt umgeben. Die Mehrheit dieser Mikroben ist harmlos, aber aktuelle Schätzungen gehen davon aus, dass es rund 1400 Mikroben gibt, die Krankheiten beim Menschen verursachen können. Schädliche Mikroben werden als Pathogene bezeichnet und umfassen Viren, Bakterien, Pilze, Protozoen und Helminthen. Es wird angenommen, dass die Hälfte aller Menschen, die bisher gelebt haben an Infektionskrankheiten gestorben sind und selbst heute sterben noch 17 Millionen Menschen pro Jahr durch Infektionskrankheiten. Immer wieder treten neue Krankheitserreger auf und Arzneimittelresistenzen werden leider zu einem zunehmenden Problem. Es sind jedoch nicht nur die Pathogene, die unsere Gesundheit gefährden, sondern auch andere Krankheiten wie Krebs, Herz- und Stoffwechselerkrankungen, die manchmal auch von unserem Lebensstil beeinflusst werden können.

Wie also bleiben wir gesund, bekämpfen Infektionen und erholen uns wieder von ihnen? Der Körper verfügt über Abwehrmechanismen, die sowohl interne als auch externe Bedrohungen erkennen und beseitigen. Das Immunsystem besteht aus verschiedenen Zelltypen und Geweben, von denen jedes spezifische Merkmale und Funktionen hat und die zusammenarbeiten, um das gemeinsame Ziel zu erreichen uns zu schützen. Der Anfang einer Infektion wird durch den angeborenen Teil des Immunsystems bekämpft, welcher „Selbst“ von „Nicht-Selbst“ unterscheidet, indem er Moleküle erkennt, die auf gesunden, körpereigenen Zellen nicht vorhanden sind. Proteine, wie das Komplement-System, antimikrobielle Peptide und Antikörper, binden Krankheitserreger sowie abnormale Zellen und hemmen diese somit. Durch das Binden von Pathogenen an verschiedene Proteine können die Krankheitserreger für andere Zellen sichtbar gemacht werden, die darauf spezialisiert sind, externe Partikel und Zellen aufzunehmen und abzubauen oder den Zelltod von infizierten Zellen zu induzieren. Wenn das angeborene Immunsystem jedoch auf komplexere Pathogene trifft, rekrutiert und aktiviert es Zellen des adaptiven Immunsystems. Schlüsselmerkmale des adaptiven Immunsystems sind seine Spezifität und sein Gedächtnis. Das adaptive Immunsystem zielt spezifisch auf Elemente ab, die für einen bestimmten Erreger einzigartig sind und kann diesen Erreger somit bei einer zweiten Begegnung schneller eliminieren. Diese spezifische, schützende Immunantwort ist abhängig von der Bekämpfung infizierter und abnormaler Zellen durch T-Lymphozyten (T-Zellen) und der Produktion von Antikörpern durch B-Lymphozyten (B-Zellen). Diese hochwirksamen, neutralisierenden Antikörper machen B-Zellen zu den Schlüsselmediatoren für lang anhaltende, schützende Immunantworten, die durch Impfstoffe induziert werden.

Da Immunantworten sehr stark sein können, ist es wichtig, dass sie ausgewogen und streng reguliert sind. Zellen müssen miteinander kommunizieren und sich gegenseitig anweisen, was zu tun ist, um die Krankheitserreger angemessen zu beseitigen und die Immunreaktion wieder zu beenden, sobald die Bedrohung beseitigt wurde. Nur so können übermäßige Kollateralschäden an körpereigenen Zellen und Geweben verhindert werden. Fehlerhaftes Verhalten vom Immunsystem kann uns anfälliger machen für Infektionen und sogar zu Immunschwächen, Autoimmunerkrankungen oder Krebs führen.

Der Schwerpunkt meiner Doktorarbeit lag darin, Immunantworten zu verstehen, die durch die Antikörper-produzierenden B-Lymphozyten vermittelt werden. Es gibt verschiedene Arten von B-Zellen, abhängig von Entwicklung, Funktion und Lokalisierung im Körper. B-1-Zellen tragen zum angeborenen Immunsystem bei, indem sie im normalen Zustand Antikörper produzieren, die Komponenten von Bakterien leicht erkennen und darauf reagieren. B-2-Zellen hingegen tragen zum adaptiven Immunsystem bei, indem sie Antikörper sekretieren, die bestimmte Pathogenkomponenten extrem spezifisch erkennen und geeignete Effektorfunktionen ausüben. Im Allgemeinen reagieren B-1-Zellen auf Antigene, die B-Zellen ohne die Hilfe von T-Zellen aktivieren können und als T-Zell-unabhängige (TI) Antigene bezeichnet werden. B-2-Zellen reagieren auf Proteinantigene, die im Allgemeinen die Hilfe von T-Zellen benötigen, um wirksame Antikörperantworten zu induzieren und werden daher als T-Zell-abhängige (TD) Antikörperantworten bezeichnet.

