Vitamin A in the interplay between ROS, autophagy and immunoglobulin production in normal B cells and B cells from patients with the immunodeficiency disorder CVID

Master thesis by
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Abstract

Background and aims: Common variable immune deficiency (CVID) is an immune disorder characterized by recurrent infections, defective B cell functions and low production of immunoglobulins (Igs). CVID-derived B cells frequently have a low response to stimulation via toll like receptors such as TLR9 and RP105, and the patients often have low levels of serum vitamin A levels. TLR9/RP105-mediated IgG production in normal B cells requires autophagy, and the vitamin A metabolite retinoic acid (RA) can augment IgG production by enhancing this autophagy. Recent research has highlighted the importance of NOX2-induced ROS as a signal transducer in B cells, and ROS has also been implicated in autophagy in various cell systems. The aims of the present thesis were: 1) Investigate a possible interplay between ROS, autophagy and Ig secretion in normal B cell stimulated via TLR9 and RP105; 2) Explore whether dysregulation of ROS and/or autophagy might contribute to the low production of IgG in B cells from CVID patients; 3) Reveal the role of RA in the interplay between ROS, autophagy and Ig production both in normal- and CVID-derived B cells.

Methods: CD19+ B cells were isolated from buffy coats or whole blood collected from CVID patients and healthy controls. The B cells were stimulated via TLR9 and RP105 in the presence or absence of RA. The levels of ROS and autophagy in the stimulated B cells were measured by flow cytometry. ELISA assays were performed to quantify Ig secretion. The results from these assays were combined to analyze for co-variations in ROS levels, autophagy and Ig secretion in normal- and CVID-derived B cells.

Results: TLR9/RP105-mediated stimulation of normal B cells increased the levels of ROS, autophagy and Ig secretion. We found a significant positive correlation between ROS and autophagy in stimulated B cells from healthy donors ($r=0.472$, $p<0.05$), but not between Ig secretion and levels of either ROS or autophagy ($p>0.05$). However, NOX2-induced ROS was found to be essential for both autophagy and Ig secretion in the normal B cells, as the inhibitor significantly reduced these levels ($p<0.05$). There was a general tendency of lowered levels of autophagy in stimulated B cells derived from CVID patients compared to normal B cells ($n=13$, $p=0.064$). In a subgroup of CVID patients, the autophagy levels were significantly reduced ($p<0.05$). The levels of ROS in CVID-derived B cells did not differ from the levels in normal B cells ($p<0.05$), but we found aberrant responses to NOX2 inhibition on ROS levels or autophagy in B cells from three of the CVID patients. RA

enhanced the levels of autophagy and Ig secretion in TLR9/RP105-stimulated B cells from both CVID patients and healthy controls, but the effects of RA was generally lower in the CVID B cells ($p<0.05$). RA did not affect the TLR9/RP105-induced ROS generation in neither normal- nor CVID-derived B cells ($p>0.05$).

Conclusion: We observed a positive correlation between ROS levels and autophagy in normal TLR9/ RP105-stimulated B cells and revealed that NOX2-induced ROS is important for both autophagy and Ig secretion in these cells. The ROS levels were not dysregulated in CVID-derived B cells. However, in TLR9/RP105-stimulated B cells from a subgroup of CVID patients characterized by more adverse disease, we revealed that reduced levels of autophagy was associated with low IgG production. RA did not enhance the ROS levels in TLR9/RP105-stimulated B cells, but enhanced autophagy and Ig secretion in B cells from both CVID patients and healthy controls.

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Abbreviations

ADH	Alcohol dehydrogenase
AID	Activation-induced cytidine deaminase
BCR	B cell receptor
BLIMP1	B lymphocyte-induced maturation protein-1
CpG-ODN	CpG oligodeoxynucleotide phosphorothionates
CRAB-II	Cellular retinoic acid binding protein II
CRABP-II	Cellular retinol-binding protein type II
CSR	Class switch recombination
CVID	Common variable immunodeficiency
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbed assay
ESID	European Society for Immunodeficiency
FBS	Fetal bovine serum
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IRF4	Interferon regulatory factor 4
LPS	Lipopolysaccharide
LRAT	Lecithin:retinol acyl transferase
LXR	Liver X Receptor
MHC	Major histocompatibility complex
μM	Micro molar
MyD88	Myeloid differentiation primary response 88
NAC	N-Acetyl Cysteine
NF-κB	Nuclear factor kappa B
nM	Nano molar
PAMP	pathogen-associated molecular patterns
PBS	Phosphate-buffered Saline
PKC	Protein kinase C
PRR	Pattern-recognition receptors
PS	Pencilin streptomycin
RA	Retinoic acid
RAE	Retinol activity equivalent
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoid acid response element
RBP	Retinol binding protein
REK	Regional Committee for Medical and Health Research in South Eastern Norway
ROH	Retinol
ROS	Reactive oxygen species
RP105	Radioprotective 105
RXR	Retinoid X receptor
SOD	Superoxide dismutase
SHM	Somatic hyper mutation
TD	T dependent
TI	T in dependent
TIR	Toll/interleukin-1 receptor
TLR	Toll like receptor
TMB	3.3´.5.5´-tetramethylbenzidine
TNF	Tumor necrosis factor

TTR	Transthyretin
x g	Times gravity

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1. Introduction

1.1 The immune system

The immune system is a host defense system developed to protect us against foreign and potentially harmful pathogens, and it is specialized to recognize and separate our own from foreign cells and tissue (1). In addition, the immune system has an important role in removing dead or damaged cells as well as defeating cancer cells (1).

The immune system is divided into the innate and adaptive immune system. Innate immunity is present from birth and is regarded as an unspecific and generic defense system. Adaptive immunity evolves as we are exposed to pathogens, and it forms the specific part of an immune response. Although these two parts of the immune systems have distinct roles, there are important bridges between the two systems (2). The tissue of the immune system are divided into primary and secondary lymphoid organs. Primary organs are the bone marrow and thymus, and are sites for the development of lymphoid cells. The secondary lymphoid organs are anatomic sites for coordination of the adaptive immune response and includes the spleen, lymph nodes and Peyer patches (2).

1.1.1 The innate immune system

The unspecific, generic part of the immune system includes multiple layers of defense. The first line of defense is the physical barrier that covers our body, including the skin and mucosal linings. The second line of defense includes immune cells such as dendritic cells, monocytes, macrophages, granulocytes and innate lymphoid cells that circulates the blood and the lymph system (3). Whereas dendritic cells are important for presenting antigens to the adaptive immune cells, macrophages and neutrophilic granulocytes are able to directly eliminate pathogens by performing phagocytosis (4, 5). Phagocytes and dendritic cells express receptors called pattern-recognition receptors (PRR) (6). PRR recognize evolutionary conserved molecular structures called pathogen-associated molecular pattern (PAMPs) that are typical for pathogens. PAMPs include structures like nucleic acids from DNA and RNA, as well as proteoglycans from the cell wall of bacteria (6). Activation of the receptor triggers the induction of cytokines and chemokines forming an immune response cascade (6). PRRs are also expressed on cells of the adaptive part of the immune system, as will be described later in this introduction.

1.1.2 Toll like receptors

One group of PRRs include Toll-like receptors (TLRs). So far, we know of 10 different TLRs (TLR1-10) in humans (7). The TLRs can form homodimers or heterodimers, and they can either be expressed on the cell surface or be associated with intracellular vesicles (8). TLR1-2, and TLR4 -6 are expressed on the cell surface and typically recognize bacterial membrane compounds, whereas the intracellular TLR3 and TLR7-9 recognize bacterial and viral nucleic acids, as well as DNA from dead cells (7). Activation of the receptors initiate an immune response (9). TLRs are expressed on cells of the innate immune system, as well as on B cells (10) and therefore TLRs are described to bridge the innate and the adaptive immune system. TLR activation leads to maturation of dendritic cells, and activation of autophagy that enhances their antigen presentation capacity (8, 11).

The TLRs are type 1 integral membrane glycoproteins consisting of three domains. The first is an extracellular N-terminal domain with leucine-rich repeats (LRRs) which recognize PAMPs (12). The second is the transmembrane domain, whereas the third is the intracellular C-terminal domain, known as the Toll/IL-1 receptor (TIR) domain. The TIR domain is essential for downstream signaling by recruiting cytosolic adaptor proteins, such as MyD88 (12) (see Figure 1). MyD88 initiates transcription of pro-inflammatory cytokines and chemokines and is a central player for downstream signaling for all TLRs, except TLR3 and TLR4 (12, 13). NF-kB is one of the key factors activated by MyD88 downstream of several TLRs (12, 13) (see Figure 1).

TLR9

Certain dendritic cells and macrophages, as well as B cells, express TLR9 in the membrane of their endosomes and endolysosomes (8). The physiological ligand for TLR9 is microbial DNA rich in unmethylated CpG motifs (14). For *in vitro* studies, the cells are cultured with CpG-oligodeoxynucleotides (CpG-ODN). The immune responses elicited by TLR9 are mediated via MyD88 (Figure 1). CpG-ODN is taken up by the cell via endocytosis. TLR9 is cleaved in the endolysosomes, and the cleaved form of TLR9 recognizes CpG-ODN that triggers MyD88-mediated activation of NF-κB, p38 and mitogen-activated protein kinases (MAPKs) (15). This results in induction of pro-inflammatory cytokines (14).

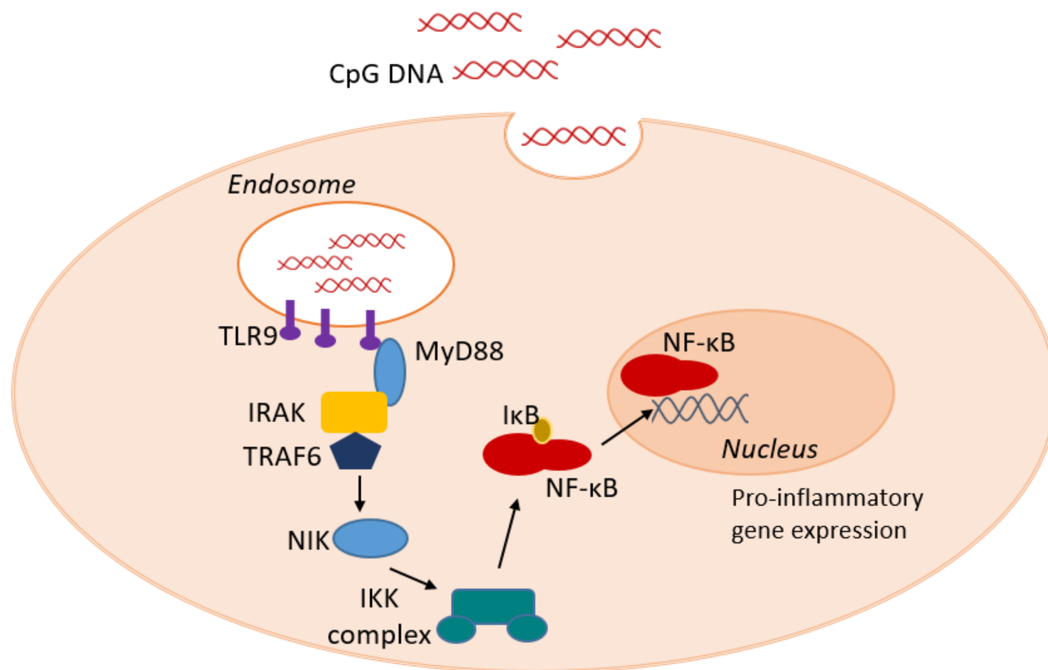

Figure 1: Signaling pathway downstream of TLR9 in B cells. CpG-ODN interact with TLR9 in endosomes and activate pathways leading to initiation of transcription mediated via NFκB.

RP105

The TLR-homolog, radioprotective 105 (RP105) and also known as CD180, belongs to the group of TLRs. RP105 is expressed on the surface of mature B-cells, but also on dendritic cells and macrophages (16). The N-terminal PAMP-recognition domain and transmembrane domain are similar to the TLRs, but they lack the C-terminal TIR domain (17). The physiological ligand of RP105 is not yet identified, but for *in vitro* studies, the cells are stimulated with antibodies directed against RP105.

1.1.3 The adaptive immune system and B cells

In contrast to the innate immune system, the adaptive immune system is specialized and specific. An important feature of the adaptive immune system is the memory of previous exposure to antigens. The immune response of the adaptive immune system is divided into the primary and secondary response, depending on whether it is the first encounter or not (1). Upon the first encounter with a pathogen, the B cells are activated and differentiated into antibody-secreting plasma cells, primarily secreting IgM (18). When the pathogen re-enters the body, the threshold for activation is lower, and the response is faster and stronger (18). A more efficient secondary immune response is then initiated, resulting in the production of IgG

or other classes of immunoglobulins as IgA and IgE (19). The more rapid secondary response is mediated by the memory cells generated during the primary immune response (20, 21).

B cells (*b*rusal or *b*one marrow-derived) are responsible for the humoral immunity of the adaptive immune system by secreting antibodies. The production of antibodies is considered as the most important role of B cells, but B cells are also crucial for antigen presentation and cytokine production (22). When located in the cell membrane, antibodies serve as the B cell receptor (BCR). Stimulation of B cells into antibody-secreting plasma cells may require help from T cells (*t*hymus-derived) (21). T cells are important for the cellular part of the adaptive immune system (23).

B cells are not only important for the adaptive immune response. As they express TLRs, they are also able to directly mediate microbial destruction (24). Therefore, B cells are important links between the innate and the adaptive immune system (25). B cells originate from hematopoietic stem cells in the bone marrow and leave the bone marrow as immature B cells (21). Immature naïve B cells express IgM on their surface. The B cells further mature in the secondary lymph organs, and when the B cells co-express IgM and IgD they are termed mature (26). Upon encounter with an antigen the B cell is activated, and dependent on the signal, the B cell may differentiate into either plasma cells or memory B cells (19). For the B cell to express Igs like IgA, IgE or IgG, the B cell undergoes somatic hypermutation and class-switching, and this takes place in germinal centers (GS) within secondary lymphoid organs (27). The differentiation of B cells into the antibody-secreting cell is a dramatic process with great changes in the metabolism and cellular structures to enable high Ig production (28, 29). Upregulation of several transcription factors such as interferon regulatory factor 4 (IRF4) and B-lymphocyte-induced maturation protein 1 (BLIMP1), and the class-switching enzyme activation-induced cytidine deaminase (AID) is essential for the development into an antibody-secreting plasma cell (20, 30). Approximately 50% of memory B cells remain non-switched and express IgM on the cell surface, whereas the other half express and secrete other isotypes of Igs (31).

Activation and differentiation of B cells

Activation of B cells mainly occur in secondary lymphoid tissues. As mentioned, activation of B cells often requires help from T cells and results in a T cell-dependent (TD) activation. However, stimulation of B cells can also be T cell-independent (TI). The B cells can be activated both via an antigen-specific BCR and via PRRs (32). Most antigens, such as proteins and glycoproteins, stimulate B cells by a TD process, and T cell help is required for

maximal antibody production. TI-stimulation of B cells can be divided into two categories; TI-1 and TI-2. TI-1 typically involves PAMP-mediated activation, such as LPS-mediated activation of TLR4 and bacterial DNA-mediated activation of TLR9, whereas TI-2 involves cross-binding of the BCR, typically by polysaccharides (33). If the stimuli signal is sufficient for activation, a cascade of signaling events will lead to differentiation and proliferation of the B cell (34). TD activation of B cells generally leads to antibody production with higher affinity to the antigen, whereas a TI response is often more rapid (35). Both TI- and TD activation results in memory lymphocytes and plasma cells, but with different life-spans (36).

For a B cell to differentiate into an IgG-producing plasma cell, several factors are required. The cytokine IL-10 is one of the key factors, and it is known to have an anti-inflammatory effect by stimulating humoral immunity and inhibit cell-mediated immunity (37-39). Endogenous IL-10 leads to proliferation and differentiation of activated B cells, as well as inducing isotype switching and increasing Ig production (40). Both mature B cells and T helper cells produce IL-10 (40).

TLR9/RP105-mediated stimulation of B cells

B cells express different PPRs, such as TLR1, TLR6-10, and RP105. TLR9 may bridge the adaptive and innate immune systems by allowing the interaction between TLR9 and the B cell receptor (BCR) in endosomes, and thereby facilitating the adaptive immune response (41). In naïve B cells, the expression of TLR9 is low, but the expression is up-regulated upon activation of the cells. Memory B cells express TLR9 at constitutively high levels, and also have a stronger response to CpG-DNA (8, 42). Activation of B cells via TLRs contribute to proliferation and differentiation into plasma cells and memory B cells (43-45).

Like TLR9, RP105 is expressed at higher levels in memory B cells than in naïve B cells (42). Activation via RP105 alone have only small effect on proliferation and Ig secretion (42). Activation of B cells via RP105 was originally found to protect the cells against DNA damage-induced apoptosis (46). However, our group has previously shown that that activation of RP105 enhances TLR9-mediated responses such as proliferation and Ig secretion (16). This synergy initiates activation of Akt and NF-κB. Activation of Akt enhances cell survival and growth, whereas activation of NF-κB inhibit apoptosis and therefor also prolongs cell survival (42).

