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Identification of new immunomodulating targets and agents in B lymphocytes

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Jury

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List of abbreviations

Ab	antibody
AID	autoimmune disease
AKT	also known as PKB
AP-1	activator protein 1
APC	antigen-presenting cell
APC (for FACS)	allophycocyanin, fluorochrome for FACS
APRIL	a proliferation-inducing ligand
BAFF	B cell-activating factor, also kown as BLyS
BCR	B cell receptor
BHK cells	baby hamster kidney cells
BLK	B lymphoid tyrosine kinase
BLyS	B lymphocyte survival factor, also known as BAFF
bp	base pairs
Breg	regulatory B cell
BTK	Bruton tyrosine kinase
CD	cluster of differentiation
CLL	chronic lymphocytic leukemia
CREB	cAMP response element-binding protein
Cre enzyme	cyclization recombination enzyme
CSNK1	casein kinase 1
CTLA4	cytotoxic T lymphocyte- associated antigen 4
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DSA	donor specific antibody
ECM	extra-cellular matrix
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GzmB	granzyme B
HRP	horse radish peroxidase
IC_{50}	half maximal inhibitory concentration

IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILK	integrin-linked kinase
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
IVIG	intravenous immunoglobulin
JAK	Janus kinase
KO	knock-out
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
ODN1826	murine TLR9 ligand
ODN2006	human TLR9 ligand
PBS	phosphate buffered saline
PD(-L)	programmed death (-ligand)
PC	plasma cell
PCR	polymerase chain reaction
PE	phycoerythrin
PE/Cy5	phycoerythrin-cyanine 5
PI3K	phosphatidylinositol-3-kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKB	protein kinase B, also known as AKT
PtdIns	phosphatidylinositol
RA	rheumatoid arthritis

RNA(i)	ribonucleic acid (interference)
RT-PCR	real time polymerase chain reaction
Ser	serine
shRNA	short hairpin ribonucleic acid
siRNA	small interference ribonucleic acid
SLE	systemic lupus erythematosus
SYK	spleen tyrosine kinase
TCR	T cell receptor
Teff	T effector cell
TGF	transforming growth factor
Th	T helper cell
Thr	threonine
TLR	toll-like receptor
TNF(R)	tumor necrosis factor (receptor)
TNP	trinitrophenyl hapten
TNP-BSA	trinitrophenyl hapten conjugated to bovine serum albumin
TNP-Ficoll	trinitrophenyl hapten conjugated to Ficoll
Treg	regulatory T cell
Tyr	tyrosine
WT	wild type

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Chapter 1 : General introduction

1.1. B cells as target for new immunomodulatory drugs

B cells play a considerable, but not yet fully understood, role as a pathogenic factor in different clinical situations such as cancer, (auto-) immune disorders, transplant rejection and graft-versus-host disease. Hence, the B cell represents a promising target for new drugs. As there is a strong unmet need to target B lymphocytes by specific drugs, there is a similar need for a broad *in vitro* immunoassay that could simplify the screening of such agents. Although *in vitro* assays have limitations, they form an approximation of the actual *in vivo* conditions and are essential to obtain insight into complex biological phenomena leading to new discoveries and predictions.

1.2. B cell biology

1.2.1. Development of B cells

B cells are produced continuously throughout life and their development and differentiation occurs in multiple phases (Figure 1.1) requiring the coordinated action of a network of cytokines and transcription factors to form the various B cell subset phenotypes[1–5] (Table 1.1). Two key processes characterize B cell maturation: 1) the generation of functional antigen-specific receptors and 2) the selection of B cells that express useful antigen receptors. B cells initially arise from the foetal liver and then from hematopoietic stem cells that originate from the bone marrow and differentiate further into multi-potent progenitor cells, then into common lymphoid progenitor cells. Hereby, several transcription factors, EA2, EBF and Pax5, are crucial in promoting B cell lineage commitment and differentiation. Developing B cells then progress through rearrangement of immunoglobulin (Ig) heavy and light chain gene segments (variable V, diversity D, joining J), expression of cluster of differentiation (CD) 19 and expression of recombination-activating genes RAG-1 and RAG-2 from pro-B to pre-B (with formation and surface expression of the pre-B cell receptor (pre-BCR) which delivers survival and further differentiation signals) to immature B cells, culminating in the expression of IgM mature B cell receptor (BCR) on the cell surface that can bind antigens. V_H, D_H and J_H rearrangements of the heavy chain (H-chain) together with the V_L-J_L rearrangements of the light chain (L-chain) gene segments produce clonally-unique, Ig variable regions that specifically bind antigen. These maturation steps depend on close interactions between

developing B cells and bone marrow stromal cells, which provide critical adhesive integrins, growth factors, chemokines and cytokines. Immature B cells that display strong reactivity to self-antigen are subjected to negative selection through deletion, receptor editing or anergy.

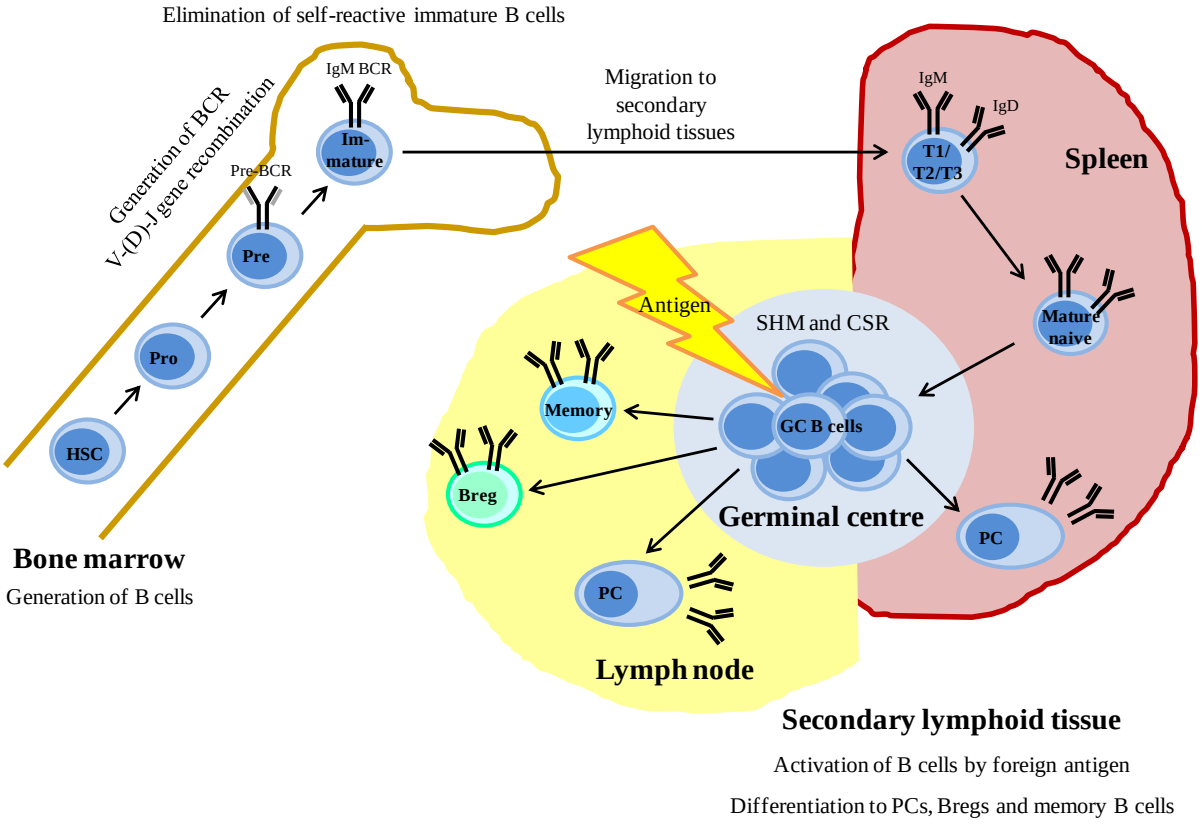

Figure 1.1. Schematic representation of B cell development.
(Pre-)BCR, (Pre-)B cell receptor; BCR, B cell receptor; Breg, regulatory B cell; CSR, class switch recombination; GC, germinal centre; HSC, hematopoietic stem cell; Ig, immunoglobulin; PC, plasma cell; SHM, somatic hypermutation; T1 / T2 / T3, transitional 1 / 2 / 3 B cell.

Immature B cells leave the bone marrow and home to secondary lymphoid tissues, preferentially to the spleen, to complete their maturation. Transitional B cells mark the crucial link between bone marrow immature and peripheral mature B cells and can be divided into T1, T2 and T3 B cells, according to surface phenotype, functional criteria and localization within the spleen. When transitional B cells in the spleen display a strong self-reactivity, they will be eliminated by clonal deletion. B cells can be separated into two lineages, B-1 and B-2[2–4], and each lineage plays distinct yet overlapping roles in humoral immunity. B-1 cells can be further sub-divided into B-1a ($CD5^+$) that provide innate protection against bacterial infections (including via production of natural antibodies) and B-1b ($CD5^-$) cells which respond to antigenic polysaccharides and other T cell-independent antigens. B-2 cells mature

further into marginal zone B cells (which are retained in the spleen) or follicular B cells (which recirculate in blood and secondary lymphoid tissues and are the major contributors to T cell-dependent immune responses) guided by BCR signals, B cell-activating factor (BAFF)[3,4], a proliferation-inducing agent (APRIL)[3,4] and expression of transcription factors, NOTCH2 (Notch family members play a role in a variety of developmental processes by controlling cell fate decisions) and Bruton tyrosine kinase (BTK)[5]. Mature B cells within the germinal centres of secondary lymphoid tissues come in contact with foreign antigens and with the help of T follicular helper cells they become triggered to proliferate, to differentiate and to undergo somatic hypermutation[3,5] (a programmed process of mutation affecting the variable regions of Ig genes giving rise to clonal variants with altered antigen affinity and specificity) and class switch recombination[3] (a recombination process in which one heavy chain constant region gene is replaced with another from a different isotype). During the germinal centre reaction, mature B cells give rise to memory B cells, regulatory B cells (Bregs), plasmablasts or plasma cells (PCs).

Subsets	**Phenotype**
▪ Pro-B cell	$CD10^+$ $CD19^+$ $CD20^-$ $CD34^+$ RAG-1/2$^+$ IgM^-
▪ Pre-B cell	$CD10^+$ $CD19^+$ $CD20^+$ $CD34^-$ RAG-1/2$^+$ IgM^- Pre-BCR
▪ Immature	$CD10^+$ $CD19^+$ $CD20^+$ $CD27^-$ $CD34^-$ IgM^+
▪ Transitional	$CD10^+$ $CD19^+$ $CD20^+$ $CD24^{high}$ $CD27^-$ $CD38^{high}$ IgM^{high} BAFF-R^+
T1[6]	$CD21^{low}$ IgD^{low}
T2[6]	$CD21^{high}$ IgD^{high}
T3[6]	$CD10^{low}$ $CD24^+$ $CD38^+$ IgM^+ IgD^+
▪ Naive mature	$CD19^+$ $CD20^+$ $CD27^-$ $CD38^+$ IgM^+ IgD^+ BAFF-R^+ $TACI^+$
▪ Marginal zone	$CD19^+$ $CD20^+$ $CD21^{high}$ IgM^+ IgD^{low}
▪ Follicular	$CD19^+$ $CD20^+$ $CD21^{-/+}$ IgM^{low} IgD^+
▪ Germinal centre	$CD19^+$ $CD20^+$ $CD38^{high}$ IgM/G/A/E^+ IgD^{low} BAFF-R^+ Bcl-6^+
▪ Plasmablast	$CD19^+$ $CD20^+$ $CD27^{high}$ $CD38^{high}$ IgM/G/A/E^+ IgD^- Blimp-1^+ $IRF4^{high}$ $XBP1^+$
▪ Plasma cell	$CD19^{low}$ $CD20^-$ $CD38^{high}$ $CD138^+$ IgM/G/A/E^+ IgD^- $TACI^+$ and/or $BCMA^+$ Blimp-1^{high} $IRF4^{high}$ $XBP1^+$
▪ Memory B cell	$CD19^+$ $CD20^+$ $CD27^+$ $CD38^-$ IgM/G/A/E^+ IgD^-
▪ Regulatory B cell[7]	$CD1d^+$ $CD5^+$ $CD19^+$ $CD20^+$ $CD24^{high}$ $CD27^{-\text{ or }+}$ $CD38^{high}$ $IL10^+$ $BANK1^+$ $GzmB^+$ Blimp-1^- $PRDM1^-$ $IRF4^-$ $XBP1^-$

Table 1.1. Overview human B cell subset phenotypes[3,4].

Cytokine IL21, which is produced mainly by T follicular helper cells, is found to be an important regulator for the generation of T follicular helper cells, germinal centre B cells and PCs through its capacity to moderate the expression of transcription factors B lymphocyte induced maturation protein 1 (Blimp-1) and B cell lymphoma-6 (Bcl-6)[8,9]. Memory B cells and PCs produce high-affinity, antigen-specific Igs, also termed antibodies (Abs) and form the basis of long-lived humoral immunity. Bregs from their part exert regulatory functions on innate and adaptive immunity to prevent that harmful excessive immune responses occur.

1.2.2. B cell actions and functional subsets

The generation of Abs is the principal function of terminally differentiated B cells, such as plasmablasts and PCs. Abs are glycoproteins belonging to the Ig superfamily and constitute, through their specificity for a single antigenic determinant, the basis of the humoral immune response by clearing pathogenic invaders and their toxic products through several mechanisms of action like complement activation, direct anti-microbial functions, Ab-dependent cellular toxicity and opsonisation[10]. The Y-shaped structure of the Ab typically consists of two identical large heavy chains and two identical small light chains, linked by disulphide bridges. The antigen-binding (Fab) fragment is composed of one constant and one variable domain of each of the heavy and the light chain. Antigen-binding sites are formed at the aminoterminal end of the variable domains of both the heavy and light chains and each arm of the Y structure binds an epitope on the antigen. The Fc region (the crystallisable fragment of the heavy chains) is the tail region that mediates interaction with effector molecules, such as complement and Fc receptors. In placental mammals, five isotypes or classes of Abs are known: IgA, IgD, IgE, IgG and IgM, as determined by the heavy chains, and they differ in their biological properties, functional locations and ability to deal with different antigens[11]. Plasmablasts are short-lived, proliferating effector cells of the early antibody response and their antibodies tend to have a weaker affinity towards their antigen compared to PCs. Unlike PCs, plasmablasts maintain CD20 expression[3,12]. PCs are long-lived, non-dividing effector cells capable of producing large amounts of high-affinity, isotype-switched Abs. Increased expression of Blimp-1 promotes the terminal differentiation of B lymphocytes into PCs[12]. Memory B cells are long-lived, quiescent B cells that recirculate through the body and can rapidly differentiate into Ab-secreting cells following re-exposure to antigen that had activated their parent B cell[3,5]. These cells contribute to humoral immunological memory that assures elimination of pathogens before the onset of clinical disease.

B cells also have the ability to serve as efficient antigen-presenting cells (APCs), which are highly specialized cells that can process antigens and display their peptide fragments on the cell surface together with co-stimulatory proteins required for activating naive T cells. While other APCs like macrophages and dendritic cells internalize antigen through pinocytosis, B cells capture antigens through their antigen-specific BCR. Even at very low concentrations, antigens can be successfully presented in this manner[13]. The antigen-binding portion of the BCR complex is a cell-surface Ig that has the same antigen specificity as the secreted Igs that

the B cell will eventually produce. Engagement of antigen and BCR induces receptor oligomerisation and downstream signalling which promotes dynamic actin cytoskeletal rearrangements enabling efficient BCR-antigen internalisation into late endosomal compartments where the foreign proteins are degraded into peptide fragments, termed epitopes, which can bind to major histocompatibility complex (MHC) molecules for recognition by the appropriate T cells. MHC antigens, in the human also called human leukocyte antigen (HLA), are divided into two subgroups: Class I and Class II. MHC Class I (MHC I) are expressed on all nucleated cells and present epitopes to cytotoxic $CD8^+$ T cells which then trigger the cell to undergo apoptosis. MHC Class II (MHC II) is normally expressed only on APCs and presents epitopes to the $CD4^+$ T helper cells (Th cells). Successful interaction primes naive Th cells, which subsequently differentiate into other subsets such as memory T cells, effector Th cells (Th1 or Th2), regulatory T cells (Tregs), etc. In a transplant procedure, MHC molecules expressed on the graft act themselves as antigens and can provoke immune response in the recipient causing transplant rejection. Thus MHC antigens strongly determine compatibility of donors for organ transplantation.

Cytokines are a large category of small proteins that play an important role in the communication between cells of multi-cellular organisms. As intercellular mediators, they regulate growth, survival, differentiation and effector functions of cells and initiate their action. They can act via an auto-, para-, or endocrine way and through specific cell-surface receptors on their target cells. B cells can actively regulate immune responses by producing distinct arrays of cytokines depending on the cellular subset they belong and on their mode of activation. B cell-derived cytokines include[14,15]: 1) Pro-inflammatory molecules, e.g. interleukin (IL) 1, IL2, IL4, IL6, IL8, interferon (IFN) γ, tumor-necrosis factor (TNF) α, lymphotoxin α (LTα); 2) Haematopoietic growth factors which are involved in the proliferation, differentiation, and survival of granulocytes, monocytes, macrophages, and bone marrow progenitor cells, e.g. granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor; and 3) Anti-inflammatory molecules, e.g. IL10, IL35 and transforming growth factor (TGF) β. The cytokine profile of B cells is influenced by the cytokine microenvironment, T cell help through CD40 signalling, and through pathogen-derived toll-like receptor (TLR) ligands[16,17]. B cells primed by Th1 cells and antigen develop into the B cell effector 1 (Be1) subset, while B cells primed by Th2 cells and antigen develop into the B cell effector 2 (Be2) subset. These Be1 and Be2 subsets produce a substantially different cytokine signature, e.g. IL4 is produced

by the Be2 subset, but not by the Be1 subset. Conversely, IFNγ is produced by Be1 cells, but only at very minimal amounts by Be2 cells[18].

Bregs are immunosuppressive cells that support immunological tolerance and are critical to dampen inflammatory responses to infections. To restrain pro-inflammatory responses, Bregs suppress the differentiation of pro-inflammatory effector cells (such as Th1 cells, cytotoxic $CD8^+$ T cells, Th17 cells, TNFα-producing monocytes and IL12-producing dendritic cells) and induce the differentiation of immunosuppressive Tregs. This is achieved through cognate interactions, mediated by MHC II and CD80/86, between Bregs and T cells and through the production of immunoregulatory cytokines IL10, IL35 and TGF β[19]. IL10 is considered the hallmark cytokine of many Breg subpopulations. Quite recently, IL35, a heterodimeric cytokine of the IL12 family, is documented as a novel suppressor molecule that is produced by a subset of Bregs distinctive from the IL10-expressing Bregs[20]. Observations in experimental models of autoimmunity (e.g. EAE[20]) and in mice with bacterial infection (such as *Salmonella*[20]) validate the hypothesis that IL10 and IL35 provide two largely separate suppressive pathways[21,22]. Different immunoregulatory B cell subsets have been described in mice and human subjects with distinct phenotypic properties. So far, no study has conclusively identified a Breg-specific transcription factor, similar to FoxP3 in Tregs. Remarkably, Bregs are found among B lymphocytes at different stages of maturation and differentiation, including early transitional B cells as well as highly differentiated plasmablasts and PCs. The inability to identify a unique transcription factor together with the heterogeneity of the phenotype of Bregs, support the idea that Bregs are not a separate lineage of B cells devoted to immunosuppression, but that they arise at various stages of B cell development in response to environmental, inflammatory stimuli[19,23,24]. B cells displaying a peculiar inhibitory profile have been described in the very rare cases of patients that show operational tolerance toward their kidney transplant (See paragraph 1.4.2.). The level of B cell-mediated suppression is controlled via a stepwise process initiated by intrinsic TLR signalling and then reinforced by signals via BCR and CD40. It sets the possibility for a negative feed-forward loop, which can allow an accelerated induction of immune responses while preventing excessive inflammation[17,25].

1.2.3. Activation and regulation of B cells

1.2.3.1. BCR signalling pathways

Engagement of an antigen with the BCR on the outer surface triggers within the B cell a complex network of regulatory signalling cascades composed of kinases and phosphatases (Figure 1.2). The outcome of the response, which can be survival, apoptosis, anergy, proliferation, and differentiation into Ab-producing cells, memory B cells or Bregs, is depending on the duration and magnitude of the BCR signalling, the maturation state of the B cell, the nature of the antigen (T cell-dependent and T cell-independent B cell activation), and the accessory signals from co-stimulatory molecules, cytokine receptors, cytoskeleton, and B cell-activating factor-receptor (BAFF-R). The BCR is composed of a membrane Ig molecule and associated Igα/Igβ (CD79a/CD79b) heterodimers[26]. Each of the Igα and Igβ subunits contain a single immunoreceptor tyrosine-based activation motif (ITAM), which is composed of two conserved tyrosine (Tyr) residues. Antigen-induced BCR aggregation and conformational changes lead to Lck/Yes novel Tyr kinase (LYN)-mediated phosphorylation of ITAMs followed by the creation of docking sites for the recruitment of B lymphoid tyrosine kinase (BLK), proto-oncogene Tyr-protein kinases FYN, LYN, spleen Tyr kinase (SYK) and BTK. SYK and BTK are crucial components to couple BCR to more distal signalling[27,28]. SYK phosphorylates and activates the adaptor molecule B cell linker protein (BLNK) which binds to BTK and phospholipase Cγ2 (PLCγ2) resulting in an optimal phosphorylation and activation of PLCγ2 to promote the generation of diacylglycerol (DAG) and the influx of calcium (Ca^{2+}) ions[26,29]. DAG and the Ca^{2+} ions activate in turn protein kinase Cβ (PKCβ), leading to activation of the RAS-RAF-MEK-ERK (also termed MAPK/ERK) pathway, and the CARMA1-, BCL-10- and MALT1-containing (CBM) complex, resulting eventually in the nuclear translocation of the p50-p65 NF-κB heterodimer. The cell surface molecule CD19 can amplify the BCR signal via activation of LYN and activation of phosphatidyl-inositol 3-kinase (PI3K). Activated PI3K phosphorylates the phospholipid phosphatidylinositol biphosphate (PIP2) resulting in the second messenger phosphatidylinositol triphosphate (PIP3). Phospholipids are a class of lipids and are a major component of all cell membranes. AKT, also called protein kinase B (PKB), is a serine/threonine (Ser/Thr) kinase that possesses a pleckstrin homology (PH) domain with high affinity for PIP3. AKT moves to the plasma membrane, binds PIP3, becomes activated by phosphoinositide-dependent kinase (PDK) 1 and PDK2-mediated phosphorylation (on Thr-308 and on Ser-473 respectively) and regulates the cell growth, cell survival, cell migration

and cell metabolism and also the NF-κB signalling. Due to its critical role in regulating diverse cellular functions, AKT is an important therapeutic target for the treatment of human cancers. BCR activation affects also other signalling pathways such as Janus kinases (JAKs) and mammalian target of rapamycin (mTOR)[26–29]. The magnitude and duration of BCR signalling are limited by negative feedback loops including those involving the Lyn/CD22/SHP-1 pathway, the Cbp/Csk pathway, SHIP, Cbl, Dok-1, Dok-3, the low-affinity inhibitory IgG Fc receptor CD32b (also named FcγRIIB), PIR-B, and internalization of the BCR. Given the central role of BCR signalling in B cell malignancies and autoimmune diseases (AIDs), therapeutic approaches to inhibit BCR signalling are targeting crucial signalling nodes.

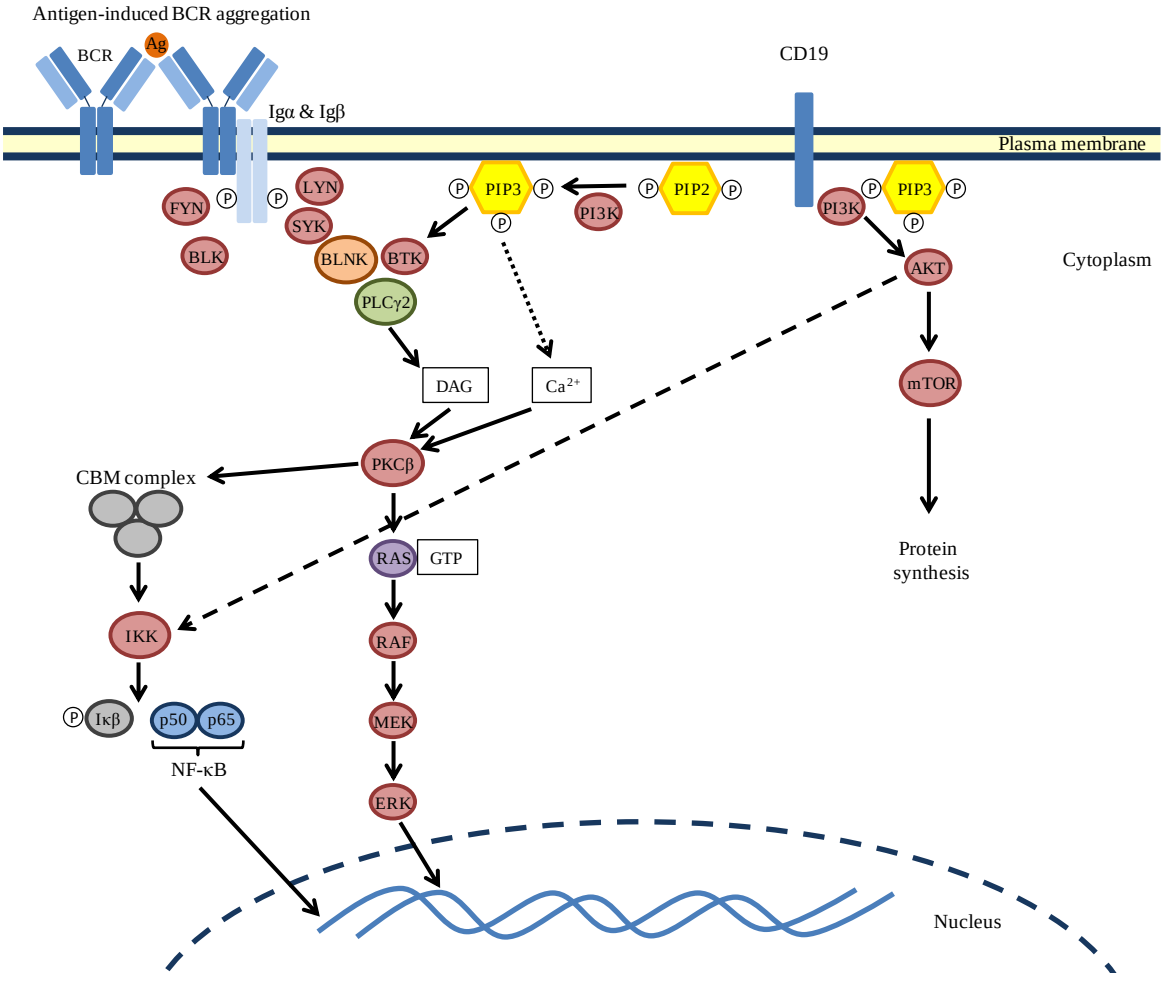

Figure 1.2. A schematic representation of BCR signalling.

1.2.3.2. Co-stimulation

In addition to the antigen-specific signal from the BCR, co-stimulation is required and involves ligand - receptor interactions between Th cells and B cells for the full activation of

the latter. These bi-directional interactions are crucial for the development of an effective immune response. The immune system provides a plethora of diverse co-stimulatory molecules and they all contribute to the quality of the immune response. The function of a particular co-stimulatory molecule, which can either be stimulatory or inhibitory, is strongly related to the timing of its action. The expression of each co-stimulatory molecule and/or its ligand is tightly regulated and dependent on the activation status of the cell[30,31].

Numerous co-stimulatory molecules have been identified playing a role in the initiation of immune responses by T and B cells and the number is still increasing. Some relevant co-stimulatory pathways with a crucial role in the B cell activation are briefly discussed below. The molecules involved in co-stimulation can roughly be classified as members from the TNF superfamily or from the Ig superfamily.

1.2.3.2.1. TNF superfamily

1.2.3.2.1.1. CD40 : CD40L pathway

Binding of CD40 on APCs (B cell, dendritic cell, macrophage) with its ligand CD40L, also termed CD154, on T cells acts in a bi-directional fashion, mediating both humoral and cellular immune responses. B cells depend on CD40 for survival, expression of co-stimulatory molecules like CD80 and CD86 to interact with T cells, germinal centre formation, memory generation, Ig class switching and production of numerous cytokines and chemokines[32–35]. CD40 signals act via adaptor proteins known as TNF-receptor (TNFR)-associated factors (TRAFs) which mediate the activation of multiple signalling pathways that regulate transcription factors. CD40 has been reported to activate the canonical and non-canonical NF-κB pathway as well as the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinases, and PI3K pathways. These pathways involve Ser/Thr kinases or Tyr kinases that regulate gene expression through phosphorylation and activation of transcription factors. Mutations in either the CD40 or CD40L gene cause the hyper IgM syndrome, termed HIGM3 or HIGM1 respectively, characterized by very low levels of serum IgG, IgA, and IgE, but normal or elevated IgM, associated with defective Ig class switching and germinal centre formation[36–38]. Aberrant expression of CD40 has been associated with various cancerous malignancies[34] and with AIDs such as multiple sclerosis (MS)[39]. CD40L is over-expressed on T and B cells in systemic lupus erythematosus (SLE)[40]. Blocking of CD40L, for instance by antagonistic CD40L mAbs (clone MR1), contributes to prolonged graft survival and seems to be synergistic when combined with immunosuppressive drugs (e.g. rapamycin) and other co-

stimulatory blockade(s)[34]. Despite promising results in non-human primates, CD40L antibodies have not yet reached clinical application because of the possible risk of thrombosis[41]. The latter may, however, change soon as non-thrombotic anti-CD40L Abs are in the stage of development (e.g. CDP7657[42]).

1.2.3.2.1.2. CD70 : CD27 pathway

The precise functional role of the CD70 : CD27 pathway in B cells remains only partially understood, but data from human MS, rheumatoid arthritis (RA) or SLE patients[43] and murine disease models of MS, RA, SLE and inflammatory bowel disease[43] document a significant role for the CD70 : CD27 surface ligand-receptor pair in providing co-stimulatory signals that regulate T cell and B cell activation and differentiation. CD27 belongs to the TNF-R family and is found on the majority of T cells, on subsets of natural killer cells, and hematopoietic stem cells. CD27 is not expressed by naive B cells, but is up-regulated in activated and antigen-experienced B cells. After the initial up-regulation CD27 can persist at high levels, making it a typical marker for memory B cells[43–45]. CD70-induced triggering of CD27 on T cells contributes to the formation of the effector T cell pool by efficient priming of T cells and the subsequent promotion of T cell survival via the canonical and non-canonical NF-κB pathways as well as the JNK pathway via TRAF2 and TRAF5 adaptor proteins[30,45,46]. CD70, characterized as the unique natural ligand of CD27, is transiently expressed on the surface of activated B cells, T cells and dendritic cells. Studies with transgenic $CD27^{-/-}$ x CD70 Tg mice that constitutively express CD70 on all B lymphocytes, but concomitantly lack CD27, indicate that CD70 engagement on B cells couples to both PI3K and MEK pathways, resulting in phosphorylation of their downstream targets AKT and ERK1/2[43] (Figure 1.3). Further, there is not much known about the signalling downstream of CD70.