Die Entwicklung einer B-Zelle bis hin zu einer Antikörper-produzierenden Plasmazelle ist sehr genau geregelt. Die Aufgabe der Plasmazellen besteht darin große Mengen von Antikörpern mit einer einzigen Spezifität zu produzieren. Dieser Weg erfordert, dass B-Zellen Merkmale aus ihrer Umgebung wahrnehmen und mit anderen Immunzellen kommunizieren. Dies geschieht über Rezeptoren auf ihrer Zell-Oberfläche, welche die Signalübertragung über intrazelluläre Proteine induzieren. Oft übertragen sich diese Signale in den Zellkern, wo sie die Genexpression verändern. Abhängig von den verschiedenen Signalen können verschiedene Parameter in der Zelle beeinflusst werden: der zelluläre Zustand von inaktiv zu aktiv, Förderung der Zellmigration, erhöhte Interaktionen zwischen Immunzellen, erhöhte Zellteilung und Unterstützung der Effektorfunktionen.

Ein wichtiger Signalweg in B-Zellen ist der NF-κB-Weg, benannt nach den NF-κB-Proteinen, die für diesen Weg von zentraler Bedeutung sind und die Genexpression bei ihrer Aktivierung verändern. Das Protein IκBNS reguliert die Aktivität von NF-κB-Proteinen im Zellkern und moduliert deren Wirkung auf die Entwicklung und Funktion von B-Zellen. Über sechs Jahre lang habe ich die Rolle von IκBNS untersucht, indem ich Mäuse verwendet habe, denen IκBNS entweder vollständig fehlt (*bumble*-Mäuse) und Mäuse, denen IκBNS nur in den B-Zellen fehlt (*nfkbid*^{B} -Mäuse).

In **Artikel I** zeigen wir, dass sich ohne IκBNS eine bestimmte Untergruppe von B-1-Zellen, die B-1a-Zellen, nicht entwickelt. B-Zellen entwickeln sich aus Stammzellen und durchlaufen mehrere Vorstufen, bevor sie vollständig ausgereift und funktionsfähig sind. Wir identifizierten ein Vorläuferstadium, das hauptsächlich B-1a-Zellen erzeugte, aber in *bumble*-Mäusen fehlte.

B-1-Zellen sind normalerweise die Hauptantwortenden auf T-Zell-unabhängige Antigene und in Übereinstimmung damit fanden wir, dass die *bumble*-Mäuse keine Antikörperantworten gegen T-Zell-unabhängige Antigene etablieren konnten.

In **Artikel II** fanden wir, dass die Aktivierung von B-Zellen aus *bumble*-Mäusen bis zu 24 Stunden relativ normal war, die Zellteilung jedoch merklich beeinflusst wurde. TACI ist ein Rezeptor, der auf der Oberfläche von B-Zellen exprimiert wird und für effiziente T-Zell-unabhängige- Antikörperantworten essentiel ist. Wir haben herausgefunden, dass TACI nicht auf der Oberfläche von *bumble*-B-Zellen exprimiert war und bei Aktivierung auch nicht effizient hochreguliert wurde. *Bumble*-B-Zellen waren auch nicht in der Lage, auf die Proteine zu reagieren, die TACI binden und aktivieren. B-Zellen, die dazu bestimmt sind, Plasmazellen zu werden, müssen ihre Genexpression verändern, um ihre zukünftige Funktion als Antikörper-sekretierende Zellen zu unterstützen. Die Proteine, die an diesen Veränderungen beteiligt sind, IRF4 und Blimp-1, wurden schnell in *bumble*-B-Zellen hochreguliert. Unabhängig davon entwickelten sich die *bumble*-B-Zellen trotzdem nicht zu Antikörper-sekretierenden Plasmazellen. Dies deutet darauf hin, dass eine „Überaktivierung“ in *bumble*-B-Zellen deren Entwicklung zu Plasmazellen negativ beeinträchtigt.

In **Artikel III** verwendeten wir die *nfkbid*$^{B-}$-Mäuse, um besser zu verstehen, inwieweit IκBNS für die B-Zell-Funktion während der T-Zell-abhängigen Antikörperantworten notwendig war. Wir immunisierten *nfkbid*$^{B-}$-Mäuse mit T-Zell-abhängigen Antigenen und stellten fest, dass diese Mäuse zwar Antikörper produzierten, jedoch ihren Antikörper-Isotyp nicht ändern konnten, welcher ihre Effektorfunktion bestimmt. Die T-Zellen verhielten sich bei diesen Mäusen normal, was darauf hindeutet, dass die Rolle von IκBNS während der T-Zell-abhängigen Immunantworten auf die Differenzierung von Plasmazellen und den Isotypwechsel in B-Zellen beschränkt ist.