1.2 Common variable immune deficiency

There are more than 300 different immunodeficiency disorders, and they are the result of impaired development and/or function of the immune system. To be considered as primary immunodeficiency, the disease must not be the result of secondary causes such as other diseases like HIV/AIDS, drug treatment, malnutrition or exposure to toxins (47). Most of the primary immunodeficiency disorders are inborn genetic disorders, and many of them are hereditary (48). The prevalence of primary immune deficiencies is unknown, as they are continually being discovered. Common variable immune deficiency (CVID) is the most commonly occurring primary immune disorder. It is a heterogeneous disease characterized by decreased immunoglobulin production, in particular of IgG and/or IgA, with increased susceptibility to infections (49). According to the diagnostic criteria established by the European Society for Immunodeficiency, the patients often respond poorly to vaccines. Other causes of hypogammaglobulinemia must be excluded (50).

The relative prevalence of CVID globally is estimated to be between 1:50 000 and 1:10 000 (51, 52), whereas the prevalence in Norway is 1:25 000, approximately 200 people (50). The onset of the disease is most often between 20 and 40 years of age (50). The etiology of CVID is still unknown. However, recent sequencing studies show that monogenic dysfunction accounts for 10% of the cases (52). So far, more than 20 genes have been associated with CVID (53, 54). The most common monogenic cause of CVID is a mutation in the gene that encodes NF-κB (47, 52).

CVID is not a curable disease. Today the most common treatment is antibody replacement therapy, in addition to symptomatic treatment of their infections and other complications (55). Although the main cause of morbidity among CVID patient is infections (51), the life expectancy of patients with CVID may be further reduced by non-infectious complications such as autoimmune diseases and cancer.

Sub-classification of CVID patients

At Oslo University Hospital, Rikshospitalet, CVID patient are sub-classified based on immunological parameters of B and T cells (56, 57), and the patients are also given a phenotype according to the severity of the disease. This is done to improve treatment, prognosis and understanding the etiology of the disease (54, 58). A low number of B cells indicates that there is an early defect in peripheral B cell differentiation, whereas the low number of switched B cells indicates that it is a defect in germinal center dependent B cell memory formation (59), making number of class-switched B cells a sensitive marker for

sufficient germinal center function (59). High percentages of CD21^{low} B cells and transitoric B cells are associated with an inflammatory phenotype, as is also low levels of class-switched memory B cells (59). Increased numbers of CD21^{low} B cells also appear to correlate with autoimmunity (60) as well as granulomatous disease (59).

Based on the severity of the disease, the patients are divided into phenotype 0 or 1. Patients that experience only infections are classified as phenotype 0. The other group of patients, that in addition to infections also have other complications such as autoimmunity, are grouped as phenotype 1. This latter group is also called the “complication-group”.

CVID and B cells

About 90% of all CVID patients display a near normal number of peripheral B cells, suggesting that the defect often occurs in the later stages of B cell differentiation (60). The main problem of CVID-derived B cells is first of all their impaired ability to produce IgG and/or IgA. Reduced numbers of switched memory B cells and plasma cells are frequently observed (44, 61).

A common feature of CVID-derived B cells is the reduced response to *in vitro* activation of TLR9 and RP105 resulting in reduced proliferation, differentiation, and Ig production compared with normal B cells (44, 52). CVID-derived B cells are also shown to express lower levels of intracellular TLR9 than normal B cells (62). The activation of TLR9 is dependent on STAT3, and STAT3 phosphorylation is shown to be impaired in B cells derived from a subgroup of CVID patients (62). STAT3 activation by TLR9 is dependent on NF-κB, and as previously mentioned, a mutation in NF-κB is the most common monogenic defect among CVID-patients (52).

Our group has previously shown that physiological concentrations of the vitamin A metabolite retinoic acid (RA) can correct several of the compromised features of CVID-derived B cells, such as TLR9/RP105-mediated proliferation and IL-10 production (15, 63). However, IgG production from the stimulated CVID-derived B cells was generally only marginally enhanced by RA (30, 63). CVID-derived B cells are shown to have lowered levels of the transcription factor IRF4, as well as of the switching factor AID, and this could explain the reduced isotype-switched Ig in CVID-derived B cells (30).

1.3 Autophagy

The term autophagy origins from the Greek words for “self-eat”. It is a process where lysosomes degrades cytoplasmic contents like organelles and misfolded proteins, and also foreign pathogens (64). There are different modes of autophagy; either chaperone-mediated, microautophagy or macroautophagy (11). Hereafter, autophagy is referring to macroautophagy.

Autophagy is initiated by the formation of an isolation membrane, called phagophore. The phagophore expands end encloses the material destined for degradation. Eventually, the isolation membrane closes and forms an autophagosome that further fuses with a lysosome for degradation of it’s content (65).

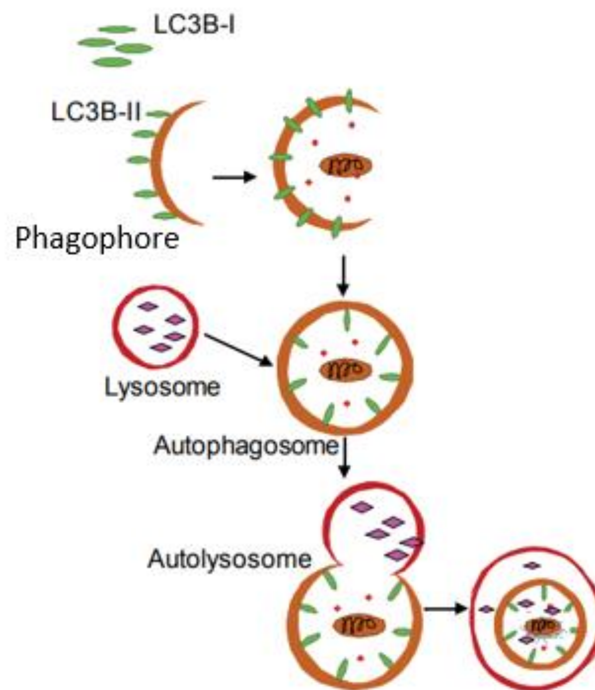

Figure 2: The process of autophagy. Starting with the initiation of an isolation membrane that further develops into an autophagosome that fuses with the lysosome to degrade it’s content. The LC3 protein localizes to the autophagosome membrane and is a common marker for autophagy.

Triggers of autophagy are inter- and extracellular stress, such as starvation, ER stress, pathogen infection, and growth factor deprivation (66). The autophagic process is essential for recycling of cellular components to promote new building blocks for the stressed cell (66), and it also functions as cellular quality control by being a selective degradation process (64, 67). Autophagy allows the cell to reutilize their own constituents for energy (68), and is generally regarded as a cell survival mechanism (11). It is also crucial for the development and differentiation of various cell types, including lymphocytes (69). A constitutive basal

level of autophagy is essential for metabolic and cellular homeostasis, and hence dysregulated autophagy may cause disease.

Autophagy depends on the expression of more than 30 different proteins, known as ATG proteins. The formation of the autophagosomes is driven by factors such as Beclin1, ULK1, and LC3 proteins (11) (see Figure 2). ULK1 is required for the initiation of the phagophore (70). The function of LC3 is to aid in the expansion and the closure of the phagophore membrane, and LC3 is translocated from the cytosol to autophagosomal membranes during the process (71). The identification of lipidated LC3 (also called LC3II) in the plasma membrane is therefore a widely used marker of autophagy.

1.3.1 Autophagy and the immune system

Autophagy seems to be critical for proper immune function in several ways. It is directly involved in elimination of microorganisms, controlling of inflammation, ensuring proper secretion of immune-mediators, regulating antigen presentation, as well as regulation of lymphocyte homeostasis (11). When the cell directly eliminates the microorganism by engulfing invading bacteria, the process is called xenophagy (72, 73).

PAMP-mediated activation of TLRs may promote autophagy and thereby prevent inflammation (11). Intracellular TLR signaling can promote phagosome-lysosome fusion for autophagic degradation of the pathogen, as well as upregulation of antimicrobial activities such as NOX2 derived ROS (11). Autophagy also delivers exogenous antigens from MHC class-II to enhance the adaptive immune system, and this process is important for maturation of T cells in the thymus to prevent autoimmunity (11).

Autophagy and B cells

Autophagy is essential for certain stages of B cell differentiation (19, 69). Whereas the absence of autophagy in early stages of lymphoid differentiation limits the survival of B cell precursors (69), autophagy seems to be unessential for the survival of resting mature B cells (73). Still, it is clear that autophagy plays a significant role in regulation of plasma cell differentiation (74, 75). It has been shown that autophagy is required for sustaining Ig production in plasma cells by limiting the ER-stress associated with the production and secretion of high levels of proteins (76). Autophagy maintains the ATP levels and thereby ensures the survival of Ig-secreting plasma cells (73). The basal level of autophagy is higher in memory B cells compared to naïve and GC B cells (19), and our group has previously shown that TLR9/RP105-mediated activation of B cells triggers autophagy (77).

1.4 Reactive oxygen species

Reactive oxygen species (ROS) are reactive molecules and free radicals derived from molecular oxygen, and with an unpaired electron in the outer shell (78). Most of the cellular ROS are produced as a byproduct of inefficient electron transfer during oxidative phosphorylation in mitochondria (79, 80). ROS has traditionally had a bad reputation associated with cell damage and is responsible for cell aging, apoptotic death, and oxidative stress. These features hold true for high levels of ROS (80). On the other hand, it is clear that ROS is required for normal development and proliferation (79). Low to intermediate levels of ROS are important as signal transducers. ROS can oxidize various signaling molecules and thereby edit post-transitional modifications of proteins that directly initiate signaling (81). One example is direct oxidation of cysteine in the active site of phosphatases (82). In addition, low concentrations of ROS protect the cells against oxidative stress and keeps up the redox-homeostasis by inducing the anti-oxidant system (78, 83, 84). How the ROS signals are interpreted by the cell, depends on the source, cell type and tissue environment (79). One of the main targets of ROS signaling is the transcription factor NF-kB (85) (see Figure 3).

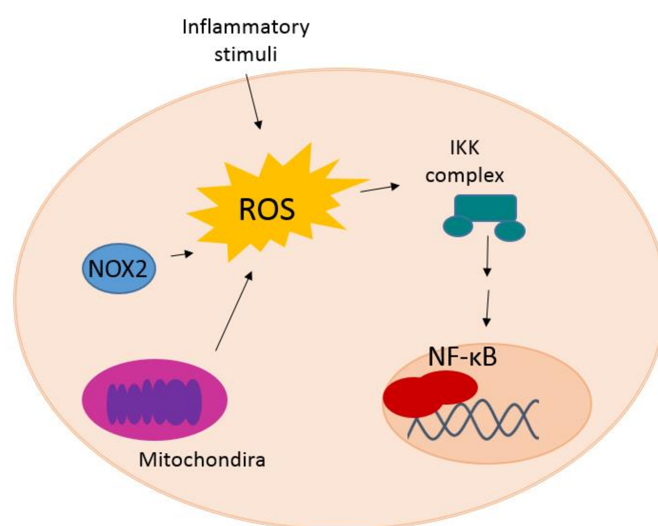

Figure 3: Illustration of ROS signaling resulting in activation of NF-κB. ROS origin from different sources, and one target for ROS is activation of NF-κB.

The major source of intracellular ROS are the mitochondria, but also other organelles as peroxisomes and ER produce ROS. Moreover, there are also enzymes that are responsible for production of ROS from oxygen called NADPH oxidase (NOX) (86, 87). The NOX-family enzymes are membrane-bound enzymes with seven unique members (24, 88). The

main NOX in lymphoid cells is NOX2 (86). NOX-derived ROS have been implicated in the regulation of cytoskeletal remodeling, gene expression, proliferation, differentiation, migration, and cell death. These processes are tightly controlled and reversible (89). NOX2-derived ROS is crucial in bacterial killing in phagocytic cells as neutrophil granulocytes and macrophages, but also B cells are shown to be able to directly mediate microbial destruction (24, 90).

Dysregulation NOX-derived ROS are associated with different chronic diseases, like atherosclerosis, hypertension, cancer and Alzheimer's disease (85). Chronic granulomatous disease a severe immunodeficiency caused by mutations in the NOX2 complex (91). A few rare inherited conditions, hypothyroidism, are also characterized by oxidative stress due to overproduction of NOX-induced ROS (85).

1.4.1 Reactive oxygen species and B cells

As already mentioned, ROS is vital for immune signaling (78). Activation of B cells via BCR or TLRs stimulates NOX2 to produce regulated levels of H_2O_2. As NOX2 is located in the exofacial side of the plasma- and ER membranes, H_2O_2 are transferred across the membrane by aquaporin 8 to exhibit the signaling effects (86) (see Figure 4).

ROS levels in stimulated B cells may derive from two different sources; NOX2-generated ROS and ROS that probably originates from mitochondria due to enhanced metabolism (92). The early NOX2-induced ROS are thought to have a signaling role, whereas the later burst might be the result of stress associated with increased Ig production. The early intracellular production of H_2O_2 has been shown to facilitate B cell proliferation (93), and the differentiation of B cells into plasma cells seems to require a finely tuned redox balance (29, 93, 94). Prolonged ROS production maintains activation of signaling molecules such as NF-kB and AKT (81)

The antioxidant N-acetylcysteine (NAC) has been reported to attenuate the activation and proliferation of B cells stimulated via BCR (87), and thereby NOX inhibitors have been reported to impair both activation and differentiation of B cells (95).

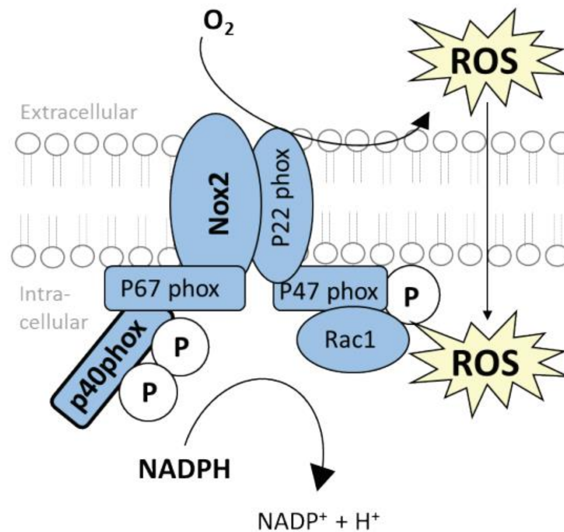

Figure 4: NOX2 enzyme complex.

1.4.2 The interplay between ROS and autophagy

There is a complex interplay between ROS and autophagy in different cell types, and ROS will often result in activation of autophagy (87). Starvation-induced autophagy is for instance shown to be mediated by ROS via oxidation of ATG4 (87, 96-98). In turn, autophagy serves as a cytoprotective negative feedback mechanism to selectively eliminate ROS (11, 19, 79, 96).

Among the different types of ROS, the superoxide is thought to be particularly important for the regulation of autophagy (98). The levels of superoxide are mainly regulated by the enzyme superoxide dismutase (SOD), that catalysis the partitioning of superoxide into either oxygen or hydrogen peroxide. Inhibition of SOD activity *in vitro* results in the induction of autophagy, probably due to enhanced superoxide levels (98).

ROS may promote autophagy through different pathways, such as the mTORC1 complex, AMPK complex, the PIK3C3 complex or via NF-κB (87, 96). NF-κB will, in turn, upregulate genes essential for autophagy (87).

1.5 Vitamin A

Vitamin A was first discovered as an essential fat-soluble factor in 1913. Later, it has been revealed that vitamin A is essential for many different biological processes such as vision, maintenance of the epithelial surfaces and mucus secretion, reproduction and cellular differentiation (99). Vitamin A deficiency is associated with higher rates of morbidity, and in the 1920's vitamin A was named the "anti-infective vitamin" after realizing the importance of vitamin A for a well function immune system (100).

Vitamin A is the collective name of all compounds that exhibit the biological activity of retinol (101). The term "retinoids" includes both naturally occurring vitamin A and synthetic analogs of retinol, with or without biological activity (102).

Animals and humans do not have the capacity for *de novo* synthesis of vitamin A, and therefore a dietary intake is essential. In the diet, vitamin A exists either as provitamin A in the form of carotenoids from plant sources or as preformed vitamin A in the form as retinyl esters from animal sources. Plants and microorganisms synthesize carotenoids that are the natural yellows, orange, red or purple pigments in colorful vegetables, fruits and flowers (103). The preformed vitamin A is mainly obtained from animal sources such as dairy products, fish, eggs, and cod liver oil (101, 103). The intake from animal sources accounts from 25 to 75% of the total vitamin A intake in a typical western diet (104). The daily requirement of vitamin A varies with age and sex, but for adults, it is in the range for 700-900 RAE (retinol activity equivalents) (104-106). RAE is the international unit for vitamin A, and 1 RAE equals the activity of 1 µg retinol (103).