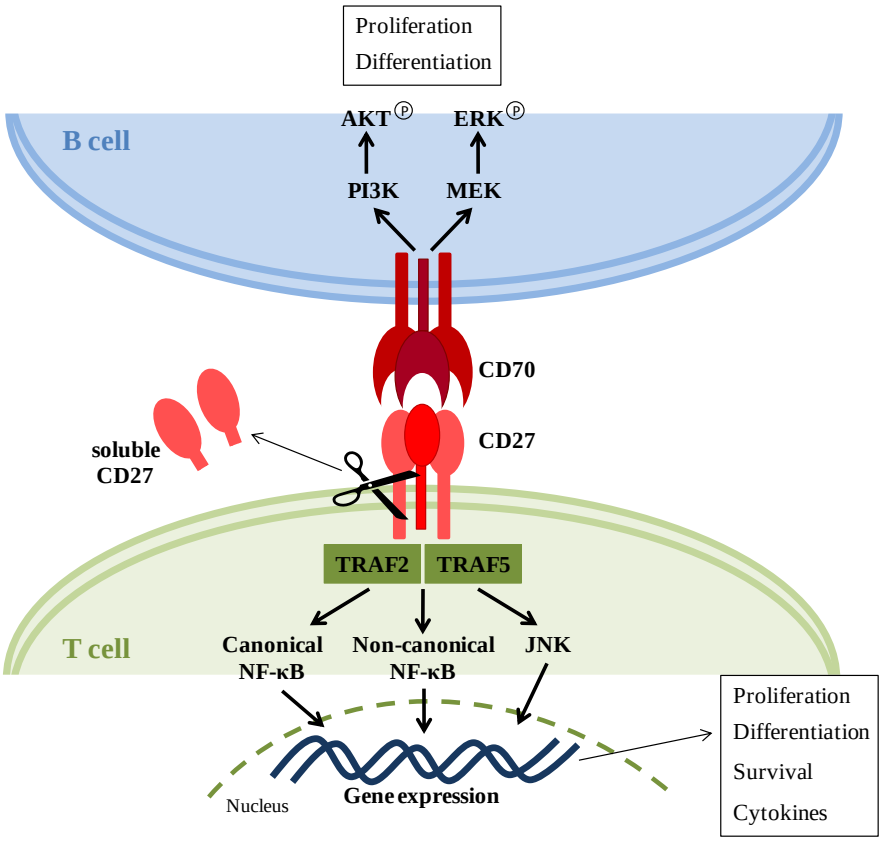

Figure 1.3. Signalling pathways mediated by CD70-CD27 interactions.

Under normal physiological conditions the expression of CD70 is tightly regulated, because continuous CD70 : CD27 interactions can lead to severe deficiencies in the immune cell composition, as observed in $CD27^{-/-}$ X CD70 Tg mice which developed increased numbers of effector $CD4^{+}$ and $CD8^{+}$ T cells, a depletion of naive T cells[47] and a IFNγ-mediated depletion in B cells[30,48]. In these transgenic mice, it was also observed that signalling through CD27 on B cells results in enhanced PC formation and increased IgG production, whereas CD70 engagement on B cells may enhance their proliferation and alter the expression of activation and maturation molecules, but may simultaneously inhibit the formation of IgG-secreting PCs. However, ligation of CD70 by soluble CD27 (membrane-bound CD27 can be cleaved through the action of matrix metalloproteases[49] forming a soluble form of CD27 in serum) resulted in enhanced IgG secretion by $CD27^{+}$ $CD70^{+}$ human memory B lymphocytes[50], suggesting that different CD70 ligands can result in qualitatively different signals with distinct functional consequences. Elevated serum level of sCD27 have been reported in AIDs (such as primary Sjögrens syndrome, SLE and MS) and in patients infected with human immunodeficiency virus type 1 and is correlated with hypergammaglobulinemia[50]. A constitutive expression of CD70 is observed on Hodgkin's lymphoma, B cell chronic

lymphocytic leukemia, and large B cell lymphomas, and on various other malignancies, implying that CD70 is an attractive candidate for cancer immunotherapy[30,43,44,46,51].

1.2.3.2.1.3. BAFF and APRIL signalling

Together with their cognate receptors, BAFF and APRIL regulate B lymphocyte homeostasis. Both ligands belong to the TNF superfamily and are predominantly produced by macrophages, neutrophils, and dendritic cells, but can also be produced by lymphoid cells, including B cells and activated T cells[52]. BAFF, also known as B lymphocyte survival factor (BLyS), plays a significant role in promoting B cell survival, maturation, proliferation and differentiation[52]. BAFF is expressed as a membrane-bound trimer, which is proteolytically cleaved to form a soluble trimer and binds with three different receptors expressed on B cells at different stages of maturation: BAFF-receptor (BAFF-R), B cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). The predominant receptor on human peripheral B cells is BAFF-R, while BCMA and TACI appear to be preferentially expressed on PCs and transitional B cells, respectively[53]. APRIL, which acts as a co-stimulator for B and T cell proliferation and supports class switching, is recognized by both BCMA and TACI[54]. Deregulation of the BAFF/APRIL system can lead to malignancies of B cells and PCs[55] and to AIDs such as SLE and Sjögren's syndrome. SLE and Sjögren's syndrome patients have high levels of BAFF which may contribute to altered differentiation of B cells[56]. An improvement of symptoms in patients with SLE can be obtained by treatment with belimumab, an anti-BAFF monoclonal antibody[57].

1.2.3.2.2. Co-stimulatory Ig superfamily

1.2.3.2.2.1. CD80/CD86 : CD28 pathway

CD80 and CD86, also named B7.1 and B7.2 respectively, are two structurally related proteins that can be found on the cell-surface of APCs, such as dendritic cells, B cells and macrophages, and are the major co-stimulatory molecules for $CD4^+$ Th cells. Both CD80 and CD86 are capable of binding the stimulatory CD28 on Th2 cells, hereby delivering to the Th2 cell the necessary second signal for its survival and activation. Upon activation, the Th2 cell up-regulates the expression of CD40L. In turn, T cell delivers the co-stimulatory signal for activation to the B cell via ligation of CD40L with CD40 on the B cell. While CD28 expression on T cells is constitutive, the expression patterns of the CD80 and CD86 molecules are variable[58,59]. In general, CD86 seems to be constitutively expressed at low levels and can

be rapidly up-regulated. Expression of CD80 can be induced on activated B cells, macrophages and natural killer cells, but the time course of induction of expression is slower than that of CD86[60]. In spite of the high degree of homology and the substantially overlapping functions, there are indications for distinct effects of CD80 and CD86 on T (regulatory[59]) cell responses[60] and on B cell activation and differentiation[61]. In studies with lipopolysaccharide (LPS)-stimulated murine B cells, it was shown that triggering through CD80 specifically inhibited the proliferation and IgG secretion while, by contrast, triggering through CD86 augmented the B cell activity. Also it was determined that CD86 had a higher relative affinity for activating CD28, while CD80 seemed to have a higher relative affinity for inhibitory cytotoxic T lymphocyte- associated antigen 4 (CTLA4)[61]. Hence, CD80 and CD86 may mediate differential signal transduction which could distinctly regulate the B cell's effector function. There is not much known about the signalling downstream of CD80 and CD86, but murine as well as human CD80/CD86 cytoplasmic tails contain potential binding motifs for different kinases[61,62], suggesting that different signalling can be initiated upon activation.
Abatacept and belatacept are inhibitors for CD80/CD86-mediated co-stimulatory signalling (See further 'CD80/CD86 : CTLA4 pathway').

1.2.3.2.3. Co-inhibitory Ig superfamily

1.2.3.2.3.1. CD80/CD86 : CTLA4 pathway

Upon activation, T cell up-regulate the negative co-stimulatory molecule CTLA4, also known as CD152. This molecule binds the same ligands as the activating CD28, namely CD80 and CD86, but with a 20-100 fold higher affinity. CTLA4 functions as an inhibitory receptor important for down-modulating the immune response through a negative feedback loop[58,63]. Ligation of CD80 and CD86 with CTLA4 also transmits outside-in signals to the APC resulting in a down-modulation in expression of CD80 and CD86[64]. Fusion protein CTLA4-Ig prevents CD28 signals by outcompeting CD28 for binding to its ligands CD80/CD86. Abatacept is such a CTLA4-Ig composed of the Fc region of the IgG1 fused to the extra-cellular domain of CTLA4 and is used in the treatment of RA. Through its binding to the CD80 and CD86 molecule, abatacept prevents the co-stimulatory signal necessary for T cell activation[65]. Second-generation CTLA4-Ig, belatacept, shows increased binding affinity compared to abatacept and is intended to ameliorate graft survival in transplantation while limiting the toxicity generated by standard immune suppressing regimens, such as calcineurin inhibitors cyclosporine A and tacrolimus[66,67].

1.2.3.2.3.2. CD83

The molecule CD83 was long considered as a specific maturation marker for dendritic cells, but is gaining more interest in studies on B cells and T cells. It belongs to the Ig superfamily and is strongly expressed on activated murine and human dendritic cells and on many immune cells upon activation, including B cells and T cells. CD83 is rapidly up-regulated on murine B cells after either TLR engagement by LPS or after BCR ligation[68–70]. Transgenic over-expression of CD83 on B cells in mice interferes with T cell-dependent and T cell-independent Ig production during infection *in vivo*[71]. Furthermore, the forced over-expression of CD83 on B cells results in increased MHC II and CD86 expression, reduced proliferation, reduced Ca^{2+} signalling, lowered Ig secretion and increased IL10 production upon *in vitro* activation by LPS[70]. Diminished CD83 expression in CD83-mutant mice gives a reduction in IL10 release and a slight increase in Ig secretion which are not correlated to impaired B cell function[70]. So CD83 might act as a negative regulator and is assumed to be involved in the down-modulation of B cell responses. Thereby CD83 interferes with early BCR-mediated signalling events such as phosphorylation of LYN and SYK, consequently leading to reduced activation of distal effectors such as ERK1/2[72] and Ca^{2+} influx[70]. How CD83 mediates its inhibitory effects and the nature of the putative CD83 ligand are not yet known, but are currently under investigation.

1.2.3.2.3.3. PD-L1/2 : PD-1 pathway

Programmed cell death (PD)-1 shares homology with CTLA4 and CD28 and is expressed on activated $CD4^+$ and $CD8^+$ T cells as well as on activated B cells, natural killer cells and macrophages. Programmed cell death-ligand 1 (PD-L1, B7-H1) and programmed cell death-ligand 2 (PD-L2, B7-DC) interact with PD-1[73]. PD-L1 is constitutively expressed on T cells, B cells, myeloid cells and dendritic cells and can be up-regulated upon activation. In addition, PD-L1 is also expressed by non-hematopoietic cells (e.g. microvascular endothelial cells), non-lymphoid organs (e.g. heart, lung, muscle and placenta), and a variety of tumours. PD-L2, however, is restricted to macrophages, mast cells and dendritic cells. The presence of immunoreceptor Tyr-based inhibition motif (ITIM) in the intracellular domain of PD-1 and the observation of an autoimmune phenotype with SLE-like glomerulonephritis and progressive arthritis in PD-1 KO mice[74] postulated a role for the PD-1 pathway in negatively regulating immune responses. However, the regulation of alloimmunity by the PD-1/PD-L1/2 pathway proved to be complex due to the differences in tissue distribution of the ligands for PD-1, suggesting that PD-L1 and PD-L2 may exert different functions in immune regulation,

and the presence or absence of CD28 co-stimulation seems to have a considerable impact on the functionality of the PD-1 pathway[73,75,76].

1.2.3.3. Toll-like receptors

Throughout vertebrate evolution, TLRs have remained highly conserved, and represent a critical link between the innate and the adaptive immunity. As sentinels of the host innate immune system, they recognize specific molecular patterns on invading microorganisms, so-called pathogen-associated-molecular-patterns (PAMPs), and damage-associated molecular pattern molecules that are released by stressed cells undergoing necrosis. In humans, ten TLRs have been characterized. TLRs 1, 2, 4, 5, 6 and 10 are expressed at the cell membranes and recognize bacterial, fungal and parasite-derived ligands. TLRs 3, 7, 8 and 9 are expressed in the endosomes and recognize viral and bacterial RNA and DNA[77]. Except for TLR3, the TLRs (TLR7/8/9 signalling is represented in figure 1.4) are coupled to the adaptor protein myeloid differentiation primary response gene-88 (MyD88) which starts a signalling cascade through MyD88/IL-1 receptor-associated kinase (IRAK) 1/IRAK4/ TRAF complexes ending up in the activation of MAP kinases (JNK, p38 MAPK) and of transcription factors NF-κB, interferon regulatory factors (IRFs), cAMP response element-binding protein (CREB) and activator protein 1 (AP-1), leading to the induction of several inflammatory genes. TLRs are expressed by immune cells like macrophages, dendritic cells, polymorphonuclear cells, and mast cells as well as T and B cells of the adaptive immune system, and by epithelial, endothelial and mesenchymal cells of various organs and tissues. These cells all have characteristic TLR expression patterns which can direct their specific behaviour[77].

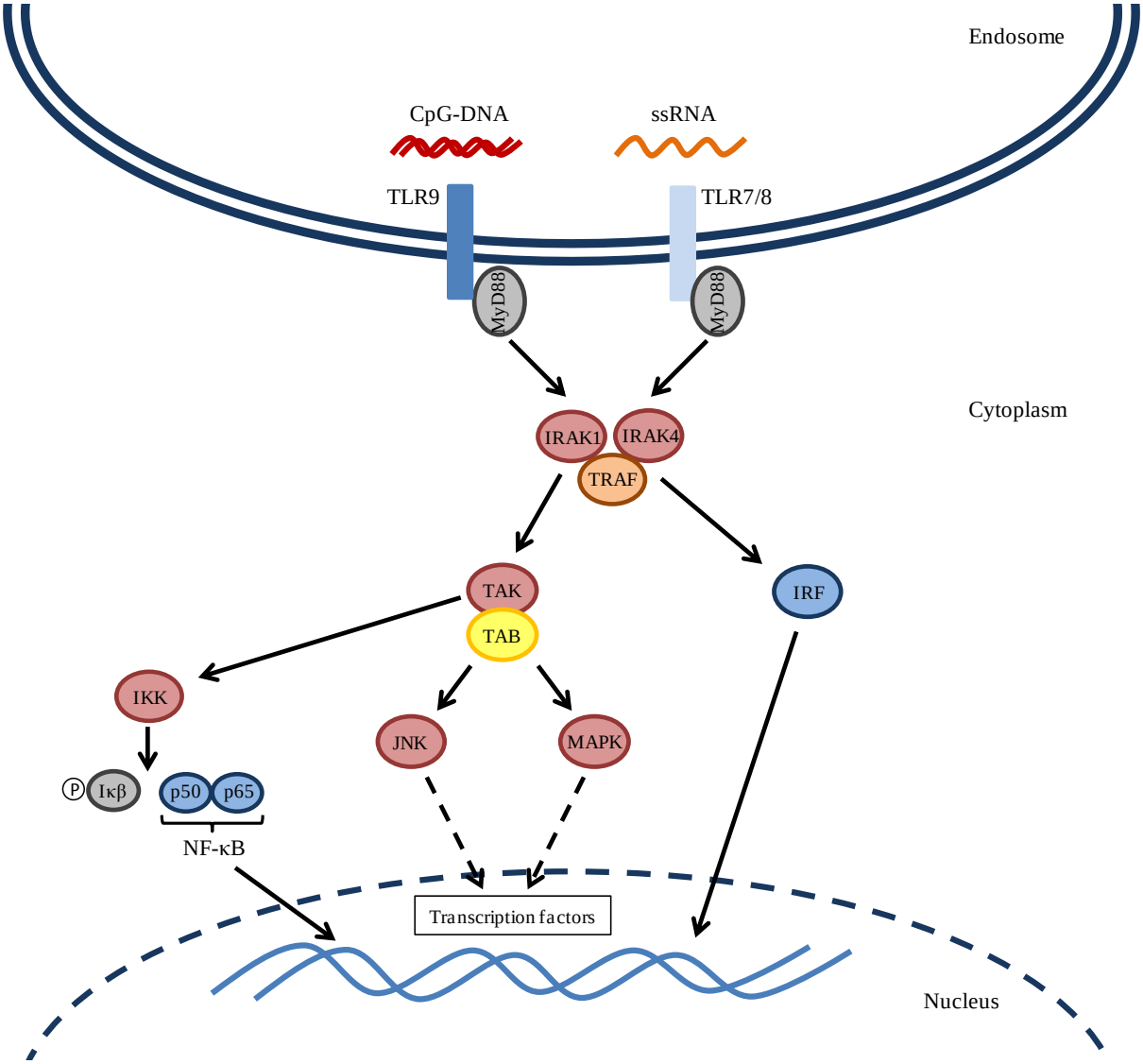

Figure 1.4. A schematic representation of TLR7/8/9 signalling.

In contrast to naive murine B cells, human B cells seem to lack significant expression of TLR4, the receptor for LPS[78,79]. Human B lymphocytes mainly express endosomal TLR7 and TLR9. Human TLR7 is often grouped together with TLR8 for sharing a similar structure, genomic localization and common ability to detect single-stranded RNA (ssRNA), although TLR8 has a function on its own in the initiation of specific pro-inflammatory responses to viruses and bacteria and is predominantly expressed on monocytes[80]. Human TLR9, which is constitutively expressed in the endosomes of B cells and plasmacytoid dendritic cells, is essential for responses to bacterial and viral CpG-containing DNA[81,82]. The level of TLR9 expression is dependent on the state of B cell maturity and activation[83–85].

TLRs have been implicated to play a significant role in various major diseases, including B cell malignancies and B cell-related AIDs. Recent studies have revealed that TLR9 is overexpressed in peripheral blood B cells isolated from patients with active SLE[86,87].

In transplantation, TLRs can facilitate allograft rejection. During the operation, surgical stress and ischemia reperfusion injury can provoke the release of endogenous (e.g. derived from apoptotic or necrotic cells, heat shock proteins and degradation products of extracellular proteins) and exogenous (e.g. commensal bacteria in skin, small bowel and lung) ligands that can activate TLRs which in turn initiate innate immunity and adaptive immune responses[77]. Although *in vivo* evidence for TLR-mediated suppression in transplantation is scarce, TLR signalling may also enhance regulatory loops controlling early inflammatory events post-transplantation and ultimately promote allograft acceptance[77,88].

At the moment, the use of TLR ligands as vaccine adjuvants forms a topic of great interest for clinical application in inflammatory and infectious diseases and cancer[87,89].

1.3. B cell-related pathologies and current treatment(s)

1.3.1. Cancer

Many different B cell malignancies have been described, e.g. non-Hodgkin's lymphoma, Hodgkin's lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma and follicular lymphomas[26]. B cell lymphomas are divided into low and high grade, typically corresponding to indolent, slow-growing lymphomas and aggressive lymphomas, respectively. Generally, the aggressive lymphomas are curable, but require intensive treatments. Indolent lymphomas respond to treatment and are kept under control (in remission) with long-term survival. Treatment, which includes radiation and chemotherapy, depends on the specific type of B cell malignancy as well as stage and grade. Inhibitors of PI3K (idelalisib[90]), SYK (fostamatinib[27], entospletinib[91]), and BTK (ibrutinib[27]) have shown promising results in clinical trials for treatment of B cell neoplasms, such as B cell chronic lymphocytic leukaemia, B cell non-Hodgkin's lymphoma and diffuse large B cell lymphoma[26].

1.3.2. Autoimmune diseases

AIDs arise from an abnormal immune response of the body against substances and tissues normally present in the body and are after cancer and atherosclerosis the third major cause of morbidity and mortality[92]. In the United States, 23.5 million up to 50 million citizens suffer from an AID (the numbers continue to increase!) and it is estimated that 80 to 100 different forms of AIDs exist. AIDs have no clear cause for their development, they are difficult to

diagnose due to their slow evolution and they are chronic and life-long diseases for which a solid treatment method does not exist. The annual direct health care costs increase to more than US$ 100 billion (National Institutes of Health www.nih.gov and American Autoimmune Related Disease Association www.aarda.org). Treatment for AIDs typically consists of immunosuppressive medication that reduces the activation or efficacy of the immune system. Through their different functions, B cells can contribute to the development of AIDs:

1) **Secretion of autoAbs**

   The presence of autoAbs in the peripheral circulation can be detected in many AIDs. Immune complexes composed of autoAbs and autoantigens can trigger inflammation through activation of the complement system and through binding with Fc-receptors inducing Ab-dependent-cell-mediated cytotoxicity. AutoAbs can also stimulate or inhibit the function of different kinds of receptors with ligands as hormones, neurotransmitters or peptides[93].

   Natural autoAbs can have a protective function by, for instance, clearing dying and aging cells which may otherwise elicit a pathogenic autoimmune response. Another mechanism of protection is the blockage of pathogenic autoAbs to react with self-antigen[93].

2) **Presentation of autoantigens and T cell activation**

   B cells function as very efficient APCs by capturing the autoantigen, even at very low concentrations, through their BCR and present it complexed with MHC to Th cells for activation.

3) **Secretion of pro-inflammatory cytokines**

   Activated B cells can produce various pro-inflammatory cytokines, including IL2, IL4, IL6, IFNγ and TNFα, which can activate other effector immune cells and provide feedback stimulatory signals for further B cell activation.

4) **Formation of ectopic germinal centres**

   During periods of chronic inflammation B cells aid in the formation of ectopic germinal centres within inflamed tissues. These structures are similar to the germinal centres in secondary lymphoid organs (e.g. spleen, lymph nodes, tonsil, Peyer's patches), however, their function and potential pathogenic role remains to be determined. It is plausible that ectopic germinal centres have a role in the maintenance of immune pathology, because the PCs residing within the ectopic germinal centres secrete autoAbs[93].

Three prominent AIDs are discussed below.

1.3.2.1. Rheumatoid arthritis

RA is a disorder that primarily affects the joints of musculo-skeletal system causing significant pain and physical disability, but also serious co-morbidities such as increased risk of cardiovascular disease, serious infections, lung cancer, lymphoma and premature death. The list of RA-associated autoAbs continues to expand. The most widely studied autoAb systems are rheumatoid factors (autoAbs against Fc portion of IgG) and Abs against citrullinated peptides. But the contribution of B cells to RA pathogenesis goes beyond autoAb production. Antigen presentation, delivery of co-stimulatory signals and secretion of cytokines[94,95], including IL1α, IL6, IL12, IL23 and TNFα, induce the activation and differentiation of effector T cells. The synovial membrane, a layer of connective tissue that lines the cavities of joints, tendon sheaths, and bursae, forms a preferential environment for the T cell and B cell interactions. Destructive inflammatory synovitis is the consequence of the infiltration of macrophages, T cells and B cells into the synovial membrane. B cells also participate in the activation of osteoclasts, bone cells that break down bone tissue, leading to destruction of bone and cartilage. Specific subsets of B cells can function as regulatory elements through the provision of IL10, the B10 cells. However, their suppressive function is partly impaired during active disease and their frequency in the systemic circulation is reduced compared to healthy individuals[94].

Therapy for RA consists generally of a combination of several medications. Disease modifying anti-rheumatic disease drugs (DMARDs) consist of anti-inflammatory agents that slow down the disease progression. Many different drugs with different mechanisms of action can be used as DMARDs in the treatment of RA, but some are used more often than others. The most prevalent: methotrexate, azathioprine and leflunomide are well-known anti-proliferative agents with inhibitory effects on DNA-synthesis; adalimumab (anti-TNF monoclonal Ab) and etanercept (fusion protein TNF inhibitor) neutralize pro-inflammatory TNFα; abatacept (CTLA4-Ig fusion protein) is a B cell - T cell co-stimulatory signal inhibitor; rituximab (anti-CD20 monoclonal Ab) causes B cell depletion; tofacitinib (an inhibitor of JAK1 and JAK3) interferes the JAK - STAT signalling pathway; and (hydroxy-) chloroquine increases pH within intracellular vacuoles and alters processes such as protein degradation by acidic hydrolases in the lysosome, assembly of macromolecules in the endosomes, and post-translation modification of proteins in the Golgi apparatus, resulting in a diminished formation of peptide-MHC protein complexes required to stimulate $CD4^+$ T cells[96]. Non-steroidal anti-inflammatory drugs (NSAIDs) and (cortico-) steroids are anti-inflammatory medicine used to relieve pain and reduce inflammation.

The collagen-induced arthritis mouse model is the most commonly studied autoimmune model of RA wherein autoimmune arthritis is induced through immunization with an emulsion of complete Freund's adjuvant and type II collagen[97].

1.3.2.2. Systemic lupus erythematosus

SLE is a chronic multi-organ AID characterized by a complex multi-factorial pathogenesis and a great clinical polymorphism. B cells carry out central roles in the pathogenesis of SLE through a combination of Ab-dependent and -independent actions. A hallmark is the production of autoAbs (anti-dsDNA, anti-cardiolipin, anti-Ro Abs) and the deposition of immune complexes resulting in glomerulonephritis and proteinurea, congenital heart block and thrombosis[98]. The Ab-independent role is related to the T cell and B cell crosstalk, including the presentation of self-antigens and the delivery of co-stimulatory signals, and the elevated production of cytokines IL6, IL10 and IFNα.

The most current treatment of SLE is directed toward easing the symptoms by decreasing inflammation and level of autoimmune activity with NSAIDs and (hydroxy-) chloroquine for those with mild symptoms and corticosteroids and immunosuppressive agents such as mycophenolate mofetil, cyclophosphamide, azathioprine, and methotrexate, for those with a more severe disease status. SLE patients show an excess of circulating BAFF leading to aberrancies in the B cell homeostasis. Treatment with belimumab, a fully human anti-BAFF monoclonal Ab, or with atacicept, a TACI-Ig fusion protein that neutralizes both BAFF and APRIL, is a manner to restore the B cell homeostasis[57]. In contrast to the success of B cell depletion with rituximab for the treatment of RA, randomized, placebo-controlled trials of rituximab failed to meet their primary or secondary clinical endpoints for renal and non-renal SLE[99,100]. IL10 serum levels are significantly elevated in human SLE and correlate with disease activity. Increased IL10 production accounts for the impairment of T lymphocyte function and the maintenance of B cell's hyperactivity and autoAb secretion. Secretion of autoAbs was significantly reduced in the presence of neutralizing IL10 specific monoclonal Abs and led to marked improvement in participants of a small clinical trial[101]. The importance of CD32b (FcγRIIB), the sole inhibitory member of the FcγR family with expression on B cells, plasmablasts and long-lived PCs and an important role in the down-regulation of Ab-production[102], is exemplified by the finding that B cells from SLE patients express lower levels of CD32b on their surface due to polymorphisms in their CD32b promoter[103] or in the receptor itself[104,105]. Agonistic CD32b Abs could show therapeutic potential, but their efficacy could be compromised by the decreased expression of CD32b in SLE B cells and PCs[100].

Epratuzumab, an anti-CD22 B cell modulator, holds great promise as novel therapeutic for patients suffering from moderate-to-severe SLE[106].

There are numerous murine (spontaneous and induced) models of SLE and all of them portray their own iterations of lupus-like diseases with a subset of symptoms akin to those observed in human SLE, namely, autoAb production, lymphoid activation and hyperplasia, and lupus nephritis. The classic models of spontaneous lupus include the F1 hybrid between the New Zealand Black and New Zealand White strains (NZB/W F1) and its derivatives, the MRL/lpr, and BXSB/Yaa strains whereas induced models include the pristane-induced model and the chronic graft-versus-host-disease models[107].

1.3.2.3. Multiple sclerosis

MS is a progressive inflammatory and demyelinating disease of the human central nervous system in which the insulating myelin sheath of nerve cells in the brain and spinal cord are damaged. Nerve impulses travelling to and from the brain and spinal cord are distorted or interrupted, causing a wide variety of autonomic, visual, motor and sensory problems.

The role of B cells in the pathogenesis of MS is diverse: autoantigen presentation (for instance myelin oligodendrocyte glycoprotein and myelin basic protein); secretion of autoAbs in cerebrospinal fluid; formation of ectopic lymphoid follicles. Generally, MS patients have normal numbers of circulating B cells and PCs, but show an increased number of B cells, mainly memory B cells and plasmablasts, in the central nervous system[108]. Plasmablasts persist in the cerebrospinal fluid throughout the course of MS and correlate with intrathecal IgG synthesis and with active inflammatory parenchymal disease. MS lesions, known as ‘plaques’, may form in central nervous system’s white matter in any location and contain B cells and PCs, but also both $CD8^+$ and $CD4^+$ T cells permitting interactions between B cells and T cells which result in the activation of T cells and their differentiation into Th1, Th2, or Th17 cells and in the T cell-dependent B cell activation with detrimental consequences[108]. Cytokines TNFα, IL1, IL2, IL4, IL6 and IL10 are identified within MS lesions and especially BAFF is found to be strongly elevated in the demyelinating MS lesions and might be involved in the formation of lymphoid follicle-like meningeal structures[108,109]. Increased APRIL expression in astrocytes has been noted in the brain of MS patients[110].

Several therapeutic substances are developed to abolish the migration of immune cells, generally T cells, across the blood-brain barrier toward the central nervous system: therapy with cytokine interferon-β1 leads to a reduction of neuron inflammation by decreasing the number of inflammatory cells (through inhibition of T cell activation and proliferation,

apoptosis of auto-reactive T cells, induction of Tregs) that cross the blood-brain barrier and by increasing the production of nerve growth factor[111]; humanized monoclonal Ab natalizumab binds to α4β1-integrin on leukocytes and blocks its interaction with VCAM-1 on endothelial cells; and pro-drug fingolimod works as a functional antagonist on the sfingosine 1-phosphate receptor type 1 on lymphocytes. Glatiramer acetate is a random polymer composed of 4 amino acids found in myelin basic protein and shows an initial strong promiscuous binding to major histocompatibility complex molecules and consequent competition with various myelin antigens for their presentation to T cells. Anti-CD20 rituximab, ocrelizumab and ofatunumab, anti-CD52 alemtuzumab and anti-BAFF agents belimumab and tabalumab aim at a depletion of pathogenic effector B and T lymphocytes[108,109]. Treatment with recombinant fusion protein atacicept, designed to neutralize the high levels of BAFF and APRIL, gave unexpectedly increased disease activity[108] and was withdrawn from clinical MS trials. Teriflunomide (dihydroorotate dehydrogenase inhibitor) and mitoxantrone (type II topoisomerase inhibitor) are anti-proliferative agents. Dimethyl fumarate, derived from the organic acid fumaric acid, holds great promise for modern immunotherapy in MS with a broad efficacy, good safety and satisfying tolerability, but its (main) mechanism of action has not yet been clarified.

Experimental autoimmune encephalomyelitis is a $CD4^+$ T cell-mediated AID characterized by perivascular $CD4^+$ T cell and mononuclear cell inflammation and subsequent primary demyelination of axonal tracks in the central nervous system, leading to progressive hind-limb paralysis. It is generally considered to be a relevant animal model for MS[112].

1.4. Solid organ transplantation

Organ transplantation is the treatment of choice when a patient has a defective or severely injured vital organ. Vital physiologic functions are restored through the surgical substitution of the defective organ of the patient, the recipient, by a healthy organ from a donor. However, solid organ transplantation is not risk-free. Serious short-term and long-term complications can occur, either related to the transplantation procedure itself or to the immunosuppressive drugs to prevent the rejection of the allograft.

The significant development of immunosuppressive drug therapies has had a major impact on the outcome of clinical solid organ transplantation, mainly by decreasing the incidence of acute rejection episodes and improving short-term patient and graft survival. Amongst the immunosuppressive drugs there are corticosteroids like prednisolone and hydrocortisone, calcineurin inhibitors like cyclosporine A and tacrolimus, mTOR inhibitors like rapamycin, and anti-proliferatives like azathioprine and mycophenolic acid. Monoclonal Abs selective

against immune components can also be used in immunosuppressive therapy. Unfortunately, long-term results remain disappointing because of chronic allograft dysfunction and the necessary chronic use of immunosuppressive drugs leads to two categories of complications: (1) drug-related adverse effects such as hypertension, cardiovascular complications, renal dysfunction, hyperlipidemia and diabetes mellitus; and (2) higher susceptibility to bacterial, fungal and viral infections and increased risk of developing a wide range of cancers due to the reduced function of the naturally-occurring immune system in the body[113,114]. Hence, immunosuppressants, although they are necessary to take, can have a serious impact on the quality of life of the patient. Furthermore, the worldwide acute shortage of human donor grafts (USA: 30 973 transplants from 15 064 donors have been performed while more than 121 000 candidates were on the waiting list in the year 2015[115]) makes the situation for people waiting for (another) transplant very problematic. It urges to find new strategies to optimize the current therapies and also to find other ways to meet the demand of organs (either by non-human derived organs for xeno-transplantation or by bio-engineered grafts from stem cells). The current "one size fits it all" strategy will certainly be substituted, because immunosuppression needs to be managed according to the quality of the transplanted organ, patient co-morbidity profile, underlying graft inflammatory status, and degree of cellular and/or humoral sensitization[116].

