Connecting B cell differentiation pathways and antibody deficiencies

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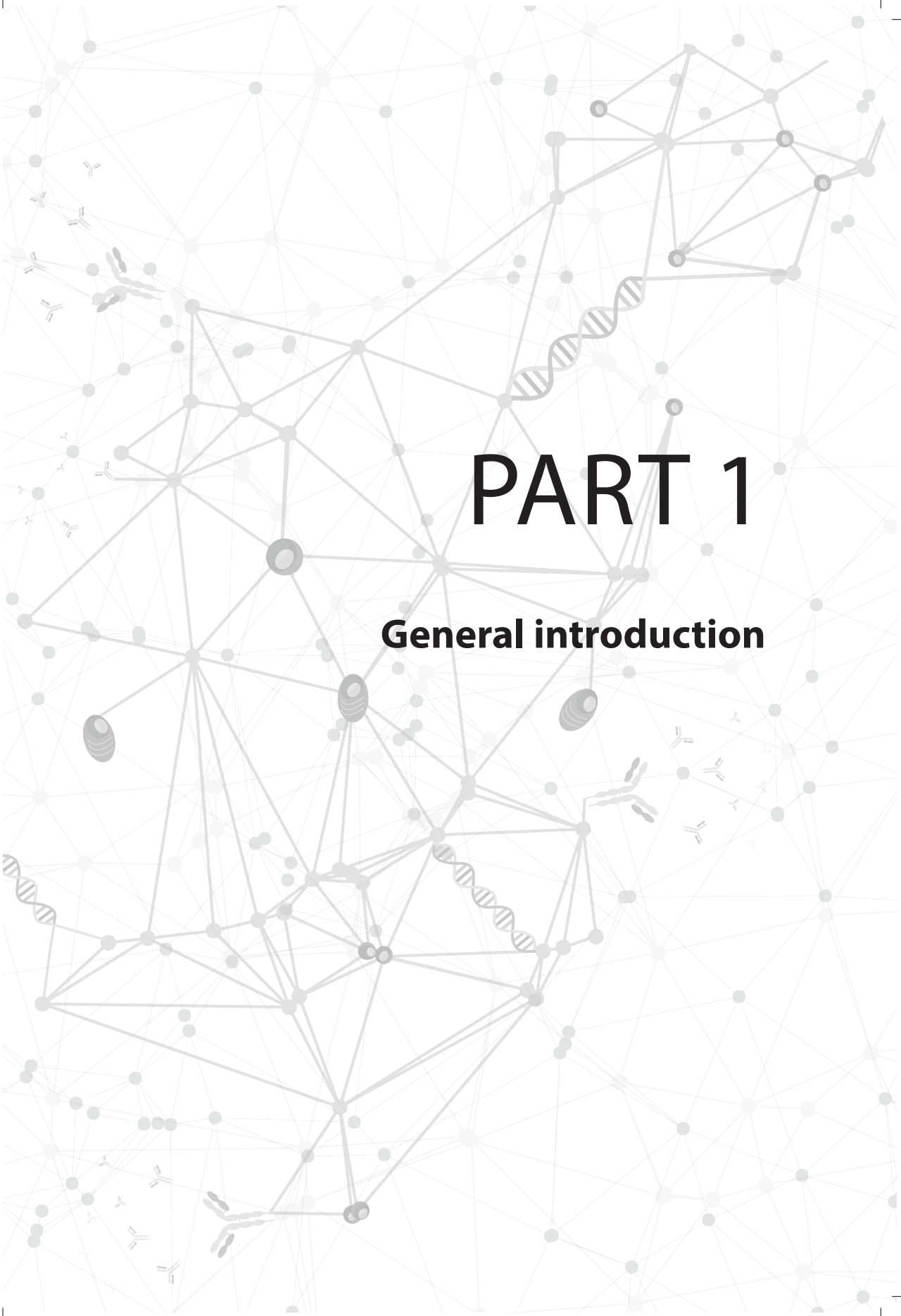


PART 1

General introduction

GENERAL INTRODUCTION

The human body consists of a collection of tissues and organs, each with its own unique composition of different cell types and extracellular matrices. To maintain integrity of all of these tissues and organ systems it is vital that potential threats from both the outside, in the form of foreign substances and infectious micro-organisms and from the inside, like (pre-)malignant cells, are recognized and neutralized. This work is done by a specialized and highly diverse organ system called the immune system.

The immune system consists of a cellular compartment of leucocytes circulating in the peripheral blood and a set of solid tissues called the primary (bone marrow and thymus) and secondary (spleen, lymph nodes and mucosal associated lymphoid tissue) lymphoid organs, that facilitate the generation, differentiation and maturation of the immune cells (Figure 1a). The large number of different cell types and tissues, each with its own function, makes the immune system a highly dynamic system that requires strict regulation. The cells of the immune system arise from hematopoietic stem cells in the bone marrow and are divided into the myeloid lineage and the lymphoid lineage. Precursor cells of the myeloid lineage can develop into erythrocytes, monocytes, granulocytes and thrombocytes.[1] The common lymphoid progenitors give rise to B lymphocytes (B cells), T lymphocytes (T cells), natural killer cells (NK cells) and dendritic cells.[2] Erythrocytes and thrombocytes are not part of the immune-system. The others cells together are the leucocytes, or white blood cells (WBCs) which are divided into the innate (granulocytes, macrophages, monocytes, dendritic cells and NK cells) and the adaptive system (T cells and B cells). Innate immune cells recognize pathogens using germ-line encoded receptors for conserved molecular patterns which are specific for microorganisms.[3, 4] Its response is fast, not antigen specific and does not require prior sensitization; then, innate cells do not form immunological memory. The adaptive immune system is complementing the innate system in many ways: pathogens are recognized by antigen-specific, somatically generated receptors that can be adapted to increase specificity, but require a few days to be generated. Additionally, the adaptive system can form an immunological memory and thus protect from recurrent infections.[5, 6] Together, these two cellular systems form a solid defense to pathogens.

If one of the components of the immune system is lacking or unable to fulfil its function due to an inborn defect, this is called a primary immunodeficiency (PID).[7-9] A large group of PIDs comprises the Primary Antibody Deficiencies (PAD), which are caused by reduced or absent production of antibodies (also referred to as immunoglobulin (Ig)) by the B cells. Defects in any stage of B cell development, differentiation, activation and maturation can lead to PAD.[10-13] This thesis will focus on defects at different stages of B cell development and how those can lead to PAD. In relation to that, it is essential to study normal precursor B cell development in bone marrow, B cell receptor repertoire formation, B-cell receptor

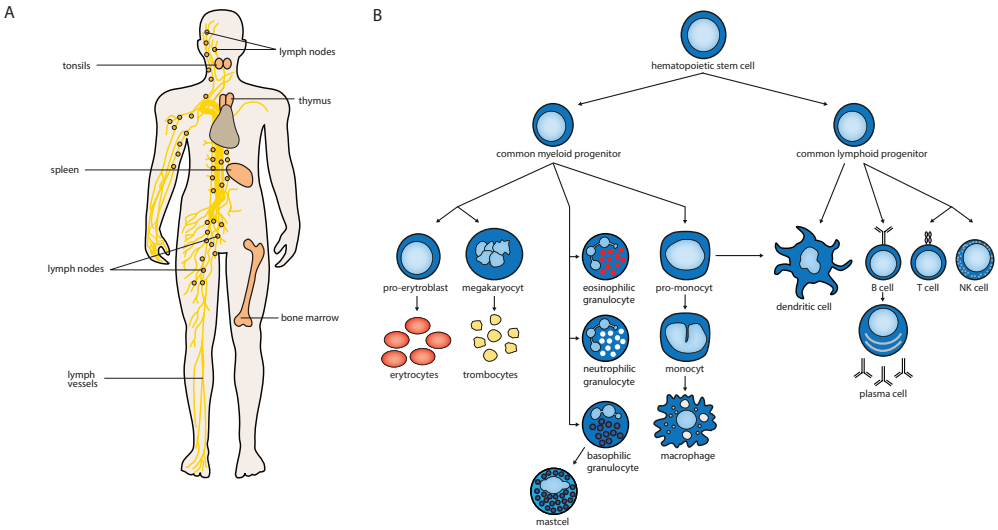


Figure 1.
Systematic overview of the immune system. **A.** The primary and secondary lymphoid organs
B. Overview of the hematopoietic cells divided into the myeloid lineage and the lymphoid lineage

mediated signaling and B cell intrinsic signaling in response to stimulation. To this extent, different techniques can be employed, many of which have been improved or even newly developed within the past few years. We aimed to make optimal use of available techniques and set-up our own assays to study B cell development and its defects in the context of PAD.

NORMAL B CELL PRECURSOR DEVELOPMENT IN HUMAN BONE MARROW

From stem cell to B cell

The B cells arise from multipotent hematopoietic stem cells that reside in the bone marrow.[14] These cells are long lived and self-renewing, and have the capacity to give rise to pluripotent hematopoietic progenitor cells. Because of their self-renewing capacity, the stem cells can provide a life-long supply of pluripotent progenitor cells, which in turn can give rise to different cell types within a specific cell lineage. In this way, pluripotent cells are formed that give rise to the myeloid lineage and the lymphoid lineage. It is important to keep in mind that this process is most studied in mice, and although most processes and factors have the same function in human bone marrow, some might have different importance and effects.[15, 16] Upon further differentiation into their lineage, cells become more committed to their specific fate. This is regulated by of combinations of transcription factors that at the same time promote one lineage and suppress others.[17] However, experiments in mice

have shown plasticity and dedifferentiation upon transcriptional reprogramming.[18, 19] One of the key transcription factors which is vital for hematopoiesis is PU.1 (Figure 2) which has a broad, but graded expression pattern.[20] Different PU.1 expression levels have a critical role in defining differentiation of different lineages; an artificial moderate PU.1 level promotes B cell development ,[21] while loss of PU.1 in mice prevents development of all lymphoid cells.[22] Another broadly expressed factor is the zinc finger transcription factor IKAROS,[23] which expression is required for the progression into lymphoid lineages[24] and which is involved in transcriptional control of the rearrangement of IgH and IgL loci during later stages of B cell development.[25] While PU.1 and Ikaros promote a lymphoid fate, the myeloid differentiation is being inhibited by the expression of E2A and IL7R.[18, 26, 27] This transcriptional program forms the common lymphoid progenitor (CLP), in which induction of recombination activating genes 1 and 2 (RAG1 and RAG2)[28] restricts the cell further to either a T or a B cell faith.[29, 30] In mice, expression of Ly6D marks the branching of T and B cells, with Ly6D$^+$ cells being called: B cell biased lymphoid progenitors,[31] in which the B cell specific transcription factor network is initiated. Key players in this network are Early B-cell factor 1 (Ebf1), essential for B cell specification and commitment[30, 32] and paired box protein 5 (PAX5), completing commitment to the B cell lineage.[33, 34] PAX5 is a critical B cell lineage commitment factor that induces the expression of multiple B cell specific markers such as CD79a (also known as Igα), which can be detected in the cytoplasm of pro-B cells, and CD19, which is first expressed in the pre-B stage.[18] These cells however, have been

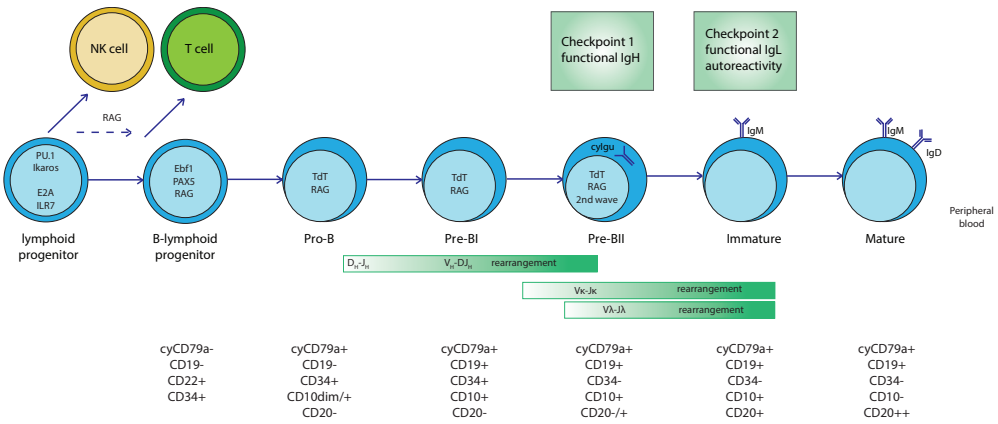


Figure 2.
B cell precursor differentiation stages in the human bone marrow. Important transcriptionfactors are indicated in the cell nuclei at individual differentiation stages. The bottom panel indicated the immunphenotype per differentiation stage.

shown to retain some plasticity in mice.[35] If Pax5 was deleted after the first B cell stages in conditional knock-out mice, those cells dedifferentiated and committed to the T-cell lineage.[36] This indicates the dual role of transcription factors like PAX5: supporting the one lineage, while suppressing another one. Once committed to the B-cell lineage, the goal of the B cell precursor (BCP) is to form a functional B cell receptor, a process that started already with the induction of RAG1 and RAG2 at the common lymphoid progenitor stage. Further progression in development and differentiation is for a large part regulated by and dependent on how well the cell succeeds to form a B cell receptor.

Structure of the B-cell receptor

The B-cell receptor (BR) of Immunoglobulin (IG) consists of two heavy chains (IGH) and two light chains, either IGκ or IGλ.(Figure 3) All chains have a variable and a constant domain. The variable domain of the IGH is encoded by a combination of one V, one D and one J gene, the variable domain of the IGκ or IGλ in encoded by a combination of one V and one J gene. The variable region is important for antigen recognition. It consists of complementary determining regions (CDRs), which bind the antigen, and frame-work regions (FRs), which are needed for proper folding and configuration of the protein. IGH also comprises a constant region, for which 9 genes are available on the IGH locus (Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2). Different constant regions have different effector functions.

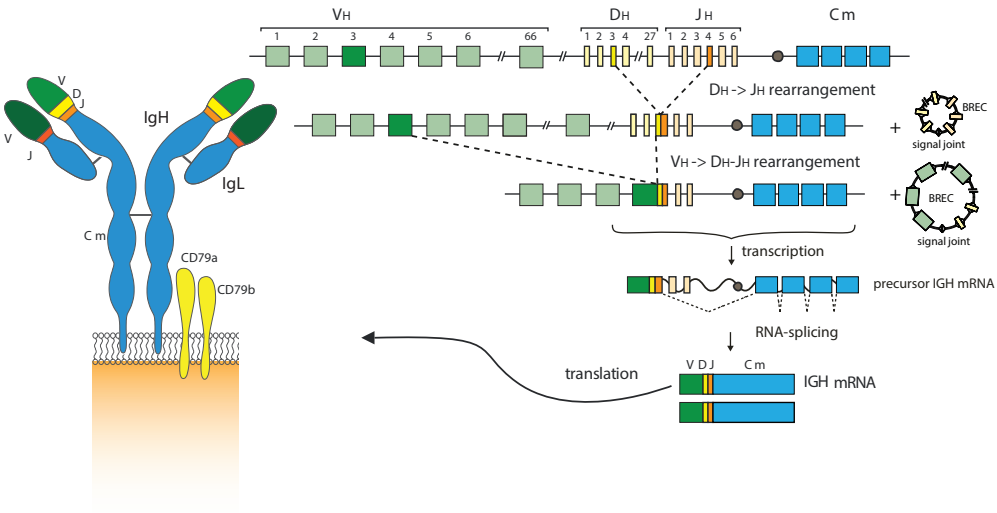


Figure 3.
Structure of the B cell receptor (BR). The left panel is a schematic representation of the BR, consisting of two heavy and two light chains and accompanied by CD79a and CD79b upon expression on the plasma membrane. The right panel is a schematic representation of the process of V(D)J-recombination.

V(D)J recombination

The V, D, and J genes need to be combined to form a functional BR (Figure 3). This process of VDJ-recombination takes place in the early B cell development in bone marrow. Ordering of the recombination process is controlled by multiple mechanisms, amongst which a specific transcriptional network,[37] localization in the nucleus[38] and epigenetics that control the accessibility of the chromatin.[39, 40] During the pro-B cell stage first DH-JH incomplete rearrangements of both the IGH loci takes place.[41, 42] The lymphocyte specific RAG1 and RAG2 recognize the recombination signal sequences (RSSs) that are flanking the coding regions and induce single strand nicks between the coding segment and the RSS. This results in a hairpin structure on the coding end and a blunt end at the RSS-end.[43] Since this happens at both the D and the J genes, all genes in between are cut out and ligated into signal joints. The resulting DNA double strand breaks are recognized by the non-homologous end joining machinery. The DNA-dependent protein kinase (DNA-PK) complex is recruited to the DNA end, to protect it from exonuclease activities.[44] Next, Artemis is recruited to this complex and phosphorylated so it will open the hairpin of the coding ends.[45] At this point nucleotides can be removed by exonuclease activity and the protein deoxynucleotidyl transferase (TdT) can add non-templated nucleotides.[46] These deletions and random nucleotides create junctional diversity. Finally, both ends are ligated by a protein complex consisting of DNA ligaseIV (LIG4), XRCC4 and XRCC4-like factor (XLF).[44] In the preB-I stage, one allele performs VH-DJH recombination in the same way as was done for the DH-JH recombination. When this results in an unproductive sequence, the second allele is rearranged.[40, 47] After formation of a VDJ-exon, this is transcribed and spliced to the Cμ exon. The resulting heavy chain can be expressed as pre-BR together with the surrogate light chain. After recombination of a functional IGH, RAG is re-expressed and the light chain V and J segments are rearranged.[48, 49] Besides different combinations of V and J, the light chain can either be formed from the kappa or lambda gene, resulting in many different light chains that can be formed. The multitude of VDJ-combination and IGH-IGL combinations, create an enormous diversity in different BR molecules that can potentially be formed.[50] This is referred to as the BR-repertoire of the naive B cells.

Checkpoints in precursor B-cell development

In addition to producing a functional BR, committed B cells need to go through multiple rounds of proliferative expansion, to form the large pool of B cells that is required for good humoral immunity. These two processes must be tightly regulated to prevent genomic instability, which can lead to malignancies. Furthermore, only cells with a functional, non-autoreactive BR must proliferate to ensure a proper function of B cells at the phase of antigenic selection. Once recombination of the heavy chain is successful, a cytoplasmic form of the BR heavy chain can be expressed and detected.[51] This heavy chain can pair with

a surrogate light chain (composed of the λ-like and VpreB proteins) and be expressed on the surface in a complex together with Igα (also known as CD79a) and Igβ (also known as CD79b)[52] that form a heterodimer. The expressed pre-BR provides a docking site for Lyn and Syk (Figure 4), two kinases that can recruit and activate SLP-65 (also known as B cell linker (BLNK)) and BTK, which in turn activates PLCγ2 by phosphorylation. It is important to note that this process and most importantly the function of BTK and IL7R-mediated signaling, is different in mice and man. In humans, deficiency in BTK results in a lack of peripheral B cells, whereas BTK-KO, even though there is an impairment in pre-B-cell development, do have peripheral B cells.[53] Although different proteins expressed on bone marrow stromal cells have been proposed to serve as a ligand,[54, 55] establishment of a feeder-cell free *in vitro* system in which human stem cells can be differentiated into functional B cells, has shown pre-BR signaling without a ligand is possible.[56] Successful signaling via this cascade induces allelic exclusion of the second IGH allele,[47] it induces proliferation via the MAPK pathway[57] and it represses RAG expression[58] to prevent DNA double strand breaks occurring during proliferation. After a couple of rounds of cell division, the IgK-locus is activated and RAG protein expression is upregulated to induce VJ-rearrangement of the BR light chain.[39, 48] Not all cells succeed to signal via expression of a pre-BR, either because they did not rearrange a functional heavy chain, or their heavy chain was not able to pair with a light chain, or other

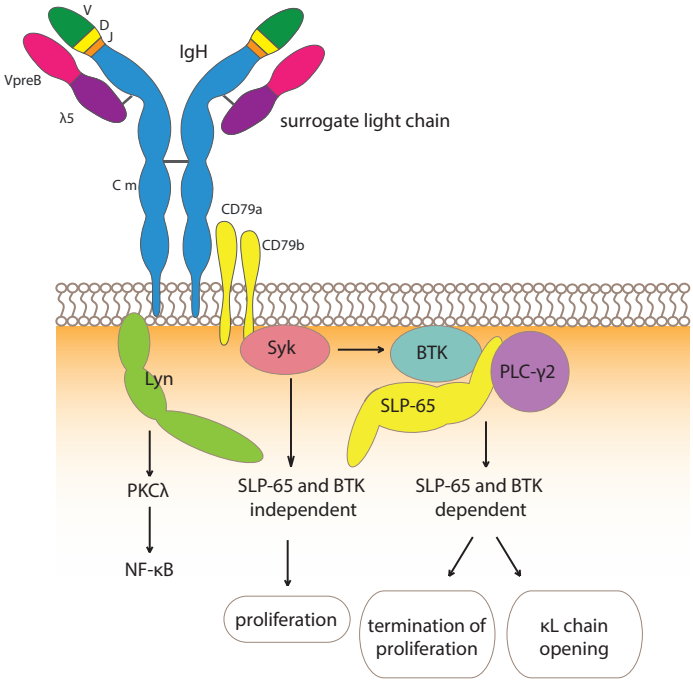


Figure 4.
Pre-BR signaling via multiple intracellular signaling molecules in BCP in human bone marrow.

components of the signaling cascade are missing.[59, 60] Without signaling via this cascade, cells will die. Thus, the cells that have rearranged a functional heavy chain that can pair with the surrogate light chain and can that be expressed to induce pre-BR signaling are positively selected for at this pre-BR checkpoint (Figure 2).[61]

A second checkpoint in precursor B cell development takes place during the immature B-cell stage (Figure 2), when cells have recombined a functional light chain and can express a BR in the form of IgM on their surface.[29] Cells that express a functional light chain that is able to pair with the cells' heavy chain and can be expressed on the surface, down regulate RAG1 and RAG2 expression and stop light chain rearrangement.[49] However, if the expressed surface-IgM signals above a certain threshold (because the BR responds to membrane bound or soluble auto-antigens), this might be indicative of auto reactivity. Then, negative selection of cells that experience high BR mediated signaling is initiated. Experiments with transgenic autoreactive mouse models indicated that high-affinity interactions lead to elimination of the cells (clonal deletion),[62] and low-affinity interactions and soluble antigens result in anergy of the cells or in receptor editing.[63-65] During the latter process the light chain loci are further rearranged under continuous RAG expression, continuing with the second Kappa allele or the Lambda alleles until a functional light chain is formed, that together with the heavy chain does not result in an autoreactive BR.

NORMAL B CELL DEVELOPMENT IN THE HUMAN PERIPHERAL BLOOD AND SECONDARY LYMPHOID ORGANS

After formation of a functional, non-autonomously signaling, non-autoreactive BR that can be expressed on the cell surface, the B cell is ready to egress from the bone marrow to the peripheral blood. The B cell is now considered mature, however, since it has not encountered antigen yet, it is still called naive. Further maturation of the B cell, from this stage onwards, will be antigen dependent (Figure 5).

The peripheral blood

The earliest stage of B cell development in peripheral blood is the transitional B cell, that has just exited the bone marrow. These are B cells that express IgM and IgD on their surface, but still have some phenotypic markers that indicate their recent exit from the bone marrow, like increased expression of CD38 and high expression of CD24.[66] Some additionally consider low expression of CD21 and increased expression of CD10 to define these cells phenotypically.[67, 68] The $CD10^{+}$ CD21lo fraction has been suggested to represent regulatory B cells[67] a cell type that is found and studied in mice.[69] After the transitional

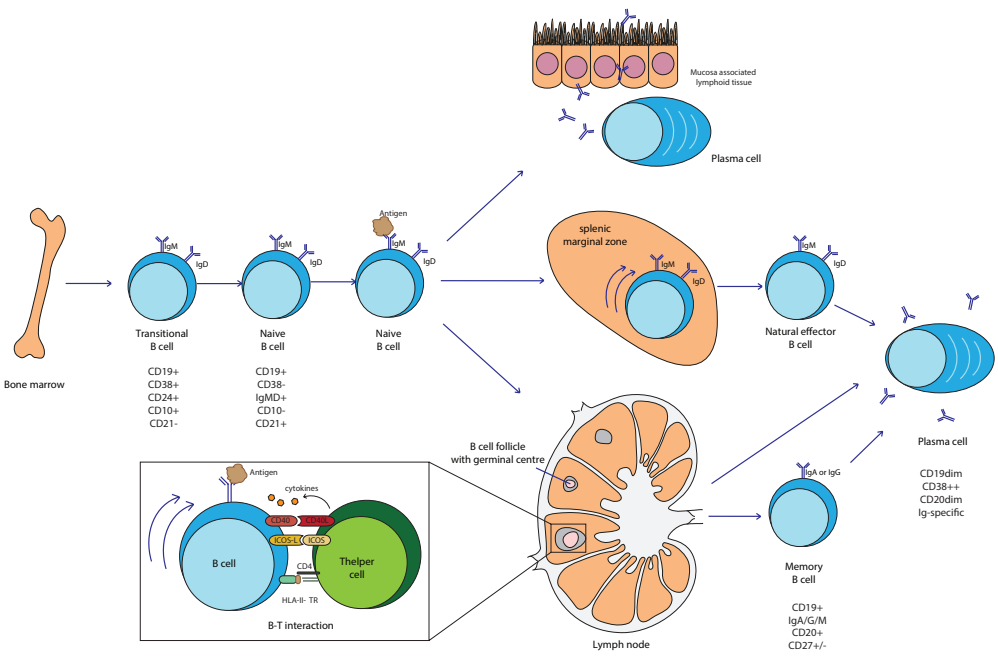


Figure 5.
B cell differentiation in the peripheral blood and secondary lymphoid organs.

stage, expression of CD21 is high on the B cells, although a small proportion of CD21lo peripheral B cells can be found in healthy controls. Their biological role has been much disputed, and high numbers of CD19hiCD21lo cells are found in a variety of diseases including systemic lupus erythematosous,[70] HIV,[71] hepatitis C,[72] Downs' syndrome,[73] and CVID.[74] Recently Keller *et al.* have shown, that high SYK expression is a common feature of CD21lo B cells independent of the underlying disorder.[75] In addition, Unger *et al.* found a possible relation between IFN-γ related auto-immunity in CVID and a high number of CD21lo cells,[76] indicating a common mechanism by which these cells arise in patients.

However, in healthy B cell development most B cells in the peripheral blood gain CD21 at the naive B cell stage. Naive B cells are defined by their expression of IgD and the lack of CD27 on their surface.[77] They patrol the peripheral blood and lymphoid organs in search for the antigen that specifically binds to their BR. After recognizing and binding antigen, the naive B cell moves to the follicles of one of the secondary lymphoid organs for further maturation. Here they will gain their effector function, either becoming an antigen secreting plasma cell or a memory B cell.[78] In the mucosal associated tissue (MALT), mainly IgA-secreting plasma blasts can be formed, which produce IgA that can be excreted, for example into the lumen of the small bowel.[79] Memory cells enter the

bloodstream again after maturation in the lymph node or spleen, they express only one of the immunoglobulin isotypes, IgM, IgA, IgG, or IgE. Many of these cells additionally express CD27, which was considered to be the hallmark of memory B cells.[80] However, over the past years, several studies have shown the presence of CD27- memory B cells, which have an activated phenotype and carry molecular signs of antigen experience.[81, 82] Also plasma cells can be detected in the peripheral blood, those are characterized by high expression of CD38 and CD27 and a reduced expression of CD19 and CD20. Plasma cells are formed in the secondary lymphoid tissues and from there move via the peripheral blood to the bone marrow, where they can live in their niche for years,[83] while secreting antibodies that circulate in the peripheral blood.

The lymph node

Once a naive B cell has encountered an antigen that binds specifically to its BR, the cell moves to the follicles of secondary lymphoid organs for further maturation to increase affinity of the BR for the specific antigen. Depending on the type of antigen, affinity maturation takes place in the lymph node (T cell dependent antigens) or the spleen (T-cell independent antigens). In order to facilitate the complex process of affinity maturation, specialized structures called germinal centers (GCs), arise in the lymph nodes upon immunization. Germinal centers consist of a light zone, where selection takes place, and the dark zone, where the B cells undergo clonal expansion, Ig-class switch recombination (CSR) and somatic hypermutation (SHM).[84] In the light zone the antigenic signal is maintained by antigen presenting follicular dendritic cells (fDCs).[85] The B cell is further stimulated by $CD4^+$ T cells via interactions such as CD40-CD40ligand interaction (Figure 5)[86] and production of IL-21[87] and other cytokines. Especially the stimulation via IL-21, induces CSR in *in vitro* cultures, promoting naive B cells to switch to IgG_1 and IgG_3.[88] In patients with defects in IL21-IL21R signaling, plasma blast formation and CSR are defect.[89] Vice versa, the B cell can stimulate and feedback to the $CD4^+$ T cell via ICOS-lCOSL interaction and by MHC-II-TR interaction. In the dark zone the positively selected B cells undergo extensive proliferation,[90] to form a large pool of B cells with specificity for the same antigen. Gitlin *et al.* showed that the affinity for the antibody determined the level of proliferation;[91] cells with the highest affinity selectively expand.[92] Affinity is further increased by CSR and SHM. Both these processes are controlled by the key regulator Activation Induced Deaminase (AID).[93] During CSR, the constant region of the IgH is replaced by another isotype. This is essential since the different isotypes have different properties, such as affinity, half-life, ability to dimerize, ability to bind to Fc receptors and activate the complement system. SHM takes place at the variable region of the IgH and IgL chains. Single nucleotide mutations are induced by AID in both the CDR and the FR of the Ig. Since these mutations can either enhance or reduce affinity, B cells move back to the light zone to again be selected by the

fDCs and T cells. To ensure only the most specific B cells are selected and expanded, cells go through multiple rounds of cycling between the light and dark zone. Imaging studies have confirmed this bi-directionally movement of the cells.[94] Eventually the B cells will migrate back into the peripheral blood as highly specific memory B cells or plasma cells.

The spleen

Some naive B cells mature further in the spleen. Blood is filtered by the spleen, and blood borne antigens are filtered, concentrated and captured by macrophages and dendritic cells.[95] In B cell follicles in the splenic marginal zone, the fDCs present antigen to the maturing B cells, but this time without T-cell help.[96] Specific lipid or carbohydrate structures of blood-borne antigens evoke this response[97] and B cells are additionally activated via Toll like receptors or via extensive cross-linking of the BR due to the repetitive nature of the antigens. This route of affinity maturation can lead to the formation of natural effector cells, which are still IgD^+, but have a memory and effector function after maturation in the spleen. A similar T-cell independent response can also take place in mucosa associated lymphoid tissue (MALT) which is found for example around the bronchi, and in the intestinal wall.[81] In all these tissues, B cell follicles can be formed that histologically highly resemble the GC. Affinity maturation is supported by binding of soluble BAFF and APRIL to TACI[98, 99] to promote B cell survival.

SIGNALING IN B-CELL DEVELOPMENT

B-cell receptor mediated signaling

The receptors from every individual B cell will recognize one specific epitope of an antigen. Once this antigen in encountered, the receptor starts signaling, enabling the B cell to respond to the antigen. Upon antigen binding, tyrosine residues in CD79a and CD79b are phosphorylated by Lyn and Syk, two Src family kinases. This results in recruitment of different kinases, like the previously discussed BTK, and adaptor proteins such as SLP-65 (or BLNK). In a positive feedback loop, amplified by the tyrosine kinases Lyn and Syk, BR complexes aggregate into microclusters.[100] The CD19-complex or B cell receptor co-complex (BR-co complex) amplifies this signal and promotes the formation of the microclusters.[101] The signal is propagated via multiple pathways. Through phospholipase C-γ2 (PLC- γ2) and BTK calcium ($Ca2^+$) influx is initiated and signaling via DAG results in activation of protein kinase C. Additionally PI3K-AKT signaling is initiated, which will be discussed in more detail later. This results in many different signals, that promote survival, proliferation and migration of the B cell to secondary lymphoid tissue, to undergo further maturation into an antibody producing plasma cell or a memory B cell. Dependent on the

presence or absence of other signals via co-stimulatory receptors, cytokine receptors and survival signals (such as BAFF, and APRIL), cells will have a specific response to antigenic stimulation.

The B-cell co-receptor complex

The BR-co complex amplifies signals through the B cell receptor thereby lowering the threshold for antigenic signaling. The complex consists of CD19, CD21, CD81 and CD225 (Figure 6). CD19 is a transmembrane protein that has an extracellular domain and a cytoplasmic tail that harbors multiple tyrosine kinase residues. It is a member of the immunoglobulin superfamily and it is expressed on all B cell stages except for pro-B cells in bone marrow and long lived plasma cells.[101] The long cytoplasmic tail enables recruitment of tyrosine kinases and signaling via PI3K.[102] Dependence on CD19 mediated signaling was shown in the mid-nineties by studies in mice,[103] and later by the description of CD19 deficient patients.[104-106] CD81 is a tetraspanin, a transmembrane protein which is critically important for the expression of CD19 on the plasma membrane.[107, 108] The third member of the BR-co receptor complex is CD21, also known as complement receptor 2 (CR2) or EBV receptor.[109-111] This protein is expressed on B cells after the transitional stage and follicular dendritic cells.[112] In humans, this is a 145kDA protein that consists of 15 short consensus repeats, a transmembrane domain ad a short cytoplasmic tail.[113] CD21 binds C3d-opsonized immune complexes and hereby enables responses to low dose antigens. In mice, it is encoded by the Cr2 locus, which also encodes the complement receptor 1 (CR1) otherwise known as CD35. Results from mice have shown that CD21/CD35 knock-out leads

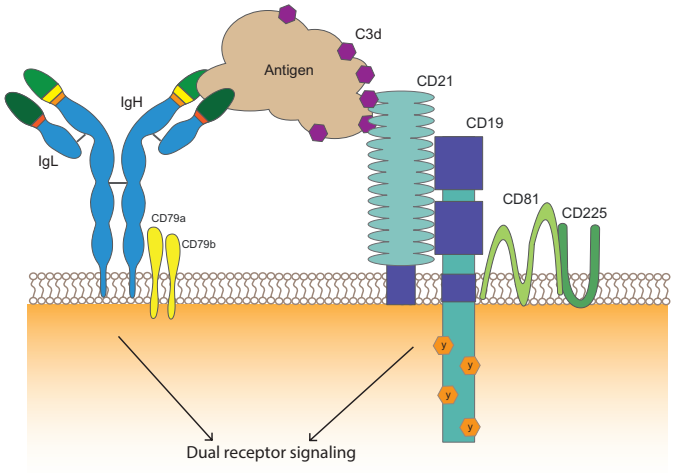


Figure 6.
Dual receptor signaling via the BR and the CD19-complex. The BR recognized epitopes of the antigen, while the CD21-molecule functions as a receptor for complement factor C3d, together initiating dual receptor signaling.

to decreased specific antigenic responses and increased susceptibility to auto-immune diseases.[114, 115] The last member of the BR co-complex is CD225, also named Interferon-induced transmembrane protein 1 (IFITM1), is supposed to have a function in antiviral immunity, however, its function within the BR co-complex in unknown.

Signaling via the PI3K- AKT pathway

Phosphoinositide 3-kinases (PI3Ks) are expressed in all mammalian cell types.[116] PI3K signaling contributes to many cellular processes like cell cycle progression, cells growth, survival and migration and intracellular transport.[117] PI3Ks can be divided into Classes (based on their lipid substrate and structural features) and isoforms. In mammals, class I is the most studied and understood isoform. All proteins in this class are heterodimers, consisting of a regulatory and a catalytic subunit. The regulatory subunit provides stabilization, inactivation of kinase activity in the basal state and recruitment to pTyrosine residues for the catalytic subunit and is therefore highly important in the regulation of signaling and activity of total heterodimer.[118] Class I PI3Ks can be activated via Ras and other small-GTPases[118] or via G-protein coupled receptors[119, 120] and tyrosine kinase-associated receptors.[121] As a result of these multiple options for activation, PI3K signaling is often found to integrate multiple signals; for example linking BR-signaling with TLR signaling on B cells, to integrate adaptive and innate immune responses[122] and integrating BR signals and BAFF-R signals to promote mature B cell survival.[123]

Upon activation, PI3Ks generate PtdIns(3,4,5)P_3 (PIP_3) lipids by phosphorylation of PtdIns(3,4)P_2 or PtdIns(4,5)P_2 (PIP_2). Its antagonist is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is often found inactivated in malignant cells. Proteins recruited to the membrane though PIP3, are referred to as PI3K-effectors.[124, 125] One of those effector is AKT, which is activated by phosphorylation via phosphoinositide-dependent kinase 1 (PDK1). Via multiple pathways AKT is important for diverse cellular processes,[126] including cell growth and metabolism via the mTOR1 signaling cascade[127-129] and regulation of transcription via the Forkhead Box Subgroup O (FoxO) transcription factors.[130, 131] PTEN and PI3K are often found to be mutated in both solid and non-solid malignancies[132] resulting in increased metabolism and proliferation together with reduced apoptosis in the cancer cells.

PI3K-AKT signaling in B cells

In lymphocytes, the most abundant isoform of PI3K is the so-called PI3Kδ, consisting of the p110δ catalytic subunit, dimerized with the p85α regulatory subunit. Expression of this isoform seems to be restricted to leukocytes.[133] It has been shown that signaling via PI3Kδ is critical for development, survival and activation of B cells. Most of this knowledge is derived from mouse knock-out experiments, and so far one case of p85α deficiency has

been described in humans,[134] but the conservation of these proteins and their function between man and mice suggest a similar role in development. In B cells, the pathway can be activated in many ways: via the tyrosine residues on CD19, via BR-mediated signaling, via TLR signaling, via CD40-CD40L interaction and via BAFF-R engagement (Figure 7). In mice, it was shown that PI3K suppresses expression of Rag via Foxo, which binds to the promotors of Rag1 and Rag2.[130, 135] Hereby, PI3K signaling enables "tonic" signaling and proliferation of preB cells in the bone marrow. In both p110δ and p85α deficient mice, nearly normal BCP development was seen, although at a later stage B cells developed abnormally.[136, 137] Experiments with single and double knock-out mice showed that, at least in mice, p110α can compensate for the lack of p110δ in BCP in bone marrow. However, mice deficient for both p110α and p110δ had a complete block at the pre-B cell stage with an elevated fraction of cells that contain two rearranged heavy chain alleles, due to a failure to inhibit Rag expression.[138] Furthermore, upon pre-BR signaling PI3K is activated to induce proliferation, and deficient mice showed reduced numbers of immature B cells.[139] In the naive mature B cells in peripheral blood, PI3K signaling was shown to integrate BR signaling and BAFF-R signals, in such a way that mature B cells that lost their BR were fully rescued by activation of PI3K signaling.[123] PI3K promotes Ca^{2+} mobilization and nuclear translocation of NFκB, thereby enabling survival of mature B cells in the absence of antigenic stimulation.[140] Upon antigen encounter, PI3K is activated via BR signaling and CD19 signaling. Studies in CD19 and PI3Kδ double deficient mice have shown that combined action of the two protein is required for survival and differentiation of B cells.[141] Besides its role in survival and proliferation, PI3K has also been shown to suppress AID, which is required for SHM and CSR, indicating a role in affinity maturation in the lymph node.[142, 143] A recent report by Chen *et al.* showed that it is the signaling balance between PTEN and PI3K that regulates CSR.[144] However, whether this is regulated via PI3K-AKT signaling directly or via FOXO, remains to be investigated. In addition, Sander *et al.* showed that PI3K-signaling and the transcription factor FOXO1 are essential antagonistic regulators within the germinal center, controlling polarization and cellular selection.[84] In summary, PI3K signaling is required throughout the total B cell development from bone marrow until final CSR and memory formation in the lymph node.

PI3K-AKT signaling in T cells

In T cells PI3K signaling can be induced via T cell receptor mediated signaling, co-stimulatory signals via CD28, the IL2 receptor and diverse chemokine receptors.[145] Via AKT-mTOR signaling this cascade is important for the regulation of T cell activation and differentiation. Additionally, Foxo regulates expression offor example CD62L and CCR7, which are important secondary lymphoid tissue homing factors for T cells. Studies in diverse knock-out mice[136, 137, 146] have indicated that besides mTOR signaling, PI3K also

feeds into Tec mediated activation of NF-AT, NFκB and MAPK signaling in T cells, also affecting activation in an mTOR independent fashion.[147] Using PTEN knock-out mice, it was shown that balance in PI3K signaling is important to maintain homeostasis and lineage stability in Treg cells.[148] Furthermore, PTEN loss in $CD4^+$ T cells, enhanced their helper function, without inducing auto-immunity or lymphoma.[149] Opposed to this, mice without p110δ, showed reduced T dependent antibody responses[150] indicating loss of T cell help to B cells. Regarding the $CD8^+$ T cells, it was shown that p110δ is required for primary and memory antiviral responses and responses against intracellular bacteria in mice.[151] These studies suggest that dysregulation of PI3K signaling can lead to disturbed activation, differentiation and function in all major T cell subsets.

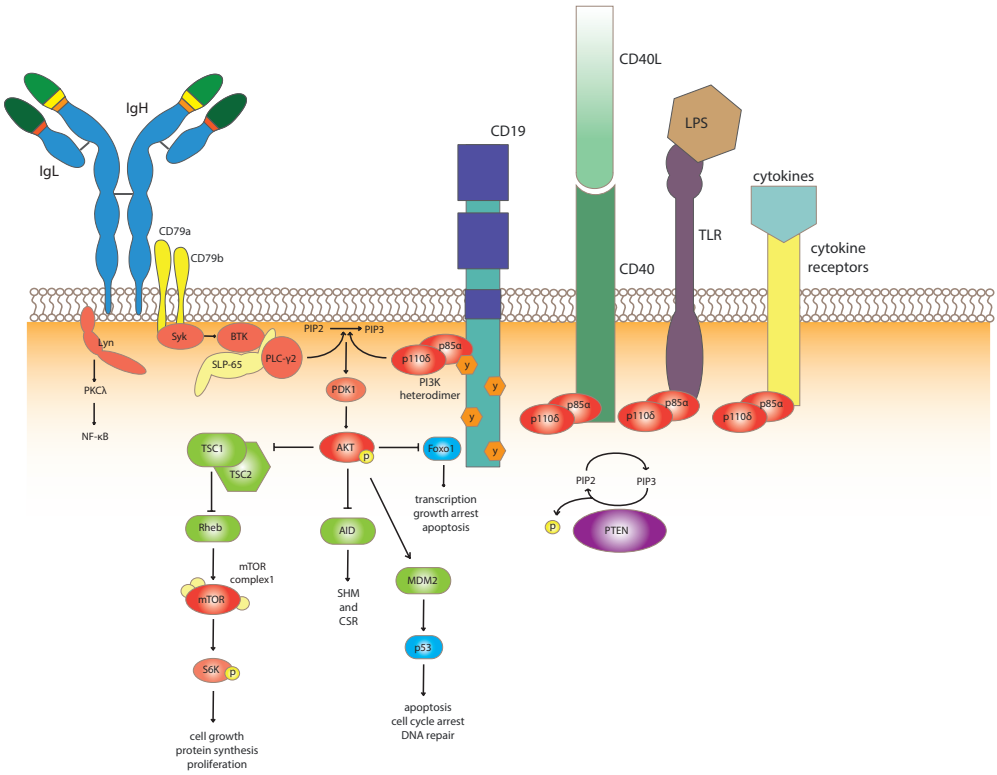


Figure 7.
BR-signaling induces activation of the PI3K-AKT signaling cascade. There are multiple receptors on the B cell plasma membrane that can activate this cascade.

PRIMARY ANTIBODY DEFICIENCIES

When an inborn defect occurs at any stage of B cell development, differentiation, activation, maturation or signaling, this can lead to reduced numbers (or absence) of B cells or to reduced or incorrect function of the B cells, which in turn results in reduced or absent production of antibodies.[10-13] This heterogeneous group of diseases is called Primary antibody deficiencies (PAD). Common variable immunodeficiency (CVID) is the most diagnosed PAD.[152] Unfortunately, in the majority of CVID cases (90-95%) no genetic defect can be found.[9, 11, 153] In other groups of patients, genetic defects that can explain the occurrence of the disease have been found.[8] Examples of those will be discussed here, grouped by the stage of B-cell development that is affected by the defect.

Defects that affect precursor B-cell development

Over the years, multiple genetic defects have been identified that cause a (partial) stop in B cell precursor differentiation in the bone marrow, often resulting in reduced numbers or even lack of B cells in the peripheral blood. Depending on the mechanism that is affected by the mutation patients can have different clinical phenotypes.

Defects in B cell commitment

Already at the stage of B-cell commitment, mutations in key-transcription factors have been found. Recently, patients with heterozygous mutations in IKAROS, causing loss of B cells in the peripheral blood and decreased early B-cell precursors in bone marrow, have been described.[154, 155] Furthermore, 4 patients with E2A mutations have been described.[156] The mutations found in these patients affected only one of the two transcription factors (namely E47) the gene encodes for, leaving the other (E12) unharmed. And thus, the phenotype in patients was less severe than in E2A KO mice, which have a complete block in B cell development. The patients did have $CD19^+$ B cells in the peripheral blood, but these were all BR-.[157] In line with this, one patients was described carrying a homozygous premature stop codon in exon 6 of PIK3R1,[134] which results in the absence of p85α but normal expression of the p50α and p55α regulatory subunits of PI3K. In bone marrow aspirates <0,1% $CD19^+$ B cells could be detected, suggesting a B lineage commitment problem.

V(D)J-recombination defects

In a group of patients with defects in both T- and B cells, mutations are found in genes that are involved in V(D)J recombination, like *RAG1* and *RAG2,*[158, 159] *Artemis,*[45, 160, 161] and *DNA-PKcs.*[162, 163] Since V(D)J-recombination cannot be performed in absence of any of these proteins, cells cannot form a functional heavy chain, and B cell precursor differentiation is

stopped at the first checkpoint in the pre-B-I stage. This classically leads to absence of B cells and T cells in the peripheral blood and the clinical phenotype of Severe Combined Immunodeficiency (SCID). Only homozygous or compound heterozygous loss-of-function mutations lead to disease. However, it was shown that some patients with *RAG* mutations can present with less severe phenotypes of combined immunodeficiency.[164] In addition, mutations in *Ligase4 (LIG4)* have been described to cause SCID,[165] whereas other patients with *LIG4* mutations have a broader clinical phenotype with primordial dwarfism.[166]

Defects in (pre-)BR expression and signaling

There are cases of patients that lack one of the BR-components due to a mutation, like a mutated Igu-heavy chain (*IGHM* gene),[167, 168] or a mutated surrogate light chain protein lambda5/14.1 (*IGLL1* gene).[169] They cannot express a BR, which leads to a stop at the pre-B-I stage and absence of B cells in the peripheral blood. Other patients cannot express their BR because of a lack of CD79a,[170, 171] leading to a comparable immunological and clinical phenotype: agammaglobulinemia. These causes are all autosomal recessive and therefore very rare. A more frequent cause of agammaglobulinemia is X-linked agammaglobulinemia (XLA), caused by mutations in *Bruton`s tyrosine Kinase (BTK*).[172-174] About 85% of cases of agammaglobulinemia can be explained by a defect of this protein,[175] which results in defective pre-BR signaling and pre-B cells die from neglect. In some cases, the defect has a leaky phenotype and a few mature B cells can be found in the peripheral blood. A similar disease causing mechanism was found in a patient without peripheral B cells and with a stop at the pre-B-I stage, that was found to have a mutation in SLP-65, which is also critical for (pre-) BR-signal transduction.[176]

Patients without defined genetic defect in precursor B-cell differentiation

One last group of patients that have been described to have abnormal precursor B cell differentiation are a subgroup patients hypogammaglobulinemia, especially patients with common variable immunodeficiency (CVID). Some of these patients have low B cell numbers in the peripheral blood, suggesting a defect arising in bone marrow. Indeed people have described aberrancies in the B cell precursor subsets of CVID patients.[177-180] Their findings suggest problems with autonomous proliferation in the pre-B-II stage in a group of CVID patients, but also a role for B-cell extrinsic factors, like the niche provided by the bone marrow stroma, that could have reduced function in CVID. Additionally, it has been suggested that a group of CVID patients might have problems in V(D)J-recombination,[181] since in these patients, also the T cell compartment is affected and in their bone marrow, pro-B and pre-B-I cells form the majority of B cell precursors.

Disturbed peripheral B cell maturation

Over the past decades, different genetic defects in factors that control peripheral B cell development have been discovered. By affecting different processes, all of these defects lead to antibody deficiencies.[10, 13, 78] To indicate how different genetic mutations can affect B cell development, some defects are described here.

Survival defects

In order to survive in the periphery, naive B cells are dependent on signaling via B-cell activating factor receptor (BAFF-R), a member of the TNF alpha family receptors, upon binding of BAFF, a soluble factor in the serum. Human patients with BAFF-R deficiency[182] display a strong B cell lymphopenia with relatively increased numbers of transitional B cells are reductions in more mature B cell compartments. This results in reduced serum IgM and IgG levels, but normal IgA levels. IgA^+ plasma cells were found in the gut of these patients. Transmembrane activator and CAML interactor (TACI) is a related receptor from the same family and mutations impair the development of IgA- and IgG-secreting plasma cells and promote lymphoproliferation.[183] However, TACI mutations are not always penetrant and mutations can be found in healthy relatives of patients PAD-patients with the same mutation.

Defects in activation, migration and BR-signaling

Upon antigenic stimulation, the signal from the BR is processed intracellularly. In the case of NFKB and NEMO deficiencies, signaling though the BR via NEMO and NFKB is defective, resulting in reduced activation of the B cells. However, dependent on the nature of the mutation, the disease phenotype can be very broad and include other cells and tissues in which this signaling cascade is required.[184] Augmentation of BR signaling is done by the B cell receptor co-complex, also called CD19 complex. Several patients with deficiencies in proteins in this complex like CD19,[104, 105, 185-187] CD81,[188] and CD21[189-191] have been described, all mutations lead to hypogammaglobulinemia and reduced formation of memory B cells, although the phenotype is less severe in the CD21 deficient cases. When factors that are required for correct homing to the lymph node are deficient, B cell maturation is hampered. This has been described for patients with leucocyte adhesion defects type III (LAD-III)[192] that developed hypogammaglobulinemia, due to defect homing to the lymph nodes. After arrival in the lymph node, B cells require T cell help via inducible co-stimulator (ICOS), which is expressed on follicular T-helper cells. Homozygous deletion of this protein results in impaired T cell help to follicular B cells resulting in late onset CVID.[193]

CSR and SHM deficiencies

B cells require co-stimulation to perform CSR. One essential signal comes from the cytokine IL21. Homozygous loss-of-function mutations in IL21R have been recently described to cause reduced serum IgG and poor vaccination responses.[194] Another essential signal is T-cell help in the form of CD40-CD40L interaction. Defects in CD40L result in X-linked hyper IgM syndrome (HIGM).[195] Autosomal recessive forms of HIGM can be caused by mutations in *AID*[196] *UNG* and *MSH6*, the latter two are both vital proteins for DNA repair. In all these defects, B cells cannot switch to IgA of IgG. In the case of AID deficiency, not only CSR in defective, but also targeting of SHM is reduced, thus this defect results in reduced SHM as well. A recent field of interest are mutations in PI3K-AKT signaling,[197] which will be discussed separately.

PI3K-PTEN disbalance due to mutations

Although PI3K-AKT signaling has been studied in the context of lymphocyte development for decades, only recently germline mutations were found to cause an antibody deficiency due to deregulation of this pathway. In 2006, one case was reported of a boy who suffered from low B cell numbers and agammaglobulinemia.[198] He was one patient of a cohort in which by sequencing of *PIK3CD* mutations were sought that could explain the clinical phenotype. Later in 2012 a case of agammaglobulinemia was described as to be caused by deficiency of p85α.[134] But only by the end of 2013, mutations in PI3K were found to cause a disease entity that is commonly referred to as Activated PI3Kδ syndrome (APDS).[199] This disease is sometimes also referred to as p110δ activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency (PASLI).[200] Patients have mutations in *PIK3CD*[199, 200] (referred to as APDS1) or *PIK3R1*[201, 202] (referred to as APDS2), resulting in hyper activation of PI3Kδ. This leads to disturbed T cell differentiation, increases in short lived effector T cells, but reduced memory T cell formation. Additionally both via reduced T cell help and B cell intrinsic defects, B cells fail to mature into class switched plasma cells that secrete high affinity antibodies and B cell memory is reduced. Although the clinical phenotype of the disease is highly variable, cohort studies[203, 204] have indicated that patients suffer from recurrent respiratory tract infections, failure to control EBV and CMV infections, progressive airway damage, lymphadenopathy and have an increased risk of lymphoma`s.[197] A group of patients that share the mechanism of disease are patients with loss of function mutations in PTEN. A subgroup of those patients also suffers from infections and was found to have hypogammaglobulinemia.[205, 206] Both groups of patients indicate the need of tight regulation of the PI3K-AKT pathway in human B and T cell immunology.

OVERVIEW OF THIS THESIS

In this thesis we aimed to study how specific defects lead to disturbances in B cell development and repertoire formation and thereby cause antibody deficiencies. In order to study antibody deficiencies, good insight in B cell development is crucial, but at the same time, patients with specific defects can give new insights in which factors are important for B-cell developmental processes. This work contributes to our understanding of genotype-phenotype correlations and with that can help individualize diagnostic work-up, and give better prognostic predictions. Furthermore, this leads the way into the field of personalized medicine also in the context of rare diseases, the importance of which is underlined in the "Nationale wetenschaps agenda, Q081". Studying these two fields at the same time is therefore highly valuable, and not only for patients with antibody deficiencies, but also for our general understanding of B cell biology and therefore for other immunological diseases and processes such as malignancies, auto-immunity, immune dysregulation and regeneration after stem cell transplantation.

Part two of this thesis focusses on normal BCP development in bone marrow and how this results in a broad repertoire of naive B cells. Using a newly established flow cytometry panel combined with novel analysis methods, we studied B cell development in healthy bone marrow and used bone marrow from patients with known genetic defects as controls. The output of naive B cells with a broad repertoire is the final goal of BCP development in bone marrow. Therefore, we used next generation sequencing to study the repertoire in naive B cells of healthy controls and patients, since we hypothesized that in some CVID patients this repertoire would be reduced. Part three of this thesis focusses on deficiencies in the BR-co-complex. We have described the second patient with CD21 deficiency. We compared the immunobiology of CD21 deficiency to CD19 deficiency, because the clinical phenotype of the two deficiencies is different, we wanted to understand more about the effect on B cell development in both deficiencies. In part four, we shift to dysregulations in PI3K-AKT signaling, studying the effect of PTEN on humoral immunity and the effect of PI3Kdelta mutations on B cell development and $CD8^+$ T cell exhaustion. Since some patients with PHTS have an antibody deficiency, we hypothesized that their B and T cell phenotype and repertoire could be affected by the mutation. Furthermore, we hypothesized that the antibody deficiency seen in APDS patients is not only due to reduced T-cell help, but also an intrinsic defect in B cells adds to the phenotype. Additionally, we asked ourselves: could the increased expression of PD-1 and the increased apoptosis rate om lymphocytes in patients with APDS be explained by exhaustion of the T cell compartment? In part 5, the general discussion, the importance of our findings is reviewed and future perspectives are discussed.

The studies described in this thesis help understand why disturbances in specific genes and processes lead to antibody deficiencies. They showed that B cell precursor development in bone marrow is not a simple linear process, but rather a complex interplay of processes that make every individual cell follow its own route of maturation into a naive B cell. Furthermore, these studies showed that subtle differences in protein function can explain phenotypical differences between different disease entities in CD19-complex deficiencies. Showing how genotype-phenotype correlations can be a tool in patient-prognostics. These studies also showed that the balance in the PI3K/PTEN-AKT signaling cascade is critically important for both humoral and anti-viral immunity, since disturbances lead to antibody deficiencies and reduced viral immunity due to exhaustion. This is especially important in the context of new, personalized, specific treatments for these patients.

REFERENCES

1. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.
2. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-72.
3. Medzhitov, R. and C.A. Janeway, Jr., *Innate immune recognition and control of adaptive immune responses.* Semin Immunol, 1998. **10**(5): p. 351-3.
4. Janeway, C.A., Jr. and R. Medzhitov, *Introduction: the role of innate immunity in the adaptive immune response.* Semin Immunol, 1998. **10**(5): p. 349-50.
5. Pancer, Z. and M.D. Cooper, *The evolution of adaptive immunity.* Annu Rev Immunol, 2006. **24**: p. 497-518.
6. Cooper, M.D. and M.N. Alder, *The evolution of adaptive immune systems.* Cell, 2006. **124**(4): p. 815-22.
7. Geha, R.S., et al., *Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee.* J Allergy Clin Immunol, 2007. **120**(4): p. 776-94.
8. Bousfiha, A., et al., *The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies.* J Clin Immunol, 2015. **35**(8): p. 727-38.
9. Gathmann, B., et al., *The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008.* Clin Exp Immunol, 2009. **157 Suppl 1**: p. 3-11.
10. Driessen, G. and M. van der Burg, *Educational paper: primary antibody deficiencies.* Eur J Pediatr, 2011. **170**(6): p. 693-702.
11. Driessen, G.J., et al., *B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency.* Blood, 2011. **118**(26): p. 6814-23.

12. van der Burg, M., et al., *Dissection of B-cell development to unravel defects in patients with a primary antibody deficiency.* Adv Exp Med Biol, 2011. **697**: p. 183-96.
13. van der Burg, M., et al., *New frontiers of primary antibody deficiencies.* Cell Mol Life Sci, 2012. **69**(1): p. 59-73.
14. LeBien, T.W., *Fates of human B-cell precursors.* Blood, 2000. **96**(1): p. 9-23.
15. Ghia, P., et al., *B-cell development: a comparison between mouse and man.* Immunol Today, 1998. **19**(10): p. 480-5.
16. Nodland, S.E., et al., *IL-7R expression and IL-7 signaling confer a distinct phenotype on developing human B-lineage cells.* Blood, 2011. **118**(8): p. 2116-27.
17. Matthias, P. and A.G. Rolink, *Transcriptional networks in developing and mature B cells.* Nat Rev Immunol, 2005. **5**(6): p. 497-508.
18. Busslinger, M., *Transcriptional control of early B cell development.* Annu Rev Immunol, 2004. **22**: p. 55-79.
19. Cobaleda, C., W. Jochum, and M. Busslinger, *Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors.* Nature, 2007. **449**(7161): p. 473-7.
20. Iwasaki, H., et al., *Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation.* Blood, 2005. **106**(5): p. 1590-600.
21. DeKoter, R.P. and H. Singh, *Regulation of B lymphocyte and macrophage development by graded expression of PU.1.* Science, 2000. **288**(5470): p. 1439-41.
22. McKercher, S.R., et al., *Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities.* EMBO J, 1996. **15**(20): p. 5647-58.
23. Nichogiannopoulou, A., et al., *Defects in hemopoietic stem cell activity in Ikaros mutant mice.* J Exp Med, 1999. **190**(9): p. 1201-14.
24. Ng, S.Y., et al., *Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells.* Immunity, 2009. **30**(4): p. 493-507.
25. Reynaud, D., et al., *Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros.* Nat Immunol, 2008. **9**(8): p. 927-36.
26. Nutt, S.L. and B.L. Kee, *The transcriptional regulation of B cell lineage commitment.* Immunity, 2007. **26**(6): p. 715-25.
27. Zandi, S., D. Bryder, and M. Sigvardsson, *Load and lock: the molecular mechanisms of B-lymphocyte commitment.* Immunol Rev, 2010. **238**(1): p. 47-62.
28. Oettinger, M.A., et al., *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.
29. Hardy, R.R. and K. Hayakawa, *B cell development pathways.* Annu Rev Immunol, 2001. **19**: p. 595-621.
30. Roessler, S. and R. Grosschedl, *Role of transcription factors in commitment and differentiation of early B lymphoid cells.* Semin Immunol, 2006. **18**(1): p. 12-9.
31. Inlay, M.A., et al., *Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development.* Genes Dev, 2009. **23**(20): p. 2376-81.

32. Lin, H. and R. Grosschedl, *Failure of B-cell differentiation in mice lacking the transcription factor EBF.* Nature, 1995. **376**(6537): p. 263-7.
33. Medvedovic, J., et al., *Pax5: a master regulator of B cell development and leukemogenesis.* Adv Immunol, 2011. **111**: p. 179-206.
34. Nutt, S.L., A.G. Rolink, and M. Busslinger, *The molecular basis of B-cell lineage commitment.* Cold Spring Harb Symp Quant Biol, 1999. **64**: p. 51-9.
35. Nutt, S.L., et al., *Commitment to the B-lymphoid lineage depends on the transcription factor Pax5.* Nature, 1999. **401**(6753): p. 556-62.
36. Busslinger, M., et al., *Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 6129-34.
37. van Zelm, M.C., et al., *Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression.* J Immunol, 2005. **175**(9): p. 5912-22.
38. Rother, M.B., et al., *Nuclear positioning rather than contraction controls ordered rearrangements of immunoglobulin loci.* Nucleic Acids Res, 2016. **44**(1): p. 175-86.
39. Stadhouders, R., et al., *Pre-B cell receptor signaling induces immunoglobulin kappa locus accessibility by functional redistribution of enhancer-mediated chromatin interactions.* PLoS Biol, 2014. **12**(2): p. e1001791.
40. Selimyan, R., et al., *Localized DNA demethylation at recombination intermediates during immunoglobulin heavy chain gene assembly.* PLoS Biol, 2013. **11**(1): p. e1001475.
41. Ghia, P., et al., *Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci.* J Exp Med, 1996. **184**(6): p. 2217-29.
42. Ehlich, A., et al., *Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development.* Cell, 1993. **72**(5): p. 695-704.
43. Weterings, E. and D.C. van Gent, *The mechanism of non-homologous end-joining: a synopsis of synapsis.* DNA Repair (Amst), 2004. **3**(11): p. 1425-35.
44. van Gent, D.C. and M. van der Burg, *Non-homologous end-joining, a sticky affair.* Oncogene, 2007. **26**(56): p. 7731-40.
45. Le Deist, F., et al., *Artemis sheds new light on V(D)J recombination.* Immunol Rev, 2004. **200**: p. 142-55.
46. Kallenbach, S., et al., *Three lymphoid-specific factors account for all junctional diversity characteristic of somatic assembly of T-cell receptor and immunoglobulin genes.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 2799-803.
47. Corcoran, A.E., *Immunoglobulin locus silencing and allelic exclusion.* Semin Immunol, 2005. **17**(2): p. 141-54.
48. Geier, J.K. and M.S. Schlissel, *Pre-BCR signals and the control of Ig gene rearrangements.* Semin Immunol, 2006. **18**(1): p. 31-9.
49. Hikida, M. and H. Ohmori, *Rearrangement of lambda light chain genes in mature B cells in vitro and in vivo. Function of reexpressed recombination-activating gene (RAG) products.* J Exp Med, 1998. **187**(5): p. 795-9.

50. Meffre, E., et al., *Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development.* J Clin Invest, 2001. **108**(6): p. 879-86.
51. Loken, M.R., et al., *Flow cytometric analysis of normal B lymphoid development.* Pathol Immunopathol Res, 1988. **7**(5): p. 357-70.
52. Espeli, M., et al., *Initiation of pre-B cell receptor signaling: common and distinctive features in human and mouse.* Semin Immunol, 2006. **18**(1): p. 56-66.
53. Middendorp, S., G.M. Dingjan, and R.W. Hendriks, *Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice.* J Immunol, 2002. **168**(6): p. 2695-703.
54. Bradl, H. and H.M. Jack, *Surrogate light chain-mediated interaction of a soluble pre-B cell receptor with adherent cell lines.* J Immunol, 2001. **167**(11): p. 6403-11.
55. Gauthier, L., et al., *Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 13014-9.
56. Kraus, H., et al., *A feeder-free differentiation system identifies autonomously proliferating B cell precursors in human bone marrow.* J Immunol, 2014. **192**(3): p. 1044-54.
57. Schlessinger, J., *SH2/SH3 signaling proteins.* Curr Opin Genet Dev, 1994. **4**(1): p. 25-30.
58. Hardy, R.R., et al., *Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow.* J Exp Med, 1991. **173**(5): p. 1213-25.
59. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nat Rev Immunol, 2009. **9**(3): p. 195-205.
60. Berkowska, M.A., et al., *Checkpoints of B cell differentiation: visualizing Ig-centric processes.* Ann N Y Acad Sci, 2011. **1246**: p. 11-25.
61. Melchers, F., *Checkpoints that control B cell development.* J Clin Invest, 2015. **125**(6): p. 2203-10.
62. Nemazee, D.A. and K. Burki, *Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes.* Nature, 1989. **337**(6207): p. 562-6.
63. Lang, J., et al., *B cells are exquisitely sensitive to central tolerance and receptor editing induced by ultralow affinity, membrane-bound antigen.* J Exp Med, 1996. **184**(5): p. 1685-97.
64. Retter, M.W. and D. Nemazee, *Receptor editing occurs frequently during normal B cell development.* J Exp Med, 1998. **188**(7): p. 1231-8.
65. Chen, C., et al., *Deletion and editing of B cells that express antibodies to DNA.* J Immunol, 1994. **152**(4): p. 1970-82.
66. Sims, G.P., et al., *Identification and characterization of circulating human transitional B cells.* Blood, 2005. **105**(11): p. 4390-8.
67. van de Veen, W., et al., *Role of regulatory B cells in immune tolerance to allergens and beyond.* J Allergy Clin Immunol, 2016. **138**(3): p. 654-65.
68. Perez-Andres, M., et al., *Human peripheral blood B-cell compartments: a crossroad in B-cell traffic.* Cytometry B Clin Cytom, 2010. **78 Suppl 1**: p. S47-60.
69. Mizoguchi, A. and A.K. Bhan, *A case for regulatory B cells.* J Immunol, 2006. **176**(2): p. 705-10.

70. Wehr, C., et al., *A new CD21low B cell population in the peripheral blood of patients with SLE.* Clin Immunol, 2004. **113**(2): p. 161-71.
71. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.
72. Doi, H., S. Tanoue, and D.E. Kaplan, *Peripheral CD27-CD21- B-cells represent an exhausted lymphocyte population in hepatitis C cirrhosis.* Clin Immunol, 2014. **150**(2): p. 184-91.
73. Verstegen, R.H., et al., *Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help.* Pediatr Res, 2010. **67**(5): p. 563-9.
74. Warnatz, K., et al., *Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia.* Immunobiology, 2002. **206**(5): p. 502-13.
75. Keller, B., et al., *High SYK Expression Drives Constitutive Activation of CD21low B Cells.* J Immunol, 2017. **198**(11): p. 4285-4292.
76. Unger, S., et al., *The TH1 phenotype of follicular helper T cells indicates an IFN-gamma-associated immune dysregulation in patients with CD21low common variable immunodeficiency.* J Allergy Clin Immunol, 2017.
77. Comans-Bitter, W.M., et al., *Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations.* J Pediatr, 1997. **130**(3): p. 388-93.
78. Pieper, K., B. Grimbacher, and H. Eibel, *B-cell biology and development.* J Allergy Clin Immunol, 2013. **131**(4): p. 959-71.
79. Berkowska, M.A., et al., *Circulating Human CD27-IgA$^+$ Memory B Cells Recognize Bacteria with Polyreactive Igs.* J Immunol, 2015. **195**(4): p. 1417-26.
80. Agematsu, K., et al., *B cell subpopulations separated by CD27 and crucial collaboration of CD27$^+$ B cells and helper T cells in immunoglobulin production.* Eur J Immunol, 1997. **27**(8): p. 2073-9.
81. Berkowska, M.A., et al., *Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways.* Blood, 2011. **118**(8): p. 2150-8.
82. Fecteau, J.F., G. Cote, and S. Neron, *A new memory CD27-IgG$^+$ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation.* J Immunol, 2006. **177**(6): p. 3728-36.
83. Kometani, K. and T. Kurosaki, *Differentiation and maintenance of long-lived plasma cells.* Curr Opin Immunol, 2015. **33**: p. 64-9.
84. Sander, S., et al., *PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones.* Immunity, 2015. **43**(6): p. 1075-86.
85. Victora, G.D. and M.C. Nussenzweig, *Germinal centers.* Annu Rev Immunol, 2012. **30**: p. 429-57.
86. Nonoyama, S., et al., *B cell activation via CD40 is required for specific antibody production by antigen-stimulated human B cells.* J Exp Med, 1993. **178**(3): p. 1097-102.
87. Nutt, S.L. and D.M. Tarlinton, *Germinal center B and follicular helper T cells: siblings, cousins or just good friends?* Nat Immunol, 2011. **12**(6): p. 472-7.
88. Pene, J., et al., *Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells.* J Immunol, 2004. **172**(9): p. 5154-7.

89. Moens, L. and S.G. Tangye, *Cytokine-Mediated Regulation of Plasma Cell Generation: IL-21 Takes Center Stage*. Front Immunol, 2014. **5**: p. 65.
90. Rajewsky, K., *Clonal selection and learning in the antibody system*. Nature, 1996. **381**(6585): p. 751-8.
91. Gitlin, A.D., Z. Shulman, and M.C. Nussenzweig, *Clonal selection in the germinal centre by regulated proliferation and hypermutation*. Nature, 2014. **509**(7502): p. 637-40.
92. Oropallo, M.A. and A. Cerutti, *Germinal center reaction: antigen affinity and presentation explain it all*. Trends Immunol, 2014. **35**(7): p. 287-9.
93. Kracker, S. and A. Durandy, *Insights into the B cell specific process of immunoglobulin class switch recombination*. Immunol Lett, 2011. **138**(2): p. 97-103.
94. Allen, C.D., et al., *Imaging of germinal center selection events during affinity maturation*. Science, 2007. **315**(5811): p. 528-31.
95. Heesters, B.A., et al., *Antigen Presentation to B Cells*. Trends Immunol, 2016. **37**(12): p. 844-854.
96. Weill, J.C., S. Weller, and C.A. Reynaud, *Human marginal zone B cells*. Annu Rev Immunol, 2009. **27**: p. 267-85.
97. Mond, J.J., et al., *T cell independent antigens*. Curr Opin Immunol, 1995. **7**(3): p. 349-54.
98. He, B., et al., *The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88*. Nat Immunol, 2010. **11**(9): p. 836-45.
99. He, B., et al., *Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL*. Immunity, 2007. **26**(6): p. 812-26.
100. Cheng, P.C., et al., *A role for lipid rafts in B cell antigen receptor signaling and antigen targeting*. J Exp Med, 1999. **190**(11): p. 1549-60.
101. Carter, R.H. and D.T. Fearon, *CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes*. Science, 1992. **256**(5053): p. 105-7.
102. Tuveson, D.A., et al., *CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase*. Science, 1993. **260**(5110): p. 986-9.
103. Engel, P., et al., *Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule*. Immunity, 1995. **3**(1): p. 39-50.
104. van Zelm, M.C., et al., *An antibody-deficiency syndrome due to mutations in the CD19 gene*. N Engl J Med, 2006. **354**(18): p. 1901-12.
105. Kanegane, H., et al., *Novel mutations in a Japanese patient with CD19 deficiency*. Genes Immun, 2007. **8**(8): p. 663-70.
106. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation*. J Allergy Clin Immunol, 2014.
107. Miyazaki, T., U. Muller, and K.S. Campbell, *Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81*. EMBO J, 1997. **16**(14): p. 4217-25.
108. Maecker, H.T. and S. Levy, *Normal lymphocyte development but delayed humoral immune response in CD81-null mice*. J Exp Med, 1997. **185**(8): p. 1505-10.

109. Aegerter-Shaw, M., et al., *Expansion of the complement receptor gene family. Identification in the mouse of two new genes related to the CR1 and CR2 gene family.* J Immunol, 1987. **138**(10): p. 3488-94.
110. Iida, K., L. Nadler, and V. Nussenzweig, *Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody.* J Exp Med, 1983. **158**(4): p. 1021-33.
111. Weis, J.H., et al., *A complement receptor locus: genes encoding C3b/C4b receptor and C3d/Epstein-Barr virus receptor map to 1q32.* J Immunol, 1987. **138**(1): p. 312-5.
112. Carroll, M.C. and D.E. Isenman, *Regulation of humoral immunity by complement.* Immunity, 2012. **37**(2): p. 199-207.
113. Moore, M.D., et al., *Molecular cloning of the cDNA encoding the Epstein-Barr virus/C3d receptor (complement receptor type 2) of human B lymphocytes.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9194-8.
114. Marchbank, K.J., et al., *Expression of human complement receptor 2 (CR2, CD21) in Cr2-/- mice restores humoral immune function.* J Immunol, 2000. **165**(5): p. 2354-61.
115. Rettig, T.A., et al., *Evasion and interactions of the humoral innate immune response in pathogen invasion, autoimmune disease, and cancer.* Clin Immunol, 2015. **160**(2): p. 244-254.
116. Vanhaesebroeck, B., et al., *Phosphoinositide 3-kinases: a conserved family of signal transducers.* Trends Biochem Sci, 1997. **22**(7): p. 267-72.
117. Vanhaesebroeck, B., et al., *The emerging mechanisms of isoform-specific PI3K signalling.* Nat Rev Mol Cell Biol, 2010. **11**(5): p. 329-41.
118. Inukai, K., et al., *Five isoforms of the phosphatidylinositol 3-kinase regulatory subunit exhibit different associations with receptor tyrosine kinases and their tyrosine phosphorylations.* FEBS Lett, 2001. **490**(1-2): p. 32-8.
119. Stoyanov, B., et al., *Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase.* Science, 1995. **269**(5224): p. 690-3.
120. Hirsch, E., et al., *Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation.* Science, 2000. **287**(5455): p. 1049-53.
121. Okkenhaug, K. and B. Vanhaesebroeck, *PI3K-signalling in B- and T-cells: insights from gene-targeted mice.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 270-4.
122. Otipoby, K.L., et al., *The B-cell antigen receptor integrates adaptive and innate immune signals.* Proc Natl Acad Sci U S A, 2015. **112**(39): p. 12145-50.
123. Srinivasan, L., et al., *PI3 kinase signals BCR-dependent mature B cell survival.* Cell, 2009. **139**(3): p. 573-86.
124. Fruman, D.A., *Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling.* Curr Opin Immunol, 2004. **16**(3): p. 314-20.
125. Lemmon, M.A., *Membrane recognition by phospholipid-binding domains.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 99-111.
126. Hers, I., E.E. Vincent, and J.M. Tavare, *Akt signalling in health and disease.* Cell Signal, 2011. **23**(10): p. 1515-27.
127. Benhamron, S. and B. Tirosh, *Direct activation of mTOR in B lymphocytes confers impairment in B-cell maturation andloss of marginal zone B cells.* Eur J Immunol, 2011. **41**(8): p. 2390-6.