In **Artikel IV** haben wir mit den Erkenntnissen aus Artikeln I-III eine Methode entwickelt, mit der wir die Aktivierung von B-Zellen und deren Entwicklung zu Plasmazellen in menschlichen B–Zellen untersuchen können. Das Testen der Plasmazellen-Entwicklung in Patienten mit verschiedenen Immunerkrankungen würde mehr Einblicke in die Entwicklung der Krankheit liefern. Dies wäre hilfreich für die Diagnose oder sogar für die Identifizierung möglicher Ziele für therapeutische Ansätze.

Zusammengefasst tragen die in dieser Doktorarbeit vorgestellten Ergebnisse zum Verständnis von Prozessen bei, die für wirksame Antikörper-Reaktionen von grundlegender Bedeutung sind und eine direkte Relevanz für Immunerkrankungen haben.

7.5 Español

Desde que nacemos y cada día durante el resto de nuestras vidas, nos encontramos rodeados de microbios en el ambiente. Aunque la mayoría de microbios no son perjudiciales, algunas estimaciones apuntan a que hay alrededor de 1400 microbios que causan enfermedades a los

humanos (Microbiology by numbers, 2011). Los microbios que son perjudiciales son llamados agentes patógenos, y éstos incluyen virus, bacterias, hongos, protozoos y helmintos. Se cree que la mitad de todos los seres humanos que han existido han muerto a causa de enfermedades infecciosas, e incluso ahora, 17 millones de persones mueren cada año a causa de éstas. Nuevos patógenos están surgiendo constantemente y la resistencia a fármacos que desarrollan se está convirtiendo desafortunadamente en un problema cada vez mayor. No solamente los agentes patógenos suponen un riesgo para nuestra salud, sino que otras enfermedades como el cáncer, enfermedades cardíacas y trastornos metabólicos son originados en nuestro propio cuerpo, en ocasiones influenciadas por nuestro estilo de vida.

Luego, ¿cómo podemos mantenernos saludables y recuperarnos de infecciones? El cuerpo cuenta con un mecanismo de defensa capaz de reconocer y eliminar agresiones tanto internas como externas. El sistema inmune está compuesto por diversos tipos celulares y tejidos, cada uno de ellos con funciones y características propias, que colaboran para alcanzar su meta común que es mantenernos a salvo. De la etapa más temprana de la infección se encarga el sistema inmune innato, el cual distingue entre lo propio y lo ajeno a través del reconocimiento de moléculas que no suelen encontrarse en células y tejidos sanos. Proteínas, como por ejemplo el complemento, péptidos antimicrobianos y los anticuerpos se unen e inhiben patógenos y células que sean anormales. Esta unión también sirve para marcar los agentes patógenos para células que están especializadas en absorber y degradar moléculas externas y células que inducen muerte celular. A la hora de lidiar con agentes patógenos complejos, las células del sistema inmune innato reclutan y activan células del sistema inmune adaptativo, el cual destaca por su especificidad y memoria: el sistema inmune adaptativo reconoce elementos que son únicos para cada agente patógeno en particular y tiene la capacidad de eliminarlo rápidamente al encontrase de nuevo con él. Esta respuesta específica y protectora se debe por un lado a la eliminación de las células infectadas y anormales por parte de las células T y, por otro lado, a la producción de anticuerpos por parte de las células B. Gracias a su producción de anticuerpos neutralizantes altamente efectivos, las células B son mediadores clave en las respuestas inmunes protectoras a largo plazo que inducen las vacunas.

Las respuestas inmunes pueden llegar a ser extremadamente potentes y es por este motivo que es necesario que estén correctamente equilibradas y altamente reguladas. Las células deben ser capaces de mantener una comunicación e informarse entre ellas, de responder a los agentes patógenos de forma adecuada y de finalizar la respuesta inmune tan pronto como haya sido eliminado el peligro para evitar un exceso de daños colaterales a las células y tejidos del huésped. Defectos en el sistema inmune nos hacen vulnerables frente a infecciones y susceptibles a desarrollar inmunodeficiencias, enfermedades autoinmunes o incluso cáncer.