The focus of B cell research in clinical transplant immunology has been mostly directed to neutralize or avoid the production of donor-specific Abs (DSAs) which play an important role in humoral rejection. However, the role of B cells in organ transplantation is not restricted to the production of Abs. Moreover, B cells can also act as beneficial players in the tolerance of the allograft[117].

1.4.1. Contribution of B cells to rejection of the allograft

As a consequence of a sensitization event before transplantation (e.g. blood transfusion, previous transplantation or pregnancy), pre-formed circulating DSAs, directed against donor-specific HLA, can be present in the recipient's immune system. These DSAs cause via complement-dependent or -independent pathways early morbidity and graft loss in early (hyper-) acute antibody-mediated rejection, mostly occurring a few days to weeks after transplantation. At later time points after transplantation, chronic antibody-mediated rejection, due to *de novo* Ab production, may occur alone or in combination with cellular reactions and results in irreversible structural damage[118]. The protocols used for pre-transplant desensitization and post-transplant treatment of (hyper-) acute antibody-mediated rejection are

based on elimination or reduction of circulating Abs, inhibition of residual Abs, and suppression or depletion of B cells[119]. Plasmapheresis and intravenous immunoglobulin (IVIG) are techniques to remove directly harmful alloAbs from the patient's plasma. The chimeric anti-CD20 Ab, rituximab, is a well-known B cell depleting agent, but its effect in the desensitization is limited. AlloAb titers remain generally unaltered, because PCs, which lack CD20, and memory B cells, which have only low expression of CD20, are not affected by rituximab. New strategies to deplete alloAb-producing B cells involve 26S proteasome inhibitor bortezomib and anti-CD52 Ab alemtuzumab. Bortezomib can strongly reduce circulating PC numbers through apoptosis induction[120,121]. Alemtuzumab provides a high spectrum depletion of circulating B cells as CD52 is expressed from the pro-B to the short-lived PC stages. Eculizumab[122], an anti-C5 Ab, diminishes complement-mediated pathogenesis. Splenectomy can be performed in highly sensitized patients in whom desensitization therapy has failed.

B cells can act as APCs which deliver the required multiple signals for activation and differentiation of the recipient T cells. Recipient $CD4^+$ T cells usually acquire helper function and can on their turn influence B cell activation, differentiation and Ab production by the production of cytokines[123–125]. At later stages of graft rejection B cells can infiltrate into the graft tissue and form ectopic germinal centres or so-called tertiary lymphoid tissue. These structures direct various B cell and T cell responses, including the induction of effector functions, Ab generation, affinity maturation, Ig class switching and clonal expansion[126]. In the end, all these actions, classified under the process of chronic rejection, lead to destruction and rejection of the donor graft. Chronic rejection is generally considered irreversible and poorly amenable to treatment.

1.4.2. Contribution of B cells to tolerance of the allograft

Emerging data from both experimental animal models[127–133] and clinical trials indicate that Bregs can act as beneficial players in the protection and acceptance of the allograft.

Operational tolerance is a very rare clinical situation in which the patient shows a stable graft function without clinical features of rejection in the absence of any immunosuppressive drugs for more than a year[134,135]. Extensive research was done on the characterization of the B cells of operationally tolerant kidney transplant patients. Apparently, as determined in a comparative study between healthy people and kidney-transplanted individuals with operational tolerance, individuals with stable graft function under immunosuppression and individuals with chronic rejection, the state of operational tolerance essentially involves the

preservation of a healthy B cell compartment profile and not a higher activation or expansion of B cells[136]. Phenotypically, peripheral blood B cells of tolerant patients display an inhibitory profile[137], defined by an increased expression of BANK1 (a negative modulator of CD40-mediated AKT activation)[138,139], CD1d (usually characterizing for cells with regulatory phenotype; it presents lipid antigens to natural killer T cells)[140–142] and CD5 (negatively regulates BCR signalling)[143], as well as a decreased ratio of CD32a/CD32b[144–146]. Particularly, tolerant patients display a higher absolute number of GzmB-expressing B cells with a PC-like phenotype and suppressive properties. They can inhibit *in vitro* the proliferation of effector $CD4^+CD25^-$ T cells in a contact- and GzmB-dependent manner[147].

Healthy volunteer	Tolerant kidney patient	Stable graft under immunosuppression	Chronic rejection under immunosuppression
B cells (% and total number)	Maintenance of B cells	Decrease in B cells	Trend ↘
B cell populations (%)			
▪ Naive	Unchanged	↘	Not determined
▪ Transitional	Unchanged	↘	↘
▪ Memory	Unchanged	Similar	Not determined
▪ Regulatory	Unchanged	↘	↘
▪ Plasma	Trend ↘	↗	↗
▪ $GzmB^+$ B cells	↗	Unchanged	Not determined
Inhibitory profile			
▪ CD32b	Similar	↘	↘
▪ BANK1	Trend ↗	Trend ↘	Trend ↘
▪ CD1d	↗	Unchanged	Similar
▪ CD5	Trend ↗	↘	↘

Table 1.2. Comparison in B cell phenotypic profiles between tolerant kidney patients, patients under immunosuppression with stable kidney graft function, patients under immunosuppression with deteriorating kidney graft function and healthy volunteers. The table is the summary of observations in kidney transplant patients from different clinical studies[136,137,148,149]. The conventional immunosuppression therapy consists of calcineurin inhibitors and corticosteroids combined with azathioprine or mycophenolate mofetil (the differences in therapy between the different clinical studies are rather small).

The expression of GzmB by B cells is not accompanied by perforin expression, as it is the case with T cells and natural killer cells, and is regulated by IL21. Activation of human B cells by IL21 and BCR or TLR engagement in the presence of CD40 ligation results in their differentiation into PCs. However, in the absence of CD40 ligation they differentiate into GzmB-expressing B cells[150–152], which are phenotypically and functionally distinct from B10 cells[151,153]. Stimulation of murine B cells with various B cell activators in combination with IL21 did not give expression of GzmB[154]. Hence, mouse models are unlikely to provide more insight into the physiological and pathological relevance of GzmB expression in human B

cells. But the peripheral blood B cells in a rat model of cardiac allograft tolerance displayed a very similar inhibitory profile as the B cells in the tolerant kidney transplant patients[149]: expression of GzmB, increased expression of BANK1 and IL21, and decreased ratio of CD32a/CD32b. So this rat model can be of use as surrogate to study function and therapeutic potential of B cells in transplantation tolerance.

Operationally tolerant patients display a reduced number of circulating PCs compared to stable kidney graft patients who are still under immunosuppression. Genetic analysis showed that B cells of tolerant patients had a lowered expression of anti-apoptotic genes and a lowered expression of transcription factors PRDM1, IRF4 and XBP1 which are implicated in the end step of differentiation into PCs[148]. In an *in vitro* two-step culture model of B cell differentiation[155], the B cells from tolerant patients displayed an increased susceptibility to apoptosis in the late step of differentiation into PCs compared to the stable graft patients under immunosuppression[148]. Figure 1.5 gives a schematic overview of the unique B cell profile in tolerant kidney transplant patients.

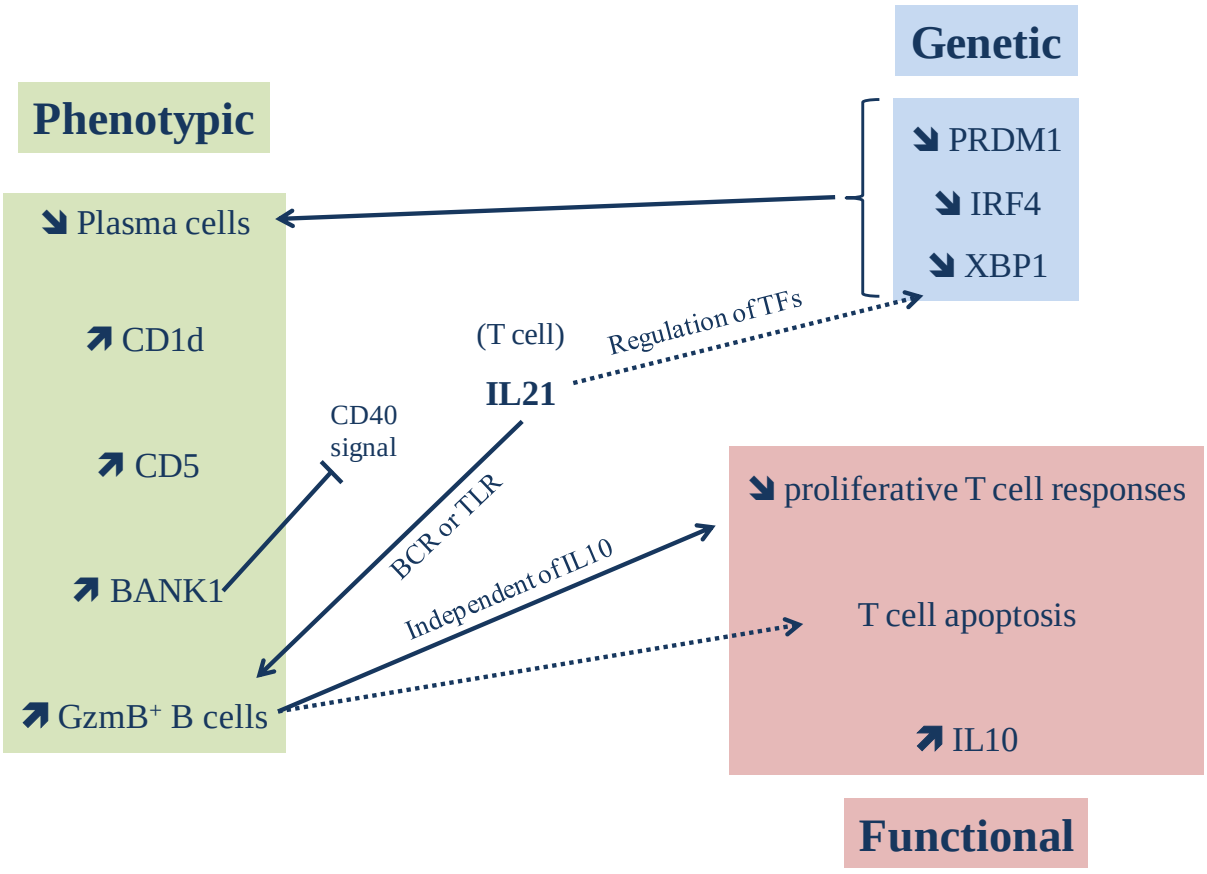

Figure 1.5. B cell phenotypic profile in operationally tolerant kidney patients.
TF: transcription factor.

Whether the Breg phenotype observed in operationally tolerant kidney transplant patients is the cause or the result of graft survival without immunosuppression remains to be determined. Evidence of a regulatory role of B cells in transplantation tolerance also raises questions about the impact of B cell-depleting therapies in transplant recipients. These agents might affect the induction and function of Bregs and potentially have detrimental effects on long-term graft outcomes. A clinical trial with renal transplant patients has shown an increased risk for acute cellular rejection following depletion of B cells by treatment with rituximab prior to transplantation, which could be due to a loss of Bregs[156].

Operational tolerance is more frequently reported in liver transplantation (immunosuppressive drugs can be completely withdrawn in up to 20 % of liver transplant recipients[157]). But B cells from tolerant liver transplant recipients do not harbour the inhibitory profile observed in tolerant kidney transplant patients. The tolerant liver transplant individuals exhibit significantly greater numbers of circulating potentially Treg subsets ($CD4^+CD25^+$ T cells and $V\delta1^+$ T cells) than either non-tolerant patients or healthy individuals. And they exhibit a gene expression signature characterized by up-regulation of genes associated with γδ T cells and natural killer receptors, suggesting a role γδ T cells and natural killer cells in the maintenance of liver allograft tolerance[157]. Hence, the process of transplant tolerance might depend on the organ that is being transplanted.

1.5. Need for better medicines to treat immune disorders and transplant rejection

Current immunosuppressive drugs or agents used in immunotherapy have improved the life expectancy of patients, but, unfortunately, they also exhibit important toxic side effects due to their mechanism of action and their necessary chronic use in combination. Their non-selective activity causes immunodeficiency in the patient, resulting in increased susceptibility to infections and decreased cancer immunosurveillance. Immunosuppressive drugs can interact with other medicines and hereby affect their metabolism and action.

The scale of the global drug-markets is gigantic and hits several US$ billions in annual sales and spending (for instance, a study in 2007 suggested that worldwide market of drugs used for the prevention of organ allograft rejection was US$ 6.6 billion[158]). In spite of the increasing levels of investments in pharmaceutical research and development, the output of new molecules and biologicals entering clinical development and reaching the market is disappointingly low[159,160]. It is clear that in drug discovery one approach does not fit all and that the strategies for screening to identify new drug compounds are constantly evolving.

Also, many major barriers to the development of new therapeutics are not scientific, but rather logistical, financial and procedural ones[158].

The majority of the marketed immunotherapeutic drugs are focused on controlling the activity of T cells (e.g. calcineurin inhibitors [cyclosporine A, tacrolimus]; mTOR inhibitors [sirolimus, everolimus]; co-stimulation blocking antibodies [belatacept, abatacept] or CD3 antagonistic antibody [muromonab]) while it is becoming clear that B cells contribute to the pathogenesis of various immune disorders and to transplant rejection. Only a few molecules against B cells, mainly biologics, are available or under development. Strategies of B cell therapeutics comprise: 1) targeting B cell-specific surface markers, 2) depleting key survival factors, 3) disrupting critical intercellular interactions or 4) inhibiting crucial intracellular functions or signalling.

1.5.1. Targeting B cell-specific surface markers

Rituximab is a chimeric mouse-human monoclonal Ab directed against the surface protein CD20, an activated glycosylated phosphoprotein expressed on the surface of all B cells, but not on either early pro-B cells and PCs. Rituximab is very successful in depleting CD20-expressing (normal and pathological) B cells and is therefore used to treat diseases characterized by overactive, dysfunctional or excessive numbers of B cells. However, PCs and memory B cells remain untouched. Rituximab has proved its therapeutic value in the treatment of several B cell malignancies and of AIDs such as RA and MS (but not in SLE). Some adverse events are associated with the use of rituximab: mild to moderate infusion reactions, higher incidence of infections, neutropenia and development of human anti-chimeric Abs[161]. Nonetheless, the efficacy and success of rituximab has led to some other anti-CD20 monoclonal Abs being developed: ocrelizumab, ofatumumab and obinutuzumab.

Epratuzumab is a fully humanized Ab directed against CD22 (also known as Siglec-2), a B lymphocyte-restricted type I transmembrane sialoglycoprotein (a combination of sialic acid and glycoprotein) of the Ig superfamily. CD22 is expressed at low levels on immature B cells and at higher levels on mature IgM^+, IgD^+ B cells whereas it is absent on differentiated PCs. The antigen is also found highly expressed on most malignant mature B cells. The exact mechanism of action of CD22 is not entirely clear, but it is an inhibitory co-receptor that negatively regulates BCR signalling, hereby preventing overactivation of the immune system[162]. Promising results have been obtained with epratuzumab in clinical trials with SLE-patients[106]. Clinical trials are now ongoing to assess its long-term safety and efficacy.

Daratumumab is a human monoclonal antibody that specifically targets CD38, a type II transmembrane glycoprotein that is highly expressed on PCs and multiple myeloma cells, but weakly expressed on other lymphoid cells, other myeloid cells and non-haematopoietic cells. Clinical trials show encouraging data for daratumumab in the treatment of refractory multiple myeloma through its capacity to substantially deplete malignant PCs[163]. And the hope is that this agent can be used in an even wider range of blood cancers[164]. Data on the efficacy of daratumumab in patients with AIDs are not yet available.

Alemtuzumab is a drug used in the B cell chronic lymphocytic leukemia (B-CLL), cutaneous T cell lymphoma and T cell lymphoma under the trade names Campath, MabCampath and Campath-1H, and in the treatment of MS as Lemtrada. It is also used in some conditioning regimens (in induction therapy for instance) for bone marrow transplantation, kidney transplantation and islet cell transplantation[165]. It is a monoclonal Ab that binds to CD52, a protein present on the surface of mature lymphocytes, but not on the stem cells from which these lymphocytes are derived. After treatment with alemtuzumab, these CD52-bearing lymphocytes are destroyed, causing profound lymphocyte depletion. A complication of therapy with alemtuzumab is that it significantly increases the risk for opportunistic infections, in particular, reactivation of cytomegalovirus. An important side-effect of alemtuzumab therapy is the significant increase in circulating BAFF[166] which might cause an increased frequency of Ab-mediated rejection or the development of (B cell-mediated) autoimmunity, e.g. thyroid AIDs, immune thrombocytopenia, Goodpasture syndrome are substantial adverse events associated with alemtuzumab treatment[167]. The levels of circulating BAFF can be controlled by using belimumab.

1.5.2. Depletion of key survival factors

Belimumab, a fully human anti-BAFF monoclonal Ab, specifically recognizes and inhibits the stimulatory activity of BAFF. Atacicept is a TACI-Ig fusion protein that neutralizes both BAFF and APRIL. Local and systemic overexpression of BAFF and APRIL is implicated in the pathogenesis of several AIDs including MS, SLE and RA[108]. This can be treated by administration of belimumab or atacicept. In clinical trials with SLE and RA patients, atacicept seemed to be well tolerated, but may be associated with injection-site reactions. However, increased inflammatory activity in the “ATAMS” (ATAcicept in Multiple Sclerosis) trial led to the suspension of atacicept trials in MS. Reasons for the unexpected increase in disease activity are unknown, but are under investigation[108].

1.5.3. Disruption of intercellular interactions

Abatacept and belatacept are fusion proteins composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA4 and bind to the CD80 and CD86 molecule, blocking the interaction between CD80/86 and CD28 co-stimulatory pathways. They are used to treat AIDs, like RA, by suppressing T cell function. Belatacept is also approved for use in organ transplantation.

1.5.4. Inhibition of intracellular functions or signalling

Small molecules inhibiting various BCR-associated kinases display encouraging clinical effects. Ibrutinib, an orally administered, covalent inhibitor of BTK, delivered a breakthrough in the treatment of B cell malignancies including mantle cell lymphoma, CLL, diffuse large B cell lymphoma and multiple myeloma. Ibrutinib or other BTK-inhibitors might show potential in the treatment of AIDs like RA and SLE. Clinical trials with fosfamatinib (targeting SYK) and idelalisib (targeting PI3Kδ) are ongoing[28]. But it must be emphasized that the 'BCR inhibitors' probably target multiple pathways interconnected with BCR, e.g. chemokine- and integrin-signalling[28].

Bortezomib is a potent, selective small molecule inhibitor of the mammalian 26S proteasome, a large protein complex that degrades misfolded proteins, cell-cycle regulatory proteins, transcription factors and inhibitory molecules in order to maintain cellular homeostasis.
Bortezomib is an N-protected dipeptide (pyrazinoic acid, phenylalanine and leucine with a boronic acid instead of a carboxylic acid). The boron atom in bortezomib binds the catalytic site of the 26S proteasome with high affinity and specificity and inhibits its proteolytic activity. Inhibition of proteasome activity results in the accumulation of misfolded proteins and affects multiple signalling cascades within the cell and sensitizes cells to programmed cell death. Rapidly dividing tumour cells appear to be more sensitive to proteasome inhibition. Moreover, bortezomib appears to increase the sensitivity of cancer cells to traditional anti-cancer agents (e.g., gemcitabine, cisplatin, paclitaxel, irinotecan, and radiation). In clinic, bortezomib is used for treatment of relapsed multiple myeloma, a cancer of PCs. Bortezomib possesses also apoptosis-inducing activity against non-transformed PCs[168,169]. The clinical potential of bortezomib in transplantation (to treat Ab-mediated rejection [169–171]) and AIDs (e.g. SLE[100,172]) is under investigation. Gastro-intestinal effects and asthenia (weakness) are the most common adverse events, but bortezomib has also been associated with peripheral neuropathy.

A visual overview of the prominent co-stimulatory and -inhibitory (cell surface) molecules is given in figure 1.6 together with the immunomodulatory therapeutics that target them.

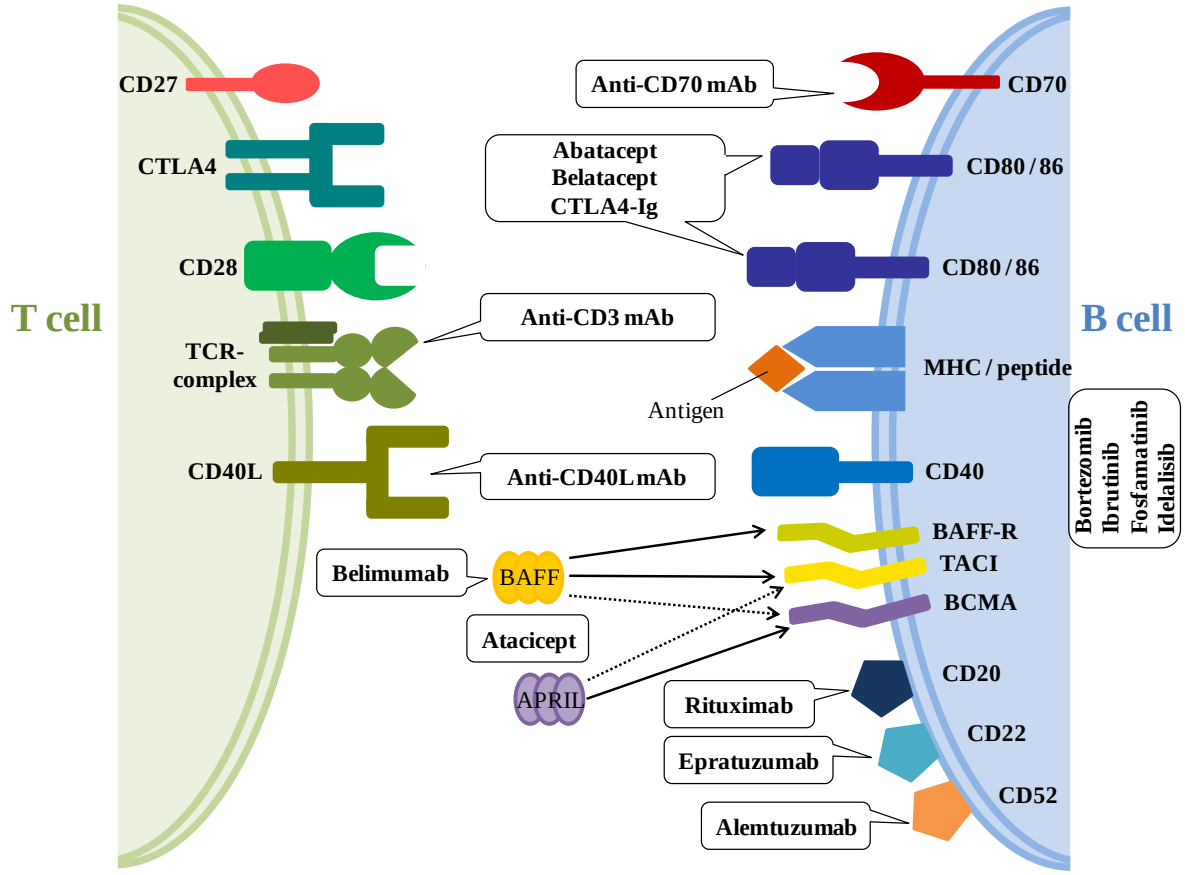

Figure 1.6. Illustration of prominent co-stimulatory/-inhibitory molecules and (B cell-specific) immuno-modulatory therapeutics.

Over time, tremendous progress has been made in understanding the mechanisms of immune responses. The challenge remains to selectively interfere with undesired, harmful immune responses responsible for autoimmunity or transplant rejection while keeping a flawless response to infectious agents.

Chapter 2 **: Aim of the study**

Considering the dramatic numbers of people who suffer from an AID, or have a transplanted organ or are on the waiting list for a transplant, there is an urgent need to develop new medicine to cope with the undesired immune responses. It is well-established that B cells play a substantial role in the pathogenesis of many AIDs and in the rejection of transplants. Still, there are only a few B cell-specific immunomodulatory agents, mainly biologics like monoclonal Abs, available in the clinic. Despite their great specificity, the biologics are limited in their therapeutic actions that usually comprise the neutralization of pathogenic Abs or the depletion of certain subsets of B lymphocytes via binding with B cell-specific surface markers. Due to their size, biologics cannot cross the cell membrane and, hence, cannot influence directly intra-cellular processes and signalling pathways. B cells contribute to immune responses not solely by producing Abs, but also by communicating with distant and adjacent Th cells in order to orientate them in their actions. The direct communication between a B lymphocyte and a neighbouring Th cell happens by binding of receptors with their matched ligands on the cell surface. Receptor-ligand binding triggers signalling processes that lead to an alteration in behaviour (either activation or suppression in activity) of the interacting cells. The expression of the various cell membrane receptors and ligands reflect the cell's activation or regulatory status and can be increased or decreased in response to (micro-) environmental conditions leading to an altered sensitivity for cellular interplay. Relevant (new) targets in the B cell activation pathway can be identified when focussing on changes in expression of activation and co-stimulatory molecules on the B cell's surface. Subsequently, these identified targets can advance the understanding of B cell regulation as well as the creation and/or discovery of improved or even first-in-class medicines for more fine-tuned therapeutic intervention.

In this project, we aimed to set up a B cell screen whereby the expression of cell surface markers forms the read-out in order to identify potential novel targets in B cell activation signalling pathways and to test new small molecule inhibitors for possible modulatory effects on B cell activation.

For the establishment of the *in vitro* B cell screen, we needed to select and implement a robust, physiologically relevant B cell activation model. Keeping in mind that reproducibility and stability are essential for repetitive and large-scale screening, we examined several

immortalized human B cell lines for their responsiveness, as determined by the expression of several relevant cell surface markers, upon stimulation with the stimulator that was selected for having the broadest immunostimulatory effects on human primary B cells. The B cell line with the most similar expression profile to that of primary B cells was taken as a reliable, homogeneous substitute of the primary B cell.

Afterwards, a lentiviral vector-based RNA interference library was screened on the established human B cell activation model in order to identify critical B cell genes coding for potential new targets involved in B cell activation. To maintain the idea of a first line screening assay, the read-out consisted of analyzing the expression of two cell surface markers with an important role in B cell - Th cell interactions.

The identified candidate targets of the lentiviral vector library were further validated and characterized for their specificity, selectivity and druggability by means of *in vitro* phenotypic screens and of *in vivo* studies using commercially available small molecule inhibitors and by the generation of animal models with specific genetic deletion of the candidate target.

Chapter 3 **Comparative in vitro immune stimulation analysis of primary human B cells and B cell lines**

The selection and implementation of a robust, physiologically relevant B cell activation model is published in Journal of Immunology Research:

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Research Article

Comparative In Vitro Immune Stimulation Analysis of Primary Human B Cells and B Cell Lines

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B cell specific immunomodulatory drugs still remain an unmet medical need. Utilisation of validated simplified in vitro models would allow readily obtaining new insights in the complexity of B cell regulation. For this purpose we investigated which human B lymphocyte stimulation assays may be ideally suited to investigate new B lymphocyte immunosuppressants. Primary polyclonal human B cells underwent in vitro stimulation and their proliferation, production of immunoglobulins (Igs) and of cytokines, and expression of cell surface molecules were analysed using various stimuli. ODN2006, a toll-like receptor 9 (TLR9) agonist, was the most potent general B cell stimulus. Subsequently, we investigated on which human B cell lines ODN2006 evoked the broadest immunostimulatory effects. The Namalwa cell line proved to be the most responsive upon TLR9 stimulation and hence may serve as a relevant, homogeneous, and stable B cell model in an in vitro phenotypic assay for the discovery of new targets and inhibitors of the B cell activation processes. As for the read-out for such screening assay, it is proposed that the expression of activation and costimulatory surface markers reliably reflects B lymphocyte activation.

1. Introduction

Current immunotherapeutic drugs have improved the life expectancy of patients, but they still exhibit important side effects. Furthermore, the number of new immunotherapeutic small molecule medicines and biologicals entering clinical development is in decline despite increasing levels of investments in the drug industry [1–3]. Moreover, the majority of the marketed immunotherapeutic drugs are focused on controlling the activity of T cells (e.g., calcineurin inhibitors [cyclosporine A, tacrolimus]; mTOR inhibitors [sirolimus, everolimus]; costimulation blocking antibodies [belatacept, abatacept]; CD3 antagonistic antibody [muromonab]; or CD25/IL2-R antagonistic antibodies [basiliximab, daclizumab]). Nevertheless, B cells are equally important players in the immune response, but presently there are only very few drugs available to target them. The effector functions of B cells are diverse. Production of Igs assures the clearance of invading pathogens and dying cells [4, 5]. B cells are efficient antigen-presenting cells capturing antigen with their antigen-specific B cell receptor (BCR) and presenting the epitopes, bound to major histocompatibility complex (MHC) molecules, to the appropriate T cells. Through the secretion of cytokines [6, 7] and the expression level of various cell surface markers, activated B cells can establish an effective intercellular communication with other effector cells to obtain a more directed and controlled immune response. The strength of the B cell lies not only in its versatility of actions, but also in its ability to adapt its phenotype in response to (micro)environmental variables. B

cells play a considerable, but not yet fully understood, role as a pathogenic factor in different clinical situations such as cancer [8], autoimmune disorders [9–11], transplant rejection [12–16], and graft-versus-host diseases [17–19].

At the present time, there are only very few B cell specific immunomodulatory agents (e.g., bortezomib, rituximab, and belimumab) available in the clinic and they are mainly depleting agents. Hence, there is an unmet need for new drugs in this field.

Exploration of B cell regulation models could lead to the identification of relevant new targets or molecular agents with potential as B cell drugs. The goal of the present study was to investigate a series of B cell stimuli and human B cell lines to identify an in vitro model which is suitable to explore B cell immune activation and readily applicable for screening and drug development.

2. Materials and Methods

2.1. Cell Culture Media. Complete RPMI 1640 culture medium consisted of RPMI 1640 with 10% foetal calf serum (FCS, HyClone® Thermo Scientific, United Kingdom) and 5 μg/mL gentamicin sulphate antibiotics. Complete DMEM culture medium consisted of DMEM (Dulbecco's Modified Eagle's Medium) with 10% heat-inactivated FCS and 5 μg/mL gentamicin sulphate antibiotics. Cell culture media and gentamicin sulphate antibiotics were purchased from BioWhittaker® Lonza (Verviers, Belgium).

2.2. Cells and Cell Lines. Blood samples of healthy volunteers were collected at the Red Cross of Mechelen, Belgium. Each donor consents to the use of his blood for research purposes. Human peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation of the heparinized venous blood over Lymphoprep™ (Axis Shield PoC AS; density 1,077 ± 0.001 g/mL). Highly purified naive peripheral human B cells were separated from fresh human PBMCs on magnetic columns by positive selection using CD19 magnetic beads according to the manufacturer's instructions (MACS Miltenyi Biotech, Leiden, Netherlands). The purity of the isolated naive B cells was ≥95% as analysed by flow cytometry. Cells were suspended at the desired concentration in complete culture medium. Human B cell lines Daudi, Raji, Ramos (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Germany), Namalwa, RPMI 8866 (European Collection of Cell Cultures, ECACC, England), and RPMI 1788 (Global Bioresource Center ATCC, USA) were used. The cell lines were maintained in culture flasks (TPP, Switzerland) as suspension cultures in complete RPMI 1640 culture medium at 37°C and 5% CO_2.