128. Laplante, M. and D.M. Sabatini, *mTOR signaling in growth control and disease.* Cell, 2012. **149**(2): p. 274-93.
129. Shimobayashi, M. and M.N. Hall, *Making new contacts: the mTOR network in metabolism and signalling crosstalk.* Nat Rev Mol Cell Biol, 2014. **15**(3): p. 155-62.
130. Dengler, H.S., et al., *Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation.* Nat Immunol, 2008. **9**(12): p. 1388-98.
131. Szydlowski, M., E. Jablonska, and P. Juszczynski, *FOXO1 transcription factor: a critical effector of the PI3K-AKT axis in B-cell development.* Int Rev Immunol, 2014. **33**(2): p. 146-57.
132. Lien, E.C., C.C. Dibble, and A. Toker, *PI3K signaling in cancer: beyond AKT.* Curr Opin Cell Biol, 2017. **45**: p. 62-71.
133. Vanhaesebroeck, B., et al., *P110delta, a novel phosphoinositide 3-kinase in leukocytes.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4330-5.
134. Conley, M.E., et al., *Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K.* J Exp Med, 2012. **209**(3): p. 463-70.
135. Amin, R.H. and M.S. Schlissel, *Foxo1 directly regulates the transcription of recombination-activating genes during B cell development.* Nat Immunol, 2008. **9**(6): p. 613-22.
136. Fruman, D.A., et al., *Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha.* Science, 1999. **283**(5400): p. 393-7.
137. Okkenhaug, K., et al., *Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice.* Science, 2002. **297**(5583): p. 1031-4.
138. Ramadani, F., et al., *The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development.* Sci Signal, 2010. **3**(134): p. ra60.
139. Tze, L.E., et al., *Basal immunoglobulin signaling actively maintains developmental stage in immature B cells.* PLoS Biol, 2005. **3**(3): p. e82.
140. Limon, J.J. and D.A. Fruman, *Akt and mTOR in B Cell Activation and Differentiation.* Front Immunol, 2012. **3**: p. 228.
141. Kovesdi, D., S.E. Bell, and M. Turner, *The development of mature B lymphocytes requires the combined function of CD19 and the p110delta subunit of PI3K.* Self Nonself, 2010. **1**(2): p. 144-153.
142. Omori, S.A. and R.C. Rickert, *Phosphatidylinositol 3-kinase (PI3K) signaling and regulation of the antibody response.* Cell Cycle, 2007. **6**(4): p. 397-402.
143. Heltemes-Harris, L.M., et al., *Activation-induced deaminase-mediated class switch recombination is blocked by anti-IgM signaling in a phosphatidylinositol 3-kinase-dependent fashion.* Mol Immunol, 2008. **45**(6): p. 1799-806.
144. Chen, Z., et al., *Imbalanced PTEN and PI3K Signaling Impairs Class Switch Recombination.* J Immunol, 2015. **195**(11): p. 5461-5471.
145. Okkenhaug, K. and D.A. Fruman, *PI3Ks in lymphocyte signaling and development.* Curr Top Microbiol Immunol, 2010. **346**: p. 57-85.

146. Okkenhaug, K., et al., *The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells.* J Immunol, 2006. **177**(8): p. 5122-8.
147. Gamper, C.J. and J.D. Powell, *All PI3Kinase signaling is not mTOR: dissecting mTOR-dependent and independent signaling pathways in T cells.* Front Immunol, 2012. **3**: p. 312.
148. Huynh, A., et al., *Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability.* Nat Immunol, 2015. **16**(2): p. 188-96.
149. Soond, D.R., et al., *Pten loss in CD4 T cells enhances their helper function but does not lead to autoimmunity or lymphoma.* J Immunol, 2012. **188**(12): p. 5935-43.
150. Rolf, J., et al., *Phosphoinositide 3-kinase activity in T cells regulates the magnitude of the germinal center reaction.* J Immunol, 2010. **185**(7): p. 4042-52.
151. Gracias, D.T., et al., *Phosphatidylinositol 3-Kinase p110delta Isoform Regulates $CD8^+$ T Cell Responses during Acute Viral and Intracellular Bacterial Infections.* J Immunol, 2016. **196**(3): p. 1186-98.
152. Chapel, H., et al., *Common variable immunodeficiency disorders: division into distinct clinical phenotypes.* Blood, 2008. **112**(2): p. 277-86.
153. Yazdani, R., et al., *Comparison of various classifications for patients with common variable immunodeficiency (CVID) using measurement of B-cell subsets.* Allergol Immunopathol (Madr), 2016.
154. Goldman, F.D., et al., *Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the Ikaros gene.* Pediatr Blood Cancer, 2012. **58**(4): p. 591-7.
155. Kuehn, H.S., et al., *Loss of B Cells in Patients with Heterozygous Mutations in IKAROS.* N Engl J Med, 2016. **374**(11): p. 1032-1043.
156. Boisson, B., et al., *A recurrent dominant negative E47 mutation causes agammaglobulinemia and BCR(-) B cells.* J Clin Invest, 2013. **123**(11): p. 4781-5.
157. Dobbs, A.K., et al., *Agammaglobulinemia associated with BCR(-) B cells and enhanced expression of CD19.* Blood, 2011. **118**(7): p. 1828-37.
158. Notarangelo, L.D., A. Villa, and K. Schwarz, *RAG and RAG defects.* Curr Opin Immunol, 1999. **11**(4): p. 435-42.
159. Noordzij, J.G., et al., *The immunophenotypic and immunogenotypic B-cell differentiation arrest in bone marrow of RAG-deficient SCID patients corresponds to residual recombination activities of mutated RAG proteins.* Blood, 2002. **100**(6): p. 2145-52.
160. Noordzij, J.G., et al., *Radiosensitive SCID patients with Artemis gene mutations show a complete B-cell differentiation arrest at the pre-B-cell receptor checkpoint in bone marrow.* Blood, 2003. **101**(4): p. 1446-52.
161. van der Burg, M., et al., *B-cell recovery after stem cell transplantation of Artemis-deficient SCID requires elimination of autologous bone marrow precursor-B-cells.* Haematologica, 2006. **91**(12): p. 1705-9.
162. van der Burg, M., J.J. van Dongen, and D.C. van Gent, *DNA-PKcs deficiency in human: long predicted, finally found.* Curr Opin Allergy Clin Immunol, 2009. **9**(6): p. 503-9.
163. van der Burg, M., et al., *A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining.* J Clin Invest, 2009. **119**(1): p. 91-8.

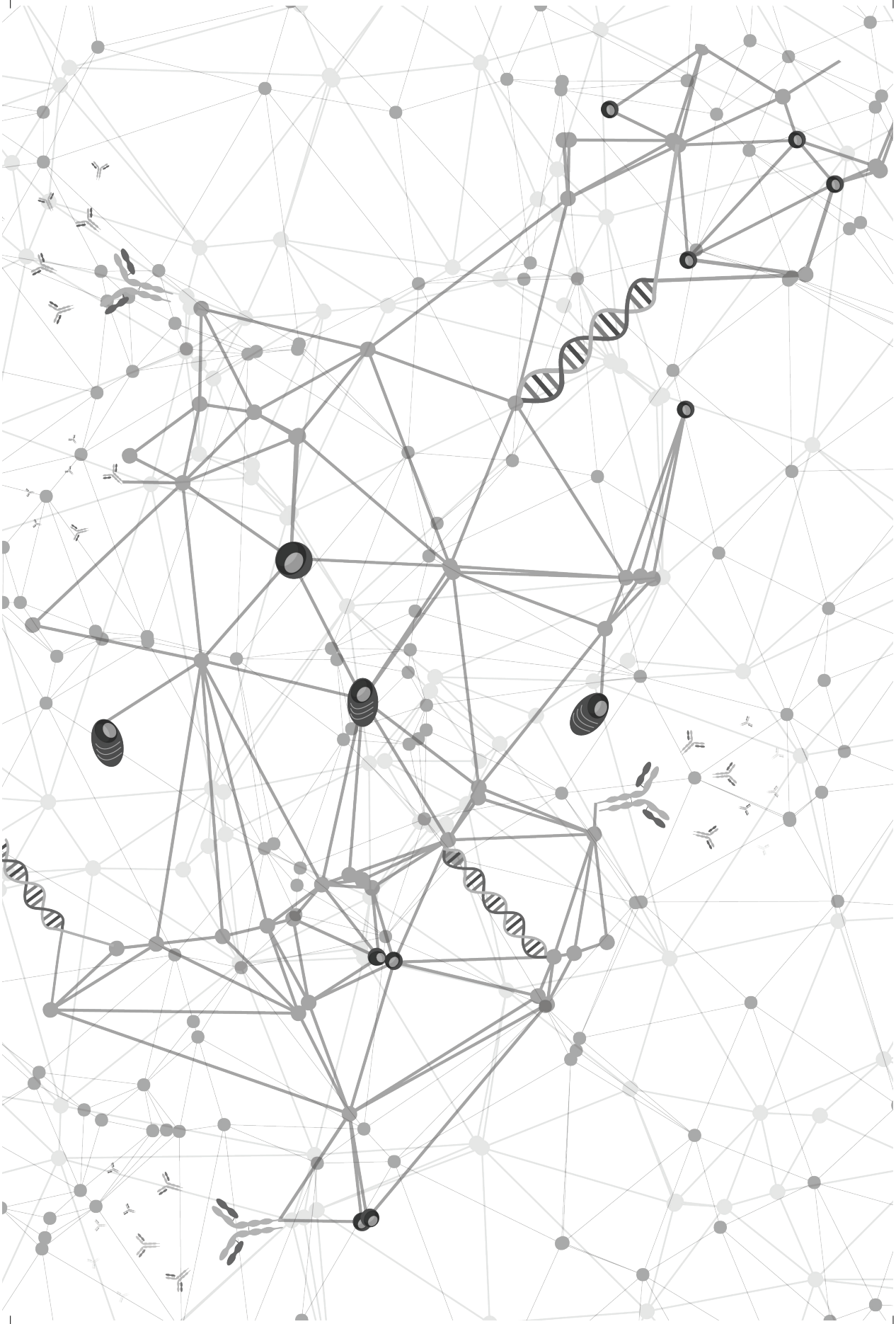
164. H, I.J., et al., *Similar recombination-activating gene (RAG) mutations result in similar immunobiological effects but in different clinical phenotypes.* J Allergy Clin Immunol, 2014. **133**(4): p. 1124-33.
165. van der Burg, M., et al., *A new type of radiosensitive T-B-NK$^+$ severe combined immunodeficieny caused by a LIG4 mutation.* J Clin Invest, 2006. **116**(1): p. 137-45.
166. H, I.J., et al., *Clinical spectrum of LIG4 deficiency is broadened with severe dysmaturity, primordial dwarfism, and neurological abnormalities.* Hum Mutat, 2013. **34**(12): p. 1611-4.
167. Yel, L., et al., *Mutations in the mu heavy-chain gene in patients with agammaglobulinemia.* N Engl J Med, 1996. **335**(20): p. 1486-93.
168. Lopez Granados, E., et al., *Clinical and molecular analysis of patients with defects in micro heavy chain gene.* J Clin Invest, 2002. **110**(7): p. 1029-35.
169. Minegishi, Y., et al., *Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia.* J Exp Med, 1998. **187**(1): p. 71-7.
170. Minegishi, Y., et al., *Mutations in Igalpha (CD79a) result in a complete block in B-cell development.* J Clin Invest, 1999. **104**(8): p. 1115-21.
171. Wang, Y., et al., *Novel Igalpha (CD79a) gene mutation in a Turkish patient with B cell-deficient agammaglobulinemia.* Am J Med Genet, 2002. **108**(4): p. 333-6.
172. Noordzij, J.G., et al., *Composition of precursor B-cell compartment in bone marrow from patients with X-linked agammaglobulinemia compared with healthy children.* Pediatr Res, 2002. **51**(2): p. 159-68.
173. Tsukada, S., et al., *Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia.* Cell, 1993. **72**(2): p. 279-90.
174. Vetrie, D., et al., *The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases.* Nature, 1993. **361**(6409): p. 226-33.
175. Conley, M.E., et al., *Genetic analysis of patients with defects in early B-cell development.* Immunol Rev, 2005. **203**: p. 216-34.
176. Minegishi, Y., et al., *An essential role for BLNK in human B cell development.* Science, 1999. **286**(5446): p. 1954-7.
177. Anzilotti, C., et al., *Key stages of bone marrow B-cell maturation are defective in patients with common variable immunodeficiency disorders.* J Allergy Clin Immunol, 2015. **136**(2): p. 487-90 e2.
178. Pearl, E.R., et al., *B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficiency states.* J Immunol, 1978. **120**(4): p. 1169-75.
179. Ochtrop, M.L., et al., *T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency.* Blood, 2011. **118**(2): p. 309-18.
180. Lougaris, V., et al., *Correlation of bone marrow abnormalities, peripheral lymphocyte subsets and clinical features in uncomplicated common variable immunodeficiency (CVID) patients.* Clin Immunol, 2016. **163**: p. 10-3.
181. H, I.J., et al., *Strategies for B-cell receptor repertoire analysis in primary immunodeficiencies: from severe combined immunodeficiency to common variable immunodeficiency.* Front Immunol, 2015. **6**: p. 157.

182. Warnatz, K., et al., *B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13945-50.
183. Castigli, E., et al., *TACI is mutant in common variable immunodeficiency and IgA deficiency.* Nat Genet, 2005. **37**(8): p. 829-34.
184. Courtois, G. and T.D. Gilmore, *Mutations in the NF-kappaB signaling pathway: implications for human disease.* Oncogene, 2006. **25**(51): p. 6831-43.
185. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation.* J Allergy Clin Immunol, 2014. **134**(1): p. 135-44.
186. Artac, H., et al., *B-cell maturation and antibody responses in individuals carrying a mutated CD19 allele.* Genes Immun, 2010. **11**(7): p. 523-30.
187. van Zelm, M.C., et al., *Antibody deficiency due to a missense mutation in CD19 demonstrates the importance of the conserved tryptophan 41 in immunoglobulin superfamily domain formation.* Hum Mol Genet, 2011. **20**(9): p. 1854-63.
188. van Zelm, M.C., et al., *CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency.* J Clin Invest, 2010. **120**(4): p. 1265-74.
189. Thiel, J., et al., *Genetic CD21 deficiency is associated with hypogammaglobulinemia.* J Allergy Clin Immunol, 2012. **129**(3): p. 801-810 e6.
190. Wentink, M.W., et al., *CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities.* Clin Immunol, 2015. **161**(2): p. 120-127.
191. Rosain, J., et al., *CD21 deficiency in 2 siblings with recurrent respiratory infections and hypogammaglobulinemia.* J Allergy Clin Immunol Pract, 2017. **5**(6): p. 1765-7
192. Suratannon, N., et al., *Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in FERMT3.* Pediatr Allergy Immunol, 2016. **27**(2): p. 214-7.
193. Grimbacher, B., et al., *Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency.* Nat Immunol, 2003. **4**(3): p. 261-8.
194. Kotlarz, D., et al., *Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome.* J Exp Med, 2013. **210**(3): p. 433-43.
195. Allen, R.C., et al., *CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome.* Science, 1993. **259**(5097): p. 990-3.
196. Revy, P., et al., *Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2).* Cell, 2000. **102**(5): p. 565-75.
197. Lucas, C.L., et al., *PI3Kdelta and primary immunodeficiencies.* Nat Rev Immunol, 2016. **16**(11): p. 702-14
198. Jou, S.T., et al., *Identification of variations in the human phosphoinositide 3-kinase p110delta gene in children with primary B-cell immunodeficiency of unknown aetiology.* Int J Immunogenet, 2006. **33**(5): p. 361-9.
199. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science, 2013. **342**(6160): p. 866-71.

200. Lucas, C.L., et al., *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency.* Nat Immunol, 2014. **15**(1): p. 88-97.
201. Deau, M.C., et al., *A human immunodeficiency caused by mutations in the PIK3R1 gene.* J Clin Invest, 2014. **124**(9): p. 3923-8.
202. Lucas, C.L., et al., *Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K.* J Exp Med, 2014. **211**(13): p. 2537-47.
203. Coulter, T.I., et al., *Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study.* J Allergy Clin Immunol, 2017. **139**(2): p. 597-606
204. Elkaim, E., et al., *Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study.* J Allergy Clin Immunol, 2016. **138**(1): p. 210-218 e9.
205. Browning, M.J., et al., *Cowden's syndrome with immunodeficiency.* J Med Genet, 2015. **52**(12): p.856-9
206. Driessen, G.J., et al., *Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency.* J Allergy Clin Immunol, 2016. **138**(6): p. 1744-47

PART 2

B cell precursor development and naive repertoire formation



Chapter 2.1

Delineating human B cell precursor development with genetically identified PID cases as a model

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ABSTRACT

B cells arise from hematopoietic stem cells in bone marrow. Identification and characterization of the different precursor B-cell subsets contributes to understanding normal B-cell development. During differentiation, B cells form a functional B-cell receptor via the process of V(D)J-recombination. Changes in rearrangement status guide the differentiation of B cell precursors (BCP). In addition to changes in rearrangement status and expression of BR molecules, BCP undergo other immunophenotypic changes in their consecutive stages. We studied BCP differentiation in human bone marrow using samples from healthy controls together with patients with known genetic defects in V(D)J recombination or pre-B cell receptor (preBR) signalling to further unravel immunophenotypic changes and to determine the effect of specific differentiation blocks caused by the specific genetic defects.

We designed a 10-color flow cytometry panel, to study human BCP development in bone marrow, that enables reliable gating of previously described populations, based on B-cell receptor-related markers. However, when we included information from other markers, we found heterogeneity within the preB-I and preB-II populations, during which V(D)J recombination takes place with expression of surface markers that seems asynchronous to the expression of cylgμ. Next Generation Sequencing of complete IGH rearrangements in sorted cell populations was performed to determine the rearrangement status at DNA level.

Our data indicate that BCP differentiation is not a single linear route of differentiation, but rather a complex process of V(D)J)recombination-driven checkpoints, divergence, parallel pathways and convergence to form a unique and functional B cell receptor. Understanding this process requires an integrated approach of hallmark protein expression analysis with DNA rearrangement status on a single-cell or on a small homogeneous cell population level.

INTRODUCTION

B cells arise from hematopoietic stem cells in bone marrow and develop in a stepwise manner.[1, 2] Identification and characterization of the different precursor B-cell subsets contributes to understanding normal B-cell development.[3, 4] Expression of transcription factor PAX5 marks commitment to the B cell lineage, which promotes expression of B-cell specific genes such as CD79a (also called Igα) and CD19 while supressing B-lineage inappropriate genes. Since CD79a (cyCD79a) expression is one of the first signs of B cell lineage commitment, cyCD79a$^+$ CD19$^-$ cells are in the human nomenclature defined as pro-B cells followed by expression of CD19 in pre-B-I cells. During this stage, V(D)J recombination of the immunoglobulin (Ig) heavy chain locus is initiated by recombination activating genes (RAG1 and RAG2).[5-11] If this rearrangement process results in a functional protein, Igμ is expressed in the cytoplasm (cyIgμ), which marks the pre-BII-stage. If rearrangement of the first allele does not result in a productive Igμ molecule, the second allele will be rearranged. Igμ is expressed together with the surrogate light chains λ14.1 and VpreB on the plasma membrane as pre-B-cell receptor (preBR).[12, 13] Upon preBR signalling, a cascade of events is induced: downregulation of the recombination machinery to ensure allelic exclusion, proliferation followed by opening of the Ig light chain (IGL) locus, which is being rearranged under the influence of a second expression wave of RAG1 and RAG2.[10, 14] After successful IGL rearrangement, a functional B cell receptor (BR) in the form of IgM is expressed, marking progression to the immature B-cell stage. Once, next to IgM, IgD is expressed on the plasma membrane, the immature B-cell becomes a naive mature B-cell and moves to the peripheral blood.

In addition to changes in rearrangement status and expression of Ig molecules, B-cell precursors (BCP) undergo also other immunophenotypic changes in their consecutive stages. Pro-B cells express stem cell markers such as CD34 and CD10, whereas later stages start to express specific B-cell markers such as CD19 and CD20. Additionally, cells that are in their rearrangement process express TdT in two waves, one during the heavy chain rearrangement and one during the light chain rearrangement which ensures junctional diversity by random addition of non-templated nucleotides. Expression of these markers and immunoglobulin or BR-molecules can be studied using flow cytometry.[15]

Most knowledge about B cell development in bone marrow came from mouse studies, however detailed insight into normal human precursor B-cell development is important to identify and unravel pathophysiological processes in hematological malignancies and Primary Immune Deficiencies (PID), which are caused by genetic defects.[16] Furthermore, precursor B-cell analysis in genetically identified PID can help elucidating the role of specific genes in precursor B-cell development,[13, 17] because absence or dysfunction of essential proteins cause a full or incomplete block at specific developmental stages.[18-20]

Here, we studied BCP differentiation in human bone marrow, using samples from healthy controls together with patients with genetic defects in V(D)J recombination or preBR signalling, to further unravel immunophenotypic changes and to determine the effect on differentiation blockades caused by specific genetic defects. We developed a 10-color panel together with a new analysis strategy using principle component analysis and viSNE analysis[21] that allowed more detailed analysis of previously described cell populations. This 10-color panel was first validated against our previously used 4-color panel.[7, 18, 22] Secondly, we analysed DNA rearrangement status and the gating strategy based on BR-related markers (cyCD79a, cyIgμ, IgM, IgD, CD19) was compared with gating based on membrane markers such as CD10 and CD20 as is also done in literature.[23-25] Gating based on BR-related markers allowed us to define the crucial steps of B cell development better than gating based on surface markers alone and we argue that intracellular markers are required to delineate BCP development. We detected considerable heterogeneity in marker expression, especially in in the pre-BI and pre-BII stages, suggesting that B cell development in bone marrow is not a linear process, but rather a complex asynchronous network with points at which maturation diverges or converges and parallel pathways, that is dictated by V(D)J-recombination-driven checkpoints. We propose that our immunophenotyping panel can be used in multi-center studies with the standardization stringency developed by the EuroFlow consortium,[26] thus allowing to mutually compare the data-files generated on patients with primary immunodeficiency with those of individuals with undisturbed B cell development.

MATERIALS AND METHODS

Bone marrow and peripheral blood samples

Bone marrow samples from healthy controls were left over from healthy children who donated bone marrow for transplantation into a diseased sibling or were collected from patients that had a bone marrow biopsy to rule out other diseases then lymphoid PID. In general, bone marrow samples were considered normal when no malignant cells were detected in combination with normal BCP differentiation upon standard diagnostic testing. Patient bone marrow samples were collected for PID-diagnostics and analyzed in this study with informed consent and according to the guidelines of the local medical ethics committee.

Flow cytometric immunophenotyping of bone marrow

10-color flow cytometric immunophenotyping of bone marrow samples was performed on a LSR Fortessa (BD BioSciences, San Jose, CA, USA) with instrument setting according

to EuroFlow SOP [26] extended to 10-colors in two EuroFlow laboratories. After bulk lysis according to EuroFlow SOP[26, 27] cells were stained for surface markers and intracellular markers in two consecutive steps. The next antibodies were used for extracellular staining: IgM-BV510 (MHM-88) CD38-BV605 (HIT2), CD20-PB (2H7, all Biolegend, San Diego, CA, USA), CD34-APC (8G12), IgD-PeCF594 (IA6, both BD Biosciences), CD19-PC7 (J3-119, Beckman Coulter, Fullerton, CA, USA) and CD10-APC-C750 (Cytognos, Salamanca, Spain). Cells were fixed and permeabilized using the Fix&Perm reagent kit (An der Grub, Vienna, Austria) according to manufacturer's instructions. The next antibodies were used for intracellular staining: IgM-PerCPcy5.5 (MHM-88, Biolegend), TdT-FITC (HT6, Supertechs, Rockville, MD, USA) and CD79a-PE (HM47 Beckman Coulter) (See Table 1 for complete panel).

Patient bone marrow samples were analyzed in parallel with a diagnostic 4-color panel as described before,[7, 18] for comparison of the new 10-color panel to the 4-color diagnostic panel (gold standard). An overview of the antibodies used in all panels can be found in Supplemental Table 1.

The data was analyzed with Infinicyt software Version 1.8 (Cytognos, Salamanca, Spain). Principle component analysis was performed with the Infinicyt software. This method calculates the most discriminating projections based on selected parameters, into a single Automated Population Separator (APS) bi-dimensional graph. Multiple APS graphs (APS1, APS2 etc) can be generated, depending on which parameters contribute more or less to the principle components on the X-axis and Y-axis. ViSNE projection[21] was calculated using Cytobank (Cytobank, Inc, Santa Clara, CA, USA).[28] This method generates a 2D dotplot in which the X- and Y-axis are defined by virtual parameters called tSNE1 and tSNE2, in which all events are projected integrating information on all selected parameters. In a viSNE plot the distance of one event to other events represents how similar events are, with the most similar events plotting closest to each other.[21]

For repertoire analysis, two bone marrow samples without malignant cells and with normal BCP differentiation (determined with standard diagnostic testing) were enriched for B cells using a RosetteSepp human B-cell enrichment cocktail (Stem cell Technologies, Vancouver, Canada) according to manufacturer's instructions and as described before.[29] Subsequently, the B-cell enriched samples were frozen in liquid nitrogen and thawed prior to sorting. The sorting was done with the same antibodies that were used in the 10-color flow-cytometry tube and performed on an Aria-III FACS-sorter (BD Biosciences). After sorting the cells were washed and DNA was isolated using a direct lysis method as described before.[30] The IGH rearrangements were amplified in a 2-step PCR reaction and sequenced by NGS. IGH rearrangements were amplified (35 cycles) using the forward VH1-6 FR2 and reverse JH consensus BIOMED-2 primers which were extended with Illumina P5 and P7 adapter sequence.[31] Subsequently, PCR products were purified by gel extraction (Qiagen, Valencia, CA), followed by a nested PCR reaction (12 cycles) to include the sample-specific

indices and Illumina sequencing adapters using primers from the Illumina TruSeq Custom Amplicon Index Kit (Illumina, San Diego, CA, USA). The PCR concentration was measured using the Quant-it Picogreen dsDNA assay (Invitrogen, Carlsbad, CA). The libraries were analyzed by NGS (221 base pair paired-end) on the MiSeq platform (Illumina, San Diego, CA, USA) with use of an Illumina MiSeq Reagent Kit V3, according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Paired sequences were aligned using paired-end read merger (PEAR),[32] and the fastq files were converted to fasta files.[33] Subsequently, the sequences were trimmed to remove the primer sequenced and were uploaded in IMGT/High-V-Quest,[34] and subsequently the IMGT output files were analysed in the ARGalaxy tool (https://bioinf-galaxian.erasmusmc.nl/argalaxy).[35] For the analysis only a single sequence per clone (defined as same V gene, same J gene and the nucleotide sequence of the CDR3 region) were included. In-frame rearrangements are defined to have an in-frame rearrangement without a stop codon. Unproductive rearrangements are either out-of-frame rearrangements or in-frame rearrangements with a stop codon.

RESULTS

Subset definition based on B cell receptor related markers is consistent between different panels

To study human bone marrow we designed a 10-color flow cytometry panel in a single tube (Table 1), to make optimal use of available material and integrate information about both intracellular and extracellular markers on each individual cell. We tested this 10-color panel against our validated 4-color diagnostic panel.[7, 18] Bone marrow samples from healthy controls and PID patients were analysed using both flowcytometry panels. B cells and BCP were defined as cytoplasmicCD79a$^+$ (cyCD79a$^+$). The five major populations (pro-B, pre-BI, preB-II, immature and mature B cells (Figure 1A)) were gated based on CD19, nTdT, cyIgμ, IgM and IgD expression (B-cell receptor (BR)-related markers) (Figure 1B and supplementary methods). This was related to the subset distribution of the 4-color panel, that we used as a gold standard. Since IgMD$^+$ cells (mature B cells) can also be detected in peripheral blood, we do not consider them a precursor stage. In ten independent samples

Table 1. Set-up of fluorochromes and epitopes of the 10-color EuroFlow BCP tube

Fluoro-chrome	PB	BV510	BV605	FITC	PE	PE-CF594	PerCP-Cy5,5	PE-Cy7	APC	Alexa 750
Target	CD20	IgM	CD38	TdT	CD79a	IgD	cyIgM	CD19	CD34	CD10
clone	2H7	MHM-88	HIT2	HT6	HM47	IA6	MHM-88	J3-119	8G12	HI10a
volume (undiluted)	1 µl	1.3 µl	1 µl	10 µl	5 µl	3 µl	2.5 µl	5 µl	2.5 µl	5 µl

Shaded fields indicate intracellular markers

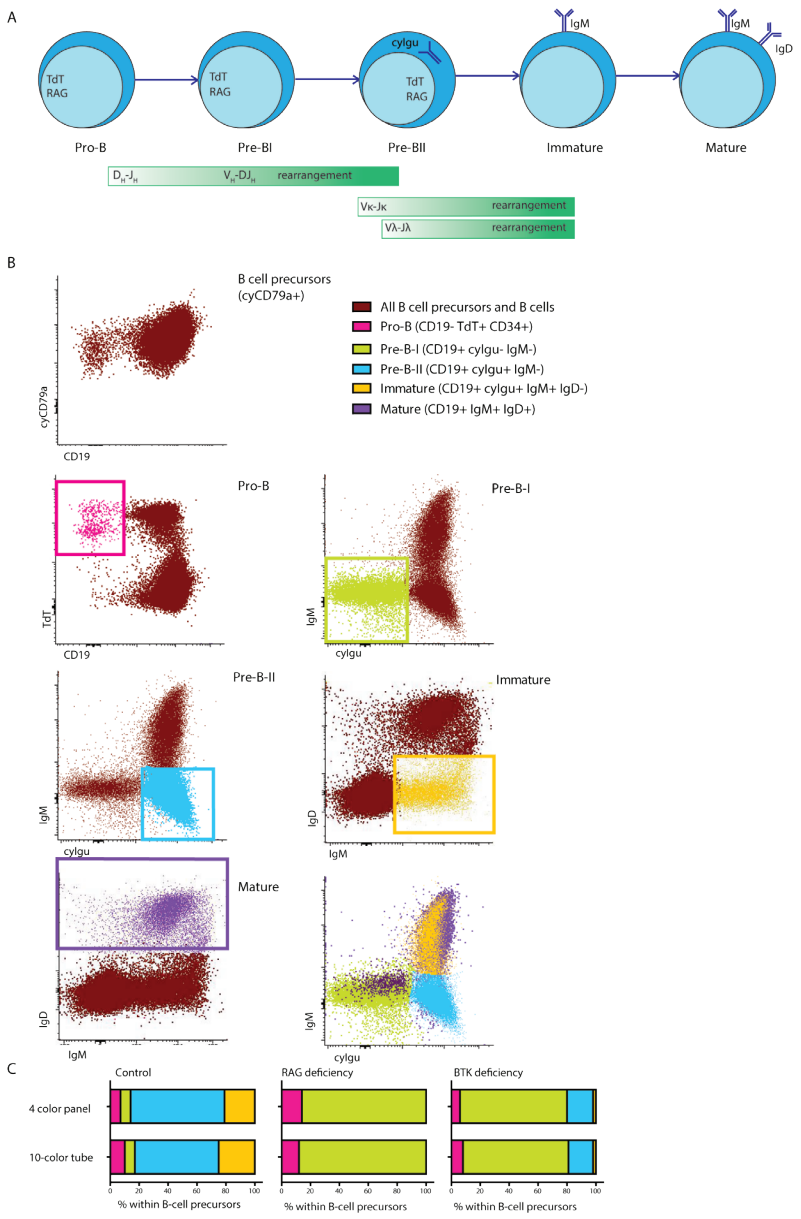

Figure 1. Major BCP subsets in human bone marrow.
A. Schematic representation of the BCP subsets in human bone marrow, the green bars indicate when recombination processes take place. **B**. Population definition based on BR-related markers. All cyCD79a expressing cells are considered BCP or B cells. Pro-B cells are defined as CD19$^-$TdT$^+$, pre-BI cells are defined as CD19$^+$ cylgμ$^-$ IgM-, pre-BII cells are defined as CD19$^+$ cylgμ$^+$ IgM-, immature B cells are defined as CD19$^+$ IgM$^+$ IgD$^-$and mature B cells are defined as CD19$^+$ IgM$^+$ IgD$^+$. **C**. BCP subset distribution in the same sample that was acquired in parallel with two different panels. Population definition was in both cases done as indicated above.

(n= 4 controls and 6 patient samples) both panels revealed the same precursor B-cell subset distribution. Three representative cases are shown: one of normal BCP development, a RAG deficient patient and a BTK deficient patient (Figure 1C). This indicates that gating based on BR-related markers is consistent between both panels and gives comparable results in both healthy controls and PID patients with BCR signaling and a V(D)J recombination defect.

Population definition based on only BR-related markers, shows heterogeneity within the population

When using only the BR-related markers (cyCD79a, CD19, cylgμ, IgM and IgD) we defined 4 subsets of BCP. However, when we took the other markers into account (TdT, CD34, CD10, CD20 and CD38) we found that these populations are not homogeneous. Especially pre-BI and pre-BII cells showed heterogeneity in the non-BR related markers (Figure 2A). In the pre-BI population, cells were mainly TdT^+ and $CD34^+$, but some cells have lost one or both markers, while at the same time being positive for CD10 and negative for CD20, indicating they are not switched memory B cells from peripheral blood. This was also observed for the loss of CD10 and CD38 and the gain of CD20, indicating that there could be more different subsets within this population (Figure 2A). Pre-BII cells, on the other hand, were $cylg\mu^+$ and mainly $CD34^-$ and TdT^-, but some pre-B-II cells still express CD34 and TdT. The expression of CD20 was highly heterogeneous in this population and, the expression was gradual (Figure 2A). To study this heterogeneity in more detail, we performed a viSNE analysis on each separate population (Figure 2B). After performing the viSNE analysis, we indicated the intensity of the individual non-BR-related markers for each cell. Within the pre-BI population, a clearly separate group of cells is found that is TdT^- $CD34^-$. Vice versa, in the pre-BII population a small population of $CD34^+$ TdT^+ cells was found. In both populations a gradual expression pattern of CD20 was detected, which was also present in the immature B cells.

Asynchronous marker expression in pre-B stages is found in PID patients

The heterogeneity within populations that we observed seemed to be the highest in the pre-BI and pre-BII stages. To further examine the relation between expression of non-BR-related markers and cylgμ, we compared pre-BI and pre-BII cells from controls to patients with a RAG deficiency and a BTK deficiency. We found that a fraction of the pre-BI cells in controls, loses CD34 and/or TdT expression and some upregulate CD20 (Figure 3A). In the RAG deficient patient, TdT expression remains intact, but again we see loss of CD34 and gain of CD20. Apparently, cells can upregulate CD20 in the absence of a functionally rearranged heavy chain. The same upregulation occurs in case of a BTK-deficiency. In addition, in the pre-BII cells of controls and BTK deficient patients, some of the cells,

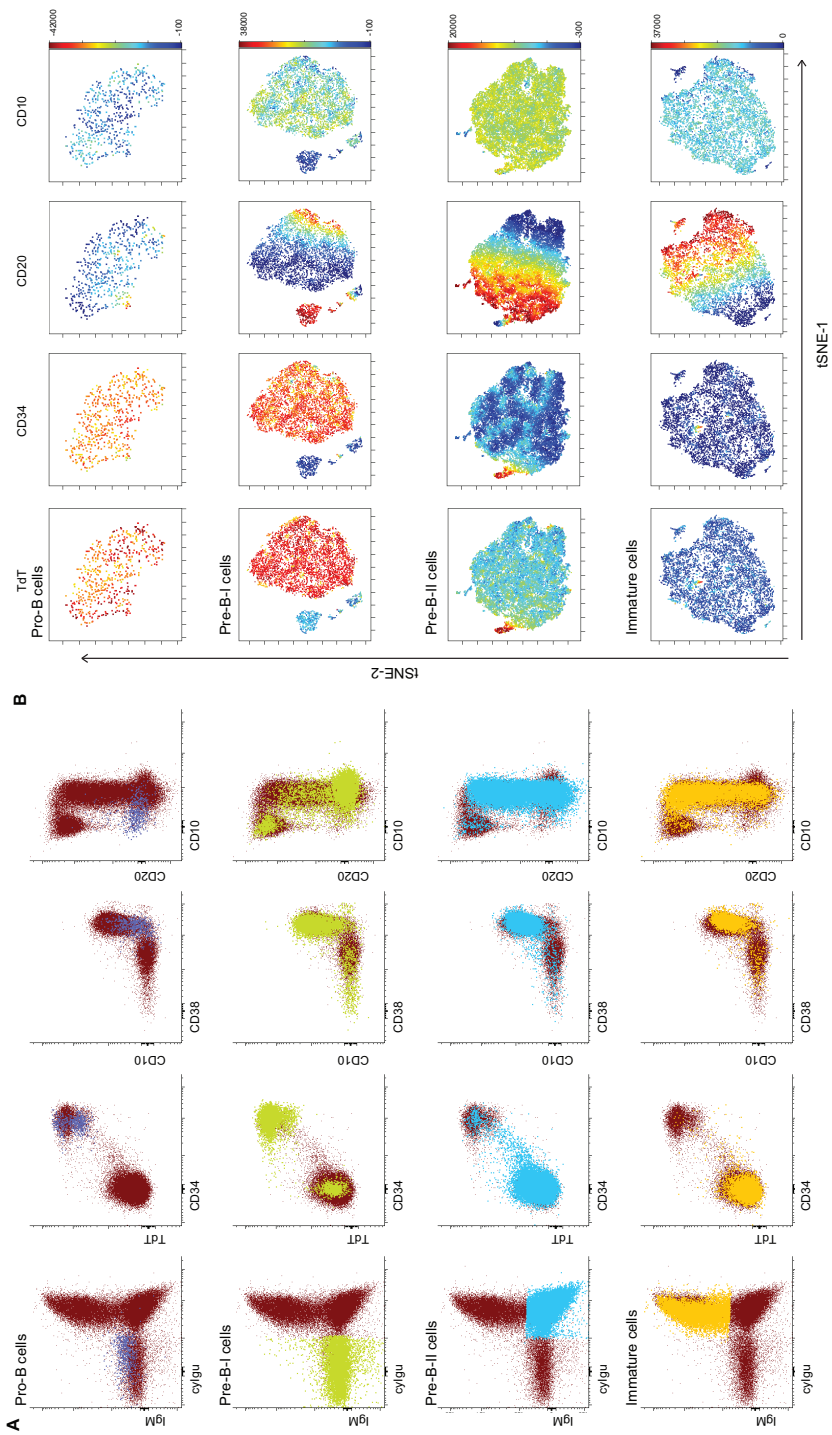

Figure 2. Heterogeneity within BCP subsets.
A. Dotplots of a representative normal bone marrow sample, showing all cyCD79a$^+$ B cells in bone marrow. Populations of interest are highlighted in a different color: pro-B cells in pink, Pre-B-I cells in green, Pre-B-II cells in blue, immature cells in orange. **B**. Results of a viSNE analysis of individual B-cell precursor subsets, represented by tSNE-1 versus tSNE-2 plots. The color scale represents the intensity of the stain for individual markers: TdT, CD34, CD20, CD10 and CD38.

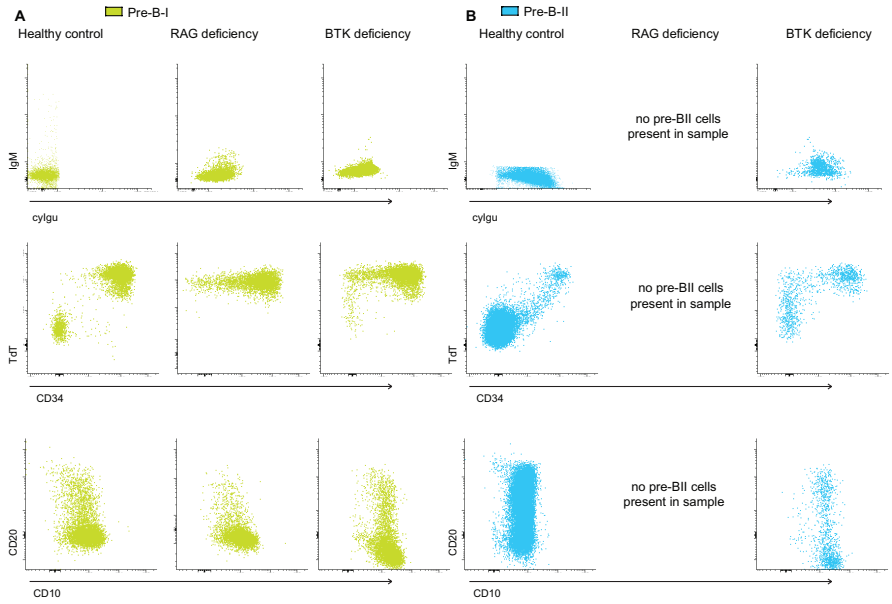

Figure 3. Pre-BI and pre-BII marker expression in control and patient samples.
A. Dotplots representing pre-BI cells from a control sample, and two patient samples, indicating the uncoupling of expression of cylgμ and surface markers like CD34, CD20 and CD10. In both the control and the patients, cells without cylgμ can lose CD34 expression and gain CD20 expression. **B**. Dotplots representing pre-BII cells from a control sample, and two patient samples. In both the control and the patients, cells with cylgμ expression can still express CD34 and TdT, and CD20 expression is heterogeneous.

although expressing cylgμ, still have CD34 and/or TdT expression. Those cells seem to have prolonged TdT expression. Since RAG deficient patients do not have pre-BII cells, we could not compare this subset for these patients. Thus, we observed that surface marker expression can behave asynchronously with the progress in BR formation. Additionally, RAG or BTK deficient cells can lose CD34 expression, without downregulating TdT.

Integration of all markers into one analysis strategy.

After we observed that marker expression can be asynchronous, we integrated all markers into one analysis strategy that was based on population gating using 2D dotplots (Supplemental Figure 1). However since the process of BCP differentiation seems gradual at some stages and some markers have gradual expression instead of clear positive and negative populations, not all populations can be clearly defined. To integrate data from multiple markers on each individual cell a principle component analysis based multidimensional view (APS graph) was generated based on a reference data set of 5 samples of healthy control bone marrow samples in Infinicyt software.[36] In the APS1 view (Figure 4A) the cells are separated in a fashion that seems to follow their maturation from less mature pro-B cells in the lower right to mature B cells at the left of the diagram. In

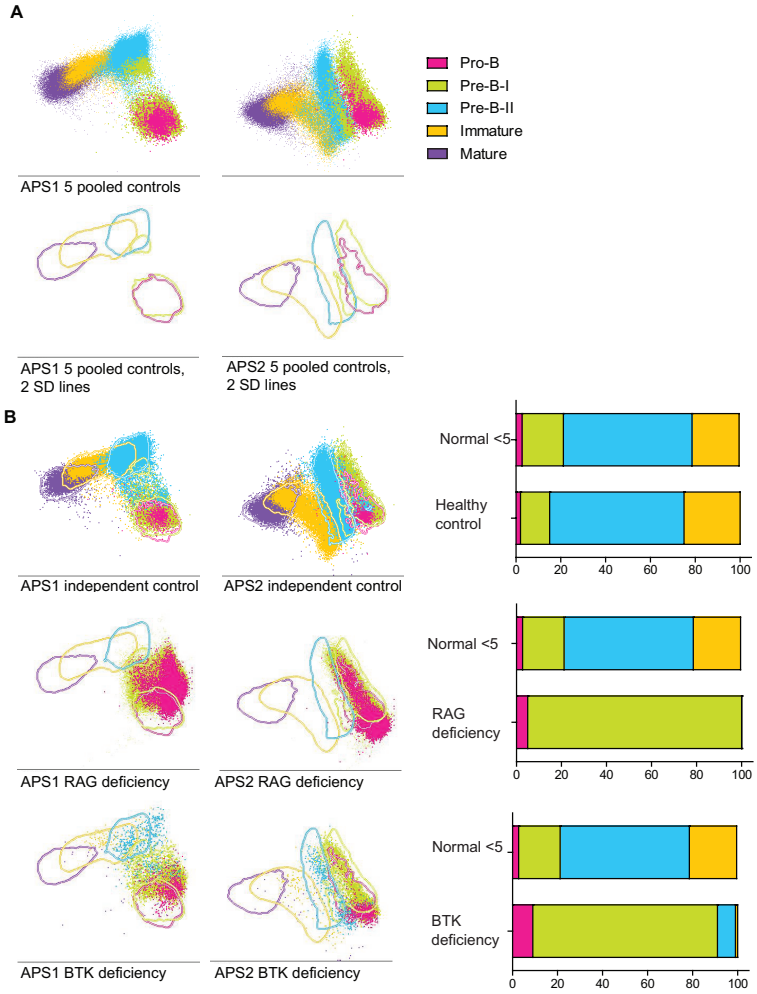

Figure 4. Supervised APS view of BCP populations in healthy bone marrow (n=5) indicated by lines at 2SD intervals.
A. BCP populations in a pool of 5 healthy bone marrow samples that were used to create the APS view. Lines indicate the 2 SD range of each population, dot indicate individual cells. The different populations are indicated by the different colors. **B**. 2 SD lines of BCP populations derived from a pool of 5 healthy donors, dots indicate total BCP from a sixth healthy donor, a RAG deficient patient and a BTK deficient patient, plotted against the reference pool. Bars indicate the BCP subset distribution of each sample, compared to age matched controls.

the APS2 diagram, again the order of maturation is visible, but now the populations with heterogeneous CD20 expression are more stretched out, indicating the gradual expression of this marker. In both diagrams, the populations as we identified them previously seem to overlap, indicating a gradual differentiation. To indicate the position of each population in these diagrams, we plotted the 2SD line of each population as a reference. Using the 5 healthy donor samples as a reference, we plotted in the BCP cells from a sixth, independent,

healthy donor (Figure 4B) into the same principal components (fixed, supervised APS plot). The events fall within the ranges of the other 5 healthy controls. However, if we plot the BCP events from a patient with a RAG deficiency or a BTK deficiency, we can see where a blockade arises. Using a reference of 5 healthy samples, showed at which points blockades are located in patients with genetic defects. However, in this representation, some cells fall outside the 2SD lines of both pre-BI and pre-BII. This is the case in both the control and the patient samples. A combination of the APS plot and the bar graph gives the best impression about the position of the differentiation block, the relative subset distribution and whether the combination of expression of all markers in patients deviates from controls.

Downregulation of CD34 and TdT between pre-BI and pre-BII stage.

When we focused on the cells that were neither within the 2SD interval of the pre-BI cells nor in the pre-BII 2SD interval in healthy controls (in controls between 1 and 3% of all BCP cells), we found that they are also present in normal BCP differentiation, although not as many as in the patient samples (Figure 5A). These cells are defined as pre-BI or pre-BII based on the absence or presence of cylgμ but the expression of TdT, CD34, CD10 and CD20 is asynchronous with their cylgμ status (Figure 5B). We can divide the pre-BI cells in true pre-BI cells that are $CD19^+$ $cylg\mu^-$ $CD34^+$ TdT^+ (pre-BI +/+) and another, more heterogeneous group that is $CD19^+$ $cylg\mu^-$ but where CD34 and or TdT expression is negative (pre-BI -/-). These cells are not switched memory B cells coming from peripheral blood, because they all express both CD10 and CD38. The same split can be made within the pre-BII cells, dividing them in pre-BII cells that are $CD19^+$ $cylg\mu^+$ $CD34^-$ TdT^- (pre-BII -/-) and a heterogeneous group that is $CD19^+$, $cylg\mu^+$ but that still have CD34, TdT or both these markers (pre-BII +/+). In the APS views that are based on all BCPs, these cells end up between pre-BI and pre-BII. If we create an APS view of only the pre-BI and pre-BII stages, we can examine how heterogeneous these populations are (Figure 5C,D).

Rearrangement status classifies the intermediate stages

To further dissect this, we sorted the pre-B populations into four populations: pre-BI +/+ ($CD19^+$ $cylg\mu^-$ $CD34^+$ TdT^+), pre-BI -/- ($CD19^+$ $cylg\mu^-$ $CD34^-$ TdT^-), pre-BII -/- ($CD19^+$ $cylg\mu^+$ $CD34^-$ TdT^-) and pre-BII +/+ ($CD19^+$ $cylg\mu^+$ $CD34^+$ TdT^+). We isolated DNA from these subsets and sequenced complete IGH rearrangements using next-generation sequencing. We found that in the pre-BI +/+ cells, the majority of complete rearrangements is unproductive with an in-frame: unproductive ratio of 1:9, which is in line with the observation that these cells do not express cylgu, yet. In the pre-BII -/- cells, the in-frame: unproductive ratio is 4:1, with approximately 80% of rearrangements in frame, which is in line with the observation that these cells all express cylgu. In the pre-BI -/- population, the in-frame: unproductive ratio is 3:1, and in the pre-BII +/+ population, this ratio is also 3:1. In these

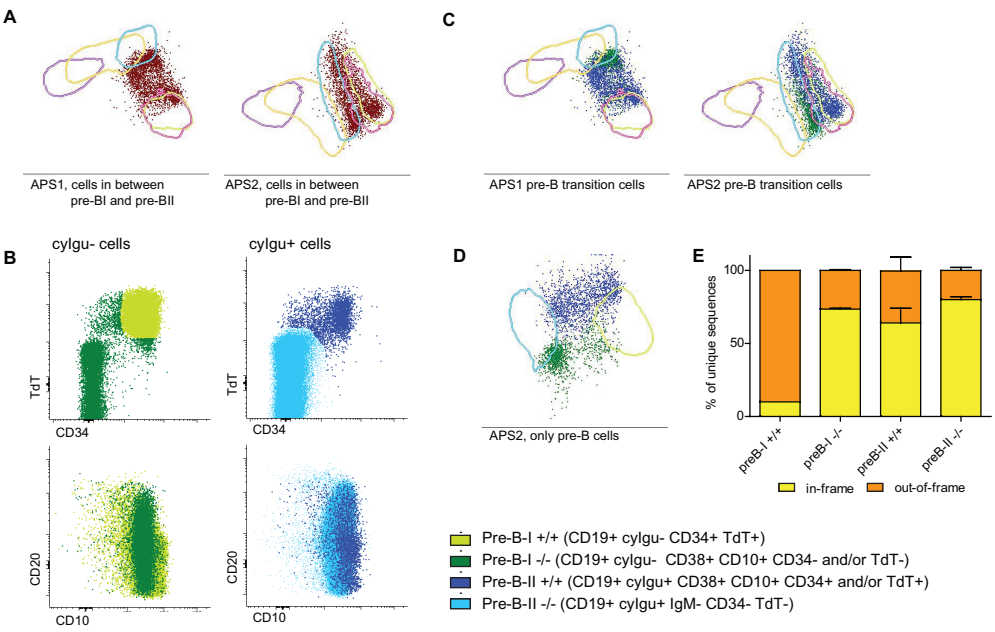

Figure 5. Further dissection of the pre-B compartment.
A. APS views indicating the cells that are outside the 2SD lines of the pre-BI and pre-BII populations of healthy controls. **B**. Expression of CD34, TdT, CD10 and CD20 within cylgμ$^-$ (in green) and cylgμ$^+$ cells. All cells here are CD19$^+$ CD10$^+$ CD38$^+$ indicating that they are bone marrow derived B cells. **C**. APS views indicating the cells that are outside the 2SD lines of the pre-BI and pre-BII populations of healthy controls, color coded as in B. **D**. APS view supervised on pre-BI and pre-BII cells, separating the transitioning pre-B cells further.**E**. Distribution of in-frame and out-of-frame DNA-rearrangements in sorted pre-B subpopulations.

populations, the relative amount of in-frame complete rearrangements is approximately the same, however, in one population the cells do not express cylgu, whereas they do in the other populations. This indicates that in the pre-BI -/- cells in-frame rearrangements are present, but they are either not productive (i.e. not leading to a functional protein) or they are not (yet) expressed. The pre-BII +/+ already express cylgu, but did not yet down-regulate CD34 and TdT.

DISCUSSION

In this study, we validated a 10-color flow cytometry panel, to study human BCP development in bone marrow of immune deficient patients in more detail on crucial developmental thresholds than what was previously done. We could reliably gate populations as they were gated and described before, based on BR-related markers. In our standardized measurements we could superimpose PID samples over healthy controls

and describe abnormalities form normal development found in patients. Unexpectedly, when we included information from additional markers, we found heterogeneity within especially the preB-I and preB-II populations, during which V(D)J recombination takes place with expression of surface markers that seems asynchronous to the expression of cyIgμ. NGS analysis of complete IGH rearrangements in sorted populations was used to determine the rearrangement status at the DNA level.

We studied BCP differentiation in the human bone marrow, using healthy control samples and PID patients with proven genetic defects in either V(D)J-recombination or pre-BR-signalling as a control. We showed that BR-related marker based population definition is consistent over samples and different panels, but this results in heterogeneous populations when other markers, like CD34, TdT and CD20 are considered. Upon more in-depth study of expression patterns of these markers, we found that in some specific populations, expression of these markers is asynchronous to the process of BR-formation. This effect is more visible in patients with defects in V(D)J-recombination. Specifically, we found that CD20 can be upregulated in the absence of cyIgμ expression and that cells can lose TdT expression and CD34 expression without having expression of heavy chain protein in the cytoplasm.

We combined the population-gating based strategy that is often used in flow cytometry with principle component analysis. We showed that the populations of precursor B-cells seem to overlap, indicating a continuous process rather that a step-wise differentiation. This is in line with asynchronous marker expression that we see between surface markers e.g. each cell seems to up-and –down regulate its phenotype markers at its own pace. Even more, some phenotype markers that were previously thought to be V(D)J-recombination dependent, seem to progress even in the absence of cyIgμ expression, as indicated by loss of CD34 and gain of CD20 in patients with genetic defects in V(D)J-recombination. Some of the cellular phenotypes that we found in controls are more common in genetically defined patient samples. This indicates that, even though cells cannot successfully rearrange their IgH-locus (RAG deficiency[10, 37]), they will still lose CD34 expression as if they are progressing to the next stage. In addition to that, we found that CD20 expression is gradually increasing over the course of several stages. However, CD20 expression is heterogeneous in many populations. Especially in patient samples, we often detected high CD20 expression in populations that were assumed to be early in B-cell differentiation. Even though the exact role of CD20 on B cells is not found yet, it is still a useful marker indicating B-cell development and if highly expressed in combination with loss of CD10 and CD38, indicating maturity of the B cells. CD20 expression does not seem to be related with BR-rearrangement.

Both in PID patients and in control samples we identified cells outside the reference borders of the defined populations (e.g. pre-BI and pre-BII) in the APS plots, although the numbers were much lower in control samples Especially in the RAG deficient patient this

is striking, because it seems as if some cells can progress in surface marker expression by expressing CD20 and loosing CD34, without having a functionally rearranged heavy chain. This further supports the idea that expression of CD20 and CD34 is not in all cells strictly linked to heavy chain rearrangement status.

To further dissect this, we sorted preB-I and preB-II cells and further divided these populations based on their CD34 and TdT expression. DNA extracted from these cell populations was used to study complete IGH-rearrangements using NGS. In preB-I +/+ cells we showed that almost around 90% of detectable rearrangements are unproductive. Since these cells do not express cylgu yet, we hypothesize that these cells have not yet rearranged an in-frame IGH, and thus they still express CD34 and TdT. In preB-II cells that are $CD34^{-}$ TdT^{-}, around 75% of detectable rearrangements were in-frame. These cells all express cylgu, and some have an unproductive rearrangement on one allele combined with an in-frame rearrangement on their second allele, which explains the 25% of unproductive rearrangements in this population. The preB-II +/+ cells also contain approximately 75% of in-frame rearrangements. Possibly, these cells have only just completed their in-frame rearrangement, started expressing cylgμ and still need to downregulate CD34 and TdT. However, our data is not sufficient to conclude this. Also, we detected around 75% in-frame rearrangements in pre-Bi -/- cells. These cells have an in-frame heavy chain rearrangement on the DNA level, however, they do not express cylgu protein. To further investigate this, single-cell analysis on DNA, RNA and protein level might give further insight in how and why V(D)J-recombination status and phenotypic marker expression are linked.

In conclusion, we have introduced a new 10-color reference approach consisting of standardized cytometry staining and analytical tools for the analysis of BCP in human bone marrow. Furthermore, our data indicate that BCP differentiation is not a single linear route of differentiation, but rather a complex process of V(D)J)recombination-driven checkpoints, divergence, parallel pathways and convergence to form a unique and functional B cell receptor. Understanding the process of BCP differentiation requires an integrated approach of single-cell DNA, RNA and protein analysis applied to a model systems with genetically defined differentiation steps and blocks.

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REFERENCES

1. Ghia, P., et al., *B-cell development: a comparison between mouse and man.* Immunol Today, 1998. **19**(10): p. 480-5.
2. LeBien, T.W., *Fates of human B-cell precursors.* Blood, 2000. **96**(1): p. 9-23.
3. Loken, M.R., et al., *Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development.* Blood, 1987. **70**(5): p. 1316-24.
4. Loken, M.R., et al., *Flow cytometric analysis of normal B lymphoid development.* Pathol Immunopathol Res, 1988. **7**(5): p. 357-70.
5. Busslinger, M., *Transcriptional control of early B cell development.* Annu Rev Immunol, 2004. **22**: p. 55-79.
6. Ghia, P., et al., *Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci.* J Exp Med, 1996. **184**(6): p. 2217-29.
7. van Zelm, M.C., et al., *Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression.* J Immunol, 2005. **175**(9): p. 5912-22.
8. Meffre, E., et al., *Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development.* J Clin Invest, 2001. **108**(6): p. 879-86.
9. Geier, J.K. and M.S. Schlissel, *Pre-BCR signals and the control of Ig gene rearrangements.* Semin Immunol, 2006. **18**(1): p. 31-9.
10. Oettinger, M.A., et al., *RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination.* Science, 1990. **248**(4962): p. 1517-23.
11. van Gent, D.C., et al., *Initiation of V(D)J recombinations in a cell-free system by RAG1 and RAG2 proteins.* Curr Top Microbiol Immunol, 1996. **217**: p. 1-10.
12. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nat Rev Immunol, 2009. **9**(3): p. 195-205.
13. Espeli, M., et al., *Initiation of pre-B cell receptor signaling: common and distinctive features in human and mouse.* Semin Immunol, 2006. **18**(1): p. 56-66.
14. Stadhouders, R., et al., *Pre-B cell receptor signaling induces immunoglobulin kappa locus accessibility by functional redistribution of enhancer-mediated chromatin interactions.* PLoS Biol, 2014. **12**(2): p. e1001791.
15. van Lochem, E.G., et al., *Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts.* Cytometry B Clin Cytom, 2004. **60**(1): p. 1-13.
16. Gathmann, B., et al., *The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008.* Clin Exp Immunol, 2009. **157 Suppl 1**: p. 3-11.
17. Schiff, C., et al., *Autosomal primary immunodeficiencies affecting human bone marrow B-cell differentiation.* Immunol Rev, 2000. **178**: p. 91-8.
18. Noordzij, J.G., et al., *Composition of precursor B-cell compartment in bone marrow from patients with X-linked agammaglobulinemia compared with healthy children.* Pediatr Res, 2002. **51**(2): p. 159-68.

19. Noordzij, J.G., et al., *Radiosensitive SCID patients with Artemis gene mutations show a complete B-cell differentiation arrest at the pre-B-cell receptor checkpoint in bone marrow.* Blood, 2003. **101**(4): p. 1446-52.
20. Pearl, E.R., et al., *B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficiency states.* J Immunol, 1978. **120**(4): p. 1169-75.
21. Amir el, A.D., et al., *viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia.* Nat Biotechnol, 2013. **31**(6): p. 545-52.
22. Anzilotti, C., et al., *Key stages of bone marrow B-cell maturation are defective in patients with common variable immunodeficiency disorders.* J Allergy Clin Immunol, 2015. **136**(2): p. 487-90 e2.
23. Dulau Florea, A.E., et al., *Abnormal B-Cell Maturation in the Bone Marrow of Patients with Germline Mutations in PIK3CD.* J Allergy Clin Immunol, 2016.
24. Lougaris, V., et al., *Correlation of bone marrow abnormalities, peripheral lymphocyte subsets and clinical features in uncomplicated common variable immunodeficiency (CVID) patients.* Clin Immunol, 2016. **163**: p. 10-3.
25. Kohn, L.A., et al., *Human lymphoid development in the absence of common gamma-chain receptor signaling.* J Immunol, 2014. **192**(11): p. 5050-8.
26. Kalina, T., et al., *EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols.* Leukemia, 2012. **26**(9): p. 1986-2010.
27. Flores-Montero, J., et al., *Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma.* Leukemia, 2017. **31**(10): p. 2094-2103.
28. Kotecha, N., P.O. Krutzik, and J.M. Irish, *Web-based analysis and publication of flow cytometry experiments.* Curr Protoc Cytom, 2010. **Chapter 10**: p. Unit10 17.
29. Pelak, O., et al., *Lymphocyte enrichment using CD81-targeted immunoaffinity matrix.* Cytometry A, 2017. **91**(1): p. 62-72.
30. van der Burg, M., et al., *Standardization of DNA isolation from low cell numbers for chimerism analysis by PCR of short tandem repeats.* Leukemia, 2011. **25**(9): p. 1467-70.
31. van Dongen, J.J., et al., *Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936.* Leukemia, 2003. **17**(12): p. 2257-317.
32. Zhang, J., et al., *PEAR: a fast and accurate Illumina Paired-End reAd mergeR.* Bioinformatics, 2014. **30**(5): p. 614-20.
33. Blankenberg, D., et al., *Manipulation of FASTQ data with Galaxy.* Bioinformatics, 2010. **26**(14): p. 1783-5.
34. Alamyar, E., et al., *IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS.* Methods Mol Biol, 2012. **882**: p. 569-604.
35. H, I.J., et al., *Antigen Receptor Galaxy: A User-Friendly, Web-Based Tool for Analysis and Visualization of T and B Cell Receptor Repertoire Data.* J Immunol, 2017. **198**(10): p. 4156-4165.

36. Costa, E.S., et al., *Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping.* Leukemia, 2010. **24**(11): p. 1927-33.
37. Notarangelo, L.D., A. Villa, and K. Schwarz, *RAG and RAG defects.* Curr Opin Immunol, 1999. **11**(4): p. 435-42.

SUPPLEMENTAL TABLES AND FIGURES

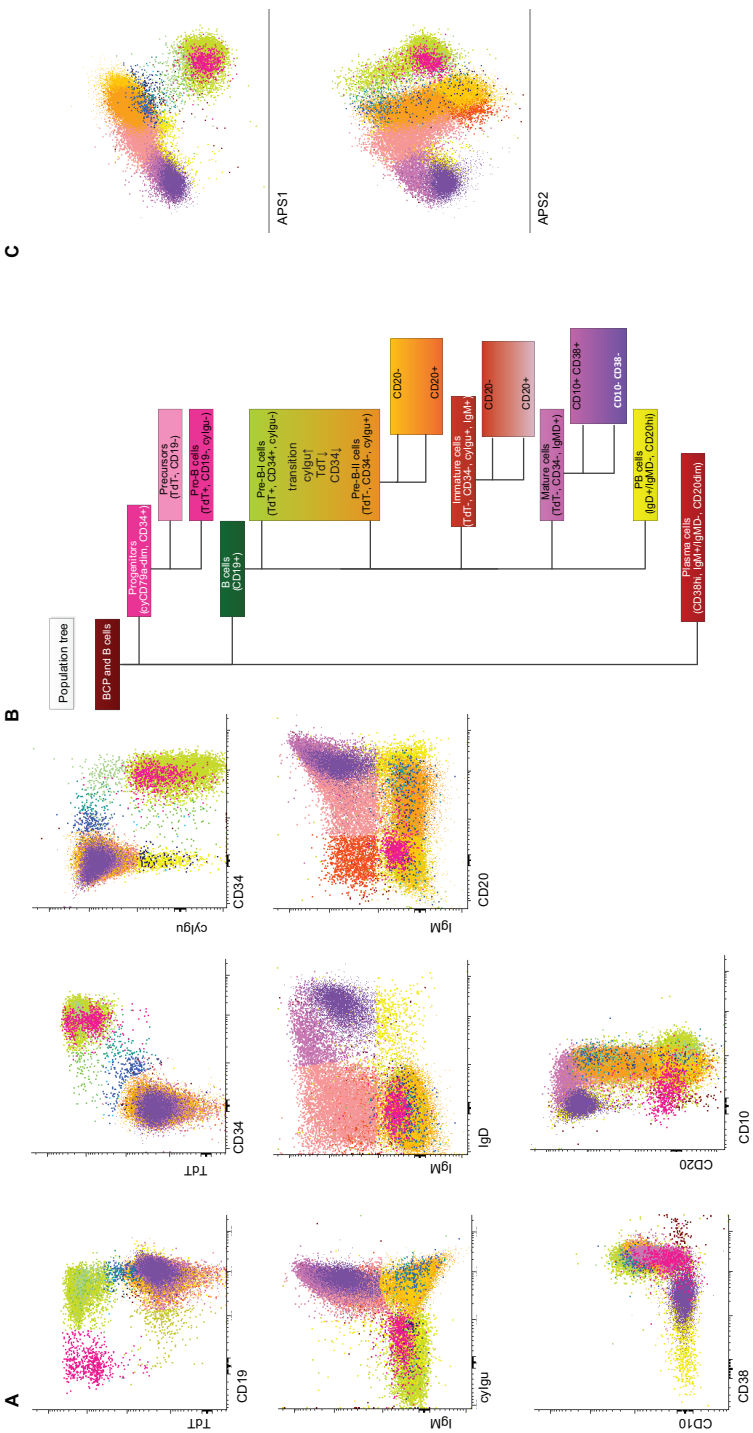
Supplemental Table 1. Markers of the different panels

4 color panel (10 tubes)			10 color tube
CD10	CD36	cylgμ	cyCD79a
CD34	CD3	TdT	TdT
IgD	CD16/CD56	cyCD79a	cylgμ
CD19	CD13/CD33	VpreB	CD19
CD20	CD71		CD34
IgM			CD20
CD138			CD10
CD45			IgM
CD22			IgD
			CD38

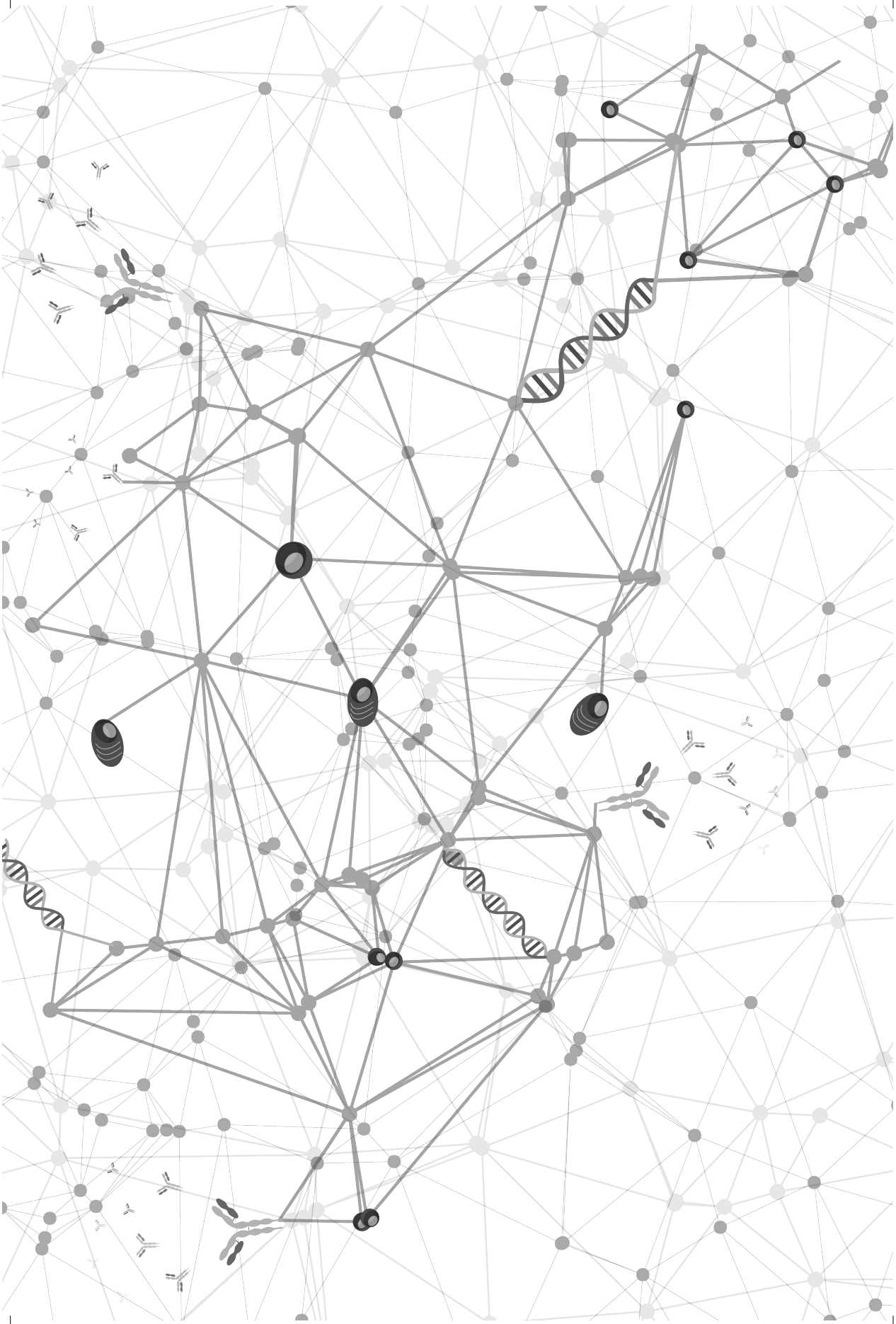
Supplemental Table 2. Expression of markers and population definition

	cyCD79a	CD34	TdT	CD19	CD10	CD38	cyIgM	IgM	IgD	CD20
All BCP	+									
pro-B	+	+	+	-	+	+	-	-	-	
pre-BI	+	+	+	+	+	+	-	-	-	
pre-BII	+	-	-	+	+	+	+	-	-	
immature	+	-	-	+	+	+	+	+	-	
mature	+	-	-	+			+	+	+	
peripheral blood	+	-	-	+	-	-				++
plasma blasts	+	-	-	dim/-	-	++		double – or single +		-

Empty field indicates that this marker is not used for gating this population



Supplemental Figure 1.



Chapter 2.2

Strategies for B-cell receptor repertoire analysis in primary immunodeficiencies: from severe combined immunodeficiency to common variable immunodeficiency

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ABSTRACT

The antigen receptor repertoires of B and T cells form the basis of the adaptive immune response. The repertoires should be sufficiently diverse to recognize all possible pathogens. However, careful selection is needed to prevent responses to self or harmless antigens. Limited antigen receptor repertoire diversity leads to immunodeficiency, whereas unselected or misdirected repertoires can result in autoimmunity. The antigen receptor repertoire harbors information about abnormalities in many immunological disorders. Recent developments in next generation sequencing allow the analysis of the antigen receptor repertoire in much greater detail than ever before. Analyzing the antigen receptor repertoire in patients with mutations in genes responsible for the generation of the antigen receptor repertoire will give new insights into repertoire formation and selection. In this perspective we describe strategies and considerations for analysis of the naive and antigen selected B-cell repertoires in primary immunodeficiency (PID) patients with a focus on severe combined immunodeficiency (SCID) and common variable immunodeficiency (CVID).