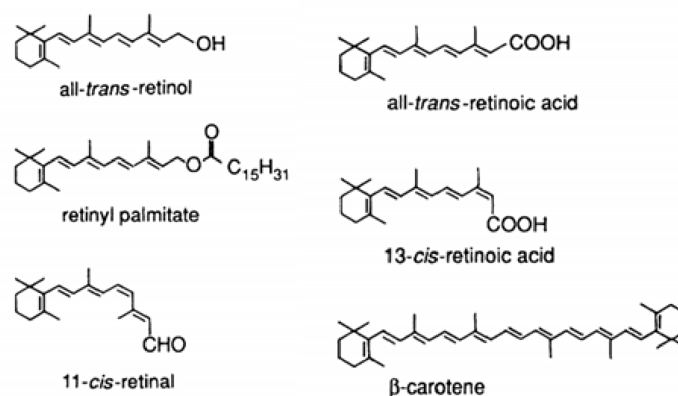

Figure 5: Structural formulas of β-carotene and some retinoids (101).

1.5.1 Deficiency and toxicity of vitamin A

The vitamin A status is estimated by measuring the plasma retinol concentration. Concentrations below 0.7 µmol/L indicates vitamin A deficiency (VAD), whereas a concentration below 0.35 µmol/L is considered as severe VAD (107). VAS is a health problem in some parts of the world, especially in developing countries with a diet primarily based on plants. WHO considers VAD a moderate to a severe health problem (108). VAD is one of the leading causes of preventable blindness among children (107), and it is also associated with an increased risk of severe infection and infection-related deaths (101). Approximately 124 million children suffer from vitamin A deficiency (109), and it is

estimated that 1-2 million deaths each year of children in the age 1-4 year could be prevented by ensuring a proper vitamin A status (110). In addition to infections and ocular symptoms, VAD is associated with anemia, impaired tissue function, as well as insufficient cell development and growth (107). As infections impair the absorption of vitamin A as well as increase its metabolism, there is a vicious cycle between VAD and infections (107). Interestingly, CVID patients are known to have low serum levels of vitamin A (111). Whether or not this is due to the high infection rate in these patients is not established, but it is currently the leading hypothesis.

Vitamin A is a fat-soluble compound, and excess vitamin A is stored in the liver. High dietary intake of vitamin A or intake of drugs containing large amounts of certain retinoids, may lead to hypervitaminosis. A daily intake even marginally above the recommendations are associated with embryonic malformations, reduced bone mineral density and therefore also an increased risk for hip fractures (103).

1.5.2 Vitamin A metabolism

Vitamin A is fat-soluble, and the absorption into enterocytes and subsequent formation of chylomicrons are therefore facilitated by dietary fat (112, 113). All retinyl esters of vitamin A and some carotenoids are converted to retinol before entering the enterocytes (101). Retinol enters the enterocytes by carrier-mediated transport, but it can also be taken up by passive diffusion - depending on the dosage (103). Inside the enterocytes, retinol binds to cellular retinol binding protein (CRBP-II) and is re-esterified into retinyl ester by the enzyme lectin retinol acetyltransferase (LRAT) (101, 114, 115). The retinyl esters are subsequently packed into chylomicrons.

The chylomicrons leave the enterocytes via the lymphatic system and are released into the circulation. After delivering fatty acids to fat- and liver cells, the chylomicrons now called chylomicron remnants, are taken up by the parenchymal liver cells (i.e hepatocytes) (116). There, the retinyl esters are hydrolyzed to retinol and bound to retinol binding protein (RBP) (117). Retinol-RPB secreted from the hepatocytes is taken up and re-esterified for storage in liver stellate cells (101, 117, 118) Upon demand, the retinol-RBP complex is released from the stellate cells (116). In the circulation retinol-RBP also binds transthyretin (TTR), thereby reducing the filtration of retinol in the kidneys (99, 117). The retinol-RPB complex can be taken up by target cells via the RBP-receptor after detachment of TTR (101). Inside the cells, retinol is oxidized to retinal and subsequently to all-trans- or 9-cis retinoic acid in a two-step process catalyzed by alcohol dehydrogenase (ADH) and retinal dehydrogenase (RALDH),

respectively (101, 103) (see Figure 6). Retinoic acid is transported into the nucleus bound to cellular retinoic acid binding protein II (CRABP-II), where it can perform its action (101, 119, 120). All-trans RA is hereafter named RA.

1.5.3 Mechanism of action of RA

The main function of RA in mammalian cells is to act as a transcription factor, which makes RA an example of direct nutrient-mediated regulation of gene expression (121). There are two families of nuclear receptors that bind RA; the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (122). These receptors are intracellular nuclear receptors. There are different isotypes of the RAR and RXR receptor; RARα, RARβ, and RARγ or RXRα, RXRβ and RXRγ encoded by separate genes (122, 123). The RARs and RXRs form homo- or heterodimers, and as such, they bind to specific retinoic acid response elements (RAREs). Upon binding of RA, coactivators are recruited to the RAR/RXR complexes, resulting in RNA polymerase II-mediated transcription (122). More than 500 genes are transcribed by RA, both directly and indirectly. Of these, 27 are direct targets to the classical RAR-RXR-RARE complex. RXR form heterodimers with other nuclear receptors, such as LXR, VDR, PPAR, and thereby vitamin A regulate target genes of these receptors as well.

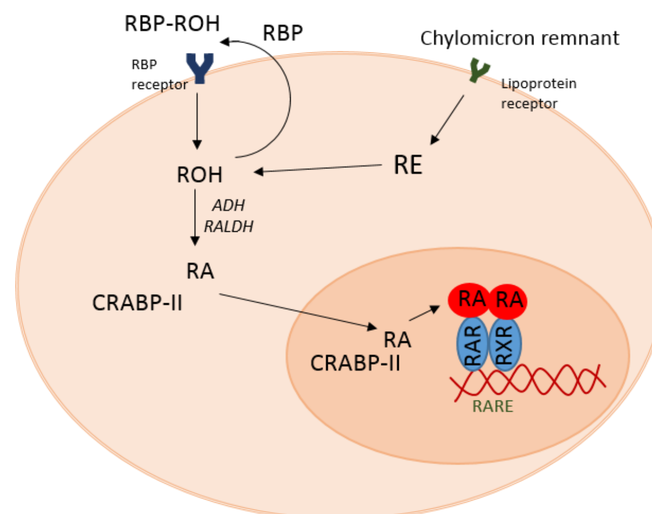

Figure 6: Mechanism of action of vitamin A. Retinol enters the cell via RBP receptor, and is transported into the nucleus by CRABP-II where it exhibits its effect through RAR and RXR.

Even though most effects of RA are mediated through transcription, other means of action have also been demonstrated. RA can for instance target PKCα directly (122). Further, RA is

also described to have antioxidant properties, because RA, as well as other vitamin A metabolites, are able to autoxidize when O_2 tension increases (124).

1.5.4 Vitamin A and the immune system

Multiple reviews have covered the research documenting the importance of RA for both innate and adapted immunity (125-127). An approach for understanding the importance of vitamin A for the immune system has been to assess the consequence of VAD. A much investigated consequence of VAD is impaired mucosal immunity. The receptivity for infections due to the impaired barrier in the respiratory, gastrointestinal and urogenital tracts increases when vitamin A levels are low (128). RA is shown to be essential for proper differentiation and function of epithelial- and mucus-producing goblets cells, as well as for increasing IgA produced by B cells in mucosal epithelial barriers. This illustrates the importance of vitamin A for mucosal immunity (128, 129).

RA has a vital role in the adaptive immune system, and the importance of RA for T cell function is particularly well explored (103, 130). A hallmark of VAD is the lack of a proper antibody defense against TD antigens (131). Naïve T cells can differentiate into Th1 or Th2 effector T cells, and RA is known to favor the differentiation into Th2 cells. Th2 cells are important for mediating a humoral immune response, whereas Th1 cells lead to increased cell-mediated response (132). The antibody response of B cells is enhanced by RA, both as a result of TD- activation, and as in the experiments in this thesis, also TI- activation of the cells (133). An important feature of RA is also to promote homing of lymphocytes. The term, homing of lymphocytes concerns the migration of lymphocytes into lymphoid organs or to non-lymphoid tissues where they first encountered the antigen (33). Homing relies on the expression of a homing receptor on the lymphocytes, and RA is shown to be responsible for the induction of the receptor (33).

It should also be mentioned that RA enhances the immune responses elicited by T cytotoxic cells, macrophages and innate lymphoid cells (ILC) such as NK cells (127). In 2014, there were two important discoveries regarding vitamin A and the immune system. Spencer and coworkers (134) revealed that VAD in mice as expected was associated with general enhanced infection risk in the animals. However more interestingly, they found that VAD simultaneously favored the elimination of the nutrient-consuming helminths in the animals. This was due to the increased frequency of ILC2 cells in the VAD mice. The increased production of ILC2s may therefore compensate for the well-established reduced adaptive immune response related to VAD that acts as primary sensors for nutrient

deficiency. Taken together, this reveals the importance of RA in the bridge between the innate and the adaptive immune system (134). In another study, Van de Pavert and coworkers (135) showed RA levels *in utero* determine lymph nod size and immune response in the adult offspring, as the formation of secondary lymphoid organs in embryogenesis depends on specific cells regulated by RA. Thereby, retinoid levels *in utero* determine the resistance to infections even in the adult offspring.

Vitamin A exhibits its regulatory role primarily through the metabolite RA (110). The local concentration of RA depend on both the levels of RA in plasma and the cells ability to metabolize retinol to RA. HSC, dendritic cells, macrophages, and mucosal epithelial cells are capable of converting retinol to RA. Other cells, such as B cells, will depend on the uptake of RA from plasma or cell to cell transfer from neighboring cells (136).

Vitamin A and B cells

Whereas it is well established that vitamin A has an important role in B cell development and for B-cell functions *in vivo*, the mechanisms are still not fully understood (33). RA is important for B cell development in adult bone marrow, illustrated as both VAD and RAR antagonists decrease the rate of B lymphopoiesis (137).

The effect of RA on the proliferation of B cells seems to depend on both the B cell subset and on the type of stimulation (126). Our group previously showed that whereas B cells stimulated via BCRs are inhibited by RA (138), stimulation via TLR9 alone or together with RP105 is enhanced by RA (42, 63). The RA-mediated enhancement of TLR9/RP105-activated B cells resulted in enforced production of both IgM and IgG, due to both IRF4-mediated transcriptional events and ULK1-induced autophagy (30, 77)

2 Aims

Our lab has previously shown that RA increases the immunoglobulin (Ig) production as well as the rate of autophagy in B cells stimulated via TLR9 and RP105 (16, 77). B cells from CVID patients are known to have low proliferative capacity and IgG production, and we have previously shown that RA can improve some of these features (15, 63). Since ROS levels in many cell systems are related to autophagy (87, 139), the aims of the present thesis are as follows:

1. Investigate the interplay between Ig production, autophagy, and ROS levels in stimulated B cells.
2. Explore whether low production of IgG in B cells from CVID patients may involve dysregulated autophagy and/or ROS levels.
3. Reveal the role of RA in the regulation of ROS levels and autophagy in normal and CVID-derived B cells.

3 Methods

Note: Detailed descriptions of solutions and chemicals are found in the Supplementary section.

3.1 Isolation of B cells

When handling blood and blood products, it was important to take safety precautions to avoid infections. The procedures were performed in a sterile workstation with vertical airflow, with double bench coat and double gloves. To ensure sterile conditions, ethanol (70%) was used to sterilize the needed equipment, and pipette tips and Eppendorf tubes were autoclaved.

3.1.1 Isolation of B cells from buffy coat

The buffy coats were provided by the Blood Bank at Oslo University Hospital, Ullevål, and contains lymphocytes, granulocytes, monocytes, and some blood platelets. Buffy coat is the remaining parts after density-gradient centrifuging to separate red blood cells and plasma (140). The donors are healthy volunteers. Magnetic beads coated with antibodies against CD19 (Dynabeads®CD19) were used to isolate the B cells, and the procedure is based on the method developed by Funderud and coworkers (141). CD19 a surface marker expressed on mature B cells, and CD19 is not expressed by plasma cells.

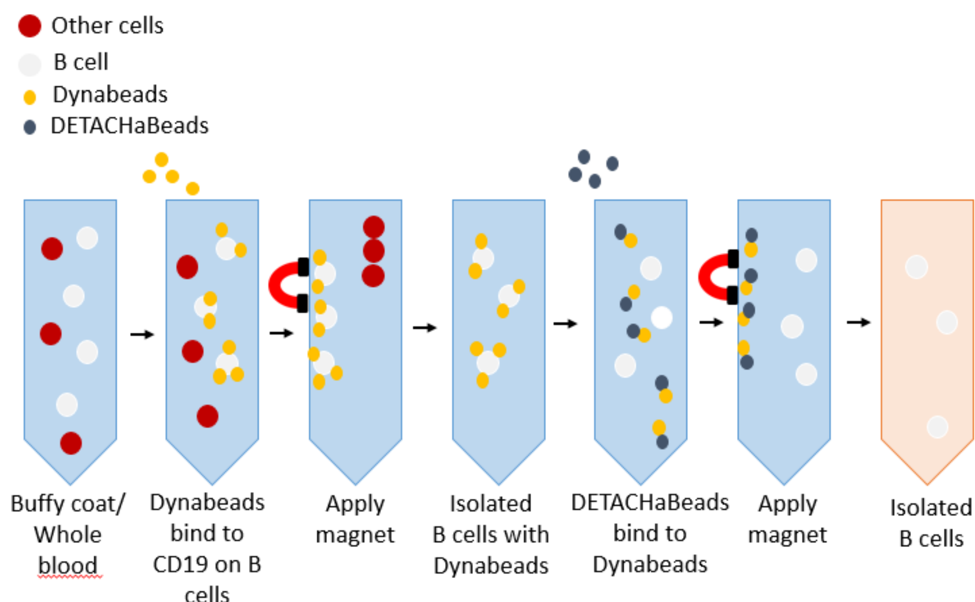

Figure 7: Isolation of B cells by CD19 Dynabeads. Dynabeads bind to the CD19 receptor on B cells, and by applying a magnet, the B cells bound to beads are separated from the remaining cells. Later, DETACHaBeads bind to DynaBeads and release the B cells from the beads. By using a magnet, the purified B cells are separated from the beads.

Procedure

RMPI medium supplemented with penicillin and streptavidin (PS) (25ml) - hereafter called medium, was transferred to a cell culture flask (75cm^3) together with 0,5M EDTA (500 µl). A bag containing the buffy coat was opened with a sterile scissors and the content was carefully added to the culture flask. Magnetic CD19 Dynabeads (350 µl) were added, and thereafter the culture flask was placed for rotation on a Rock-and roller for 40 minutes at 4˚C. The flask was placed on a magnet, and the supernatant was discarded after 5 minutes (see Figure 7). The flask was removed from the magnet, and medium (10 ml) was added and mixed with the B-cells still attached to the Dynabeads. The content was transferred to a sterile 10 ml tube placed on a second magnet. The B cells with beads were then washed 5 times with medium (10 ml), by repeating placing the tubes on the magnet for 2 min and discarding the supernatant. After the last washing step, the B cells were resuspended in medium (1 ml), transferred to an Eppendorf tube and placed on a magnet for 1.5 minutes. The supernatant was again removed. Culture medium (medium supplemented with 10 % fetal bovine serum (FBS)) (200 µl) and DETACHaBeads (200 µl) were added to detach the Dynabeads from the cells. The Eppendorf tube was placed for rotation on a Rock-and roller for 45 minutes at 4℃. Thereafter medium (1 ml) was added to the cells, and the tube was placed on a magnet for 1.5 minute. The B cells are now in the supernatant, detached from the beads. The supernatant was transferred to a second Eppendorf tube placed on a magnet. After 1.5 minute, the supernatant were transferred to a 14 ml Falcon tube placed on ice. The cells were collected by centrifugation at 4°C for 6 minutes at 400 x g and counted on an automated cell counter or by flow cytometry (see section 3.1.3). The yield of CD19+ B cells from buffy coats was typically 5-15 x10^6 cells.

3.1.2 Isolation of B cells from whole blood

Samples of whole blood were obtained from CVID patients admitted to Department of Clinical Immunology and Infection Medicine at Oslo University Hospital, Rikshospitalet, and from healthy volunteers at Institute of Basic Medical Science at the University of Oslo. The controls were intended to match the patients by age and gender. All participants signed a consent form prior to sampling.

Procedure

The isolation procedure of B cells from whole blood was similar to isolation from buffy coat, except for a few steps. Whole blood was collected in 6-7 sodium heparin tubes, each containing 6 ml. The blood was transferred to a cell culture flask (75cm^3), and medium (15

ml) and 0,5M EDTA (340 µl) were added. The magnetic CD19 Dynabeads (175µl) were washed twice prior to adding them to the cell culture flask. The succeeding procedure is identical to the one used for buffy coats, except for adjustment of the volumes according to the expected number of cells. The yield of B cells from whole blood samples is typically 0.5-5 x 10^6.