2.3. Pharmacological Agents. Pharmacological inhibitors AZD-5363, ibrutinib, and MK-2206.2HCl were purchased from SelleckChem (Munich, Germany); chloroquine, LY-294,002, Mirin, SAHA, and TPCA-1 from Sigma-Aldrich (Diegem, Belgium); STAT3 inhibitor VII from Calbiochem, Merck Millipore (Overijse, Belgium); bortezomib and dasatinib from LC Laboratories (Woburn, MA, USA).

2.4. In Vitro Stimulatory Conditions. Human primary B cells were stimulated with a variety of conventional in vitro stimulatory conditions. The following stimulatory reagents were used: oligodeoxynucleotide 2006 (ODN2006, InvivoGen, Toulouse, France); resiquimod, lipopolysaccharide (LPS), and pokeweed (Sigma-Aldrich, Diegem, Belgium); anti-CD40 (Bioceros BV, Utrecht, Netherlands); anti-IgM (Jackson ImmunoResearch, Suffolk, United Kingdom); pansorbin (Calbiochem, Merck Millipore, Overijse, Belgium); 2,4,6-trinitrophenyl hapten conjugated to bovine serum albumin (TNP-BSA) and 2,4,6-trinitrophenyl hapten conjugated to Ficoll (TNP-Ficoll) (Biosearch technologies, Novato, California, USA); recombinant human IL4 and IL21 (MACS Miltenyi Biotec, Leiden, Netherlands); recombinant human IL2 and IL10 (BioLegend, ImTec Diagnostics NV Antwerp, Belgium). Primary B cells were stimulated with the reagents at different concentrations and the most optimal reagent concentrations were then used for further experiments. Following in vitro stimulatory conditions were applied in duplicate: 0,1 μM ODN2006; 1 μM resiquimod; 1 μg/mL LPS; 5 μg/mL anti-CD40 alone or in combination with 20 ng/mL IL4 or with 12.5 ng/mL IL21; 5 μg/mL anti-IgM with or without 20 ng/mL IL4; 1/10 000 pansorbin with 100 units/mL IL2 and 50 ng/mL IL10; 1 μg/mL pokeweed; 91 μg/mL TNP-BSA; or 91 μg/mL TNP-Ficoll. Human cell lines were stimulated for 24 hours in presence of 0.1 μM ODN2006.

2.5. Ig Production. For assessment of Ig production, freshly isolated human $CD19^+$ B cells were plated at 25 000 cells per well in a 384-well plate (Perkin Elmer, Zaventem, Belgium) in 55 μL complete DMEM medium. After 7 days of stimulation, supernatant was taken for analysis of IgG and IgM with the AlphaLISA human IgG and IgM kits according to the manufacturer's instructions (Perkin Elmer, Zaventem, Belgium). Analysis was performed with the EnVision™ 2103 Multilabel Reader (Perkin Elmer, Zaventem, Belgium). For IgG production, less than 5-fold increase was considered as a weak effect of the stimulus, 5- to 20-fold increase as a moderate effect, and more than 20-fold increase as a strong effect. For IgM production, less than 5-fold increase was considered as a weak effect of the stimulus, 5- to 10-fold increase as a moderate effect, and more than 10-fold increase as a strong effect.

2.6. Cytokine Production. Freshly isolated human $CD19^+$ B cells were plated at 50 000 cells per well in a 96-well plate in 220 μL complete DMEM medium and activated by different stimulatory conditions. Analysis of IL6 and IL8 was performed after 2 days with the AlphaLISA human IL6 and IL8 kits according to the manufacturer's instructions (Perkin Elmer, Zaventem, Belgium). Analysis was performed with the EnVision 2103 Multilabel Reader (Perkin Elmer, Zaventem, Belgium). For IL6 production, less than 5-fold increase was considered as a weak effect of the stimulus, 5- to 20-fold increase as a moderate effect, and more than 20-fold increase as a strong effect. For IL8 production, less than 5-fold increase was considered as a weak effect of the stimulus, 5 to 10-fold increase as a moderate effect, and more than 10-fold increase as a strong effect.

2.7. B Cell Proliferation. Freshly isolated human $CD19^+$ B cells were plated at 50 000 cells per well in a 96-well plate in 220 μL complete DMEM medium and activated by different stimulatory conditions. Ten μCi ^{3}H thymidine (Perkin Elmer, Zaventem, Belgium) was added in the wells for the last 18 hours of 3 days of incubation. The cells were harvested on glass filter paper in 96-well format (Perkin Elmer, Zaventem, Belgium). After drying, radioactivity was counted in a scintillation counter (TopCount, Perkin Elmer, Zaventem, Belgium). Less than 5-fold increase in proliferation was considered as a weak effect of the stimulus, 5 to 20-fold increase as a moderate effect, and more than 20-fold increase as a strong effect.

2.8. Flow Cytometry. Freshly isolated human $CD19^+$ B cells and cells of human B cell lines were plated at 50 000 cells per well in a 96-well plate in 220 μL complete DMEM medium and analysed for their cell surface markers by a 3-color Becton Dickinson FACSCalibur apparatus after 24 hours of stimulation as described above. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin-cyanine 5- (Pe-Cy5-) labelled antibodies to CD40, CD69, CD70, CD80, CD83, CD86, MHC class I, and MHC class II were purchased from BioLegend (ImTec Diagnostics NV, Antwerp, Belgium). Cells were washed twice with cold phosphate buffered saline (PBS) and then incubated 30 minutes at 4°C with fluorochrome-conjugated antibodies diluted in cold PBS. After incubation and two washing steps with cold PBS, cells were suspended in PBS with 2% paraformaldehyde and analysed. Less than 1.5-fold increase in mean fluorescence intensity (MFI) was considered as a weak effect of the stimulus, 1.5- to 2-fold increase was considered as a moderate effect and more than 2-fold increase as a strong effect.

2.9. Human Mixed Lymphocyte Reaction. Freshly isolated human PBMCs were the responder cells and growth-inhibited RPMI 1788 cells served as stimulator cells in the human mixed lymphocyte reaction (MLR) assay. To block their proliferation RPMI 1788 cells were treated with 96 nM mitomycin C (mitomycin C Kyowa®, Takeda Belgium, Brussels, Belgium) for 20 minutes at 37°C. After three washes with RPMI 1640 containing antibiotics, the stimulator cells were then diluted at the desired cell concentration in complete RPMI 1640 medium. The responder human PBMCs were cocultivated with the stimulator cells, ratio responder/stimulator of 8/3, in complete RPMI 1640 culture medium for 6 days at 37°C and 5% CO_2. DNA synthesis of the responder cells was assayed by the addition of 10 μCi ^{3}H thymidine (Perkin Elmer, Zaventem, Belgium) during the last 18 hours of culture. The cells were harvested on glass filter paper in 96-well format (Perkin Elmer, Zaventem, Belgium). After drying, radioactivity was counted in a scintillation counter (TopCount, Perkin Elmer, Zaventem, Belgium).

2.10. WST-1 Viability Assay. Analysis of cytotoxic and cytostatic compounds on Namalwa cell line was done with the WST-1 viability assay. The cell proliferation reagent WST-1 (Roche Diagnostics, Mannheim, Germany), a soluble tetrazolium salt, was used for the spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well plate format. Quantification of the formed formazan was done by a scanning multiwall spectrophotometer. Compounds were added at different doses to the cell lines. After 48 hours of incubation at 37°C and 5% CO_2, Triton® X-100 (0.5% final; Fluka Biochemika) was added in the control wells and 10 μL of WST-1 reagent was added in each well. Cells were incubated for 2 to 4 hours at 37°C and 5% CO_2. The absorbance of the formazan dye was measured by the EnVision 2103 Multilabel Reader (Perkin Elmer, Zaventem, Belgium) at 540 nM.

2.11. Statistical Analysis. Comparison of activation marker upregulation on B cells versus Namalwa was performed by *t*-test analysis of induction ratios. p values less than 0.05 are considered as significant.

3. Results

3.1. In Vitro Immune Stimulation. B cells act in a specific manner according to the nature and the strength of the stimulatory signal they receive. Natural stimulatory conditions in vivo can be simulated in vitro. Several in vitro stimulatory conditions were tested on purified human primary B cells in order to find the stimulus that induces the clearest and broadest immunostimulatory effects.

3.1.1. Phenotypic Outcome of Various In Vitro Stimulatory Conditions on Primary Human B Cells. Table 1 gives an overview of different stimuli tested on primary human B cells and their effect on various phenotypic responses at different time points after initiation of the stimulation. Stimulation of B cells with the hapten-modified T-independent antigen TNP-Ficoll had neither effect on proliferation and production of Igs or cytokines nor on the expression of cell surface markers.

TNP-BSA, considered as a T cell/CD40L dependent stimulus, resulted in moderate induction of B cell proliferation and of Ig and IL6 production. The production of IL8 was more strongly induced. Both CD69 and MHC class I expression showed a moderate augmentation following TNP-BSA stimulation.

Anti-IgM antibodies elicit aggregation of the BCR as it happens in vivo after antigen ligation. On their own, anti-IgM antibodies proved to be weak inducers of in vitro B cell activation. However, in combination with IL4 there was a high proliferation rate and IL6 production; production of IgG augmented and activation markers CD69 and CD83 were strongly upregulated whereas the expression of CD40 was only moderately increased. Besides this, there was no or only a limited effect of anti-IgM with IL4 on the other cell surface markers. The production of IgM could not be measured when anti-IgM antibodies were used for the stimulation of human B cells.

Agonistic anti-CD40 antibodies mimic the ligation CD40-CD40L which stimulates the clonal expansion and differentiation of B cells. The agonistic anti-CD40 antibodies

Table 1: Immune effects at various time points after initiation of stimulation.

Stimulus	Read-out												
	CD40	CD69	CD70	CD80	CD83	CD86	MHC I	MHC II	IL6	IL8	Proliferation	IgG	IgM
	24 hours								2 days		3 days	7 days	
TNP-Ficoll	+	+	+	+	+	+	+	+	+	+	+	+	+
TNP-BSA	+	++	+	+	+	+	++	+	++	+++	++	++	++
Anti-IgM	+	++	+	+	++	+	+	+	+	+	+	+	NA
Anti-IgM + IL4	++	+++	+	+	+++	+	+	+	+++	+	+++	++	NA
Anti-CD40	NA	++	+	+	++	++	+	+	+	+	+	++	+
Anti-CD40 + IL4	NA	+++	+	++	+++	+++	+	+	+++	+	+++	++	+++
Anti-CD40 + IL21	NA	+++	+	++	+	+++	++	+	+	+	+++	+++	+++
IL21	+	+++	+	+	+	+	+	+	+	+	+	++	++
LPS	+	+	+	+	++	+	+	+	++	++	+	+	+
Resiquimod	+++	+++	++	++	++	+++	+	+	+++	+ + +	++	++	+++
ODN2006	+++	+++	++	+++	+++	+++	++	+	+++	++	+++	+++	+++
Pansorbin + IL2 + IL10	+	+++	+++	++	+++	+++	+	+	+++	+++	+++	+++	+++
Pokeweed	+	+	+	+	+	+	+	+	++	+++	++	+	+++

In four independent experiments, different in vitro stimulatory conditions were tested on freshly isolated human B cells; the strength of their effect on proliferation, Ig and cytokine production, and expression of cell surface markers is represented in the table (+++ = strong effect, ++ = moderate effect, + = weak effect, and NA = not analysed). For the definition of weak, moderate, or strong effect, see Section 2.

we used in the assays were by themselves only a weak stimulus for primary B cells with poor effects on proliferation, Ig, and cytokine production. However, when combined with IL4 or IL21, moderate to strong effects on proliferation, Ig production, and expression of several cell surface markers were obtained. The expression of CD40 could not be analysed due to the agonistic anti-CD40 antibodies used for the stimulation. The secretion of IL6, but not of IL8, was strongly increased with the combination of agonistic anti-CD40 antibodies with IL4. IL21, which is a very potent inducer of terminal B cell differentiation in humans [20], increased by itself strongly the expression of CD69, and was a moderate inducer of IgG and IgM production. There was no effect recorded on cytokine production nor on the expression of the other cell surface markers.

Next, three TLR agonistic ligands were tested for their potential as B cell stimulator. LPS is the major component of the outer membrane of Gram-negative bacteria and is a ligand of TLR4. LPS did not show any effect, besides a small increase of IL6 and IL8. This unresponsiveness was not unexpected, because human B cells, unlike murine B cells, lack significant TLR4 expression [21]. The small effect on IL6 and IL8 is probably due to an effect on the very small percentage of contaminating non-B cells.

Resiquimod belongs to the class of imidazoquinolinamines and is a synthetic agonist of TLR7/TLR8. It turned out to be a potent B cell activator with strong effect on the expression of various activation and costimulatory cell surface markers and on the production of IgM, IL6, and IL8. Only a moderate effect was observed on cellular proliferation and on IgG production.

ODN2006 belongs to the class B CpG ODNs which are synthetic oligonucleotides which contain unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs) and are recognized by human TLR9. ODN2006 showed more potency than resiquimod in the cellular proliferation and in the IgG production. However, the production of IL8 was less apparent than with resiquimod. Most cell surface markers were strongly upregulated upon stimulation with ODN2006.

Pansorbin cells are heat-killed, formalin-fixed *Staphylococcus aureus* cells which have a coat of protein A and can activate B cells through cross-linking of surface Igs [22]. In combination with IL2 and IL10, pansorbin provoked a strong boost in B cell proliferation and in Ig and cytokine production. There were no effect on the markers CD40 and MHC class I and class II and a moderate effect on CD80, but expression of CD69, CD70, CD83, and CD86 was strongly elevated.

Pokeweed mitogen is a carbohydrate-binding lectin, isolated from the pokeweed plant *Phytolacca americana*, with stimulatory effects on B cells presumably attributed to the cross-linking of glycoproteins on the B cell surface [23]. Pokeweed showed stimulatory effect on B cell proliferation and on the production of IgM, IL6, and IL8. But IgG production was not induced and expression of the cell surface markers hardly changed.

From all the stimulation conditions tested, ODN2006 was selected as the stimulus of choice, because it is able to induce B cell proliferation, IgM and IgG secretion, cytokine release, and activation markers upregulation.

3.1.2. Comparative Analysis of Various Human B Cell Lines in Their Cell Surface Marker Expression after ODN2006 Stimulation. Primary B cells are not convenient for repetitive assays because of the disparity between the different human blood donors and because of the heterogeneity of B cell subpopulations after isolation and purification of the B cells from peripheral blood. Using a homogeneous, immortalized B cell line would circumvent many of these drawbacks. As ODN2006 appeared to have the most broad stimulatory

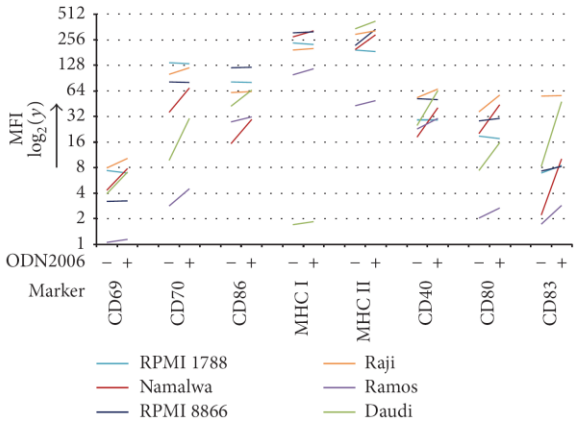

Figure 1: Flow cytometry analysis of cell surface markers on human B cell lines after stimulation with 0.1 μM ODN2006 for 24 hours. This graph is a representative of three independent experiments. Each colored line in the graph represents a human B cell line and displays the change in expression (MFI, y-axis) of the different cell surface markers (marker, x-axis) between naive ("−," x-axis) and ODN2006-stimulated ("+," x-axis) cells.

effect on polyclonal B cells, six different human B cell lines, Daudi, Namalwa, Raji, Ramos, RPMI 1788, and RPMI 8866, were investigated for their reactivity upon in vitro ODN2006 stimulation. Since, depending on their development stage, cancerous B cell lines frequently display aberrancies in Ig and cytokine production, the focus was placed on the expression of the cell surface markers.

The six human B cell lines were evaluated by flow cytometry for their expression of CD40, CD69, CD70, CD80, CD83, CD86, and MHC class I and class II before and after 24 hours of stimulation with the TLR9 agonist ODN2006. Results are presented in Figure 1. Stimulation of both RPMI 1788 and RPMI 8866 with ODN2006 had hardly any effect on all investigated markers. Between the four Burkitt lymphoma cell lines Daudi, Namalwa, Raji, and Ramos, Daudi and Namalwa showed a very dynamic response for most of the markers after ODN2006 stimulation. Daudi, however, did not express the marker MHC class I; therefore Namalwa was chosen as our cell line model of B cells.

Next, it was investigated if, for the same ODN2006 stimulation, Namalwa behaved as polyclonal human B cells (Figure 2) and could be used as a reliable model. For some of the expression markers (CD40, CD83) expression was homogeneous in both polyclonal and Namalwa B cells and clearly further increased after ODN2006 stimulation. Other markers (CD69, CD70, CD80, and CD86) were much less homogeneous at baseline in polyclonal than in Namalwa B cells but did in both cases clearly increase after ODN2006 stimulation. For CD69 and CD86, the induction was more pronounced in B cells than in Namalwa cells (CD69: 6.4-fold for B cell versus 2.0 for Namalwa, p value 0.044, CD86: 5.2-fold versus 2.0, resp., p value 0.032). On the opposite, CD70 was more induced on Namalwa cells than on B cells (2.1-fold versus 1.8, p value 0.035). As for MHC antigen expression, this was generally very high in Namalwa and B cells at basal level precluding further strong increase after ODN2006 stimulation. All together Namalwa and polyclonal B cells show a similar pattern in the induction of activation markers after TLR9 stimulation.

3.2. Characterization of Immunosuppressive Drugs or New Inhibitors Using ODN2006-Stimulated Namalwa Cells or Primary B Cells. To verify if we could detect B cell immunosuppressants through the model of ODN2006-stimulated Namalwa and to determine if there is a consensus between the polyclonal primary B cell Ig production and the expression of activation and costimulatory markers on Namalwa, some established pharmacological agents were investigated simultaneously in functional assays (MLR, B cell IgG secretion assay) and in flow cytometric assay of markers on ODN2006-stimulated Namalwa, a cytotoxicity counterscreen being performed on Namalwa (Table 2).

Both bortezomib, a selective small molecule inhibitor of the mammalian 26S proteasome, and dasatinib, an inhibitor of multiple tyrosine kinases, were cytotoxic on Namalwa cells as shown by the very low IC_{50}-values in the WST-1 viability assay. This precluded further examination in functional B cell assays.

Ibrutinib, a selective Bruton's-tyrosine kinase (BTK) inhibitor, used in clinic as an anticancer drug targeting B cell malignancies, impeded very potently IgG production by ODN2006-stimulated B cells but showed much less effect in MLR. All cell surface markers, except for CD69 and CD80, were significantly downregulated by ibrutinib at nontoxic concentration.

Chloroquine is a weak base which accumulates in acidic compartments, like the endosomes, and inhibits TLR9 signalling by preventing its cleavage and activation. It reduced strongly the production of IgG by peripheral B lymphocytes and decreased to a similar extent the expression of activation and costimulatory markers on Namalwa cells.

LY-294,002, a phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor, inhibited the production of IgG of polyclonal B cells and the expression of CD86 on Namalwa confirming the involvement of PI3K in B cell activation processes.

MK-2206.2HCl and AZD-5363, both AKT1/2/3 inhibitors, inhibited IgG production by peripheral B cells and suppressed overexpression of CD40, CD70, CD80, CD83, and CD86 upon ODN2006 activation on Namalwa cells. In contrast, CD69 experienced a boost in expression induced by both agents. MK-2206 was more potent on IgG, CD80, and CD83 than AZD-5363 suggesting a difference in pharmacodynamics or in specificity-properties of the compound.

TPCA-1 is a human IκB kinases (IKK) antagonist that impedes the nuclear localization of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). It significantly decreased the expression of the activation and costimulatory markers on Namalwa cells and the IgG production by peripheral B cells. Nonetheless, TPCA-1 is not a B cell-selective modulator as it appeared to be a potent compound

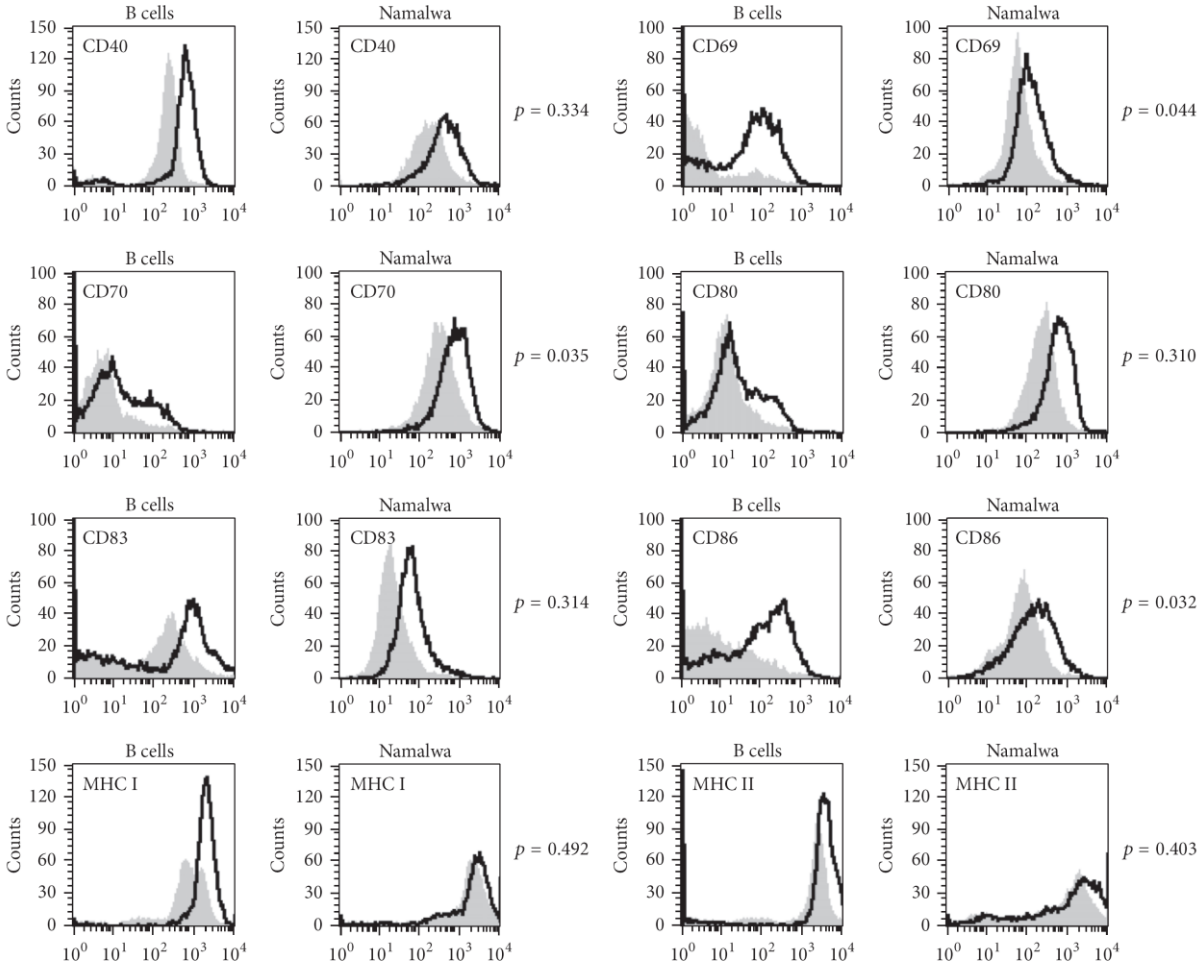

Figure 2: Comparative flow cytometric analysis of cell surface markers shows a close resemblance in expression profile between human peripheral B cells and Namalwa. The histogram plots from one out of four independent experiments show the expression of the cell surface markers before (grey region) and after 24 hours of stimulation (black line) with 0.1 μM ODN2006. Comparison of activation marker upregulation on B cells versus Namalwa was performed by *t*-test analysis of induction ratios for each marker from four independent experiments.

in MLR, conforming to NF-κB also being described in T cell activation pathways.

STAT3 inhibitor VII, antagonist of transcription factor signal transducer and activator of transcription 3 (STAT3), demonstrated hardly any effect in the performed assays, except that it moderately impeded the upregulation of costimulatory marker CD86.

Suberoylanilide hydroxamic acid (SAHA) is a potent, reversible pan-histone deacetylase inhibitor. It inhibits both class I and class II HDACs, altering gene transcription and inducing cell cycle arrest and/or apoptosis in a wide variety of transformed cells. Although SAHA proved to be slightly cytotoxic for Namalwa and impeded IgG production, it did not show potency on the expression of cell surface markers. Similarly, Mirin, a DNA repair targeting agent showed an effect on IgG secretion by ODN2006 stimulated B cells but had no effect on activation markers on Namalwa cells after TLR9 stimulation.

Table 2 shows that IgG secretion in ODN2006 activated B cells and activation markers upregulation on ODN2006 stimulated Namalwa share several signalling pathways (NF-kB, tyrosine kinases, and serine/threonine kinases) while others are not shared (HDAC, DNA repair).

4. Discussion

As there is a strong unmet need to target B lymphocytes by specific immunosuppressive drugs, there is a similar need for a broad in vitro immunoassay that could simplify the screening of such potential agents. Although they have limitations, in vitro assays form an abstract approximation of the actual in vivo conditions and are essential to obtain insight into

Table 2: Characterization of pharmacological inhibitors in phenotypic assays.

Chemical	Target	PBMC	Primary B cell	Namalwa						
		MLR	IgG production	Viability WST-1	CD40	CD69	CD70	CD80	CD83	CD86
Bortezomib	Proteasome: 30–100	35	90	10	—	—	—	—	—	—
Ibrutinib	BTK: 0.5	4 000	<1	1 800	690	↗	670	> 3 000	730	<30
Chloroquine	TLR 3, 7, 8, and 9: 560	> 10 000	95	> 10 000	70	90	1 000	60	210	<30
Dasatinib	SRC: 0.55 BCR/ABL: 3 LYN: 8.5 Other TK	15	55	20	—	—	—	—	—	—
LY-294,002 hydrochloride	PI3K: 3 000	5 100	670	4 600	2 800	> 3 000	2 500	2 200	> 3 000	450
MK-2206 2HCl	AKT1/2/3: 8/12/65	3 500	25	2 700	1 500	↗	1 200	1 100	120	<30
AZD-5363	AKT1/2/3: 3/7/7	2 600	600	3 000	1 500	↗	850	> 3 000	2 200	<30
TPCA-1	IKK-2: 17.9 IKK-1: 400	360	450	2 800	210	550	480	700	550	<30
STAT3 inhibitor VII	STAT3: 170	8 500	> 10 000	> 10 000	> 3 000	> 3 000	> 3 000	2 800	> 3 000	1 000
SAHA	Class I & II histone deacetylase (HDAC): <86 HDAC1: 13.7	5 000	440	2 100	> 3 000	↗	> 3 000	1 300	> 3 000	> 3 000
Mirin	Mre11-Rad50-Nbs1 (MRN) complex (DNA-repair) Cell-free: 12 000 Human: 66 000	> 10 000	1 400	> 3 000	> 3 000	> 3 000	> 3 000	> 3 000	> 3 000	> 3 000

The table shows the IC_{50} (nM) of the inhibitors on the molecular target and in the different phenotypic assays: MLR with human PBMCs, IgG production by human B cell stimulated by ODN2006, and surface markers expression on ODN2006-stimulated Namalwa. Cytotoxic counterscreen (WST-1) was performed on Namalwa cells. ↗: indicates an increase in expression.

complex biological phenomena leading to new discoveries and predictions. That assay has to involve a stimulus which leads to broad phenotypic changes in a stable B cell model. For this purpose, we first compared 13 different stimuli for their capacity to activate human primary B cells by looking at several activation outcomes: cellular proliferation, IgM and IgG secretion, cytokine release, and upregulation of different activation markers. To the best of our knowledge this is the first report comparing these stimuli on several activation outcomes. Amongst the various stimuli investigated, in vitro stimulation with TLR9 agonist ODN2006 resulted in the "broadest" phenotypic change profile in human polyclonal B cells. The pattern recognition receptor TLR9 recognizes the CpG motifs in oligodeoxynucleotides as pathogen-associated molecular patterns due to their abundance in microbial genomes and their rarity in vertebrate genomes, except in the mitochondrial DNA [24]. TLR9 signalling mediates the activation of both innate and adaptive humoral and cellular immunity against viral and bacterial infections by promoting cellular proliferation and differentiation into antibody-secreting cells, upregulating molecules involved in immune cellular interactions, and increasing secretion of proinflammatory (IL6, TNFα, and type I interferons) and immune regulatory (IL10) cytokines [25, 26]. The molecular pathways triggered by TLR9 activation involved, as confirmed with the pharmacological agents we assessed, NF-κB, PI3K, tyrosine, and serine/threonine kinases [27].

The heterogeneity between the different human blood donors, the heterogeneity after isolation and purification from peripheral blood, the limited yield of the purification, and the short longevity make primary B cells less optimal for repeatable assays. So a monoclonal population of dividing cells that could substitute the primary B cell would stabilise and facilitate the assay by overcoming the aforementioned limitations. The Namalwa cell line appeared as the best model to mimic the primary B cells when the expression of cell surface markers is used as read-out.

In vitro and in vivo studies have indicated that cell surface markers CD40, CD70, CD80, and CD86 are more than just costimulatory molecules for activation of $CD4^+$ T cells. Indeed, they are also key molecules in the signalling for the regulation of Ig production, particularly of IgG. CD80/CD86 activation plays a key role in regulating the IgG1-production by previously activated B cells [28, 29]. Naive B cells from patients with common variable immunodeficiency are markedly impaired in upregulating the costimulatory

molecules CD86 and CD70 upon BCR cross-linking and the expression remained reduced even in the presence of autologous helper $CD4^+$ T cells. The insufficient upregulation of these two crucial costimulatory molecules could explain the poor class switching and, hence, reduced Ig serum levels, except for IgM [30, 31]. Similarly, inadequate CD40-CD40L interactions, as depicted in the X-linked immunodeficiency with hyper-IgM syndrome, cause defects in Ig class switching, a central process to antigen-dependent B cell maturation and to the generation of memory B cells and plasma cells [32].

Several pharmacological agents were assessed to challenge our assay in the prediction of B cell targeting agents. Cytotoxic compounds were identified by the Namalwa WST-1 viability assay (bortezomib and dasatinib) and excluded from further investigation. Compounds able to suppress overexpression of cell surface markers on Namalwa cells after the ODN2006 challenge in a range of concentration lower than the IC_{50} detected in the viability assay can be considered as hits. The costimulatory marker CD86 appears to be the most sensitive marker.

We confirmed the involvement of BTK, PI3K/AKT, and NF-κB pathways in our assay. Inhibitors of these pathways were also able to block the production of IgG by human primary B cells after ODN2006 stimulation, confirming the suitability of the assay to detect inhibitors of IgG production. Chloroquine was also found to be a potent inhibitor both in the Namalwa activation markers assay and in the B cell IgG assay. This is not surprising as chloroquine is a very proximal inhibitor interfering with the first step of the TLR9 activation [33].

Some compounds active in the IgG production assay can be missed in our screening (false negatives), as illustrated by Mirin or SAHA. This can be explained by the fact that both compounds target the DNA recombination step during the Ig class switch, a process that does not occur in the Namalwa cell line that secretes constitutively IgM [34]. It must be realized that, as for many screening assays, results can be "falsely positive" and, hence, that a new compound may not work in primary human B cells, because the pathway it blocks in Namalwa may be not pertinent or may be bypassed in primary human B cells. To exclude "false positivity," hits selected after the screening must be confirmed in other assays like the B cell IgG assay potentially with different stimuli to validate them as broad B cell inhibitors.