Key words: V(D)J recombination, next generation sequencing, CVID, immunodeficiency, repertoire

GENERATION OF THE T-AND B-CELL REPERTOIRE

V(D)J recombination of immunoglobulin and T-cell receptor loci

The antigen receptor repertoire is defined as the total set of different B-cell (BR) or T-cell receptors (TRs). The loci encoding these receptors consist of multiple variable (V), diversity (D) and joining (J) genes, which can be recombined via V(D)J recombination to ensure the enormous diversity of the antigen receptors. V(D)J recombination starts with induction of DNA double strand breaks (DSBs) by the recombination activating gene products RAG1 and RAG2 between the coding element and the recombination signal sequence (Figure 1A).[1] The DNA ends that contain the recombination signal sequence, the so-called signal ends, are blunt DNA ends, which can be ligated directly to form the signal joint. The other ends (called the coding ends because they contain the coding sequence of the Ig or TR gene) are blocked by a covalent phosphodiester bond between the top and the bottom strand of the DNA. These DNA hairpins are recognized, processed and repaired by the non-homologous end joining pathway (NHEJ) (Figure 1A). First, the Ku70/80 heterodimer forms a ring around the DNA end that can migrate into the DNA after initial binding. Ku70/80 bound to a DNA end can then attract the DNA dependent protein kinase catalytic subunit (DNA-PK_{CS}), which acquires protein kinase activity upon DNA end binding. DNA-PK_{CS} autophosphorylation induces a conformational change in the DNA-bound complex of Ku70/80 and DNA-PK_{CS}, collectively called the DNA-PK complex.[2,3] After this conformational change, Artemis opens the DNA hairpins.[4,5] If the ends are compatible, they can be ligated by ligase IV, which forms a stable complex with XRCC4. XLF (XRCC4 like factor), which has also been called 'Cernunnos'.[6] Before ligation, non-templated (N) nucleotides can be inserted by TdT or deleted via exonuclease activity (Figure 1B).[7,8] In addition to the NHEJ components, several other factors are required to ensure efficient ligation of so-called 'difficult breaks' or 'complex DNA damage'. Extensive analysis of DSB repair kinetics revealed that these DSBs are mainly localized to heterochromatin and require opening of the closed chromatin structure in order to be repaired by NHEJ during the G1 phase of the cell cycle or HR in the G2 phase. Chromatin opening probably requires the initial phosphorylation of histone H2AX, the ATM kinase, the MRE11/RAD50/NBS1 complex and several enzymes necessary for ubiquitin addition near the DSB, including RNF8 and RNF168.[9-11]

Antigen receptor repertoire diversity

The total diversity of the unique TRs and BRs is the sum of combinational diversity based on the usage of different combinations of V, (D) and J genes and junctional diversity due to nucleotide deletions by exonuclease activity, non-templated (N) nucleotide insertions that are introduced by the enzyme terminal deoxynucleotidyl transferase (TdT) and the presence of palindromic (P) nucleotides that arise due to asymmetric hairpin opening by

Artemis (Figure 1B). In addition, combination of the different chains (TRα and TRβ, TRγ and TRδ, or heavy and light chains) also contributes to the overall diversity. The immunoglobulin heavy chain (IGH) locus consist of 38-46 functional VH, 25 DH and 6 JH genes resulting in a combinational diversity of >5.7x10^3. In combination with the 200 possible Igκ and 124 Igλ rearrangements, this results in a combinational diversity of >1.8 x10^6. The total diversity of the BR is estimated to result in a total BR repertoire of >10^{12}.

STRATEGIES FOR B-CELL RECEPTOR REPERTOIRE ANALYSIS

Naive versus antigen-selected B-cell receptor repertoire

The B-cell repertoire in peripheral blood can be divided into the naive and antigen-selected repertoire (Figure 1C). The naive repertoire, present in the transitional and naive mature B cells, has not encountered antigen and most closely resembles the initial repertoire formed by V(D)J recombination during precursor B-cell differentiation. The naive repertoire can be regarded as the end-stage product of V(D)J recombination. However, it should be

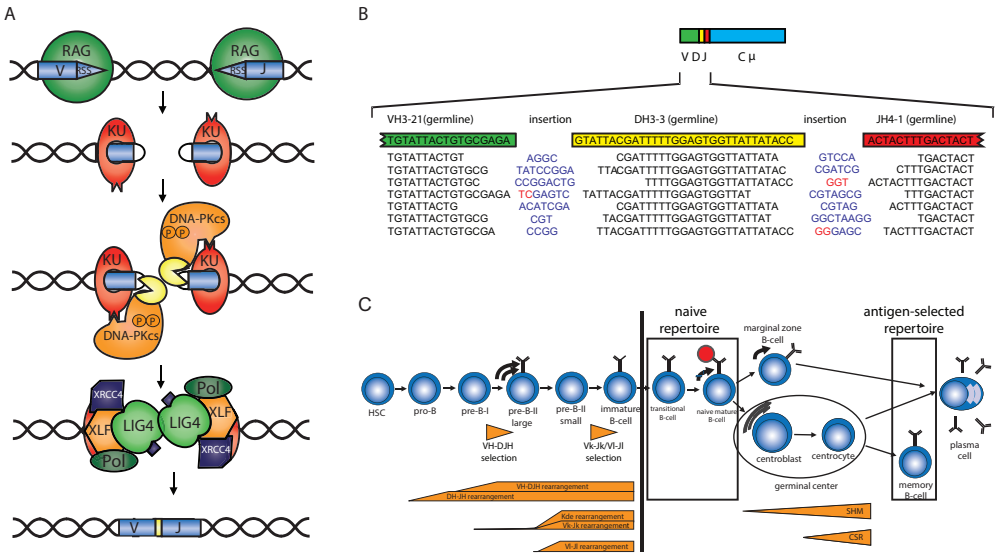


Figure 1. V(D)J recombination and B cell development
A. Schematic overview of V(D)J recombination. DNA double strand breaks are introduced by the RAG proteins, subsequently the DNA is processed and ligated by DNA repair proteins from the NHEJ pathway. **B.** Examples of VDJ junctions showing junctional diversity by nucleotides that are removed, non-templated (N) nucleotides (blue) that are added, or presence of palindromic (P) nucleotides (red). **C.** The B-cell repertoire in peripheral blood can be divided into the naive repertoire and the antigen-selected repertoire.

noted that by analyzing the naive repertoire only those B cells can be studied that reach the naive mature B-cell stage. For studying disturbances in the V(D)J recombination itself, it can be better to analyze complete V(D)J or incomplete DJ rearrangements in bone marrow (see below). Analysis of the naive repertoire is preferably performed on DNA or RNA obtained from sorted naive B-cells. Alternatively, IGM transcripts can be analyzed in total RNA from peripheral blood mononuclear cells, of which only the unmutated sequences are taken into account. Usually, sequences with 98% V region identity are considered to be unmutated, since PCR and sequence errors might occur.[12]

The antigen-selected repertoire is the repertoire of cells that encountered antigen, i.e. of memory B-cells. This repertoire is different from the naive repertoire, because it has undergone SHM with subsequent selection in the germinal center. The antigen selected repertoire can be analyzed in sorted memory B-cells or by sequencing of IGG and IGA transcripts from RNA isolated from peripheral blood mononuclear cells.

In summary, for BR analysis it is important to select and sort the correct B-cell population depending on the research question.

Qualitative characteristics of the repertoire

BR repertoire encloses a lot of information about different processes of B-cell development. The V, D and J usage and junction composition, defined as the number of N- and P- nucleotides, deletions and the length of the CDR3 region, provide information about the V(D)J recombination process.

Increased numbers of P-nucleotides[13] or deletions,[14] and decreased numbers of N-nucleotides are indications for a NHEJ defect.[15] In addition, skewing in the usage of V and J genes can be observed, as is the case in the TR alpha repertoire in both Xlf knockout mice and an XLF-deficient patient.[16] Vera *et al*. hypothesized that the reduced thymocyte lifespan does not allow the T cells to undergo multiple waves of VαJα rearrangements that can be needed for positive selection of the T cells. Finally, several characteristics like increased usage of certain auto-reactive VH genes, the charge of the CDR3 and increased length of the CDR3 have been associated with autoimmunity or impaired selection in patients with primary immunodeficiencies.[17-20] Patients with CD19 and CD40L deficiency lack selection against long CDR3 and VH4-34, which is known to encode intrinsically self-reactive cold agglutinin antibodies that recognize carbohydrate antigens on erythrocytes.[20] Similarly, a patient with RAG deficiency and autoimmunity has been described who lacked selection against these inherited autoreactive features and in addition had skewing of the CDR3 repertoire for rearrangements with a certain CDR3 length.[21]

Productive and unproductive IGH repertoire

The BR rearrangements can be amplified from DNA or from RNA. Rearrangements amplified from RNA are mostly functional (also called productive), which means that they code for a functional Ig protein. Amplification of rearrangements from DNA allows analysis of both productive and unproductive rearrangements, which have not been selected. The latter is interesting because analysis of productive and unproductive IGH rearrangements in naive B cells in controls shows that the productive rearrangements in naive B cells have a lower number of total N-nucleotides (13.8 vs 20.2nt) consequently leading to a shorter complementarity determining region (CDR) 3 length (Figure 2A). This might be explained by the fact that in bone marrow only B-cells are selected with a shorter CDR3 region. This indicates that analysis of unproductive rearrangements could give additional information about the V(D)J recombination process and selection.

The antigen-selected repertoire is mostly studied at RNA level, by amplifying *IGG*, *IGA* or *IGE* transcripts with primers located in or upstream of the V gene and in the constant gene. In addition to studying above mentioned characteristics of rearrangements, somatic hypermutations (SHM) can be studied.[20]

Diversity of the repertoire

For several years, conventional cloning and sequencing were the golden standard to study BR rearrangements. However, only small numbers (20-100) of rearrangements could be studied, which give a limited overview on the diversity of the total repertoire. Therefore, the current next generation sequencing (NGS) approaches, which enables to study thousands of rearrangements, give new opportunities for studying the diversity of the repertoire. Although the potential BR repertoire is estimated to be over 10^{12}, this number exceeds the total number of different B cells present in an individual. When analyzing the diversity of the repertoire by NGS, only a small fraction of the total B-cell population is analyzed. When studying the antigen receptor repertoire diversity, it is of great importance to take into account the number of B cells that was used to generate the repertoire data. If you start with 100,000 B cells, one cannot expect to find more than 100,000 different productive rearrangements. Since every B cell contains only one DNA copy of a productive rearrangement, the total number of productive rearrangement can be estimated when using a fixed DNA input of sorted B-cells. Every cell contains around 6pg of DNA, so 100ng DNA represent approximately 16,667 cells, which also equals the number of productive rearrangements that can be expected. In general every B cell contains only one productive rearrangement, however multiple B cells with the same rearrangements can be present in the B cell pool being studied.

When using RNA for the repertoire analysis it is difficult to estimate the number of cells, because every B cell contains multiple RNA copies of the same rearrangement, and plasma

cells contain more RNA copies of the rearrangement compared to other B-cell subsets. So, when multiple copies of the same rearrangement are found, these can be derived from the same B cell, two independent B cells, or they can be a technical duplication. One should therefore be careful interpreting the data, and for repertoire calculations it is best to only take the unique productive rearrangements in to account, unless randomly barcoded primers to identify individual B cells are used.[22]

In literature different methods for diversity calculations are used. Species Richness, and Shannon entropy are frequently used methods.[23, 24] The Species Richness takes into account the number of different rearrangements in a sample, whereas the Shannon entropy also takes the frequency of the rearrangement into account.[23] The latter methods are usually performed on rearrangements derived from an individual PCR amplification. Boyd *et al.* used a slightly different method in which occurrences of rearrangements between six individual PCR within the same individual was measured.[25] These occurrences, also called coincidences, could only be derived from clonally related B cells. Immunocompetent individuals should have little coincidences since they have a diverse repertoire, while immunocompromised individuals are likely to have more coincidences since they have a more restricted repertoire.

B-CELL RECEPTOR REPERTOIRE ANALYSIS IN SCID

In patients with primary immunodeficiencies antigen receptor repertoire formation or selection can be disturbed, resulting in restricted antigen receptor repertoire diversity. Depending on the genetic defect this can result in severe immunodeficiency as is the case in SCID patients with defects in RAG1/RAG2 or in components of the NHEJ pathway with severely impaired V(D)J recombination.[6, 14, 26-29] These genetic defects not only result in a quantitative V(D)J recombination defect, but also in a qualitative defect. Because of the qualitative V(D)J recombination defects, there are no peripheral B-cells or the number of B-cells is too low for reliable analysis. In this case the repertoire can be studied by analyzing incomplete or complete BR rearrangements in bone marrow derived precursor B cells. NHEJ defects leads to aberrant formation of V(D)J junctions and greatly impact junctional diversity, which implies that NHEJ defects cause a quantitative and qualitative antigen receptor repertoire defect. For example, mutations in *Artemis* and *DNA-PKcs*,[13, 29] which affect hairpin opening, result in a significant increase in the number of P-nucleotides, whereas defects in *XRCC4* result in significantly reduced numbers of N-nucleotide insertions.[15]

Also other genetic defects, like ATM and NBS deficiency, result in reduced diversity of the naive B cell repertoire. These proteins are not directly involved in the V(D)J recombination process itself, but are important for sensing of the DNA double strand breaks and for keeping

the two DNA ends together during V(D)J recombination. As the result of less efficient V(D)J recombination, patients have reduced numbers of naive B cells in the periphery, which can in addition show an increased proliferation history which results in even more restriction of the repertoire. This is what we have shown in patients with mutations in *ATM* causing Ataxia Telangiectasia (AT), which is involved in DSB sensing and juxtaposition of DNA ends, impair antigen receptor repertoire diversity.[30] Similar observations have been done in patients with the Nijmegen Breakage Syndrome (NBS).[31]

B-CELL RECEPTOR REPERTOIRE ANALYSIS IN CVID

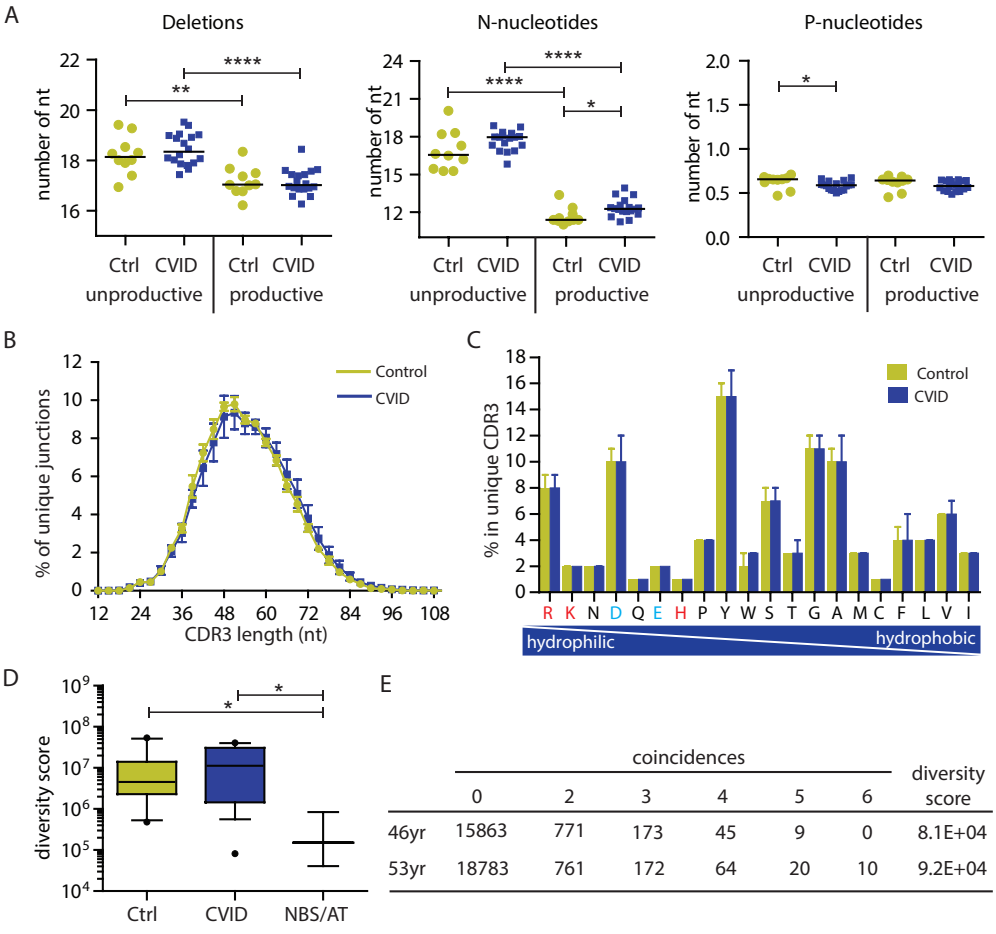
No qualitative defect in the naive IGH repertoire of CVID patients

CVID is the most common primary antibody deficiency characterized by hypogammaglobulinemia and poor response to vaccination resulting in infections and in some patients to non-infectious complications including autoimmunity, lymphoproliferative disease, malignancies and granulomas. In the majority of patients (95%), the genetic defect is unknown, but several studies have demonstrated defects in B-cell differentiation. Therefore, CVID patients could have a restricted BR repertoire underlying the disease.

Driessen *et al*. showed that a subgroup of CVID patients also have a reduced number of naive B cells, with an increased proliferation history, like the AT and NBS patients.[32] Therefore, we hypothesized that a subgroup of patients with CVID might have a reduction in the diversity of the naive IGH repertoire, possibly caused by less efficient V(D)J recombination. We studied the naive IGH repertoire in 18 patients described by Driessen *et al*.[32] and 10 healthy controls. We sorted naive B cells and amplified the VH-JH junctions in six independent PCRs using 100ng DNA per PCR. For the analysis of the qualitative parameters we combined the rearrangements of all six individual PCRs and selected the productive or unproductive unique rearrangements based on the V and J gene and the amino acid sequence of the CDR3.

All of the qualitative parameters we studied were similar to healthy controls in this group of CVID patients. The composition of the junctions in both productive and unproductive rearrangements was normal (Figure 2A). The only differences were a slightly higher number of N-nucleotides (average difference of 0.7 nucleotides) in productive rearrangements, and a very small decrease (on average 0.04 nucleotides) in the number of P-nucleotides in unproductive rearrangements. However, this did not result in a shorter CDR3 length (Figure 2B). Similar to controls, the productive rearrangements had significantly less deletions and N-nucleotide insertions, indicating selection for shorter CDR3 length. Furthermore, the CDR3 length distribution and composition of hydrophilic

and hydrophobic amino acids was also similar to controls (Figure 2B and 2C). These data suggests that these CVID patients do not have a qualitative defect in the naive repertoire.



E

	coincidences						diversity score
	0	2	3	4	5	6	
46yr	15863	771	173	45	9	0	8.1E+04
53yr	18783	761	172	64	20	10	9.2E+04

Figure 2. Naive B-cell repertoire in control and CVID patients
The naive B-cell repertoire was measured in 10 controls (**C**) and 18 CVID patients, resulting in total 293,216 unique productive rearrangements for control and 539,220 for CVID, and 127,261 unique unproductive rearrangements for control and 305,402 for CVID. **A.** Junction characteristics of CVID patients are similar to controls. Average number of total number of deletions, N-nucleotides and P-nucleotides are indicted per patient. **B.** Similarly, the CDR3 length distribution (mean with SEM) of IGH rearrangements is comparable to controls. In addition, the frequency of amino acids in the CDR3 (median with range) is also comparable. The positively charged amino acids are indicated in red and the negatively charged in blue. **D.** The diversity of the naive B-cell repertoire in CVID patients is comparable to controls, however one patient has a very restricted repertoire similar to patients with Nijmegen breakage syndrome (NBS) and Ataxia Telangiectasia (AT). Data is shown in box and whiskers (10-90 percentile). **E.** The repertoire of this patient remains very restricted over time. * p<0.05, ** p<0.01, *** p<0.001,**** p<0.0001.

Diversity of the naive IGH repertoire is normal in most CVID patients

To measure the diversity of the naive IGH repertoire we used the method proposed by Boyd *et al*.[25] Based on the occurrence of rearrangements between six independent PCRs the diversity of the repertoire can be calculated. We expressed the diversity of the repertoire in a diversity score, in which a low value indicate a restricted repertoire and a high value represents a more diverse repertoire. Both in the control as in the CVID patients there is some spread in the diversity of the repertoire (Figure 2D). However, one CVID patient clearly had a more restricted repertoire that the controls. This repertoire was as restricted as patients with NBS and AT deficiency (Figure 2D). To assess if this reduction in the repertoire is stable over time, the repertoire was analyzed at a second time point seven years later (Figure 2E). At both 46 and 53yr of age this patient had a very restricted repertoire. At the second time point there were even 10 rearrangements present in all individual PCRs, indicating that in the small sample of blood that we took at least six B cells had the same IGH rearrangement. Interestingly, this patient has a family history of breast cancer indicative for a possible DNA repair defect. These data show that patients with a restricted repertoire can be identified using this method, however, most CVID patients have a very diverse naive repertoire. Based on the B-cell patterns identified by Driessen *et al*, it is expected that most of the CVID patients will have problems after the naive B-cell stage. It will therefore we very interesting to also study the antigen-selected repertoire in patients with CVID.

FUTURE PERSPECTIVES

Developments in NGS give possibilities to study the antigen receptor repertoire of patients at a very detailed level than ever before. Different strategies can be followed to address many research questions related to the pathophysiology of PID. In this perspective, we focused on SCID and CVID, but it has already been proven valuable for many other PIDs.[33] The next challenge will be linking antigen receptor repertoire data to antigen reactivity and specificity, which might in future be linked to specific clinical features.

METHODS

Repertoire analysis using next generation sequencing

Naive B cells were sorted from peripheral blood from 18 CVID patients and 10 healthy controls. DNA was isolated using direct lysis and IGH rearrangements were amplified and sequenced using Roche 454 sequencing as previously described.[30] In short IGH

rearrangements were amplified from in a multiplex PCR using the forward VH1-6 FR1 and reverse JH consensus BIOMED-2 primers.[34] PCR products were purified by gel extraction (Qiagen, Valencia, CA) and Agencourt AMPure XP beads (Beckman Coulter). Subsequently, the PCR concentration was measured using the Quant-it Picogreen dsDNA assay (Invitrogen, Carlsbad, CA). The purified PCR products were sequenced on the 454 GS junior instrument according the manufacturer's recommendations. Sequences were demultiplexed based on their multiplex identifier sequence and trimmed using the IGGalaxy tool.[35] Fasta files were uploaded in IMGT/High-V-Quest,[36] and subsequently the IMGT output files were analyzed in the IGGalaxy tool.[35] Information on junction characteristics, CDR3 length and composition were extracted from the data provided by IMGT. The repertoire diversity score was calculated by dividing 1 to the clonality score, which is given by the IGGalaxy tool and is based on the calculation of Boyd *et al*.[25]

Statistics

Significance differences were calculated using the two-tailed Mann-Whitney test in the GraphPad Prism program (GraphPad Software, Inc.).

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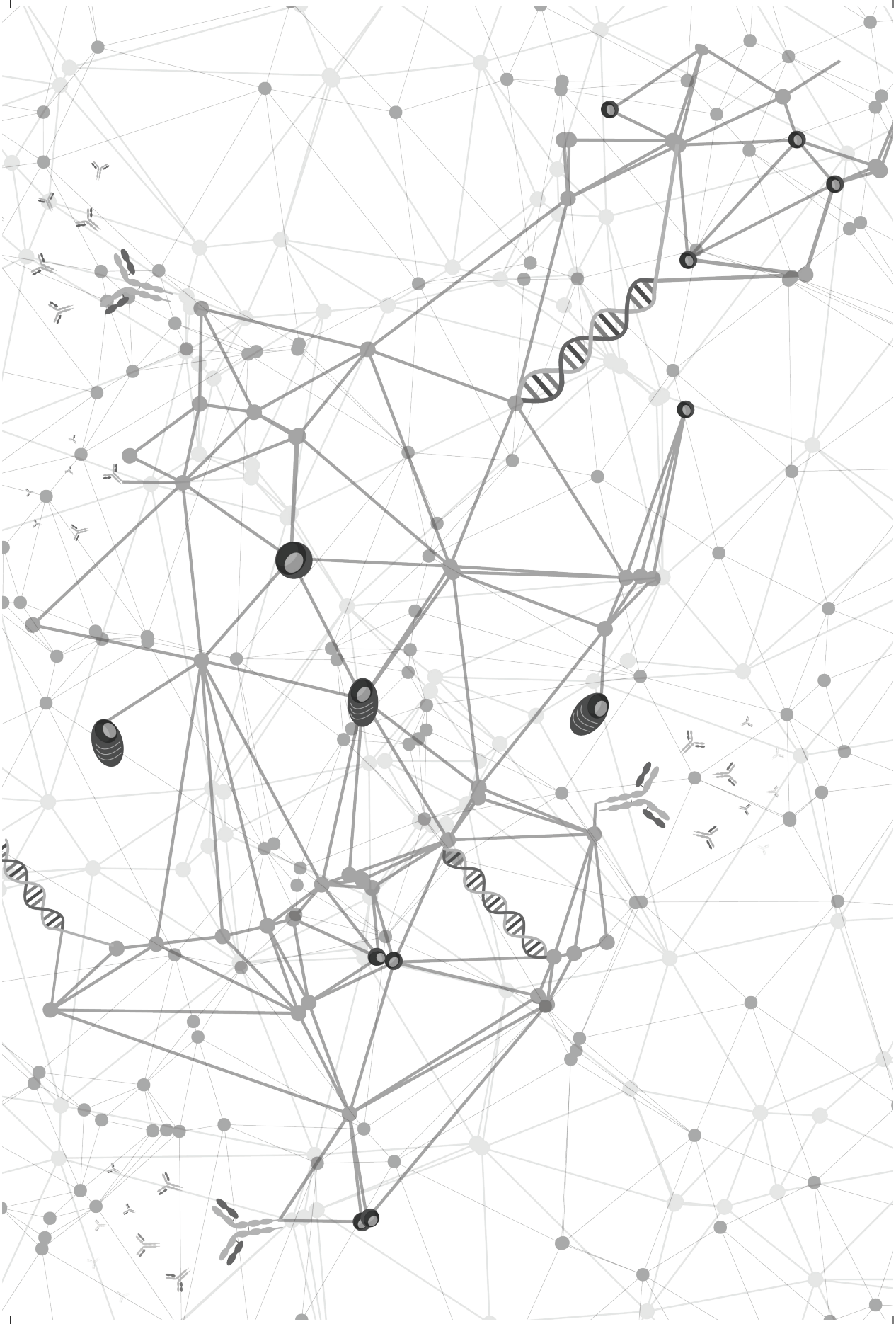
REFERENCES

1. McBlane, J.F., et al., *Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps.* Cell, 1995. **83**(3): p. 387-95.
2. Smith, G.C. and S.P. Jackson, *The DNA-dependent protein kinase.* Genes Dev, 1999. **13**(8): p. 916-34.
3. Weterings, E. and D.C. van Gent, *The mechanism of non-homologous end-joining: a synopsis of synapsis.* DNA Repair (Amst), 2004. **3**(11): p. 1425-35.
4. Douglas, P., et al., *Identification of in vitro and in vivo phosphorylation sites in the catalytic subunit of the DNA-dependent protein kinase.* Biochem J, 2002. **368**(Pt 1): p. 243-51.
5. Ma, Y., et al., *Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination.* Cell, 2002. **108**(6): p. 781-94.
6. Ahnesorg, P., P. Smith, and S.P. Jackson, *XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining.* Cell, 2006. **124**(2): p. 301-13.

7. Kallenbach, S., et al., *Three lymphoid-specific factors account for all junctional diversity characteristic of somatic assembly of T-cell receptor and immunoglobulin genes.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 2799-803.
8. Landau, N.R., et al., *Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector.* Mol Cell Biol, 1987. **7**(9): p. 3237-43.
9. Burma, S., et al., *ATM phosphorylates histone H2AX in response to DNA double-strand breaks.* J Biol Chem, 2001. **276**(45): p. 42462-7.
10. Mailand, N., et al., *RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins.* Cell, 2007. **131**(5): p. 887-900.
11. Stucki, M., et al., *MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks.* Cell, 2005. **123**(7): p. 1213-26.
12. Ghia, P., et al., *ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia.* Leukemia, 2007. **21**(1): p. 1-3.
13. van der Burg, M., et al., *Defective Artemis nuclease is characterized by coding joints with microhomology in long palindromic-nucleotide stretches.* Eur J Immunol, 2007. **37**(12): p. 3522-8.
14. van der Burg, M., et al., *A new type of radiosensitive T-B-NK+ severe combined immunodeficiency caused by a LIG4 mutation.* J Clin Invest, 2006. **116**(1): p. 137-45.
15. Murray, J.E., et al., *Mutations in the NHEJ Component XRCC4 Cause Primordial Dwarfism.* Am J Hum Genet, 2015. **96**(3): p. 412-24.
16. Vera, G., et al., *Cernunnos deficiency reduces thymocyte life span and alters the T cell repertoire in mice and humans.* Mol Cell Biol, 2013. **33**(4): p. 701-11.
17. Pascual, V., et al., *Nucleotide sequence analysis of the V regions of two IgM cold agglutinins. Evidence that the VH4-21 gene segment is responsible for the major cross-reactive idiotype.* J Immunol, 1991. **146**(12): p. 4385-91.
18. Silberstein, L.E., et al., *Variable region gene analysis of pathologic human autoantibodies to the related i and I red blood cell antigens.* Blood, 1991. **78**(9): p. 2372-86.
19. Meyers, G., et al., *Activation-induced cytidine deaminase (AID) is required for B-cell tolerance in humans.* Proc Natl Acad Sci U S A, 2011. **108**(28): p. 11554-9.
20. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation.* J Allergy Clin Immunol, 2014. **134**(1): p. 135-44.
21. IJspeert, H., et al., *Similar recombination-activating gene (RAG) mutations result in similar immunobiological effects but in different clinical phenotypes.* J Allergy Clin Immunol, 2014.
22. Busse, C.E., et al., *Single-cell based high-throughput sequencing of full-length immunoglobulin heavy and light chain genes.* Eur J Immunol, 2014. **44**(2): p. 597-603.
23. Michaeli, M., et al., *Immunoglobulin gene repertoire diversification and selection in the stomach - from gastritis to gastric lymphomas.* Front Immunol, 2014. **5**: p. 264.
24. Yu, X., et al., *Human syndromes of immunodeficiency and dysregulation are characterized by distinct defects in T-cell receptor repertoire development.* J Allergy Clin Immunol, 2014. **133**(4): p. 1109-15.

25. Boyd, S.D., et al., *Measurement and clinical monitoring of human lymphocyte clonality by massively parallel VDJ pyrosequencing.* Sci Transl Med, 2009. **1**(12): p. 12ra23.
26. Buck, D., et al., *Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly.* Cell, 2006. **124**(2): p. 287-99.
27. Moshous, D., et al., *Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency.* Cell, 2001. **105**(2): p. 177-86.
28. Schwarz, K., et al., *RAG mutations in human B cell-negative SCID.* Science, 1996. **274**(5284): p. 97-9.
29. van der Burg, M., et al., *A DNA-PKcs mutation in a radiosensitive T-B- SCID patient inhibits Artemis activation and nonhomologous end-joining.* J Clin Invest, 2009. **119**(1): p. 91-8.
30. Driessen, G.J., et al., *Antibody deficiency in patients with ataxia telangiectasia is caused by disturbed B- and T-cell homeostasis and reduced immune repertoire diversity.* J Allergy Clin Immunol, 2013. **131**(5): p. 1367-1375 e9.
31. van der Burg, M., et al., *Loss of juxtaposition of RAG-induced immunoglobulin DNA ends is implicated in the precursor B-cell differentiation defect in NBS patients.* Blood, 2010. **115**(23): p. 4770-7.
32. Driessen, G.J., et al., *B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency.* Blood, 2011. **118**(26): p. 6814-23.
33. Picard, C. and A. Fischer, *Contribution of high-throughput DNA sequencing to the study of primary immunodeficiencies.* Eur J Immunol, 2014. **44**(10): p. 2854-61.
34. van Dongen, J.J., et al., *Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936.* Leukemia, 2003. **17**(12): p. 2257-317.
35. Moorhouse, M.J., et al., *ImmunoGlobulin galaxy (IGGalaxy) for simple determination and quantitation of immunoglobulin heavy chain rearrangements from NGS.* BMC Immunol, 2014. **15**(1): p. 59.
36. Alamyar, E., et al., *IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS.* Methods Mol Biol, 2012. **882**: p. 569-604.



Chapter 2.3

Precursor B-cell development in bone marrow of Good Syndrome patients

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ABSTRACT

Background and aims: Good syndrome (GS) is a rare immunodeficiency presenting with thymoma and antibody deficiency. Severe recurrent infections and autoimmune manifestations are common in GS patients. Although GS pathogenesis is largely unclear, the severe reduction of peripheral B cells suggests impaired differentiation of B cell precursors (BCP) in bone marrow (BM).

Methods: We analyzed BM samples from 6 GS patients, 15 healthy donors (HD) and 19 genetically confirmed agammaglobulinemia patients. BCP differentiation in different stages (ProB, PreB-I, PreB-II, Immature) was studied using flow cytometry.

Results: GS patients showed a severe reduction of BCP ($CD22^+$) but highly increased T cells numbers ($CD3^+$). GS patients had a significantly higher frequency of ProB cells and PreB-I cells compared to HD. PreB-II cells and Immature cells were significantly decreased in GS patients. Agammaglobulimia patients showed a relative expansion of ProB and PreB-I cells compared to HD, with a significant reduction later in differentiation at the pre-BII and immature stages. GS and agammaglobulinemia patients showed a different type of arrest in BCP development. GS patients generally showed a relative increase in ProB cells, together with a decrease in PreB-I cells compared to agammaglobulinemia patients.

Conclusions: The absence of peripheral blood B cells in GS patients is accompanied by very low numbers of BCP in the BM of these patients. The BCP that are present show an arrest in differentiation mainly after ProB stage, which is different from patients with monogenic agammaglobulinemia, in whom a block is found after PreB-I stage, which is linked to the absence of pre-B cell receptor signaling.

CAPSULE SUMMARY

Good syndrome is a rare immunodeficiency presenting with thymoma, hypogammaglobulinemia and almost absent B cells. To investigate the origin of the B-cell lymphopenia in these patients, we studied B cell differentiation in the bone marrow of Good syndrome patients. We found very low numbers of (prescursor)B cells in bone marow of Good syndrome patients which showed a differentiation arrest after the pro-B-cell stage, which is different from other patients with agammaglobulinemia with a defect in preB-cell receptor signaling.

TO THE EDITOR

Good syndrome (GS)[1] is a rare immunodeficiency presenting with thymoma, consisting of neoplastic thymic epithelial cells and non-neoplastic maturing thymocytes, hypogammaglobulinemia and almost absent B cells.[2-3] Both sexes are equally affected and age of onset is between 40 and 70 years. Patients suffer from severe recurrent infections with mainly encapsulated bacteria and opportunistic pathogens.[1, 3] Several reports indicate a poor prognosis and survival in GS patients due to the high susceptibility for severe bacterial infections and because of autoimmunity.[3]

Although GS pathogenesis is largely unclear, the severe reduction of peripheral B cells suggests impaired differentiation of B cell precursors (BCP) in bone marrow (BM). In this study we analyzed the clinical and immunological phenotype of 9 GS patients . We included BM samples from 6 GS patients and clinical data for 3 additional GS patients. Additionally, BM samples from 15 healthy donors (HD) (age >10 years) and 19 patients with agammaglobulinemia (X-linked agammaglobulinemia, μ-chain-, CD79- or BLNK deficiency) were included. These agammaglobulinemia patients all have a defect before the pre-BII cell stage because of defects pre- B-cell receptor signalling. This study was performed according to the guidelines of the Medical Ethics Committee of the Erasmus MC and was approved by the regional ethical committee in Stockholm.

Clinical and immunological characteristics of the GS patients are shown in Table 1. All 9 GS patients (4 females, 5 males) had undergone thymectomy and 8 had histologically confirmed thymoma. The predominant infections were similar to what was previously described in GS patients.[2-4] Those comprise mainly respiratory tract infections and in some cases opportunistic infections were recorded. All patients were treated with immunoglobulin substitution therapy. One patient suffered from autoimmune pure red cell aplasia two years after thymectomy.

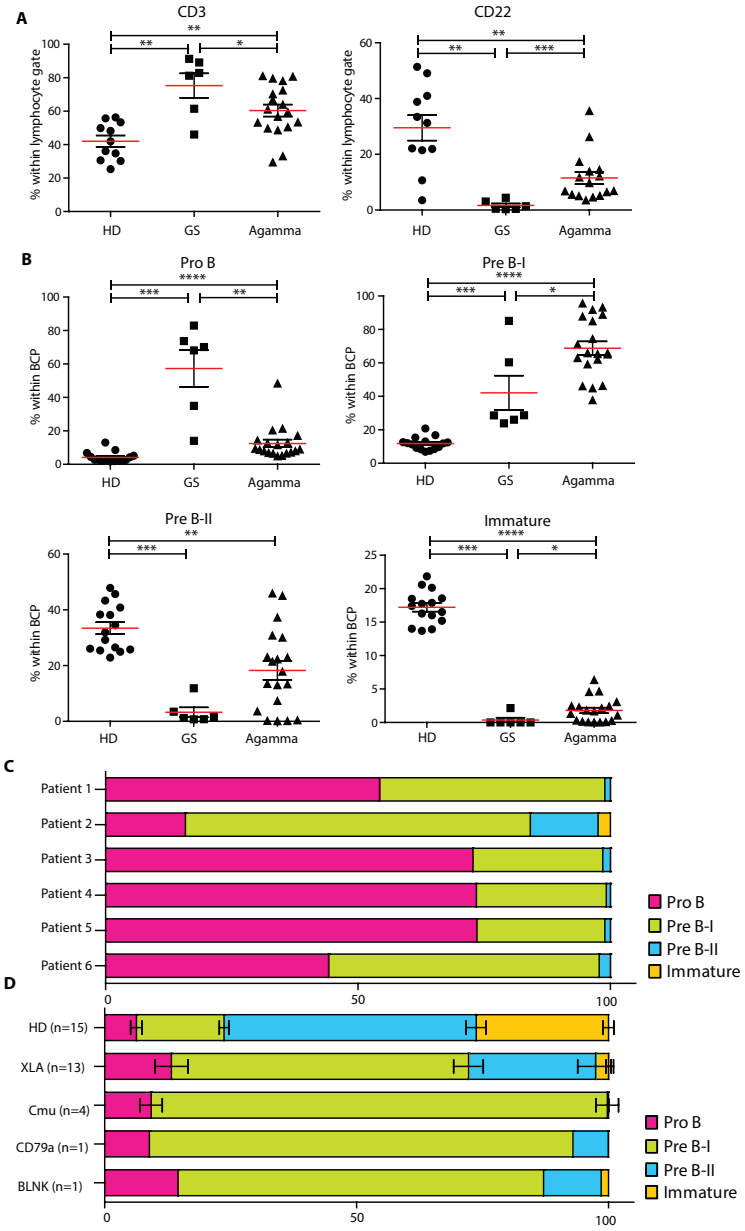


Figure 1.
Immunophenotyping of BCP in BM of Healthy donors (HD) (n=15), Good syndrome (GS) (n=6) and agammaglobulinemia (n=19; for subtypes see D panel) patients. **A**. Relative frequencies of $CD3^+$ T cells and $CD22^+$ B cells in BM samples. $CD22^+$ B cells were CD3/D13/CD33/CD36/CD16/$CD56^-$). **B**. Relative frequencies of BCP populations: proB ($CD19^-$cylgμ$^-$), PreB-I ($CD19^+$ cylgμ$^-$), PreB-II ($CD19^+$ cylgμ$^+$$IgM^-$) and Immature B cells ($CD19^+$ vylgμ$^+$ IgM^+ IgD^- $CD10^+$ $CD38^+$). **C**. Subset distribution of BCP subsets in BM for all individual GS patients. **D**. Subset distribution of BCP subsets in BM for HD (pooled) and agammaglobulinemia patients divided on genetic diagnosis.

Concurrent peripheral blood analysis (Supplementary Table 1) in GS patients revealed a severe reduction of peripheral blood B cells, 5 patients had a mild reduction in T cells counts, 4 patients had reduced levels of $CD4^+$ T cells and 3 patients had lower $CD8^+$ counts (Supplementary Table 1).

BCP maturation stages in BM samples were studied using flow cytometry, as previously described.[5] Only in 6 out of 9 patients precursor B-cells were present. The relative sizes of 4 different populations (ProB, PreB-I, PreB-II, Immature) were expressed as percentages of total BCP. Mature B cells were excluded from the calculations. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, Ca); Mann-Whitney test was used.

GS patients showed a severe reduction of BCP ($CD22^+$) but highly increased frequency of T cells ($CD3^+$) compared to HD (P=0.0015; P=0.0057 respectively) and agammaglobulinemia patients (P=0.0006; P=0.0492 respectively) (Figure 1 A).

GS patient had a significantly higher frequency of ProB cells ($CD19^-$ $CD34^+$ TdT^+ $cyIg\mu^-$) and PreB-I cells ($CD19^+$ TdT^+ $CD34^+$ $cyIg\mu^-$) compared to HD (P=0.0005 and P=0.0005 respectively) (Figure 1B). However, relative frequencies of PreB-II cells ($CD19^+$ $CD34^-$ TdT^- $cyIg\mu^+$) and Immature cells ($CD19^+$ $cyIg\mu^+$ IgM^+ IgD^-) were significantly decreased in GS patients compared to HD (P=0.0005 and P=0.0005 respectively). Thus, there is a significantly reduced number of BCP in the BM of GS patients and the BCP that are present are mainly ProB cells and PreB-I cells.

The composition of the BCP compartment in GS patients differs from the agammaglobulinemia patients (Figure 1B). Agammaglobulimia patients showed a relative expansion of ProB and PreB-I cells compared to HD (P<0.0001 and P <0.0001 respectively), combined with a significant reduction later in differentiation (Pre B-II and Immature cells P=0.0016 and P <0.0001 respectively) due to a defect in preB-cell receptor signaling, which precludes further differentiation.

GS and agammaglobulinemia patients showed a different type of arrest in BCP differentiation (Figure 1 C, D). In GS patients there was a trend towards a relative increase in mainly the ProB cells (P=0.0013), together with a decrease in PreB-I cells compared to agammaglobulinemia patients (P=0.0170). Also the immature B cell subsets are significantly reduced in BM from GS patients (P=0.0111). The arrest in GS patients seems to occur after the ProB stage, whereas in agammaglobulinemia patients a differentiation arrest occurs after the Pre B-I stage.

GS has been compared to common variable immunodeficiency (CVID) previously. In both diseases BAFF-R and TACI mutations have been identified.[6] To our knowledge, this is the first comparison to patients with monogenic causes of agammaglobulinemia who also have a block in BCP development in bone marrow.

Table 1.
Clinical and immunological characteristics of the Good Syndrome patients. Thymoma pathology was according to WHO Classification of Thymic Epithelial Tumors (1999).

Patient	Age/ sex	Pathology Thymoma	IgG levels (g/l) at diagnosis	IgA	IgM	Infections	Therapy/ Thymectomy	Other morbidity/ Specific symptoms
ID473 Patient 1	62/M	Type B2	4.8	0.14	<0.30	Recurrent airway infections: haemophilus	Valaciclovir Chemotherapy: Cisplatinum/ Etoposide IVIg [a] / Yes	Recurrent Herpes simplex Loss of CD36 on the monocytes Mannan-binding lectin deficiency Vena cava superior syndrome
ID550 Patient 2	65/M	Type AB	5.6	1.63	0,44	Recurrent airway infections: haemophilus	Radiation IVIg/ Yes	
ID0559 Patient 3	67/F	Type AB	0.24	<0.06	0.05	Rarely airway infection	SCIg [b]/ Yes	Chronic skin/mouth rash: GVH due to thymoma or lichen ruber. Kidney failure.
ID0823 Patient 4	60/M	Type AB	2.7	<0.10	<0.10	Bronchitis 2-3 times/year Skin mycosis	SCIg/ Yes	Bronchiectasies. Pure red cell anemia for two years after thymectomy
ID0824 Patient 5	51/M	PAD not available	1.6	0.10	<0.04	Bronchitis/pneumonia frequently. Skin mycosis	SCIg/ Yes	Bronchiectasies.
ID0825 Patient 6	56/F	Type AB	4.4	0.5	<0.04	Pneumonias before SCIg. Then rarely infections.	SCIg/ Yes	Mastectomy due cancer mam. Bleeding disorder (mild). Bronchiectasies (minor).

Table 1.
Continued

Patient	Age/ sex	Pathology Thymoma	IgG levels (g/l) at diagnosis	IgA	IgM	Infections	Therapy/ Thymectomy	Other morbidity/ Specific symptoms
ID0826 Patient 7	73/F	Type AB	0.33	0.03	<0.04	Upper and lower airway infections before SCIg. Reduced after treatment.	SCIg/ Yes	Chronic bronchitis. Myasthenia gravis that disappeared after thymectomy.
ID307 Patient 8	46/F	Unknown	1.3	<0.05	<0.1	Recurrent upper airway	IVIg	Increased growth hormone Acromegalic appearance
ID327 Patient 9	71/M	Type A	2.0	0.50	0.31	CMV Pneumocystis carinii Persistent Noro virus Pneumonia: haemophilus	IVIg/ Yes	Skin carcinoma, basalioma, hepatosplenomegaly, liver fibrosis, lymphadenopathy

[a] IVIg, intravenous immunoglobulin substitution therapy; [b]SCIg, subcutaneous immunoglobulin substitution therapy

Previous studies on the T cell compartment of GS patients revealed frequent $CD4^+$ lymphopenia,[4] a case with increasing γδ T cells[7] and a case presenting $CD8^+$ T cell large granular lymphocyte leukemia with an exhausted phenotype.[8] The clonal expansion of $CD8^+$ T lymphocytes in bone marrow had been described and associated with B-lymphopoiesis deficiency. The authors hypothesized this was due to a $CD8^+$ T cell-driven immune response against BCP.[9] Other hypotheses have been proposed to explain GS pathogenicity, such autoimmune destruction of B cells mediated by autoantibodies.[4] Pathogenic antibodies are commonly present in GS patients, for example anti-acetyl choline receptor antibodies causing myasthenia gravis.[2] The aberrant thymic microenvironment may predispose for self-activation of thymocytes against autoantigens, and thus evoke a T-cell response against B cells. Additionally, murine models have shown a role for interferon-like cytokines such as Limitin, which is produced by bone marrow stromal cells. This was hypothesized to be involved in BCP differentiation, promoting cell cycle arrest upon impaired differentiation.[4] Aberrancies in BM stromal environment could cause a maturation arrest of the BCP.

In conclusion, this study demonstrates that the absence of peripheral blood B cells in GS patients is reflected in very low numbers of BCP in the bone marrow of these patients. The BCP that are present show an arrest in differentiation mainly after ProB stage, which is different from a selected series of patients with defined monogenic agammaglobulinemia, in whom a block is found after PreB-I stage, which is linked to the absence of pre-B cell receptor signaling.

REFERENCES

1. Good, R.A., et al., *Thymic tumor and acquired agammaglobulinemia: a clinical and experimental study of the immune response.* Surgery, 1956. **40**(6): p. 1010-7.
2. Kelesidis, T. and O. Yang, *Good's syndrome remains a mystery after 55 years: A systematic review of the scientific evidence.* Clin Immunol, 2010. **135**(3): p. 347-63.
3. Jansen, A., et al., *Prognosis of Good syndrome: mortality and morbidity of thymoma associated immunodeficiency in perspective.* Clin Immunol, 2016. **171**: p. 12-17.
4. Agarwal, S. and C. Cunningham-Rundles, *Thymoma and immunodeficiency (Good syndrome): a report of 2 unusual cases and review of the literature.* Ann Allergy Asthma Immunol, 2007. **98**(2): p. 185-90.
5. van der Burg, M., et al., *New frontiers of primary antibody deficiencies.* Cell Mol Life Sci, 2012. **69**(1): p. 59-73.
6. Lougaris, V., et al., *BAFF-R mutations in Good's syndrome.* Clin Immunol, 2014. **153**(1): p. 91-3.
7. Tadic, D., et al., *Good's syndrome with increasing gammadelta T-lymphocyte subpopulation: A case report.* Vojnosanit Pregl, 2015. **72**(11): p. 1039-43.

8. Caperton, C., S. Agrawal, and S. Gupta, *Good syndrome presenting with CD8(+) T-Cell large granular lymphocyte leukemia.* Oncotarget, 2015. **6**(34): p. 36577-86.
9. Masci, A.M., et al., *Clonal expansion of CD8+ BV8 T lymphocytes in bone marrow characterizes thymoma-associated B lymphopenia.* Blood, 2003. **101**(8): p. 3106-8.

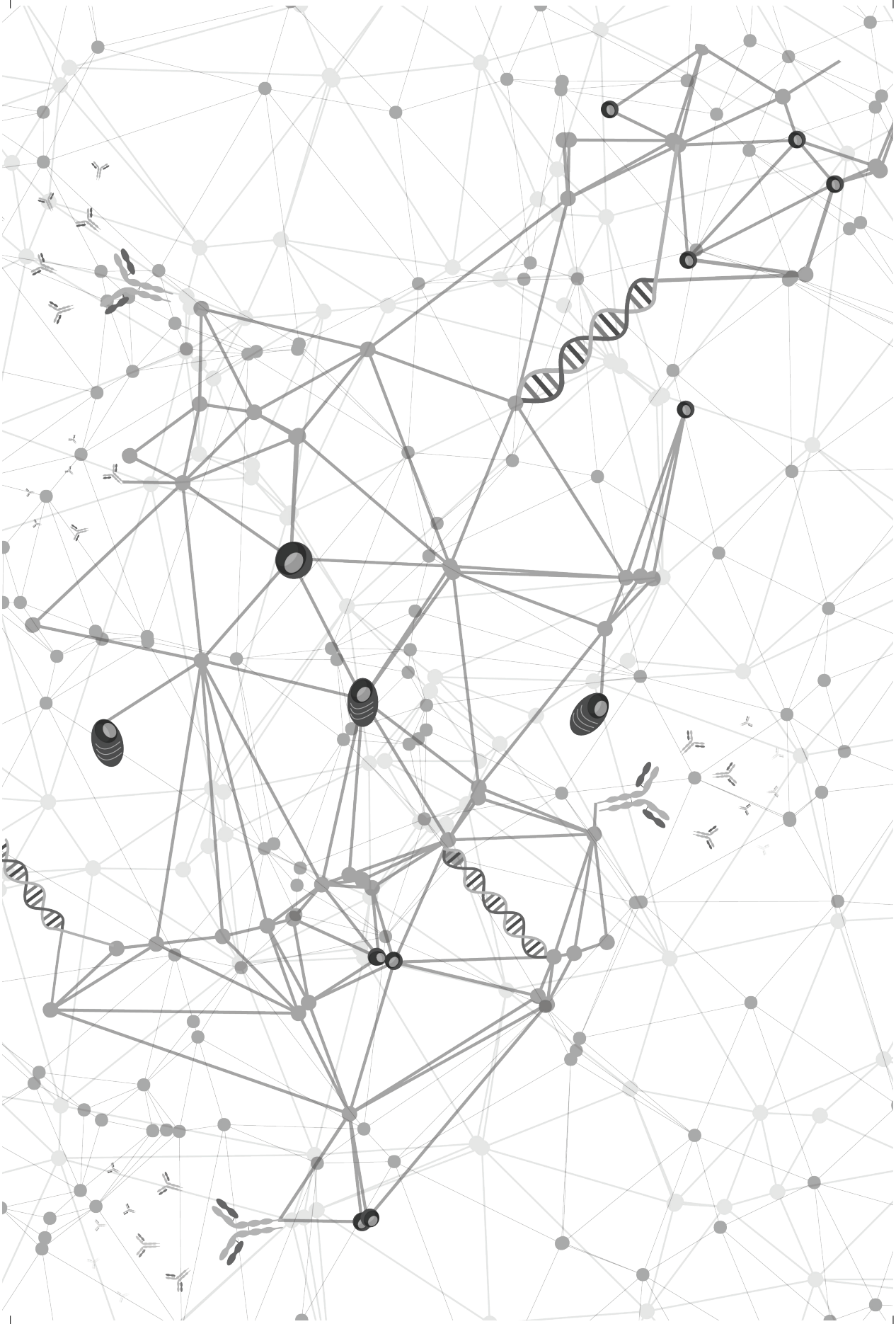
Supplementary Table 1. Phenotypic analysis of peripheral blood in Good syndrome patients.

	ID473 Patient 1	ID550 Patient 2	ID559 Patient 3	ID823 Patient 4	ID824 Patient 5	ID825 Patient 6	ID826 Patient 7	ID307 Patient 8	ID327 Patient 9	Normal value adults
White blood cell count	12.8	7.00	3.50	3.00	3.40	8.70	11.60	4.80	2.70	
Lymphocytes	Nd	1.14	1.09	1.14	0.51	2.69	4.52	1.18	0.80	1.0-2.8
CD19$^+$ B cells	0.00	0.01	0.00	<0.01	<0.01	<0.01	<0.01	0.10	0.07	0.1-0.5
- Igκ/Igλ ratio	Nd	1.28	Nd	0.50	Nd	Nd	Nd	0.90	1.47	
- IgM$^+$IgD$^-$ [a]	Nd	3.2	Nd	Nd	Nd	Nd	Nd	3.20	3.20	
- IgM$^+$IgD$^+$ [a]	Nd	91.4	Nd	Nd	Nd	Nd	Nd	93.90	96.60	
- CD21$^-$ [a]	Nd	26.2	Nd	Nd	Nd	Nd	Nd	13.10	67.70	
Immature B cells [a] (IgM$^+$/IgD$^-$)	Nd	3.2	Nd	Nd	Nd	Nd	Nd	3.20	3.20	
Transitional B cells [a] (CD38^{high}/CD24^{high})	Nd	0	Nd	Nd	Nd	Nd	Nd	0.80	0.20	3-50
Naïve mature B cells [a] (IgD$^+$/CD27$^-$)	Nd	91	Nd	Nd	Nd	Nd	Nd	64.40	83.20	57-447
Marginal Zone/ Natural effector B cells [a] (IgD$^+$/CD27$^+$)	Nd	1.8	Nd	Nd	Nd	Nd	Nd	31.50	15.40	'9-88
Switched Memory B cells [a] (IgD$^-$/CD27$^+$)	Nd	2.2	Nd	Nd	Nd	Nd	Nd	2.90	0.60	13-122
IgG [a]	Nd	0.8	Nd	Nd	Nd	Nd	Nd	1.20	0.40	
IgA a	Nd	1.4	Nd	Nd	Nd	Nd	Nd	1.20	0.00	
CD3$^+$ T cells	6.77	0.55	0.61	0.96	0.34	2.27	3.86	0.61	0.67	0.7-2.1
CD4$^+$ T cells	1.11	0.26	0.42	0.17	0.13	0.51	1.56	0.38	0.29	0.3-1.4
Naïve [b]	Nd	5.7	29.50	2.60	8.10	7.10	5.20	21.40	13.30	
Memory [b]	Nd	51.5	27.00	14.90	26.70	14.40	31.50	32.80	30.80	
Effector [b]	Nd	11.4	1.20	2.60	3.40	2.40	3.50	4.10	1.00	
CD8$^+$ T cells	5.74	0.23	0.16	0.72	0.19	1.63	2.06	0.18	0.33	0.2-0.9
Naïve [b]	Nd	8.5	17.30	9.80	8.30	36.50	11.50	9.60	15.60	
Memory [b]	Nd	15.9	5.60	41.10	31.10	20.20	27.40	8.80	19.60	
Effector [b]	Nd	16.2	2.00	18.50	14.60	10.50	12.30	6.50	1.90	
NK cells	0.13	0.23	0.27	0.04	0.06	0.16	0.33	0.09	0.02	0.09-0.6

[a] % of CD19$^+$ B cells; [b] % of CD3$^+$T cells; Nd= Not determined

PART 3

Deficiencies in the B cell co-receptor complex



Chapter 3.1

Deficiencies in the CD19 complex

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Submitted manuscript

ABSTRACT

Signaling via the CD19-complex, consisting of CD19, CD81, CD21 and CD225, is critically important for B-cell development, differentiation and maturation. In this complex, each protein has its own distinct function. Over the past decade, 15 patients with antibody deficiency due to deficiencies in the CD19 complex have been described. These patients have deficiencies in different complex-members. All deficiencies were caused by either homozygous or compound heterozygous mutations. Although all patients had antibody deficiencies, the clinical phenotype was different per deficient protein. We aimed to provide an overview of what is known about the function of the different complex-members, knowledge from mouse-studies and to summarize the clinical phenotypes of the patients. Combining this knowledge together can explain why deficiencies in different members of the same complex, result in disease phenotypes that are alike, but not the same.

Keywords: Primary antibody deficiencies, CD19, CD21, CD81, B-cell receptor co-complex

Highlights: Thus far 15 patients with deficiencies in the CD19-complex have been described

CD19/CD81 deficiency seems to cause a more severe phenotype in humans

Mouse models indicate that CD21 is more redundant for protective antibody responses than CD19 or CD81.

These patients can shed light on the function of the different complex-members in humans

INTRODUCTION

Human B cells are commonly identified by the expression of CD19 on their surface. This protein is part of the CD19 complex, also referred to as B cell receptor co-complex, consisting of CD19, CD81, CD21 and CD225. Being first described in 1991[1] this complex functions as a co-stimulatory element to amplify antigenic signaling via the B-cell receptor (BR). Furthermore, it bridges innate and adaptive immunity by signaling complement.[2] The complex was shown not only to amplify BR-mediated signaling, but also to prolong the signal, by stabilizing the BR in plasma membrane lipid rafts and blocking internalization of the BR.[3] The different proteins all have their own unique function within the complex, which is illustrated by mouse experiments in which single elements are knocked-out or inhibited.[4-6] These experiments have shown that especially correct balance between the different elements of the complex is vital for optimal function of the complex. Over the past decade, multiple patients with mutations in elements of the CD19 complex have been described.[7-13] All of these patients suffer from hypogammaglobulinemia and most of them have recurrent infections. However, different deficiencies lead to different disease phenotypes and specifically to different levels of disease severity. Studying the immunobiology of the B cells in these patients has increased our knowledge on the function of the individual proteins and the complex as a whole in human B cell immunology. Here, we summarize phenotypic and immunobiological characteristics of patients with CD19 complex deficiencies and link the phenotype that was described to the function of the different proteins in the complex.

FUNCTIONAL CHARACTERISTICS

CD19, is exclusively expressed on B cells.[14] Already in bone marrow, early B cell precursors start expressing CD19 under the influence of the B cell-fate promoting transcription factor PAX5.[15, 16] From the cytoplasmic Igμ-negative preB-I stage onwards, CD19 expression can be detected during B cell precursor development in bone marrow.[17] After this stage CD19 expression is one of the hallmarks of B cells. In the peripheral blood, it remains on the cell surface until differentiation into plasma cells. This member of the Ig-superfamily is a 556 amino acid, 61 kD protein encoded on chromosome 16. Its cytoplasmic tail serves as a specialized adaptor protein to recruit multiple kinases including Lyn, ERK, Vav and PI3K upon BR-mediated signaling.[3] Especially PI3K signaling was shown to be important for CD19-signal transduction.[18]

The tetraspanin CD81 is crucial for the expression of CD19 on the plasma membrane in human B cells. Similar to other tetraspanins it has four hydrophobic transmembrane

domains with which it can cross the plasma-membrane four times, these domains flank short amino and carboxyl cytoplasmic termini and a small and a large extracellular loop. It is encoded on chromosome 11, and widely expressed on the surface of many cells, including lymphocytes, brain and liver cells.[19, 20] CD81 associates with cell-specific partners such as CD19 (and indirectly CD21) on B cells, whereas in T cells it associates with CD4 and CD8.21 In addition it can associate with other, ubiquitously expressed molecules in a range of cell types. As for the B cells, the 25 kD protein serves as the anchor of the CD19 complex.

The third member of the CD19 complex is CD21, or complement receptor 2 (CR2), also known as EBV receptor.[22, 23] Being a complement receptor it facilitates the capture, binding, uptake, presentation and clearance of C3d-opsonized immune complexes.[24] Additionally it lowers the threshold to signal low-dose antigens and it helps in the induction of tolerance and generation of immunological memory and differentiation of B cells. Functioning as a complement receptor and engaging in CD19-complex mediated antigenic signaling, CD21 forms a bridge between the complement system and the adaptive immune system. The 145 kDa protein consists of 15 short consensus repeats, a transmembrane domain and a short cytoplasmic tail.[22, 25] The 19 exons of the CD21 gene are encoded on chromosome 1. It is expressed on follicular dendritic cells and B cells,[2] where it is first detected on naive B cells in the peripheral blood. In healthy individuals, the majority of B cells express CD21,[26] but in several diseases including HIV infection, CVID, systemic lupus erythematosus and Down syndrome, increased frequencies of $CD19^{+}$ $CD21^{-}$ cells can be detected.[27-30]The last member of the CD19 complex is CD225 or Interferon-induced transmembrane protein 1 (IFITM1), previously also known as Leu-13. The IFITM1 gene is located on chromosome 11 and encodes a 125 amino acid, 14kD protein. It is predicted to cross the membrane several times with its two transmembrane domains.[31] It additionally has a short N-terminal and C-terminal domain and a short cytoplasmic tail. It has a broad expression and is largely known to function in cellular adhesion and migration, and in many species is has a function in anti-viral immune response signaling.[32] Within the CD19 complex, it is associating directly to CD81, and via CD81 it is associating with CD21 and CD19. However, so far, the significance of these interaction is not clear.[33] No deficiencies of this protein in humans have been reported so far, neither are mouse models. However, based on the broad expression of the protein and its family members, and its as yet unknown function on B cells suggest that it is unlikely that a deficiency would lead to a phenotype with an isolated immune deficiency. Therefore, we will not further discuss this protein.

MOUSE KNOCK-OUT MODELS

After identification of the different components of the CD19 complex, mouse models were generated to study the functions of the different proteins in B cell biology. Independent studies in CD19$^{-/-}$ mice showed the importance of CD19 mediated signaling for differentiation in response to antigens.[5, 34] Additional studies in CD19 and PI3Kδ double deficient mice have shown that combined action of the two proteins is required for survival and differentiation of B cells.[35] CD19 KO mice had seemingly normal B cell development in bone marrow, and normal numbers of B220^{+} B cells in peripheral blood, however these were mainly of a naive phenotype. Serum Ig-levels of CD19 KO mice were markedly decreased, indicating a defect in B cell differentiation after antigenic stimulation. Especially the response to T-cell dependent antigens was defective in those mice. The cytoplasmic domains of mouse and human CD19 are highly homologous and human CD19 could replace mouse CD19 function in CD19$^{-/-}$ mice.[36]

CD81 was studied in three independently generated CD81 KO mouse-models, all of which showed reduced CD19 expression and impaired activation of B cells.[37-39] Next to that, reduced serum immunoglobulin in response to T-cell dependent antigens was observed, very similar to CD19 KO mice. CD81 KO mice expressed strongly reduced levels of CD19 on the B cell membrane, which is in line with the observation that CD19 is dependent on CD81 for expression on the plasma membrane. Because of the expression of CD81 in tissues other than lymphocytes, these mice additionally showed defects in brain and germ-cells.

Since in mice CD21 (CR2) and CD35 (CR1) are encoded by the same gene through alternative splicing, the first studies were done in CD21/CD35$^{-/-}$ mice.[40] These mice have normal levels of serum IgM and IgG and normal B- and T cell subset distributions. However, upon challenge with a T-cell dependent antigen, decreased titers of antigen specific immunoglobulins were found.[4] This is consistent with studies that used anti-mouse-CD21/CD35 antibodies to block function of the complement receptors.[6] Additionally, the CD21/CD35 KO mice had an increased susceptibility for autoimmune diseases.[41] These results led to the conclusion that CD21 lowers the threshold for signaling by binding to complement. Later studies with expression of human CD21 on CD21/CD35$^{-/-}$ mouse cells, showed that human CD21 can take over mouse CD21 function, indicating a high level of homology between mouse and human CD21-mediated signaling.[42]

To study the interaction between CD19 and CD21 within the CD19 complex, CD21/35$^{-/-}$ mice were generated that either lack or overexpress CD19.[43] It was shown that expression levels of both proteins regulate one another, but that CD19 expression regulates IgM and IgD expression independent of CD21. Overall, CD19 function was dominant over CD21 function and that CD19 regulates B cell signal transduction independent of CD21 activation.

Overall, mouse models showed that all members of the CD19 complex are important to transduce antigenic signaling in B cells, especially for T-cell dependent antigens. Although the signaling is optimal when all proteins are expressed in the correct balance and functioning together as one complex, CD19 signaling seems more critical than CD21 signaling.

ANTIBODY DEFICIENCIES IN PATIENTS

In 2006, we described the first group of patients with mutations in the CD19 complex.[7] Over the past decade, additional patients have been described and so far, 10 patients with CD19 deficiency have been reported (Table 1).[7, 8, 10, 44, 45] All these patients have homozygous or compound heterozygous mutations in CD19, leading to absence of the protein on the plasma membrane of B cells. The phenotype of these patients is quite homogeneous, all suffered from moderate to severe recurrent respiratory tract infections, starting in childhood. Some had additional bacterial infections including diarrhea, skin infections, meningitis and conjunctivitis. All patients were reported to have hypogammaglobulinemia in the form of low IgG serum titers, and in many of them additional low levels of serum IgA and/or IgM were found. In the majority of patients, vaccination responses are reduced and some develop IgA-related nephropathy.[46]

Additionally, we reported one CD81 deficient patient, who is the only patient with CD81 deficiency described so far.[9] Her clinical phenotype is much alike the CD19 deficient patients, with quite severe recurrent infections and IgA-related nephropathy and vasculitis; she was diagnosed with Henoch-Schönlein purpura before the diagnosis of CD19 deficiency was made. In this girl, the disease was caused by a homozygous splice site mutation that lead to complete absence of CD81 expression. In addition, no CD19 expression was found on her B cells due to the disruption of the complex by loss of CD81. Whereas in CD81 KO models, some residual expression of CD19 was detected on B cells,[37-39] human CD81 deficiency resulted in complete absence of CD19 on the B cell surface.

In all CD19 deficient patients and the CD81 deficient patient, $CD20^+$ B cell counts were normal, but expression of CD19 was undetectable or barely detectable. Furthermore, CD21 expression was reduced on the B cells of these patients, indicating that without CD19, the whole complex is disrupted and less CD21 is available on the cells surface. In family members who were heterozygous for the mutations, there was reduced CD19 expression on the surface of the B cells, however, these individuals had no clinical signs or symptoms of immune deficiency, indicating that reduced levels of CD19 do not cause disease. Upon antigenic stimulation, calcium influx was reduced or even absent in CD19 and CD81 deficient patients. This indicates that there is an early defect in activation. Additionally,

Table 1. Patient characteristics

patient[ref]	Mutation (protein)	Muta-tion (DNA)	M/F	age at onset (yr)	age at diagn. (yr)	consanguinity	country of residence	clinical history	immunophenotyping	serum Ig-levels	vaccination response
CD19 1.1[7]	p.R325AfsX4/ p.R325AfsX4	c.972insA	F	1	10	yes	Turkey	recurrent URTI and LRTI, meningitis, hematuria	undetectable CD19, reduced CD21, decreased memory B cells	IgG.M low, IgA normal	reduced
CD19 1.2[8]			M	0.5	12	yes	Turkey	recurrent URTI and LRTI		IgG/A low, IgM normal	reduced
CD19 2.1[7]	p.N463RfsX3/ p.N463RfsX3	c.1384delGA	M	7	35	no	Colombia	childhood: recurrent URTI, adult: recurrent LRTI, bacterial conjunctivitis, gastritis	barely detectable CD19, reduced CD21, decreased memory B cells	IgG low, IgA low, IgM low	reduced
CD19 2.2[7]			F	6	33	no	Colombia	childhood: recurrent URTI, adult: recurrent LRTI, herpes zoster infection, bacterial conjunctivitis, diarrhea		IgG/A/M low	reduced
CD19 2.3[7]			F	5	49	no	Colombia	childhood: recurrent URTI, LRTI, skin abcesses, adult: recurrent LRTI, diarrhea, bacterial conjunctivitis		IgG/A low, IgM normal	reduced
CD19 3[10]	p.A316DfsX5/ p.X	c.947-1G>T/del gene	M	5	8	no	Japan	pyolonefritis, recurrent URTI, gastritis	undetectable CD19, reduced CD21, decreased memory B cells	IgG/A/M low	reduced
CD19 4[45]	p.W52C / p.W52C	c.156G>C	M	6	6	yes	Morocco	recurrent URTI and LRTI		IgG/M low, IgA normal,	reduced
CD19 5[46]	p.P488PfsX15 / p.P488PfsX15	c.1464delC	F	infant	11	unk	Kurdish	recurrent URTI giardiasis, meningitis	barely detectable CD19, reducedCD21, decreased memory B cells	IgG/M low, IgA normal,	reduced
CD19 6[46]	G551GfsX25/ G551GfsX25	c.1653ins23bp	F	13	31	yes	Morocco	child: failure to thrive, hematuria, adult: end-stage renal failure, IgA nephropathy, recurrent RTI	barely detectable CD19, reduced CD21, normal rel frequency memory B cells	IgG low, IgA/M normal	present
CD19 7[44]	p.A316DfsX5/ p.A316DfsX5	c.947-1G>T	M	infant	11	unk	France	recurrent URTI and LRTI, COPD	undetectable CD19, reduced memory B cells	IgG/M low, IgA normal	reduced
CD81[9]	p.E188MfsX13 / p.E188MfsX13	c.561+1G>A	F	infant	6	yes	Morocco	recurrent URTIs and LRTIs, glomerulonefritis, arthralgia, IgA-vasculitis, failure to thrive, hepatomegaly, trombocytopenia	undetectable CD19, reduced CD21, decreased memory B cells	IgG low, IgA normal, IgM low	reduced
CD21 1[11]	p.I274_E409del / p.W766X	c.1225+1G>C/ c.2297G>A	M	6	26	no	Germany	<6 y: URTI, 6-26 asymptomatic, 26y: URTI, LRTI, diarrhea, fever	CD21 expression absent, decreased class switched memory B cells	IgG/A low, IgM normal	poly-saccharide low
CD21 2[13]	p.R142X/ p.I926SfsX14	c.424C>T/c.2777_78delTA	M	13	13	no	Netherlands	no infections		IgG/A/M low	normal
CD21 3[12]	p.T209HfsX10 / p.T209HfsX10	c.243delC	F	7	14	yes	France	recurrent URTI and LRTI since early childhood		IgG low/ normal, IgA/M normal	protein: low
CD21 4[12]			M	5	11	yes	France	recurrent URTI since early childhood		IgG/A normal, IgM subnormal	protein+ polysaccharide low

URTI= upper respiratory tract infection, LRTI= lower respiratory tract infection, del= deletion

they all had very few class switched memory B cells, and experiments showed that the number of somatic hypermutations in the few memory B cells that were formed in these patients, is reduced.[47] This indicates a defect in the early phase of memory formation. This is further supported by the observation that the memory B cells compartment of CD19 deficient patients is more affected than the plasma cell compartment.[47] Overall, the clinical phenotype that was found in human patients with a CD19 or CD81 defect resembled the phenotype of the CD19 KO mice, which clearly indicated the necessity of CD19 for a good humoral immune response.

In addition to the CD19 deficient patients, over the past six years, four patients with CD21 deficiency have been reported.[11-13] All of them had low IgG serum levels, but interestingly, one of them did not suffer from infections, and one patients has been asymptomatic for 20 years. The phenotype overall seems milder than the phenotype of the CD19 deficient patients, with the infections being less severe and more restricted to the respiratory tract. However, the first patient that was described by Thiel *et al*, developed more serious infections in his twenties, so it could very well be possible that the other patients will develop more serious infections when they reach adulthood. Experimental data suggest that reduced memory formation and reduced IgG levels in CD21 deficiency result from a suboptimal threshold for activation rather than an incapability to be activated, as seems the case for the CD19 deficiency. The data gathered from these patients is in line with the function of CD21 as a linker between BR and CD19 by signaling the complement that is bound to opsonized antigen, while the BR is activated by the same antigenic-complex. Also, mouse data previously suggested that CD21 is more redundant than CD19 in the complex function, which was shown by comparison of Ca-influx assays of CD19-deficient and CD21-deficient patients.[13] These data indicate that a lack of CD21 can be overcome if CD19 is still present, but the other way around, CD21 cannot make up for a lack of CD19.

Overall, 10 patients with CD19 deficiency, one with CD81 deficiency and four with CD21 deficiency have been described. All of these patients have hypogammaglobulinemia and all but one suffer from recurrent infections. However, CD19/CD81 deficiency seems to cause a more severe phenotype with overall earlier onset and higher morbidity, which is in line with the data from mouse studies, which showed that CD21 is more redundant for protective antibody responses than CD19 or CD81. Calcium influx following BCR stimulation is absent or strongly reduced in CD19/CD81 deficient patients, but was shown reduced only in a complement dependent way in CD21 deficiency. This difference in response indicates that CD19 deficiency results in an activation problem, whereas CD21 deficient cells can still response, given that the stimulus is strong enough. Therefore, the different function of the components of the CD19 complex and their interlinked expression pattern in which CD19 is more dominant can explain the heterogeneous phenotypes of patients with different genetic deficiencies. Prolonged follow-up of these patients can improve

our understanding of the natural course of these deficiencies and provide more detailed information on the redundancy of the various elements in specific infections.