Mi tesis se ha centrado en entender las respuestas inmunes mediadas por células B productoras de anticuerpos. Existen distintos tipos de células B en base a cómo éstas se desarrollan, su función y la posición que ocupan en el cuerpo. Las llamadas células B-1 contribuyen al sistema inmune innato al producir anticuerpos en condiciones normales que fácilmente reconocen y responden a componentes de las paredes celulares bacterianas. Las células B-2 contribuyen al

sistema inmune adaptativo al secretar anticuerpos especializados en reconocer componentes específicos de los agentes patógenos y también en ejercer determinadas funciones efectoras. En general, las células B-1 responden a antígenos que activan células B sin la ayuda de las células T, los cuales son denominados antígenos independientes de célula T (*T cell-independent*, TI). Las células B responden a antígenos proteicos que generalmente requieren de la ayuda de las células T para inducir respuestas de anticuerpos efectivas, y son por lo tanto denominadas respuestas de anticuerpos dependientes de célula T (*T cell-dependent*, TD).

El proceso por el cual una célula B se convierte en célula plasmática cuyo único cometido es el de secretar grandes cantidades de anticuerpos con una única especificidad es altamente preciso. Este proceso requiere que la célula B integre las señales que le llegan de su ambiente y que se comunique con otras células inmunitarias a través de receptores que tiene en su superficie que inducen una señalización a través de proteínas intracelulares. Todo esto supone una serie de cambios en el estado de la célula que pasa de inactivo a activo, promoviendo la migración celular, posibilitando interacciones entre células inmunitarias y la proliferación celular que expande la magnitud de la respuesta en curso y apoya sus funciones efectoras.

Una importante vía de señalización en las células B es la vía NF-κB, la cual recibe el nombre de las proteínas NF-κB, que son elementales en esta vía y ven su expresión génica alterada cuando ésta es activada. La proteína IκBNS regula la actividad de las proteínas NF-κB en el núcleo y modula su efecto en el desarrollo y función de las células B. Durante seis años, he estudiado el papel que juega IκBNS mediante el uso de ratones que son totalmente deficientes en IκBNS, los ratones *bumble*, y ratones que son deficientes en IκBNS solamente en sus células B, los ratones *nfkbid*$^{B-}$.

En el **artículo I**, reportamos que sin IκBNS hay un subconjunto de células B-1, las células B-1a, que no se desarrollan. Las células B se originan a partir de células madre que pasan por diversas etapas precursoras antes de ser completamente maduras y funcionales. Hemos identificado una etapa precursora que principalmente genera células B-1a pero que sin embargo estaba ausente en ratones *bumble*. Las células B-1 responden principalmente a antígenos TI y, coherentemente, observamos que ratones *bumble* eran incapaces de establecer una respuesta de anticuerpos contra antígenos TI.

En el **artículo II**, observamos que la activación de las células B en ratones *bumble* era relativamente normal en las primeras 24 horas pero que la división celular estaba notablemente afectada. TACI es un receptor expresado en la superficie de las células B y es esencial para llevar a cabo respuesta de anticuerpos TI. Vimos que TACI no se expresaba en la superficie de células B *bumble* y que su expresión tampoco se regulaba al alza eficientemente después de haber sido las células activadas. Las células B *bumble* además eran incapaces de responder a las proteínas que se unen a TACI y lo activan. Las células B que están cometidas a convertirse en células plasmáticas necesitan modificar su expresión génica para adaptarse a su futura función como células secretoras de anticuerpos. La expresión de las proteínas involucradas en

estas modificaciones, IRF4 y Blimp-1, era rápidamente inducida en células B *bumble*. A pesar de todo, células B *bumble* no se convertían en células plasmáticas. Esto sugiere que la "sobreactivación" de células B *bumble* es perjudicial para el proceso de conversión a células plasmáticas.

En el **artículo III**, usamos los ratones *nfkbid*$^{B-}$ para entender en qué medida IκBNS era necesario para una correcta función de las células B durante las respuestas de anticuerpos TD. Inmunizamos ratones *nfkbid*$^{B-}$ con antígenos TD y observamos que, aunque estos ratones son capaces de producir anticuerpos, no pueden cambiar el isotipo de éstos, que es el componente del anticuerpo que determina su función efectora. Las células T tenían un comportamiento normal en estos ratones, sugiriendo así que el rol de IκBNS en las respuestas TD está limitado a la diferenciación en célula plasmática y la conmutación de isotipo en las células B.

En el **artículo IV**, con el conocimiento que adquirimos en los artículos **I-III**, desarrollamos un método para evaluar el proceso de activación de la célula B y generación de células plasmáticas en células B humanas. Investigar la generación de células plasmáticas en pacientes que padecen enfermedades inmunes nos permitiría obtener una información sobre cómo estas enfermedades se desarrollan. Esto sería beneficioso para el proceso diagnóstico e incluso a la hora de identificar candidatos adecuados para intervenciones terapéuticas.

En resumen, el trabajo presentado en esta tesis contribuye al conocimiento que tenemos de los procesos fundamentales para las respuestas de anticuerpos efectivas y que son de especial relevancia en enfermedades inmunes.

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