3.1.3 Estimation of cell numbers by automated cell counting

Due to the small size of B cells, not all automated cell counters are suitable for counting these cells. In the present thesis, we routinely used the Countess™ Automated Cell Counter or the Novocyte Flow Cytometer for counting the B cells. When counting the cells on the Countess™ Automated Cell Counter, 10 µl of the cell culture was transferred into the counting chamber slide for analysis, whereas prior to counting the B cells by flow cytometry, the samples were diluted 5 times in PBS.

3.2 Stimulation of B cells

Purified B cells in culture will rapidly undergo apoptosis if they are not properly stimulated (142). The cells were diluted in cell culture medium to a density of 0.25 million cells/ml and stimulated with CpG-ODN (2µg/ml) and anti-RP105 (1µg/ml) in the presence or absence of RA (1-100 nM). The cells were incubated in a humidified CO_2 incubator at 37℃. To prevent oxidation, the stock solution of RA was protected from light and flushed with nitrogen, and the light in the work station was turned off during procedures involving RA.

In certain experiments, NOX2 was inhibited by adding VAS (2.5 µM) to the cell cultures 30 minutes prior to the stimulants.

3.3 Flow cytometry

The flow cytometer is an instrument that rapidly can analyze a large number of single cells in a solution. The cells pass through a laser at a certain beam length, and the cells will scatter light depending on the cell size (forward scatter, FSC) and granularity (side scatter, SSC). The cells are gated to separate the living and dead cells, and all the measurements are performed on living cells (see Figure 24 in the Supplementary section). The flow cytometer used in this thesis is a NovoCyte® Flow Cytometer equipped with the NovoExpress®software. Approximately, 12 000 B cells were analyzed per sample. The flow cytometer was used for analyzing cell numbers, autophagy, and ROS levels.

3.3.1 Analysis of autophagy

CytoID green detection reagent (CytoID) was used to measure autophagy by flow cytometry. This fluorescent dye is incorporated into pre-autophagosomes, autophagosomes, and autolysosomes and thereby measures autophagic vacuoles and monitors the rate of autophagy (143). CytoID is excited at 488nm, and the fluorescence is detected at 530/30. Autophagy was measured at 48 and 96 hours.

Procedure

Prior to analyses, 75 000 cells from each sample were collected by centrifugation at 0.5 x g for 5 minutes at 4℃. CytoID was diluted 1:1000 prior added to the cells (250 µl). After incubation for 30 minutes at 37℃, the cells were collected by centrifugation, washed once in of ice cold PBS (500 µl), before resuspended in PBS (300 µl). Thereafter, the cells were transferred to flow-tubes for analyses. Before running the samples on the flow cytometer, the instrument lasers were tested by an internal quality control.

3.3.2 Analysis of ROS

ROS levels were measured by staining the cells with the CellROX deep red reagent (CellROX) or the H_2DCFDA dye. H_2DCFDA is excited at 488nm and the fluorescence is detected at 530/30. CellROX is essentially nonfluorescent but will exhibit a fluorescence signal upon oxidation (144). CellROX is excited at 640nm, and the fluorescence is detected at 675/30. For simultaneous analysis of autophagy and ROS, the cells were co-stained with both CellROX and CytoID, as the fluorescence signals from these dyes do not interfere with each other.

The H_2DCFDA procedure

H_2DCFDA was added to the B cells 10 minutes prior to stimulation. The cells were kept in the CO_2 incubator for 2, 5, 24 or 48 hours, before centrifugation at 0.5 x g for 5 minutes. The collected cells were re-suspended in ice-cold PBS (300 µl) and transferred to flow tubes for analyses of ROS levels.

The CellROX procedure

The procedure for measuring ROS by staining the cells with the CellROX is similar to the procedure for measuring autophagy by CytoID, and the two reagents were often combined. The CellROX reagent was diluted 1:1000 in ice-cold PBS, and 250 µl were added to

approximately 75 000 cells. The subsequent steps are identical to the procedure for CytoID-staining.

3.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a well-known technique for quantifying proteins like cytokines and immunoglobulins secreted from cells in culture plates. The method is based on using labeled antibodies specific for the target substances to be quantified. There are several versions of the ELISA assay. The sandwich ELISA assay is regarded as the most sensitive one (145) and is the version we have used in this thesis. As shown in Figure 8, the target protein is captured between two specific antibodies. The capture antibodies are bound to the culture plates and bind the target protein in the sample, and the detection antibody binds to the target protein at another site. The detection antibody is conjugated to a tag, which in our case is horseradish peroxidase (HRP). The HRP enzyme oxidizes the substrate 3.3'.5.5'-tetramethylbenzidine (TMB), that results in the development of a blue color. The reaction is stopped by sulfuric acid, and the change in pH will shift the color from blue to yellow, proportional to the amount of target protein in the sample. The quantification is performed by a spectrophotometer at a wavelength of 450nm and based on the known standard curve we are able to estimate the concentrations of the target protein. In the present study, ELISA was used to measure the levels of secreted IgG and IgM.

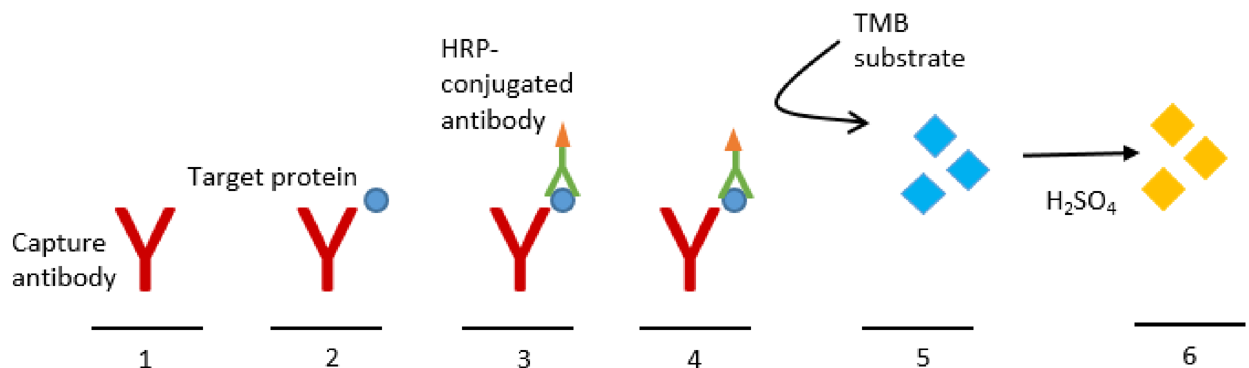

Figure 8: The principles of sandwich ELISA. The capture antibody captures the target protein, and a second antibody conjugated to HRP binds the target at another site. HRP oxidizes the TMB substrate to a product with blue color, which turns yellow upon addition of sulfuric acid. The intensity of the color reflects the amount of target protein.

Procedure

After 96 hours of stimulation, the B cells were subjected to centrifugation at 0.5 x g for 4 minutes. The supernatants were transferred to Eppendorf tubes and kept at -80 ^{o}C ready for analyses.

The ELISA Starter Accessory Kit was used according to the manufacturer's protocol. The capture antibody was diluted 1:100 in ELISA coating buffer and 100 µl of the diluted capture antibody was added to each well on a 96 well Elisa plate. The plate was incubated for 1 hour at room temperature or overnight at 4℃ and thereafter washed 3 times with ELISA washing buffer. ELISA blocking buffer (120µl) was added to each well. After 30 minutes, the blocking buffer was removed, and the plate was washed 3 times with ELISA washing buffer.

The standards and samples were added to the wells, 100 µl per well. The standards used for estimating the concentration of IgG and IgM were 1000, 500, 250, 125, 63.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml. For estimation of IgM concentrations, some of the samples were diluted, 1:30 or 1:40, in sample diluent prior to analysis, whereas some of the samples for IgG measurements were diluted between 1:4 and 1:6 prior analysis. After 1.5 hours at room temperature, the plate was washed five times in the ELISA washing buffer. The HRP-conjugated detection antibody was diluted 1:160 000, and 100 µl was added per well. The plate was incubated at 1 hour in room temperature, and thereafter the plate was washed another five times. 100 µl of the TMB substrate solution was added per well, and the reaction was stopped after 5 minutes by adding 100 µl H_2SO_4. A photometer (Multiscan EX) measured the absorbance at a wavelength of 450 nm, and the concentrations of IgG or IgM were calculated from the standard curves by using the software available at www.myassays.com.

3.5 Statistical analysis

The statistical analysis of the data is performed using IBM SPSS Statistics 25. The B cell responses vary substantially between donors, both within the group of normal donors and between CVID patients. When testing for normality, normal distribution could not be assumed, therefore non-parametric methods are used. Differences between the controls and patients are analyzed by Mann Whitney U-test. For differences within the groups, the analyses are performed using the paired method Wilcoxon signed rank test. Spearman's Rho test is used for analyzing correlations. All analyses are performed on both absolute and relative values. P values less than 0.05 are considered statistically significant and are marked with an asterisk. GraphPad Prism 7 and Adobe Illustrator are the software programs used for constructing the figures, whereas the tables are made in Microsoft Excel 2016.

4 Results

In the first part of the result section, we present pilot experiments destined for establishing the methodology for measuring the levels of ROS and autophagy in B cells, and to determine time points for these measurements. The initial experiments required a large number of cells, and these experiments were therefore performed on B cells isolated from buffy coats.

In the second and main part of the results section, we compare B cells isolated from whole blood of healthy controls and CVID patients. We study the effects of RA on ROS, autophagy and Ig levels in stimulated B cells from both groups. We also relate our results to different subgroups of CVID patients. B cells derived from whole blood contains a limited number of cells, thereby restricting the number of experiments that could be performed on each blood donor.

4.1 Pilot experiments on analyses of ROS and autophagy and Ig production in stimulated normal B cells

Our group has previously shown that stimulation of normal B cells via TLR9 and RP105 initiates downstream events like proliferation and differentiation into Ig secreting cells (16, 30, 42). As presented in section 1.4.2, there seems to be an interplay between autophagy and ROS levels in many cell systems (146). In light of our previous finding that RA-induced Ig production in normal B cells involves autophagy (16), we here aimed to reveal a possible interplay between ROS levels, autophagy and Ig production in TLR9/RP105-stimulated B cells.

4.1.1 ROS levels are induced upon activation of TLR9 and RP105 in normal B cells

To investigate the effect of RA on ROS generation in TLR9/RP105-stimulated B cells, the cells were stained with H_2DCFDA or CellROX and analyzed by flow cytometry as described in the Methods section. As presented in Figure 9, stimulation of the cells with the combination of CpG-ODN and anti-RP105 for 24 hours enhanced the levels of ROS. Concentrations of RA between 1 and 100nM had no effects on ROS levels, either alone or in the presence of the stimulants.

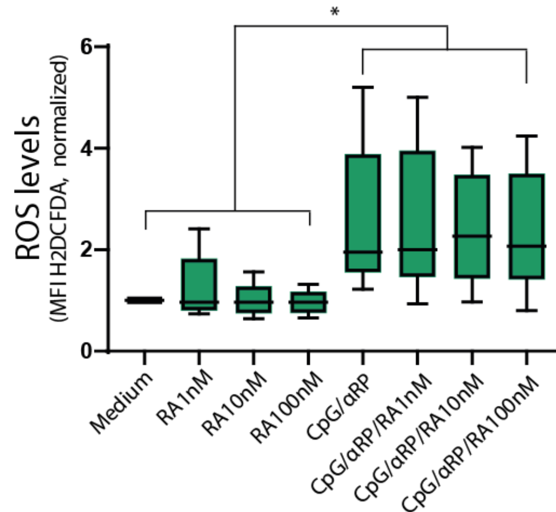

Figure 9: The effect of RA on ROS production in normal B cells stimulated via TLR9 and RP105. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured for 24 hours in the presence or absence (medium) of combinations of CpG-ODN (CpG, 2µg/ml), anti-RP105 (αRP,1µg/ml) and RA (1nM, 10 nM or 100nM). The ROS levels were detected by H_2DCFDA-staining and analyzed by flow cytometry. The results are presented as median fluorescence intensity (MFI) of 5 experiments normalized to the values in unstimulated cells. The whiskers represent the range of the results. *$p<0.05$ Wilcoxon signed rank test

In order to test the kinetics of the ROS production in stimulated B cells, as well as assessing the potential effect of RA over time, we measured the ROS levels at different time points after stimulation. As presented in Figure 10A, we observe an increase in ROS levels already 2 hours after TLR9/RP105-stimulation. The elevated ROS levels remained stable for 48 hours before declining at 72 hours (Figure 10B). RA had no effects on the ROS levels in the stimulated B cells on any of the time points.

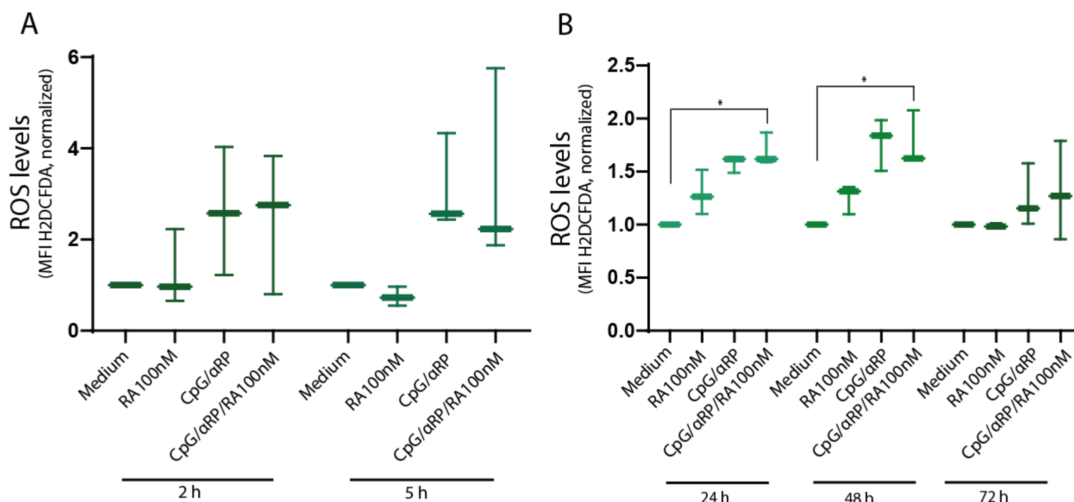

Figure 10: ROS levels in normal B cells at different hours after stimulation. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured for 2 and 5 hours (panel A) or for 24, 48 and 72 hours (panel B) in the presence or absence (medium) of combinations of CpG-ODN (CPG, 2µg/ml), anti-RP105 (αRP, 1µg/ml) and RA (100nM). The ROS levels were detected by H_2DCFDA-staining and analyzed by flow cytometry. The results are presented as median fluorescence intensity (MFI) of 3 experiments normalized to the values in unstimulated cells. The whiskers represent the range of the results. *$p<0.005$ Wilcoxon signed rank test

Due to stable ROS levels up to 48 hours in stimulated B cells, we routinely chose to measure ROS levels at this time point throughout the main experiments. In Figure 11, we verified that staining the cells with H_2DCFDA or CellROX resulted in the same estimated ROS levels.

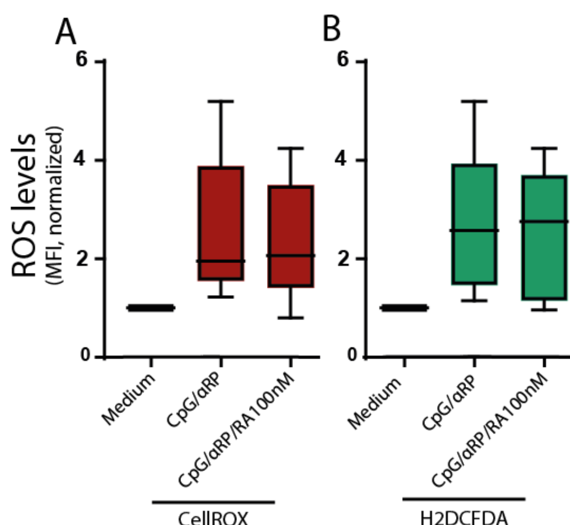

Figure 11: Comparison of ROS levels detected by H_2DCFDA and CellROX. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured for 24 hours in the presence or absence (medium) of combinations of CpG-ODN (CPG, 2µg/ml), anti-RP105 (αRP, 1µg/ml) and RA (100nM). ROS levels were detected by staining with H_2DCFDA (panel A) or by CellROX (panel B) and analyzed by flow cytometry. The results are presented as median fluorescence intensity (MFI) of 3 experiments normalized to the values in unstimulated cells. The whiskers represent the range of the results. *$p<0.005$ Wilcoxon signed rank test

It has been proposed that vitamin A metabolites may have antioxidant properties (124). In order to rule out the possibility that the missing effects of RA on ROS levels could be the net result of RA as a B cell stimulant counteracted by RA as an anti-oxidant, we measured the effects of RA on ROS levels in H_2O_2-treated B cells after 2 hours. Antioxidant effects are assumed to be direct and rapid. Based on the two experiments presented in Figure 12, 100 nM of RA did not reduce the ROS levels generated by H_2O_2. We therefore conclude that TLR9/RP105-mediated stimulation of B cells enhance the ROS production in normal B cells and that RA does not affect these ROS levels.