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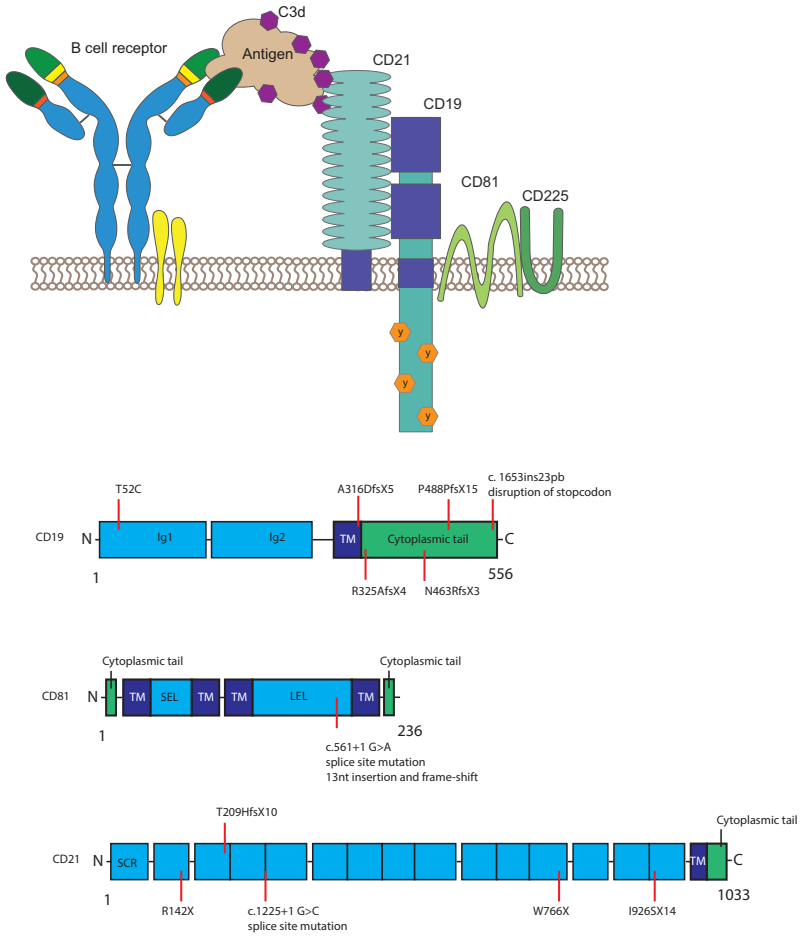


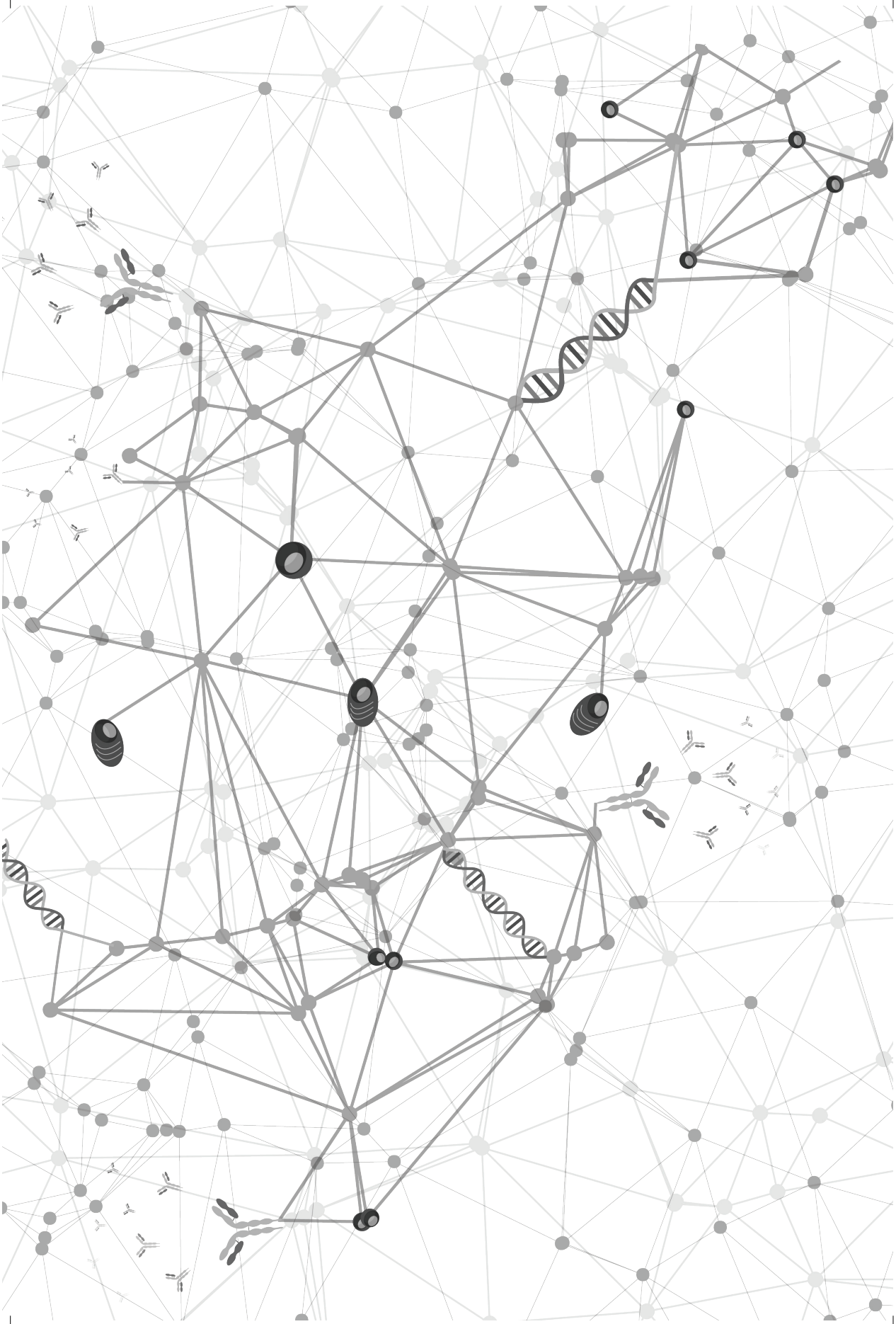
Figure 1.
The CD19 complex and mutations that cause antibody deficiencies. **A.** The CD19 complex consists of CD19, CD81, CD21 and CD225 and signals in conjunction with the B cell receptor, to reduce the threshold for antigenic stimulation. **B.** Positioning of mutations identified in patients that lead to absent or dysfunctional protein when present in a homozygous or compound heterozygous fashion.

REFERENCES

1. Matsumoto, A.K., et al., *Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes.* J Exp Med, 1993. **178**(4): p. 1407-17.
2. Carroll, M.C. and D.E. Isenman, *Regulation of humoral immunity by complement.* Immunity, 2012. **37**(2): p. 199-207.
3. Cherukuri, A., et al., *The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts.* Immunity, 2001. **14**(2): p. 169-79.
4. Molina, H., et al., *Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3357-61.
5. Engel, P., et al., *Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule.* Immunity, 1995. **3**(1): p. 39-50.
6. Hebell, T., J.M. Ahearn, and D.T. Fearon, *Suppression of the immune response by a soluble complement receptor of B lymphocytes.* Science, 1991. **254**(5028): p. 102-5.
7. van Zelm, M.C., et al., *An antibody-deficiency syndrome due to mutations in the CD19 gene.* N Engl J Med, 2006. **354**(18): p. 1901-12.
8. Artac, H., et al., *B-cell maturation and antibody responses in individuals carrying a mutated CD19 allele.* Genes Immun, 2010. **11**(7): p. 523-30.
9. van Zelm, M.C., et al., *CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency.* J Clin Invest, 2010. **120**(4): p. 1265-74.
10. Kanegane, H., et al., *Novel mutations in a Japanese patient with CD19 deficiency.* Genes Immun, 2007. **8**(8): p. 663-70.
11. Thiel, J., et al., *Genetic CD21 deficiency is associated with hypogammaglobulinemia.* J Allergy Clin Immunol, 2012. **129**(3): p. 801-810 e6.
12. Rosain, J., et al., *CD21 deficiency in 2 siblings with recurrent respiratory infections and hypogammaglobulinemia.* J Allergy Clin Immunol Pract, 2017.
13. Wentink, M.W., et al., *CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities.* Clin Immunol, 2015. **161**(2): p. 120-127.
14. Carter, R.H. and D.T. Fearon, *CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes.* Science, 1992. **256**(5053): p. 105-7.
15. Medvedovic, J., et al., *Pax5: a master regulator of B cell development and leukemogenesis.* Adv Immunol, 2011. **111**: p. 179-206.
16. Busslinger, M., *Transcriptional control of early B cell development.* Annu Rev Immunol, 2004. **22**: p. 55-79.
17. van Lochem, E.G., et al., *Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts.* Cytometry B Clin Cytom, 2004. **60**(1): p. 1-13.
18. Okkenhaug, K. and B. Vanhaesebroeck, *PI3K-signalling in B- and T-cells: insights from gene-targeted mice.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 270-4.

19. Shoham, T., et al., *Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments.* Mol Cell Biol, 2006. **26**(4): p. 1373-85.
20. Feneant, L., S. Levy, and L. Cocquerel, *CD81 and hepatitis C virus (HCV) infection.* Viruses, 2014. **6**(2): p. 535-72.
21. Levy, S., *Function of the tetraspanin molecule CD81 in B and T cells.* Immunol Res, 2014. **58**(2-3): p. 179-85.
22. Weis, J.H., et al., *A complement receptor locus: genes encoding C3b/C4b receptor and C3d/Epstein-Barr virus receptor map to 1q32.* J Immunol, 1987. **138**(1): p. 312-5.
23. Moore, M.D., et al., *Molecular cloning of the cDNA encoding the Epstein-Barr virus/C3d receptor (complement receptor type 2) of human B lymphocytes.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9194-8.
24. Fearon, D.T., et al., *Immunoregulatory functions of complement: structural and functional studies of complement receptor type 1 (CR1; CD35) and type 2 (CR2; CD21).* Prog Clin Biol Res, 1989. **297**: p. 211-20.
25. Jacobson, A.C. and J.H. Weis, *Comparative functional evolution of human and mouse CR1 and CR2.* J Immunol, 2008. **181**(5): p. 2953-9.
26. Comans-Bitter, W.M., et al., *Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations.* J Pediatr, 1997. **130**(3): p. 388-93.
27. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.
28. Warnatz, K., et al., *Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia.* Immunobiology, 2002. **206**(5): p. 502-13.
29. Wehr, C., et al., *A new CD21low B cell population in the peripheral blood of patients with SLE.* Clin Immunol, 2004. **113**(2): p. 161-71.
30. Verstegen, R.H., et al., *Defective B-cell memory in patients with Down syndrome.* J Allergy Clin Immunol, 2014. **134**(6): p. 1346-1353 e9.
31. Jia, R., et al., *The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity.* PLoS One, 2015. **10**(3): p. e0118794.
32. Bailey, C.C., et al., *IFITM-Family Proteins: The Cell's First Line of Antiviral Defense.* Annu Rev Virol, 2014. **1**: p. 261-283.
33. Diamond, M.S. and M. Farzan, *The broad-spectrum antiviral functions of IFIT and IFITM proteins.* Nat Rev Immunol, 2013. **13**(1): p. 46-57.
34. Rickert, R.C., K. Rajewsky, and J. Roes, *Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice.* Nature, 1995. **376**(6538): p. 352-5.
35. Kovesdi, D., S.E. Bell, and M. Turner, *The development of mature B lymphocytes requires the combined function of CD19 and the p110delta subunit of PI3K.* Self Nonself, 2010. **1**(2): p. 144-153.
36. Sato, S., et al., *CD19 expression levels regulate B lymphocyte development: human CD19 restores normal function in mice lacking endogenous CD19.* J Immunol, 1997. **158**(10): p. 4662-9.
37. Maecker, H.T. and S. Levy, *Normal lymphocyte development but delayed humoral immune response in CD81-null mice.* J Exp Med, 1997. **185**(8): p. 1505-10.

38. Tsitsikov, E.N., J.C. Gutierrez-Ramos, and R.S. Geha, *Impaired CD19 expression and signaling, enhanced antibody response to type II T independent antigen and reduction of B-1 cells in CD81-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10844-9.
39. Miyazaki, T., U. Muller, and K.S. Campbell, *Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81.* EMBO J, 1997. **16**(14): p. 4217-25.
40. Molina, H., et al., *A molecular and immunochemical characterization of mouse CR2. Evidence for a single gene model of mouse complement receptors 1 and 2.* J Immunol, 1990. **145**(9): p. 2974-83.
41. Rettig, T.A., et al., *Evasion and interactions of the humoral innate immune response in pathogen invasion, autoimmune disease, and cancer.* Clin Immunol, 2015. **160**(2): p. 244-54.
42. Kulik, L., et al., *Human complement receptor type 2 (CR2/CD21) transgenic mice provide an in vivo model to study immunoregulatory effects of receptor antagonists.* Mol Immunol, 2011. **48**(6-7): p. 883-94.
43. Hasegawa, M., et al., *CD19 can regulate B lymphocyte signal transduction independent of complement activation.* J Immunol, 2001. **167**(6): p. 3190-200.
44. Skendros, P., et al., *Misdiagnosed CD19 deficiency leads to severe lung disease.* Pediatr Allergy Immunol, 2014. **25**(6): p. 603-6.
45. van Zelm, M.C., et al., *Antibody deficiency due to a missense mutation in CD19 demonstrates the importance of the conserved tryptophan 41 in immunoglobulin superfamily domain formation.* Hum Mol Genet, 2011. **20**(9): p. 1854-63.
46. Vince, N., et al., *Defects in the CD19 complex predispose to glomerulonephritis, as well as IgG1 subclass deficiency.* J Allergy Clin Immunol, 2011. **127**(2): p. 538-541 e1-5.
47. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation.* J Allergy Clin Immunol, 2014. **134**(1): p. 135-44.



Chapter 3.2

CD21 and CD19 deficiency: two defects in the same complex leading to different disease modalities

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ABSTRACT

Purpose: Deficiencies in CD19 and CD81 (forming the CD19-complex with CD21 and CD225) cause a severe clinical phenotype. One CD21 deficient patient has been described. We present a second CD21 deficient patient, with a mild clinical phenotype and compared the immunobiological characteristics of CD21 and CD19 deficiency.

Methods: CD21 deficiency was characterized by flowcytometric immunophenotyping and sequencing. Real-time PCR, *in vitro* stimulation and next generation sequencing were used to characterize B-cell responses and affinity maturation in $CD21^{-/-}$ and $CD19^{-/-}$ B cells.

Results: A compound heterozygous mutation in *CD21* caused CD21 deficiency. $CD21^{-/-}$ B cells responded normally to *in vitro* stimulation and *AID* was transcribed. Affinity maturation was less affected by CD21 than by CD19 deficiency.

Conclusions: Both CD21 and CD19 deficiency cause hypogammaglobulinemia and reduced memory B cells. CD19 deficiency causes a more severe clinical phenotype. B-cell characteristics reflect this, both after *in vitro* stimulation as in affinity maturation.

Keywords: CD21, CD19, CD81, hypogammaglobulinemia, primary antibody deficiency

INTRODUCTION

Genetic defects leading to primary antibody deficiencies (PAD) have been described in both the B cell co-receptor complex and the complement cascade.[1, 2] Previously 12 patients have been described with deficiencies in CD19 and CD81, both part of the B cell co-receptor complex.[3-9] Thus far, only a single patient with a deficiency in CD21, also part of this complex, has been described.[10] All of these patients exhibit hypogammaglobulinemia and impaired vaccination responses and suffer from recurrent infections. However, age of disease onset and severity of infections are variable.

CD19, CD81 and CD21 form, together with CD225, the B cell co-receptor complex (Figure 1) that enhances B-cell receptor (BR) mediated signaling.[11-14] In this complex, CD81 (a tetraspanin) is essential for CD19 expression on the B-cell membrane.[4, 15] CD19 has a cytoplasmic tail with multiple tyrosine-kinase residues, needed for intracellular signaling.[11, 13, 16, 17] CD21 is also known as complement receptor 2 (CR2) or EBV receptor.[18-20] It is expressed on both B cells and follicular dendritic cells.[21] The 145 kDa protein consists of 15 short consensus repeats, a transmembrane domain and a short cytoplasmic tail. [20, 22] The 19 exons of the *CD21* gene are encoded on chromosome 1q32.[20] CD21 facilitates complement binding via C3d-opsonized immune complexes and responses to low dose antigens.[18, 20]. Functioning as a complement receptor, CD21 is involved in antigen uptake and presentation, clearance of immune complexes and apoptotic cells, induction of tolerance, generation of immunological memory, and survival, activation, and differentiation of B cells.[21, 23, 24] Studies in mice have been done with CD21/CD35 knock-out models, since these proteins are encoded by the same Cr2 locus.[25] Results from these studies indicate that CD21/CD35 deficiency leads to decreased specific antigenic antibody responses[26-29] and increased susceptibility to autoimmune diseases.[30] In several immunological diseases such as HIV and autoimmune disorders increased $CD21^{low/-}$ B-cell populations can be found.[31-34]

Since CD81 is required for expression of CD19 on the plasma membrane, patients with CD19 and CD81 deficiencies show a highly similar phenotype with recurrent ear-nose-throat and respiratory infection starting early in childhood.[3-9] Most patients develop accompanying skin and gastro-intestinal infections. Upon flowcytometric analysis, all patients have normal B-, T- and NK-cell numbers, but reduced transitional and memory B-cell numbers. Both CD81 deficiency and CD19 deficiency result in reduced frequencies of somatic hypermutations (SHM).[7] BR activation upon antigenic *in vitro* stimulation is impaired in CD19 and CD81 deficient cells. This emphasizes the necessity of CD19 and CD81 in the co-receptor complex to enable BR signaling.

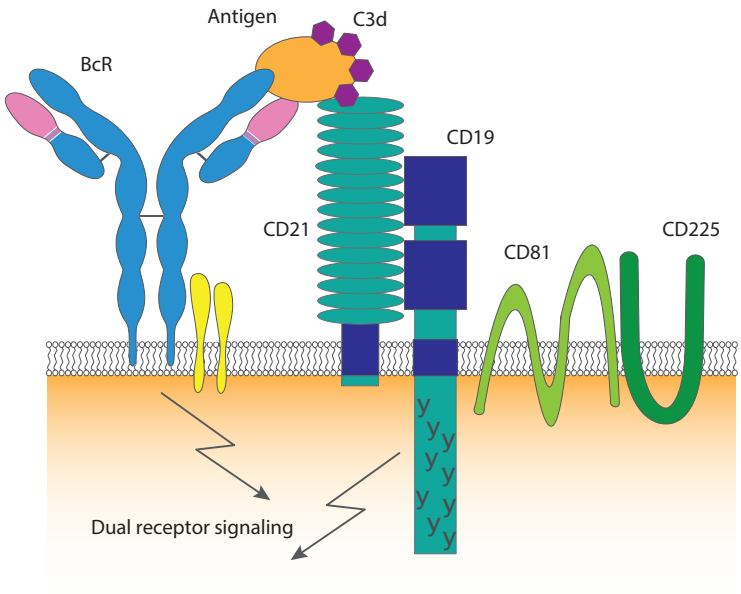

Figure 1.
Schematic representation of the B cell co-receptor complex, in which CD81 is vital for CD19 expression on the cell membrane,[15] CD19 has an intracellular tail with multiple tyrosine-kinase residues[11, 16] and CD21 is able to connect to the BR via antigen bound complement.[18-21] Co-activation of the BR and B cell co-receptor complex, leads to dual receptor signaling.[45].

The first patient described with a CD21 deficiency[10] suffered from recurrent upper respiratory tract infections in early childhood. In his early twenties, this patient developed recurrent infections, including respiratory tract and gastrointestinal infections. He had hypogammaglobulinemia mainly affecting immunoglobulin (Ig)G; IgA levels were slightly reduced and IgM levels were normal. Vaccination responses to protein antigens were normal, but the response to pneumococcal polysaccharide vaccination was moderately impaired. Flowcytometric analysis revealed normal B- T and NK-cell numbers, but reduced memory B cells. BR mediated signaling was affected in a complement-dependent manner in case of sub-optimal stimulation but unaffected upon strong stimulation. This underlines the complement receptor function of CD21 as an enhancer of B cell co-receptor signaling.

Here we describe a second patient (13-year old, male) with a compound heterozygous CD21 deficiency resulting in hypogammaglobulinemia. We compared the clinical and immunobiological characteristics of CD21 deficiency with CD19 deficiency. We show that CD21 deficient B cells have a normal BR mediated signaling upon maximal stimulation *in vitro*, but slightly reduced SHM frequency and class switch recombination (CSR).

METHODS

Cell samples and ethical approval

Peripheral blood was obtained from the CD21 deficient patient, age-matched healthy controls and both parents of the patient with informed consent and according to the guidelines of the local Medical Ethics Committees.

Flowcytometric immunophenotyping

Eight-color flowcytometric immunophenotyping of peripheral blood was performed on a Canto II (BD BioSciences, San Jose, CA, USA). Data were analyzed using FACS Diva (BD Biosciences) and Infinicyt software (Cytognos, Salamanca, Spain). The following antibodies were used: CD19-PerCP-Cy5.5 (SJ25C1), CD21-PECy7 (B-ly4), IgD-biotin (IA6-2), CD27-APC (L128), CD38-APC-H7 (HB7; all from BD Biosciences), CD24-PB (SN3; Exbio, Prague, Czech Republic), CD45-PO (HI30; Invitrogen, Life Technologies, Carlsbad, CA, USA), IgG-PE (K0103-41437) and IgA-FITC (IS11-8E10; both Miltenyi, Bergisch Gladbach, Germany), polyclonal IgD-FITC and IgM-PE (Southern Biotechnologies, Birmingham, AL, USA). CD21 absence was determined using the following four CD21 antibodies: CD21-APC (CR2; BD Biosciences), CD21-PB (LT21; Exbio), CD21-PE (LB21; Serotech, Hercules, CA, USA), and CD21-PerCP (Bu32; BioLegend, San Diego, CA, USA).

Molecular analysis

DNA was isolated from blood granulocytes after separation using Ficoll Hypaque (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Sequence analysis of *CD21* was performed following PCR-amplification of the coding regions with TaqGold™ (Life Technologies), followed by direct sequencing on an ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems, Bleiswijk, The Netherlands). Primer sequences are available upon request. Sequences were analyzed with CLC DNA-workbench software (CLCBio, Aarhus, Denmark) and compared to the NCBI reference sequence (NG_013006).

Sorting of B-cell subsets and Ig transcript analysis

Naive, natural effector and memory B cells from 4 healthy controls and the patient were sorted from post-Ficoll mononuclear cells on a FACS Aria I (BD Biosciences) using the following antibodies: CD19-PerCP-Cy5.5 (SJ25C1), CD27-APC (L128), CD3-FITC (SK7; all from BD Biosciences) and polyclonal IgD-PE (Southern Biotechnologies). mRNA was extracted using Genelute mammalian total RNA Kit (Sigma-Aldrich, Saint Louis, MO, USA) and converted into cDNA. Taqman based RQ-PCR with gene specific primers and probes (sequences available upon request) was used to measure *CD19*, *CD21*, *CD81*, *CD79A* and

PAX5 transcript levels as described before.[3, 4] Expression levels were normalized to *ABL* and *PAX5*.

Ca^{2+} flux analysis

Post-Ficoll PBMCs from the CD21-deficient patient and a healthy control were used to determine free intracellular Ca^{2+} levels before and after stimulation with anti-IgM as described previously.[35] Ca^{2+} influxes for the CD19 deficient cells were determined previously (Patient CD19-1.1).[3, 4]

In vitro stimulation

PBMCs were cultured in 24-well plates ($2x10^6$ PBMCs per well) in 1 ml of IMDM culture medium, supplemented with 10% FCS, penicillin (100U/ml) and streptomycin (100µg/ml), at 37˚C and stimulated with anti-IgM (10µg/ml) and anti-CD40 (10µg/ml) and either hIL-4 (10ng/ml) or hIL-10 (10ng/ml) as described previously.[7] After 3 and after 6 days of culture cells were harvested and RNA was isolated. This RNA was used to synthesize cDNA. Taqman based RQ-PCR with gene specific primers and probes (sequences available upon request) was used to measure *CD19, AID* and *ABL* transcription levels as described before.[7] *AID* levels were normalized to *ABL* and *CD19* levels and compared to expression levels in unstimulated cells to calculate the fold increase in transcript levels.

Analysis of SHM and CSR

cDNA was prepared following RNA isolation of post-Ficoll PBMCs of patients and age-matched healthy controls. This cDNA was used to amplify *IGA* and *IGG* transcripts using VH1-6 consensus BIOMED-2 primers[36] and Cγ (3'Cγ-CH1,[37]) and Cα (IGHA-R,[38]) primers. The primers were adapted for 454 sequencing by adding the forward A, or the reverse B adaptor, the 'TCAG' key and multiplex identifier (MID) adaptor. PCR products were purified by gel extraction with the QIAQuick gel extraction kit (Qiagen, Valencia, CA, USA) and Agencourt AMPure XP beads (Beckman Coulter, Fullerton CA, USA). Subsequently, the PCR concentration was measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen). The purified PCR products were sequenced on the 454 GS Junior instrument using the GS Junior sequencing kit XL+, sequencing kit and PicoTiterPlate kit (454 Life Sciences, Roche, Brandford CT, USA) according to the manufacturers recommendations. Using the Antigen Receptor Galaxy Tool[39] sequences were demultiplexed based on their MID sequence and quality checked. FASTA files were uploaded in IMGT HighV-Quest.[40] For all sequences the subclass of constant region was determined. Uniqueness of sequences was determined by V gene usage, amino acid sequence of the CDR3 and C gene usage. Only unique, productive sequences were used to calculate the frequency of mutated nucleotides in the V_H gene (from CDR1 until FR3).

In addition, *IGG* and *IGA* transcripts were amplified and analyzed as described previously.[41, 42] The mutation frequency was determined for the V_H segment, with exclusion of FR1, of each unique transcript.

Statistical analysis

Differences in mutation frequencies in SHM were analyzed using the nonparametric Mann-Whitney U-test (1 tailed) and transcript expression differences were analyzed by the two-tailed T test for independent samples ($P<0.05$ was considered significant) in the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

RESULTS

Clinical and immunological presentation/ case report

Our patient was a 13-year old boy, the second child of non-consanguineous Dutch parents without family history of recurrent infections or autoimmune diseases. He was referred to the department of Pediatrics of the University Medical Centre Groningen for evaluation hypogammaglobulinemia, which was discovered in the work-up for a possible auto-immune origin of myalgia and rigidity. He did not have a history of recurrent infections. No abnormalities were found upon physical examination. Total serum IgG was 4.4 g/l (ref. 7-16 g/l for age).IgG subclass analysis showed reduced IgG1 (2.5 g/l), reduced IgG2 (0.5 g/l) and absent IgG4 (<0.1 g/l), whereas IgG3 was normal (0.2 g/l). IgA levels were reduced (0.3 g/l (ref. 0.7-4g/l)), as were IgM levels (0.3 g/l, (ref. 0.4-2.3g/l)). Serum IgE levels were in the high normal range (109 g/l). He had been vaccinated according to the Dutch National Vaccination Program and his antibody titers were within the normal range, however additional booster vaccinations with subsequent determination of antibody levels has not been done. Autoimmune antibodies were negative. A C1q binding test was performed to analyze presence of immune complexes; no abnormalities were found. Analysis of B- and T- cell subsets revealed normal B- T- and NK-cell numbers with slightly increased naive B cells and reduced memory B cells (Table 1). However, none of the B cells expressed CD21 (Figure 2A).

After three years of follow-up, now 16 years old, the patient is in good health and has not experienced any (serious) infection. There has been no further reduction in his Ig levels.

Flowcytometric immunophenotyping

The absence of CD21 expression on B cells was confirmed using different antibody-fluorochrome combinations with multiple CD21 clones. Furthermore, following fixation and permeabilization, CD21 could not be detected intracellularly either (data not shown).

Table 1. Distribution of B-cell subsets in our CD21 deficient patient at 13 years of age

B cell subsets	%	Absolute count (cells/µl)	Normal values (10-16 jr)	
Transitional B ($CD38^{high}/CD24^{high}$)	9,5	52	4-108	Within $CD19^+$ gate
Naive Mature ($CD38^{dim}/CD24^{dim}/IgD^+/CD27^-$)	81,0	**446**	87-390	..
MZ/Natural effector ($CD38^{dim}/CD24^{dim}/IgD^+/CD27^+$)	5,3	29	7-90	..
Memory ($CD38^{dim}/CD24^{dim}/IgD^-/CD27^+$)	1,6	**9**	10-76	..
IgM	23,0		5-32%	Within memory B-lymphocytes
IgG	39,5		25-74%	....
IgA	37,5		14-47%	
Plasmablasts	0,3			Within $CD19^+$ gate

Analysis of parental peripheral blood showed a decreased expression of CD21 on the surface of B cells in both parents, as compared to healthy controls (Figure 2A). Expression of CD19 on the cell membrane was increased in the CD21-deficient patient and to a lesser extent in his parents (Figure 2B). CD81 expression was normal in both the patient and his parents, as was CD35 (complement receptor 1) expression (data not shown).

Molecular analysis

Sequence analysis of all 19 exons and splice sites of the *CD21* gene revealed the presence of two heterozygous mutations (Figure 2C, D): a nonsense mutation in exon 2 (c.424C>T; p. Arg142Stop), and a two-nucleotide deletion in exon 15 leading to a frame shift and a premature stop codon (c.2777_2778delTA; p.Ile926SerfsX14). Both mutations lead to premature truncations of the protein in such way that the protein lacks its transmembrane and cytoplasmic domain. Analysis of the parents revealed that the mother was heterozygous for the mutation in exon 2 and the father was heterozygous for the exon 15 mutation (Figure 2C). We concluded that these two truncating mutations lead to absence of CD21 expression on the cell membrane and in the cytosol of B cells.

Transcript levels of CD19, CD81 and CD21

To study the nature of the increased membrane expression of CD19 on CD21-deficient cells, we determined the transcript levels of CD19, CD21 and CD81 in CD27-IgD^+ naive, $CD27^+IgD^+$ natural effector and $CD27^+$IgD- memory B cells of 4 healthy controls and the patient (Figure 3A). In all subsets from the patient, *CD21* transcripts were severely reduced. *CD19* transcript levels in all the patient's subsets were comparable to normal. *CD81* and *CD79A* (anchor protein for the BR) transcript levels in the patient, were in normal ranges in all subsets. The increased expression of CD19 did not seem to result from altered transcriptional regulation.

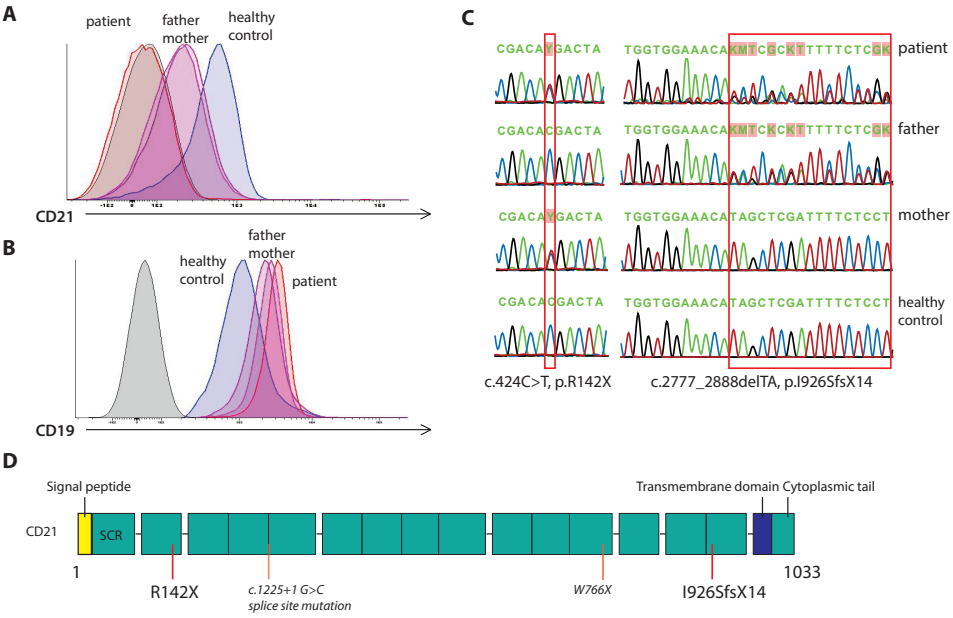

Figure 2.
CD21 deficiency due to a compound heterozygous mutation. **A.** CD21 membrane expression on B cells of the patient (in red), his parents (in purple), a healthy control (in blue) and CD19 negative lymphocytes from the healthy control (in grey). **B.** CD19 membrane expression on B cells of the patient (in red), his parents (in purple),a healthy control (in blue) and CD19 negative lymphocytes from the healthy control (in grey). **C.** Compound heterozygous mutations in the *CD21* gene of the patient. Both parents carry one of both mutations. **D.** Schematic representation of CD21 with 15 short consensus repeats (SCR). Position of the mutations found here (R142X and I926SfsX14) and the mutations found previously (c.1225+1 G>C and W766X, in Italics)[10] are depicted.

BR signaling

To study the effects of CD21 absence on BR signaling, we analyzed Ca^{2+} influx following stimulation of the patient's B cells with anti-IgM. CD21-deficient B cells showed a normal initial Ca^{2+} flux from the rough endoplasmatic reticulum into the cytoplasm, which is indicative for a normal response upon stimulation with high amounts of anti-IgM (Figure 3B). In contrast, cells from CD19-deficient patients showed a defective Ca^{2+} flux after maximal stimulation (Figure 3B).[3, 4] Thus, signaling via BR upon stimulation is normal in CD21 deficiency, but impaired in CD19 deficiency.

In vitro stimulation

We studied whether the absence of CD21 affected the induction of *AID* transcription, since AID is a major regulator in affinity maturation after antigenic stimulation.[43] We stimulated total PBMCs from the CD21-deficient patient *in vitro* for 3 or 6 days with anti-CD40, anti-IgM and either hIL-4 or hIL-10 to induce transcription of *AID*. Using

real-time quantitative PCR the levels of *AID* transcripts were determined after stimulation and compared to unstimulated cells. To correct for the amount of B cells present in each sample, we normalized for *CD19* transcripts. *AID* transcription was upregulated in B-cells of healthy controls after 3 days of stimulation and was further enhanced after 6 days of stimulation. Induction of *AID* transcription in B cells from the CD21-deficient patient was less than the normal control after 3 days of stimulation. After 6 days of stimulation, the patient cells reached the same levels in *AID* transcripts as the healthy control cells after 3 days (Figure 3C). These results show that *AID* transcription can be induced in CD21-deficient B cells, but at slower pace.

SHM analysis

To study the effect of CD21-deficiency on the frequency of SHM, we prepared PBMC derived cDNA from the CD21-deficient patient, age-matched CD19-deficient patients (6-12 years) and aged-matched healthy controls (6-14 years). *IGG* and *IGA* transcripts were amplified in a PCR reaction with specific primers and PCR products were sequenced using next-generation sequencing. The proportion of unique productive sequences in the CD21 deficiency is comparable to the healthy controls but lowered in the CD19 deficiency (data not shown). SHM frequencies in transcripts of the CD21-deficient patient were slightly lower than those of age-matched controls (Figure 4A), but significantly higher than in CD19-deficient patients.

These findings are in line with previous analysis of these transcripts in CD19 deficiency.[3,4]

Analysis of CSR

To study Ig CSR, we determined the *IGG* and *IGA* constant region subclass usage in amplified *IGG* and *IGA* transcripts prepared from PBMC derived cDNA. Compared to the healthy control, usage of downstream *IGG* regions (*IGG2* and *IGG4*) was reduced in CD21 deficiency, resulting in increased *IGG1* usage. In CD19 deficiency this increased *IGG1* usage is even more pronounced (Figure 4B). *IGA* usage in CD21 deficiency was comparable to normal, while in CD19 deficiency both *IGA1* and *IGA2* transcripts were overall strongly reduced and could only be amplified by conventional cloning and sequencing (Figure 4B).

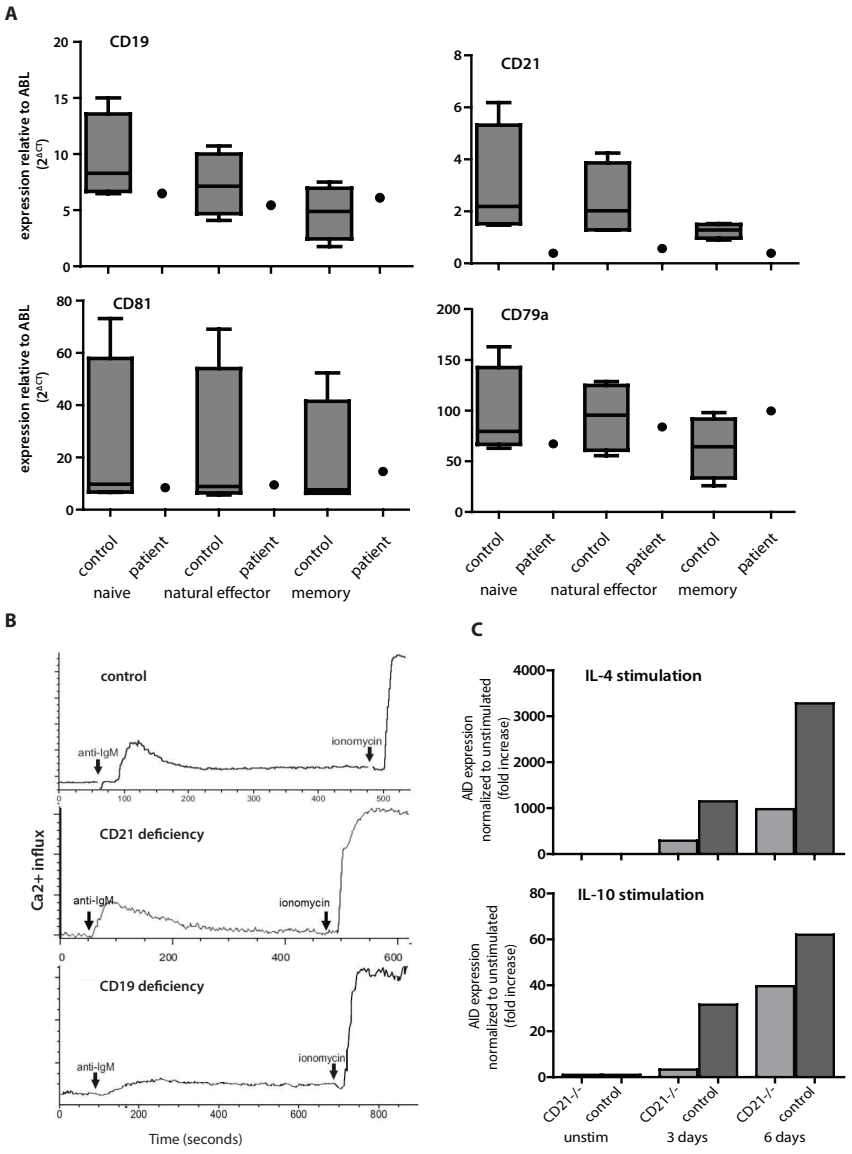

Figure 3.
Transcription of CD19-complex members and response to *in vitro* stimulation. **A.** Expression levels of *CD19*, *CD21*, *CD81* and *CD79A* in naive, natural effector and memory B cells of controls (n=4) and the CD21-deficient patient, normalized to *ABL*. **B.** Ca^{2+} influx in CD21 deficient B cells resembles influx in healthy control cells upon maximal stimulation with anti-IgM. Ca^{2+} influx in CD19-deficient cells is severely reduced upon maximal stimulation with anti-IgM. Response to ionomycin is the same in all samples. Ca^{2+} influx for the CD19 deficiency was performed by Van Zelm *et al*, previously.[3] **B**. *AID* transcription in CD21-deficient B cells and healthy control B cells after *in vitro* stimulation with anti-IgM, anti-CD40 and either hIL-4 or hIL-10. Expression after 3 and 6 days was normalized to *ABL* and compared to unstimulated cells from the same samples. *CD19* expression levels were used to correct for the amount of B cells, present in the sample. In CD21-deficient B cells, *AID* transcription can be induced.

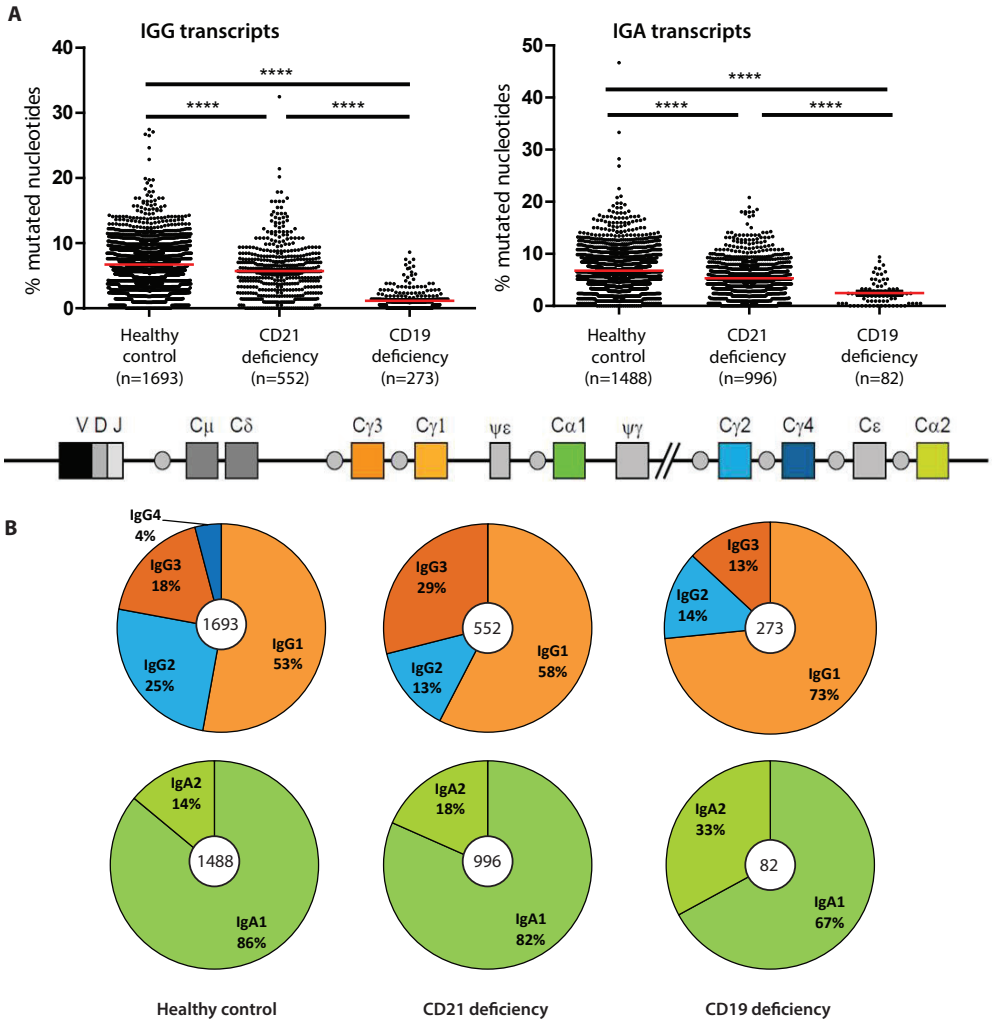

Figure 4.
SHM and CSR in CD21 and CD19 deficiency compared to in aged matched donors (all samples between 6 years and 14 years of age), data from multiple donors was combined for controls (n=4) and CD19 deficiency (n= 4). **A**. SHM frequency determined in unique productive sequences N represents the number of unique productive sequences that were analyzed. **B**. Usage of IG constant gene segments determined for unique productive sequences. In CD19 deficiency no NGS data for IgA was available, sequences were all derived from cloning and sanger sequencing. The number in the center of the plots represents the number sequences that were analyzed.

DISCUSSION

We describe the second patient with a CD21 deficiency caused by two compound heterozygous mutations in the *CD21* gene. Both mutations cause a premature stop-codon resulting in truncated proteins both lacking their transmembrane and cytoplasmic domains.

In contrast to the first described CD21-deficient patient, this patient has no remarkable infections so far, despite his hypogammaglobulinemia. However, at the age of 13, the previously described patient was asymptomatic as well, developing recurrent infections only during early adulthood.[10] Both patients exhibit hypogammaglobulinemia, with normal vaccination responses. This indicates that their immune system is capable of mounting a seemingly normal immune response. With time, however, infections have occurred in the first patient, resulting in the need for intravenous immunoglobulin (IVIG) substitution. This in contrast with CD19 and CD81 deficiencies leading to (severe) recurrent infections already in childhood, requiring frequent hospital admission, surgery for chronic sinusitis and IVIG at early age.[3-9] The CD19-deficient patients often have splenomegaly and impaired vaccination responses, indicating that defects in CD19-complex can seriously impair B-cell function.

CD21 deficiency, like CD19 deficiency, does not lead to a decrease in absolute B-, T- and NK- cell numbers. CD21 deficiency leads to increased naive mature B cells and slightly decreased memory B cells, whereas CD19 deficiency and CD81 deficiency result in a more prominent decrease in transitional (CD81) and memory B cells (both CD19 and CD81). It seems that in CD21 deficiency an immune response is mounted upon encounter of an antigenic stimulus, but with reduced memory formation, whereas in CD19 and CD81 deficiency the immune response is severely hampered. This is in line with the observation that in CD19 and CD81 deficiency, signaling upon BR stimulation is impaired, while calcium influx upon maximal stimulation is unaffected in CD21 deficiency. Previously, Thiel *et al* showed that calcium influx in CD21 deficiency is impaired only in a complement dependent manner.[10] Thus, hypogammaglobulinemia in CD19 and CD81 deficiency can, at least partially, be explained by defective BR signaling, whereas this is intact in CD21 deficiency.

Interestingly, the CD21-deficient B cells show an increase in CD19 expression on the plasma membrane which was not reflected on transcript level. This increased CD19 expression on the membrane is also seen in $CD21^-$ cells found in auto-immune diseases[32-34] and on $CD21^{low/-}$ cells in healthy individuals, although in health very few of those cells can be detected. Increased CD19 expression was also found in CD21/CD35 deficient mice.[44-46] In contrast, in CD19 deficiency, CD21 expression is decreased on the plasma membrane. This suggests that CD19 and CD21 expression levels are inter-connected. The formation

of a regulatory loop between CD19, CD21 and C3 has previously been postulated in mouse studies, which also showed that CD19 can function independent of complement activation.[44, 45] Our results indicate that this is not due to deregulated transcription. Possibly, the increased CD19 expression is compensating for the loss of CD21 whereas in case of loss of CD19, CD21 cannot compensate and might even be down regulated.

CD21 deficient B cells respond normal to maximal stimulation, but in the patient we see a profound hypogammaglobulinemia and a reduction in class switched memory B cells. After *in vitro* stimulation, *AID* transcription could be induced in patients B cells (largely naive B cells). The delay that we see in the patient sample could be caused by the reduced number of memory B cells in the patient sample compared to the healthy control, which show fast induction of *AID* after stimulation. It seems that overstimulation or prolonged exposure to antigenic stimuli can induce a proper response, but short or weak stimulation does not evoke a response. This would be consistent with CD21 acting as a receptor for complement: lowering the threshold for a response to stimulation.[18-21, 47]

Although the number of memory B cells is reduced in CD21 deficiency, they have a close to normal frequency of SHM and show a normal ability for CSR, although they seem to switch preferentially to the more upstream constant genes. This contrasts with the CD19 deficiency where SHM frequency is substantially lower and where the skewing towards *IGG1* is more pronounced. We hypothesize that CD21 negative B cells need strong or prolonged stimulation to evoke an antigenic response. However, when this stimulation is strong enough, the cells will respond with almost appropriate affinity maturation. CD19-deficient B cells cannot respond to even these strong stimuli, resulting in disturbed affinity maturation.

In our patient IgE levels are, opposed to IgA and IgG levels in a high-normal range. It seems that class-switching to IgE is not hampered, showing that class-switch machinery functions correctly and the reduced usage of downstream genes is rather a reflection of an impaired secondary germinal center response than inability of CD21 deficient cells to switch to distantly located constant regions.

Thus, in CD21 deficiency the hypogammaglobulinemia and impaired memory formation seem to result from a defect in signaling threshold rather than from an intrinsic inability to form memory cells whereas in CD19 and CD81 deficiency the clinical syndrome can be attributed to a defect in B-cell memory formation already during an early phase of memory formation.

CONCLUSIONS

In conclusion, CD21 deficiency results in reduced numbers of memory B cells. However, the fraction of productive unique sequences is normal and SHM frequency is only slightly reduced. CSR is intact but shows a slight preference for switching to upstream genes. An increased signaling threshold seems to cause of an impaired immune response, resulting in hypogammaglobulinemia and reduced memory B cells. This might partially be restored by increased CD19 expression. Prolonged or repeated antigen exposure can probably result in normal antigen specific memory formation. In contrast, in CD19 deficiency impaired BR signaling is seen and therefore a defect in the early phase of memory formation with a more sever clinical phenotype. Whereas CD21 and CD19 belong to the same protein complex, absence of CD21 gives a milder immunological and clinical phenotype than CD19 deficiency.

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REFERENCES

1. Geha, R.S., et al., *Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee.* J Allergy Clin Immunol, 2007. **120**(4): p. 776-94.
2. Gathmann, B., et al., *The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008.* Clin Exp Immunol, 2009. **157 Suppl 1**: p. 3-11.
3. van Zelm, M.C., et al., *An antibody-deficiency syndrome due to mutations in the CD19 gene.* N Engl J Med, 2006. **354**(18): p. 1901-12.
4. van Zelm, M.C., et al., *CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency.* J Clin Invest, 2010. **120**(4): p. 1265-74.
5. Kanegane, H., et al., *Novel mutations in a Japanese patient with CD19 deficiency.* Genes Immun, 2007. **8**(8): p. 663-70.

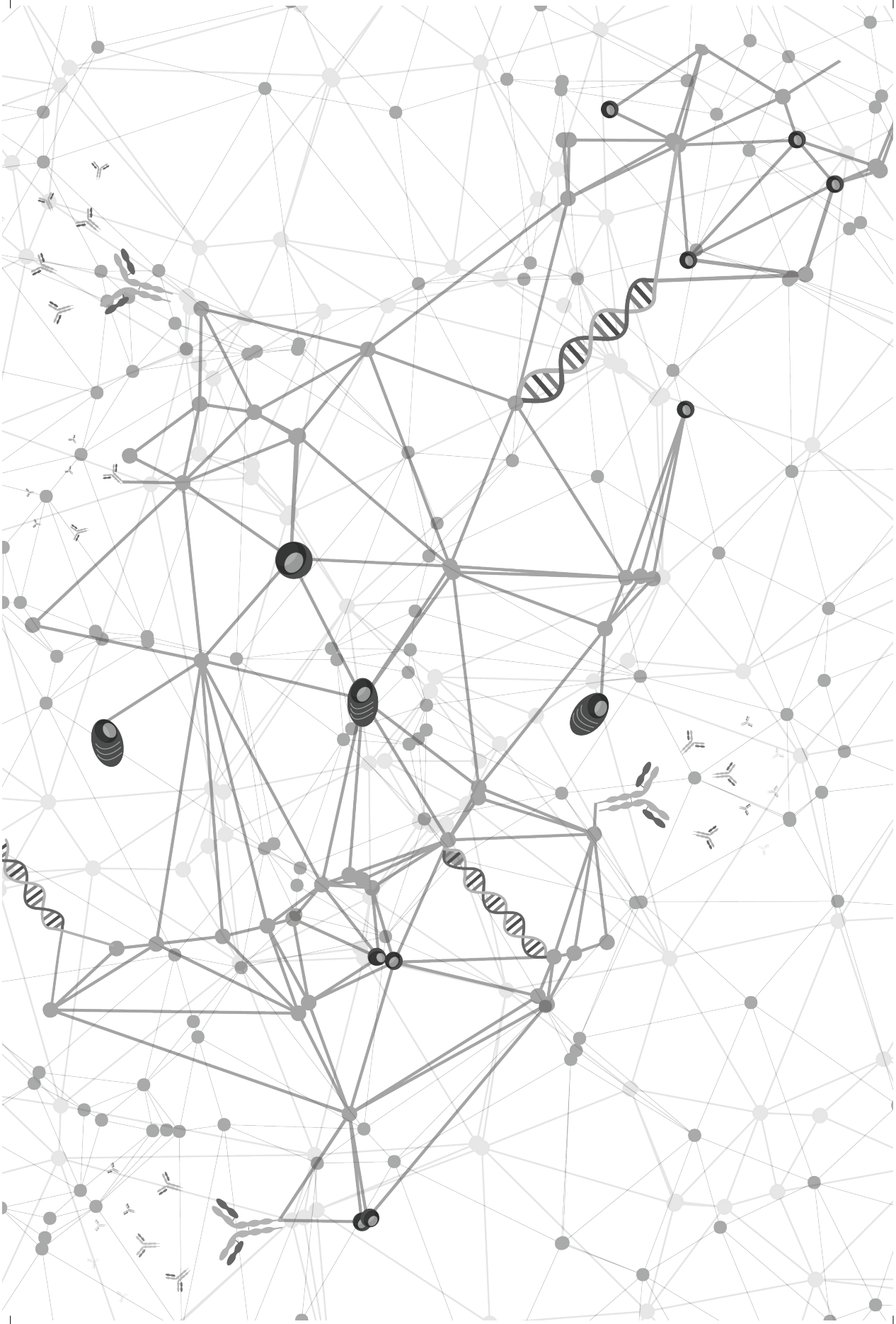
6. Artac, H., et al., *B-cell maturation and antibody responses in individuals carrying a mutated CD19 allele.* Genes Immun, 2010. **11**(7): p. 523-30.
7. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation.* J Allergy Clin Immunol, 2014. **134**(1): p. 135-44.
8. van Zelm, M.C., et al., *Antibody deficiency due to a missense mutation in CD19 demonstrates the importance of the conserved tryptophan 41 in immunoglobulin superfamily domain formation.* Hum Mol Genet, 2011. **20**(9): p. 1854-63.
9. Vince, N., et al., *Defects in the CD19 complex predispose to glomerulonephritis, as well as IgG1 subclass deficiency.* J Allergy Clin Immunol, 2011. **127**(2): p. 538-541 e1-5.
10. Thiel, J., et al., *Genetic CD21 deficiency is associated with hypogammaglobulinemia.* J Allergy Clin Immunol, 2012. **129**(3): p. 801-810 e6.
11. Carter, R.H. and D.T. Fearon, *CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes.* Science, 1992. **256**(5053): p. 105-7.
12. van Noesel, C.J., A.C. Lankester, and R.A. van Lier, *Dual antigen recognition by B cells.* Immunol Today, 1993. **14**(1): p. 8-11.
13. Sato, S., et al., *Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19.* J Immunol, 1997. **159**(7): p. 3278-87.
14. Tedder, T.F., M. Inaoki, and S. Sato, *The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity.* Immunity, 1997. **6**(2): p. 107-18.
15. Shoham, T., et al., *Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments.* Mol Cell Biol, 2006. **26**(4): p. 1373-85.
16. Bradbury, L.E., V.S. Goldmacher, and T.F. Tedder, *The CD19 signal transduction complex of B lymphocytes. Deletion of the CD19 cytoplasmic domain alters signal transduction but not complex formation with TAPA-1 and Leu 13.* J Immunol, 1993. **151**(6): p. 2915-27.
17. Tuveson, D.A., et al., *CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase.* Science, 1993. **260**(5110): p. 986-9.
18. Aegerter-Shaw, M., et al., *Expansion of the complement receptor gene family. Identification in the mouse of two new genes related to the CR1 and CR2 gene family.* J Immunol, 1987. **138**(10): p. 3488-94.
19. Iida, K., L. Nadler, and V. Nussenzweig, *Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody.* J Exp Med, 1983. **158**(4): p. 1021-33.
20. Weis, J.H., et al., *A complement receptor locus: genes encoding C3b/C4b receptor and C3d/Epstein-Barr virus receptor map to 1q32.* J Immunol, 1987. **138**(1): p. 312-5.
21. Carroll, M.C. and D.E. Isenman, *Regulation of humoral immunity by complement.* Immunity, 2012. **37**(2): p. 199-207.
22. Moore, M.D., et al., *Molecular cloning of the cDNA encoding the Epstein-Barr virus/C3d receptor (complement receptor type 2) of human B lymphocytes.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9194-8.
23. Erdei, A., et al., *Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions.* Mol Immunol, 2009. **46**(14): p. 2767-73.

24. Twohig, J., et al., *Defective B cell ontogeny and immune response in human complement receptor 2 (CR2, CD21) transgenic mice is partially recovered in the absence of C3.* Mol Immunol, 2007. **44**(13): p. 3434-44.
25. Molina, H., et al., *A molecular and immunochemical characterization of mouse CR2. Evidence for a single gene model of mouse complement receptors 1 and 2.* J Immunol, 1990. **145**(9): p. 2974-83.
26. Ahearn, J.M., et al., *Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen.* Immunity, 1996. **4**(3): p. 251-62.
27. Molina, H., et al., *Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2.* Proc Natl Acad Sci U S A, 1996. **93**(8): p. 3357-61.
28. Pozdnyakova, O., et al., *Impaired antibody response to group B streptococcal type III capsular polysaccharide in C3- and complement receptor 2-deficient mice.* J Immunol, 2003. **170**(1): p. 84-90.
29. Marchbank, K.J., et al., *Expression of human complement receptor 2 (CR2, CD21) in Cr2-/- mice restores humoral immune function.* J Immunol, 2000. **165**(5): p. 2354-61.
30. Rettig, T.A., et al., *Evasion and interactions of the humoral innate immune response in pathogen invasion, autoimmune disease, and cancer.* Clin Immunol, 2015. **160**(2): p. 244-254.
31. Doi, H., S. Tanoue, and D.E. Kaplan, *Peripheral CD27-CD21- B-cells represent an exhausted lymphocyte population in hepatitis C cirrhosis.* Clin Immunol, 2014. **150**(2): p. 184-91.
32. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.
33. Suryani, S., et al., *Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells.* Blood, 2010. **115**(3): p. 519-29.
34. Wehr, C., et al., *A new CD21low B cell population in the peripheral blood of patients with SLE.* Clin Immunol, 2004. **113**(2): p. 161-71.
35. Muggen, A.F., et al., *Basal Ca(2+) signaling is particularly increased in mutated chronic lymphocytic leukemia.* Leukemia, 2015. **29**(2): p. 321-8.
36. van Dongen, J.J., et al., *Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936.* Leukemia, 2003. **17**(12): p. 2257-317.
37. Tiller, T., et al., *Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning.* J Immunol Methods, 2008. **329**(1-2): p. 112-24.
38. Berkowska, M., *Generation of an Immunocompetent B-cell repertoire*, in *Immunology*. 2012, Erasmus University: Rotterdam. p. 175.
39. Moorhouse, M.J., et al., *ImmunoGlobulin galaxy (IGGalaxy) for simple determination and quantitation of immunoglobulin heavy chain rearrangements from NGS.* BMC Immunol, 2014. **15**(1): p. 59.
40. Alamyar, E., et al., *IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS.* Methods Mol Biol, 2012. **882**: p. 569-604.
41. Driessen, G.J., et al., *B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency.* Blood, 2011. **118**(26): p. 6814-23.

42. van Zelm, M.C., et al., *Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation.* J Allergy Clin Immunol, 2014.
43. Revy, P., et al., *Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2).* Cell, 2000. **102**(5): p. 565-75.
44. Hasegawa, M., et al., *CD19 can regulate B lymphocyte signal transduction independent of complement activation.* J Immunol, 2001. **167**(6): p. 3190-200.
45. Barrington, R.A., et al., *Uncoupling CD21 and CD19 of the B-cell coreceptor.* Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14490-5.
46. Haas, K.M., et al., *Complement receptors CD21/35 link innate and protective immunity during Streptococcus pneumoniae infection by regulating IgG3 antibody responses.* Immunity, 2002. **17**(6): p. 713-23.
47. Shishido, S.N., et al., *Humoral innate immune response and disease*. Clin Immunol, 2012. **144**(2): p. 142-58.

PART 4

Dysregulation of the PI3K-PTEN balance



Chapter 4.1

A mediastinal mass in a young child

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CASE PRESENTATION

A three-year-old girl was referred to a pediatric pulmonologist for dyspnea and recurrent upper respiratory tract infections (RTIs). The patient was born full term to unrelated Dutch parents after an uneventful pregnancy and birth. The year before presentation she had suffered from a pneumonia and over 10 upper RTIs. Apart from the recurrent RTIs, which started in infancy, her medical history was not significant and did not include allergies or eczema. An adenotonsillectomy was performed at the age of two years, and she was treated with multiple antibiotic regimens and inhalation therapy with salbutamol and corticosteroids, with no relieve of symptoms.

PHYSICAL EXAMINATION FINDINGS

A dyspneic girl was seen. She had a small stature (-1,7 SD for age), but her weight was appropriate for her age and height. Chest examination revealed mild intercostal retractions and bilateral rhonchi, crackles and wheezing. Examination of the heart and abdomen showed a regular cardiac rate without murmurs and a non-tender abdomen with enlargement of spleen and liver. The patient did not have palpable lymph nodes.

DIAGNOSTIC STUDIES

A chest X-ray showed bilateral pulmonary infiltrations. A spirometry controlled in- and expiratory high resolution chest CT-scan showed compression of the left main bronchus by an undefined subcarinal mediastinal mass as well as diffuse bronchiectasis with atelectasis (Figure 1A). A chest MRI confirmed the mediastinal mass and indicated it was not a cyst. It also showed bilateral hilar lymphadenopathy. Ultra-sound of the abdomen showed hepatosplenomegaly without lymphadenopathy or other abnormalities. Examination of the trachea by bronchoscopy revealed tracheal deviation to the right, due to compression, nodular hyperplasia of the bronchial mucosal surface and purulent secretion. Pathological evaluation of the broncho-alveolar lavage fluid showed no signs for malignancy, however, it was highly cellular, with 44% T cells (ref. 5.9%±7.7) and only 8% B cells (ref. <5%).

Microbiological examination of sputum was positive for *Streptococcus pneumoniae*. Cultures where negative for (atypical) mycobacteria. Serum levels of immunoglobulins were normal, but the antibody response to pneumococcal polysaccharide vaccination was insufficient. Also, despite previous vaccination, antibodies against poliovirus, pneumococcal conjugate vaccination and *H. Influenzae* were not detected in serum. The

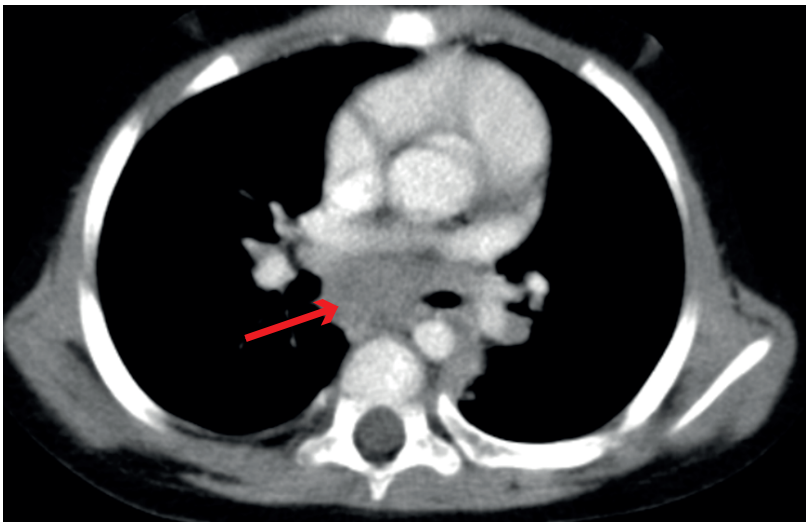


Figure 1.
Compression of the left main bronchus by an undefined subcarinal mediastinal mass (indicated with an arrow) and diffuse bronchiectasis with atelectasis on the chest CT-scan of a 3-year-old with recurrent RTIs.

patient was further evaluated by a pediatric immunologist. Upon immunological evaluation reduced memory B-cell subsets were found in the peripheral blood (Table 1), compatible with the possibility of an antibody deficiency. To rule out malignancy and to provide decompression of the trachea, the mediastinal mass was removed by uncomplicated thoracoscopic surgery, under full anesthesia without any complications. Macroscopically, the mass seemed to consist multiple lobular lymph nodes. Pathological review confirmed the mass to be lymphatic tissue with dysplastic germinal centers. No malignant cells were found. Other diagnostic tests revealed no signs of viral, bacterial, (atypical) mycobacterial or yeast infections or most common immunological diseases.

Question: What is the diagnosis in this young girl?

Genetic evaluation 3 years after presentation revealed a mutation in *PIK3CD*, exon 13 (c.1537G>A) resulting in an amino acid substitution p.E525K.

Diagnosis: Activated PI3Kdelta syndrome (APDS)

DISCUSSION

Over the past years, multiple patients with gain-of-function mutations in PI3Kdelta have been described. These patients suffer from Activated PI3Kdelta syndrome (APDS) and share clinical characteristics such as recurrent RTIs, bronchiectasis, hepatosplenomegaly, generalized lymphadenopathy and antibody deficiency. Because 96% of APDS patients

Table 1. Immunophenotyping of peripheral blood, at the time of presentation at the immunologist

Immunophenotyping of lymphocyte subsets	%	Absolute count ($x10^9$/L)	Normal values (2-5 yr)
White Blood cell count		5,4	
Lymphocytes	19,3	1,0	1.7-6.9
$CD3^+$ T-lymphocytes		0,84	0.9-4.5
$CD16.56^+CD3^-$ NK-cells		0,27	0.1-1.0
$CD19^+$ B-lymphocytes		0,23	0.2-2.1
B-cell subsets			
Transitional B ($CD38^{high}/CD24^{high}$)	60,0	138	24-333
Naive Mature ($CD38^{dim}/CD24^{dim}/IgD^+/CD27^-$)	28,6	66	170-1691
MZ/Natural effector ($CD38^{dim}/CD24^{dim}/IgD^+/CD27^+$)	5,8	13	16-226
Memory ($CD38^{dim}/CD24^{dim}/IgD^-/CD27^+$)	1,5	3	20-149
IgM	28%		3-32%
IgG	36%		13-63%
IgA	36%		4-42%
Plasmablast	3,9		
T-cell subsets			
$CD4^+$ T-lymphocytes	29,9	0,3	0.5-2.4
- Naive ($CD45RO^-CCR7^+CD27^+CD28^+$)	6,4		
- Central Memory ($CD45RO^+CCR7^+CD27^+CD28^+$)	5,8		
- Effector Memory ($CCR7^-$)	87,5		
$CD8^+$ T-lymphocytes	57,5	0,5	0.3-1.6
- Naive ($CD45RO^-CCR7^+CD27^+CD28^+$)	2,8		
- Central Memory ($CD45RO^+CCR7^+CD27^+CD28^+$)	0,5		
- Effector Memory ($CCR7^-$)	95,0		
CD4/CD8 ratio	0,5		

suffer from recurrent RTIs, many patients will be examined by the pulmonologist first. Infections are not the only thoracic problem in APDS patients. Most patients have pulmonary damage including bronchial wall thickening, tree-in-bud opacities and/or bronchiectasis, which are seen on chest CT-scans from a young age onwards and which are not in all cases related to the severity of RTIs. Additionally, lymphadenopathy is a common problem in APDS because of the fast proliferation but poor differentiation of B and T cells. Lymphadenopathy can present in the form of nodular lymphoid hyperplasia in the mediastinum and intestines, and in the mucosal tissue of the bronchus. Mediastinal lymphadenopathy may cause severe obstruction of the airways, increasing the risk of RTIs

and secondary pulmonary damage. Because of the wide spectrum of possible clinical manifestations, including bronchiectasis, lymphoproliferation and auto-immune disease, APDS patients need a thorough pre-operative screening.

Recent cohort studies show that, besides infections, patients with APDS often have auto-immunity (34% of patients) and lymphoid malignancy (13% of patients). Furthermore, viral infections can have a more severe course. Especially EBV and CMV infections are poorly controlled. This may result in severe illnesses such as encephalitis and disseminated infections. Recurrent EBV infections also increase the risk for lymphoma. Therefore, it is crucial that these patients are carefully monitored by both the pulmonologist and immunologist.

Immune phenotyping revealed an inversed CD4/CD8 ratio and increased transitional B-cells. APDS is caused by dysregulation of the PI3K-AKT pathway. Heterozygous gain-of-function mutations in two genes (*PIK3CD* and *PIK3R1*), which together encode the heterodimer PI3Kdelta, cause hyperactivation of the PI3K-AKT-pathway in patient's lymphocytes.

Analysis of the phosphorylation status of AKT can show increased activation of the PI3K-AKT-pathway. This has been described in APDS patients, and was confirmed in our patient by phospho-flow. The hyper-activation causes fast proliferation and differentiation of naive T-cells into short-lived effector T-cells, skewing the subset distribution of $CD4^+$ and $CD8^+$ T-cells and reducing the memory T-cell subset. Within the B-cell compartment, there is increased apoptosis and reduced memory formation, leading to antibody deficiencies.

Patients with antibody deficiencies are treated with immunoglobulin replacement therapy and preventive anti-microbial agents. Since APDS not only causes an immune deficiency but also dysregulation, some of the symptoms respond well to steroid-treatment. However, specifically in young children, long-term use of prednisone and related drugs can cause serious side effects. Therefore, other treatment options are currently being investigated. The mammalian target of rapamycin (mTOR) signaling pathway is downstream of PI3K-AKT signaling. In APDS, dysregulated mTOR signaling is one of the driving mechanisms of disease, causing fast proliferation and differentiation and preventing memory formation in the immune system. Rapamycin inhibits mTOR signaling and is therefore considered as a possible treatment to reduce immune dysregulation. Rapamycin is currently used by both pediatric and adult immunologists in the treatment of APDS. Additionally, specific PI3Kdelta-inhibitors might be of use to inhibit PI3K-AKT hyper activation. The first results of trials with these inhibitors as a treatment for APDS in adults are promising. Furthermore, inhalation therapy with PI3Kdelta-inhibitors might prevent extensive pulmonary damage.

CLINICAL COURSE

One-and-a-half years after removal, the mediastinal mass was back to its original size. Meanwhile, the patient received subcutaneous immunoglobulin substitution and maintenance antibiotics, although these did not entirely prevent RTIs. Her symptoms responded well to steroid treatment and she became prednisone dependent. After the discovery of APDS in 2013, the patient's DNA was sequenced revealing an APDS-causing mutation. Treatment with rapamycin was started and prednisone could be tapered and stopped. The frequency of RTIs decreased and she rarely needed antibiotic treatment. She and her mother reported she was more energetic and additionally her height growth increased. Physical examination revealed no more hepatosplenomegaly. She is carefully monitored by the pulmonologist and immunologist, because of her current pulmonary damage, future risks of auto-immunity and malignant transformation, and for the effects of long-term rapamycin treatment.

CLINICAL PEARLS

Patients with APDS commonly suffer from recurrent RTIs (96%) and therefore will mainly present first at the pulmonologist

A mediastinal mass as part of generalized lymphadenopathy may obstruct the airways in APDS, increasing the risk of pulmonary infections and persistent severe damage

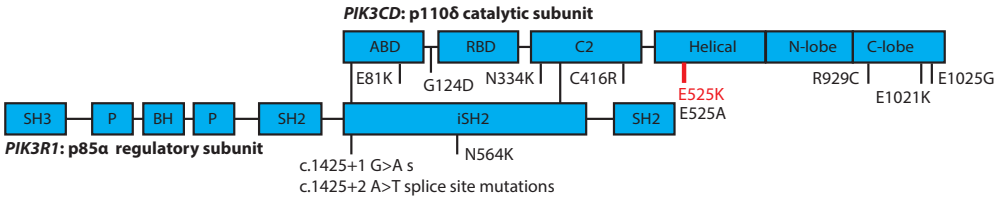
Patients who suffer from APDS induced pulmonary problems benefit from dual treatment with immunoglobulin and immune suppression

Rapamycin is a treatment option in children with APDS who are steroid dependent. Steroid treatment in especially in young children can have severe effects on growth and development. In adults, PI3Kdelta inhibitors can be considered.

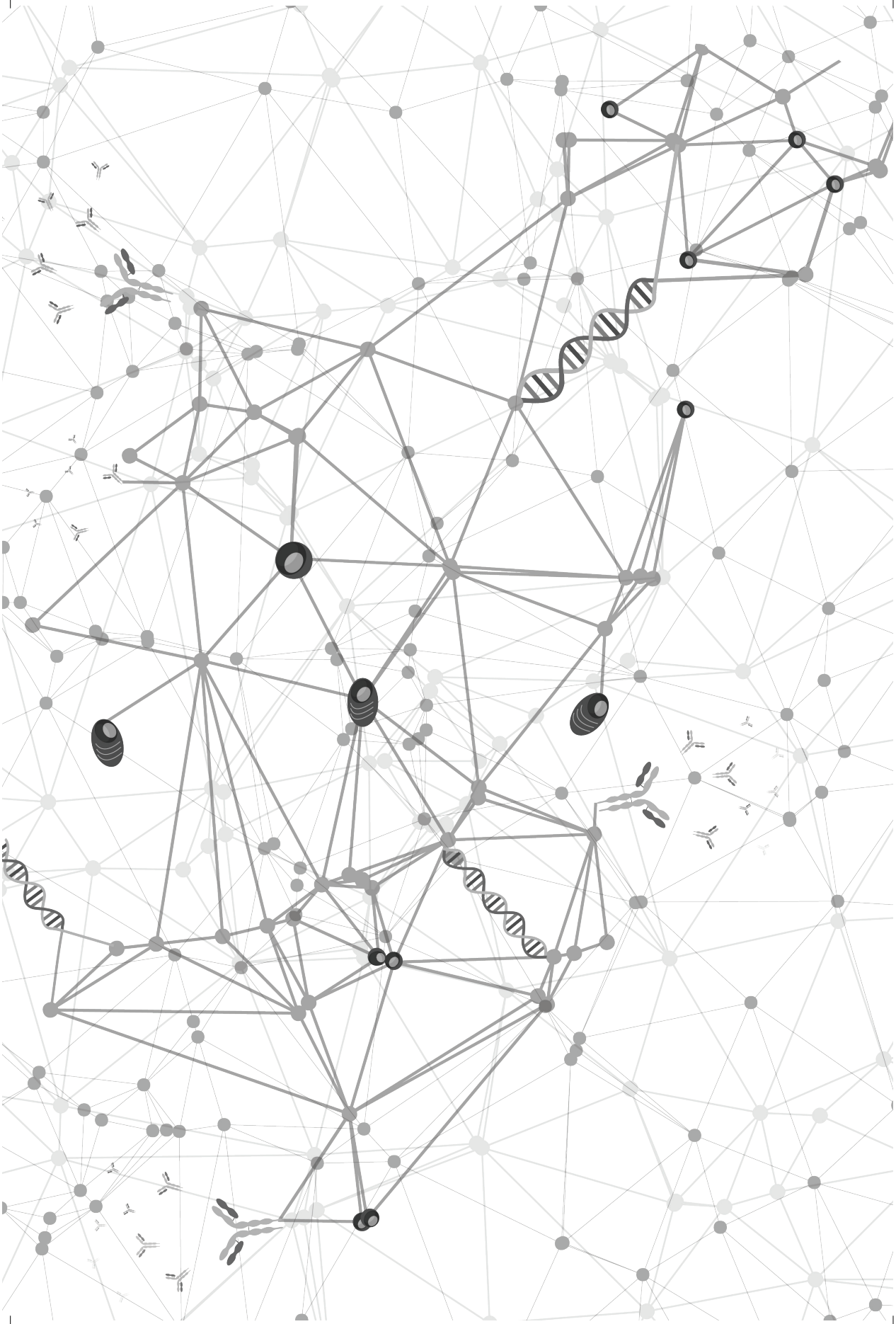
SUGGESTED READING

1. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage*. Science, 2013. **342**(6160): p. 866-71.
2. Lucas, C.L., et al., *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency*. Nat Immunol, 2014. **15**(1): p. 88-97.
3. Kracker, S., et al., *Occurrence of B-cell lymphomas in patients with activated phosphoinositide 3-kinase delta syndrome*. J Allergy Clin Immunol, 2014. **134**(1): p. 233-6.