In conclusion, the limitations inherent to the use of human primary B cells in repetitive and large-scale experiments have been circumvented through the Namalwa B cell line. The described in vitro immunoassay with ODN2006-stimulated Namalwa cells and with flow cytometric readout of the activation and costimulatory cell surface markers can serve as a potent and robust first-line screening to identify potential new B cell active compounds or to refine mechanisms of action of known immunomodulators.

Abbreviations

BCR: B cell receptor
BTK: Bruton's tyrosine kinase
CD: Cluster of differentiation
DMEM: Dulbecco's Modified Eagle's Medium
FCS: Foetal calf serum
Ig: Immunoglobulin
IKK: IκB kinase
IL: Interleukin
LPS: Lipopolysaccharide
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
MLR: Mixed lymphocyte reaction
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
ODN: Oligodeoxynucleotide
PBMC: Peripheral blood mononuclear cell
PBS: Phosphate buffered saline
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
STAT3: Signal transducer and activator of transcription 3
TLR: Toll-like receptor
TNP: 2,4,6-Trinitrophenyl hapten.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Chapter 4 : Detection of potential new B lymphocyte targets using RNAi screening on ODN2006-stimulated Namalwa

4.1. Introduction

The developed *in vitro* immunoassay with ODN2006-stimulated Namalwa cells, as previously described, may be suitable to serve as a robust first line screening to identify potential B cell active compounds or to elucidate mechanisms of action of known immunosuppressive drugs.

However, there is not an established pharmacological agent available for every target-molecule encoded in the human genome. Furthermore, small molecule agents are commonly not specific for one target, but can display off-target effects at a similar or lower potency.

Specific inhibition of a single target can be obtained via genetic silencing. RNA interference (RNAi) is a cellular mechanism that decreases the function of a gene through the degradation of its associated messenger RNA (mRNA). The introduction of short hairpin RNA (shRNA), an artificial RNA molecule with a tight hairpin turn, into mammalian cells through infection with replication-incompetent lentiviral vectors allows for stable integration of shRNA and long-term inhibition of the targeted gene[173].

Kinases are interesting targets for the development of therapeutic agents, because they catalyze phosphorylation, a process wherein phosphate groups are transferred from high-energy phosphate-donating molecules, e.g. ATP, to specific substrates such as a protein, a lipid, a carbohydrate or an amino acid. The phosphorylation state of a molecule can affect its activity, reactivity, and its ability to bind other molecules. In eukaryotic cells, kinases are organized in signalling cascades which are typically initiated by various receptors which further pass their signals through various downstream effectors and regulate many essential cellular processes. Aberrant regulation of kinases plays a causal role in many diseases ranging from cancer to inflammatory diseases, diabetes, and infectious diseases[174–177].

The MISSION LentiExpress Human Kinases library is a broad library of lentiviral vectors carrying specific shRNA sequences targeting 501 human protein kinases and was selected to perform the genetic knock-out studies in order to identify new potential targets in B cell activation pathways and to gain more insight in the regulation of B cell activation.

4.2. Materials and methods

4.2.1. Pharmacological reagents

Following agents were tested for their effects in functional *in vitro* assays: HS38, IC261, NVP-BHG712 from Sigma-Aldrich (Diegem, Belgium); OSU-T315 from CalBiochem, Merck Millipore (Overijse, Belgium); Ki8751 and ML281 from Tocris Bioscience (Bristol, United Kingdom).

4.2.2. Lentiviral shRNA human kinase library

MISSION® LentiExpress™ Human Kinases library from Sigma-Aldrich (Diegem, Belgium) contained 3.200 different lentiviruses carrying shRNA sequences targeting 501 human protein kinase genes. Each gene was represented by a clone set that consisted of 3 to 10 individual constructs targeting different regions of the gene sequence. The lentiviral particles were provided in a pre-arrayed set ready-to-use format in 96-well plates with 5 000 transfection units per well. In addition to the kinase shRNA particles, each plate included positive and negative controls to monitor the transduction efficiency. The puromycin-resistance gene within the vector constructs allowed selection of the successfully transduced Namalwa cells by the antibiotic puromycin.

Transduction of Namalwa cells, suspended in antibiotics-free DMEM-medium with 10 % FCS, with the lentiviral constructs was performed at a multiplicity of infection of 10 (5 000 transfection units per 500 cells) in the presence of 8 µg/mL protamine sulphate (Leo pharma, Lier, Belgium) to increase transduction rates. After 3 days of incubation, puromycin (0,25 µg/mL, InvivoGen, Toulouse, France) was added and a further incubation of 10 days gave an appropriate cell number of transduced Namalwa cells. Thereafter the Namalwa cells underwent stimulation with TLR9 agonist ODN2006 (Invivogen, Toulouse, France) for 24 hours and were then analysed by flow cytometry (Figure 4.1).

Figure 4.1. Screening of the lentiviral shRNA human kinase library on human B cell line Namalwa.

4.2.1. Flow cytometry

FITC-labelled antibody to CD70 from Becton Dickinson Biosciences (Erembodegem, Belgium) and allophycocyanin (APC)-labelled antibody to CD80 from BioLegend (ImTec Diagnostics N.V., Antwerp, Belgium) were used for flow cytometric analysis of the lentiviral human kinase library on a Becton Dickinson FACSCanto™ II apparatus.

PE- or PE/Cy5-labelled antibodies to CD70 and CD80 from BioLegend (ImTec Diagnostics N.V., Antwerp, Belgium) were used for flow cytometric analysis of small molecule inhibitors by a 3-colour Becton Dickinson FACSCalibur™ apparatus.

4.2.2. Characterization of pharmacological reagents

Pharmacological reagents were tested at different concentrations within the range of 0.0001 to 10 µM in several functional *in vitro* assays: flow cytometry on Namalwa cells, human B cell IgG assay, MLR and cytotoxicity assays on cell lines Jurkat, Namalwa (both from European Collection of Cell Cultures, ECACC, England), RPMI 1788 and RAW264.7 (both from Global Bioresource Center ATCC, USA). The assays were performed as previously described (See 'Materials and methods' in Chapter 3).

4.3. Results

4.3.1. Selection of read-out parameters for library screening

For the screening of the large LentiExpress Human Kinases library, it was decided to restrict the read-out to two cell surface markers, because analysis of more markers would complicate the processing of all data and would impair the concept of a first line screening. The expression of various markers on naive and on ODN2006-stimulated Namalwa cells were discussed previously and represented in Figures 1 and 2 of article in Chapter 3.

CD69 was not suited as read-out parameter, because on Namalwa cells it had a low base-line expression with only a limited increase after stimulation with ODN2006. Although CD40 showed already a strong base-line expression on human peripheral B cells and on Namalwa cells, it gained a strong further increase after stimulation. However, CD40 was not selected as read-out parameter for the shRNA KO experiments, because in a second stage after the initial screening of the library we would like to rely on agonistic CD40 antibodies for B cell activation. The expression of CD70, CD80, CD83 and CD86 augmented well on peripheral B cells and Namalwa cells in response to ODN2006. Hereby, the up-regulation in expression of CD70 was more pronounced on Namalwa cells than on peripheral B cells. CD86, on the other hand, was more strongly induced on peripheral B cells. CD80 and CD83 increased more

uniformly in expression on Namalwa cells than on peripheral B cells. So far, there is only limited knowledge about the signalling pathways upon CD70 and CD80/CD86 triggering. Simultaneously with detecting new targets in B cell activation, the screening of the lentiviral vector shRNA library may provide interesting information about kinases involved in inducing or controlling the expression of CD70 and CD80/CD86 on B cells. Although CD70 and CD80/CD86 are not exclusively expressed by B cells, they are pragmatic indicators for the B cell's activation status. On Namalwa cells CD80 showed a more pronounced and less diffuse up-regulation in expression than CD86 upon ODN2006 stimulation, which is more convenient for the detection of inhibition in expression. Hence, CD80 was selected together with CD70 as phenotypic read-out parameters for the initial screening of the lentiviral vector shRNA library.

4.3.2. Detection of protein kinases with a possible role in B cell activation

Electroporation or lipid transfection using transfection reagents such as Lipofectamine® RNAiMAX (Invitrogen™, Thermo Fisher Scientific, Niederlebert, Germany) and HiPerFect Transfection Reagent (Qiagen, Antwerp, Belgium) were not effective to achieve gene silencing in human peripheral B cells, nor in Namalwa cells. The lentiviral particles of MISSION® LentiExpress™ shRNA libraries (Sigma-Aldrich, Diegem, Belgium) effectively transduced Namalwa cells. Using this method, a permanent silencing of the targeted gene can be achieved through the stable integration of the viral vector, encoding specific shRNA sequences, into the host genome. Transduced Namalwa cells were then stimulated by ODN2006 for 24 hours and flow cytometric analysis on markers CD70 and CD80 was performed. If the knock-out of a certain protein kinase led to an inhibition in up-regulation of CD70 and of CD80, it was hypothesized that this protein kinase may play an important role in B cell activation. The LentiExpress™ library provided 3 - 10 shRNA clones per kinase target and we relied on the reproducibility between the different shRNA clones targeting the same kinase to refine the list of potential candidates. Figure 4.2 shows a global representation of the library screening. Up to now, the human kinome contains 538 protein kinase genes[178] of which 501 are targeted in the lentiviral shRNA human kinase library.

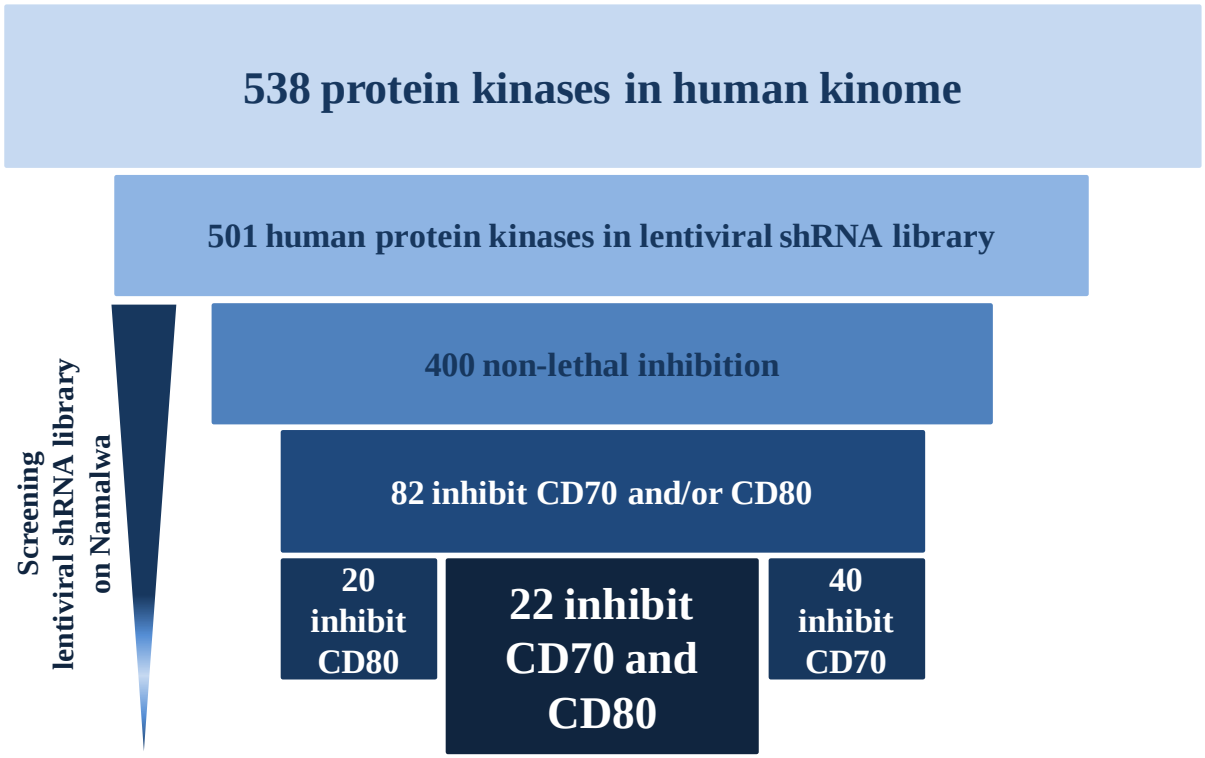


Figure 4.2. Screening of the lentiviral shRNA human protein kinase library

4.3.2.1. Lethal kinases

Five hundred and one kinases were evaluated in our screening and the inhibition of 101 protein kinases in the library proved to be lethal for Namalwa cells. Amongst them there were several kinases which have been demonstrated to be essential for B cell's survival (e.g. BTK and BLK) or for the cellular viability in general (e.g. cyclin-dependent kinases (CDKs), glycogen synthase kinase 3 beta (GSK3β), IκB kinases beta, gamma and epsilon (IKK-β/γ/ε), several MAP2Ks and MAP3Ks). Quite surprising at first notion, zeta-chain associated protein kinase 70 kDa (ZAP70) proved to play a role for Namalwa's viability. The cytoplasmic Tyr kinase ZAP70 from the SYK family was originally described to be present in T cells and natural killer cells and to be committed to signalling pathways following TCR stimulation[179,180]. Afterwards, ZAP70 has been reported in normal mouse B lineage cells and in human normal and malignant B lymphocytes wherein it shows a redundant function with the related SYK during B cell development and activation[181,182].

Hence, the screen was able to detect several kinases out of the library that are described to be essential for the B cell's viability. This could also imply that our group of lethal kinase inhibition target kinases that have not yet been reported before as vital for the B cell and, hence, might be interesting new targets for B cell depletion. However, as the attention in the

present research went to targets that predominantly influence B cell activation and not B cell viability, we did not further concentrate on these targets.

4.3.2.2. Non-lethal kinases without effect on CD70 and CD80 expression

The silencing of 400 kinases was not lethal for Namalwa cells, but the majority of them, 318 kinases in total, did not show a significant inhibition of the up-regulation of CD70 and CD80 in response to ODN2006 stimulation. They were regarded as "non-active, non-lethal" kinases. The group of 318 "non-active" kinases included the known AKT1/2/3, FYN, LYN and SYK, which are positioned in the early events of signalling pathways that coordinate cellular proliferation, survival and metabolism. Silencing of their expression can probably be bypassed in order to maintain viability and metabolism. AKT1/2/3, FYN, LYN and SYK are not (closely) involved in the TLR9-signalling pathway, clarifying why the expression of markers CD70 and CD80 on Namalwa cells remained unaltered.

4.3.2.3. Non-lethal kinases with effect on CD70 and/or CD80 expression

Deletion of 82 non-lethal kinases decreased the up-regulation of CD70 and/or CD80. Hence, they are regarded as "active" kinases. Amongst these, blockade of 40 kinases inhibited only CD70, whereas 20 other kinases seemed to be involved only in CD80 expression, suggesting a (partially) different regulation in expression for these two markers. Finally, genetic suppression of 22 kinases led to the inhibition of the up-regulation of both CD70 and CD80. The initial focus to detect small molecule kinase inhibitors with immunosuppressive effects on human B cells was put on the latter 22 "candidate target" kinases.

4.3.2.4. Candidate target kinases

The shRNA suppression screen identified 22 kinases which may play a relevant role in broad B cell activation since inhibition of these kinases abrogated the ODN2006-induced up-regulation of the surface molecules CD70 and CD80, both shown to be important in providing co-stimulatory signals for T and B cell activation. Remarkable was the diversity of the known biological processes in which the 22 candidate target kinases have been described to be involved, notably intracellular signal transduction, angiogenesis, cell shape, cellular adhesion and motility, mitosis and vesicle-mediated transport of the Golgi apparatus. This may indicate that many events and pathways intervene in the ODN2006 - CD70/CD80 activation axis.

4.3.3. Validation of the candidate target kinases with small molecule drugs

To validate the 22 "candidate target" kinases as interesting targets or new therapeutics, several *in vitro* experiments were performed using small molecules shown to inhibit them on

the protein level. For only 6 of the 22 "candidate target" kinases, small molecule inhibitors were reported in the literature (Table 4.1): casein kinase 1 δ (CSNK1D)[183], death-associated protein kinase 1 (DAPK1)[184], ephrin type-B receptor 4 (EPHB4)[185], integrin-linked kinase (ILK)[186] kinase insert domain receptor (KDR)[187] and Ser/Thr kinase 33 (STK33)[188].

The screening assay was repeated with the available small molecule inhibitors to check their effect on CD70 and CD80 expression. Two compounds successfully inhibited the up-regulation of CD70 and CD80, namely NVP-BHG712, targeting EPHB4, and OSU-T315, targeting ILK. Furthermore, the compounds were tested in the human B cell IgG production assay (wherein B cells were stimulated by ODN2006) and only OSU-T315 potently suppressed the IgG production *in vitro*. In human MLR (to analyse the effects on T cell proliferation), OSU-T315 showed a moderate activity while NVP-BHG712 displayed much more potency than it did in the B cell IgG assay. Both compounds showed moderate intrinsic cell toxicity in the WST-1 assays on human B cell lines Namalwa and RPMI1788, T cell line Jurkat, and murine macrophage cell line RAW264.7.

IC_{50} (µM)

			Namalwa (*)							
		(xx)			(**)	(***)	(x)	(x)	(x)	(x)
Target	**Chemical**	**Product IC_{50}**	**Inhibition CD70**	**Inhibition CD80**	**IgG B cell**	**MLR**	**WST-1 Jurkat**	**WST-1 Namalwa**	**WST-1 RPMI 1788**	**WST-1 RAW264.7**
CSNK1D & E	IC261	1	**> 10**	**> 10**	**6.16**	**0.62**	**0.55**	**1.24**	**0.59**	**4.14**
DAPK1	HS38	0.2	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**
EPHB4	NVP-BHG712	0.025	**0.85**	**2.50**	**8.80**	**0.86**	**5.52**	**6.39**	**5.13**	**7.32**
ILK	OSU-T315	0.6	**0.74**	**0.24**	**0.53**	**3.53**	**5.58**	**4.60**	**4.04**	**5.30**
KDR	Ki8751	0.0009	**> 10**	**> 10**	**> 10**	**0.89**	**> 10**	**> 10**	**0.90**	**> 10**
STK33	ML 281	0.014	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**	**> 10**

Table 4.1. Small molecule inhibitors of the identified target kinases were tested at different concentrations, maximum 10 µM and minimum 0.0001 µM, in several *in vitro* experiments. The values are calculated averages of three independently performed experiments. (*) Flow cytometric analysis of CD70 and CD80 on Namalwa after 24 hours incubation with ODN2006 and small molecule inhibitor; (**) Human B cell IgG production assay; (***) MLR with human PBMCs; (^{x}) WST-1 viability assay on human cell lines Jurkat, Namalwa and RPMI 1788 and murine cell line RAW264.7; (^{xx}) Product IC_{50} mentioned by the supplier. (■ = Strong effect, ■ = Moderate effect, ■ = Weak effect). For the definition of weak, moderate or strong effect, see section "materials and methods".

Hence, only ILK-inhibitor OSU-T315 effectively suppressed the up-regulation of markers CD70 and CD80 (IC_{50} 0.74 µM and 0.24 µM, respectively) and also impeded the IgG production by primary B cells (IC_{50} 0.53 µM) with IC_{50}-values comparable to the protein inhibition IC_{50}-value of 0.6 µM. The moderate toxicity and moderate activity in MLR indicate

that inhibition of ILK rather selectively impacts on B cell activity. Hence, the role of ILK on B cell activity and its potential as B cell target warranted further and more profound research.

4.4. Discussion

The objective of the present chapter was to identify new B cell targets via first-line screening of a large lentiviral vector-based shRNA library on human B cell line Namalwa and to validate these by small molecule inhibitors.

Kinases were chosen as class of target molecules to search for new B cell targets, because, firstly, they generate important signalling cascades for the regulation of the cell's metabolism and viability and, secondly, their catalytic activity in phosphorylation can be manipulated by pharmacological drugs.

A lentiviral vector-based shRNA library containing lentiviral particles designed to knock-out permanently and efficiently the genes of 501 human protein kinases was screened. The screening revealed firstly a rough distinction between lethal and non-lethal protein kinases. In the lethal group, there were kinases which are known to be essential in (B) cell viability, like BLK, BTK, CDKs, GSK3β, IKKs, ZAP70 and several MAP2Ks and MAP3Ks. But there were also several unfamiliar kinases in the lethal group which have not been reported before as vital for the B cell. This information may contribute to the development of new and possibly more specific B cell-depleting drugs after further investigations. An interesting result of the screening is that SYK is classified in the "non-active" kinases while ZAP70 is placed in the "lethal" kinases. The redundancy of ZAP70 with SYK, as mentioned earlier, is apparently not applicable in Namalwa. Perhaps Namalwa does not express SYK and, consequently, ZAP70 acquires a substantial role in viability of Namalwa. So far there is no information available about expression of SYK in Namalwa cells. Western blot or PCR analyses have not been performed to investigate that issue.

The focus of the research was to identify targets which influence B cell activation in order to find B cell immunomodulatory drugs. Hereby, the expression of cell surface markers CD70 and CD80 was used as read-out for the library screening. The group of non-lethal kinases were subdivided in "non-active" kinases, meaning there was no effect seen on the markers CD70 and CD80, and "active" kinases, meaning the up-regulation of CD70 and/or CD80 upon ODN2006 stimulation had been impeded when these kinases were deleted. It must be mentioned that the absence of any effect does not necessarily mean that the kinases do not have a function in the TLR9-mediated activation pathway of the B cell. Indeed, ODN2006 is a broad stimulator with diverse phenotypic effects on B cells and it may be that some kinases

are on a position in the signalling pathway that is not involved in CD70 and CD80 expression, but still play a role, for instance, in Ig or cytokine production. Throughout evolution, signalling cascades of kinases have gained a high degree of robustness, which is achieved through redundancy at various levels, like compensatory pathways and feedback loops[178]. Each of the shRNA clones in the library targets one specific kinase. But closely related variants of the targeted kinase, so-called isoforms, are not inhibited in expression and can compensate for the loss of the targeted kinase. Also non-isoform kinases can take over from the deleted kinase. Moreover, the importance of a kinase depends on cell or tissue type and on (micro-environmental) circumstances.

The group of "active", non-lethal kinases was divided further in three sub-groups: 1) only CD70 was affected, 2) only CD80 was affected, and 3) both CD70 and CD80 were affected. The fact that kinases were identified in all groups indicates that CD70 and CD80 have a (partially) different regulation for their expression. The sub-groups of kinases that affect the expression of only CD70 or of only CD80 can provide important information about the position and role of the kinases involved within the B cell's signalling pathway(s) and about the particular regulation of CD70 and CD80. For example, the "selective CD70 blockers" may be interesting because 1° the CD70 pathway is a promising one for immunology and immune deficiencies, 2° a more selective blockade may also be clinically safer and 3° the other "selective" blockers active on the CD80 pathway already have more competitors in clinical development (the whole collection of CD80/86 : CD28 interfering mAbs).

The suppressive effect of a genetic KO or of a small molecule inhibitor may be located at various levels of the activation pathways (Figure 4.3). Antagonism at the level of ligand - receptor ligation (Figure 4.3 a) can abort the complete signalling pathway from the very beginning leading to a general non-responsiveness upon stimulation. Chloroquine is a weak base that perturbs the acidic pH in endosomes and lysosomes and is commonly used as an antagonist for endosomal TLRs[189]. Inhibition of key molecules like IRAKs, p38 MAPK, JNK and IKK-complex in the proximal signalisation from TLR9 to transcription factors (e.g. NF-κB, IRFs, CREB, AP-1) can still have exhaustive consequences in the progress to B cell activation. The B cell can then be impeded in all or in several of its functions (Figure 4.3 b and b'). In contrast, interference of more distal molecules downstream the signalling pathway can result in a more specific functional/phenotypic aberrance, but can still permit some space for redundancy and alternative pathways to preserve the functional activity/phenotype (Figure 4.3 c, c', c'' and c'''). Specific functional defects or restrictions appear through suppression

of molecules at the very distal or terminal position of signalling pathways (Figure 4.3 d, d', d'' and d''').

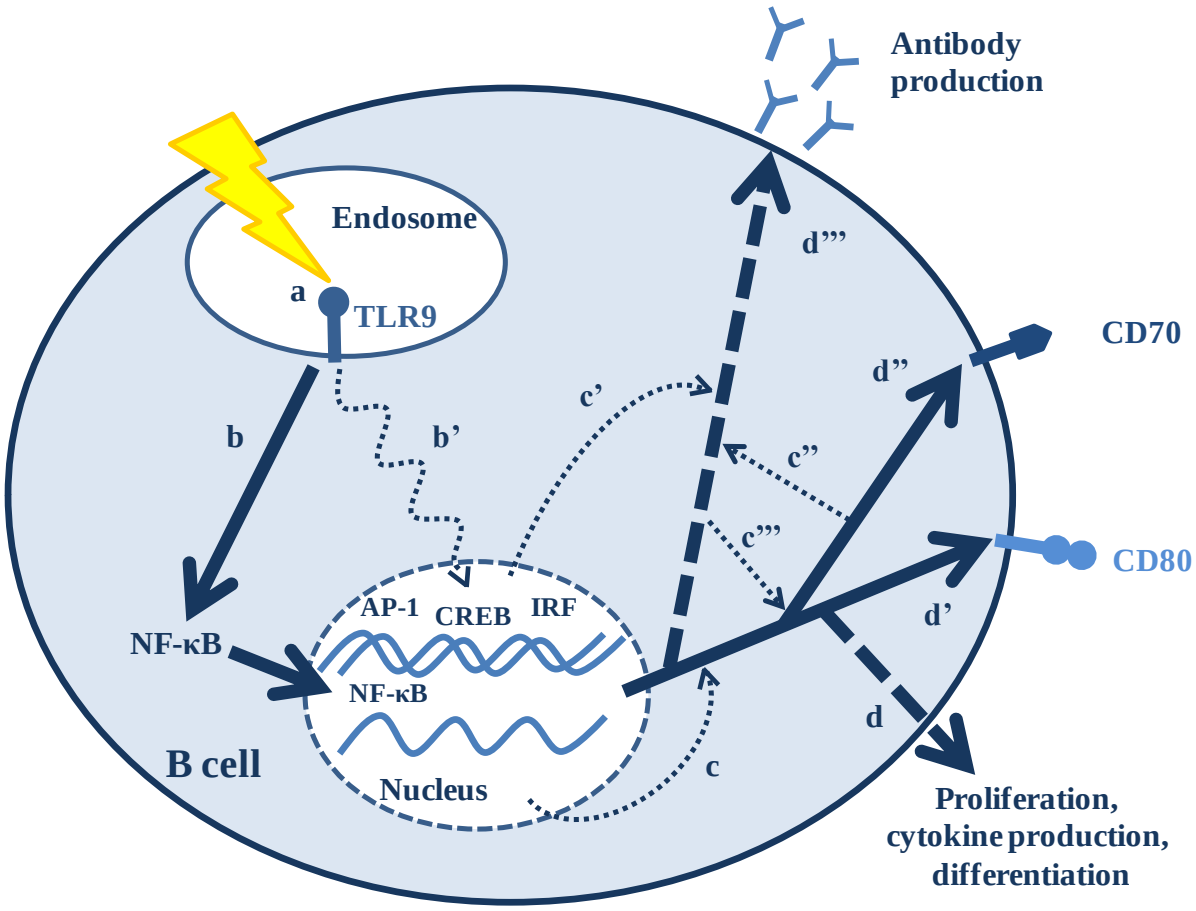


Figure 4.3. ODN2006 - CD70/CD80 activation axis

After the initial screening, we focused on the sub-group of "candidate target" kinases that altered both CD70 and CD80, because we assumed that these suppressing kinases may have the highest likelihood of profound *in vivo* effects. For 6 of the 22 "candidate target" kinases, small molecule inhibitors were available for further validation. From the small molecules available, only two pharmacological agents, NVP-BHG712 (active against EPHB4) and OSU-T315 (active against ILK), were able to reproduce the *in vitro* suppression of CD70 and CD80 expression, as seen with the shRNA-transduced Namalwa cells. As for OSU-T315, it also effectively inhibited IgG production by human primary B cells. Its effect on MLR was probably a reflection of the general anti-proliferative effect at higher concentrations as detected in the WST-1 assays. Altogether, the preliminary data of the shRNA suppression screen and of the *in vitro* experiments with the small molecule drug OSU-T315 confirm that ILK might indeed play an important role in the B cell activation cascade. The fact that the OSU-T315 compound has a relatively high IC_{50} in an *in vitro* radiometric kinase assay (0.6

μM)[190] makes it, in addition, an interesting chemical hit susceptible to further chemical improvements.

It is quite striking that 4 of the 6 tested small molecule inhibitors did not confirm the suppression of markers CD70 and CD80. Several elements may explain this. First, it must be notified that for the library screening the Namalwa cells were incubated during 14 days with the shRNA lentiviral particles for obtaining successful transduction giving a complete abrogation of the kinase in question, while the small molecule inhibitors were incubated together with ODN2006-stimulated Namalwa cells for just 24 hours. This much shorter incubation time of the small molecules and a lower inhibitory efficacy of these molecules compared to the shRNA-mediated deletion could have impeded suppressive activity on CD70 and CD80. Repetition of the screening with a pre-incubation of Namalwa cells with the inhibitors, especially the non-toxic inhibitors, over a longer timeframe prior to ODN2006-stimulation might still reveal molecules with activity in the ODN2006-CD70/CD80 signalling axis. Next, the kinase selectivity and inhibitory activity of the small molecules are usually determined by cell-free *in vitro* kinase assays. Consequently, these molecules can be less active due to their pharmacokinetic or -dynamic properties or their inhibitory activity may be bypassed or compensated within the cellular context. In addition, the observed cytotoxicity of a few molecules can be caused by off-target effects.

IC261, targeting both CSNK1D and CSNK1E, proved to be a cytotoxic molecule, as determined by the low IC_{50}-values in WST-1 on human cell lines (less explicit on murine RAW264.7) and in MLR. CSNK1D has an important function in cell proliferation and is described to be overexpressed in choriocarcinoma and pancreatic carcinoma[191,192]. In the lentiviral shRNA library screening, the inhibition of CSNK1D did not result in death of Namalwa cells. Only the genetic KO of the highly related isoform CSNK1E was lethal for Namalwa. Like CSNK1D, CSNK1E has a role in regulating cell division and overexpression of CSNK1E is associated with various cancers (breast, ovary, lung, colon, bladder, kidney, prostate, salivary glands, etc.)[191]. The difference in outcome between the shRNA library and the *in vitro* assays might probably be due to IC261's inhibitory effects on CSNK1E, rather than on CSNKID. While potent in WST-1 and MLR, IC261 displayed a moderate effect in the B cell IgG assay. IgG-producing B cells are much less active in proliferation and possibly for that reason IC261 was less effective in B cell IgG assay.

HS38 and ML 281, inhibitors of DAPK1 and STK33 respectively, were inactive in all the performed assays. The KDR-inhibitor Ki8751 was potent in MLR and was cytotoxic for RPMI 1788, but showed furthermore no activity.