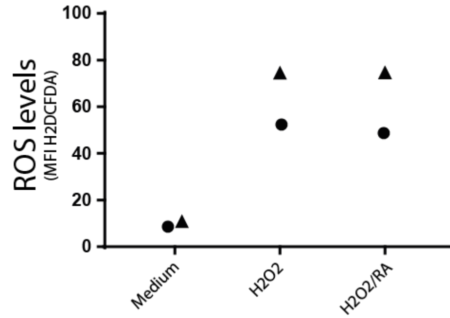

Figure 12: RA has no pro- or antioxidant effect in B cells. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured with or without H_2O_2 (200 µL/ml) and RA (100nM) for 2 hours, and ROS levels were detected by H_2DCFDA-staining and analyzed by flow cytometry. The results represent ROS levels from two different donors.

4.1.2 Autophagy is induced in normal B cells upon activation via TLR9 and RP105

As shown in Figure 13, TLR9/RP105-mediated stimulation of the B cells significantly increased the levels of autophagy. At 48 hours, however, RA had no additional effect on autophagy. Previous results from our lab have shown that RA enhances the level of autophagy in TLR9/RP105-stimulated B cells after 96 hours (77). As these previous results were based on LC3-detection, it was important to verify the results by analyzing autophagy by CytoID staining at 96 hours. As shown in Figure 13, the levels of autophagy in the stimulated B cells further increased from 48 to 96 hours, and RA had the expected enhancing effects on TLR9/RP105-mediated autophagy at 96 hours.

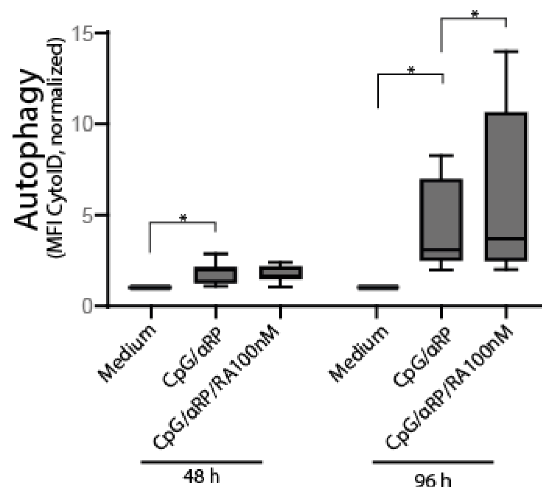

Figure 13: Autophagy in B cells after 48 and 96 hours. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured in presence or absence (medium) of combinations of CpG-ODN (CpG, 2µg/ml), anti-RP105 (αRP, 1µg/ml) and RA (100nM) for 48 and 96 hours. Autophagy was detected by staining the cells with CytoID, and the results are presented as the median fluorescence intensity (MFI) of 5 experiments normalized to the values in unstimulated cells. The whiskers represent the range of the results $*p<0.005$ Wilcoxon signed-rank test.

4.1.3 Correlation between ROS and autophagy in stimulated B cells

In the previous experiments, we observed relatively low levels of ROS and autophagy in the unstimulated B cells. Upon stimulation of the cells, both ROS- and autophagy levels increased. Other researchers in our lab had in independently measured ROS and autophagy levels in B cells for other purposes than the present project. We collected all these measurements (including our own analyses) of ROS and autophagy in B cells stimulated for 48 hours via TLR9 and RP105. As presented in Figure 14, we found a significant positive correlation between ROS and autophagy in these cells, indicating that cells with high levels of ROS also have high levels of autophagy at 48 hours.

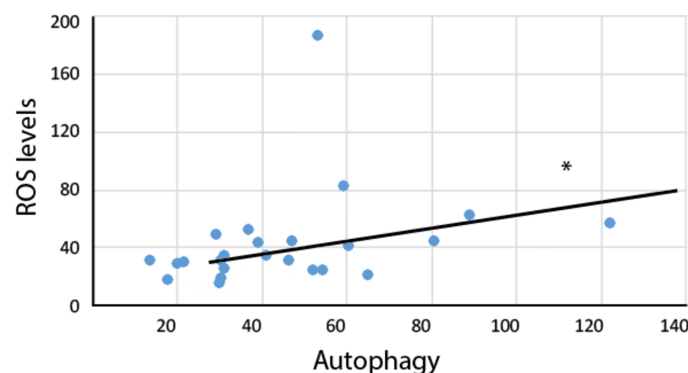

Figure 14. Correlation between ROS and autophagy in stimulated B cells. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured with CpG-ODN (CpG, 2µg/ml), anti-RP105 (αRP, 1µg/ml) and RA (100nM) for 48 hours. ROS and autophagy were measured as in legends to figures 10 and 13. n=24, R=0,472, *p<0.05 Spearman's Rho.

4.2 The interplay between ROS, autophagy and Ig production in normal versus CVID-derived B cells

Having now established the optimal timing for measuring ROS levels and autophagy, we now proceeded to the main part of the project. Here, the aim was to compare the impact of RA on the interplay between ROS, autophagy and Ig levels in normal versus CVID-derived B cells. All the subsequent experiments were therefore performed on B cells isolated from samples of whole blood.

4.2.1 Characteristics of the population

Blood samples from 16 CVID patients and healthy gender- and age-matched controls were collected. Of the 16 blood samples received from CVID patients and controls, three from each group were discarded due to the low yield of B cells after isolation.

The characteristics of the study population are presented in Table 1. When comparing age, gender and number of B cells isolated from the patients and controls, there were no statistically significant differences between the two groups.

Controls				CVID			
ID	Gender	Birth year	B-cell number(x 10^6)	ID	Gender	Birth year	B-cell number(x 10^6)
K1	Male	1983	3,12	P1	Male	1986	2,60
K2	Female	1978	5,80	P2	Male	1985	5,40
K3	Male	1974	2,48	P3	Male	1954	5,92
K4	Male	1975	5,44	P4	Male	1972	2,12
K5	Female	1994	1,68	P5	Male	1989	1,68
K6	Female	1995	5,76	P8	Male	1986	5,72
K7	Male	1968	1,66	P9	Female	1951	1,34
K9	Male	1983	6,40	P11	Female	1987	5,20
K10	Female	1973	0,65	P12	Female	1990	2,40
K11	Female	1993	1,20	P13	Female	1953	2,02
K12	Female	1982	1,90	P14	Female	1964	0,65
K13	Male	1965	2,20	P15	Female	1966	3,40
K14	Female	1991	1,12	P16	Female	1965	1,16
Average	7 Male, 7 Female	1981	3,03	Average	7 Male, 7 female	1973	3,05

Table 1: The distribution of age, gender and B cell numbers of the participants included in the study. There are no statistical differences between the two groups regarding gender, age or B cells number. $p>0.05$ Mann-Whitney U-test

The CVID patients were further divided into two subgroups based on immune-phenotypes, presented in Table 2. Patients with phenotype 1 are considered to have more complications associated with their disease, and this sub-group is characterized as having a more severe immunodeficiency (58, 59). Low numbers of Ig class-switched B cell is a common feature of CVID-patients (147), and all patients included in this study were below the normal range of class-switched B cells. As mentioned in the introduction, high levels of CD21^{low} and transitory B cells seem to associate with chronic inflammation (148).

Immunophenotypes	ID	Phenotype	CD19+	CD19+ (%)	Class switch (%)	Transitoriy B-cells	CD21 low
	1	1	0	0	1	0	0
	2	1	0	0	1	0	1
	3	0	0	0	1	0	0
	4	1	0	1	1	1	1
	5	1	1	0	1	1	0
	8	1	0	0	1	1	0
	9	0	0	0	1	1	0
●	11	1	0	0	1	1	1
▲	12	0	1	0	1	1	0
▼	13	0	0	0	1	0	1
	14	1	1	1	1	1	0
◆	15	1	0	0	1	1	1
■	16	0	0	0	1	0	1
Number	13	8	3	2	13	8	6

Table 2: Characterization of CVID patients based on immune-phenotypes. Each patient is characterized based on a score of 0 or 1. Score 0 implies that the patient is within the reference interval, whereas a score 1 denotes that the patient's values are outside the reference interval. CD19+ is based on the total number of CD19+ B cells, and the reference interval is 100-400 x10^9/L. The normal range of the percentage of CD19+ cells is 4.9-18.4%. The reference interval for the percentage of class-switched B cells range from 6.5 to 29.1 %, and the reference interval for transitory B cells is 0.6-3.4%. The normal reference interval for the percentage of CD21^{low} is 0.9-7.6%. The symbols indicated in the left column, refer to the symbols the donors were given in Figures 19-21.

3.2.2 Normal ROS levels in stimulated B cells from CVID patients

Having shown that stimulation of normal B cells via TLR9 and RP105 results in the induction of ROS (Figures 9 and 10), we here investigated the regulation of ROS in B cells from CVID patients and healthy controls after 48 hours of stimulation. Analyses of ROS were performed by flow cytometry of cells stained with CellROX. As shown in Figure 15, stimulation of the cells by CpG-ODN and RP105 increased the levels of ROS in both normal- and CVID-derived B cells. Although the ROS levels in TLR9/RP105-stimulated CVID-derived cells appeared as slightly higher than in the stimulated normal cells, we did not find significant differences between the two groups. In line with our initial experiments on normal B cells (Figure 9), RA did not increase ROS in B cells from either CVID patients or healthy controls, rather a minimal inhibition of the ROS levels. Taken together, ROS seems to be normally regulated in CVID-derived B cells at this timing.

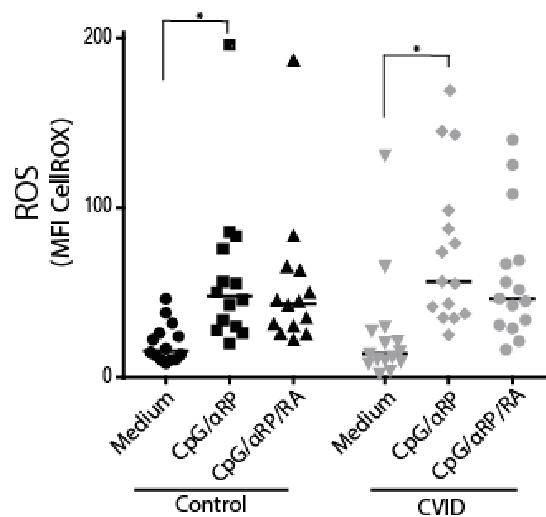

Figure 15: Normal regulation of ROS in CVID-derived B cells. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13 for 48 hours. The cells were stained by CellROX, and ROS levels were analyzed by flow cytometry as described in Methods. The center lines represent the median fluorescence intensity (MFI) of values from controls (n =13) or CVID patients (n =13), and each symbol represents one donor. * $p<0.005$ Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

4.2.3 Autophagy levels tend to be reduced in stimulated B cells from CVID patients

To compare the regulation of autophagy in normal and CVID-derived B cells, the cells were stimulated via TLR9 and RP105 for 48 or 96 hours, and the effects of RA were monitored. Autophagy was measured by staining the cells with CytoID, and the autophagy was measured by flow cytometry. The results presented in Figure 16 show a tendency (p=0.064) of reduced autophagy in TLR9/RP105-stimulated B cells from CVID patients compared to controls. As

previously noted in the initial experiments on normal B cells (Figure 13), we found again that RA significantly increased the autophagy induced by TLR9/RP105-stimulation after 96 hours of stimulation. The effect of RA was somewhat reduced in the CVID-derived B cells compared to the effect on normal cells (Figure 16).

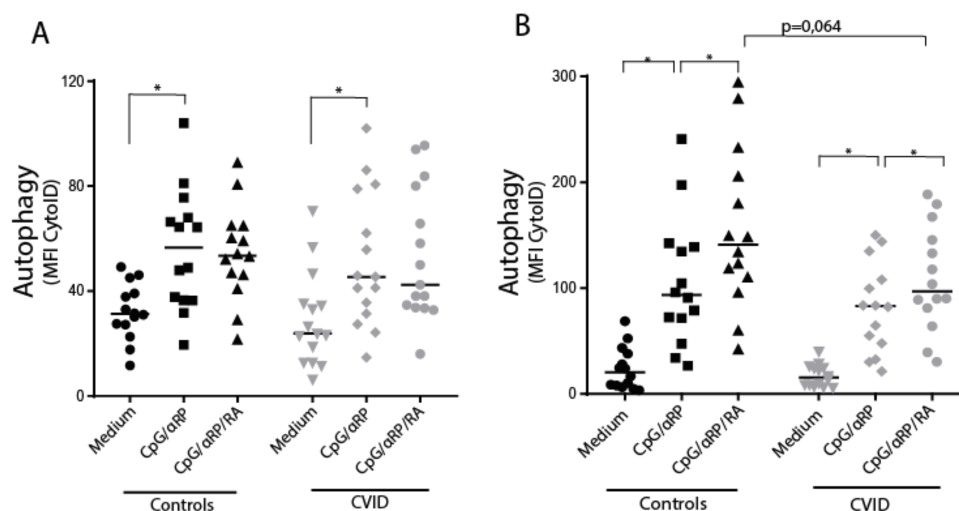

Figure 16: Autophagy in CVID-derived B cells. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13. The cells were stained with CytoID at 48 hours (panel A) or 96 hours (panel B), and autophagy was analyzed by flow cytometry as described in Methods. The center lines represent the median fluorescence intensity (MFI) of values from controls (n=13) or CVID patients (n=13), and each symbol represent one donor. * $p<0.005$ Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

4.2.4 Reduced IgG production in CVID-derived B cells

To address the interplay between ROS levels, autophagy, and Ig production, we measured Ig secretion in the TLR9/RP105-stimulated B cells. Secretion of IgG was analyzed by ELISA as described in the Methods section, and as expected the levels of secreted IgG were significantly lower in stimulated B cells from CVID patients than in B cells from controls (Figure 17A). Although statistically significant, the effect of RA on TLR9/Rp105-induced IgG on CVID-derived B cells was more modest than in the normal cells.

In line with previous results from our group (63), the levels of IgM did not differ between stimulated B cells from CVID patients and healthy controls (Figure 17B). RA increased the IgM production to the same extent in B cells from both study groups.

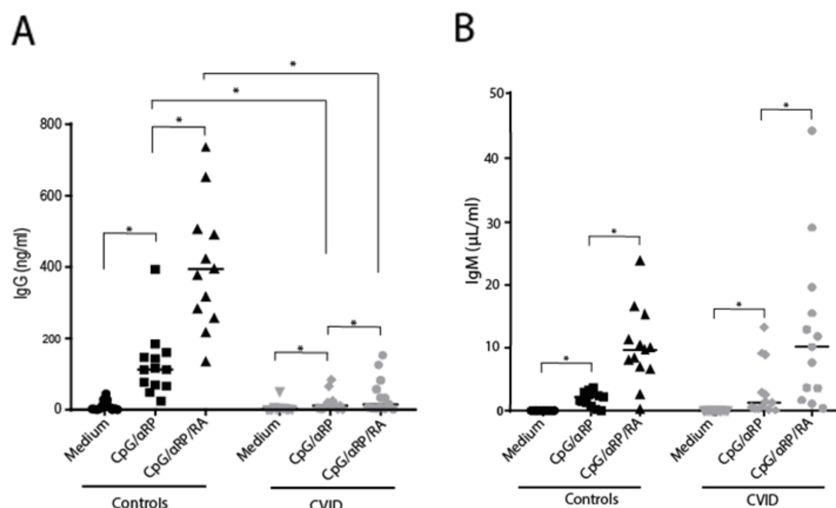

Figure 17: Ig production in stimulated normal- and CVID-derived B cells. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13. Supernatants were collected after 96 hours, and IgG secretion (panel A) and IgM secretion (panel B) were analyzed by ELISA as described in Methods. The center lines represent the median values of controls (n =13) and CVID-patients (n =13), and each symbol represents one donor. * p<0.005 Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups

4.2.5 Correlation analyses between ROS, autophagy and Ig production in normal- and CVID-derived B cells

Because we observed a positive correlation between ROS and autophagy in the initial experiments (see Figure 14), we here looked for a correlation between these events in the two study groups of blood donors. As shown in Figure 18, there is a tendency for a positive correlation between ROS and autophagy levels in B cells both from controls and patients, but the correlations were not statistical significance in any of the groups. An explanation for why we obtained significant correlations in Figure 14 and not in Figure 18, might be the larger number of donors included in Figure 14.