4. Elkaim, E., et al., *Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study*. J Allergy Clin Immunol, 2016. **138**(1): p. 210-218 e9.
5. Coulter, T.I., et al., *Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study*. J Allergy Clin Immunol, 2017. **139**(2): p.597-606.e4.
6. Wentink, M., et al., *Genetic defects in PI3Kdelta affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infections*. Clin Immunol, 2017. **176**: p. 77-86
7. Heurtier, L., et al., *Mutations in the adaptor-binding domain and associated linker region of p110delta cause Activated PI3K-delta Syndrome 1 (APDS1)*. Haematologica, 2017. **102**(7):p. e278-e281
8. Rao, V.K., et al., *Effective 'Activated PI3Kdelta Syndrome'-targeted therapy with the PI3Kdelta inhibitor leniolisib.* Blood, 2017. **130**(21):p. 2307-2316



Supplemental figure.
Known APDS causing mutations and their location in the PI3Kdelta heterodimer



Chapter 4.2

Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency

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ABSTRACT

PTEN deficiency in humans is associated with PTEN Hamartoma Tumor Syndromes (PHTS). It causes increased activation of the PI3K/Akt pathway and we here show that the humoral immune response is deregulated resulting in CSR and SHM deficiency. This is clinically apparent in part of the PHTS patients as antibody deficiency. In conclusion, PI3K/Akt signaling is important for the human B-cell response to antigens and deregulated signaling is implicated in the pathophysiology of antibody deficiency.

Keywords: PTEN, PI3K, Akt, AID, PHTS, Cowden, macrocephaly, immunodeficiency, Common Variable Immunodeficiency Disorders, B-cell, Activated PI3K Delta Syndrome, PIK3CD, hypogammaglobulinemia, primary antibody deficiency, somatic hypermutation, class switch recombination.

TO THE EDITOR

Autosomal dominant germline mutations in PTEN are associated with PTEN Hamartoma Tumor Syndromes (PHTS), including Cowden syndrome, characterized by hamartomas, malignant tumors, macrocephaly and neurodevelopmental delay.[1] Immunodeficiency has recently been reported in PHTS,[2] but the mechanism of disease is not clear. Therefore we performed a detailed study of the peripheral B-cell development of nine patients with PHTS, to explore the role of PI3K/Akt signalling in the humoral immune response in these patients (details are described in the supplemental methods).

We included nine patients bearing heterozygous germline mutations in PTEN (clinical and genetic details are given in Table E1, Figure E1 and methods in the Online repository). Three patients suffered from hypogammaglobulinemia fulfilling the Common Variable Immunodeficiency Disorders (CVID) diagnostic criteria, of whom one has been previously reported as a case of hypogammaglobulinemia and macrocephaly.[3] Peripheral B-cell subset distribution was studied by analysing the relative and absolute B-cell subset size of two naive and three memory B-cell subsets (Figure 1). PHTS patients were compared to 45 age matched healthy controls. Absolute counts of transitional B-cells were increased (Figure 1A). Data of three memory B-cells subsets showed that absolute counts of class switched $CD27^+IgG^+$ memory B-cells, which originate from a T-cell dependent differentiation pathway,[4] were decreased in PHTS (Figure 1B). We observed a trend towards a more severe reduction of switched memory B-cell subsets in patients with hypogammaglobulinemia (data not shown). In summary, the T-cell dependent B-cell response is deregulated in PHTS.

Analysis of peripheral T-cell subsets revealed that the absolute counts of naive, memory and effector $CD4^+$ and $CD8^+$ T-cells were comparable to healthy controls (Figure E2 in the Online Repository).

CSR was studied at the molecular level by analysing the IGG and IGA subclass distribution of IGH transcripts (Figure 2). We compared the IGG and IGA subclass distribution between patients and controls by Chi-square analysis. In PHTS IgG subclass distribution differed significantly from controls (P=0.002), which was the result from an increase of CSR to IgG_1 and a decrease to IgG_2. CSR distribution seemed more clearly affected in PHTS patients with hypogammaglobulinemia compared to PHTS without hypogammaglobulinemia (data not shown). Similar results were obtained for CSR to IgA_2, which was significantly reduced in PHTS (P<0.0001). So, mutations in PTEN cause abnormalities in CSR to distal IgG and IgA subclass regions.

The SHM frequency was determined by mutational analysis of the IGG and IGA transcripts (Figure 2C). Mutational analyses showed a significant decreased SHM frequency in both IGG and IGA transcripts of PHTS patients, indicating that SHM is affected in PTEN deficiency. In mice levels of AID expression are regulated by PI3K/Akt signalling.[5-7] We

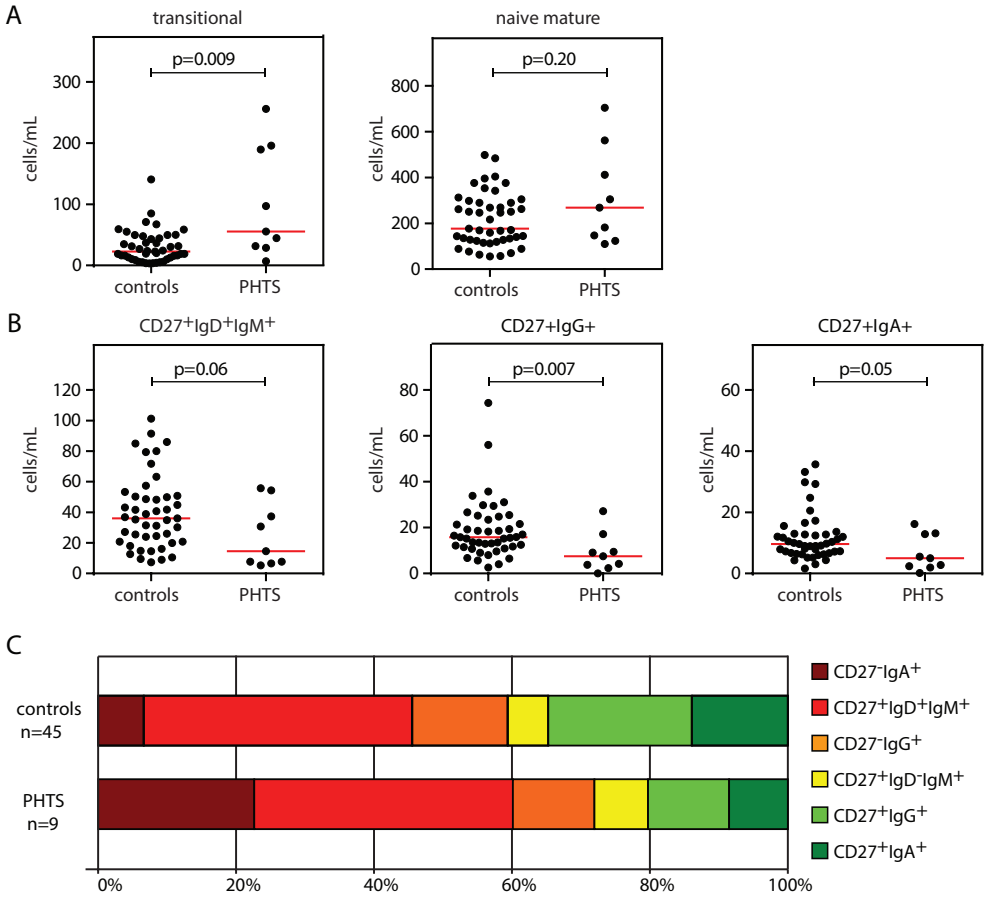

Figure 1. Naive and memory B-cell subsets in PHTS patients.
Absolute counts of transitional and naive mature B cells (**A**) and of three memory B-cell subsets (**B**). **C**. relative distribution of six memory B-cell subset. Data are compared to age-matched normal controls using the Mann-Whitney test. Individual data points are displayed and bars indicate medians. Significant values are indicated: P<0.01 after Bonferroni correction for multiple comparison. PHTS; PTEN hamartoma Tumor Syndrome.

hypothesize that in PHTS patients decreased SHM is caused by a deregulating effect of Akt signalling on AID expression and function, which has to be established in future studies. PTEN deficiency results in abnormalities in B-cell development indicative of CSR deficiency and SHM deficiency, which is clinically apparent as hypogammaglobulinemia in part of the PHTS patients. Our data provide evidence that increased PI3K/Akt signaling deregulates the human humoral immune response, which is in accordance with recent observations in patients with autosomal dominant activating mutations in the PIK3CD gene, coding for the P110δ subunit of PI3K.[8,9] These patients suffer from recurrent infections and have a variable severe antibody deficiency, similar to PHTS patients. The clinical outcome of an individual patient most likely depends on inter-individual differences in any of the multiple factors

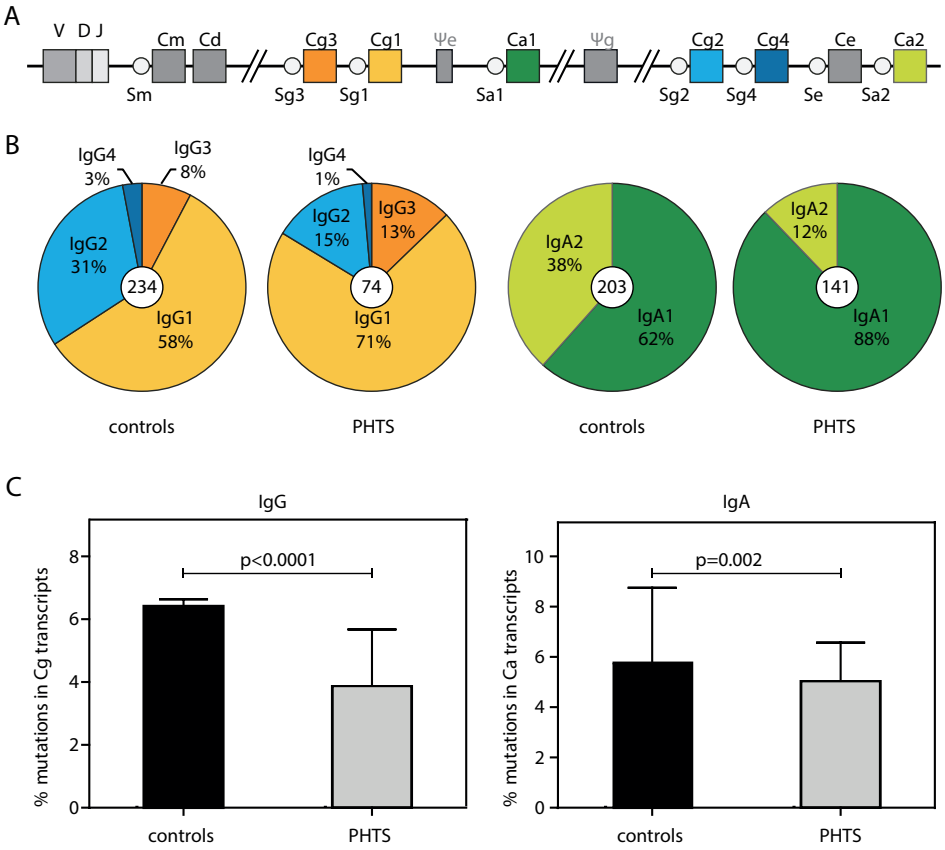

Figure 2. IgA and IgG class switching and somatic hypermutation in *IGH* transcripts of PHTS patients.
A. Schematic representation of the constant regions in the *IGH* locus and frequencies of *IGG* and *IGA* transcripts in PHTS (n=9) were compared to controls (n=8). In the center of each plot the number of analyzed transcripts is depicted. **B.** Somatic hypermutation analysis of *IGG* and *IGA* transcripts; the number of analysed transcripts is indicated. Grey bars represents the 9 PHTS patients and black bars controls.

4.2

that regulate PI3K/Akt signaling and the complex interplay between immune function, (auto)antigen exposure, and the timing and cell types involved in immune activation.

PHTS patients with hypogammaglobulinemia fulfilled the CVID diagnostic criteria. We explored whether mutations in PTEN were present in a cohort of 42 CVID patients, but no mutations could be detected. PHTS patients show similarities to patients with activation mutations in PIK3CD gene:[8, 9] a variable antibody deficiency, a tendency to develop malignancies, respiratory infections and as far as immunologically findings are concerned, increased Akt activity, an increase of the proportion of transitional B cells and a decreased proportion of memory B cells. Differences are also present, such as the absence of hamartomas and macrocephaly in patients with activating mutations of the PIK3CD gene, which can be explained by the fact that P110δ is lymphoid specific. Despite

these differences, our study and the study of Angulo *et al*.[8] and Lucas *et al*.[9] show that PI3K/Akt signaling is important for the human B-cell response to antigens and that deregulated signaling is implicated in the pathophysiology of antibody deficiency. We propose that deregulated PI3K/Akt signaling is an attractive disease causing mechanism to be further explored in CVID patients, because heterozygous germline mutations in PTEN mutations have been associated with auto-immunity, lymphoproliferation, an increased risk to develop malignancies and intestinal nodular interstitial hyperplasia, which are all commonly encountered in CVID.

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REFERENCES

1. Marsh, D.J., et al., *Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation.* Hum Mol Genet, 1998. **7**(3): p. 507-15.
2. Browning, M.J., et al., *Cowden's syndrome with immunodeficiency.* J Med Genet, 2015. **52**(12): p. 856-9.
3. Cogulu, O., et al., *Two cases of macrocephaly and immune deficiency.* Clin Dysmorphol, 2007. **16**(2): p. 81-4.
4. Berkowska, M.A., et al., *Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways.* Blood, 2011. **118**(8): p. 2150-8.
5. Omori, S.A. and R.C. Rickert, *Phosphatidylinositol 3-kinase (PI3K) signaling and regulation of the antibody response.* Cell Cycle, 2007. **6**(4): p. 397-402.
6. Suzuki, A., et al., *Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination.* J Exp Med, 2003. **197**(5): p. 657-67.
7. Werner, M., E. Hobeika, and H. Jumaa, *Role of PI3K in the generation and survival of B cells.* Immunol Rev, 2010. **237**(1): p. 55-71.
8. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science, 2013. **342**(6160): p. 866-71.
9. Lucas, C.L., et al., *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency.* Nat Immunol, 2014. **15**(1): p. 88-97.

PTEN SUPPLEMENT, ONLINE REPOSITORY

METHODS

Patients

We included nine patients bearing heterozygous germline mutations in *PTEN* gene. Three patients suffered from hypogammaglobulinemia and six did not have a clinically apparent antibody deficiency. Data of these patients were compared to 45 age matched normal controls. Furthermore, we screened 42 CVID[1] patients for the presence of mutations in *PTEN*. The research was approved by the Medical Ethical Committee of the Erasmus MC.

Patient histories

Patient 1 is a 43 year old woman who suffered from recurrent upper and lower respiratory tract infections from childhood and was diagnosed with hypogammaglobulinemia at the age of 12 years. Immunoglobulin replacement was initiated. At the age of 30 a hemithyroidectomy was performed because of nodular hyperplasia. At the age of 31 a melanoma in situ was removed. Thereafter, she suffered from several episodes of lobar pneumonia resulting in bronchiectasis despite immunoglobulin replacement. Switched memory B-cells were virtually absent. Furthermore, she was treated for a candida esophagitis. Dermatologic evaluation showed keratotic plugs hand palms, lipoma's and café-au-lait maculae. At the age of 40 the diagnosis of Cowden disease was made, based on the clinical history, including macrocephaly, and heterozygous mutations in *PTEN*. *Patient 2* is 12 years old and is a daughter of patient 1. She was known with developmental delay and macrocephaly, without specific diagnosis. Cerebral MRI showed cortical dysplasia. She appeared to have the same *PTEN* mutation as her mother and the diagnosis of Cowden disease was made at the age of 10 years. She suffered from recurrent ENT infections in childhood, which improved after adenotomy. Screening for immunodeficiency revealed IgA deficiency, IgG_2 deficiency and specific polysaccharide antibody deficiency (decreased response to pneumococcal polysaccharide vaccination; response to serotype 1,3,4,5,23 measured 4 weeks after Pneu23 vaccination <0.1 mg/ml). Within two years of follow up, she developed a mild hypogammaglobulinemia at the age of 12 years. She is not receiving immunoglobulin replacement therapy, because of a stable clinical condition. She is suffering from recurrent upper respiratory tract infections during the winter periods, which respond well to antibiotic treatment.

Patient 3 is a 6 year old boy, who has previously been published as a case of macrocephaly and hypogammaglobulinemia (case 2).[2] This child of non-consanguineous parents suffered from recurrent febrile episodes from the age of three months. Several dysmophic features

were observed, including macrocephaly. There was a mild developmental delay and brain MRI revealed delay in myelinisation of the periventricular white matter. He suffered from frequent upper respiratory tract infections. At the age of 21 month a diagnosis of hypogammaglobulinemia was made, in the presence of a decreased response to tetanus (<100IU/ml). Immunoglobulin replacement was initiated, after which the frequency and severity of infections improved. At the age of 6 years, a de novo heterozygous *PTEN* mutation was detected.

Patients without antibody deficiency:

Patient 4 Macrocephaly, delayed motor and metal development.

Patient 5 Macrocephaly, delayed motor and metal development.

Patient 6 Macrocephaly, delayed motor and metal development, periventricular white matter abnormalities on cerebral MRI.

Patient 7 Macrocephaly, delayed motor and metal development, lipoma's.

Patient 8 Macrocephaly, resection of sigmoid carcinoma.

Patient 9 Macrocephaly, resection borderline ovarian tumor, fibroadenoma breast.

Flow cytometry

Six-color flow cytometric immunophenotyping of peripheral blood was performed on a CantoII (BD Biosiences) and data were analyzed using FACS Diva software (BD Biosiences). The following monoclonal antibodies were used: CD19-PerCP-Cy5.5, CD19-PE-Cy7, CD19-APC (all SJ25C1), CD5-APC (L17F12), CD45-PerCP (2D1), CD19-APC (SJ25C1), CD38-PE, CD38-APC and CD38-PE-Cy7 (HB7), CD27-APC (L128), CD3-PerCP-Cy5.5 (SK7) and CD8-APC-Cy7 (SK1) all from BD Biosciences, polyclonal IgD-FITC, IgD-PE and IgM-PE (all from Southern Biotechnologies), polyclonal IgG-FITC (Kallestad), IgA-FITC and IgA-PE (IS11-8E10; Miltenyl Biotech), CD24-FITC (gran-B-ly-1; Sanquin), CD21-PE (LB21; Serotech), CD45RO-FITC (UCHL1; DAKO), CD4-PC7 (SFCI12T4D11) and CD45-RA-RD1 (2H4; all from Beckman Coulter). The cell counts of the peripheral B-cell subsets (transitional B-cells, naive mature B-cells, and six memory B-cell subsets) and T-cell subsets were compared to age matched controls.

Analysis of SHM and CSR

SHM were analyzed as described previously.[3] In short in V_H3-Cα, V_H4-Cα, V_H3-Cγ, and V_H4-Cγ transcripts were amplified, cloned into pGEM-T easy vector (Promega, Madison, WI) and sequenced using Sanger sequencing on the ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems). Sequences were analyzed with the International ImMunogGeneTics database (IMGT, http://imgt.cines.fr) V-quest analysis tool in order to assign the V, D and J segments but also for the identification of SHM's. The mutation frequency and *IGHV* isotype and subclass distribution was determined for V_H gene of each transcript in 7 controls and 9

patients. The number of transcripts and mutations frequency per individual can be found in Online repository Table E2.

Statistical analysis

Statistical analysis was performed with Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA, USA). Whenever two groups with continuous outcomes were compared, the Mann-Whitney test was applied. For categorical variables the $\chi 2$ or Fisher's exact tests were used. For groups with unequal variances, an unpaired t-test with Welsh correction was applied. Statistical significance was set at two sided $P<0.05$. In case of multiple comparisons we performed Bonferroni correction for multiple testing.

REFERENCES

1. Driessen, G.J., et al., *B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency.* Blood, 2011. **118**(26): p. 6814-23.
2. Cogulu, O., et al., Two cases of macrocephaly and immune deficiency. Clin Dysmorphol, 2007. 16(2): p. 81-4.
3. IJspeert, H., et al., *Artemis splice defects cause atypical SCID and can be restored in vitro by an antisense oligonucleotide*. Genes Immun, 2011. **12**(6): p. 434-44.

SUPPLEMENTAL FIGURES AND TABLES

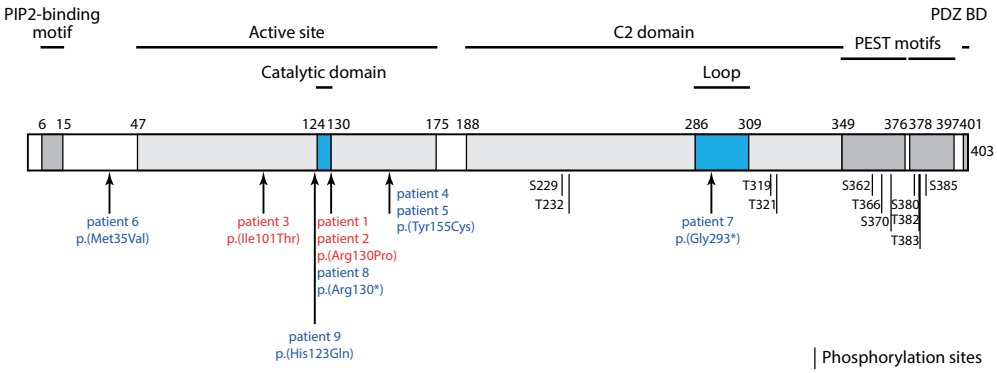


Figure E1. A structural overview of the PTEN protein with type of mutations
The overview includes distinct domains, phosphorylation sites and the locations as well as the type of mutations within the patient cohort. Red fonts indicate patients with hypogammaglobulinemia, blue fonts patients without hypogammaglobulinemia

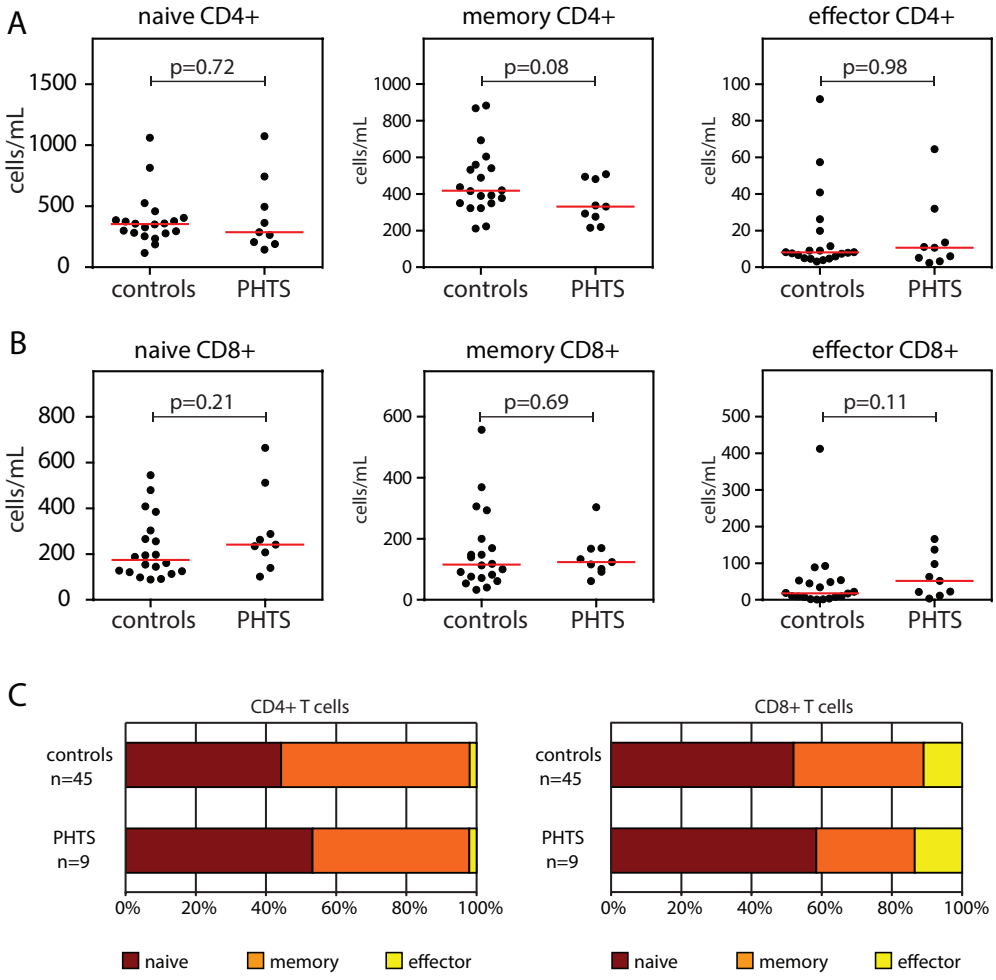


Figure E2. Naive, memory and effector T-cell subsets in PHTS patients.
Absolute numbers of $CD4^+$ (**A**) and $CD8^+$ (**B**) T-cell subsets. **C**. Relative distributions of $CD4^+$ and $CD8^+$ T-cell subsets. Naïve T-cells ($CD45RA^+CD27^+$); memory T-cells ($CD45RA^-CD27^+$) and effector T-cells ($CD45RA^+/-CD27^-$).

Table E1. Characteristics of PHTS patients

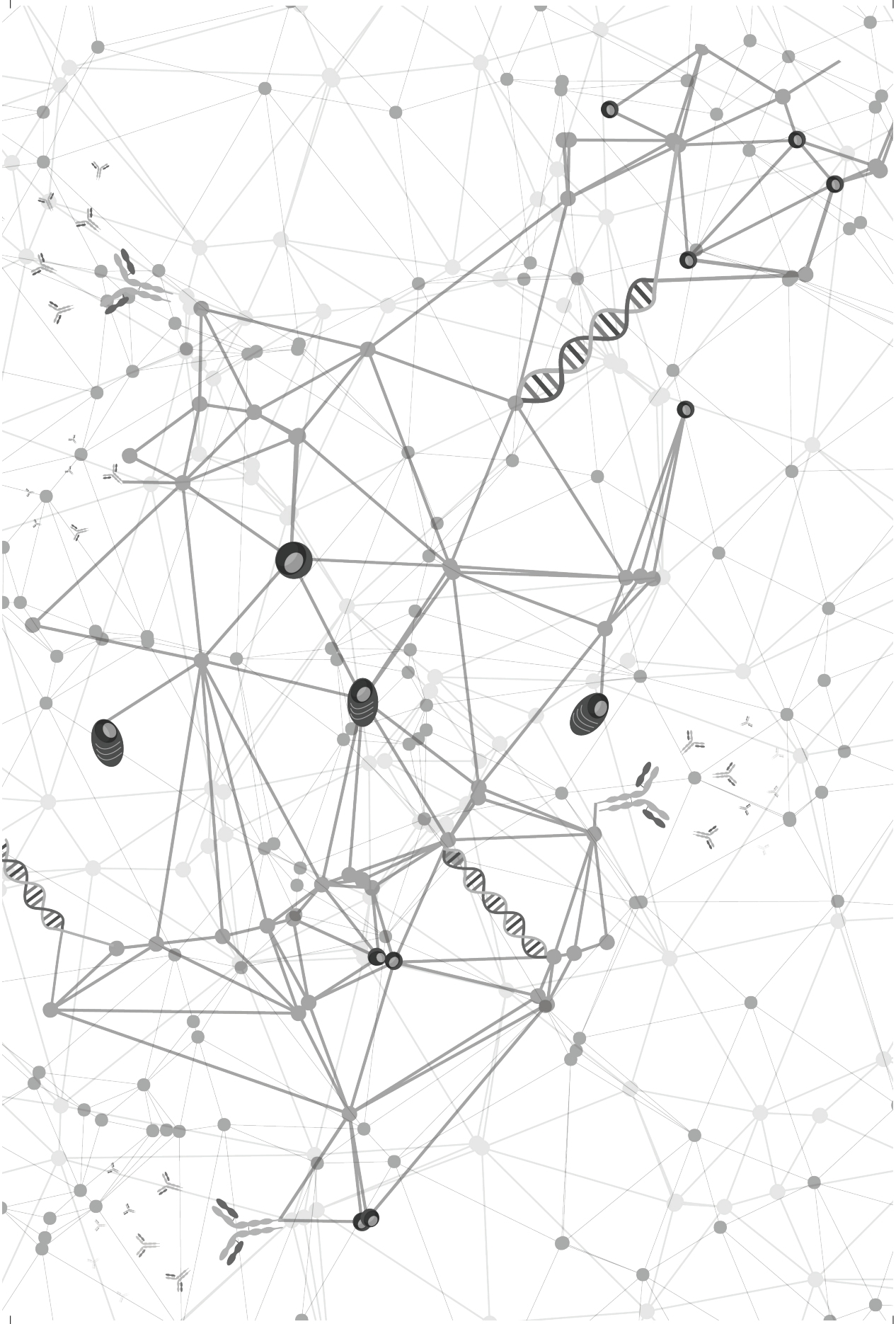
Pat.	Sex	Age	PTEN Mutation	Clinical diagnosis	B-cells*	T-cells*	CD4*	CD8*	CD4/CD8 ratio	NK cells*	IgG g/L	IgA g/L	IgM g/L
1	F	43	c.389G>C p. Arg130Pro	PHTS, hypogamma	0.16 (0.1-0.4)	1.37 (0.7-2.1)	0.58 (0.3-1.4)	0.71 (0.2-1.2)	0.82	0.06 (0.09-0.6)	4.7 (6,0–12,3)	<0.07 (0.3-1.6)	0.30 (0,5–2,0)
2	F	12	c.389G>C p.Arg130Pr0	PHTS, hypogamma	0.38 (0.2-2.1)	1.18 (0.8-3.5)	0.53 (0.4-2.1)	0.51 (0.2-1.2)	1.03	0.26 (0.07-1.20)	5.5 (6,0–12,3)	0.16 (0.3-1.6)	0.65 (0,5–2,0)
3	M	6	c.302T>C p.Ile101Thr	PHTS, hypogamma	0.41 (0.2-1.6)	1.77 (0.7-4.2)	0.93 (0.3-2.0)	0.68 (0.3-1.8)	1.38	0.36 (0.09-0.9)	1.5 (4,0–11,0)	0.07 (0,1–1,6)	0.69 (0,5–1,8)
4	M	6	c.464A>G p.Tyr155Cys	PHTS	1.01 (0.2-1.6)	3.20 (0.7-4.2)	1.69 (0.3-2.0)	0.95 (0.3-1.8)	1.78	1.29 (0.09-0.9)	9.0 (4,0–11,0)	0.80 (0.3-1.6)	0.90 (0,5–1,8)
5	M	37	c.464A>G p.Tyr155Cys	PHTS	0.18 (0.1-0.4)	1.01 (0.7-2.1)	0.68 (0.3-1.4)	0.28 (0.2-1.2)	2.41	0.43 (0.09-0.6)	14.0 (7,0–16,0)	2.60 0,7–4,0	1.80 (0,4–2,3)
6	M	8	c.103A>G p.Met35Val	PHTS	0.76 (0.2-1.6)	2.25 (0.7-4.2)	1.25 (0.3-2.0)	0.85 (0.3-1.8)	1.48	0.16 (0.09-0.9)	10.0 (4,0–11,0)	0.70 (0.3-2.0)	0.50 (0.5-2.0)
7	M	7	c.877G>T p.Gly293X	PHTS	0.91 (.2-2.6)	1.50 (0.7-4.2)	0.97 (0.3-2.0)	0.42 (0.3-1.8)	2.32	0.33 (0.09-0.9)	7.0 (4,0–11,0)	0.50 (0.3-2.0)	1.70 (0.5-2.0)
8	F	18	c.388C>T p.Arg130X	PHTS	0.20 (0.1-0.4)	1.28 (0.7-2.1)	0.78 (0.3-1.4)	0.23 (0.2-1.2)	1.83	0.11 (0.09-0.6)	12.4 (7,0–16,0)	0.82 0,7–4,0	1.68 (0,4–2,3)
9	F	32	c.369C>G p.His123Gln	PHTS	0.23 (0.1-0.4)	1.07 (0.7-2.1)	0.70 (0.3-1.4)	0.31 (0.2-1.2)	2.27	0.12 (0.09-0.6)	13.7 (7,0–16,0)	1.12 0,7–4,0	1.58 (0,4–2,3)

Pat.; Patient number, M; Male, F; Female, PHTS; PTEN Hamartoma Tumor Syndrome, hypogamma; hypogammaglobulinemia. Mutation nomenclature is according to HGVS guidelines (http://www.hgvs.org). *) . Age related normal values are indicated between brackets. Age related normal values of lymphocyte subsets are adapted from Comans-Bitter et al Pediatr. 1997 Mar;130(3):388-93. Lymphocyte subset counts are given as 109/L; bold values are below the normal range. Immunoglobulin levels of levels of patient 1 and 3 are before immunoglobulin replacement was started.

Table E2. Number of analysed IgG and IgA transcripts and percentage SHM per sample

	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	% SHM IgG	% SHM IgA
Control 1	12	6	0	4	20	9	7.2	6.2
Control 2	25	6	0	0	23	7	5.9	4.8
Control 3	19	8	1	0	14	4	7.1	6.3
Control 4	14	4	4	1	23	2	6.1	7.7
Control 5	25	18	3	2	21	19	6.5	7.1
Control 6	20	19	4	0	21	33	4.9	5.2
Control 7	16	12	6	0	24	9	7.9	7.6
Control 8	43	17	6	0	27	23	6.2	4.3
Patient 1	1	0	7	0	0	0	0.6	nd
Patient 2	12	0	1	0	10	0	3.4	4.8
Patient 3	14	0	3	0	7	1	2.8	4.3
Patient 4	14	3	1	0	9	3	4.2	4.1
Patient 5	11	1	1	0	9	0	6.8	6.1
Patient 6	12	1	2	0	12	0	5.9	3.2
Patient 7	18	5	3	0	7	3	3.3	3.3
Patient 8	3	2	0	0	4	1	4.2	8.5
Patient 9	15	9	0	2	8	2	3.4	5.9

SHM; somatic hypermutation; nd: not determined



Chapter 4.3

Genetic defects in PI3Kδ affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infections

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ABSTRACT

Background: Mutations in *PIK3CD* and *PIK3R1* cause activated PI3K-δ syndrome (APDS), by dysregulation of the PI3K-AKT pathway.

Methods: We studied precursor and peripheral B-cell differentiation and apoptosis via flowcytometry. Furthermore, we performed AKT-phosphorylation assays and somatic hypermutations and class switch recombination analysis.

Results: We identified 13 patients of whom 3 had new mutations in *PIK3CD* or *PIK3R1*. Patients had low total B-cells numbers with increased frequencies of transitional B cells and plasmablasts, while the precursor B-cell compartment in bone marrow was relatively normal. Basal AKT phosphorylation was increased in lymphocytes from APDS patients and natural effector B cells where most affected. PI3K mutations resulted in altered SHM and CSR and increased apoptosis.

Conclusions: The B-cell compartment in APDS patients is affected by the mutations in PI3K. There is reduced differentiation beyond the transitional stage, increased AKT phosphorylation and increased apoptosis. This B-cell phenotype contributes to the clinical phenotype.

Keywords: B cells, PI3Kδ, APDS, B-cell differentiation, apoptosis

INTRODUCTION

Gain of function mutations in *PIK3CD* and *PIK3R1* have been described to cause activated PI3K-δ syndrome (APDS),[1] which is also referred to as p110 delta activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency (PASLI).[2] The disease is characterized by recurrent respiratory tract infections, progressive airway damage, EBV and CMV viremia and lymphopenia due to dysregulation of the PI3K-AKT pathway in T cells.[1-6] Earlier studies have described hyperactivity in the PI3K-AKT pathway in patient T cells, resulting in increased proliferation and fast differentiation into short living effector T-cells. This results in reduced memory formation and reduced numbers of $CD4^+$ T cells, resulting in ineffective control of infections. Moreover, patients have been reported to suffer from B-cell lymphomas,[7] primary sclerosing cholangitis[8] and hyper IgM syndrome.[9, 10] These latter reports suggest B cell involvement in APDS. Additionally one patient has been described, who lacked the pα-subunit of PI3K, which caused absence of B-cell lineages and agammaglobulinemia.[11] Moreover, the first APDS patient described, was identified in a cohort of children with primary B-cell immunodeficiencies.[12] Recent studies on patients with loss of function mutations in Phosphatase and tensin homolog (PTEN), the oppositional counterpart of PI3K, have indicated that hyperactivation of the PI3K-AKT pathway leads to reduced antibody production and T cell lymphopenia.[13, 14] This indicates that besides T cells, the B cells can be heavily affected in APDS.

PI3K is critically important in B-cell development and function.[15-22] It can be activated via multiple surface receptors like the B-cell receptor (BCR),[21] CD19 and TLRs[19, 22] and in turn, PI3K can activate AKT via induction of PIP3.[16, 17, 23] Phosphorylated AKT regulates multiple downstream effectors,[24] executing different cellular functions such as regulation of metabolism, growth and proliferation (via mTOR),[25-27] and transcriptional regulation (via FoxO).[28, 29] Furthermore, mice lacking PTEN show that the PI3K-AKT signaling cascade is needed for proper induction of AID, which is the main regulator of somatic hyper mutation and class switch recombination.[18, 30, 31] All of these downstream effectors of AKT have specific functions in the different developmental stages of B cells, indicating that dysregulation of this signaling network has major impact on B-cell development, homeostasis and maturation.[32]

We describe 13 patients with genetically confirmed APDS, suffering from recurrent infections. Our aim was to specifically investigate the precursor and peripheral B-cell compartment in these patients. We show a skewed distribution of the B-cell subsets in peripheral blood, while the composition of the precursor B-cell compartment was relatively normal. Additionally, we show that the pAKT/AKT ratio is disturbed in various B-cell subsets and that B-cell memory formation is hampered. Furthermore, B cells from APDS patients

show an increased rate of apoptosis. All of this contributes to hypogammaglobulinemia and recurrent infections.

MATERIALS AND METHODS

Cell samples and ethical approval

From all patients DNA from blood granulocytes, after separation using Ficoll Hypaque, was available for Sanger sequencing and mutation confirmation. Frozen bone marrow samples were available from three patients, fresh bone marrow was available from one patient. Frozen peripheral blood mononuclear cells were available in variable amounts from six patients. From some patients, additional material could be collected during clinical visits. Peripheral blood was obtained from the patients and (age matched) healthy controls, with informed consent and according to the guidelines of the local Medical Ethics Committees. Clinical data were provided by treating physicians.

Flowcytometric immunophenotyping of peripheral blood and bone marrow

Eight-color flowcytometric immunophenotyping of peripheral blood was performed on a Canto II (BD Biosciences, San Jose, CA, USA). Data were analyzed using FACS Diva (BD Biosciences) and Infinicyt software (Cytognos, Salamanca, Spain). Antibodies against the following markers were used: CD16-PE (3G8), CD4-PerCP-Cy5,5 (SK3), CD3-APC (SK7), CD38-FITC (HB7), CD21-APC (B-ly4), TCRgd-PE-Cy7 (11F2), IgG-PE (G18-145) (all BD Biosciences), CD27-BV421 (O323) IgD-FITC (IA6-2), IgM-PerCP-Cy5,5 (MHM-88), IgM-BV510 (MHM-88), CD5-PE (UCHT-2), IgD-PerCP-Cy5,5 (IA6-2) (all Biolegend, San Diego, CA, USA) CD45-PO (HI30), IgE-FITC (H15701) (all Invitrogen, Carlsbad, CA, USA), CD8-FITC (UCH-T4), CD56-PE (C5.9) (both Cytognos), CD45RA-APC-Alexa750 (2H4), CD19-Pe-Cy7 (J3-119), CD24-APC-Alexa750 (ALB9), CD38-APC-Alexa750 (LS198-4-3) (all Beckman Coulter, Fullerton, CA, USA) IgA-FITC (IS11-8E10), IgA-PE (IS11-8E10) (both Miltenyi, Bergisch Gladbach, Germany).

Bone marrow mononuclear cells were firstly isolated using Ficoll Hypaque (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions, and frozen in liquid nitrogen. Prior to staining, cells were thawed and washed. Flowcytometric immunophenotyping of bone marrow samples was performed on a LSR Fortessa (BD BioSciences). For extracellular staining we used antibodies against the following markers: IgM-BV510 (MHM-88), CD38-BV605 (HIT2), CD20-PB (2H7, all Biolegend), CD34-APC (8G12), IgD-PeCF594 (IA6, both BD BioSciences), CD19-PC7 (J3-119, Beckman Coulter, Fullerton, CA, USA) and CD10-APC-C750 (Cytognos). For intracellular staining we used antibodies against IgM-PerCPcy5.5 (MHM-88, Biolegend), TdT-FITC (HT6, Supertechs, Rockville, MD, USA) and CD79a-PE (HM47 Beckman Coulter). We analyzed the data in Infinicyt software

Version 1.8 (Cytognos). Annexin and 7-AAD staining was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) together with the antibodies for extracellular staining of the bone marrow, according to manufacturer's instructions.

Molecular analysis

Sequence analysis of *PIK3CD* and *PIK3R1* was performed following PCR-amplification of the regions that harboured previously described mutations with TaqGold™ (Life Technologies) or Expand long template PCR system (Roche, Basel, Swiss), followed by direct sequencing on an ABI Prism 3130 XL fluorescent sequencer (Applied Biosystems, Bleiswijk, The Netherlands). Primer sequences are available upon request. Sequences were analyzed with CLC DNA-workbench software (CLCBio, Aarhus, Denmark) and compared to the NCBI reference sequences (NM_005026 and NM_181523).

Phospho-flow

For phospho-flow analysis, freshly isolated or previously frozen and thawed PBMC`s were rested for 1 hour in medium without FCS and stained with anti-CD20-BV421 (2H7, Biolegend) prior to stimulation with anti-IgM (Southern Biotech, Birmingham, AL, USA) and anti-CD19 (J3-119, Beckman Coulter). PMA was used as positive control. Cells were fixed using Cytofix (BD Biosciences) buffer according to manufacturer's protocol. After fixation, cells were washed and incubated with anti-CD19-PC7 (HIB19, Biolegend) for 15 minutes at room temperature prior to permeabilization with Perm Buffer III (BD-Biosciences) according to manufacturer's protocol. Cells were then stained with antibodies against pAKT-Alexa488 (55/PKBa/Akt) or AKT-alexa488 (M89-61) (both BD Biosciences),CD3-BV711 (UCHT-1, BD Biosciences), CD27-APC (L128, BD Biosciences), CD56-BV510 (HCD56, Biolegend), and IgD-PE (SBA) for 30 minutes at room temperature and analysed on an LSRII flow cytometer (BD Biosciences). Different T cell and B-cell subsets were analysed for AKT and pAKT expression using Infinicyt software. pAKT/AKT ratio`s where calculated using mean fluorescent intensities.

SHM and CSR analysis

SHM and CSR were analyzed as described previously.[33, 34] In short, *IGA* and *IGG* transcripts were amplified from PBMC-derived cDNA of patients, data for healthy controls were collected previously.[34] These transcripts were purified and sequenced using 454 sequencing. Using IMGT HighV-Quest[35] and an extended version the Antigen Receptor Galaxy Tool.[36, 37] Sequences were analyzed for AID targeting, SHM and CSR.

Analysis of apoptosis after in vitro stimulation

Frozen PBMCs from patients (n=3) and healthy controls (n=5) where thawed and cultured 24-well plates (1x10^6 PBMCs per well) in 1 ml of RPMI $^+$ HEPES culture medium, supplemented with 10% FCS, penicillin (100U/ml) and streptomycin (100µg/ml) at 37˚C and stimulated with either anti-Ig antibodies (mix of anti-IgM, anti-IgA and anti-IgG, 10ug/ml) and anti-CD19 antibody (2ug/ml) or with PMA (20nM). Unstimulated cells where used as a negative control. Cells where harvested after 3, 6 and 24 hours. For the last time-point 2 patient samples and 4 healthy control samples could be included. The cells where stained with tryphane blue and counted in a Countess II FL (Life-technologies), to determine the number of living cells per well. Before culturing and at each time-point and stimulation condition flow cytometric analysis of the cells was performed using antibodies against the following targets: CD27-BV421 (O323), IgM-BV510 (MHM-88), CD38-BV605 (HIT2), Annexin-V-APC (all Biolegend), Fixable Viability Dye eFluor® 520 (eBioscience), CD20-PE (L27), IgD-PeCF594 (IA6, both BD BioSciences), CD24-APC-Alexa750 (ALB9), CD19-PC7 (J3-119, both Beckman Coulter). After staining, cells were fixed in 1% paraformaldehyde prior to flow cytometric analysis on a LSR Fortessa (BD BioSciences). Analysis was performed using Infinicyt software Version 1.8 (Cytognos).

Statistical analysis

Relative distributions of B-cell subsets were analyzed using a two-tailed T-test, ($p<0.05$ was considered statistically significant) and pAKT/AKT ratio`s, AID targeting frequencies, and apoptosis rates were compared using the non-parametric Mann-Whitney U test ($p<0.05$ was considered statistically significant) in the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

RESULTS

Mutation analysis and patient identification

We identified 13 patients from nine different families (Table 1). Nine patients were heterozygous for the previously described E1021K (c.3061G>A) mutation,[1, 2] one carried the previously described E525K (c.1537G>A) mutation,[2] and one patient a novel R929C (c.27845C>T) mutation (Figure 1). This variant is mapped to the catalytic domain of p110δ, like the E1021K mutation. This patients has a comparable clinical phenotype as patients with proven pathogenic mutations and pAKT was increased in all lymphocyte subsets (as discussed below), thus we concluded that this mutation is disease causing. We identified one patient with a new heterozygous N564K (c. 1692C>G) mutation in *PIK3R1* (1), which is located in the SH2 domain of *PIK3R1* coding for the p85α regulatory subunit. This mutation

Table 1. Patient characteristics

Pt number	Age (y)	M/F	Mutation	WBC (x10^9/L)	T/B/NK cells numbers (abs, x10^9/L) [46]	Ig-levels[47]	Vaccination responses	Hepato / splenomegaly	CT-chest results	treatment	other
PI3K-1	3	M	E1021K	8.0	T cells:1.22 B cells: 0.07 ↓ NK cells: 0.17	IgG↓ IgA↓ IgM↑	unk	hepatosplenomegaly resolved after HSCT	no bronchiectasis	IVIG, AB HSCT (2014)	after transplant: full chimerism, T-and B cell reconstitution
PI3K-2.1	8	F	E1021K	5.5	T cells: 0.99 B cells: 0.20 NK cells: 0.33	IgG= IgA= IgM=	S. pneum↓		Bronchial wall thickening, air trapping	AB	
PI3K-2.2	32	M	E1021K	4.3	T cells: 0.72 B cells: 0.09 ↓ NK cells: 0.1	IgG= IgA= IgM=	S.pneum ↓		bronchial wall thickening lymphadenopathy	AB (prophylaxis) IvIg	MALT lymphoma gl. parotis → radioTx
PI3K-2.3	37	F	E1021K	7.6	T cells: 2.1 B cells: 0.05↓ NK cells: 0.07 ↓	IgG↓ IgA↓ IgM↑	S.pneum ↓ HiB =		bronchial wall thickening	SCIG (HyQvia) Tacrolimus AB (prophylaxis)	dysgerminoma + chemotherapy, → renal insufficiency → renal transplant
PI3K-3	10	M	E1021K	4.5	T cells 0.66 B cells 0.03 ↓ NK cells 0.12	IgG = IgA ↓ IgM↑	DTP= HiB = S.pneum ↓	no evident splenomegaly at 10y	No bronchiectasis	AB (prophylaxis)	chronic EBV infection
PI3K-4.1	20 *	M	E1021K	1.4	T cells: 0.17↓ B cells: 0.01↓ NK cells: 0.03↓	IgG↓ IgA↓ IgM↑	S.pneum ↓	splenomegaly	Airtrapping, no bronchiectasis	IVIG, Prednisone, Cellcept, Plaquenil AB (prophylaxis)	liver cirrhosis with PH, SLE-like disease, recurrent EBV infections
PI3K-4.2	43	F	E1021K	9.6	T cells: 0.43↓ B cells: 0.09↓ NK cells: 0.15	unk	unk	unk	unk	unk	unk
PI3K-5.1	25	M	E1021K	4.3	T cells: 0.5↓ B cells: 0.03↓ NK cells: 0.05↓	IgG ↓ IgA= IgM↑	Normal on IVIG		*Bronchiectasis* and *atelectasis*	IVIG	
PI3K-5.2	31	F	E1021K	3.0	T cells: 0.98 B cells: 0.04↓ NK cells: 0.03↓	IgG = IgA ↑ IgM=	No revac.		*Bronchiectasis* and *atelectasis*	IVIG	
PI3K-6	4	F	E525K	5.4	T cells: 0.84 ↓ B cells: 0.23 NK cells: 0.27	IgG= IgA= IgM=	S. pneum↓	hepatosplenomegaly	Atelectasis, lympadenopathy, bronchial wall thickening	AB, SCIG, sirolimus, prednisolone	
PI3K-7	44	M	R929C	4.4	T cells: 0.77 B cells: 0.06 ↓ NK cells:0.17	IgG ↓ IgA= IgM=	S. pneum↓			IvIg	Epilepsy Hemochromatosis
PI3KR1-1	6	M	N564K	6.7	T cells: 0.99 B cells: 0.24 NK cells: 0.26	IgG = IgA = IgM =	Normal after routine vacc in childhood		Diffuse abnormalities	AB IVIG	Tracheomalacy and double aortic arch macrocephaly
PI3KR1-2	15	M	c. 1425+2	10.3	T cells:1.76 B cells: 0.09 ↓ NK cells: 0.21	unk	unk	unk	unk	unk	Short stature, submandibular lymphadenopathy, colitis ulcerosa

Patients are numbered according to gene with mutation- number of the family, number within family. Age: at time of WBC, * age at time of BM sampling was 5y, Gender: M = male, F = female, unk = unknown, AB = antibiotics, PH = portal hypertension, S.pneum = S. pneumonia, HiB: H. influenza type B, DTP = diphtheria, tetanus, poliomyelitis, Ig/vaccination response
"=" means : is in normal range; ↓/↑ decreased/increased compared to normal age-matched range

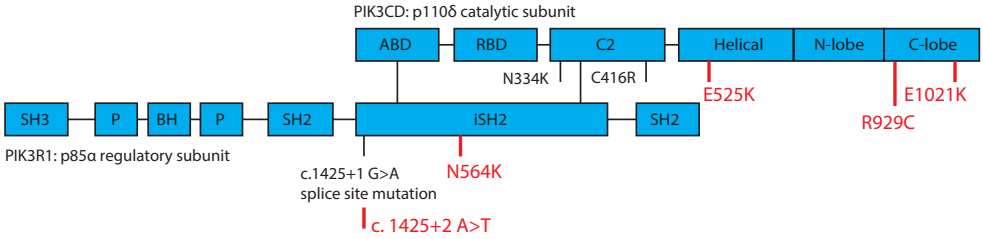

Figure 1.
Schematic representation of the PIK3CD and PIK3R1 proteins and their major domains. Indicated in red are the mutations found in this study.

is predicted to influence binding to p110δ. Again, clinical phenotype and pAKT status matched APDS patients. Additionally, we identified one patient with a mutation in a splice-site of PIK3R1: (c.1425+2A>T), which is the same site that harbors a previously described mutation.[4]

Clinical phenotype

All patients were diagnosed with an immune deficiency prior to mutational analysis. Our cohort consists of both children and adults (age range: 3y- 43y) (Table 1). Five patients are female and eight are male. All patients suffered from both upper and lower respiratory tract infections. Most infections were found to be caused by *S. pneumonia* and *H. influenza*. For 11 patients Ig-levels were determined. IgG was either normal (n=6/11) or decreased (n=5/11). IgA levels were reduced in four out of 11 patients and IgM levels were either normal (n= 6/11) or increased (n= 5/11). In seven patients vaccination responses against S. pneumoniae (polysaccharide vaccine) were tested; none of them responded to this vaccination. Where other vaccination responses were determined, these were normal. In 11 patients CT-chest results were available, showing bronchiectasis in two out of 11 patients, atelectasis in three out of 11 patients and thickening of the bronchial wall in five out of 11. Evident splenomegaly (confirmed by either ultrasound or CT-scan) was reported in three patients, and another patient has had episodes with splenomegaly. Most patients were treated with Ig-substitution (either subcutaneously or intravenously) (n=8) and/or prophylactic antibiotics (n=7). Two patients receive immunosuppressive drugs to treat their immune dysregulation and one patient is treated with tacrolimus because of a renal transplant. Patient PI3K-1 was treated successfully with a hematopoietic stem cell transplantation. Since then, his hepatosplenomegaly resolved. Patient PI3KR1-1 who harbors a heterozygous mutation in the SH2 domain of *PIK3R1*, coding for the p85α regulatory subunit, additionally suffers from macrophephaly, tracheomalacy and a double aortic arch in the context of megalencephaly-capillary malformation syndrome (MCAP).

B-cell differentiation in peripheral blood and bone marrow

For all patients, data on total lymphocyte counts was available. In 9 out of 13 patients fresh PBMC were available for phenotypical analysis. Total B cell numbers were normal (n=3/13) or decreased (n= 10/13) and total T cell numbers were normal (n=9/13) or decreased (n=4/13) (Table 1). Upon extended B-cell subset analysis we observed a relative increase in transitional (IgMD$^+$, CD38$^+$, CD24$^+$, CD27$^-$) B cells (upto 60%) in the majority of our patients (Figure 2A). In addition, we found a relative increase in plasma blasts (CD38^{hi}, CD27^{hi}) in all patients (Figure 2A), which were almost exclusively IgM positive. The naive (IgM/IgD$^+$, CD27$^-$), natural effector (IgD$^+$, CD27$^+$) and memory B-cell subsets (IgD$^-$, CD27$^+$) where relatively low in most patients (Figure 2A). Further dissection of the memory B cell compartment showed that the distribution of both non-switched memory B cells (IgM$^+$ and/or IgD$^+$, CD27$^+$) and switched memory B cells (IgA$^+$ or IgG$^+$, CD27$^+$ or CD27$^-$) was highly variable among patients, with a marked decrease in IgG$^+$ CD27$^+$ memory B cells (Figure 2B).CD27$^-$ switched memory B-cell subsets[38] where not different between patients and controls. Considering the IgA$^+$ CD27$^+$ memory B-cell subsets, we can distinguish patients with either normal or reduced numbers, however, division of the patients in these two groups is not associated with the type of mutation, age or another factor.

From 4 patients, bone marrow samples were available (3 frozen previously, 1 fresh sample) for flow cytometric immunophenotyping. cyCD79a expression was used as B-cell marker. Upon first analysis of proB cells (CD19$^-$ cyIgM$^-$, IgMD$^-$), preB-I cells (CD19$^+$ cyIgM$^-$, IgMD$^-$), preB-II cells (CD19$^+$, cyIgM$^+$, IgMD$^-$) and immature B cells (CD19$^+$, cyIgM$^+$, IgM$^+$ IgD$^{dim/+}$), relative B-cell precursor subsets distribution was comparable between patients and controls (n=3) (Figure 2C). However, upon extended analysis, including CD34, TdT and CD20 expression, both the preB-I and the preB-II cells can be dissected in smaller populations. Here, differences in population sizes between controls and patients were observed (Supplemental Figure 1A, B), especially the CD34$^-$ TdT$^+$ subsets with in the preB-I subset is increased in 3 out of 4 patients. We also found that the CD20 relative distribution of CD20$^+$ and CD20$^-$ cells is disturbed in preB-I cells from patients (Supplemental Figure 1C).Furthermore, we found an increase in CD19$^-$ B cells in patients. On average 1,1% (range: 0,0%- 2,2%) of CD19$^-$ cells was found in controls; in patients this ranged from 3,3% up to 36.6%. These cells were not pro-B cells and did not express CD38, and thus were not plasma cells. Upon staining with Annexin V combined with 7-AAD, cells that were positive for both markers showed a dim CD19 expression (Supplemental Figure1D), indicating that the CD19$^-$ cells might be apoptotic cells.

pAKT in B-cell subsets

To determine the effect of the mutations in PI3K on AKT phosphorylation, we set up a phospho-flow assay to determine the ratio of pAKT/AKT in the patients and controls.

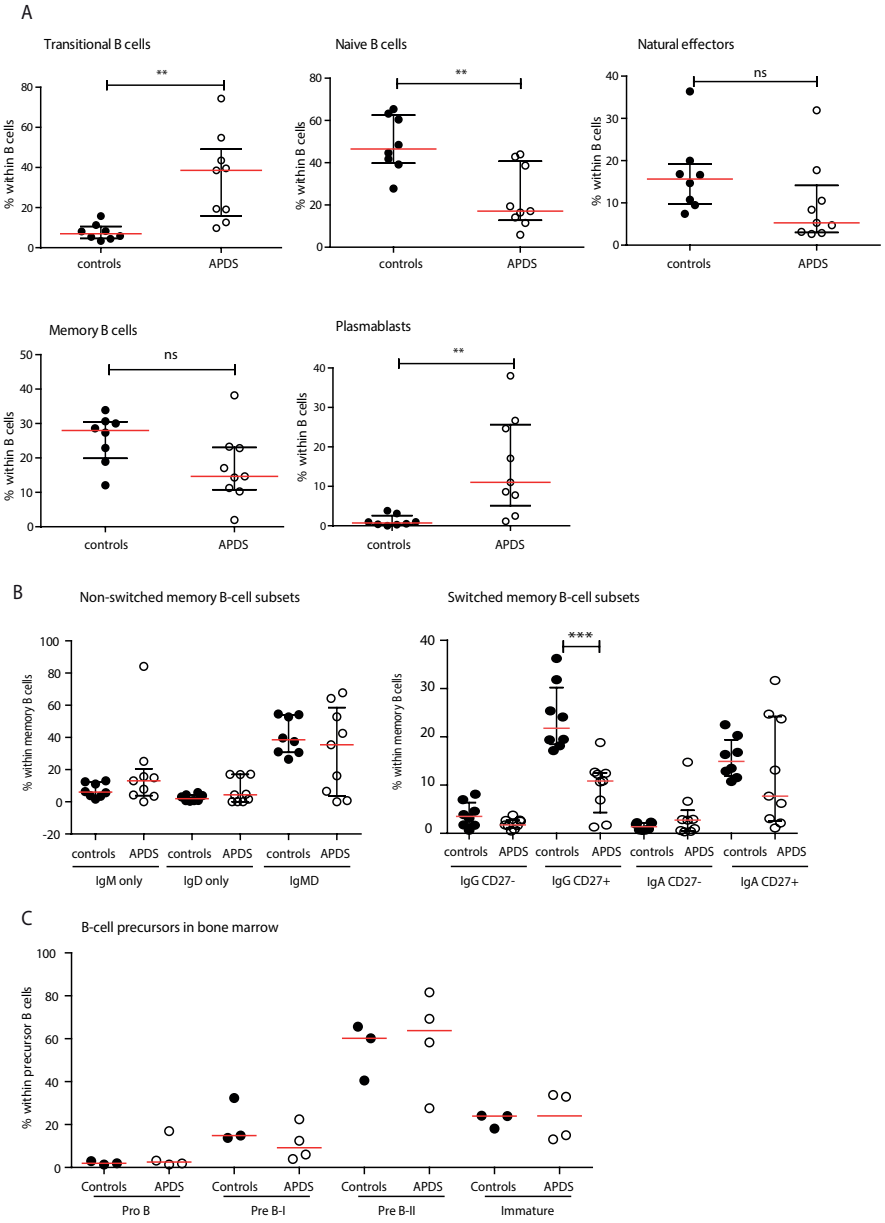

Figure 2. Immunophenotyping of B cells in peripheral blood (control n=8, APDS n=7) and bone-marrow (control n=3, APDS n=4).
A. Transitional, naïve and natural effector B cells from patients are significantly decreased, compared to controls, plasmablasts are relatively increased. (medians and interquartile range are indicated) **B**. Within the memory B cell compartment, specifically the IgG^+ $CD27^+$ subset is decreased in APDS patients. (medians and interquartile range are indicated) **C**. relative distribution of proB cells ($CD19^-$ $cyIgM^-$, $IgMD^-$), preB-I cells ($CD19^+$ $cyIgM^-$, $IgMD^-$), preB-II cells ($CD19^+$, $cyIgM^+$, $IgMD^-$) and immature B cells ($CD19^+$, $cyIgM^+$, IgM^+ $IgD^{dim/+}$) is comparable between patients and controls. (red lines represent the median).

We could discriminate B, T and NK cells, and within the B cells, 4 subsets: $CD27^-$ IgD^+ naive B cells, $CD27^+$ IgD^+ natural effector B cells, $CD27^+$ IgD^- memory B cells and $CD27^-$ IgD^- B cells. We determined levels of pAKT and AKT in all subsets, in order to compare the pAKT expression to the total AKT expression. The total AKT expression was lower in the patients (Figure 3A). We found an increase in the pAKT/AKT ratio in the unstimulated cells from the patients, which was most prominent in the $CD27^+$ IgD^+ fraction (Figure 3B). There was no difference between patients with mutations that were previously described and patients with new mutations. Upon stimulation with B-cell specific stimuli (anti-CD19 with anti-IgM) or PMA, control and patient cells showed increased pAKT levels, confirming the specificity of our assay (Supplemental Figure 2).

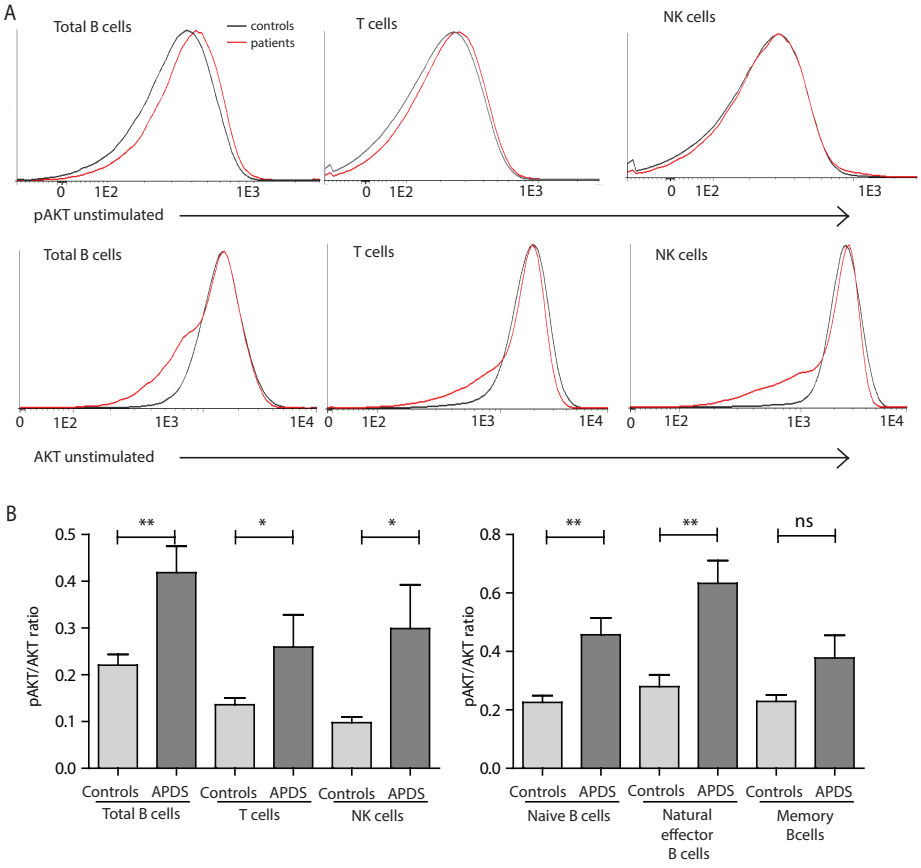

Figure 3. Phospho-flow analysis of APDS lymphocytes and control lymphocytes.
A. Normalized histograms pf pAKT expression of unstimulated total B cells, T cells and NK cells from pooled healthy controls (dark grey) and APDS patients (red). **B**. pAKT/AKT ratios of unstimulated samples from healthy controls (n= 8) and APDS patients (n=8). B-cell subsets where defined as: naive (IgD^+, $CD27^-$), natural effector (IgD^+ $CD27^+$) and memory B cells (IgD^- $CD27^+$) (error bars represent SEM).

SHM and CSR analysis

We studied the effect of PI3K mutations on SHM in sequences from IGG and IGA in 7 patients and compared them to a cohort of healthy controls.[34] In one patient (PI3K-4.1), we were unable to obtain IGG transcripts; this is consistent with the lack of IgG memory B cells in this patient. In another patient (PI3KR-2) we could not amplify any IGA transcripts. In one patient (PI3K-3) we could not amplify any IGA or IGG transcripts. There were no differences between patients and controls in CDR3-length or VDJ-gene family usage. The frequency of SHM is on average lower in patients compared to controls, although not significantly. When patients are compared to controls based on age, most patients are in the low-normal range. However, between individual patients the results are highly variable, with a few patients that have markedly decreased SHM frequencies (Figure 4A). There was no difference in the ratios of replacement/silent mutations, neither in the frame work regions, nor in the CDR-regions indicating normal antigen selection. We determined the constant gene region usage in all sequences and compared each individual patient to 4 age-matched controls. In class switch recombination within IgA subclasses there seemed to be a trend in the patients towards reduced IgA2 usage. Within the IgG subclasses, there is a reduction in IgG2 and IgG4, the more distal gene segments, in the patients (Figure 4B). Although our data on SHM and CSR suggest reduced ability of AID to initiate these processes, the targeting of AID is not significantly different between patients and controls (Figure 4C).

Apoptosis after in vitro stimulation

Since we observed abnormalities in CSR in some of the APDS patients, we aimed to study CSR *in vitro* in two patient samples and healthy control samples. However, upon stimulation of naive B cells *in vitro*, all of the patients cells where dead after three days in culture (data not shown). Therefore, we stimulated total PBMCs from three patients and five healthy controls for a short period *in vitro* with B cell specific stimulation (anti-CD19 and anti-Ig antibodies) and aspecific stimulation (PMA), to see if stimulation induced apoptosis in different B-cell subsets. After three and six hours of culture, we found reduced numbers of live cells in the wells containing the patients PBMCs (Figure 5a). After 24 hours, cell numbers where reduced in all wells, including the control wells. We stained the cells for Annexin V and a fixable viability dye combined with surface markers to enable detection of early apoptotic cells (Annexin V^+, viability dye^-) and late apoptotic cells (Annexin V^+, viability dye^+) within different B cells subsets. At all time-points, the number of both early and late apoptotic cells was increased in the patients B cells (Figure 5b and c) and in the patients non-B lymphocytes (data not shown). The apoptosis rate was not associated with stimulation. Within the B cells, we identified transitional B cells ($CD38^{hi}$, $CD24^{hi}$) naïve B cells ($CD27^-$, IgM^+ and/or IgD^+), natural effector B cells ($CD27^+$, IgD^+) and memory B cells ($CD27^+$,

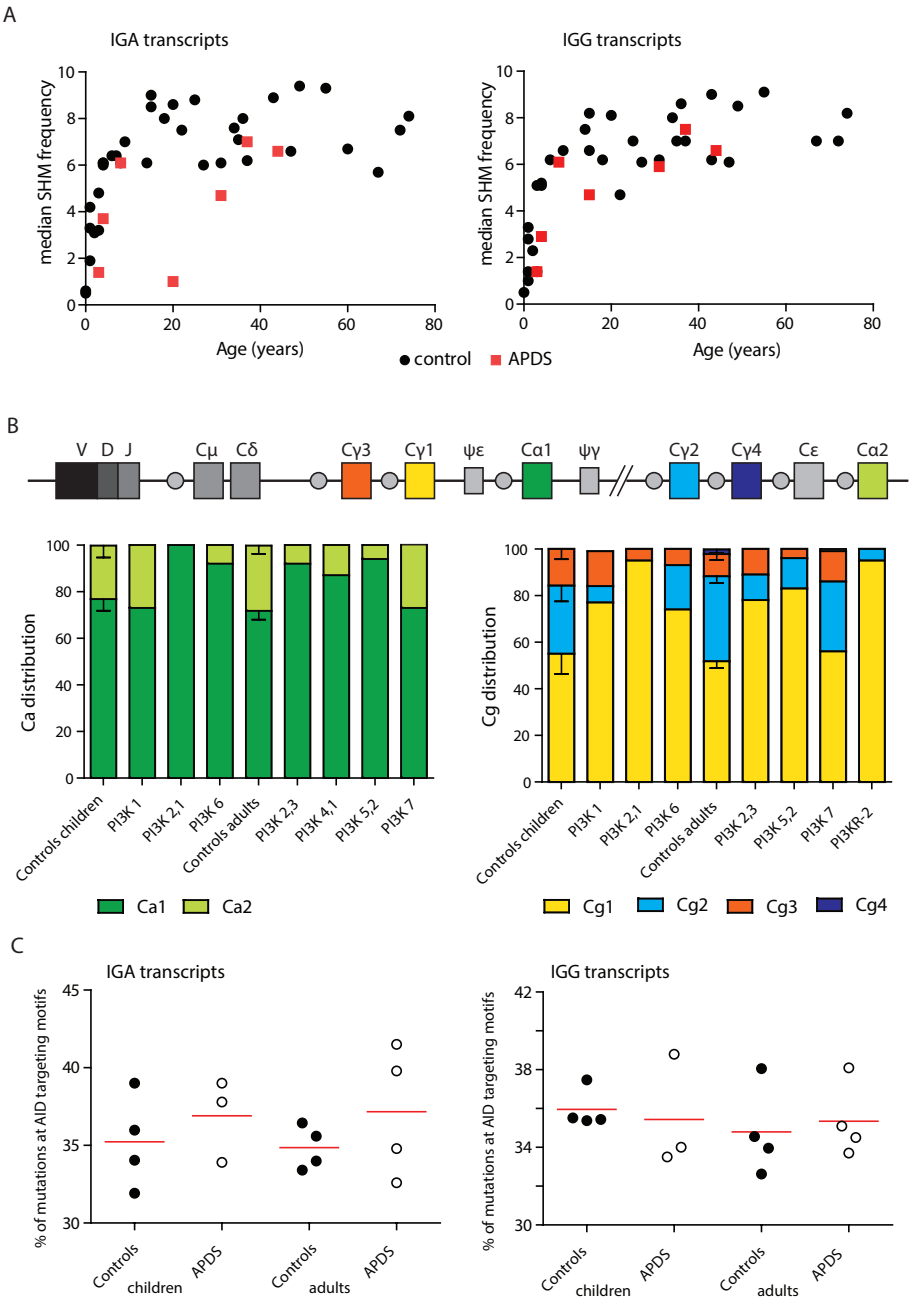

Figure 4. Analysis of SHM frequency, CSR and AID targeting in patients and healthy controls.
A. Median SHM frequency of controls (black dots) and APDS patients (red squares) **B**. Schematic overview of IGH locus and relative distribution of Ca and Cg subclass usage in patients and age matched healthy controls. Error bars in healthy controls represent SEM **C**. AID targeting represented by the percentage of SHM at AID targeting motifs in the patients and age matched healthy controls. (red lines represent the median).