In the previous chapter, several clinical immunosuppressants and pharmacological reagents with known molecular mechanism of action have been tested *in vitro* on ODN2006-activated Namalwa and were analysed through one-on-one analysis of cell surface markers CD40, CD69, CD70, CD80, CD83 and CD86 by flow cytometry (See Table 2 of article in chapter 3). In addition to what is already mentioned in the discussion of that chapter, it was afterwards noticed that several immunosuppressants showed a stronger suppressive activity on the expression of CD86 than of CD80. Moreover, moderately toxic immunosuppressants that displayed a strong inhibitory effect on the human B cell IgG production, showed on Namalwa cells a similarly potent inhibitory activity on the expression of CD86. This was observed with ibrutinib (BTK), LY-294 (PI3K), MK-2206 (AKT1/2/3) and AZD-5363 (AKT1/2/3). These data might indicate that there is a correlation between the IgG production and the expression of CD86, even though we tested a selected group of immunosuppressants. Although they are highly similar, the co-stimulatory molecules CD80 and CD86 may, nonetheless, differ from each other in their contribution to T cell responses and in their regulation of expression, as suggested in literature[59,60,193]. The characterization of clinical immunosuppressants and pharmacological reagents was actually performed after the complete screening of the lentiviral shRNA human kinase library which had CD70 and CD80 as read-out parameters. If it was known before the start of the shRNA library screening about this possible interrelationship between IgG production and CD86 expression, we could have replaced CD80 by CD86 as read-out parameter.

Due to the very large size of the shRNA library, verification of genetic knock-out by western blot or by polymerase chain reaction (PCR) could not be performed. However, we relied on the reproducibility between the different shRNA clones targeting the same kinase to conclude whether a kinase did have a role in B cell immunology or not.

In conclusion, the screening of the lentiviral shRNA human protein kinase library on ODN2006-stimulated Namalwa cells has proved its value through its ability to pick several kinases known to be essential for B cell's viability, but has also revealed several interesting outcomes that require further investigations. Our obtained data can form the initiation of a whole array of more profound studies leading to a better understanding about the roles and position of the different protein kinases in the B cell's functioning.

Chapter 5 **Integrin-linked kinase as a candidate target in B cell activation**

5.1. Introduction

The screening of the lentiviral human protein kinase shRNA library on TLR9-stimulated Namalwa cells gave 22 "candidate target" kinases that were presumed to have a role both in the B cell's ODN2006-CD70 and the ODN2006-CD80 activation pathways. The further validation was initially confined to only 6 of these "candidate target" kinases due to limited availability of small molecules described in the literature to inhibit these kinases on the protein level. Only two chemical inhibitors could potently suppress the expression of both CD70 and CD80 in ODN2006-stimulated human B cells as observed in the shRNA library screening. Because of the encouraging data that OSU-T315 gave in the functional *in vitro* assays (See table 4.1), we further concentrated on ILK as a potential new B cell drug target.

ILK is an evolutionarily conserved and ubiquitously expressed protein in the focal adhesions, which are large macromolecular assemblies through which mechanical force and regulatory signals are transmitted between the extra-cellular matrix (ECM) and the interacting cell, and is assumed to play an essential role in the integrin-matrix interactions, in microtubule dynamics, and in some signalling pathways (e.g. AKT, GSK3β, Rac1)[194]. ILK is composed of three structurally distinct domains (Figure 5.1): an N-terminal ankyrin repeat domain (ARD) with five ankyrin repeats, a central pleckstrin homology-like domain (PH) and a C-terminal kinase-like domain. Structure-based alignment sequencing and crystallographic studies show that ILK displays an atypical protein kinase domain. However, the lack of key conserved residues is not sufficient to predict whether a kinase domain has catalytic activity or not, as it is the case with Ca^{2+}/calmodulin-activated Ser/Thr kinase (CASK), an apparent pseudokinase with the capacity of phosphoryl transfer[195–197]. In skin, bone and central nervous system, ILK regulates processes that are related to cell adhesion, migration and proliferation. By contrast, cells of the immune and cardiovascular systems rely on the adaptor and "kinase" capabilities of ILK for maintenance of immune cell trafficking, cell survival, and tissue function. Hence, the precise cellular processes regulated by ILK are dependent on contextual cues (e.g. cell type, tissue, pathology like tumorigenic cells). The potential "kinase" activity of ILK is stimulated by integrins and soluble mediators, including growth factors and chemokines, and is regulated in a PI3K-dependent manner, Figure 5.1 (1), whereby ILK binds PIP3 via the PH-domain. After its activation, ILK exerts control over several downstream effectors, in

particular AKT and GSK3β (2). ILK rather regulates the phosphorylation of AKT at Ser-473 and hereby Rictor, a member of the mTORC2 complex (reckoned as PDK2), facilitates that phosphorylation event (3). In addition to its catalytic function, the C-terminal kinase-like domain of ILK also interacts with integrins, paxilin, parvins and various actin-binding adaptor proteins (4) hereby establishing a linkage of integrins (5) to the actin cytoskeleton. Via the N-terminal ARD domain with ankyrin-repeats, ILK interacts directly with several key proteins like PINCH (6) (which recruits ILK to the focal adhesion sites and connects ILK via NCK2 indirectly with growth factor receptors), ILKAP (7) (which negatively regulates ILK-signalling) and membrane receptors like SPARC and T-cadherin (8) (which contribute to outside-in signalling, cytoskeleton reorganisation and activation of small GTPases). Small molecule OSU-T315 is expected to disrupt the interaction of AKT with its binding site on ILK (9). Additionally, ILK also localizes to the centrosomes where it might aid in the assembly of the mitotic spindle through its interaction with tubulins and tubulin-binding proteins[198].

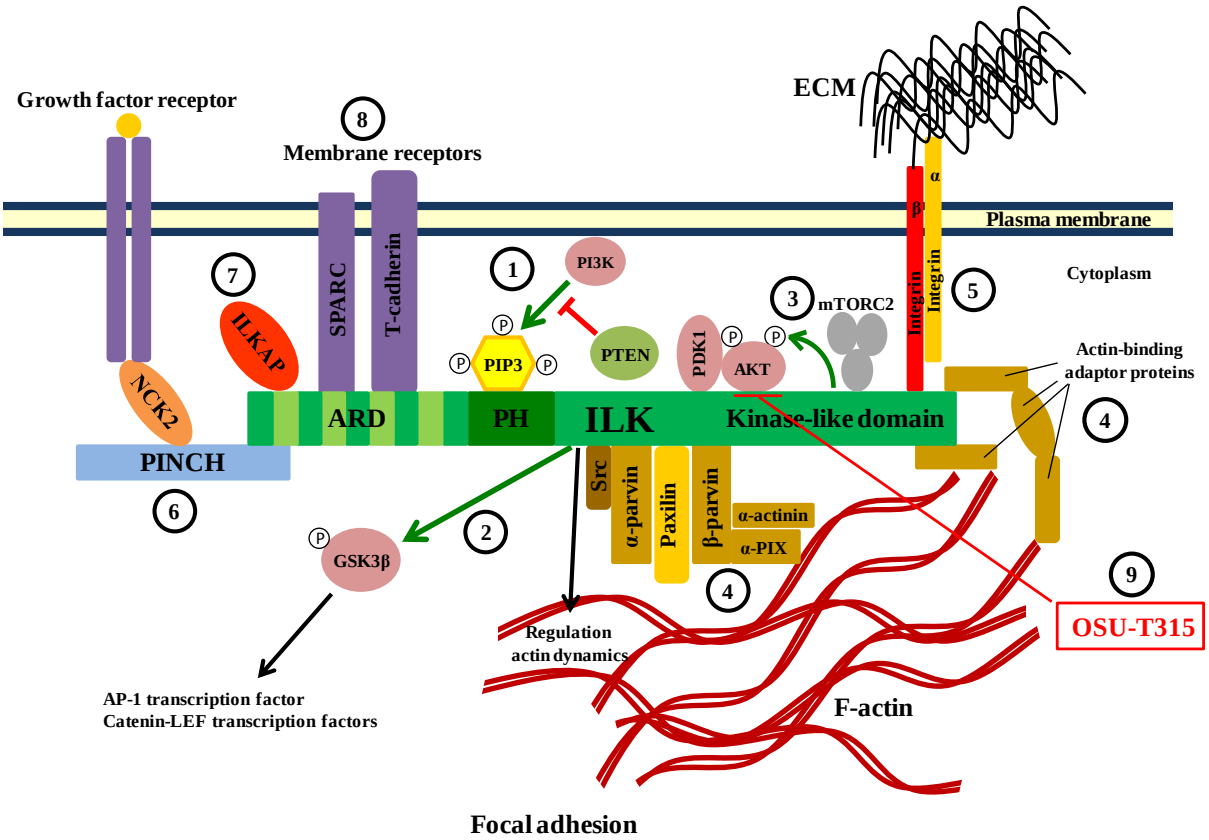


Figure 5.1. A schematic representation of the ILK interactome, based on the picture from Paul C. McDonald et al.[199].

Whether ILK is a functional kinase remains a contentious issue and the exact function of ILK remains unclear due to its intrinsic kinase function that is not always easily detected or demonstrated. Following arguments from literature argue against ILK as a functional kinase: ILK-deficient murine fibroblasts, chondrocytes or keratinocytes did not show impaired AKT and GSK3β phosphorylation as analysed by western blot[197,200]. Analysis on the structure of human ILK showed that the catalytic domain of ILK lacks critical consensus sequences of Ser/Thr kinases. There is an ATP-binding pocket present, but the unusual structural arrangement enholds a strong clue that hydrolysis of ATP does not occur[201]. Thus, ILK cannot perform catalysis as a conventional kinase. On the other hand, following arguments from literature support ILK being a functional Ser/Thr protein kinase: Pure human ILK showed kinase activity *in vitro* using a peptide or protein substrate and could directly phosphorylate several substrates (AKT, GSK3β, MYPT-1, myelin basic protein, MLC-20 and αNAC)[202]. HEK-293 cells which were transfected with ILK-specific siRNA showed suppressed phosphorylation of AKT and of GSK3β[203]. Ablation of ILK in murine heart, skeletal muscle, nervous system or macrophages resulted in abrogated AKT phosphorylation as analysed by western blot[197]. Suppression of ILK in various types of human normal and malignant cells led to decreased phosphorylation of AKT and GSK3β[190,199,202,203]. In ILK-deficient thymic T cells from Lck-Cre^{+} / $ILK^{flox/flox}$ mice there was a decrease in AKT phosphorylation compared to those from Lck-Cre^{+} / $ILK^{WT/WT}$ mice in response to stimulation with CXCL12[204], a chemokine with the ability to orchestrate directional migration of a wide spectrum of cells to selected tissues. As a *bona fide* protein kinase ILK may gain activity under very (tissue-) specific conditions (e.g. upon post-translational modification and/or allosteric activators)[197,202,205]. The relative contributions of its kinase and adaptor functions may depend on tissue- and stage-specific developmental requirements, as well as consideration of normal versus pathological conditions.

In the field of oncology, several investigations were conducted on ILK. Elevated ILK protein levels and activity have been associated with the oncogenesis and tumour progression of many types of malignancies (prostate, ovary, breast, colon, pancreas, stomach and liver), indicating that ILK represents a potential target for new cancer treatments[199,206–209]. OSU-T315 was described as a novel, orally bio-available ILK inhibitor with high *in vitro* potency against a panel of prostate and breast cancer cell lines. In an *in vitro* radiometric kinase assay, OSU-T315 displayed a dose-dependent inhibitory effect with an IC_{50} of 0.6 µM on the phosphorylation of myelin basic protein, a known ILK substrate, by immunoprecipitated human ILK[186]. OSU-T315 proved its *in vivo* anti-tumour efficacy as a single oral agent by

suppressing PC-3, a human prostate cancer cell line, in a xenograft tumour growth model in athymic nude mice at 50 mg/kg/day[186]. The anti-tumour effect was ascribed to induction of apoptosis in cancerous cells.

Relatively recently, studies are focusing on the interplay of the ECM and the cytoskeleton in B cell signalling and activation, e.g. resulting into stronger interactions with other immune cells (the 'immunological synapse'), formation and function of BCR micro-clusters, in antigen internalisation and in availability of additional co-stimulatory signals. Hence, the cellular cytoskeleton is emerging as a key player at several stages of B cell activation, including the initiation of receptor signalling[28,210–212]. From that point of view ILK might influence the B cell's function via its central position in connecting the ECM with the actin-cytoskeleton. Hence, the present study was undertaken to further substantiate the role of ILK in B cell biology by investigating the effects of compound OSU-T315 on human B cells and in *in vivo* tests and by selectively knocking out the ILK gene in the B cells of mice.

5.2. Materials and methods

5.2.1. Characterization of small molecule compound OSU-T315

Primary human B cells were stimulated *in vitro* with 0.1 µM ODN2006 (InvivoGen, Toulouse, France), 1 µM resiquimod (Sigma-Aldrich, Diegem, Belgium) or with 91 µg/mL TNP-BSA (Biosearch technologies, Novato, California, USA) in the absence or presence of different concentrations of compound OSU-T315 (CalBiochem, Merck, Millipore, Overijse, Belgium) within the range of 0.0001 to 10 µM. Several read-out were used: cell surface markers expression at 24 hours analyzed by flow cytometry, cytokine production at 48 hours analyzed by AlphaLISA (Perkin Elmer, Zaventem, Belgium), cellular proliferation at 72 hours using ^{3}H thymidine (Perkin Elmer, Zaventem, Belgium) incorporation and IgG and IgM production at 1 week analyzed by AlphaLISA. These assays were performed as previously described (Van Belle K et al., J Immunol Res 2016, 2016:5281823; PMID: 28116319).

5.2.2. Generation of a mouse line with B cell-specific deletion of ILK

Female ILK$^{flox/flox}$ mice with C57Bl6 background, wherein the ILK encoding gene sequence is flanked by LoxP, were generated by the research group of Prof. Dr. René St.-Arnaud (McGill University and Shriners Hospital for Children, Montreal, Canada) and were kindly provided by Prof. Dr. Christa Maes (KULeuven, Belgium). Male CD19$^{Cre/Cre}$ mice with C57Bl6 background, wherein the gene sequence encoding for the cell surface marker CD19 is replaced by the gene sequence encoding for the enzyme Cre recombinase, were kindly

provided by Prof. Dr. Jan Cools (KULeuven, Belgium). The male $CD19^{Cre/Cre}$ mice and female $ILK^{flox/flox}$ mice were placed in a breeding program to obtain offspring with a B cell-specific deletion of ILK, the $CD19^{Cre/WT}$ / $ILK^{flox/flox}$ mice. Animal care was according the guidelines for laboratory animals from the KULeuven and all experiments were approved by the Ethical Committee for Animal Science of KULeuven (ethical committee, P075-2010 and P107-2015). Through a genotypic approach with PCR and a phenotypic approach with flow cytometry, the mice with B cell-specific deletion of ILK, genotype $CD19^{Cre/WT}$ / $ILK^{flox/flox}$, were identified and were selected for the *in vivo* proof of concept experiments.

5.2.3. Identification of mice with B cell-specific deletion of ILK

5.2.3.1. Genotypic approach with PCR

PCR was used to genotype the newly bred mice. Lysis of a piece of the tail of each mouse was performed by overnight incubation at 55 °C with lysis-buffer (100 mM Tris-HCl at pH 7.5, 200 mM NaCl, 0.5 % SDS and 1 mM EDTA) and proteinase K (0.16 g/L, Roche Diagnostics, Mannheim, Germany). A pellet of tail lysate was obtained after centrifugation and the supernatant was taken. Isopropanol was added to the supernatant and the DNA was collected. After drying, the DNA was solved in TE-buffer (10 mM Tris-HCl at pH 7.5 and 1 mM EDTA at pH 8) and incubated overnight at room temperature for complete solubilisation.

5.2.3.1.1. Identification of mice with CD19-Cre allele(s)

Mice with a CD19-Cre positive genotype were identified with the SYBR Green dye-based real-time PCR (RT-PCR) amplification and detection method. The final concentrations of the components in the PCR-mix for CD19-Cre were as follows: 1x SYBR® Green PCR master mix (Applied Biosystems, Halle, Belgium), 0.8 µM oligonucleotide primers and 1 µL of DNA sample for total volume of 20 µL. With the set of primers CD19-Cre sense (primer sequence 5’ GCGGTCTGGCAGTAAAAACTATC 3’; Sigma-Aldrich, Diegem, Belgium) and CD19-Cre anti-sense (primer sequence 5’ GTGAAACAGCATTGCTGTCACTT 3’; Sigma-Aldrich, Diegem, Belgium) there was only amplification of DNA when there is a CD19-Cre allele. PCR cycling was performed using the StepOnePlus RT-PCR System (Applied Biosystems, Halle, Belgium) with initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 40 seconds, the final extension step was done at 72 °C for 3 minutes. The amplified DNA was separated on a 2 % agarose gel. After staining of the agarose gel with ethidium bromide the DNA bands were visualized with UV light. A band of 100 base pairs (bp) indicated the mice with a CD19-Cre allele. Mice with

homozygous CD19-WT genotype did not give a band on the agarose gel, as there was no DNA amplification. Distinction between the homozygous and the heterozygous CD19-Cre mice was done by flow cytometry (See paragraph 5.2.3.2.).

5.2.3.1.2. Identification of mice with floxed ILK allele(s)

The genotypic distinction between $ILK^{WT/WT}$, $ILK^{flox/WT}$ and $ILK^{flox/flox}$ mice was done by PCR. The final concentrations of the components in the PCR-mix for floxed ILK were as follows: 25 U/mL GoTaq® G2 Flexi DNA polymerase (Promega), 1x Green GoTaq® Flexi buffer (Promega), 2 mM $MgCl_2$, 200 µM dNTP's, 0.4 µM oligonucleotide primers and 1 µL of DNA sample for total volume of 25 µL. We used the set of primers ILK Jax sense (primer sequence 5' GACCAGGTGGCAGAGGTAAG 3'; Sigma-Aldrich, Diegem, Belgium) and ILK Jax anti-sense (primer sequence 5' GCTTTGTCCACAGGCATCTC 3'; Sigma-Aldrich, Diegem, Belgium) to identify the mice with a homozygous floxed ILK genotype ($ILK^{flox/flox}$). PCR cycling was performed using the GeneAmp® PCR System 9700 (Applied Biosystems) with the same program as for the identification of CD19-Cre allele(s). The amplified DNA was separated on a 2 % agarose gel. After staining of the agarose gel with ethidium bromide the DNA bands were visualized with UV light. The size of the wild type (WT) ILK allele was 245 bp and of the floxed ILK allele was 270 bp. Mice that were homozygous for the WT ILK allele showed a DNA band of 245 bp. Mice that were homozygous for the floxed ILK allele showed a DNA band of 270 bp. Heterozygous $ILK^{flox/WT}$ mice were identified by the observation of two DNA bands on the agarose-gel: a band of 245 bp from the WT allele and a band of 270 bp from the floxed allele.

5.2.3.2. Phenotypic approach with flow cytometry

5.2.3.2.1. Identification of homo- and heterozygous CD19-Cre mice

Since $CD19^{Cre/WT}$ (with one CD19-Cre allele) and $CD19^{Cre/Cre}$ mice (with two CD19-Cre alleles) could not be distinguished from each other by separation of the PCR-products on agarose-gel, the distinction between $CD19^{WT/WT}$, $CD19^{Cre/WT}$ and $CD19^{Cre/Cre}$ mice was done by flow cytometry on murine blood. Blood was taken from the newly bred mice by eye puncture. Murine blood cells were washed with cold PBS and were incubated 30 minutes at 4 °C with FITC-labelled anti-murine CD19 (Biolegend, ImTec Diagnostics N.V., Antwerp, Belgium) and PE/Cy5-labelled anti-murine CD45R/B220 (Biolegend, ImTec Diagnostics N.V., Antwerp, Belgium). CD45R/B220, commonly used as a pan-B cell marker, was included in the double staining to allow visualization of B cells in the blood of mice with

lowered (heterozygous CD19-Cre genotype) or completely deleted (homozygous CD19-Cre genotype) expression of CD19. After staining, the blood cells were washed twice with cold PBS and incubated for 20 minutes at 4 °C with fixation/permeabilization solution (BD Cytofix/Cytoperm™, BD Biosciences, Erembodegem, Belgium). After two washes with permeabilization/wash buffer (BD Cytofix/Cytoperm™, BD Biosciences, Erembodegem, Belgium), the cells were suspended in cold PBS and analysed with the 3-color Becton Dickinson FACSCalibur apparatus.

5.2.4. Protein detection and western blot analysis

Single-cell suspensions of murine splenocytes were prepared by manual disruption from total spleen of an ILK wild type (WT) mouse (genotype $CD19^{WT/WT}$ / $ILK^{flox/flox}$), an ILK knock-out (KO) mouse (genotype $CD19^{Cre/WT}$ / $ILK^{flox/flox}$) and of a hetero mouse (genotype $CD19^{Cre/WT}$ / $ILK^{flox/WT}$) and highly purified B lymphocytes were isolated by immunomagnetic positive selection according to manufacturer's instructions (STEMCELL Technologies, EasySep™ Mouse CD19 positive selection kit II, Grenoble, France). Cells were lysed and proteins were separated on NuPAGE® Novex® 4-12 % Bis-Tris gel (Invitrogen™, Thermo Fisher Scientific, Niederlebert, Germany) and electrotransferred onto polyvinylidene fluoride membrane (PVDF membrane, Invitrogen™, Thermo Fisher Scientific, Niederlebert, Germany) and incubated with specific antibodies. Immunoreactive proteins were detected by Bio-Rad Imager (Bio-Rad laboratories N.V., Temse, Belgium) and normalized to the β-actin content. The used antibodies were: monoclonal rabbit anti-ILK (4G9; 1/1 000; Cell Signaling Technology®, Bioké, Leiden, The Netherlands), monoclonal rat anti-CD20 (AISB12; 1/500; Affymetrix®, eBioscience®, Vienna, Austria), anti-β actin (I-19; 1/1 000, Santa Cruz Biotechnology, Heidelberg, Germany), polyclonal (goat anti-rabbit, rabbit anti-goat and rabbit anti-rat) Ig/HRP (Dako, Heverlee, Belgium).

5.2.5. Mice deficient in mature B cells

Homozygous mutant B6.129S2-Ighmtm1Cgn/J mice, also known as muMt-, lack mature B cells. There is no expression of membrane-bound IgM because a neomycin resistance cassette disrupted one of the membrane exons of the gene encoding immunoglobulin heavy chain of the class mu (IgM). Mutant animals with C57Bl6 background were purchased at The Jackson Laboratory (France).

5.2.6. ODN1826 *in vivo* assay

Mice were stimulated subcutaneously with ODN1826 (InvivoGen, Toulouse, France; murine TLR9 agonist) at 60 µg/head. Naive control mice were injected with sterile PBS. Two hours post-stimulation blood was collected by eye puncture. Analysis of IL6 and TNFα in the plasma was done with the AlphaLISA murine IL6 and TNFα kit according to manufacturer's instructions (Perkin Elmer, Zaventem, Belgium). In case of treatment of mice with (small molecule) compound, which was administered 30 minutes before ODN1826-stimulation, the inhibitory effect was calculated with following formula:

$$\% \text{ inhibition} = \left(\frac{Analyte._{Vehicle group} - Analyte_{Treated group}}{Analyte_{Vehicle group} - Analyte_{Naive group}} \right) \times 100$$

Analyte stands for the concentration of the analyte (ng/mL) contained in the sample.

5.2.7. TNP-BSA *in vivo* assay

ILK WT mice (genotype $CD19^{WT/WT}$ / $ILK^{flox/flox}$), ILK KO mice (genotype $CD19^{Cre/WT}$ / $ILK^{flox/flox}$) and ILK hetero mice (genotype $CD19^{Cre/WT}$ / $ILK^{flox/WT}$) were stimulated with T cell-dependent antigen TNP-BSA (50 µg/head) solved in complete Freund's adjuvant (Sigma-Aldrich, Diegem, Belgium) by injection, subcutaneously. Blood was collected by eye puncture before stimulation and on days 7, 10, 14, 21 and 28 post-stimulation. On day 28, the mice underwent for a second time stimulation with TNP-BSA (50 µg/head, solved in complete Freund's adjuvant) and blood samples were taken on days 35 and 42.

Analysis of anti-TNP IgG and IgM in the collected sera was done by ELISA procedure with sheep anti-mouse IgG (H/L):horse radish peroxidise (HRP) and with goat anti-mouse IgM:HRP (both from Bio-Rad AbD Serotec, Oxford, United Kingdom), respectively. Peroxidase activity was detected by adding 3,3',5,5'-tetramethyl-benzidine liquid substrate (Sigma-Aldrich, Diegem, Belgium). Reaction was stopped by adding 1 M HCl, forming a yellow reaction product. Absorbance was measured by the ELISA reader at 450 nM.

5.2.8. TNP-Ficoll *in vivo* assay

ILK WT mice (genotype $CD19^{WT/WT}$ / $ILK^{flox/flox}$), ILK KO mice (genotype $CD19^{Cre/WT}$ / $ILK^{flox/flox}$) and ILK hetero mice (genotype $CD19^{Cre/WT}$ / $ILK^{flox/WT}$) were immunised intraperitoneally with T cell-independent antigen TNP-Ficoll (25 µg/head) solved in PBS. Blood was collected by eye puncture before stimulation and on days 5, 7, 10, 14 and 21 post-stimulation. Analysis of anti-TNP IgM in the collected sera was done by ELISA procedure with goat anti-mouse IgM:HRP (Bio-Rad AbD Serotec, Oxford, United Kingdom).

Peroxidase activity was detected by adding 3,3',5,5'-tetramethyl-benzidine liquid substrate (Sigma-Aldrich, Diegem, Belgium). Reaction was stopped by adding 1 M HCl, forming a yellow reaction product. Absorbance was measured by the ELISA reader at 450 nM.

5.2.9. *In vivo* xeno-antibody assay with BHK-570 cells

ILK WT mice, ILK KO mice and ILK hetero mice were stimulated with intraperitoneally injected baby hamster kidney (BHK) 570 cells (5.10^6 cells in RPMI-medium, 200 µL/head; Global Bioresource Center ATCC®, USA) and the production of IgM and IgG against the hamster cells was analysed on days 3, 5, 7 and 10 post-immunisation. Naive ILK WT mice were included as a control group. For analysis of the IgM and IgG titer in the murine sera, 5.10^5 BHK-570 cells were dispatched per well in a 96-well V-bottom plate and 5 µL of serum sample was added. After incubation of 30 minutes on ice, BHK-570 cells were washed two times with PBS. Secondary antibody (goat anti-mouse IgM antibody-PE or goat anti-mouse IgG antibody-FITC, Biolegend, ImTec Diagnostics N.V. Antwerp, Belgium) was added and incubated for 30 minutes on ice and protected from light. After 3 washes with PBS, IgM and IgG were assessed by flow cytometry with the 3-color Becton Dickinson FACSCalibur apparatus.

5.2.10. *In vitro* murine B cell activation assays

Single-cell suspensions of splenocytes were prepared by manual disruption from total spleens of ILK WT mice, ILK KO mice and ILK hetero mice and highly purified B lymphocytes were isolated by immunomagnetic positive selection according to manufacturer's instructions (STEMCELL Technologies, EasySep™ Mouse CD19 positive selection kit II, Grenoble, France). The purity of the isolated murine B cells was ≥ 95 % as analysed by flow cytometry. Splenic B cells were suspended at the desired concentration in complete DMEM culture medium and activated by 5 µg/mL LPS. The production of cytokines IL6 and TNFα was analysed 2 days post-stimulation and the proliferation rate was measured 3 days post-stimulation.

5.2.11. LPS survival assay

ILK WT mice, ILK KO mice and ILK hetero mice were challenged intraperitoneally with 200 µL LPS-solution of 0.150 mg/mL, 0.500 mg/mL or 1.500 mg/mL (30 µg LPS/head, 100 µg LPS/head or 300 µg LPS/head, respectively). Body weight and survival rate were followed up during 8 days.

5.2.1. Statistical analysis

Statistical analyses for different experiments were performed using GraphPad Prism software and p values were calculated by two-tailed Student t-test. P values less than 0.05 are considered as significant.

5.3. Results

5.3.1. Assessment of the immunomodulatory activity of OSU-T315 on B cells

5.3.1.1. OSU-T315 effectively inhibits in vitro B cell activation

To investigate the potential role of ILK in the B cell's functions, several phenotypic *in vitro* experiments with compound OSU-T315 were performed on human primary B cells.

First, we investigated the effects of OSU-T315 on human primary B cells that were stimulated by ODN2006. Analysis of the cell surface markers CD69, CD70, CD80 and CD86 demonstrated that OSU-T315 was able to suppress in a dose-dependent manner the up-regulation of markers CD69, CD70, CD80 and CD86. The IC_{50} was about 0.5 µM for each marker (Figure 5.2).

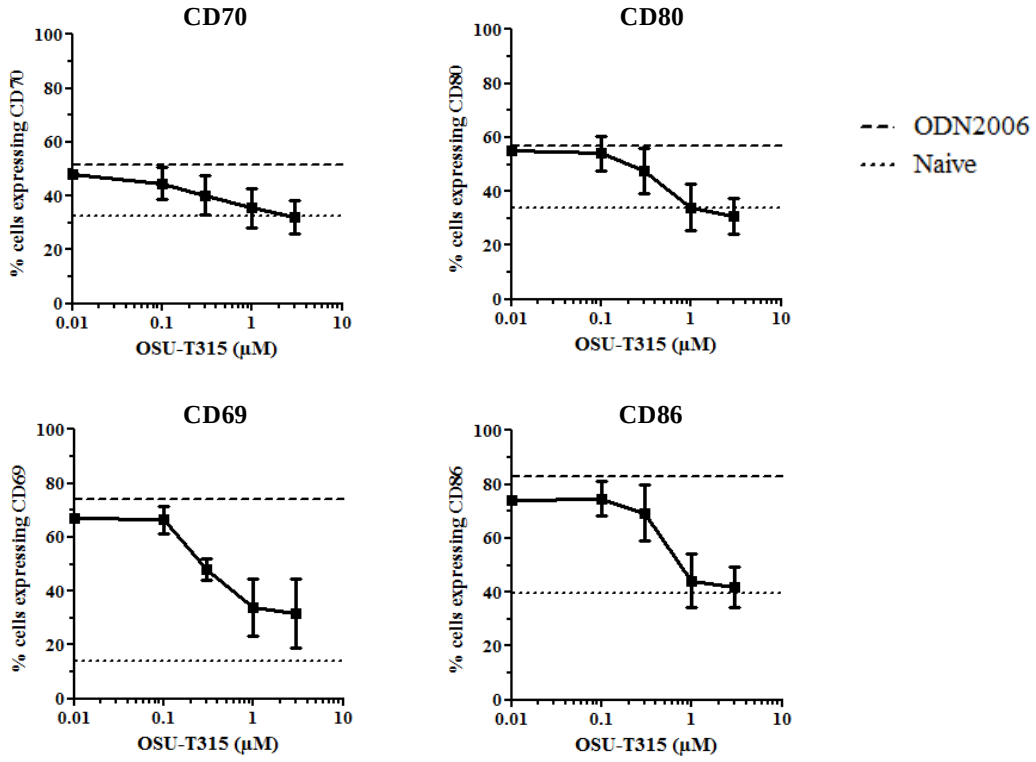


Figure 5.2. Effect of OSU-T315 on activation and co-stimulatory markers after ODN2006 stimulation. Purified human primary B cells were stimulated with 0.1 µM ODN2006 and simultaneously treated with OSU-T315 at different concentrations and were analysed 24 hours later by flow cytometry. Graphs represent the data from 5 independent experiments.

Also the production of IgG, IgM and IL6 in response to ODN2006 stimulation was significantly decreased when OSU-T315 was applied at concentrations between 0.1 - 1 µM (Figure 5.3 A, B and C, respectively). Similarly, OSU-T315 inhibited the proliferation of activated B cells with comparable efficacy (Figure 5.3 D). The IC_{50} value in the IgG assay was around 0.2 µM and in the IgM, IL6 and proliferation assays the IC_{50} values were around 0.5 µM, as with the cell surface markers.