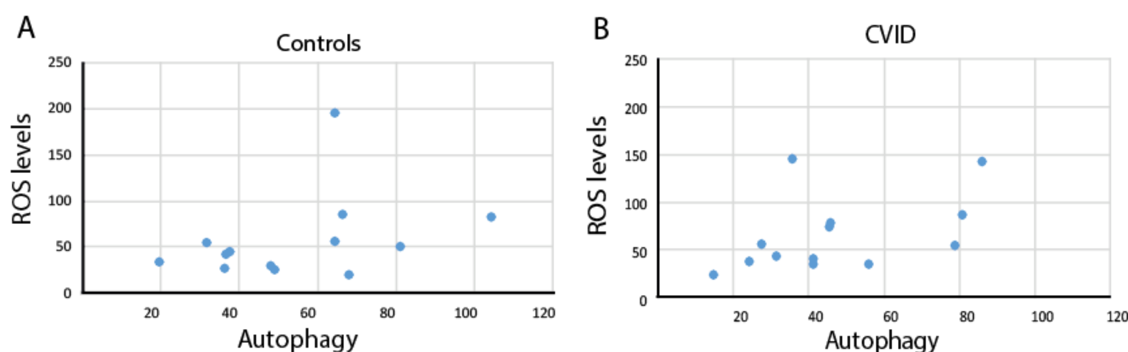

Figure 18: Correlation between ROS and autophagy in stimulated B cells from CVID patients. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13 for 48 hours. ROS- and autophagy-levels were measured by flow cytometry as described in Methods. The correlation was estimated by Spearman's Rho. Panel A: p=0.405 (n=13). Panel B: p=0.108 (n=13).

We found no statistically significant correlations between ROS levels and IgG secretion, or between autophagy and IgG secretion in either of the study groups (data not shown).

4.2.6 The effect of NOX2 inhibition on ROS, autophagy, and Ig-production in CVID-derived B cells

In light of the vital role of NOX2-induced ROS in activation of B cells (34, 86, 92), we extended the experiments to explore the effect of the NOX2 inhibitor VAS-2870 (VAS) on the interplay between ROS, autophagy and Ig levels in B cells from 5 CVID patients and 5 healthy controls. To facilitate tracking of the donors in Figures 19-21, each donor is given a specific symbol that is used in all the figures (see Table 2).

The dose of VAS was chosen based on reports in the literature and separate experiments in our lab. VAS significantly inhibited the ROS levels in the stimulated B cells from healthy controls (Figure 19). VAS also tended to inhibit the ROS-induction in the stimulated CVID-derived B cells, but the inhibitory effect was not statistically significant (Figure 19). VAS only marginally reduced the ROS levels in B cells from patient #11 and #13. To verify this unexpected finding, a new blood sample was collected from patient #11, and the analyses gave nearly identical results (data not shown). This result indicates that the ROS levels in healthy controls mainly origin from NOX2, whereas the ROS levels observed in the CVID patients probably origin from other sources.

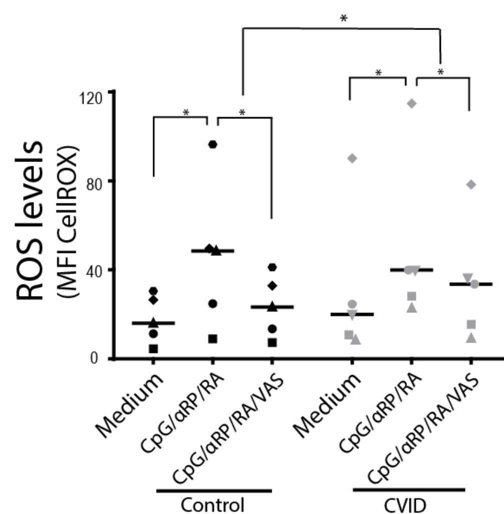

Figure 19: The effects of VAS on ROS levels in stimulated B cells from normal and CVID-derived B cells. CD19+ B cells (0.25 x 10^6 cells/ml) were cultured in the presence and absence (medium) of combinations of CpG-ODN (CpG, 2µg/ml), anti-RP105 (αRP, 1µg/ml), RA (100nM) and VAS (2.5 µM) for 24 hours. The cells were stained with CellROX, and ROS levels were analyzed by flow cytometry. The center lines represent the median fluorescence intensity (MFI) of values from controls (n=5) and CVID-patients (n=5), and each symbol represents one donor. * $p<0.005$ Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

The abnormal effect of VAS on CVID-derived B cells became even more notable when exploring its effect on autophagy. Inhibition of NOX2 in normal stimulated B cells significantly reduce the levels of autophagy. As shown in Figure 20, the inhibiting effects of VAS were generally lower in the CVID-derived B cells, and the autophagy in the B cells derived from patient #12 was even dramatically enhanced by VAS.

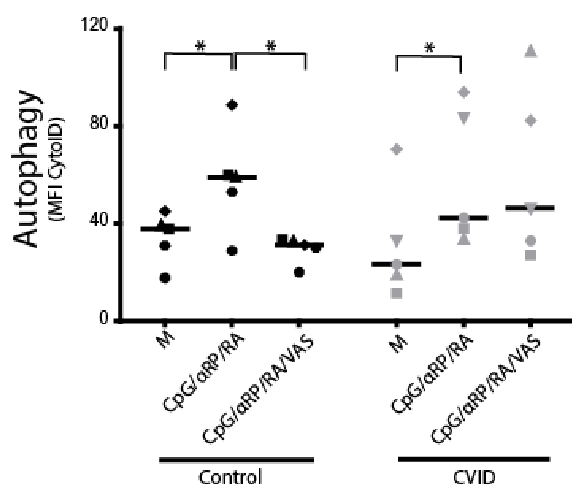

Figure 20: Effect of VAS on autophagy in normal- and CVID-derived B cells. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 19. The cells were stained with CytoID, and autophagy was analyzed by flow cytometry at 48 hours. The center lines represent the median fluorescence intensity (MFI) of values from controls (n=5) and CVID-patients (n=5), and each symbol represents one donor. * $p<0.005$ Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

Finally, we revealed that the inhibitory effect of VAS on both IgG- and IgM production was significantly reduced in the stimulated CVID-derived B cells as compared to B cells from the healthy donors (Figure 21).

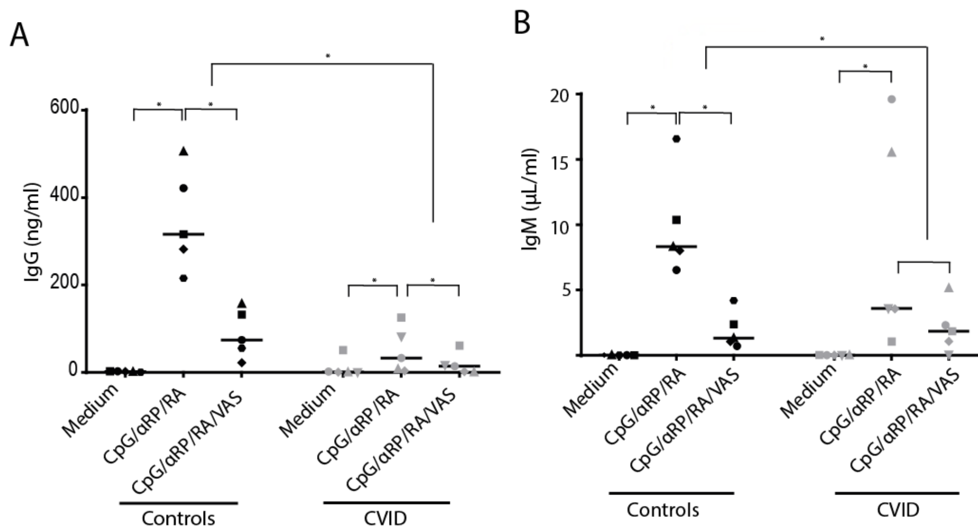

Figure 21: The effect of VAS on Ig production in normal- and CVID-derived B cells. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 19. The supernatants were harvested after 96 hours, and IgG (panel A) and IgM (panel B) were quantified by ELISA assays. The center lines represent the median values of controls (n=5) and CVID-patients (n=5), and each symbol represents one donor. * $p<0.005$ Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

Taken together, the results presented in Figure 19-21 demonstrate that NOX2 is required for induced autophagy and Ig secretion in normal TLR9/RP195-stimulated B cells. However, NOX2 seems to be dysregulated in CVID-derived B cells, as illustrated by the reduced effects of VAS on ROS, autophagy and Ig levels in these cells.

4.2.7 ROS, autophagy and Ig secretion within different subgroups of CVID patients

It is well established that CVID patients are highly diverse both in terms of etiology, clinical presentation, and prognosis (49, 58). In clinical practice, the CVID patients are therefore sub-grouped based on both clinical and immunological parameters. As shown in Table 2, one of the classification criteria is based on whether the patients experience infections only (phenotype 0) or whether they experience other complications in additions to their frequent infections (phenotype 1 = complication group). Of the 13 CVID patients included in the study, 8 patients belonged to the “complication” group.

Compared to normal B cells, we did not see any differences in ROS levels in either subgroup (data not shown). However, both in terms of induced autophagy (Figure 22) and IgG production (Figure 23), we observed significantly reduced levels in the “complication” group compared to B cells from healthy controls. Furthermore, there was a significantly lower level of induced IgG in the “complication” group compared to the phenotype 0 group (Figure 23). Since 6 of the 8 patients in the “complication” group also had high levels of transitory B

cells (see Table 2), we observed the same tendency when analyzing autophagy and Ig levels in the patients based on this criterion (data not shown). Taken together, our results suggest that stimulated B cells from the subgroup of CVID patients with the more adverse disease have lower autophagy- and IgG levels compared to normal B cells and B cells from the non-complication CVID patients.

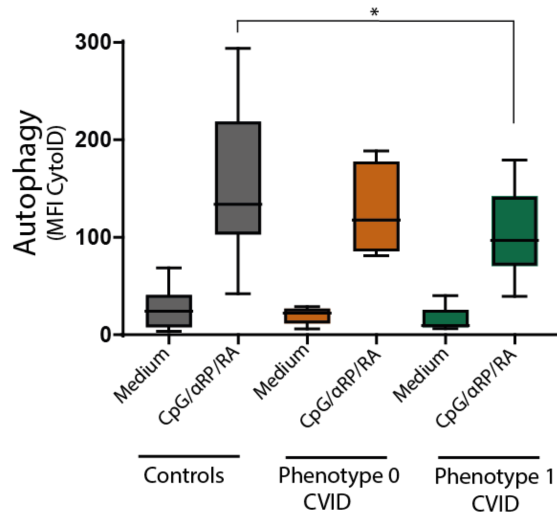

Figure 22: The levels of autophagy in B cells from different subgroups of CVID patients. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13. The cells were stained with CytoID and autophagy was analyzed by flow cytometry after 96 hours. The center lines represent the median fluorescence intensity (MFI) of values from controls (n=13), phenotype 1 CVID patients (n=8) and phenotype 0 CVID patients (n=5). * p<0.005 Wilcoxon singed rank test used within each group, and Mann Whitney U-test used between the two groups.

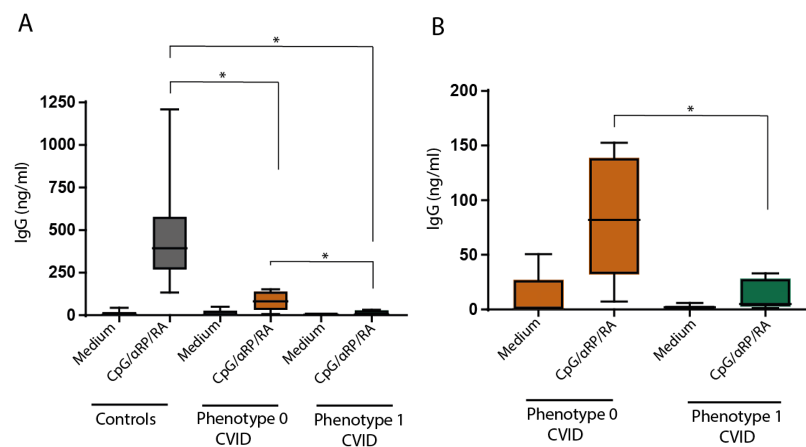

Figure 23: IgG production in B cells from different subgroups of CVID patients. CD19+ B cells from CVID patients and controls were cultured and stimulated as described in figure 13. The supernatants were collected for ELISA analysis of IgG levels after 96 hours. The center lines represent the median of values from controls (n =13), phenotype 1 CVID patients (n =8) and phenotype 0 CVID patients (n=5). *p<0.05 Wilcoxon signed rank test

These results imply that the severely reduced IgG production in B cells from CVID patients with complications could be a consequence of the low level of autophagy in these cells. Furthermore, the results may suggest that the complications associated with this group of CVID patients might be linked to the severely low levels of autophagy and/or of IgG.

5 Discussion

5.1 Methodological considerations

5.1.1 Peripheral blood B cells

In the present thesis, we have studied peripheral blood B cells isolated either from buffy coats (used in the pilot experiments) or from whole blood (used in the main part of the experiments). The big advantage of using peripheral blood B cells compared to established B cell lines is that peripheral blood B cells are normal resting cells naturally synchronized in G_0. B cell lines are usually derived from cancer cells or by EBV-transfection (149), making the cells prone to undergo mutations. In contrast to normal B cells, the B cell lines are also constitutively diving cells that only depend on factors obtained from the culture medium. The disadvantage of using peripheral blood B cells is the variable biological responses between B cells from different blood donors, making it necessary to perform experiments on cells from many donors in order to draw firm conclusions. When in addition the number of cells obtained from each buffy coat or blood sample is limited, this makes experiments on peripheral blood B cells both time consuming and expensive.

The B cells are isolated from buffy coats or peripheral blood by the protocol developed by Funderud and coworkers (141). The method is based on magnetic beads coated with the B cell antibody anti-CD19, and it results in highly viable cells (> 95 %) with a purity > 98 %. CD19 is a commonly used marker for B cells, as it is expressed in pre-B cells and until the terminal differentiation into plasma cells (150). The cells obtained from isolation are therefore mainly naïve B cells, but also memory B cells. High purity of the isolated B cells ensures that the results are not influenced by contaminating cells. On the other hand, the high purity of the cells implies that the B cells are separated from their natural environment and interaction with other cells naturally present in the blood.

5.1.2 B cells from CVID patients and healthy controls

We obtained blood samples from CVID patients admitted to Oslo University Hospital, Rikshopitalet, in connection with their routine follow-ups and Ig replacement therapies. The physicians were responsible for selecting the CVID patients to be included in our study, and subgrouping of the patients was not taken into consideration when including the participants. Only patients known to have at least moderate numbers of B cells were included in the study, causing a possible selection bias. However, the number of B cells are generally not severely altered in CVID patients compared to healthy controls (58, 59), and as shown in Table 1, the

number of B cells from the CVID patients in our study did not significantly differ from that of the healthy blood donors. CVID patients with ongoing infections were excluded from the study.

The healthy controls were recruited from the Institute of Basic Medical Sciences at the University of Oslo, and the healthy donors were age and gender-matched with the CVID patients (see Table 1). Blood samples from patients and controls were as far as possible collected on the same day, to minimize the experimental difference between controls and patients.

The subgrouping of the CVID patients was based on certain reference values for clinical features and immune-phenotyping (59). As shown in Table 2, all the patients included in our study have low levels of class-switched B cells. This is a rather common feature of CVID patients (59, 151), and it may explain the very low IgG production from CVID-derived B cells in our study. Noteworthy, 6 of the 8 CVID patients belonging to the "complication-group" also had high levels of transitory B cells, in accordance with the general assumption that the patients with abnormal levels of transitory B cells have an more inflammatory profile (59).

5.1.3 Activation of B cells *in vitro*

Purified peripheral blood B cells can be activated *in vitro* in various ways, including via BCR, CD40 and various TLRs (152). In the present study, we stimulated the B cells via the innate toll like receptors TLR9 and RP105. The natural ligands of TLR9 are microbiotic DNA characterized by CpG motifs with phosphodiester backbones (153). To avoid that these phosphodiester bindings are cleaved by nucleases in cell cultures, we used commercially modified CpG-ODN with stable phosphodiester backbones (154). The natural ligand for RP105 remains unknown, and we therefore used anti-RP105 for activation of the receptor. This is a well-established method (155), previously reported by us (16, 42) and others (42) to synergize with stimulators of TLR9. Yamazaki *et al* described the synergy between RO105 and TLR9 stimulation only to apply to naïve B cells, but our lab demonstrated that the synergic effect also applied to memory B cells (16, 42). Stimulation of B cells via TLR9 and RP105 results in polyclonal activation of the cells (16, 42). According to results from our lab (Karin Gilljam, personal communication), approximately 70 % of the normal B cells become positive for the activation marker CD80, when the cells are stimulated via TLR9 and RP105.

5.1.4 Inhibitors of ROS

Increased levels of ROS can be both the cause and result of B cell stimulation (19). As described in section 1.4.2 of the introduction, stimulation of B cells results in early activation of NOX2-generated H_2O_2 that acts as a signaling molecule (figure 10) (86). Later, as the cells differentiate into Ig producing plasma cells, the ROS levels are mainly a result of enhanced metabolism and ER-stress (156). In order to reveal the role of ROS in normal and CVID-derived B cells, we used the NOX2 inhibitor VAS2870 (VAS). This is a well-established and validated inhibitor of NOX2, without an intrinsic antioxidant activity (157).

As an alternative way of inhibiting ROS levels, we initially also used the antioxidant N-Acetyl Cysteine (158). NAC is a general antioxidant that is expected to reduce ROS levels independent of the source. NAC clearly inhibited the ROS levels induced in stimulated B cells measured by H_2DCFDA (data not shown). However, NAC interfered with the CellROX staining that we used for combining ROS analyses with CytoID-analyses of autophagy (data not shown), and we therefore used only VAS as a ROS-inhibitor in the experiments presented in the thesis.