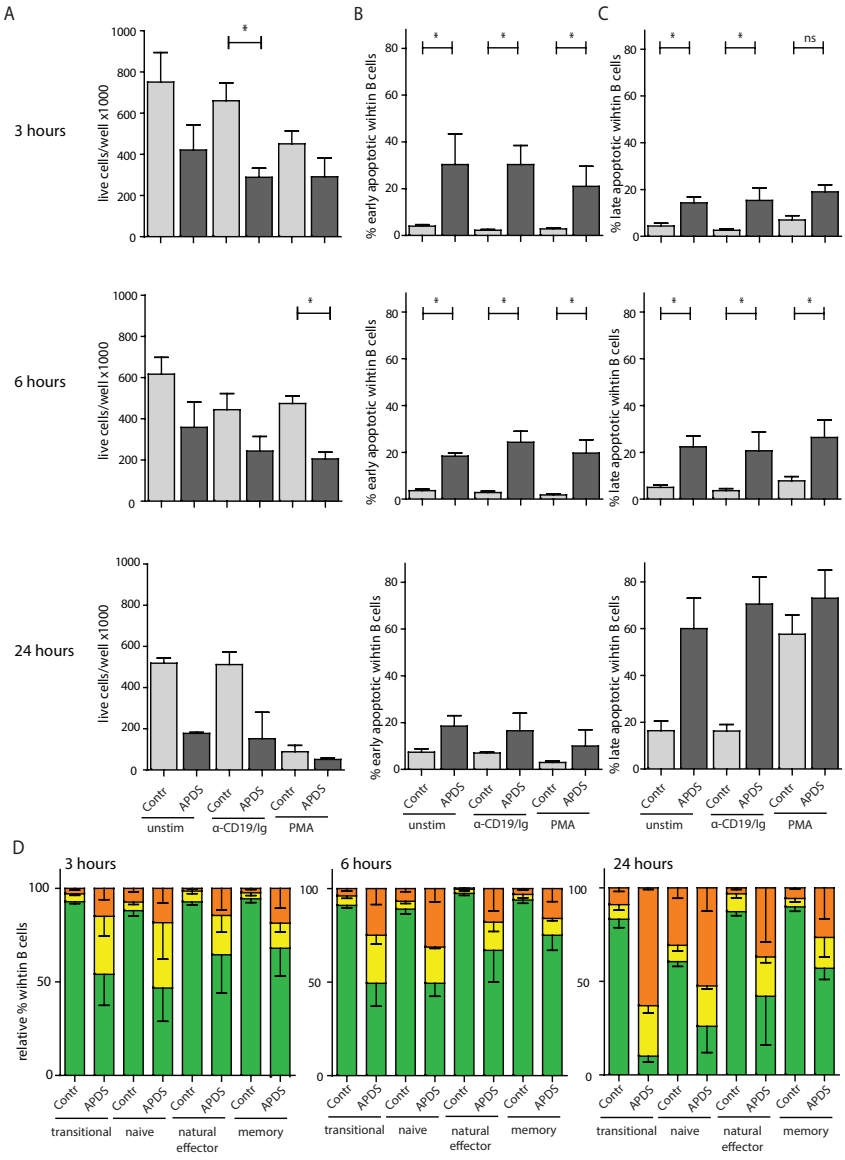

Figure 5. Analysis of *in vitro* apoptosis in B cells in patients (n=3 for 3 and 6 hours, n=2 for 24 hours) and healthy controls (n=5 for 3 and 6 hours, n=4 for 24 hours).
A. Number of life cells per well in controls (light grey) and patients (dark grey) after 3, 6 and 24 hours. **B+C**. Mean relative amount of early apoptotic cells (Annexin V^+, viability dye^-) and late apoptotic cells (Annexin V^+, viability dye^+) within the total B cell compartment in controls and patients after 3, 6 and 24 hours. (error bars represent the SEM). Patients samples where significantly different from the control samples ($P<0.05$, determined by Mann Whitney) but stimulation did not increase apoptosis rates. **D**. Distribution of life cells (green) early apoptotic cells (yellow) and late apoptotic cells (orange) within different B cell subsets in patients and controls at 3, 6 and 24 hours without stimulation in vitro. B-cell subsets where defined as: transitional ($CD38^{hi}$, $CD24^{hi}$), naive (IgD^+, $CD27^-$), natural effector (IgD^+ $CD27^+$) and memory B cells (IgD^- $CD27^+$) (error bars represent SEM).

IgD$^-$). At all time-points we observed more early and late apoptotic B cells in the patients samples (Figure 5d, Figure S3) than in the control samples. This effect was stronger in the transitional and naïve B cell subsets than in the natural effector and memory subsets.

DISCUSSION

In our cohort we identified 11 patients with known mutations in *PIK3CD* and two patients with new mutations in *PIK3R1*. Total peripheral blood B cells were reduced, and peripheral blood B-cell subsets showed a skewing towards transitional B cells and increased plasma blasts in most patients. In bone marrow, increases in CD19$^-$ cells and in CD34$^-$ TdT$^+$ cyIgμ$^-$ cells were detected. Phospho-flow analysis showed increased pAKT/AKT ratios in unstimulated patient cells, which was observed in all B-cell subsets. Levels of SHM were decreased in some patients, and CSR seemed skewed in the majority of the analyzed patients.

Patients have difficulties producing sufficient amounts of functional memory B cells and plasma cells. This is reflected in the absent response to vaccination with the polysaccharide S. Pneumoniae vaccine, a T-independent response. However, in a few patients in our cohort other vaccine responses (all T-dependent) were tested and those were found to be normal. This is in line with previous publications, in which the response to Tetanus toxoid was found to be normal.[1, 5, 32]

The phenotype and functional consequences for PI3KCD or PI3KR1 deficiency is described as highly variable.[5] In line with this, we find a great inter patient variability in the B cell compartment of our patients. In combination with the low number of PI3KR1 patients in our cohort, we cannot draw conclusions on what discriminates these two groups of patients. Until now, no major differences have been reported.[38]

Mutations in PI3K can affect maturation and differentiation of B cells and in many stages of development.[39] In the bone marrow, stepwise development is regulated by many transcriptional activators that in some instances depend on BR-signaling. PI3Kδ has been shown to play an important but non-essential role at the pre-B-cell receptor checkpoint in bone marrow.[40] P110δ deficient mice show a normal B-cell development[41] since p85α was shown to be able to take over the role of P110δ role in autonomous pre-BR signaling.[42] However, in these patients over-active signaling because of constitutive activation of the PI3K-AKT pathway may cause disturbance at the check-point. This might lead to cells routing to alternative maturation pathways because of increased proliferation signals and increased tonic signaling at the pre-B I stage might cause increased apoptosis. We hypothesize that the increase of CD34$^-$ TdT$^+$ cells that we observe in our patients, might be

the result of alternative maturation pathways. The loss of CD19 expression could be one of the first markers of apoptotic cells.

After differentiation in the bone marrow, transitional B cells move to the peripheral blood, where they can encounter antigens. In APDS patients, the number of transitional B cells is relatively increased, mainly because the numbers of all of the other subsets are decreased. This might indicate that cells do not easily pass the phase of antigenic stimulation. This might be due to an already hyperactive metabolic state of cells that not have encountered antigen yet, as indicated by our phospho-flow results. This may lead to cell death rather than differentiation.

A relation between PI3K and the induction of AID has been described in mice and in patients with PTEN mutations before.[14, 18, 29] According to our data, AID targeting seems to be intact, however, SHM frequencies are either reduced or in the lower normal range and there is reduced usage of the downstream *IGG* genes (*IGG2* and *IGG4*). Switching to these gene segments is seen more often when B cells enter the germinal center for a second time, to undergo a second round of affinity maturation. Our data suggest that cells can switch to IgA and IgG in a primary response, but only few cells enter the germinal center for a second time. This is supported by the finding that the $CD27^-$ memory compartment, which is formed in the first response to an antigen, of our patients is comparable to normal, but the $CD27^+$ IgG^+ compartment is significantly reduced.

Previously, increased spontaneous apoptosis rates were observed in subsets of CVID patients.[43-45] To test if apoptosis could contribute to the reduced memory B-cell formation in APDS, we stimulated cells from APDS patients *in vitro* and assessed the relative amounts of early and late apoptotic cells. Our data indicate increased apoptosis in all B-cell subsets of APDS patients, which is not dependent on stimulation. Although memory cells from APDS patients also show increased apoptosis, it seems that the naive compartment (transitional B-cells and naive B-cells) are more prone to apoptosis than the memory compartment (natural effector B-cells and memory B-cells).

CONCLUSIONS

We conclude that the novel mutations in *PIK3CD* and *PIK3R1* we found in our cohort lead to APDS with a comparable clinical phenotype as has been described earlier and with comparable increased pAKT status in multiple B-cell subsets. Our data show that the clinical phenotype is not only due to reduced effector function of $CD8^+$ T cells. There are reduced effector and memory B-cell compartments, which have aberrancies in SHM and CSR. The reduced memory formation might be caused by increased apoptosis of the B

cells. All of this contributes to the hypogammaglobulinemia and subsequent infections that are seen in these patients.

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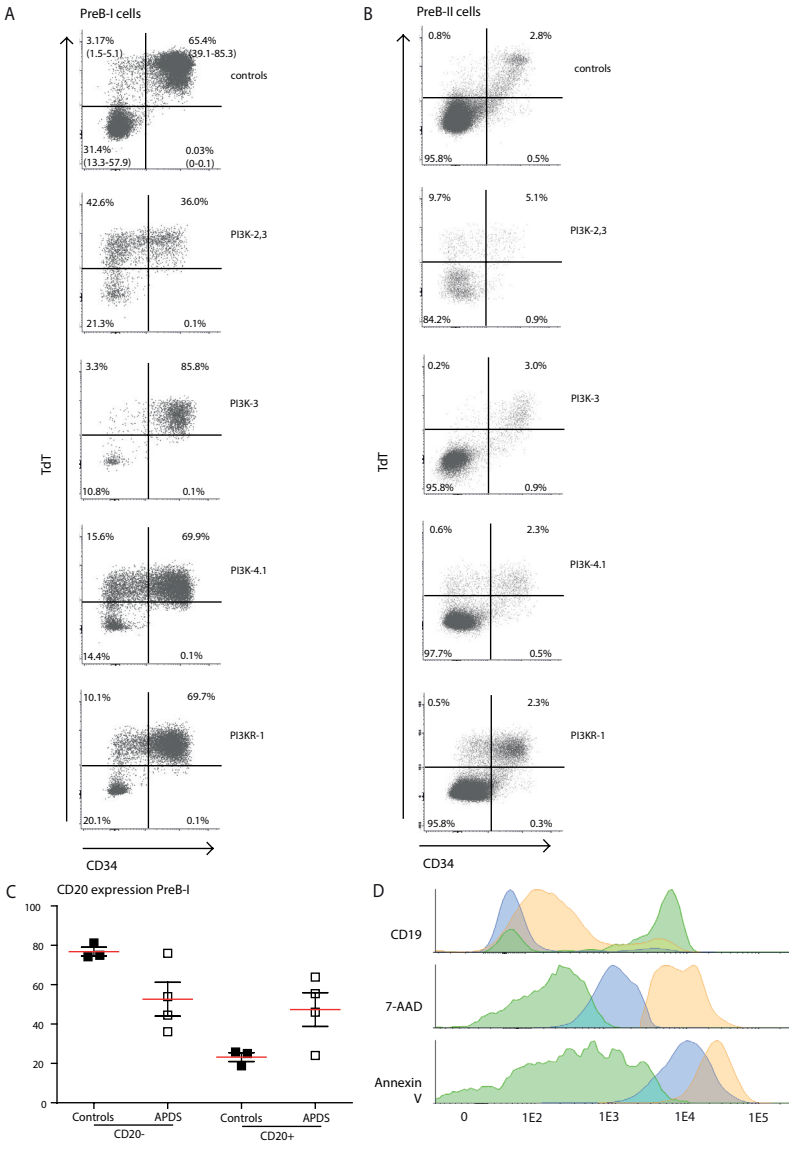
REFERENCES

1. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science, 2013. **342**(6160): p. 866-71.
2. Lucas, C.L., et al., *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency.* Nat Immunol, 2014. **15**(1): p. 88-97.
3. Deau, M.C., et al., *A human immunodeficiency caused by mutations in the PIK3R1 gene.* J Clin Invest, 2014. **124**(9): p. 3923-8.
4. Lucas, C.L., et al., *Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K.* J Exp Med, 2014. **211**(13): p. 2537-47.
5. Coulter, T.I., et al., *Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study.* J Allergy Clin Immunol, 2016.
6. Martinez-Saavedra, M.T., et al., *Gain-of-function mutation in PIK3R1 in a patient with a narrow clinical phenotype of respiratory infections.* Clin Immunol, 2016.
7. Kracker, S., et al., *Occurrence of B-cell lymphomas in patients with activated phosphoinositide 3-kinase delta syndrome.* J Allergy Clin Immunol, 2014. **134**(1): p. 233-6.
8. Hartman, H.N., et al., *Gain of Function Mutations of PIK3CD as a Cause of Primary Sclerosing Cholangitis.* J Clin Immunol, 2014.
9. Crank, M.C., et al., *Mutations in PIK3CD can cause hyper IgM syndrome (HIGM) associated with increased cancer susceptibility.* J Clin Immunol, 2014. **34**(3): p. 272-6.
10. Lougaris, V., et al., *Altered germinal center reaction and abnormal B cell peripheral maturation in PI3KR1-mutated patients presenting with HIGM-like phenotype.* Clin Immunol, 2015. **159**(1): p. 33-6.

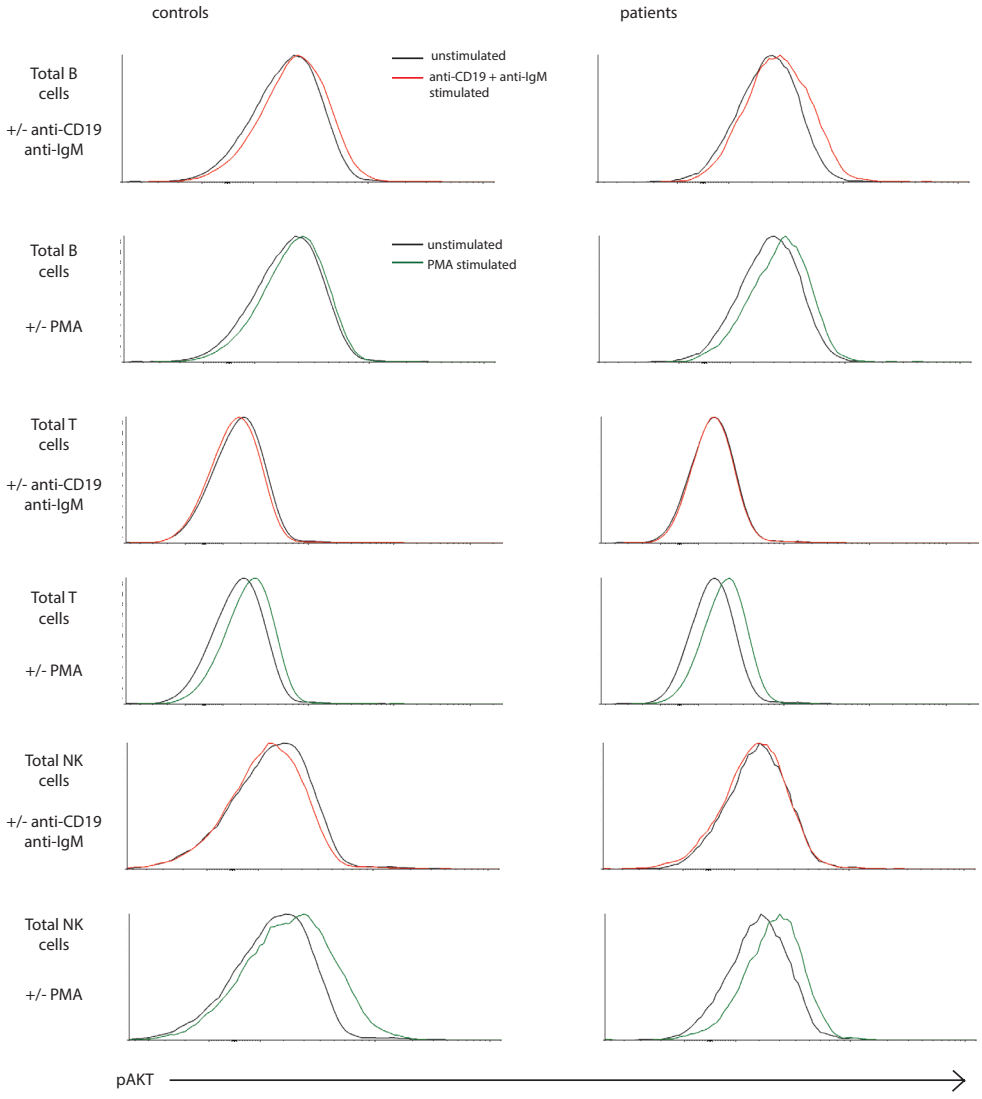
11. Conley, M.E., et al., *Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K.* J Exp Med, 2012. **209**(3): p. 463-70.
12. Jou, S.T., et al., *Identification of variations in the human phosphoinositide 3-kinase p110delta gene in children with primary B-cell immunodeficiency of unknown aetiology.* Int J Immunogenet, 2006. **33**(5): p. 361-9.
13. Browning, M.J., et al., *Cowden's syndrome with immunodeficiency.* J Med Genet, 2015.
14. Driessen, G., *Autosomal dominant germline mutations in PTEN impair clas switch recombination and somatic hypermutation and are associated with CVID like hypogammaglobulinemia*. 2016.
15. Kovesdi, D., S.E. Bell, and M. Turner, *The development of mature B lymphocytes requires the combined function of CD19 and the p110delta subunit of PI3K.* Self Nonself, 2010. **1**(2): p. 144-153.
16. Okkenhaug, K. and B. Vanhaesebroeck, *PI3K in lymphocyte development, differentiation and activation.* Nat Rev Immunol, 2003. **3**(4): p. 317-30.
17. Okkenhaug, K. and B. Vanhaesebroeck, *PI3K-signalling in B- and T-cells: insights from gene-targeted mice.* Biochem Soc Trans, 2003. **31**(Pt 1): p. 270-4.
18. Omori, S.A., et al., *Regulation of class-switch recombination and plasma cell differentiation by phosphatidylinositol 3-kinase signaling.* Immunity, 2006. **25**(4): p. 545-57.
19. Omori, S.A. and R.C. Rickert, *Phosphatidylinositol 3-kinase (PI3K) signaling and regulation of the antibody response.* Cell Cycle, 2007. **6**(4): p. 397-402.
20. Otipoby, K.L., et al., *The B-cell antigen receptor integrates adaptive and innate immune signals.* Proc Natl Acad Sci U S A, 2015. **112**(39): p. 12145-50.
21. Srinivasan, L., et al., *PI3 kinase signals BCR-dependent mature B cell survival.* Cell, 2009. **139**(3): p. 573-86.
22. Werner, M., E. Hobeika, and H. Jumaa, *Role of PI3K in the generation and survival of B cells.* Immunol Rev, 2010. **237**(1): p. 55-71.
23. Fruman, D.A., A.B. Satterthwaite, and O.N. Witte, *Xid-like phenotypes: a B cell signalosome takes shape.* Immunity, 2000. **13**(1): p. 1-3.
24. Hers, I., E.E. Vincent, and J.M. Tavare, *Akt signalling in health and disease.* Cell Signal, 2011. **23**(10): p. 1515-27.
25. Benhamron, S. and B. Tirosh, *Direct activation of mTOR in B lymphocytes confers impairment in B-cell maturation andloss of marginal zone B cells.* Eur J Immunol, 2011. **41**(8): p. 2390-6.
26. Laplante, M. and D.M. Sabatini, *mTOR signaling in growth control and disease.* Cell, 2012. **149**(2): p. 274-93.
27. Shimobayashi, M. and M.N. Hall, *Making new contacts: the mTOR network in metabolism and signalling crosstalk.* Nat Rev Mol Cell Biol, 2014. **15**(3): p. 155-62.
28. Dengler, H.S., et al., *Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation.* Nat Immunol, 2008. **9**(12): p. 1388-98.
29. Szydlowski, M., E. Jablonska, and P. Juszczynski, *FOXO1 transcription factor: a critical effector of the PI3K-AKT axis in B-cell development.* Int Rev Immunol, 2014. **33**(2): p. 146-57.

30. Suzuki, A., et al., *Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination.* J Exp Med, 2003. **197**(5): p. 657-67.
31. Janas, M.L., et al., *The effect of deleting p110delta on the phenotype and function of PTEN-deficient B cells.* J Immunol, 2008. **180**(2): p. 739-46.
32. Lucas, C.L., et al., *PI3Kdelta and primary immunodeficiencies.* Nat Rev Immunol, 2016.
33. Wentink, M.W., et al., *CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities.* Clin Immunol, 2015. **161**(2): p. 120-127.
34. H, I.J., et al., *Evaluation of the Antigen-Experienced B-Cell Receptor Repertoire in Healthy Children and Adults.* Front Immunol, 2016. **7**: p. 410.
35. Alamyar, E., et al., *IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS.* Methods Mol Biol, 2012. **882**: p. 569-604.
36. Moorhouse, M.J., et al., *ImmunoGlobulin galaxy (IGGalaxy) for simple determination and quantitation of immunoglobulin heavy chain rearrangements from NGS.* BMC Immunol, 2014. **15**(1): p. 59.
37. H, I.J., et al., *Strategies for B-cell receptor repertoire analysis in primary immunodeficiencies: from severe combined immunodeficiency to common variable immunodeficiency.* Front Immunol, 2015. **6**: p. 157.
38. Berkowska, M.A., et al., *Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways.* Blood, 2011. **118**(8): p. 2150-8.
39. Dulau Florea, A.E., et al., *Abnormal B-Cell Maturation in the Bone Marrow of Patients with Germline Mutations in PIK3CD.* J Allergy Clin Immunol, 2016.
40. Herzog, S., M. Reth, and H. Jumaa, *Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling.* Nat Rev Immunol, 2009. **9**(3): p. 195-205.
41. Okkenhaug, K., K. Ali, and B. Vanhaesebroeck, *Antigen receptor signalling: a distinctive role for the p110delta isoform of PI3K.* Trends Immunol, 2007. **28**(2): p. 80-7.
42. Ramadani, F., et al., *The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development.* Sci Signal, 2010. **3**(134): p. ra60.
43. Saxon, A., et al., *B cells from a distinct subset of patients with common variable immunodeficiency (CVID) have increased CD95 (Apo-1/fas), diminished CD38 expression, and undergo enhanced apoptosis.* Clin Exp Immunol, 1995. **102**(1): p. 17-25.
44. Yazdani, R., et al., *Role of apoptosis in common variable immunodeficiency and selective immunoglobulin A deficiency.* Mol Immunol, 2016. **71**: p. 1-9.
45. Yazdani, R., et al., *Comparison of various classifications for patients with common variable immunodeficiency (CVID) using measurement of B-cell subsets.* Allergol Immunopathol (Madr), 2016.

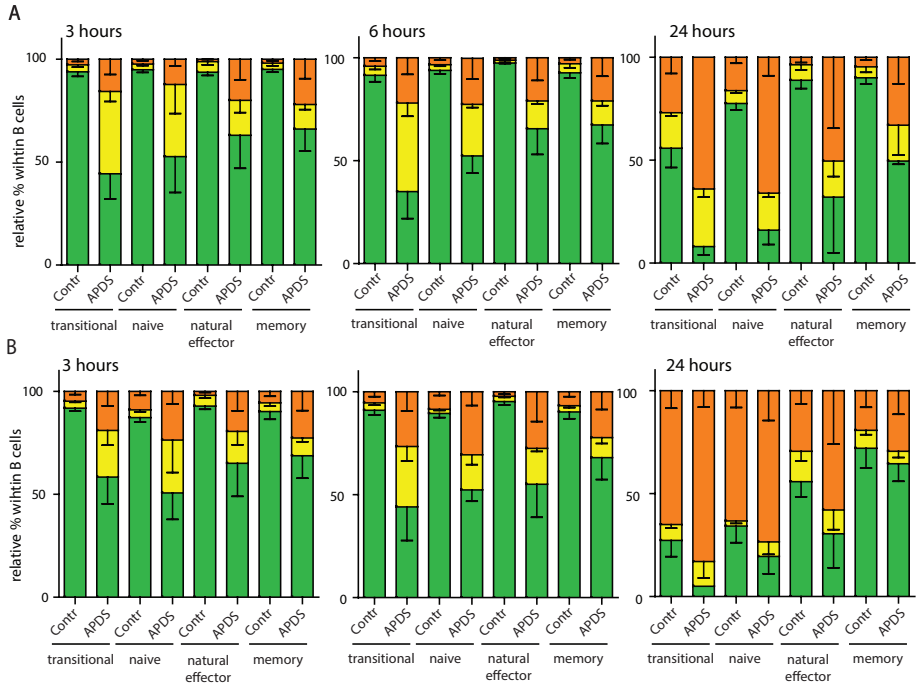
SUPPLEMENTAL FIGURES



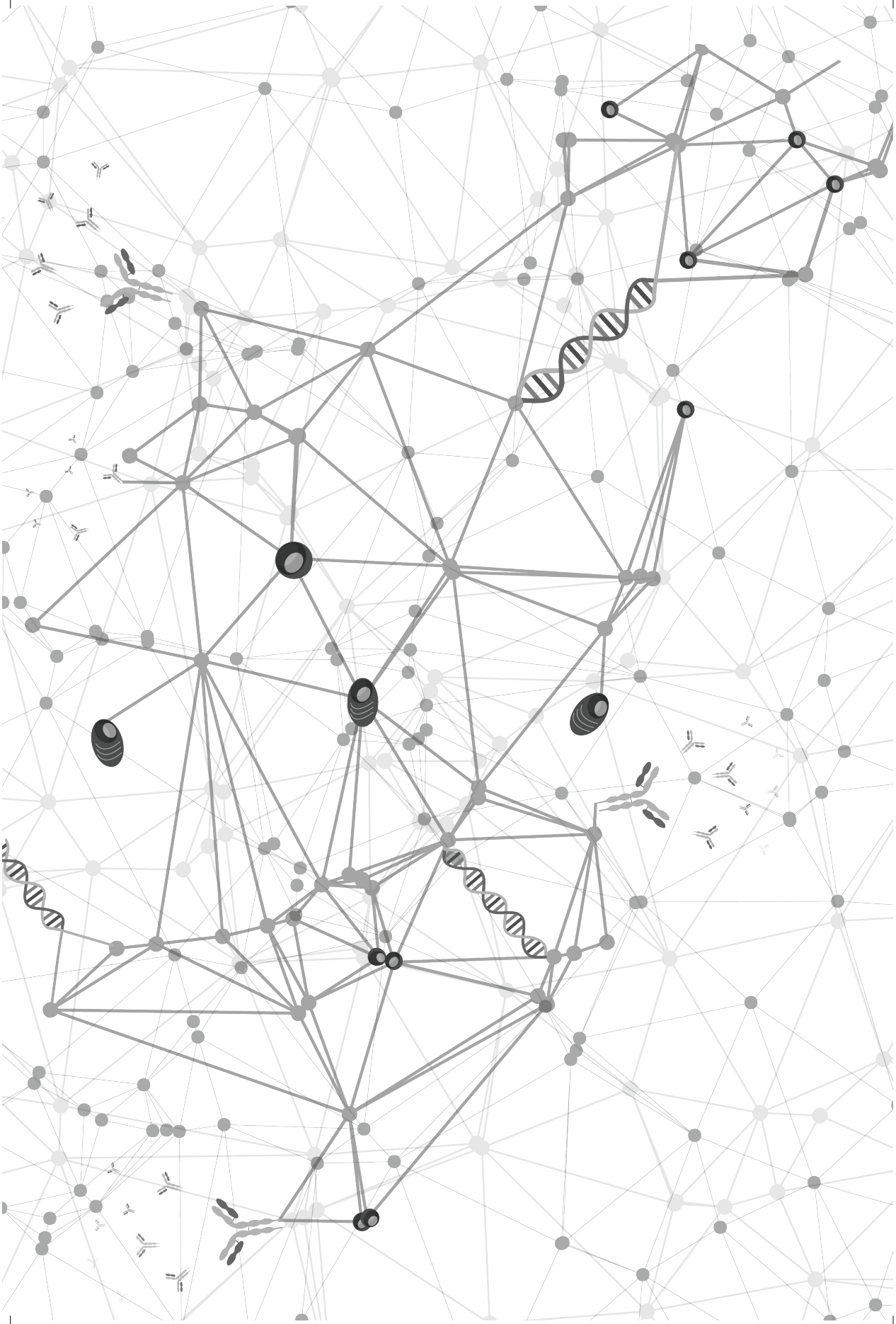
Supplementary figure 1. Abnormal populations in bone marrow.
A+B. Subsetting of preB-I cells (A) and preB-II cells (B) based on CD34 and TdT expression. Patient samples show an increase in $CD34^{-}$ TdT^{+} cells, compared to healthy controls. Healthy control plots consists of pooled data from three healthy controls, mean percentage is indicated with the range in brackets. **C**. CD20 expression in preB-I cells. Patient samples have a higher percentage of $CD20^{+}$ preB-I cells. **D**. Expression of CD19, Annexin V and 7-AAD in live B cells (green), early apoptotic B cells (blue) and late apoptotic B cells (orange), represented as histograms that are normalized for population size.



Supplementary figure 2.
Phospho-flow analysis of pAKT after stimulation. Normalized histograms of pAKT expression of unstimulated (grey), CD19/IgM stimulated (red) and PMA stimulated (green) total B cells, T cells and NK cells of pooled healthy controls and APDS patients. All subsets respond to aspecific stimulation with PMA, but only the B cells respond to anti-CD19/anti-IgM stimulation.



Supplementary figure 3.
Distribution of life cells (green) early apoptotic cells (yellow) and late apoptotic cells (orange) within different B cell subsets in patients and controls at 3, 6 and 24 hours with B cell specific stimulation (anti CD19 + anti-Ig antibodies) or aspecific stimulation (PMA) in vitro. B-cell subsets where defined as: transitional (CD38hi, CD24hi), naive (IgD^+, $CD27^-$), natural effector (IgD^+ $CD27^+$) and memory B cells (IgD^- $CD27^+$) (error bars represent SEM).



Chapter 4.4

Exhaustion of the CD8$^+$ T cell compartment in patients with mutations in PI3Kdelta

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ABSTRACT

Pathogenic gain-of-function mutations in the gene encoding phosphoinositide 3-kinase delta (PI3Kδ) cause Activated PI3Kδ Syndrome (APDS), a disease characterized by humoral immunodeficiency, lymphadenopathy and an inability to control persistent viral infections including EBV and CMV. Understanding the mechanisms leading to impaired immune response is important to optimally treat APDS patients. Immunosenescence of $CD8^+$ T cells was suggested to contribute to APDS pathogenesis. However, the constitutive activation of T cells in APDS may also result in T cell exhaustion. Therefore, we studied exhaustion of the $CD8^+$ T cell compartment in APDS patients and compared them to healthy controls and HIV patients, as a control for exhaustion. The subset distribution of the T cell compartment of APDS patients was comparable to HIV patients with decreased naive $CD4^+$ and $CD8^+$ T cells and increased effector $CD8^+$ T cells. Like in HIV^+ patients, expression of activation markers and inhibitory receptors CD160, CD244 and PD-1 on $CD8^+$ T cells was increased in APDS patients, indicating exhaustion. EBV-specific $CD8^+$ T cells from APDS patients exhibited an exhausted phenotype that resembled HIV-specific $CD8^+$ T cells in terms of inhibitory receptor expression. Inhibition of PD-1 on EBV-specific $CD8^+$ T cells from APDS patients enhanced *in vitro* proliferation and effector cytokine production. Based on these results, we conclude that total and EBV-specific $CD8^+$ T cells from APDS patients are characterized by T cell exhaustion. Furthermore, PD-1 checkpoint inhibition may provide a possible therapeutic approach to support the immune system of APDS patients to control EBV and CMV.

Keywords: Activated PI3Kdelta syndrome, APDS, p110δ, PI3K, $CD8^+$ T cells, exhaustion, PD-1, CD160, CD244, checkpoint inhibition;

INTRODUCTION

The phosphoinositide 3-kinase – AKT (PI3K-AKT) signalling pathway is involved in many crucial cellular processes including regulation of metabolism, proliferation, apoptosis, cell cycle regulation and protein synthesis.[1-3] In human lymphocytes, the PI3Kδ isoform, a heterodimer consisting of the catalytic subunit p110δ (encoded by *PIK3CD*) and regulatory subunit p85α (encoded by *PIK3R1*), is essential for both B cell and T cell development and maturation.[4-7] For $CD8^+$ T cells PI3Kδ has been shown to be essential for optimal immune responses to pathogens.[8, 9]

Over the past years, patients with gain-of-function (GOF) mutations in PI3Kδ have been described.[10-12] These patients suffer from a specific form of primary immune deficiency called Activated PI3Kδ Syndrome (APDS).[13, 14] This disease is characterized by disturbed humoral immunity resulting in hypogammaglobulinemia, recurrent respiratory tract infections an absent response to polysaccharide vaccination, pulmonary damage, lymphadenopathy, hepatosplenomegaly, an increased risk for haematological malignancies and an inability to control persistent viral infections such as Epstein-Barr virus (EBV) and Cytomegalovirus (CMV).[13, 14] Immunophenotypically, these patients have decreased numbers of total $CD4^+$ and especially naive $CD4^+$ T cells together with increased $CD8^+$ effector T cells. Furthermore, they have a relative increase in their transitional B cells accompanied by reduced memory B cells.[15] Several studies indicated that the effector function of their T cells is defective, causing an inability to control chronic viral infections including CMV and EBV.[13,14]

Impaired T cell effector function can be caused by different mechanisms, one of which is senescence.[16, 17] Hallmarks of senescence are permanent cell cycle arrest[18, 19] and resistance to apoptosis.[20, 21] Importantly, senescent T cells are metabolically and functionally active and retain their cytotoxic functions and ability to produce and secrete cytokines.[22,23] Reduced telomere length and surface-expression of CD57 were used to define senescent T cells. However, to reliably distinguish senescence from other causes of T cell impairment additional markers like senescence-associated β-galactosidase and cyclin-dependent kinase inhibitor 2A (p16Ink4A) can be used.[24, 25] Although senescence is age-dependent, other factors such as CMV-infection can contribute to senescence.[26]

Exhaustion of T cells due to chronic antigenic stimulation is another mechanism leading to impaired T cell effector functions. T cell exhaustion was first described in chronic viral infections such as Lymphocytic Choriomeningitis Virus (LCMV) infection in mice[27-29] but is also recognized as an underlying mechanism in immunological failure in human viral infections including HIV infection30-33 and tumors.34-36 Exhaustion is a hierarchical process[37, 38] by which $CD8^+$ T cells first lose their proliferative capacity and IL-2 secretion, followed by diminished secretion of effector cytokines such as tumour necrosis factor (TNF)

α and interferon (IFN)γ and eventually they become sensitive to apoptosis, which leads to the loss of these cells.[29, 38, 39] Simultaneously, these cells upregulate several inhibitory receptors including programmed death receptor (PD)-1, CD160, and CD244 which, when co-expressed, indicate later stages of exhaustion.[40-43] These inhibitory receptors are considered to play a central role in exhaustion. Blocking these inhibitory receptors on exhausted $CD8^+$ T cells can restore or improve their function in chronic viral infections as shown *in vitro*[41, 42, 44-46] and *in vivo*.[40, 47-51] Additionally, inhibitory receptor blockade was introduced into the clinic to re-activate exhausted T cells in cancer.[52, 53]

Previously, total and virus-specific $CD8^+$ T cells in APDS patients were shown to have upregulated CD57 expression, and reduced proliferative capacity. These findings were interpreted as T cell senescence.[12, 54-56] However, patients' lymphocytes also exhibited an increased rate of apoptosis compared to healthy controls,[11, 15, 54] which is not in line with the resistance to apoptosis that has been ascribed to T cell senescence.[21] Increased apoptosis sensitivity is associated with exhaustion rather than senescence.[39, 57] Additionally, APDS patient T cells have been reported to express more PD-1, a receptor associated with T cell activation and exhaustion.[12, 54, 55] The PI3Kδ pathway is critical for TCR signalling in $CD8^+$ T cells[58] and chronic antigen stimulation alone is sufficient to lead to $CD8^+$ T cell exhaustion.[59] This raises the question whether GOF mutations in PI3Kδ lead to changes in the activation of T cells which might pre-dispose for T cell exhaustion rather than or in addition to immune senescence. Understanding the mechanisms leading to impaired immune response in APDS patients is a requirement to define the best treatment options for these patients that can support the control of viral infections, which could in turn reduce virus-related morbidities in these patients.

To elucidate the role of exhaustion in APDS patients, total $CD8^+$ T cells and $CD4^+$ T cells from APDS patients were phenotypically characterized and compared to T cells from healthy individuals and HIV-infected patients (HIV^+ patients). We have included PBMC from HIV infected patients since it is well established that HIV infection leads to exhaustion of HIV-specific $CD8^+$ T cells but not CMV-specific $CD8^+$ T cells in HIV infected patients[39, 41] and can therefore serve as a positive control for exhaustion. Furthermore, virus-specific $CD8^+$ T cells in all three groups were characterized and the effect of PD-1 blockade on proliferation and effector functions was investigated. Our findings indicate that indeed $CD8^+$ T cells from APDS patients are more similar to the ones from HIV^+ patients and exhibit characteristics of exhaustion. Importantly, we show that blocking PD-1 signalling can increase virus-specific $CD8^+$ T cell proliferation and cytokine production. Our findings suggest that $CD8^+$ T cells in APDS patients undergo exhaustion and that this may contribute to the impaired control of persistent viral infections like EBV and CMV. These findings raise the possibility of checkpoint inhibition as a treatment strategy to support APDS patients to control recurrent or chronic viral infections.

MATERIALS AND METHODS

Cell samples and ethical approval

This study was carried out in accordance with the recommendations of Erasmus MC Medical Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Erasmus MC Medical Ethics Committee.

Ten APDS patients were included with a median age of 27 years (range 6 - 44 years), and a gender ratio of six males to four females. Nine of the patients have a mutation in the PIK3CD gene (seven patients: E1021K mutation, one patient: S312R mutation, one patient R929C mutation)[15] and one patient has a mutation in the PIK3R1 gene (N564K). From the patients including in this study 9/10 received immunoglobulin substitution therapy and 4/10 received prophylactic antibiotics. None of the patients received steroids or immune modulating drugs at the time of sampling. None of the APDS patients had active EBV or CMV infection at the time of sampling. Three of the APDS patients are EBV-antibody positive and 2 are EBV-antibody negative, for the other patients the EBV status is not known. CMV status is not known from these APDS patients. From the healthy controls 9 are EBV positive. The five HIV-infected patients included have a median age of 42 years (range 35 to 46), gender ratio is 1 female to 4 males, three patients have undetectable viral loads (below 20 HIV copies/ml), and two have detectable viral loads (295 and 4900 copies/ml, respectively). The median CD4 count is 300 cells/µl (range 50 – 630 cells/µl) and four out of the five patients are on antiretroviral therapy. The ten healthy control individuals included have a median age of 27 years (range 18 – 51) and a gender ratio of 5 males to 5 females.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll-Hypaque (GE Healthcare life sciences) density centrifugation, frozen in freezing media (90% FBS/10% DMSO) and stored in liquid nitrogen until used. Clinical data were provided by treating physicians. Due to availability of material, not all tests could be performed on all samples.

Flow cytometric immunophenotyping

PBMC were thawed, rested for 30-60 min at 37°C and stained with previously determined optimal amounts of tetramers and antibodies. For phenotyping of surface antigens, 0.8 - 1x10^6 cells were washed with Facs wash (FW, Hanks buffered saline solution (HBSS, Corning) 3% fetal bovine serum (FBS, Gibco), 0.02% NaN_3), stained with tetramer/antibody mix for 30 min at 4°C, washed two times with FW and fixed with 1% paraformaldehyde. Anti-HLA-A2-PE antibodies (clone BB7.2) were used to identify HLA-$A2^+$ donors. Virus-specific $CD8^+$ T cells were identified by using APC- or PE-conjugated HLA class I A*0201-β2-microglobulin tetramers loaded with HIV Gag p17 77-85 (SLYNTVATL) peptide, HIV Pol

476-484 (ILKEPVHGV) peptide, EBV peptide (GLCTLVAML), and CMV peptide (NLVPMVATV) (all tetramers were prepared in the lab). The following directly conjugated monoclonal anti-human antibodies were used: CD3-BV421 (UCHT1), CD4-BV650 (SK3), CD8-BV786 (RPA-T8), CD45RA-APC-H7 (HI100), CCR7-PE-CF594 and Alexa Fluor 700 (CD197, 150503), PD-1-BV711 (CD279, EH12.1), CD160-Alexa Fluor 488 (clone BY55), CD244-PE (eBioC1.7, eBioscience), HLA-DR-BV605 (G46-6), CD38-PE-Cy7 (HIT2), CD57-BV605 (NK-1), TNFa-FITC (MAb11, eBioscience), and IFNg-PECy7 (4S.B3, eBioscience). All antibodies were purchased from BD Biosciences unless otherwise indicated. When AnnexinV-PerCP-Cy5.5 was used to exclude dead cells 2.5 mM $CaCl_2$ was added to all solutions.

Between 1-4 x 10^5 events were collected per sample within 24 hours after staining on a LSRFortessa (BD Biosciences, 4 lasers, 18 parameters) and analyzed using FlowJo software (version 9.9.4, Tree Star). Data are represented as frequency within a defined population or as Median Fluorescence intensity (MFI).

In vitro proliferation

To determine proliferative capacity of proliferation-dye-labeled virus-specific $CD8^+$ T cells, thawed PBMC were incubated with 0.1 µM of CellTrace Far Red Cell stain (Invitrogen) in PBS for 20 min at 37°C and free dye was removed by adding RPMI-10%FBS and incubating for 5 min at 37°C. Cells were spun down and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and added to 24-well plates in a concentration of 1x10^6 PBMC/ml. To inhibit PD-1/PDL-1 interaction, 10 µg/ml of anti-PD-L1 antibody (CD274, MIH1, eBioscience) or isotype control (Mouse IgG1, eBioscience) was added and cells were incubated for 30 min at 37°C. Virus-specific peptide in a concentration of 1µg/ml was added then in appropriate wells: Gag peptide (SL9, SLYNTVATL, ANASPEC), Pol peptide (IV9, ILKEPVHGV, ANASPEC), CMV peptide (pp65, NLVPMVATV, ANASPEC), EBV peptide (BMLF1, GLCTLVAML, ANASPEC), EBV peptide pool (PepMix EBV BMLF1, JPT) or media alone (no peptide stimulation). Purified anti-CD28 (1µg/ml, clone CD28.2, BD Biosciences) and anti-CD49d (1µg/ml, clone 9F10, BD Biosciences) was added to all wells. Cells were incubated for 5 days at 37°C in a 5%CO2 incubator. Cells were harvested on day 5, counted and resuspended in RPMI-10%FBS/1µg/ml Brefeldin A (GolgiPlug, BD Biosciences)/anti-CD28 (1µg/ml)/anti-CD49d (1µg/ml) and incubated for 6 hours to determine cytokine production of these cells. For intracellular staining for cytokines, PBMC were first stained for surface antigens, fixed and permeabilized (Cytofix/Cytoperm, BD Bioscience), incubated with the antibodies (see above) for 60 min, washed two times with Perm/Wash Buffer (BD Biosciences) and fixed with 1% paraformaldehyde.

Statistical analysis

All data sets were tested for normal distribution using the D'Agostino-Pearson omnibus test. Relative distributions of T cell subsets and comparisons of population frequencies data were analyzed using either the non-parametric Mann-Whitney U test or a T-test, dependent on whether or not the data were normally distributed ($p<0.05$ was considered statistically significant). When more than two data sets were analyzed in the same test, the Kruskall-Wallis test was performed, combined with a Dunn`s multiple comparisons test. Correlations were calculated using a Spearman model for correlation, since in the majority of groups at least one population was not normally distributed. Statistics were performed using the GraphPad Prism program (GraphPad Software, San Diego, CA, USA). Populations frequencies per group are represented as mean with standard error of the mean (SEM)

RESULTS

Patient characteristics

We included 10 patients with APDS. From one patient (Pt1) two samples were included, one taken at age 7, and one collected at age 25. For analysis of total $CD4^+$ and $CD8^+$ T cells, we only included the adult sample. For analysis of virus-specific $CD8^+$ T cells, we included samples from both time-points. Most of the patients have been described before.[15] The majority of patients (n= 7) carried the previously described E1021K mutation in *PIK3CD,*[11, 12] one carried a R929C mutation in *PIK3CD* and one carried a N564K[15] GOF mutation in *PIK3R1*. One patient carried a missense variant c.935C>G (NM_005026.3) resulting in an amino acid change p.S312C (NP_005017.3) in *PIK3CD*. This latter variant is also found in the general population with a minor allele frequency of around 2% (SNP reference: rs61755420)[60] and can therefore not be classified as disease causing. However, this patient does suffer from antibody deficiency and auto-immunity and increased phosphorylation of AKT in lymphocyte subsets was found (M.W.J.Wentink, unpublished data). Therefore, we decided to study the effect of this variant together with patients with known disease causing mutations. Two of the patients were HLA-A2-positive (including the patient with samples available as a child and an adult) and EBV-specific $CD8^+$ T cells were analyzed.

We compared the PBMC from APDS patients to PBMC from 10 healthy controls and 5 HIV^+ patients.

The phenotype of T cells in APDS patients is distinct from healthy controls and resembles HIV-infected patients

Previous studies on the T cell compartment of APDS patients showed a reduction of the naive $CD4^+$ and $CD8^+$ T cells and an increase in the $CD8^+$ T cell effector memory population.[11-13]

In our patient cohort, we observed a comparable skewing of the T cell populations. The frequencies of $CD45RA^+CCR7^+$ naive $CD4^+$ T cells were significantly decreased in APDS patients compared to healthy controls and comparable with the frequencies in HIV^+ patients (Figure 1A, B). $CD45RA^+CCR7^+$ naive $CD8^+$ T cells were significantly reduced in our APDS patient cohort compared to the healthy controls although not as profound as observed in HIV^+ patients (Figure 1C). Within memory $CD4^+$ T cells, an increase in $CD45RA^-CCR7^+$ central memory (CM) cells was found for the APDS and the HIV^+ patients compared to healthy controls (Figure 1D). The $CD45RA^-CCR7^-$ effector memory (EM) $CD4^+$ T cell frequencies were not significantly different when healthy controls, APDS and HIV^+ patients were compared (Figure 1D). Frequencies of CM $CD8^+$ T cells were comparable between healthy controls and APDS patients as were the $CD45RA^+CCR7^-$ effector memory re-expressing CD45RA (EMRA) $CD8^+$ T cell populations (Figure 1E). A significant increase was found for the $CD45RA^-CCR7^-$ effector memory (EM) $CD8^+$ T cell population for APDS patients and HIV^+ patients when compared to healthy controls. These findings indicate that both patient populations show comparable reduction of naive T cells and increased EM $CD8^+$ T cells (Figure 1E).

To examine the effect of the GOF mutations on chronic activation we determined the expression of several chronic activation markers on T cells from healthy controls, APDS patients and HIV^+ patients. The frequency of $CD38^{bright}CD8^+$ T cells was increased in APDS patients compared to healthy controls, however HIV^+ patients had an even higher percentage of $CD38^{bright}$ $CD8^+$ T cells (Figure 2A). Although the frequency of $CD38^{bright}$ $CD4^+$ T cells was also higher in APDS patients compared to healthy controls this was not significant (Figure 2D). HLA-DR was examined as a second marker of chronic activation and indeed a significant increase was found for HIV^+ patients within the $CD8^+$ T cell population. HLA-DR expression was significantly increased on $CD8^+$ T cells (Figure 2B) and $CD4^+$ T cells (Figure 2E) from the APDS patients compared to healthy controls.

We studied the expression of CD57 on $CD8^+$ and $CD4^+$ T cells, since this was reported to be increased in a subset of APDS patients. We found that in APDS patients 34.9±5.0% (mean ± SEM) of $CD8^+$ T cells express CD57, compared to 25.2%±4.0% in healthy controls and 56.4%±5.2% in HIV^+ patients (Figure 2C). This indicates that although $CD57^+$ $CD8^+$ T cells are increased in APDS patients, this is not significantly different from the frequency in healthy controls and lower than the frequency in HIV^+ patients. A small but non-significant increase of $CD57^+$ cells was also found within the $CD4^+$ T cell population of APDS patients compared to healthy controls (Figure 2F). Overall, the expression of activation markers and CD57 indicate that T cells from APDS patients tend to be more activated than healthy controls and are therefore more alike T cells from HIV^+ patients.

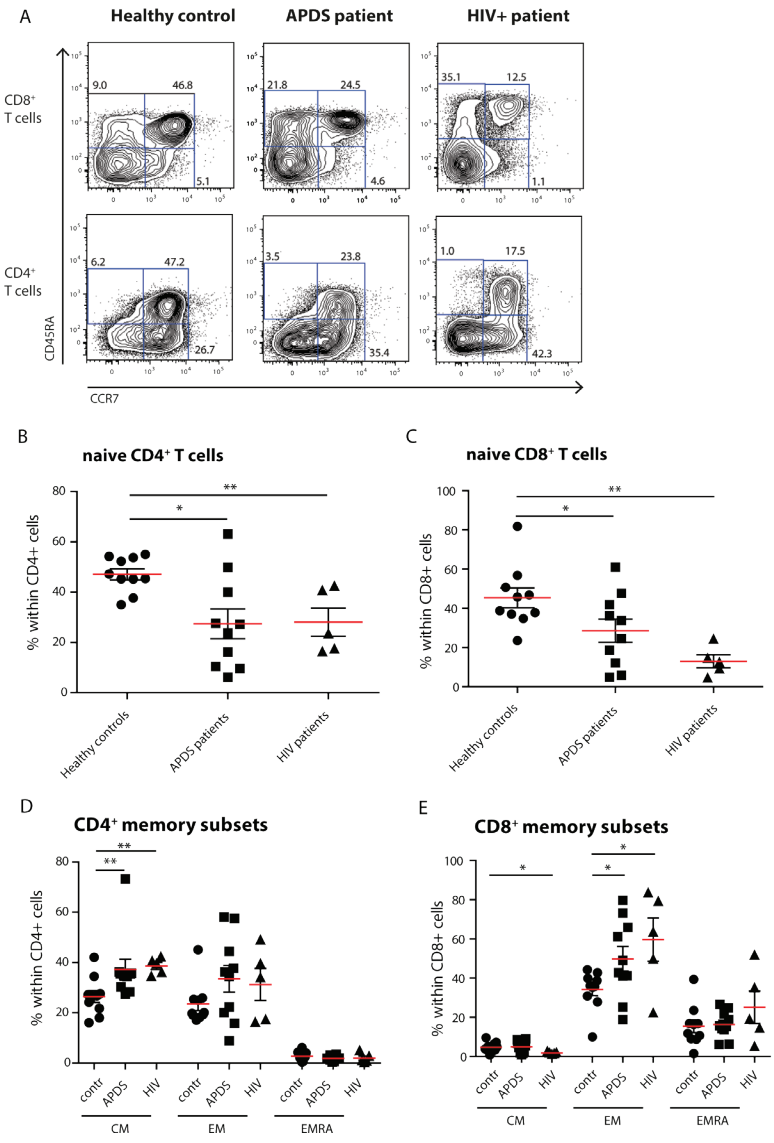

Figure 1.
Immunophenotyping of the T-cell compartment of controls (black dots), APDS patients (black squares) and HIV+ patients (black triangles). Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group. (* p<0.05, **p<0,005). **A.** Representative dot plots of controls and patients indicating naive ($CD45RA^+CCR7^+$), CM ($CD45RA^-CCR7^+$), EM ($CD45RA^-CCR7^-$) and EMRA ($CD45RA^+CCR7^-$) $CD8^+$ and $CD4^+$ T cell subsets. Numbers depict frequency of cell populations. **B. and C.** The frequency of naive $CD4^+$ T cells **(B)** and $CD8^+$ T cells **(C)** is reduced in APDS patients and HIV+ patients compared to controls. **D.** The frequency of CM $CD4^+$ T cells is increased in APDS patients and HIV+ patients. The frequency of the different memory $CD4^+$ T cells in controls, APDS patients and HIV+ patients is shown. **E.** The frequency of EM $CD8^+$ T cells is increased in APDS patients and HIV+ patients. The different memory $CD8^+$ T cell subpopulations are shown for controls APDS patients and HIV+ patients.

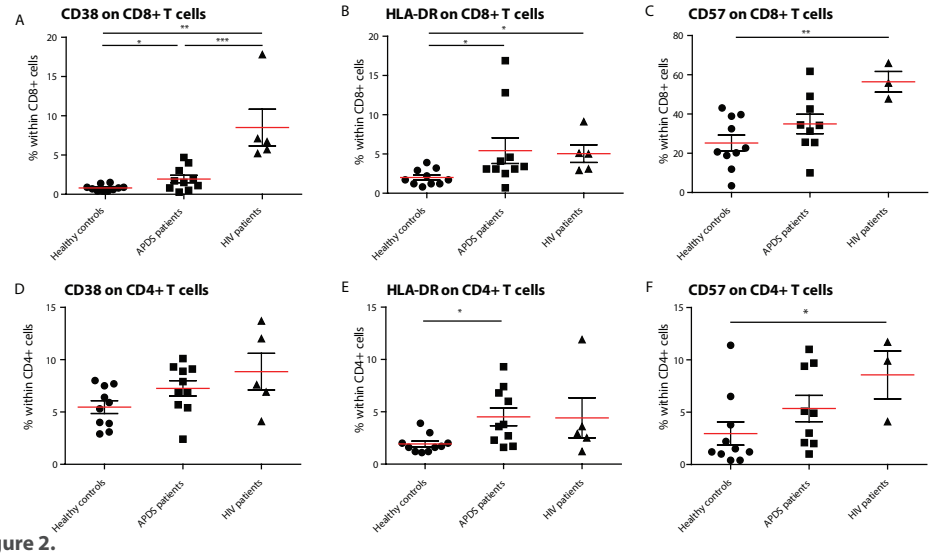

Figure 2.
Expression of CD38, HLA-DR and CD57 on CD8$^+$ and CD4$^+$ T cells from controls (black dots), APDS patients (black squares) and HIV$^+$ patients (black triangles). Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group. (* p<0.05, **p<0,005). **A.** The frequency of CD38^{bright} cells is increased in APDS patients and HIV$^+$ patients compared to controls. The frequencies of CD38^{bright} cells within CD8$^+$ cells are shown for healthy controls, APDS and HIV$^+$ patients. **B.** The frequency of HLA-DR$^+$ cells is increased in APDS patients and HIV$^+$ patients compared to controls. Frequencies of HLA-DR$^+$ cells within CD8$^+$ T cells are shown. **C.** Frequency of CD57$^+$ CD8$^+$ T cells is not increase in APDS patients. Frequencies of CD57$^+$ cells within CD8$^+$ cells shown for healthy controls, APDS patients and HIV$^+$ patients. **D.** Frequency of CD38$^+$ CD4$^+$ T cells is comparable in healthy controls, APDS patients and HIV$^+$ patients. **E.** Frequency of HLA-DR$^+$ CD4$^+$ T cells is increased in APDS patients. Frequency of HLA-DR$^+$ cells within CD4$^+$ T cells shown for heathy controls, APDS patients and HIV$^+$ patients. **F.** Frequency of CD57$^+$CD4$^+$ T cells is not increased in APDS patients compared to healthy controls. Frequency of CD57-expressing cells within CD4$^+$ T cells shown.

APDS patients have increased inhibitory receptor expression on CD8$^+$ T cells

Since exhaustion is a gradual process in which cells over time co-express multiple inhibitory receptors, we studied both the single expression of PD-1, CD160 and CD244 and co-expression of these three receptors on CD8$^+$ T cells (Figure 3A-E). As we observed with the activation markers, the expression profile of inhibitory receptors within the APDS patients is very heterogeneous, with some patients in the range of controls and others in the range of HIV$^+$ patients. We observed a significantly higher percentage of CD8$^+$ T cells from APDS patients expressing CD160 compared to controls (mean 38%±6.0% and 21%±4.5%, respectively). This frequency in APDS patients was closer to HIV$^+$ patients (mean 50%±6.8%) (Figure 3B). The frequency of CD244-expressing CD8$^+$ T cells within APDS patients and HIV$^+$ patients was significantly increased compared to healthy controls (Figure 3C). PD-1 was also found increased on CD8$^+$ T cells in APDS patients compared to controls (mean 38.8%±6.5% and 23.6%±6.9%, respectively) (Figure 3D). Most importantly, we observed a significant increase in the frequency of CD8$^+$ T cells expressing all three inhibitory receptors, PD-1, CD244, and CD160 (PD-1$^+$CD160$^+$CD244$^+$), in the APDS patients

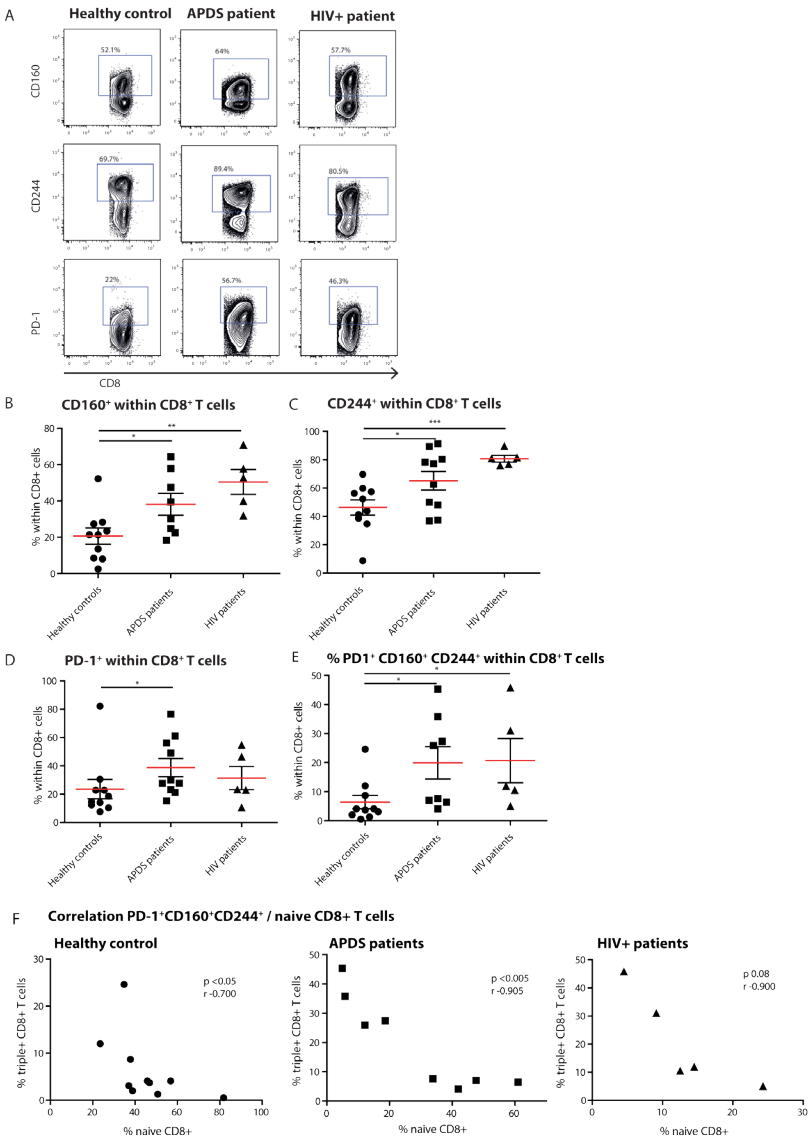

Figure 3.
Expression of inhibitory receptors on CD8⁺ cells from controls (black dots), APDS patients (black squares) and HIV⁺ patients (black triangles). Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group. (* p<0.05, **p<0,005). **A.** Representative dotplots of controls and patients indicating populations that were considered positive for inhibitory receptors. **B.** CD160 expression is increased on CD8⁺ T cells from APDS patients and HIV⁺ patients compared to controls. **C.** CD244 expression is increased on CD8⁺ T cells from APDS patients and HIV⁺ patients compared to controls. **D.** PD-1 expression is increased on CD8⁺ T cells from APDS patients but not HIV⁺ patients compared to controls. **E.** The frequency of PD-1⁺ CD160⁺ CD244⁺ CD8⁺ T cells is increased in APDS patients and HIV⁺ patients compared to controls. **F.** The frequency of PD-1⁺ CD160⁺ CD244⁺ CD8⁺ T cells is negatively correlated with the frequency of naive CD8⁺ T cells in controls and APDS patients.

which was 20%±5.6% compared to 6%±2.3% PD-1$^+$CD160$^+$ CD244$^+$ CD8$^+$ T cells in healthy controls. This frequency of PD-1$^+$CD160$^+$CD244$^+$ CD8$^+$ T cells in APDS patients is comparable to the one observed in HIV$^+$ patients (21%±7.6%).

We examined whether a history of EBV infection influences the expression of inhibitory receptors on CD8$^+$ T cells. From the APDS patients, EBV status information was available from 5 individuals, with 3 being EBV-antibody positive and 2 being EBV-antibody negative. We compared the expression of inhibitory receptors on CD8$^+$ T cells from these two groups to the expression of the inhibitory receptors on the CD8$^+$ T cells in the 9 EBV-positive healthy controls. We found that the frequency of CD160$^+$, CD244$^+$, PD-1$^+$ and CD160$^+$CD244$^+$PD-1$^+$ CD8$^+$ T cells is highest in the EBV$^+$ APDS patients (CD160$^+$CD8$^+$: 57%±4.9%; CD244$^+$CD8$^+$: 87%±3.4%; PD-1$^+$CD8$^+$: 61%±8.2%; CD160$^+$CD244$^+$PD-1$^+$CD8$^+$: 36%±5.6%), but lower in the EBV- APDS patients and the EBV$^+$ healthy controls (CD160$^+$CD8$^+$: 26%±3.8%, 22%±4.7%; CD244$^+$CD8$^+$: 43%±5.3%, 48%±5.9%; PD-1$^+$CD8$^+$: 18%±3.0%, 25%±7.6%; CD160$^+$CD244$^+$PD-1$^+$CD8$^+$: 5.6%±1.5%, 7.0%±2.5% for EBV$^-$ APDS patients and EBV$^+$ healthy control, respectively). Thus, EBV antibody positivity is accompanied by increased inhibitory receptor expression on CD8$^+$ T cells.

We next analyzed whether reduced naive CD8$^+$ T cell frequency and PD-1$^+$CD160$^+$CD244$^+$ CD8$^+$ T cells correlate in healthy controls and APDS patients. Although a negative correlation was already observed for healthy controls (Figure 3F), the correlation between the frequencies of PD-1$^+$CD160$^+$CD244$^+$ CD8$^+$ T cells and naive CD8$^+$ T cells was highly significant in APDS patients (Figure 3F). For the HIV$^+$ patients, this relationship was not significantly correlated. These results indicate that total CD8$^+$ T cells from APDS patients have increased co-expression of inhibitory receptors similar to what is observed in HIV$^+$ patients. Furthermore the negative correlation of PD-1$^+$CD160$^+$CD244$^+$ CD8$^+$ T cells and naive CD8$^+$ T cells indicates that exhaustion in this compartment is associated with the skewed subset distribution.

Virus-specific CD8$^+$ T cells from APDS patients exhibit an exhaustion phenotype

To further compare exhaustion in APDS patients to exhaustion due to HIV infection, we analyzed virus-specific CD8$^+$ T cells in both patient groups and healthy controls. HIV-specific CD8$^+$ T cells from HIV$^+$ patients are highly sensitive to apoptosis, present with a skewed memory phenotype, have proliferative defects and show increased expression of inhibitory receptors.[29, 38, 39] However, in the same HIV$^+$ patients, CMV-specific CD8$^+$ T cells are not impaired. Using peptide-loaded HLA-A2 tetramers, virus-specific CD8$^+$ T cells were analyzed from three APDS-patients (EBV-specific CD8$^+$ T cells), five HIV$^+$ patients (HIV Gag- or Pol-specific CD8$^+$ T cells, CMV-specific CD8$^+$ T cells) and four healthy controls (EBV-specific CD8$^+$ T cells). Within the three APDS samples, are two different donors with one

donor being represented as a child (7 years, red square in Figure 4) and as an adult (25 years, open square).

The virus-specific CD8$^+$ T cells in all four groups have a predominantly CD45RA$^-$CCR7$^-$ EM phenotype (Figure 4A) which is lowest in CMV-specific CD8$^+$ T cells from HIV$^+$ patients since these cells have the highest frequency of more differentiated EMRA (Figure 4A). No clear difference was observed for the frequencies of the chronic activation markers CD38 and HLA-DR when the four virus-specific CD8$^+$ T cell groups were compared (Figure 4B). All virus-specific cells have higher frequencies of CD38$^+$ and HLA-DR$^+$ cells than total CD8$^+$ T cells (Figure 2A, B) from the same groups. CD57 expressing cells seem to be more abundant within EBV-specific CD8$^+$ T cells from APDS patients and CMV-specific CD8$^+$ T cells from HIV patients than in EBV-specific CD8$^+$ T cells from healthy controls and HIV-specific CD8$^+$ T cells from HIV patients.

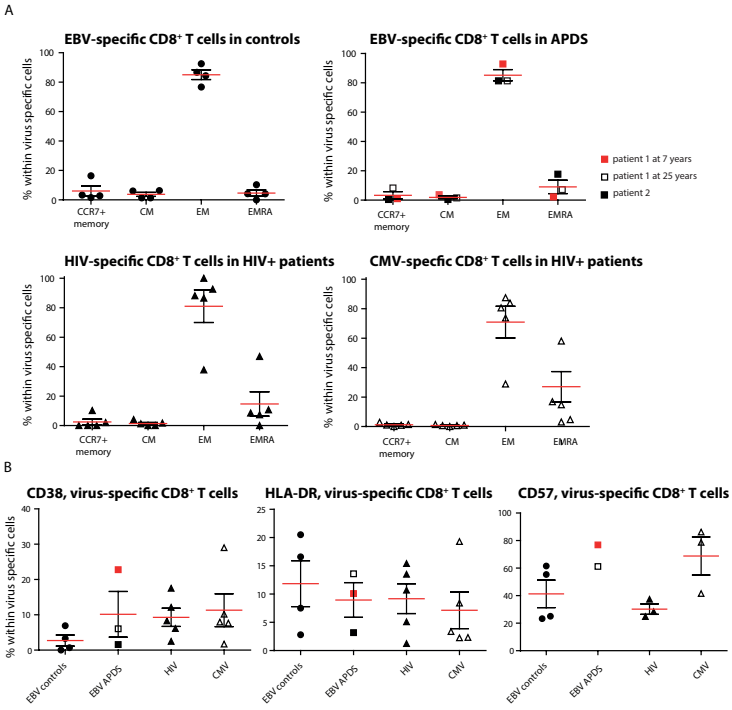

Figure 4.
Phenotyping and activation marker expression on EBV-specific CD8$^+$ T cells from controls (black dots), EBV-specific CD8$^+$ T cells from APDS patient 1 at the age of 7 years (red square), at the age of 25 years (open square), APDS patient 2 (black square), HIV-specific CD8$^+$ T cells from HIV$^+$ patients (black triangles) and CMV-specific CD8$^+$ T cells from HIV$^+$ patients (open triangles). Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group. (* $p<0.05$, ** $p<0,005$). **A.** Frequency of memory subpopulations (CM, EM and EMRA, CCR7$^+$ memory (CD45RA$^+$CCR7$^+$)) within virus-specific CD8$^+$ T cells shown for healthy controls (EBV-specific CD8$^+$ T cells), APDS patients (EBV-specific CD8$^+$ T cells) and HIV$^+$ patients (HIV- and CMV specific CD8$^+$ T cells). **B.** Frequency of CD38$^+$ (left), HLA-DR$^+$ (center), and CD57$^+$ (right) cells shown within virus-specific CD8$^+$ T cells.

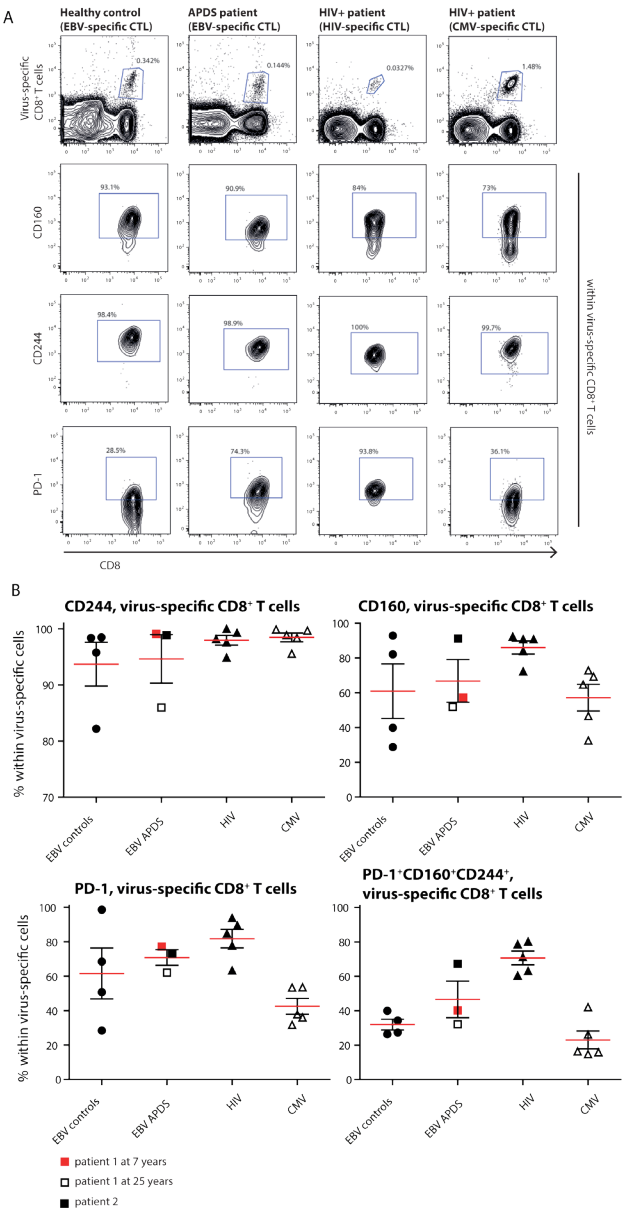

Figure 5.
Inhibitory receptor expression on virus-specific cells from controls and patients. **A.** Representative dotplots of healthy controls and patients indicating identification of virus specific cells and inhibitory receptors positive cells within virus specific populations. **B.** Inhibitory receptor expression on EBV-specific cells from controls (black dots), EBV-specific cells from APDS patient 1 at the age of 7 years (red square), at the age of 25 years (open square), APDS patient 2 (black square), HIV-specific cells from HIV$^+$ patients (black triangles) and CMV-specific cells from HIV$^+$ patients (open triangles). Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group.

To assess exhaustion in the virus-specific $CD8^+$ T cell population, the expression of PD-1, CD160 and CD244 was analyzed (Figure 5). The mean percentage of $CD244^+$ $CD8^+$ T cells was above 90% in all virus-specific $CD8^+$ T cell groups. Frequency of CD160 expression slightly increased on HIV-specific $CD8^+$ T cells compared to the other groups. The frequency of PD-1 expressing $CD8^+$ T cells was lowest in CMV-specific $CD8^+$ T cells and EBV-specific $CD8^+$ T cells from healthy controls but higher within EBV-specific $CD8^+$ T cells from APDS patients and HIV-specific $CD8^+$ T cells from HIV patients. We found that within the HIV-specific $CD8^+$ T cells 71%±4.0% express all three inhibitory receptors, and within the EBV-specific $CD8^+$ T cells from APDS patients 47%±10.6% express all three inhibitory receptors. EBV-specific cells from controls and CMV-specific cells from HIV^+ patients have a lower frequency of $PD\text{-}1^+$ $CD160^+$ $CD244^+$ populations (32%±3.2% and 23%±5.2%, respectively). Compared to overall $CD8^+$ T cells, all virus-specific $CD8^+$ T cells show increased expression of inhibitory receptors.

PD-1 blockade can enhance in vitro proliferation of virus-specific cells in APDS and HIV^+ patients

Although both senescent and exhausted cells do not proliferate, the mechanism leading to the replicative impairment is different. In senescence, a cell cycle arrest causes the inability of cells to replicate whereas in exhaustion receptor-ligand interaction (such as between the inhibitory receptor PD-1 and its ligand PD-L1) leads to inhibition of TCR-signaling and therefore inhibition of proliferation. Thus, blocking the interaction between receptor and ligand through checkpoint inhibitors could lead to an increase in proliferation of exhausted cells, as shown for HIV-specific $CD8^+$ T cells from HIV^+ patients[44-46] but not in senescent cells. We stimulated PBMC from HIV^+ patients and APDS patients with virus-specific peptide in the presence of blocking anti-PD-L1 or isotype control antibodies for five days prior to analysis of proliferation and effector function. As reported previously,[44-46] inhibition of PD-1/PD-L1 interaction increased proliferation of HIV-specific $CD8^+$ T cells in 2 out of the 4 tested PBMC samples from HIV^+ patients by ~2-fold (Figure 6). When PBMC from APDS patients were stimulated with EBV-peptides in the presence of anti-PD-L1 antibodies, proliferation was increased in all three samples compared to samples stimulated with peptide in the presence of an isotype control. Not only did we observe improved proliferation of these cells but also increased effector function as demonstrated by the frequency of cytokine producing cells (Figure 6). The patient with the highest proliferative and effector cytokine response was also the patient with the highest frequency of $PD\text{-}1^+$ and $PD\text{-}1^+CD160^+CD244^+$ EBV-specific $CD8^+$ T cells. These findings indicate that PD-1 signal inhibition not only increased proliferation but also enabled these cells to release effector cytokines including IFNγ and TNFα. These results suggest that exhausted EBV-specific $CD8^+$ T cells from APDS patients can be functionally restored through checkpoint inhibitors, resulting in increased proliferation and effector functions.