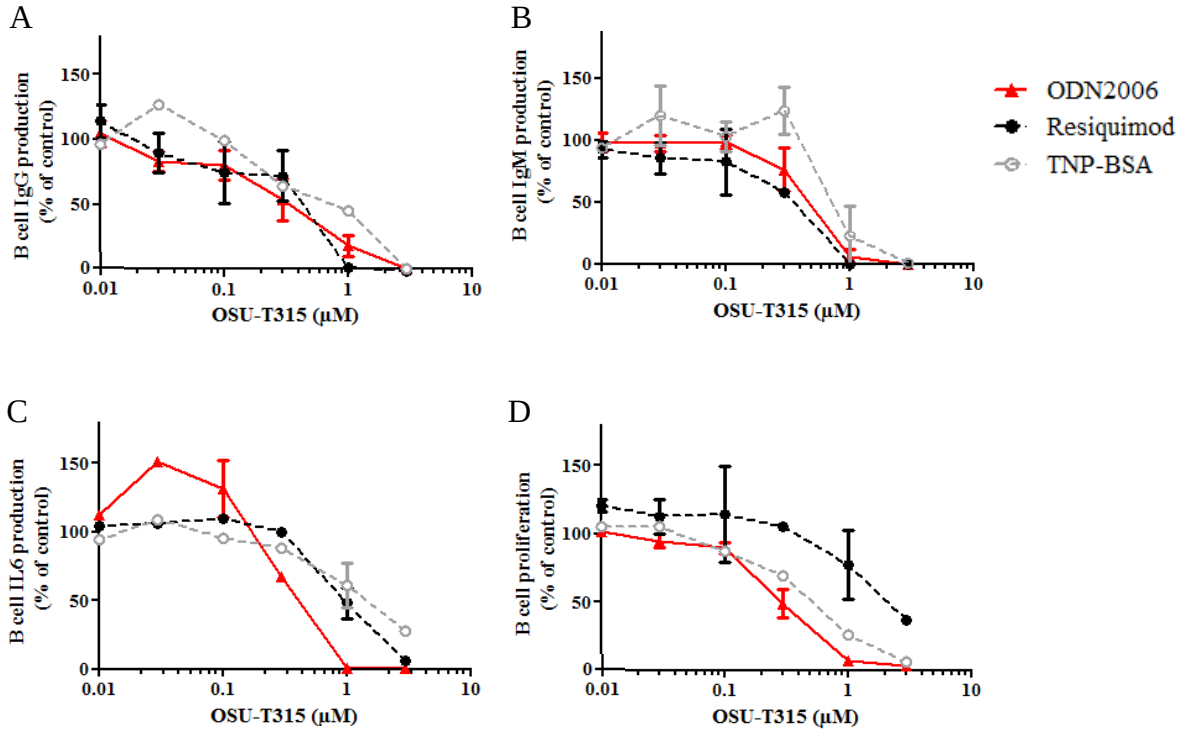


Figure 5.3. Effect of OSU-T315 on the production of IgG (A), IgM (B) and IL6 (C) and on B cell proliferation (D). Highly purified human primary B cells were cultured in the presence of a range of stimuli (0.1 µM ODN2006, 1 µM resiquimod or 91 µg/mL TNP-BSA). IL6 was analysed after 2 days of incubation, proliferation was measured by ^{3}H thymidine incorporation after 3 days of incubation and analysis of IgG and IgM was performed after 7 days of incubation. Graphs represent the data from 2 independent experiments.

OSU-T315 was also investigated on human B cells stimulated with resiquimod, a synthetic agonist of TLR7/TLR8, or with TNP-BSA. The inhibition of the IgG and IgM production by OSU-T315 after stimulation with resiquimod was comparable to ODN2006 (IC_{50} values 0.3 µM and 0.4 µM, respectively) (Figure 5.3 A-B). For IL6 the IC_{50} value was around 1 µM (Figure 5.3 C). OSU-T315 displayed less potency in the B cell proliferation assay (Figure 5.3 D), but that was probably due to the fact that resiquimod is a relatively moderate stimulator of B cell proliferation (See Table 1 of article in chapter 3). Also resiquimod-induced up-regulation of the cell surface markers was suppressed by OSU-T315 in a dose-dependent manner (data not shown). OSU-T315 inhibited TNP-BSA-triggered B cell proliferation

(Figure 5.3 D) with an IC_{50} value of 0.6 µM. Less inhibitory effect by OSU-T315 was noticed in the TNP-BSA-induced IgG, IgM and IL6 production, with IC_{50} values of 1.5 µM, 1 µM and 1 µM, respectively (Figure 5.3 A, B and C, respectively). TNP-BSA was not able to induce the cell surface markers (See Table 1 of article in chapter 3).

5.3.1.2. OSU-T315 inhibits in vivo IL6 production induced by ODN1826

The data from the phenotypic *in vitro* assays indicated that ILK may have a role in the activation of B cells. Next, we investigated the *in vivo* effects of OSU-T315 treatment on IL6 and TNFα production after TLR9 stimulation with ODN1826 (the murine counterpart of ODN2006). Naive control mice and stimulated control mice were both treated with vehicle and upon stimulation a clear up-regulation in IL6 and TNFα production was observed in the stimulated control mice ($p < 0.0001$) (Figure 5.4).

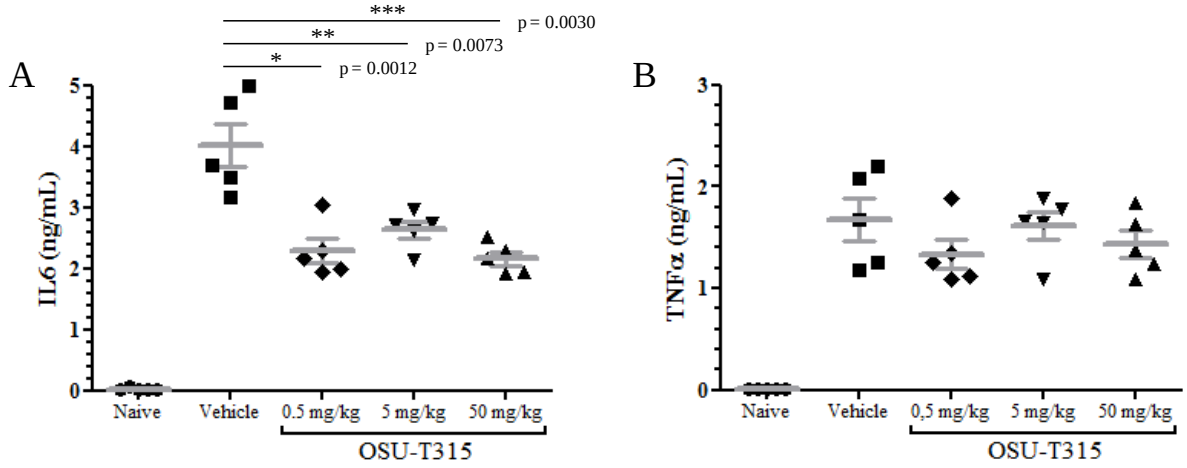


Figure 5.4. Effect of OSU-T315 on IL6 and TNFα production *in vivo*. Thirty minutes before TLR9 stimulation with ODN1826, C57Bl6 mice were treated orally with OSU-T315 at the indicated doses. The naive and stimulated control mice received vehicle. Five mice were included in each group. Two hours post-stimulation IL6 (A) and TNFα (B) were measured in the plasma. The graphs show data from one representative of the three independently performed experiments.

The mice that were treated orally with OSU-T315 showed a significantly lower production of IL6 in the *in vivo* experiments (Figure 5.4 A). In contrast to the *in vitro* assay with human B cells, a clear dose effect of OSU-T315 was not seen *in vivo* over the range of doses tested (0.5 mg/kg to 50 mg/kg). The average inhibition of IL6 production was 30 %. The absence of a dose effect could be due to IL6 production by (non-) hematopoietic cells that responded to ODN1826 stimulation, but were not affected by OSU-T315's activity. OSU-T315 did not influence the *in vivo* TNFα production upon ODN1826 stimulation (Figure 5.4 B).

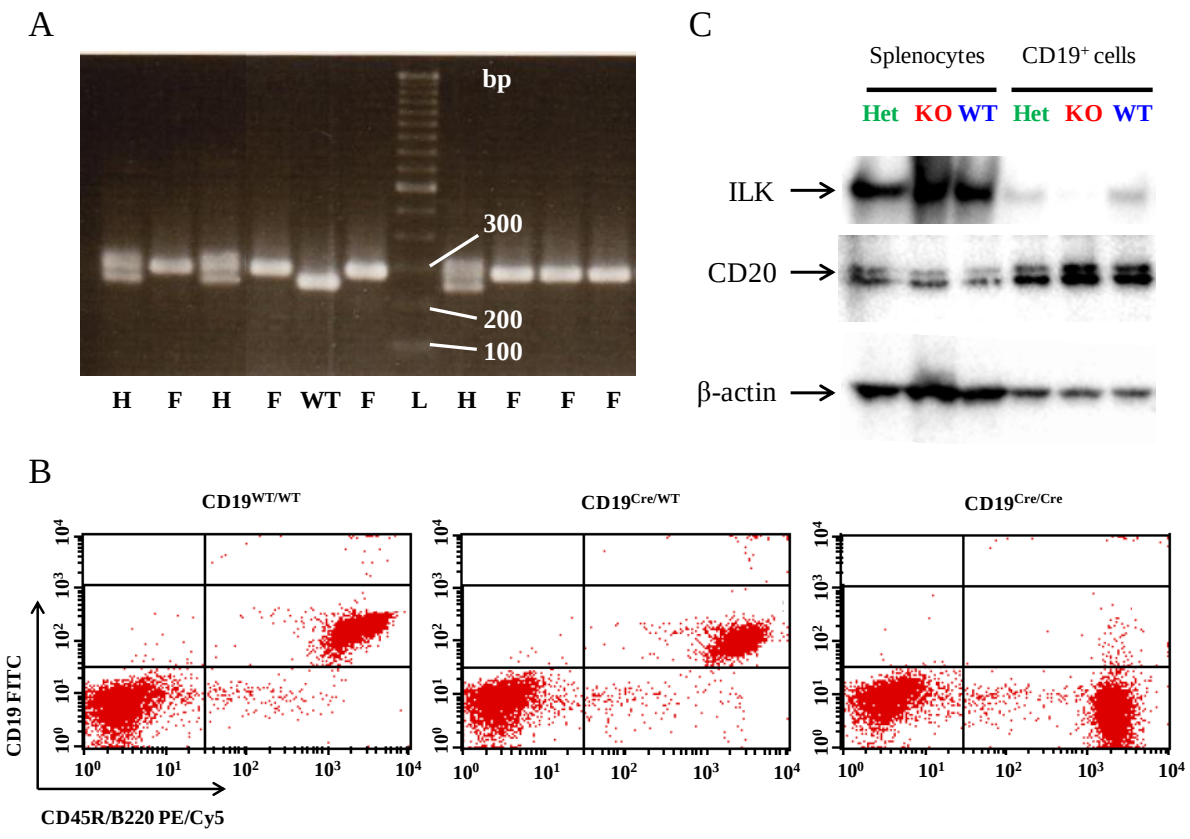
The preliminary results with OSU-T315 confirmed that ILK could be a promising target in the modulation of some B cell activity. To further validate ILK as a B cell target, we created mice with B cell-specific deletion of ILK.

5.3.2. Investigation on the importance of ILK on B cell biology *in vivo*

To investigate the significance of ILK *in vivo*, we created mice with B cell-specific deletion of ILK by means of the Cre/LoxP system. Cre recombinase enzyme, whose expression was under control of the CD19-promotor, excised the "floxed" ILK gene exclusively in the B lymphocytes.

5.3.2.1. Identification of ILK WT, hetero and KO mice

CD19 is a transmembrane protein on B lymphocytes that serves as a co-stimulatory molecule for amplifying proximal BCR signalling. It is described that a complete abrogation in expression of CD19 in mice causes defective late B cell differentiation and decreased Ab responses[213,214]. In human patients, CD19 deficiency not only causes common variable immunodeficiency, but also favours the development of autoimmunity[215]. For that reason, we preferred the mice with $CD19^{Cre/WT}$ / $ILK^{flox/flox}$ genotype with a partial deletion of CD19 over the ones with $CD19^{Cre/Cre}$ / $ILK^{flox/flox}$ genotype with a complete deletion of CD19 as our mice with B cell-specific knock-out for research. By means of PCR and flow cytometry, we were able to select the mice of interest (Figure 5.5). Separation and visualisation of the PCR products of tail DNA on agarose gel permitted us to identify the $ILK^{WT/WT}$ (band of 245 bp), $ILK^{flox/WT}$ (bands of 245 bp and 270 bp) and $ILK^{flox/flox}$ (band of 270 bp) mice (Figure 5.5 A). Flow cytometric analysis of cell surface markers CD19 and CD45R/B220 on blood samples of the earlier identified $ILK^{flox/flox}$ mice (Figure 5.5 B) enabled us to determine homozygous $CD19^{WT/WT}$ mice, homozygous $CD19^{Cre/Cre}$ mice and heterozygous $CD19^{Cre/WT}$ mice amongst the $ILK^{flox/flox}$ mice. Mice with $CD19^{Cre/Cre}$ genotype did not display CD19 on the outer surface of the B cells. Heterozygous mice still expressed CD19 on the outer surface of the B cells, but at a lower density compared to $CD19^{WT/WT}$ mice which translated in lower mean fluorescence intensity (MFI). The similar percentages of gated $CD45R/B220^{+}$ cells with equal MFI demonstrated that there was no loss in the B cell population in the $CD19^{Cre/WT}$ and $CD19^{Cre/Cre}$ mice compared to the $CD19^{WT/WT}$ mice.



Representative values / Mouse	CD19		CD45R/B220	
	% gated	MFI	% gated	MFI
CD19$^{WT/WT}$	40	202	40	2720
CD19$^{Cre/WT}$	45	105	45	2665
CD19$^{Cre/Cre}$	2	-	49	2505

Figure 5.5. Identification of ILK WT, hetero, KO mice through PCR (A) and flow cytometry (B) and confirmation of B cell-specific deletion of ILK by western blot (C). (A) Amplified DNA from mouse tails was separated and visualized on a 2 % agarose gel to identify the floxed ILK genes: L, TrackIt^{TM} 100 bp DNA ladder; WT, wild type ILK$^{WT/WT}$; H, heterozygote ILK$^{flox/WT}$; F, homozygote ILK$^{flox/flox}$. (B) Blood from the homozygote ILK$^{flox/flox}$ mice was double-stained with CD19 (FITC) and CD45R/B220 (PE/Cy5) to determine for homozygous CD19$^{WT/WT}$, heterozygous CD19$^{Cre/WT}$ and homozygous CD19$^{Cre/Cre}$. (C) Western blot was performed on splenocytes and on splenic B cells from mice identified as being ILK wild type (WT; genotype CD19$^{WT/WT}$ / ILK$^{flox/flox}$), ILK hetero (Het; genotype CD19$^{Cre/WT}$ / ILK$^{flox/WT}$) or ILK knock-out (KO; genotype CD19$^{Cre/WT}$ / ILK$^{flox/flox}$).

B cell-specific deletion of ILK in ILK KO mice was confirmed by western blot analysis (Figure 5.5 C). High expression of ILK was observed in the splenocytes of WT (wild type; CD19$^{WT/WT}$ / ILK$^{flox/flox}$), Het (hetero; CD19$^{Cre/WT}$ / ILK$^{flox/WT}$) and KO (knock-out;

$CD19^{Cre/WT}$ / $ILK^{flox/flox}$) mice, but ILK was not detected in the B lymphocytes of KO mice. Hetero mice showed a lower expression of ILK compared to WT mice.

5.3.2.2. The in vivo production of IL6 and TNFα upon ODN1826 stimulation is not influenced by the B cell-specific deletion of ILK

In all three groups of ILK mice (ILK WT, ILK hetero and ILK KO), stimulation with ODN1826 resulted in a clear increase of IL6 and TNFα production. Under naive circumstances, all three groups of mice showed an average level of IL6 at 10^{-5} ng/mL and an average level of TNFα at 10^{-2} ng/mL.

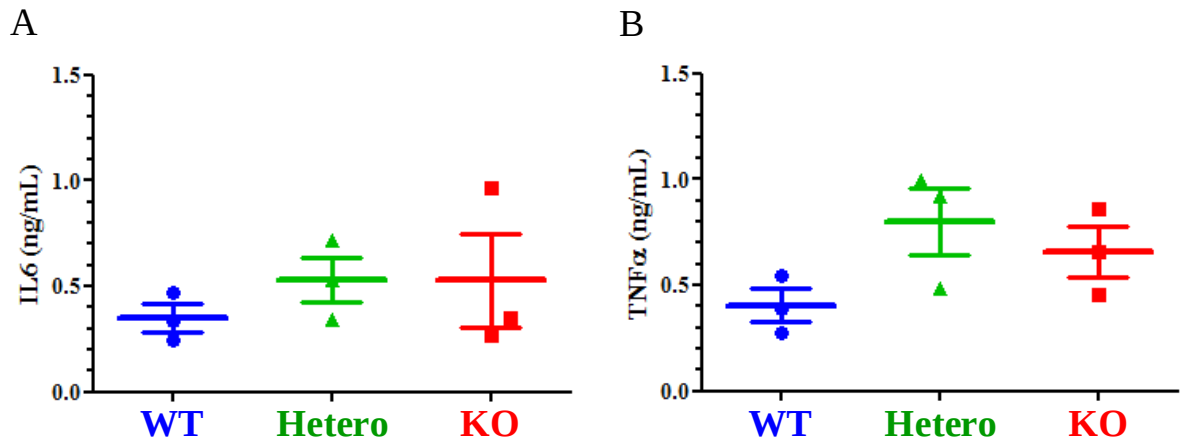


Figure 5.6. ODN1826 *in vivo* assay on mice with B cell-specific ILK deletion. IL6 (A) and TNFα (B) production was measured two hours post-stimulation in the plasma of ILK WT mice (blue, 3 mice), ILK hetero mice (green, 3 mice) and ILK KO mice (red, 3 mice). The graphics show data from one representative of the three independently performed experiments.

After stimulation, there was no significant difference in the level of IL6 (Figure 5.6 A) and of TNFα (Figure 5.6 B) between the three groups of ILK mice. Neither a lowered expression nor a complete deletion of the ILK protein in the murine B lymphocytes led to a change in production of both pro-inflammatory cytokines. This could indicate that ILK may not be involved in B cell-mediated IL6 and TNFα production upon TLR9 stimulation in our model.

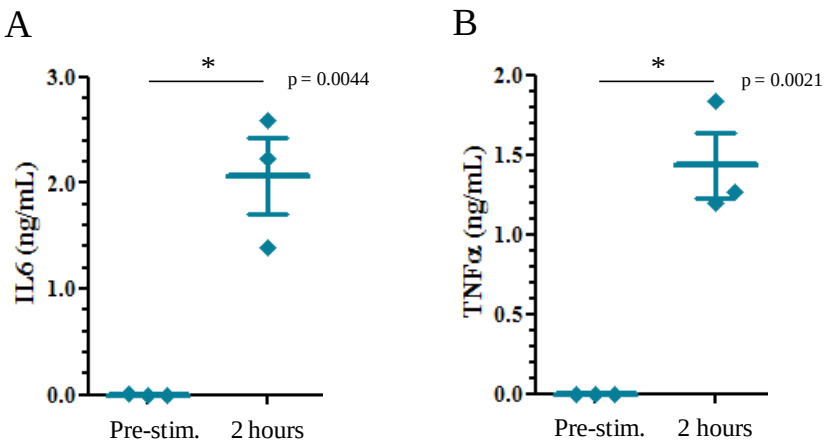


Figure 5.7. ODN1826 *in vivo* assay on B cell-deficient mice. IL6 (A) and TNFα (B) production was measured in the plasma of 3 B cell-deficient mice before ODN1826 stimulation and 2 hours post-stimulation.

We used a strain of B cell-deficient mice (Ighmtm1Cgn/J) to examine if other immune cells besides B cells can be induced by ODN1826 to raise the level of IL6 and TNFα in the plasma. The animals showed a clear augmentation of both cytokines (Figure 5.7 A and B), demonstrating that B lymphocytes are not the main producers of IL6 and TNFα. For instance, T cells, macrophages and some non-immune cells such as muscle[216] can secrete IL6 in response to stimulatory environmental conditions. Consequently, B cell-specific deletion of ILK might not have any notable influence on IL6 and TNFα production induced via TLR9.

5.3.2.3. The Ig-production against TNP-BSA and TNP-Ficoll is not influenced by the deletion of ILK

For triggering of *in vivo* Ig-production, we had to switch to TNP-hapten conjugate as stimulator, because we could not obtain an adequate induction of Ig-production with ODN1826. ILK WT, ILK hetero and ILK KO mice were immunised with TNP-BSA and the production of IgG and IgM was followed over time. This TNP-BSA *in vivo* assay has been carried out two times and a different outcome was obtained in the IgG production (Figure 5.8).

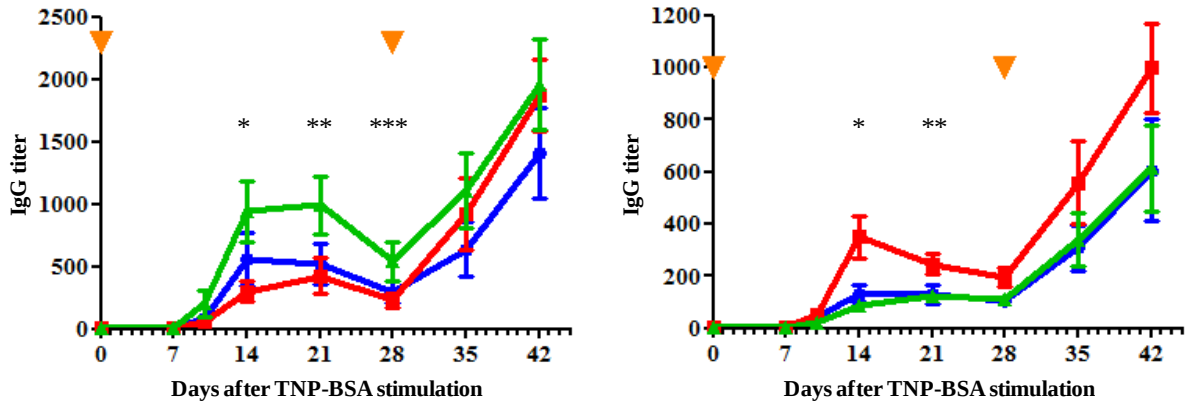


Figure 5.8. IgG production in mice with B cell-specific ILK deletion after TNP-BSA immunisation. Graphics display data of two independently performed experiments. The level of IgG was measured over time in the sera of ILK WT mice (blue, 8 mice), ILK hetero mice (green, 6 mice) and ILK KO mice (red, 8 mice). TNP-BSA challenges are indicated with reversed orange triangles.

In the first experiment (left graphic), it was quite remarkable that ILK hetero mice had the highest anti-TNP IgG in their sera. On days 14, 21 and 28 after the first immunisation, there was a significant difference between ILK hetero mice and ILK KO mice ($p = 0.0048$, $p = 0.0188$ and $p = 0.0389$, respectively), even if the increase was only by a factor 2. No significant difference was measured between ILK WT mice and ILK hetero mice and between ILK WT mice and ILK KO mice. ILK KO mice displayed during the first 28 days after the primary immunisation the lowest level of IgG, but the IgG production seemed to be very potently induced after reboost on day 28 hereby reaching similar levels as the ILK hetero mice. On days 35 and 42 there was no significant difference in IgG level between the three groups of mice. In the second experiment (right graphic), lower IgG values were measured compared to the first experiment. Here, ILK KO mice had the highest IgG titer after the first immunisation and displayed after reboost a strong augmentation in IgG production (day 14: ILK KO mice versus ILK WT mice, $p = 0.0270$, and ILK KO mice versus ILK hetero mice, $p = 0.0300$; day 21: ILK KO mice versus ILK WT mice, $p = 0.0416$, and ILK KO mice versus ILK hetero mice, $p = 0.0202$).

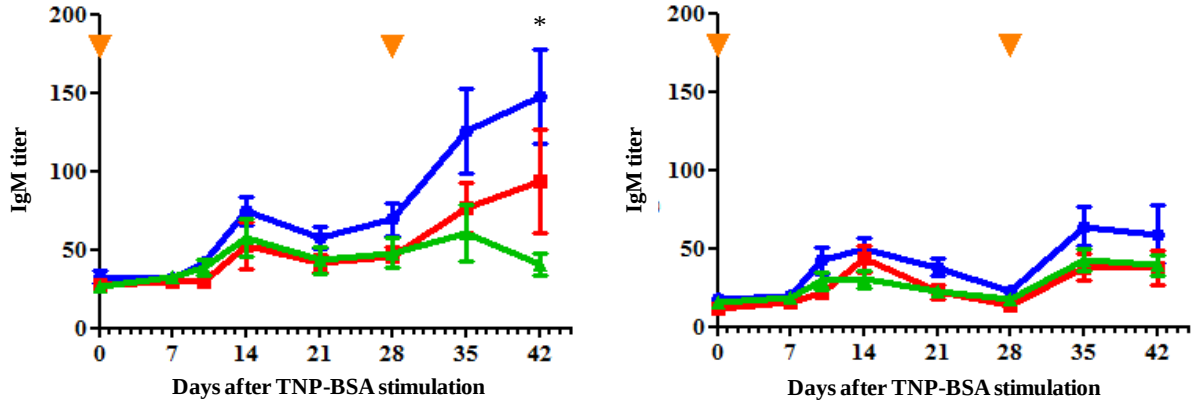


Figure 5.9. IgM production in mice with B cell-specific ILK deletion after TNP-BSA immunisation. Graphics display data of two independently performed experiments. The level of IgM was measured over time in the sera of ILK WT mice (blue, 8 mice), ILK hetero mice (green, 6 mice) and ILK KO mice (red, 8 mice). TNP-BSA challenges are indicated with reversed orange triangles.

Regarding IgM production (Figure 5.9), the results of the two tests were more similar. TNP-BSA gave low IgM levels in all three groups of mice. In the first experiment (left graphic), ILK WT mice showed higher values of IgM in their sera after reboost than ILK KO mice and ILK hetero mice (day 42, $p = 0.0198$), but that was not reproduced in the second experiment (right graphic) in which the three groups of mice showed more comparable, but low values.

In a following *in vivo* experiment, ILK WT, ILK hetero and ILK KO mice were immunised with TNP-Ficoll and the production of IgM was followed over time. The production of IgM was more potently induced by TNP-Ficoll, reaching a peak at 5 days after the immunisation and showing a diminution afterwards (Figure 5.10).

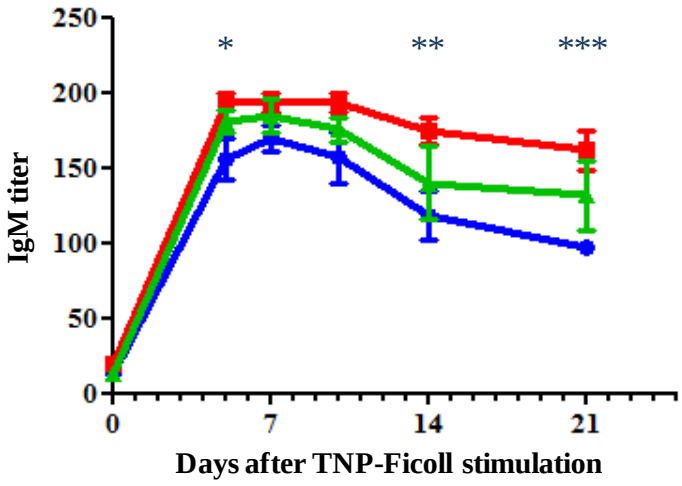


Figure 5.10. IgM production in mice with B cell-specific ILK deletion after TNP-Ficoll immunisation. IgM production was measured over time in the sera of ILK WT mice (blue, 6 mice), ILK hetero mice (green, 4 mice) and ILK KO mice (red, 6 mice) after immunisation with TNP-Ficoll.

The ILK KO mice had the highest IgM titer of the three groups and that remained over time more or less at the same high level as on day 5. ILK WT mice displayed the lowest level of IgM and ILK hetero mice had an IgM titer between the two other groups. ILK KO mice differed significantly in IgM titer with ILK WT mice on days 5, 14 and 21 ($p = 0.0260$, $p = 0.0117$ and $p = 0.0014$, respectively), but not with ILK hetero mice.

5.3.2.4. Deletion of ILK has no significant impact on the anti-BHK-570 xeno-antibody production

In the previous *in vivo* experiments, mice were immunised with a TNP-hapten conjugate to induce Ig-production. Here, we examined if the B cell-specific deletion of ILK might affect the IgG and IgM production against baby hamster kidney cells BHK-570.

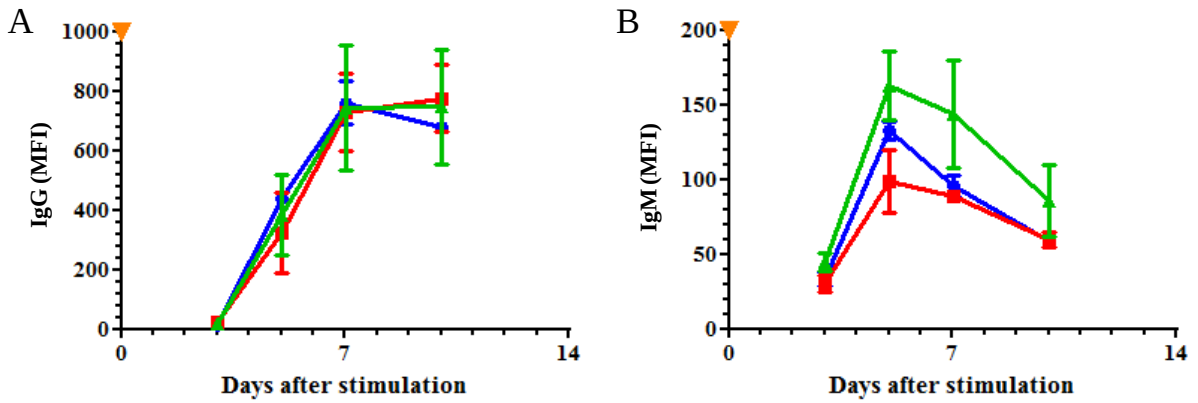


Figure 5.11. Xeno-antibody production in mice with B cell-specific ILK deletion against BHK-570 cells. IgG (A) and IgM (B) production was measured by flow cytometry in the sera of ILK WT mice (blue, 3 mice), ILK hetero mice (green, 3 mice) and ILK KO mice (red, 3 mice) after immunisation with BHK-570 cells (orange reversed triangle).

The production of IgG against BHK-570 (Figure 5.11 A) was potently induced and the level of IgG was very similar in the three groups. Thus, lowering or deletion of ILK expression did not affect the xeno-IgG production. The level of IgM against BHK-570 (Figure 5.11 B) was less strongly up-regulated compared to IgG, but there was some more divergence between the three groups. ILK hetero mice showed a higher level of IgM in their sera compared to ILK WT and ILK KO mice, but the difference was not statistically significant due to quite big variation in values between the 3 ILK hetero mice. Experiment has been performed only one time.

5.3.2.5. In vitro splenic B cell activation experiments

In previous *in vivo* experiments, alterations in IgG, IgM, IL6 and TNFα were investigated in the peripheral blood. Subsequently, we examined if the deletion of ILK might have a more profound effect on the activity of splenic B lymphocytes. Additionally, we investigated if there is a difference in the inhibitory action of compound OSU-T315. ODN1826, resiquimod, TNP-BSA, TNP-Ficoll and LPS were tested on WT mouse splenic B lymphocytes to see which stimulator gave good induction in proliferation and production of IL6 and TNFα. Apparently, LPS was the best stimulator, the other stimuli had no or hardly any effect on the murine B cells. Splenic B cells of ILK WT, ILK hetero and ILK KO mice underwent stimulation with LPS and OSU-T315 was added at different doses. Experiment has been performed only one time.