5.1.5 Retinoic acid as an active vitamin A metabolite

Retinoic acid (RA) is the most active vitamin A metabolite in the immune system (103, 126). As presented in section 1.5.4, B cells are incapable of oxidizing retinol to RA and therefore depend on RA from neighboring dendritic cells or from the circulation (33, 103). The physiological concentration of RA in the circulation is usually about 3-13 nM, whereas the concentration of retinol-RBP is strictly regulated within a range from 1 to 3 μM (101, 138). In the present *in vitro* experiments, we generally used 100nM of RA to stimulate the cells. Concentrations of RA between 100 and 1000 nM can be reached when RA is used for pharmacological purposes, as in cancer treatment (159). However, in our experiments, RA at concentrations as low as 1 nM had nearly the same effects on autophagy and IgG production as had 100 nM (data not shown). Still, we routinely used 100 nM RA throughout the experiments, to enable comparisons with previous results from our lab.

5.1.6 Analyses of ROS and autophagy by flow cytometry

ROS levels were measured by flow cytometry analyses of cells stained by either H_2DCFDA or CellROX. As shown in the initial experiments (Figure 11), the two methods estimated the comparable levels of ROS. Viable cells were separated from dead cells in the flow analyses by gating the cells based on granularity (SSC) and size (FSC) of the cells (Supplementary Figure 24). Based on the aberrant response to NOX2 inhibition, it would have been of interest

to stain the B cells MitoSOX Red Mitochondrial Superoxide Indicator from Invitrogen. MitoSOX stains mitochondria-derived ROS (160), and could therefore provide insight about the origin of ROS in both normal- and CVID-derived B cells.

Autophagy was quantified by flow analyses of cells stained with CytoID. We recently demonstrated that CytoID gives the same estimation of autophagy as methods based on the estimation of autophagosome formation and LC3-analyses detected by western blotting (161). The advantages of using CytoID analysis for measuring autophagy in B cells, is that it is quantitative, requires few cells, and that it can be combined with analyses of ROS by CellROX-staining of the cells.

5.1.7 Analyses of Ig production by ELISA

ELISA was used for quantifying IgG- and IgM secretion. The quantifications of Ig levels in a sample was based on a standard curve included in each experiment, and the samples should ideally fall within the linear area of the standard curve. Therefore, the samples were frequently diluted prior to analysis.

As ELISA measures the total amount of Ig in a given sample, it is highly dependent on the number of IgG- or IgM-secreting cells. Equal numbers of B cells were seeded in the culture plates, but the cell numbers after several days vary depending on the stimulation. Upon stimulating the cells with CpG-ODN, anti-RP105 and RA for 96 hours, the cells have divided 3-4 times, both CVID-derived and normal B cells (data not shown). To determine whether a given estimation of Ig levels in a sample is the result of enhanced Ig production per cell or due to the enhanced number of Ig secreting cells, one can alternatively perform ELISPOT analyses. Based on ELISPOT analyses previously performed in our lab, it was concluded that the enhanced levels of IgG in TLR9/RP105-stimulated B cells was due to enhanced secretion per cell and not due to the enhanced proliferation (16, 63). Ideally, we would have liked to perform ELISPOT analyses also on the CVID-derived B cells. However, due to the very low levels of induced IgG in these cells, this was not possible. Given the reduction of IgG levels in the stimulated CVID-derived B cells, we assume that it is both the production of IgG per cell that is reduced in these cells, as well as the reduced number of IgG-secreting cells.

5.1.8 Ethical considerations

When collecting blood from human donors, there are ethical aspects to consider. Although blood sampling is an invasive procedure, it is well tolerated by patients and volunteers. The blood volume collected from CVID patients (36 ml) is considered not to affect the disease of

the patients. However, any procedure involving breaking the natural skin barrier increases the risk of infections. For immune deficient persons like CVID patients, this can be particularly harmful. Therefore, blood samples from CVID patients in the present study were only collected in connection with routine withdrawal of blood samples for disease monitoring purposes. The patients and healthy controls included in the study, all signed a written informed consent form prior to the donation of the blood.

The project was approved by the Regional Committee for Medical and Health Research in South Eastern Norway (Supplementary 8.3), and the study adhered to the principles defined by the Declaration of Helsinki.

5.1.9 Statistical analysis

Due to high variations in B cell response both between normal blood donors and between the control group and the CVID group, non-parametric (distribution-free) tests have been used for analysis in the present study (see section 3.4). The major disadvantage of these tests is the low power and therefore the need for many observations.

There are two major pitfalls regarding the hypothesis testing statistic. Either to reject a true null hypothesis, type 1 error, or the less severe, to not reject a false null hypothesis, type II error. The most common reason for type II errors is too few observations as the basis for the statistical calculations. This type of error might explain why significant correlations were only observed on buffy-coat derived B cells (n=22, see figure 14), and not on B cells from whole blood samples (n=13, see figure 18).

In all the calculations included in the study, the level of significance is set to 5%, implying that it is a 5 % chance for the conclusion being wrong. Although some statisticians consider the level of 5 % as being too lenient, it is the standard level of significance used in biomedical research. It should, however, be emphasized that statistically significant results not necessarily imply that the result is clinical or even biological important.

5.2 Discussion of the results

5.2.1 Interplay between Ig production, autophagy, and ROS levels in stimulated B cells.

Our lab has previously demonstrated that activation of normal peripheral blood B cells via TLR9 and RP105 results in increased levels of IgG and IgM and that the Ig production involves autophagy (77, 162). In light of these results and the many links between ROS and autophagy in various cell systems (87, 96, 139), we here aimed to reveal the interplay between ROS levels, autophagy, and IgG in peripheral blood B cells. Upon activating the B cells via TLR9 and RP105, we observed that the ROS levels increased already after 2 hours. ROS are short-lived molecules (80), but we observed that the levels of ROS in B cells remained stable between 2 and 48 hours (Figure 10). After 72 hours, the ROS levels started to decline. We suspected that the early ROS generation was mediated by activation of NOX since TLR-stimulation in various cell types have been shown to activate NOX2 (82). In line with this notion, we demonstrated that the NOX2-inhibitor VAS significantly reduced the ROS levels induced after 24 hours (Figure 19). However, we did not observe that VAS reduced the levels of ROS after 48 hours of B cell stimulation (data not shown). This lost effect of VAS could be due to the inhibitor losing its effect over time. However, it is more likely that the enhanced ROS levels after prolonged stimulation of B cells origin from other sources than NOX2-activation. In support of our results, Weeler and coworkers demonstrated that BCR-mediated stimulation of B cells results in early NOX2-derived ROS, followed by later ROS derived from mitochondrial respiration (92). The enhanced mitochondrial-derived ROS level is probably the results of increased metabolism required for the extensive proliferation linked to B cell differentiation and Ig production (19).

TLR9/RP105-mediated stimulation of the B cells promoted autophagy. Enhanced autophagy was noted after 48 hours of stimulation, but was even more pronounced after 96 hours (Figure 13). Hence, the induced autophagy seemed to succeed in the induction of ROS. In the pilot experiments, we noted a significant positive correlation between ROS levels and autophagy measured after 48 hours of stimulation. ROS from different sources may promote different types of autophagy. Whereas mitochondria-generated ROS is thought to initiate stress-induced autophagy, NOX2-mediated ROS selectively activates bactericidal autophagy (139). In our experiments, the B cells are stimulated with CpG-ODN mimicking bacterial DNA, possibly initiating NOX2-mediated bactericidal autophagy. On the other hand, it is plausible that the mitochondrial ROS induced upon prolonged TLR-stimulation of the B cells may be linked to the ER-stress-induced autophagy known to sustain the Ig production in

plasma cells (76). We observed that VAS reduced the autophagy of normal B cells after 48 hours. This finding supports the involvement of NOX2-mediated ROS (Figure 20).

Despite the observed correlation between ROS and autophagy in our initial experiments on buffy coat-derived B cells, and the known involvement of autophagy in Ig production (74, 77), we did not find any correlation between ROS and IgG levels in our study. This suggests that although NOX2-mediated ROS is a signaling molecule required for activating the B cells (86), the later mitochondrial-derived ROS levels might not be required for Ig production. This is in line with previous findings of Ogura and coworkers on BCR-stimulated murine B cells. They found that mitochondrial ROS, in fact, reduces the humoral immune response in mice (163), and they suggest that mitochondrial ROS attenuate B-cell activation through a reduction in CD19 expression known to be important for BCR-induced signaling (163).

5.2.2 Regulation of ROS and autophagy in CVID-derived B cells

CVID patients suffer from common infections and complication such as respiratory- and gastrointestinal diseases, as well as autoimmune diseases and increased incidence of cancers such as B cell lymphomas (49, 52). It is therefore of great interest to reveal mechanisms that may explain these defects. As described in the introduction, TLR9-mediated B cell responses such as activation and isotype-switched Ig production are diminished in CVID patients. With the severely reduced IgG production in the CVID-derived B cells, we expected that ER-stress-mediated (mitochondrial) ROS would be reduced at late time points in these cells. However, this turned out not to be the case. Instead, we found the ROS levels measured at 48 hours to be comparable in normal- and CVID-derived B cells (Figure 15). This might at least partly be explained by the sustained high levels of IgM in B cells from CVID patients, as we know that the majority of B cells are IgM^+ B cells.

Based on the dysregulated response to NOX2 inhibition on ROS levels in CVID-derived B cells, it would have been interesting to compare the ROS levels also after 2 hours of TLR-stimulation. This would have enabled us to address possible differences in NOX2-induced ROS levels in B cells from the two study groups. However, the limited number of B cells obtained from blood samples restricted the number and types of experiments that could be performed. We routinely chose 48 hours, since we at this time point simultaneously could measure ROS and autophagy in the same samples. From the results we obtained by comparing the ROS levels at 48 hours, dysregulated ROS production does not seem to explain the defects in CVID-derived B cells. Kovács *et al* (24) described that certain murine B cells are able to

perform phagocytosis, and that NOX2-deficient murine B cells have restricted ability to kill engulfed pathogens (24). Therefore, dysregulated NOX2 may not only affect downstream signaling in CVID-derived B cells, but also affect microbial killing that will further increase their receptivity for infections.

It has been reported that patients with immunodeficiency diseases and cancer have reduced levels of antioxidants (164). However, it seems that the levels of ROS in CVID patients vary from one cell type to another. Monocytes derived from CVID-patients have enhanced levels of ROS compared to monocytes derived from healthy donors, whereas CVID-derived peripheral mononuclear cell (including lymphocytes and granulocytes) showed decreased ROS levels compared to healthy controls (165, 166). The levels of the important antioxidant glutathione were reduced in CVID-derived monocytes, possibly accounting for the higher levels of ROS in these cells (164, 167). The level of glutathione is not altered in CVID B cells (167), supporting our finding of similar levels of ROS in stimulated normal and CVID-derived B cells.

We observed that TLR9/RP105-stimulated B cells from CVID patients tended to have decreased autophagy compared to normal B cells (Figure 16). Since autophagy is known to be important for eliminating mitochondria-induced ROS (168), one would expect that reduced autophagy would result in higher levels of ROS. As already discussed, we did not find support for altered levels of ROS in CVID-derived B cells stimulated via TLR9 and RP105 for 48 hours. Interesentaly, we observed that whereas VAS severely reduced the level of autophagy in normal B cells, the inhibiting effect of VAS on autophagy in CVID-derived B cells was diminished (Figure 20). In one of the patients (#12), VAS even increased the autophagy. This could indicate that, compared to normal B cells, NOX2-mediated autophagy is less important than mitochondrial stress-induced autophagy in CVID-derived B cells. This notion is supported by the inability of VAS to inhibit the ROS levels in B cells from two of the 5 CVID patients (#11 and #13). The NOX2 inhibitor reduced the level of IgG in both normal and CVID-derived B cells, without significantly reducing the IgM levels in the CVID B cells. Again, this illustrates that some CVID patients have altered NOX2 function compared to normal B cell that is affecting their immune responses.

Whole-genome sequencing of European CVID patients have revealed that mutations in NF-κB subunit 1 is the most common known monogenic cause of CVID (52). This genotype is associated with worse prognosis in terms of Ig production and number of switched B cells, though highly variable penetrance (16). NOX2 signaling has been implicated in the activation of NF-κB (169). The abnormal VAS responses in B cells from

certain CVID patients imply that CVID patients without mutations in NF-κB subunit 1, instead might have defects in upstream regulators of NF-κB. NF-κB is known to upregulate genes essential for autophagy, and this could explain the reduced autophagy observed (170, 171). Defective NF-κB signaling might explain the observed tendency of reduced autophagy in CVID B cells, as well as the chronic inflammation frequently observed in these patients (172). Future experiments should address whether CVID patients without mutations in NF-κB itself, might have dysregulated NOX2 to deregulate NF-κB signaling.

Reduced autophagy in CVID patients with complications

Although we found a clear tendency of reduced autophagy in stimulated B cells from the whole population of CVID patients compared to the normal B cells, the effect was not statistically significant. However, when the autophagy levels were related to the different subgroups of CVID patients, we observed interesting differences (Figure 22). The levels of autophagy in stimulated B cells from the "complication group" of CVID patients were remarkably and significantly lower than in B cells from the other subgroups. Failures in the autophagic machinery are known to increase susceptibility to inflammatory- and autoimmune-diseases and for immune disorders in general (11), supporting the notion that reduced autophagy was linked to the CVID patients with most such complications. Accordingly, we also observed that this subgroup of CVID patients was the one with significantly lowest levels of IgG. Taken together, the results support the notion that dysregulated autophagy may be an underlying mechanism accountable for immune deficiency and autoimmunity characteristic for the "complication-group" of CVID patients.

Of the 13 CVID patients included in the present study, 8 of them belonged to the "complication-group" (phenotype 1 subgroup). Of these 8 patients, 6 of them also had high levels of transitory B cells associated with a more inflammatory profile (59). We observed that B cells from the 6 patients with high levels of transitory B cells tended to have both lower levels of autophagy and IgG compared to B cells from the other CVID patients. These results imply that the traits associated with phenotype 1 also are associated with high levels of transitory B cells.

5.2.3 The role of RA in the regulation of ROS levels and autophagy in normal and CVID-derived B cells

Previous research from our lab has demonstrated the ability of RA to enhance TLR9/RP105-mediated stimulation of both normal- and CVID-derived B cells (77, 159). Having shown that

TLR9/RP105-mediated stimulation of B cells from both CVID patients and healthy controls enhances the levels of ROS (Figure 15), we anticipated that RA might further increase the ROS levels in the stimulated B cells. However, this turned out not to the case. RA did not augment the TLR9/RP105-induced ROS levels, and in fact, showed a modest decreasing effect. Since vitamin A in other cell systems has exhibited anti-oxidant activity (173), we addressed the possibility that the missing effect of RA on ROS levels in stimulated B cells was due to possible anti-oxidant activity. However, we did not detect anti-oxidant activity of RA in our B cell cultures, supporting previous reports indicating that RA is a less potent antioxidant than other vitamin A metabolites. Das and co-workers ranked the antioxidant activities of vitamin A metabolites as retinol ≥ retinal > retinoic acid (124, 174).

Despite the inability of RA to augment the TLR9/RP105-induced ROS levels in normal B cells, we confirmed the previous observations from our lab that RA enhances both autophagy and Ig secretion. The effect of RA on autophagy was not detected until after 96 hours, in line with the notion that the effect requires the transcription of ULK1 (77). As mentioned, RA mainly exhibits its effect by being a transcription factor, and that could explain the delayed effect observed. It is interesting to note that RA also was able to enhance the TLR9/RP105-induced autophagy in CVID-derived B cells, although to a lesser extent than in normal B cells (Figure 16). It was particularly intriguing to find that the level of TLR9/RP105-induced autophagy in the presence of RA was significantly lower in the group of CVID patients with complications compared to the other CVID-derived B cells. This might signify that this "complication group" require higher levels of RA to obtain favorable responses.

Clinical use of vitamin A in the treatment of CVID patients

It has been suggested that low serum levels of vitamin A may have a role in the pathogenesis of CVID patients (111). It is well established that intestinal inflammation may lead to poor absorption of vitamin A, resulting in low serum levels of vitamin A (111). However, Aukrust and coworkers found that intestinal inflammation could only account for the low serum levels of vitamin A in a minority of the CVID patients (111). Since it is not anticipated that the vitamin A levels in the diet differs between CVID patients and healthy individuals, the main current hypothesis is that CVID patients have a higher turnover rate of vitamin A due to a general increased rate of infections (111). A varied diet provides enough vitamin A to maintain a concentration of approximately 10 nM RA in the serum of healthy individuals. From our *in vitro* results, concentrations of RA as low as 1nM enhances autophagy and Ig

levels in TLR9/RP105-stimulated B cells, implying that even low concentrations of RA could have a positive effect on B cell responses in healthy people. However, as it seems that CVID patients have a higher turnover of RA, a higher intake of vitamin A rich food than the recommendations might have beneficial effects.