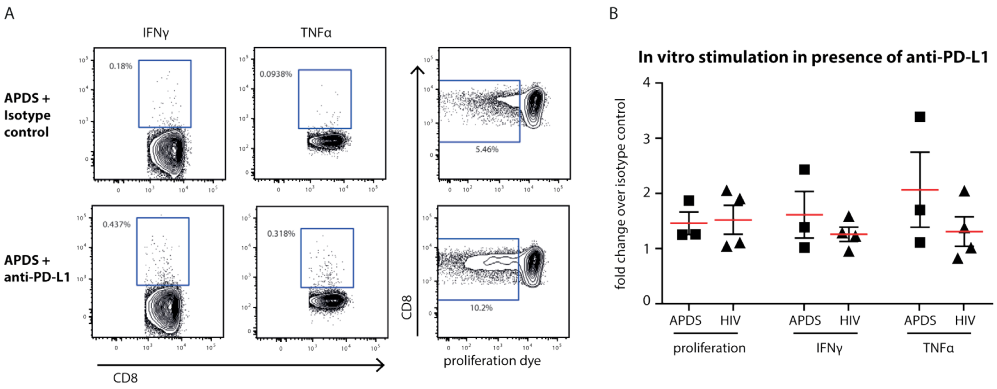

Figure 6.
Proliferation and effector cytokine production *in vitro* of EBV-specific CD8⁺ T cells from APDS patients and HIV-specific CD8⁺ T cells from HIV⁺ patients after peptide stimulation in the presence of anti-PD-L1 antibodies or isotype controls. **A.** Representative dot plots showing cytokine-producing and proliferated CD8⁺ T cells after 5 day peptide stimulation of PBMC from an APDS patient in the presence of isotype control (upper panel) or anti-PD-L1 monoclonal antibodies (lower panel). Gates indicate cells positive for IFNγ, TNFα or cells with diluted cell-trace proliferation dye. **B.** Anti-PD-L1 antibody treatment resulted in increased proliferation and production of TNFα and IFNγ upon peptide stimulation in both APDS and HIV⁺ patients. Pooled data showing fold change of the frequency of either cells undergoing proliferation or cytokine producing cells in the presence of anti-PD-L1 antibody over isotype control. Cells were stimulated with viral peptide for 5 days in the presence of monoclonal antibodies. Red lines and brackets indicate the mean and standard error of the mean (SEM) of each group.

DISCUSSION

How T cell defects due to GOF mutations of PI3Kδ contribute to morbidity in APDS patients is not fully understood. The goal of this study was to determine whether the GOF mutations in PI3Kδ (causing APDS) lead to exhaustion of CD8⁺ T cells. Furthermore, we determined whether virus-specific CD8⁺ T cells against recurrent or persistent infections such as EBV are exhausted in APDS patients and if immune-checkpoint blockade can rejuvenate exhausted CD8⁺ T cells in APDS patients. We compared CD8⁺ T cells from APDS patients to healthy controls and HIV⁺ patients, which served as a control for antigen induced exhaustion of CD8⁺ T cells. Understanding the effect these mutations have on the immune system is central to further treatment strategies to support these patients in controlling chronic infections like EBV and CMV to reduce morbidity and mortality.

In line with previous reports,[13, 14] we found significantly reduced frequencies of naive CD4⁺ and CD8⁺ T cells in APDS patients. Loss of naive T cells is thought to be caused by hyperactivation of the mTOR pathway induced by PI3Kδ mutations. This leads to increased glycolysis, proliferation and differentiation into short lived effector cells.[11, 12] The APDS patients showed a skewed CD8⁺ T cell subset distribution, with increased EM CD8⁺ T cells, which we also found in HIV⁺ patients. Impaired control of viral infection could be an

underlying mechanism causing the increased effector memory pool in HIV$^+$ patients. In APDS patients chronic and/or recurrent viral infections such as EBV and CMV on top of the GOF mutation is likely to contribute to this phenotype. Interestingly, although the effector memory population is also slightly increased in CD4$^+$ T cells, it is the central memory population which is significantly higher in both patient groups.

To further examine the effect of PI3Kδ GOF mutations on the total CD4$^+$ and CD8$^+$ T cell compartments, we analysed the expression of the chronic activation markers CD38 and HLA-DR. Especially HLA-DR was shown before to correlate with immune activation and disease progression in HIV infection.[61] This is the first time that these chronic activation markers are analysed on T cells from APDS patients. HLA-DR expression was significantly increased on CD8$^+$ and CD4$^+$ T cells in APDS patients compared to healthy controls and it is comparable to what is observed in HIV$^+$ patients. Our findings indicate that a subpopulation of CD4$^+$ and CD8$^+$ T cells in APDS patients are chronically activated. However, APDS patients show great heterogeneity, with only a small part of T cells expressing the chronic activation markers, our findings thus suggest that the mutation alone does not lead to a chronic activation of T cells. One exhaustion-inducing factor could perhaps be infection with a persistent virus; we therefore analysed the expression of CD38 and HLA-DR on CD8$^+$ T cells specific for persistent viruses. We did not find significant differences in the expression of activation markers on virus-specific cells between controls, APDS patients and HIV$^+$ patients. Thus, we cannot at this moment conclude that infections with persistent viruses contribute to the higher frequency of HLA-DR$^+$ T cells in APDS patients.

Some APDS patients have increased CD57 expression on CD8$^+$ T cells and previously this was interpreted as increased senescence.[12, 54, 55] The surface marker CD57 is commonly used as a senescence marker, but T cells in HIV$^+$ patients also express increased CD57, indicating that exhaustion and senescence can co-exist in patients.[62-64] In our study cohort, the mean CD57$^+$ T cell frequency in APDS patients is only slightly increased compared to healthy controls and much lower than that of HIV$^+$ patients. However, the CD57 expression in the APDS cohort is very heterogeneous, with some patients comparable to controls and others in the range of HIV$^+$ patients. CD57 expression levels were not correlated with age, gender or mutation in our patient cohort. Neither was this correlated with clinical characteristics like lymphadenopathy, auto-immunity, malignancies, the expression of other markers we studied here or with B-cell phenotypes. Our results suggest that senescence and exhaustion might not be totally separate processes, but rather two intertwined cellular states that can occur together in specific types of disease like APDS.

To further assess exhaustion in APDS patients, we analysed the expression of the inhibitory receptors CD160, CD244 and PD-1. We have included PBMC from HIV infected patients since it is well established that HIV infection leads to exhaustion of HIV-specific CD8$^+$ T cells but not CMV-specific CD8$^+$ T cells in HIV infected patients[39, 41] and can therefore

serve as a positive control for exhaustion. We found that CD244$^+$, CD160$^+$ and PD-1$^+$ CD8$^+$ T cells are significantly increased in APDS patients. CD8$^+$ T cells expressing all three inhibitory receptors (PD-1$^+$CD160$^+$CD244$^+$), which would indicate the most exhausted state, were also significantly increased in APDS patients and to a similar degree as in HIV-infected individuals. We did not find any correlates for the PD-1$^+$ CD160$^+$ CD244$^+$ CD8$^+$ T cell population in APDS patients when age, gender, type of mutations, B cell phenotype, or any other marker we have described were analysed. When we separated the APDS patients in EBV-antibody positive and EBV-antibody negative, we found that the highest expression of the inhibitory receptors including the concurrent expression of all 3 inhibitory receptors was observed in EBV$^+$ APDS patients. However, EBV$^-$ APDS patients had a frequency of inhibitory receptor expressing CD8$^+$ T cells which was similar to the one from EBV$^+$ healthy controls. These findings suggests that chronic EBV infection or antigen stimulation in APDS patients contributes to the exhaustion of CD8$^+$ T cells.

Within virus-specific CD8$^+$ T cells, we found increased expression of inhibitory receptors in all subpopulations. Overall, the expression of inhibitory receptors on CD8$^+$ T cells from APDS patients has more similarities with HIV$^+$ patients, supporting the idea that the PI3Kδ GOF mutations may contribute to exhaustion. This was supported by the highly significant negative correlation between the frequency of naive and PD-1$^+$CD160$^+$CD244$^+$CD8$^+$ T cells, indicating that the hyper-activation leading to reduced naive T cells, may also be responsible for the increased expression of inhibitory receptors on T cells. Our findings do not imply that signalling mechanisms leading to exhaustion is identical in APDS and HIV infection although chronic antigen exposure may play an important role in both APDS and HIV patients as has been suggested in mouse studies.[59]

Commonly, exhaustion is studied in the context of chronic antigen stimulation due to chronic viral infections and cancer. In APDS we see a similar increase of exhausted CD8$^+$ T cells especially in patients that are EBV-antibody positive implying that chronic antigen stimulation contributes to T cell exhaustion. Chronic TCR stimulation is sufficient to lead to CD8$^+$ T cell exhaustion.[59] Since PI3Kδ participates in TCR signalling in T cells,[58] a cell-intrinsic chronic activation due to the hyperactivity of the PI3K-AKT signaling pathway could be a factor promoting the exhausted phenotype in the absence of specific chronic antigen in APDS. Since not all APDS patients' T cells show a significant increase in inhibitory receptor expression, the PI3Kδ mutations may be necessary but not sufficient for exhaustion. One mechanism to lower the threshold for exhaustion in APDS could be PI3K-induced epigenetic modifications. Several studies have shown epigenetic alterations in exhausted T cells.[65, 66] The altered gene expression patterns induced by epigenetic modifications are rather stable resulting in permanent exhaustion independent of the level of remaining antigen.[67, 68] Demethylation of the PD-1 promotor region in exhausted CD8$^+$ T cells allows sustained expression of PD-1. That PI3K can indeed alter epigenetic modifications

was indicated in a study in mouse embryonic stem cells, suggesting that de novo DNA methyltransferases are downregulated due to PI3K-induced AKT, leading to reduced DNA methylation of imprinted loci.[69] Therefore PI3Kδ GOF mutations could promote the demethylation of inhibitory receptors or reduce the threshold for such demethylation, thus facilitating the exhaustion in $CD8^+$ T cells.

Over the past years, PD-1 blockade has been a subject of research in both HIV and cancer treatment. By blocking inhibitory receptors, exhausted $CD8^+$ T cells can regain effectors functions and provide anti-viral or anti-tumor immunity. However, upregulation of inhibitory receptors on activated T cells has also a physiological function: they function as negative regulators, downregulating the immune response after successful control of infections.[70, 71] They are important to prevent autoimmunity and pathological responses leading to tissue damage. In the absence of inhibitory receptors an increased risk for autoimmunity and immunopathology was reported.[72-74] Upregulation of inhibitory receptors in APDS could be a protective mechanism to prevent damage through an overly activated immune system. This hypothesis is supported by a study which showed how PD-1 signaling can prevent activation of PI3K and AKT phosphorylation, thereby preventing proliferation.[75] This benefit of preventing autoimmunity or inflammation in APDS comes at a cost of impairing immunity to viruses. Indeed, we have seen an increase in proliferation and effector cytokine secretion in virus-specific $CD8^+$ T cells from APDS patients *in vitro* in the presence of PD-L1 blocking antibodies. The increase of proliferation and cytokine production we have observed in the presence of a PD-L1 blocking antibody is moderate, and this raises the question of biological significance. Since we have observed that the patient with the highest expression of PD-1 showed the highest increase in proliferation and cytokine-producing EBV-specific $CD8^+$ T cells this suggests that the more responsive patients to PD-1 blockade will be the highest expressers. To more significantly improve the effector functions in all patients a combination of blocking several checkpoint inhibitors may be required, as indicated by PD-1/CTLA-4 blocking in cancer studies.[76, 77] Furthermore, the increase of effector function is similar when EBV-specific $CD8^+$ T cells from APDS patients are compared with HIV-specific T cells from HIV-infected patients and within the range reported previously for HIV-specific $CD8^+$ T cells.[41, 44, 45] Thus, short term treatment of patients with checkpoint inhibitors during a re-current EBV^- and/or CMV infection could be a means to augment efficacy of exhausted virus-specific $CD8^+$ T cells and thus reduce EBV- and CMV related morbidity. Because inhibitory receptor upregulation may possibly be beneficial in APDS by preventing excessive inflammation or autoimmunity, manipulating this pathway has to be done with caution, and ideally it should be combined with long-term treatment that can reduce the hyperactivation of the pathway, for example selective PI3K-inhibition.[78]

In summary, we have shown that $CD8^+$ T cells from APDS patients have an exhausted phenotype and upregulated expression of inhibitory receptors. This exhaustion of the $CD8^+$ T cell compartment contributes to the deficient anti-viral capacities of the $CD8^+$ T cell compartment. Blocking the PD1-PD1L interaction could be a target for treatment in these patients during recurrent or persistent viral infections.

AUTHORS AND CONTRIBUTORS

Y.M.M. and M.W.J.W. performed experiments and analyzed data. V.A.S.H.D., G.J.D., P.M.v.H., and J.M.v.M., contributed to study design and data analysis. Study was conceived and designed by P.D.K., M.v.d.B, Y.M.M. and M.W.J.W. Manuscript was written by P.D.K., Y.M.M. and M.W.J.W. All authors have read and approved the manuscript.

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REFERENCES

1. Okkenhaug K, Vanhaesebroeck B. P*I3K in lymphocyte development, differentiation and activation*. Nat Rev Immunol. 2003;**3**(4):317-30.
2. Okkenhaug K, Vanhaesebroeck B. P*I3K-signalling in B- and T-cells: insights from gene-targeted mice*. Biochem Soc Trans. 2003;**31**(Pt 1):270-4.
3. Wymann MP, Pirola L. *Structure and function of phosphoinositide 3-kinases*. Biochim Biophys Acta. 1998;**1436**(1-2):127-50.
4. Ramadani F, Bolland DJ, Garcon F, Emery JL, Vanhaesebroeck B, Corcoran AE, et al. *The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development*. Sci Signal. 2010;**3**(134):ra60.
5. Srinivasan L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA, et al. *PI3 kinase signals BCR-dependent mature B cell survival*. Cell. 2009;**139**(3):573-86.

6. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. *The emerging mechanisms of isoform-specific PI3K signalling*. Nat Rev Mol Cell Biol. 2010;**11**(5):329-41.
7. Vanhaesebroeck B, Welham MJ, Kotani K, Stein R, Warne PH, Zvelebil MJ, et al. *P110delta, a novel phosphoinositide 3-kinase in leukocytes*. Proc Natl Acad Sci U S A. 1997;**94**(9):4330-5.
8. Pearce VQ, Bouabe H, MacQueen AR, Carbonaro V, Okkenhaug K. *PI3Kdelta Regulates the Magnitude of CD8+ T Cell Responses after Challenge with Listeria monocytogenes*. J Immunol. 2015;195(7):3206-17.
9. Gracias DT, Boesteanu AC, Fraietta JA, Hope JL, Carey AJ, Mueller YM, et al. *Phosphatidylinositol 3-Kinase p110delta Isoform Regulates CD8+ T Cell Responses during Acute Viral and Intracellular Bacterial Infections.* J Immunol. 2016;**196**(3):1186-98.
10. Jou ST, Chien YH, Yang YH, Wang TC, Shyur SD, Chou CC, et al. *Identification of variations in the human phosphoinositide 3-kinase p110delta gene in children with primary B-cell immunodeficiency of unknown aetiology*. Int J Immunogenet. 2006;**33**(5):361-9.
11. Angulo I, Vadas O, Garcon F, Banham-Hall E, Plagnol V, Leahy TR, et al. P*hosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science. 2013;3**42**(6160):866-71.
12. Lucas CL, Kuehn HS, Zhao F, Niemela JE, Deenick EK, Palendira U, et al. *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency*. Nat Immunol. 2014;**15**(1):88-97.
13. Coulter TI, Chandra A, Bacon CM, Babar J, Curtis J, Screaton N, et al. *Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study*. J Allergy Clin Immunol. 2017;**139**(2):597-606.
14. Elkaim E, Neven B, Bruneau J, Mitsui-Sekinaka K, Stanislas A, Heurtier L, et al. *Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study.* J Allergy Clin Immunol. 2016;**138**(1):210-8 e9.
15. Wentink MWJ. *Genetic defects in PI3Kδ affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infection*. Clin Immunol. 2017;**176**:77-86.
16. Miller RA. *The aging immune system: primer and prospectus*. Science. 1996;**273**(5271):70-4.
17. Cambier J. *Immunosenescence: a problem of lymphopoiesis, homeostasis, microenvironment, and signaling*. Immunol Rev. 2005;**205**:5-6.
18. Rodier F, Campisi J. *Four faces of cellular senescence*. J Cell Biol. 2011;**192**(4):547-56.
19. Campisi J, d'Adda di Fagagna F. *Cellular senescence: when bad things happen to good cells*. Nat Rev Mol Cell Biol. 2007;**8**(9):729-40.
20. Spaulding C, Guo W, Effros RB. *Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation*. Exp Gerontol. 1999;**34**(5):633-44.
21. Salminen A, Ojala J, Kaarniranta K. A*poptosis and aging: increased resistance to apoptosis enhances the aging process.* Cell Mol Life Sci. 2011;**68**(6):1021-31.
22. Akbar AN, Henson SM. *Are senescence and exhaustion intertwined or unrelated processes that compromise immunity?* Nat Rev Immunol. 2011;**11**(4):289-95.

23. Kaech SM, Wherry EJ. *Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection.* Immunity. 2007;**27**(3):393-405.
24. Hall BM, Balan V, Gleiberman AS, Strom E, Krasnov P, Virtuoso LP, et al. A*ging of mice is associated with p16(Ink4a)- and beta-galactosidase-positive macrophage accumulation that can be induced in young mice by senescent cells*. Aging (Albany NY). 2016;**8**(7):1294-315.
25. Campisi J. *Aging, cellular senescence, and cancer*. Annu Rev Physiol. 2013;**75**:685-705.
26. Koch S, Larbi A, Ozcelik D, Solana R, Gouttefangeas C, Attig S, et al. *Cytomegalovirus infection: a driving force in human T cell immunosenescence.* Ann N Y Acad Sci. 2007;**1114**:23-35.
27. Gallimore A, Glithero A, Godkin A, Tissot AC, Pluckthun A, Elliott T, et al. *Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes*. J Exp Med. 1998;**187**(9):1383-93.
28. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. *Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells*. Nature. 1993;**362**(6422):758-61.
29. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. V*iral immune evasion due to persistence of activated T cells without effector function.* J Exp Med. 1998;**188**(12):2205-13.
30. Goepfert PA, Bansal A, Edwards BH, Ritter GD, Jr., Tellez I, McPherson SA, et al. *A significant number of human immunodeficiency virus epitope-specific cytotoxic T lymphocytes detected by tetramer binding do not produce gamma interferon*. J Virol. 2000;**74**(21):10249-55.
31. Shankar P, Russo M, Harnisch B, Patterson M, Skolnik P, Lieberman J. *Impaired function of circulating HIV-specific CD8(+) T cells in chronic human immunodeficiency virus infection*. Blood. 2000;**96**(9):3094-101.
32. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, et al. *Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection.* J Virol. 2007;**81**(8):4215-25.
33. Radziewicz H, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, et al. *Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression.* J Virol. 2007;**81**(6):2545-53.
34. Lee PP, Yee C, Savage PA, Fong L, Brockstedt D, Weber JS, et al. *Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients*. Nat Med. 1999;**5**(6):677-85.
35. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. *Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates*. J Clin Oncol. 2010;**28**(19):3167-75.
36. Kim PS, Ahmed R. *Features of responding T cells in cancer and chronic infection*. Curr Opin Immunol. 2010;**22**(2):223-30.
37. Fuller MJ, Zajac AJ. *Ablation of CD8 and CD4 T cell responses by high viral loads*. J Immunol. 2003;**170**(1):477-86.
38. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. *Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment.* J Virol. 2003;**77**(8):4911-27.

39. Mueller YM, De Rosa SC, Hutton JA, Witek J, Roederer M, Altman JD, et al. *Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells.* Immunity. 2001;**15**(6):871-82.
40. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. *Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection*. Nat Immunol. 2009;**10**(1):29-37.
41. Yamamoto T, Price DA, Casazza JP, Ferrari G, Nason M, Chattopadhyay PK, et al. S*urface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection.* Blood. 2011;**117**(18):4805-15.
42. Peretz Y, He Z, Shi Y, Yassine-Diab B, Goulet JP, Bordi R, et al. *CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction*. PLoS Pathog. 2012;**8**(8):e1002840.
43. Raziorrouh B, Schraut W, Gerlach T, Nowack D, Gruner NH, Ulsenheimer A, et al. *The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function*. Hepatology. 2010;**52**(6):1934-47.
44. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, et al. P*D-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection.* J Exp Med. 2006;**203**(10):2281-92.
45. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. *PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression*. Nature. 2006;**443**(7109):350-4.
46. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, et al. *Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction.* Nat Med. 2006;**12**(10):1198-202.
47. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature. 2006;**439**(7077):682-7.
48. Gardiner D, Lalezari J, Lawitz E, DiMicco M, Ghalib R, Reddy KR, et al. *A randomized, double-blind, placebo-controlled assessment of BMS-936558, a fully human monoclonal antibody to programmed death-1 (PD-1), in patients with chronic hepatitis C virus infection.* PLoS One. 2013;**8**(5):e63818.
49. Fuller MJ, Callendret B, Zhu B, Freeman GJ, Hasselschwert DL, Satterfield W, et al. *Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1).* Proc Natl Acad Sci U S A. 2013;**110**(37):15001-6.
50. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, et al. *Enhancing SIV-specific immunity in vivo by PD-1 blockade.* Nature. 2009;**458**(7235):206-10.
51. Gay CL, Bosch RJ, Ritz J, Hataye JM, Aga E, Tressler RL, et al. *Clinical Trial of the Anti-PD-L1 Antibody BMS-936559 in HIV-1 Infected Participants on Suppressive Antiretroviral Therapy*. J Infect Dis. 2017;**215**(11):1725-33.
52. Pardoll DM. *The blockade of immune checkpoints in cancer immunotherapy*. Nat Rev Cancer. 2012;**12**(4):252-64.
53. Sharma P, Allison JP. *The future of immune checkpoint therapy*. Science. 2015;**348**(6230):56-61.
54. Lucas CL, Chandra A, Nejentsev S, Condliffe AM, Okkenhaug K. *PI3Kdelta and primary immunodeficiencies*. Nat Rev Immunol. 2016;**16**(11):702-14.

55. Lucas CL, Zhang Y, Venida A, Wang Y, Hughes J, McElwee J, et al. *Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K*. J Exp Med. 2014;**211**(13):2537-47.
56. Heurtier L, Lamrini H, Chentout L, Deau MC, Bouafia A, Rosain J, et al. *Mutations in the adaptor-binding domain and associated linker region of p110delta cause Activated PI3K-delta Syndrome 1 (APDS1)*. Haematologica. 2017;**102**(7):e289-e281.
57. Petrovas C, Chaon B, Ambrozak DR, Price DA, Melenhorst JJ, Hill BJ, et al. D*ifferential association of programmed death-1 and CD57 with ex vivo survival of CD8+ T cells in HIV infection*. J Immunol. 2009;**183**(2):1120-32.
58. Okkenhaug K, Bilancio A, Farjot G, Priddle H, Sancho S, Peskett E, et al. I*mpaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice.* Science. 2002;**297**(5583):1031-4.
59. Bucks CM, Norton JA, Boesteanu AC, Mueller YM, Katsikis PD. *Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion.* J Immunol. 2009;**182**(11):6697-708.
60. Aken BL, Achuthan P, Akanni W, Amode MR, Bernsdorff F, Bhai J, et al. *Ensembl 2017*. Nucleic Acids Res. 2017;**45**(D1):D635-D42.
61. Giorgi JV, Detels R. *T-cell subset alterations in HIV-infected homosexual men: NIAID Multicenter AIDS cohort study.* Clin Immunol Immunopathol. 1989;**52**(1):10-8.
62. Pereira BI, Akbar AN. *Convergence of Innate and Adaptive Immunity during Human Aging*. Front Immunol. 2016;**7**:445.
63. Wherry EJ. *T cell exhaustion*. Nat Immunol. 2011;**12**(6):492-9.
64. Wherry EJ, Kurachi M. *Molecular and cellular insights into T cell exhaustion*. Nat Rev Immunol. 2015;**15**(8):486-99.
65. Youngblood B, Oestreich KJ, Ha SJ, Duraiswamy J, Akondy RS, West EE, et al. *Chronic virus infection enforces demethylation of the locus that encodes PD-1 in antigen-specific CD8(+) T cells.* Immunity. 2011;**35**(3):400-12.
66. Pauken KE, Sammons MA, Odorizzi PM, Manne S, Godec J, Khan O, et al. *Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade*. Science. 2016;**354**(6316):1160-5.
67. Angelosanto JM, Blackburn SD, Crawford A, Wherry EJ. *Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection*. J Virol. 2012;**86**(15):8161-70.
68. Utzschneider DT, Legat A, Fuertes Marraco SA, Carrie L, Luescher I, Speiser DE, et al. *T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion.* Nat Immunol. 2013;**14**(6):603-10.
69. Popkie AP, Zeidner LC, Albrecht AM, D'Ippolito A, Eckardt S, Newsom DE, et al. P*hosphatidylinositol 3-kinase (PI3K) signaling via glycogen synthase kinase-3 (Gsk-3) regulates DNA methylation of imprinted loci.* J Biol Chem. 2010;**285**(53):41337-47.
70. Chen L, Flies DB. *Molecular mechanisms of T cell co-stimulation and co-inhibition*. Nat Rev Immunol. 2013;**13**(4):227-42.

71. Odorizzi PM, Wherry EJ. *Inhibitory receptors on lymphocytes: insights from infections*. J Immunol. 2012;**188**(7):2957-65.
72. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. *Tissue expression of PD-L1 mediates peripheral T cell tolerance*. J Exp Med. 2006;**203**(4):883-95.
73. Okazaki T, Honjo T. *The PD-1-PD-L pathway in immunological tolerance.* Trends Immunol. 2006;**27**(4):195-201.
74. Francisco LM, Sage PT, Sharpe AH. *The PD-1 pathway in tolerance and autoimmunity.* Immunol Rev. 2010;**236**:219-42.
75. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol. 2005;**25**(21):9543-53.
76. Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, et al. *Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial.* Lancet Oncol. 2016;**17**(11):1558-68.
77. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. C*ombined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma.* N Engl J Med. 2015;**373**(1):23-34.
78. Rao VK, Webster S, Dalm V, Sediva A, van Hagen PM, Holland S, et al. *Effective 'Activated PI3Kdelta Syndrome'-targeted therapy with the PI3Kdelta inhibitor leniolisib.* Blood. 2017;**130**(21):2307-16.

PART 5

General discussion

GENERAL DISCUSSION

In this thesis we aimed to study how specific genetic defects influence signaling cascades in B-cells and thereby lead to disturbances in B cell development and repertoire formation eventually causing antibody deficiencies. To study how mutations and disturbances lead to disease, knowledge of normal B cell development and B cell intrinsic processes is crucial to embed newly obtained results from patients. We employed different techniques including multi-color flow cytometry, next generation sequencing, and *in vitro* stimulation assays to study B-cell differentiation and signaling in the context of healthy controls and patients with different causes of antibody deficiency.

This General Discussion, highlights how the knowledge obtained in our studies can be used for further research on for example B cell precursor development (**chapter 2.1 and 2.2**). The effect of PI3Kδ gain-of-function mutations on B cell differentiation was studied in **chapters 4.1 and 4.3** and in that light, other types of mutations than the classical loss-of-function mutations will we reviewed. Additionally there will be a focus on other etiological mechanisms that might cause antibody deficiencies like epigenetics. In in extension to that the likelihood of polygenetic causes and how pathway analysis might help to elucidate this as a cause for immune deficiency or dysregulation will be discussed. Finally the current vision on CD21lo B cells and how they compare to CD21deficient cells (**part 3**) will be reviewed as a well as and how these cells might link to exhaustion (**chapter 4.4**).

BONE MARROW B-CELL PRECURSOR DEVELOPMENT

Bone marrow studies described in this thesis (**part 2**) are just the beginning of more extended experiments that can shed light on normal and abnormal B-cell development in bone marrow. Not only can this data be used in the diagnostic practice of PID, additionally, using data from genetically identified cases, clues for diagnosis in unsolved cases can be obtained. Furthermore, by employing other techniques next to the flow cytometric immunophenotyping and immune repertoire sequencing as described here, more knowledge on BCP differentiation can be obtained.

Asynchronous marker development reflects multiple differentiation routes between BCP checkpoints

In our BCP differentiation studies in **chapter 2.1**, we employed a new flow cytometric panel developed by the EuroFlow PID consortium to study BCP differentiation in healthy bone marrow and we used genetically defined PID cases as a control for absence of recombination and (pre-)BR signalling. We found that expression pattern of some surface

markers is asynchronous to cylgμ expression This suggests that marker development is partially asynchronous to the process of BR formation. Additionally, we found that population based gating is not ideal for analysing this continuous process of differentiation, especially in the stages in which marker development is most asynchronous. We hypothesize that some cells, deviate from the most common route, because they are unable to form a functional B cell receptor, or because they need more time to compose a functional allele. V(D)J rearrangement is a stepwise process in which first the D segment is linked to the J segment.[1-3] This occurs commonly on both alleles, forming incomplete rearrangements. V-DJ rearrangements takes place on one allele at a time. When the first attempt (on allele one) is successful, the second allele will not be rearranged. However, in case rearrangement of the first allele results in an unproductive sequence, or if the produced heavy chain cannot pair with the surrogate light chain, the second allele needs to be rearranged. Hence, rearranging a functional IGH-allele takes more time. If the rearrangement of the second allele fails, the cell will become apoptotic. This means that different cells, depending on the speed with which they can produce a functional heavy chain, will follow a different molecular route. We hypothesize that this is reflected to some extent in phenotypic markers that can be measured by flow cytometry. Some of the routes can, at least partially, be explained by molecular processes that are linked to V(D)J recombination and (pre-)BR signalling.

This can be further studied using extended phenotyping techniques like cytometry time-of-flight (cyTOF), which enables integration of up-to 40 markers on a single cell.[4] We have put together a cyTOF panel, that integrates the markers present in our flow-panel, with more extended phenotypic markers, apoptosis and proliferation markers and several transcription markers (Table 1). This enables more extensive sub-setting of populations and prediction of which populations have increased apoptosis and therefore might be part of a maturation route that leads to a dead end. We tested the flow cytometry panel and the cyTOF panel in parallel on the same samples and found that we could reliably gate the same populations with the cyTOF approach as with the flow cytometry approach (Figure 1). This indicates that the cyTOF approach can be a good extension of our flow cytometry studies. The data sets that can be obtained with this panel can be used with advanced analysis tools to predict differentiation routes of B cells in bone marrow. Others have used the Wanderlust algorithm to model BCP-differentiation.[5] This model takes into account the continuity of BCP differentiation, circumventing population based gating. However, it also assumes a non-branching model of BCP differentiation, which is not in line with our observations. Therefore, it is interesting to adapt the Wanderlust algorithm in such a way that branching in the form of divergence based on differences in V(D)J-recombination and convergence at the BCP-checkpoints can be modelled to integrate the continuous differentiation and asynchronous development.

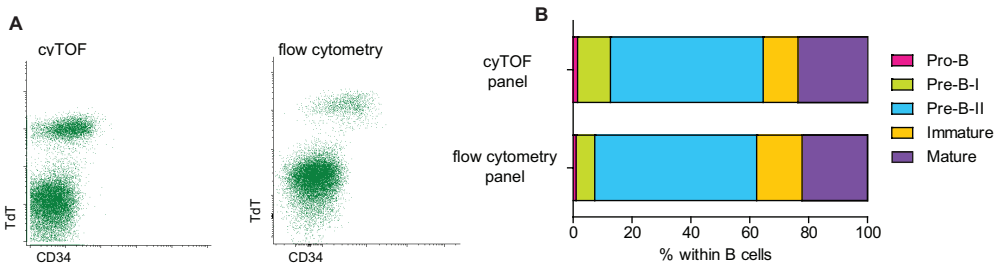

Figure 1.
Comparison between flow cytometry and cyTOF in parallel on the same human bone marrow sample. **A.** Dotplots showing TdT and CD34 expression of BCP measured by cyTOF and flow cytometry. **B.** Gating of the main BCP populations acquired cyTOF and flow results in approximately the same subset distribution.

The single-cell protein expression data that can be obtained by flow cytometry or cyTOF should ideally be extended with single cell RNA-expression data and DNA-sequencing of IG-loci to understand the link between V(D)J-recombination status, transcriptome and protein expression. These type of studies can give more in-depth knowledge on how different processes in BCP development are linked and interacting to form a diverse pool of functional B cells that provide complete humoral immunity.

Table 1. cyTOF panel for BCP development in human bone marrow

Markers in flow panel	Phenotyping	Apoptosis	Proliferation and survival	Cell cycle regulation	Differentiation and migration		
CD19	CD34	CD45	CD9	i- Caspase 3	i- PAX5	i-cPARP	CXCR5
IgM	CD38	CD24	CD44	i- Caspase 7	BCL-6	BCL-6	CXCR4
IgD	i-CD79a	CD22	CD27	BCL-2	CD135		HLA-DR
CD20	i-Igμ	IgKappa	i-CD79b		Ki-67		CD73
CD10	i-TdT	IgLambda					

i- indicated intracellular markers

Bone marrow studies in PAD; Good syndrome and CVID

In **chapter 2.3** bone marrow precursor B cells were studied in patients with Good syndrome.[6] These patients suffer from thymoma and hypogammaglobulinemia and in most cases have a complete absence of B cells in the peripheral blood.[7] We found that the bone marrow of these patients is highly B-lymphopenic as well, and that the subset distribution and the type of arrest is different than in other patients with agammaglobulinemia. The arrest in Good syndrome patients seems to occur after the Pro B stage, whereas in agammaglobulinemia patients a differentiation arrest occurs after the Pre B-I stage, indicating a different disease causing mechanism in the two groups of patients. Good

syndrome patients are one group of PAD patients in whom studying the bone marrow can help resolve these disease causing mechanisms. But there are other groups of patients with PAD in which bone marrow studies can be useful. One of the most frequently diagnosed PAD is common variable immunodeficiency (CVID). A highly heterogeneous disease in which a defect arises at some point in B-cell differentiation. Driessen *et al.* proposed a model for classification of CVID patients based on the underlying pathophysiological mechanism.[8] This model uses a combined approach of flow cytometric and molecular techniques to identify 5 patterns of CVID-causing defects. In pattern 1, patients have low numbers of transitional and memory B cells and an increased number of cell divisions in the naive B cells, which likely reflects proliferation to compensate for the reduced bone marrow output. In this group of patients a defect that arises already in bone marrow B cell development is proposed to cause the disease. Different studies have previously identified groups of CVID patients with defects in BCP development. Ochtrop *et al.* found a partial defect in BCP differentiation at the pre-BI to pre-BII stage in CVID patients with low numbers of transitional B cells.[9] Results from a study by Anzilotti *et al.* showed that all investigated CVID patients had BCP in all major stages of development, consistent with the observation that they had peripheral blood B cells, however reduced numbers of pre-BII large and immature cells (the last two stages of BCP development) were found in the patients.[10] This suggests that in a group of CVID patients, a defect in the pre-BI to pre-BII checkpoint is the cause of disease. Additionally, a B-cell extrinsic defect, such as in the bone marrow stroma or cytokine milieu, might contribute to the disease. Another explanation could be a defect that is not B-cell unique, since some patients additionally have T cell abnormalities. The most recent study in bone marrow of CVID patients was performed by Lougaris *et al.*[11] and confirmed that in one third of CVID patients, a problem arises in the bone marrow, however, this study did not show the underlying pathological mechanism. Additional studies in CVID studies have indicated that possibly, some patients have problems in DNA-repair.[12] This hypothesis can be further studied using a combined approach of flow cytometry and repertoire analysis. Based on these observations, we hypothesized that some CVID patients might have reduced efficiency of V(D)J-recombination causing reduced bone marrow output of naive B cells. This could be reflected in a reduced diversity of the naive repertoire. In **chapter 2.2** we studied the naive repertoire of 18 CVID patients. We found no qualitative defect in the naive IGH repertoire of CVID patients, and the diversity was not reduced in 17 out of 18 patients. However, one of them had a profound reduced diversity which was stable over the years. This indicates that repertoire analysis can be a useful tool to identify CVID patient that might have a recombination problem. In our studies we so far did not study precursor B-cell development in CVID patients using flow cytometry. However, our new panel and understanding of BCP can be used to study the etiology of disease by linking specific phenotypical patterns that are seen in genetically defined cases

to specific pathways. In this way, we might be able to understand which pathways are affected in patients with CVID. Of course, while performing such studies, it is crucial to recognize and emphasize the heterogeneity within the disease that is called CVID. This means that studying the bone marrow is most useful in patients with early defects in B cell development and that it is likely that within a group of patients, different causal mechanisms can play a role. Moreover, in many patients the interplay between multiple mechanisms can cause variability in immune biology. Therefore it is crucial to integrate multiple analysis strategies for these types of studies, like flow cytometric phenotyping, repertoire analysis, RNAsequencing and analysis of epigenetic regulation in healthy controls, genetically defined PID cases and CVID patients. By this approach, we can better understand the pathways that lead to disease in CVID.

DISTURBED SIGNALING PATHWAYS CAUSING ANTIBODY DEFICIENCIES

In **part 3** of this thesis deficiencies in the CD19 complex and specifically in CD21 have been described. In addition, dysregulation of the PI3K-AKT signaling by mutations in PI3K and PTEN was studied in **part 4**. All these pathways are interlinked in the B cell. Here, we discuss how disruption of CD19-complex signaling and PI3K signaling lead to antibody deficiency. Additionally we will discuss the current view of increased CD21^{lo} B cells, since these cells are found in conditions of PI3K-signaling dysbalance.

The connection between B-cell receptor signaling, CD19-complex signaling and PI3K signaling

The goal of each BCP is to recombine and express a functional BR to be able to signal antigen once it enters the peripheral blood. The BR can forward a signal via multiple signaling cascades (Figure 2). Via tyrosine kinases LYN and SYK, Bruton`s Tyrosine Kinase (BTK) can be activated, which can in turn activate Phospholipase C Gamma 2 (PLCγ2) and thus induce Mitogen-Activated Protein Kinase (MAPK) and Nuclear Factor Kappa B (NF-κB).[13, 14] These factors can both translocate to the nucleus to induce transcriptional changes that promote B cell survival and differentiation. Furthermore, BR-mediated signaling will enhance release of intracellular calcium, also referred to as calcium influx, via PLCγ. Additionally, Via SHP-2, a protein tyrosine phosphatase, and by LYN directly, PI3K-AKT signaling can be initiated. PI3K signaling again stimulates the increase of intracellular calcium and NFκB. BR-signaling upon antigen binding is enhanced by the CD19 complex, consisting of CD19, CD21, CD81 and CD225. The intracellular domain of CD19 contains multiple tyrosine residues and hereby can activate PI3K signaling, enhancing the BR-initiated signaling cascade. This effect is rather strong, which is reflected by the observation that CD19 induced signaling could rescue BR-deficient naive B cells.[15]

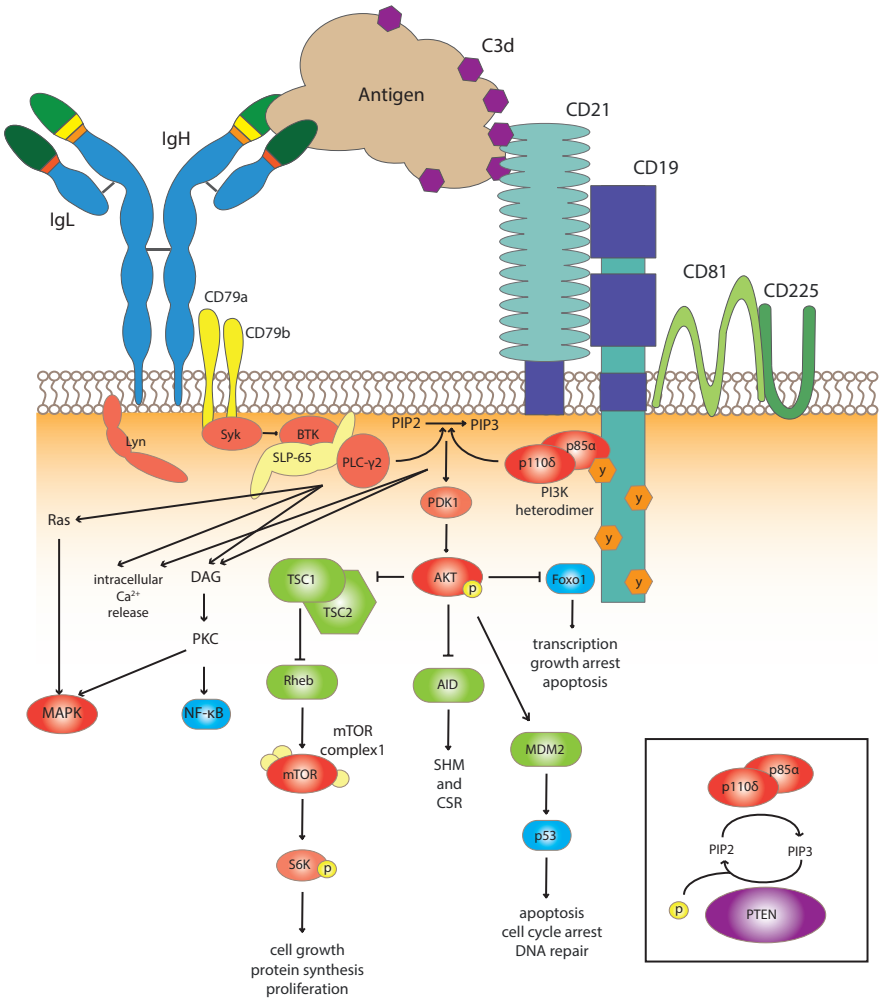

Figure 2.
Linking BR, CD19 complex and PI3K signaling.

CD21 in the CD19 complex

Signaling via the CD19 complex is critically important for B cell development, differentiation and maturation.[16] The complex functions as a co-stimulatory element to amplify antigenic signaling and it bridges innate and adaptive immunity by signaling complement[17] via CD21, which is complement receptor 2. The complex does not only amplify the BR-signal but also prolongs the signal.[18] CD19 has a cytoplasmic tail with multiple tyrosine kinase residues to induce for example PI3K signaling and CD81 is a tetraspanin, which is critically important for the expression of CD19 on the surface. Therefore it is no surprise that a deficiency in this complex results in PAD. In this thesis the

second CD21 deficient patient is described in **chapter 3.1**. We found that CD21 deficiency results in hypogammaglobulinemia with a rather mild phenotype compared to CD19 deficiency. Since CD19 deficiency and CD81 deficiency as a cause for PAD were described before by our group, we were able to compare the differences between the different disease modalities in more detail in **chapter 3.2**. These patients with specific defects in the CD19-complex are highly interesting since their disease phenotype can be used to study the function of the different proteins in human B cells.

In our CD21 deficient patient (**chapter 3.1**), two compound heterozygous mutations resulted in complete absence of CD21 protein on the surface of B cells. In his parents, who both carried one of the mutations, CD21 expression was reduced by half compared to healthy controls. This decreased expression did not result in any clinical or immunobiological phenotype. Expression of CD19 was increased in the patient and to lesser extent in his parents. Although no CD21 transcripts could be detected at mRNA level, the increased plasma membrane expression of CD19 was not reflected at transcript level. The increased CD19 surface expression is also found in $CD21^{lo}$ cells in auto-immune diseases[19, 20] and in Cd21/Cd35 deficient mice.[21, 22] Vice versa, in CD19 deficient patients, CD21 surface expression is reduced, indicating that CD21 and CD19 expression are interlinked. This was previously suggested in mouse studies as well, where it was shown that even though CD21 and CD19 are closely connected, CD19 function was dominant over CD21 function and regulates B cell signal transduction independent of CD21.[23] Our data from both human CD21 deficiency and human CD19 deficiency indicate that not only function but also expression of the two proteins is connected albeit not at transcript level.

In **chapter 3.2** we further investigated the differences and similarities between patients with different deficiencies in the CD19 complex. In total 15 patients with PAD due to deficiencies in the CD19 complex have been described. All deficiencies were caused by either homozygous or compound heterozygous mutations and although all patients had an antibody deficiency, the clinical phenotype was different per deficient protein. Thus far, 10 patients with CD19 deficiency have been reported[24-28] and 1 patient with CD81 deficiency.[29] These patients suffer from (severe) recurrent infections and all of them had hypogammaglobulinemia. Many of them additionally had low serum levels of IgA and/or IgM and reduced responses to vaccination. The four patients with CD21 deficiency that have been described in literature, had a less severe phenotype. Although they all had hypogammaglobulinemia, the infections that were found in these patients were less severe and one of them did not have infectious complications at the time of analysis.[30-32] Calcium influx assays that were performed by us and others indicated that in CD21 deficiency, B cells can be activated though the BR as long as the signal is strong enough, in contrast to CD19 deficient or CD81 deficient B cells that to not respond even to high doses of stimulation. Thiel *et al*. showed that in CD21 deficiency, calcium influx is only impaired

in a complement dependent manner.[30] Thus, the hypogammaglobulinemia in CD19 and CD81 deficiency can at least partially, be explained by defective BR-signaling and therefore by an activation problem. In CD21 deficiency, the activation is intact and perhaps even compensated for by increased CD19 expression, but our results from *in vitro* stimulation experiments indicate that induction of activation induced deaminase (AID) is delayed. AID is a key factor for somatic hypermutation (SHM) and class switch recombination (CSR) in B cells. AID is activated, via PI3K-AKT signaling (Figure 2). It could be that because of the lack of CD21, the PI3K-AKT signaling pathway is less activated, resulting in a slower signal transduction and delayed AID induction. In CD19 and CD81 deficiency however, this signaling cascade is not induced at all, resulting in more pronounced absence of SHM and CSR.

Overall, CD19 and CD81 deficiency result in a more severe clinical phenotype than CD21 deficiency. This can be explained by the interaction of the different proteins and their different functions within the CD19-complex.

Activated PI3Kδ syndrome: APDS

Over the past years, multiple series of patients with gain-of-function mutations in PI3Kδ have been described.[33-37] These patients suffer from Activated PI3Kδ syndrome (APDS) and share clinical characteristics such as recurrent respiratory tract infections, bronchiectasis, hepatosplenomegaly, generalized lymphadenopathy and antibody deficiency. Immune phenotypically an inversed CD4/CD8 ratio and increased transitional B-cells are commonly found. The first reports on this disease showed that hyperactivation of the PI3K-AKT signaling pathway in T cells resulted in constitutive activation of naive cells, fast differentiation into short-lived effector $CD8^+$ T cells and reduced memory formation.[33-35]

In **chapter 4.1** of this thesis, we report a young girl with APDS, who presented with a mediastinal mass. She had recurrent respiratory tract infections, severe pulmonary damage and generalized lymphadenopathy with an enlarged spleen and liver. The mediastinal mass consisted of lymph adenal tissue with dysplastic germinal centers. She was treated with corticosteroids and became prednisone-dependent, which resulted in reduced growth in length. After discovery of APDS, a pathogenic mutation in *PIK3CD* (E525K) was found, confirming the diagnosis APDS. She started on rapamycin treatment and prednisone could be weaned. Upon follow-up the infectious load reduced dramatically, reducing the need for antibiotic treatment and she gained . This case illustrates the importance close collaboration between pulmonologists and immunologists. Furthermore, the clinical improvement in this girl shows that rapamycin can be a possible treatment for patients with APDS.

APDS is not the only disease in which PI3K-signaling is dysregulated. *PTEN* (phosphate and tensin homologue deleted on chromosome 10) is the antagonist of PI3K (Figure 2).

Autosomal dominant germline mutations in *PTEN* are associated with PTEN deficiency resulting in three partly overlapping clinical syndromes which together are referred to as PTEN Hamartoma Tumor Syndromes (PHTS). Although in mice PTEN deficiency was linked to reduced AID induction and reduced CSR over a decade ago,[38] in humans, this connection was not made until 2015 when we and other described PHTS patients with hypogammaglobulinemia.[39, 40] These patients are described in **chapter 4.2**, where we identified three patients with heterozygous *PTEN* mutations who suffered from PTEN deficiency and hypogammaglobulinemia. Not all patients with PHTS develop hypogammaglobulinemia and thus, we performed study of the peripheral B-cell development of these three patients with hypogammaglobulinemia and of patients with PHTS without hypogammaglobulinemia. We demonstrated that patients with PTEN deficiency have CSR and SHM deficiency irrespective of hypogammaglobulinemia. This is most likely because of PI3K/AKT mediated inhibition of AID, which is present in all patients. However, other factors, like the encounter of micro-organisms or perhaps SNPs in other genes contributing to B cell development might be different between individual patients and thus explain why some develop hypogammaglobulinemia while others do not. This clinical heterogeneity is also seen in patients with APDS.

The hypogammaglobulinemia in APDS patients was originally thought to result from decreased $CD4^+$ T cell help to B cells. However, the PI3K-AKT signaling pathway is critical for B cell development and maturation and therefore, in **chapter 4.3** we focused on B cell differentiation in APDS patients. We identified 13 patients of whom 3 had new mutations in *PIK3CD* or *PIK3R1*. We studied the peripheral blood B cell compartment and BCP differentiation in BM. P110δ deficient mice show a normal BCP development[41] since p85α was shown to be able to take over the role of p110δ role in autonomous pre-BR signaling.[42] However, in APDS patients over-active signaling because of constitutive activation of the PI3K-AKT pathway in BCP in bone marrow may cause disturbance at the pre-BR check-point. This might lead to cells routing to alternative maturation pathways because of increased proliferation signals and increased tonic signaling at the pre-B I stage might cause increased apoptosis. Indeed we found an increase of $CD34^-$ TdT^+ BCP in our patients, which we hypothesize to be the result of alternative maturation pathways. Also, we found reduced CD19 expression in a subset of BCP cells, which could reflect early apoptotic cells. The increase in transitional B cells in the peripheral blood that we found in APDS patients could indicate that cells do not easily pass the phase of antigenic stimulation, which might be due to an already hyperactive metabolic state of the cells. This is in line with our phospho-flow results, where we found increased constitutive phosphorylation of AKT, especially in the naive and natural effector B cells and to lesser extent in memory B cells. This increased activation may lead to cell death rather than differentiation, as indicated by

the increased apoptosis rate that we found upon *in vitro* culture and stimulation of APDS lymphocytes.

This constitutive activation and apoptosis were also found in the T cells of APDS patients by us and others. Continuous activation of T cells can induce senescence and/or exhaustion of $CD8^+$ T cells. The increased CD57 expression that was previously described in a subgroup of APDS patients was associated with senescence of the $CD8^+$ T cell compartment.[34] However, the increased apoptosis rate and previous reports of increased expression of PD-1 could also indicate a role for exhaustion in causing reduced function of the $CD8^+$ T cells in APDS. In **chapter 4.4** we therefore investigated the expression of activation markers and inhibitory receptors as markers for exhaustion on total $CD8^+$ T cells and virus-specific $CD8^+$ T cells in patients with APDS. We compared them to healthy controls and patients with HIV, who served as a model for exhaustion. $CD8^+$ T cells expressing all three inhibitory receptors ($PD\text{-}1^+$ $CD160^+$ $CD244^+$), which would indicate the most exhausted state, were significantly increased in APDS patients and to a similar degree as in HIV-infected individuals. This increased inhibitory receptor expression was not correlated with gender, age, type of mutation, B cell phenotype or with other phenotypic markers on T cells that we have investigated. The frequency of $PD\text{-}1^+$ $CD160^+$ $CD244^+$ T cells was negatively correlated with the amount of naive $CD8^+$ T cells, we suggest that the hyper-activation leading to reduced naive T cells, may also be responsible for the increased expression of inhibitory receptors on T cells. Overall, the expression of inhibitory receptors on $CD8^+$ T cells from APDS patients has more similarities with HIV^+ patients, supporting the idea that the PI3Kδ GOF mutations may contribute to exhaustion. Blocking inhibitory receptors on exhausted $CD8^+$ T cells can restore or improve their function in chronic viral infections.[43, 44] Inhibitory receptor blockade was introduced into the clinic to re-activate exhausted T cells in cancer.[45, 46] In our study, we showed that blocking PD-1 signalling can increase virus-specific $CD8^+$ T cell proliferation and cytokine production of APDS EBV-specific $CD8^+$ T cells. However, upregulation of inhibitory receptors on activated T cells has a physiological function: they downregulate the immune response after successful control of infections[47, 48] to prevent autoimmunity and pathological responses leading to tissue damage. In APDS, upregulation of inhibitory receptors could be a protective mechanism to prevent damage. This hypothesis is supported by a study which showed how PD-1 signaling can prevent activation of PI3K and AKT phosphorylation, thereby preventing proliferation.[49] The increase in proliferation and effector cytokine secretion in virus-specific $CD8^+$ T cells from APDS patients that we found *in vitro* was already detectable after 5 days, indicating that, short term treatment of patients with PD1/PD-L1 blockade might work. During a re-current EBV- and/or CMV infection, inhibitory receptor blockade could augment efficacy of exhausted virus-specific $CD8^+$ T cells and thus reduce EBV- and CMV

related morbidity and mortality. However, because of the increased risk of autoimmunity and hyper activation, manipulating this pathway has to be done with caution.

Although mutations in *PIK3δ* and *PTEN* have only recently been found to cause PAD, somatic PI3K and PTEN mutations are found in a variety of solid tumors.[50] The vast majority of the PI3K mutations arise in *PIK3CA*, the gene encoding p110α (the catalytic subunit of PI3Kα) and are found in carcinoma`s and in some B-cell lymphoma`s *PIK3CD* mutations are found. The most common "hotspots" of oncogenic *PIK3CA* mutations are H1047R and E542K/E545K, which respectively are located in the C-terminal kinase domain and the helical domain. Both these and other less common mutations (e.g. N345K and C420R) result in activation of PI3Kα through increased lipid binding and/or increased basal activation.[51] P110α and p110δ share ~72% sequence homology. Germline APDS-causing *PIK3CD* mutations are located in the same PI3K domains as somatic oncogenic *PIK3CA* mutations, and in some cases even corresponding amino acids are affected amino acid (Figure 3). They result in the same activation of the PI3K-AKT signaling pathway, albeit in different cell types. Heurtier *et al.* recently published two APDS-causing mutations in *PIK3CD* (E81K and G124D) that cause increased PI3K-AKT signaling and the clinical phenotype of APDS.[37] These same two mutations were later found in other patients by Takeda *et al*, confirming the findings of the earlier paper.[52] Some of the oncogenic *PIK3CA* mutations are also found as mosaic mutations occurring during development or germline mutations,[53] in which case they result in the megalencephaly-capillary malformation-polymicrogyria syndrome (MCAP).[54] This syndrome can also be caused by mutations in *PIK3R1* and *PIK3R2*, as was the case in one of our APDS2 patients (PI3KR-1) in **chapter 4.3**.[36] In this boy, one *PIK3R1* mutation caused both APDS and MCAP which underlines the notion that gain-of-function mutations in *PIK3CD*, *PIK3CA* and *PIK3R1* likely share the same activation mechanisms. These mechanisms probably are also affected in patients with germline PTEN-mutations, whom have been described by us (in **chapter 4.2**) and others to have PI3K-signaling mediated hypogammaglobulinemia next to their PTEN hamartoma tumor syndrome

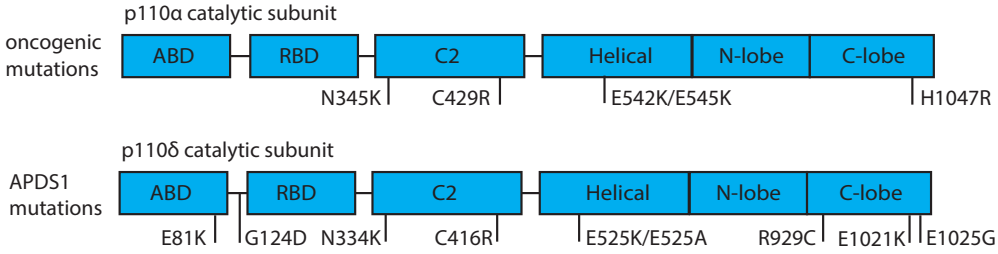

Figure 3.
Schematic representation of locations of mutations in *PIK3CA* and *PIK3CD*. Oncogenic mutations in *PIK3CA* map to the same domains and in some cases to corresponding amino-acids as APDS-causing *PIK3CD* mutations.

(PHTS) phenotype.[39, 55] Both patients with MCAP and PHTS have neurological abnormalities and in some cases they suffer from developmental delay.

Because of the extended knowledge on PI3K in the field of oncology, there are multiple possible options for treatment of APDS known; most of these drugs already existed before the disease was described (Figure 4). These strategies are now under investigation. We have suggested short-term treatment with PD1/PD-L1 blocking agents as a possible treatment option in **chapter 4.4**. Extended studies *in vitro* are needed to prove our results, before these can be applied in the clinics. These treatment strategies are already available in cancer treatment and we would recommend trialling them in APDS patients to control recurrent viral infections on a short-term basis. Another readily available treatment option for patients with APDS is rapamycin (also known as sirolimus). This drug has a long history as immune suppressive drug used to treat kidney transplant patients. Over the past years, it has been used in both adult and children`s clinics to treat patients hyperactivation of mTOR signaling, for example patients with tuberous sclerosis complex.[56, 57] Rapamycin inhibits mTOR and thereby changes the metabolic state of cells, reducing proliferation. Since part of the APDS phenotype was attributed to increased mTOR activity, many APDS patients that were prednisone dependent have started to use rapamycin, like the patient we described in **chapter 4.1** and some of the others described in **chapter 4.3 and 4.4**. The success of this treatment strategy can be evaluated in the next couple of years, when more patients have been treated over extended periods of time. Ideally, this should be done in trials where this treatment regime is tested against standard treatment to prove which treatment is best in which case. A third option, that is currently under investigation is treatment with specific PI3Kδ-inhibiting compounds. This was suggested already when APDS was discovered as an ideal form of personalized medicine.[58] A specific PI3Kδ-inhibitor is expected to treat the disease at the level of the hyperactive protein and, because of specificity, have fewer side effects. In a recent study, 6 patients were treated with the specific PI3Kδ-inhibitor leniolisib.[59] The patients in this study all had improvement of their clinical symptoms and laboratory parameters and the drug was well tolerated. This study is now extended to include more patients and treat them over a longer period to investigate long-term outcome. Another option for treatment of APDS could be hematopoietic stem cell transplantation (HSCT). One of the patients we described in chapter 4.3 has been transplanted years before we found the genetic defect leading to his disease. He was diagnosed with a combined immunodeficiency as a child and received a HSCT at a young age. He had a good engraftment and infections ceased. However, since APDS has a highly heterogeneous phenotype HSCT might not be the treatment of choice in all APDS patients given the risks and possible complications of HSCT.

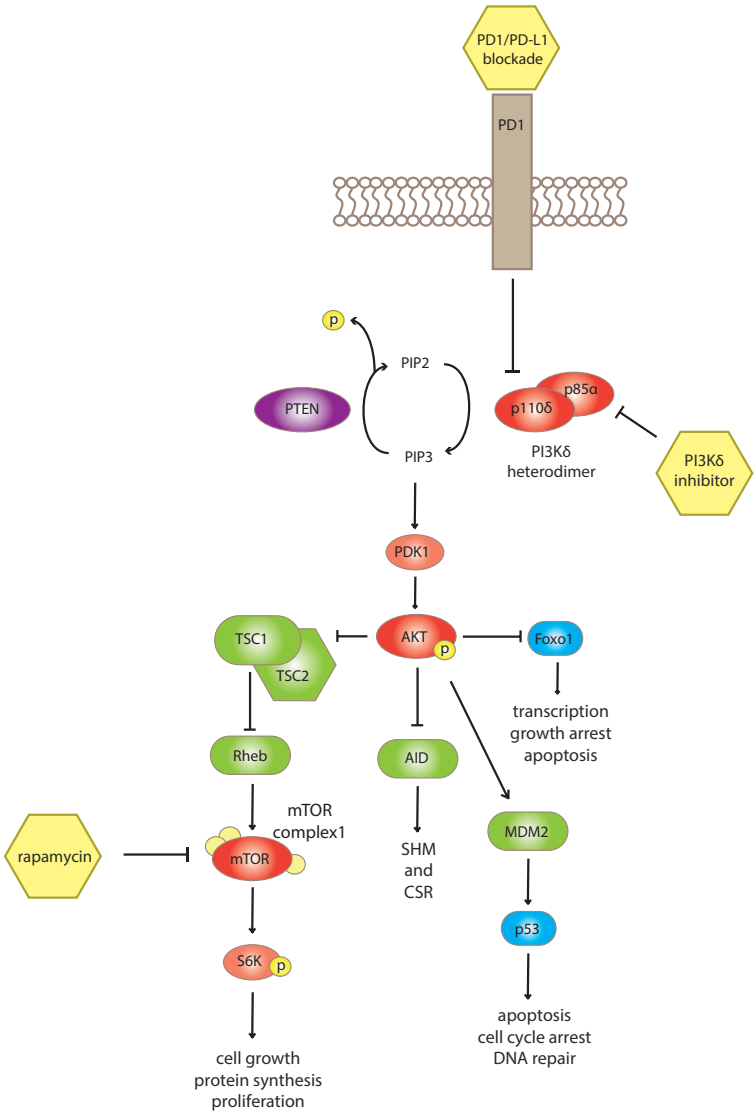

Figure 4.
Possible compounds to treat APDS.

CD21^{lo} B cells, what are they?

The CD21 protein has been of interest in the field of PAD for decades, because CD21^{lo} cells have been suggested to play a role in a subgroup of patients with CVID that have increased CD21^{lo} cells. To date, the role and function of these cells in physiology and disease etiology has not completely been elucidated, although many studies have been performed. In our patients with APDS, which were described in **chapter 4.3**, and in the

patients with PTEN mutations described in **chapter 4.2**, we found increased CD21^{lo} cells in a subgroup of patients (unpublished data, Figure 5). For years CD20^{lo} B cells have been known to be increased in several auto-immune diseases like SLE[20] but also in patients with CVID who suffer from auto-immune cytopenias.[60] This was linked to the finding that CD21 can function as a receptor for DNA,[61] and thus, down regulation might lead to decreased sensitivity of the B cells and reduced auto-antibodies. Recently, patients with Down syndrome were shown to have a high frequency of these cells,[62-64] but in these patients, this finding was linked to increased apoptosis in the lymphocyte compartment.

Rakhmanov *et al.* studied CD21^{lo} B cells in CVID patients and concluded that they to some extent *in vitro* resemble innate B cells, or B1 B cells that are found in mice.[65] They hypothesized that in CVID patients, this subset is abnormally expanded. In HIV patients CD21^{lo} B cells are expanded as well.[66] This is thought to be a sign of B-cell exhaustion occurring during chronic infections. The "exhausted-like" phenotype of CD21^{lo} B cells in chronic infection was additionally found in patients infected with hepatitis C,[67] where the authors found that the CD21^{lo} B cells were hypo proliferative but retained some antibody secreting capacity. This hypothesis is supported by the finding that CD21^{lo} B cells in CVID express a variety of inhibitory receptors and do not proliferate *in vitro* in response to stimuli.[65, 68] A study by Visentini *et al*, confirmed the reduced replicative potential of CD21^{lo} B cells from CVID patients but furthermore showed that telomeres are shortened in the B and T cells of their study-cohort.[69] This was explained as an increase in replicative senescence in the lymphocytes if these patients. However, telomere shortening can also be found in patients with HIV and PD-1 blockade increased telomerase activity in HIV specific CD8^{+} T cells,[70] indicating that senescence and exhaustion are two mechanisms that are not necessarily mutually exclusive. In our study on CD8^{+} T cell exhaustion in patients

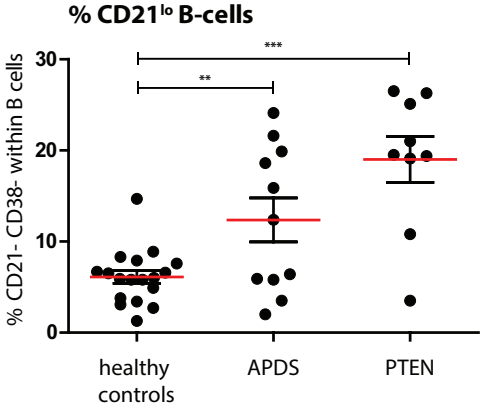

Figure 5.
CD21^{lo} B cells in healthy adults, APDS patients and PTEN patients. Both in the APDS patients and PTEN patients a subgroup of patients has increased CD21^{lo} B cells.

with APDS (**chapter 4.4**) we concluded that both senescence (as described previously) and exhaustion probably play a role in the decreased immune function in these patients. This would fit the notion that also the B cell compartment is affected in an exhaustion-like manner by PI3K-mutations, resulting in an increase in $CD21^{lo}$ cells. The decreased CD21 expression was recently linked to high expression of SYK, which presumably results from chronic activation in inflammatory environments.[71] The idea of this exhausted-B-cell-hypothesis is, that B cells become less sensitive to stimulation, hence the threshold for activation in increased by downregulation of CD21. And this can be a possible mechanism to protect the cell (and the immune system) from hyper activation, thus reducing severe inflammation or auto-reactivity. In APDS patients, both the intrinsic hyper activation of B cells resulting from the mutations and the inflammatory milieu that is caused by recurrent infections might cause CD21 down-regulation on B cells. However, this has to be elucidated further in more extensive studies.

Many diseases that are associated with increased numbers of $CD21^{lo}$ B cells are also associated with dysregulation of PI3K-signaling. Perhaps extended studies of $CD21^{lo}$ B cells in patients with APDS or PTEN mutations can reveal a common mechanism for exhaustion within the B and T cell compartment, since we have shown that exhaustion is present in the $CD8^{+}$ T cell compartment of APDS patients (**chapter 4.4**) and they have increased $CD21^{lo}$ B cells. Our studies in a CD21 deficient patient (**chapter 3.1**) showed that the loss of CD21 per se does not lead to complete unresponsiveness, but rather to an increased threshold for activation. However, B cells from a CD21 deficient patient do not represent $CD21^{lo}$ cells that are found in other diseases, since the origin of the reduced or absent CD21 expression in different.

CAUSES FOR ANTIBODY DEFICIENCY; MONOGENETIC DEFECTS AND BEYOND

In this thesis we have investigated how specific defects disturb signaling and B cell differentiation ultimately resulting in an antibody deficiency. But for many patients, we still cannot explain why they suffer from antibody deficiencies. In search for disease etiology of PAD, many turn to genomics, searching for genetic defects that can explain the disease phenotype. And although over the years over 300 monogenetic causes for PID have been described,[72, 73] in most antibody deficiencies no genetic defects can be found, even when the whole genome is sequenced. It is likely that in many PAD patients, disease etiology is complex including genetic, epigenetic, regulatory and environmental factors.[74] The position of genetics and some of the other possible disease modifying mechanisms and contributing factors will be discussed here.

Discovery of new genetic defects, Next-Generation Sequencing and targeted sequencing

The availability of next generation sequencing techniques has massively changed the field of genetic PID research in the past few years. Screening for genetic mutations by sequencing all coding regions (whole exome sequencing, WES) or even whole genomes (WGS) is done in PID patients in whom no clear candidate genes are likely to cause the immune deficiency. However, success rates depend highly on the a priori chance that a genetic defect is causing the disease. This chance increases in cases with familial history of PID or in patients that have a syndrome-like phenotype with multiple specific features other than infections due to an antibody deficiency. In some cases, the clinical and immunological phenotype clearly indicates a candidate gene. Then, it is faster and more effective to Sanger sequence the candidate gene. WES/WGS takes a couple of weeks to months, before the results are known. Children with severe immune deficiencies, who need treatment fast, cannot always wait for WES/WGS results. Therefore it is vital to combine genetic diagnostics with flow cytometric immunophenotyping in an early stage, so that severe deficiencies can be recognized fast (i.e within days). A point that should also be taken in to account is that with WES or WGS also regions will be sequenced that are not of interest for the question, but that can hold variants that predispose for neurodegenerative disease or increased risk for certain malignancies. It is therefore important to decide what to do with this information before the assay is run, and patients and their parents should be carefully counselled by a clinical geneticist before this type of diagnostic testing is done.

In searching for APDS patients (**part 4** of this thesis), we have sequenced locations of known mutations in *PIK3CD* and *PIK3R1* of a cohort of >100 patients with CVID or hyper IgM syndrome (HIGM). In this cohort, we found one APDS patient. When we sequenced PAD patients who were selected based on high transitional B cells combined with reduced memory B cells and increased $CD8^+$ effector T cells, hepatosplenomegaly or lymphadenopathy and clinical symptoms (respiratory tract infections or EBV/CMV recurrence) most patients (4 out of 5 sequenced in one cohort) were found to have a *PIK3CD* or *PIK3R1* mutation. This indicates that combining clinical phenotype with immune phenotyping and targeted sequencing can be a strong method to identify mutations in patients. However, this only works if you know what you are looking for.

In the research context, WGS and WES approaches are used to search for new gene defects in groups of patients with PID. For example CTLA4-deficiency, APDS and LRBA-deficiency were discovered to a certain extent because of efforts to sequence cohorts of PID patients. As such, it has proven to be an effective tool in discovery of new mutations in new candidate genes. However, finding new mutations always requires carefully conducted functional testing to link the specific defect to the disease phenotype. And, not unimportantly, in the majority of PAD patients that were sequenced in these cohorts, no candidate variants were found. In a study by Maffucci *et al*, 50 (highly pre-selected) CVID

patients were subjected to WES.[75] In 30% of them, a possible candidate gene was found, 14% had a TACI variant that possibly was predisposing for CVID and in 56% no variant was found that could be linked to disease. Using this approach can also reveal new mutations in known genes that lead to a different clinical phenotype, a phenomenon that is referred to as phenotypic extension. Therefore, WGS and WES approaches can be useful to discover new disease causing genes in PAD if combined with functional testing of newly found variants.

Single gene errors: loss of immunity by gain-of-function

Immune deficiency is often related with loss-of-function of a certain immune-element, immune-cell or specific gene. New genetic defects in PI3Kδ and STAT3 have shown that not only deficiencies but also increased protein function can lead to disease.

In **chapter 4.3** we focused on the effect of gain-of-function PI3Kδ mutations (APDS) on the B cells. We found that besides a relative increase in the transitional B cells and reduced memory B cells, there is increased apoptosis of the lymphocytes and B cells specifically *in vitro*. It will be interesting to further investigate which mechanisms that induce apoptosis are affected by gain-of-function PI3Kδ mutations. Furthermore, in **chapter 4.4** we examined the expression of inhibitory receptors on $CD8^+$ T cells of APDS patients and controls as a measure for exhaustion of the $CD8^+$ T cell compartment. We found that in APDS patients, a relatively large proportion of total $CD8^+$ T cells express multiple inhibitory receptors at the same time, indicating that this compartment has features of exhaustion. Some of these inhibitory receptors signal via the PI3K-AKT signaling pathway, and down regulation could be induced via negative feedback to prevent overstimulation and premature cell death in the case of these patients. However, not all mutations in PI3Kδ lead to gain-of-function. Thus far, one patient has been described to suffer from immune deficiency due to homozygous mutations in *PIK3R1*, that caused a loss-of-function of the p85α regulatory subunit which is the abundant form in lymphocytes.[76] This patient suffered from colitis and had no B cells in the peripheral blood. In bone marrow, a very early block in BCP with normal numbers of $CD19^-$ pro-B cells was found. After this stage, hardly any $CD19^+$ cells could be detected. The patient's T cells were unaffected. Analysis showed lowered levels of p110δ indicating that p85α is needed to stabilize p110δ. This patient did not have abnormalities in other organ systems; her disease phenotype was markedly different from APDS. Until now, no other patients have been described to have a loss of function of p110δ, which is expected to follow a recessive trait, and therefore will be rare. However, p110δ KO mice have been generated which showed normal BCP development in bone marrow, normal numbers of $CD4^+$ and $CD8^+$ T cells and a reduction of specifically B1 B cells and marginal zone splenic B cells.[77] The mice had impaired antibody responses and reduced *in vitro* proliferation with increased spontaneous apoptosis. These results indicate that PI3Kδ LOF whether it is

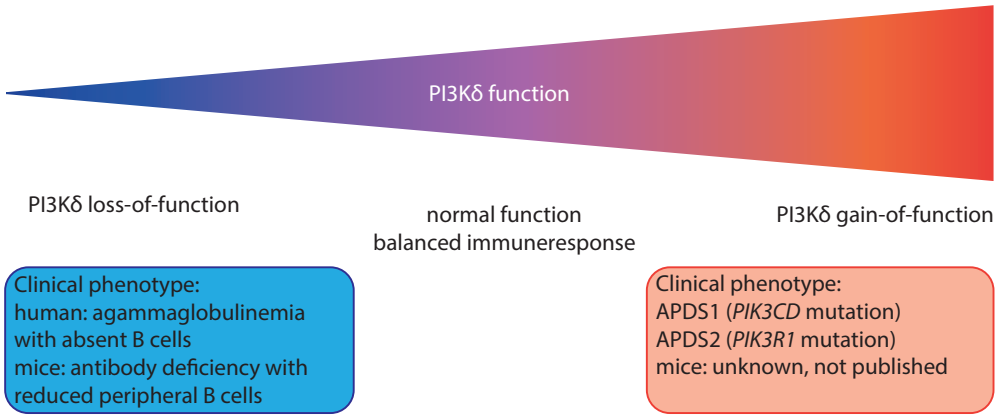

Figure 6.
PI3K dysbalance.

through *PIK3CD* mutations or *PIK3R1* mutations is likely to result in a B cell defect either in bone marrow or early in peripheral blood. (Figure 6)

Another example of a gene in which both gain-of-function and loss-of-function mutations are found in STAT3. In, loss of STAT3 function causes autosomal dominant Hyper-IgE syndrome (AD-HIES).[78-80] Gain-of-function mutations in STAT3 cause severe multi-organ autoimmunity.[81-83] These examples illustrate that immunodeficiency is more than simply loss of a protein and that in some cases, loss-of-balance because of gain-of-function should be considered.