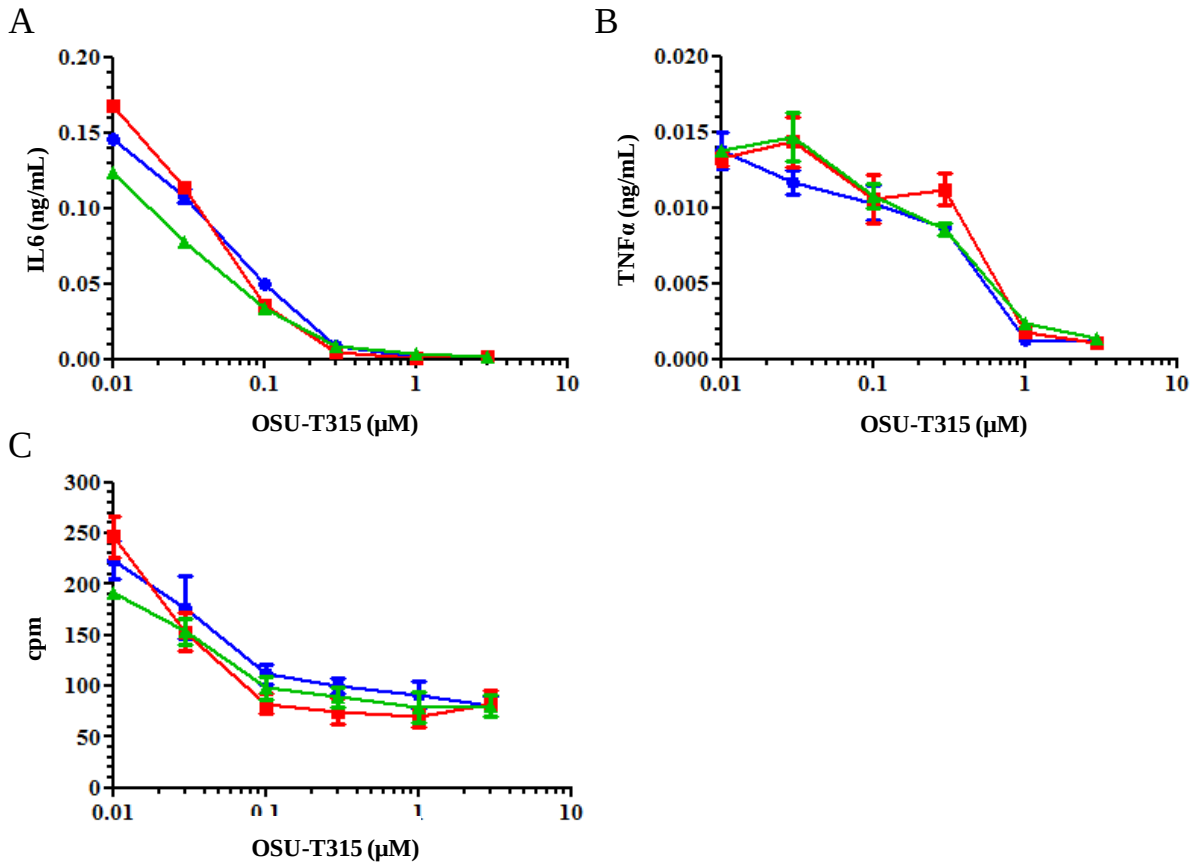


Figure 5.12. Effect of OSU-T315 on the IL6 (A) and TNFα (B) production and the proliferation (C) of splenic B cells from ILK WT (blue), ILK hetero (green) and ILK KO (red) mice upon stimulation with LPS.

Generally, the production of IL6 (Figure 5.12 A) and TNFα (Figure 5.12 B) and the B cell proliferation (Figure 5.12 C) were not substantially altered by a lowered or deleted expression of ILK in the B lymphocytes. OSU-T315 strongly lowered the proliferation (IC_{50} µM: ILK WT = 0.227; ILK hetero = 0.143; ILK KO = 0.103) and the secretion of IL6 (IC_{50} µM: ILK

WT = 0.081; ILK hetero = 0.055; ILK KO = 0.059) and TNFα (IC_{50} µM: ILK WT = 0.469; ILK hetero = 0.485; ILK KO = 0.686), but there was no considerable difference in potency of the compound between the three groups of mice.

5.3.2.6. Deletion of ILK possibly gives a higher sensitivity to LPS-induced septic shock

We investigated if mice with a lowered or completely deleted expression of ILK in B lymphocytes were more sensitive to LPS-induced sepsis (Figure 5.13).

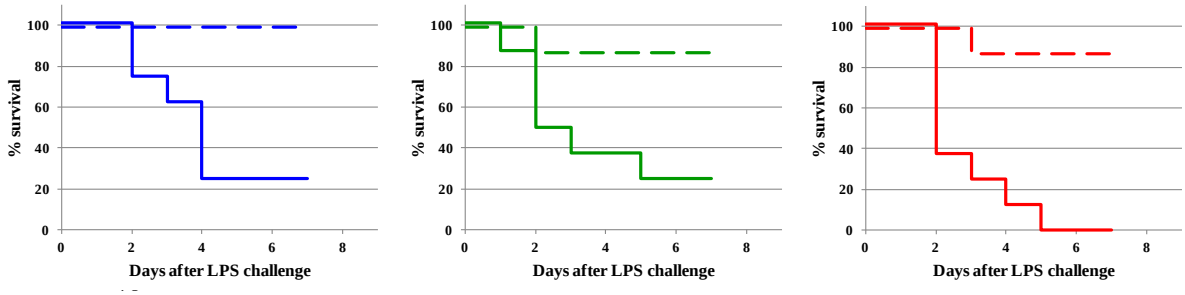


Figure 5.13. LPS survival assay. ILK WT mice (blue), ILK hetero mice (green) and ILK KO mice (red) were challenged by 2 different doses of LPS (100 µg, long dashed line; 300 µg, solid line) and were followed up during 7 days. Eight mice were used per tested dose of LPS.

At dose 300 µg of LPS, ILK KO mice seemed to have a higher lethality rate than ILK WT mice and ILK hetero mice, however, it is not decisive to say that the deletion of ILK in B lymphocytes makes mice more vulnerable to LPS-induced septic shock.

All obtained *in vitro* and *in vivo* ILK data are brought together in table 5.1 on the next page.

ILK inhibition	Species	Experimental condition			Read-out	Results
		In vitro /in vivo	Cell type or treatment *in vivo*	Stimulus		
OSU-T315	Human	*In vitro*	Namalwa	ODN2006	CD markers	IC_{50}: CD40 = 270; CD69 = 630; CD70 = 740; CD80 = 240; CD83 = 620; CD86 = 300
					WST-1	IC_{50}: 4 600
			Primary B cells	ODN2006	CD markers	IC_{50}: CD40 = 490; CD69 = 274; CD70 = 454; CD80 = 527; CD83 = 726; CD86 = 459
					IgG and IgM	IC_{50}: IgG = 205; IgM = 510
					IL6	IC_{50}: 555
					Proliferation	IC_{50}: 415
				Resiquimod	IgG and IgM	IC_{50}: IgG = 260; IgM = 365
					IL6	IC_{50}: 1 095
					Proliferation	IC_{50}: ≥ 3 000
				TNP-BSA	IgG and IgM	IC_{50}: IgG = 985; IgM = 965
					IL6	IC_{50}: 1 505
					Proliferation	IC_{50}: 650
	Mouse	*In vivo*	Oral treatment OSU-T315 after stimulation	ODN1826	IL6	IL6 ↓, but no dose-dependent effect
					TNFα	TNFα remained unaltered
Genetic KO in CD19$^+$ cells	Mouse	*In vivo*	Stimulation	ODN1826	IL6 and TNFα	No difference between WT mice and mice with ILK KO
				TNP-BSA	IgG and IgM	No difference between WT mice and mice with ILK KO
				TNP-Ficoll	IgM	No difference between WT mice and mice with ILK KO
				BHK-570	Xeno-IgG and IgM	No difference in xeno-Ab production between WT mice and mice with ILK KO
			Septic shock induction	LPS	Survival rate	Survival rate was similar between WT mice and mice with ILK KO
Genetic KO in CD19$^+$ cells and OSU-315	Mouse	*In vitro*	Splenic B cells	LPS	IL6 and TNFα	IL6 IC_{50}: ILK WT = 81, ILK hetero = 55 and ILK KO = 59
						TNFα IC_{50}: ILK WT = 469, ILK hetero = 485 and ILK KO = 686
					Proliferation	Proliferation IC_{50}: ILK WT = 227, ILK hetero = 143 and ILK KO = 103

Table 5.1. Overview of the obtained *in vitro* and *in vivo* ILK data. The measuring unit of the IC_{50}-values is nM.

5.4. Discussion

Based on various *in vitro* tests on the human Namalwa B cell line and on human primary B cells using different techniques (shRNA-mediated deletion or small molecule inhibitor), it was shown that ILK may be important for B lymphocyte immune activation by various stimuli such as ODN2006, resiquimod and TNP-BSA. Indeed, inactivation or inhibition of ILK suppressed several *in vitro* B lymphocyte responses (such as CD69, CD70, CD80 and CD86 expression, IgG, IgM or IL6 production as well as proliferation). The potency of ILK inhibitor OSU-T315 was validated by IC_{50} values of generally 0.5 µM.

When investigated *in vivo* in mice, OSU-T315 treatment resulted in significant suppression of ODN1826-induced IL6 production, however, a clear dose response effect could not be demonstrated. *In vivo* production of TNFα was not affected by OSU-T315 treatment. Unfortunately, none of the stimuli used on human B cells gave an adequate activation of murine splenic B cells to compare effects of OSU-T315 in comparable models. To know if ILK blockade may work *in vivo* and not only *in vitro* as shown in the shRNA experiments, we created mice with B cell-specific ILK KO. In these mice and using various B cell stimulation experiments, the absence of the ILK gene did not reveal a suppressive effect in various B cell phenomena such as ODN1826-induced IL6 or TNFα production (but as proven *in vivo* with B cell-deficient mice, IL6 and TNFα are not exclusively produced by B lymphocytes), TNP-BSA- or TNP-Ficoll-induced IgG or IgM production and xeno-antibody production. When using B cells from these mice *in vitro*, there were no defects shown either. The latter may suggest that the role of ILK in human and murine B cells is not fully overlapping and needs deeper investigation to clarify its role and to check if mice are an acceptable model to study B cell biology. This is further supported by the fact that the stimuli we used in the present study to stimulate human B cells *in vitro* do not work in mice and, vice versa those used in mice do not work in humans. Another possible explanation could be that the deletion of a target can induce an alternative “rescue”-pathway to compensate for the absence of the target and that could have happened in our ILK KO mice.

Small molecule OSU-T315, which is modelled from the scaffold that docks into AKT-binding site of ILK, was originally designed to specifically disrupt the interaction of AKT with its binding site on ILK[217]. For complete activation, AKT needs to be phosphorylated on Thr-308 by PDK1 and on Ser-473 by PDK2. The identity of PDK2 has been elusive for quite a while and ILK was regarded as a putative PDK2[217]. In literature, western blot analysis on the

phosphorylation of AKT on Ser-473 versus Thr-308 in PC-3 cells, a human prostate cancer cell line, and in MDA-MB-231 cells, a human breast cancer cell line, demonstrated that OSU-T315 selectively suppressed AKT phosphorylation on Ser-473, not on Thr-308, in a dose-dependent manner and this was accompanied by parallel decreases in the phosphorylation levels of GSK3β and MLC, two downstream targets of ILK[207]. As a positive control, shRNA-mediated KO of ILK in PC-3 cells modulated the phosphorylation of these signalling proteins in a manner similar to that of OSU-T315[207]. Together, the findings suggested that OSU-T315 might mediate Ser-473 AKT dephosphorylation through inhibition of ILK or through interaction with ILK. However, the mTORC2 complex, composed of mTOR, Rictor, and Sin1, has now been reckoned as PDK2 and not ILK[218,219]. In particular, Rictor, which interacts directly with ILK, facilitates the phosphorylation of AKT by ILK[218,220,221].

Very recently, a novel mechanism of action of OSU-T315 was characterized in studies on survival and proliferation pathways in human CLL. OSU-T315, which showed a preferential cytotoxicity toward CLL cells compared with normal B and T cells, inhibited AKT signalling independently of ILK by preventing the translocation of AKT to lipid rafts[217] which are specialized membrane micro-domains for the interactions and regulations of membrane-localized kinases. The activation of the PI3K/AKT pathways initiates at the plasma membrane where PIP3 recruits AKT to lipid rafts near the plasma membrane, leading to its subsequent phosphorylation and activation by PDK1 and PDK2. OSU-T315 displaced AKT from lipid rafts and impaired in that manner the AKT phosphorylation induced by a variety of signalling pathways including BCR, CD40L, TLR9 and integrin, and not through inhibition of ILK as expected[217]. This could imply that our obtained phenotypic outcomes in the *in vitro* and *in vivo* experiments with OSU-T315 may rather be the result of off-target effects. In the *in vitro* experiment with LPS-stimulated murine B cells, OSU-T315 displayed a similar potency in lowering proliferation and secretion of IL6 and TNFα between ILK WT, ILK hetero and ILK KO mice, supporting the hypothesis that OSU-T315 has other actions besides blocking ILK.

Since ILK is an evolutionarily conserved and ubiquitously expressed protein, there are many studies performed on complete or conditional deletion of ILK in various organisms and cells to find out more about its functions. A complete deletion of ILK leads to lethality in mice[200], *Xenopus laevis*[222], *Drosophila* flies[223] and *Caenorhabditis elegans* worms[224]. Using the Cre/loxP recombination system, the mouse ILK-encoding gene has been deleted in several cell types and organs (fibroblasts[200], immortalized macrophages[203], vascular endothelial cells[225], vascular smooth muscle cells[226], chondrocytes[227], heart[228], mammary epithelial cells[229]). One mouse model described the phenotype of a T cell-specific deletion of ILK, Lck-

Cre^+ / $ILK^{flox/flox}$ mice[204]. These mice showed neither gross developmental abnormalities nor thymus defects. Thymocytes from the T cell-specific ILK KO mice were phenotypically indistinguishable from control WT thymocytes, but there was enhanced susceptibility to cell death upon stress conditions like serum deprivation. ILK-deficient T cells showed an aberrant response to the chemokines CXCL12 and CCL19, indicating that ILK might have a role in leukocyte chemotaxis and immune cell trafficking. But ILK deletion did not affect T cell proliferation. Normal numbers and proportions of $CD4^+$ and $CD8^+$ peripheral T cells were observed in the mice with T cell-specific deletion of ILK. These observations seem to be compatible with our earlier data describing rather weak effect of compound OSU-T315 in MLR (Table 4.1).

In conclusion, we wanted to investigate if ILK would prove value as B cell target for treatment of (B cell-related) immunological disorders and pathologies. The human *in vitro* experimental data gave a first impression of ILK as being a central target for interference in different B cell signalling pathways and formed the onset of *in vivo* investigations about ILK in the immunology. However, with the obtained murine *in vivo* and *in vitro* data, it is likely that small molecule OSU-T315 might have another way of action than inhibiting ILK and that, under the tested conditions, ILK might not play a notable role in B cell activity.

Chapter 6 **: General discussion and future perspectives**

As increasing knowledge is gathered about the pathology of diseases, there is, as a consequence, an increasing demand for more specific, effective and affordable drugs to deal with the anomalies in the immune responses while leaving the normal responses undisturbed. In reality, however, there is a steep decline in the approval rate for novel molecular entities. The rate of attrition, as drug-candidates move through clinical trials, is still critically high and can be partly attributed to the disconnection between human physiology and the *in vitro* screening regimen which is usually at the beginning of the drug development chain. T cells have long been considered as main targets for the treatment of immune disorders and the majority of marketed immunomodulators are directed towards T cells. In this work, we contributed to the field of drug discovery by establishing an *in vitro* screening assay for the identification of (new) B cell-specific targets that would be valuable in the development of urgently needed innovative (small molecule) medicine for treatment of immune disorders and transplant rejection.

Strategies to identify potential drug candidates are diverse in approach and all have their strengths and weaknesses. For the set up of our *in vitro* screening assay, we had to consider all together several pragmatic issues, including: first line screening assay, possibility to automation, costs, time span of the assay, a clear and manageable protocol, robustness and reproducibility, etc. We went for the idea of a first line screening assay with a multiplexed read-out to characterize best the hits after the first screening. Further, we needed to decide what has to be screened: a chemical library of small molecule agents to identify lead molecules with modulatory effects on B cells or a siRNA library to identify (new) relevant B cell targets. The strength of siRNA is that it interferes efficiently with the expression of specific genes with limited off-target effects in opposite to small molecules of a chemical library which are rarely well-characterized in specificity and potency. The choice for screening a siRNA library, consequently, implied that we had to look for a human B cell line that is susceptible to siRNA transfection because primary cells are very difficult to transfect. Above all, the human B cell line had to serve as a reliable substitute for the primary B cell. Nonetheless, the use of a cell line is more appropriate to ensure robustness, reproducibility and continuity of the screening as cell lines are more homogeneous and they are easy to cultivate allowing collection of large amounts of cells. The B cell’s versatility of actions and

its ability to adapt in response to changes in the (micro-) environment make the process of B cell-specific drug discovery challenging. There is a plethora of *in vitro* stimulatory conditions for human B lymphocytes described in literature, ranging from a single agent stimulator to complex stimulator-mixtures, each inducing different responses. To maintain as best as possible the robustness and reproducibility of the screening, we screened various elementary B cell stimulators on human polyclonal B cells. TLR9-agonist ODN2006 appeared to have the most extensive stimulatory effect on the human B cells. Since cancerous B cell lines frequently display abnormal Ig and cytokine production, we focussed on the expression of cell surface markers to find a stable substitute for primary B cells. Almost all investigated cell surface markers were receptors with a considerable role in B cell activation, because we searched for targets involved in activation pathways for the development of drugs that can modulate B cell's effector functions. Human Burkitt's lymphoma Namalwa displayed a dynamic immunophenotypic profile upon ODN2006 stimulation with a close resemblance to that of human B lymphocytes. For read out of the screening, we focussed on the expression of cell surface markers CD70 and CD80, because the signalling pathways of these two markers are partially overlapping and partially divergent. That would permit us to detect for common, possibly proximal targets in CD70 and CD80 signalling and for more distal, divergent targets in either CD70 signalling or in CD80 signalling.

To detect (new) B cell targets with the siRNA screening, we first focussed on protein kinases because they are important mediators of signalling cascades for the regulation of the cell's metabolism and their catalytic activity can be manipulated by pharmacological drugs. Other appealing drug targets include lipid kinases, phosphatases, esterases, proteases, ion channels, receptors (G protein-coupled receptors, nuclear hormone receptors), structural proteins, membrane transport proteins and nucleic acids.

Via screening of the lentiviral shRNA human kinase library, we have identified several lethal kinases causing cell death when deleted in expression and various non-lethal kinases with suppressive effect on the up-regulation in expression of CD70, CD80 or both markers.

Twenty-two kinases affected the expression of both CD70 and CD80 and we focussed first on these "candidate target" kinases in order to possibly hit more different pathways and as such yield broader biological effects. For 6 "candidate target" kinases, there was a known chemical inhibitor available and these inhibitors were analysed in several *in vitro* functional assays. OSU-T315, compound described as inhibitor of ILK, potently suppressed the up-regulation in expression of both CD70 and CD80 on Namalwa cells after ODN2006 stimulation and

showed *in vitro* potential as a suppressor of human B cell activity. OSU-T315 actively lowered the *in vivo* IL6-production in common C57Bl6 mice upon TLR9-mediated immunisation, but a dose response effect was not observed as seen *in vitro* with human B cells. Although the data from *in vivo* experimentation with OSU-T315 are quite limited, it would be interesting to test OSU-T315 out in preclinical models, such as for RA (collagen-induced arthritis mouse model), MS (experimental autoimmune encephalomyelitis mouse model) or SLE, to see if it has a therapeutic effect. As there was only a limited stock available of the compound, we could not execute *in vivo* experiments at long(er) term to investigate, for instance, the effects of OSU-T315 on Ig production and to evaluate its mechanism of action, the pharmacokinetic and pharmacodynamic properties and possible toxic side-effects.

We have successfully generated mice with specific deletion of ILK in B lymphocytes. Our goal with this strain was to gain insight about the relevance of ILK in the actions of B lymphocytes within a whole, living organism. Throughout all performed experiments with ILK KO mice, the deletion of ILK did not produce B cell-modulating effects similar to those observed with OSU-T315 *in vivo* and *in vitro* on human B cells. The discrepancy can be explained by the fact that inhibition of a target by means of drugs is not exactly the same as by specific genetic KO. Off-target effects, pharmacokinetic and pharmacodynamic characteristics of small molecule agents can give differences in phenotypic outcome, especially *in vivo* in a whole organism. On the other hand, depletion of a target by means of genetic deletion can induce an alternative "rescue"-pathway to compensate for the absence of the target. Although the data are not yet complete to cover the whole story of ILK and there are differences between *in vitro* and *in vivo* studies and between human and mouse, we can say, based on our obtained data with the ILK KO mice, that ILK may not play an important role in B lymphocyte activity. Or perhaps ILK is not a valid B cell target in mice and other than mouse experiments need to be executed to investigate if ILK blockade may work in humans *in vivo*. Indications of possible side-effects or other actions of OSU-T315 besides blocking of ILK came from the *in vitro* experiment with LPS-stimulated splenic murine B cells wherein it was observed that the inhibitory activity of OSU-T315 on the proliferation and the production of IL6 and TNFα did not differ between ILK WT mice, ILK heterozygote mice and ILK KO mice. Exploration of the mechanism of action of OSU-T315 on the B cell would be interesting to understand its specific target.

Because of the promising data that OSU-T315 gave in the *in vitro* functional assays, our focus in this work went to its target ILK. The atypical catalytic domain of ILK may be

advantageous and prove value for the development of more specific drugs, because most of the described protein kinase inhibitors are directed to the ATP-binding site within the catalytic domain which is strongly conserved in the human kinome. The high degree of homology between protein kinase family members and, additionally, the required high affinities of the inhibitors to compete with the high *in vivo* ATP concentrations leads to a high prevalence of secondary target effects[178,230,231]. Most inhibitors have the potential to inhibit multiple targets (e.g. dasatinib). There is increasing interest in identifying kinase inhibitors that do not compete with ATP.
In earlier studies, ILK was investigated in various organisms and many cell types, but it was not yet studied in B cells. Our work provides first data about ILK in the context of B cells.

The screening of the lentiviral shRNA library resulted in a first categorization of the human protein kinases. A first subdivision is in lethal kinases and non-lethal kinases and the non-lethal kinases can be further categorized in kinases that can suppress either CD70 or CD80, kinases that can suppress both CD70 and CD80 and kinases that have no effect at all. The listing of the kinases for each category was not given for intellectual property reasons. As mentioned earlier, for only a few "candidate target" kinases there was a chemical inhibitor available. So, most kinases from that group could not be further examined for their potential as B cell target through chemical inhibition. The CRISPR/Cas9 technology, which stands for 'Clustered Regularly Interspaced Short Palindromic Repeats' and 'CRISPR-associated nuclease 9'[232], is a quite new tool for targeted genome editing and can provide the solution. Currently, the CRISPR/Cas9 system is regarded as the simplest (making it faster and more cost effective), most versatile and precise method of genetic manipulation with a wide range of applications in many cell lines, organisms and even primary cells and basically consists of two key molecules that introduce a change into DNA: the enzyme Cas9, which acts as a pair of "molecular scissors" that can cut the 2 strands of DNA at a specific location, and a piece of RNA, called guide RNA (gRNA), that 'guides' Cas9 to the right part of the genome[233]. Several huge gRNA libraries have already been created, indicating that the technology is gaining a prominent position in genome engineering. Hence, genetic screening of human B cells by means of the CRISPR/Cas9 technology might be the way to explore further and more profoundly on the role and function of our "candidate target" kinases in human B cell activation. This research can be extended to the other kinase groups (CD70 only, CD80 only and lethal) to obtain a sub-categorization of the kinases.

Also interesting for further refinement of the protein kinase groups would be the use of other read-out parameters. In the discussion of Chapter 4, we postulated a correlation between IgG production and expression of CD86. Maybe CD86 can be a more sensitive parameter to detect targets with a preferably B cell modulatory activity. Unfortunately, the Namalwa cell line is incapable to produce IgG, so the proposed CD86-IgG parallel cannot be explored in more detail by means of the shRNA library screening on Namalwa. Screening on inhibitory receptors can provide more information about the regulatory pathways of B cell activity with the idea of favouring expansion, development and activation of Bregs instead of inhibiting activated effector B cells and that could be an elegant way to control B cell activity in pathological conditions. CD22, CD32b and CD72 act as negative regulators of BCR signalling and hold great potential for therapy of AIDs and for treatment in organ transplantation. CD22, also known as Siglec-2, is a member of the sialic acid-binding immunoglobulin-like lectin (Siglec) family[162] and is exclusively expressed on mature B cells and to a lesser extent on some immature B cells. The humanized monoclonal anti-CD22 antibody epratuzumab is subject of various clinical trials for the treatment of moderate-to-severe SLE[162,234]. Apparently, binding of epratuzumab induces co-localization of CD22 with the BCR, independent of BCR engagement, hereby promoting the inhibitory function of CD22 on BCR-mediated signalling and thereby modulating B cell activity with inhibition of B cell activation, but without total B cell depletion[106]. CD32b or FcγRIIB is expressed on B cells, plasmablasts and long-lived PCs and is the sole inhibitory member of the FcγR family with an important role in the down-regulation of Ab-production[102]. It is noticed that B cells from SLE patients have either a lower expression of CD32b or they express a dysfunctional form of the receptor at an increased frequency[102,103,105]. CD72 was quite recently identified as an inhibitory receptor that is dominantly expressed in B cells[102]. CD72-deficient mice spontaneously develop SLE-like disease (production of autoAbs to various nuclear components and development of immune complex nephritis) and CD72 polymorphism is strongly associated with human SLE, hence, making CD72 a novel meaningful target in the treatment of SLE[162]. The quite unfamiliar CD83 is assumed to be an inhibitory receptor and has gained attention for investigations in B cells and T cells to further explore its mechanism of action for immune regulation. Just like CD22, CD83 is a member of the Siglec-family[235]. Hence, it can be very interesting to see if a deletion of a protein kinase or other target would increase or further decrease the expression of inhibitory receptors, such as CD22, CD32b, CD72 and CD83, indicating a more regulatory status or a hyper-active status, respectively.

Counter-screen(s) on human cell lines derived from non-B cells, for instance T cells, macrophages and dendritic cells, upon appropriate stimulatory conditions and with relevant read-out parameters can further determine the B cell specificity of the identified protein kinases. The group of “lethal” kinases should be included in the additional (counter-) screens to reveal new B cell (or even malignancy)-specific targets.

Full exploitation of our developed screening assay can lead to the identification of B cell targets that, consequently, stimulate the development of original drugs.

Chapter 7 **Summary**

It becomes more and more clear that B lymphocytes play an important pathogenic role in various immune mediated disorders as well as in transplant rejection. Still to date, there are not many B cell-specific immunosuppressive drugs available.

The present study was undertaken first, to identify an *in vitro* B lymphocyte stimulation assay able to optimally and most broadly reproduce polyclonal human B cell biology and, next, to use it to detect new B lymphocyte specific targets allowing for the discovery of new B lymphocyte specific small molecule drugs.

From 13 different known *in vitro* B cell stimuli, it was found that stimulation with the TLR9-agonist ODN2006 resulted in the clearest and most broad *in vitro* activation of polyclonal human B cells as measured with various criteria. Human Burkitt's lymphoma Namalwa was selected as a more homogeneous substitute for primary B cell and the expression of cell surface markers CD70 and CD80 was chosen as read-out.

Next the ODN2006/Namalwa assay was used to identify potential, new B lymphocyte drug targets by selectively eliminating 501 different human protein kinases using a lentiviral library carrying interfering RNA able to functionally "knock-out" the various protein kinases in the Namalwa cells. The whole screening identified 60 protein kinases involved either in the CD70 pathway or in the CD80 pathway as well as 22 other kinases suppressing both cell surface marker pathways. From none of the latter 22 kinases, a role in B cell activation has been described until today. For only 6 of them, a small molecule inhibitor of the respective kinase activity was available, but only one of these compounds showed significant *in vitro* effects on the activation of Namalwa cells as well as of polyclonal human B cells. OSU-T315 showed potential as suppressor of human B cell activity and, hence, the focus was laid on this compound and its target ILK.

Mice with B cell-specific ILK depletion have been successfully generated and were of great value for investigating the role and function of ILK in the B cell biology of the living organism. The exploratory *in vivo* knock-out experiments may not have confirmed ILK's potential function in B cell activation, but can motivate for further investigation of ILK and of all the other categorized kinases. Exploration of the "candidate target" shortlist is still ongoing.

Chapter 8 **Samenvatting**

Het wordt meer en meer duidelijk dat B lymphocyten een belangrijke rol hebben in de pathogenese van verscheidene immuunziekten en in de afstoting van getransplanteerde organen. En toch, tot op heden zijn er maar een beperkt aantal immunosuppressiva beschikbaar die gericht zijn op de B lymphocyten.

Deze studie was ondernomen, ten eerste, om een *in vitro* B lymphocyte stimulatie-test te vinden die zo optimaal en ruim mogelijk de biologie van polyclonale humane B cellen weergeeft en, vervolgens, om deze stimulatie-test te gebruiken voor detectie van nieuwe B cel-specifieke doelwitten die kunnen bijdragen tot de ontdekking van nieuwe B cel-specifieke, moleculaire geneesmiddelen.

Van 13 verschillende *in vitro* B cel stimuli bleek stimulatie met de TLR9-agonist ODN2006 te resulteren in de meest uitgesproken en meest algemene *in vitro* activering van polyclonale humane B cellen zoals het was gemeten met verscheidene criteria. Humane Burkitt's lymphoma Namalwa was geselecteerd als meer homogene vervanger van de primaire B cel en de expressie van celoppervlakte merkers CD70 en CD80 was gekozen als read-out.

Vervolgens werd de ODN2006/Namalwa test gebruikt om potentiële, nieuwe B lymphocyte drug doelwitten te identificeren door 501 verschillende humane proteïne kinasen selectief uit te schakelen in de Namalwa cellen met behulp van een heuse bibliotheek van lentivirale partikels met interfererende RNA moleculen. Het screenen van de hele bibliotheek identificeerde 60 proteïne kinasen die betrokken zijn in ofwel de CD70 pathway ofwel in de CD80 pathway, maar ook 22 proteïne kinasen die beide celoppervlakte merkers onderdrukten.

Van die laatst genoemde 22 kinasen was er tot dan toe geen rol in de B cel activering beschreven. Voor 6 kinasen was er een klein moleculaire inhibitor beschikbaar, maar slechts één van deze inhibitoren beïnvloedde aanzienlijk de *in vitro* activering van zowel Namalwa cellen als polyclonale humane B cellen. OSU-T315 toonde potentieel als onderdukker van humane B cel activiteit en, aldus, de aandacht werd gevestigd op deze inhibitor en zijn doelwit ILK.

Muizen met een B cel-specifieke uitschakeling van ILK werden succesvol gecreëerd en waren waardevol voor het onderzoek naar de rol en functie van ILK in de B cel biologie van het levend organisme. De explorerende *in vivo* knock-out experimenten hebben misschien niet de potentiële functie van ILK in de B cel activering geverifieerd, maar kunnen de motivatie

vormen voor verdere exploratie van ILK en van al de andere gecategoriseerde kinasen. Het onderzoek van de “kandidaat doelwit” lijst is lopende.

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Curriculum vitae

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Thesis: "First analysis of mice with liver-specific elimination of N-WASP (Alb-Cre N-WASP$^{flox/flox}$) mice." (Promotor: Dr. Jolanda Van Hengel)
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Scientific publications

K. Van Belle, J. Herman, L. Boon, M. Waer, B. Sprangers & T. Louat. "Comparative *in vitro* immune stimulation analysis of primary human B cells and B cell lines." Journal of Immunology Research, vol. 2016, Article ID 5281823, 2016. doi:10.1155/2016/5281823.

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Congresses

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