Previous studies have explored the possibility of using vitamin A in the treatment of CVID patients (175, 176). Aukrust and coworkers revealed that vitamin A supplementation to vitamin A deficient CVID patients improved IgG secretion from PBMCs *in vitro*, enhanced IgA secretion *in vivo*, and shifted the cytokine profile to the more anti-inflammatory IL-10 levels and reduced TNFα. Saxon and coworkers improved the differentiation of circulating B cells by *in vivo* administrating 13-cis RA to CVID patients (30) but found enhanced levels of Igs in only a few of the patients (30). Interestingly, Saxon suggested that RA was not effective alone, but required stimulation of the B cells (30). This is supported by our *in vitro* results, demonstrating that effects of RA on autophagy and Ig secretion in both normal and CVID-derived B cells require co-stimulation via TLR9 and RP 105 (Figure 16 and 17).

Since vitamin A is a fat-soluble vitamin with potential toxic effects at high concentrations, excessive use should be avoided (113, 173, 177). In the present study, we have shown that the B cell responses vary considerably between donors. Our group has previously hypothesized that CVID patients with low responses to RA in terms of IgG are the one with $IRF4^{high}AICD^{low}$- expressing B cells (30). It would be interesting to correlate this subgroup of CVID patients to the "complication" subgroup identified in the present study. It is possible that these CVID patients might need higher levels of RA to obtain favorable responses in terms of induction of autophagy and IgG. Before clinical testing can be initiated, further *in vitro* studies on a larger number of patients are required. Effects of different vitamin A metabolites should also be tested. 13-cis RA is for instance commonly used in cancer treatment, due to its improved stability in the body (176, 178, 179).

6 Conclusions

In the present master thesis, we have addressed the potential interplay between ROS levels, autophagy and Ig production in TLR9/RP105-stimulated normal B cells and B cells from CVID patients. The role of RA in these processes has also been explored.

The conclusions related to the specific aims of the thesis are:

1. A positive correlation between ROS levels and autophagy was revealed in normal TLR9/ RP105-stimulated B cells. There was no significant correlation between Ig secretion and ROS levels or autophagy in these cells, but NOX2-induced ROS was essential for both autophagy and Ig secretion in the TLR9/RP105-stimulated normal B cells.
2. Reduced autophagy was associated with low IgG production in B cells from a subgroup of CVID patients characterized by a more severe disease. There were no differences in the ROS levels between stimulated B cells from CVID patients and healthy controls. However, aberrant responses to NOX2-inhibition were revealed in B cells from certain CVID-patients.
3. RA did not enhance the ROS levels in TLR9/RP105-stimulated B cells, but enhanced autophagy and Ig secretion in stimulated B cells from both CVID patients and healthy controls.

Taken together, the present results suggest a critical role of autophagy in RA-induced IgG production in TLR9/RP105-stimulated B cells from a subgroup of CVID-patients characterized by a serious disease. More studies are needed to get a better understanding of ROS as a signal transducer in B cells. In light of the dysregulated response to NOX2-inhibition, it would be particularly important to explore the impact of different sources of ROS on autophagy and Ig secretion in both normal- and CVID-derived B cells. The presented results indicate that RA has beneficial immune modulating effects both in normal- and in CVID-derived B cells. However, in order to enable clinical use of RA for CVID patients, it will be important to optimize doses and forms of RA to balance beneficial and possible toxic effects.

7 References

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8 Supplementary

8.1 Detailed description of solutions and chemicals

Chemicals	**Manufacturer**	**Location**
CpG oligonucleotides phosphorothionate (CpG-ODN)	Enzo Life Science	New York, USA
All-trans retinoic acid (RA)	Sigma-Aldrich	Missouri, USA
ELISA Blocking Buffer	Bethyl Laboratories	Texas, USA
ELISA coating Buffer	Bethyl Laboratories	Texas, USA
ELISA Horseradish Peroxidase (HRP) conjugate	Bethyl Laboratories	Texas, USA
ELISA Sample Diluent	Bethyl Laboratories	Texas, USA
ELISA standard IgG	Bethyl Laboratories	Texas, USA
Elisa Standard IgM	Bethyl Laboratories	Texas, USA
Elisa Wash solution	Bethyl Laboratories	Texas, USA
Ethanol (70% solution)	University of Oslo	Oslo, Norway
Ethylenediaminetetraacetic acid (EDTA) 0.5 M	Sigma-Aldrich	Missouri, USA
Fetal Bovine Serum (FBS)	Bionordika	Oslo, Norway
Sulfuric acid solution (H2SO4) 2N	Sigma-Aldrich	Missouri, USA
NovoClean Solution 5X	AH Diagnostics	Oslo, Norway
NovoRinse Solution 5X	AH Diagnostics	Oslo, Norway

NovoFlow Sheath Fluid (1X)	AH Diagnostics	Oslo, Norway
VAS2870	Sigma	Missouri, USA
CellROX Oxidative Stress Reagents Green	Enzo Life Science	New York, USA
H2DCFDA (H2-DCF, DCF)	Invitrogen	California, USA
CYTO-ID® Autophagy detection kit	Enzo Life Science	New York, USA
NovoCyte® QC Particles (Generation 2)	ACEA	San Diego, USA
RPMI 1640	Bionordika/Lonza	Oslo, Norway
Dynabeads® CD19 Pan B	Invitrogen	California, USA
DETACHaBEAD® CD19	Invitrogen	California, USA
Penicillin Streptomycin (PS)	Thermo Fischer	Massachusetts, USA
Serum FBS South America, ultra-low endotoxin	Bionordika	Oslo, Norway
Hydrogen peroxide solution	Sigma	Missouri, USA

Table 3: List of chemicals

Antibodies	**Manufacturer**	**Location**
Purified anti-human CD180 (RP105) antibody	BioLegend	California, USA
Dynabeads CD19	Invitrogen	California, USA
Elisa Capture antibody for IgG	Bethyl Laboratories	Texas, USA
Elisa Capture antibody for IgM	Bethyl Laboratories	Texas, USA

Table 4: List of antibodies

Solutions	
I	**Medium (RPMI/PS)**
	10 ml PS
	500 ml RPMI 1640 medium
II	**Culture medium (RPMI/PS/FBS)**
	50 mL FBS
	10 mL PS
	500 mL RPMI 1640 medium
III	**PBS**
	1 PBS tablet
	200 mL deionized water
IV	**Blocking Buffer (ELISA)**
	1 packet TBS with 1% BSA
	100 ml deionized water
V	**Coating Buffer (ELISA)**
	1 carbonate-bicarbonate buffer capsule
	100 mL deionized water
VI	**Sample Diluent (ELISA)**
	10 mL TBS
	90 mL deionized water
	50 ul Tween 20

	1g BSA
VII	**Washing solution (ELISA)**
	100 mol 10 x TBS
	500 µl Tween 20
	900 ml dH2O

Table 5: List of solutions

Equipment	Manufacturer	Location
96 well Microtiter Plates (for ELISA)	Bethyl Laboratories	Texas, USA
Cell culture flasks (75 cm3)	Thermo Fisher Scientific	Massachusetts, USA
Cell culture plates (6,12,24, or 48 wells)	Thermo Fisher Scientific	Massachusetts, USA
Counting slides	Bio-Rad	California, USA
Eppendorf tubes, 1.5mL	BrandTech	Connecticut, USA
Falcon tubes, 14 mL	BD Falcon	New Jersey, USA
Falcon tubes, 50 mL	BD Falcon	New Jersey, USA
Magnet for cell culture flasks, 75 cm3	Homemade (UiO)	Oslo, Norway
Magnet for Eppendorf tubes	Invitrogen	California, USA
Magnet for Falcon tubes, 14 mL	Invitrogen	California, USA
Parafilm "M" ® Laboratory Film	Bemis Company	Wisconsin, USA
Pipete tips (5, 10, 25 mL)	BD Falcon	New Jersey, USA
Pipette tips (0.01, 0.02, 0.2, 1mL)	VWR	Pennsylvania, USA
Plastic wrap	Lyreco	Nord, France
Sterile Scissors	Rocket Medical	Hertfordshire, UK

Table 6: List of equipment

Instruments	Manufacturer	Location
Eppendorf Centrifuge 5424	Eppendorf	Hamburg, Germany
Forma Steri-Cycle Co2 incubator	Thermo Fisher Scientific	Massachusetts, USA

NovoCyte flow cytometer	ACEA	San Diego, USA
Multiscan EX	Thermo Fisher Scientific	Massachusetts, USA
Reax top, vortexer	Heidolph	Nuremberg, Germany
Rockn Roller	Labinco	Breda, Netherlands

Table 7: List of instruments

8.2 Illustration from NovoExpress

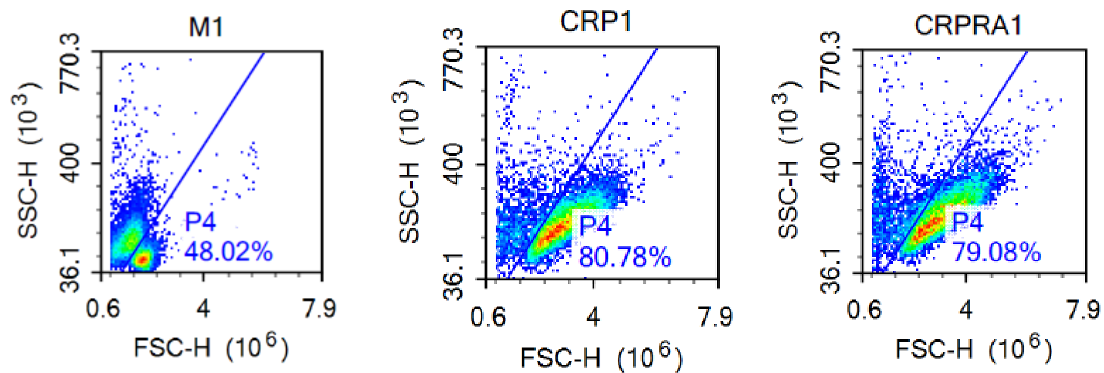

Figure 24: Illustration of the gating of viable and dead using the NovoExpress Software. Viable cells are gated in P4. M1 = unstimulated cells; CRP1 = B cells stimulated via TLR9 and RP105; CRPRA1 = B cells stimulated via TLR9and RP105 in the presence of RA.

8.3 Approvals from REK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Hege Holde Andersson	22845514	28.11.2017	2017/2296/REK sør-øst B
			Deres dato:	Deres referanse:
			15.11.2017	

Vår referanse må oppgis ved alle henvendelser

Til Heidi Kiil Blomhoff
UiO, Institutt for medisinske basalfag

2017/2296 Regulering av celledeling og apoptose i lymfocytter

Forskningsansvarlig: Universitetet i Oslo
Prosjektleder: Heidi Kiil Blomhoff

Vi viser til søknad om prosjektendring datert 15.11.2017 for ovennevnte forskningsprosjekt. Søknaden er behandlet av sekretariatet i REK sør-øst på delegert fullmakt fra REK sør-øst B, med hjemmel i helseforskningsloven § 11.

De omsøkte endringene er beskrevet i skjema for prosjektendringer og gjengis her i sin helhet:
«B-cellene som isoleres fra de normale blodgiverne lagres ikke, men benyttes umiddelbart i forsøk. Vi får ingen opplysninger om kjønn, alder eller andre forhold når vi mottar blodproduktene (buffycoats og fullblod). På bakgrunn av dette, mener vi det ikke lenger er riktig å opprettholde en biobank knyttet til prosjektet.»

I tillegg søkes det om en forlengelse av prosjektperioden til 31.12.2027.

Komiteens vurdering
Komiteen har ingen innvendinger til de omsøkte endringene.

Vedtak
Komiteen har vurdert endringsmeldingen og godkjenner prosjektet slik det nå foreligger med hjemmel i helseforskningsloven § 11.

Godkjenningen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i endringsmeldingen.

Klageadgang
Komiteens vedtak kan påklages til Den nasjonale forskningsetiske komité for medisin og helsefag, jf. Forvaltningslovens § 28 flg. Eventuell klage sendes til REK Sør-øst. Klagefristen er tre uker fra mottak av dette brevet.

Vi ber om at alle henvendelser sendes inn via vår saksportal: http://helseforskning.etikkom.no eller på e-post til post@helseforskning.etikkom.no.

Vennligst oppgi vårt referansenummer i korrespondansen.

Besøksadresse:
Gullhaugveien 1-3, 0484 Oslo

Telefon: 22845511
E-post: post@helseforskning.etikkom.no
Web: http://helseforskning.etikkom.no/

All post og e-post som inngår i saksbehandlingen, bes adressert til REK sør-øst og ikke til enkelte personer

Kindly address all mail and e-mails to the Regional Ethics Committee, REK sør-øst, not to individual staff

Med vennlig hilsen

Knut W. Ruyter
avdelingsdirektør
REK sør-øst

Hege Holde Andersson
komitésekretær

Kopi til: *l.t.helgesen@medisin.uio.no*

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Hege Holde Andersson	22845514	30.03.2012	2012/521/REK sør-øst B
			Deres dato:	Deres referanse:
			25.03.2012	

Vår referanse må oppgis ved alle henvendelser

Stig S Frøland
Postboks 4950 Nydalen
0424 Oslo

2012/521 B Patogenetisk betydning av kronisk inflammasjon ved immunsviktsykdommer

Vi viser til innsendt prosjektendringsskjema for ovennevnte studie mottatt 25.03.2012.

Forskningsansvarlig: Oslo universitetssykehus
Prosjektleder: Stig S Frøland

Prosjektomtale
Sykdommer hvor immunsvikt spiller en vesentlig rolle, blir stadig viktigere. Det dreier seg om medfødte, evt arvelige, immunsviktsykdommer, om HIV-infeksjon og om følger av visse behandlingsformer, bl.a. immundempende midler og transplantasjon. Det er av vesentlig betydning å kartlegge hvordan immunsvikt oppstår ved disse sykdommene. Det er holdepunkter for at kronisk betennelse (inflammasjon) kan bidra til immunsviktutviklingen. Kartlegging av sentrale komponenter i betennelsesprosessen ved immunsvikt kan gi helt nye angrepspunkter for behandling og forebygging av immunsvikt.Prosjektet tar sikte på studier av kronisk betennelse ved HIV-infeksjon, medfødt immunsvikt av typen CVID, den alvorlige soppsykdommen Aspergillose og Wegeners granulomatose. Etter samtykke fra pasientene vil det bli samlet inn blod- og vevsprøver tatt som ledd i ordinær diagnostikk og behandling. Disse undersøkes med tanke på betennelsesmekanismer. Enkelte genetiske undersøkelser vil også inngå i analysene.

De omsøkte endringene er beskrevet i skjema for prosjektendringer og dreier seg om at Kristian Bjøro blir ny prosjektleder for prosjektet. Per Morten Sandset blir ny ansvarshavende for forskningsbiobanken.

Komiteens vurdering
Komiteens leder har på delegert fullmakt vurdert endringssøknaden. REK sør-øst B har ingen forskningsetiske innvendinger til prosjektet slik det nå foreligger.

Vedtak
Komiteen har vurdert endringsmeldingen og godkjenner prosjektet slik det nå foreligger med hjemmel i helseforskningsloven § 11.

Godkjenningen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i endringsmeldingen.

Dersom det skal gjøres vesentlige endringer i prosjektet i forhold til de opplysninger som er gitt i søknaden, må prosjektleder sende endringsmelding til REK.

Besøksadresse: Gullhaug torg 4A, Nydalen, 0484 Oslo
Telefon: 22845511
E-post: post@helseforskning.etikkom.no
Web: http://helseforskning.etikkom.no/
All post og e-post som inngår i saksbehandlingen, bes adressert til REK sør-øst og ikke til enkelte personer
Kindly address all mail and e-mails to the Regional Ethics Committee, REK sør-øst, not to individual staff

Forskningsprosjektets data skal oppbevares forsvarlig, se personopplysningsforskriften kapittel 2, og Helsedirektoratets veileder for «Personvern og informasjonssikkerhet i forskningsprosjekter innenfor helse- og omsorgssektoren».

Prosjektet skal sende sluttmelding på eget skjema, senest et halvt år etter prosjektslutt, jf. helseforskningsloven § 12.

REKs vedtak kan påklages til Den nasjonale forskningsetiske komité for medisin og helsefag, jfr. helseforskningsloven § 10, 3 ledd og forvaltningsloven § 28. En eventuell klage sendes til REK sør-øst. Klagefristen er tre uker fra mottak av dette brevet, jfr. forvaltningsloven § 29.

Vi ber om at alle henvendelser sendes inn via vår saksportal: http://helseforskning.etikkom.no eller på e-post til: post@helseforskning.etikkom.no.

Vennligst oppgi vårt referansenummer i korrespondansen.

Med vennlig hilsen,

Stein Opjordsmoen Ilner (sign.)
Dr. med.
Overlege, professor

Hege Holde Andersson
komitésekretær

Kopi til: per.morten.sandset@ous-hf.no
kristian.bjorlo@ous-hf.no
Oslo universitetssykehus