Polygenic causes and pathway analysis

In the search for monogenic causes for antibody deficiencies, mutations in TACI[84, 85] have been described for years. However, some of the variants that were found can also be found in healthy relatives of patients.[84, 85] This led to the idea that these mutations are disease contributing or predisposing factors but solely do not cause disease. It is likely to think that in a patients multiple less-favorable variants in the same pathway could eventually cause disease. This mechanism is called a polygenetic cause for disease. This disease causing mechanism has been postulated for CVID, which is the most common PAD and therefore often studied. Orange *et al*, performed genotyping with >600.000 single nucleotide polymorphisms (SNPs) in 360 CVID patients.[86] This data was used to perform genome wide association analysis which revealed multiple novel susceptibility loci. The authors hypothesize that CVIDs are collections of diverse mechanisms leading to complex disease. This hypothesis was further underlined by a study performed by Van Schouwenburg *et al*, in which WGS was applied to a cohort of CVID patients. Additionally, they performed transcriptomic analysis by means of RNA-sequencing.[12] Their results indicate that in some

CVID cases, the origin of disease is perhaps polygenic. By bio-informatic analysis, several cellular pathways were found that can be linked to B cell differentiation and development and as such are likely candidate pathways to be affected by CVID.

One of the pathways that is potentially disrupted in antibody deficiencies is the PI3K-AKT signaling pathway. As discussed extensively in **part 4** of this thesis, this signaling cascade is crucial for both T and B cell development and function. Besides mutations in *PIK3CD* and *PIK3R1* that are reported to cause the disease called APDS,[87, 88] mutations in *PTEN* can also cause disturbed B cell maturation and hypogammaglobulinemia.[39, 55] These are examples of monogenetic mutations that directly cause a disease phenotype. However, it is not unlikely that variants in different components of the pathway exist and that an unfavorable combination of variants can cause a comparable disease phenotype. There is one variant in *PIK3CD* that has been found in multiple patients with an APDS-like phenotype (unpublished data). It concerns a missense variant c.935C>G (NM_005026.3) resulting in an amino acid change p.S312C (NP_005017.3) in *PIK3CD*. This variant is also found in the general population with a minor allele frequency of ~2% (SNP reference: rs61755420)[89] and therefore it is unlikely that it causes disease by itself. However, phospho-flow analysis of AKT phosphorylation in a patient carrying this variant revealed that pAKT/AKT ratio is increased compared to healthy controls without this SNP (Figure 7)(unpublished data). Furthermore, this patient suffers from antibody deficiency and auto-immunity. We studied the effect on the $CD8^+$ T cells of this variant together with patients with known disease causing mutations in **chapter 4.4**, and found that the $CD8^+$ T-cell phenotype was alike the APDS patients with disease causing mutations and not alike controls. This variant is likely disease modifying, like TACI mutations in CVIDs. Extended analysis of patients and

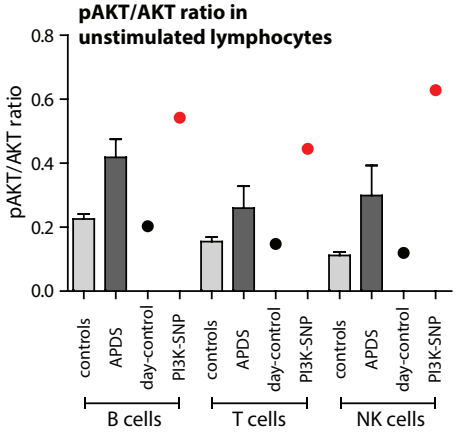

Figure 7.
pAKT/AKT ratio of a patient with the PI3Kδ S312C variant (PI3K-SNP, red dot) compared to a cohort of healthy controls (light grey bar), known APDS-patients (dark grey bar) and an age matched healthy control that was ran in the same experiment (day control, black dot).

controls with this variant could shed light on the clinical relevance of this variant and other genetic and epigenetic variants that could together cause disease. Given that PI3K signaling is important in many phases of B cell development[90-92] (Figure 8) and that PI3K-PTEN balance is linked to AID-expression,[93-96] it is likely that more variants in this pathway will be discovered that contribute to or partially cause antibody deficiencies.

Other signaling pathways in B cell differentiation and development can also be candidates for more extensive analysis to find new polygenetic, pathways defects, such as B cell receptor signaling itself, NFκB or CD19-complex signaling. It is not unlikely to think that suboptimal function of two or more kinases that are involved in these pathway might result in defective B cell survival or maturation.

Epigenetics in the context of PAD

Genetic defects are mistakes in the DNA-sequence itself. Those can affect protein function directly or affect gene expression by mutations in promotor regions. Moreover, premature stop-codons can lead to nonsense mediated RNA-decay,[97] heavily influencing gene expression. Gene expression can also be influenced by accessibility and structure of the DNA , and alterations could potentially also cause disease by altering gene expression. This is studied in the field of epigenetics. Epigenetics is the study of heritable traits that are not confined within the DNA sequence.[98] Commonly, epigenetics refers to changes in DNA-structure that affect gene activity and expression, but also microRNA`s, a form of small non-coding RNA`s, can influence gene expression.[98] These changes are heritable in the sense that daughter cells will have the same imprint, but some can also be heritable over organisms. Most studied in this field are DNA-methylation and post-translational histone-modifications, two mechanisms that together change the ability of nuclear factors to access the DNA. This either allows or blocks transcription and expression of genes and therefore epigenetics are crucial in immune cell development[99-101] and thus could be of importance in PID pathogenesis.[102, 103]

For the studies on BCP development that are described in this thesis we mainly made use of flow cytometry, repertoire analysis and to some extent *in vitro* stimulation assays. The panel for flow cytometry we developed and described in **chapter 2.1** and our method for repertoire analysis described in **chapter 2.2** could be extended with epigenetic studies to further elucidate the interplay between genetics, epigenetics and protein expression in the developing BCP cells in BM.

DNA methylation occurs at cytosines in CpG dinucleotides, which are known to play a regulatory role. CpG dense regions are referred to as CpG islands, which are found often in promotor and enhancer regions and are thus hotspots for functionally relevant DNA methylation changes.[104] Early stages of B cell development have a specific DNA demethylation signature, as shown by Lee *et al*, who studied pre-B-stages in human bone

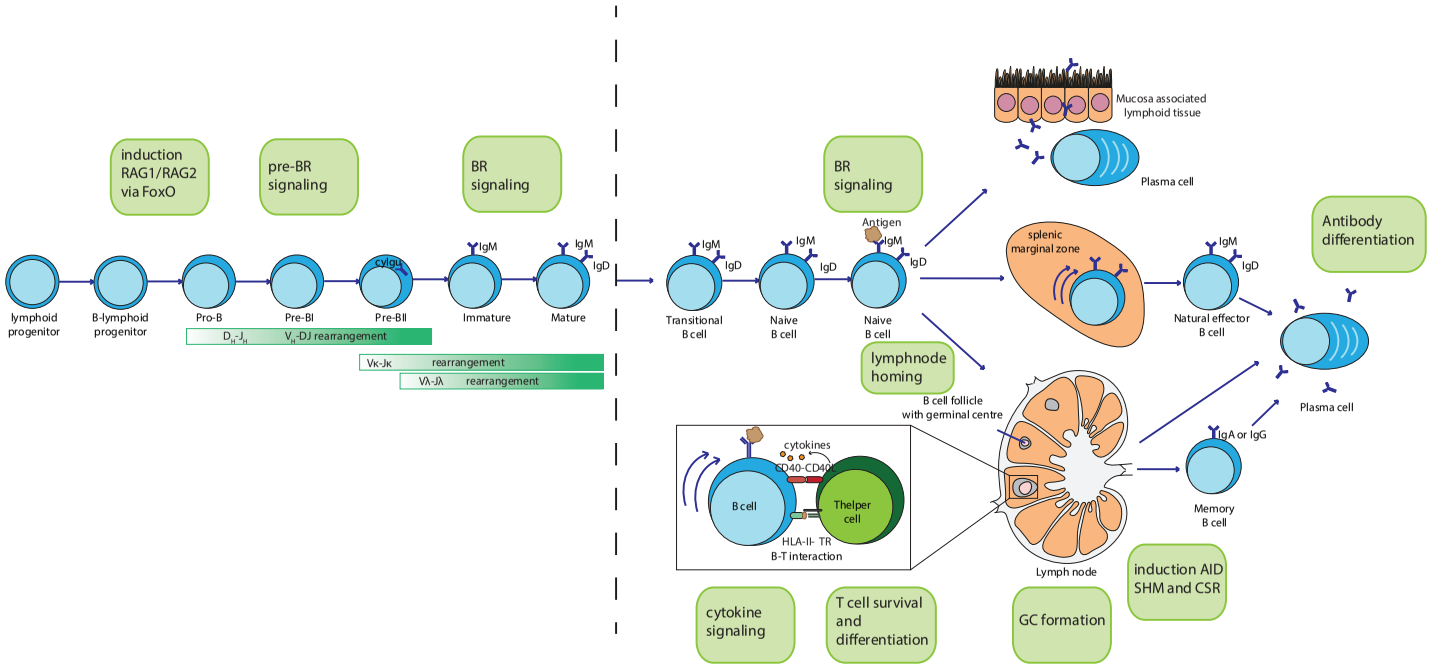

Figure 8.
Processes in B cell differentiation that require PI3K-AKT signaling. BR formation via V(D)J recombination is induced by RAG which is under transcriptional control of FoxO, which in turn is regulated via PI3K-AKT signaling, Pre-BR signaling/BR signaling/cytokine signaling are all connected to PI3K-AKT signaling. Some lymphnode homing factors are under the control of FoxO, homing to lymph node and formation of germinal centers is partially controlled via this pathway. SHM&CSR in initiated by AID, which is controlled by PI3K-AKT signaling.

marrow and found demethylation events were often associated with transcription factors that are crucial for early B cell development such as *EBF1* and *PAX5*.[105] Also in the peripheral blood B cell compartment, distinct DNA-methylation profiles were found for naive and memory subsets and these correlated with stimulation induced profiles that were found.[106] Moreover, many epigenetic changes mapped to transcription factor binding sites and this corresponded with changes in the transcriptome between naive B cells versus stimulated B cells, memory B cells and plasma cells. This indicates that epigenetics play an important role in the differentiation and maturation of B cells.

Previously, it was shown that the epigenetic profile of several leukemia types could refine patients stratification.[107] Since the epigenetic marks reflect both cell intrinsic factors (e.g. genetic alterations)[99] and cell extrinsic factors,[108] DNA methylation profiles were used to indicate the cell-origin of for example chronic lymphocytic leukemia.[109] Although not often studied, recent studies by the group of Ballestar in Barcelona, have shown that epigenetic profiles are changed in B cells of CVID patients. In a study from 2015 they compared monozygotic twins discordant for CVID and studied a cohort of CVID patients. They showed B-cell specific DNA methylation alterations in genes relevant to B-cell function such as, *PIK3CD*, *TCF3, BCL21L1* and *RPTOR* in sorted B-cell subsets of CVID patients.[110] However, as noted by them and others,[103, 111] this field is still little studied in primary immune deficiencies and more studies that combine different high-throughput analysis methods are needed to elucidate the role of epigenetics in PAD disease etiology.

When studying exhaustion of the $CD8^+$ T cells compartment in APDS, our results indicated that not all patients showed the same degree of exhaustion and we hypothesized that probably having the mutation alone does not induce exhaustion (**chapter 4.4**). It was indicated in a study in mouse embryonic stem cells that PI3K can indeed alter epigenetic modifications. The authors showed that *de novo* DNA methyltransferases are downregulated due to PI3K-induced AKT, leading to reduced DNA methylation of imprinted loci.[112] Demethylation of the PD-1 promotor region in exhausted $CD8^+$ T cells allows sustained expression of PD-1. Therefore PI3Kδ GOF mutations could promote the demethylation of inhibitory receptors or reduce the threshold for such demethylation, thus facilitating the exhaustion in $CD8^+$ T cells. In APDS but also in other forms of PAD, this is an interesting field to study disease etiology.

Another method that could be applied in the field of PAD is the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq),[113] a technique that can measure genome-wide chromatin accessibility and thus define which regulatory elements in a given cell are active. In ATAC-seq, probes are used to label "open-chromatin" in such a way that these areas can be sequenced and mapped back to total genome. In this way, low cell numbers (between 10.000 and 50.00 cells) allow sequencing and bio-informatic reconstruction of a chromatin fingerprint of a given cell subset. In this way patients can be

compared with controls and subsets within one individual can be compared to each other (Figure 9). Rendeiro *et al.* recently showed the use of this technique in profiling primary chronic lymphocytic leukemia samples[109] and associated the epigenetic landscape to gene regulatory networks. It will be interesting to apply these types of analysis with transcriptome analysis in B cell subsets from bone marrow and peripheral blood in both healthy individuals and patients with PAD do dissect disease contributing mechanisms that lead to defective B cell development and reduced production of antibodies.

B-cell extrinsic factors contributing to PAD

Besides an origin intrinsic to the B cell (be it genetic, polygenetic or epigenetic), factors in the external milieu of the developing B cell can also contribute to disturbed development of the B cells in antibody deficiencies. As discussed already before in this chapter, disturbances in the bone marrow niche or cytokine milieu are hypothesized to contribute to CVID development.[10] In addition to this, Pott *et al.* recently raised the question whether autoantibodies against BAFF, APRIL or IL21, which are crucial for peripheral B cell development) could form an alternative pathogenesis for antibody-deficiencies.[114] The presence of autoantibodies against cytokines has been recognized for decades, but they were usually regarded as an epiphenomenon rather than disease causing. However, more recent studies have shown presence of specific disease-causing autoantibodies in pure red cell aplasia.[115] Additionally, anti-IL6 antibodies were found in a patient with recurrent staphylococcal infections[116] and anti-IL17A, anti-IL17F and anti-IL22 antibodies were found in patients with chronic mucocutaneous candidiasis.[117] This indicates that autoantibodies can be of specific interest in the field of immune deficiencies. Therefore, the authors screened for auto-antibodies against BAFF, APRIL and IL-21 in >200 PAD patients. They found presence of these antibodies was more abundant in their patient cohort than in

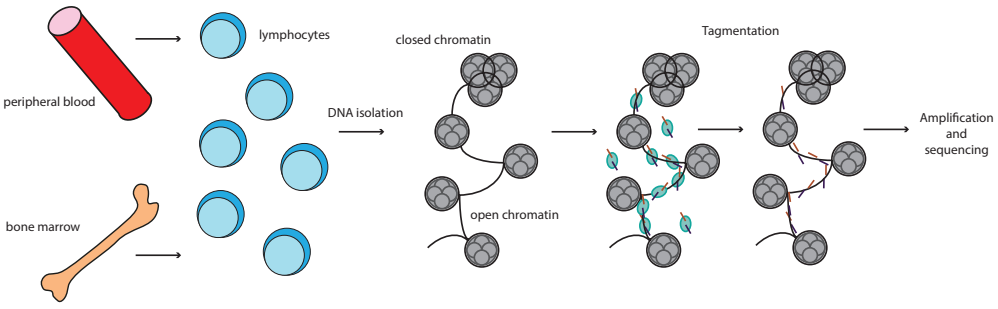

Figure 9.
Adapted from Rendeiro *et al.*[109] ATAC sequencing. Lymphocytes, total B cells or B cells subsets are isolated from peripheral blood or bone marrow. DNA is isolated and the open chromatin is tagmented with Tn5 transposomes. After tagmentation, the open chromatin is selectively amplified and sequenced.

controls. However, they could not show that functionally these autoantibodies inhibit the function of BAFF, APRIL or IL21, and thus, their role in disease formation remains uncertain.

Another recent field of interest in the studies of immune dysregulation is the microbiome. The microbiota in the gut contributes to health and disease in the gut. But because of the interaction of the microbiota and the immune cells in the intestinal mucosa, the microbiome is essential for immune regulation and homeostasis.[118] In HIV patients, is was shown that impaired immune function can lead to microbial translocation, causing a systemic immune activation.[119] A similar mechanism has been proposed to play a role in the immune deregulatory component of CVID;[120] because of impaired immune response, microbial translocation can take place and the immune system in turn over responds driving dysregulation and associated complications. The association between gut microbiome and CVID was reviewed recently by Berbers *et al.* [121] They conclude that there is evidence of increased microbial translocation in CVID patients with immune dysregulation complications, and that the composition of the microbiome is likely different in this group of patients, although up to now, few studies that specifically addressed these questions in antibody deficiencies have been conducted. Therefore, the microbiome may contribute to the disease phenotype in antibody deficiencies, but more research is needed to confirm the observations that were done previously.

Besides commensal bacteria in the gut, that influence the immune-system, another group of micro-organisms that has for years been recognized as immune-cell-influencing are the persistent viral infections of which EBV and CMV are two often studied examples. EBV is known to play a role in $CD8^+$ T cell exhaustion[122, 123] and can be driving factor in certain B-cell lymphoma`s.[124] Several groups of PAD patients, especially if also T cell function is hamered, are known to have trouble to control EBV[87, 125] and as such an EBV infection is an external factor influencing their risk for lymphoma development. Additionally, recurrent or chronic infections with EBV could lead to exhaustion-phenotypes in these patients, which in turn could further decrease their control of other infections. We studied this in our cohort of APDS patients in **chapter 4.4**. However, the number of EBV-positive patients was too low to draw firm conclusions and more extensive experiments are needed to resolve this question. CMV is considered one of the contributors to immunosenescence[126] and a recent report showed that CMV-infection caused dysregulation in a CVID patient which eventually led to development of lymphoid interstitial pneumonia.[127] On the other hand, a study by Kuntz *et al.* demonstrated that CVID patients have normally differentiated virus-specific $CD8^+$ T cells.[128] In their study CMV infection however, was associated with advanced $CD8^+$ T cell differentiation. Overall, their CVID patients had a more senescent phenotype than controls. But they could not conclude from their data that the virus-specific response was different in the patient group. They did state that preventive vaccination for persistent viral infection might be preferable in CVID patients since this might prevent virus-infection

related complications. Taken together, patients with immune deficiencies more often suffer from recurrent infections with persistent herpes viruses, and those in turn contribute to increased senescence and exhaustion and thus to decreased immune function. This makes them an extrinsic factor that plays a role in symptom pathogenesis.

CONCLUDING REMARKS

In this thesis we aimed to study antibody deficiencies and how specific defects disturb B cell development. Our studies of BCP differentiation in human bone marrow indicate that this is not a single linear route of differentiation, but rather a complex process that requires many mechanisms including V(D)J-recombination-driven checkpoints, divergence, parallel pathways and convergence to form a unique and functional B cell receptor. Our studies indicate that different phenotypes in different CD19-complex deficiencies are related to the function of the protein in the complex. PI3K signaling is a connecting element between the B cell receptor and CD19-complex signaling and vice versa misbalanced PI3K signaling can result in increased $CD21^{lo}$ cells. PI3K/PTEN imbalance additionally results in T cell and B cell intrinsic problems, reflected in defective humoral immune responses and an inability to control persistent viral infections. This knowledge is not only important for patients with antibody deficiencies, but also for our general understanding of B cell biology and therefore for other immunological diseases and processes such as malignancies, auto-immunity, immune dysregulation and regeneration after stem cell transplantation. The future of PAD disease etiology research is connecting multiple–omics approaches to investigate differentiation pathways and signaling cascades and connect genetic and functional defects to immunophenotypical and clinical findings.

REFERENCES

1. Ghia, P., et al., *Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain gene loci.* J Exp Med, 1996. **184**(6): p. 2217-29.
2. Ehlich, A., et al., *Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development.* Cell, 1993. **72**(5): p. 695-704.
3. van Zelm, M.C., et al., *Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression.* J Immunol, 2005. **175**(9): p. 5912-22.
4. Ornatsky, O., et al., *Highly multiparametric analysis by mass cytometry.* J Immunol Methods, 2010. **361**(1-2): p. 1-20.

5. Bendall, S.C., et al., *Single-cell trajectory detection uncovers progression and regulatory coordination in human B cell development.* Cell, 2014. **157**(3): p. 714-25.
6. Good, R.A., et al., *Thymic tumor and acquired agammaglobulinemia: a clinical and experimental study of the immune response.* Surgery, 1956. **40**(6): p. 1010-7.
7. Jansen, A., et al., *Prognosis of Good syndrome: mortality and morbidity of thymoma associated immunodeficiency in perspective.* Clin Immunol, 2016. **171**: p. 12-17.
8. Driessen, G.J., et al., *B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency.* Blood, 2011. **118**(26): p. 6814-23.
9. Ochtrop, M.L., et al., *T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency.* Blood, 2011. **118**(2): p. 309-18.
10. Anzilotti, C., et al., *Key stages of bone marrow B-cell maturation are defective in patients with common variable immunodeficiency disorders.* J Allergy Clin Immunol, 2015. **136**(2): p. 487-90 e2.
11. Lougaris, V., et al., *Correlation of bone marrow abnormalities, peripheral lymphocyte subsets and clinical features in uncomplicated common variable immunodeficiency (CVID) patients.* Clin Immunol, 2016. **163**: p. 10-3.
12. van Schouwenburg, P.A., et al., *Application of whole genome and RNA sequencing to investigate the genomic landscape of common variable immunodeficiency disorders.* Clin Immunol, 2015. **160**(2): p. 301-14.
13. Courtois, G. and T.D. Gilmore, *Mutations in the NF-kappaB signaling pathway: implications for human disease.* Oncogene, 2006. **25**(51): p. 6831-43.
14. Woyach, J.A., A.J. Johnson, and J.C. Byrd, *The B-cell receptor signaling pathway as a therapeutic target in CLL.* Blood, 2012. **120**(6): p. 1175-84.
15. Srinivasan, L., et al., *PI3 kinase signals BCR-dependent mature B cell survival.* Cell, 2009. **139**(3): p. 573-86.
16. Matsumoto, A.K., et al., *Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes.* J Exp Med, 1993. **178**(4): p. 1407-17.
17. Carroll, M.C. and D.E. Isenman, *Regulation of humoral immunity by complement.* Immunity, 2012. **37**(2): p. 199-207.
18. Cherukuri, A., et al., *The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts.* Immunity, 2001. **14**(2): p. 169-79.
19. Suryani, S., et al., *Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells.* Blood, 2010. **115**(3): p. 519-29.
20. Wehr, C., et al., *A new CD21low B cell population in the peripheral blood of patients with SLE.* Clin Immunol, 2004. **113**(2): p. 161-71.
21. Hasegawa, M., et al., *CD19 can regulate B lymphocyte signal transduction independent of complement activation.* J Immunol, 2001. **167**(6): p. 3190-200.
22. Haas, K.M., et al., *Complement receptors CD21/35 link innate and protective immunity during Streptococcus pneumoniae infection by regulating IgG3 antibody responses.* Immunity, 2002. **17**(6): p. 713-23.

23. Barrington, R.A., et al., *Uncoupling CD21 and CD19 of the B-cell coreceptor.* Proc Natl Acad Sci U S A, 2009. **106**(34): p. 14490-5.
24. van Zelm, M.C., et al., *An antibody-deficiency syndrome due to mutations in the CD19 gene.* N Engl J Med, 2006. **354**(18): p. 1901-12.
25. Kanegane, H., et al., *Novel mutations in a Japanese patient with CD19 deficiency.* Genes Immun, 2007. **8**(8): p. 663-70.
26. Artac, H., et al., *B-cell maturation and antibody responses in individuals carrying a mutated CD19 allele.* Genes Immun, 2010. **11**(7): p. 523-30.
27. Skendros, P., et al., *Misdiagnosed CD19 deficiency leads to severe lung disease.* Pediatr Allergy Immunol, 2014. **25**(6): p. 603-6.
28. van Zelm, M.C., et al., *Antibody deficiency due to a missense mutation in CD19 demonstrates the importance of the conserved tryptophan 41 in immunoglobulin superfamily domain formation.* Hum Mol Genet, 2011. **20**(9): p. 1854-63.
29. van Zelm, M.C., et al., *CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency.* J Clin Invest, 2010. **120**(4): p. 1265-74.
30. Thiel, J., et al., *Genetic CD21 deficiency is associated with hypogammaglobulinemia.* J Allergy Clin Immunol, 2012. **129**(3): p. 801-810 e6.
31. Wentink, M.W., et al., *CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities.* Clin Immunol, 2015. **161**(2): p. 120-127.
32. Rosain, J., et al., *CD21 deficiency in 2 siblings with recurrent respiratory infections and hypogammaglobulinemia.* J Allergy Clin Immunol Pract, 2017.
33. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science, 2013. **342**(6160): p. 866-71.
34. Lucas, C.L., et al., *Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency.* Nat Immunol, 2014. **15**(1): p. 88-97.
35. Lucas, C.L., et al., *Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K.* J Exp Med, 2014. **211**(13): p. 2537-47.
36. Wentink, M., et al., *Genetic defects in PI3Kdelta affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infections.* Clin Immunol, 2017. **176**: p. 77-86.
37. Heurtier, L., et al., *Mutations in the adaptor-binding domain and associated linker region of p110delta cause Activated PI3K-delta Syndrome 1 (APDS1).* Haematologica, 2017.
38. Omori, S.A., et al., *Regulation of class-switch recombination and plasma cell differentiation by phosphatidylinositol 3-kinase signaling.* Immunity, 2006. **25**(4): p. 545-57.
39. Browning, M.J., et al., *Cowden's syndrome with immunodeficiency.* J Med Genet, 2015.
40. Driessen, G.J., et al., *Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency.* J Allergy Clin Immunol, 2016.
41. Okkenhaug, K., K. Ali, and B. Vanhaesebroeck, *Antigen receptor signalling: a distinctive role for the p110delta isoform of PI3K.* Trends Immunol, 2007. **28**(2): p. 80-7.

42. Ramadani, F., et al., *The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development.* Sci Signal, 2010. **3**(134): p. ra60.
43. Petrovas, C., et al., *PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection.* J Exp Med, 2006. **203**(10): p. 2281-92.
44. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.
45. Pardoll, D.M., *The blockade of immune checkpoints in cancer immunotherapy.* Nat Rev Cancer, 2012. **12**(4): p. 252-64.
46. Sharma, P. and J.P. Allison, *The future of immune checkpoint therapy.* Science, 2015. **348**(6230): p. 56-61.
47. Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nat Rev Immunol, 2013. **13**(4): p. 227-42.
48. Odorizzi, P.M. and E.J. Wherry, *Inhibitory receptors on lymphocytes: insights from infections.* J Immunol, 2012. **188**(7): p. 2957-65.
49. Parry, R.V., et al., *CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms.* Mol Cell Biol, 2005. **25**(21): p. 9543-53.
50. Chalhoub, N. and S.J. Baker, *PTEN and the PI3-kinase pathway in cancer.* Annu Rev Pathol, 2009. **4**: p. 127-50.
51. Burke, J.E., et al., *Oncogenic mutations mimic and enhance dynamic events in the natural activation of phosphoinositide 3-kinase p110alpha (PIK3CA).* Proc Natl Acad Sci U S A, 2012. **109**(38): p. 15259-64.
52. Takeda, A.J., et al., *Novel PIK3CD mutations affecting N-terminal residues of p110delta cause activated PI3Kdelta syndrome (APDS) in humans.* J Allergy Clin Immunol, 2017. **140**(4): p. 1152-1156 e10.
53. Fruman, D.A., et al., *The PI3K Pathway in Human Disease.* Cell, 2017. **170**(4): p. 605-635.
54. Riviere, J.B., et al., *De novo germline and postzygotic mutations in AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes.* Nat Genet, 2012. **44**(8): p. 934-40.
55. Driessen, G.J., et al., *Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency.* J Allergy Clin Immunol, 2016. **138**(6): p. 1744-1747 e5.
56. Hoogeveen-Westerveld, M., et al., *Functional assessment of variants in the TSC1 and TSC2 genes identified in individuals with Tuberous Sclerosis Complex.* Hum Mutat, 2011. **32**(4): p. 424-35.
57. Wentink, M., et al., *Functional characterization of the TSC2 c.3598C>T (p.R1200W) missense mutation that co-segregates with tuberous sclerosis complex in mildly affected kindreds.* Clin Genet, 2012. **81**(5): p. 453-61.
58. Conley, M.E. and D.A. Fruman, *Genetics. Can cancer drugs treat immunodeficiency?* Science, 2013. **342**(6160): p. 814-5.
59. Rao, V.K., et al., *Effective 'Activated PI3Kdelta Syndrome'-targeted therapy with the PI3Kdelta inhibitor leniolisib.* Blood, 2017.
60. Warnatz, K., et al., *Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia.* Immunobiology, 2002. **206**(5): p. 502-13.

61. Asokan, R., et al., *Human complement receptor 2 (CR2/CD21) as a receptor for DNA: implications for its roles in the immune response and the pathogenesis of systemic lupus erythematosus (SLE).* Mol Immunol, 2013. **53**(1-2): p. 99-110.
62. Gemen, E.F., et al., *Increased circulating apoptotic lymphocytes in children with Down syndrome.* Pediatr Blood Cancer, 2012. **59**(7): p. 1310-2.
63. Verstegen, R.H., et al., *Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help.* Pediatr Res, 2010. **67**(5): p. 563-9.
64. Verstegen, R.H., et al., *Defective B-cell memory in patients with Down syndrome.* J Allergy Clin Immunol, 2014. **134**(6): p. 1346-1353 e9.
65. Rakhmanov, M., et al., *Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells.* Proc Natl Acad Sci U S A, 2009. **106**(32): p. 13451-6.
66. Moir, S., et al., *Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals.* J Exp Med, 2008. **205**(8): p. 1797-805.
67. Doi, H., S. Tanoue, and D.E. Kaplan, *Peripheral CD27-CD21- B-cells represent an exhausted lymphocyte population in hepatitis C cirrhosis.* Clin Immunol, 2014. **150**(2): p. 184-91.
68. Isnardi, I., et al., *Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones.* Blood, 2010. **115**(24): p. 5026-36.
69. Visentini, M., et al., *Telomere-dependent replicative senescence of B and T cells from patients with type 1a common variable immunodeficiency.* Eur J Immunol, 2011. **41**(3): p. 854-62.
70. Lichterfeld, M., et al., *Telomerase activity of HIV-1-specific CD8+ T cells: constitutive up-regulation in controllers and selective increase by blockade of PD ligand 1 in progressors.* Blood, 2008. **112**(9): p. 3679-87.
71. Keller, B., et al., *High SYK Expression Drives Constitutive Activation of CD21low B Cells.* J Immunol, 2017. **198**(11): p. 4285-4292.
72. Bousfiha, A., et al., *The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies.* J Clin Immunol, 2015. **35**(8): p. 727-38.
73. Picard, C., et al., *International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity.* J Clin Immunol, 2018. **38**(1): p. 96-128.
74. Kienzler, A.K., C.E. Hargreaves, and S.Y. Patel, *The role of genomics in common variable immunodeficiency disorders.* Clin Exp Immunol, 2017. **188**(3): p. 326-332.
75. Maffucci, P., et al., *Genetic Diagnosis Using Whole Exome Sequencing in Common Variable Immunodeficiency.* Front Immunol, 2016. **7**: p. 220.
76. Conley, M.E., et al., *Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K.* J Exp Med, 2012. **209**(3): p. 463-70.
77. Clayton, E., et al., *A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation.* J Exp Med, 2002. **196**(6): p. 753-63.
78. Holland, S.M., et al., *STAT3 mutations in the hyper-IgE syndrome.* N Engl J Med, 2007. **357**(16): p. 1608-19.
79. Minegishi, Y., et al., *Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome.* Nature, 2007. **448**(7157): p. 1058-62.

80. Vogel, T.P., J.D. Milner, and M.A. Cooper, *The Ying and Yang of STAT3 in Human Disease.* J Clin Immunol, 2015. **35**(7): p. 615-23.
81. Flanagan, S.E., et al., *Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease.* Nat Genet, 2014. **46**(8): p. 812-4.
82. Haapaniemi, E.M., et al., *Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3.* Blood, 2015. **125**(4): p. 639-48.
83. Milner, J.D., et al., *Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations.* Blood, 2015. **125**(4): p. 591-9.
84. Castigli, E., et al., *TACI is mutant in common variable immunodeficiency and IgA deficiency.* Nat Genet, 2005. **37**(8): p. 829-34.
85. Poodt, A.E., et al., *TACI mutations and disease susceptibility in patients with common variable immunodeficiency.* Clin Exp Immunol, 2009. **156**(1): p. 35-9.
86. Orange, J.S., et al., *Genome-wide association identifies diverse causes of common variable immunodeficiency.* J Allergy Clin Immunol, 2011. **127**(6): p. 1360-7 e6.
87. Coulter, T.I., et al., *Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study.* J Allergy Clin Immunol, 2017. **139**(2): p. 597-606 e4
88. Elkaim, E., et al., *Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study.* J Allergy Clin Immunol, 2016. **138**(1): p. 210-218 e9.
89. Aken, B.L., et al., *Ensembl 2017.* Nucleic Acids Res, 2017. **45**(D1): p. D635-D642.
90. Otipoby, K.L., et al., *The B-cell antigen receptor integrates adaptive and innate immune signals.* Proc Natl Acad Sci U S A, 2015. **112**(39): p. 12145-50.
91. Sander, S., et al., *PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones.* Immunity, 2015. **43**(6): p. 1075-86.
92. Dulau Florea, A.E., et al., *Abnormal B-Cell Maturation in the Bone Marrow of Patients with Germline Mutations in PIK3CD.* J Allergy Clin Immunol, 2017. **139**(3): p. 1032-1035
93. Suzuki, A., et al., *Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination.* J Exp Med, 2003. **197**(5): p. 657-67.
94. Omori, S.A. and R.C. Rickert, *Phosphatidylinositol 3-kinase (PI3K) signaling and regulation of the antibody response.* Cell Cycle, 2007. **6**(4): p. 397-402.
95. Chen, Z., et al., *Imbalanced PTEN and PI3K Signaling Impairs Class Switch Recombination.* J Immunol, 2015. **195**(11): p. 5461-5471.
96. Compagno, M., et al., *Phosphatidylinositol 3-kinase delta blockade increases genomic instability in B cells.* Nature, 2017. **542**(7642): p. 489-493.
97. Hug, N., D. Longman, and J.F. Caceres, *Mechanism and regulation of the nonsense-mediated decay pathway.* Nucleic Acids Res, 2016. **44**(4): p. 1483-95.
98. Berger, S.L., et al., *An operational definition of epigenetics.* Genes Dev, 2009. **23**(7): p. 781-3.
99. Bock, C., et al., *DNA methylation dynamics during in vivo differentiation of blood and skin stem cells.* Mol Cell, 2012. **47**(4): p. 633-47.

100. Ngalamika, O., et al., *Epigenetics, autoimmunity and hematologic malignancies: a comprehensive review.* J Autoimmun, 2012. **39**(4): p. 451-65.
101. Dogra, P., et al., *Generating long-lived CD8(+) T-cell memory: Insights from epigenetic programs.* Eur J Immunol, 2016. **46**(7): p. 1548-62.
102. Stagi, S., et al., *Epigenetic control of the immune system: a lesson from Kabuki syndrome.* Immunol Res, 2016. **64**(2): p. 345-59.
103. Rodriguez-Cortez, V.C., et al., *Dissecting Epigenetic Dysregulation of Primary Antibody Deficiencies.* J Clin Immunol, 2016. **36 Suppl 1**: p. 48-56.
104. Eden, S. and H. Cedar, *Role of DNA methylation in the regulation of transcription.* Curr Opin Genet Dev, 1994. **4**(2): p. 255-9.
105. Lee, S.T., et al., *A global DNA methylation and gene expression analysis of early human B-cell development reveals a demethylation signature and transcription factor network.* Nucleic Acids Res, 2012. **40**(22): p. 11339-51.
106. Lai, A.Y., et al., *DNA methylation profiling in human B cells reveals immune regulatory elements and epigenetic plasticity at Alu elements during B-cell activation.* Genome Res, 2013. **23**(12): p. 2030-41.
107. Oakes, C.C., et al., *DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia.* Nat Genet, 2016. **48**(3): p. 253-64.
108. Scott, R.S., *Epstein-Barr virus: a master epigenetic manipulator.* Curr Opin Virol, 2017. **26**: p. 74-80.
109. Rendeiro, A.F., et al., *Chromatin accessibility maps of chronic lymphocytic leukaemia identify subtype-specific epigenome signatures and transcription regulatory networks.* Nat Commun, 2016. **7**: p. 11938.
110. Rodriguez-Cortez, V.C., et al., *Monozygotic twins discordant for common variable immunodeficiency reveal impaired DNA demethylation during naive-to-memory B-cell transition.* Nat Commun, 2015. **6**: p. 7335.
111. Li, J., et al., *Understanding the genetic and epigenetic basis of common variable immunodeficiency disorder through omics approaches.* Biochim Biophys Acta, 2016. **1860**(11 Pt B): p. 2656-63.
112. Popkie, A.P., et al., *Phosphatidylinositol 3-kinase (PI3K) signaling via glycogen synthase kinase-3 (Gsk-3) regulates DNA methylation of imprinted loci.* J Biol Chem, 2010. **285**(53): p. 41337-47.
113. Buenrostro, J.D., et al., *Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position.* Nat Methods, 2013. **10**(12): p. 1213-8.
114. Pott, M.C., et al., *Autoantibodies against BAFF, APRIL or IL21 - an alternative pathogenesis for antibody-deficiencies?* BMC Immunol, 2017. **18**(1): p. 34.
115. Casadevall, N., et al., *Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin.* N Engl J Med, 2002. **346**(7): p. 469-75.
116. Puel, A., et al., *Recurrent staphylococcal cellulitis and subcutaneous abscesses in a child with autoantibodies against IL-6.* J Immunol, 2008. **180**(1): p. 647-54.
117. Puel, A., et al., *Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I.* J Exp Med, 2010. **207**(2): p. 291-7.
118. Kabat, A.M., N. Srinivasan, and K.J. Maloy, *Modulation of immune development and function by intestinal microbiota.* Trends Immunol, 2014. **35**(11): p. 507-17.

119. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
120. Perreau, M., et al., *Exhaustion of bacteria-specific CD4 T cells and microbial translocation in common variable immunodeficiency disorders.* J Exp Med, 2014. **211**(10): p. 2033-45.
121. Berbers, R.M., et al., *Microbial Dysbiosis in Common Variable Immune Deficiencies: Evidence, Causes, and Consequences.* Trends Immunol, 2017. **38**(3): p. 206-216.
122. Macedo, C., et al., *EBV-specific CD8+ T cells from asymptomatic pediatric thoracic transplant patients carrying chronic high EBV loads display contrasting features: activated phenotype and exhausted function.* J Immunol, 2011. **186**(10): p. 5854-62.
123. van Baarle, D., et al., *Progressive telomere shortening of Epstein-Barr virus-specific memory T cells during HIV infection: contributor to exhaustion?* J Infect Dis, 2008. **198**(9): p. 1353-7.
124. Kim, H.J., et al., *Epstein-Barr Virus-Associated Lymphoproliferative Disorders: Review and Update on 2016 WHO Classification.* J Pathol Transl Med, 2017. **51**(4): p. 352-358.
125. Boztug, H., et al., *NF-kappaB1 Haploinsufficiency Causing Immunodeficiency and EBV-Driven Lymphoproliferation.* J Clin Immunol, 2016. **36**(6): p. 533-40.
126. Koch, S., et al., *Cytomegalovirus infection: a driving force in human T cell immunosenescence.* Ann N Y Acad Sci, 2007. **1114**: p. 23-35.
127. Zdziarski, P., A. Gamian, and G. Dworacki, *A case report of lymphoid intestitial pneumonia in common variable immunodeficiency: Oligoclonal expansion of effector lymphocytes with preferential cytomegalovirus-specific immune response and lymphoproliferative disease promotion.* Medicine (Baltimore), 2017. **96**(23): p. e7031.
128. Kuntz, M., et al., *Analysis of bulk and virus-specific CD8+ T cells reveals advanced differentiation of CD8+ T cells in patients with common variable immunodeficiency.* Clin Immunol, 2011. **141**(2): p. 177-86.

PART 6

Addendum

Abbreviations

Summary

Samenvatting

Dankwoord

Curriculum Vitae

PhD Portfolio

Publications

ABBREVIATIONS

AD-HIES	autosomal dominant Hyper-IgE-syndrome
AID	activation induced deaminase
APDS	activated PI3K delta syndrome
APRIL	a proliferation inducing ligand
APS	automated population separator
ATACseq	assay for transposase-accessible chromatin using sequencing
ATM	ataxia-telangiectasia mutated
BAFF	B cell activating factor
BCP	B cell precursor
BLNK	B cell linker
BM	bone marrow
BR	B cell receptor
BTK	Brutons tyrosine kinase
CCR	C-C chemokine receptor
CDR	complementary determining region
CMV	cytomegalo virus
CR1/2	complement receptor 1/2
CSR	class switch recombination
CVID	common variable immune deficiency
cyTOF	cytometry time-of-flight
DBS	double strand breaks
DC	dendritic cell
DNA-PKcs	DNA dependent protein kinase catalytic subunit
DS	Down syndrome
Ebf1	early B cell factor 1
EBV	Epstein-Barr virus
FOXO	Forkhead box subgroup O
FR	framework region

GC	germinal center
GOF	gain-of-function
GS	Good syndrome
HD	healthy donor
HIGM	hyper IgM syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HR	heterochromatin
ICOS	inducible co-stimulator
IFITM1	interferon induced transmembrane protein 1
IFN-γ	interferon gamma
Ig	immunoglobulin, antibody
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
IVIG	intravenous Ig substitution
KO	knock-out
LAD	lymphocyte adhesion defect
LOF	loss-of-function
MALT	mucosa associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MCAP	megalencephaly capillary malformation syndrome
MFI	mean fluorescent intensity
mTOR	mammalian target of rapamycin
N nucleotide	non-templated nucleotide
NEMO	nuclear factor-kappaB essential modulator
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NHEJ	non-homologous end-joining
P nucleotides	palindromic nucleotide
PAD	primary antibody deficiency

PASLI	p110 delta activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency
PAX5	paired box protein 5
PC	principle component
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PHTS	PTEN hamartoma tumor syndrome
PI3K	phosphatidyl inositol 3-kinase
PID	primary immune deficiency
preBR	pre-B cell receptor
PTEN	phosphate and tensin homologue deleted on chromosome 10
RAG	recombination activating genes
RSS	recombination signal sequences
SCID	severe combined immune deficiency
SCIG	subcutaneous Ig substitution
SD	standard deviation
SEM	standard error or the mean
SHM	somatic hypermutation
SLE	systemic lupus erythematosous
SNP	single nucleotide polymorfism
TACI	transmembrane activator and CAML interactor
TdT	terminal deoxynucleotidyl transferase
TLR	toll like receptor
TNF	tumor necrosis factor
TR	T cell receptor
WES	whole exome sequencing
WGS	whole genome sequencing
XLA	X linked agammaglobulinemia
XLF	XRCC4 like factor
XRCC4	X-ray repair cross-complementing protein 4

SUMMARY

The human body is a collection of tissues and organs, which are all composed of cells and extracellular matrices that work together to form the total organism. Potential threats from both the outside, in form of foreign substances and infectious micro-organisms and from inside, like (pre-)malignant cells, are recognized and neutralized by a specialized and highly diverse organ system called the immune system. The immune system consists of lymphoid organs (e.g. bone marrow, lymph nodes, spleen) and the white blood cells: leukocytes. Leucocytes can be divided in the cells of the innate immune system and the cells of the adaptive system, the latter are called lymphocytes or T cells and B cells.

B cells arise in bone marrow were they differentiate from hematopoietic stem cells via multiple precursor stages into precursor B cells and mature B cells, that enter the peripheral blood. In the bone marrow, each individual B cells forms its own unique B cell receptor, via the process of V(D)J-recombination.

To ensure the quality B cell receptors, B cells have to pass checkpoints during which their receptor is tested for expression and signaling. B cells that cannot pass the checkpoint will become apoptotic. The total pool of different B cell receptors forms the naive B cell repertoire. Because of the variety of possible antigens, a broad repertoire of B cell receptors is vital for proper humoral immunity. Upon antigen encounter, the naive mature B cells differentiate into memory B cells or plasma cells, which can produce antibodies. This process takes place in the secondary lymphoid organs, where the naive B cells undergo affinity maturation and proliferation with or without help from $CD4^+$ T cells.

When B cells are unable to develop into functioning plasma cells due to an inborn defect too few antibodies can be formed, resulting in a primary antibody deficiency (PAD). Patients with a PAD suffer from recurrent infections, but additionally can have auto-immunity, lymphoproliferation or malignancies. Some patients have a defect in the B cell precursor stages in the bone marrow due to a defect in an early transcription factor or in the pre B-cell receptor or its downstream signaling molecules. Other processes that can be disturbed in PAD are B cell survival, antigenic activation, class switch recombination, memory formation or even excretion of antibodies from plasma cells. In a part of PAD patients causative genetic defects have been described, but in other and the genetic cause or etiology are still unknown.

Studying the etiology and immune biology of PAD requires extensive knowledge on B cell development. Connecting B cell differentiation to disease causing genetic defects or specific clinical phenotypes of PAD leads to new insights into pathophysiology, prognosis and even provides new treatment options for patients. Moreover, knowledge that is obtained by studying these diseases teaches us about protein function, developmental checkpoints and signaling cascades in B cell development.

The aim of this thesis was to study B cell differentiation in the context of antibody deficiencies and how specific deficiencies affect signaling cascades that are crucial for this normal development. We followed the line of B cell development, starting in the bone marrow until memory and plasma cell formation after antigenic stimulation. Part 1 outlines normal B cell development and recent findings regarding this development in the **General Introduction**. Additionally, different genetic defects that cause antibody deficiencies are discussed and placed in the context of B cell development. This underlines why it is crucial to study B cell development, together with studying antibody deficiencies.

Part 2 of this thesis focuses on developmental processes in bone marrow, studying the expression of phenotypic markers by flow cytometry in **chapter 2.1.** Together with the EuroFlow consortium we developed a new flow cytometry panel and a specific analysis strategy, that gives insight into how processes that guide B cell precursor development are linked to phenotypic expression patterns. Here we found that although the start and the end-point of BCP development are defined, the route to get to the end-point can be different for individual cells, with asynchronous marker expression while passing the checkpoints that are dictated by IgH and IgL rearrangements. In **chapter 2.2** we studied the naive BR repertoire of controls and patients with common variable immunodeficiency (CVID) using Next Generation sequencing of the B cell receptor loci. We found that the naive BR repertoire of patients with CVID was comparable to healthy controls. Both the diversity of the repertoire and the junction characteristics of the CVID samples were in the same range as healthy controls, indicating that although the numbers of B cells might be low in CVID patients, the naive cells that are selected for the periphery have a normal BR repertoire.

Naive B cells need to be activated by antigen, and this signal is amplified by the CD19 complex (described in Part 3). In **chapter 3.1** we discuss the function of the CD19 complex and described human patients with deficiencies in this complex that have been reported in literature. **Chapter 3.2** is dedicated to a patient with CD21 deficiency that we described and compared to previously described CD19 deficiency. Here we found that depending on which protein is affected, the phenotype and disease severity are slightly different, and this is related to the function of the protein in the complex.

A recent discovery in the PAD field are the dysregulations of the PI3K/PTEN-AKT signaling pathway. Part 4 focuses on disturbances in this pathway, starting with a case report of a young girl with a mediastinal mass who was finally diagnosed with Activated PI3Kδ syndrome (APDS) in **chapter 4.1**. This disease is characterized by recurrent infections, progressive airway damage, EBV and CMV viremia and lymphopenia due to dysregulation of the PI3K-AKT pathway in T cells and B cells. This report is an example of how finding a genetic diagnosis leads to personalized treatment, furthermore it underlines the importance of multidisciplinary diagnostics in complicated cases. **Chapter**

4.2 describes patients with heterozygous loss-of function mutations in PTEN, that leads to PTEN hamartoma tumor syndrome (PHTS). We found that a subset of these patients suffer from hypogammaglobulinemia and that this is partially due to the dysregulation of PI3K/PTEN AKT signaling in the T and B lymphocytes. This dysregulation was also observed in patients who did not show overt hypogammaglobulinemia, but it indicates that immune deficiencies should be considered as possible clinical phenotype in patients with PHTS. We continued these studies in **chapter 4.3**, studying patients with APDS. We identified PI3KCD and PIKR1 mutations in patients of our PAD cohort and studied their peripheral B cell compartment. We found that the B cells of APDS patients display a specific phenotype with a high frequencies of transitional B cells and reduced memory B-cells despite relatively normal levels of somatic hypermutation. Furthermore, B cells from these patients were extremely sensitive to apoptosis, which probably contributes to the clinical phenotype. We performed more studies on APDS patients in **chapter 4.4**, where we found that $CD8^+$ T cells of these patients, show an exhausted phenotype rather than a senescent phenotype. Additionally, blocking the PD-1 PD-L1 interaction could restore cytokine production and proliferation of EBV-specific $CD8^+$ T cells in these patients, suggesting that drugs that interfere with this interaction might be useful to treat chronic viral infections in these patients.

Finally in part 5 (**chapter 5 General discussion)**, we highlight interesting findings of our studies and additionally shed light on how our findings can lead to better understanding of PAD and B cell differentiation. We discuss how PI3K signaling forms a link between the B cell receptor and CD19-complex signaling. Furthermore, recent ideas on CD21lo cells are discussed and related to CD19-complex deficiencies and APDS. Disease- causing mechanisms other than the classical loss-of-function mutations are summarized including gain-of-function mutations, polygenic causes and B cell extrinsic factors that contribute to disease. Furthermore we suggest new tools such as cyTOF, epigenetic studies and single cell analysis will further enhance knowledge on B cell biology and PAD.

And in the end, it is repertoire: the development of B cells via multiple pathways in bone marrow and the checkpoint that cells have to pass ensure a competent B cell repertoire, which we studied in more detail using NGS. Both in the CD21 deficient patients and in the PTEN and APDS patients, we studied the antigen experienced memory and plasma cell repertoire, since these mutations affect activation and maturation of the B cells after antigenic encounter and are specifically linked to activation of AID in the case of PI3K-PTEN dysregulation. Altogether, we studied how specific defects lead to disturbances in B cell development and repertoire formation and thereby cause antibody deficiencies.

SAMENVATTING

Het menselijk lichaam is een verzameling weefsels en organen, die op hun beurt allemaal bestaan uit cellen en extra-cellulaire matrixen die samenwerken om het totale organisme te vormen. Mogelijke bedreigingen van zowel buitenaf, in de vorm van vreemde stoffen of infectieuze micro-organismen én van binnenuit, zoals (pre-) maligne cellen, worden herkend en geneutraliseerd door een gespecialiseerd orgaansysteem dat het immuunsysteem wordt genoemd. Het immuunsysteem bestaat uit de lymfoïde organen (zoals beenmerg, lymfklieren en de milt) en de witte bloedcellen: leukocyten. Leukocyten kunnen onderverdeeld worden in de cellen van het aangeboren systeem en de cellen van het adaptieve immuunsysteem. Deze laatsten worden lymfocyten genoemd, ofwel T cellen en B cellen.

B cellen ontstaan in het beenmerg, waar ze differentiëren vanuit bloedvormende (ook wel hematopoietische) stamcellen. Via meerdere voorloper-stadia ontwikkelen ze zich tot voorloper-B cellen en later naïeve B cellen die naar het perifere bloed migreren. In het beenmerg vormt iedere individuele B cel zijn eigen, unieke B cel receptor via het V(D) J-recombinatie proces.

Om een goede kwaliteit van de B cel receptoren te waarborgen moeten de voorloper B cellen een aantal checkpoints passeren. Hier wordt getest of de receptoren op de juiste wijze tot expressie kunnen worden gebracht op de celmembraan en of ze op de juiste manier kunnen signaleren. B cellen die niet slagen voor deze tests worden apoptotisch. De totale pool van verschillende B cel receptoren vormt het naïeve B cel repertoire. Een breed repertoire aan verschillende B cel receptoren is nodig om te zorgen dat ieder mogelijk antigeen herkend kan worden. Bij het signaleren van antigeen differentiëren de naïeve B cellen in geheugen B cellen of plasmacellen, die antistoffen kunnen produceren. Dit proces vindt plaats in de secundaire lymfoïde organen, waar de naïeve B cellen affiniteits-maturatie en proliferatie ondergaan, met of zonder hulp van $CD4^+$ T cellen.

Indien de B cellen niet in staat zijn om functionele plasma cellen te vormen door een aangeboren defect, kunnen te weinig antistoffen worden gevormd, wat resulteert in een primaire antistofdeficiëntie: PAD. Patiënten met PAD krijgen recidiverende infecties, maar zij kunnen ook lijden aan auto-immuniteit, lymfo-proliferatieve aandoeningen of maligniteiten. Sommige van deze patiënten hebben een blokkade in de voorloper B cellen in het beenmerg door een defect in een vroege transcriptiefactor, in de B cel receptor zelf of in de signaleringsmoleculen die nodig zijn voor B-cel signalering. Andere processen die verstoord kunnen zijn in patiënten met PAD zijn B-cel overleving, activatie door antigenen, klasse switch recombinatie, vorming van geheugen cellen of zelf het uitscheiden van de antistoffen door de plasma cellen. In een deel van de patiënten met PAD wordt de ziekte

veroorzaakt door genetische defecten, fouten in het DNA, maar in veel van hen is nog geen genetisch defect gevonden en is soms zelfs de etiologie van de ziekte onbekend.

Het bestuderen van de etiologie en de immuno-biologie van PAD vereist uitgebreide kennis over normale B cel ontwikkeling. Het verbinden van B cel differentiatie met ziekte-veroorzakende genetische defecten of specifieke klinische fenotypes geeft nieuwe inzichten in pathofysiologie, prognose en nieuwe behandelingsopties voor PAD patiënten. Verder kan het bestuderen van deze ziekten ons meer leren over eiwit-functie, ontwikkelings-checkpoints en signaleringscascades in B cel ontwikkeling.

Het doel van dit proefschrift was het bestuderen van B cel ontwikkeling in de context van antistof deficiënties en hoe specifieke defecten signaleringscascades beïnvloeden die nodig zijn voor normale ontwikkeling. Dit proefschrift volgt de lijn van B cel ontwikkeling, beginnend in het beenmerg tot aan de geheugen B cel en plasma cel formatie na antigene activatie.

Deel 1 belicht normale B cel ontwikkeling en recent onderzoek betreffende deze ontwikkeling in de Algemene introductie. Verder worden hier verscheidene genetische defecten besproken, die leiden tot antistof deficiënties. Deze defecten worden geplaatst in de context van de normale B cel ontwikkeling. Dit onderstreept hoe belangrijk het is om B cel ontwikkeling samen met antistof deficiënties te bestuderen.

Deel 2 focust op B cel ontwikkeling in het beenmerg, waarbij in hoofdstuk 2.1 wordt beschreven hoe humane B cel ontwikkeling in het beenmerg met behulp van flowcytometrie kan worden onderzocht. In samenwerking met het EuroFlow Consortium hebben we een nieuw flowcytometrie panel ontwikkeld met een eigen analyse strategie. Samen geeft dit inzicht in hoe de sturende processen in B cel voorloper ontwikkeling samenhangen met fenotypische expressie patronen. Hier vonden we dat alhoewel het begin en eindpunt bekend zijn, de route tussen deze twee punten verschillend kan zijn voor iedere individuele cel, waarbij asynchrone expressie van markers kan bestaan terwijl de cellen de checkpoints passeren die worden gevormd door de status van zware- en lichte keten rearrangering. In hoofdstuk 2.2 hebben we het naïeve B cel receptor repertoire bestudeerd in gezonde controles en patiënten met Common Variable Immunodeficientie (CVID). Hiervoor hebben we Next Generation Sequencing technieken gebruikt om de B cel receptor loci te sequencen. We vonden dat het naïeve B cel receptor repertoire van onze patiënten groep vergelijkbaar was met dat van gezonde controles. Zowel de diversiteit als de junctie-karakteristieken van de CVID patiënten bevonden zich in dezelfde range als de gezonde controles. Dit indiceert dat hoewel de aantallen B cellen in het perifere bloed laag zijn in CVID patiënten, de cellen die geselecteerd worden in het beenmerg een min of meer normaal repertoire lijken te vormen.

Naïeve B cellen moeten worden geactiveerd door antigen, dit signaal wordt versterkt door het CD19-complex, beschreven in deel 3. In hoofdstuk 3.1 bespreken we de functie

van het CD19 complex en beschrijven we de humane patiënten met deficiënties in dit complex die tot nu toe in de literatuur beschreven zijn. Hoofdstuk 3.2 is gewijd aan een patiënt met CD21-deficiëntie die wij beschreven en vergeleken met de eerder beschreven CD19-deficiëntie. Hier vonden we dat afhankelijk van welk eiwit is aangedaan, het fenotype en de ernst van de ziekte enigszins kunnen verschillen en dat dit samenhangt met de functie van de verschillende eiwitten binnen het complex.

Een recente ontdekking in het PAD-veld vormen de ziekten die veroorzaakt worden door disregulatie van de PI3K/PTEN-AKT signaleringsroute. Deel 4 focust op verstoringen in deze signaleringsroute, te beginnen met een casusbeschrijving van een jong meisje met een massa in het mediastinum, die uiteindelijk werd gediagnosticeerd met Activated PI3Kδ Syndrome (APDS) in hoofdstuk 4.1. Deze ziekte wordt gekenmerkt door recidiverende infecties, progressieve schade aan de luchtwegen, EBV en CMV viraemie en lymfopenie veroorzaakt door disregulatie van de PI3K-AKT signalering in T cellen en B cellen. Deze casusbeschrijving is een voorbeeld van hoe het vinden van een genetische diagnose kan leiden tot gepersonaliseerde behandeling en daarnaast onderschrijft deze casus het belang van multidisciplinaire diagnostiek in gecompliceerde casus. Hoofdstuk 4.2 beschrijft patiënten met heterozygote loss-of-function mutaties in PTEN, welke leiden tot PTEN hamartoma tumor syndroom (PHTS). Wij vonden dat een subgroep van deze patiënten lijdt aan een antistofdeficiëntie en dat dit deels veroorzaakt wordt door disregulatie in de PI3K/PTEN-AKT signalering in de T en B cellen van deze patiënten. Deze disregulatie werd ook gezien in patiënten zonder klinisch evidente antistofdeficiëntie, maar het impliceert dat immuundeficiënties mogelijk een onderdeel kunnen vormen van het klinisch fenotype van patiënten met PHTS. We zetten onze studies voort in hoofdstuk 4.3, met het bestuderen van patiënten met APDS. We vonden mutaties in PIK3CD en PIK3R1 in patiënten in ons PAD cohort en bestudeerden hun B cellen in het perifere bloed. We vonden dat de B cellen van patiënten met APDS een specifiek fenotype vertonen met een hoge frequentie van transitionele B cellen en verminderde geheugen B cellen, ondanks relatief normale frequenties van somatische hypermutaties. Verder waren de B cellen van deze patiënten extreem gevoelig voor apoptose, wat waarschijnlijk bijdraagt aan het klinisch fenotype van de patiënten. In hoofdstuk 4.4 hebben we nog meer studies gedaan bij APDS patiënten, hier vonden we dat $CD8^+$ T cellen van deze patiënten een zogenaamd "exhausted" (uitgeput) fenotype vertonen, meer dan een "senescent" (slapend) fenotype. Verder vonden we dat het blokkeren van PD-1 PD-L1 interactie het cytokine producerend vermogen en prolifererend vermogen van EBV-specifieke T cellen kon herstellen in deze patiënten. Dit suggereert dat medicijnen die interfereren met deze PD-1 PD-L1 interactie mogelijk van nut kunnen zijn om virale infecties bij deze patiënten te bestrijden.

Tenslotte wordt in hoofdstuk 5, de algemene discussie, aandacht besteed aan de meest interessante bevindingen uit dit proefschrift en ook wordt hier beschreven hoe deze

bevindingen leiden tot nieuwe inzichten in PAD en B cel ontwikkeling. Er wordt beschreven hoe PI3K een verbinding vormt tussen de B cel receptor en het CD19-complex. Verder worden recente ideeën omtrent CD21lo cellen beschreven en deze worden gerelateerd aan CD19-complex deficiënties en APDS. Ziekte-veroorzakende mechanismen die anders zijn dan klassieke loss-of-function mutaties worden beschreven, zoals gain-of-function mutaties, polygenetische oorzaken en B-cel extrinsieke factoren die kunnen bijdragen aan het ontwikkelen van een antistof deficiëntie. Ook wordt dieper ingegaan op nieuwe mogelijkheden zoals cytometrie time-of-flight (cyTOF), epigenetische studies en analyse van individuele cellen om meer inzicht te krijgen in B cel biologie en PAD.

En uiteindelijk is het repertoire: het ontwikkeling van B cellen via meerdere routes in het beenmerg en de checkpoints die ze moeten passeren om te komen tot een competent B cel repertoire, wat we in detail bestudeerden met NGS. Zowel in de CD21 deficiënte patiënten als in de APDS en PTEN patiënten hebben we het antigeen-geselecteerde repertoire bestudeerd, omdat deze mutaties invloed hebben om activatie en maturatie van de B cellen na het herkennen van antigeen, met name in het geval van PI3K/PTEN disregulatie, wat is gelinkt aan de activatie van AID. Samengevat hebben we bestudeerd hoe specifieke defecten leiden tot verstoringen in B cel ontwikkeling en repertoire formatie, waardoor ze antistof deficiënties veroorzaken.

DANKWOORD

De enige verstandige manier van opvoeden bestaat eruit een voorbeeld te zijn, desnoods een waarschuwend voorbeeld - Albert Einstein

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Dear Marta, thank you for being part of my committee. I remember us sitting in the back seat of a car, crossing the beautiful country side around Prague and discussing going back to the clinics after years in the lab. You told me geriatrics was also one of your choices of specialization and that you thought I was making the right choice going back to the clinics. Thank you for your advice, both the scientific and work-related part and the personal advice.

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Dear Martin, Quentin and the rest of the Salamanca lab. Thank you for teaching me how to work with Infinicyt and helping me set-up analysis strategies for bone marrow. I am still learning and the strategies are still evolving, but I think this will be an ongoing process. I have really enjoyed the week I have spent with you in Salamanca, gracias!

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- *Bij twijfel: doe het allebei* - D.A. de Man

CURRICULUM VITAE

Marjolein Wentink was born in Puttershoek on the 30th of March 1987. In 2005 she graduated from St. Monfort College Rotterdam, after completing VWO-gymnasium, profile: "Natuur en gezondheid" (science and health). She studied medicine at Erasmus MC, Rotterdam and in parallel did a research master Molecular Medicine at the same institute. In 2009 she obtained her doctoraal diploma in Medicine after completing an internship entitled: *Expression of Splice-Variants of the Testis-Specific Protein of the Y-Chromosome in Carcinoma in Situ of the Testis Compared to Normal Testis* in the lab of prof. L. Looijenga, Experimental Pathology, Erasmus MC. Subsequently she studied Tuberous sclerosis complex under supervision of dr. M. Nellist and dr. J.A. Kievit at the department of Clinical genetics, Erasmus MC, Roterdam. She defended her master thesis *"Functional analysis of TSC2 variants associated with Tuberous sclerosis complex"* in 2010 to obtain her master degree in Molecular Medicine. From 2011 until 2013 she completed medical internships in different hospitals to finish her MD degree, which she obtained in April 2013. In April 2013 she started her PhD-project entitled: *"Immune repertoire in the picture"* under supervision of dr. M. van der Burg and Prof. dr. J.J.M. van Dongen at the department of Immunology, Erasmus MC, Rotterdam. After completion of her PhD project she resumed clinical work as an MD and she is currently working at the Amphia Hospital in Breda as a resident in Clinical Geriatrics.

PHD PORTFOLIO

Name PhD student:	Marjolein Wentink
Erasmus MC department:	Immunology
Research school:	Molecular Medicine
PhD-period:	April 2013-October 2017
Promotor:	Prof. dr. J.J.M. van Dongen
Co-promotor:	Dr. M. van der Burg

PhD training

Courses

2013	NGS data analysis, MGC, University Medical Center Utrecht
2014	Advanced Immunology Course, MolMed, Erasmus MC
2016	Biomedical English writing, Erasmus MC
2016	Course on R, MolMed, Erasmus MC

Workshops

2014	MS Excel Basic and MS Excel Advanced , MolMed
2014	Photoshop and Illustrator workshop, MolMed
2014	Research integrity, Erasmus MC

Traject deel-BKO, Desiderius School, Erasmus MC (finished)

2016	Teach the teacher I
2014	workshop "individuele begeleiding"
2017	workshop "feedback geven"

Seminars and symposia

2013-2017	seminars and mini-symposia at the department of Immunology
2013-2017	weekly journal club at the department of Immunology
2013	EuroFlow PID juniors, visit to CLIP, Prague, Czech republic
2014	EuroFlow Infinicyt Workshop, Salamanca, Spain
2016	Symposium Medical Immunology, De Doelen, Rotterdam
2017	ESID juniors Spring School, Sv. Jan Pod Skalou, Czech Republic

National conferences

2013 Dutch Society for Immunology (NVVI) annual meeting
Poster: CD21 deficiency and CD19 deficiency: two defects in the same complex lead to different disease modalities

2013 Werkgroep Immuundeficienties (WID)
Oral presentation: CD21 deficientie

2014 Dutch Society for Immunology (NVVI) annual meeting
Poster: CD21 deficiency and CD19 deficiency: two defects in the same complex lead to different disease modalities

2015 Molecular Medicine Day, Rotterdam
Oral presentation: CD21 deficiency and CD19 deficiency: two defects in the same complex lead to different disease modalities

2015 Dutch Society for Immunology (NVVI) annual meeting
Poster: Genetic defects in PI3Kδ affect B-cell differentiation and maturation via multiple AKT-dependent pathways

2016 Molecular Medicine Day, Rotterdam
Poster: Genetic defects in PI3Kδ affect B-cell differentiation and maturation via multiple AKT-dependent pathways

2017 Werkgroep Immuundeficienties (WID)
Oral presentation: Immunological aspects of APDS

International conferences

2015 European Congress of immunology, Vienna, Austria
Oral presentation: CD21 deficiency and CD19 deficiency: two defects in the same complex lead to different disease modalities

2015 IPIC conference, Budapest, Hungary

2016 Keystone, Systems Immunology, Big Sky, Montana, United Stated
Poster: Genetic defects in PI3Kδ affect B-cell differentiation and maturation via multiple AKT-dependent pathways

2016 European Society for Immune Deficiencies (ESID) Biennial Meeting, Barcelona, Spain
Poster: Using geneticaly defined PID cases to unravel normal precursor B-cell differentiation in bone marrow

EuroFlow PID meetings

2013 Rotterdam, The Netherlands, *oral presentation: Precursor B-cell labelings, EuroFlow*

2014	Milan, Italy, *oral presentation: 10-12 color flow cytometry for Precursor B cells in Bone Marrow*
2015	Madrid, *oral presentation: Precursor B-cell labelings BM EuroFlow PID Workpackage*
2015	Zurich, Swiss, *oral presentation: Using genetically defined PID cases to unravel normal precursor B-cell differentiation in bone marrow*
2016	Rome, Italy, *oral presentation: PI3K project introduction and hypothesis*
2016	Leiden, The Netherlands *no presentation*
2016	Prague, Czech Republic, *oral presentation Using genetically defined PID cases to unravel normal precursor B-cell differentiation in bone marrow*
2017	The Hague, The Netherlands, *oral presentation: Flowcytometric immunophenotyping of normal bone marrow and PID cases, the EuroFlow approach*

Memberships

2013-2017	Junior member of the European Society for Immunodeficiencies
2013-17	Member of the Dutch Society of Immunology

Teaching activities

2013-2017	Histology practicals, 1st and 2nd year medical students, and Clinical Technology
2015+2016	Keuze onderwijs Primaire immuundeficienties, 2nd year medical students, *Lecture: PI3K signalering defecten* *Lecture: B cell development*
2017	Keuze onderwijs Primaire immuundeficienties, 2nd year medical students, *Practical: PID cases*
2016+2017	Minor Biomedical research in practise *Lecture: PI3K signaling defects*
2017	Summer course Master Infection and Immunity, *Lecture: CVID and APDS*
2014-2016	supervision of 2nd year medical students for systematic review writing
2014	supervision 2 week lab-rotation minor students
2014	supervision internship master Life Science and Technology Arko Boogert *Title: Activity of AKT in CVID*
2015	supervision internship master Molecular Medicine, Margot Bongenaar *Title: Dysregulation of Phosphoinositide 3-kinase/AKT signaling in common variable immune deficiency*
2016	supervision internship master Medicine, Eveline de Leeuw *Title: BACH2 overexpression could lead to disharmonic B-cell development*

Funds

2015	European Federation of Immunological Societies, ECI travel grant to attend European Conference of Immunology
2015	Dutch Society for Immunology (NVVI) travel grant, to support 1 week working visit to University of Salamanca, Spain (project: B cell precursor development)
2016	Erasmus Trustfonds travel grant to attend Keystone symposium: Systems Immunology
2017	European Federation of Immunological Societies, short-term fellowship to support 8 weeks to visit lab of dr. Tomas Kalina, Prague Czech Republic (project: cyTOF to study B cell differentiation in bone marrow)

PUBLICATIONS

1. **Wentink MWJ**, Mueller YM, Dalm VASH, Driessen GJ, van Hagen PM, van Montfrans JM, van der Burg M, Katsikis PD. Exhaustion of the CD8(+) T cell compartment in patients with mutations in phosphoinositide 3-kinase delta. Front Immunol. 2018;9:446.

2. Blanco E, Perez-Andres M, Sanoja-Flores L, **Wentink M**, Pelak O, Martín-Ayuso M, Grigore G, Torres-Canizales J, López-Granados E, Kalina T, van der Burg M, Arriba-Méndez S, Santa Cruz S, Puig N, van Dongen JJM, Orfao A. Selection and validation of antibody clones against IgG and IgA subclasses in switched memory B-cells and plasma cells. J Immunol Methods. 2017. pii: S0022-1759(17)30079-0.

3. **Wentink M**, Dalm V, Lankester AC, van Schouwenburg PA, Schölvinck L, Kalina T, Zachova R, Sediva A, Lambeck A, Pico-Knijnenburg I, van Dongen JJ, Pac M, Bernatowska E, van Hagen M, Driessen G, van der Burg M. Genetic defects in PI3Kδ affect B-cell differentiation and maturation leading to hypogammaglobulineamia and recurrent infections. Clin Immunol. 2017;176:77-86.

4. Driessen GJ, IJspeert H, **Wentink M**, Yntema HG, van Hagen PM, van Strien A, Bucciol G, Cogulu O, Trip M, Nillesen W, Peeters EA, Pico-Knijnenburg I, Barendregt BH, Rizzi M, van Dongen JJ, Kutukculer N, van der Burg M. Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency. J Allergy Clin Immunol. 2016;138(6):1744-1747.e5.

5. Ekong R, Nellist M, Hoogeveen-Westerveld M, **Wentink M**, Panzer J, Sparagana S, Emmett W, Dawson NL, Malinge MC, Nabbout R, Carbonara C, Barberis M, Padovan S, Futema M, Plagnol V, Humphries SE, Migone N, Povey S. Variants within TSC2 exons 25 and 31 are very unlikely to cause clinically diagnosable tuberous sclerosis. Hum Mutat. 2016;37(4):364-70.

6. Suratannon N, Yeetong P, Srichomthong C, Amarinthnukrowh P, Chatchatee P, Sosothikul D, van Hagen PM, van der Burg M, **Wentink M**, Driessen GJ, Suphapeetiporn K, Shotelersuk V. Adaptive immune defects in a patient with leukocyte adhesion deficiency type III with a novel mutation in FERMT3. Pediatr Allergy Immunol. 2016;27(2):214-7.

7. **Wentink MW**, Lambeck AJ, van Zelm MC, Simons E, van Dongen JJ, IJspeert H, Schölvinck EH, van der Burg M. CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities. Clin Immunol. 2015;161(2):120-7.

8. IJspeert H, **Wentink M**, van Zessen D, Driessen GJ, Dalm VA, van Hagen MP, Pico-Knijnenburg I, Simons EJ, van Dongen JJ, Stubbs AP, van der Burg M. Strategies for B-cell receptor repertoire analysis in primary immunodeficiencies: from severe combined immunodeficiency to common variable immunodeficiency. Front Immunol. 2015;6:157.

9. **Wentink M**, Nellist M, Hoogeveen-Westerveld M, Zonnenberg B, van der Kolk D, van Essen T, Park SM, Woods G, Cohn-Hokke P, Brussel W, Smeets E, Brooks A, Halley D, van den Ouweland A, Maat-Kievit A. Functional characterization of the TSC2 c.3598C>T (p.R1200W) missense mutation that co-segregates with tuberous sclerosis complex in mildly affected kindreds. Clin Genet. 2012;81(5):453-61.

10. Hoogeveen-Westerveld M, **Wentink M**, van den Heuvel D, Mozaffari M, Ekong R, Povey S, den Dunnen JT, Metcalfe K, Vallee S, Krueger S, Bergoffen J, Shashi V, Elmslie F, Kwiatkowski D, Sampson J, Vidales C, Dzarir J, Garcia-Planells J, Dies K, Maat-Kievit A, van den Ouweland A, Halley D, Nellist M. Functional assessment of variants in the TSC1 and TSC2 genes identified in individuals with Tuberous Sclerosis Complex. Hum Mutat. 2011;32(4):424-35.